Natividad Garrido Mesa

Minocycline in inflammatory bowel disease: far beyong an antibiotic

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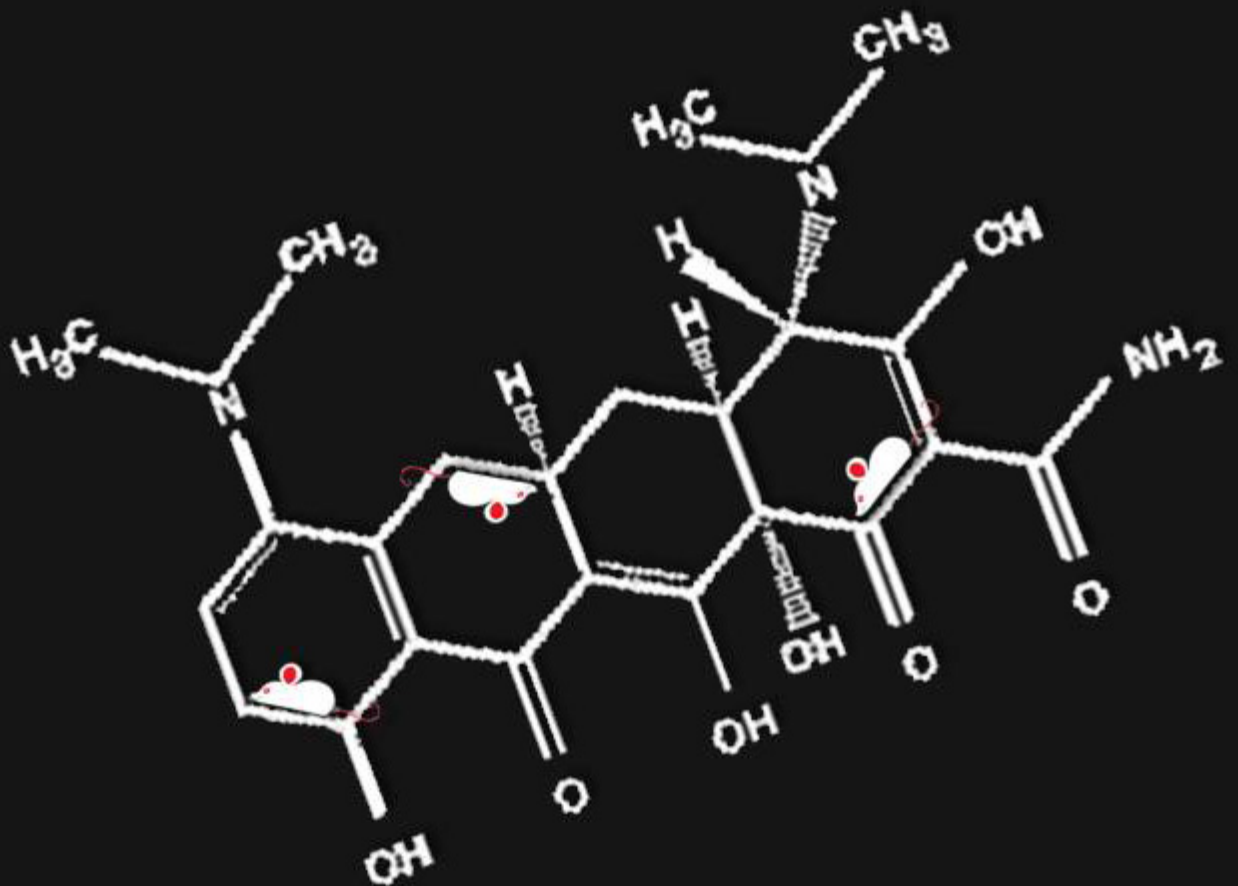

Tesis Doctoral

Natividad Garrido Mesa

Departamento de Farmacología

Universidad de Granada

MINOCYCLINE IN INFLAMMATORY BOWEL DISEASE: FAR BEYOND AN ANTIBIOTIC.

Tesis Doctoral

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Universidad de Granada.

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Minocycline in inflammatory bowel disease: far beyond an antibiotic

Tesis doctoral para aspirar al grado de doctor presentada por

Natividad Garrido Mesa

Bajo la dirección de los Doctores

Antonio Zarzuelo Zurita
Julio Juan Gálvez Peralta
María Elena Rodríguez Cabezas

Granada, 2011

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DEPARTAMENTO DE FARMACOLOGIA

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Certifica: que la Tesis Doctoral titulada "Minocycline in inflammatory bowel disease: far beyond an antibiotic", ha sido realizada por la Licenciada en Farmacia Dña. Natividad Garrido Mesa, en los laboratorios de este Departamento.

Y a los efectos legales se firma la siguiente constancia en Granada, a 24 de noviembre de 2011.

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A mi familia.

When you make the finding yourself – even if you're the last person on Earth to see the light – you'll never forget it.

Carl Sagan

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1. Introducción.

El término Enfermedad Inflamatoria Intestinal (EII) comprende dos patologías: la enfermedad de Crohn y la colitis ulcerosa. Ambas enfermedades se caracterizan por una inflamación crónica y recurrente del tracto intestinal, de etiología desconocida, en la que se alternan periodos de exacerbación de los síntomas, seguidos de intervalos más o menos prolongados de remisión de los mismos [1, 2].

Aunque hasta el momento se desconocen los mecanismos responsables de la iniciación y perpetuación en el tiempo del proceso inflamatorio intestinal, es aceptado que en su fisiopatología están implicados factores genéticos, ambientales e inmunológicos (Figura 4). Así, numerosos estudios han propuesto que, en personas genéticamente predispuestas, una activación exagerada y descontrolada del sistema inmune intestinal frente a un determinante antigénico desconocido, puede desencadenar la aparición de la respuesta inflamatoria intestinal exacerbada [3]. Esta respuesta inmunológica genera numerosos mediadores de carácter pro-inflamatorio (citocinas, eicosanoides y metabolitos reactivos derivados del oxígeno o del nitrógeno) que actúan de forma sinérgica y simultánea promoviendo la amplificación y cronificación del proceso inflamatorio intestinal [4 - 8].

Asimismo, son numerosos los estudios que sugieren que la microbiota entérica juega un papel clave en el inicio y desarrollo de la EII [9]. Se propone que existe un desequilibrio en las concentraciones luminales de determinadas bacterias en pacientes con EII, proceso llamado disbiosis, de forma que el incremento en la proporción de bacterias potencialmente dañinas (*E. coli* enteroinvasivo, *Bacteroides fragilis*, y anaerobios totales) se acompaña de la disminución de bacterias potencialmente protectoras de la función intestinal (lactobacilos y bifidobacterias) [10 - 14].

El papel que la microbiota intestinal desempeña en la patogénesis de la EII se ve reforzado por numerosas observaciones. En pacientes de EII, el número de bacterias asociadas a la capa mucosa intestinal se encuentra incrementado [11, 15]. Además, las zonas del intestino con mayor carga bacteriana (íleon distal y colon) son los lugares más frecuentemente afectados por el proceso inflamatorio en la EII [8]. En base a esto, el tratamiento dirigido a reducir la carga bacteriana en el lumen intestinal, mediante la administración de antibióticos (metronidazol o ciprofloxacino), ha resultado efectivo induciendo la remisión en pacientes con EII [16, 17]. La evidencia más contundente, deriva de modelos experimentales que muestran cómo el proceso inflamatorio intestinal que se desarrolla de forma espontánea en distintos animales transgénicos, no tiene lugar cuando los animales son mantenidos en condiciones libres de gérmenes [18 - 22].

Por lo tanto, una estrategia terapéutica potencialmente útil en el control de estas enfermedades consistiría en restablecer el equilibrio en la microbiota del lumen intestinal, lo que podría obtenerse mediante la administración de determinados antibióticos y/o probióticos [23].

En base a esto, algunos antibióticos de amplio espectro, como el metronidazol o el ciprofloxacino, han sido ampliamente utilizados en el tratamiento de la enfermedad de Crohn [23 - 26] . Además, recientemente se ha descrito su capacidad de inducir la remisión en pacientes con colitis ulcerosa, bien mediante una triple terapia antibiótica [27] o asociados a corticoides [28]. No obstante, ha sido en el tratamiento de la *pouchitis* donde sin duda han demostrado su máxima eficacia, constituyendo de hecho unas de las primeras líneas de tratamiento [29, 30]. Sus efectos beneficiosos en la EII han sido clásicamente atribuidos a su acción antimicrobiana, por la que modificarían la población bacteriana del lumen intestinal, y prevendrían la adhesión y posterior traslocación de bacterias potencialmente patógenas hacia el tejido intestinal.

Como consecuencia, la instauración de una respuesta inmune exacerbada se vería reducida [31 - 33] (Figura 6).

Sin embargo, estudios más recientes han puesto de manifiesto que, además de su actividad antimicrobiana, algunos antibióticos pueden presentar propiedades inmunomoduladoras, afectando así tanto a la respuesta inmune innata como a la adquirida [34, 35, 37] (Figura 6). En concreto, la minociclina, una tetraciclina semisintética de segunda generación, ha demostrado poseer propiedades inmunomoduladoras, anti-apoptóticas y anti-inflamatorias al margen de su acción antimicrobiana [38]. Tales propiedades han resultado de gran interés en numerosas enfermedades como el acné vulgaris, la periodontitis, artritis reumatoide, esclerosis múltiple, asma, isquemia, y daños medulares entre otras, así como en diversas enfermedades neurodegenerativas (Parkinson, Alzheimer, enfermedad de Huntington) [38 - 45] (Figura 2). Los mecanismos responsables de su actividad inmunomoduladora no son aún del todo conocidos, pero de entre sus múltiples acciones destacan su actividad antioxidante, la inhibición de distintas enzimas implicadas en el proceso inflamatorio (óxido nítrico sintasa (NOS) (*Nitric Oxide Synthase)*, metaloproteinasas de la matriz extracelular (MMPs) *(Matrix Metalloproinase)* , ciclooxigenasa (COX), fosfolipasa A2 (PLA_2)) y la regulación de la muerte, proliferación y activación de células inmunocompetentes (microglia, linfocitos T, macrófagos, neutrófilos) [46 - 54] (Figura 3).

Es importante destacar que todas estas acciones sobre la respuesta inmune, al colaborar con su acción antimicrobiana, pueden resultar clave en el tratamiento de las infecciones, tal y como se ha propuesto en diversas afecciones sistémicas o localizadas en el aparato respiratorio [37, 55]. Sin embargo, son escasos los estudios que han descrito el impacto que los antibióticos pueden tener en la respuesta inmune intestinal [56].

Esto adquiere una especial relevancia en el caso de la EII, ya que la posibilidad de disponer de un compuesto como la minociclina, en el que se combina la actividad antimicrobiana junto con un efecto inmunomodulador, podría constituir un gran avance en el tratamiento de estas enfermedades intestinales. Sin embargo, algunos estudios han puesto de manifiesto que la supresión del tratamiento antibiótico generalmente conlleva la reactivación del proceso inflamatorio, lo que implicaría su utilización durante periodos prolongados de tiempo, suponiendo un riesgo por la aparición de reacciones adversas [17].

En cuanto al uso de probióticos en la EII, la evidencia más significativa viene de ensayos clínicos en los que se ha evaluado la efectividad del probiótico *Escherichia coli* Nissle 1917 o de la mezcla de probióticos VSL#3 [57, 58], y que muestran su utilidad como tratamientos para el mantenimiento del estado de remisión y la prevención de las recaídas, más que como tratamiento inductor de la remisión [59]. Los mecanismos propuestos como responsables de los beneficios derivados de su uso en la EII incluyen: la producción de compuestos antibacterianos y la reducción del pH, acciones por las que modifican la composición de la microbiota intestinal, inhibiendo el crecimiento de bacterias nocivas; su capacidad de desplazar a estas bacterias de su sitio de unión al epitelio; la mejora de la función de barrera intestinal; y la modulación de la respuesta inmune de la mucosa del hospedador [60, 61] (Figura 7). Sin embargo, no todos los probióticos comparten las mismas propiedades, cada uno posee mecanismos de acción individuales, y es el estado del hospedador el que va a condicionar la elección de la especie o cepa óptima [62].

Por lo tanto, teniendo en cuenta la etiología de la EII, sería interesante desarrollar estrategias terapéuticas que reinstauren el equilibrio en la microbiota intestinal, corrigiendo así la disbiosis, y que al mismo tiempo, controlen la

respuesta inmune alterada que promueve el proceso inflamatorio intestinal a largo plazo.

A pesar de que el uso combinado de antibióticos y probióticos ya ha sido propuesto en el contexto de la EII con la intención de crear con los antibióticos un nicho microbiológico que pueda ser posteriormente ocupado por los probióticos [56, 63], sólo algunos estudios apoyan esta estrategia [64, 65], y ninguno de ellos contempla los beneficios derivados de la modulación de la respuesta inmune.

En base a esto se propusieron dos objetivos principales:

1) Evaluación del efecto antiinflamatorio intestinal de la minociclina en distintos modelos experimentales de colitis, con la intención de estudiar el papel de sus propiedades inmunomoduladoras y antibióticas en el efecto global alcanzado. Para ello la minociclina, administrada de forma oral, fue ensayada como tratamiento preventivo y curativo en el modelo de colitis experimental por ácido trinitrobencenosulfónico (TNBS) en ratas, y sus efectos como tratamiento crónico se estudiaron en el modelo del sulfato de dextrano sódico (DSS) *(Dextran Sulfate Sodium)* en ratones (Figuras 8 y 9). De forma adicional, su acción inmunomoduladora directa se comprobó mediante estudios *in vitro* sobre la línea epitelial de mucosa colónica (Caco-2) y la línea celular macrofágica RAW 264.7, dos tipos celulares implicados activamente en la respuesta inmune intestinal.

2) Caracterización de los efectos derivados de la asociación del antibiótico minociclina y el probiótico *Escherichia coli* Nissle 1917 en un modelo de colitis experimental con recidivas inducida por DSS en ratones. Con ésto se pretende establecer una estrategia terapéutica que restaure el balance en la microbiota entérica al tiempo que equilibre la respuesta inmune, ejerciendo un efecto curativo; una vez alcanzado el estado de remisión, el mantenimiento del tratamiento probiótico actuará previniendo la reactivación del proceso

inflamatorio tras la interrupción del tratamiento antibiótico. Para ello se siguió un protocolo de tratamiento curativo: una vez establecida la colitis, se administró diariamente el antibiótico durante una semana, con el objetivo de controlar el proceso inflamatorio. Tras este periodo, se procedió a administrar el probiótico, cuya administración se mantuvo hasta el final de la experiencia con la intención de mantener el estado de remisión. Dos semanas después del inicio de los tratamientos, los ratones fueron sometidos a un segundo ciclo de DSS, obteniéndose de esta forma una reactivación del proceso inflamatorio que permitiera la evaluación del impacto de la asociación en la prevención de la reactivación de la inflamación intestinal en comparación con los dos tratamientos por separado. Distintos grupos de animales fueron sacrificados a distintos tiempos (7, 14, 21 y 28 días), evaluándose la evolución del proceso inflamatorio en el tiempo (Figura 10).

Durante el desarrollo de las experiencias se controlaron diariamente una serie de parámetros generales, que incluyen el consumo diario de comida de los animales, la evolución del peso corporal, y la aparición de heces diarreicas y sanguinolentas por visualización de restos perianales [66]. Tras el sacrificio de los animales, el colon fue extraído en su totalidad, se recogieron los contenidos luminales para la realización de los correspondientes estudios microbiológicos [67] y las alteraciones intestinales fueron caracterizadas en base a parámetros macroscópicos, microscópicos y bioquímicos, evaluando el efecto antiinflamatorio intestinal de los diferentes tratamientos administrados.

El daño macroscópico fue valorado en función de la relación peso/longitud del colon. En el modelo del TNBS se asignó un índice de daño macroscópico (IDM) de acuerdo con el criterio descrito por Bell y col. (1995) [66] (Tabla 1). Para la evaluación microscópica, las muestras de colon fueron teñidas con hematoxilina-eosina y los cambios histológicos fueron evaluados según el criterio establecido por Stucchi y col. (2000) (Tabla 3). Por último, las determinaciones bioquímicas

incluyeron la valoración del estado oxidativo colónico, mediante la determinación de la actividad del enzima mieloperoxidasa (MPO) [68], el contenido de glutation (GSH) total [69] y la expresión proteica del enzima oxido nítrico sintasa inducible (iNOS) intestinal mediante Western blot [70]. Asimismo se valoró la producción de citocinas pro-inflamatorias como el factor de necrosis tumoral (TNF) (*Tumour Necrosis Factor)* α o las interleucinas (IL) -1β y -6 mediante técnicas de ELISA. Finalmente, se procedió al análisis de la expresión génica, mediante PCR cualitativa y cuantitativa, de distintos marcadores del proceso inflamatorio. Esto incluye citocinas como TNFα, IL-1β, IL-6, IL-17 e IL-2; mediadores quimiotácticos como la proteína quimioatrayente de monocitos MCP-1 *(Monocyte Chemoatractant Protein)*, la molécula quimioatrayente de neutrófilos inducidos por citoquina CINC-1 (*Cytokine-Induced Neutrophil Chemoattractant*) y la proteína inflamatoria de macrófagos MIP (*Macrophage Inflammatory Protein*) -2; la molécula de adhesión intercelular ICAM (*Inter-Cellular Adhesion Molecule*) -1; y marcadores de la función barrera intestinal como mucinas (MUC-2 y MUC-3), *trefoil factor* (TFF) -3, y proteínas de las uniones estrechas del epitelio como *Zonula occludens* (ZO) -1 (Tablas 4 y 5).

2. Resultados.

La actividad inmunomoduladora de la minociclina fue puesta de manifiesto en los estudios *in vitro*, en los que, a diferencia de la tetraciclina o el metronidazol, fue capaz de inhibir de forma concentración-dependiente la producción de IL-8 por células Caco-2 estimuladas con IL-1β, así como la acumulación de nitritos en el medio de cultivo de macrófagos RAW 264.7 estimulados con lipopolisacárido bacteriano (LPS) (Figuras 11 y 12).

In vivo, la minociclina, siguiendo un protocolo de tratamiento curativo, demostró un evidente efecto antiinflamatorio intestinal en el modelo del TNBS en rata a las dos dosis ensayadas (20 y 40mg/kg), de acuerdo con la reducción, en

relación a los animales colíticos no tratados, de los valores de los índices de daño macroscópico y microscópico (Figuras 14 y 15) correspondientes a los animales que recibieron este antibiótico. Sin embargo, ninguno de los otros antibióticos ensayados, tetraciclina y metronidazol, mostró tal efecto antiinflamatorio (Figuras 14 y 15).

Los grupos de animales tratados con minociclina o con tetraciclina mostraron una reducción en la actividad MPO, un buen marcador de la infiltración granulocítica [68, 71] que se encuentra incrementado como consecuencia del proceso inflamatorio. El estrés oxidativo fue contrarrestado igualmente por ambos tratamientos, como muestra la parcial recuperación de los niveles de glutation total, uno de los principales compuestos implicados en la respuesta antioxidante fisiológica [72] que se vio disminuido en los animales colíticos sin tratamiento (Figura 16). Igualmente, ambos antibióticos disminuyeron la producción de TNFα e IL-1β, dos de las principales citocinas involucradas en el proceso inflamatorio [73, 74], si bien el efecto conseguido por la minociclina fue significativamente mayor que el mostrado por la tetraciclina (Figura 17). El metronidazol, a pesar de reducir significativamente la producción de TNFα, no modificó ninguno de los otros parámetros estudiados (Figura 17). La expresión génica de mediadores quimiotácticos de neutrófilos y macrófagos como CINC-1 y MCP-1 y de la molécula de adhesión leucocitaria ICAM-1, fue igualmente reducida por las dos tetraciclinas (Figura 18).

Además, la minociclina afectó significativamente a la expresión del enzima iNOS, principal productor de óxido nítrico (NO) *(Nitric Oxide)*, mediador pro-inflamatorio que juega un papel clave en la patogénesis de la EII [75] (Figura 17). Asimismo, redujo la expresión génica de IL-17, citocina pro-inflamatoria crítica para el desarrollo de la colitis inducida por TNBS [76] y fue capaz de mejorar la función barrera intestinal aumentando la expresión de mucinas (MUC-2) y otros factores esenciales para la integridad del epitelio intestinal (TFF-3) (Figura 18).

El análisis microbiológico reveló que la minociclina también fue el único de los tres antibióticos estudiados que consiguió restablecer parcialmente la ratio entre bacterias beneficiosas (lactobacilos y bifidobacterias)/ baterías potencialmente patógenas (aerobios totales y enterobacterias), drásticamente disminuido en los animales colíticos (Figura 19).

Sin embargo, la administración de minociclina de forma previa a la inducción del daño no fue capaz de prevenir el desarrollo del proceso inflamatorio intestinal, a pesar de que sí que incrementó ligeramente el cociente bacteriano (Figura 20). Estos resultados demostrarían que su actividad antimicrobiana *per se*, no es suficiente para la consecución de un efecto antiinflamatorio, a diferencia de lo que se ha descrito previamente para otros antibióticos [23, 32, 33]. Por lo tanto, podemos sugerir que el efecto antiinflamatorio mostrado en este modelo se debe a algo más que a una acción meramente antimicrobiana, y probablemente derive de la actividad inmunomoduladora atribuida a la minociclina.

El efecto curativo de la minociclina fue confirmado en el modelo del DSS en ratones, en el que se administró como tratamiento crónico durante 21 días, periodo tras el cual los ratones tratados con minociclina experimentaron una mayor recuperación del daño intestinal (disminución del índice de actividad de la enfermedad, menor relación peso/longitud del colon, menor score histológico), asociada a una reducción en la producción de citocinas pro-inflamatorias como IL-1β, TNFα e IL-6 (Figuras 21 y 22).

En conclusión, podemos señalar que la minociclina muestra un efecto antiinflamatorio intestinal en diferentes modelos experimentales de EII, el cual puede ser atribuido a la asociación de su actividad antimicrobiana y sus propiedades inmunomoduladoras.

Una vez demostrado el efecto antiinflamatorio intestinal de este antibiótico,

se procedió al estudio de los efectos derivados de su asociación con el probiótico *E. coli* Nissle 1917, en un modelo de colitis con recidivas en ratones, intentando reproducir la naturaleza recurrente de la EII humana, y considerando las restricciones de la administración crónica de antibióticos.

Los resultados de este estudio mostraron que la asociación de la minociclina con el probiótico presenta un mayor efecto antiinflamatorio intestinal que ambos tratamientos de forma aislada. Igualmente, la reactivación del proceso inflamatorio se vio atenuada en el grupo de animales que habían recibido minociclina seguida de *E. coli* Nissle 1917, de acuerdo a los menores valores de índice de actividad de la enfermedad correspondientes a los animales que recibieron ambos tratamientos (Figura 23).

Estos efectos fueron corroborados histológica (Figura 25) y bioquímicamente. La asociación de tratamientos mostró una mayor efectividad al reducir la expresión génica de citocinas pro-inflamatorias (TNFα, IL-1β, IL-2), mediadores quimiotácticos (MIP-2, MCP-1, ICAM-1) y enzimas como iNOS y MMP-9 (enzima del grupo de las proteasas cuya expresión se encuentra altamente incrementada en el intestino inflamado [6]). Asimismo, también provocó un mayor aumento en la expresión de MUC-3, constituyente de la capa mucosa que recubre el intestino, y de ZO-1, proteína transmembrana componente de las uniones estrechas intercelulares implicada en la preservación de la integridad del epitelio [77]. Estos efectos fueron constatados a los distintos tiempos en que se evaluó el daño colónico (Figuras 26, 28, 29 y 30). Así, tras una semana de tratamiento, la minociclina fue capaz de recuperar el tejido colónico de forma similar a como lo hizo en los tratamientos posteriores. Tras estos siete días, el tratamiento antibiótico fue interrumpido con el objetivo de "evitar" los efectos adversos de su administración prolongada, siendo sustituido por la administración del probiótico, con la que se pretendía mantener el estado de remisión así como prevenir el daño tras la reactivación. El tratamiento con *E. coli*

Nissle 1917 fue capaz de promover la recuperación del tejido colónico, y tras el segundo periodo de inducción de colitis mediante DSS, los animales que habían recibido la asociación de los dos tratamientos experimentaron una exacerbación del daño inferior a la de los animales que recibieron cada tratamiento por separado, a la vista de los resultados de los estudios macroscópicos, microscópicos e inmunológicos. Al final del estudio, los animales del grupo que recibió ambos tratamientos, mostraron una recuperación casi completa del proceso inflamatorio intestinal.

Por último, la disbiosis que caracterizaba a los animales colíticos fue parcialmente restaurada con todos los tratamientos (Figura 27). Por tanto, podemos sugerir que este efecto sobre la microbiota, aunque podría influir en la consecución del efecto antiinflamatorio, no explicaría el mayor beneficio alcanzado con la asociación, sino que éste derivaría más probablemente de la combinación de las propiedades inmunomoduladoras de ambos. En conclusión, el suplemento con *E. coli* Nissle 1917 al tratamiento con minociclina aumenta la recuperación del daño intestinal y contribuye a prevenir la reactivación de la colitis experimental.

3. Conclusiones.

A la vista de los resultados de la presente tesis doctoral podemos concluir que:

- La minociclina, a diferencia de otros antibióticos utilizados en el tratamiento de la enfermedad inflamatoria intestinal, ejerce un efecto inmunomodulador directo. Esto se evidencia *in vitro* tanto en células epiteliales colónicas, disminuyendo la producción de IL-8, como en macrófagos, reduciendo la producción de nitritos.

- La minociclina, administrada como tratamiento curativo, presenta un efecto antiinflamatorio intestinal en el modelo de colitis experimental aguda por TNBS en ratas. En este efecto participan su capacidad de restaurar el equilibrio en la microbiota intestinal y sus propiedades inmunomoduladoras. En relación a su acción inmunomoduladora, ésta afecta tanto a la respuesta inmune innata, al preservar la integridad de la barrera intestinal y disminuir la producción de citocinas proinflamatorias, como a la respuesta inmune adaptativa, mediante la inhibición de la expresión de citocinas de células T colaboradoras (Th) (T *helper*) 1 y Th17.

- El efecto antiinflamatorio intestinal de la minociclina también se manifiesta en un modelo de progresión crónica de colitis inducida por DSS en ratones.

- La asociación del antibiótico minociclina con el probiótico *Escherichia coli* Nissle 1917 constituye una buena estrategia para el mantenimiento del estado de remisión, así como en la prevención de la reactivación del proceso inflamatorio, en un modelo de colitis experimental con recidivas. La combinación se traduce en un efecto sinérgico sobre los distintos marcadores evaluados, en comparación con los efectos observados con los tratamientos aislados.

Introduction

MINOCYCLINE

Tetracyclines are bacteriostatic antibiotics discovered by Benjamin M. Duggar in 1947. They are active against a wide range of aerobic and anaerobic gram-positive and gram-negative bacteria, being considered as broad-spectrum antibiotics. They are also effective against other microorganisms, including *Rickettisa*, *Chalmiydia* spp., *Mycoplasma pneumoniae*, and *Plasmodium* spp. Their chemical structure consists of a tetracyclic naphthacene carboxamide ring with substituents at different positions. They have been extensively used to treat many infectious diseases, but these uses have led to increased bacterial resistance to them, and thus, their use have been limited to infectious caused by *Rickettisae*, *Chalmiydiae* and *Mycoplasma*, as well as to the treatment of chronic conditions as acne and respiratory tract infections, in which atypical microorganisms that are also increasingly resistant to other antibiotics, are frequent [38], [78]. The mechanism of action involved in the antibiotic properties of tetracyclines is related to their ability to bind to the 30S ribosomal subunit of bacteria and inhibit protein synthesis. In an attempt to improve the efficacy of this group of antibiotics, structural changes have been developed, like those modifying ring D through carbon locations C7-C9. This was the basis of the higher efficacy obtained with the semisynthetic compounds minocycline and doxycycline [79].

Minocycline (7-dimethylamino-6-dimethyl-6-deoxytetracycline) is a second-generation, semi-synthetic tetracycline analog that has been in use for over 30 years [80]. It is effective against both gram-positive and gram-negative bacteria and it was approved by the US Food and Drug Administration (FDA), being indicated for acne vulgaris, some sexually transmitted diseases and rheumatoid arthritis [81, 82]. It shows a better pharmacokinetic profile than its parent, tetracycline, when used orally. Indeed, minocycline is absorbed rapidly and completely even in aged population, has a longer half-life when compared

to the first-generation tetracyclines and shows excellent tissue penetration with near 100% bioavailability [78, 83 - 86]. In addition, it is a highly lipophilic molecule that can easily go through brain-blood barrier [87], thus promoting its accumulation into the cerebrospinal fluid and central nerve system (CNS) cells [85, 88, 89], and enabling its use in the treatment of many CNS diseases [80, 90, 91]. More strikingly, minocycline has a good safety record when used chronically. Long-term treatment with minocycline up to 200 mg/day, the highest dosage recommended by US FDA, is generally safe and well tolerated in humans, being just only in the early stages after minocycline administration when its known and most common side-effects can appear, including nausea and mild dizziness [92, 93]. However, the reported potential vestibular toxicity has limited its extensive use in human infections [78].

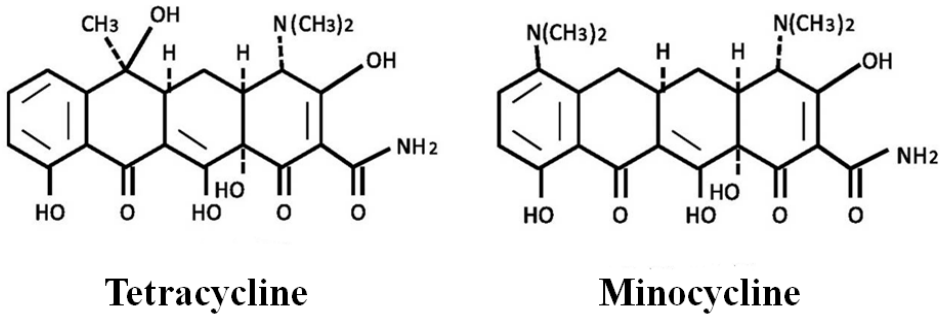

Figure 1. Chemical structure of tetracycline and minocycline.

The antibiotic properties of tetracyclines were initially described in the late 1940s, but more recently numerous studies have focused on their non-antibiotic properties. In fact, it has been reported that tetracyclines can exert a variety of biological actions, including anti-inflammatory and antiapoptotic activities, inhibition of proteolysis, as well as suppression of angiogenesis and tumour metastasis, that are independent of their antimicrobial activity [38, 54, 94 - 96]. These

observations specifically concern to minocycline since it has recently been found to have multiple non-antibioitic biological effects [89, 97], being potentially beneficial for diseases with an inflammatory background including rosacea, bullous dermatoses, neutrophilic diseases, pyoderma gangrenosum, sarcoidosis, aortic aneurysms, cancer metastasis, periodontitis, and autoimmune disorders such as rheumatoid arthritis and scleroderma (reviewed in: [38, 98 - 101]). Of note, minocycline has also emerged among the tetracycline derivatives as the most effective one regarding neuroprotection. This effect has been confirmed in various experimental models (reviewed by [102, 103]) of brain injury, including ischemia [41, 88, 104], traumatic brain injury [105], neuropathic pain [106 - 110] and glutamate-induced neurotoxicity [111] as well as in several neurodegenerative conditions like Parkinson's disease [39, 40, 112], Huntington's disease [43, 113], amyotrophic lateral sclerosis [114], Alzheimer's disease [115], multiple sclerosis [116 - 118] and spinal cord injury (SCI) [80, 94, 119 - 121]. All these preclinical studies have led to the evaluation of minocycline in clinical trials in patients with neuronal disease, showing promising neuroprotective properties in humans [122]. Moreover, other studies have evidenced its ability to inhibit malignant cell growth [123] and to prevent bone resorption [124].

Many of these studies describing the beneficial effects of minocycline as an anti-inflammatory, immunomodulatory and neuroprotective drug, have also proposed some of the mechanisms that can be involved, among these: a) inhibitory effects on enzyme activities, like inducible nitric oxide synthase (iNOS) [49, 125] matrix metalloproteinases (MMPs) [94] or phospholipase A_2 (PLA_2) [126]; b) reduction of protein tyrosine nitration due to its peroxynitrite scavenging properties [127], c) inhibition of caspase-1 and caspase-3 activation [43]; d) enhancement of Bcl-2 derived-effects, thus protecting cells against apoptosis [91, 102, 128] e) reduction of p38 mitogen-activated protein kinase (MAPK) phosphorylation [129] and f) inhibition of poly(ADP-ribose) polymerase (PARP) -1 [130]. It is interesting

to note that the well-known ability of tetracyclines to bind Ca^{2+} and Mg^{2+} may account for some of these biological activities via the chelation of these cations and their transport into intracellular compartments [131]. The growing interest in this compound has led to evaluate the therapeutic efficacy of minocycline in many other experimental models of diseases, such as diabetes [91, 132 - 134], fragile X syndrome (FXS) [135], cardiac ischemia [51] and human immunodeficiency virus (HIV) infection [136, 137].

Therefore, the special feature of minocycline to combine immunomodulatory and antimicrobial properties makes it an interesting therapeutic approach for diseases in which both a deregulated immune response and a microbial aetiology are involved, like inflammatory bowel disease.

1. Minocycline effects in non-infectious diseases.

The reported immunomodulatory properties of minocycline have encouraged the evaluation of this tetracycline in different experimental models of diseases with an inflammatory background, and which are not directly related with the existence of a responsible bacterial pathogen. The pathologies in which minocycline has been tested comprise a wide range of different conditions affecting the CNS, including neurodegenerative diseases, and the cardiovascular and respiratory systems, as well as systemic immune conditions like rheumatoid arthritis and skin dermatitis.

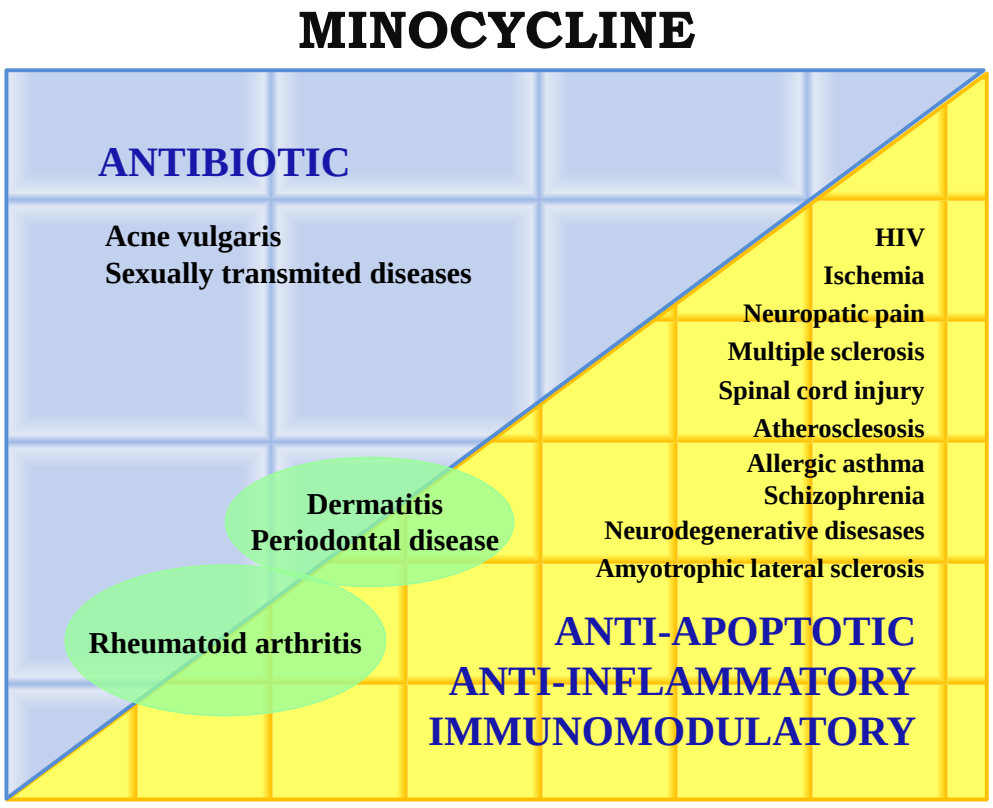

Figure 2. Clinical potential of minocycline.

1.1. Dermatitis.

Minocycline was first reported to be therapeutically effective in non-infectious forms of dermatitis in open clinical studies [138]. Since then, it has been clinically used to treat various skin disorders, such as inflammatory acne, rosacea, bullous dermatoses and neutrophilic dermatoses [38, 139]. These favourable effects on cutaneous inflammation, reported as well for other tetracyclines, were partially explained by the reduction of neutrophil chemotaxis [140, 141], but recently, new modulatory effects have been described for second-generation tetracyclines that might explain their greater effectiveness in treating these inflammatory skin disorders. In this regard, Ishikawa et al. (2009) [142] recently reported that minocycline, at concentrations of 5 or 10 μM, quite similar to the therapeutic concentrations that can be obtained with these drugs in serum [143], reduced the protease-activated receptor (PAR) 2-mediated production of IL-8 and thus attenuates the proinflammatory process in epidermal keratinocytes. They proposed that the well-known ability of tetracyclines to chelate Ca^{2+} can contribute to these effects, since PAR2 activation transiently increases intracellular Ca^{2+} levels in keratinocytes, which triggers the downstream binding of nuclear factor-kappaB (NF-κB) to DNA [144, 145].

1.2. Periodontal disease.

The periodontal disease, in addition to its well-known microbial aetiology, is characterized by the onset of an inflammatory process. The pharmacological profile of tetracyclines, which combines antimicrobial and anti-inflammatory properties, makes these antibiotics suitable for their potential use in this condition. In fact, they have shown to be effective in reducing the parameters of periodontal disease progression and promoting periodontal healing when used as adjunctive therapy [146]. In addition, their anti-apoptotic and matrix stimulatory actions may also account for their potency [101]. At levels conventionally detected

in plasma and gingival crevicular fluid, minocycline causes significant stimulation of osteoblastic cells, whereas long term exposure of these cells to tetracyclines results in a proportional increase in mineralised bone matrix [147]. Additionally, as *in vitro* assays have shown, minocycline topical application implies minimal cellular damage, since it does not affect cell survival and protein expression of human gingival fibroblasts, epithelial cells and periodontal ligament fibroblasts [148].

1.3. Rheumatoid arthritis.

In the last four decades, many studies have been focused on the effects of minocycline on rheumatoid arthritis, an immune-inflammatory condition whose etiopathogenesis is not fully understood at present.

The first clinical trial evaluating the effects of a tetracycline for rheumatoid arthritis was reported by Skinner et al. in 1971[149]. Since an antimicrobial effect could benefit against a putative and long postulated infectious involvement in rheumatoid arthritis, 250 mg of tetracycline hydrochloride per day were given to 7 patients over one year; however, no benefit was noted in this study. Later on and based on the studies performed by Golub et al. (1983) [150] who revealed the anticollagenase activity of minocycline, many assays re-evaluated the effects of tetracyclines in rheumatoid arthritis. Thus, in 1987, minocycline was reported to inhibit human synovial collagenase from rheumatoid tissue from patients, and this effect was associated with a decreased loss of alpha collagen components and reduced formation of alpha A digestion fragments [151], being this corroborated in assays performed in sinovial cultures *in vitro*. Moreover, tetracyclines have been reported to inhibit the synthesis and/or activity of cartilage proteinases both *in vivo* and *in vitro* [152].

The results reported in experimental models of rheumatoid arthritis have confirmed the beneficial effects initially proposed for minocycline. Greenwald et

al. (1992) [99] described that tetracyclines by themselves did not show anti-inflammatory effects in the adjuvant arthritis model of rheumatoid arthritis in rats, but they did reduce the levels of collagenase activity in the inflammed tissue. However, the same study revealed that when tetracyclines were combined with non-steroidal anti-inflammatory drugs, a synergic effect was noted, as evidenced by total inhibition of degradative enzyme activity as well as normalization of radiologic bone damage. In other study, Zernicke et al. (1997) [153] reported that tetracycline administration could reverse the deleterious effect of adjuvant disease on the mechanical strength of the femur in rats.

All these studies prompted the development of clinical trials with different tetracyclines, including minocycline. Initially, two double-blind, placebo-controlled trials were run: the study developed by Kloppenburg, M. et al (1994) [154] and the MIRA trial (Minocycline in Rheumatoid Arthritis), initiated by the National Institutes of Health [155]. They first demonstrated that minocycline possessed clinically useful anti-inflammatory properties in patients with rheumatoid arthritis, being superior to placebo; whereas the second revealed some methodological problems, and no clear conclusions could be drawn from it.

Langevitz et al. (2000) [156] summarized the results of two previous open trials and 3 double-blind controlled studies, concluding that minocycline might be beneficial in patients with rheumatoid arthritis, especially when given early in the disease course or in patients with a mild disease, since it showed beneficial effects with respect to joint swelling and/or tenderness, laboratory parameters, patient assessment, etc. A meta-analysis from 2003 that summarized the results from clinical trials up until 2002 confirmed these beneficial effects and added that toxicity in general was mild [93].

However, and despite all these promising results as well as the FDA-approval of semi-synthetic tetracyclines for rheumatoid arthritis, Greenwald

acknowledged in a recent review [157] that the weak anti-inflammatory properties of tetracyclines are easily surpassed by many other agents. Nevertheless, the potential of tetracyclines in osteoarthritis still seems attractive and minocycline *in vitro* inhibition of cartilage degradation represents a solid rationale for forward progress.

1.4. Central Nervous System pathologies.

In recent years, minocycline has been shown to be especially beneficial in animal models of CNS diseases. Many of these studies were initially based on its ability to inhibit microglia activation, a process that has been reported to have deleterious effects on neurogenesis and neuronal survival, being responsible for neuronal and/or glial damage and death. This inhibitory effect would justify the potential effectiveness of this antibiotic in the treatment of neuroinflammation and/or neurodegenerative disorders [39, 43, 88, 104, 114, 158, 159].

In fact, different *in vitro* studies have reported the ability of minocycline to block lipopolysaccharide (LPS)-stimulated inflammatory cytokine secretion and Toll-like-receptor (TLR)-2 surface expression in the BV-2 microglia-derived cell line and on brain microglia isolated from adult mice. It also attenuated mRNA expression of inflammatory genes including interleukin (IL)-6, IL-1β, major histocompatibility complex (MHC) II, and TLR2 [160, 161]. This ability to mitigate cytokine expression in the brain during systemic inflammatory events may be useful in preventing cognitive and behavioral deficits. According to this, minocycline has been reported to attenuate sickness behavior and anhedonia associated with LPS-induced neuroinflammation, paralleled with a decrease in neuroinflammatory markers in the hippocampus and cortex, such as IL-6 and IL-1β secretion, MHC II, TLR2, IL-1β and IL-6 mRNA levels and LPS-induced indolamine 2,3-dioxygenase (IDO) mRNA expression [161]. These data are consistent with another report showing a causal relationship between IDO

activity and acute depressive effects in adult CD-1 mice. In this report, the ability of minocycline to block IDO induction prevented depressive-like immobility [162]. In addition, they observed that while minocycline pretreatment attenuated LPS-induced brain IL-1β production, it had no effect on plasma IL-1β levels, suggesting that minocycline has anti-inflammatory properties within the brain that account for improving recovery from sickness and reducing the frequency of neurobehavioral complications.

- ***Cerebral ischemia.***

Inflammation has been also recognized as a significant contributing mechanism in cerebral ischemia. Different studies have shown that inflammatory cells infiltrate into the ischemic brain area, where they promote the production of several proinflammatory mediators [163 - 165]. Minocycline has been reported to act as a neuroprotective agent in models of both global and focal ischemia due to its anti-inflammatory properties. In a gerbil model of forebrain ischemia minocycline was able to prevent microglial activation, reducing infarct size and increasing the survival of hippocampal neurons, even when the treatment was started after the ischemic insult; these effects were accompanied by a reduction of IL-1β converting enzyme (ICE), ciclooxygenase (COX) -2, and iNOS mRNA levels in the affected brain regions [104, 88]. Koistinaho M, et al (2005) [41] showed that this effect of minocycline seemed to be MMP dependent, since this compound protected against permanent cerebral ischemia in wild type mice, but not in MMP-9-deficient mice. Moreover, Hong Park (2011) [166] reported that minocycline, similarly to other MMP inhibitors, was effective in treating neuroinflammation following experimental photothrombotic cortical ischemia, showing a similar protective effect on permanent stroke to that observed in previous reports [41, 167] and being this effect clearly attributed to MMPs inhibition. In these studies, both pre- and post-ischemic minocycline treatment significantly reduced the infarct size as well as the expression of neuroinflammatory

mediators, including the monocyte chemotactic protein (MCP) -1, tumour necrosis factor (TNF) α and IDO in the ischemic cortex.

- ***Neurodegenerative diseases.***

The potential efficacy of minocycline in the treatment of different neurodegenerative conditions (Parkinson's, Alzheimer's and Huntington's diseases) has been also proposed. In this context, Chen et al (2000) [43] evaluated the effects of minocycline in the transgenic R6/2 mouse model of Huntington's disease, and reported that minocycline delayed disease progression and mortality. This was associated to the inhibition of caspase-1 and caspase-3 expression as well as to a reduction of iNOS activation, preventing the detrimental role that these enzymes have been proposed to exert in Huntington's disease [168]. Moreover, the generation of the endogenous huntingtin cleavage fragment was significantly inhibited and mature IL-1β levels in brains were lowered in minocycline-treated mice.

The anti-inflammatory effects of minocycline can also account for the beneficial effects observed in both *in vitro* and in animal models of Parkinson's disease. Minocycline was found to prevent nigrostriatal dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease, an effect related with the prevention of dopamine depletion in the striatum and in the nucleus accumbens; these effects were associated with marked reductions in iNOS and caspase-1 expressions [39]. In a different study, the preventive effects of minocycline against the demise of nigrostriatal dopaminergic neurons were related to a prevention of MPTP-induced activation of microglia, and inhibition of mature IL-1β formation and nicotinamide adenine dinucleotic phosphate (NADPH)–oxidase and iNOS activation [169]. In addition, *in vitro* studies using primary cultures of mesencephalic and cerebellar granule neurons (CGN) and glia confirmed that

minocycline inhibits 1-methyl-4-phenylpyridinium (MPP+)-mediated iNOS expression and nitric oxide (NO) -induced neurotoxicity. This effect was related to the inhibition of p38 MAPK activation in CGN [39]. All together, these results suggest that minocycline blocks MPTP neurotoxicity *in vivo* by indirectly inhibiting MPTP/MPP+-induced glial iNOS expression and neurotoxicity, most likely by inhibiting the phosphorylation of p38 MAPK.

Similarly, the pharmacological profile of minocycline makes it interesting for the treatment of Alzheimer's disease. Two prominent characteristics of Alzheimer's disease are basal forebrain cholinergic degeneration and neuroinflammation, characterized by glial activation, and the release of proinflammatory cytokines; in addition, amyloid β peptide (Aβ)-induced neuronal cell death has been proposed as the causative factor for the decline of cognitive ability observed in Alzheimer's disease [170]. However, synaptic failure and impairment of cognitive function could also precede neuronal degeneration [171]. This may be the result of prolonged endoplasmic reticulum (ER) stress, as it has been proposed to occur in several neurodegenerative disorders [172 - 174]. In response to stress signals, the inhibition of general translation may occur [175], which is evidenced after the phosphorylation of eukaryotic initiation translation factor 2 alpha (eIF-2a) [176]; however, this factor can also promote mRNA translation of the transcriptional modulator ATF4 [177], which impairs synaptic plasticity and behavioral learning [178]. Aβ has been also reported to phosphorylate eIF-2a via protein kinase (PK) R activation in neuronal cells [179, 180]. Moreover, increased phosphorylation of eIF2a is observed in Alzheimer's disease patients' brains and may result in impairment of cognitive functions by decreasing the efficacy of de novo protein synthesis required for synaptic plasticity. The first report describing the effects of minocycline in a model of Alzheimer's disease appeared in 2004, describing the beneficial effects of minocyline in the Alzheimer's disease experimental model induced by i.c.v. injection of the

immunotoxin mu p75-saporin to mice, in which the antibiotic ameliorated the cholinergic cell loss and reduced the simultaneous activation of microglia and astrocytes that takes place after the administration of the immunotoxin, together with a dowregulation in the transcription of proinflammatory mediators and the mitigation of cognitive impairment [181]. Minocycline treatment has also been reported to suppress microglial production of IL-1β, IL-6, TNF, and nerve growth factor (NGF) in amyloid precursor protein (APP) transgenic mice, but it did not affect Aβ deposition in this model [182]. Morevoer, Choi (2007) [115] described that minocycline was able to attenuate eIF-2a phosphorylation and caspase-12 activation in $A\beta_{1-42}$ - treated or APP-CTs-transfected differentiated PC 12 neuronal cells. The increases in p-eIF2a were also attenuated by minocycline administration in two animal models: $A\beta_{1-42}$ infused rats and Tg2576 mice, in which minocycline reduced neuronal cell death, improved cognitive impairment and attenuated the deficits in learning and memory [115].

Similarly, minocycline treatment was able to correct behavioral impairments, lower inflammatory markers and levels of Aβ trimers in an early, pre-plaque inflammatory process in Alzheimer's disease-like transgenic rat model [183]. This study showed that accumulation of Aβ is sufficient to provoke cognitive impairment and biochemical alterations in the cerebral cortex and hippocampus in the absence of amyloid plaques, together with an up-regulation of proinflammatory markers such as MHC-II, iNOS and COX-2; responses that were successfully arrested by minocycline [183].

It is interesting to note that the interaction of CD40 with CD40L has been described to enable microglial activation in response to Aβ [184], being this CD40-CD40L interaction a possible target for therapeutic intervention in Alzheimer's disease. In addition, and more recently, 5-lipoxygenase (5-LO) has been reported to play a role in the formation of Aβ in the brain [185]; and in consequence, the pharmacological inhibition of this enzyme may also be beneficial in the treatment

and prevention of Alzheimer's disease, as shown using a specific 5-LO inhibitor and in a transgenic model of Alzheimer's disease lacking the enzyme [186, 187]. Minocycline has also been reported to inhibit 5-LO activation (Song Y 2006) and to decrease CD40L expression [188], in addition to its inhibitory effects on Aβ formation and microglial activation in the brain, thus it might be a therapeutic approach to prevent or delay the onset of Alzheimer's disease.

However, controversial studies have also reported the lack of benefit, and even the occurrence of detrimental effects, of minocycline in some neurodegenerative conditions. For example, minocycline exacerbated MPTP-induced damage to dopaminergic neurons [189], worsened hypoxic–ischemic injury in a neonatal mouse model [190] and had deleterious effects in an animal model of Huntington's disease [191].

- ***Multiple sclerosis.***

Increasing clinical and experimental evidence suggest that minocycline, alone or combined with other drugs, could ameliorate the severity and progression of multiple sclerosis. This has been mainly based on the promising results obtained in an established animal model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE). In 2002, two different groups described for the first time the ability of minocycline to attenuate the clinical and histological severity of EAE, an effect associated with decreased inflammation and the inhibition of microglial activation [116, 117]. Popovic et al. (2002) [117] reported that administration of minocycline, following a curative protocol, suppressed ongoing disease activity and limited disease progression in EAE induced in Dark Agouti rats. Similarly, Brundula et al (2002) [116] described that minocycline, most probably due to its ability to inhibit MMP activity, could delay the onset of clinical symptoms and attenuate the severity of the

neuroinflammation that occurred in EAE, even when it was administered after the onset of the clinical signs.

Later on, Nikodemova et al. (2007) [192] reported that minocycline treatment, when started at the onset of the symptoms, considerably decreased the severity of the clinical course of the disease by reducing both the number and the size of the lesions. This effect was associated with a reduced migration of macrophages from the periphery into the CNS, thus resulting in a decreased leukocyte infiltration into the parenchyma of the spinal cord and decreased microglia MHC II expression and proliferation. Moreover, these neuroprotective effects were improved if high minocycline concentrations were locally delivered into the CNS [193].

The efficacy showed by minocycline in these experimental models has led to consider it as an appropriate candidate for combination therapy in multiple sclerosis. In fact, Luccanini et al. (2008) [194] reported that combined treatment of minocycline and atorvastatin (a statin effective in the treatment of multiple sclerosis in animal models) in comparison with each drug alone, resulted in a greater reduction in disease severity, in both the acute and chronic phases of the disease, along with attenuation of inflammation, demyelination and axonal loss. One of the advantages of this combination would be the administration of lower doses of each drug, thus reducing the risk of side effects.

The success of this antibiotic in the treatment of multiple sclerosis experimental models has prompted its evaluation in phase I/II clinical trials in humans, which confirmed the beneficial effects and showed that it was safe and well-tolerated [158, 195, 196]. The results revealed that minocycline significantly reduced relapse rates, MRI active lesions and local brain atrophy. These trials also showed that the clinical response to minocycline was accompanied by

beneficial immune changes that are likely desirable in the control of multiple sclerosis.

The mechanisms responsible for the pharmacological actions of minocycline in EAE and multiple sclerosis are most probably related to its influence on T cell activity, its ability to inhibit microglial activation and its neuroprotective effects. *In vitro* studies have revealed that minocycline inhibited the antigen processing for presentation to human T cells [197], T cell proliferation and production of inflammatory cytokines [198, 199] and T cell transmigration across a fibronectin matrix barrier, most likely due to the inhibition of MMPs. *In vivo* assays have described that this antibiotic promoted immune differentiation from a type 1 helper T-cell (Th1) towards a type 2 helper T-cell (Th2) phenotype, thus modulating the susceptibility to EAE [117]. Regarding microglial activation, Popovic et al. (2002) [117] found in relapsing–remitting EAE that activated microglia were absent in rats treated with minocycline. Moreover, minocycline inhibits microglia MHC II expression and the subsequent reactivation of T cells, which resulted in the attenuation of the clinical severity of EAE [192] and reduced infiltration of T lymphocytes into the CNS parenchyma [116, 117]. In addition, direct antioxidant potential of minocycline attenuated reactive oxygen species (ROS)-mediated neuronal and axonal destruction *in vitro* [200], and its ability to chelate Ca2+ may prevent activation of calpains and preserve axonal integrity, as observed in minocycline-treated EAE rats [80, 201 - 203].

- ***Amyotrophic lateral sclerosis.***

The pathogenesis of amyotrophic lateral sclerosis has been related to an up-regulation of the expression and increased activity of different proinflammatory signals, including caspases -1 and -3, iNOS and p38 MAPK [204 - 206]. For this reason, and based on its pharmacological profile, there is a rationale for minocycline to have a positive impact in this disease. Supporting this, minocycline has been

reported to delay disease onset and extend survival in an experimental model of amyotrophic lateral sclerosis: transgenic mice expressing the mutant human SOD1G93A transgene. In this model, minocycline inhibited mitochondrial permeability transition (MPT)-mediated cytochrome c (Cyt *c*) release, a mechanism of action that was confirmed *in vitro* both in cells and in isolated mitochondria [114]. Similar findings have been reported by Kriz et al. (2002) [207] and Van Den Bosch et al. (2002) [208], who showed that minocycline delayed the onset of motor neuron degeneration and muscle strength decline, and increased the longevity of amyotrophic lateral sclerosis mice. In addition, *in vitro* studies revealed that minocycline reduced apoptosis of cultured neurons from patients with motor neuron diseases, including amyotrophic lateral sclerosis [209].

However, despite these promising results from these animal models, a multicentre, randomised placebo-controlled phase III trial revealed a harmful effect of minocycline in patients with amyotrophic lateral sclerosis, which deteriorated significantly faster than the placebo control group [210].

1.5. Neuropathic pain.

The contribution of glial cells (microglia and astrocytes) to the initiation of neuropathic pain sensitization and peripheral nerve injury-induced neuropathic pain has been well characterized [211 - 213]. As commented previously, minocycline is able to inhibit microglia activation in various pathological conditions, which consequently lowers expression of proinflammatory cytokines, without affecting astroglia and neurons [104, 214], and this may justify its reported ability to reverse neuronal sensitization in neuropathic animal models when applied to the spinal cord [106, 214 - 216].

In fact, several studies have revealed that both systemic (intraperitoneal) and local (intrathecal) administration of minocycline could exert antinociceptive effects on experimental neuropathic pain induced by peripheral nerve injury,

inflammation or SCI [106, 107, 121, 215 - 225]; of note, the beneficial effects showed by minocycline in these conditions are clearly improved when its administration was performed as early as possible, especially during the initiation stage [110, 215, 219].

Similarly, minocycline has been recently found to reverse microglial reactivity and thermal hyperalgesia secondary to sciatic neuropathy, when injected in the ventral posterolateral thalamus [109]. Special attention has been paid to the effects of minocycline in the development of diabetic neuropathy. Pabreja et al. (2011) [226] recently reported that, although chronic administration of minocycline did not alter diabetic hyperglycemia, it significantly prevented cold allodynia and thermal hiperalgesia in diabetic rats. This beneficial effect was associated with decreased levels of proinflammatory cytokines and an attenuated oxidative stress balance in the spinal cord of these diabetic animals, observations that were also reported by Raghavendra et al. (2003) [106] and Ledeboer et al. (2005) [219]. Moreover, the beneficial effects of minocycline in diabetes were associated with the prevention of retinal complications, most probably due to the inhibiton of diabetes-induced cytokine and cytotoxin production [133] in agreement with this, Wang et al. (2005) [227] showed that minocycline inhibited the up-regulation and increased release of IL-1β, TNFα and NO caused by bacterial LPS in retinal microglia. Moreover, Cai et al. (2011) [134] investigated the neuroprotective mechanisms of minocycline against diabetic brain injury, reporting its ability to improve the behavioral deficits caused by the altered glucose metabolism in diabetic rats, and down-regulating the increased β-amyloid protein in the hippocampus through the inhibition of the NF-KB pathway activation and its upstream signal transduction molecules, and the attenuation of oxidative stress.

1.6. Spinal cord Injury.

Since the neuropathic pain and motor weakness that result from microglia activation are believed to trigger nociceptive hypersensitivity in spinal cord

injury (SCI), it is feasible that minocycline could be a rational approach in the treatment of the complications of SCI [228]. This has been supported by different studies showing that minocycline may reduce neuropathic pain after SCI, however, only few data exist regarding the ability of minocycline to promote motor recovery after SCI, leaving this indication controversial. In rodent models of SCI, minocycline administration significantly improved both hindlimb function and strength, reduced gross lesion size in the spinal cord and induced axonal sparing. Minocycline-treated mice demonstrated superior behavioural recovery than those that received the approved treatment for acute SCI in humans, methylprednisolone [229]. In rats with SCI, minocycline inhibited the release of Cyt *c* from mitochondria, markedly enhancing long-term hindlimb locomotion [120]. More recently, Saganová et al. (2008) [230] showed that both short- and long-term treatment with minocycline had a neuroprotective effect on the spinal cord rostral to the injury epicentre, although these effects were not observed at caudal sites and did not result in any overall improvement in motor outcome. Conversely, data from Teng et al. (2004) [120] had indicated before that minocycline had a protective effect on white matter and motor neuron number at sites both rostral and caudal from the lesion epicenter [120].

Minocycline was also shown to improve functional recovery after SCI through the inhibition of the production of pro-NGF by microglia, thereby reducing oligodendrocyte death and apoptosis after traumatic SCI. It also inhibited the expression of p75 neurotrophin receptors and the activation of Ras homolog gene family member A (RhoA) after SCI [231]. Furthermore, Festoff et al. (2006) reported that minocycline might also exert a neuroprotective effect in SCI by reducing microgliosis and inhibiting caspase expression [119].

Recently, a new study has reported the effects of minocycline on motor neuron recovery and neuropathic pain in a rat model of thoracic SCI [121], revealing that, at post-operative day 2, the locomotore score was higher and the mechanical

hiperalgesia was reduced in animals treated with minocycline in comparison with the corresponding controls. The attenuation of neuropathic pain behaviour and motor recovery correlated with reductions in microglia and astrocytes activation, respectively.

1.7. Ischemia.

The ability of minocycline to limit tissue damage in the setting of ischemia has been documented in kidney, heart, lung and neural cells both *in vitro* and *in vivo* [80, 102, 232 - 234]. When considering stroke, it is interesting to note that blood-brain barrier disruption after stroke can worsen ischemic injury by increasing oedema and causing haemorrhage. It has been reported that minocycline, probably due to its ability to inhibit microglia activation, was able to attenuate infarct volume and neurological deficits in mice after experimental stroke, as a result of a marked reduction in blood-brain barrier disruption and haemorrhage [235]. Based on this preliminary evidence, clinical studies in patients with stroke have been performed. Lampl et al. (2007), [122] reported that the oral administration of minocycline (200mg) for 5 days, with a therapeutic window of 6-24 h after stroke onset, promoted a better outcome in comparison to placebo. This beneficial effect can be associated with a significant blunting of ischemic tissue oxidative stress, consistently with previous reports where tetracyclines have shown to reduce tissue oxidative stress [236]. Moreover, and supporting these observations, minocycline could attenuate oxygen–glucose deprivation-induced high mobility group box 1 protein (HMGB1) release and HMGB1-induced cell death in ischemic neuronal injury in PC12 cells [237].

In another ischemic condition, like the myocardial ischemia-reperfusion (I/R), in which the injury has been associated with the activation of MMPs and serine proteases, minocycline can protect myocardium from ischemic injury, as evidenced from studies using *ex vivo* heart systems and cultured cardiac

myocytes [51]. These cardioprotective effects were also attributed to minocycline actions on apoptotic cell pathways. However, the first *in vivo* study that described the cardioprotection exerted by minocycline showed that pre- and post-treatment of rats subjected to I/R with this antibiotic yielded a significant reduction in infarct size, an effect that was accompanied by a reduction in MMP-9 activity and oxidative stress [238]. This study also confirmed previous results describing that the accumulation of tetracyclines in infarcted myocardium was directly related to the degree of tissue damage [239, 240]. In fact, minocycline accumulated in myocardium several fold above plasma levels, being this accumulation higher in ischemic vs. normal myocardium. Thus, it is possible that part of the cardioprotective effects of minocycline may be derived from high tissue levels of the compound, which allows for notable effects on its targets, as MMP-9 inhibition and ROS-scavenging properties, together with its anti-apoptotic effects.

In a more recent study, the protective effect of minocycline against myocardial ischemia and I/R injury was attributed to the inhibition of HMGB1 expression, a protein which has been also found to act as an early mediator of inflammation and cell damage during I/R injury [241, 242].

Poly (ADP-ribose) polymerase (PARP)-1 inhibition has been also proposed as a possible mechanism explaining minocycline cardioprotective activity [243]. During myocardial I/R injury there is an increase in reactive oxygen and nitrogen species that leads to oxidative DNA damage and activation of nuclear repair enzymes such as PARP-1, which promotes DNA repair under normal conditions, but that could lead to cell death if excessive [130, 244, 245]. In cultured adult rat cardiac myocytes in which I/R injury was simulated using oxygen-glucose deprivation; minocycline, at micromolar concentrations, significantly reduced cell death, the biochemical markers of PARP-1 activation and prevented mitochondrial permeability transition [243].

Moreover, minocycline has also been reported to be effective in preventing ischemia-induced ventricular arrhythmias in rats. The incidence of ventricular fibrillation, the duration and the number of episodes of ventricular tachycardia plus unidentifiable and low voltage QRS complexes, and the severity of arrhythmias were significantly reduced by minocycline treatment [246]. In this study, the authors postulated that the anti-arrhythmic effect of minocycline may be associated with activations of phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway and mitochondrial K_{ATP} channels, which are known to participate in the anti-arrhythmic effect of ischemic or pharmacological preconditioning during myocardial ischemia [247 - 250].

1.8. Atherosclerosis.

Different studies have proposed that minocycline exerts beneficial actions in preventing atherosclerosis, most probably related with its cyto-protective effects in vascular cells. Accordingly, it has been shown to protect against diabetic microvascular complications [133] and, in regard to macrovascular disease, to reduce neointima formation following an acute vascular injury of the rat carotid artery [251]. In these studies, a reduction in the number of vascular smooth muscle cells (VSMC) has been seen after minocycline treatment, which has been attributed to an inhibition of MMP activity and cytokine-induced VSMC migration [251 - 253]. Moreover, minocycline has been shown to inhibit vascular endothelial growth factor- induced MMP-9 mRNA transcription and protein activation in human aortic VSMC *in vitro* [251, 253]. More recently, Shahzad et al. (2011) [254] showed that minocycline reduces plaque size and vascular stenosis in diet-induced atherosclerosis through a PARP-1 and $p27^{Kip1}$ dependent mechanism. *In vitro* assays revealed that minocycline reduced the proliferative process in different cell types, like human aortic smooth muscle cells (HASMC) or murine primary aortic VSMC. All these data can justify the lower number of VSMC observed within atherosclerotic plaques of ApoE−/− HFD mice [254]. These

authors also established that the antiproliferative effect of minocycline in VSMC depends on p27Kip1, since it induced its expression both *in vitro* in VSMC and HASMC, as well as in atherosclerotic plaques when analyzed *ex vivo*. Of note, the knock down of p27Kip1 in primary mouse aortic cells abolished the antiproliferative effect of minocycline. In addition, minocycline reduced PAR formation, a marker of PARP-1 activity, in plaques of the truncus brachiocephalicus of ApoE−/− HFD mice, and markedly reduced PARP-1 expression, in particular in low density lipoprotein (LDL) treated HASMC. These results are in accordance with the fact that expression of p27Kip1 can be regulated by PARP-1[255] and the previously reported ability of minocycline to inhibit PARP-1 at very low concentrations [130].

1.9. Human immunodeficiency virus infection.

Several studies have shown the ability of minocycline to inhibit HIV activation, proliferation, and viral replication of microglia, macrophages, and lymphocytes *in vitro*, as well as to promote the production of immune activators by these cells [47, 192, 256]. Similarly, it has also been shown to reduce virus infection and immune responses in experimental models [47, 56, 136, 257 - 259].

One of the first studies describing this potential use of minocycline in HIV infection was performed in a simian immunodeficiency virus (SIV) macaque model of HIV-associated neurological [47]. Minocycline-treated SIV-infected macaques were noted to have less severe encephalitis, reduced expression of CNS inflammatory markers and reduced axonal degeneration. In addition, the authors found that treatment with minocycline significantly decreased virus load in the cerebrospinal fluid and plasma, viral RNA and cytotoxic lymphocyte infiltration into the brain. *In vitro* assays revealed that minocycline also decreased p38 activation and HIV replication in primary human lymphocytes, in association with a reduction in MCP-1/CCL2 production [47].

These findings led an acquired immunodeficiency syndrome (AIDS) Clinical Trials Group to study whether minocycline might improve performance in cognitively impaired HIV-infected subjects. (http://clinicaltrials.gov/ct2/show/NCT00361257).

In this setting, Szeto et al. (2010) [136] demonstrated that minocycline has significant anti-HIV effects in primary human CD4+ T cells. Antibiotic treatment reduced single-cycle replication, reactivation from a primary CD4+ T cell–derived model of HIV latency, and viral RNA expression after de novo infection with reference strain HIV NL4–3. These results described for the first time the ability of minocycline to decrease *ex vivo* virus expression from the resting CD4+ reservoirs of HIV-infected patients during highly active antiretroviral treatment (HAART), and that the anti-HIV effects of minocycline apply to both laboratory and clinical strains of HIV [47].

Minocycline displayed many effects on CD4+ T cells that impair HIV by reducing permissiveness and reactivation from latency. It altered T cell activation, blunting changes in expression of activation/proliferation markers and cytokine secretion, which are critical for activation pathways that regulate HIV replication. But minocycline also affected HIV replication, since it decreased dose-dependently the level of productive virus, acting before translation of HIV proteins, and by inhibiting DNA integration or transcription. All these data support that minocycline would be effective as a novel maintenance therapy in combination with HAART [136].

In addition to the effects of minocycline on CD4+T cells and viral replication, the decreased monocyte/macrophage activation caused by the antibiotic can also play a neuroprotective role on SIV-AIDS, although this has not been well-defined yet Ratai et al. (2010) [259] studied the neuroprotective effects of minocycline in a nonhuman primate model of accelerated neuroAIDS, and

reported that none of the minocycline treated animals developed SIVE, defined as the accumulation of monocyte/macrophages, virally infected cells, and multi-nucleated giant cells in the CNS. More recently, Campbell et al. (2011) [137] have concluded that not only its effects on T cells, but also the inhibition of monocyte activation correlates with neuronal protection in SIV NeuroAIDS. These authors observed that the reduction of viral replication in CD14+ monocytes *in vitro* after minocycline treatment was directly related to CD16+ expression in these cells, thus impairing their trafficking into the brain. Therefore, there was a correlation between expansion of activated monocytes and neuronal protection with minocycline. This may result in decreased replication or abundance of CD14+CD16+ target cells for HIV and SIV *in vivo*, as shown in a rapid model of SIV-neuropathogenesis in rhesus macaques. In this model, minocycline treatment resulted in neuronal protection: it reduced the activation of monocytes, their accumulation in-lymph nodes of treated animals, and inhibited the expression of several markers critical for monocyte traffic and function (CCR2, CD163, CD11b, and CD64). These results indicate that the antiviral effects of minocycline are linked to its ability to reduce activation of monocytes and their permissiveness to viral infection.

Unfortunately, despite all the mentioned above, a small pilot study reported that minocycline failed to modulate cerebrospinal fluid HIV infection or immune activation in chronic untreated HIV-1 infection [260]. This study suggested that the effects of minocycline were not sufficient to impact chronic HIV in the absence of antiretroviral treatment. Furthermore, the results from a clinical trial conducted by Sacktor et al. (2011) [261] revealed that although minocycline was safe and well-tolerated in individuals with HIV-associated cognitive impairment, cognitive improvement was not observed, rolling out the usefulness of minocycline neuroprotective properties in the treatment of this HIV-infection associated complication. Therefore, there seems little justification for treating

chronic HIV infection with minocycline instead of combination antiretroviral drugs. However, given the effects of this tetracycline reported *in vitro* and in experimental models [47, 136, 137] , there still may be reason for further study, for example in well-treated patients in which the level of immunoactivation is partially attenuated or in combination with antiretroviral drugs.

1.10. Allergic asthma.

A randomized, double-blind, placebo-controlled, crossover study performed by Daoud et al. (2008) [44], showed that treatment of allergic asthmatic patients with minocycline significantly improved their asthma symptoms, reduced oral steroid requirements and improved their spirometric outcomes, findings that indicate the potential usefulness of minocycline for treating asthma.

The mechanisms involved in the beneficial effects of minocycline in allergic asthmatic patients were investigated later on by Joks et al. (2010) [262]. They found that antibiotic treatment suppressed ongoing human and murine immunoglobulin (Ig) E responses while IgM, IgG and IgA responses were not affected. In addition, Joks et al. (2010) [262] also reported that minocycline strongly suppressed *in vitro* induction of memory IgE antibody forming cells responses of spleen and mesenteric lymph node cells from BPO-KLH sensitized mice. This suppression was dose- dependent and IgE isotype specific [262]. More recently, it has been described that minocycline suppressed *in vitro* IgE production by peripheral blood mononuclear cells (PBMCs) from asthmatic subjects, whereas there was no change in IgE levels in PBMC cultures from non-asthmatic subjects. They also found that both CD4+ and CD8+ T are required for minocycline mediated suppression of IgE responses by PBMC of allergic asthmatic humans, because if either CD4+ or CD8 T cells were depleted from PBMC before minocycline administration no longer suppression was obtained. These

minocycline-mediated decreases in IgE responses were associated with suppression of p38 MAPK in T lymphocytes of these patients [263, 264].

1.11. Miscellaneous.

- ***Schizophrenia.***

Minocycline has been recently found to be effective as an adjunct to antipsychotics in people with schizophrenia [265 - 269] as well as in predictive animal models of psychosis [270, 271]. Similarly to lamotrigine, minocycline may act synergistically with clozapine on the glutamateric system, specifically through the GluR1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) subtype [272]. In a recent study, Kelly et al. (2011) [273] suggests that adjunctive minocycline to clozapine is safe and effective in patients with schizophrenia who continue to have symptoms despite adequate clozapine treatment. Patients had improvements in their symptoms and reported "just feeling better". Moreover, a double blind placebo controlled clinical trial conducted to evaluate the effects of this combination [269] showed that minocycline treatment was associated with improvement in negative symptoms and executive functioning, supporting the beneficial effect of minocycline add-on therapy in early-phase schizophrenia.

- ***Autism.***

Fragile X syndrome (FXS) is the most common genetic determinant of cognitive impairment and autism spectrum disorders [274, 275]. Minocycline has been recently revealed as a new possible FXS drug treatment, as evidenced by different studies involving either experimental animals or humans. In a mouse model of FXS, minocycline promoted the maturation of hippocampal dendritic spines towards normal morphology, both *in vitro* and *in vivo*, and repressed anxiety and memory defects, related to a specific inhibition of MMP-9 [276]. In addition, Siller and Broadie (2011) [277] have shown in a Drosophila model of FXS

that neural circuit architecture defects were alleviated by minocycline treatment, as well as by genetic removal of MMPs. They treated Fmr1 (dfmr1) null Drosophila with minocycline and found that it effectively restored the normal synaptic structure. Minocycline treatment prevented both structural over-elaboration and synaptic developmental defects caused by dFMRP loss in a wide range of circuits. Their results support minocycline as a promising potential FXS treatment and confirm that it might act via MMP inhibition, as previously reported by Bilousova et al. (2009) [276]. In fact, minocycline has been effective in FXS patients. In an openlabel add-on trial on FXS patients, it was reported that a wide variety of symptoms were improved by minocycline treatment, including irritability, stereotypy, hyperactivity and inappropriate speech subscales [135]. In addition, this was associated with a good tolerability, since the most common side effect of minocycline treatment was limited to gastrointestinal problems [278].

- ***Cancer.***

The degradation of the extracellular matrix by MMP is a critical phenomenon in cancer invasion and metastasis. Considering the potent MMP inhibitory activities of tetracyclines, their anticancer potential has been studied in a variety of cancers, including melanoma, lung, breast and prostate cancers [279]. In fact, minocycline has been shown to inhibit *in vitro* invasion and experimental pulmonary metastasis in a subline of mouse renal adenocarcinoma (MRAC-PM2) cells. In addition, intraperitoneal administration of minocycline reduced the number of metastatic nodules in the lung when MRAC-PM2 cells were injected intravenously to mice. Minocycline also suppressed type IV collagenolytic activity of these cells, which can contribute to suppression of their metastatic potential [123]. The potential of tetracyclines in cancer therapy takes on an added dimension in the bone regarding their natural osteotropism, which would allow them to be highly effective in the inhibition of MMPs produced by osteoclasts or tumour cells in the bone [280]. Moreover, when combined with celecoxib,

minocycline inhibited the osseous metastasis of breast cancer in nude mice, by increasing tumour-cell death and decreasing tumour expression of MMP-9 and vascular endothelial growth factor (VEGF) [281].

In addition, minocycline has been recently shown to be a promising new candidate for adjuvant therapy against malignant gliomas, since it reduced glioma growth both *in vitro* and in an experimental mouse model, an effect that was associated with a strongly attenuated expression of membrane type 1 matrix metalloprotease (MT1-MMP) in glioma associated microglia [282]. Furthermore, minocycline has been described to inhibit tumour growth in the xenograft tumour model of C6 glioma cells. This effect was associated with an induction of autophagic cell death, although minocycline still induced cell death through the activation of caspase-3 when autophagy was inhibited [283].

Moreover, minocycline may benefit patients undergoing standard cancer chemotherapy by alleviating drug-induced gut damage. In a model of 5-fluorouracil (5-FU) -induced small intestinal mucositis, minocycline protected mice from gut injury. Body weight loss, diarrhoea, and villi measurements were improved by minocycline treatment, which also repressed the expression of TNFα, IL-1β, and iNOS, decreased the apoptotic index, and inhibited PARP-1 activity in the mouse small intestine. In addition, minocycline treatment appeared to enhance the antitumor effects of 5-FU in tumour CT-26 xenograft mice [284].

- ***Osteoporosis.***

In ovariectomized aged rats, a model for postmenopausal osteoporosis, minocycline was able to both increase bone formation and decrease bone loss in trabecular bone, showing a similar efficacy to that obtained with estrogen therapy [285]. In a subsequent study, minocycline treatment prevented the decrease in bone mineral density induced after the ovariectomy, and abolished the

detrimental effects induced in the femoral trabecular bone area [286]. In this study, minocycline showed dual effects, since it modestly reduced bone resorption and substantially stimulated bone formation. In addition, minocyline was found to stimulate the colony-forming efficiency of marrow stromal cells derived from ovariectomized rats, possibly explaining the effect of this compound on increased bone formation. Of note, in a rat model of synchronized osseous remodelling, minocycline impaired very significantly the disorganization of both the osteoid seam and the layer of osteoblasts, preserved the synthetic activity of osteoblasts, inhibited interstitial collagenase activity and thus bone resorption [287].

2. Mechanisms involved in the anti-inflammatory and immunomodulatory activity of minocycline.

There are many studies that have focused on the elucidation of the mechanisms involved in the non-antibiotic properties of minocycline. Despite all these data, the exact molecular mechanisms underlying the immunomodulatory and anti-inflammatory activities of minocycline are still poorly understood. They include anti-oxidant activity, the inhibition of several enzymes, anti-apoptotic activity, regulation of cell proliferation and the impairment of leucocytes function [46 - 48, 53, 198].

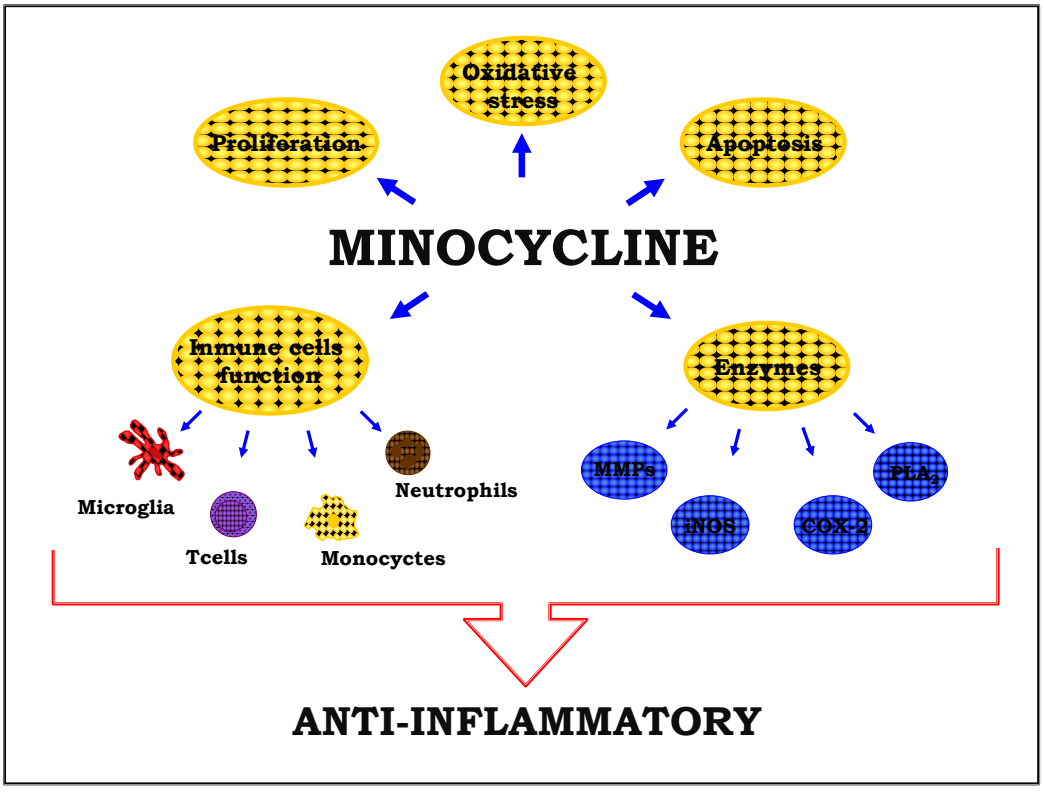

Figure 3. Mechanisms involved in the anti-inflammatory activity of minocycline.

2.1. Antioxidant properties.

Tetracyclines are particularly versatile in their ability to combat oxidative stress and scavenge free radicals [48]. These antioxidant properties are consistent with their chemical structure, multi-substituted phenol ring, similar to that of vitamin E, and thus belonging to the class of phenolic antioxidants [288]. In fact, minocycline has demonstrated direct antioxidant effects in several cell-free mixed-radical assays [236, 289], being its radical scavenging activity comparable with that of α-tocopherol and independent of Fe^{2+}chelation. In detail, minocycline has been shown to be very effective in quenching H_2O_2 [288] and in scavenging superoxide [235] and peroxynitrite due to a direct interaction with these free radicals [127]. Depending on the assay used, minocycline had a half maximal inhibitory concentration (IC50) value of 3–40 μM, being 200-300 times and 10 times more potent than tetracycline and doxicycline, respectively. In addition, minocycline was also found to be more potent (200- fold) than tetracycline in inhibition of lipid peroxidation [288, 289]. This superior scavenging ability of minocycline is likely due to the presence of a diethyamino group on the phenolic carbon, which is unique to minocycline among tetracyclines and provides improved steric hindrance [236].

Excess reactive oxygen species (ROS) are produced under many pathological conditions, leading to the oxidative destruction or dysfunction of many cellular constituents [290]. In this context, the antioxidant properties of minocycline might be particularly relevant in the management of periodontal diseases, myocardial infarcts and inflammatory conditions, and for its neuroprotective effect [236].

2.2. Anti-apoptotic activity.

Minocycline has been reported to prevent cell death by at least two mechanisms: attenuation of innate and adaptive immunity (which will be discussed below) and blockade of apoptotic cascades [128], causing a general anti-inflammatory effect and providing cell protection in a variety of experimental models [80]. When considering its effects on apoptosis, it has been shown to interfere with different processes, including inhibition of both caspase-dependent and -independent cell death [43, 91, 114, 120, 223, 291].

1) Caspase-dependent pathway

In the caspase-dependent pathway, caspase 3, the Bcl family, and cytochrome *c* (Cyt *c*) are known to play important roles. This apoptotic response can be initiated by two distinct but convergent pathways: the mitochondrial and death-receptors pathways, both of them leading to the final activation of the caspase cascade. The latter, is activated when death ligands, including TNF-Related Apoptosis-Inducing Ligand (TRAIL), Fas Ligand (FasL), and TNFα bind to cell surface death receptors, whose ligation causes the downstream activation of caspases. After death receptors activation, a balance is established between pro-apoptotic (Bax, Bid, Bim, Puma, Bak, or Bad) and anti-apoptotic (Bcl-xL, Bcl-2, Bcl-W, Mcl-1, A1, or Boo/Diva) proteins of the Bcl-2 family, which controls the mitochondrial apoptosis pathway. Initiators of the mitochondrial pathway include increased intracellular oxidative stress, DNA damage, unfolded protein response, or deprivation of growth factors, which ultimately lead to increased mitochondrial permeability, facilitating the release of pro-apoptotic proteins, like Cyt *c*, that ultimately results in the activation of caspases trough the apoptosome, into the cytosol. Another of these proteins, second mitochondria-derived activator of caspase (Smac/DIABLO), antagonizes the cytosolic inhibitors of

apoptosis proteins (IAP), thus allowing the activation of caspases and progression of apoptosis [292, 293].

Minocycline has been shown to significantly reduce the expression of caspase-1 (or ICE) and caspase-3 [51, 88, 104, 114]. In addition to inhibiting caspase expression, it has also been reported to exert several synergistic actions that dramatically prevent the induction of their activity. Related to this, minocycline prevents the release of Smac/DIABLO from mitochondria at both mRNA and protein level. This protein binds to IAP, preventing it from engaging and inhibiting caspases, and thus leading to the induction of apoptosis [51].

Moreover, minocycline has also been reported to protect mitochondria by regulating the balance between the pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family. It induces the up-regulation of antiapoptotic Bcl-2 and Bcl-xl, which accumulate in mitochondria and interact with death-promoting molecules including Bax, Bak, and Bid. These effects seem to be essential for minocycline cytoprotective activity, since down-regulation of Bcl-2 in the cells abolished its effects [294]. Simultaneously, minocycline decreases the expression of the pro-apoptotic proteins Bax, Bak, Bid [294, 295] and Fas [296]. It also reduces the up-regualtion of p53, which activates the expression of Bax in response to apoptotic stimuli. These result in the prevention of the formation of the mitochondria outer membrane permeabilization pore, and consequently prevents Cyt *c* and SMACs proteins release, leading to the blockage of the downstream caspase activation that execute the apoptotic program [114, 120, 227, 295 - 297].

Most recently, minocycline has been reported to physically interact with and inhibit apoptotic protease activator factor-1(Apaf-1), a key protein in the formation of the apoptosome, a multiprotein complex involved in caspase activation [298]. Minocycline treatment inhibited Apaf-1-induced activation of caspase-3. In addition, Apaf-1 inhibition influenced the cellular levels of the anti-

apoptotic protein Bcl-xL, which appeared restored after minocycline treatment. Moreover, minocycline was not able to inhibit cell death or caspase activation in Apaf-1 knockout mouse embryonic fibroblasts, therefore its protective effect seemed to require the presence of Apaf-1 in the cell [299].

In addition, several groups have reported that minocycline directly block pathophysiologically related and stress-induced Cyt *c* release from mitochondria, both *in vitro* and *in vivo* [43, 88, 91, 114, 294]. In these reports, the authors proposed that the inhibition of Cyt *c* release by minocycline might involve direct and indirect inhibition of the MPT, promoting its stabilization [91, 114, 120, 295, 296]. However, subsequent studies have reported that minocycline did not inhibit Ca^{2+}-induced Cyt *c* release, so this effect could not be mediated through direct inhibition of calcium-induced MPT [202].

Contrary to all the mentioned above, studies performed by Jordan's group on cerebral granular cells using a malonate-induced model of apoptosis reported that minocycline was not cytoprotective in the concentration range of 10–100 μM [300]. The authors presented data indicating that it was not able to prevent Bcl-2 down-regulation by malonate. However, in the set of experiments conducted by this group on isolated mitochondria, the authors showed that minocycline is protective against Ca^{2+}-induced mitochondrial swelling, and suggested that this effect might be mediated through dissipation of the mitochondrial membrane potential ($\Delta\Psi$) and inhibition of Ca^{2+} uptake [301].

Regarding the death-receptor pathway, although minocycline impaired Fas-FasL interaction by decreasing the expression of Fas [296], it did not affect TNFα-induced caspase-3 activity [299], suggesting that the extrinsic receptor-mediated apoptosis activation is not modulated by minocycline.

Finally, minocycline has also been reported to elicit anti-apoptotic effects by opposite modulation of extracellular signal-regulated kinase (ERK) 1/2

(restored ERK1/2) and p38 MAPK (inhibited activation) [302]. Moreover, minocycline prevents p38- dependent activation and stimulation of apoptosis by abolishing the down-regulation of ERK1/2 activity. In addition, minocycline was found to exert neuroprotection against glutamate-induced apoptosis in cerebellar granule neurons by sustaining the activation of the PI3-K/Akt-mediated survival pathway [43, 120, 214, 223, 291]. Similarly, in retinal ganglion cells minocycline increased the phosphorylation of MAPKs and Akt, leading to a better survival of these neurons (Maier 2007). In contrast, in the study performed by Corsaro et al. (2009), Akt increased activity seemed not to be required for minocycline effects [302].

2) Caspase-independent pathway

The caspase-independent pathway, which is considered as "regulated necrosis", is mediated by PARP-1, and plays a key role in cell death and survival under stress conditions [303]. When activated by DNA damage, PARP-1 consumes NAD+ to form branched poly(ADP-ribose) on target proteins. Poly(ADP-ribose) formation on enzymes involved in DNA repair appears to facilitate this process [304, 305] and to regulate gene transcription through interactions with transcription factors, notably NF-κB [306 - 308]. Therefore, it also plays a key role on inflammation.

Minocycline has been reported to directly suppress PARP-1 and downstream apoptosis-inducing factor (AIF) in this pathway. It inhibits the activation of PARP-1in the cell nucleus, followed by down-regulation of JNK and AIF in the cytoplasm. Therefore, AIF translocation into the nucleus is decreased and thus nuclear DNA fragmentation it triggers, resulting in cell death prevention [91, 130, 243, 254]. This activity was first reported by Alano et al. (2006) [130] who found that when PARP-1 activation was induced in cortical neuron cultures by different genotoxic agents, neuronal death was reduced by minocycline treatment. Biochemical markers of PARP-1 activation, neuronal NAD+ depletion and poly(ADP-ribose) formation were also blocked by minocycline. In a cell-free

assay using recombinant PARP-1, minocycline acted as a mimetic of the essential co-factor, NADH, competitively inhibiting this enzyme. Moreover, comparing several tetracycline derivatives, they suggested that the potency as PARP-1 inhibitor correlates with potency as neuroprotective agent, being minocycline potency close to that of PJ34, one of the most potent PARP inhibitors available [309]. So, in some settings, neuroprotective and anti-inflammatory effects of minocycline may be attributable to PARP-1 inhibition [130]. In fact, this effect has been lately confirmed by other authors and has been shown to mediate the beneficial effects exerted by minocycline in experimental models of different diseases, including atherosclerosis [254], ischemia–reperfusion injury [243], intestinal mucositis [284] and stroke [310].

2.3. Regulation of proliferation.

Minocycline effects on proliferation have been reported for various cell types. The first studies performed by Kloppenburg et al., (1995, 1996) [198, 199] revealed that minocycline was able to decrease T cell proliferation and the production of inflammatory cytokines *in vitro*. Since then, different studies have supported these observations and have reported the ability of minocycline to inhibit proliferation of microglia, macrophages, and lymphocytes both *in vitro* and in experimental models of different diseases [47, 111, 192, 256, 311, 312].

In addition, minocycline has also been shown to affect the proliferation of other cells. For example, treatment of HASMC with minocycline reduced proliferation and promoted an arrest in the G1 phase of the cell cycle [254, 251]. An antiproliferative effect of minocycline was also observed in murine primary aortic VSMC [254]. In human bone marrow osteoblastic cells, minocycline showed dual effects: at low concentrations, it significantly increased proliferation, and induced a significant increase in the number of active osteoblastic cells; on the other hand, high concentrations of the antibiotic led to a dose-dependent

deleterious effect on cell proliferation [147]. Endothelial cell proliferation has also been shown to be affected by minocycline, being this inhibition associated with collagenase inhibition. *In vitro* proliferation of cells comprising the microvascular wall was similarly affected by minocycline treatment. While bovine retinal microvascular endothelial cells proliferation was significantly reduced by minocycline, bovine retinal pericytes were only minimally affected and neonatal rat brain astrocytes completely unaffected. Therefore, minocycline selectively inhibits endothelial cell growth, which constitutes a potential mechanism of the anti-angiogenic activity of minocycline [313]. Mechanistically, as stated before, minocycline potently inhibits PARP-1 [130, 254], an enzyme that partially regulates cell cycle progression [314], which provides a potential mechanistic link between minocycline and regulation of proliferation.

2.4. Enzymes inhibition.

- ***Matrix metalloproteinases.***

Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent proteolytic enzymes that degrade the structural proteins in the extracellular matrix. They are involved in many physiological processes including embryogenesis, tissue remodelling and inflammation [315].They can be subdivided based on substrate specificities into the collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7, MMP-10 and MMP-11), and membrane- type MMPs (MT-MMPs) [316]. Increased MMP activity is associated with the pathophysiology of various diseases, specially with those characterized by an important inflammatory response, including many neurological diseases [317], blood-brain barrier injuries [318], heart remodelling [319], rheumatoid arthritis [320] inflammatory bowel diseases [6] and tumour metastasis [321].

The ability to inhibit MMPs activity is a property shared by most of the tetracyclines family [53]. The mechanism by which tetracyclines inhibit these enzyme activities has not been completely elucidated, but it is believed that they exert their anti-proteolytic effects via direct effects on the enzyme and indirect inhibition of their expression [322, 323].

Direct inhibition appears to be mediated by tetracyclines interaction with metal ions within the enzyme [324]. Interestingly, it appears to be dependent on the chelation of structural metals rather than the chelation of the active site Zn^{2+} [325]. *In vitro* assays have revealed that this inhibition could be partially reversed by addition of Ca^{2+} or Zn^{2+} to the reaction mixture [150, 322, 326]. Moreover, Chang et al. (1996) [327] showed that in skin extracts from diabetic rats *in vitro*, minocycline selectively inhibited some MMPs (collagenase, gelatinase) but not elastase and beta-glucuronidase, suggesting that direct and indirect mechanisms might be involved. Indirect mechanisms involved the inhibition of both MMPs mRNA expression and synthesis [328, 329]. In addition, since MMP transcription is induced by proinflammatory mediators and several growth factors, including NO, IL-1β, IL-6, TNFα, epidermal growth factor, and others [330], the ability of tetracyclines to target these mediator are likely important for the reduction in MMP expression observed [49].

The effectiveness of tetracycline inhibition against MMPs depends on the tetracycline and the type of MMP considered. Regarding minocycline, it has been reported to inhibit the levels and activities of various MMPs both *in vitro* and *in vivo*, including the gelatinases MMP-9 and MMP-2, the collagenases MMP-1, 8 and13, and the stromelysin MMP-3 [94, 123, 150, 151, 331], thus preventing pathogenic tissue destruction.

MMP-9 is the most abundantly expressed protease in inflamed intestine [6]. *In vitro* incubation of minocycline, at concentrations as low as 0.1 μg/ml, with

recombinant MMP-2 or MMP-9 impaired enzymatic activity, being MMP-9 more sensitive. In consequence, minocycline seems to be likely more selective for MMP-9 at lower concentrations [332]. Moreover, *in vivo* studies have confirmed the ability of minocycline to reduce this enzyme expression in diverse experimental models, including mouse colitis [328], EAE [116] , experimental stroke [332] or blood-retinal barrier damage [333], in which minocycline delayed the course and severity of these diseases, effects that were associated to MMP inhibition.

Other MMPs have also been reported to be down-regulated by minocycline. In bovine articular chondrocytes cultured in alginate gel beads in presence of IL-1β, minocycline reduced collagenase activity and mRNA expression of MMP-1; however, it was not able to decrease MMP-3 production, while tetracycline was. However, a study performed with fibroblast collagenase, stromelysin, and gelatinase A showed that, at the micromolar range, minocycline was found to only inhibit stromelysin [334]. Tissue inhibitor(s) of metalloproteinase-1 (TIMP-1), plasminogen activators (PA), and PA inhibitor-1, which are all involved in the ultimate regulation of MMP activity, are not affected by minocycline treatment, so the inhibition of MMPs by minocycline seems to occur mainly via down-regulation of the respective gene expression [329].

Several studies have suggested that metalloprotease inhibition, as that exerted by tetracyclines, can be clearly involved in the inhibition of on tumour progression [335], bone resorption [124], and angiogenesis [336], and may be responsible for the anti-inflammatory properties of a given compound [337].

Clinically, MMP inhibition with tetracyclines has proven itself in periodontal disease and rosacea, and may well become an acceptable therapy for preventing aneurysm enlargement, plaque rupture, acute respiratory distress syndrome, and other conditions [157].

- ***Inducible nitric oxide synthase.***

Similarly to MMPs inhibition, the inhibition of iNOS activity is also a common feature of most tetracyclines. Minocycline was found to be a more potent iNOS inhibitor than the others in stimulated murine macrophages [49]. Initially, it was thought that minocycline inhibited iNOS activity as a consequence of its Ca^{2+}-chelating properties, via a direct inhibition at the enzyme level (as reported for the metalloproteases). However, Amin et al. (1996) [49] showed that, unlike acetylating agents or competitive inhibitors of L-arginine, minocycline do not directly inhibit the catalytic activity of iNOS. Its inhibitory effect may be due to an effect at transcriptional and/or translational level, which would account for the decreased protein and specific activity of the enzyme, and the subsequent reduction in NO production [43, 49, 88, 104, 111]. Additionally, *in vitro* assays with RAW macrophages suggested that minocycline has no effect on transcription itself, but it renders the iNOS mRNA susceptible to degradation [49]. These effects on iNOS and nitrite accumulation in RAW cells LPS-stimulated were found to be not nonspecific [49] and dose-dependent [338]. Moreover, it has been recently shown that minocycline can down-regulate the expression of iNOS, while up-regulating the expression of endothelial NOS (eNOS), in vascular dementia [339].

The overexpression of NOS in a variety of autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, ulcerative colitis and Crohn's disease) and in several classic inflammatory symptoms (erythema and vascular leakiness) has led to propose that the modulation of NO synthesis and action could represent a new approach to the treatment of inflammatory and autoimmune conditions, as showed for minocycline in experimental colitis [328] and rheumatoid arthritis [49]. The deleterious effects of NO overproduction are also involved in several CNS diseases, accounting for minocycline neuroprotective effects showed in Parkinson's disease [39], cerebral ischemia [88, 104], and

Huntington's disease [43]. In addition, considering the multifunctional condition of NO, by inhibiting NOS activity, minocycline can accomplished additional protective effects on inflammation, since NO is known to mediate several catabolic activities of IL-1β [340] and to potentiate matrix degradation by the up-regulation of MMPs [341].

- ***Cyclooxygenase-2.***

It is well known that the synthesis and release of prostaglandins (PGs) are increased in inflammation, mainly derived from the inducible form of cyclooxygenase COX-2. In experimental models of CNS diseases, such us brain ischemia and Alzehimer's disease, minocycline has been shown to reduce COX-2 and, subsequently, PGE_2 production [88, 183]. In addition, minocycline is known to inhibit p38 MAPK activation [342], which is involved in COX-2 expression [343]. Surprisingly, some *in vitro* studies have suggested that minocycline increased COX-2 activity instead of inhibiting it [49, 344]. Therefore, the reduction of COX-2 expression and PGE_2 production observed after minocycline treatment *in vivo* experiments might result from an amelioration of the inflammatory response. For example, in brain ischemia, minocycline inhibited the induction of ICE mRNA [88], thus decreasing IL-1β production, which is thought to contribute to COX-2 induction in this disease [345].

- ***Secretory phospholipase A_2***

Secretory phospholipase A_2 ($sPLA_2$) plays an important role in inflammatory processes. For example, non-pancreatic $sPLA_2$ has been implicated in the pathogenesis of articular inflammation in rheumatoid arthritis and in rosacea, whereas pancreatic PLA_2 contributes to the tissue damage associated with acute pancreatitis. *In vitro*, minocycline was shown to inhibit, in a calcium-independent way, both pancreatic and non-pancreatic PLA2 [126]. More recently, minocycline was reported to interfere with the conformation of the active-site

Ca2+-binding loop of PLA2 of the Indian cobra, preventing Ca2+ binding and blocking the active site from substrate entrance, resulting in inhibition of the enzyme. Especially, the dimethylamino group at position C7 of minocycline might play a major role in this interaction, making hydrophobic connections with residues of PLA2 belonging to the hydrophobic tunnel and the interfacial binding site [346]. In addition, tetracyclines present the advantage of easily penetrate membrane cell walls, which is a problem of some other inhibitors of PLA2 that have a zwitterionic or highly charged nature.

2.5. Effects on immune cells

- ***Microglial cells.***

Different studies have reported that minocycline inhibits the activation of microglial cells, the macrophage-like cells of the CNS that regulate immune reactivity within the brain, and thus decreases the production of microglia derived inflammatory mediators [97, 161]. One of the first evidences that minocycline prevents microglial activation was provided by Yrjanheikki et al. (1998) [104] in a model of forebrain ischemia. Since then, this activity has been reported as well in many other *in vivo* and *in vitro* experiments [111, 169, 201, 214, 347].

In vivo, the protection afforded by minocycline in various models of CNS diseases was correlated with this inhibitory effect on microglia activation, i.e. in brain ischemia [104], multiple sclerosis [116, 117], Parkinson's disease [169, 312], neuropathic pain [110] and neuroinflammation [89].

In vitro, minocycline has also shown to inhibit microglial activation in response to a broad range of stimuli, such as β-amyloid, excitotoxins such as glutamate or NMDA or kainate, interferon (IFN)γ, LPS and even cerebrospinal fluid from patients with motor neuronal disease [111, 119, 192, 209, 214, 342]. Moreover, this compound was also able to prevent microglia proliferation and the production of

its related proinflammatory mediators, including NO, IL-1β, IL-6, TNFα, and NGF [182, 214]. In aggregating brain cell cultures, Defaux et al. (2011) [342] reported that minocycline-induced decreased reactivity of microglial cells activated by IFNγ and LPS was accompanied by a promotion of remyelination and an enhanced maturation and increased survival of oligodendrocyte precursors. In addition, in primary rat microglial cells minocycline decreased IFNγ-induced MHCII expression, proteins and mRNA levels in association with decreased mRNA expression of co-activator class II transactivator (CIITA), a key regulator of MHC II expression [192]. By decreasing MHC II expression, minocycline decreases antigen presentation capacity of CNS resident microglia, a mechanism that may underlie the pleiotropic effects of minocycline in CNS infectious diseases.

Mechanistically, the exact molecular mechanisms behind this activity are not yet fully understood. One of the mechanisms that mediate minocycline anti-inflammatory and neuroprotective activity might be the inhibition of the NF-κB pathway. Minocycline prevents the degradation of the inhibitory subunit of IκBα, thereby reduces NF-κB translocation to nucleus and its activation, resulting in decreased transcription of proinflammatory mediators, such as citokynes, COX-2 and iNOS [88, 111, 160]. Moreover, minocycline was shown to inhibit NF- κB binding to DNA in HIV-1-infected microglia [256].

In addition, several studies have suggest that at least some of the anti-inflammatory effects of minocycline may be mediated via interference with the MAPKs pathways, whose activation is essential for proinflammatory gene expression [39, 111, 169, 209]. Minocycline has been shown to inhibit the activation of p38 MAPK [47, 111, 160]. Nikodemova et al. (2006) [160] showed that, in addition to p38 inhibition, minocycline also has a strong ability to reduce the activation of P44/42 (ERK1/2) and p54/46/ (c-Jun N-terminal protein kinase (JNK) 1/2) MAPKs in response to LPS. It was able to attenuate the increase in phosphorylation of these kinases, but it did not modify their basal level of

phosphorylation. Importantly, although minocycline inhibited the activation of all these MAPKs in response to LPS, these effects appeared to depend upon the stimulus used for their activation, implying that minocycline targets signal transduction protein(s) that are stimulus specific. For example, minocycline differentially affects oxidative stress (H_2O_2) and 2¢,3¢-O-(4-benzoylbenzoyl)-adenosine 5¢-triphosphate (BzATP)-stimulated MAPK activation. It was unable to inhibit the activation of any of the MAPKs (p38, ERK1/2 and JNK1/2) in response to stimulation with H_2O_2, and it had differential effects on BzATP-stimulated MAPKs: ERK1/2 and JNK1/2 activation were significantly decreased, whereas p38 activation was not affected. In addition, when MAPK activation was stimulated by phorbol myristate acetate (PMA), minocycline did not affect ERK1/2 activation but it inhibited JNK1/2 and p38 phosphorylation. Therefore, each stimulus probably utilizes different signalling molecules to mediate MAPK activation, and only some of them might be target(s) of minocycline [160].

Minocycline also impairs activation of PKC, which is involved in activation of MAPK [348]. Minocycline inhibited IFNγ- induced PKCα/βII phosphorylation and the subsequent nuclear translocation of IFNγ regulatory factor (IRF-1) that controls glial response to IFNγ, thus explaining the observed decreased in MHCII expression [192]. However, minocycline can also affect proteins involved in JNK1/2 or p38 activation residing downstream of PKC considering that not all MAPKs whose activation is dependent on PKC were affected by minocycline [160]. Furthermore, the inhibition of ICE [88, 111] also accounts for minocycline anti-inflammatory effects. During inflammation, ICE acts as an inhibitor of spontaneous neutrophil apoptosis through the processing of IL-1β, therefore, its inhibition reduces neutrophil proinflammatory activity [349].

- ***Peripheral blood mononuclear cells.***

In vitro studies have revealed the ability of minocycline to inhibit the proliferative response of human peripheral blood mononuclear cells (PBMC) to mitogenic stimulation. Three decades ago, Banck and Forsgren (1979) [350] showed that there was a significant depression of the mitogenic responses of both B and T lymphocytes after minocycline treatment. In addition, minocycline appreciably reduced DNA synthesis in PMBC at concentrations just above the therapeutic range [351]. In isolated human peripheral blood lymphocytes stimulated with IL-1β, minocycline suppressed the mitotic response, and similar effects were observed when mononuclear cell fractions were stimulated with phytohaemagglutinin (PHA). Stimulation of lymphocytes in whole blood cultures with PHA in the presence of minocycline revealed a similar suppression of the mitotic response [352]. On the other hand, the cytokine production has been also shown to be modified by minocycline treatment, being dependent on the stimulus used. The addition of minocycline to whole-blood cultures stimulated with LPS revealed a dose-dependent increase in TNFα and IL-6 production. In contrast, minocycline dose-dependently inhibited TNFα and IFNγ production induced by PHA stimulation, whereas IL-6 production was hardly affected. These effects on whole-blood samples were reproduced when using isolated PBMCs [199].

- ***T cells.***

Many of the anti-inflammatory and inmmunomodulatory activities of minocycline can be attributed to its effects on T cells. Suppressive effects of minocycline on T-cell proliferation and activation have been long described [117, 188, 198, 199, 352, 353]. Minocycline also consistently attenuates activation-induced increases in T cell size dose-dependently [136]. These authors have also suggested that minocycline reduced T cell turnover, since the pretreatment of CD4+ T cells *in vitro* with this antibiotic resulted in decreased levels of proliferating (Ki67+) and

activated (HLADR+) cells, and increased levels of circulating naïve (CD45RA+) cells after activation [136].

Minocycline effect on T cell activation was associated with an inhibitory effect on cytokine production, as reported by Kloppenburg et al. (1995, 1996) [198, 199] who observed that supernatants from minocycline-treated T cells 24 h after activation showed significant decreases in IL–2, IFNγ and TNFα. This effect was found to be signal dependent. Primary peripheral T cells, as well as human T-cell clones, developed an impaired proliferative response and a decreased production of the cytokines IFNγ and TNFα in response to anti-CD3 when pretreated with minocycline *in vitro*. When T cells were stimulated by a calcium-independent way, i.e. with PMA and anti-CD28, minocycline exerted an inhibitory effect on TNFα production, associated with a decrease in the levels of TNFα mRNA. However, if cells were stimulated in a calcium-dependent manner, i.e. PMA and the calcium ionophore A23187, a dose-dependent effect of minocycline on IFNγ and TNFα production was observed, but the decrease in TNFα production was not accompanied by a decrease in the levels of TNFα mRNA. On the other hand, contradictory data were reported by Popovic et al. (2002) [117], who showed that minocycline had no effect on T cell proliferation and IFNγ production in rodents.

T cells surface markers expression has also been reported to be affected by minocycline treatment, which suppressed CD25 (IL-2 receptor), CD40L and HLADR activation markers expression [50, 136, 188].

As commented previously, minocycline is able to impair T cell-microglia interaction. The pre-treatment of either T cells or microglial cells from human brains or from the U937 cell line with minocycline resulted in a decreased TNFα production in the subsequent co-culture. Minocycline pre-treatment of T cells also promoted an increase in IL-10 production in these co-cultures. However, when minocycline was added at the time of the co-culture, TNFα production was

not modulated. These data suggested that minocycline, despite interfering with microglia activation, directly acts on T cells as well, reducing their activation state and impairing its ability to interact with microglia [188]. Furthermore, minocycline pre-treatment also decreased the number of T cells clustering around-microglia, an effect associated to the above mentioned down-regulation of CD40L expression in T cells surface, which contributes to their reduced ability to adhere and engage microglia [188]. Therefore, minocycline seems to target the CD40-CD40L pathway, which is involved in several inflammatory processes [188]. In addition, the antigen presentation capacity of professional antigen presenting cells (APCs), a necessary process for the activation of CD4+ T cells in the periphery, has been reported to be affected by minocycline as well [192, 197].

The mechanism by which minocycline interfere with T cells activation has been recently shown to be mediated trough selective suppression of nuclear factor of activated T cells 1 (NFAT1) transcriptional activation [50]. NFAT is a key regulatory factor in T cell activation. Minocycline was found to increase NFAT1 rephosphorylation, which reduces its nuclear translocation after several hours of activation, and thus decreases its transcriptional activity. Two potential mechanisms were suggested for this effect: increased activity of glycogen synthase kinase (GSK) 3 and attenuated intracellular Ca^{2+} flux. Regarding GSK3, minocycline was found to decrease the inhibition of this NFAT kinase, thus enhacing NFAT1 rephosphorylation. However, the effects of minocycline were found to be synergistic with PMA-mediated deactivation of NFAT1, supporting a direct action on Ca^{2+} signalling. However, direct chelation of available Ca^{2+} and direct molecular interaction with calcineurin were ruled out. In contrast, minocycline was found to attenuate intracellular Ca^{2+} flux in the first five minutes after stimulation through a mitochondria-based mechanism, by reducing store-operated Ca^{2+} entry (SOCE) through the plasma membrane. By this way, it suppresses activation-induced intracellular Ca^{2+} flux over time, which is

consistent with the reduced NFAT1 dephosphorylation and its increased nuclear export several hours after activation, providing a more plausible mechanism for enhanced NFAT1 phosphorylation. Considering previous reports, the authors suggested that minocycline may act by reducing the capacity of mitochondria to buffer Ca^{2+}, resulting in decreased SOCE in CD4+ T cells. It reduced the levels of sustained extracellular Ca^{2+} entry, which is consistent with an accelerated return of intracellular Ca^{2+} to basal levels. In fact, minocycline exerts several actions on mitochondria that support this hypothesis [301, 354].

In general, the suppressive effects of minocycline on T cell activation and NFAT1 transcriptional activity were found to be dose-dependent, starting its effects at concentrations as low as 5 µg/mL and being the optimal 20 µg/mL [136]. However, the concentrations achieved in sera of patients during routine oral dosing are below this threshold (1-2 µg/mL), hence only a partial blunting of T cell responses would be expected. However, plasma concentrations above 20 µg/mL have shown no short-term toxicity [355], so higher doses are viable to increase suppression of T cell activation if necessary. In addition, complete inhibition of NFAT would require concentrations exceeding 40 µg/mL, making the danger of total immunosuppression remote [355]. Considering the dose-dependent and NFAT specific nature of minocycline effects, it could be used in an immunomodulatory manner, equilibrating the immune response to a desired point by therapeutic dose monitoring. In consequence, and in view of its safety and excellent bioavailability, minocycline could be a therapeutic candidate for autoimmune and inflammatory diseases such as HIV, inflammatory bowel disease (IBD) or rheumatoid arthritis, where the goal of therapy is not immunosuppression, but a new equilibrium for the immune response [50].

- ***Monocytes/Macrophages.***

The studies evaluating the effects of minocycline on monocytes have provided contradictory results. Contrasting its effects on lymphocytes, minocycline has been described to enhance cytokine secretion by monocytes [352, 199]. This capacity has been demonstrated for IL-1β, TNFα and IL-6 production. In LPS-stimulated monocytes the dose-dependent increase in TNFα production was associated to an enhancement of TNFα mRNA synthesis. On the contrary, other studies have reported that minocycline was able to inhibit LPS-induced activation of macrophages both *in vitro* and *ex vivo*, being this effect characterized by decreased production and release of proinflammatory mediators derived from iNOS, COX-2 and MMPs activities [49, 356]. Recently, a proteomic analysis of the J774 cell line in response to LPS with or without a minocycline pre-treatment has suggested that minocycline does not inhibit complete macrophage activation, since a number of LPS-induced functions remained unaffected. Interestingly, there were also some proteomic changes in response to minocycline in the absence of LPS, such us induced expression of heat shock protein 71, aldose reductase and olfactory receptor 1204 [338].

Finally, minocycline has also been reported to impair the antigen presentation capacity of professional APCs, such as dendritic cells (DCs). It has been shown to impair *in vitro* antigen processing for presentation to T cells by peripheral blood APCs [197] and to inhibit INFγ-independent MHC II expression, in association with a decreased PKCα phosphorylation, in macrophages [192].

- ***Neutrophils.***

A reduction of neutrophil chemotaxis by minocycline has been traditionally proposed to explain the favourable effects of tetracyclines on cutaneous inflammation in humans [140, 141]. Lately, minocycline has been shown to indeed reduce the number of neutrophils that migrate into the sites of inflammation in

rat models of intracerebral haemorrhage [357] and to significantly decreased MPO release from human neutrophils [289].

INFLAMMATOY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disorder of the gastrointestinal tract that comprises two major conditions: Crohn's disease (CD) and ulcerative colitis (UC). Both forms of IBD significantly impair quality of life, and require prolonged medical and/or surgical interventions. What make it particularly challenging is its still unknown cause, its unpredictable presentations and symptoms, the less than optimal treatments, and a continuous rise in its incidence and prevalence in many areas of the world [358].

Histologically, UC is characterized by non-transmural inflammation that is restricted to the colon, beginning at the rectum and spreading proximally in a continuous fashion, frequently involving the periappendiceal region. Tipically, the inflammatory changes are limited to the mucosa and submucosa with cryptitis and crypt abscesses. Patients usually present with bloody diarrhoea, passage of pus, mucus, or both, and abdominal cramping during bowel movements [359, 360]. On the other hand, in CD the inflammation of the gastrointestinal mucosa is transmural and can discontinuously affect the any part of the gastrointestinal tract, from the mouth to the anus, affecting most commonly the terminal ileum or the perianal region. Typical presentations include the development of complications including strictures, abscesses, or fistulas. The microscopic features of CD comprise thickened submucosa, transmural inflammation, fissuring ulceration and non-caseating granulomas. The clinical presentation is largely dependent on disease location and can include diarrhoea, abdominal pain, fever, clinical signs of bowel obstruction, as well as passage of blood or mucus or both [359, 360].

These complex conditions are considered as multifactorial and, although their aetiology is not fully understood, it has been proposed that, in genetically susceptible individuals, there is an altered and chronic activation of the immune

and inflammatory cascade against unknown environmental stimulus, which triggers the disease [3].

1. Aetiology.

Over the years many theories have been proposed to explain IBD pathogenesis, ranging from infectious to psychosomatic, social, metabolic, vascular, genetic, allergic, autoimmune and immune-mediated [361 - 365]. There is now a general consensus that IBD is the result of the combined effects of four basic components: global changes in the environment, the input of multiple genetic variations, alterations in the intestinal microbiota, and aberrations of innate and adaptive immune responses. There is also agreement on the conclusion that none of these four components can by itself trigger or maintain intestinal inflammation but it is their integration and reciprocal influence what determines whether IBD will emerge and with which clinical phenotype [366].

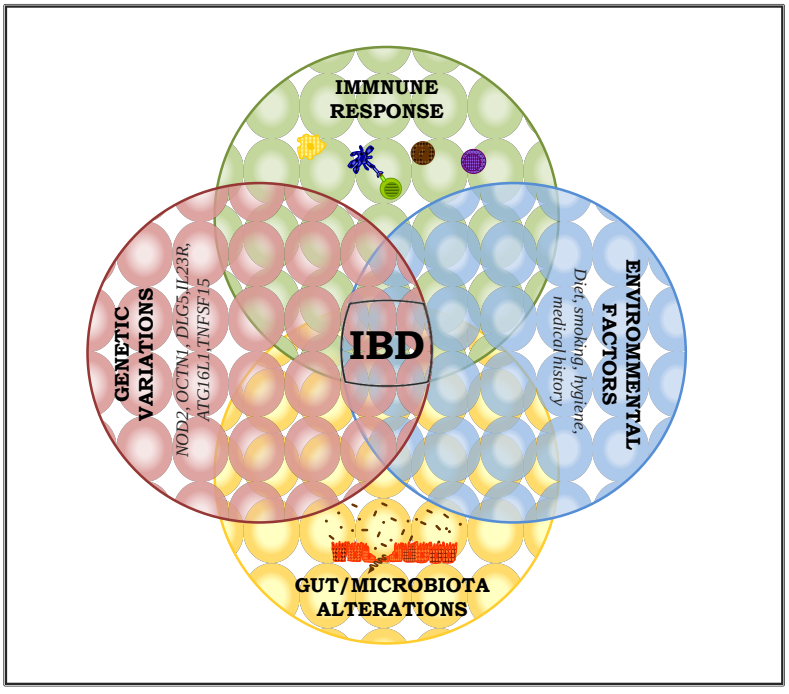

Figure 4. Factors involved in Inflammatory Bowel Disease (IBD).

1.1. Environmental influences.

There is wealth of evidence showing that both forms of IBD are increasingly recognized worldwide [367]. Epidemiological studies have considered a large number of risk factors for developing IBD, such us cigarette smoking, diet, oral contraceptives, vaccination history and other drugs, appendectomy, infections, water supply, social circumstances and perinatal and childhood factors. However, with the exception of cigarette smoking, none of the other factors is supported by direct evidence to be considered true risk factors.

Another feature that has been brought to explain the increasing evidence of IBD is the 'hygiene hypothesis', which proposes that the lack of proper exposure to common infections early in life negatively affects the development of the immune system, which becomes less 'educated' and less prepared to deal with multiple new challenges later in life [368]. This is indirectly supported by evidence of improved health and acquisition of western societies habits in parts of the world where IBD is emerging [369, 370].

1.2. Genetic variations.

The well-known familial occurrence of IBD has suggested for a long time that this condition could have a genetic basis [371]. In the last years, high number of susceptibility loci has been associated with either CD [372] or UC [373]. Genome-wide association studies (GWAS) have identified 99 non-overlapping genetic risk loci. Although the genetic component is stronger in CD than in UC, and despite their distinct clinical features, approximately 30% of these IBD-related genetic loci are shared between both conditions, indicating that these diseases engage common pathways [374, 375]. Analyses of the genes and genetic loci implicated in IBD show several pathways that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, ROS

generation, autophagy, regulation of adaptive immunity, ER stress and metabolic pathways associated with cellular homeostasis.

Nucleotide-binding oligomerization domain-containing protein 2 (*NOD2),* or caspase recruitment domain family member 15 (*CARD15),* located on chromosome (Chr) 16, was the first specific gene to be associated with IBD, in particular with ileal CD in white (but not oriental) populations [376, 377]. Genetic variants of NOD2 are deficient in signalling pathways that allow the response from the intestinal immune system to muramyldipeptide (MDP), resulting in reduced activation of NF-κB [378]. Moreover, mutations in NOD2 result in decreased production of antibacterial defensins by Paneth cells [379, 380]. Therefore, patients with the *NOD2* mutation have defective clearance of intracellular bacteria in intestinal epithelia [381] and also impaired immune responsiveness to bacterial components [382]. The inflammation developed in *CARD15* mutation murine models has also been suggested to be driven by altered TLR activation of NF-κB [382]. Low concentrations of MDP could impair generation of IL-8, TNFα and IL-1β, similarly to the deficient signalling through TLRs when the CD-associated variants are present. This diminished early innate immune response could lead to inadequate microbial clearance and eventually result in the characteristic inflammation of CD [383]. In fact, homozygous people for a NOD2 variant may have a ≥ 20-fold increase in susceptibility to CD, and heterozygotes are also at increased risk. However, it is important to note that only ≈20% of CD patients are homozygous for NOD2 variants [3, 384]. Later, the organic cation transporter 1 on Chr 5 *(OCTN1*), was identified as another susceptibility gene [385] and some evidence suggests that the disks large homolog 5 gene (*DLG5),* on Chr 10, could be a third [386]. In addition, polymorphisms of the *TLR4* gene have been reported in both CD and UC [387], further reinforcing the notion of potentially defective innate immunity pathways in these patients in the recognition and response to bacteria.

Variants of the *IL-23 receptor (IL23R)* gene were found in both CD and UC [388], being in this case protective [389]. IL-10R signalling components have also been implicated, including *IL10RA* polymorphisms, *SAT3*, *TYK2*, *JAK2* and *IL10* itself [390].

An unsuspected role for autophagy in IBD was recently described, implicating two component genes, *ATG16L1* and *IRGM* [391 - 393]. Similarly, genetic variants that perturb mechanisms that protect against ER stress can signal apoptosis and can affect intestinal homeostasis in IBD [394].

In addition to coding variants, non-coding single nucleotide polymorphisms (SNPs) have shown to confer susceptibility to CD, like the SNPs in TNFSF15 [395 - 397]. Furthermore, genetic changes may affect transcription-factor-binding sequences, locus accessibility, translational efficiency and *trans*-regulators such as non-coding RNAs and microRNAs (miRNAs). In this regard, IBD-implicated loci contain more than 10 miRNA-encoding sequences and 39 large intervening non-coding RNAs (lincRNAs), supporting the notion that regulation of gene expression by miRNAs and lincRNAs may be mechanistically relevant in IBD [398].

1.3. Disturbances in the innate and adaptive immune responses.

The physical barrier of the intestinal epithelium is complemented by a well-evolved mucosal innate immune system, which is poised to defend against pathogenic incursions, and limit inflammatory responses to maintain a state of hyporesponsiveness to commensal bacteria. However, it is also the effector arm that mediates intestinal inflammation. The altered immune response that takes place in IBD may be caused by a loss of barrier function, which overwhelms normal immune regulation, as well as dysfunction of regulatory mediators of the intestinal immune system [3]. Both T cell mediated and humoral responses have

been implicated in this breakdown of immunologic tolerance to commensal bacteria in the intestine [399, 400].

Innate immunity

CD and UC are traditionally viewed as predominantly T-cell-driven processes but recent evidence suggests that innate immune responses could also play an important role, at least in CD, in initiating the inflammatory cascade and the subsequent pathological adaptive immune responses [3]. Supporting this notion, patients with innate immunodeficiencies tend to develop IBD and similarly, patients with CD have defective innate immune responses, including attenuated macrophage activity *in vitro*, and impaired neutrophil recruitment and exogenous *Escherichia coli* clearance *in vivo* [401].

The intestine network of dendritic cells (DCs) and macrophages are involved in local innate immune phenomena but also have an important role in shaping adaptive immunity in response to intestinal environmental antigens [402, 403]. Under homeostatic conditions, both DCs and macrophage populations have specific adaptations that promote tolerance, being conditioned by the mucosal microenvironment to express a non-inflammatory phenotype. Intestinal resident macrophages, and as highly phagocytic cells, clear apoptotic cells and debris and contribute to wound repair of the epithelium [404, 405]. They have adaptations to prevent excessive inflammatory responses towards the intestinal flora, including expression of inhibitors of NF-κB signalling that permit bactericidal activity in the absence of TLR-driven proinflammatory cytokine production [405], IL-10 production and maintenance of forkhead box P3 (Foxp3) among colonic regulatory T (Treg) cells [406]. Intestinal DCs are highly specialized APCs that can function to provide protection and defence, induce tolerance or mediate inflammation [407]. For example, Treg-cell differentiation can be promoted by tolerogenic DCs [408]. By contrast, DCs expressing E-cadherin are a

proinflammatory subset that promotes Th17-cell differentiation [409]. Bacterial flagellins can stimulate TLR5 in hyporesponsive DCs from lamina propria and induce the release of proinflammatory mediators [410]. CD11chigh CD103+ DCs are dispersed throughout the lamina propria, taking up pathogenic and commensal bacteria, innocuous antigens or apoptotic IECs. After maturation, they migrate to the draining mesenteric lymph node, where they initiate adaptive responses focused on the intestine, preferentially inducing FoxpP3+ Treg cells [403], [411]. However, during intestinal inflammation, they acquire inflammatory properties, such as the ability to produce IL-6 and drive Th1 responses [412]. CD103– CX3CR1+ APCs comprise a heterogeneous population of DCs and macrophages. CD11c+CX3CR1+ DCs are adjacent to the intestinal epithelium where they sample antigens and bacteria [403, 411]. CX3CR1+ APCs accumulate in response to microbiota-derived signals [413], and promote colonic Th17 responses in response to commensal-derived ATP [411].

During infection, however, responses shift to a more inflammatory nature, which can lead to immune pathology when dysregulated. Focusing on IBD and experimental colitis, there is an increase in DCs and macrophage populations displaying an activated phenotype, with enhanced expression of microbial receptors, that contributes to intestinal pathology through the potent proinflammatory effects of the cytokines that they secrete, particularly TNFα and IL-6 [362, 411, 414]. In CD they produce more IL-23 and TNFα than those in normal and UC mucosa, and contribute to the production of IFNγ by local T cells [415]. Acute and chronic mouse colitis models were also associated with a marked increase in recruited monocyte-derived DCs and macrophages that produced IL-12, IL-23 and TNFα and showed enhanced TLR responsiveness [409, 416, 417].

Neutrophil accumulation within epithelial crypts and in the intestinal mucosa directly correlates with clinical disease activity and epithelial injury in IBD. Activated neutrophils produce reactive oxygen and nitrogen species, and

myeloperoxidase within intestinal mucosa, which induce oxidative stress that participates in the intestine damage associated to these conditions [4]. Neutrophils also contribute to the resolution of inflammation, by synthesizing anti-inflammatory mediators such as lipoxin A_4. Studies showing impaired secretion of lipoxin A4 in mucosal tissues from UC patients support the relevance of such mechanisms in IBD [418].

In addition, innate leukocyte populations, including γδ T cells, natural killer T (NKT) cells and natural killer (NK) cells, can secrete Th1 and Th17 cytokines such as IFN-γ, IL-17a and IL-22 [419 - 422], thus also contributing to intestinal inflammation.

Adaptive immunity

- ***T cells.***

The activated immune response characteristic of IBD is dominated by mucosal CD4+ lymphocytes. CD has a predominant Th1 type cytokine profile with elevated IFNγ and IL-2, whereas UC was associated with a predominant Th2 type - like cytokine profile, characterized by increased amounts of transforming growth factor β (TGFβ), IL-5 and IL-13 (but without the altered expression of IL-4, the other prototypic Th2 cytokine) [8]. After the identification and characterization of the new Th subset, the IL-17-producing Th17 cells, in CD mucosa [423, 424], recent evidence suggests that observations attributed to alterations in Th1 and Th2 populations can actually result from the down-regulating effects of their products on this new helper cell population. In addition, the microbiota has an important role in the preferential localization of Th17 cells in the gut [425, 426]. Regarding the relative enrichment of Th17 cells at mucosal sites, together with the increased levels of Th17 cytokines in the inflamed gut [420, 427], tissue destruction might therefore actually be mediated by these Th17 cells subset [428, 429]. Early studies have also suggested a role for IL-13-producing NKT cells in UC [362].

In addition to helper-cell activation, reduced numbers of regulatory T cells (Treg) characterized by CD4+, CD25+ and Foxp3, which produce IL-10 and/or TGFβ, might be equally important. These cells monitor the immune response and prevent an excessive and potentially harmful immune activation [430, 431]. Although there are various T-cell populations, Foxp3+ Treg cells and Foxp3− IL-10-secreting CD4+ T cells are particularly important in the intestine [432]. Foxp3+ Treg cells are abundant in the small intestine and colon, where they control potentially deleterious responses to dietary and microbial stimuli [432]. They are usually generated in the thymus, but the intestine is also a preferential site for TGFβ-dependent induction of Foxp3+ Treg cells [432]. Microbiota also have a role in promoting intestinal Treg-cell responses, since Treg-cell accumulation in the colon is reduced in germ-free mice and can be increased by particular indigenous bacteria [433]. Deletion or loss-of-function mutations in the gene encoding Foxp3 result in inflammatory disease in mice and humans, often accompanied by intestinal inflammation [432].

Interestingly, induced Treg-cell and Th17-cell populations seem to be reciprocally regulated in the intestine. Although TGFβ is required for the differentiation of both populations, the presence of signal transducer and activator of transcription (STAT) 3-mediated signals (such as IL-6 or IL-23) promotes Th17 cells at the expense of Foxp3+ Treg cells [427, 434]. This mechanism allows the inflammatory response to override Treg-cell induction in the presence of proinflammatory stimuli, promoting intestinal effector T-cell responses and host defence. In fact, mice with a *Stat3* deletion in Foxp3+ Treg cells develop aggressive colitis owing to uncontrolled Th17 responses [435]. This system is delicately balanced but sometimes can lead to Treg-cell instability. For example, high-level T-bet expression in the presence of acute intestinal infection drives Treg cells into an inflammatory IFN-γ-secreting phenotype [436]. Transcription factors that direct Th1-cell or Th17-cell responses, such as T-bet or retinoic-acid-

receptor-related orphan receptor-γt (RORγt), respectively, were shown to be essential for T-cell-mediated colitis [362, 437].

- ***B cells.***

Antibody production in active IBD is increased both in the circulation and at the mucosal levels. There are alterations on IgM, IgG and IgA synthesis and secretion by both peripheral blood as well as by mucosal mononuclear cells in both UC and CD [438]. In the involved mucosa there is an increased production of monomeric IgA, which is normally predominant in the circulation [439]). The patterns of antibody class production differ in UC and CD, particularly in regard to IgG production: in UC there is a disproportional increase in IgG1 secretion, while in CD IgG1, IgG2 and IgG3 are increased [440]. At the moment limited attention is being given to B cells in IBD but renewed interest may occur if new biologics that specifically induce B cell depletion, like rituximab [441] turn out to be effective in its management.

Inflammatory mediators

Inflammation is further amplified by a broad spectrum of inflammatory mediators including additional cytokines, chemokines, leukotrienes and prostaglandins.

Chemokines mediate the recruitment of leucocyte effector populations to the sites of immune reaction and tissue injury [442]. In chronic inflammatory diseases like IBD, aberrant leukocyte chemoattraction occurs, characterised by an excessive recruitment of inflammatory cells into the injured intestine [361]. Chemokines tightly control leukocyte adhesion to and migration across the endothelium [443] but they are also able to trigger multiple inflammatory actions including leukocyte activation, granule exocytosis, production of metalloproteinases for matrix degradation, and up-regulation of the oxidative

burst [444, 445]. Some of the chemokines whose expressions are consistently increased during the active phases of IBD are: IL-8 and its receptor, MCP -1 and -3, and macrophage inflammatory proteins (MIP) [446 - 449]. Similarly, the up-regulated expression in IBD of different adhesion molecules, such as the inter-cellular adhesion molecule (ICAM) -1, the lymphocyte function-associated antigen (LFA) -1, the macrophage 1 antigen (Mac-1), the vascular cell adhesion molecule (VCAM)-1, the very late antigen (VLA) -4 and P- and E-selectins, promotes the recruitment of granulocytes and lymphocytes through blood vessels. In addition, these adhesion molecules also facilitate cell interactions, like those between lymphocytes and APCs or among lymphocytes, thereby sustaining the immune-inflammatory response [450]. Of note, ICAM-1 is pivotal for the influx of neutrophil granulocytes into colonic mucosa, and gene analyses have found polymorphisms in the gene encoding ICAM-1, indicating that it may be involved in the pathogenesis of UC [451].

The cytokines that control T-cell differentiation and regulation have been identified as central points of potential intervention to control the inflammatory response. IL-12, IL-18 and IL-23 each have a crucial function in Th1 differentiation and chronic activation, whereas other cytokines, such as TNFα, IL-1β and IL-6 augment the inflammatory response by recruiting other cells and enhancing the production of inflammatory mediators [452].

IL-2, or the T cell growth factor, is the major cytokine that is produced during the primary response of Th cells. This cytokine acts through its receptor (IL-2R) to activate signalling molecules that are involved in cell proliferation [453]. *IL-2 KO* mice have been reported to develop intestine inflammation that is dependent on T cells and the presence of intestinal flora [21]. They were shown to be deficient in $CD4^+CD25^+$ Treg cells, thus promoting the expansion of organ-specific T cells, one of the major causes for UC [454, 455].

IL-23 is induced by pattern recognition receptors (PRRs), whose sustained activation drives chronic intestinal inflammation. ER stress can also synergize with TLR signals to selectively increase IL-23 expression by DCs [394], which is constitutively expressed in a small population of DCs present in the lamina propria [415]. It was initially linked to the preferential expression of Th17 responses, but it can promote a wide range of pathological responses in the intestine, mediated either by T cells or by excessive innate immune activation. IL-23-mediated enhancement of Th1 and Th17 responses is consistent with the increased levels of IFN-γ, IL-17 and IL-22 observed in the chronically inflamed intestine [420, 427]. Furthermore, studies in several mouse IBD models have used selective targeting of the IL-23 p19 subunit to demonstrate that IL-23 plays a key part in chronic intestinal pathology [420]. T-cell-intrinsic IL-23R signals favour the expression of pathogenic proinflammatory T-cell responses in several ways, including enhanced proliferation of effector T cells, reduced differentiation of Foxp3+ Treg cells and the emergence of IL-17+IFN-γ+CD4+ T cells [427] as found in the inflamed lamina propria of patients with CD [456].

The differential activation of IL-12 p40 and Th1-cell responses was associated with CD. In mice lacking the *Il12b* gene or given neutralizing antibodies directed against IL-12 p40 or IFN-γ it was observed a colitis-attenuating effect [362]. Moreover, in phase II clinical trials, anti-IL-12-p40 monoclonal antibodies have shown clinical efficacy in a subset of patients with CD [457].

Th17 cells produce several cytokines, including IL-17A, IL-17F, IL-21 and IL-22 [420], which have diverse effects on intestinal inflammation. For example, in acute DSS colitis, IL-17A has a protective role, whereas IL-17F seems to exacerbate disease [458]. However, studies performed in T-cell-transfer colitis models suggest that IL-17A and IL-17F can have redundant proinflammatory effects in the gut [437]. In mice with *Stat3*-deficient Treg cells, the neutralization of

IL-17A attenuated chronic colitis [459] and decreased innate immune colitis after *H. hepaticus* infection [460].

IL-22, which is mainly produced by ILCs expressing the transcription factor RORγt [461, 462], is down-regulated in CD [463]. It is emerging as an important cytokine in epithelial homeostasis, showing protective activity in different models of colitis through its stimulatory effect on antimicrobial and reparative processes. In intestinal epithelial cells (IECs), IL-22 signalling drives the production of antimicrobial peptides (AMPs) and promotes epithelial regeneration and healing by activating the transcription factor STAT3 [464]. Consistent with this, IL-22 administration attenuated disease severity in the DSS and T-cell receptor-α (*Tcra*−/−) mouse IBD models, by restoring goblet cells and mucus production [419]. By contrast, other studies support a pathogenic role for IL-22 in IBD, as its expression is increased in patients with CD, and high serum IL-22 levels correlate with increased disease activity and susceptibility-associated *IL23R* polymorphisms [419].

Although less extensively studied, IL-21 may also regulate intestinal inflammation, through effects on Th17 cells and the production of MMPs [420].

The association of IBD with polymorphisms in *NLRP3* and *IL18RAP* and the central role for inflammasomes and NOD-like receptors (NLRs) in auto-inflammatory diseases [465] have rekindled interest in the potential roles of IL-1β and IL-18 in IBD. Their levels are increased in IBD [362, 466], and *Il18*−/− mice were resistant to colitis induced by trinitrobenzene sulphonic acid [467], suggesting their contribution to intestinal pathology. This hypothesis is consistent with the ability of IL-1β and IL-18 to promote Th17 and Th1 responses, respectively [466, 468].

TGFβ is present at high concentrations in the intestine and has a crucial involvement in modulating the immune response [469]. For example, it stimulates intestinal IgA responses, thus reinforcing intestinal homeostasis [470]. Deletion of

Tgfb1 in mice leads to a fatal inflammatory disease. In addition, T cells from IBD patients are refractory to TGFβ anti-inflammatory actions [471]. During inflammation, TGFβ in the presence of proinflammatory cytokines such as IL-6 promotes the development of inflammatory Th17 responses [472].

IL-10 is produced by a wide range of leukocytes, including T cells, B cells and myeloid cells. The colon contains large numbers of CD4+ IL-10+ cells, mainly Foxp3+, whose IL-10 production is required to prevent intestinal inflammation. In fact, IL-10$^{-/-}$ mice spontaneously develop colitis [432]. Intestinal bacteria can promote the activity of colonic Treg cells by inducing IL-10 production [473]. Foxp3−IL-10+ CD4+ cells are more heterogeneous since most effector Th-cell subsets produce IL-10 after chronic immune stimulation [474]. Myeloid sources of IL-10 are also important in some settings, as shown in an adoptive transfer model of colitis in which IL-10 production by intestinal macrophages promoted Foxp3 Treg-cell function [406]. This cytokine controls chronic intestinal inflammation partly through direct anti-inflammatory effects on myeloid cells [432]. Evidence for the role of IL-10 in human IBD comes from findings that mutations in the IL-10 receptor genes *IL10RA* and *IL10RB* lead to severe early-onset IBD [390].

1.4. Gut/Intestinal microbiota alterations.

Intestinal homeostasis depends on complex interactions between the microbiota, the intestinal epithelium and the host immune system. Breakdown in the pathways that control this homeostasis may precipitate the chronic inflammatory pathology found in IBD.

Intestinal barrier

The intestinal barrier, which protects the individual from potential bacterial threats, consists on the biofilm, the mucous layer, and the epithelium and innate immune defences. A defect in this barrier, which is commonly found in IBD, can lead to persistent activation of the immune system, initiating early events in its pathogenesis. Of note, murine models with defects in barrier function on a genetic basis manifest chronic colitis [385].

The intestinal barrier is improved by a pre-epithelial layer formed primarily of mucus glycoproteins, trefoil peptides, IgA and AMPs. The mucus layer is generated by goblet cells, and it is a protective polysaccharide bilayer rich in cationic proteins, composed by secreted mucins overlying a dense inner glycocalyx of membrane-anchored mucins and is inaccessible to most bacteria [475, 476]. Decreased levels of goblet cells, leading to reduced mucin secretion, are a hallmark of human IBD, and some UC patients show defective intestinal O-glycosylation. Moreover, mice lacking the major mucin protein MUC2 have shown to develop spontaneous colitis [477]. In addition to providing a biophysical barrier, mucus forms a matrix that allows the retention of high concentrations of antimicrobial molecules, such as defensins and secretory IgA, which defend against pathogenic bacteria and control the composition of the gut microbiota contributing to local homeostasis [478]. These AMPs, including lysozymes, defensins, cathelicidins, lipocalins, C-type lectins and secreted PLA_2 are constitutively produced by many IEC, whereas others are secreted in an inducible fashion by Paneth cells [479]. This inducible production is regulated by TLR and NOD2 signals triggered by commensal flora. Paneth cell dysfunction and impaired defensins secretion may also contribute to IBD susceptibility, as observed in CD patients [379 - 481] and in mice deficient in several CD-associated genes [393, 391].

The intestinal mucosa exists in a functional equilibrium with the complex luminal content. Within this mucosa, the intestinal epithelium represents a huge surface area lined by a single layer of columnar IECs, which absorbs nutrients, constitute a solid physical barrier, and also performs several other functions crucial for intestinal homeostasis, such as sampling of the intestinal microenvironment, sensing of both beneficial and harmful microbes, AMPs secretion and induction and modulation of immune responses in underlying lamina propria [475]. The dynamic crosstalk between intestinal epithelial cells (IECs), intestinal microbes and local immune cells represents one of the main features of intestinal homeostasis and of IBD pathogenesis [476, 475].

Abnormal intestinal permeability has been observed in IBD patients. Genes within several IBD-associated loci indicate a role for barrier integrity in disease predisposition, e.g. truncated forms of the adherens junction protein E-cadherin (CDH1) are associated with CD [482], activation of the G protein Gα12 (encoded by GNA12) leads to phosphorylation of the tight junction proteins ZO-1 and ZO-2, resulting in destabilization of cell junctions in epithelial cell lines [483]. The protein tyrosine phosphatase family member PTPN2 protects against IFN-γ-induced epithelial permeability; concordantly, Ptpn2-deficient mice show increased susceptibility to experimental colitis [484, 485]. Defects in epithelial regeneration have been associated with IBD as well. Genetic studies have shown that several transcription factors involved in epithelial regeneration, such as HNF4A and NKX2-3, which control crypt cell proliferation and IEC differentiation respectively, are related to IBD [486 - 488]. STAT3, whose deletion in IECs affects epithelial repair [464], is activated in epithelial cells from patients with IBD.

In addition to limiting bacterial translocation across the mucosal barrier, IECs promote intestinal homeostasis by regulating innate and adaptive immune responses. In the healthy intestine, cytokines constitutively expressed by IECs, such as thymic stromal lymphopoietin, IL-25 and TGFβ, limit DCs production of

the p40 subunit of IL-12 and IL-23 and promote IL-10 secretion, impeding the priming of Th1-cell responses and instead favouring the induction of Treg- and Th2-cell responses [475, 489]. They also produce intestinal alkaline phosphatase, which can mediate LPS detoxification. Conversely, after sensing pathogenic invasion or damage, IECs can elaborate the secretion of proinflammatory chemokines, such as IL-8, which have an important role in alerting the immune system to microbial attack [490]. Breakdown in these mechanisms could lead to pathological intestinal inflammation. IECs also exert a strong influence on local antibody responses by producing factors such as TGFβ, B-cell activating factor (BAFF, also known as TNFSF13B) and a proliferation-inducing ligand (APRIL, also known as TNFSF13), which promote class-switching of B cells towards the production of IgA [491] and mediating the transport of secretory IgA into the mucus layer, where it has a complementary role to innate defences in limiting the penetration of commensal bacteria across the epithelium [491, 492].

The intestinal mucosa monitors microbial ligands using PRRs, and microbial metabolites using G-protein-coupled receptors (GPCRs) and solute carriers. For example, short-chain fatty acids (SCFAs), generated by some microflora constituents, can act on neutrophils with notable proresolving effects on inflammation [493]. These SCFAs are decreased in UC, and other genes implicated in microbial ligands monitoring have been also described as IBD-risk loci [494].

Gut microbiota

The gut microbiota confers beneficial effects to the host, including helping to metabolize nutrients, modulate immune responses and defend against pathogens. However, dysregulation of normal homeostatic relationships between gut bacteria and host immune responses can lead to intestinal inflammation. An increasing amount of evidence suggests that intestinal bacteria may have a role in

the pathogenesis of inflammatory bowel disease (IBD), providing the stimulus for the aberrant immune response that leads to chronic inflammation in genetically susceptible people [3, 17, 23, 358, 366, 495]. The exact pathogenic mechanisms are not yet clear, but possibilities include persistent infection with a specific pathogen, altered luminal microbiota causing chronic inflammation, increased exposure to normal luminal bacteria caused by abnormal mucosal barrier function, or abnormally aggressive immune response to normal luminal contents such as in the setting of NOD2 polymorphisms [8, 16, 56].

The possibility that IBD is a chronic inflammatory response directed against microbial agents has been considered since the initial reports describing the disease. Over many years repeated attempts have been made to identify a causative agent, but most potential candidates have been dismissed due to the lack of reproducible scientific evidence. One agent that raised a great deal of controversy is *Mycobacterium avium* spp. *paratuberculosis* [496, 497]. The adherent-invasive *Escherichia coli* is still under active investigation. It is frequently present in close association with the ileal mucosa in patients with CD and exploits host defects in phagocytosis and autophagy to promote chronic inflammation in the susceptible host [362, 498]. *Bacteroides fragilis* biofilm was also investigated, as it was found to be the predominant feature in mucosal microbiota [11]. Recent studies have highlighted that intestinal inflammation can confer a selective growth advantage to certain pathogens, including *Salmonella typhimurium* [499] or *Ruminococcus* strains in IBD mucosa, which raises the possibility that they may contribute to the barrier defect observed in these patients, although whether their presence is cause or effect of the colitis remains unclear. However, none of the possible etiologic agents proposed have been successfully implicated in the pathogenesis of IBD [8, 10, 500, 501].

Despite the fact that an etiologic agent has not been directly implicated, the role of intestinal bacteria in IBD pathogenesis is supported by strong evidence.

Studies in animal models suggesting that the development of colitis is attenuated in the absence of normal enteric flora have provided the most compelling evidence. Regardless of the strain and genetic background or method used to induce inflammation, animals raised under germ-free conditions do not develop experimental colitis [502, 503]. In addition, spontaneous colitis occurs when commensal bacteria is introduced to the intestines of these mice [18 - 22]. Purified bacterial products have also been able to initiate and perpetuate experimental colitis [504].

Another generally accepted principle is that immunological tolerance to commensal bacteria is lost in patients with IBD [399, 505]. Luminal microbiota shapes development and function of the mucosal immune system in a tightly correlated manner, however, dysregulation of normal homeostasis can lead to intestinal inflammation. The fact that local immune system mounts an inflammatory response against intestinal microbes anti-microbiota has been demonstrated by the presence of panels of serum antibodies against a variety of microorganisms (now used as disease biomarkers) [506].

Then the question that arises is to whether the immune response is directed at the gut flora as a whole, specific subgroups or strains or selected microbes. In IBD patients the number of bacteria associated with the mucosa layer is dramatically increased [11, 15]. In addition, IBD is associated with an imbalance in the composition of the intestinal microbiota, termed dysbiosis, which may promote inflammation [14, 507]. A key unresolved issue is whether dysbiosis represents a primary or secondary predisposing factor for IBD and recent studies have indicated that dysbiosis is influenced by both the host genotype [508] and IBD phenotype [509]. Different studies of luminal bacterial composition in IBD patients showed they have reduced diversity and number of protective bacteria such as lactobacilli and bifidobacteria, while aggressive bacteria such as *Bacteroides*, adherent/invasive *Escherichia coli* and enterococci are increased [11 - 13, 507]. It is

interesting that commensals belonging to the *Clostridiales* order, such as *Faecalibacterium* and *Roseburia*, were significantly reduced in patients with ileal CD [508], [509]. These genera are potent sources of short-chain fatty acids, such as butyrate, and have protective effects in mouse colitis models (Maslowski, 2011). In addition, these clostridial groups promote the accumulation of Treg cells in the mouse colon [473], *F. prausnitzii* stimulates IL-10 production in peripheral blood mononuclear cells [510] and *Bacteroides fragilis*, produces polysaccharide A, which suppresses IL-17 production and promotes the activity of IL-10-producing CD4+ T cells in mice [511], all of them promote an anti-inflammatory status. Dietary factors may also affect microbiota composition, leading to alterations in intestinal immune homeostasis [512], for example, mice fostered on milk lacking sialyl(α2,3)lactose develop a distinct microbiota that confers transmissible resistance to DSS colitis [513].

In addition, the distal ileum and the colon, which represent the major sites of inflammation in IBD, are the areas with the highest bacterial concentrations [23] and enteric bacteria or their products have been found within the inflamed mucosa of patients with CD [9]. Moreover, in human studies of patients with CD and a diverting ileostomy, reinfusion of luminal contents from their ileostomy into the excluded normal ileal loop is able to trigger a recurrence of the disease within a few days [514, 515]. It is also well known that diversion of faecal stream in these patients determines a decrease in disease activity, with disease recurrence occurring after restoration of faecal stream [516].

2. The rationale for using antibiotics and probiotics in the treatment of IBD.

The primary therapeutic goals in IBD are related to improve patient quality of life by inducing and maintaining remission, preventing and treating complications and restoring nutritional deficits [359, 517]; surgical intervention is required in those who are non-responders to medical treatment. The current pharmacological treatment includes these major categories:

- Anti-inflammatory drugs, mainly 5-aminosalicylic acid (mesalazine), which include mesalazine formulations and oral pro-drugs (sulfasalazine, olsalazine and balsalazide); and corticosteroids such as hydrocortisone, budesonide, and beclomethasone.
- Immunosuppressants: Ciclosporin, tacrolimus, methotrexate, azathioprine and antimetabolites (mercaptopurine).
- Biologic agents: Antibodies that block a variety of cytokines central to T-cell activation and other strategies to modify the pathophysiological process, being most of them under current development.
    - Anti-TNFα antibodies: Infliximab (a chimeric monoclonal antibody), Adalimumab (fully human), Certolizumab pegol (pegylated anti-TNF antibody FAb´ fragment), Etanercept, (a fully human dimeric fusion protein), Onercept (recombinant form of the human soluble p55 TNF receptor binding protein), CDP571 (humanised IgG_1 monoclonal antibody).
    - Fontolizumab, humanised anti-interferon-γ antibody.
    - IL10: Tenovil (rHIL-10), bacterial vectors that deliver IL-10 in the intestine (*LL-Thy12* – IL-10 secreting *Lactococcus* spp).
    - Blockade of T cells: Visilizumab (humanized anti-CD3 antibody), Basiliximab and Daclizumab (monoclonal antibodies blocking CD25), Abatacept (anti-CTLA-4-Ig).

- Blockade of cell migration and adhesion: Natalizumab (monoclonal α4-integrin antibody), MLN02 (monoclonal antibody that targets α4β7-integrin).

Unfortunately, adverse effects, an inconvenient dosing schedule and/or prohibitive price may limit their long term use [518]. For this reason, the development of new therapies that combine efficacy, convenient dosing and lower side effects is an important target in human IBD therapy.

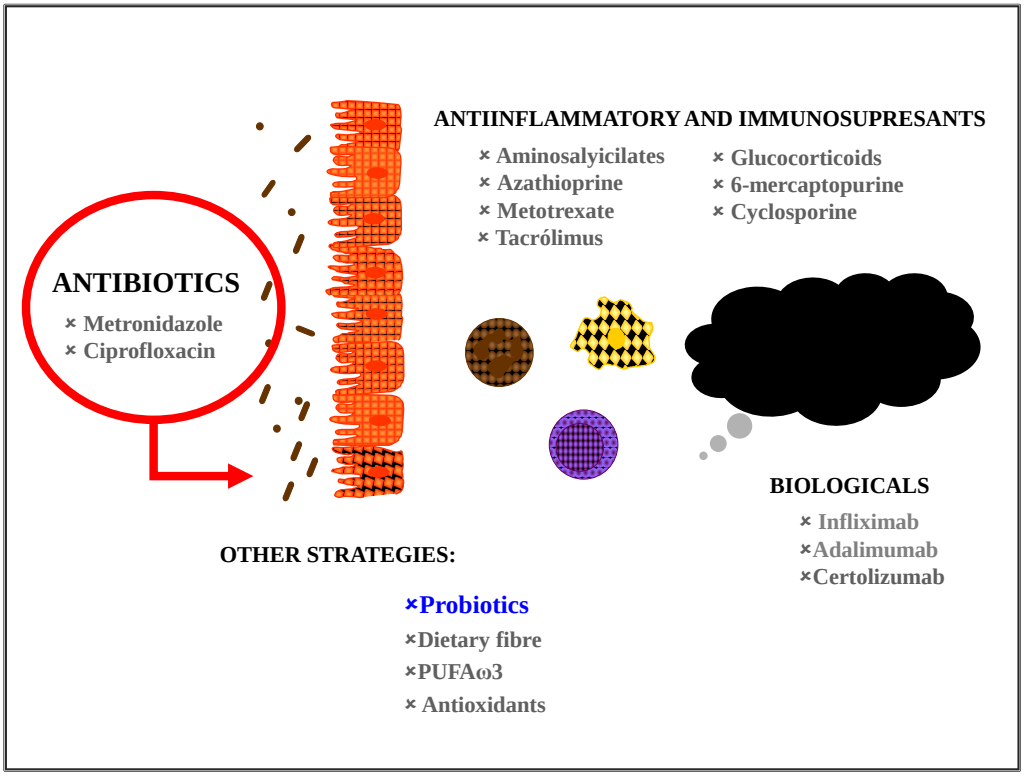

Figure 5. Current pharmacological treatment of IBD.

As described before, there is increasing experimental data that supports a role for luminal bacteria in the initiation and progression of these intestinal conditions; probably related to an imbalance in the intestinal microflora, with a relative predominance of aggressive bacteria and an insufficient amount of protective species [1, 519]. Despite the relevance of the gut microbiota in IBD, therapy has been mainly focused on suppressing the immune system rather than on restoring the composition of the altered gut microbiota, a strategy that may be achieved through the use of antibiotics or the administration of probiotics or prebiotics [56, 366, 495].

Although antibiotics have been long used in the treatment of human IBD, their benefit in primary or adjunctive treatment has not been well established in randomized controlled trials [16, 56]. However, in clinical practice, they are frequently used. Since no pathogen has been specifically targeted, broad-spectrum antibiotics, mainly metronidazole and ciprofloxacin, are most frequently used [17]. In a recent systematic review and meta-analysis about antibiotic therapy in IBD, it is concluded that some antibiotics, alone or in combination, may induce remission in active CD and UC [520]. Although their use in CD as a primary therapy is poorly documented, there is good evidence that ciprofloxacin, metronidazole or their combination is effective in Crohn's colitis and ileocolitis. In open-label study, their association with corticosteroids (prednisone) resulted more effective than antibiotics alone in the treatment of active CD. It was also noted that antibiotic therapy was more beneficial to patients with involvement of the colon [25]. In UC, their use is not supported by the available studies and large trials with broad-spectrum agents are required [16, 521]. In contrast, a randomized controlled trial performed in 1998 suggested that ciprofloxacin may be beneficial as an adjunctive to conventional treatment with mesalamine and prednisone for active UC [522]. More recently, remission has been described to be induced in active UC, either by a triple antibiotic therapy [27] or by

a synergistic association of antibiotics and corticosteroids [28]. However, they play an essential role in treating the septic complications of IBD, such as intra-abdominal and perianal abscesses, fistulae and fissures, bacterial overgrowth, peritonitis, and toxic megacolon [16, 56]. In fact, treatment with antibiotics, specifically metronidazole and ciprofloxacin, is well established in patients with pouchitis, the most common long-term complication of ileal pouch-anal anastomosis for UC, representing the mainstay of therapy in this setting [29, 30].

Classically, the beneficial effect exerted by the antibiotics in the treatment of IBD has been mainly attributed to their antimicrobial properties [31, 32, 523]. Theoretically, they have the potential to alter the course of IBD in several ways [10, 16, 56, 524]: decreasing luminal bacteria overgrowth, altering the composition of micrtaoflora to favour beneficial bacteria, decreasing bacterial tissue invasion and/or translocation and/or systemic dissemination, reducing proinflammatory bacterial toxins and eradicating bacterial antigenic triggers. However, different studies have recently reported the ability of many antibiotics to modulate both the innate and the adaptive immune responses by acting directly on different inflammatory cells [34, 35, 37], an activity that increases their potential as therapeutic options for IBD (Figure 6).

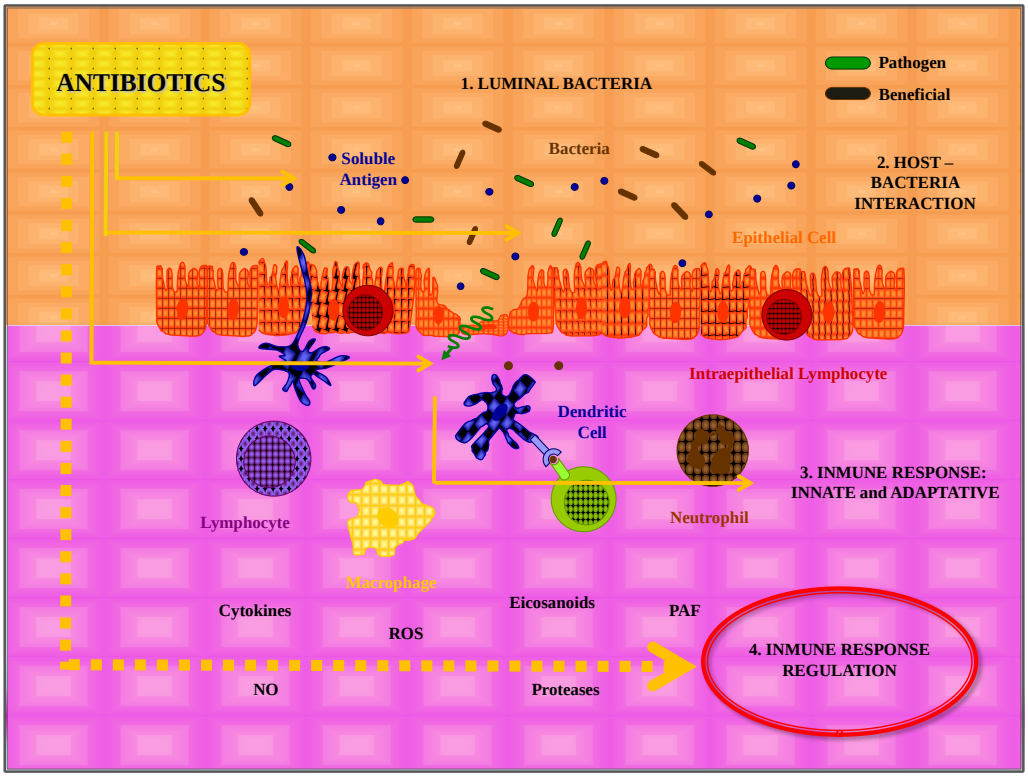

Figure 6. Mechanisms of action of antibiotics in IBD.

The use of probiotics in IBD therapy is even more controversial. Probiotics are live organisms, usually bacteria, used as a therapeutic agent to benefit the health of the host by altering the microbial balance. Their administration in IBD was proposed to promote a balanced colonic microbial environment and thus probably help in both prevention and control of this disease. Despite the large number of probiotics that have shown beneficial effects in experimental models of intestinal inflammation [525], the studies describing their efficacy in human IBD, mainly UC and pouchitis, are less abundant. The strongest evidence comes from clinical trials conducted with *Escherichia coli* Nissle 1917 [57] or with the probiotic mixture VSL#3 [58], revealing their greater usefulness in maintaining the disease in remission and preventing the relapses rather than in inducing remission, when studied in more severe active forms of IBD [59].

Escherichia coli Nissle 1917 is the most extensively studied probiotic in IBD. It was purportedly isolated from a German soldier in World War I who had withstood a severe outbreak of gastroenteritis that devastated his unit [526]. This non-pathogenic bacteria has been demonstrated to be able to displace pathogenic *E. coli* [527] and is also suggested to down-regulate intestinal T-cell expansion through TLR2 signalling [528].

VSL#3, a mixture of eight bacteria (predominantly *S. thermophilus*, to a lesser extent *B. breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. casei* and *L. bulgaricus*) has demonstrated to increase IL-10 as well to decrease T-cell production of IFNγ and seemed to be effective in maintaining remission of chronic pouchitis in patients with prior UC [58].

Other probiotics studied include a mixture of bifidobacteria and lactobacilli [529], which prevented the relapse of UC showing an equivalent effect to mesalazine in maintaining remission, *Lactobacillus GG* [530, 531] and *Saccharomyces boulardii* (Florastor; Biocodex) [532, 533], although with limited and equivocal results.

Different mechanisms have been proposed to be involved in the therapeutic effects exerted by probiotics. First, these bacteria can suppress the growth of enteric pathogenic bacteria and their epithelial binding and subsequent invasion, maybe due to their ability to decrease luminal pH via production of SCFAs [534] and to promote the secretion of bactericidal proteins [535, 536] and/or stimulate mucin production. Second, these micro-organisms improve epithelial and mucosal barrier function by decreasing mucosal permeability, producing SCFAs and enhancing mucus production, thus increasing barrier integrity [537, 538]. An finally, probiotics have been reported to exert immunoregulatory activities, either by inducing protective cytokines (IL-10 and TGFβ), or by inhibiting proinflammatory cytokines (TNFα), in the intestinal mucosa [60, 61, 539 - 541].

However, it is important to note that not all probiotic bacteria have similar therapeutic effects; each may have individual mechanisms of action, and the host condition may determine which probiotic species and even strains may be optimal [62].

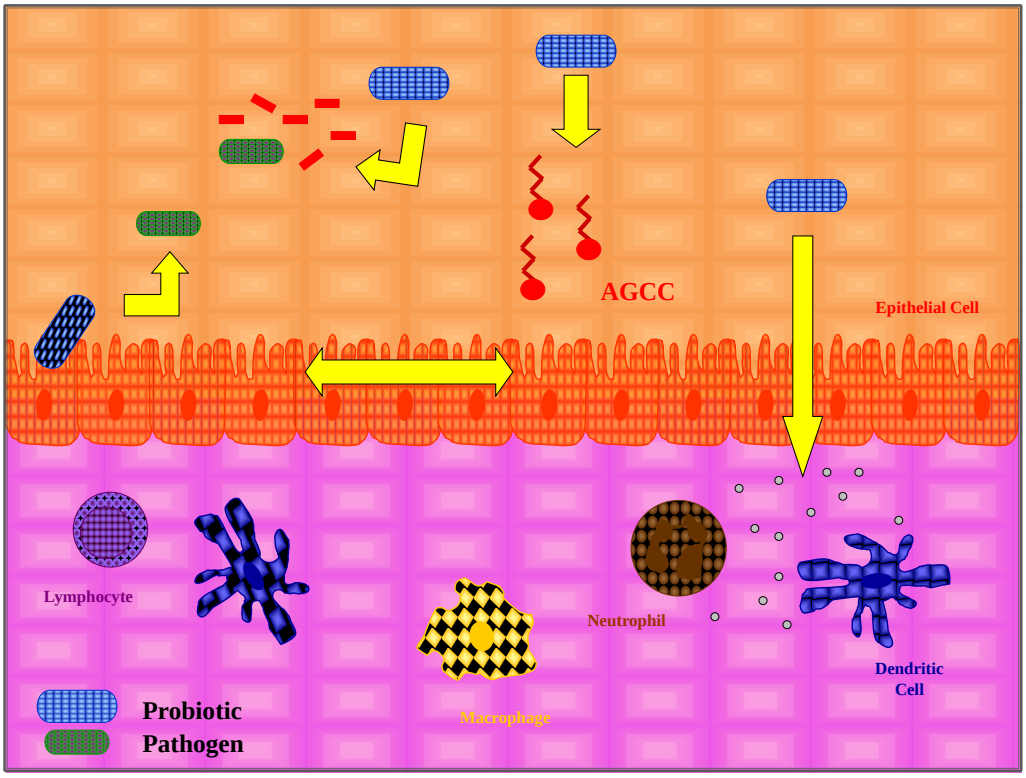

Figure 7. Mechanisms of action of probiotics in IBD.

Aims

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract that comprises two major conditions: Crohn's disease and ulcerative colitis. Although the pathogenesis of IBD remains elusive, the altered and chronic activation of the immune and inflammatory cascade that occurs in genetically susceptible individuals against unknown environmental stimulus may play a key role. There is increasing experimental data that supports a role for luminal bacteria in the initiation and progression of these intestinal conditions; probably related to an imbalance in the intestinal microflora, with a relative predominance of aggressive bacteria and an insufficient amount of protective species [1, 5-19].

For this reason, the manipulation of enteric flora through the administration of antibiotics and/or probiotics has been shown to be an important approach in controlling the disease.

In fact, it has been reported that remission may be achieved after treatment with antibiotics in intestinal inflammation [24 - 26]. More recently, remission has been described to be induced in active UC, either by a triple antibiotic therapy [27] or by a synergistic association of antibiotics and corticosteroids [28]. Classically, the beneficial effect exerted by the antibiotics in the treatment of IBD has been mainly attributed to their antimicrobial properties [31 - 33]. More recently, different studies have reported the ability of many of them to modulate both the innate and the adaptive immune responses by acting directly on different inflammatory cells [34 - 37]. In particular, minocycline, a semi-synthetic second-generation tetracycline, has been shown to possess anti-apoptotic, immunosuppressive and anti-inflammatory properties in several pathological conditions such as acne vulgaris, periodontitis, rheumatoid arthritis, asthma, scleroderma, neural ischemic damage, Parkinson´s disease, spinal cord injury and Huntington disease [38 - 45]. Therefore, the use of a single compound like minocycline that combines

both immunomodulatory and antimicrobial activities could be very interesting in the pharmacological treatment of IBD. Unfortunately, several studies have reported that discontinuation of antibiotic therapy results in a high relapse rate, suggesting a need for long-term therapy and then increasing the risk of drug side effects [17].

Regarding the use of probiotics in IBD therapy, the strongest evidence comes from clinical trials conducted with *Escherichia coli* Nissle 1917 or with the probiotic mixture VSL#3 [57, 58], revealing their greater usefulness in maintaining the disease in remission and preventing the relapses rather than in inducing remission, when studied in more severe active forms of IBD [59]. Different mechanisms have been proposed to participate in their beneficial effects, including the suppression of the growth of enteric pathogenic bacteria and their epithelial binding and subsequent invasion, the immunoregulatory activity, as well as the improvement of the intestinal barrier function by decreasing mucosal permeability [60, 61]. However, it is important to note that not all probiotic bacteria have similar therapeutic effects; each may have individual mechanisms of action, and the host condition may determine which probiotic species and even strains may be optimal [62].

Considering IBD etiology, it is interesting to develop combined approaches that would restore the local ecological conditions in the gut lumen, thus correcting dysbiosis, and, at the same time, would reinstate the altered immune response that takes place in the inflamed intestine in the long term. Although the combination of antibiotics and probiotics has been proposed to play a role in the treatment of IBD, with the rationale of opening a microbial niche with the antibiotics that the probiotics can then occupy [56, 63], there are only few studies supporting this strategy [64, 65] and none of them have focused on the immunomodulatory effects derived from it.

Based on all the above, we proposed two main objectives:

1) To test the intestinal anti-inflammatory effect of orally administered minocycline in different models of colitis, in order to study the role of its immunomodulatory properties and its antibiotic activity on the global beneficial effect achieved. For this purpose, orally administered minocycline was assayed as prophylactic or curative treatment in the trinitrobencenesulphonic acid (TNBS) acute colitis model in rats, and its curative effects as a chronic treatment were tested in the dextrane sodium sulfate (DSS) model in mice. Additionally, *in vitro* studies were performed to evaluate the direct immunomodulatory properties of minocycline, both in intestinal epithelial cells (Caco-2 cells) and macrophages (RAW264.7 cells), two cell types actively involved in the intestinal immune response.

2) To characterize the beneficial effects derived from the association of the antibiotic minocycline and the probiotic *Escherichia coli* Nissle 1917 in an experimental model of reactivated colitis in mice. We aimed to simulate a combined approach that would reinstate the altered immune response that takes place in the inflamed intestine, displaying curative activity, and restore the local ecological conditions in the gut lumen. Once remission is achieved, sustained probiotic administration would prevent the reactivation of the experimental colitis after antibiotic therapy interruption. For this purpose, a curative treatment protocol was followed: once DSS experimental colitis had been established, minocycline was initially administered for seven days; then, the antibiotic treatment was substituted by the probiotic administration, which continued until the end of the experiment in order to maintain the remission. 14 days after the beginning of the treatment, mice were subjected to a second cycle of DSS intake for five days, thus promoting a relapse in the colonic inflammatory process,

which would allow to evaluate the impact of the association in preventing the reactivation, in comparison with each individual treatment.

Materials & Methods

IN VITRO STUDIES

Caco-2 cells (human colon adenocarcinoma cells) and RAW 264.7 cells (mouse macrophages) were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS and 2 mM L-glutamine, in a humidified 5% CO_2 atmosphere at 37°C. Caco-2 cells were seeded onto 24-well plates at a density of 5×10^5 cells per well and grown until formation of a monolayer. Then, they were pre-incubated for 24 hours with different concentrations of each antibiotic (minocycline, tetracycline or metronidazole) ranging from 2 to 100 μM. Afterwards, cells were stimulated with IL-1β (1 ng/mL) for 20 hours. Untreated unstimulated cells and untreated cells were used as negative and positive controls. Then the supernatants were collected, centrifuged at 10000 *g* for 5 min and stored at −80°C until IL-8 determination by ELISA (Biosource, Invitrogen™) was performed. RAW 264.7 cells were seeded onto 24-well plates at a density of 5×10^5 cells per well and grown until confluence. They were cultured for 1 hour with each of the antibiotics described above and then stimulated with LPS (100 ng/mL); similarly, positive and negative controls were also included. After 20 h, supernatants were collected and centrifuged at 10000 *g* for 5 min, and nitrite levels were measured using the Griess assay [542], in which Griess reagents (0.1 % N-(1-naphthy)ethylenediamine solution and 1% sulphanilamide in 5% (v/v) phosphoric acid solution) convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance at 550 nm due to this azo chromophore accurately determines nitrite concentration. Cell viability was examined by the MTT-test following the manufacturer's instructions [543], and it was not affected by antibiotic treatments.

IN VIVO STUDIES

All the studies were carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health.

All the animals were housed in makrolon cages, maintained in an air-conditioned atmosphere with a 12 h light-dark cycle, and they were provided with free access to tap water and food.

1. Trinitrobenzene sulphonic acid model of rat colitis.

Female Wistar rats (180 - 200 g) obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) were randomly assigned to 6 groups (n=10). Four of them received antibiotic treatment: minocycline (20 or 40 mg/kg), tetracycline (80 mg/kg) or metronidazole (40 mg/kg). The antibiotics were dissolved in 2 mL of distilled water and administered daily by oral gavage. An untreated TNBS control group and a non-colitic group were also included for reference.

Colonic inflammation was induced in control and treated groups as previously described [67], by the administration of 10 mg of TNBS dissolved in 0.25 mL of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. Two different treatment protocols were performed: preventive and curative (Figure 8). In the preventive protocol, the antibiotic administration was started one week before TNBS instillation and continued up to the day before of the sacrifice of the rats, which took place two days after the colitis induction. In the curative protocol, the antibiotic was administered from the day of the colitis induction until the day before of the sacrifice of the rats, seven days after the induction of the colonic damage. All the rats were sacrificed with an overdose of halothane.

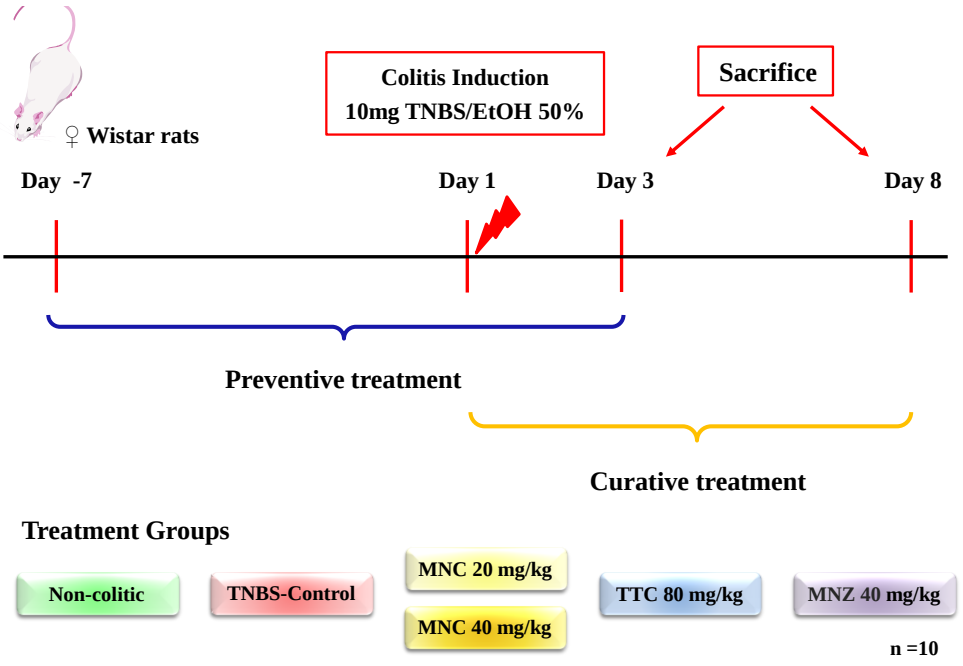

Figure 8. Experimental design in the TNBS model of rat colitis. Minocycline (MNC), tetracycline (TTC), metronidazol (MNZ)

Animal body weights, occurrence of diarrhoea and water and food intake were recorded daily throughout all the experiments. Once the animals were sacrificed, the colon was removed aseptically and placed on an ice-cold plate, longitudinally opened and luminal contents were collected for the microbiological studies. Afterwards, the colonic segment was weighed and its length measured under a constant load (2 g). Each colon was scored for macroscopically visible damage on a 0-10 scale by two observers unaware of the treatment, according to the criteria described by Bell et al. (1995) [66] (Table 1).

Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered formaldehyde for the histological studies. Equivalent colonic segments were also obtained from the non-colitic group. The

remaining colon samples were subsequently sectioned in different longitudinal fragments to be used for biochemical determinations or for RNA isolation.

Table 1. Criteria for assessment of macroscopic colonic damage in rat TNBS induced colitis.

Score	Criteria
0	No damage
1	Hyperemia, no ulcers
2	Linear ulcer with no significant inflammation
3	Linear ulcer with inflammation at one site
4	Two or more sites of ulceration/inflammation
5	Two or more major sites of ulceration and inflammation or one site of ulceration/inflammation extending along the length of the colon
6-10	If damage covers along the length of the colon, the score is increased by 1 for each additional centimeter of involvement

Described by Bell et al., (1995).

2. Dextran sodium sulfate model of mouse colitis.

Female C57BL/6J mice (7-9 weeks old; approximately 20 g) obtained from Harlan (Barcelona, Spain) were randomly assigned to two different groups: non-colitic (n=10) and DSS colitic groups (n=20). The colitis was induced by adding DSS (36-50 KDa, MP Biomedicals, Ontario, USA) in the drinking water at the concentration of 3% for a period of 5 days, after which DSS was removed [544]. Then, colitic mice were divided in two groups of 10 animals each: minocycline treated group, which received orally 30 mg/kg per day of minocycline dissolved in 200 µL of distilled water, and control mice which were given the vehicle (Figure 9). Mice from the non-colitic group were administered tap water during the whole experiment. All mice were sacrificed 21 days after the beginning of the experiment.

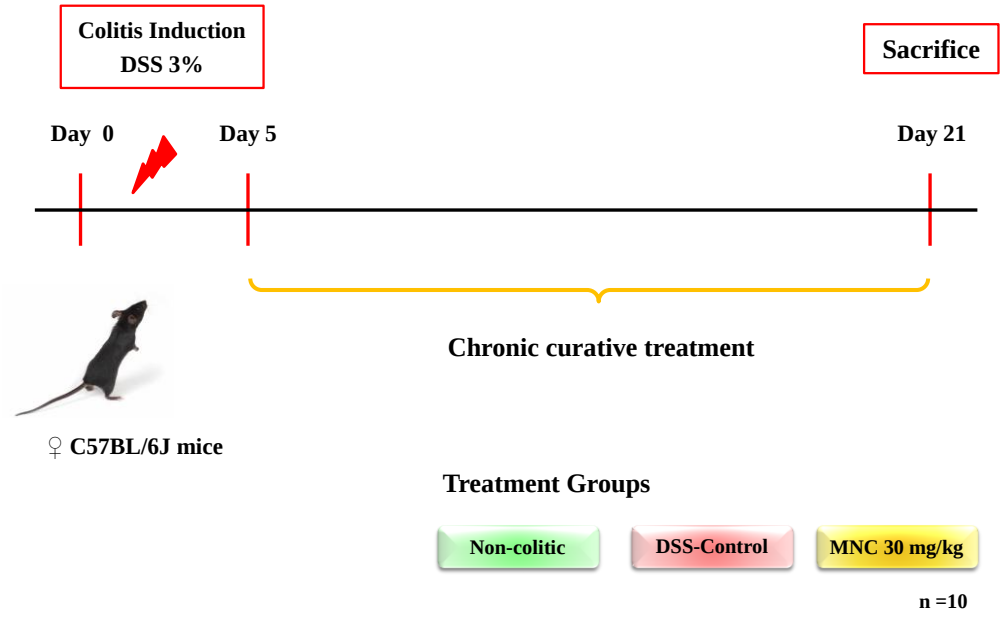

Figure 9. Experimental design in the DSS model of mouse colitis.

Minocycline (MNC)

Animal body weight, the presence of gross blood in the faeces and stool consistency were evaluated daily for each mouse by an observer unaware of the treatment. These parameters were each assigned a score according to the criteria proposed previously by Cooper et al. (1993) [545] (Table 2) and used to calculate an average daily disease activity index (DAI). Once the animals were sacrificed, the colon was removed aseptically and weighed, and its length was measured under a constant load (2 g). Representative whole gut specimens (0.5 cm length) were taken from the distal inflamed region and were fixed in 4% buffered formaldehyde for histological studies; equivalent colonic segments were also obtained from the non-colitic group. The remaining colonic tissue was subsequently sectioned in longitudinal fragments for biochemical determinations and RNA isolation.

Table 2. Scoring of disease activity index (DAI).

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

DAI value is the combined scores of weight loss, stool consistency, and rectal bleeding divided by 3. Adapted from Cooper et al., (1993).

3. Dextran sodium sulfate model of reactivated mouse colitis.

Female C57BL/6J mice (7-9 weeks old; approximately 20 g) obtained from Janvier (St Berthevin Cedex, France) were randomly assigned to different groups: non-colitic (n=10) and DSS colitic groups (n=140). As stated above, the colitis was induced by adding DSS (3% w/v) in the drinking water for a period of 5 days [544]. Then, colitic mice were divided in two groups of 60 animals each: control mice, which were given distilled water (200 µL), and minocycline treated group, which received orally 30 mg/kg per day of minocycline dissolved in of 200 µL of distilled water for seven days. At this time point, half of the mice from each group (control and minocycline-treated), received the probiotic *Escherichia coli* Nissle 1917 (O6:K5:H1) (Ardeypharm GmbH, Herdecke, Germany) at doses of 5 x 10^8 colony forming units (CFUs) per mouse (suspended in 200 µL of distilled water), until the end of the experiment. Fourteen days after, all colitic mice received a new cycle of DSS for other five days. Concurrently with DSS administration, all the mice that had received minocycline previously were orally, and daily, treated again with the same doses of antibiotic. Mice from the different groups (n=10) were sacrificed 7, 14, 19 and 26 days after the removal of the first cycle of DSS (Figure 10).

Animal body weight, the presence of gross blood in the faeces and stool consistency were evaluated daily and an average daily DAI was calculated. Once the animals were sacrificed, the colon was removed, the contents were collected aseptically and the organ was weighed and measured under a constant load (2 g). As described before, representative whole gut specimens were taken for the histological studies and the remaining colonic tissue was subsequently sectioned in different longitudinal fragments for RNA isolation.

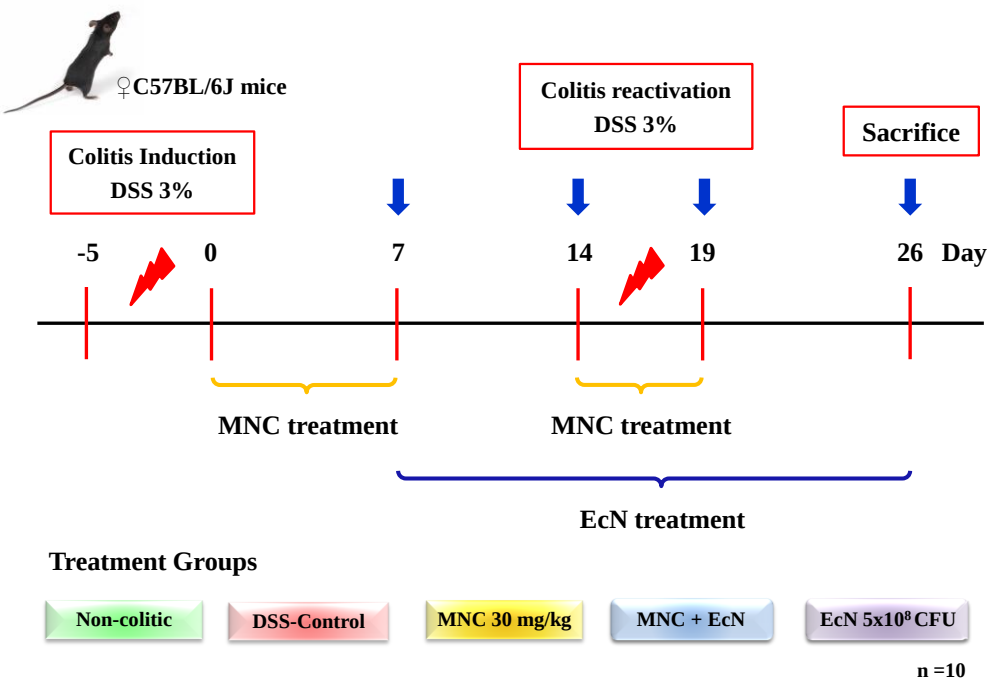

Figure 10. Experimental design in the DSS model of reactivated mouse colitis.

Minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN)

4. Evaluation of the intestinal inflammatory process.

4.1. Histological studies.

Colonic cross-sections were selected and embedded in paraffin. Full-thickness sections of 5 μm were obtained at different levels and stained with haematoxylin and eosin. The histological damage was evaluated by a pathologist observer, who was blinded to the experimental groups, according to the criteria described in table 3 (modified from Camuesco et al. (2004) [70]).

4.2. Biochemical determinations in colonic tissue.

- ***Mieloperoxidase activity.***

Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al. (1984) [68]. Colonic specimens where homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0) and MPO activity in supernatant was measured and calculated from the absorbance (at 460 nm) changes that resulted from decomposition of H_2O_2 in the presence of O-dianisidine; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25ºC.

- ***Glutathione content.***

Total glutathione (GSH) content was quantified with the recycling assay described by Anderson (1985) [69] in which it is sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase (Boehringer Mannheim, Barcelona, Spain). The rate of 2-nitro-5-thiobenzoic acid formation is monitored at 412 nm and the glutathione present was evaluated by comparison of that result with a standard curve, and the results were expressed as nmol/g wet tissue.

Table 3. Scoring criteria of full-thickness distal colon sections.

Mucosal epithelium and lamina propia
Ulceration: none (0); mild surface (0-25%) (1); moderate (25-50%) (2); severe (50-75%) (3); extensive-full thickness (more 75%) (4).
Polymorphonuclear cell infiltrate
Mononuclear cell infiltrate and fibrosis
Edema and dilation of lacteals
Crypts
Mitotic Activity: lower third (0); mild mid third (1); moderate mid third (2); upper third (3)
Dilations
Goblet cell depletion
Submucosa
Polymorphonuclear cell infiltrate
Mononuclear cell infiltrate
Edema
Vascularity
Muscular layer
Polymorphonuclear cell infiltrate
Mononuclear cell infiltrate
Edema
Infiltration in the serosa

Scoring scale: 0, none; 1 slight; 2, mild; 3, moderate; 4, severe. Maximum score: 59. Adapted from Camuesco et al., (2004).

- ***Cytokine production.***

Colonic samples for TNFα, IL-1β and IL-6 determinations were immediately weighed, minced on an ice-cold plate and suspended (1:5 w/v) in a lysis buffer containing 20 mM HEPES (pH 7.5), 10 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 40 mM β-glycerophosphate, 2.5 mM magnesium chloride, 1% Igepal®, 1 mM dithiothreitol, 500μM phenylmethanesulfonyl fluoride, 1μg/mL aprotinin, 1μg/mL leupeptin, 1μg/mL iodoacetamide and 2 mM sodium orthovanadate. The tubes were placed in an orbital rotor (4ºC) for 20 min and centrifuged at 9,000 g for 10 min at 4ºC; the supernatants were frozen at -80ºC until assay. The cytokines were quantified by enzyme-linked immunoabsorbent assay (R&D Systems Europe, Abingdom, UK) and the results were expressed as pg/g wet tissue.

- ***Inducible nitric oxide synthase protein expression.***

The iNOS Western blot from tissue was performed as described by Comalada et al. (2005) [546]. Equal amounts of protein from tissue samples (150 μg) were separated on 7.5 % SDS-PAGE. iNOS antibody (Transduction Laboratories, BD Biosciences, Madrid, Spain) was used at a dilution of 1/3000. A primary antibody against β-actin was used as loading control. Peroxidase-conjugated anti-rabbit IgG were used as secondary antibodies. Then, ECL (Perkin Elmer™, Life Sciences, Boston, USA) detection was performed.

4.3. Analysis of gene expression in colonic tissue.

- ***Analysis of gene expression in rat colonic samples by RT-PCR.***

Total RNA from rat colonic samples was extracted using TRIzol® Reagent (Invitrogen Life Technologies), following the manufacturer's instructions, and RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000

Spectrophotometer. 2μg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). Semiquantitative polymerase chain reaction (PCR) was performed using specific primers (Table 4). Reverse transcriptase-PCR *(*RT-PCR) analysis was performed at cycles below 35 using primers for the housekeeping gene β-actin for comparative reference. PCR reaction was performed using Go Taq® DNA Polymerase (Promega) in accordance with the manufacturer's recommendations. The PCR mixtures were denatured at 95 ^{o}C for 3 min, followed by 22 to 35 cycles of denaturation at 94°C for 1 min, annealing at 55-60°C for 45 s and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were analysed by electrophoresis in a 1% agarose gel containing ethidium bromide.

Table 4. Primer sequences used in PCR assays in rat colonic tissue.

Gene	Sequence (5′–3′)	Annealing temperature (^{o}C)
IL-17	F: TGGACTCTGAGCCGCATTGA R: GACGCATGGCGGACAATAGA	60
IL-6	F: CTTCCCTACTTCACAAGTC R: CTCCATTAGGAGAGCATTG	60
MCP-1	F: CACTATGCAGGTCTCTGTCACG R: CTGGTCACTTCTACAGAAGTGC	61
CINC-1	F: GGCAGGGATTCACTTCAAGA R: GCCATCGGTGCAATCTATCT	60
ICAM-1	F: AGGTATCCATCCATCCCACA R: AGTGTCTCATTGCCACGGAG	60
MUC-2	F: GCTCAATCTCAGAAGGCGACAC R: CCAGATAACAATGATGCCAGAGC	59
TFF-3	F: ATGGAGACCAGAGCCTTCTG R: ACAGCCTTGTGCTGACTGTA	59
β-actin	F: AATCGTGCGTGACATCAAAG R: ATGCCACAGGATTCCATACC	60

- ***Analysis of gene expression in mouse colonic samples by RT-qPCR.***

Total RNA from colonic samples was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer and 2µg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK).

Real time quantitative PCR (qPCR) amplification and detection was performed on optical-grade 96-well plates in a 7500 real-time-PCR System (PE Applied Biosystems, CA, USA). Each reaction was composed of 25 µL of FastStart Universal SYBR Green Master (ROX) Mix (Roche Applied Science, Indianapolis, IN), each amplification primer at a concentration of 0.3 µmol/L, 25 ng of cDNA from the RT reaction and PCR-grade water up to a final volume of 50 µL.

The thermal cycling program consisted of an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at annealing temperature (55-62^{o}C). Fluorescence was measured at the end of the annealing period of each cycle to monitor the progress of amplification, and dissociation curves were added to confirm the specificity of the amplification signal in each case. To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. For each sample, both the housekeeping and target genes were amplified in triplicate and the mean was used for further calculations. The mRNA relative quantitation was done using the ΔΔCt method. The specific primers used are indicated in table 5.

Table 5. Primer sequences used in real-time qPCR assays in mouse colonic tissue.

Gene	Sequence (5′–3′)	Annealing temperature (°C)
TNFα	F: AACTAGTGGTGCCAGCCGAT R: CTTCACAGAGCAATGACTCC	56
IL-1β	F: TGATGAGAATGACCTGTTCT R: CTTCTTCAAAGATGAAGGAA	55
IL-2	F: CTTCAAGCTCCACTTCAAGCT R: CCATCTCCTCAGAAAGTCCACC	60
MIP-2	F: CAGTGAGCTGCGCTGTCCAATG R: CAGTTAGCCTTGCCTTTGTTCAG	62
MCP-1	F: AGCCAACTCTCACTGAAG R: TCTCCAGCCTACTCATTG	60
ICAM-1	F: GAGGAGGTGAATGTATAAGTTATG R: GGATGTGGAGGAGCAGAG	60
iNOS	F: GGCAGAATGAGAAGCTGAGG R: GAAGGCGTAGCTGAACAAGG	55
MMP-9	F: TGGGGGGCAACTCGGC R: GGAATGATCTAAGCCCAG	60
MUC-3	F: CGTGGTCAACTGCGAGAATGG R: CGGCTCTATCTCTACGCTCTCC	62
ZO-1	F: GGGGCCTACACTGATCAAGA R: TGGAGATGAGGCTTCTGCTT	56
GAPDH	F: CATTGACCTCAACTACATGG R: GTGAGCTTCCCGTTCAGC	55

5. Microbiological analysis of the colonic contents.

- ***Analysis of rat colonic luminal contents.***

Luminal content samples were weighed, homogenized and serially diluted in sterile peptone water. Serial 10-fold dilutions of homogenates were plated on specific media for lactobacilli (MRS media, Oxoid) or bifidobacteria (MRS media supplemented with 0.5 mg/L dicloxacilin, 1 g/L LiCl and 0.5 g/L L-Cysteine hydrochloride) and incubated under anaerobic conditions for 24-48 h at 37ºC. Enterobacteria were also determined by using specific Count Plates Petrifilm (3M, St. Paul, MN). After incubation, the final count of colonies was reported as $\log_{10}$ CFUs per gram of material.

- ***Analysis of mouse colonic luminal contents.***

The bacterial DNA present in colonic content samples was characterized using real time qPCR as reported previously [547] . For DNA extraction, samples were diluted 1:10 (w/v) in PBS. DNA was extracted from 2 mL of the dilution using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 20 µL of buffer AE (provided in the kit), and the purified DNA extracts were stored at -20ºC. For bacterial DNA analysis, a series of genus-specific primer pairs were used (Table 6). PCR amplification and detection was performed as described above. In this case, each reaction mixture (50 µL) was composed of 25 µL of FastStart Universal SYBR Green Master (ROX) Mix (Roche Applied Science, Indianapolis, IN), 0.5 µL of each specific primer at a concentration of 30 µM and 4 µL of template DNA. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from pure cultures with a bacterial population ranging from 2 to 9 $\log_{10}$ CFUs, as determined by plate counts. One strain belonging to each of the bacterial genera or groups targeted in this study was used to construct the standard curve. More specifically, the strains from which the DNA was extracted

were the following: *Bifidobacterium longum* CECT 4551, *Clostridium* coccoides DSMZ 933, *Bacteroides fragilis* DSMZ 2151 and *Lactobacillus salivarius* CECT 2197. They were obtained from the Spanish Collection of Type Cultures (CECT) or from the German Collection of Microorganisms and Cell Cultures (DSMZ).

Table 6. Primer sequences used for microbiological analysis in real-time qPCR assays in the colonic contents.

Target bacterial group	Sequence (5′–3′)	Annealing temperature (°C)
Bacteroides group	g-Bfra-F:ATAGCCTTTCGAAAGRAAGAT g-Bfra-R: CCAGTATCAACTGCAATTTTA	50
Clostridium cluster XIVa–XIVb	g-Ccoc-F: AAATGACGGTACCTGACTAA g-Ccoc-R: TTTGAGTTTCATTCTTGCGAA	50
Bifidobacterium group	g-Bifid-F: CTCCTGGAAACGGGTGG g-Bifid-R: GTGTTCTTCCCGATATCTACA	50
Lactobacillus group	Lab 159: GGAAACAG(A/G)TGCTAATACCG Lab 677: CACCGCTACACATGGAG	61

6. Antibiotic sensitivity test.

Escherichia coli Nissle 1917 (O6:K5:H1) sensitivity to minocycline was tested as follows. *E.coli* was grown overnight in Tryptic Soy Broth (TSB) (Oxoyd, Hampshire, United Kingdom) at 37 ^{o}C continuously agitated. Test tubes with different concentrations of minocycline ranging from 0.2 to 200 μM in TSB were inoculated with the fresh *E.coli* culture. They were incubated at 37 ^{o}C in continuous agitation and after 20 hours bacterial cell density was examined measuring optical density (OD) at 600 nm and minimum inhibitory concentration of antibiotic was determined.

7. Statistics.

All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and *post hoc* least significance tests. Differences between proportions were analysed with the chi-squared test. Non-parametric data (DAI values and histological score) were analyzed using the Mann-Whitney U-test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P<0.05$.

8. Reagents.

All chemicals, including the antibiotics minocycline, tetracycline and metronidazole, were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated.

Results

IN VITRO EFFECTS OF MINOCYCLINE IN CACO-2 AND RAW 264.7 CELLS

In order to characterise the involvement of the immunomodulatory properties of minocycline in its intestinal anti-inflammatory effect, we first checked its immunomodulatory activity in two *in vitro* models of cells involved in the intestinal immune response. The human colon adenocarcinoma cell line Caco-2 was used as a model of intestinal epithelial cells. Incubation of these cells with IL-1β induces the secretion of IL-8, a proinflammatory chemokine that is released by intestinal epithelial cells and increases inflammatory cells migration from the blood stream into the mucosa and submucosa during chronic IBD, enhancing intestinal tissue destruction [444]. The pre-treatment of these epithelial cells with minocycline resulted in a concentration-dependent reduction in IL-8 production, when compared with the control cells, whereas neither tetracycline nor metronidazole significantly affected it at the different concentrations assayed (Figure 11).

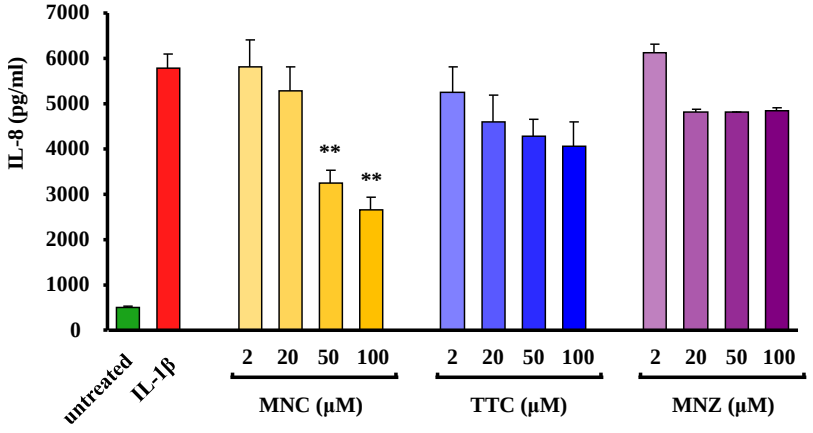

Figure 11. Effects of minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) on IL-8 production in Caco-2 cells stimulated with IL-1β (1 ng/ml). Data are expressed as means ± SEM. ** $P<0.01$ *vs.* untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

Previous studies have shown that minocycline decreases NO production in LPS stimulated murine macrophages [49], for this reason, we also evaluated the effect of different concentrations of minocycline on LPS-stimulated RAW 264.7 cells, a cell line of mouse macrophages. We found that minocycline pre-treatment inhibited LPS induced nitrite accumulation in the culture media, whereas neither tetracycline nor metronidazole could decrease it (Figure 12).

These results clearly evidenced a direct effect of minocycline on the immune response elicited by these cell types, which was independent from its antimicrobial effect.

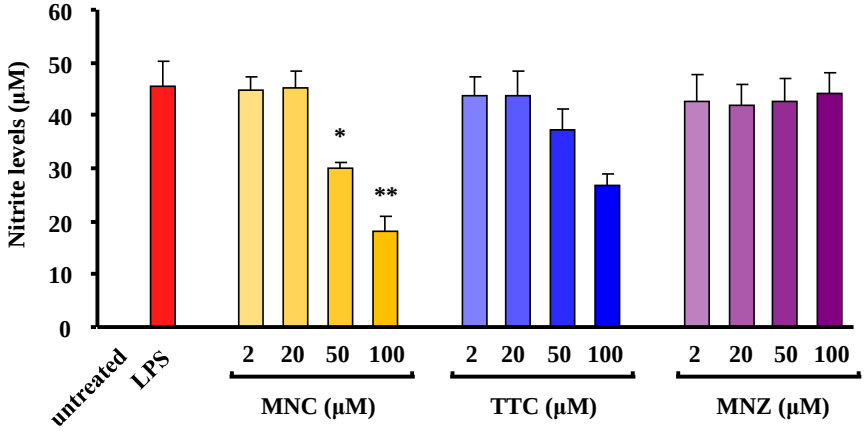

Figure 12. Effects of minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) on nitrite accumulation induced in RAW 264.7 cells after LPS (100 ng/ml) stimulation. Data are expressed as means ± SEM. * $P<0.05$ and ** $P<0.01$ *vs.* untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

CURATIVE EFFECT OF MINOCYCLINE IN ACUTE AND CHRONIC MODELS OF RODENT COLITIS

1. Minocycline anti-inflammatory effect on TBNS rat colitis.

To evaluate the potential intestinal anti-inflammatory effect of minocycline *in vivo*, we tested whether oral administration of minocycline, could ameliorate the intestinal damage in the TNBS model of rat colitis, a widely used model of intestinal inflammation that resembles CD pathology [548]. The antibiotics minocycline, tetracycline and metronidazole, the two latter used as controls, were administered starting the same day of the colitis induction, since previous studies have reported that antibiotic pre-treatment may reduce the development of experimental colitis mainly by modifying the intestine microbiota [23, 32, 33].

The intestine inflammatory process induced by TNBS was associated to a decrease in rat body weight when compared to non-colitic rats, most probably due to the anorexia and the presence of diarrhoea in the colitic animals. No significant differences were found among colitic groups in the body weight throughout the seven days period of the experiment (Figure 13).

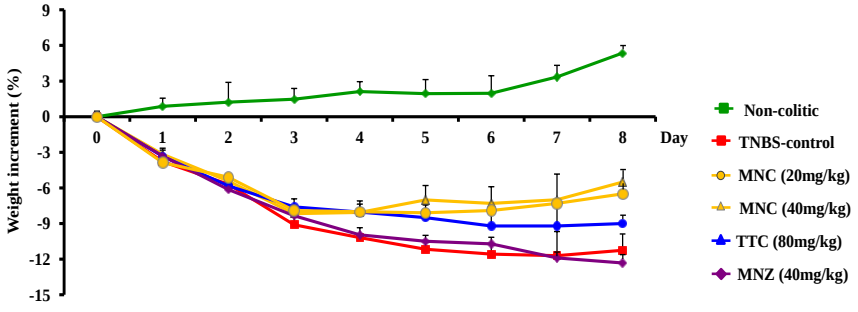

Figure 13. Body weight evolution from TNBS colitic rats treated with minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) following a curative treatment protocol. Data are expressed as median (range).

Macroscopic and microscopic analysis of the colonic samples showed that only minocycline, and not tetracycline or metronidazole, had an evident anti-inflammatory effect. Both doses of 20 and 40 mg/kg of minocycline significantly ameliorated the macroscopic colonic damage when compared to the colitic control group, since a significant reduction in the extension of inflamed/necrotic colonic tissue was observed, thus resulting in a significant reduction in damage score (Figure 14).

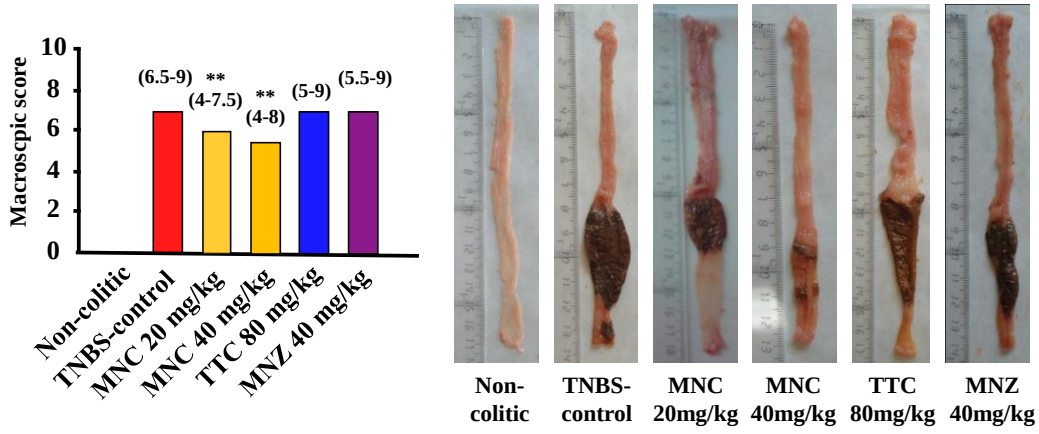

Figure 14. Colonic macroscopic damage score from TNBS colitic rats treated with minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) following a curative treatment protocol, according to the criteria described in table 1. Data are expressed as median (range); $^{**}P<0.01$ *vs.* TNBS control group. Representative colonic segments, showing the intestine anti-inflammatory effects of the treatments.

Microscopically, the histological studies confirmed this effect (Figure 15). Colonic samples from the TNBS control group revealed severe transmural disruption of the normal architecture of the colon, characterised by extensive ulceration and inflammation involving all the intestinal layers. The epithelial ulceration of the mucosa affected over 75% of the surface, and it was associated with diffuse leukocyte infiltration, mainly composed of neutrophils in the mucosa layer and, to a lesser extent, lymphocytes in the submucosa.

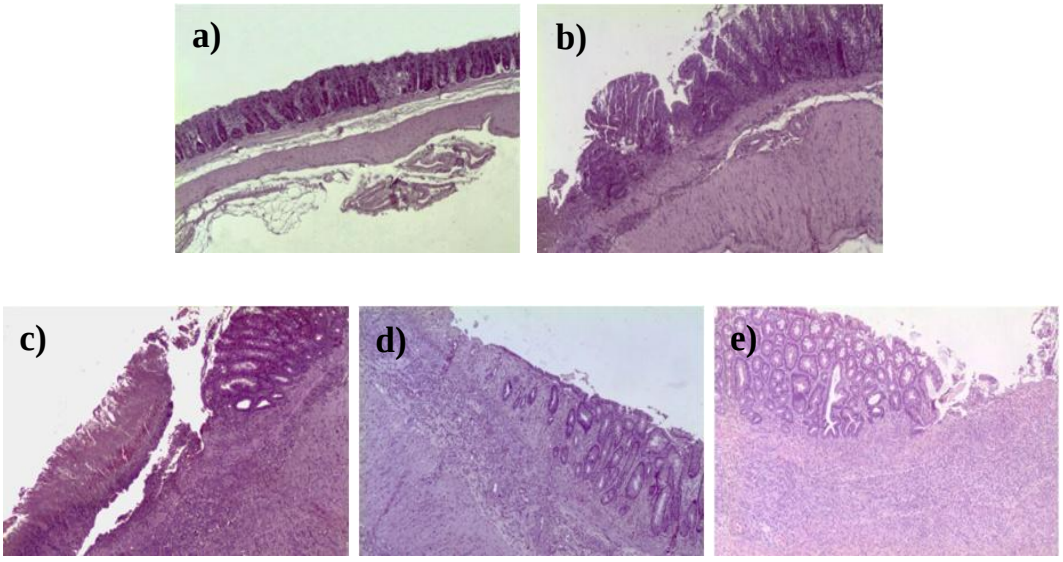

Figure 15. Histological sections of colonic mucosa stained with haematoxylin and eosin, showing the anti-inflammatory effect of the treatments: a) Non-colitic; b) TNBS-control; c) Minocycline (40 mg/kg); d) Tetracycline (80 mg/kg); e) Metronidazole (40 mg/kg) and f) microscopic score assigned according the criteria described in table 3. Data are expressed as means ± SEM. $^{*}P<0.05$ *vs.* TNBS control group.

The inflammatory process was also associated with crypt hyperplasia and dilation, and moderate to severe goblet cell depletion. However, histological analysis of the colonic specimens from rats treated with minocycline revealed a pronounced recovery with a significantly reduced score in comparison to untreated rats. The transmural involvement of the lesions was reduced and most of the samples showed a restoration of the epithelial cell layer. Only a maximum of 50% of the epithelium was affected in contrast to the extensive ulceration observed in non-treated animals. In addition, the goblet cells appeared replenished with their mucin content and dilated crypts were scarcely observed.

The improvement in colonic histology was accompanied by a reduction in the inflammatory infiltrate, which was slight to moderate with a patchy distribution, although neutrophils were also the predominant cell type. However, colonic samples from tetracycline- or metronidazole-treated groups showed similar degree of ulceration and goblet cell depletion to that observed in the TNBS control group.

The biochemical analysis revealed that minocycline significantly reduced the colonic MPO activity, a marker of neutrophil infiltration that was enhanced in the TNBS control group (Figure 16A), confirming the microscopic observations. However, tetracycline was also able to reduce this enzyme activity, although it showed no significant anti-inflammatory effect when macroscopically evaluated. In addition, a colonic depletion of the antioxidant peptide glutathione was observed in control colitic rats in comparison with healthy rats, which was partially counteracted by either minocycline or tetracycline administration, although the levels did not reach

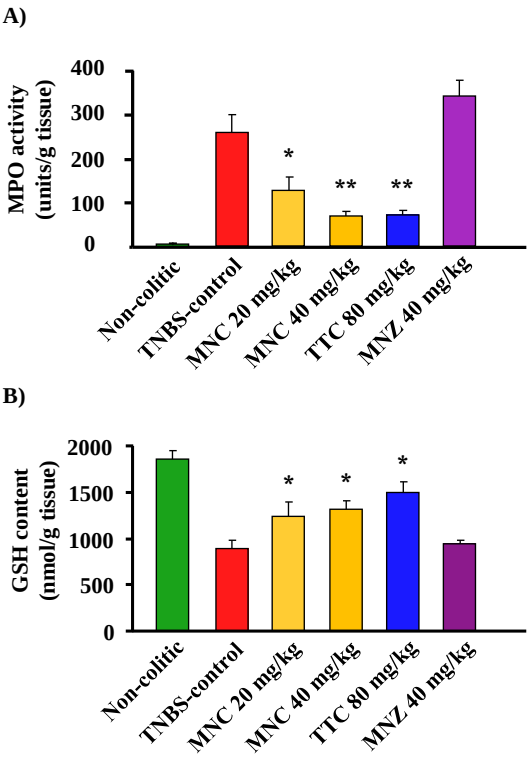

Figure 16. Effects of minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) in TNBS rat colitis following a curative treatment protocol. A) Colonic myeloperoxidase (MPO) activity and B) Colonic glutathione (GSH) content. Data are expressed as means ± SEM. $^{*}P<0.05$; $^{**}P<0.01$ *vs.* TNBS control group.

those obtained in healthy rats (Figure 16B). No significant differences were observed in the metronidazole-treated colitic rats either in colonic MPO activity (Figure 16A) or in glutathione content (Figure 16B), in comparison with the corresponding control colitic group.

The colonic inflammatory status was also characterised by increased levels of colonic TNFα and IL-1β, when quatified by ELISA, together with a higher colonic iNOS protein expression, analyzed by western blotting, in comparison with non-colitic animals. The treatment of colitic rats with minocycline for 7 days reduced the colonic levels of both proinflammatory cytokines ($P < 0.05$ vs. colitic control group) and colonic iNOS expression. Tetracycline also reduced TNFα and IL-1β, although in a lesser extent than minocycline ($P < 0.05$). Neither tetracycline nor metronidazole affected colonic iNOS expression (Figure 17).

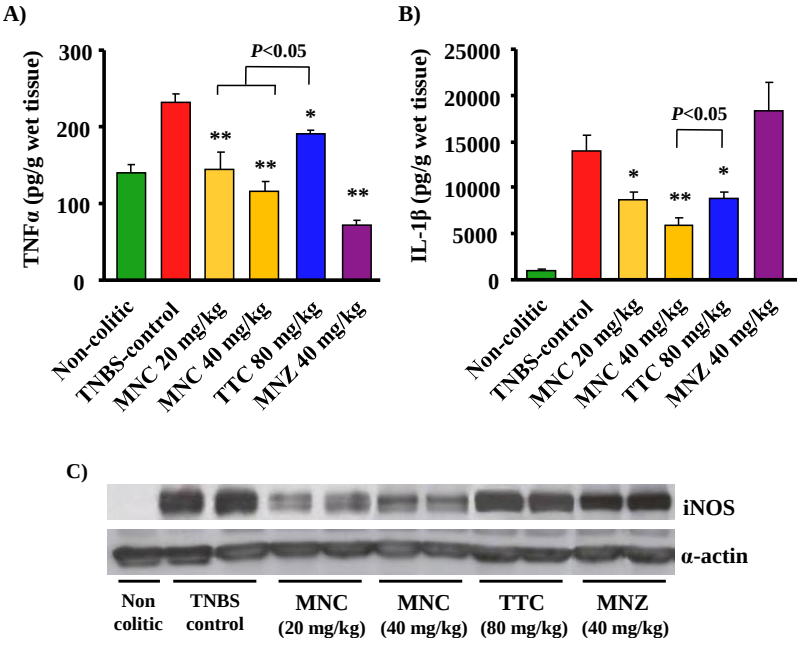

Figure 17. Effect of minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) on colonic A) TNFα and B) IL-1β production in TNBS colitis in rats quantified by ELISA (means ± SEM; *P<0.05 and ** P<0.01 *vs.* TNBS control group) and C) iNOS expression examined by western blot.

Other proinflammatory markers were analysed in the colonic samples to characterise the differences between the two tetracyclines assayed. For this purpose, IL-17 and IL-6 cytokines expression was studied by RT-PCR. The data revealed an up-regulation of the expression of these cytokines in control colitic rats. Whereas both tetracyclines exerted a similar inhibitory effect on IL-6 expression, the ability of minocycline to down-regulate IL-17 was again greater than that showed by tetracycline (Figure 18). IL-17 plays a critical role in the development of TNBS-induced colitis [76] and accordingly, the ability of minocycline to down-regulate its expression seems to be one of the responsible mechanisms for its greater anti-inflammatory effect in this model of colitis.

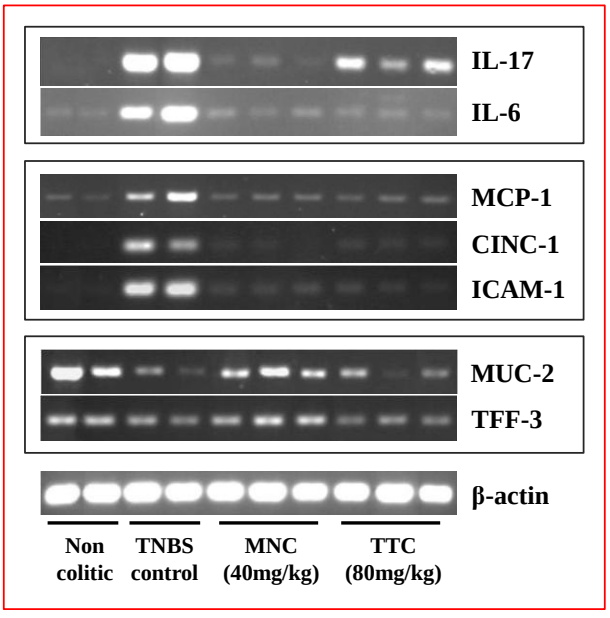

Figure 18. Effects of minocycline (MNC) and tetracycline (TTC) on gene expression of the cytokines IL-6 and IL-17, the chemokines MCP-1 and CINC-1, the adhesion molecule ICAM-1 and on the mediators of the colonic barrier function MUC-2 and TFF-3, analysed by RT-PCR.

The TNBS colonic inflammatory process was also associated with increased expressions of the chemokines monocyte chemotactic protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant-1 (CINC-1) as well as of the intercellular adhesion molecule-1 (ICAM-1). We could not find any difference between minocycline and tetracycline regarding these mediators, since both

antibiotics clearly down-regulated them in the same manner (Figure 18). When the colonic barrier function was studied, the colitic status involved an impaired expression of the mucin MUC-2 and trefoil factor-3 (TFF-3), thus revealing a defect in the colonic integrity. In this regard, only minocycline reversed the decline of colonic mucus thickness during colitis through an up-regulation of the expression of both MUC-2 and TFF-3 (Figure 18).

Colonic bacterial profile was modified in the inflammatory process. The non-pathogenic (lactobacilli and bifidobacteria) / potentially pathogenic (enterobacteria) bacterial ratio was reduced in the TNBS-control animals and remained altered 7 days after the TNBS instillation. Whereas minocycline-treated colitic rats showed a significantly increased ratio between non-pathogenic and potentially pathogenic bacteria compared to control colitic rats, tetracycline and metronidazole treatments only reduced enterobacteria counts, without increasing bifidobacteria and lactobacilli counts (Figure 19). This points out that both the immunomodulatory effects of minocycline and its ability to modulate the intestinal microbiota play an important role in the amelioration of the inflammatory process.

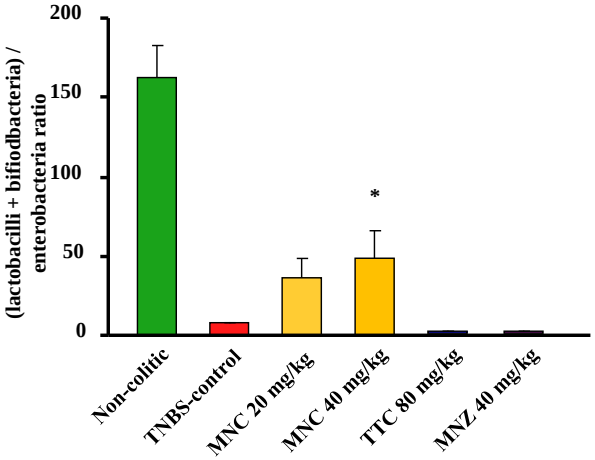

Figure 19. Effects of the antibiotics minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) on the bacterial profile in the colonic luminal contents in TNBS rat colitis when administered following a curative treatment protocol. Data are expressed as means ± SEM. **P*<0.05 *vs.* TNBS control group.

- ***Minocycline has no prophylactic effect in TNBS rat colitis.***

In order to study a possible preventive effect of minocycline the colitis development in TNBS rat colitis, this antibiotic was assayed following a prophylactic treatment, similarly to what has been previously described for other antibiotics on experimental colitis [23, 32, 33]. With this aim, rats were given minocycline, or the two control antibiotics, for 1 week before TNBS instillation and the administration continued until the sacrifice of the animals, i.e. 2 days after the colitis induction. In this experimental setting, minocycline could not prevent the development of the colitis (Figure 20A), even though it promoted a partial restoration of the non-pathogenic (lactobacilli and bifidobacteria) / potentially pathogenic (enterobacteria) bacterial ratio, which was reduced in the TNBS-control animals (Figure 20B). Furthermore, minocycline treatment did not significantly modify MPO activity or glutathione content in comparison with untreated colitic rats, thus confirming the lack of any anti-inflammatory effect (Figures 20C and D). However, metronidazole modulation of the intestinal microbiota seemed to be efficient in preventing the intestinal damage, as evidenced by a significantly decreased macroscopic damage score when compared with untreated colitic rats ($P < 0.05$) (Figure 20A). No beneficial effect was observed in the group of colitic rats treated with tetracycline (Figure 20A).

These results suggest that minocycline, only by modulating the intestinal microbiota, is not able to prevent intestinal inflammation, and therefore, it is the conjunction of both its antimicrobial and immunomodulatory properties what makes it an effective anti-inflammatory agent in rat colitis.

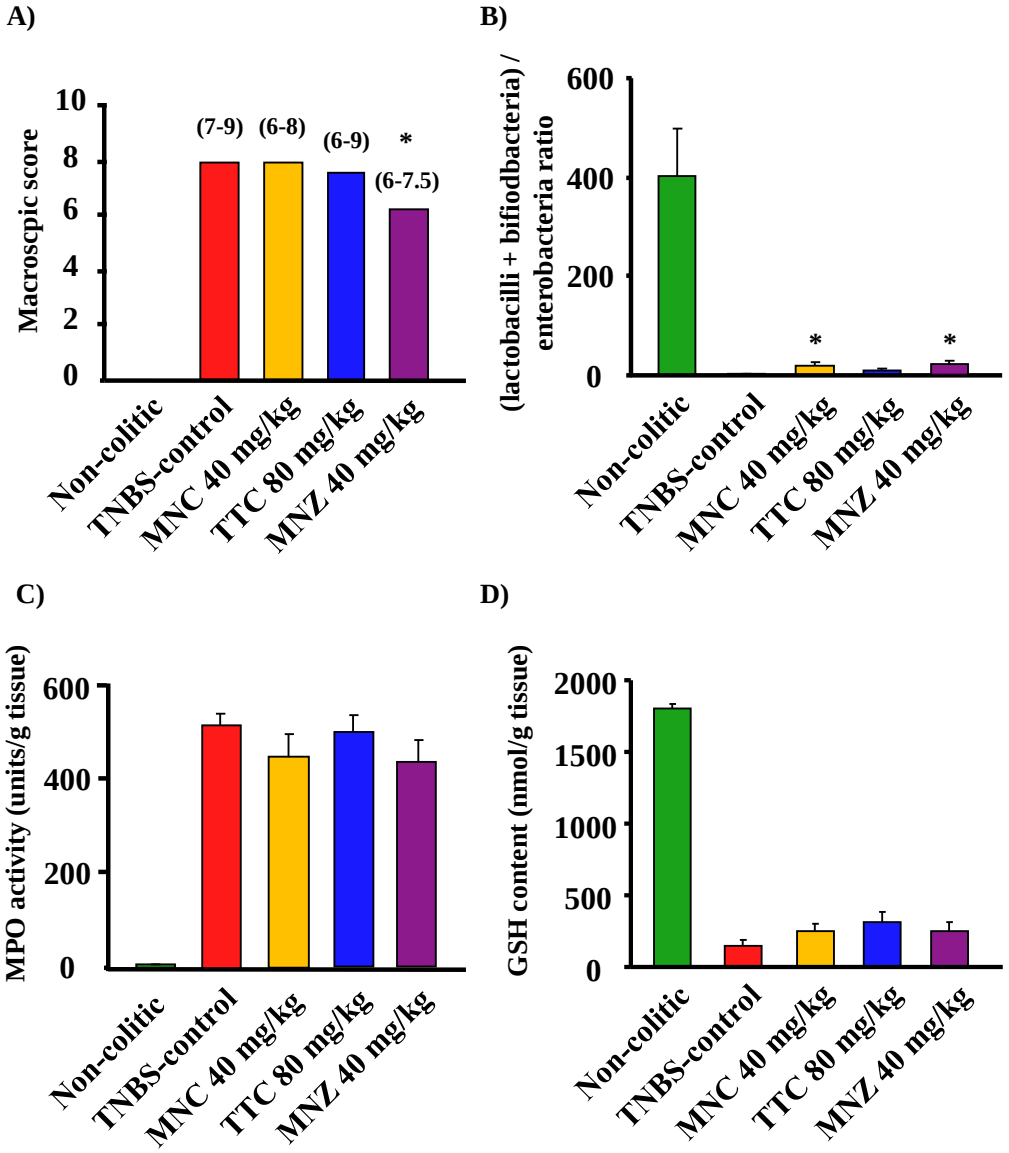

Figure 20. Effects of minocycline (MNC), tetracycline (TTC) and metronidazole (MNZ) in TNBS rat colitis following a preventive treatment protocol. A) Colonic macroscopic damage score, assigned for each rat according to the criteria described in table 1. Data are expressed as median (range). B) Bacterial profile. C) Colonic myeloperoxidase (MPO) activity. D) Colonic glutathione (GSH) content. Data are expressed as means ± SEM. **P*<0.05 *vs.* TNBS control group.

2. Effects of minocycline administration on DSS-induced mice colitis.

Once the anti-inflammatory effect of minocycline was confirmed in the acute model of TNBS-colitis in rats, we evaluated its activity in the chronic phase of the DSS model of colitis in mice, a well established model with resemblance to human UC [549]. For this purpose, the antibiotic was administered to colitic mice for 21 days after the removal of the DSS from the drinking water. The results revealed that oral minocycline treatment improved the recovery of DSS colitic mice, reducing body weight loss and disease activity index (DAI) during the time-course of the experiment in comparison to untreated colitic mice (Figure 21A). The macroscopic evaluation of the colonic segments confirmed this beneficial effect; minocycline-treated mice showed a significantly reduced colonic weight/length ratio in comparison with control mice (Figure 21B); a parameter that has been suggested to be directly correlated with the severity of the colonic damage in the DSS-induced colitis [550].

Microscopically, DSS-induced colitis was characterised by mucosa epithelial ulceration (typically affecting more than 50% of the surface), marked crypt hyperplasia accompanied by goblet cell depletion (Figure 21C). A chronic inflammatory cell infiltration into the lamina propria was observed and oedema was evidenced between the mucosa and muscularis layers of the intestine. In contrast, most of the samples of the minocycline treated group showed an almost full recovery of the inflammatory process. Mucosal architecture was preserved, only a few animals showed an inflammatory infiltrate of mononuclear cells in the lamina propria, and a minor oedema in the submucosa. The evaluation of the damage resulted in a significant reduction in the microscopic score compared with the untreated control group (Figure 21D).

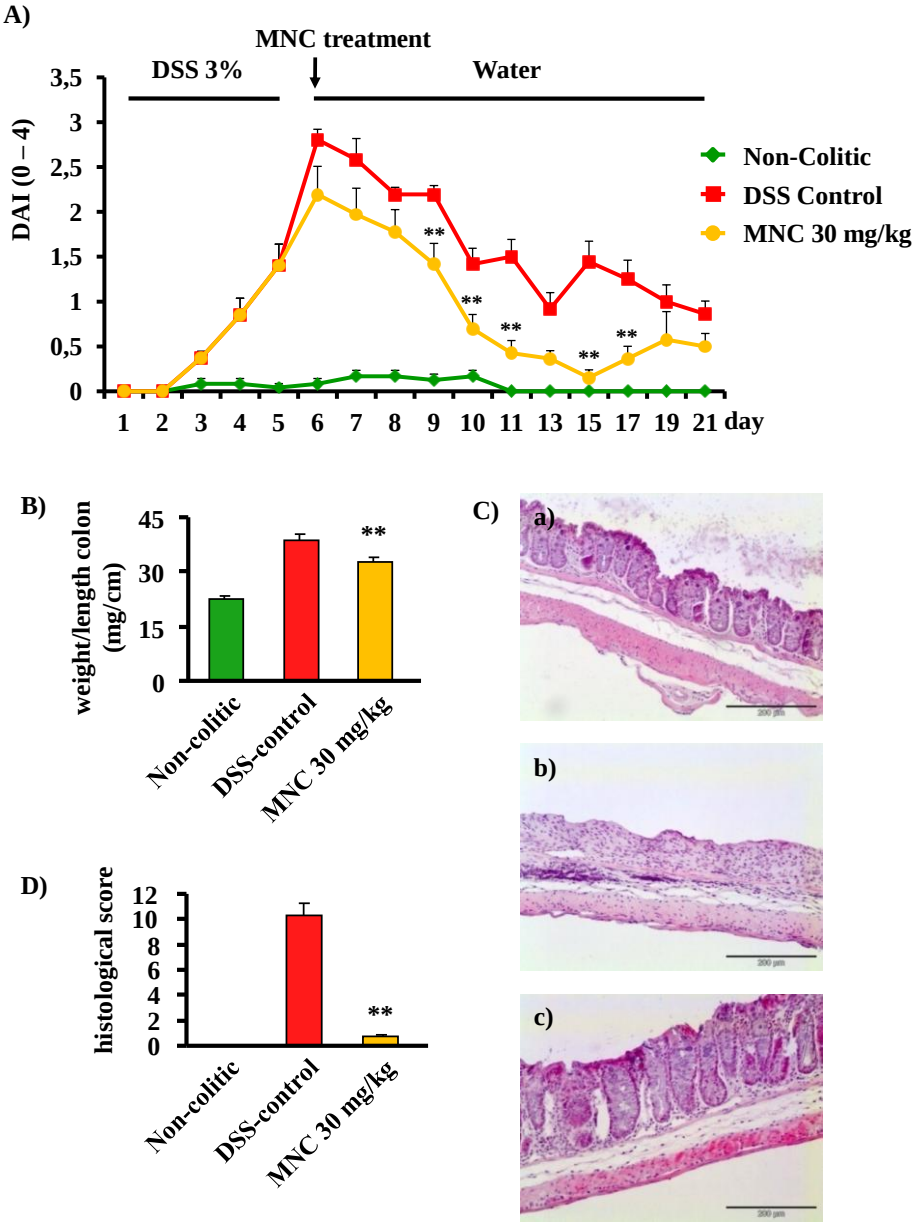

Figure 21. Effects of minocycline in DSS mice colitis. A) Disease activity index (DAI) values over the 21-day experimental period, based on the criteria proposed in table 2. * $P<0.05$ and ** $P<0.01$ *vs.* DSS control group. B) Colonic weight/length ratio, expressed as means ± SEM. C) Histological sections of colonic specimens stained with haematoxylin and eosin showing the recovery of the inflammatory process achieved after the antibiotic treatment. a) Non-colitic group; b) DSS-control group; c) Minocycline (MNC) (30 mg/kg) treated group. D) Histological score assigned according the criteria described in table 3; data are expressed as means ± SEM.

Furthermore, the biochemical analysis of the colonic segments confirmed the effects of minocycline on the immune response. The antibiotic treatment reduced the expression of the proinflammatory cytokines TNFα, IL-1β and IL-6, whose production was significantly increased in colitic mice in comparison with the non-colitic group (Figure 22).

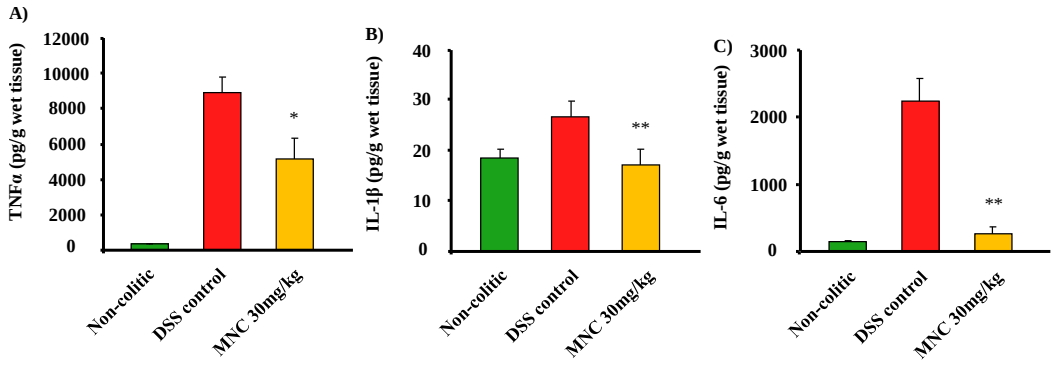

Figure 22. Effect of minocycline on colonic production of (A) TNFα, (B) IL-1β and (C) IL-6 in DSS mice colitis quantified by ELISA (n=10) (means ± SEM). * $P<0.05$ and ** $P<0.01$ *vs.* DSS control group.

ANTI-INFLAMMATORY EFFECTS OF THE ASSOCIATION OF MINOCYCLINE AND *E. COLI* NISSLE 1917 IN DSS-INDUCED REACTIVATED MICE COLITIS

Our second aim was to evaluate the potential of this association, mimicking the relapsing nature of human IBD and considering the therapeutic restrictions for long term antibiotic administration.

1. Evaluation of the colonic inflammatory status at day 7 after DSS removal.

As expected, the administration of 3% (w/v) DSS dissolved in the drinking water for 5 days to mice resulted in a progressive increase in DAI values, due to the body weight loss and the excretion of diarrheic/ bleeding faeces. Oral minocycline treatment promoted the recovery of DSS colitic mice, as evidenced by the significant decrease observed in the DAI during the 7 days following DSS administration in comparison with untreated colitic mice (Figure 23), mainly associated with an improvement in the weight loss, rather than in the amelioration of faeces consistency.

Macroscopically, the inflammatory process in the untreated control group was related to a significant shortening of the colonic length in comparison with healthy mice (Figure 24), but this was not evidenced in minocycline-treated animals at this time point.

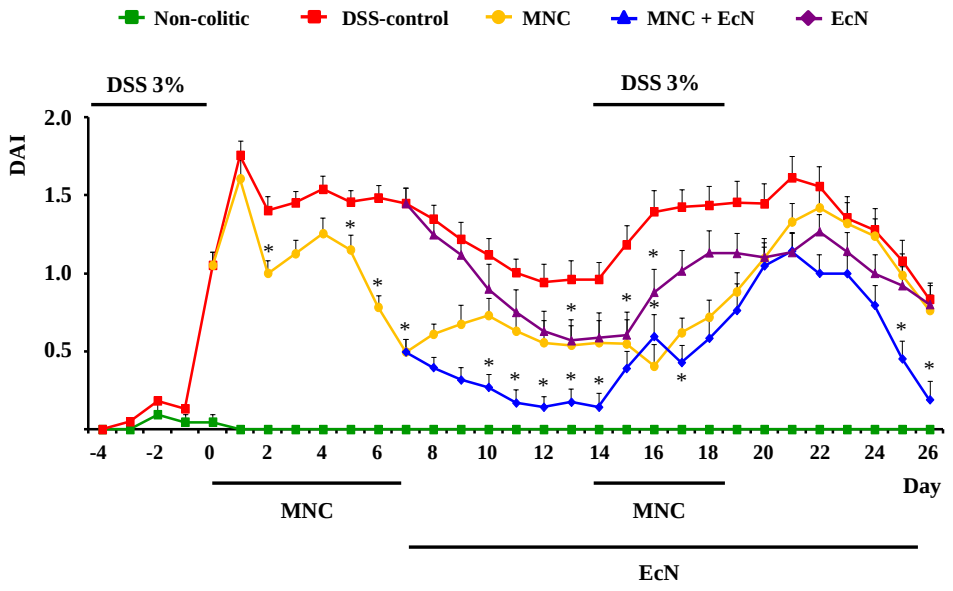

Figure 23. Effect of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) on Disease Activity Index (DAI) values in DSS mice colitis over the 31-day experimental period, based on the criteria proposed in table 2.*P<0.05 vs. DSS control group.

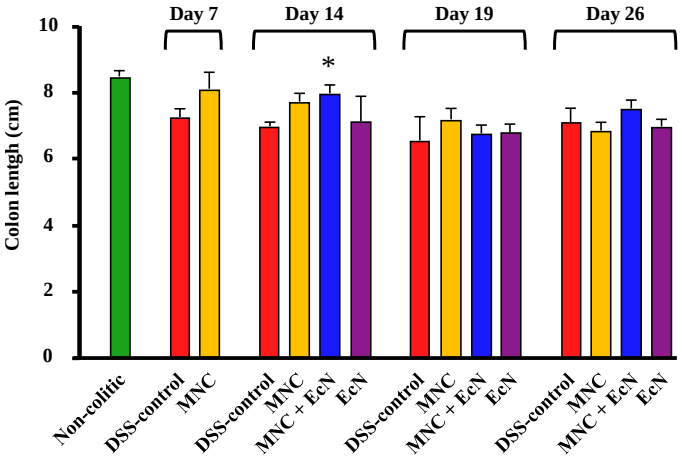

Figure 24. Effect of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) on colonic length of DSS colitic mice at the different time points. *P<0.05 vs. DSS control group; all colitic groups significantly differ from non-colitic group, except MNC at day 7 and MNC + EcN at day 14.

Histologically, 7 days after DSS removal, the colonic specimens from control colitic mice were characterized by mucosa epithelial ulceration, that typically affected more than 75% of the surface, and marked crypt hyperplasia with goblet cell depletion. A chronic inflammatory cell infiltration into the lamina propria was also observed, and oedema was evidenced between the mucosa and muscularis layers of the intestine. The microscopic score assigned, expressed as median (range) was 17 (13–20). Minocycline-treated colitic mice showed a significant improvement of the altered colonic histology associated with the inflammatory process, the mucosal epithelium appeared restored, and there was a lower inflammatory infiltrate of mononuclear cells in the lamina propria and slight edema in the submucosa. This resulted in a significant reduction in the microscopic score compared with the untreated control group, showing a value of 9 (5–10) ($P < 0.05$ vs. colitic control) (Figure 25).

The qPCR analysis of the mRNA expression of different inflammatory markers in the colonic segments also corroborated the intestinal anti-inflammatory effect exerted by minocycline at this time point. The colonic inflammation induced by DSS was characterized by an increased expression of the proinflammatory cytokines TNFα, IL-1β and IL-2, the chemokines MIP-2 and MCP-1, the adhesion molecule ICAM-1 and the enzymes iNOS and MMP-9, as well as by decreased expression of some biochemical markers of the epithelial integrity, including MUC-3 and zona occludens-1 (ZO-1) (Figure 26). Mice treated with minocycline showed a significant restoration of the expression of the different markers when compared with the untreated colitic group (Figure 26).

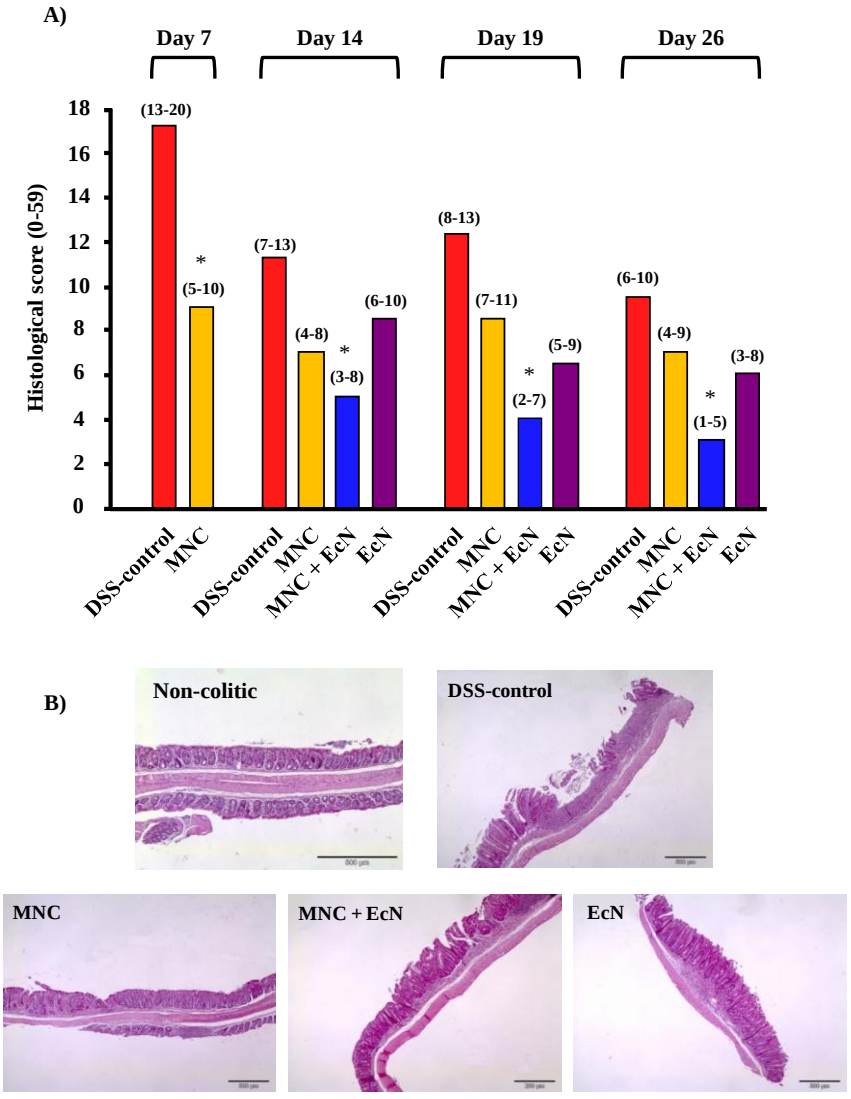

Figure 25. Histological analysis of the colonic segments from DSS colitic mice treated with minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) at the different time points. (A) Microscopic score assigned according the criteria described in table 3; data are expressed as median (range); *P<0.05 vs. DSS control group. (B) Representative histological sections of colonic segments stained with hematoxylin and eosin, showing the intestine anti-inflammatory effect of the treatments at day 26.

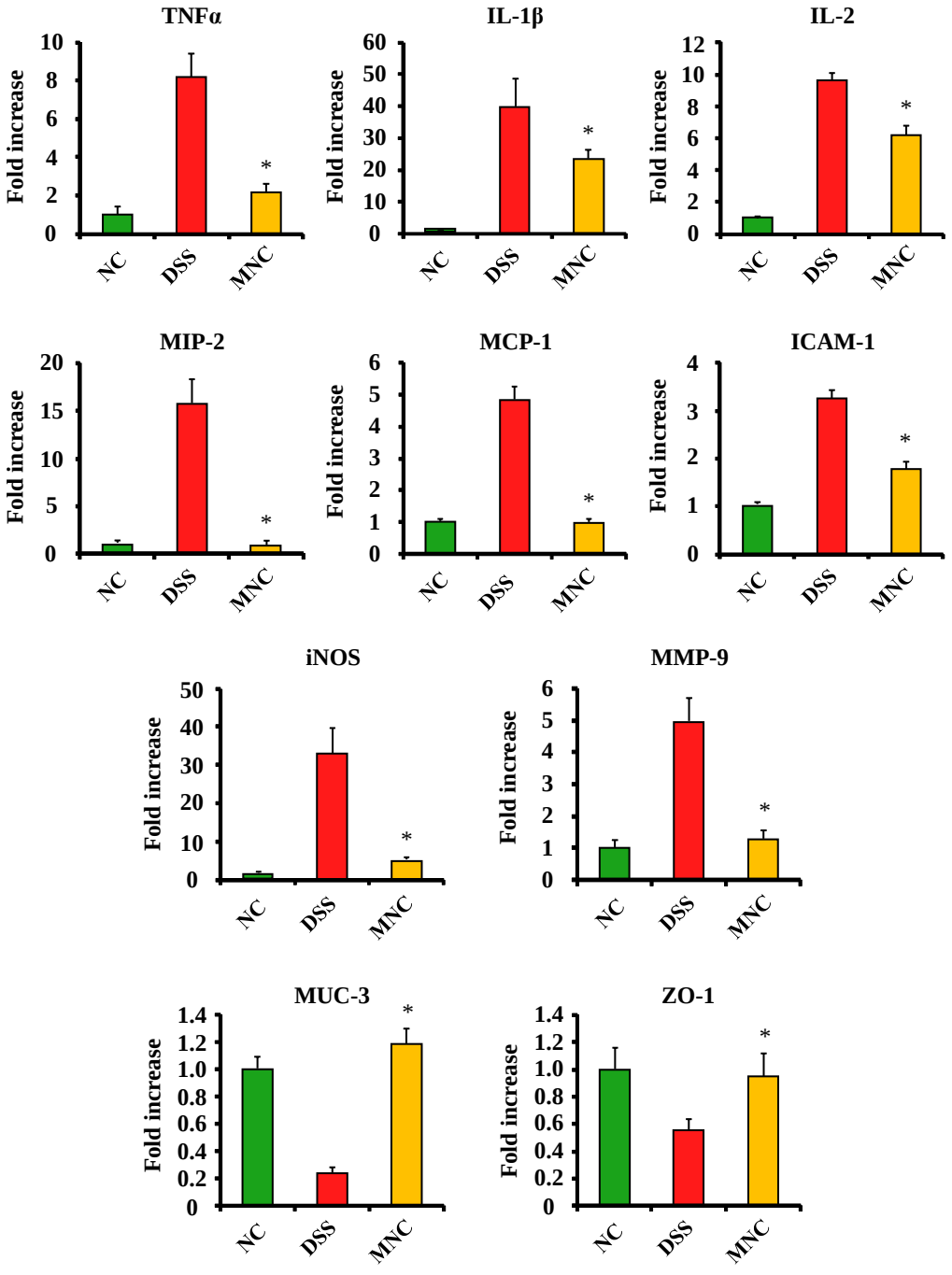

Figure 26. Biochemical evaluation of the effects of minocycline (MNC) after 7 days of treatment; mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; *P<0.05 *vs.* DSS control group.

In addition, the microbiota composition of the colonic contents was modified as a result of the inflammatory process. The ratio between non-pathogenic (lactobacilli and bifidobacteria) and potentially pathogenic (bacteroides and clostridia) bacteria was reduced in the DSS-control animals when compared with non-colitic mice. The beneficial effect observed in minocycline-treated colitic mice was associated with a significant increase in this ratio in comparison with untreated control mice (Figure 27).

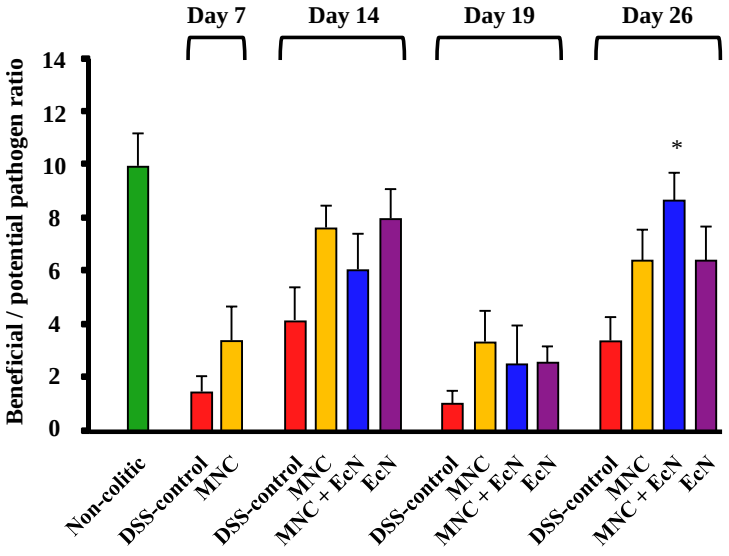

Figure 27. Effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) on the bacterial profile of the colonic contents from DSS colitic mice. Data are expressed as means ± SEM of the ratio between non-pathogenic (lactobacilli and bifidobacteria) and potentially pathogenic (bacteroides and clostridia). *P<0.05 *vs.* DSS control group.

2. Evaluation of the colonic inflammatory status at day 14 after DSS removal.

Seven days after DSS removal, the administration of minocycline was interrupted and half of the mice from both colitic groups (treated or not with the antibiotic) received a daily dose of the probiotic *E. coli* Nissle 1917 until the end of the experiment, in an attempt to maintain the ameliorated status achieved after minocycline treatment. DAI time-course evaluation during the following 7 days revealed that probiotic administration improved the recovery of the colitic mice when compared with DAI values showed by those mice without probiotic treatment, that remained constantly high (Figure 23). During this period, in addition to a reduction in the body weight loss, probiotic supplementation to minocycline treatment did have a positive impact on faeces consistency. When the colonic length was measured, only the group that received the antibiotic followed by the probiotic showed no statistical differences with healthy mice as well as a significant increase in comparison with untreated control mice (Figure 24).

The histological study confirmed the beneficial effect of the probiotic in colitic mice 14 days after DSS removal. Although in the control colitic mice the colonic damage was partially recovered, the intestine segments still showed ulceration of the mucosa affecting 50% of the surface. The inflammatory infiltrate was slight to moderate, mainly composed by mononuclear cells, and there were a moderate crypt hyperplasia and goblet cell depletion. The microscopic score value assigned to these mice was 11 (7–13). *E. coli* Nissle 1917 administration improved the recovery of the colonic tissue in all colitic mice, treated or not with minocycline. The mucosal layer appeared almost completely preserved, with goblet cells full of mucin content and only a slight crypt hyperplasia. It is remarkable that those mice previously treated with the antibiotic showed a lower microscopic score (5 (3–8)) than those without minocycline treatment (8.5 (6–10))

($P < 0.05$). Of note, the group of colitic mice that only received minocycline also showed a lower microscopic score value (7 (4–8)) than the control mice ($P < 0.05$) (Figure 25). No statistical differences were observed among the different treatments at this time point.

When the biochemical markers were analyzed, the expression of IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS and MMP-9 still remained increased in control colitic mice when compared with healthy mice, whereas MUC-3 and ZO-1 expressions persisted reduced (Figure 28). In this regard, the association of minocycline and *E. coli* Nissle 1917 was the most effective of the treatments assayed, since the reduction in the expression of IL-1β, IL-2, MIP-2, MCP-1, ICAM-1 and iNOS was greater than in the groups of mice that received a single treatment. Similarly, the expression of ZO-1 was significantly increased when compared with the other colitic mice, without showing differences with the non-colitic group. However, no significant modifications were observed among the three treated groups when MMP-9 was considered (Figure 28).

When the colonic microbiota was analyzed, the beneficial/potential pathogen bacteria ratio was still significantly reduced in control colitic mice in comparison with the non-colitic group. All treated groups revealed an increase in this ratio; and, although the values were not statistically different from the untreated colitic control group, no significant differences were either observed when compared with healthy mice (Figure 27).

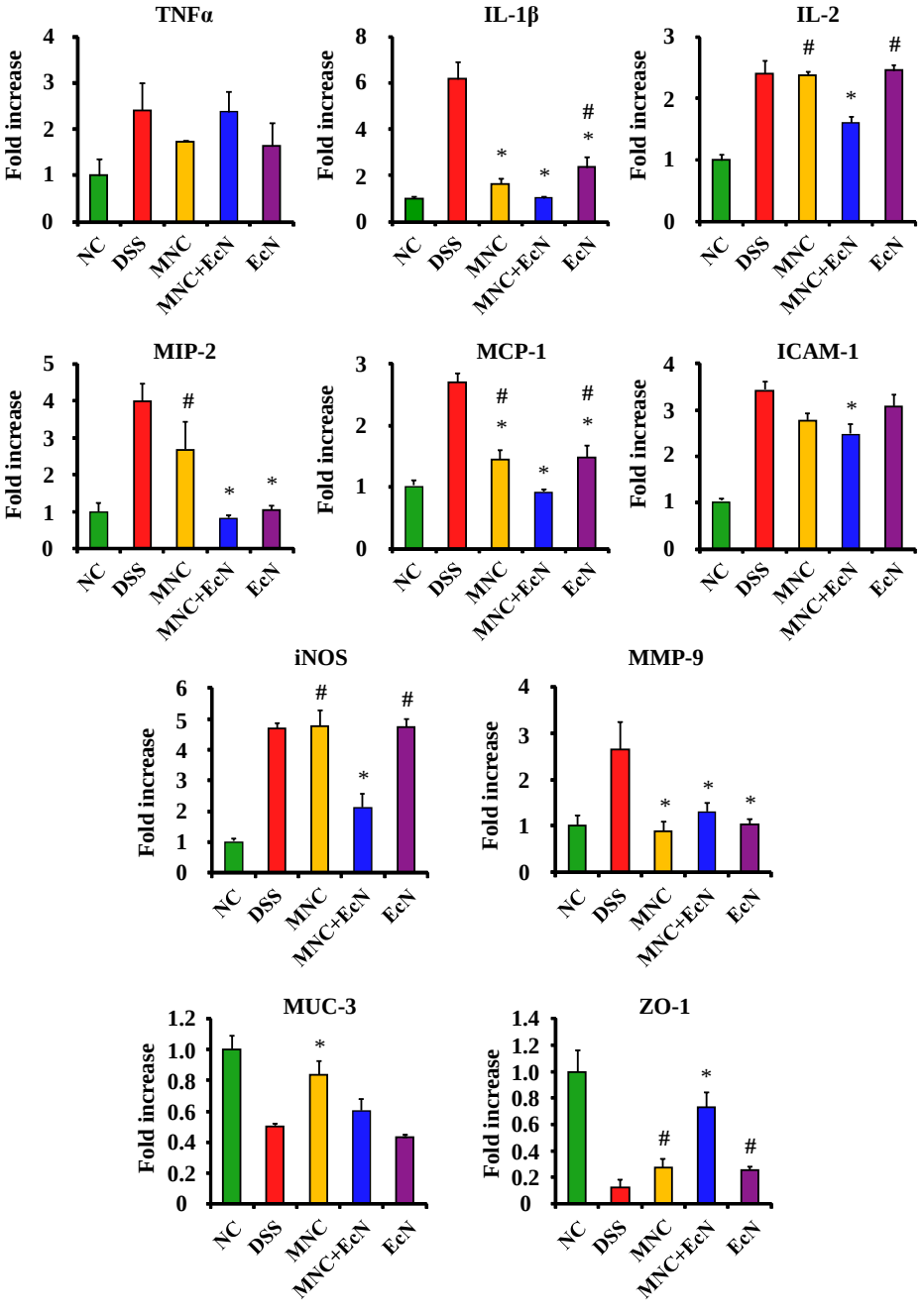

Figure 28. Biochemical evaluation of the effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) at day 14; mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; *P<0.05 *vs.* DSS control group, # P<0.05 *vs.* MNC + EcN treated group.

3. Evaluation of the colonic inflammatory status after colitis reactivation.

The exacerbation of the intestine inflammatory process after the second cycle of DSS was evidenced by a progressive increase in the DAI values in all groups, although it was more moderate than that obtained during the first colitis onset. The mice previously treated with minocycline again received the antibiotic during the second exposure to DSS. All treated mice showed lower DAI values when compared with untreated colitic mice, being these differences more evident in those treated with the antibiotic than in the mice that only received the probiotic (Figure 23). When the mice were sacrificed at the end of this period, the histological evaluation showed no significant differences between those mice which received either minocycline or the probiotic alone when compared with control colitic group. However, the combined treatment improved colonic histology as evidenced by the lower histological damage score obtained (Figure 25).

As expected, the colitis reactivation was associated with an increased expression of all the proinflammatory makers assayed, together with a decreased expression of MUC-3 and ZO-1 (Figure 29). The combination of minocycline and the probiotic resulted to be again the most effective treatment, since it decreased all the proinflammatory mediators studied, whereas individual treatments were only able to significantly modify some of them. Regarding the expression of the mediators involved in the intestine barrier integrity, the concurrent administration of the antibiotic and the probiotic significantly increased colonic ZO-1 expression in comparison with untreated colitic mice. However, none of the treatments significantly modified colonic MUC-3 expression (Figure 29).

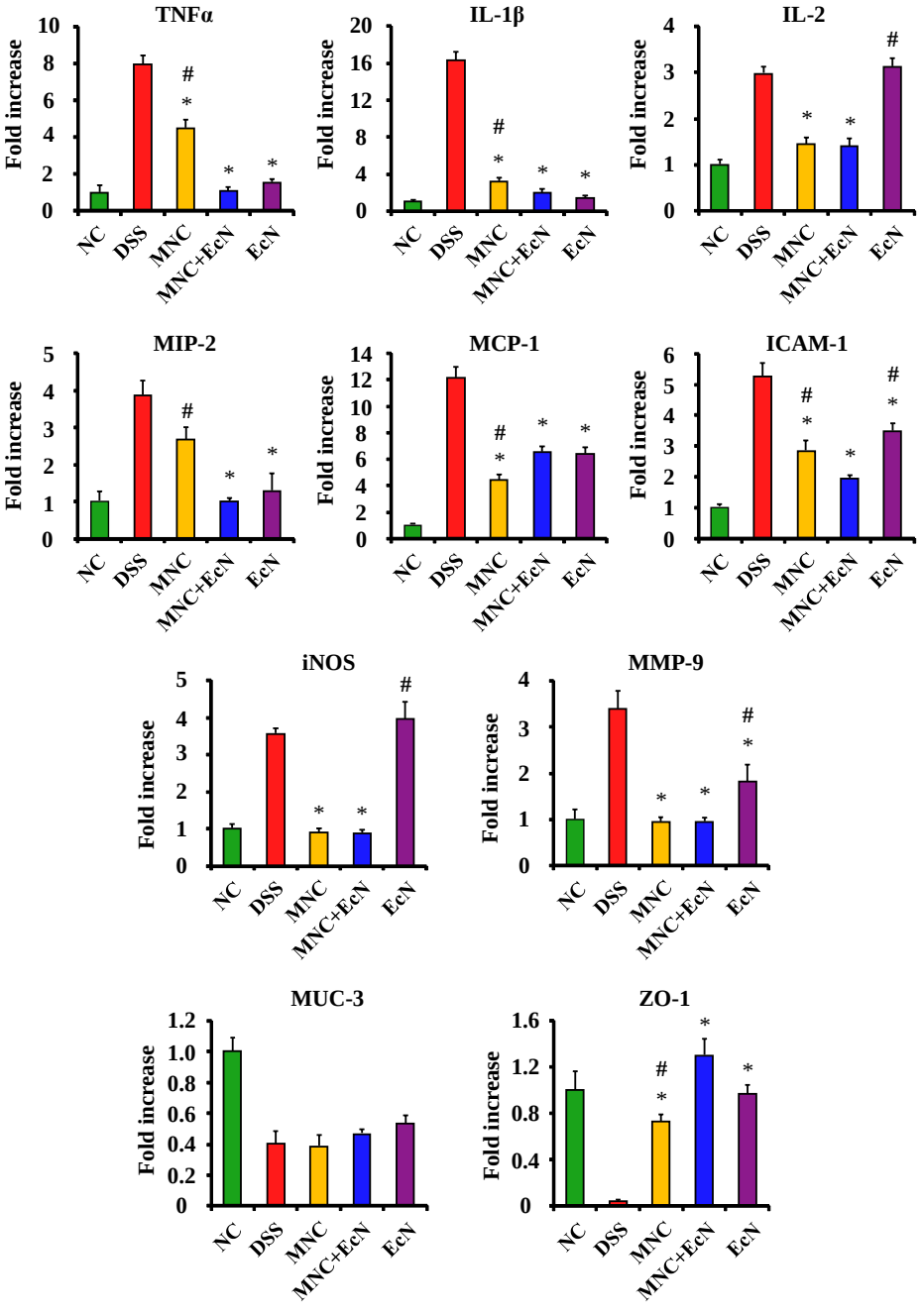

Figure 29. Biochemical evaluation of the effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) after colitis reactivation (day 19); mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; *P<0.05 *vs.* DSS control group, # P<0.05 *vs.* MNC + EcN treated group.

Furthermore, the reactivation of the colonic inflammatory process was associated with an alteration in the microbiota composition. However, the administration of either the antibiotic, the probiotic or their combination, partially restored the non-pathogenic/potential pathogenic ratio (Figure 27).

After these 5 days, minocycline treatment was stopped, and *E. coli* Nissle 1917 administration continued until the sacrifice of the mice, 7 days later, i.e. 31 days after starting the experiments, being the colonic inflammatory status evaluated once more. After this period, the mice that received the combination of treatments throughout the study recovered almost completely. Their DAI evolution showed significantly lower values than the other colitic mice, as a result of the amelioration of both body weight loss and faeces consistency, and no significant differences were seen in comparison with the healthy mice at the end of the study (Figure 23). Microscopically, these animals showed the lowest histological score (Figure 25) and the qPCR analysis revealed that the administration of minocycline and *E. coli* Nissle 1917 significantly decreased the expression of the proinflammatory mediators studied (TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1 MMP-9 and iNOS), while increasing MUC-3 and ZO-1 expressions (Figure 30). Finally, the ratio between beneficial and potential pathogen bacteria, that remained reduced in the control colitic group in comparison with healthy mice, was increased in all the treated groups, but even more in the group that received the combination of both treatments (Figure 27).

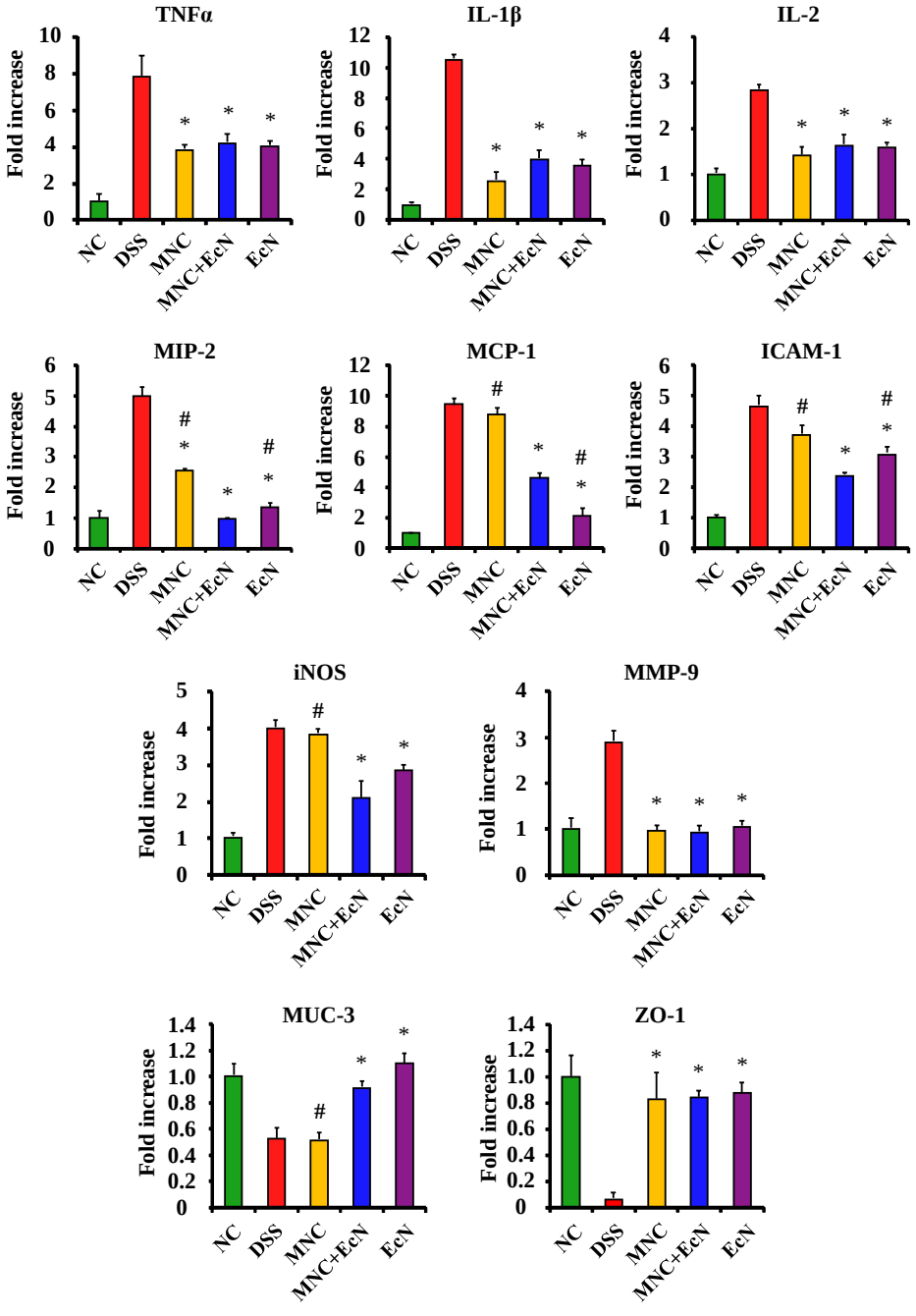

Figure 30. Biochemical evaluation of the effects of minocycline (MNC), *Escherichia coli* Nissle 1917 (EcN) and their association (MNC + EcN) at the end of the study (day 26); mRNA expression of TNFα, IL-1β, IL-2, MIP-2, MCP-1, ICAM-1, iNOS, MMP-9, MUC-3 and ZO-1 was quantified by real-time PCR, and fold increases are expressed as means ± SEM; *P<0.05 *vs.* DSS control group, # P<0.05 *vs.* MNC + EcN treated group.

4. Evaluation of *E. coli* Nissle 1917 sensitivity to minocycline.

Escherichia coli Nissle 1917 was sensitive to the antibiotic from concentrations of 2 µM (Figure 31).

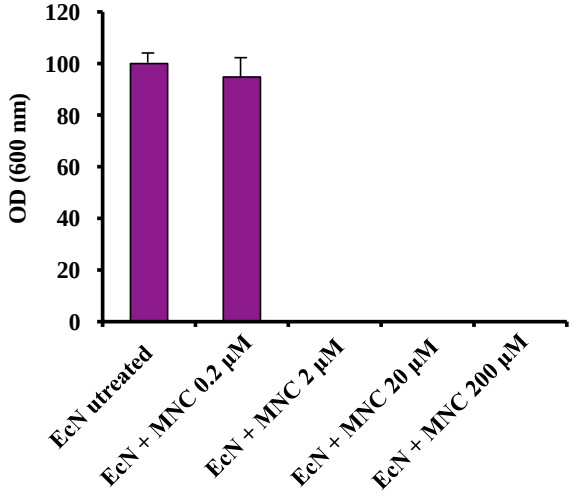

Figure 31. Escherichia coli Nissle 1917 (EcN) sensitivity to minocycline (MNC). Optical density (OD) measured at 600nm; data are expressed as means ± SEM.

Discussion

Minocycline is a broad-spectrum antibiotic that has been shown to display inmunomodulatory properties besides its antimicrobial effect [551]. Inflammation importantly participates in host defenses against infections and injury, but it also contributes to the pathophysiology of many chronic diseases like inflammatory bowel disease (IBD). Common effector mechanisms of inflammation contribute to tissue injury, oxidative stress, remodeling of the extracellular matrix, angiogenesis, and fibrosis in diverse target tissues [552]. Therefore, it is unsurprising that minocycline, a compound with such pleiotropic effects, e.g. reduction in proinflammatory cytokine levels, inhibition of MMPs and iNOS, anti-angiogenic and anti-oxidant activity, and impairment of immune cells activation and migration, displayed potent anti-inflammatory effects in colitis experimental models.

Moreover, although the aetiology of IBD is not completely elucidated, recent studies have attributed a key role to the intestine microbiota in its pathogenesis. However, no specific microorganism has been clearly involved as a causative agent of this pathology; most probably, this intestinal condition may be due to an aberrant immunological reaction against an unknown antigen from the intestinal lumen, most probably from the intestine microbiota, in a susceptible host [56, 74, 553], and/or due to an altered luminal microbiota composition leading to dysbiosis [554, 555].

Accordingly, and based on the pathogenic role of these two factors, either the exacerbated immune response in the gut or the dysbiosis in the intestinal lumen can be considered as appropriate targets for the management of IBD. In fact, it has been previously reported that agents that reduce gut bacterial load or block inflammatory cascades have potential in the prevention and treatment of IBD [517, 556]. On one hand, conventional IBD treatments including corticosteroids, salicylates and immunosuppressants, as well as the more recent biological

therapies (infliximab and adalimumab) are used with the aim of controlling the inflammatory events that drive the disease [517]. On the other hand, antibiotic treatment, mainly metronidazole and ciprofloxacin, has been long used in the treatment of human IBD, even though a bacterial etiologic agent has not been directly implicated in this intestinal condition [8, 10, 500]; and more recently, there has been a increasing interest in different therapies focused on restoring the balance in the altered microbiota, i.e. through the administration of probiotics, prebiotics or synbiotics [557, 558].

Therefore, the development of therapeutic strategies that combine an immunomodulatory activity and the ability to restore the luminal microbial balance in the intestine could be very interesting in the management of IBD. Although it was recently demonstrated that the anti-inflammatory effects of minocycline are independent of its bacteriostatic activity [89], the fact that it is able to combine these two effects, i.e. to modify intestinal microbiota and to modulate the immune response, confers an increased interest on minocycline regarding IBD treatment. This hypothesis was supported by the present study, since minocycline showed anti-inflammatory effect in two well established experimental models of IBD: the TNBS model in rats and the DSS-induced colitis in mice.

The TNBS-induced rat colitis is typified by colonic transmural damage caused by the hapten TNBS and it has been traditionally used as a model to study human CD [71, 548]. The anti-inflammatory effect of minocycline in this model was evidenced either histologically, associated with a reduction in the inflammatory infiltrate, and biochemically, by a reduction of the main proinflammatory cytokines involved in the pathogenesis of IBD. Of note, this beneficial effect was only exerted when the antibiotic was administered following a curative treatment protocol, promoting the recovery of the inflamed tissue, while no preventive effect was observed when it was administered for 7 days

before TNBS-colitis induction. These results point out that the anti-inflammatory effect exerted by minocycline in these experimental models cannot only be attributed to a modulation of the intestinal microbiota, as previously described for other antibiotics such as metronidazole, ciprofloxacin, vancomycin-imipenem or the association of neomycin and metronidazole, which were able to prevent the intestinal damage in experimental models of rodent colitis [32, 33].

In fact, although tetracycline and minocycline have a similar antimicrobial spectrum [98], tetracycline did not show the same efficacy in ameliorating the intestinal inflammation, as evidenced histologically (both macroscopically and microscopically), even though it affected some of the parameters studied. Therefore, minocycline must display additional effects that contribute to the amelioration of the inflammatory process. Furthermore, metronidazole, a broad-spectrum antibiotic which has been widely used in human IBD therapy [23], was indeed able to prevent TNBS-induced intestinal inflammation, similarly to what reported before [32, 33]. However, even though it modulated of the intestinal microbiota, it could not ameliorate the colitis when its administration started once it was established. In consequence, the distinctive immunomodulatory properties ascribed to minocycline [234], and corroborated in our *in vitro* studies, would definitively participate in its ability to reduce colonic inflammation.

Actually, as the results of the present study show, minocycline exerted differential effects from tetracycline when assayed *in vivo* that could explain its higher activity in reducing intestinal inflammation. As expected from the well known anti-oxidant activity of the tetracycline family [288], both minocycline and tetracycline decreased the colonic oxidative stress caused by the TNBS, as evidenced by decreased MPO activity and increased GSH content. In addition, both compounds decreased the expression of different chemotactic mediators like CINC-1, MCP-1 and ICAM-1 in the inflamed tissue, although the inflammatory infiltrate was lower in minocycline-treated rats. The inflamed

muscle layer is initially infiltrated by monocytes, which then differentiate and develop into muscularis-resident macrophages, that express MCP-1 for further recruitment of monocytes [559]. CINC, a chemokine analogous to human IL-8, is highly selective for neutrophil movement into the inflammatory sites [560]. It has been shown that the regulation of CINC production is critically involved in the control of neutrophil infiltration in intestinal damage [561]. The similar reduction in this mediators exerted by both tetracyclines *in vivo* could be derived from the reduction in the oxidative stress, rather than from a direct activity. However, the *in vitro* experiments performed revealed that minocycline did have a direct effect on chemokines production, as evidenced by the reduced the release of the chemokine IL-8 by the intestinal epithelial cells Caco-2, whereas tetracycline, did not significantly affect it. This seems to be crucial for the minocycline effect, and could partly explain the higher reduction in the inflammatory infiltrate observed in the colitic rats treated with minocycline than in those receiving tetracycline, as well as its greater efficacy in reducing colonic inflammation.

Other mechanisms may also explain the differences displayed by both tetracyclines. One of them is related to the ability of minocycline, and not shown by tetracycline, to inhibit colonic iNOS expression in the inflamed intestine, thus avoiding the deleterious effect that NO overproduction exerts on the colonic tissue in these intestinal conditions [562]. Sustained high NO production in the colon, especially when mediated by iNOS, is strongly associated with the progression of human IBD and experimental colitis [7, 563]. Moreover, blockade of iNOS expression using specific inhibitors ameliorates the severity of experimental colitis and iNOS knockout mice display a phenotype that is resistant to both DSS- and TNBS-induced colonic injury [75, 564 - 566]. In this study we found that minocycline ability to *in vivo* decrease iNOS protein was in agreement with the decrease NO production observed in our *in vitro* studies, supporting previous results obtained both *in vitro* and *in vivo* [43, 49, 125]. Our *in vitro* assays

showed that minocycline was able to inhibit NO production by RAW 264.7 murine macrophages, while tetracycline was not; confirming a direct effect of minocycline on macrophages, one of the main sources of NO in inflammatory conditions [70].

Although to a lesser extent, minocycline also showed greater efficacy than tetracycline in decreasing the production of TNFα and IL-1β, two of the main proinflammatory cytokines that contribute to intestinal inflammation [517]. Characteristically, innate immune responses are activated during the progression of IBD and up-regulate the expression of proinflammatory cytokines and chemokines. In our colitis model, minocycline not only suppressed colonic infiltration of neutrophils and macrophages, but also suppressed intestinal expression of these proinflammatory mediators.

In addition, minocycline displayed a higher protective role by blocking inflammatory processes downstream of the initial T cell activation. The differences observed between minocycline and tetracycline on the Th17 pathway could also justify the higher anti-inflammatory efficacy of minocycline. In the deregulated immune response that takes place in IBD, the Th17 pathway has recently received an increasing interest, since its related cytokines, IL-23 and IL-17, have been described to play a key role in the development of chronic intestinal inflammation [5, 567]. IL17 contributes to neutrophil migration, expansion and function, and it enhances DC maturation, T cell priming, and the production of inflammatory mediators from different cell types [568, 569]. Furthermore, IL-17 can synergize with other cytokines to stimulate the release of additional proinflammatory cytokines, thus being essential for the maintenance of the inflammatory response in the intestine [570, 571]. Experimental colitis has also been reported to be associated with an increased IL-17 production [572]; in fact, in TNBS rodent colitis, the infiltration of CD4 lymphocytes and neutrophils, mostly driven by a Th1/Th17 cytokine response, is one of the predominant features in the

initiation and perpetuation of the inflammatory process [76]. The ability of minocycline to down-regulate IL-17, whereas tetracycline showed no effect, seems to be significant for its intestinal anti-inflammatory effect.

Furthermore, the TNBS colitis was associated with a reduction in the expression of MUC-2, the primary constituent of the mucus layer in the colon [573], and TFF-3, a bioactive peptide that is involved in epithelial protection and repair [574], similarly to that described in the DSS experimental model of rat colitis [575]. Minocycline administration, but not tetracycline, reversed this reduction, thus preserving the mucus-secreting layer that covers the epithelium and acts as a physical barrier protecting its integrity. The impairment in the epithelial barrier function is considered as one of the initial steps in intestinal inflammation since it may facilitate the access of antigens from the intestinal lumen, triggering the exacerbated immune response [576, 577]. Human IBD has been associated with a defective colonic mucus layer and a reduced number of goblet cells [578]. Moreover, a previous study in a MUC-2 knockout model has confirmed that MUC-2 deficiency led to a mild increase of inflammatory cells in the colon, together with an abnormal morphology and ulceration of epithelial cells, effects that, according to the histological analysis, were also reversed by minocycline administration in our study [579].

The noticeable immunological activity of minocycline was reinforced by its remarkable effect in restoring a balanced intestinal microbiota. Similarly to previous reports, the present study shows that the inflammatory process was accompanied by an alteration of the colonic microbiota, which leads to dysbiosis [580]. A significant increase in the counts of enterobacteria in comparison with healthy rats was observed, and these were significantly down-regulated by both tetracyclines, thus reducing the bacterial load in the intestinal mucosa and contributing to the amelioration of the exacerbated inflammatory response. However, although tetracycline administration was able to decrease these

bacterial counts to normal, this effect was not enough to promote an evident recovery from the intestinal inflammation, as significant as the one achieved after minocycline treatment. Furthermore, the counts of lactobacilli and bifidobacteria, which were down-regulated in colitic rats from control group when compared with healthy rats, only appeared increased after treatment with minocycline. It has been shown that these microorganisms positively affect the intestinal barrier function by decreasing mucosal permeability [538], and are able to modulate the immune response [539], so they are used as probiotics in these intestinal conditions. In consequence, the ability showed by minocycline to promote the restoration of these beneficial bacteria levels may contribute to its higher efficacy observed in this experimental model of rat colitis.

In summary minocycline displays a better anti-inflammatory profile than the other antibiotics tested, probably derived from its ability to restore the luminal microbiota imbalance that may occur in IBD, to inhibit iNOS and IL-17 expressions in the inflamed tissue and to promote intestinal membrane integrity. Furthermore, the differences between the three antibiotics regarding to their immunomodulatory activity, which seems not to be related to their antibiotic effect, have been clearly manifested in the vitro studies.

This anti-inflammatory effect of minocycline was confirmed in a different model of experimental IBD, the DSS-induced colitis in mice. DSS-induced acute or chronic colitis is characterized by direct mucosal/submucosal damage, which is particularly severe in the distal colon and thus mimics human UC [549, 548]. In this case, we performed a chronic administration of minocycline up to 21 days in order to evaluate its long-term effects in intestinal inflammation. After this period, colonic weigh/length ratio, which is inversely associated with the severity of DSS-induced colitis [550], was decreased in minocycline-treated mice, and colonic mucosa appeared almost completely restored, which is in accordance with the reduced levels found for the proinflammatory cytokines studied. The

overall impact of minocycline treatment on these mice resulted in a decreased DAI evolution through the study.

From all these studies we concluded that the combination of both immunomodulatory and antimicrobial properties of minocycline constitute an advantage in the treatment of acute and chronic intestinal inflammation in IBD experimental models.

Given the relapsing nature of both CD and UC, IBD therapy has mainly focused in two different goals, firstly, to induce remission in the active episodes, and then to maintain this status and avoid future relapses. Minocycline has been shown to ameliorate experimental intestinal inflammation; however, previous studies in human have revealed that antibiotic treatment discontinuation seemed to be related to an exacerbation of the disease [17]. In addition, long-term administration of antibiotics, although it was found to be safe for minocycline [92, 93], is restricted by the appearance of bacteria resistance and increased risk of drug side effects. Therefore, the chronic antibiotic administration that could be required in IBD therapy is limited [17]. So, the new question that arose was how to avoid long-term administration of minocycline while maintaining the experimental remission initially achieved.

To find out an answer, we proposed a new therapeutic strategy: Minocycline treatment during the acute phase, followed by probiotic administration to maintain the remission and prevent the relapses. In order to evaluate the effectiveness of our hypothesis, we developed a reactivated-colitis model in mice, in which the animals were subjected to two separated cycles of DSS, starting the second cycle once the colonic inflammation was in process of recovery.

Probiotics have been reported to prevent intestinal inflammation in several experimental models, due to the restoration of the intestine microbial balance and, in some cases, due to their peculiar immunomodulatory activity. *E coli* Nissle 1917 was chosen for being one of the best known probiotics, when considering its safety as well as its beneficial effects in gastrointestinal conditions like diarrhoea. In fact, is one of the few probiotics that have definitively shown their efficacy in human IBD [57].

The results obtained in these assays confirmed our previous findings, since minocycline was able to promote the recovery of DSS-induced colitis in mice when it was administered for 1 week after colonic damage induction. Again, this beneficial effect was associated with an amelioration of the altered immune response, as evidenced by a decrease in the expression of the proinflammatory cytokines TNFα, IL-1β and IL-2, produced during the primary immune response; and the chemokines MIP-2 and MCP-1 and the adhesion molecule ICAM-1, thus resulting in a reduced migration of leucocytes into the injured intestine. Moreover, the enzymes iNOS and MMP-9 were as well down-regulated by minocycline treatment. As in human IBD, expression of MMPs is also implicated in the pathogenesis of experimental colitis [6, 581, 582, 583]. It has been suggested that these enzyme activities have a pathogenic role in elevating proteolysis of the mucosa, which results in ulceration, inflammation, and fistula formation [584]. Of the MMPs, MMP-9 is the most abundantly expressed protease in inflamed intestine [6] and here, minocycline was able to inhibit its expression, as reported for other experimental animals and *in vitro* [38], which can result in the decreased T cell transmigration to the intestine wall and the decreased mucosal damage evidenced histologically.

In addition, the treatment with this antibiotic improved the defensive mechanisms of the intestine epithelial barrier, whose architecture appeared restored in the histological studies. Biochemically, minocycline counteracted the

reduced expression of the mucin MUC-3, one of the primary constituents of the mucus layer in the colon [573], and of ZO-1, a transmembrane protein that maintains tight junctions integrity [77], similarly to that previously described in other models of experimental colitis [585]. Finally, the administration of minocycline was also able to restore the balance in the intestinal microbiota, which was altered in the DSS-induced inflammatory process, in accordance to that reported in the TNBS model of rat colitis.

At this time, after 1 week of treatment, the administration of minocycline to colitic mice was stopped in order to avoid the risk of side effects. The DAI evolution during the following 7 days showed constant values for these mice, being lower than those of the untreated colitic group. Nevertheless, the absence of an additional recovery in these mice after the interruption of the treatment contrasts with that achieved in the same model of colitis when the antibiotic treatment was maintained longer (for 21 days). For this reason, we evaluated if the subsequent administration of the probiotic *E. Coli* Nissle 1917 could have a positive impact in these treated mice. This probiotic is sensitive to the antibiotic, as confirmed in the sensitive assay performed. Therefore, the possibility of resistance transmission to other bacteria would be avoided. Moreover, the beneficial effect showed by this probiotic was not compromised when it was combined with minocycline. In fact, the probiotic treatment caused a significant improvement of DAI values, regardless of whether the mice were previously treated with the antibiotic or not. These results support previous studies that reported the beneficial effects of this probiotic in intestinal inflammation, both in experimental models [586] and in human IBD [57], ascribed to its ability to interfere with the intestine microbiota and to modulate the intestine immune response. Indeed, the evaluation of the colonic inflammatory status 1 week after confirmed this beneficial effect. The histological damage score in those mice treated with the probiotic alone was similar to that obtained in the group previously treated only

with minocycline. However, the group of mice that received the combined therapy, i.e. first the antibiotic followed by the probiotic, showed a greater improvement in the colonic histology when compared with the other experimental groups, revealing the additional beneficial effect of the association, which was confirmed in the biochemical analysis.

The immunomodulatory properties reported for both minocycline [38] *and E. coli* Nissle 1917 [528, 586] could clearly contribute to reduce the expression of some of the proinflammatory markers. However, these effects were increased when the antibiotic treatment was followed by the administration of the probiotic, since all the mediators assayed were clearly improved. As a result, the vicious cycle generated by the different mediators involved in IBD pathogenesis, responsible for maintaining the chronic inflammatory response, can be blocked, thus facilitating the recovery of the inflamed colonic tissue. The decreased levels of the proinflammatory cytokines TNFα, IL-2 and IL-1β can lead to decreased T cell proliferation and to a down-regulation of the expression of the chemokines MIP-2 and MCP-1, as well as of the adhesion molecule ICAM-1, which cause a reduced inflammatory cell infiltrate in the colonic tissue. As a consequence, the expression of enzymes like iNOS and MMP-9 is reduced, since they are mainly synthesized by inflammatory cells, particularly T cells, macrophages and polymorphonuclear leucocytes [70, 587]. Since minocycline also directly inhibits these enzymes, it is difficult to affirm if this effect is either cause or consequence; anyway, the down-regulation in their activity should clearly contribute to the beneficial effect observed for minocycline in this experimental model of intestine inflammation.

Furthermore, the combination of both treatments also restored intestine integrity by increasing the expression of colonic MUC-3 and ZO-1, as previously shown for the antibiotic (in the TNBS model of rat colitis) or the probiotic [588] when administered separately. As commented before, this can be of special

relevance since the altered epithelial barrier function can be considered as one of the key pathogenic mechanisms in intestinal inflammation because it may facilitate the access of antigens from the intestinal lumen, thus promoting the exacerbated immune response that occurs in this condition [576, 577].

In order to study the impact of the combined therapy in the prevention of the relapses that usually take place in human IBD, colitic mice were submitted to a second cycle of DSS. This resulted in a worsening of the intestine inflammatory process with increased DAI values, as a result of the body weight loss and the presence of diarrhoea in these mice. All the treatments assayed attenuated the aggravation of the inflammatory status and lately promoted the recovery of the mice, but the group that received both the antibiotic and the probiotic displayed a lower colonic damage, as evidenced by DAI evolution and histologically, appearing at day 26 almost fully recovered. This was also associated with a significant down-regulation of the expression of all the biochemical proinflammatory markers evaluated, and again, the treatment was able to preserve the intestine integrity, which was affected in the reactivation of the colitis.

When the microbiota was evaluated throughout the experiment, all treatments were able to restore the ratio between beneficial and potential pathogen bacteria, which was modified due to the colitic process, in a similar way. Although this activity may have a role in the greater effect observed with the combined therapy, the absence of clear differences among the treated groups may point out that is their ability to regulate the altered immune response and to preserve intestine integrity what might justify the greater anti-inflammatory effect displayed with the association of both treatments.

Therefore, the supplementation of minocycline treatment with the probiotic *E. coli* Nissle 1917 improves the recovery of the intestinal damage and prevents the reactivation of experimental colitis, supporting our hypothesis and the potential use of this new therapeutic strategy in the treatment of human IBD.

Finally, in view of the variety of actions displayed by minocycline in these *in vitro* and *in vivo* experimental models, we could hypothesise possible mechanisms of action responsible for the benefits derived from its administration in rodent colitis. A direct effect on the innate immune response seems to be involved, as supported by the *in vitro* inhibition of the proinflammatory activity of epithelial cells and macrophages after minocycline pretreatment. This is in agreement with the *in vivo* data, i.e. minocycline inhibited iNOS protein expression, reduced MPO activity, increased protective epithelial factors, such us mucins and other proteins involved in the intestinal barrier integrity, and a minor inflammatory infiltrate of immune cells was also observed in those animals treated with minocycline. In addition minocycline has been reported to directly inhibit several proinflammatory enzymes, such as iNOS and matrix metalloproteinases, an activity that was also evidenced in our studies. Inhibition of these enzymes could explain the improvement achieved in colonic tissue, since their aggressive actions may be avoided.

Maybe as a consequence of the decline in the innate response, the adaptive response was therefore also diminished, but a direct effect of minocycline on the immune cells that mediate this response cannot be ruled out. Although it has not been checked in this thesis, minocycline has been reported to affect T cell differentiation and proliferation [198, 199], thus, some of the effects observed in our studies i.e. IL-2 and IL-17 decreased expressions, might be derived from the ability of minocycline to decrease NFAT transcriptional activity [50] and to impair T cells – APCs interaction [188, 192, 197], all these resulting in a decreased adaptive response that would help in the recovery from the intestinal inflammation.

In a different setting, its antibiotic activity can be naturally involved in the beneficial effect. Thus, minocycline increased the number of protective species in the intestinal lumen, which in turn implies the beneficial actions that these bacteria may exert, including: suppression of the growth, epithelial binding and subsequent invasion of enteric pathogenic bacteria [534 - 536]; improvement of epithelial and mucosal barrier function [537, 538] and regulation of the immune response in the intestinal mucosa [60, 61, 539 - 541].

Conclusions

1. Minocycline, unlike other antibiotics used in inflammatory bowel disease therapy, exerts a direct immunomodulatory effect, evidenced *in vitro* in colonic epithelial cells and macrophages, in which it decreased the production of interleukin 8 and nitrite respectively.

2. Minocycline, administered as a curative treatment, displays an intestinal anti-inflammatory effect in the experimental model of acute colitis induced by TNBS in rats. In this effect are involved both its ability to restore the intestinal microbiota balance and its immunomodulatory properties. Regarding its immunomodulatory activity, it has an effect on the innate immune response, preserving intestinal barrier integrity and decreasing the production of proinflammatoy cytokines; and on the adaptive immune response, inhibiting the expression of Th1 and Th17 cytokines.

3. The intestinal anti-inflammatory effect of minocycline is also manifested in the DSS-induced model of mouse colitis.

4. The association of the antibiotic minocycline and the Probiotic *Escherichia coli* Nissle 1917 represents a good strategy in maintaining remission and in preventing the reactivation of the inflammatory process in a reactivated colitis model. This combination exerted a synergistic effect on the different markers evaluated, in comparison with the effect displayed by the independent treatments.

Conclusiones

1. La minociclina, a diferencia de otros antibióticos utilizados en el tratamiento de la enfermedad inflamatoria intestinal, ejerce un efecto inmunomodulador directo. Esto se evidencia *in vitro* tanto en células epiteliales colónicas, disminuyendo la producción de interleucina 8, como en macrófagos, reduciendo la producción de nitritos.

2. La minociclina, administrada como tratamiento curativo, presenta un efecto antiinflamatorio intestinal en el modelo de colitis experimental aguda por TNBS en ratas. En este efecto participan su capacidad de restaurar el equilibrio en la microbiota intestinal y sus propiedades inmunomoduladoras. En relación a su acción inmunomoduladora, ésta afecta tanto a la respuesta inmune innata, al preservar la integridad de la barrera intestinal y disminuir la producción de citocinas proinflamatorias, como a la respuesta inmune adaptativa, mediante la inhibición de la expresión de citocinas del tipo Th1 y Th17.

3. El efecto antiinflamatorio intestinal de la minociclina también se manifiesta en un modelo de progresión crónica de colitis inducida por DSS en ratones.

4. La asociación del antibiótico minociclina con el probiótico *Escherichia coli* Nissle 1917 constituye una buena estrategia para el mantenimiento del estado de remisión, así como en la prevención de la reactivación del proceso inflamatorio, en un modelo de colitis experimental con recidivas. La combinación se traduce en un efecto sinérgico sobre los distintos marcadores evaluados, en comparación con los efectos observados con los tratamientos aislados.

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Abbreviations

5-FU	5-Fluorouracil
5-LO	5-Lipoxygenase
AIDS	Acquired immunodeficiency syndrome
AMP	Antimicrobial peptide
Apaf-1	Apoptosis protease-activating factor-1
APC	Antigen presenting cell
APP	Amyloid β precursor protein
ATP	Adenosine TriPhosphate
Aβ	Amyloid β peptide
CARD15	Caspase recruitment domain family member 15
CD	Crohn's disease
CFU	Colony forming units
CGN	Cerebellar granule neuron
Chr	Chromosome
CINC-1	Cytokine-induced neutrophil chemoattractant - 1
CNS	Central nervous system
COX	Cyclooxigenase
Cyt c	Cytochrome c
DAI	Disease activity index
DC	Dendritic cell
DSS	Dextran sulfate sodium
EAE	Experimental autoinmune encephalomyelitis
EcN	*Escherichia coli* Nissle 1917
eIF-2a	Eukaryotic initiation translation factor 2 alpha
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
Foxp3	Forkhead box P3

FXS	Fragile X syndrome
GADPH	glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GSK	Glycogen synthase kinase
GWAS	Genome-wide association studies
HAART	Highly active anti-retroviral therapy
HASMC	Human aortic smooth muscle cells
HIV	Human immunodeficiency virus
HMGB1	High-mobility group protein B1
I/R	Ischemia-reperfusion
IAP	Inhibitor of apoptosis protein
IBD	Inflammatory bowel disease
ICAM-1	Inter-Cellular Adhesion Molecule - 1
ICE	Interleukin 1beta converting enzyme
IDO	Indoleamine 2, 3-dioxygenase
IEC	Intestinal epithelial cell
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible NOS
JNK	c-Jun N-terminal protein kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoatractant protein
MDP	Muramyldipeptide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproinase

MNC	Minocycline
MNZ	Metronidazole
MPO	Mieloperoxidase
MPT	Mitochondrial permeability transition
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MUC	Mucin
NADPH	Nicotinamide adenine dinucleotic phosphate (reduced)
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappaB
NGF	Nerve growth factor
NK	Natural killer cell
NKT	Natural Killer T cell
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOS	Nitric oxide synthase
OD	Optical density
PAR	Protease-activated receptor
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PG	Prostaglandin
PHA	Phytohaemagglutinin
PK	Protein kinase
PMA	Phorbol myristate acetate
PRR	Pattern-recognition receptor
qPCR	Real time quantitative PCR
RORγt	Retinoic-acid-receptor-related orphan receptor-γt

ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase - PCR
SCFA	Short-chain fatty acid
SCI	Spinal cord injury
SIV	Simian immunodeficiency virus
Smac	Second mitochondria-derived activator of caspases
SNPs	Single Nucleotide Polymorphism
SOCE	Store-operated calcium entry
STAT	Signal transducer and activator of transcription
TFF	Trefoil factor
TGFβ	Transforming growth factor β
Th	T helper cell
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TTC	Tetracycline
UC	Ulcerative colitis
VSMC	Vascular smooth muscle cells
ZO	Zonula occludens protein

Anexo

Mi incorporación al grupo de investigación "Farmacología de productos naturales" ha supuesto que, de forma paralela a la realización del trabajo presentado en esta tesis doctoral, haya podido participar en otros proyectos, cuyos resultados han sido publicados en prestigiosas revistas científicas a nivel internacional. Asimismo, mi formación predoctoral se ha visto complementada con la realización de cursos, asistencia a congresos, y distintas estancias en otros centros de investigación.

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ESTANCIAS EN OTROS CENTROS.

- Centro: **Beth Israel Deaconess Medical Center**. Boston (USA). Investigador responsable: Cornelis Terhorst MD PhD (Chief división of Immunology). 15 Julio – 8 Octubre 2010.

- Centro: **Instituto de Biomedicina y Biotecnología de Cantabria** (CSIC-Universidad de Cantabria) Santander (España). Investigador rsponsable: Dr. Ramón Merino Pérez (Científico titular CSIC). 4-14 Julio 2011.

- Centro: **Institut d'investigació Germans Trias i Pujol/CIBERehd**. Badalona (España). Investigador responsable: Dr. Josep Mañé Almero. 1-30 Abril 2010.

- Centro: **Leiden University Medical Center.** Leiden (Holanda). Investigador responsable: Gijs Van den Brink MD PhD (Head of the laboratory of the Department of Gastroentelogy & Hepatology). 1 Junio – 1 Octubre 2009.

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- **Curso "Metodología docente: Aprendizaje Basado en Problemas",** Departamento de Farmacología de la Universidad de Granada. Fundación A. Esteve. Vicerrectorado para la Garantía de la Calidad de la UGR en el marco de la II Convocatoria para la realización de actividades de formación docente en Centros, titulaciones y Departamentos. (16 horas; 2011)

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- **Young Investigator Meeting**. Association of National European and Mediterranean Societies of Gastroenterology. (Viena, 2009).

- **Certificado de aptitud pedagógica (CAP).** Universidad de Granada. 2007/2008.

- **Premio joven investigador** (Pharma Nutrition 2011).

- Colaboradora como revisora de la revista "Pharmacological Research".

- Colaboradora como revisora de la revista "British Journal of Pharmacology".

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… Y para el investigador no existe alegría comparable a la de un descubrimiento, por pequeño que sea....

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Ahora sí… This is it.

"En un partido hay momentos en que la pelota golpea el
borde de la red y durante una fracción de segundo
puede seguir hacia delante o caer hacia atrás.

Con un poco de suerte sigue adelante y ganas…"