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IMMUNOMODULATORY TETRACYCLINES:

Facing the complexity of intestinal inflammation

Tesis doctoral para aspirar al grado de doctor presentada por

José Garrido Mesa

Bajo la dirección de los Doctores

Julio Juan Gálvez Peralta

Natividad Garrido Mesa

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Autor: José Garrido Mesa
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RESUMEN

La aparición del sistema digestivo es una pieza clave en la aparición de organismos pluricelulares, proporcionando la energía necesaria para evolucionar y adquirir nuevas y más complejas funciones. Para ello, el interior del intestino se moldea para formar una enorme superficie en contacto con millones de bacterias. Desde el inicio, los microorganismos han contribuido a la evolución de este complejo sistema y al desarrollo de sus funciones. Es por ello que el intestino también aloja a la población más potente de células inmunes, que se entrena para diferenciar a potenciales patógenos entre la abundancia de microorganismos comensales. El sistema inmune intestinal cumple con la delicada tarea de mostrar una amplia tolerancia al mismo tiempo que una rápida capacidad de respuesta ante el menor indicio de amenaza. Este equilibrio no sólo se mantiene gracias él, la barrera epitelial y mucosa también juega un importante papel, limitando el acceso de antígenos, al igual que el ecosistema intestinal, que compite con especies potencialmente patógenas, limitando su crecimiento. Estas tres barreras contribuyen al mantenimiento de la homeostasis intestinal, permitiendo al intestino llevar a cabo su función fisiológica.

Este equilibrio complejo se ve amenazado por múltiples factores, y cuando el balance se rompe, se desencadena una respuesta inflamatoria con el objetivo de volver a restaurar la homeostasis intestinal. Estos mecanismos desarrollados durante miles de años de evolución, nos protegen de amenazas como parásitos intestinales, infecciones por bacterias patógenas, sustancias tóxicas presentes en los alimentos… Sin embargo, la ausencia de dichas amenazas priva al sistema inmune de dicho entrenamiento, cambios en los estilos de vida y alimentación condicionan la función de la barrera ecológica y la acumulación de alteraciones genéticas que debilitan los mecanismos de barrera, confieren cierta susceptibilidad al desarrollo de inflamación intestinal, incluso en ausencia de una amenaza real. En este contexto, la inflamación se perpetúa, interfiriendo con la función intestinal y causando un daño irreversible al tejido. Es el caso de la enfermedad inflamatoria intestinal (IBD), un conjunto de patologías de etiología desconocida que se agrupan en dos grandes categorías, la enfermedad de Crohn y la colitis ulcerosa. En la primera, la inflamación puede aparecer en cualquier zona del intestino, aunque generalmente afecta a colon e íleon, y afecta a las capas más internas de la pared intestinal, causando fístulas, fibrosis y estenosis. En la colitis ulcerosa, el daño se limita a la mucosa, que se extiende de forma continua y ascendente desde el recto. En ambos casos, los episodios de inflamación aparecen de forma repentina, causando diarrea, dolor abdominal y malestar general. Sin embargo, la gravedad de los síntomas puede variar

muchísimo, siendo leve en algunos individuos mientras que en otros la inflamación progresa severamente, llegando a requerir la eliminación quirúrgica de zonas afectadas debido a la falta de tratamientos efectivos. El arsenal terapéutico disponible para el tratamiento de la enfermedad inflamatoria intestinal se basaba principalmente en el uso de anti-inflamatorios no esteroideos derivados del ácido salicílico, corticoides e inmunosupresores. Dado el amplio número de mediadores inflamatorios involucrados, la llegada de terapias biológicas prometía conseguir un mejor control de este proceso patológico; sin embargo, debido a la complejidad de la enfermedad, hasta ahora únicamente los anticuerpos frente a TNFα han mostrado un beneficio claro.

Dada la falta de éxito y la creciente incidencia de esta patología, la IBD es un campo de investigación activa en busca de un mayor conocimiento y estrategias terapéuticas que consigan controlar la inflamación intestinal. Entre las diferentes opciones, y dado el importante papel que juega la microbiota en el inicio y desarrollo de la patología, la utilización de antibióticos siempre se ha considerado una posibilidad, aunque no ha mostrado ser realmente útil en todos los grupos de pacientes. Sin embargo, algunos antibióticos han mostrado poseer propiedades adicionales de interés terapéutico, como es el caso de algunos miembros de la familia de las tetraciclinas, objetivo de esta tesis doctoral.

Esta línea de investigación fue iniciada en el departamento de farmacología de la Universidad deGranada, encontrando que la minociclina ejercía un efecto anti-inflamatorio beneficioso en modelos experimentales de inflamación intestinal. La minociclina es una de las tetraciclinas más estudiadas por sus propiedades adicionales beneficiosas en otras patologías con un cierto componente inflamatorio. En los estudios desarrollados por este grupo, se observó que el efecto beneficioso se debía, en parte, a la presencia de dichas propiedades inmunomoduladoras. Se diseñó a su vez una estrategia terapéutica combinando el tratamiento de la inflamación aguda con minociclina con el mantenimiento de la remisión de los síntomas con la administración del probiótico *E. coli* Nissle 1917. De esta forma, se consigue controlar el curso recurrente de la enfermedad sin necesidad de prolongar el tratamiento antibiótico, evitando así un impacto excesivo sobre la flora bacteriana.

A la vista de estos resultados positivos, el objetivo de esta tesis doctoral consistió en continuar con dicha investigación, comprobando si la actividad anti-inflamatoria intestinal de la minociclina estaba presente también en otros

miembros de esta familia de antibióticos e investigando el mecanismo por el cual consiguen dicho efecto, dada la amplia gama de propiedades descritas para estas moléculas.

Para conseguir dicho objetivo general, en primer lugar se comprobó que la doxiciclina también poseía dichas propiedades antiinflamatorias. La doxiciclina mostró una efectividad similar a minociclina al reducir la inflamación inducida por TNBS en rata y por DSS en ratón. Entre los mecanismos puede estar implicada su capacidad para disminuir la producción de mediadores inflamatorios, como IL-8 observado directamente en células epiteliales, o la producción de radicales libres y oxidantes, comprobado mediante su actividad in vitro disminuyendo la producción de nitritos en macrófagos. Este efecto se ve reflejado in vivo, reduciendo la expresión de citoquinas inflamatorias y los niveles de MPO y glutatión, indicativo de un menor daño oxidativo y menor daño a la barrera epitelial. Al igual que con minociclina, la asociación de doxiciclina junto con un probiótico, en este caso *S.boulardii*, consigue disminuir el episodio agudo de inflamación y mantener posteriormente la remisión, reduciendo el impacto inflamatorio sobre el tejido y haciéndolo menos susceptible al desarrollo de un nuevo brote.

Comprobada la actividad antiinflamatoria de doxiciclina y minociclina, quisimos profundizar en los posibles mecanismos responsables del efecto beneficioso de estos fármacos. Para ello se llevaron a cabo dos estudios en modelos experimentales de colitis similares a los utilizados anteriormente, el DSS y el DNBS, ambos en ratón. En estos nuevos estudios se incluyeron, además de doxiciclina y minociclina, un fármaco antibiótico de acción local (rifaximina), la tetraciclina, que carece de propiedades inmunomoduladoras tan marcadas, una tetraciclina de tercera generación, derivada de minociclina, y un corticoide (dexametasona), con propiedades inmunomoduladoras, pero sin efecto antibiótico. El efecto de los diferentes tratamientos se evaluó de forma general, sobre la evolución del proceso colítico, a nivel histológico y de expresión génica de varios marcadores de la función barrera, mediadores inflamatorios, receptores TLR y microRNAs, involucrados en la regulación de múltiples vías celulares y cuya modulación puede tener un importante efecto sobre la respuesta inflamatoria. Adicionalmente, se evaluaron los cambios en la composición de la microbiota por pirosecuenciación. Los resultados obtenidos mostraron un claro beneficio terapéutico en ambos modelos en los grupos tratados con las tetraciclinas inmunomoduladoras, doxiciclina, minociclina y tigeciclina, mientras

que la tetracilina y el resto de tratamientos no siempre consiguió modificar el curso de la inflamación.

En el modelo del DNBS, agresivo y con una alta mortalidad, las tetraciclinas inmunomoduladoras consiguieron aumentar la supervivencia y disminuir la pérdida de peso, el acortamiento colónico y el intenso daño histológico al tejido. La dexametasona no mostró ningún efecto terapéutico y los otros dos antibióticos, especialmente la tetraciclina, mejoraron ligeramente la pérdida de peso hacia al final del ensayo, tras 6 días de tratamiento. Esto se vio reflejado en una mejora general del perfil de expresión génica de este modelo, mejorando la protección de la barrera mucosa y disminuyendo varios mediadores inflamatorios. Cabe destacar el incremento que todas las tetraciclinas inducen en la expresión de Ccl2, quimiocina involucrada en el reclutamiento de macrófagos, que se encuentra disminuida en este modelo. Ninguno de los TLRs se modificó significativamente en la colitis inducida por DNBS, aunque destaca el incremento consistente inducido por las tetraciclinas inmunomoduladoras sobre la expresión de TLR6. Las alteraciones en la expresión de microRNAs son sutiles, modificándose principalmente los miR-146a, 155 y 223 en el grupo control colítico, aunque los efectos de los tratamientos no alcanzaron significancia estadística en la mayoría de los casos. La evaluación de la composición de la microbiota mostró diferencias significativas entre sanos y enfermos, y especialmente en los grupos tratados con antibióticos, indicativo una vez más de que el efecto antibiótico debe jugar un papel importante en este modelo de inflamación intestinal. Sin embargo, como muestran los marcadores inflamatorios evaluados y la propia evolución de los animales, dicho efecto aislado no es suficiente y únicamente consigue controlar la inflamación cuando se refuerza con el efecto inmunomodulador de doxiciclina, minociclina y tigeciclina.

En el modelo del DSS, se llevaron a cabo dos ensayos, uno más agresivo y prolongado, en el que se vio el efecto de los tratamientos sobre la evolución y mortalidad, y otro más controlado, sacrificando tras cuatro días de tratamiento, suficientes para que las tetraciclinas inmunomoduladoras obtuvieran un efecto significativo. En el primer ensayo, de 6 días de tratamiento, al igual que en el DNBS, se produjo una alta mortalidad, que solamente fue reducida por doxiciclina, minociclina y tigeciclina, mientras que el efecto de la tetraciclina fue muy limitado, y la dexametasona y rifaximina no consiguieron un impacto beneficioso, siendo la última incluso perjudicial. Estos efectos se mantuvieron en

el segundo ensayo, observando una mejora del índice de actividad de la enfermedad desde el primer día de tratamiento con los antibióticos inmunomoduladores que se reflejó a su vez en un daño histológico significativamente menor. La evaluación de marcadores de la barrera mostró una clara mejora de la producción de mucus y proteínas de unión intercelular, y parámetros inflamatorios como la producción de IL-6, IL-1β y la quimiocina atrayente de neutrófilos también fueron reducidas significativamente. Sin embargo, al igual que en el modelo del DNBS, las tetraciclinas inmunomoduladoras produjeron un fuerte incremento de Ccl2, que ya se encontraba incrementada en el grupo control de ratones colíticos, sugiriendo un incremento en el reclutamiento de monocitos y células dendríticas al foco inflamatorio. Dicho efecto paradójico también se observó en la expresión del miR-142, que siguió el mismo patrón, siendo incrementado en el modelo de inflamación pero más aún con las tetraciclinas inmunomoduladoras. Las variaciones en la expresión de otros microRNAs destacan principalmente el incremento de miR-150, 155 y 223, cuya expresión se vio disminuida por los fármacos con efecto inmunomodulador. Por último, cabe destacar la disminución de TLR4 en la colitis inducida por DSS, un efecto que fue contrarrestado en los grupos tratados con doxiciclina, minociclina y tigeciclina, consistente con su efecto positivo y, probablemente, ligado a una mejor reconstitución de la barrera epitelial. No se observaron modificaciones significativas de la composición de la microbiota, ni en el modelo ni con los tratamientos, lo cual apoya la ausencia de beneficio del efecto antibiótico en este modelo, aunque también pueda deberse a la menor duración del tratamiento.

Ya que la importancia real de la microbiota radica en su funcionalidad y que el impacto de los antibióticos puede resultar negativo, quisimos descartar la presencia de cambios que no fueran apreciados en la evaluación de la composición a nivel filogenético. Para ello utilizamos un modelo de transferencia fecal, con el que consiguiéramos implantar la microbiota resultante tras el proceso colítico inducido por DSS y el tratamiento farmacológico en ratones a los que previamente se había reducido su microbiota original mediante el tratamiento prolongado con un coctel antibiótico. Se transfirió la microbiota de ratones sanos, colíticos y colíticos tratados con minociclina, y se procedió a evaluar la susceptibilidad a una inflamación inducida por DSS. Inesperadamente, se observaron diferencias en la evolución de los animales: el grupo transferido con microbiota de ratones colíticos no tratados resultó más sensible a la inflamación por DSS que el grupo control al que no se realizó transferencia y que

los ratones transferidos con microbiota de ratones sanos y de ratones colíticos tratados con minociclina. Este efecto se evidenció también a nivel histológico y al evaluar algunos marcadores de la función barrera, aunque los cambios en estos fueron muy sutiles y no se detectaron variaciones en la activación de citoquinas del sistema inmune. Por tanto, estos resultados indican que, aunque moderados, los efectos de la inflamación inducida por DSS sobre la microbiota pueden predisponer a nuevos brotes inflamatorios y que el efecto antibiótico de un tratamiento con minociclina, lejos de empeorar esta situación, consigue revertirla a niveles basales.

A la vista de estos datos, nos resultó especialmente interesante el incremento de Ccl2, que indicaría un aumento de la población de macrófagos, y su papel en la respuesta inflamatoria. Por ello evaluamos el efecto in vitro de estos tratamientos sobre el cultivo primario de macrófagos derivados de médula ósea estimulados con LPS. Observado que el efecto inmunomodulador se asociaba con una disminución de la expresión de iNOS, marcador del fenotipo de activación M1, aunque tan solo la dexametasona conseguía disminuir la producción de citoquinas inflamatorias, como TNFα, IL-1β e IL-6. Es más, su producción se veía fuertemente incrementada por el efecto de las tetraciclinas inmunomoduladoras, tanto a nivel basal como tras la estimulación con LPS, indicando la presencia de un efecto divergente sobre diferentes vías de activación, un resultado que también ha sido observado por otros grupos de investigación. Aparentemente, este resultado podría ser contradictorio con el efecto anti-inflamatorio observado hasta ahora, pero dado el importante papel que llevan a cabo los macrófagos en el control del proceso inflamatorio, y en concreto en el modelo de inflamación inducido por DSS, nos propusimos profundizar en esta línea para determinar, si el mecanismo de acción de las tetraciclinas con propiedades inmunomoduladoras, pueda estar mediado por una potenciación de la función protectora de los macrófagos sobre otras que perpetúan la inflamación.

Utilizando el modelo del DSS y el tratamiento con minociclina, ya que es el fármaco más estudiado por sus efectos inmunomoduladores, evaluamos los cambios en las principales poblaciones inmunes mediante citometría de flujo. Tras cuatro días de tratamiento, comprobamos que efectivamente se produjo un incremento en las poblaciones de macrófagos y células dendríticas, cuyos precursores son atraídos al intestino inflamado por el incremento de Ccl2. De hecho, únicamente se observó una disminución en la infiltración por neutrófilos, cuya respuesta proteolítica y oxidativa es responsable en gran medida del daño

causado en el tejido inflamado. No se observaron cambios en la población de células B, la más numerosa, mientras que el tratamiento con minociclina potenció ligeramente el incremento de células T y produjo un aumento del número de eosinófilos, no existiendo diferencias entre sanos y tratados en esta población. El análisis en más profundidad reflejó cómo la minociclina, a pesar del incrementar el número de macrófagos, estos no se acumulan con el fenotipo inflamatorio que perpetúa el proceso, sino que maduraron al fenotipo de macrófagos residentes, cuyo papel en la regeneración y resolución del proceso inflamatorio puede mediar el efecto beneficioso observado a nivel global. El perfil de las poblaciones de células dendríticas no se vio alterado por el tratamiento con minociclina. El análisis de las diferentes subpoblaciones de células T colaboradoras, incrementadas en el grupo tratado, mostró una mayor abundancia de células T reguladoras y Th17, las principales implicadas en este modelo, así como de células Th2, lo cual puede estar relacionado la mayor presencia de eosinófilos y macrófagos residentes, potenciando respuesta inmune local tipo 2, asociada a la regeneración y protección de mucosas. Dicho perfil también se observó en nódulos mesentéricos, donde si inicia la respuesta inmune adaptativa, y en bazo y sangre, representativos de la respuesta sistémica, observando una intensa neutrofilia en el grupo control, que fue contrarrestada por el tratamiento. La evaluación de la expresión génica confirmó el incremento de IL-4 e IL-22, que podrían explicar los cambios observados y el efecto beneficioso sobre la protección de la barrera epitelial y mucosa, así como el incremento de la enzima implicada en la síntesis de mediadores lipídicos involucrados en la resolución del proceso inflamatorio.

Por último, dicho análisis se repitió tras dos días de tratamiento, con el objetivo de capturar los eventos iniciales desencadenados por la minociclina. Aquí no se observaron cambios en las poblaciones inmunes presentes en el intestino, aunque sí destacó un fuerte efecto sistémico, incrementando las poblaciones de la línea mieloide, neutrófilos, eosinófilos y monocitos, acelerando el perfil de inflamación observado en el grupo control a día 4. La evaluación de la expresión génica en el tejido colónico sí reveló importantes cambios. Se observó el aumento de Ccl2 y Ccl11, que explicarían la mayor presencia de macrófagos y eosinófilos observados a día cuatro, así como una potenciación de la expresión de las citoquinas IL-1β, IL-6, IL-10, IL-4, GM-CSF e IL-22. Las dos primeras coinciden con el efecto directo observado in vitro de la minociclina sobre macrófagos, potenciando estas vías inflamatorias. El aumento de IL-10 e IL-4 están implicados en la potenciación de la respuesta mediada por células T

reguladoras y Th2. Por último, GM-CSF e IL-22 son producidos por las células linfoides innatas tipo 3 (ILC3), una población recientemente descubierta y actualmente el foco de una intensa investigación, dado su papel en la respuesta inmune asociada a mucosas.

Dada la amplia gama de propiedades y acciones de las tetraciclinas inmunomoduladoras en múltiples poblaciones celulares, sería difícil y definir su efecto anti-inflamatorio intestinal mediante un único mecanismo. Su capacidad antioxidante, la regulación de procesos apoptóticos y la proliferación celular, la inhibición de enzimas implicadas en el proceso inflamatorio, como metaloproteinasas que digieren la matriz extracelular, y la modificación de la liberación de citoquinas y otros mediadores en células epiteliales, estromales e inmunes, todas ellas, contribuyen al gran efecto beneficioso observado. Pero además, el controvertido efecto pro-inflamatorio observado en macrófagos puede a su vez contribuir, y en gran medida, al control de la inflamación intestinal. La activación temprana de la respuesta inflamatoria mediada por macrófagos parece potenciar y acelerar la respuesta inflamatoria, influyendo sobre las ILC3 y mejorando la protección de la barrera epitelial mucosa. Esto contribuye a frenar rápidamente el paso de bacterias y antígenos que perpetúan la inflamación. A su vez, este efecto pro-inflamatorio cambia rápidamente hacia una respuesta alternativa tipo 2 y pro-resolutiva, mediada por el incremento de macrófagos residente, células T reguladoras y eosinófilos y Th2, que limita la llegada de neutrófilos, el daño al tejido y contribuye a la protección y regeneración de la barrera mucosa. El incremento observado en células Th17 puede ser consecuencia de esa potenciación inicial de la respuesta inmune, pero dado su papel dual en la protección de mucosas y el hecho de que este sea un modelo de inflamación aguda no desencadenada por una alteración inmune, sino por el daño a la mucosa, tampoco debería descartarse un efecto beneficioso del papel de esta población, con capacidad de producir IL-22 y el consiguiente efecto beneficioso sobre la barrera epitelial.

En conclusión, los resultados obtenidos hasta ahora apoyan fuertemente el potencial de las tetraciclinas inmunomoduladoras en el control de la inflamación aguda, pudiendo resultar muy beneficiosas para limitar el daño tisular generado por los brotes de inflamación por los que se caracteriza la IBD. De hecho, los estudios genéticos muestran que las principales alteraciones encontradas se centran en defectos de la inmunidad innata y la capacidad de proteger la homeostasis intestinal, más que en alteraciones que potencien directamente la

respuesta inflamatoria. Esto explica en gran medida el fracaso obtenido con fármacos que atacan la respuesta inmune, anti-inflamatorios y terapias biológicas, cuyo objetivo es controlar las consecuencias de la enfermedad, una vez que la inflamación y el daño han sido generados. A pesar de la utilidad de estos grupos farmacológicos, un mayor control inicial de los brotes y el mantenimiento de los periodos de remisión se podría conseguir con estrategias como la que proponemos, combinando el uso de tetraciclinas inmunomoduladoras, que rápidamente induzcan remisión, y probióticos, que mejoren y protejan la homeostasis intestinal sin atacar agresivamente al sistema inmune y los mecanismos de protección naturales, desarrollados durante miles de años de evolución.

INTRODUCTION

CHAPTER I
Inflammatory bowel disease

Proper immune function and controlled inflammatory response are necessary for an adequate regulation of homeostasis. This is of special relevance in the intestine, where regulatory immune cells are continually integrating signals from intestinal bacteria and food particles[1]. Threatening events such as uncontrolled bacterial colonization, epithelial barrier disruption or disregulated immune stimulation can lead to the breakdown of this homeostatic balance and disease onset[2], [3]. In this context, the inflammatory response, which would lead to the recovery and repair of damaged tissue, sometimes becomes the cause of increased harm and damage when insufficiently regulated. This chapter will review the current knowledge on intestinal inflammation, the potential causes and events involved in the regulations of this complex condition.

INTESTINAL INFLAMMATION

Pathologies

Pathologies are often categorized by the organs affected and the resulting clinical manifestations produced. Disease duration is also an important factor in characterizing intestinal disease with chronic diseases persisting for months or longer, and acute diseases lasting only weeks[4], [5]. In any case, uncontrolled inflammation of the intestine always imparts a systemic impact on the body[6], [7]. Acute and chronic inflammatory diseases of the intestine decrease the quality of life of people in both developing and developed countries[8], [9]. Symptoms can include diarrhea, abdominal pain, fever, nausea and vomiting. It interferes

with the intestinal function, absorption of nutrients and water, which in case of persistence can seriously compromise life.

Infectious acute intestinal inflammation:

Acute intestinal inflammation is often self-limiting and many diarrheic episodes often go unreported[10]. Despite that, 1.7 billion cases of diarrheal disease are reported globally each year[11] and acute enteritis incited by foodborne pathogens alone is estimated to affect approximately 10–20 % of the human population annually[10]. Even more important, diarrheal disease is the second leading cause of death worldwide[12], given the high mortality rates that can be found in developing countries[13]. However, acute enteritis also implies significant costs to developed societies, including the loss of worker productivity and the important economic impact on health care systems[14], [15]. The most common cause of acute intestinal inflammation is an infection in the intestinal tract. It can be caused by a variety of bacterial, viral and parasitic organisms spread through contaminated food or drinking-water, or from person-to-person. Therefore, the most effective tool to control these pathologies is the rise on sanitary and hygienic preventive actions.

Coeliac disease.

Coeliac disease is an inflammatory disorder affecting the upper small intestine due to a T cell response against dietary gluten[16]. Since the main cause leading to this pathology is well known, gluten-free diets achieve to avoid coeliac disease in susceptible individuals. Despite that, recent studies have suggested that local microbiota may also influence this inflammatory response[17], [18]. This highlights the importance of microbial species present in each anatomical compartment of the intestine to develop or influence the susceptibility to a specific disease.

Chronic intestinal inflammation

Chronic intestinal inflammatory diseases have a tremendous negative impact on the health and great costs to health care systems. Large numbers of people suffer from these conditions and rates continue to rise[19], [20]. Inflammatory bowel disease (IBD) is the most important chronic inflammatory disease. It is often linked to prior acute inflammatory disease incited by infections[21], dysregulation of the intestinal immune response and concomitant autoimmune disorders[22]. The therapeutic choice and its efficacy are compromised by the important fact that the aetiology of IBD is enigmatic[20], and different conditions are grouped under the same name due to a convergent symptomatology. The complexity of these conditions is reviewed in more detail in the next sections.

Colon cancer

Adenocarcinoma is the third most common cause of death due to malignant disease in developed countries. It is almost entirely restricted to the large intestine, especially the distal colon, which might be related to the increased number and diversity of the microbiota at this location and the inflammatory responses that microorganisms incite[23]–[25]. This, together with the higher risk of developing colon cancer observed in IBD patients, suggest an important role for intestinal inflammatory response underlying colonic carcinogenesis. The stress and impact that inflammation causes on intestinal homeostasis and the increased susceptibility to develop colorectal cancer (CRC) has been the focus of research. It has been proposed that dysregulated inflammation leads to altered microbial metabolism and constantly elevated levels of IL-22, which display proliferative effects on epithelial cells[26], [27].

Inflammatory Bowel Disease

The term "Inflammatory Bowel Disease" cluster different inflammatory pathologies of the gastrointestinal tract. It has been classically divided in two major conditions: Crohn's disease (CD) and ulcerative colitis (UC). These chronic disorders are characterized by relapsing inflammatory episodes of unpredicted nature that alternate with phases of clinical remission[28], [29]. Symptoms include diarrhea, abdominal pain and rectal bleeding, which seriously impair quality of life and require prolonged medical and/or surgical interventions. The fear of a disease relapse must also be considered, as it affects many aspects of patients' lives and has a profound impact on mental wellbeing, personal relationships and work productivity[30]. Extra-intestinal manifestations are also frequent, with possible involvement of joints, skin, eyes and kidneys[31], and long standing symptomatology is associated with increased risk of colon cancer, as pointed above[26], [27].. Moreover, IBD patients have an increased risk of developing other chronic immune pathologies, such as psoriasis, ankylosing spondylitis and primary sclerosing cholangitis[32], which is indicative of the underlying immune/autoimmune disorder.

Crohn's disease

Intestinal inflammation in Crohn's disease can affect discontinually any part of the gastrointestinal tract[33], although it commonly affects the ileocaecal region and colon. Anatomical differences in the distribution of CD reflect the heterogeneity within this disorder and the clinical presentation is largely dependent on disease location. In addition to diarrhoea, abdominal pain and fever, CD patients can development complications such as fistulas, abscesses, or strictures. This could lead to fibrotic stenosis and bowel obstruction[34], [35]. CD lesions are patchy and fibrotic, and the histological features of CD show transmural inflammation with thickened submucosa, fissuring ulceration and

non-caseating granulomas, and the large inflammatory infiltrate is predominantly mononuclear[34].

CD has been classically assigned to Th1 inflammatory response because IFNγ and macrophage-derived IL-12 are highly up-regulated in inflamed CD compared to UC mucosa. Il-12 signals through STAT4 and promotes Th1 cell polarisation by upregulation of T-bet, the Th1 master regulator/transcription factor. Indeed, T-bet and phospho-STAT4 are also abundant in mucosal T cells in CD[36], [37]. However, increased number of Th17 cells and overexpression of IL-17A have also been detected in the lamina propria of both CD and UC patients[38], [39]. It has been proposed that CD may evolved from Th1 to Th17 pathway: high levels of IL-12-induced IFN-γ characterize initial inflammation found in children with active CD and experimental models of CD, while the late and established lesions are dominated by Th17 cytokines[40]–[42]. Interestingly, lamina propria $CD14^+$ macrophages produce high amounts of IL-23[43]. IL-23-responsive ILC3 are increased in CD inflamed intestine, where they produce IL-17A and IFNγ[44].

Ulcerative colitis

Ulcerative colitis is restricted to the colon. Inflammation usually begins at the rectum and spreads proximally in a continuous fashion, frequently involving the periappendiceal region. Patients present bloody diarrhoea, passage of pus, mucus, or both, and abdominal cramping during bowel movements[34]**.** Microscopical examination shows that UC is characterized by non-transmural inflammation since immune infiltrate, dominated by neutrophils, is limited to the mucosa and submucosa with extensive epithelial damage, cryptitis and crypt abscesses[34], [35], [45].

Immunological definition of UC has traditionally classified it has an atypical Th2 mediated inflammation[46]. This was based on the findings that UC patients make more IL-5[47] and IL-13 than controls or CD patients[48], [49], released by atypical natural killer T cells. In addition, IL-13 mediates oxazolone-induced colitis and impairs intestinal epithelial cell barrier function [49], which closely resembles the inflammation observed in UC. However, these differences have proved difficult to confirm and the immunological basis of ulcerative colitis remains unclear[50].

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Epidemiology of IBD

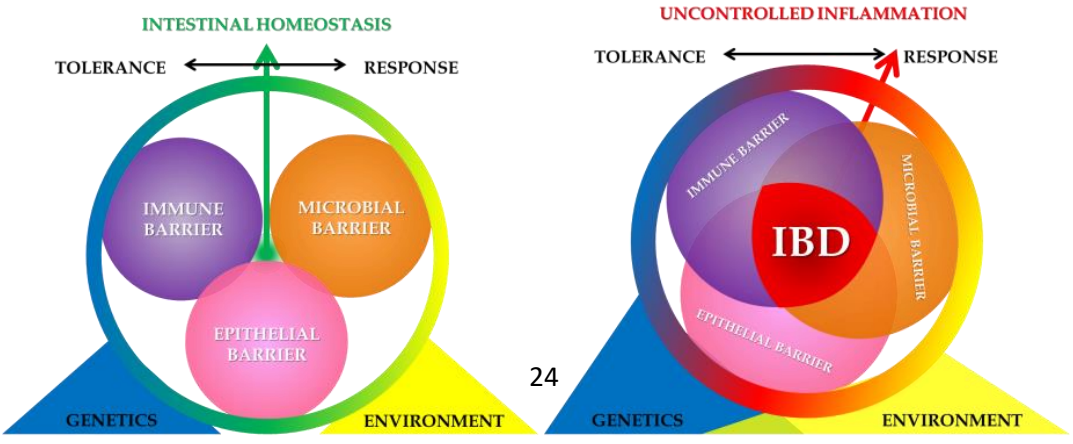
The prevalence of IBD is around 1 in 1000 people in Europe, with higher incidence rates observed in westernized and industrialized countries than in developing countries[51], [52]. However, in the recent years, there is a trend to reduce these differences, being the 2011 mean annual IBD incidence rate 14 in western Europe and 11.3 for eastern Europe[53]. Other developed countries have even higher rates: Australia have a high prevalence of IBD, with 30.3 mean annual incidence in 2011[53], [54]. In Canada, approximately 0.7 % of the population were living with inflammatory bowel disease in 2012 and diagnosis and treatment costs to the health care system were estimated at $1.2 billion [55], [56]. As observed in eastern Europe, rates in Asian countries are increasing[19]. In general, increased evidence shows an increase in IBD incidence and prevalence worldwide[52], [57]. The acquisition of western societies habits and the improvement in health and hygienic standards correlate with disease emergence within developing countries[58]–[60]. This highlights the significance of environmental factors on influencing IBD development globally[61].

Aetiology of IBD

The origin of IBD has been unknown for a long time, and even now, after

decades of intense research, the aetiology of this complex conditions is still not fully understood[33]. Numerous factors have been identified and they are generally classified within various groups: genetic susceptibilities, aberrations of innate and adaptive immune responses, alterations in the intestinal microbiota and environmental factors. However, a defined antigen or the exact contribution of the different factors remains elusive. None of the factors itself can induce intestinal inflammation. It is the confluence of them what determines whether IBD will appear and on which clinical phenotype[62]. Therefore, there is a general agreement that IBD is multifactorial disease in which genetically susceptible individuals develop an exacerbated immune response against unknown environmental triggers[63].

The convergence of genetic susceptibility and environmental influence causes the breakdown of intestinal homeostasis. The balance between tolerance and response is abrogated by their negative impact on the three barriers involved in the segregation of the host from the environment: mucosal immune barrier, mucosal epithelial barrier and microbial ecological barrier. Knowing how both factors account for flaws on these three barriers and development of modelling sistems that integrate this information could lead to a better understanding of the origin of IBD and the wide spectrum of manifestations. Genetic and environmental factors are briefly summarized below, and how alterations on the three barriers abrogate intestinal homeostasis are explained in more detail on subsequent sections



Genetic susceptibility

Familial occurrence of IBD has suggested for a long time that this condition could have a genetic basis[64]. However, initial studies describing the pathogenesis of IBD focused on elevated markers, suggesting a role for abnormal adaptive immune responses. As commented above, CD has long been considered to be driven by a Th1 response while UC has been rather associated with a non-conventional Th2 response[47]. Later on, Th17 cells, which expand in response to IL-23 and play an important role in mucosal immunity, were introduced in this paradigm[65]. Whether this profile observed in patients is truly involved in the onset of the disease or a consequence could not be assessed until advances in genetics and immunology were introduced in the field.

On the first genome scan, a link of CD with chromosome 16 was found[66]. Shortly after, the same group identified the first IBD gene variants associated with ileal CD in the *intracellular nucleotide oligomeration domain 2/caspase recruitment domain 15* (*NOD2/CARD15)*[67]. Since then, the input of multiple genetic variations, found by genome wide association studies (GWAS), moved the attention from adaptative to innate immune response displayed by both the immune system and the epithelial barrier. These genetic variants lie within functions that are crucial for intestinal homeostasis, defining major pathogenic pathways involved in IBD susceptibility: epithelial barrier integrity, innate microbial sensing, innate and adaptative immune regulation, microbial defence, ROS generation, autophagy, unfolded protein response, ER stress and metabolic pathways associated with cellular homeostasis.

Over 200 IBD susceptibility loci have been identified, which makes IBD the pathology with the most numerous genetics associations found to date[68], [69]. 163 are associated with both diseases while 37 are CD specific and 27 are UC specific indicating that these diseases engage common pathways, despite their

distinct clinical features and CD having a stronger genetic component than UC[70]–[73]. It is now evident the strong genetic contribution to these pathologies[74], and recent studies have shown that the odd ratio for developing UC or CD increases in direct proportion to the number of risk alleles that each patient carries[75].

Many of these genes encode proteins that are involved in immune responsiveness against infection[68]. Defective innate immunity pathways in the recognition and response to bacteria affect both CD and UC. *NOD2/CARD15* is strongly induced by different inflammatory stimuli, such as Muramyl dipeptide (MDP), found in bacterial peptidoglycan[76], [77]. Its recognition by the leucine rich repeat (LRR) domain of NOD2 leads to the activation of Nuclear factor-kappaB (NF-κB)[78], but mutations at this site result in decreased production of antibacterial defensins by Paneth cells[79]–[83]. The *CARD15* variants also drive altered toll-like receptor (TLR) activation of NF-κB[84]. Patients with these mutations have a reduced early innate immune response that lead to inadequate microbial clearance[84]–[86]. The notion of potentially defective innate immunity pathways in the recognition and response to bacteria is further reinforced by polymorphisms of the *TLR4* gene found in both CD and UC patients[87].

Also related with Paneth cell function[68], [71], [79], [88], [89], an unsuspected role for autophagy in IBD was recently described, implicating two component genes, *ATG16L1* and *IRGM* [90]–[92]. Autophagy is a highly conserved cellular process enables efficient degradation of intracellular content, and typically occurs in response to stress or starvation[93], [94]. Closely related to autophagy and innate immunity, XBP1 variants perturb unfolded protein response that protect against ER stress, which can lead to epithelial cell apoptosis and compromise intestinal homeostasis, as it has been observed in experimental models of colitis[92], [95]. Alterations in NOD2, ATG16L1 or XBP1 activities have all been linked to Paneth cell dysfunction, which may represent a major

convergent pathogenic pathway affecting antimicrobial responses[96]. Interestingly, recent studies have also shown that NOD2 and ATG16L1 variants can also affect DCs by inducing altered autophagy, antigen presentation and intracellular bacterial handling[97], [98].

Ulcerative colitis has shown some unique linkages to genes involved in the regulatory network of epithelial barrier function[99] and the immune response. UC is strongly associated with the polymorphisms in the HLA-DRA gene[100] and some IL-10R signalling components, including *IL10RA* polymorphisms, *SAT3*, *TYK2*, *JAK2* and *IL10* itself[101]. Although CD has lower association with these loci, loss-of-function mutations in the IL-10 receptor result in early-onset IBD with a CD-like phenotype[101], [102].

Different examples of gene variants associated with both CD and UC involved in Th1 proinflammatory immune responses include STAT1, STAT4, IL12B, IFNG, and IL18RAP[68]. Similarly, variations of IL23R, STAT3, RORC, and CCR6, linked with a greater risk in both forms of IBD, are related with Th17 immune response. SNPs in the IL23R gene have been identified and largely replicated in independent cohorts of both CD and UC patients[103], suggesting that the IL-23/Th17 axis might represent a shared inflammatory pathway in chronic intestinal inflammation. Variants of the *IL-23 receptor (IL23R)* gene were in this case protective[104]. Interestingly, the terminal ileum is a site where IL-23-producing DCs and macrophages accumulate[105], which is consistent with the idea that the tissue damage in ileal CD is driven by TH17 cell responses to the local microbiota[79], [106]. Some of these variants can also be associated with the differentiation and function of innate lymphoid cells (ILCs)[107], which are increased in the inflamed intestine in CD but not UC patients[44].

Finally, genetic changes also affect non-coding sequences with regulatory functions. The non-coding single nucleotide polymorphisms (SNPs) in TNFSF15

have shown to confer susceptibility in CD[69], [108], [109]. Interestingly, 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[110].

Environmental factors

Emergence of IBD, as well as other autoimmune and allergic disorders, in developing countries has been associated with the rise of hygienic standards[58]–[60]. This led to 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[111]. In addition, epidemiological studies have suggested numerous environmental risk factors for developing IBD[112]: air pollution, diet, drugs, stress, infections, water pollution, food additives, and lifestyle. These could have a direct effect on epithelial or immune barrier, but it could also affect disease susceptibility by modifying the composition and function of intestinal microbiota.

Specificly, increased environmental pollution correlates with increases in IBD cases [113] and direct evidence support the effect of cigarette smoking [19]. Air-borne toxic particles can reach the intestine via mucociliary clearance from the lungs, and influence gut microbiota composition [114]. The influence of diet[115], [116] is supported by the effectiveness of nutritional therapy in the treatment of CD[117], [118], and monotonous diets have shown to protect agains experimental colitis[119]. Dietary saturated fats may increase numbers of pro-inflammatory gut microbes by stimulating the formation of taurine-conjugated bile acids that

promotes growth of these pathogens[120]. Minor dietary constituents, including polyphenols, catechins, lignin, tannins and micronutrients can also influence both microbial populations and activities[121]–[123]. Among lifestyle, physiological stress and sleep loss or disturbances has been observed to precede a disease flare up[124], [125]. Sleep deprivation dysregulates the immune system, a key pathophysiologic factor in IBD[126]–[128].

EXPERIMENTAL MODELS OF COLITIS

Scientific knowledge coming from studies with tissue samples from IBD patients would provide the most reliable data; however, several difficulties in acquiring human tissue limit the use of human beings when investigating intestinal diseases. Animal models help to circumvent those limitations. They have a genetically homogeneous background, allow the development of interventionist studies and provide the convenience of collecting larger samples of any tissue. Despite that, ethical considerations should never be left aside and, no matters how similar the experimental model might be, animal results need to be supported by human studies in order to acquire reliable knowledge on this disease.

Experimental models of acute and chronic intestinal inflammation have provided invaluable information on the factors and mechanism that govern intestinal inflammation, and they are still a powerful tool to assess novel hypothesis and pharmacological studies[129]–[131]. A variety of animal models are used, from invertebrates to non-human primates. Although no single animal model is perfect, each possess unique features to explore the various aspects of intestinal injury and disease. Rodent species are the most used ones, especially mice. Therefore, subsequent information will be focused on them.

Chemicals are the most common agents used to induce intestinal injury and inflammation. They are fast, economic and effective strategies to cause inflammation and they are often considered the best methods to study the immune response associated in intestinal disease. Chemicals induce intestinal tissue injury by initially disrupting the epithelial barrier, exposing the lamina propria to intestinal contents, and stimulating pro-inflammatory cytokine activity. Each incitant has the ability to induce distinct tissue lesions accompanied by specific helper T-cell cytokine cascades during inflammation[132], [133].

Dextran sulphate sodium, Azoxymethane and haptenicing agents have been especially effective in inducing injury within the distal colon. The effectiveness of inducing colitis depends on several factors, such as molecular weight, concentration, manufacturer, and batch of the chemical[134]. The route of administration also influences the induction and severity of disease, as some chemicals work well to induce inflammation after ingestion [135], while others function best when applied directly to the site of infection, such as the rectal administration of haptenating agents[136]. As mentioned above, chemically induced models are also susceptible to variations according to genetic background, gender and other features of the animals[133], [135] as well as the composition of intestinal flora[137]. Therefore, all those considerations should be taken into account together with the specific features of the intestinal inflammation incited by each chemical agent.

TNBS/DNBS colitis

Trinitrobenzene sulfonic acid is an haptenizing agent primarily used to establish acute intestinal inflammation[138]–[140]. It needs to be solubilized in ethanol to become active, and ethanol also contributes to irritate and damage the epithelial barrier[141]. Then, the TNBS/ethanol mixture produces 'hapten

modified self-antigens' that are recognized by the host immune system and contribute to acute intestinal inflammation[136]. Rats supplemented with TNBS often lose weight, present with bloody diarrhea, and exhibit marked mucosal and transmural intestinal inflammation[142]. Similar intestinal lesions are induced in mice [143] with colon shortening, intestinal hemorrhage, crypt architecture destruction with epithelial necrosis, and transmural inflammation accompanied by an elevated Th1 immune response within the colon[141], [141], [144]. When used to induce chronic inflammation in BALB/c mice, an increase in IL-23 production changes the cytokine profile to a Th17 dominant response before ultimately switching to an IL-13 dominant immune response[40]. However, genetic background and phenotypic profile of the mouse are crutial factors to consider on this model, as well as other considerations, such as the requirement of previous sensitization steps[136]. Despite that, colitis induction not always performs well, being either unsuccessful or lethal. In order to circumvent this and other limitations, other haptenicing agents were tested. The use of dinitrobenzene sulfonic acid (DNBS) showed to produce comparable levels of colonic inflammation compared to the TNBS model[145]. It has been used since then in pharmacological and immunological studies[146], [147].

Oxazolone

Oxazolone administration produce 'hapten-like proteins' in the host intestine and induce body weight loss, diarrhea and intestinal lesions associated with a predominant Th2 immune response. In correlation with the immunolody of UC, IL-13 produced by CD1-reactive natural killer T (NKT) cells, is crucial in the pathogenesis of this experimental colitis, and elimination of NKT cells or neutralization of IL-13 prevents the development of intestinal inflammation[148], [149]. Similarly to in UC, oxazolone injury cause mucosal ulceration with loss of

epithelial cells in the large intestine, submucosal edema, and tissue haemorrhaging[133], [150]. One of its advantages is the quick injury development and rapid progression of tissue architecture alteration in comparison to other chemical agents[40], [133], [150]. BALB/c mice have shown increased tissue injury to oxazolone incitant when compared to C57BL/6 mice[150].

DSS induced colitis

DSS is very useful as a chemical model for UC-like intestinal injury used either independently or in conjunction with other chemicals[151], [152]. DSS incites inflammation by disrupting the epithelial barrier, causing vascular and mucosal injury through the exposure of the lamina propria to luminal contents and bacterial antigens[153]. This exposure triggers the activation of inflammatory pathways resulting in an increased production of the inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-10, IL-12, IFN-γ and IL-17[153], [154]. Factors involved in innate immunity are also affected, such as the expression of MyD88, TLR4 and TLR9[135]. Long-term treatment with DSS increases IL-4 and IL-5 expression, suggesting that DSS induced colitis is mediated by both type-1 and type-2 immune responses[153].

Mechanisms involved in both acute and chronic inflammation can be studied by adjusting the concentration and duration of DSS treatment: oral administration at a concentration of 1–5 % for approximately 1 week induces acute inflammation in the intestine[135], [141]. Many factors affect the susceptibility to DSS-induce inflammation such as genetic backgrounds[135], [155], molecular composition and purity of the DSS among different batches and chemical supliers[133], [134] and bacterial composition. Germ-free mice develop severe colitis whereas conventional mice with microbiota showed only minor

intestinal crypt damage and a relatively non-severe colitis with the same treatment [137], [156].

Chronic inflammation can be obtained by administering DSS for 2 months in cycled rotations of 1 week of DSS treatment followed by 2 weeks of rest[133]. Indeed, recurring administration of DSS can progress to colorectal cancer (CRC), a consequence also observed in IBD patients. The low frequency of dysplastic lesions impedes the extensive use os this model although its association with other chemical incitants can accelerate and increase the effect of DSS. Initial administration of a carcinogen such as AOM or DHM?? followed by repeated cycles of DSS makes inflamed tissue to progress to CRC with 100% incidence. Mechanisms involved in the pathophysiology of AOM/DSS induced CaCRC include increased prostaglandin E2 due to upregulation of cyclooxygenases[157], mutagenic epithelial changes caused by the O6 methylation of guanine[158] and β-catenin, TGF-β and k-ras regulated MAPK pathways[159].

Biological incitants

Biological incitants of different nature can also be used to induce both acute and chronic inflammation. It offers the advantage of using agents that naturally cause inflammation. Most are best used when studying acute inflammation, however helminth and protozoan models are better suited for chronic inflammatory studies.

Genetic models

Genetically engineered mice are particularly important in studying intestinal inflammation[135], [160], [161]. Most of the gene knockout mice have been specifically designed to investigate specific aspects of intestinal inflammation associated with innate and adaptive immune responses. However, a variety of

models are available for study. Defective barrier function in UC allows increased bacterial contact with the intestinal epithelium that triggers immune activation[162], [163]. The most straightforward model for a defective mucus barrier is Muc2$^{-/-}$ mice[164], [165], which develop spontaneous colitis and have luminal bacteria in contact with the intestinal epithelium[162], [166]. Deletion of the regulatory cytokine IL-10 also lead to spontaneous colitis[167], as well as macrophage-specific knockout of IL-10 receptor signalling[168], [169]. In TNFΔARE mice, overproduction of TNF as a result of the deletion of a regulatory element in the TNF locus induce spontaneous inflammation of the distal small intestine and colon[170]. The SAMP1/Yit mouse strain also develop ileal inflammation[171]–[173]. Both of these models display transmural terminal ileitis similar to that seen in patients with Crohn's disease.

Immunocompromised genotypes have also been very useful and include severecombined- immunodeficient (SCID) and Rag−/− mice combined with the supplementation of naive T-cells. These become activated as colitogenic T-cells upon their interaction with antigens, and result in chronic transmural inflammation in both the small and large intestine[174], [175]. Almost all of these models are dependent on the presence of the microbiota, and involve CD4+ T cells producing IL-17 and/or IFNγ[176].

MICROBIAL MISBALANCE

Homeostatic interactions between the host and the resident microbiome occur in the intestine. Commensal bacteria are important in maintaining a healthy intestine by preventing the overgrowth of pathogenic microorganisms and maintaining a quiescent intestinal immune system[177]. However, in certain circumstances, the increased exposure to the commensal bacteria can lead to an

uncontrolled immune response and intestinal injury [22], [178]. Moreover, modifications to the community structure of the intestinal microbiome can incite disease, often by the uncoordinated expression of pro-inflammatory cytokine profiles in concert with the simultaneous loss of anti-inflammatory signalling[178]–[180].

Intestinal inflammation in IBD patients mostly affects distal small intestine and colon, the areas with the highest bacterial concentrations, which is consistent with the idea that they reflect aberrant inflammatory responses to commensal bacteria[57], [62], [63], [79], [181]–[183]. As commented above, the association of IBD genetic susceptibilities with genes involved in bacterial recognition and the resultant inflammatory cascade highlights the importance of microbiota-host interactions in IBD[184]. The role of intestinal bacteria is further supported by studies in animal models of spontaneous colitis, which fail to develop intestinal inflammation when raised under germ-free conditions[185], [186], whereas spontaneous colitis occurs when commensal bacteria are reintroduced in the intestine[167], [187]–[190], and purified bacterial products have also been able to initiate and perpetuate experimental colitis[191].

Both forms of IBD have been associated with an imbalance in the composition of the intestinal microbiota[192]–[198]. Whether this dysbiosis represents a primary or secondary predisposing factor is still unresolved. Recent studies have indicated that dysbiosis is influenced by both the host genotype[199] and IBD phenotype[200]. Microbiota is dominated by 4 bacterial phyla in most mammals: *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*[201]. In patients with IBD, loss of diversity has been commonly observed within the *Bacteroidetes* and *Firmicutes*, the most prominent phyla, while it has also been reported an increase in diversity and abundance of *Proteobacteria* phylum during IBD related infections and acute enteric infections[202]–[204]. Metabolically, this represent a shift in abundance of species that utilize

carbohydrates to species that utilize proteins for energy[177], [205]. Decreased abundance of bacteria able to produce butyrate has also been observed in IBD[194], [200], [206], [207], which can be linked to species within the class *Clostridia,* implicated in immune development and maintenance of intestinal homeostasis through induction of TGFβ and promotion of FoxP3+ Treg differentiation[116], [194], [208], [209]. Aditionally, it has been reported that Butyrate signals induce IL-18 expression in IEC and this inhibited colitis-associated colon cancer (CAC)[210], [211]. Bifidobacterium-derived acetate can also promote antiapoptotic responses in IEC, which also account for a protective barrier effect[212] SCFAs can also act on neutrophils with notable proresolving effects on inflammation[213].

A generally accepted principle is that immunological tolerance to commensal bacteria is lost in patients with IBD[214], [215], being supported by the presence of serum antibodies against a variety of microorganisms[216]. The unsolved question is whether the immune response is directed against the gut microboiota as a whole or towards specific microbes or subgroups of bacteria. At present, No specific associations have been established that might account for initiation of intestinal inflammation or the distribution along the gastrointestinal tract[217]. In IBD patients the number of bacteria associated with the mucosa layer is dramatically increased[218], [219]. Recent studies highlighted that intestinal inflammation can confer a selective growth advantage to certain pathogens, including *Salmonella typhimurium*[220] or *Ruminococcus* strains, and they may contribute to the a defective barrier protection. Mucin glycan-degrading bacterial could accelerate mucin release and reduce mucus layer protection[221], which is supported by the reported increase of *Ruminococcus gnavus* and *R. torques* that have been detected in IBD patients. Other example that supports the pathogenic role of microbiota in IBD is related with adherent-invasive Escherichia coli (AIEC), mostly found in the small intestine and associated to the mucosa of

patients with ileal CD[222], which exploits host defects in phagocytosis and autophagy to promote chronic inflammation in the susceptible host[223], [224]. Similarly, *Mycobacterium avium* spp. *paratuberculosis* and their potential association with CD has also been studied[225], [226].

While aggressive bacteria increase, IBD patients have reduced diversity and abundance of protective bacteria, including lactobacilli and bifidobacteria[197], [219], [227], [228]. Commensals belonging to the *Clostridiales* order, such as *Faecalibacterium* and *Roseburia*, have been also reported to be significantly reduced in patients with ileal CD[199], [200]. Of note, these genera are potent sources of SCFA, with protective effects as stated above. However, a direct immunomodulatory activity has also been observed in these bacteria. Selective reduction of *Faecalibacterium prausnitzii* is found in UC[229], a bacteria that stimulates IL-10 production in peripheral blood mononuclear cells[230]. Finally, polysaccharide A from *Bacteroides fragilis* has shown to suppress IL-17 production and promote the activity of IL-10-producing CD4+ T cells in mice, promoting an anti-inflammatory status[231].

EPITHELIAL BARRIER

Host defence requires an accurate interpretation of the microenvironment and a precise regulation of the responses, and the intestine epithelium provides the first line of defence. IECs constitute a solid physical barrier, but they are also involve in sampling of the intestinal microenvironment, sensing of both beneficial and harmful microbes, AMPs (antimicrobial peptides) secretion and induction and modulation of immune responses in underlying lamina propria[232]. Intestinal epithelial cells respond to signals derived from the microbiota by means of their expression of PRRs. Generally, commensal

organisms are tolerated through the development of numerous mechanisms, such as masking or modification of microbial-associated molecular patterns that are usually recognized by TLRs[233] and NOD/CARD[234], and the inhibition of the NF-κB inflammatory pathway[235]. However, a defect in intestinal epithelial barrier can lead to persistent activation of the immune system. 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[232], [236].

Antimicrobial peptides

Antimicrobial peptides (AMPs) are retained in the mucus matrix to confer protection against pathogenic bacteria and control the composition of the gut microbiota[237]. Some of these AMPs, such as lysozymes, β-defensins, cathelicidins, lipocalins, C-type lectins and secreted PLA_2 are constitutively produced and secreted by IEC, as well as by Paneth cells stimulated by TLR and NOD2 signals triggered by commensal microbiota[238], [239]. In fact, Paneth cell dysfunction and impaired defensins secretion is observed in CD patients[81], [240] and in mice deficient in several CD-associated genes, which contribute to disease susceptibility[90], [92], [96], [241], [242], [243, p. 8], [244]–[246].

Mucus layer

Defective mucus layer protection is another common feature of human IBD. Decreased levels of goblet cells lead to reduced mucin secretion[162] and an impaired mucus layer allows bacteria to penetrate and directly contact with the epithelial surface, thus inciting to intestinal inflammation [166], [247], [248]. This is observed in mice lacking MUC2, the major mucin protein, which develop spontaneous colitis and have an increased risk of colorectal cancer[164], [165], [249]. Reduced MUC3, 4 and MUC5B has been also observed in the uninflamed

ileum of CD patients[250]. Therefore, reduced protection of this layer could lead to the initiation of the inflammatory response.

Upon activation of intestinal inflammation, marked reduction of mucus-filled goblet cells is commonly observed. This could be liked to induction of apoptosis and detachment of goblet cells by inflammatory signals, such as IFN and TNFα. However, their rapid reappearance after remission suggests that the impact of inflammation mainly cause the exhaustation of goblet cells by accelerated exocytosis of their content to overcome the pathogenic invasion. Additionally, autophagy plays an essential role for gobblet exocytosis, and defects in this pathway could also account for increased susceptibility in IBD patient[251]. Another mechanism involved in the loss of mucus layer protection in inflammation is the release of proteases that cleave mucins, wich can be activated by certain bacteria[162]. All these factors contribute to reduced mucus layer in intestinal inflammation, which additionaly reduce the protection of AMPs and sIgA, transported out of the intestine with fecal stream[251].

Cell monolayer

Epithelial barrier integrity is maintained by tight junctions, adherens junctions and desmosomes that form the intercellular apical junction complex. Increased intestinal permeability has long been observed in patients with both CD and UC, associated with reduced expression of their components[252]. GWAS have identified defects within some of the genes involved in epithelial barrier integrity[253]. This suggests that abnormal intestinal permeability may also represent a primary pathogenetic mechanism. The genetic susceptibilities associated with IBD affect to diferent effectors including: 1) HNF4A[254], [255], a transcription factor that regulates the assembly of the apical junction complex and crypt cell proliferation; 2) E-cadherin[256], a main component of adherens junctions and a key mediator of epithelial intercellular communication; 3)

LAMB1, a laminin expressed in the basal membrane of the intestinal epithelium; 4) the GTPase Gα12 involved in tight junction formation through interaction with ZO-1 and Src[257]; and 5) the tyrosine phosphatase PTPN2, which protects against IFN-γ-induced epithelial permeability, increasing the susceptibility to experimental colitis when absent[258], [259]. Defects in epithelial regeneration have also been identified, such as the above mentioned HNF4A, NKX2-3, a transcription factors that controls IEC differentiation[260]; or STAT3, whose deletion in IECs affects epithelial repair[261].

Sensing and communication

The IEC sensing of commensal microbiota through PAMP is required to induce many of these protective functions, as well as to control the release of mediators that recruit and activate immune cells[262]–[264]. TLR4 and CD14, involved in LPS signalling, are expressed at higher levels in the colon than in the small intestine [265], [266]. TLRs activation is required to recover from epithelial injury following DSS administration by secretion of cytoprotective factors such as IL-6, TNFα, keratinocyte chemokine-1 and heat shock proteins[267]. Reg3γ regulate IEC proliferation and is also increased upon mucosal damage[268].

In response to microbiota, IEC influence recruitment, activation and differentiation of immune cells through the production of modulatory factors[269]. Thus, the ctivation of IECs induce the secretion of IL-7 and IL-15, that regulate the proliferation of IEL, ILCs and lámina propria T cells. IEC release of NLRP3 inflammasome-mediated IL-18 has demonstrated protective effects against DSS-induced colitis and colon cancer[270], [271]. They also secrete IL-25, IL-33 and TSLP. These promote Th2 inflammation and tissue repair at barrier surfaces[272]. Some cytokines, such as TSLP, IL-25 and TGFβ, are constitutively expressed by IECs, although synthesized at low level in IBD. They have shown to limit CD11b+DCs production of IL-12/IL-23 p40 and promote IL-10 secretion,

favouring the induction of Treg and Th2 cell responses[232], [273], [274]. IL-25 has both preventive and curative effects in murine models of colitis[275].

Conversely, after sensing pathogenic invasion or damage, IECs secrete proinflammatory chemokines, such as IL-8, which have an important role in alerting the immune system to microbial attack[276]. Finally, as mentioned previously, IECs have key influence on local antibody responses by release of TGFβ, BAFF and APRIL, which induce IgA- class-switching on B cells [277] and mediate the transport of secretory IgA into the mucus layer, where it limits the penetration of commensal bacteria[277], [278].

IMMUNE RESPONSE

Dysregulation in innate and adaptive immunity contributes to the development of IBD. Loss of barrier function lead to increased contact between host microbiome and the immune compartment. In this situation, the normal immune regulation is overwhelmed, result of a dysfunction in the regulatory pathways[28]. Multiple components of the mucosal immune system are implicated in the pathogenesis of IBD. Immune response is initiated by innate players, such as macrophages, ILCs and effector neutrophils. Then, antigen-presenting cells can mediate the differentiation of naïve T-cells into effector T helper (Th)[214], [279]. Traditionally, CD and UC have been viewed as predominantly T-cell-driven processes; however, more recent evidences suggest that innate immune responses play an important role[63]. Patients with innate immunodeficiency tend to develop IBD and patients with CD have defective innate immune responses, including attenuated macrophage activity in vitro, as well as impaired neutrophil recruitment and bacterial clearance in vivo[280].

Innate immunity

The innate immunity provide a rapid non-specific response, our first line of defense against pathogens. In addition to **intestinal epithelial cells** and **myofibroblasts, innate immune cells** sense the intestinal microbiota and respond to **the recognition of PAMPs** in a stereotypicalmanner. This initiates the rapid and effective inflammatory responses against microbial invasion. It also coordinates the activation of the adaptive immune response to intestinal environmental antigens[281], [282]. It comprised macrophages, monocytes, DCs, neutrophils, eosinophils and basophils. In addition, other innate leukocyte populations, including ILCs γδ T cells, natural killer T (NKT) cells and NK cells, can secrete Th1- and Th17-derived cytokines such as IFNγ, IL-17A and IL-22, thus also contributing to intestinal inflammation[283]–[286].

Monocytes and Macrophages

Macrophages are master regulators of the homeostatic condition on the healthy intestine. As summarised in before, they have adaptations to prevent excessive inflammatory responses, including expression of inhibitors of NF-κB signalling that allow bactericidal activity in the absence of proinflammatory cytokine production[287] and their release of IL-10, which contributes to the maintenance of Treg colonic pool[288]. However, in inflammatory conditions, in both human and experimental models, it has been observed an increase accumulation of intestinal mononuclear phagocytes (iMP), macrophages and DCs, displaying an activated phenotype. Enhanced responsiveness to PRRs and their excesive activation in these conditions contribute to intestinal pathology through the potent pro-inflammatory effects of their cytokines[33], [289], [290]. In mouse models of acute and chronic colitis, increased recruitment of monocyte-derived DCs and macrophages that produced IL-12, IL-23, IL-6 and TNF-α has shown to drive the inflammatory process[291]–[293]. This has also been observed

in the mucosa of CD patients, and contribute to the production of IFNγ by local T cells[43].

Recently, Bain et. al provided a more detailed description of the process involved in macrophages recruitment and perpetuation of intestinal inflammation[294]. On the context of intestinal inflammation, recruitment $CCR2^{+}$ $CX3CR1^{+}$ $Ly6C^{hi}$ monocytes to the lamina propria is acelerated by means of increase expression of CCL2 as well as other chemokines and cell adhession molecules in the vasculature bed at the inflammed tissue/site. This process also takes place in the steady state, however, conditioned by the inflammatory milieu, these monocytes fail to acquire quiescence, a charasteristic feature of intestinal resident macrophages. Therefore, the monocyte-macrophage differentiation waterfall is arrested, favoring the accumulation of inflammatory macrophages that outnumber the resident population. These inflammatory macrophages conserve $Ly6C^{hi}$ expression, lack of MHC II marker and produce high amounts of inflammatory mediators, such as IL-1β, IL-6, IL-12, IL-23, TNFα and iNOS, in response to TLR and NLR agonists[294].

Secretion of IL-6 and NO by macrophages may influence epithelial permeability, increasing pathogen invasion[295]. IL-1β and IL-23 production in models of bacterially triggered IBD exacerbated pathology by promoting acumulation of IL-17 producing innate and adaptive leukocytes[106], [296]. IL-1β production in colonic lamina propria has been linked to response to NLRC4-triggering pathogens, but not commensals[297]. However, production of IL-1β signals upon TLR and NLR activation of CX3CR1+ mononuclear phagocytes under homeostatic condictions drive the differentiation of protective Th17 responses, and it has also been observe to reduce Th17 induction by CD103+CD11b+ DCs[298]–[301]. Thus, kinetics and context may determine whether iMP-drived Th17 response mediate protective or pathogenic effects. Interferon regulatory factors (Irf)-4 and 5 are associated with classical and

alternative macrophage activation, respectively[302]–[304]. Intestinal resident macrophages, as highly phagocytic cells, clear apoptotic cells and debris and contribute to wound repair of the epithelium[287], [305].

Dendritic cells

DCs are professional antigen presenting cells, representing key players in the crosstalk between innate and adaptive immunity. They are involved in induction of tolerance as well as mediate inflammation[306]. $CD103^+$ DCs, dispersed throughout the lamina propria, take up bacterial antigens and apoptotic intestinal epithelial cells (IECs). After maturation, they migrate to lymphoid locations, where they initiate adaptive responses focused on the intestine, preferentially inducing Foxp3+ Treg cells[281], [290], [307].

However, increased recruitment and activation of DCs in inflammation polarize their previous tolerogenic balance to the generation of effective/effector innate and adaptative immune responses. Different subsets have been idenfitied using overlapping as well as different phenotypic markers, which makes sometimes difficult to pool the conclussion arised in each study. $CD11b^+CD103^+$ DCs are the main migratory subset involved in priming Th17 responses on the intestine, in the steady state as well as inflammation[308]. Bacterial lagellin stimulation of $TLR5^+$ $CD103^+$ DCs in the small intestine promote Th17 differentiation and secretion of IL-23, which induces IL-22 production by ILC3 and subsequent epithelial upregulation of antibacterial peptides[309], [310]. Tolerogenic $CD103^+$ DCs acquire inflammatory properties in experimental colitis, accumulate in the MsnLNs and express RALDH and TGFβ, driving Th1 responses[311].

In response to microbiota-derived signals, $CX3CR1^+$ DCs accumulate adjacent to the intestinal epithelium where they sample antigens and bacteria[281], [290], [312]. Colonic Th17 responses are promoted by this subsets in

response to commensal-derived ATP[290]. Similarly, the pro-inflammatory DCs subset expressing E-cadherin promotes Th17-cell differentiation[292]. Monocyte-derived dendritic cells expressing $CD11b^{+}CD103^{-}CCR2^{+}CX3CR1^{+}$ [313] are also generated on inflammatory conditions. They can express CCR7 and migrate in the intestinal lymph, where they contribute to induction of pathogenic Th17 and Th1 immune responses via secretion of IL-12/IL-23p40[314], [315].

Neutrophils

Neutrophils are important innate effector cells, clearly involved in the inflammatory process that occurs in IBD. Neutrophil infiltrate and accumulate within epithelial crypts and in the intestinal mucosa, which directly correlates with clinical disease activity and epithelial injury. Activated neutrophils produce proinflammatory cytokines, but also reactive oxygen and nitrogen species, and myeloperoxidase, which induce oxidative stress that participates in the intestine damage associated to inflammation[316].This action contributes to maintain inflammation by inducing redox-sensitive signalling pathways and transcription factors[317]. Moreover, several inflammatory molecules generate further oxidation products, leading to a self-sustaining and autoamplifying vicious circle, which eventually impairs the gut barrier. However, neutrophils may also contribute to the resolution of inflammation by synthesis of anti-inflammatory mediators such as lipoxin A4. Impaired secretion of lipoxin A4 in mucosal tissues from UC patients support the relevance of such mechanisms in IBD[318].

Innate Lymphoid cells

Other cells with an innate immune function are also activated on inflammatory conditions, mainly by IL-23, a key cytokine in driving early responses to microbes and orchestrating the crosstalk between innate and adaptive immunity. Unconventional, innate-like T cell populations are particularly represented at mucosal sites, such as γδT cells, invariant natural

killer T (iNKT) cells, mucosal associated invariant T (MAIT) and innate lymphoid cells (ILCs). They respond to IL-23 stimulation, secrete Th1 and Th17-related cytokines, such as IFNγ, IL-17a and IL-22, and induce the recruitmen of other inflammatory cells[283], [284], [284]–[286], [319].

ILCs play an important role in mouse models of intestinal inflammation[320], [321], and IL23-responsive ILCs have also been identified in the human mucosa[322]. Intestinal inflammation in innate models of colitis is drived by ILCs through secretion of IL-17A or IFNγ depending on the experimental model[323]. Selective accumulation of CD56-cKit-Nkp44- ILC, which express IFNγ and T-bet, has been found in the inflamed intestine of CD patients[324], as well as increase in IL-17A-producing CD56− ILCs[44].

Adaptive immunity

The adaptive immune system is highly specific and it confers long lasting immunity. Normally their T cell mediated and humoral responses cooperate with the molecules and cells of the innate immune system to mount an effective immune response, which is capable of eliminating the invading pathogens. However, when barrier protection is loss and inflammatory response is perpetuated, the adaptive immune response contributes to the breakdown of immunologic tolerance to commensal bacteria in the intestine[214], [279].

T cells

Dysregulated T cell response may lead to the onset of inflammation by an excessive release of cytokines and chemokines and their multiple pathogenic effects on the immune system. Based mainly on the levels of T cell-derived

cytokines detected in IBD mucosa, several studies have associated CD and UC to different subtypes of pro-inflammatory immune responses.

Th1 and Th2

Crohn's disease has a predominant Th1 type cytokine profile, with increased mucosal levels of IFNγ, IL-2 and TNF-α. Infiltrating T cells also express Stat4 and T-bet, two transcription factors required for Th1 differentiation[325], [326]. This signature correlates with increased MΦ and DCs producing the Th1 induced IL-12[36], and other molecules, such as osteopontin, IL-15 and IL-18, which amplify Th1 cell responses[36], [327]–[329]. In ulcerative colitis, early studies suggested a role for atypical NK T cells releasing high amounts of the Th2 cytokine IL-13[33], [330], [331]. Elevated production of other type 2-like cytokines, such as TGFβ and IL-5 (but not IL-4, the other prototypic Th2 cytokine) was also detected, which led to associate UC with a predominant atypical Th2 response[330]–[333]. These cytokines are potent in vitro stimulators of intestinal mucosal effector functions, including T cell and macrophage proliferation, adhesion molecule and chemokine expression, as well as the secretion of other proinflammatory cytokines, thus generating the vicious circle that maintain the inflammatory response[186], [334]. However, regarding UC, data obtained in experimental colitis have suggested an anti-inflammatory effect of IL-13 in the gut[335]–[337], while other study showed a predominance of IL-6 and absence of IL-13 in supernatants of UC biopsies cultured ex vivo[338].

Th17

Identification and characterization of the new Th17 subset changed de Th1/Th2 paradigm in IBD[339], [340]. The microbiota has an important role in the preferential localization of Th17 cells in the gut[341], [342], where they play

an important role in host defence against extracellular pathogens, which are not efficiently cleared by Th1 or and Th2-type immunity. Th17 cells were detected in the mucosa of both CD and UC patients, as well as their secreted cytokines IL-17A, IL-17F, IL-22 and IL-26[38], [343]–[347]. Th17 cells are induced by a combination of IL-6 and TGF-β, and their expansion is promoted by IL-23[348]. IL-21 produced by Th17 cells in turn increases their expression of the IL-23 receptor, therefore potentiating the expansion of this cell subtype by a positive autoregulatory feedback loop[346]. Regarding the relative enrichment of Th17 cells at mucosal sites, together with the increased levels of Th17 cytokines in the inflamed gut[285], [349], tissue destruction might therefore actually be mediated by these Th17 cells subset[350], [351]. Indeed, these cells are involved in the proliferation, maturation and chemotaxis of neutrophils, thus contributing to the pathogenesis of these intestinal conditions[352], [353]. However, IL-17A have shown to have both pro-inflammatory or tissue-protective effects in the gut depending on the model used[354], suggesting once more that, in the complexity of intestinal inflammation, the effect of a given mediators is determined by an adecuate time-context balance.

Tregs

In addition to potentially pathogenic role of T-helper subsets, reduced regulatory function exerted by Tregs could contribute uncontrolled inflammation in IBD. Treg cells are abundant in the intestine[355]. Where they monitor the immune response and are crucially involved in the maintenance of gut mucosal homeostasis, preventing excessive and potentially harmful immune activation[356], [357]. Tregs exert their suppressive function by producing the anti-inflammatory cytokines IL-10 and TGF-β[358]. These cells are able to suppress Th0 cell proliferation both *in vitro* and *in vivo*[359] and prevents both the activation and the effector function of T cells that have escaped other

mechanisms of tolerance. Studies in IBD patients found that peripheral blood Tregs are depleted in active compared to quiescent IBD and control subjects [360]–[362]. Conversely, increased Treg can be found in the intestinal mucosa of IBD patients[363]–[365]. These showed a normal function, however, effector T cells in the lamina propria of IBD patients were unresponsive to the action of Treg. TGF-β signalling is impaired in inflamed IBD mucosa because of the up-regulation of the inhibitory molecule Smad7, required for Treg function[366], [367]. Thus, the response to Treg suppressive activity was reversed by an anti-Smad7 antisense oligonucleotide[368]. Therefore, decreased anti-inflammatory activity of Treg may be equally as important as the exacerbated effector response in contributing to perpetuate intestinal inflammation.

Tregs are characterized by expression of CD4, CD25 and Foxp3, although a population of Foxp3− IL-10-secreting CD4+ T cells is also particularly important in the intestine[355]. Foxp3+ Tregs are usually generated in the thymus, but the intestine is also a preferential site for TGFβ-dependent induction of Foxp3+ Treg cells[355] where they control potentially deleterious responses to dietary and microbial stimuli[356]. Microbiota have a role in promoting intestinal Treg responses, since their accumulation in the colon is reduced in germ-free mice and can be increased by particular indigenous bacteria[369]. Their crutial relevance if highlighted by studies where a deletion or loss-of-function mutations in the gene encoding Foxp3 result in inflammatory disease in mice and humans, often accompanied by intestinal inflammation[355].

Plasticity

T cell subsets show plasticity, certain subset can swap one in another, providing with adaptability to different requirements. Induced Treg and Th17 populations seem to be reciprocally regulated in the intestine. Although TGFβ is required for the differentiation of both populations, the presence of STAT3-

mediated signals (such as IL-6 or IL-23) promotes Th17 cells at the expense of Foxp3+ Treg cells[349], [370], [371]. This mechanism allows the inflammatory response to override Treg 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[372]. This system is delicately balanced but sometimes can lead to deregulation. For example, high-level T-bet expression in the presence of acute intestinal infection drives Treg cells into an inflammatory IFN-γ-secreting phenotype[373]. 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[33], [374].

B cells

Interaction between T and B cells leads to the production of antibodies upon the contact with T cell or DC[375], [376]. Humoral homeostasis is disrupted in IBD. The intestinal lamina propria in active IBD shows an increased presence of antibody-secreting plasma cells, which correlates with changes in the quality of non-inflammatory IgA responses, enhanced pro-inflammatory IgG production[377] and, in some cases, augments B cell expression of the pro-inflammatory cytokine IL-8[378], [379]. Monomeric IgA production is increase in the inflamed mucosa, despite this form is normally predominant in the circulation[377], [380]. These abnormalities are also present in blood, finding systemic antibody responses to various autologous and microbial antigens[381] which predates the clinical diagnosis of IBD and identifies patients with negative disease course[382]–[386]. Among the production of autologous antibodies, the presence of anti-GM-CSF in patients with CD is associated with ileal phenotype as well as a complicated behavior of the disease [387]. NOD2 KO mice treated with anti-GM-CSF antibodies develop transmural ileitis, emphasizing the crucial

role of this autoantibody in the pathophysiology of ileal CD[387]. Intrinkingly, UC and CD differ in the patterns of IgG antibody class production: in UC, secretion of IgG1 is disproportionally increase while CD involve all IgG subclasses, with IgG2 being predominant[388]. Limited attention has been given to B cells in IBD however, and negative results obtained in clininal trial for B cell depletion with the CD20-targeting rituximab[389] has not ascertained whether B cell antibody responses play a pathogenetic role[379].

The protective action of activation of B cell responses is supported by animal studies, where B cell intrinsic TLR activation and MyD88 signaling was required to provide protection from microbial dissemination following DSS-induced intestinal damage. However, this protection was mediated by IgM, not IgA, involved in mucosal protection on the steaty state[390]**.** Although their main function is the antibody production, B cells can also act as antigen presenting cells. Intestinal DC generate primary T cell responses in lymphoid tissues, whilst MΦ and B-cells contribute to polarization and differentiation of secondary T cell responses in the gut lamina propria[391]. Finally, B-1 cells have recently emerged as a first line of defense by neutralizing a wide range of pathogens and are also import regulatory players of acute and chronic inflammatory diseases by means of producing immunomodulatory molecules, such as IL-10, adenosine, GM-CSF, IL-3, and IL-35[392]. IL-10 and TGFβ produced by B cells with regulatory capacity, known regulatory B cells (Breg) is important for the generation of mucosal Tregs[393]–[399]. Bregs have shown to be able to suppress experimental colitis[400]–[402], highlighting the potential contribution of their regulatory properties.

CHAPTER II
The treatment

THERAPEUTIC ARSENAL

*S*uch a complex disease like IBD may not have a simple solution. Indeed, to date, no curative treatment has been found due to the still unknown aetiology. Current therapeutic strategies are aimed to improve patient quality of life by inducing and maintaining remission, preventing and treating complications and restoring nutritional deficits[34], [403]. The clinical course of IBD varies from mild to a chronic active form, in which remission is only reached by permanently taking medication or by taking it for a long time[404]. As a result, patients are not only burdened by IBD symptoms, but also by the side effects of the drugs they take to treat those. This, together with the inconvenient dosing schedule and/or prohibitive price in some cases, limits their long term use[405]. Therapeutic strategies for IBD patients use to associate drugs with interventions on life-style habits (). Despite those efforts, many patients remain non-responders to medical treatment and need surgical interventions. The development of new therapies that combine efficacy, convenient dosing and lower side effects is an important target in human IBD therapy. For this reason, a wide variety of drugs are currently under the scope of.

The medical management of IBD includes mainly anti-inflammatory drugs, immunosuppressant agents and biologic therapies[34]. It is noticeable that Genetic and immunological variations driving the onset of the disease seem to have a small or non-existent effect on response to treatment once inflammation is stablished[406]. However, differences in clinical response to these drugs also evidences disease heterogeneity. Current guidelines try to classify patients by

distinguishing the course, place and severity of the disease in order to select the most appropriate treatment individually[404], [407], [408]. IBD therapy traditionally initiates with mesalazine and corticosterois, then immunosupressants such as azathioprine or, in UC, calcineurin inhibitors such as cyclosporine A and tacrolimus, and finally the biological agents targeting TNFα, following an step-up aproach[409], [410].

A step-down therapy, using the most effective treatment in order to reach an effective remission as soon as possible, has also been proposed in the field of IBD[404], [406]. While some patients develop serious complications, others may have an indolent course. The identification of biomarkers able to predict poor outcome in IBD would enable patients with worse prognosis to receive more potent therapies, whereas those with milder disease would avoid unnecessary immunosuppression[406].

Therapeutic strategies for IBD patients use to associate drugs with interventions on life-style habits such as nutrition. For example, patients with severe UC or CD benefit from short term parenteral or additional high calorie nutrition[411]. An adequate balance of trace elements and vitamins is also essential for successful therapy[404].

Despite those efforts, many patients remain non-responders to medical treatments and need surgical interventions. The progression of intestinal lesions in CD may lead to the development of complications such as fistulae, abscesses, and strictures. These might require surgical intervention and a protective ileostoma can be very useful in supporting conservative treatment. Likewise, The proximal extent of disease in UC tends to progress and refractory UC can only be successfully treated by an operation (e.g. a colectomy with an ileoanal pouch anastomosis)[404], [412]. In fact, within 7 years of initial diagnosis, the resection rate for CD is at 29% and colectomy rate in UC is 12.5%[413].

5-Aminosalicylates

5-aminosalicylic acid (mesalazine) formulations and its oral pro-drugs (sulfasalazine, olsalazine and balsalazide) function as anti-inflammatory drugs, in which their free radical scavenging properties seem to play a role. They are considered as bowel-specific drugs, since the release of the active moiety, 5-ASA, essential for the therapeutic effect, takes place in the gut lumen upon the action of the intestinal microbiota.Aminossalicylates are recommended for mild to moderate UC with an oral dosage of no less than 3 g/d. In left-sided disease, intrarectal formulations (like enema, foam or suppositories) are more effective than the oral route. In contrast, aminosalicylates are not very successful in maintaining remission in CD [414], [415]. They are generally well tolerated. Most common side-effects, such as headache, are not severe. However some cases of nephritis, pancreatitis and hair loss have been reported and therefore nephritic and hepatic monitoring is required[404].

Corticoids

Corticoids are the anti-inflammatory drugs per excellence. In patients with moderate to severe UC or CD, corticosteroids are effective for the induction of remission[416]–[418]. Before the initiation of steroid treatment, the presence of an abscess should be excluded. Parenteral administration of corticoids in severe disease as soon as possible is key for an anti-inflammatory response. Among them, budesonide achieves the best anti-inflammatory effect in ileocecal inflammation due to a special structural formulation[419]–[422]. Starting doses of 40-60 mg/d or 1 mg/kg per day orally should be tapered after inducing remission and no corticosteroid should be used for maintenance therapy due to the side-effects (e.g. Cushingsyndrome, osteoporosis or cardiomyopathy)[404], [423], [424].

When considering corticosteroid therapy, the clinical course in IBD patients has been defined as steroid responsive (44% of patients), steroid-refractory (36%), and steroid-dependent (20%), in whom disease recurs upon steroid interruption [425]. Patients with chronic active IBD and those classified as steroid-refractory are eligible for the use of immunomodulators to control inflammation and maintenain remission.

Immunosupressants

Purine antagonists: azathioprine & mercaptopurine

Azathioprine and its metabolite, 6-mercaptopurine, are immunosuppressive drugs with similar efficacy in the longterm use to treat chronic active disease Therapeutic doses are 2-3 mg/kg/day and 1.5 mg/kg/day respectively). 6-mercaptopurine is metabolized to thioguanine, the active metabolite, which is then incorporated and exerts an anti-proliferative effect on mitotically active lymphocytes [426].

Severe bone marrow toxicity with purine antagonists has been related to homozygous deficiency in thiopurine methyltransferase (TPMT), and patients should be genotyped for it before the initiation of this treatment. In addition, they can affect xxxxxx, therefore white blood cells count and liver enzymes should be monitored in all patients[427]–[429].

Folate analogues: Methotrexate

Methotrexate is another immunosupressor agent, structural analogue of folic acid, that interferes with nucleotides and DNA synthesis[430]. In chronic active

disease, remission is induced with a dose of 25 mg i.m. per week for 16 weeks, followed by a maintenance treatment of 15 mg i.m. per week. Its use in UC has provided mixed results, although it is considered in refractory or steroid-dependent patients that fail to respond to purine antagonists[426], [431].

Calcineurin inhibitors: cyclosporine and tacrolimus

Cyclosporine is a cyclic peptide that forms a complex with cycliphilin and inhibits calcineurin, a phosphatase involved in activating proinflammatory transcription factors[426]. By this mechanism, cyclosporine reduces lymphocyte proliferation and cytokine production[432]. Its use is reserved for the treatment of severe steroid-refractory cases of UC to avoid a colectomy, for which 2-4 mg/kg are administeresd intravenously. In CD, cyclosporine has been shown to be effective only in fistulizing, but not luminal disease[433]–[435]. Tacrolimus is a macrolide antibiotic with a similar mechanism of action to cyclosporine. It acts as a calceneurin inhibitor, leading to decreased IL-2 production and lymphocyte proliferation[436]. It has shown to improve fistula drainage, but not closure, although scarce data is available regarding this treatment[437].

Biological therapy

Immunological studies describing the different pathways involved in intestinal inflammation in IBD promoted the development of therapeutic strategies specifically targeting many of these mediators. Although very promising, most of the strategies developed to date have shown rather dissapointin effects, with the exception of anti-TNF therapy[438].

Anti-TNFs

Anti-TNF agents play a pivotal role in the treatment of chronic active IBD, fistulizing disease and those not responders to immunosuppressive therapy[439]–[441]. Infliximab, the first on the market, shows efficacy inducing clinical response and remission in half of patients with active CD and one third of UC patients. Therapy initiates with three infusions of infliximab (5 mg/kg i.v.) at 0, 2 and 6 weeks, followed by a maintenance treatment at the same dose every 8 wk.[442]–[444]. Contraindications and side-effects include the formation of human anti-chimeric antibodies, which occurs in 30-75% of the patients, and can be reduced by concomitant administration of immunosuppressants. Acute allergic/anaphylactic reaction or delayed hypersensitivity to the infusion have been reported, and cases of reactivation of infections, such as tuberculosis, have also been observed in clinical trials [445], [446].

Failure of biological therapies: "stratify and conquer"

Despite the several factors that contribute to the onset of IBD, once the disease is established, heterogeneity may not remain and mucosal inflammation is sustained by limited pathways. The majority of CD lesions are dependent on TNFα[447] and in primary non-responders to anti-TNF therapy theirlesions may be driven by other proinflammatory mediators. This is supported by the higher efficacy shown by ustekinumab (anti-IL-12p40) and vedolizumab (anti-β7 integrin) in anti-TNFα refractory patients[448]–[450].

Overall, many reasons may lay behind the failure of most biological therapies[406]. First of all, targets of biological therapies may not be involved in driving IBD lessions or the response may be lost due to compensatory pathways. Most clinical trials have not stratified inclusion and therefore, although treatment may be effective in subsets of patients, they failed their primary endpoints. Development of antidrug antibodies, which neutralise their actions and

accelerate their clearance[451], [452] can also lead to ineffectiveness of the antibody. Pharmacokinetics could be also influenced by other factors, such as sex, body size, serum albumin levels, and disease characteristics [453] leading to disappointing results when doses where fixed instead of based on patient's parameters[454]. Thus, stratification based on early combined measurements of diagnostic markers, the parameters mentioned above and the presence of antidrug antibodies could predict the response to the different biological therapies in IBD[406], [455] .

Antibiotics

Previous therapeutic strategies are focused on the immune system, despite important alterations in IBD also affect the other protective barriers. Changes in the microbial ecosystem have been associated with the development and evolution of intestinal inflammation, representing another therapeutic target in the management of this condition. [193], [194], [198], [456]–[460]. The composition of the gut microbiome can be modified with the use of antibiotics, probiotics or prebiotics[459].

Antibiotics have been long used in the treatment of human IBD[461] and they are still frequently used in clinical practice in primary or adjunctive treatment of fistulizing disease and post-operative management after an ileocecal resection or fistula/abscess operation for CD[462]. Besides their benefit has not been well established in randomized controlled trials[463]–[465], they have shown to be effective in almost all preclinical models of colitis[466], [467] and there is evidence indicating that some antibiotics, alone or in combination, may induce remission in active CD and UC[468].

Since no causative pathogen has been identified, broad-spectrum antibiotics,

mainly metronidazole and ciprofloxacin, are the most frequently used[469]. In CD, antibiotic therapy is more beneficial to patients with involvement of the colon[469]. In active UC, a randomized controlled trial suggested that ciprofloxacin may be beneficial as an adjunctive treatment to mesalamine and prednisone[470], and more recently, remission has been described to be induced in active UC, either by a triple antibiotic therapy[471], [472] or by a synergistic association of antibiotics and corticosteroids[473]. But probably the most essential role of antibiotics in IBD therapy is in the treatment of the septic complications of IBD, such as intra-abdominal and perianal abscesses, fistulae and fissures, bacterial overgrowth, peritonitis, and toxic megacolon [459], [464]. In addition, 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[474], [475].

The beneficial effect of antibiotics in IBD has been traditionally attributed to their antimicrobial properties[466], [476], [477] altering the composition of microbiota to favour beneficial bacteria, decreasing luminal bacteria overgrowth and tissue invasion, translocation and systemic dissemination, and reducing proinflammatory bacterial toxins and antigenic triggers[464], [465], [478], [479].

Notably, 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[472], [480]–[482]. This is of great interest in the pharmacological treatment of diseases where infectious and inflammatory factors converge[483]–[486]. However, the potential of immunomodulatory antibiotics in managing intestinal conditions, such as IBD, is still poorly documented[459], [480], [487].

Despite their utility, several studies have reported that discontinuation of

antibiotic therapy results in a high relapse rate, and long-term therapy is associated to increased risk of drug side effects and bacterial resistance[488], [489]. Additionally, antibiotic impact on microbial community has also been reported to predispose to infections, favoring the overgrowth of pathogens by disturbing the equilibrium of this ecological barrier. Antibiotic exposure is in fact the principal risk factor for Clostridium difficile infection[490]–[493]. These and other side effects have hampered the use of antibioitics as first line of treatment in IBD.

Probiotics and prebiotics

A less aggressive strategy to influence microbial communities is the use of Pharmabiotics, a term that has emerged to encompass any therapeutic exploitation of commensal bacteria: live probiotic bacteria, probiotic-derived biologically active metabolites, prebiotics, synbiotics and genetically modified commensal bacteria[494]. Prebiotics are compounds found on foods or added as supplements which promote the growth and activity of beneficial bacterial communities over others[495]. They are nondigestible oligosaccharides, such as fructooligosaccharides, galactooligosaccharides, lactulose, and inulin[496], [497], that cannot be adsorbed until they reach colon. There, specific bacteria can ferment them and produce SCFAs and lactate, modulating gut microbiota and cytokine production from immune cells located in the intestine mucosa[498]. Probiotics are life organisms, usually bacteria, that when administered in adequate amounts can provide a benefit to the health of the host by altering the microbial balance[499]. The synergistic combination of prebiotics with probiotics is what we know as "synbiotic"[497].

The use of probiotics was first proposed by Elye Metchnikoff at the beginning of the last century, suggesting that a high concentration of Lactobacilli in the intestinal lumen is important for the health and longevity of humans. These strategies, despite not inducing a persistent effect, they could be useful to recover the healthy balance lost in many pathological conditions[495]. Their use in intestinal inflammation has been long reported,including controlled trials that demonstrated their effectiveness for remission maintenance in UC[500], [501] and pouchitis[502]–[504].

One of the mechanisms behind their potential benefit is their ability to increase or restore the protective and symbiotic functions of intestinal microbiota. Specific bacteria have the ability to change their metabolic profile to support the growth of carbohydrate-reducing bacteria[495]. These micro-organisms produce bioactive molecules, such as SCFAs[505], [506]. In addition, probiotics may preferentially bind to the epithelial surface in the intestine and therein inhibit the attachment and colonization of pathogenic species. A direct competition against enteric pathogenic bacteria by means of their production of antimicrobial compounds (referencias) or decreasing luminal pH might also be involved[507]. And moreover, probiotics have also been reported to exert immunoregulatory activities. They stimulate host secretion of bactericidal proteins[508], [509] and modulate mucosal immune responses by either inducing protective cytokines or by inhibiting proinflammatory cytokines[510], [511]. However, it is important to note that each probiotic may have individual mechanisms of action, and characteristic of the host condition may determine which probiotic species and even strains may be optimal.

Despite the large number of probiotics that have shown beneficial effects in experimental models of intestinal inflammation[512], 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[500], [501], [513], the probiotic mixture VSL#3[514]–[516] and *Lactobacillus GG*[517], [518]. These studies reveal their usefulness in maintaining disease remission and preventing the relapses[519].

Escherichia coli Nissle 1917 is the most extensively studied probiotic in IBD. It was isolated from a German soldier in World War I who had withstood a severe outbreak of gastroenteritis that devastated his unit[501]. This probiotic is able to displace pathogenic *E. coli*[520] and is also suggested to down-regulate intestinal T-cell expansion through TLR2 signalling [521].

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[504], [516].

The yeast *Saccharomyces boulardii* CNCMI-745 has also been shown to modulate the gut immune response, inducing intestinal homeostasis, which supports its effectiveness in intestinal inflammatory conditions[522]. A comparative open study showed that its combination with mesalazine was significantly superior to mesalazine in maintenance of remission in CD[514] and in an open uncontrolled 4-weeks study S. boulardii induced remission in 71% of patients with mild to moderate UC[515]. It is worth pointing that many antibiotics do not affect yeasts, and some have been long used in combination in many different settings, remaining the growth, phenotype or functions of the yeast unaffected by the antibiotic [523].

IMMUNOMODULATORY TETRACYCLINES

Tetracyclines are a family of antibiotics derived from a tetracyclic naphthacene carboxamide ring. They were discovered in 1947 by Benjamin M. Duggar[524], from a mix of natural antibiotic compounds produced by species of *Streptomyces*. Since then, they have overcome pharmacokinetic difficulties and appearance of antimicrobial resistances, providing an endless source of possibilities far beyond their antibiotic properties. Undoubtedly, a promising tool to face the treatment of complex diseases that elude conventional therapies.

A family story

Discovery

Professor Duggar discovered that some species of the genus *Streptomyces*, mainly composed by soil-dwelling bacteria, were able to inhibit bacterial growth by producing antibiotic compounds[524]. These could be isolated by fermentation, providing a simple and cost effectively method, which has contributed to their extensive use[525], [526]. The first tetracycline isolated and characterized was chlortetracycline. Soon after, other natural tetracyclines were also purified, including tetracycline itself.

They are proved to be broad-spectrum bacteriostatic antibiotics, active against a wide range of aerobic and anaerobic bacteria, gram-positive and gram-negative, but also effective against other microorganisms, including *Rickettisa*, *Chalmiydia* spp., *Mycoplasma pneumoniae*, and *Plasmodium* spp. The larger spectrum of activity of these compounds, compared with the previously

discovered penicillins, together with being well tolerated by patients and easily produced, made them effective and economically valuable drugs. For this reason, they were extensively used in human therapy to treat many infectious diseases, as well as in veterinary medicine, animal growth promotion, and aquaculture, which unfortubantely, also led to the appearance and increase of bacterial resistance. Now, their use is limited to infections caused by *Rickettisae, Chalmiydiae* and *Mycoplasma,* as well as acne, respiratory tract infections and other chronic conditions, caused by atypical microorganisms with resistance to other antibiotics[485], [527].

Evolution

The tetracycline family grew up with many novel compounds obtained by chemical modification of the natural tetracyclic structure, with the aim of improving their antimicrobial spectrum or pharmacokinetic properties. Semi-synthetic second-generation tetracyclines were developed in order to increase their bioavailability and activity. Of those, minocycline and doxycycline are two of the most commonly used in clinic, as they display a strong activity and are well tolerated[528]. However, the emergence of bacterial resistance also affected them and, after four decades of extensive clinical use, tetracyclines began to decline as first-line antibiotics.

In order to overcome bacterial resistance, extensive research led to the development of glycylcyclines, the third generation of tetracyclines. Among them, tigecycline, a minocycline derivative, is the best known, although its use is still restrained to hospitals[529]. Even more, a last generation, the aminomethylcyclines, is currently under study[530]–[532], with one of its candidates, Omadacycline, entrering phase III clinical trials in 2016[533].

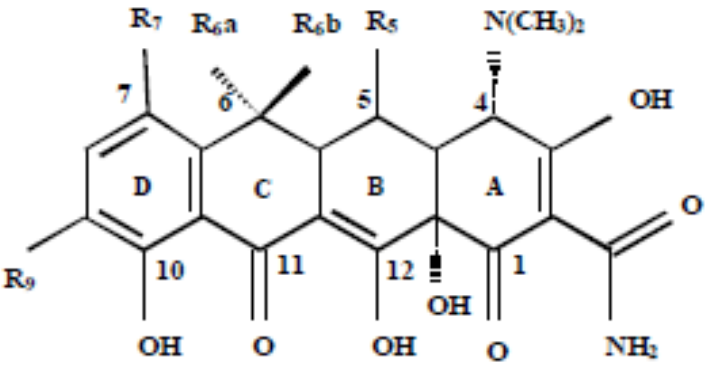
The story does not end up here. Following the discovery of their non-antibiotic properties, such as their ability to inhibit MMPs, 4-De-Dimethylamino

tetracyclines and other derivatives were designed, giving rise to a novel class of chemically modified tetracyclines (CMTs) without antibiotic properties. These compounds retain the ability to bind to non-microbial targets, facilitating their use in other pathological conditions, without the threat of increasing antibacterial resistance[534], [535].

Structure and properties: smart drugs.

Structure and properties

Tetracycline molecules are based on a linear fused tetracyclic nucleus (rings A, B, C, and D) with various functional groups attached at the upper and lower peripheral zones (Table X). The simplest tetracycline to display detectable antibacterial activity is 6-deoxy-6-demethyltetracycline and as such, this structure may be regarded as the minimum pharmacophore[525]. Chemical modifications were introduced to improve either their activity, pharmacokinetic properties or to evade bacterial resistance mechanisms[536]. Natural or synthetically introduced modifications of this structure have allowed the performance of a structural-activity study, leading to the identification and better understanding of the importance of the different regions of these compounds[537].



The tetra-cycle carboxylic skeleton is necessary for antibacterial activity. Specifically, the lower hydrophilic domain is responsible for binding to the ribosome and inhibition of protein synthesis, and substitutions at these positions lead to the loss of antibiotic activity. Other key features required for the bioactivity of all tetracyclines are the keto-enol system (positions 11, 12, and 12a) in proximity to the phenolic D ring and the 4-Dimethylamino group at position C4, as well as the natural stereochemical configurations at positions 4a and 12a (A-B ring junction) [525]. Conversely, modifications of the upper hydrophobic region and on positions 7 through 9 on the D ring produce compounds with enhanced antibacterial activity[536].

Zwitterionic form

Tetracyclines are not static molecules, changing molecular conformations as pH and other aqueous-phase factors change. Zwitterionic form (dipolar ion) change with unionized form with intra-molecular hydrogen bonding at the position 3 oxygen. This change renders the molecule more lipophilic and able to cross lipid bi-layers. Both conformations are believed to be responsible for their biological properties *in vivo*: the zwitterionic form facilitate porin transportation and affinity-binding to their biological target, while the unionized form is primarily responsible for membrane permeation and other pharmacokinetic properties[536], [537].

Chelating agents

The tetracyclines are strong chelating agents[538] and this action influences both their antimicrobial and pharmacokinetic properties. Tetracyclines circulate in blood plasma primarily as Ca^{2+} and Mg^{2+} chelates. Chelation sites include the beta-diketone system (positions 11 and 12) and the enol (positions 1 and 3) and carboxamide (position 2) groups [525]. The affinity of the interaction depends on

the type of tetracycline, metal ion, pH and presence of other metals. Their role as calcium ionophores has important biologic implications since intracellular Ca^{2+} act as a secondary messenger and affect pathways such as secretary processes, receptor activation or inhibition, cell division and metabolic reactions[536].

Lipophilicity

Second generation tetracyclines, DXC and MNC, have increased lipophilicity[539], which is directly related to their activity against Gram-positive bacteria. Lipophilicity also affects absorption and tissue distribution, and therefore they show a better pharmacokinetic profile than their parent, tetracycline, with near 100% bioavailability[536].

Pharmacokinetic profile

Absorption of tetracyclines occurs in the stomach and proximal small intestine. First generation tetracyclines have reduced absorption, in an average range of 25-60%, while second generation tetracyclines are 3 to 5 times more lipophilic and are absorbed rapidly and completely[540]. Food and the formation of insoluble complexes can affect absorption of the firsts reducing it by 50%, while they have a lower impact on doxycycline and minocycline[541]–[543]. No data is available regarding the absorption of tigecycline, although its thought to be limited[544].

Blood concentration of tetracyclines follows a plateau-shaped course, with a slow rise followed by an even slower drop(Aronson, 1980; Barza et al., 1975; Bosó-Ribelles et al., 2007; Klein and Cunha, 1995; Kramer et al., 1978; Meagher et al., 2005; Muralidharan et al., 2005a, 2005b; Rodvold et al., 2006; Saivin and Houin, 1988; Sklenar et al., 1977; Welling et al., 1975)

High wolumes of distribution illustrate their good tissue penetration, which can reach several fold concentrations in some tissues. This profile is remarkably

improved in newer generation tetracyclines, and in particular for tigecycline, which have a longer half-life and excelent tissue penetration[527], [541], [545]–[549], [549]–[552]. Minocycline on the other side, has superior lipophilicity and the ability to cross the blood-brain barrier. It can accumulate into the cerebrospinal fluid and central nerve system (CNS) cells at concentrations 3 fold higher than doxycycline, while tetracycline is is undetectable in the brain [545], [546], [553]–[555]. This enables its use in the treatment of many CNS diseases[541], [556], [557]. In general, they are poorly metabolised, being excreted unaltered in the urine and bile[540]. In particular, no metabolism has been detected for doxycycline, while tetracycline, minocycline and tigecycline do have metabolites, some of them with antibacterial activity. Most tetracyclines have enterohepatic circulation and their billiary concentration is several times higher than that in blood[558], [559].

An interesting and remarkable ability of tetracyclines is to concentrate at the site of tissue injury, which makes them look as smart drugs. Various studies have reported and took advantage of this property: radiolabeled tetracycline was used to diagnose infarcts due to its capacity to accumulate in damaged myocardium[560]–[562] and the high concentration of doxycycline achieved in the inflammatory exudate of periodontal lesions is advantageous in the treatment of periodontitis[563], [564]. Increased uptake has also been observed with increasing temperature and in specific cell types, such as neutrophils, which may partly explain the high levels observed in injured tissues[565]. The intracellular/extracellular concentration of tetracycline, doxycycline and minocycline on neutrophils can reach ratios of approximately 1.8, 7.5 or 64, respectively[566]. Tigecycline have also been found 20 to 30 times more concentrated on PMN and up to 78-times more in alveolar macrophages than in blood[550], [567]. At these levels, mass action effects could be expected at the same time[568].

Antibiotic activity

Mechanism of action

Antibiotic activity of tetracyclines involve their ability to bind to the acceptor site (A-site) at the 30S ribosomal subunit of the bacteria. This prevents the binding of aminoacyl-tRNA to the mRNA-ribosome complex[569], interrupting protein biosynthesis and killing the bacteria.

The physicochemical properties of tetracyclines play an important role in their ability to penetrate inside the bacteria, which is required for their antibiotic activity. Positively charged cation-tetracycline coordination complexes are attracted by the Donnan potential and traverse the outer membrane of gram-negative bacteria through the OmpF and OmpC porin channels[525], [538]. Then, their accumulation in the periplasm and their dissociation from the cation complex allow uncharged tetracyclines, weakly lipophilic molecules, to diffuse through the lipid bilayer of both gram-negative and positive bacteria[570].

A war against bacterial resistance

The mechanisms involved in bacterial resistance include the active efflux of the compound[571], the disruption of the tetracycline-ribosomal interaction by ribosomal protection proteins (RPPs)[572], the enzymatic inactivation of the drug through mono-hydroxylation[573] and the alteration of the target site through 16S RNA mutation[574]. The most common mechanism in gram-negative bacteria is associated with the membrane protein TetA, which exports the drug out of the bacteria. The expression of TetA is tightly regulated by the homodimeric tetracycline repressor. Binding of tetracycline–Mg2+ complex to the repressor triggers its release from the DNA and the expression of TetA[575]. Curiously, this obstacle for tetracyclines was turned into a genetic engineering

tool: since its discovery, the Tet regulatory system has become an important transcriptional regulatory mechanism widely used in genetically modified eukaryotes for scientific and biotechnological purposes[576].

Third generation tetracyclines, the glycylcyclines, have a substitution of an N-alkylglycylamido group on the D ring at position 9. This is thought to be responsible for steric hindrance, which facilitates the broader spectrum of activity and elude major resistance mechanisms. Glycylcyclines interation with the bacterial ribosome is about five times stronger than previous tetracyclines[577] and even in a different way[578], which dodge protection mechanisms[579]. Additionally, sulphonamide derivatives have been found to be more active against Gram-possitive bacteria than acetylated series, opening the door for the development of a new class of antibiotics to selectively target gram-possitive pathogens[580].

Clinical use

Despite the development of resistance by some bacterial species, tetracyclines are still effective and particularly useful in several types of infections, such as atypical pneumonias, community acquired pneumonia, rickettsial and chlamydial infections, Lyme disease, cholera, syphilis and periodontal infections[542].

Doxycycline and minocycline are the most widely used tetracyclines today. One of the most common uses of tetracyclines, and in particular minocycline, is in the treatment of acne vulgaris[581]–[584]. Here, the inflammatory reaction profoundly contributes to the pathophysiology of the disease[585] and minocycline, due to its great lipophilicity, is highly active in the pilosebaceous complex inhibiting both the growth of *Propionibacterium acnes* and the associated inflammation[582], [584]. Doxycycline is also frequently used as first line therapy in the treatment of uncomplicated genital *Chlamydia trachomatis* infections or

acute Q fever. Moreover, doxycycline may be used after initial therapy with other antibiotics has failed, as in the case of infections with penicillin resistant *Streptococcus pneumonia*[586].

The use of tigecycline is approved for skin, soft-tissue and intra-abdominal complicated infections, many of them caused by hospital-adquired multiresistant bacteria, responsible of high mortality rates[587], [588]. Although it did prove to be equivalent to other comparative antibiotic combinations, its high tolerability, excellent tissue distribution and convenient dosing make it an attractive candidate(Wenzel et al., 2005).

Non-antibiotic properties

Tetracyclines are an endless source of surprises. Along with their interesting story as antibiotics, even more striking and fascinating are their non-antibiotic actions. Some tetracyclines can specifically modulate various homeostatic mechanisms and cellular pathways in mammalian cells, openning a new chapter for tetracyclines to face the pathogenesis of diseases[568]. Extensive research, specially focused on doxycycline and minocycline, has unveiled a wide range of pharmacological effects, including immunomodulatory, anti-apoptotic and antiproliferative properties, inhibition proteolysis and suppression of angiogenesis and tumour metastasis[484], [536], [589, p.], [590]–[592]. Many studies tried to elucidate the mechanism involved in these non-antibiotic properties, which likely involves multiple pathways. This section reviews the current knowledge on such diverse yet highly beneficial properties of tetracyclines.

Antioxidant

Excessive oxidative stress leads to tissue destruction and dysfunction, and it is closely correlated with many inflammatory and pathological disorders[593], [594]. Tetracyclines are multi-substituted phenolic rings, similar to vitamin E, and as such they belong to the group of phenolic antioxidants[595]. Due to that structure, tetracyclines are particularly versatile in their ability to combat oxidative stress and scavenge free radicals[592], [596], thereby reducing pathological tissue destruction. This antioxidant effect has been demonstrated in several cell-free mixed-radical assays[597], [598] where they showed to be able to quench H_2O_2 [595] and scavenge superoxide[599] and peroxynitrite, by directly trapping free radicals[600].

Minocycline has a superior scavenging activity to doxycycline and tetracycline, comparable to that of α-tocopherol and independent of Fe^{2+}chelation[595], [598]. This superior ability is likely due to the presence of a diethyamino group on the phenolic carbon, which provides improved steric hindrance[597]. Therefore, minocycline derivatives such as tigecycline may retain this potent activity, although no studies regarding these compounds are available yet

Anti-apoptotic

Prevention of cell death by tetracyclines can result from at least two actions: modulation of innate and adaptive immunity and blockade of apoptotic cascades[557], [601]. Among the latter, tetracyclines have multiple effects on mitochondrial functioning and modulate apoptotic pathways. This effect is best described with minocycline, whose ability to cross blood-brain barrier and inhibit apoptosis on neurons has attracted the attention to control neurodegenerative diseases[556], [602]–[609], [609], [610]. The numerous mechanisms involved in

cytoprotection of minocycline are well review by Garrido-Mesa N.[611, p.], and include inhibition of both caspase-dependent and -independent cell death.

Caspase-dependent pathway: On the extrinsic pathway, cell surface death receptors activation by death ligands, such as Fas Ligand (FasL), and TNFα directly induces Casp-8 activation and establishes a balance between pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family. On the intrinsic pathway, increased intracellular oxidative stress, DNA damage, unfolded protein response or deprivation of growth factors, also induce the release of pro-apoptotic proteins. These two pathways converge on the mitochondrial pathway, triggering the release of Cytochrome *c* and Smac/DIABLO, which induce apoptosome complex formation and the subsequent activation of caspases[612], [613]. Some of the mechanisms behind the anti-apoptotic effect of minocycline that results in the prevention of neuronal damage[555], [556], [603], [608]–[610], [614] include up-regulation of the antiapoptotic proteins Bcl-2 and Bcl-xl, [614], reduction of the expression of the pro-apoptotic mediators caspase-1, caspase-3[555], [608], [610] Smac/DIABLO [608], and the pro-apoptotic proteins Bax, Bak, Bid, Fas and p53[614]–[616], and inhibition of the apoptotic protease activator factor-1(Apaf-1) [617]. However, it did not affect TNFα-induced caspase-3 activity[618], suggesting that the extrinsic receptor-mediated apoptosic pathway is not modulated by minocycline. It has also been proposed that minocycline binds to mitochondrial membranes in a Ca2+-dependent manner and forms ion channels in the inner mitochondrial membrane, inducing their depolarization and inhibiting Cyt c release[602].

Caspase-independent pathway: this route plays a key role in cell death under stress conditions, being considered as "regulated necrosis"[619]. Poly(ADP-ribose) polymerase (PARP)-1, an enzyme involved in DNA repair, mediates this apoptotic pathway through the regulation of transcription factors, notably NF-κB[620]–[623]. Minocycline was shown to competitively inhibit PARP-1 in a cell-

free assay, acting as a mimetic of the essential co-factor NADH [556], [624]–[626]. Comparing several tetracycline derivatives, it has been suggested that the potency as PARP-1 inhibitor correlates with the potency as neuroprotective agent. Doxycycline showed a similar potency as Minocycline, which was close to one of the most potent PARP inhibitors available [627]. This effect has been observed to mediate the protective effect of minocycline in different experimental models of disease, including atherosclerosis[625], ischemia–reperfusion injury[626], intestinal mucositis[628, p.], [629], [630] and stroke [631], and it could also be involved in the anti-inflammatory effects exerted by tetracyclines[624].

Regulation of proliferation

Different studies have revealed that tetracyclines are able to regulate the proliferation of various cell types both *in vitro* and in *vivo,* In human bone marrow osteoblastic cells, doxycycline and minocycline showed dual effects on cell proliferation: a dose-dependent inhibitory effect at high concentrations and increased proliferation and activation at the concentrations attained in plasma (2-3μM)[632]. A similar effect has also been observed recently in the generation of dendritic cells from bone marrow precursors under the conditioning of low concentration tetracyclines (5μM)[633]. In addition, Tetracyclines have shown to selectively inhibit endothelial cell growth, which constitutes a potential mechanism of the anti-angiogenic activity of these compounds[634]. It has also been reported that minocycline reduces vascular smooth muscle cell proliferation promoting an arrest in the G1 phase[625], [635]. Recently, the identification of novel anticancer properties on tetracycline analogues has attracted attention. Beside the potential of MMP inhibition to fight tumor metastasis, direct cytotoxic effects have been described for doxycycline, minocycline, tigecycline and CMTs in various tumor cell lines Increased susceptibility of cancerous cells to mitochondrial dysfunction has been proposed to explain their actions.[636]–[638].

Additionally, a potential mechanistic link between inhibition of PARP-1, which partially regulates cell cycle progression[624], [625], [639], might be implicated, including microglia, macrophages and lymphocytes[640]–[645]. Among tetracyclines, CMT-3 has been selected as the most promising candidate and the first one going into clinical trials[646].

Inhibition of enzymes

Matrix metalloproteinases.

The ability of tetracyclines to inhibit MMPs activity is probably their best characterized non-antimicrobial property, shared by most of the family members [484]. The mechanisms of this anti-proteolytic action involvedirect effects on the enzyme as well as inhibition of enzyme expression [647]. The first is mediated by chelation of metal ions within the enzyme[648], particularly by the interaction of tetracyclines with structural metals rather than the active site Zn^{2+} [647], [649]–[651]. The effectiveness of this inhibition depends on the tetracycline and the type of MMP considered. Doxycycline has shown higher chelating ability and MMP inhibition activity than tetracycline and minocycline[652]. Proinflammatory mediators and several growth factors regulate MMP expression[653], and therefore the ability to target these mediators can also mediate a reduction in MMP activity[654]. In addition, indirect mechanisms also comprise the inhibition of both MMPs synthesis and expression[630], [655], an action that can be context-dependent and selective for some enzyme types[656].

In general, tetracyclines have shown to inhibit, both *in vitro* and *in vivo*, the level and activity of the gelatinases MMP-9 and MMP-2, the collagenases MMP-1, 8 and 13, and the stromelysin MMP-3[590], [649], thus preventing their contribution to multiple pathological processes[657] such as tumour progression[658], angiogenesis[659], plaque formation leading to heart stroke and subsequent myocardial damage[660]–[662], bone resorption[663], [664] and

arthitis[665], [666]. Tigecycline also retaine MMP inhibition activity, which has been found to accelerate wound healing in staphylococcal-infected burns[667]. In particular, CMTs have been specially developed to potentiate this activity[534], [535], so they can be easly applied in pathological conditions where MMPs are involved. It has been proposed that the anticollagenase activity of CMTs is specific against the collagenases produced from neutrophils, but is does not affect fibroblasts, therefore they protect from connective tissue breakdown while normal tissue remodelling is not impaired[668]. CMT-3 is specifically active against MMP-2, MMP-9 and MMP-14 isoenzymes[669], and preserves other non-antibiotic properties of tetracyclines that contribute to reduce MMP production and activation, such as decreasing iNOS [670] and pro-inflammatory cytokine release[669], [671].

Regarding the gut, MMP-9 is the most abundantly expressed protease in the inflamed intestine[672], although MMP-2 has been reported to be more determinant in DSS colitis[673]. *In vitro* incubation of minocycline, at concentrations as low as 0.1 μg/ml, with recombinant MMP-2 or MMP-9 impaired their enzymatic activity[674]. Moreover, *in vivo* studies have confirmed the ability of minocycline to reduce MMP expression in experimental models of mouse colitis[628]–[630].

Inducible nitric oxide synthase.

The inhibition of iNOS is also a common feature of most tetracyclines. NOS is overexpressed in a variety of inflammatory and autoimmune conditions and NO is known to mediate several catabolic activities of IL-1β[675] and to potentiate matrix degradation by the up-regulation of MMPs [676]. Therefore, modulation of NO could be therapeutically benefitial on several conditions, as shown for minocycline in experimental colitis [630].

Among tetracyclines, tigecycline is the strongest inhibitor of NO production in stimulated murine macrophages [677], followed by minocycline and doxycycline[654]. However, the mechanism is not related to a direct inhibition of NOS catalytic activity, as initially thought, but they rather act at a transcriptional and/or translational level[555], [603], [645], [654], [678]. Additionally, some results suggest that minocycline has no effect on NOS transcription itself, but it renders the iNOS mRNA susceptible to degradation[654]. The ability to decrease iNOS has also been conserved on CMTs, contributing to their potential as host modulating agents without the associated troublesome of the antibiotic activity[670].

Lipid mediators: sPLA2, COX-2, 5-LOX.

Soluble lipid mediators play an important role in the control of inflammatory responses and are involved in the pathogenesis of inflammatory diseases [679]. Secretory phospholipase A2 (sPLA2) hidrolyses membrane phospholipids releasing fatty acids such as arachidonate, a rate-limiting step for the biosynthesis of eicosanoids[680]. Cyclooxygenases (COXs) and 5-lipoxygenase (5-LOX) are the main arachidonic acid-metabolizing enzymes involved in the production of the different inflammatory metabolites[681]–[684]. Various groups have reported that tetracyclines can interfere with the induction, activation or activity of these enzymes, therefore influencing several pathologies in which they are involved.

Doxycycline and minocycline have shown to inhibit **PLA2** in vitro, in a Ca^{2+}-independent mechanism[685]. More recently, a structure-activity study described that lipofilic tetracyclines, particularly minocycline, make hydrophobic connections and interfere with the conformation of the active-site Ca^{2+}-binding loop of PLA2, preventing Ca^{2+} binding and blocking substrate entrance to the active site [686]. CMTs have also shown to inhibit Secretory PLA2 and, in general, tetracyclines present the advantage of easily penetrating membrane cell

walls, which is a problem of some other inhibitors of PLA2[686].

Minocycline protection in experimental models of CNS diseases has also been linked to a reduction in **COX-2** and, subsequently, PGE_2 production[555], [687], an action that might result from an amelioration of the inflammatory response and p38 MAPK inhibition[688]. Contradictory results have however been reported: some studies have shown no modification of COX-2 levels[654]; others, increased COX-2 leves in chondrocytes and macrophages in vitro and increased PGE2 production by ex vivo stimulated cartilage[689]; and at the same time, CMT-3 stimulated COX-2 production while inhibited net PGE2 accumulation through specific inhibition of the enzyme[690].

Finally, *in vitro* and *in vivo* studies have shown that minocycline can inhibits **5-LOX** expression and its translocation to the nuclear membrane and activation[691], [692] This activity has been correlated to minocycline protective activity against brain inflammation and neuronal cell death and with accelerated functional recovery in the chronic phase of focal cerebral ischemia[693]. Although no mechanistic studies have been performed in this regard, it has been proposed that the ability minocycline to reduce ROS production and modulate p38 MAP kinase activity may underlay this effect.

Immune modulation

The anti-inflammatory actions of tetracyclines were first recognized upon their application to several skin diseases, but they have shown to moderate inflammatory responses in many other conditions[590], [694]. Their wide range of anti-inflammatory effects is a consequence of their even wider spectrum of activities, such as interfering with enzyme production and activity as mentioned above, modulation of cytokine release and direct regulation of immune cell function under inflammatory conditions[695]–[697]. The most relevant actions on specific immune subsets will be reviewed here.

Peripheral blood mononuclear cells.

In vitro studies have revealed the ability of tetracyclines to inhibit the proliferative response of human peripheral blood mononuclear cells (PBMC) to mitogenic stimulation[698]. Tetracyclines, at concentrations just above the therapeutic range, reduce DNA synthesis in isolated human peripheral blood lymphocytes stimulated with either IL-1β or phytohaemagglutinin (PHA)[699], [700]. On the other hand, it has also been observed that they can modify cytokine production in a stimulus-dependent way. The addition of minocycline to whole-blood cultures or isolated PBMCs 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 in this case was hardly affected[701]. Tigecycline has also been reported to modify cytokine production by PBMCs stimulated with *S. aureus* toxins in a comparative study with other antibiotics, proving to be among the most potent inhibitors of IFNγ, TNFα, IL-1β, IL-6 and IL-8 production[702]. Due to the precence of different leukocytes within PBMCs, it is difficult to interpretate these results, which might represent the general outcome of disparate effects on various populations under different stimulus.

T cells.

The immunomodulatory effect of tetracyclines on T cells may explain many of their anti-inflammatory effects observed *in vivo*. Suppressive effects on T-cell proliferation, activation and function have been long described[641], [701], [703]–[705]. Inicial studies with different tetraciclines showed the ability of doxycycline to inhibit delayed hypersensitivity responses in mice[705]. Later, the influence of tetracyclines on T cell activation was associated with an inhibitory effect on cytokine production. For example, minocycline was found to significantly decrease IL–2, IFNγ and TNFα secretion, which could in turn reduce

T cell proliferation and activation[641], [701]. Contradictory reports have however shown no effect for minocycline on T cell proliferation and IFNγ production in rodents.[704]. More recent studies have suggested that minocycline reduces T cell turnover after activation, with decreased levels of proliferating ($Ki67^+$) and activated ($HLADR^+$) cells and increased levels of circulating naïve ($CD45RA^+$) cells[706]. Reduced surface markers expression might mediate this effect, since minocycline suppressed CD25 (IL-2 receptor), CD40L and HLADR expression, as well as IL-2 production[703], [706], [707]. Intrinkingly, T cell activation was not completely abolish and other activation markers, such as CD69, were found increased[706].

The mechanism by which minocycline interfere with T cell activation has been recently shown to be mediated trough selective suppression of nuclear factor of activated T cells 1 (NFAT1) transcriptional activation, a key regulatory factor in T cell activation[707]. Minocycline was found to increase NFAT1 rephosphorylation, which reduces its nuclear translocation after several hours of activation. Two potential mechanisms were suggested for this effect: increased activity of g*lycogen synthase kinase (*GSK) 3 and attenuated intracellular Ca^{2+} flux. Regarding GSK3, the NFAT kinase, minocycline decreased its inhibition, thus enhacing NFAT1 rephosphorylation. In addition, minocycline also reduces the capacity of mitochondria to buffer Ca^{2+} in CD4+ T cells resulting in decreased store-operated Ca^{2+} entry (SOCE) through the plasma membrane. This accelerates the return of intracellular Ca^{2+} to basal levels, which is consistent with the reduced NFAT1 dephosphorylation and its increased nuclear export several hours after activation. These effects were found to be dose-dependent, starting at concentrations as low as 5 µg/mL and being the optimal 20 µg/mL[706]. Although serum concentrations achieved with routine oral dosing are below this threshold (1-2 µg/mL), increased tissue concentrations can be expected and the lack of short-term toxicity at plasma levels above 20 µg/mL[708] supports the

use of increased doses to enhance suppression of T cell activation if necessary.

Dendritic cells:

The effect of tetracyclines on dendritic cells has not been widely studied. Minocycline and doxycycline have been reported to impair antigen processing for presentation to T cells by peripheral blood APCs *in vitro*[709], and although this activity has not been evidenced *in vivo*, impairment of antigen presentation has been observed on other cell types too[642]. In addition, attenuation of type I IFN induction and IFN-stimulated IDO1 and TNF-related apoptosis inducing ligand (TRAIL) has also been observed in human plasmacytoid dendritic cells and PBMCs exposed to HIV or infectious influenza virus[710]. Conversely, a recent study has exposed that adition of tetracyclines to the conditioned medium used in the generation of DCs from bone marrow precursors modified the resulting phenotype of these cells[633]. Low concentrations of minocycline (5μM), the tetracycline displaying the highest activity, induced a tolerogenic phenotype on dendritic cells. These showed to be resistant to subsequent maturation stimuli, with impaired MHC class II restricted exogenous Ag presentation and decreased cytokine secretion. Minocycline-conditioned DCs also showed decreased ability to prime allogeneic-specific T cells, while increasing the expansion of $CD4^+CD25^+Foxp3^+$ T regulatory cells. Additionally, tetracyclines also proved a superior ability to increase cell recovery, as compared to other immunomodulatory compounds[633], [711]. The potential of minocycline-conditioned DCs was also confirmed *in vivo*, being able to prevent the clinical signs of experimental autoimmune encephalitis. Finally, enhanced generation of tolerogenic DCs after minocycline treatment was also observed *in vivo*[633].Together, these properties could be of great interest for the successful stablishment of cell therapies based on tolerogenic dendritic cells.

Monocytes and Macrophages.

Pharmacological studies focused on the effect of tetracyclines on monocytes and macrophages have provided contradictory results. Tetracycline and minocycline have been shown to enhance cytokine secretion (IL-1β, TNFα and IL-6) by monocytes in a dose-dependent manner[699], [701]. However, many studies have reported the ability of tetracyclines to inhibit LPS-induced activation of macrophages. reducing the production of iNOS, COX-2 and MMPs and the release of proinflammatory mediators[690], [712]. However, a proteomic analysis of the effect of minocycline on J774 macrophages suggested that it does not completely inhibit LPS activation in this cell line[677]. In addition, some macrophage functions were also modified by tetracycline treatment in the absence of LPS stimulation, indicating that tetracyclines have direct modulatory effects on macrophage homeostasis and that the consequences of this depend on the type of activation stimuli and the specific cell phenotype. For example, divergent results have been obtained with peritoneal and alveolar macrophages in a model of systemic inflammation induced by acute pancreatitis[713]. In this model, while minocycline reduced IL-1β, manose receptor and IL-10 expression on peritoneal macrophages, it increased their expression on alveolar macrophages, promoting lung inflammation. Upon LPS activation, IL-1β expression and NFkB activation were reduced on minocycline-treated peritoneal but not on alveolar macrophages. Therefore, tetracycline effects on specific macrophage subsets range from anti-inflammatory actions, to no effect or a potentiation of the activated phenotype.

Regarding the first, a vast body of knowledge comes from studies of the effect of minocycline in the microglia, the resident macrophages of CNS that regulate immune reactivity within the brain. Numerous reports suggest that is the inhibition of inflammation and microglia activation, together with the

previous mentioned anti-apoptotic properties, what mediates the protective effect of tetracyclines, and in particular minocycline, in many neurodegenerative diseases[714]. *In vivo* and *in vitro* experiments have reported that minocycline decreases the production of microglia-derived inflammatory mediators in response to a broad range of stimuli, such as β-amyloid, excitotoxins such as glutamate or NMDA or kainate, IFNγ, LPS[642], [645], [678], [688], [715] The mechanistic characterization of this effect has been associated with various possible pathways, including the prevention of the degradation of the inhibitory subunit of IκBα and reduction of NF-κB translocation and activation[555], [645], [714], the inhibition of p38 [645], [714], P44/42 (ERK1/2) and *p54/46/* (c-Jun N-terminal protein kinase (JNK) 1/2) MAPKs activation, and impairment of IFNγ-induced phosphorylation PKCα/βII and the subsequent nuclear translocation of IFNγ regulatory factor (IRF-1)[642], [716]. These effects appeared to depend on the stimulus used to induce activation. For example, while minocycline inhibited MAPK activation in response to LPS, it did not inhibited any of the MAPKs in response to H_2O_2. On BzATP-stimulated MAPKs, minocycline decreased the activation of ERK1/2 and JNK1/2 but not p38. On the contrary, ERK1/2 activation by PMA was not affected but JNK1/2 and p38 phosphorylation were. Of note, not all MAPKs whose activation is dependent on PKC were affected by minocycline. Since each stimulus utilizes different signalling molecules to mediate MAPK activation, only some of them might be targets of minocycline[714].

Overall, minocycline reduces microglia proliferation and the production of its related proinflammatory mediators, including NO, IL-1β, IL-6 and TNFα[715], [717]. Furthermore, the inhibition of ICE [555], [555], which acts as an inhibitor of spontaneous neutrophil apoptosis through the processing of IL-1β, also accounts for minocycline anti-inflammatory effects by reducing neutrophil proinflammatory activity[718].Minocycline has also showed to impaired T cell-

microglia interaction[703]. Minocycline targeted the CD40-CD40L pathway and diminished the ability of T cells to interact with and cluster around–microglia, thus resulting in decreased TNFα and increase IL-10 production in T cell–microglia co-cultures[703].

Neutrophils

The favourable effects of tetracyclines on cutaneous inflammation have been traditionally associated with a reduction of neutrophil chemotaxis[719], [720]. This was confirmed for minocycline in rat models of intracerebral haemorrhage, in which it reduced neutrophil migration into the sites of inflammation[721]. Although this may be a result of an improved inflammatory status caused by other means, direct effects have also been observed. Various studies have proved that tetracyclines directly inhibit the chemotactic activity of PMN cells at concentrations as low as 10 μg/ml through the chelation of Calcium ions[722]–[724]. The same mechanism also contributes to the impairment of other neutrophil functions observed with doxyclycline and minocycline at higher concentrations[723]. Decreased degranulation, MPO release [598] and ROS production (O2-, H2O2, OH.)[725], [726] account for the reduced neutrophil-mediated tissue damage observed with tetracyclines[727]. Tetracyclines have also been found to impair neutrophil phagocytosis [722], [728]. A recent study has shown that tigecycline decreased the density of complement receptors CD11b and CD35 and Fcγ receptors CD16 and CD32 on neutrophils, although no significative effect on phagocytosis or oxidative burst induced by *S. aureus* were observed[729]. Tetracyclines are found at high concentrations in neutrophils, in which they seem to accumulate and contribute to the killing of phagocytosed bacterial pathogens. This uptake of tetracyclines by neutrophils could explaining their tropism for inflammatory sites[566], [567].

RESULTS

AIM 1

The family of tetracyclines has proved to exert a wide variety of non-antibiotic actions, such as inhibition of apoptosis, regulation of cell proliferation, inhibition of MMPs, iNOS and enzymes involved in the production of lipid mediators of inflammation and direct immunomodulatory effects. These properties, together with their antibiotic activity, could be of great benefit on pathological conditions in which an altered immune response is associated with bacterial infection and dysbiosis, such as IBD. Indeed, one of the most studied tetracycline compounds, minocycline, has already been studied on experimental models of intestinal inflammation showing very promising effects. The positive results obtained with minocycline encourage further research on other tetracyclines. Considering that it shares many of the non-antibiotic properties of minocycline, the effects of doxycycline in experimental colitis are worth evaluating. Therefore, we proposed giving answer to the following question:

Does doxycycline exert anti-inflammatory activity in experimental colitis?

In order to assess this question, the effects of doxycycline on different experimental models of colitis were evaluated, and compared with those obtained with minocycline. Additionally, the combination of doxycycline with the probiotic *Saccharomyces boulardii* was also assayed as a therapeutic strategy to manage the relapsing course of IBD. The following studies were performed:

- Evaluation of doxycycline-effect *in vitro*.
- Evaluation of doxycycline effect in TNBS colitis
- Evaluation of doxycycline effect in DSS colitis
- Evaluation of the effects of the association of doxycycline with *Saccharomyces boulardii* to control the recurrence of intestinal inflammation.
- Evaluation of the effects of the association of doxycycline with *S. boulardii* or its conditioned medium *in vitro*.

1.1. DOXYCYCLINE DISPLAYS IMMUNOMODULATORY EFFECTS *IN VITRO*.

The immunomodulatory properties of doxycycline were characterized *in vitro* in two cell types involved in the intestinal immune response, epithelial cells and macrophages. The human colon adenocarcinoma cell line Caco-2 was used as a model of intestinal epithelial cells. Caco-2 cells were incubated with IL-1β for 20 h to induce the secretion of IL-8, a pro-inflammatory chemokine released by intestinal epithelial cells that increases inflammatory cell migration from the blood stream into the mucosa and submucosa, enhancing intestinal tissue destruction during chronic IBD[730]. Pretreatment of these epithelial cells with doxycycline at different concentrations (1-50 μM) resulted in a statistically significant reduction of IL-8 production upon IL-1β stimulation (Fig. 1A). Indeed, the inhibition achieved by doxycycline treatment was equivalent to that observed after minocycline treatment[629]. Previous studies have shown that second generation tetracyclines inhibit nitrite production by murine macrophages[629], therefore the effects of Doxycycline on nitrite production were evaluated in RAW 264.7 cells, a cell line of mouse macrophage. Our results confirmed previous reports, and show that doxycycline, at concentrations of 25 and 50 μM, inhibited nitrite accumulation in LPS stimulated RAW 264.7 cells, to a similar extent to minocycline (Fig. 1B). Cell viability was not affected by antibiotic treatments. These results evidence a direct effect of doxycycline on the immune response elicited by these cells, which is independent from its antimicrobial effect.

Figure 1. Immunomodulatory effects of doxycycline on **A)** IL-8 production by IL-1β-stimulated Caco-2 epithelial cells, and **B)** nitrite production by LPS-stimulated RAW 264.7 macrophages. Cells were incubated in the presence of the different treatments for 24h and then stimulated with IL-1β (1 ng/ml) or LPS (100 ng/ml) for another 24h. IL-8 concentration in the culture supernatant was determined by ELISA and nitrite concentration was measured by the Griess Assay. Data are expressed as mean ± SD. $^{*}p < 0.05$ *vs.* stimulated untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate.

Figure 1: Evaluation of doxycycline effects *in vitro*.

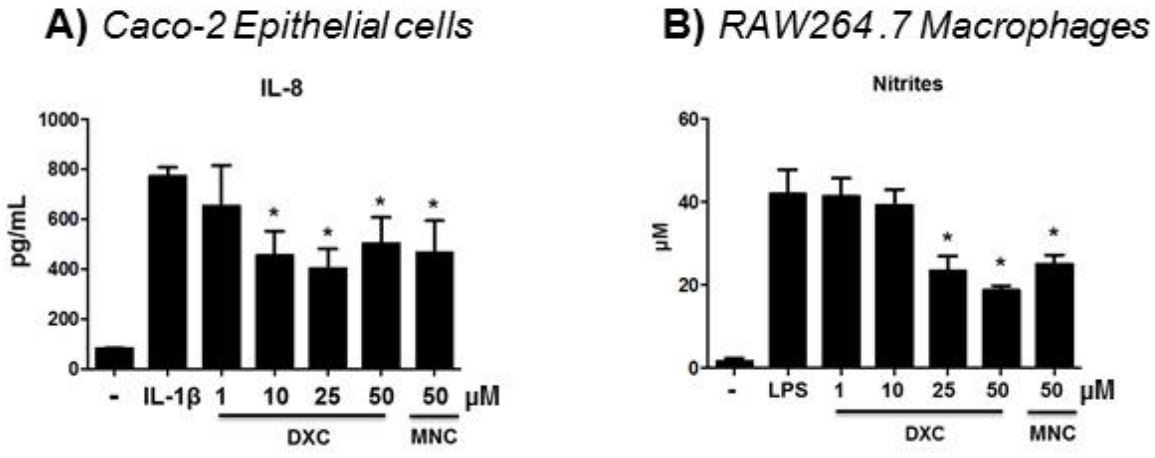


Figure 2: Evaluation of doxycycline effects in TNBS colitis.

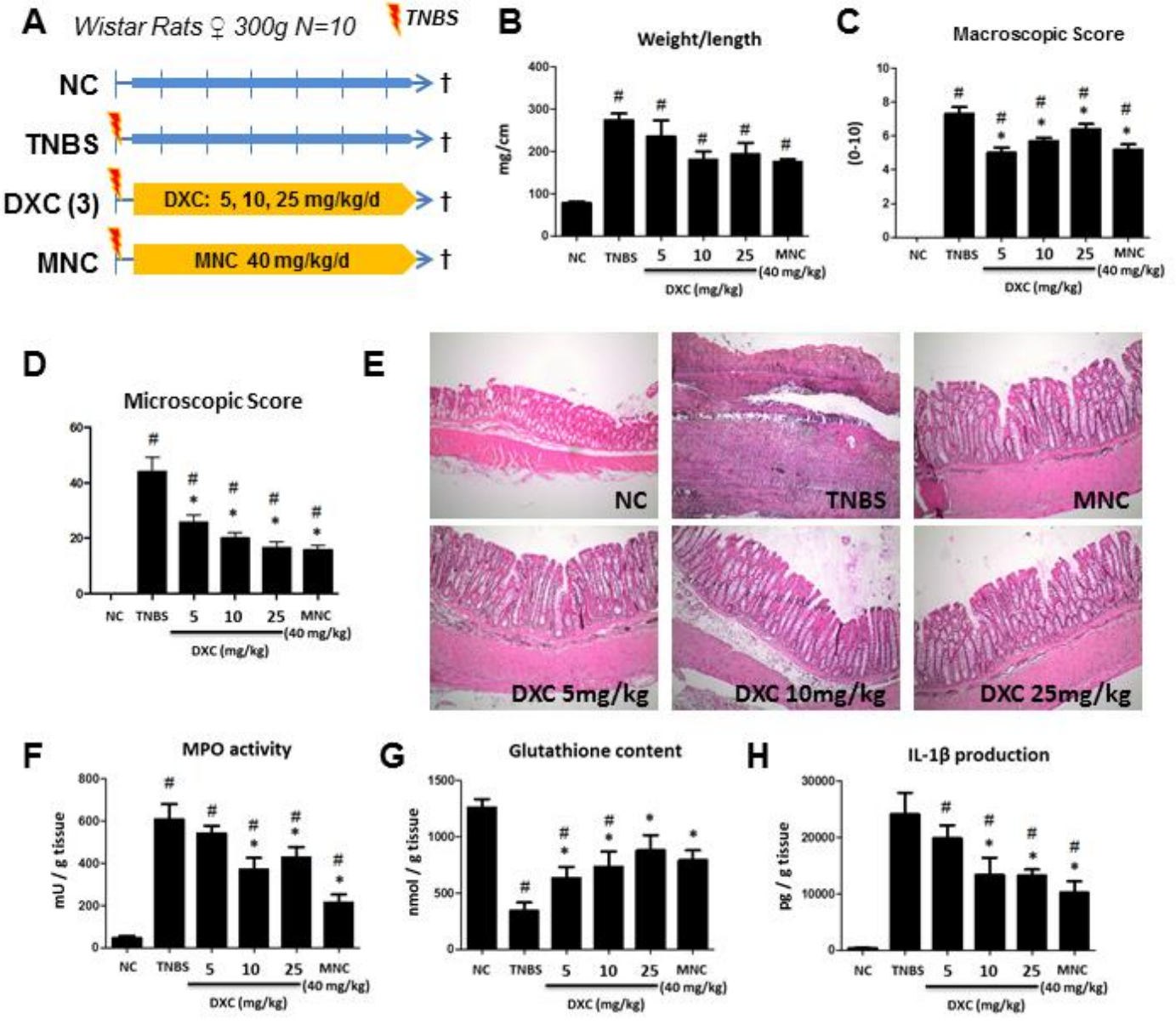


Figure 2. Intestinal anti-inflammatory effects of doxycycline in the TNBS model of rat colitis. **A)** Schematic illustration of the experimental design followed. NC: Non-colitic group, TNBS: TNBS-colitic group, DXC: doxycycline-treated colitic groups (5, 10, 25 mg/kg/d), MNC: minocycline-treated colitic group (40mg/kg/d) (n=10). **B)** Colonic weight/length ratio. **C)** Colonic macroscopic damage score, according to the criteria described by Bell et al., 1995. Data are expressed as median (range). **D)** Microscopic damage score assigned according the criteria described in (Camuesco et al., 2004). Data are expressed as median (range). **E)** Histological sections of colonic mucosa (40x) stained with haematoxylin and eosin, showing the anti-inflammatory effect of the treatments. **F)** Colonic myeloperoxidase (MPO) activity. **G)** Colonic glutathione (GSH) content. **H)** IL-1β production in colonic tissue quantified by ELISA. Data are expressed as means ± SEM unless otherwise stated. $^{*}P<0.05$ *vs.* TNBS control group. #$P<0.05$ *vs.* Non-colitic group.

1.2. DOXYCYCLINE EXERTS INTESTINAL ANTI-INFLAMMATORY EFFECTS IN TBNS RAT COLITIS.

Once the immunomodulatory properties of doxycycline were demonstrated *in vitro*, the intestinal anti-inflammatory effect of orally administered doxycycline was evaluated in the TNBS model of rat colitis(Fig. 2A), in comparison to minocycline, that was used as positive control[629]. The analysis of the colonic specimens one week after colitis induction showed that doxycycline exerted an intestinal anti-inflammatory effect. Macroscopically, doxycycline significantly reduced the colonic weight/length ratio (Fig. 2B) and the colonic damage score (Fig. 2C) when compared to the untreated colitic control group, an effect that was linked to a significant reduction in the area of inflamed/necrotic tissue, which was similar to that observed with minocycline.

The anti-inflammatory effect of doxycycline was also evidenced biochemically. Colonic MPO activity, a marker of neutrophil infiltration[731], [732] that was increased upon inflammation in colitic rats, was significantly reduced in the groups treated with 10 and 25 mg/kg of doxycycline (Fig. 2F). Colonic inflammation was also associated with a depletion of the antioxidant peptide glutathione, which was partially counteracted by doxycycline administration in a dose-dependent manner (Fig. 2G). In addition, doxycycline treatment was able to reduce the colonic production of the pro-inflammatory cytokine IL-1β, which was found increased in inflamed colonic tissue (Fig. 2H).

The improvement in all these biochemical markers of colonic inflammation was also evidenced after minocycline treatment.

Doxycycline anti-inflammatory effects were confirmed histologically (Fig. 2D & E). In this regard, the colonic tissue from the untreated colitic rats showed extensive ulceration of the mucosa that typically affected over 90% of the surface. The inflammatory process involved all the intestinal layers and it was characterized by intense leukocyte infiltration, mainly neutrophils, in the mucosa layer and, to a lesser extent, lymphocytes in the submucosa. In addition, complete goblet cell depletion was observed in these animals (Fig. 2E). The grade of lesion was considered in this group of rats as very severe, showing a histological damage score value of 44.0 ± 5.4 (mean ± SEM) (Fig. 2D), assigned following the criteria described in [733]. However, when the colonic segments from rats treated with either doxycycline or minocycline were evaluated, a clear tissue recovery was evidenced, resulting in a significantly reduced score value in comparison with untreated colitic rats (Fig. 2D & E). A restored epithelial cell layer was observed in most of the samples, with ulcers affecting a maximum of 25% of the epithelium in comparison with to the extensive ulceration observed in the TNBS control group.

1.3. DOXYCYCLINE EXERTS INTESTINAL ANTI-INFLAMMATORY EFFECTS IN DSS MOUSE COLITIS.

To corroborate the intestinal anti-inflammatory effect of doxycycline in experimental colitis, its anti-inflammatory activity was evaluated in a different model and specie, using the DSS model of mouse colitis (Fig. 3A). 3% DSS (w/v) administration to mice resulted in an acute colonic inflammatory process that induced the excretion of diarrheic/bleeding feces and severe body weight loss, features that were reflected in a progressively increasing disease activity index (DAI), a parameter used to monitor the evolution of the inflammatory process (Fig. 3B). The treatment of colitic mice with doxycycline at the doses of 7.5, 15 and 30 mg/kg/d induced an important reduction in the DAI values, an effect that was dose-dependent and more pronounced at the beginning of the treatment (Fig. 3B).

Figure 3: Evaluation of doxycycline-effects in DSS colitis.

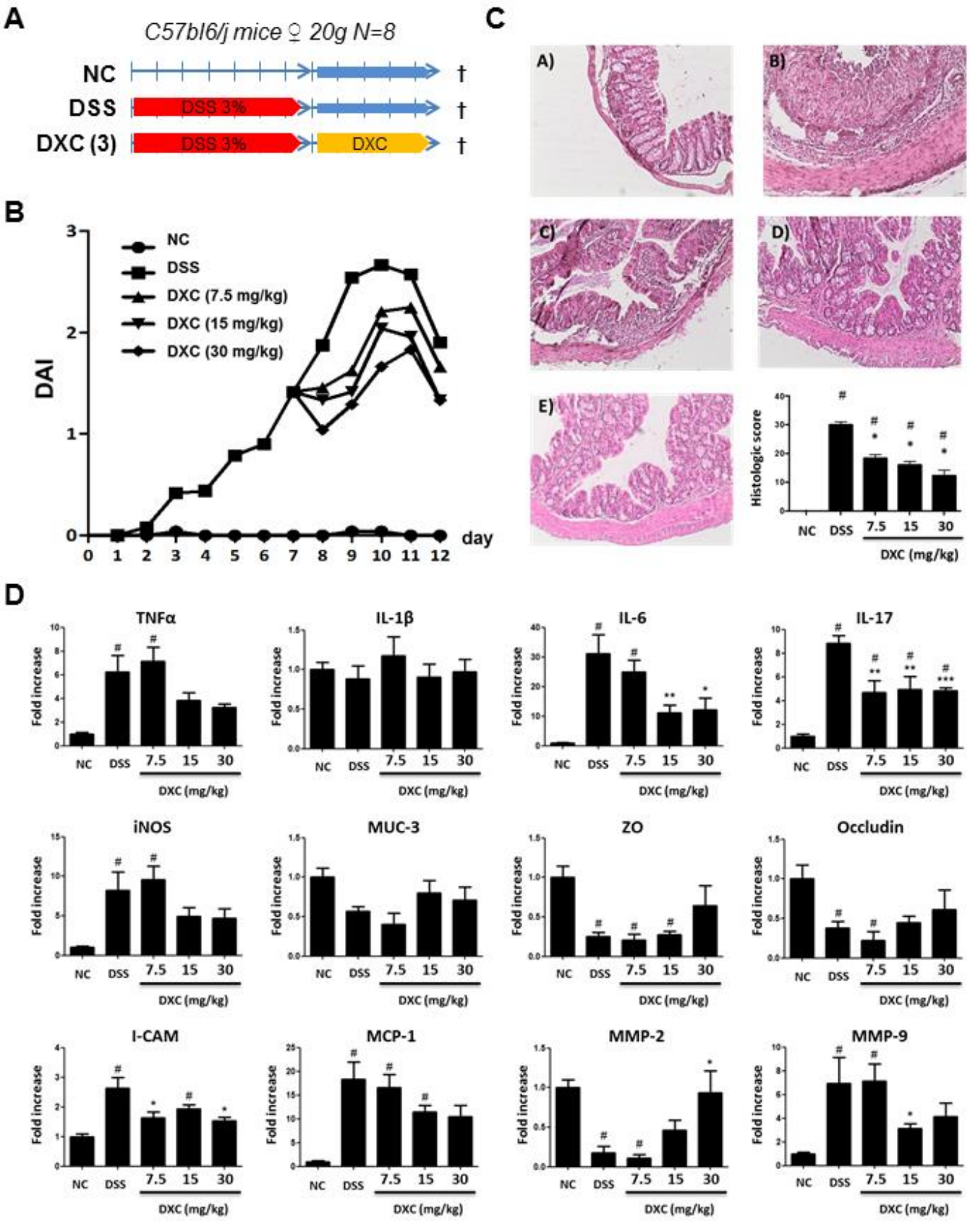


Figure 3. Intestinal anti-inflammatory effects of doxycycline in the DSS model of mouse colitis. **A)** Schematic illustration of the experimental design followed. NC: Non-colitic group, DSS: DSS-colitic group, DXC: doxycycline-treated colitic groups (7.5, 15, 30 mg/kg/d) (n=8). **B)** Disease Activity Index (DAI) values in DSS mice colitis over the 12-day experimental period, based on the criteria described in (Cooper et al., 1993). **C)** Histological sections of colonic mucosa stained with haematoxylin and eosin, showing *A)* NC group, *B)*DSS group, *C)* DXC group (7.5mg/kg/d), *D)* DXC group (10mg/kg/d) and *E)* DXC group (30mg/kg/d) (40x magnification); Microscopic damage score assigned according the criteria described in (Camuesco et al., 2004). Data are expressed as median (range). **D)** Biochemical evaluation of the effects of doxycycline (DXC) in DSS colitis; mRNA expression of TNFα, IL-1β, IL-6, IL-17, iNOS, MUC-3, ZO-1, Occludin, I-CAM-1, MCP-1, MPP-2 and MMP-9 quantified by real-time PCR. Fold increase calculated *vs.* NC group, and expressed as means ± SEM. *P<0.05, **P<0.01, ***P<0.001 *vs.* DSS control group. #*P*<0.05 *vs.* Non-colitic group.

The histological evaluation of the colonic samples confirmed the intestinal anti-inflammatory effects of the different doses of doxycycline in this model of mouse colitis (Fig.3C). Microscopically, DSS-induced colitis was characterized by epithelial ulceration (typically affecting more than 75% of the surface), intense goblet cell depletion, severe inflammatory cell infiltration in all the colonic layers and oedema between the mucosa and muscularis layers of the intestine. An average microscopic score of 30.5 ± 1.9 was assigned to control colitic mice (Fig. 3C). In contrast, the samples from the mice treated with the different doses of doxycycline showed a significant recovery of the inflammatory process. The mucosal layer was typically preserved, the inflammatory infiltrate was slight/moderate and only present in the lamina propria, and a slight oedema in the submucosa was observed (Figs. 3C). The evaluation of the colonic damage in the doxycycline - treated groups resulted in a reduced microscopic score in comparison with the untreated control group: 18.2 ± 1.9 (7.5 mg/kg), 15.9 ± 1.3 (15 mg/kg) and 12.3 ± 1.8 (30 mg/kg) (P<0.01 vs. DSS colitic group) (Fig.5F).

Biochemical analysis of the colonic tissue showed an increased mRNA expression of the pro-inflammatory markers IL-6, TNFα, IL-17, ICAM-1, iNOS and MCP-1 in response to the inflammatory process, parameters that were significantly reduced after doxycycline treatment (Fig. 3D). Doxycycline beneficial effects were also associated to a partial restoration of the mRNA levels of markers of intestinal barrier function (ZO-1, occludin and MUC-3), which were compromised during the inflammatory process. These results show that doxycycline, at doses of 15 and 30 mg/kg, also displays intestinal anti-

inflammatory activity in the DSS model of mouse colitis, and, together with the effects observed in the TNBS model of rat colitis, confirm the anti-inflammatory activity of doxycycline in the context of experimental intestinal inflammation.

1.4. ASSOCIATION OF DOXYCYCLINE WITH *SACCHAROMYCES BOULARDII* OR ITS CONDITIONED MEDIUM *IN VITRO*

The combination of immunomodulatory antibiotics and probiotics represents a therapeutic strategy that has been proposed to achieve a better control of IBD [628]. Therefore, once the intestinal anti-inflammatory activity of doxycycline was established in both the TNBS model of rat colitis and the DSS model of mouse colitis, we tested the effects of its combination with the probiotic *S. bourlardii. In vitro* experiments were initially performed to characterize the potential direct beneficial effect of this combination. With this aim, the immunomodulatory properties of the antibiotic on IL-8 and nitrite production by caco-2 and RAW 246.7 respectively were now evaluated in the presence of the viable probiotic or with the probiotic-conditioned medium (containing the compounds derived from a pre-incubation with the probiotic). No significant effects on IL-8 or nitrite production were observed after incubating the cells with either the probiotic or the conditioned medium alone. However, a slightly bigger effect was observed in both cell lines when combining doxycycline with *S. boulardii* conditioned medium (Fig. 4A & B).

Figure 4. *In vitro* immunomodulatory effects of doxycycline (DXC) 25µM, *Saccharomyces boulardii (S.b.)* 10^8 UFC/ml, *S. boulardii* conditioned medium (CM) and their association on **A)** IL-8 production by IL-1β-stimulated Caco-2 epithelial cells, and **B)** nitrite production by LPS-stimulated RAW 264.7 macrophages. Cells were incubated in the presence of the different treatments for 24h and then stimulated with IL-1β (1 ng/ml) or LPS (100 ng/ml) for another 24h. IL-8 concentration in the culture supernatant was determined by ELISA and nitrite concentration was measured by the Griess Assay. Data are expressed as mean ± SD. **P<0.01, ***P<0.001 *vs.* stimulated untreated cells. The experiments were performed three times, with each individual treatment being run in triplicate

Figure 4: Evaluation of the association DXC + *S.boulardii in vitro*

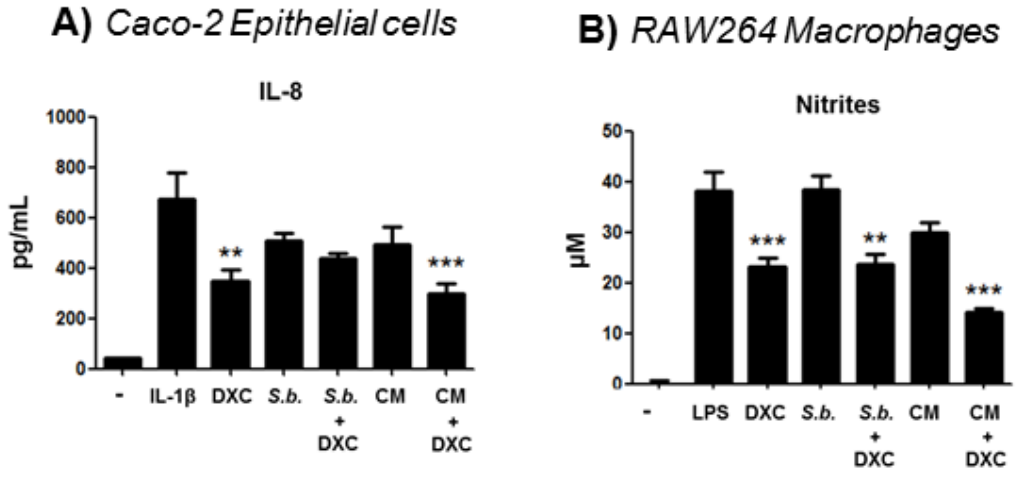


Figure 5: Evaluation of the association of DXC + *S.boulardii* in DSS-reactivated colitis

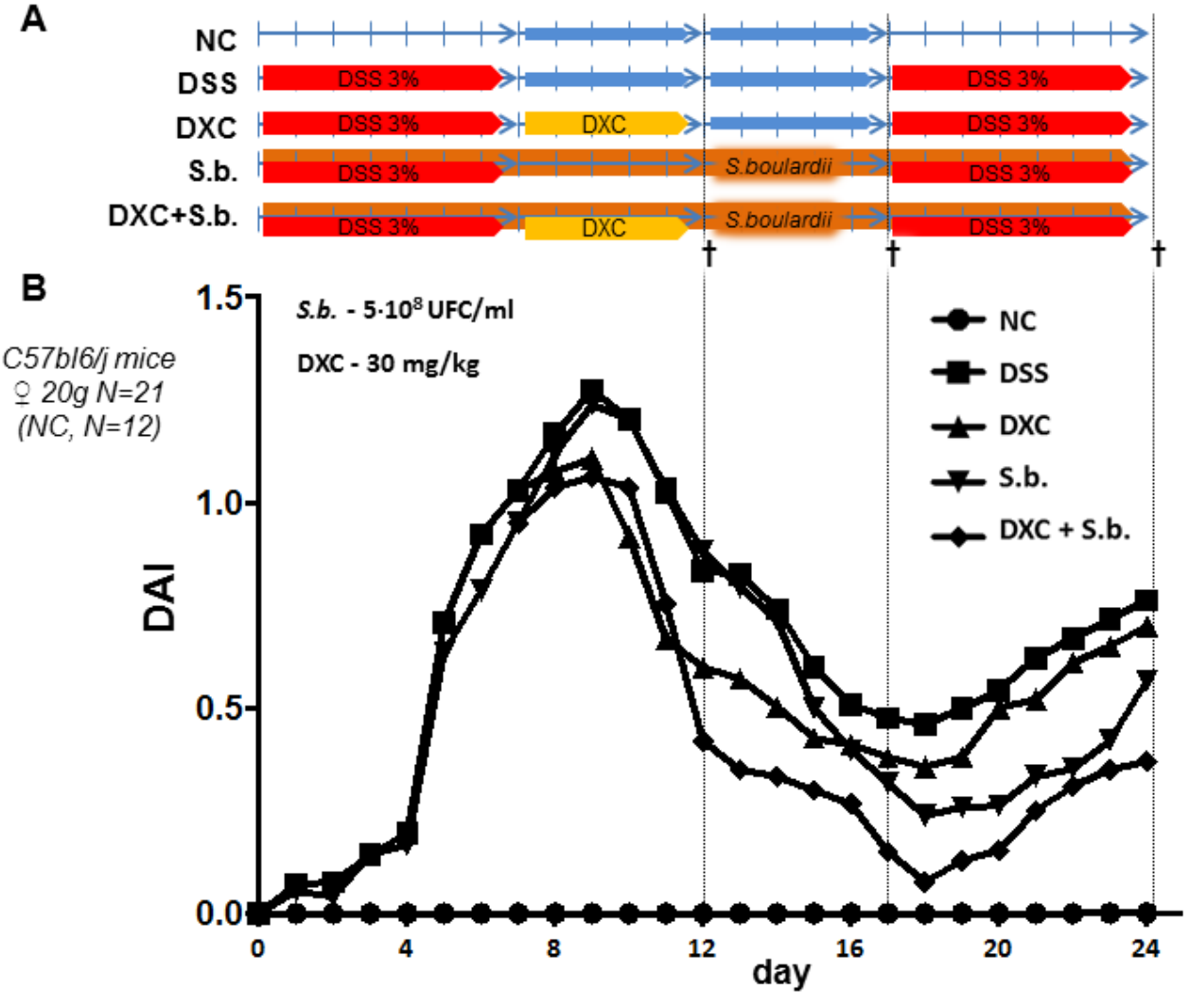


Figure 5. Intestinal anti-inflammatory effects of doxycycline (DXC), *Saccharomyces boulardii* (S.b.) and their association (DXC + S.b.) in a model of DSS-reactivated mouse colitis. **A)** Schematic illustration of the experimental design followed. NC: Non-colitic group (n=12), DSS: DSS-colitic group, DXC: doxycycline-treated colitic group (30 mg/kg/d), S.b.: *Saccharomyces boulardii* treated colitic group ($5 \cdot 10^8$ UFC/ml), DXC + S.b.: doxycycline and *Saccharomyces boulardii* treated colitic group (n=21). **B)** Disease Activity Index (DAI) mean values in DSS mice colitis over the 24-day experimental period, based on the criteria described in (Cooper et al., 1993).

1.5. ANTI-INFLAMMATORY EFFECT OF DOXYCYCLINE IN ASSOCIATION WITH *S. BOURLARDII* IN A MOUSE MODEL OF REACTIVATED COLITIS.

The strategy of combine doxycycline with the probiotic *S. bourlardii* was evaluated *in vivo* in a DSS model of reactivated colitis[628] to improve the management of the relapsing course of IBD. With this aim, 3% DSS (w/v) was administered to mice for 7 days to induce colitis, and the inflammatory process and the effects of this association were evaluated at different time points as explained below. Then, the effects of this strategy in preventing the relapses were assessed after colitis was reactivated 10 days later by a administering a second cycle of DSS (Fig. 5A).

Evaluation of the colonic inflammatory status at day 12.

Administration of 3% DSS (w/v) in the drinking water to mice for 7 days induced the development of an intestinal inflammatory process. A daily DAI evaluation was performed to follow up the evolution of the colonic inflammatory process and the impact of the treatments. Doxycycline administration, either alone or in combination with the probiotic *Saccharomyces boulardii*, promoted the recovery of the mice, as shown by the significant decrease in the DAI values observed during the first five days of treatment, (Fig. 5B). However, the group

treated with only *S. boulardii* did not show significant differences in the DAI values in comparison with the corresponding untreated colitic groups (Fig. 5B).

The histological studies confirmed these observations (Fig.6A & B). Similarly to that previously described, the colonic damage induced by DSS was characterized by severe epithelial ulceration (affecting almost all the surface), goblet cell depletion and an intense inflammatory cell infiltration into the lamina propria, together with the presence of edema between the mucosa and muscularis layers of the intestine. In contrast, most of the samples of the doxycycline-treated animals, alone or in combination with the probiotic, showed a substantial improvement in the inflammatory process. The epithelium was significantly preserved, which was associated with the recovery of the mucosa layer and the presence of goblet cell replenished with its mucin content; only a few animals showed an inflammatory infiltrate of mononuclear cells in the lamina propria, and slight edema in the submucosa (Figs. 6A & B). The evaluation of the damage showed a significant reduction in the microscopic score values of the doxycycline-treated groups compared with the untreated control group (Fig. 6A). However, the colonic specimens from *S. boulardii*-treated colitic mice did not show substantial differences when compared with those from untreated colitic mice being assigned with similar histologic scores (Fig. 6A).

Biochemically, DSS-induced inflammation was characterized by increased mRNA expression of the pro-inflammatory cytokines IL-12, IL-17, IL-1β and TNFα, the adhesion molecule ICAM-1 and the enzymes MMP-2, MMP-9 and iNOS, together with a decreased expression of the anti-inflammatory cytokine IL-10 and some proteins involved in epithelial integrity, including MUC-1 and MUC-3 (Fig. 7). Doxycycline administration to colitic mice had a significant beneficial impact on the expression of IL-17 and IL-10. Of note, and despite not having an effect on DAI values at this time point, *S. boulardii* pretreatment was able to significantly restore the altered expression of IL-17, ICAM-1, MMP-2 and IL-10. Interestingly, antibiotic administration to mice that had previously received the probiotic was the most effective therapeutic intervention in restoring the expression of some these markers, including IL-17, IL-10 and MUC-1, (Fig. 7).

Figure 6: DXC + *S.boulardii* in DSS-reactivated colitis: Histological analysis.

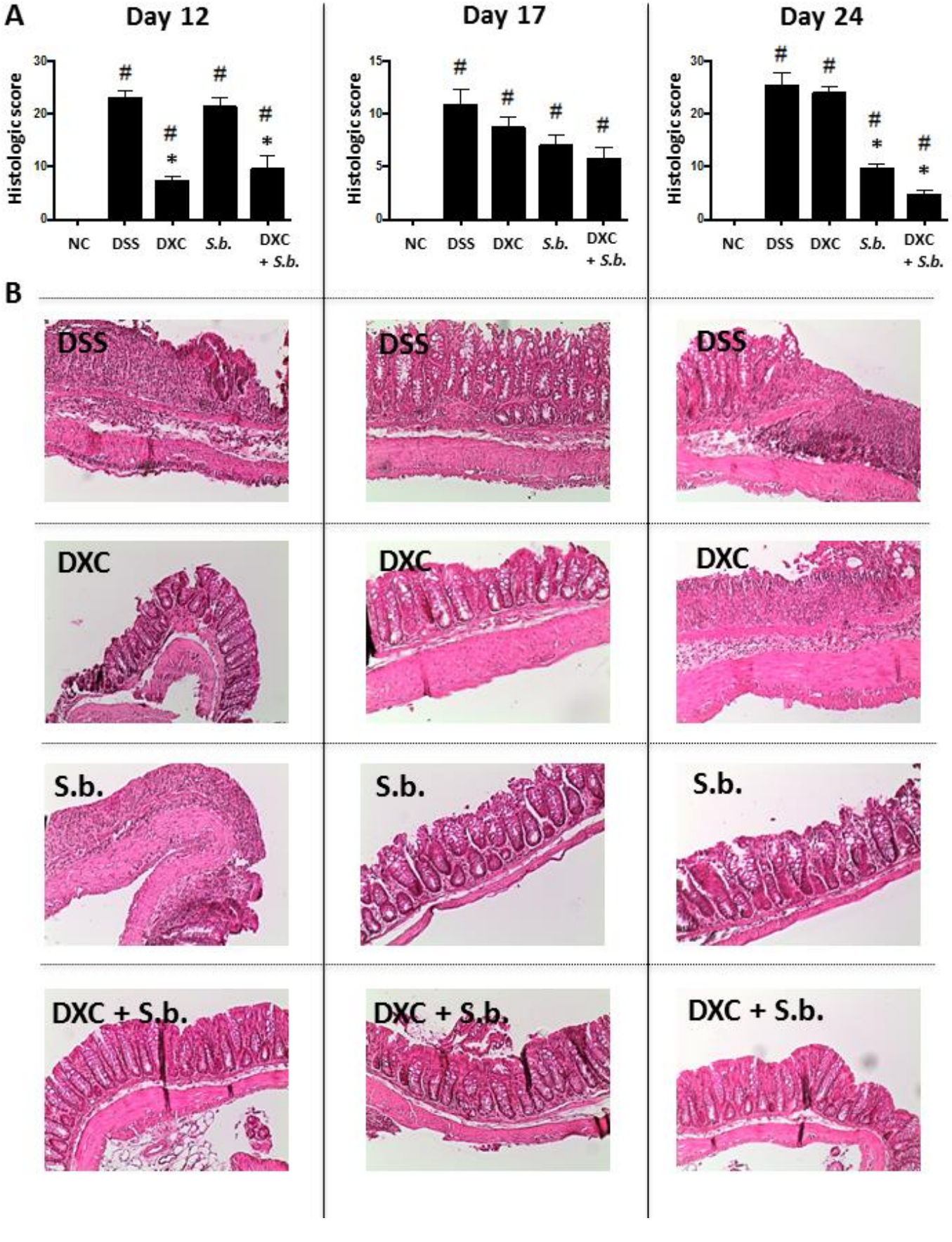


Figure 7: DXC + *S.boulardii* in DSS-reactivated colitis: Effects after 5 days of treatement (Day 12).

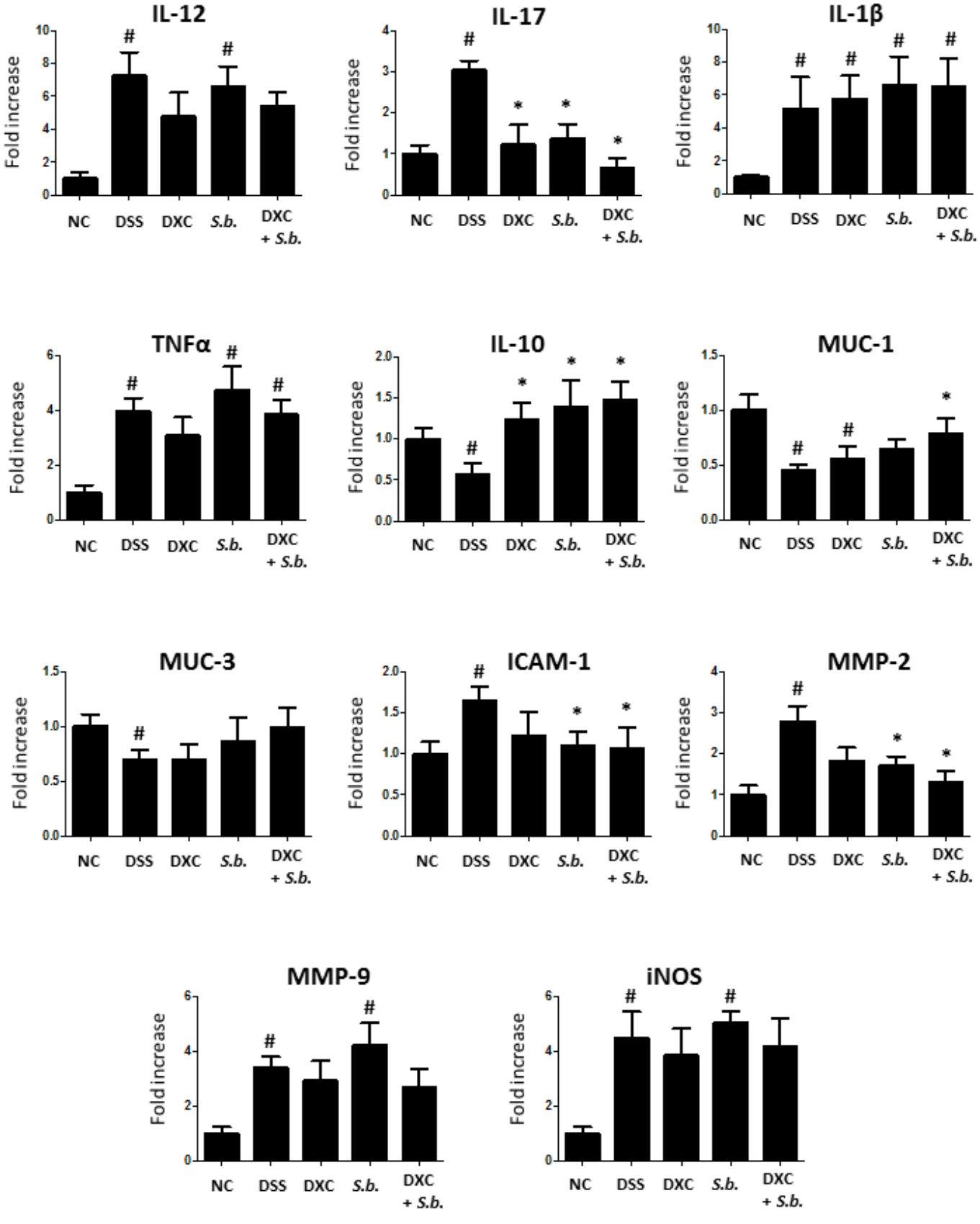


Figure 6: Histological analysis of colonic mucosa showing the effects of doxycycline (DXC), *Saccharomyces boulardii* (S.b.) and their association (DXC + S.b.) in a model of DSS-reactivated mouse colitis. **A)** Microscopic damage score at the different time points assigned according the criteria described in (Camuesco et al., 2004). Data are expressed as median (range). $^{*}P<0.05$ *vs.* DSS control group (DSS). #$P<0.05$ *vs.* Non-colitic group (NC). **B)** Representative sections of the different treatment groups and at the different time points stained with haematoxylin and eosin (40x magnification).

Figure 7: Biochemical evaluation of the effects of doxycycline (DXC), *Saccharomyces boulardii* (S.b.) and their association (DXC + S.b.) in DSS-reactivated mouse colitis after 5 days of treatement (Day 12). mRNA expression of the indicated genes quantified by real-time PCR. Fold increase calculated *vs.* NC group, and expressed as means ± SEM. *P<0.05 *vs.* DSS control group (DSS). #$P<0.05$ *vs.* Non-colitic group (NC).

Evaluation of the colonic inflammatory status at day 17.

After five days of treatment, the administration of doxycycline was suspended, but the treatment with the probiotic *S. boulardii* was continued in order to evaluate its effect in maintaining the remission. The time-course evolution of the DAI values during the following five days showed that the continued probiotic administration further improved the recovery of the mice when compared to those mice without treatment, in which the DAI values remained constantly higher (Fig. 5B). It is interesting to note that in the group that previously received doxycycline, the treatment with the probiotic at this stage did have a positive impact on the body weight loss and feces consistency, showing the lowest DAI value among all colitic groups.

At this time point, the microscopic study revealed a gradual improvement of the colonic tissue with time, that was generalized to all colitic mice, although differences could still be observed in comparison with non-colitic mice (Figs. 6A & B). The mucosa was in process of recovery: the epithelium appeared restored and the presence of goblet cells replenished with their mucin content was noted in most of the samples, although crypt hyperplasia was still evident in some of them. Similarly, the inflammatory infiltrate was less intense than a week earlier. When the microscopic score was evaluated, no differences were observed among the colitic groups), although a trend towards a lower score was appreciated in the treated groups (Fig. 6A)

Figure 8: DXC + *S.boulardii* in DSS-reactivated colitis: Effects 10 days after colitis induction (Day 17).

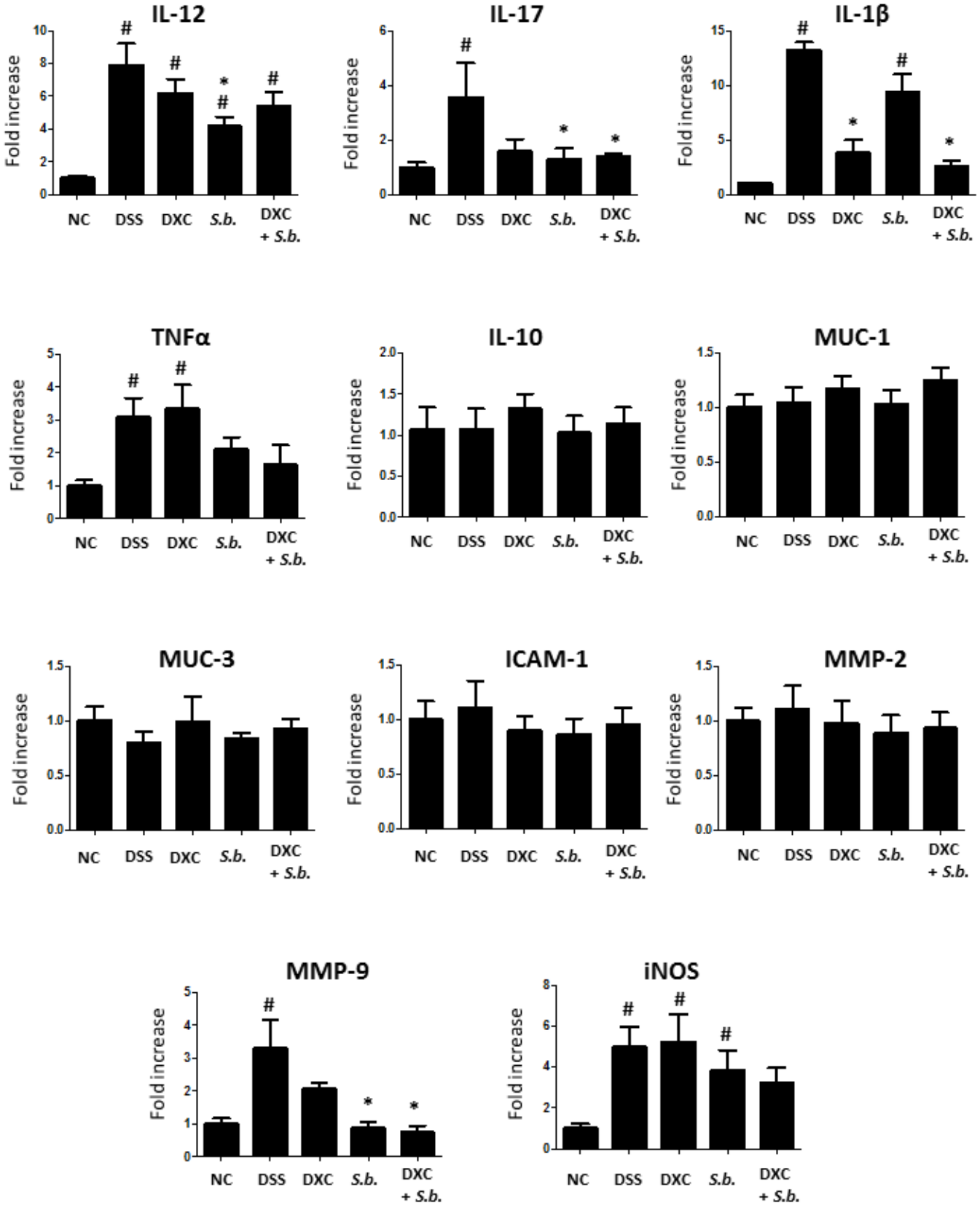


Figure 8: Biochemical evaluation of the effects of doxycycline (DXC), *Saccharomyces boulardii* (S.b.) and their association (DXC + S.b.) in DSS-reactivated mouse colitis 10 days after colitis induction (Day 17). mRNA expression of the indicated genes quantified by real-time PCR. Fold increase calculated *vs.* NC group, and expressed as means ± SEM. *P<0.05 *vs.* DSS control group (DSS). #*P*<0.05 *vs.* Non-colitic group (NC).

The analysis of biochemical markers showed that the expression of IL-12, IL-17, IL-1β, TNFα, MMP-9 and iNOS remained increased in control colitic mice when compared with healthy mice (Fig. 10). However, the in the mice treated with doxycycline followed by *S. boulardii* the expression of most of these markers of inflammation appeared restored, being this combination the one with the greatest efficacy of all the treatments assayed (Fig. 8).

Evaluation of the colonic inflammatory status at day 24, after colitis reactivation.

A relapse in the inflammatory process was provoked by the administration of a second cycle of DSS. As expected, this resulted in a progressive increase in the DAI values of all the groups, reflecting the exacerbation of the intestine inflammatory process(Fig. 5B). Although the DAI values after this cycle were more moderate than those reached after the first one, it was noted that the relapse of the inflammatory process in the group of mice that had previously received the probiotic was milder.

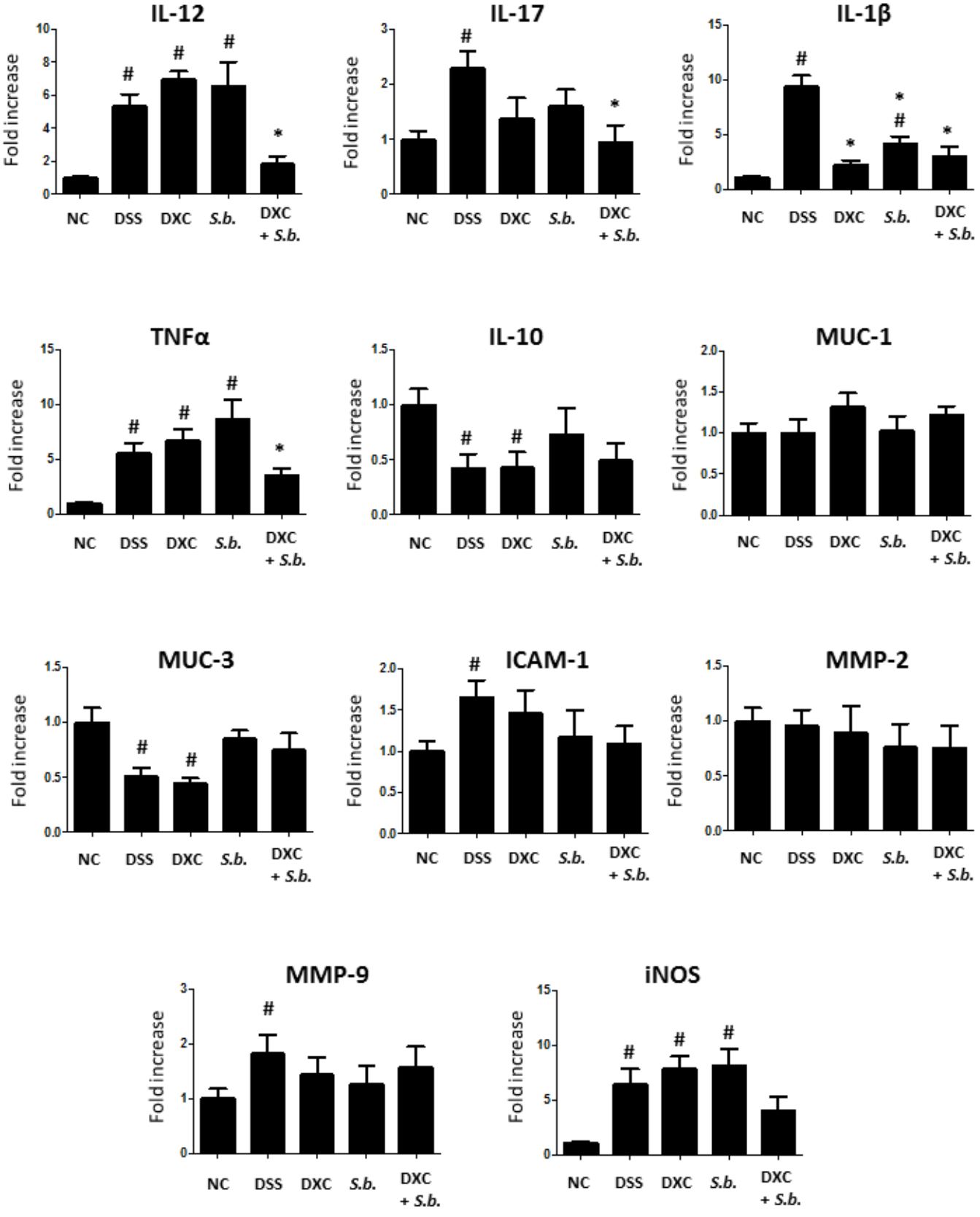
The microscopic evaluation of the colonic samples also reflected the reactivation of the inflammatory process. This was characterized by the ulceration and necrosis of the epithelial cells, affecting more than 50% of the surface in the majority of the animals. This colonic damage was associated again with intense infiltration of leukocytes in all the colonic layers and the presence of oedema was evident in most of the samples (Fig. 6A & B). The grade of lesion was considered as severe, showing a score value of 25.2 ± 2.5 (Fig. 6A). Post-reactivation, treatment of these colitic mice with doxycycline did not significantly ameliorate the damage, however, the groups receiving *S. boulardii*, alone or in

association with doxycycline, showed a significant recovery of the intestinal histology compared with the untreated control group (Figs. 6A & B). This improvement was evidenced by a significant reduction in the ulcerated surface, a preservation of the mucosal architecture, with the presence of goblet cells and a reduction in the inflammatory infiltrate, which was considered slight to moderate in most of the samples. These changes were particularly evident in the mice that received the combined therapy., As a result, the microscopic score values were significantly reduced in these treated groups in comparison with the untreated control group (Fig. 6A).

As expected, the colitis reactivation promoted an increase in the expression of most of the pro-inflammatory makers assayed, together with a decrease in the expression of IL-10 and MUC-3 (Fig. 9). Of note, the combination of doxycycline with *S. boulardii* was proved again to be the most effective of the treatments, promoting the restoration of the expression of most of the mediators studied, including TNFα, IL-1β, IL-12, IL-17 and iNOS, whereas the individual treatments were only able to significantly modify some of them (Fig. 9).

Figure 9: Biochemical evaluation of the effects of doxycycline (DXC), *Saccharomyces boulardii* (S.b.) and their association (DXC + S.b.) in DSS-reactivated mouse colitis after colitis reactivation (Day 24). mRNA expression of the indicated genes quantified by real-time PCR. Fold increase calculated *vs.* NC group, and expressed as means ± SEM. *P<0.05 *vs.* DSS control group (DSS). #*P*<0.05 *vs.* Non-colitic group (NC).

Figure 9: DXC + *S.boulardii* in DSS-reactivated colitis: Effects after colitis reactivation (Day 24).



AIM 2

So far we have proven that doxycycline and minocycline display intestinal anti-inflammatory effects in two different experimental models of colitis, most probably due to the contribution of their additional non-antibiotic activities. Previous studies have compared the activity of minocycline to that of tetracycline, which does not have such strong immune-modulatory properties [629]. However, and although minocycline was superior, some anti-inflammatory activity was still observed with tetracycline, in agreement with other studies that have reported a role for antibiotics in experimental colitis (ref antibiotics). An increased understanding of the role that the immunomodulatory properties of these antibiotics play in the management of these intestinal conditions would further support their potential applications. Therefore, to better characterize the mechanisms of action of these compounds, we proposed to assess the following question:

How do the different activities of immunomodulatory tetracyclines contribute to the control intestinal inflammation?

It is evident the great difficulty to segregate the degree of contribution of both components, antibiotic or immunomodulatory properties, to the intestinal anti-inflammatory effects of tetracyclines. In an attempt to clarify this, we compared the effects a range of compounds with different activities, ranging from the antibiotic action to immunomodulatory properties, including tetracyclines that combined the two of them.

- Immunomodulatory activity in macrophages.
- Comparative study in DNBS colitis.
- Comparative study in DSS colitis.
- Functional evaluation of the impact on intestinal microbiota.

2.1. IMMUNOMODULATORY ACTIVITY ON MACROPHAGES

One of the best characterized properties of tetracyclines is their ability to reduce iNOS enzyme activity and the subsequent NO production. Therefore, nitrite production was determined in LPS-activated RAW 246.7 macrophages after their incubation with the different compounds (rifaximin (RFX), tetracycline (TTC), doxycicline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) (Fig 10A), and concentration-response curves were obtained in order to compare their immunomodulatory activity. Dexamethasone proved to be the most potent inhibitor of NO production by LPS activated macrophages, followed by TGC, MNC and DXC, while no significant effect was observed for rifaximin and tetracycline at the concentrations assayed (Fig. 10A).

In addition, previous studies have described that immunomodulatory tetracyclines may increase the production of pro-inflammatory mediators by monocytes and alveolar macrophages, while others have shown that they inhibit the activity of peritoneal macrophages and microglia[611], [713]. With this in mind, we tested the effect of dexamethasone and the three immunomodulatory tetracyclines in a primary culture of mouse bone marrow derived macrophages (BMDM) at the concentration of 25 μM. Gene expression analysis confirmed that LPS-activation of BMDM induces iNOS Mrna (Fig. 10B). All the tested compounds significantly reduced iNOS expression, with TGC attaining the strongest effect. Surprisingly, when the three most relevant innate cytokines produced by macrophages (TNF, IL-1β and IL-6) were evaluated, the three tetracyclines did however potentiate their LPS-induced expression (Fig. 10C). Even more, an increase of the basal levels of IL-6 and IL-1β was observed. Conversely, dexamethasone inhibited the expression these cytokines. These results were confirmed when protein levels were evaluated in the culture supernatant by ELISA. Dexamethasone reduced cytokine release by LPS-activated macrophages, while the immunomodulatory tetracyclines potentiated their production, significantly increasing TNFα, IL-1β and IL-6 levels (Fig 10D).

Figure 10: Comparative study in macrophages *in vitro*.

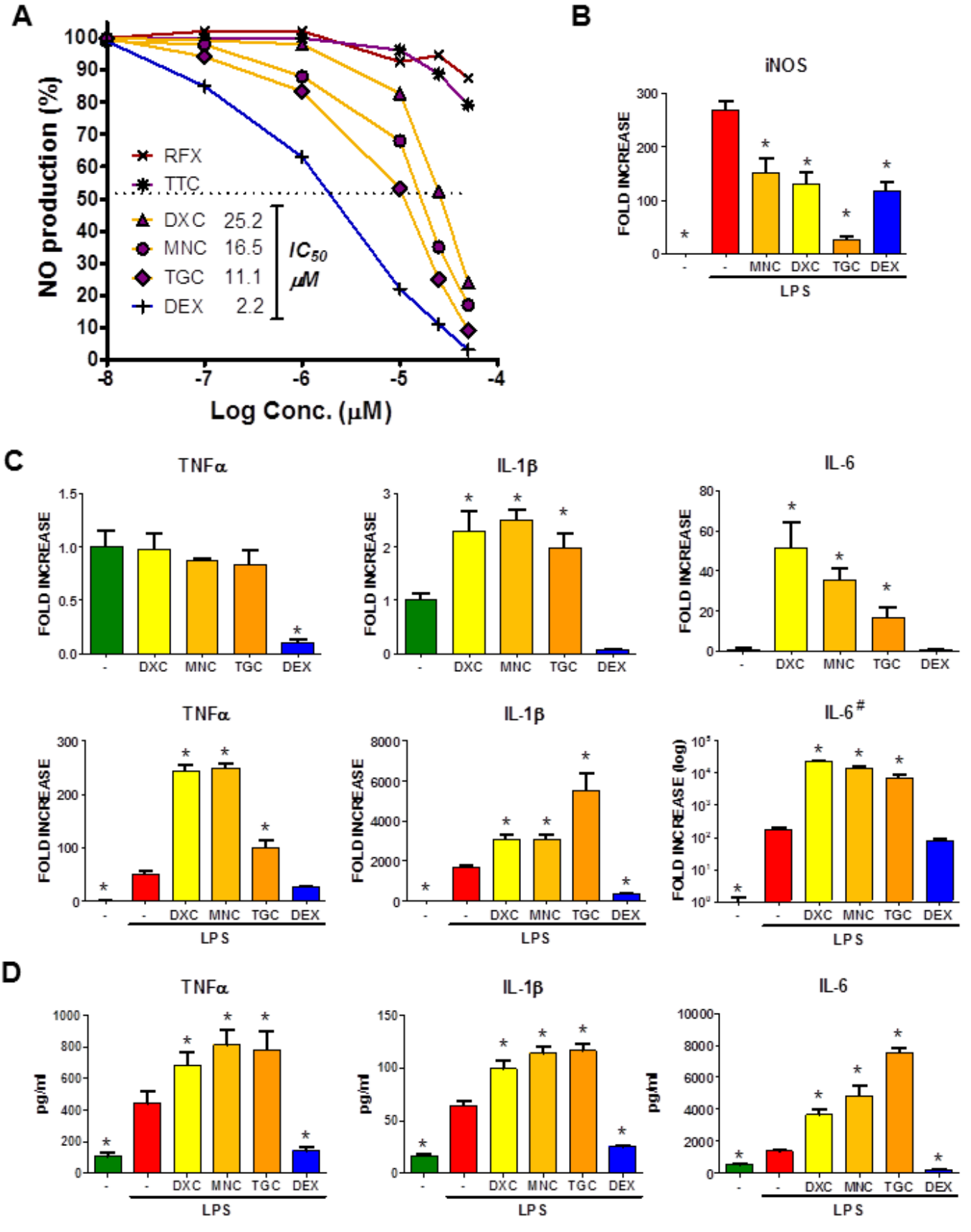


Figure 10: Comparative study of the effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) on macrophage activity *in vitro*. **A)** Nitrite production by LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were incubated with the different treatments at the indicated concentrations for 24h and then stimulated with LPS (100 ng/ml) for 24h. Nitrite concentration in the culture supernatant was measured by the Griess Assay. **B)** iNOS mRNA expression in LPS-stimulated (10ng/ml) BMDM after 24h of pre-incubation with the different treatment quantified by real-time PCR. **C)** mRNA expression of the indicated genes in LPS-stimulated (10ng/ml) BMDM after 24h of pre-incubation with the different treatment quantified by real-time PCR. **D)** TNFα, IL-1β and IL-6 concentration in the culture supernatant of LPS-stimulated (10ng/ml) BMDM after 24h of pre-incubation with the different treatment quantified by ELISA. The experiments were performed three times, with each individual treatment being run in triplicate. Data expressed as mean ± SD. Fold increase is calculated *vs.* unstimulated untreated cells. *P<0.05 *vs.* stimulated untreated cells.

2.2. COMPARATIVE STUDY ON DNBS COLITIS

The DNBS model of mouse colitis is a hapten-induced model, with similar features to TNBS model in rats[146]. Using this model, different experimental groups were stablished (Fig.11A), including both untreated Non-colitic and DNBS-colitic control groups, as well has 6 treated DNBS-Colitic groups that received respectively the following treatments: 1) rifaximin (200 mg/kg/day), a non-absorbable antibiotic, which provides a local antibiotic action; 2) tetracycline (250 mg/kg/day), included as reference of systemic antibiotic action.; 3-5) immunomodulatory tetracyclines: doxycycline (25 mg/kg/day), minocycline (50 mg/kg/day) and tigecycline (25 mg/kg/day); and 6) dexamethasone (2.4 mg/kg/day), a compound with immunomodulatory properties without the antibiotic action. In these experiments, the different treatments were administered orally 6 hours after colitis induction, and they were then given daily until the end of the study (Fig11A).

It has been previously reported that DNBS administration induces a severe damage in the colonic tissue, macroscopically characterized by the presence of fibrosis, necrosis and obstruction[145], [732]. In fact, in the present study, colitis induced a severe weight lost and high mortality rate was obtained from the third day after the DNBS instillation(Fig. 11B & C), when the inflammation is considered completely stablished in this model. The groups treated with the immunomodulatory tetracyclines showed a clear amelioration of the colonic inflammatory process. A milder body weight loss was observed in all the groups treated with immunomodulatory tetracyclines(Fig. 11C), which was already evident from the beginning of the treatment, and reached statistically significant differences compared to the untreated colitic group from day 3. The mice treated with rifaximin also showed a reduced weight loss from day 4, although the effect of rifaximin was smaller than that obtained with the tetracyclines. It is interesting to note that dexamethasone treatment did not induce any beneficial effect in terms of weight evolution in comparison with the untreated control group (Fig.11C).

The positive impact of tetracyclines, was also reflected on survival rates(Fig.11B). Statistically significant differences were found in the survival curves of TTC, DXC and MNC-treated groups when compared with that of the DNBS control group. No significant differences were observed for TGC treated group, although an early death event could have been attributed to the procedure of colitis induction. Survival rates at the end of the experiment for each group were: NC (100%), DNBS (37%), RFX (50%), TTC (75%), DXC (87%), MNC (87%), TGC (75%) and DEX (37%). Neither dexamethasone nor rifaximin were able to reduce the strong mortality caused by the DNBS-induced acute inflammation. (Fig.11B)

Upon sacrifice of the animals, the macroscopic evaluation of the colonic specimens confirmed the severe damage induced by the DNBS. The inflammatory process was characterized by the shortening and thickening of the large intestine and the presence of multiple adherences between it and the surrounding tissues. The colonic weight/length ratio was used to quantify the macroscopic damage(Fig.11D), being this ratio almost 4-times higher in the colitic control group in comparison with non-colitic mice. The four groups of colitic mice treated with tetracyclines showed a significant reduction in this ratio. However, the administration of rifaximin or dexamethasone did not significantly modify this ratio when compared with the control colitic group(Fig.11D).

Figure 11: Comparative study in DNBS colitis.

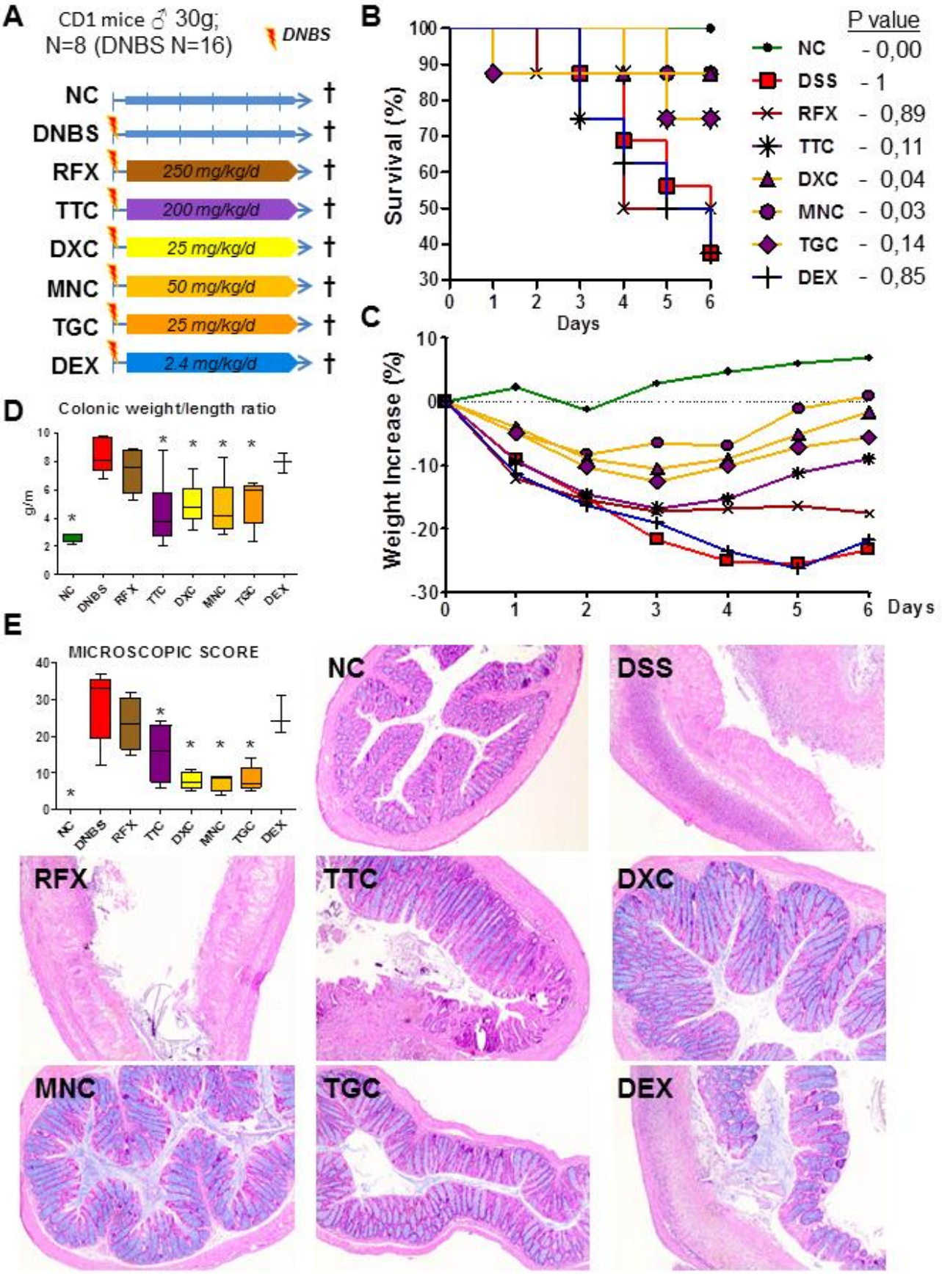
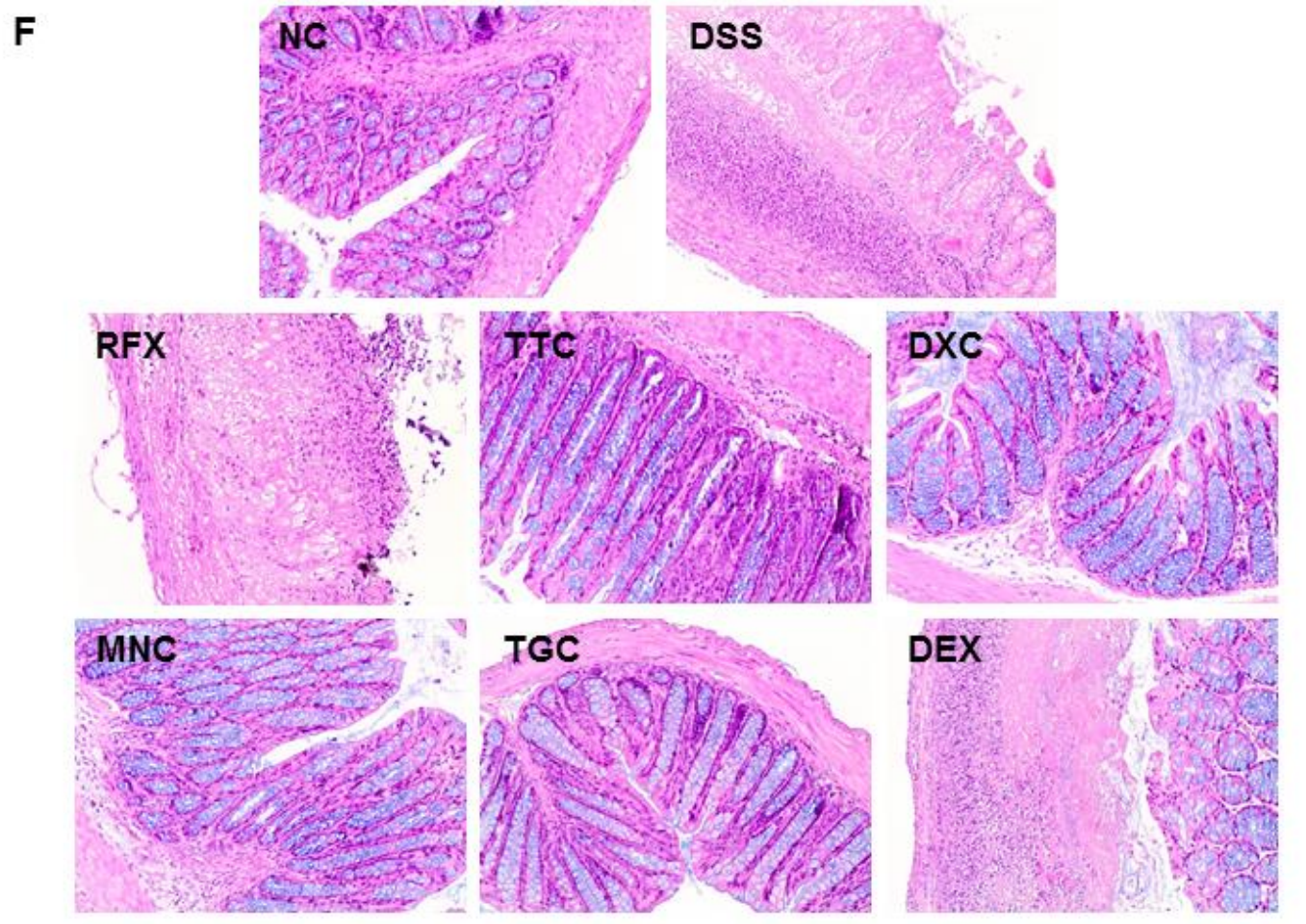


Figure 11: Comparative study in DNBS colitis.



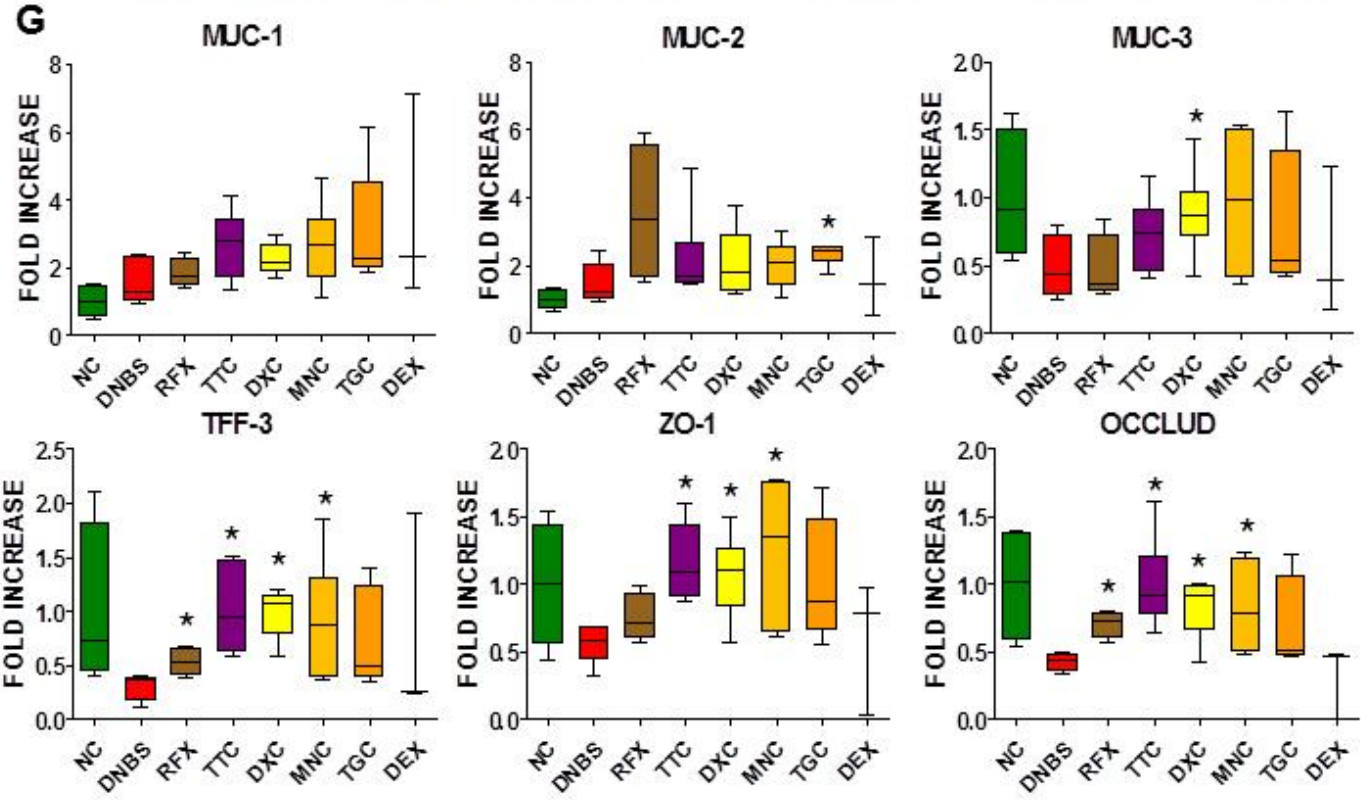


Figure 11. Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DNBS model of mouse colitis. **A)** Schematic illustration of the experimental design followed. NC: Non-colitic group, DNBS: DNBS-colitic group. **B)** Survival curves (%) and their P values *vs.* DNBS control group of the different experimental groups during the 6-days experimental period. **C)** Body weight evolution (mean % of increase) of the different experimental groups during the 6-days experimental period. **D)** Colonic weight/length ratio. **E)** Microscopic damage score assigned according the criteria described in (Camuesco et al., 2004). Representative histological sections of colonic mucosa of the different experimental groups stained with haematoxylin and eosin (40x magnification). **F)** Representative histological sections of colonic mucosa of the different experimental groups stained with haematoxylin and eosin (100x magnification). **G)** mRNA expression of mucins (MUC-1, MUC-2, MUC-3), TFF-3, ZO-1 and Occludin quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DNBS control group.

The microscopic evaluation of the colonic tissues from the different experimental groups confirmed the beneficial effects exerted by the different tetracyclines(Fig.11E & F). The colonic samples from the DNBS control group showed extensive necrosis and ulceration that affected almost the entire surface of the colon. On the most affected areas, the mucosal architecture was lost and in those areas where the epithelial layer could be recognized, goblet cells were depleted from their mucin content. In this model, the inflammatory process involved all the intestinal layers, with intense leukocyte infiltration, and thickening of the submucosa and muscularis mucosa (Fig.11E & F). Therefore, the grade of lesion in the DNBS control group was considered as very severe and it was assigned a microscopic score of 33, according to the criteria described[733]. No differences were observed between the control mice and those treated with rifaximin and dexamethasone, which also showed a severe microscopic damage. However, a reduced extension of the colonic surface appeared damaged in mice treated with tetracyclines and the visible lesions in these animals were also less severe: the mucosal layer mostly conserved the crypt architecture, with intense mucus staining in goblet cells, revealing a replenishment of their mucin content. As a result, the microscopic score values in these groups were significantly reduced in comparison with the colitic control group(Fig.11E & F).

Finally, the inflammatory status was also evaluated biochemically. With this purpose, colonic tissue was processed for RNA extraction and gene expression

was evaluated by RT-qPCR. The results revealed that the colonic expression mediators of epithelial barrier function(Fig.11G), such as TFF-3, ZO-1, and OCCLUDIN, was reduced as a consequence of the inflammatory process, and their colonic expression was significantly increased in colitic mice treated with tetracycline, doxycycline or minocycline, in comparison with untreated colitic mice. However, the gene expression of the mucins MUC-1, MUC-2 and MUC-3 was not significantly modified in this model. Despite that, the administration of tetracyclines to colitic mice showed a trend to increase their expression in comparison with the colitic control group, although the statistical differences were only obtained with TCG in MUC-2 expression or with DXC in MUC-3 expression (Fig.11G).

The colonic expression of the cytokines TNFα, IL-1β and IL-6 was significantly increased in the DNBS control group when compared with non colitic mice (Fig.12). These cytokines can be considered as the most relevant cytokines in the innate immune response in the inflamed intestine. Paradoxically, rifaximin and tetracycline significantly decreased TNFα expression, as well as dexamethasone, although the other immunomodulatory tetracyclines did not significantly modify the expression of this cytokine(Fig.12). However, all tetracyclines markedly reduced the expression of IL-1β and IL-6, although these values were not always statistically significant, probably due to the high variability among individual data in a given group. Similar results were obtained when evaluating the expression of MPP-9, an enzyme involved in tissue remodelling, and CXCL2, a chemokine that participates in neutrophil recruitment(Fig.12). Surprisingly, the expression of CCL2, involved in monocyte and dendritic cell recruitment, was reduced in the DNBS group, suggesting a reduced recruitment of those cells to the inflammatory site. The administration of tetracyclines not only restored its expression, but also increased it above the levels found in non-colitic mice(Fig.12). It is interesting to note that the immunosuppressant effects of dexamethasone, evidenced by the reduction of the colonic expression of both TNFα and IL-1β, did not seem to result in the amelioration of the intestinal inflammation induced by the DNBS, thus revealing the lack of efficacy of this glucocorticoid to control the outcome of this inflammatory response.

Figure 12: Comparative study in DNBS colitis.

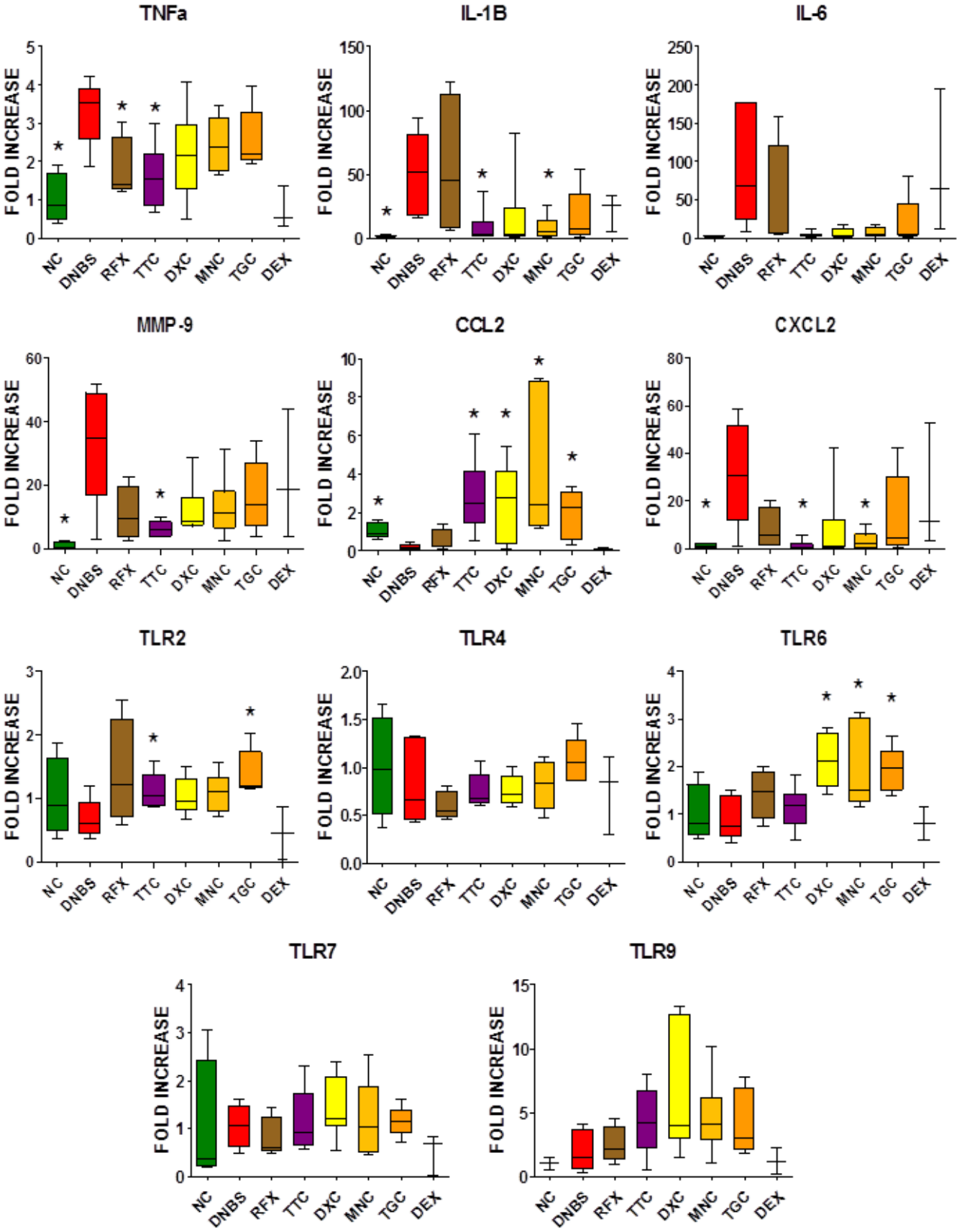


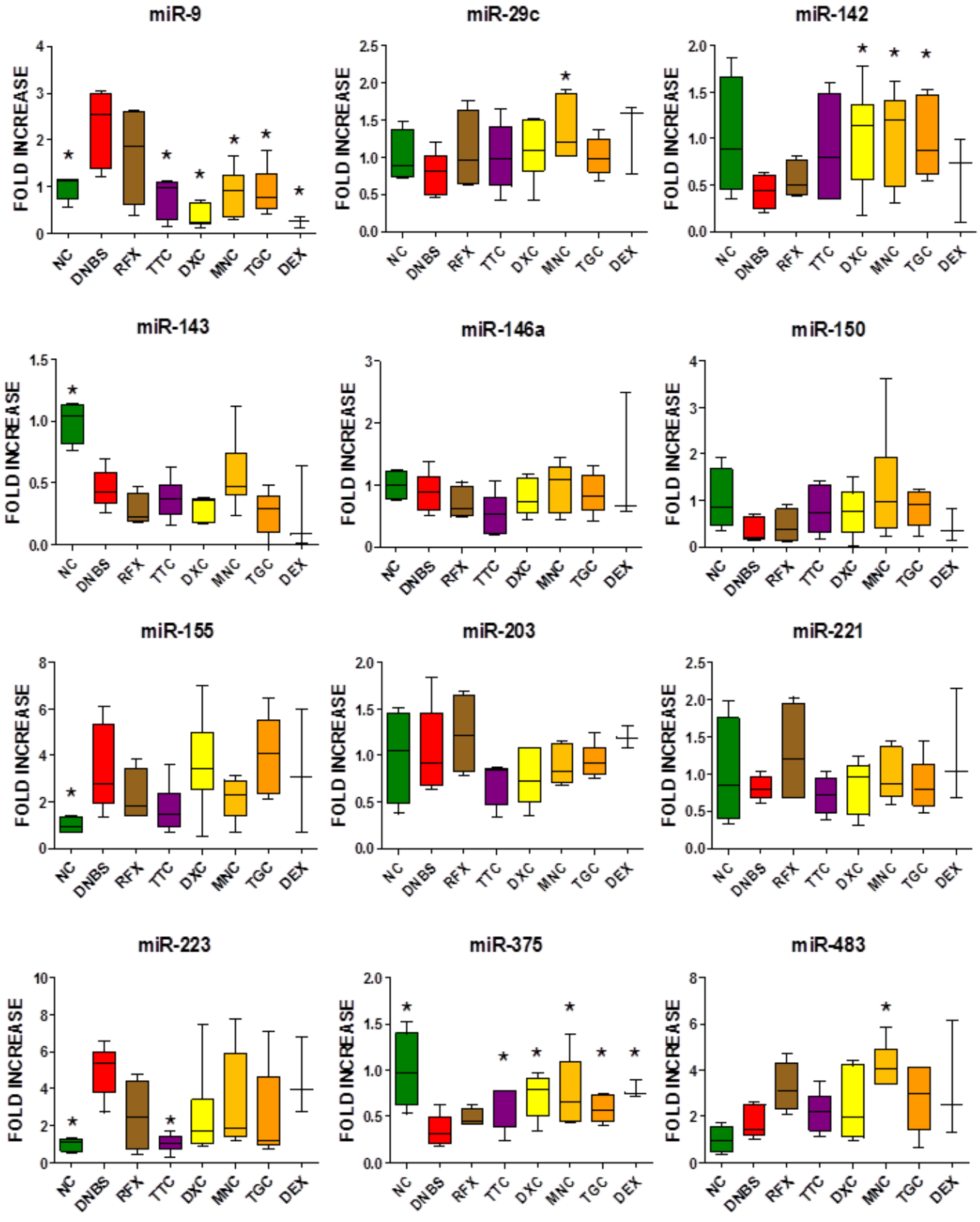
Figure 12. Gene expression analysis in the comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DNBS model of mouse colitis. NC: Non-colitic group, DNBS: DNBS-colitic group. mRNA expression of the indicated genes quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DNBS control group.

Recent studies have highlighted the role of microRNAs in the regulation of many biological processes, and their contribution to adapt cellular responses to environmental stress[734]–[736]. In fact, deregulated miRNA expression has been associated with intestinal inflammation, and differences in miRNA expression between inflamed and non-inflamed tissue can be found in IBD patients[736]. Every year, new roles are discovered for miRNAs in the regulation of intestinal epithelial barrier and the immune system, and changes on the intestinal microbiota, epithelial barrier and the immune response are linked to variations on miRNA expression. Microbial antigens signal through PRR, such as TLRs, which strongly regulated the expression of miRNAs, as well as other inflammatory mediators[87], [266]. Consequently, variations in the expression and activation of these receptors could modulate the impact that microbial signals have on the inflammatory response). Considering this, a characterization of the miRNA and TLR expression profile of the different models of colitis used in this project was performed, and the variations on this profile induced by the different treatments were analysed.

However, in this model, no significant modifications in the expression of the different TLRs were observed in the colonic tissue of colitic mice (Fig.12). Nevertheless, a trend to increase colonic TLR expression was observed in colitic mice with tetracyclines, especially on TLR6 and TLR9 expression (Fig.12).

Figure 13. micro-RNA expression analysis in the comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DNBS model of mouse colitis. NC: Non-colitic group, DNBS: DNBS-colitic group. Expression of the indicated micro-RNAs quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DNBS control group.

Figure 13: Comparative study in DNBS colitis.



Based on previous studies from our group and on the available scientific literature, a set of miRNAs was selected for its evaluation in this model of colitis (Fig.13). It is important to note that changes on miRNA expression are generally subtle, despite their biological relevance given their wide regulatory potential. The results obtained in the present study revealed that the DNBS model of colitis was characterized by an increase on miR-9, miR-155, miR-223 and miR-488 expression, while miR-142, miR-143 and miR-150 were decreased, although statistically significant differences were not always reached(Fig.13). When considering the impact of the different treatments on experimental colitis, the most pronounced effects on the miRNA expression profile characteristic of this model were: 1) tetracyclines and dexamethasone reduced the expression of miR-9; and 2) tetracyclines restored the expression of miR-142, miR-150 and miR-375 (Fig.13). Due to a high variability and lack of consistency of these changes among the groups, drawing further conclusions at this point does not seem accurate.

In order to characterize the modifications in the intestinal microbiota composition, 16S ribosomal DNA sequencing and bioinformatics alignment comparison against RDP database were performed. In this model of colitis, no statistical differences were observed in the different ecological parameters evaluated (Fig.14, table), including those related with richness (Margalef and Chao1), evenness (Simpson and Pielou) or diversity (Shannon). However, a trend was observed in colitic animals towards an increase in richness and diversity in comparison with the NC group, which was generally reverted in those colitic animals receiving ~~an~~ antibiotic treatment. In particular, a statistically significant reduction in the richness of species (Margalef) was observed with rifaximin (Fig.14, table).

In this strain of mice (CD1), Firmicutes, Bacteroidetes and Actinobacteria were the most predominant phyla(Fig.14, A2, B2, C2). Of note, significant differences were observed when Shannon diversity was evaluated separately on these three phyla(Fig.14, A1, B1, C1). When the phylum Actinobacteria was considered, although no significant differences in the Shanon index were observed in the control colitic group when compared with non-colitic mice, a marked decrease in this index was observed in those colitic mice treated with the antibiotics, in particular with RFX, DXC and MNC (Fig 14, A1). These results correlated with the abundance of Actinobacteria (Fig 14, A2), since a significant and pronounced decrease in the proportion of reads was observed in antibiotic-treated colitic groups. (Fig 14, A2). The abundance of Actinobacteria was also

decreased in DNBS control, compared to non-colitic mice, and glucocorticoid administration did not result in significant modifications in either diversity or abundance in this phylum (Fig 14, A1 and A2).

DNBS-colitis was associated with a significant increase in Bacteroidetes abundance and diversity (Fig 14 B1 and B2); however, no statistical differences in this phylum were observed between untreated and treated colitic groups. Finally, and in comparison with the non-colitic animals, the phylum Firmicutes was not altered in DNBS control group, but both diversity and abundance were increased after antibiotic treatment and statistically significant differences in the Shanon index were observed in particular after the administration of RFX, TTC and MNC (Fig 14 C1 and C2).

Then, we performed a deeper analysis to identify the main bacterial groups modified as a result of the inflammatory process and the different treatments. Mean composition of bacterial communities of each group is represented down to family level on multi-layered pie charts on Figure 15. Of note, the changes observed in the phylum Actinobacteria were mainly associated with *Bifidobacterium pseudolongum* (Fig 14, A3), which represented an average of 50% abundance in NC mice, and was significantly reduced in the DNBS control group to 14% and further reduced by antibiotics to 1.2-5.9%. Conversely, dexamethasone treatment increased the abundance of this species compared to the DNBS control group. *Bacteroides acidifaciens* was the main species involved in the increase in the phylum Bacteroidetes observed in colitic mice (Fig 14, B3). Statistically significant differences between NC and DNBS colitic groups were also found in the species *Alistipes finegoldii* and *Porphyromonas endodontalis*, from Bacteroidia, and in the Flavobacteria class, although they had lower representation on the overall community. The differences in the microbial content among the Firmicutes phylum were mainly ascribed to modifications in Bacilli and Clostridia classes. Thus, and in comparison with the NC group, the DNBS control group showed a reduction of Bacilli (data not shown) while Clostridia were significantly increased (Fig 14, C3). Treated colitic groups showed an increase in *Bacilli* abundance, although no statistical differences were observed. The differences within the *Clostridia class* were more prominent on deeper taxonomic levels. For instance, the genus *Blautia* was significantly increased in the DNBS control group and it remained elevated in colitic mice treated with DXC, MNC and TGC, while a statistically significant reduction was observed in RFX, DEX y TTC colitic groups (Fig 14, C4). Similarly, colitic control

mice had a marked increase in *Ruminococcus*, which mainly associated with an increase in *Ruminococcus obeum*, and this was reduced in all the colitic mice that received pharmacological treatment (Fig X, C5). Finally, control DNBS and DEX colitic groups showed a significant increase in *Clostridium disporicum* (Fig 14, C6), as well as in *C. lituseburense* and *C. sordellii* (data not shown), which were generally undetected in NC and antibiotic treated groups.

In order to simplify and represent the differences between groups, beta-diversity was evaluated. Hierarchical clustering analysis at order level based on the method of minimum variance of Ward is represented on Figure 16A, over a heatmap with the ten most abundant orders. The DNBS colitic mice lay together on one side while antibiotic treated mice are spread between the DNBS and distanced to healthy mice. Dissimilarity analysis at genus level was performed by the taxon-based Bray-Curtis complementary algorithm. Based on the ordination of the distance matrix, the PCA plot (Fig 16B) illustrates the differences among samples explained by the two principal components, that account for 42.7 and 26.2% of the variability observed. PC1 is associated with differences between antibiotic treated mice and the others, while PC2 mainly explains de differences between healthy and DNBS control mice, with treated groups spread through it. Therefore, we identified 3 main groups: NC group, DNBS colitic group, and an antibiotic treated group, which showed some overlap with the first two. High degree of dispersion was observed among healthy mice at this level. No differential patterns were identified among antibiotic treated colitic groups, which may indicate they have a similar overall impact on microbial communities despite the individual differences observed on specific taxons. Finally, dexamethasone treated mice can be found between DNBS and NC areas, although the low number of individuals did not allow to determine a specific pattern in the impact of this treatment.

Figure 14. Estimation of the phylogenetic diversity of the gut microbiota in Non-colitic group (NC), DNBS-colitic group (DNBS), and rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) treated groups in the DNBS model of mouse colitis. Total community α-diversity values are included in the table. Phyla and lower taxa specific measures are included in graphics: diversity **(A1)** and abundance **(A2 & A3)** within *Actinobacteria* phylum; diversity **(B1)** and abundance **(B2 & B3)** within *Bacteroidetes* phylum; diversity **(C1)** and abundance **(C2-C6)** within *Firmicutes* phylum. Data expressed as means ± SEM. *P<0.05 *vs.* DNBS control group.

Figure 14: Comparative study on DNBS colitis.

INDEX	*Margalef*	*Chao1*	*1-Simpson*	*Shannon*	*Pielou*
NC	7,6 ± 1,38	105,3 ± 24,6	0,64 ± 0,12	1,89 ± 0,38	0,46 ± 0,08
DNBS	9,0 ± 0,65	121,3 ± 14,3	0,88 ± 0,01	2,69 ± 0,11	0,63 ± 0,03
RFX	* 6,0 ± 1,00	93,2 ± 12,9	0,83 ± 0,03	2,34 ± 0,14	0,59 ± 0,02
TTC	7,8 ± 2,47	109,1 ± 38,8	0,83 ± 0,06	2,31 ± 0,48	0,55 ± 0,08
DXC	7,4 ± 0,60	86,2 ± 13,8	0,87 ± 0,01	2,53 ± 0,13	0,61 ± 0,02
MNC	8,8 ± 1,32	126,8 ± 24,6	0,85 ± 0,03	2,58 ± 0,20	0,60 ± 0,04
TGC	7,0 ± 2,21	101,4 ± 25,5	0,83 ± 0,05	2,26 ± 0,40	0,56 ± 0,06
DEX	9,9 ± 1,69	155,2 ± 14,0	0,87 ± 0,01	2,67 ± 0,22	0,60 ± 0,03

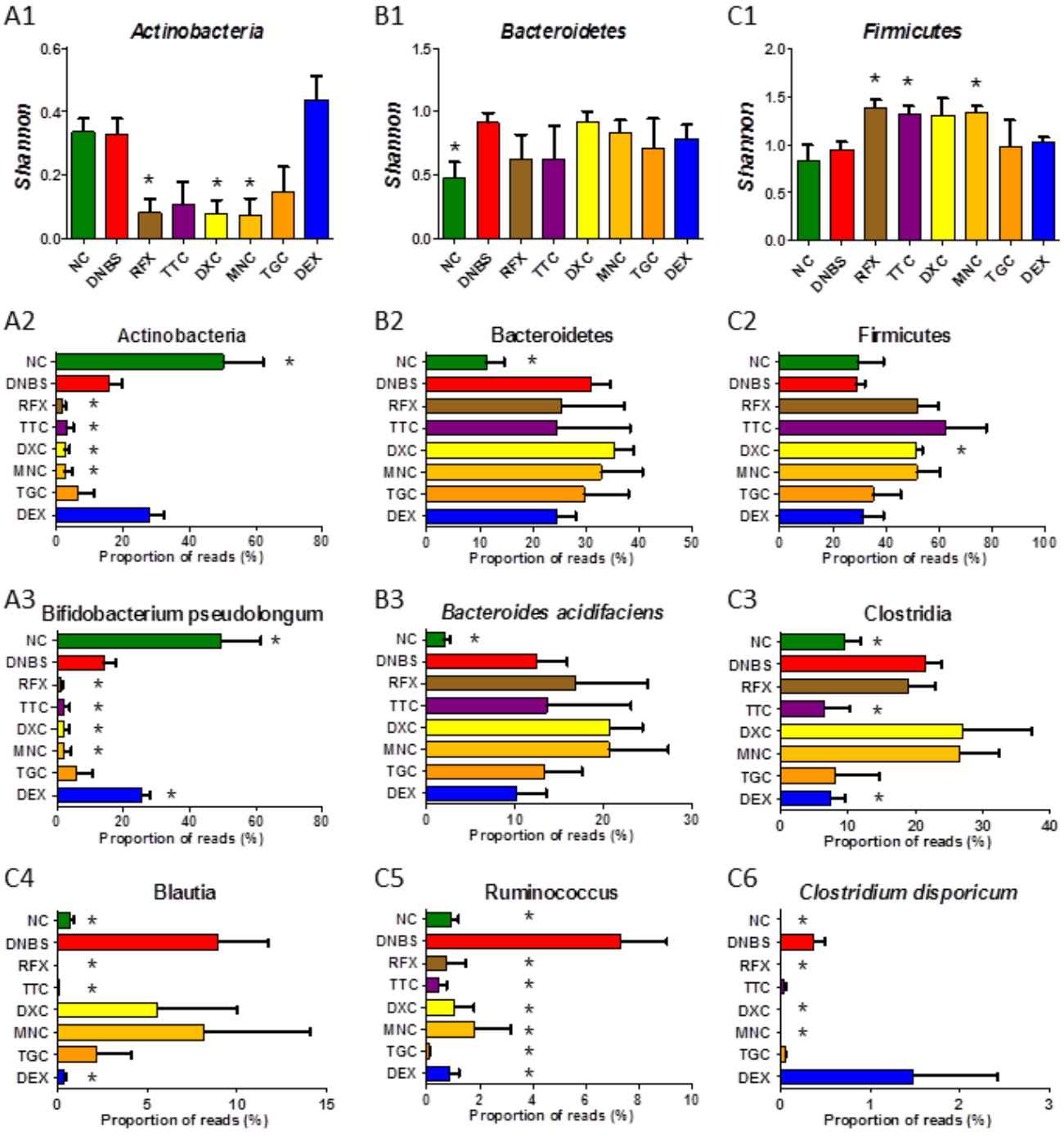


Figure 15: Comparative study on DNBS colitis.

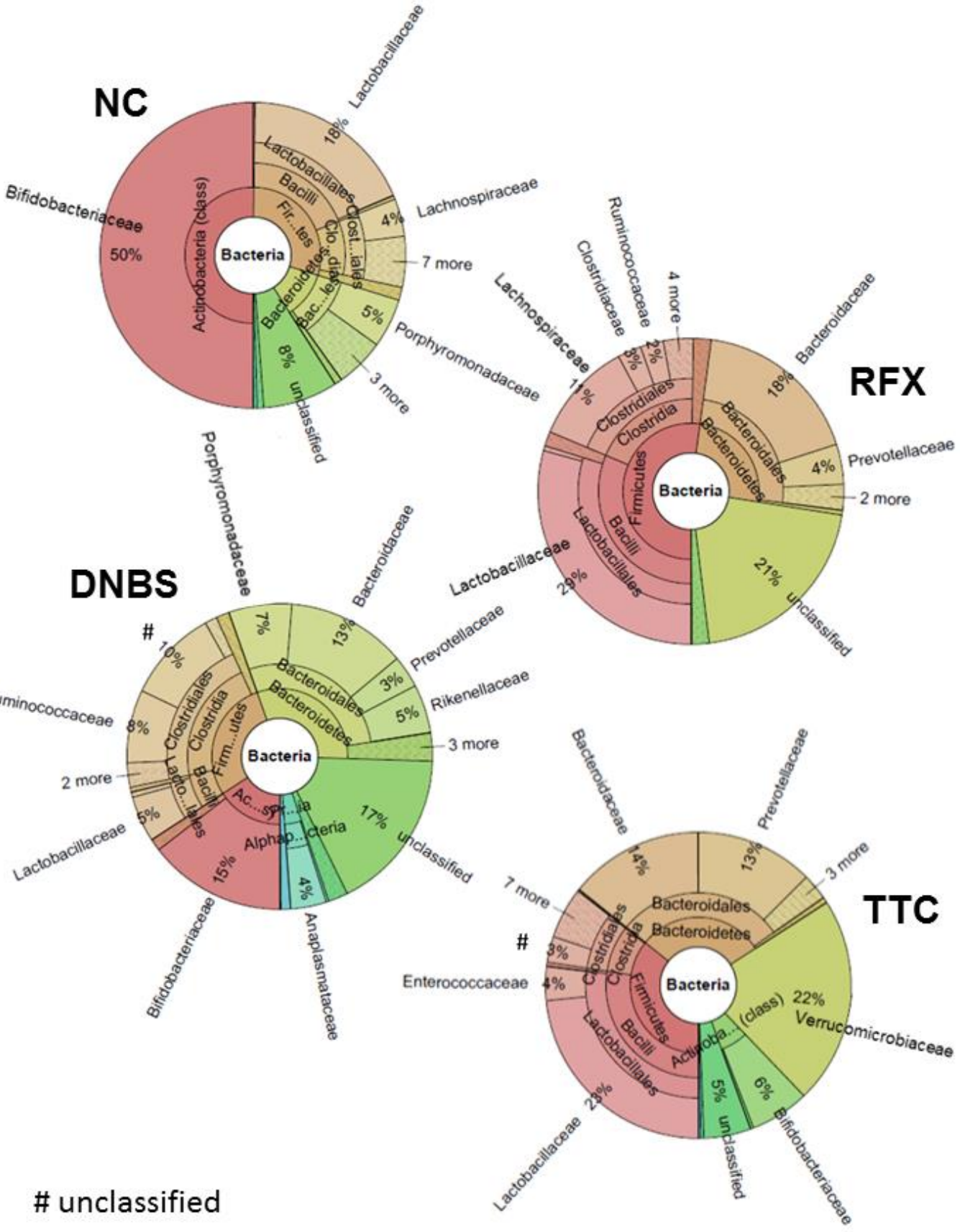


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Figure 15: Comparative study on DNBS colitis.

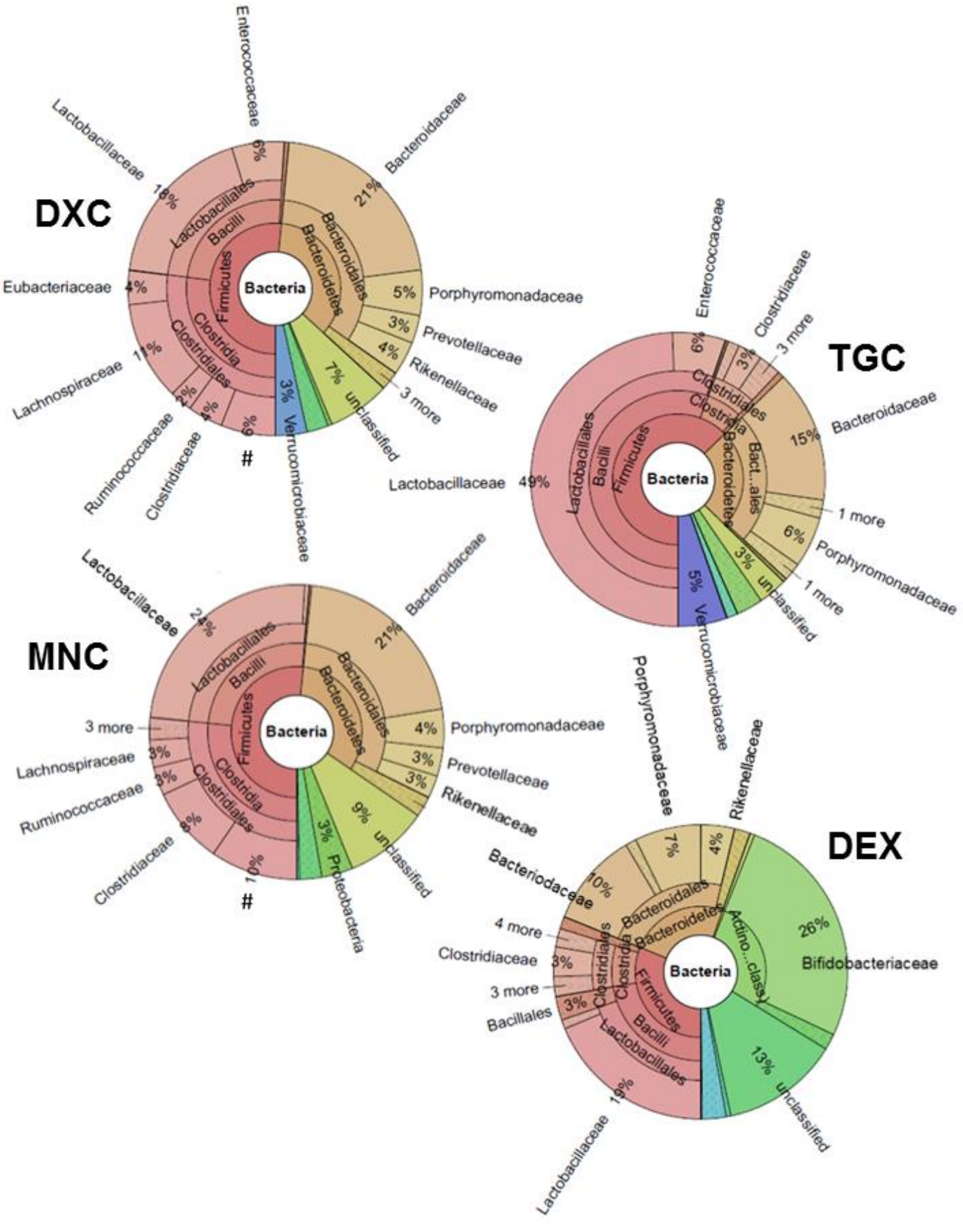


Figure 16: Comparative study on DNBS colitis.

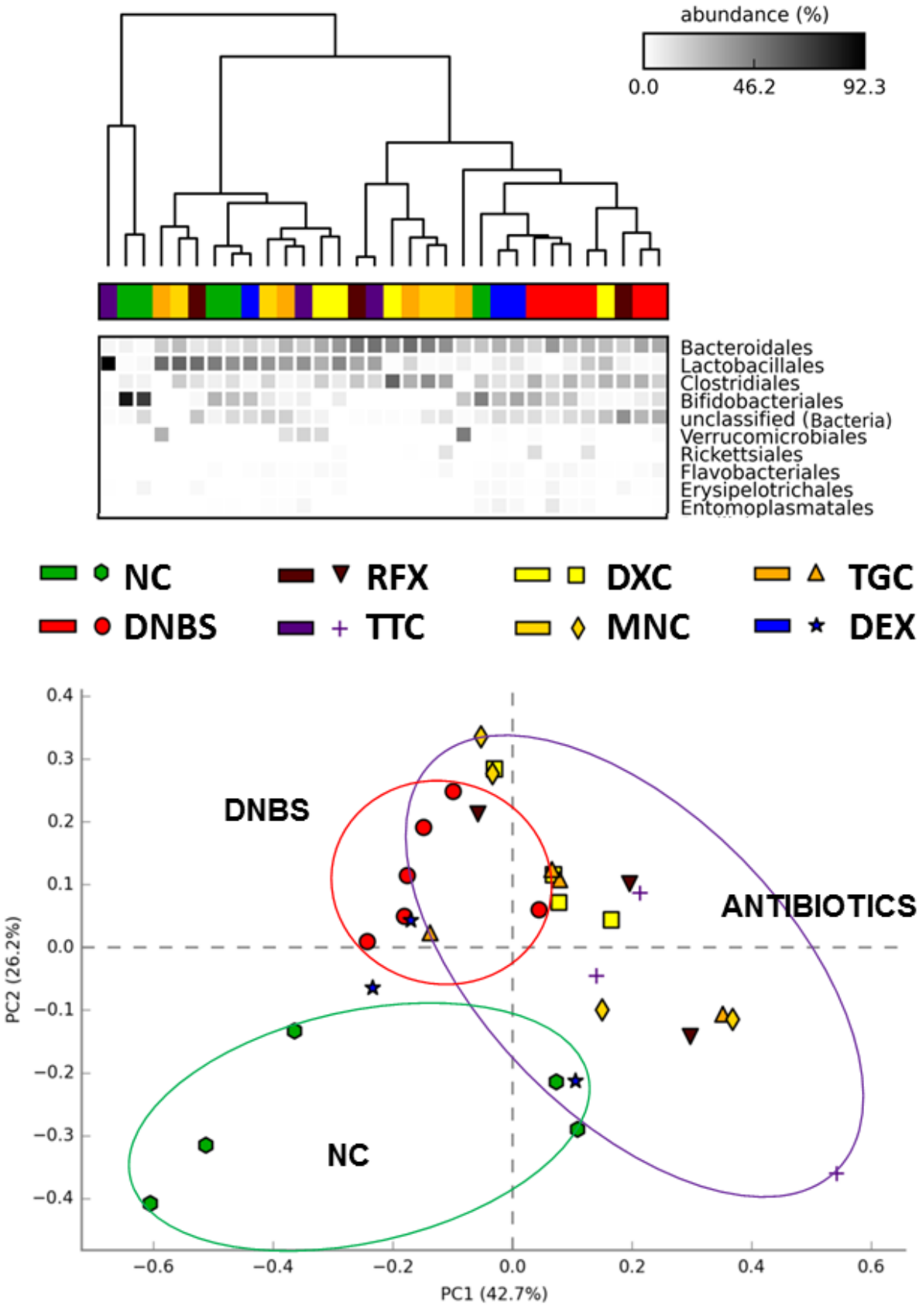


Figure 15. Microbiota composition of Non-colitic group (NC), DNBS-colitic group (DNBS), and rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) treated groups in the DNBS model of mouse colitis. Pie-charts collectively represent mean abundance of the different taxa included in *Bacteria* domain down to family level.

Figure 16. Comparison of microbiota composition between Non-colitic group (NC), DNBS-colitic group (DNBS), and rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) treated groups in the DNBS model of mouse colitis (β-diversity analysis). **A)** Heatmap with relative abundance of the 10 most abundant orders, include hierarchical clustering of samples with the method of minimum variance of Ward Phylum **B)** PCA plot representation based on the ordination of the distance matrix build with a dissimilarity analysis at genus level using the taxon-based Bray-Curtis complementary algorithm. Green ellipse includes NC samples red ellipse includes DNBS samples and purple ellipse includes sample from antibiotic-treated groups (RFX, TTC, DXC, MNC & TGC).

2.3. COMPARATIVE STUDY ON DSS COLITIS

The DSS colitis model is the most widely used experimental model of intestinal inflammation. In contrast to the DNBS model, which is hapten-induced, in this model the DSS induces a direct damage on the mucosal epithelial barrier, and the inflammatory response is subsequently activated after direct exposure of the mucosal immune system to microbial triggers[152]. The induction procedure in this model allows a greater control of the severity of the inflammatory process than in the DNBS model, depending on the period of exposure and the concentration of DSS administered in the drinking water.We took advantage of the flexibility of this model with two purposes: first, to study the impact of the treatments on the mortality rate and DAI evolution after inducing a fatal colitis; and second, to evaluate the effects of these treatments on the different disease parameters (those studied in the DNBS model) in the context of a less aggressive inflammatory process.

Fulminant colitis was induced by administration of 3% DSS for 6 days. After that, mice were treated with the different drugs, following the same design used in the DNBS model (Fig.17A). As commented previously, the evolution of the disease was assessed by daily determination of the disease activity index (DAI). After the first day of treatment, immunomodulatory tetracyclines and dexamethasone significantly prevented the weight loss(Fig.17B). This effect was maintained on the groups treated with immunomodulatory tetracyclines throughout the length of the study, whereas dexamethasone treated mice experienced an accelerated weight loss after 3 days of treatment (Fig.17B). Consequently, mice treated with immunomodulatory tetracyclines had an improved DAI evolution, with DAI values statistically reduced in these groups in comparison with the colitic control group by the end of the experiment(Fig.17C). TTC also caused a positive effect on DAI evolution especially on the last days of treatment, although no statistical differences were observed in comparison with the control colitic group. In contrast, after 4 days of treatment, dexamethasone treated mice showed similar DAI values to mice in the DSS control group. Surprisingly, rifaximin did not show any beneficial effect on the clinical parameters of the disease, showing a similar DAI evolution to that of the untreated control group(Fig.17C).

The survival curves illustrated the effects observed on the DAI evolution with the different treatments(Fig.17D). The maximum values of DAI were reached at day 8, coinciding with the initiation of the mortality events. Survival rates at the end of the experiment were: NC (100%), DNBS (30%), RFX (20%), TTC (50%), DXC (80%), MNC (100%), TGC (70%) and DEX (40%), showing that the administration of any of the immunomodulatory tetracyclines resulted in a significant reduction in the mortality rate in comparison with untreated DSS control group, whereas none of the other treatments managed to significantly reduce the mortality rates(Fig.17D). Of note, and although the mortality rate of the TTC-treated group was higher than that of the immunomodulatory tetracycline-treated groups, it was reduced compared to the untreated colitic mice suggest that systemic antibiotic activity may protect from acute inflammation, limiting bacterial translocation and dissemination through the organism. The same reasoning could explain the loss of effect of dexamethasone after the first 3 days, which could be attributed to the blockade of the protective immune response needed in these circumstances.

Figure 17: Comparative study in DSS colitis.

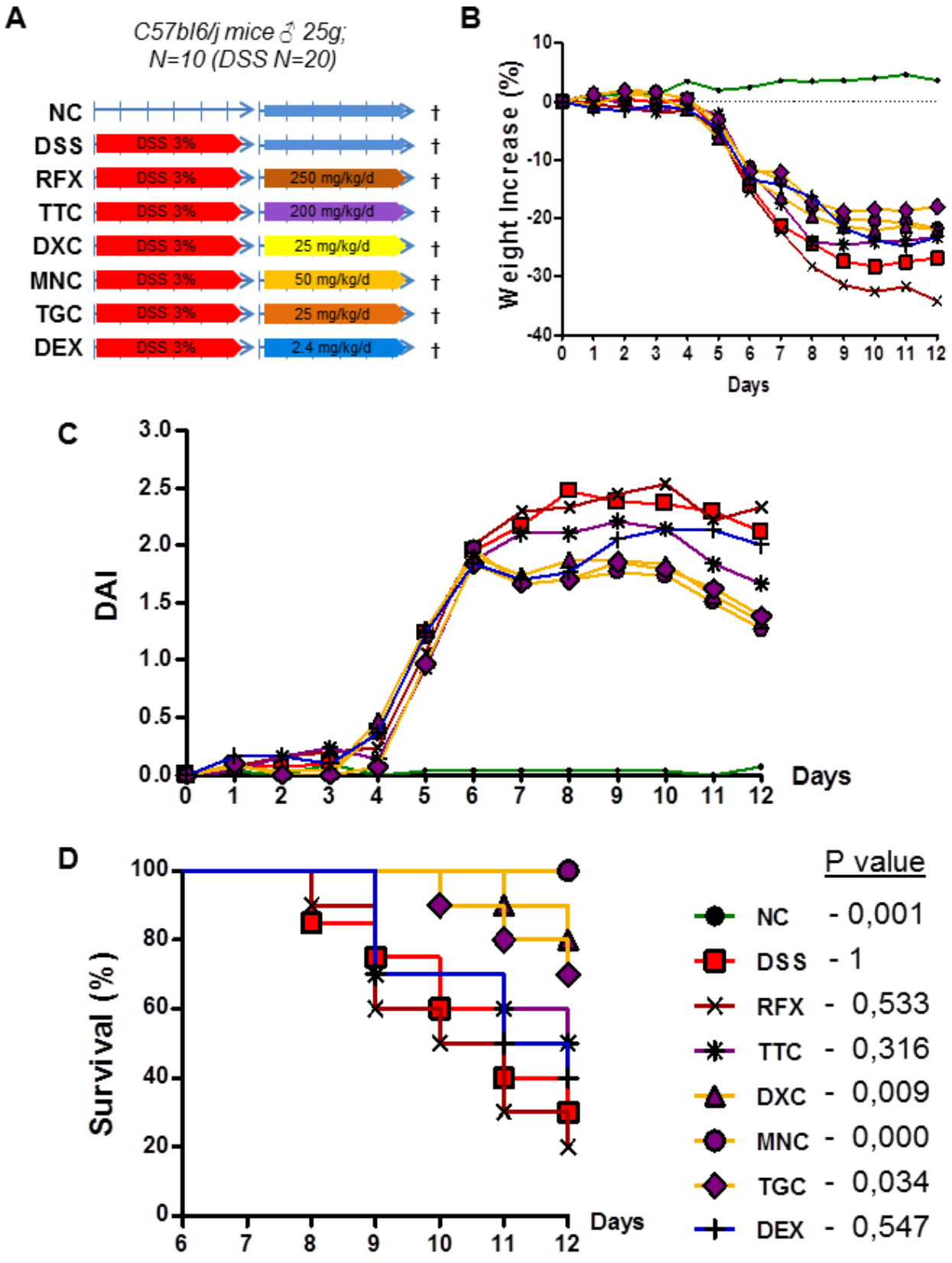


Figure 17. Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DSS model of mouse colitis. **A)** Schematic illustration of the experimental design followed. NC: Non-colitic group, DSS: DSS-colitic group. **B)** Body weight evolution (% of increase) and C) Disease Activity Index (DAI) values of the different experimental groups during the 12-days experimental period. **C)** Survival curves (%) and their P values *vs.* DSS control group of the different experimental groups during the 6-days experimental period. Data expressed as means unless otherwise stated. DAI values assigned based on the criteria described in (Cooper et al., 1993).

Based on these results, and in order to explore the mechanisms involved in the early effects displayed by the immunomodulatory tetracyclines, a second experiment was performed, in which the animals were sacrificed after 4 days of treatment. However, and in an attempt to improve the survival rates, the severity of the colitis was reduced by shortening the period of DSS administration to 5 days(Fig.18A). In this protocol, the evolution of intestinal inflammation, as represented by the DAI values, followed a similar pattern than in the previous experiment. Immunomodulatory tetracyclines ameliorated the course of the disease from the first day of administration and the initial effect of dexamethasone disappeared by the end of the experiment (Fig.18B). Similarly, rifaximin did not cause any significant improvement in the colitis status, and tetracycline did not have a clear impact on DAI evolution, perhaps due to the shorter treatment time and/or to the fact that bacterial translocation might not play such a key role in milder colitis, therefore systemic antibiotic activity does not have such a positive impact(Fig.18B).

Figure 18. Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DSS model of mouse colitis. **A)** Schematic illustration of the experimental design followed. NC: Non-colitic group, DSS: DSS-colitic group. **B)** Disease Activity Index (DAI) mean values assigned based on the criteria described in (Cooper et al., 1993), during the 9-days experimental period. **C)** Microscopic damage score assigned according the criteria described in (Camuesco et al., 2004). Representative histological sections of colonic mucosa of the different experimental groups stained with haematoxylin and eosin (40x magnification). **D)** Representative histological sections of colonic mucosa of the different experimental groups stained with haematoxylin and eosin (100x magnification). **E)** mRNA expression of mucins (MUC-1, MUC-2, MUC-3), TFF-3, ZO-1 and Occludin quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DSS control group.

Figure 18: Comparative study in DSS colitis.

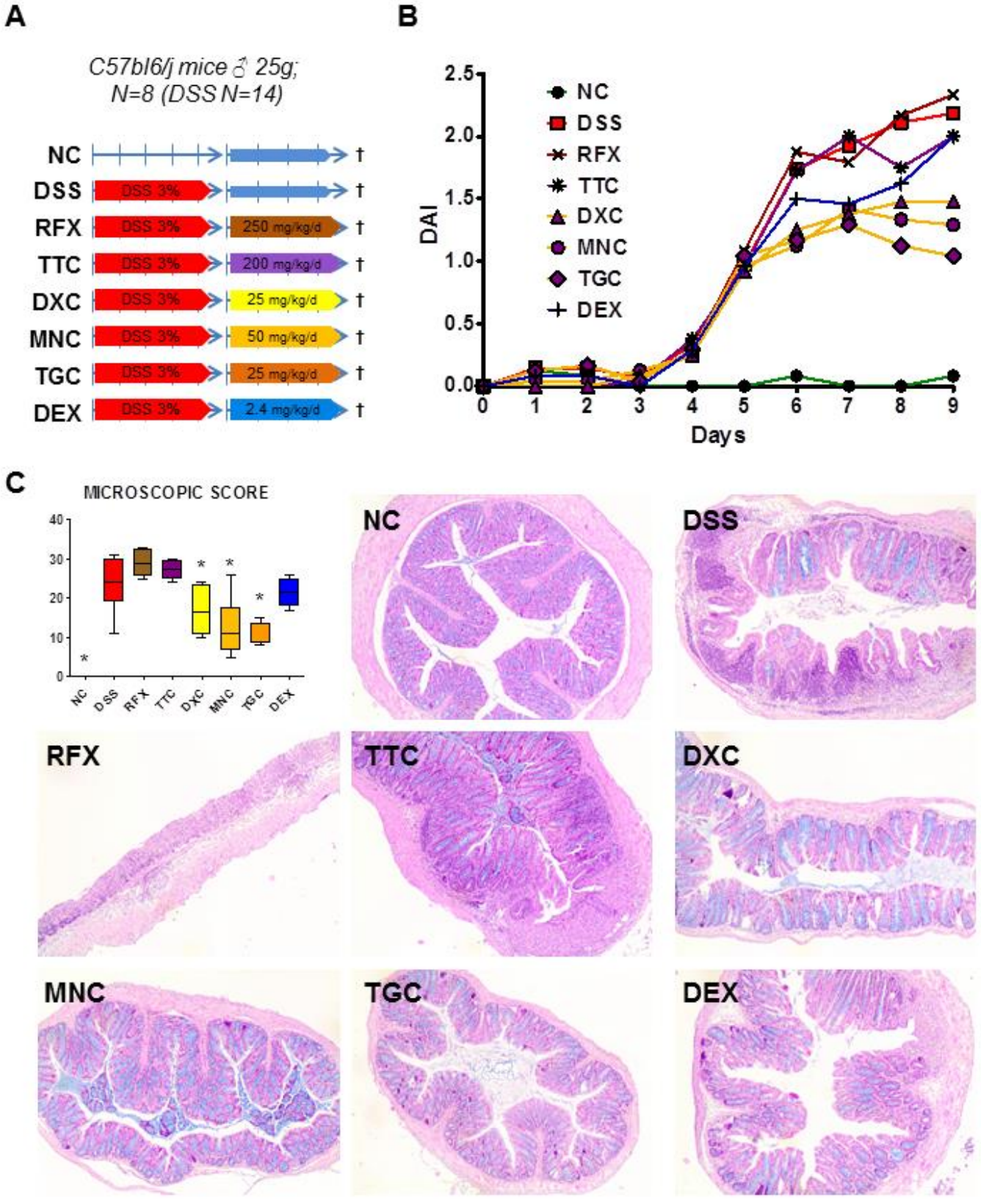
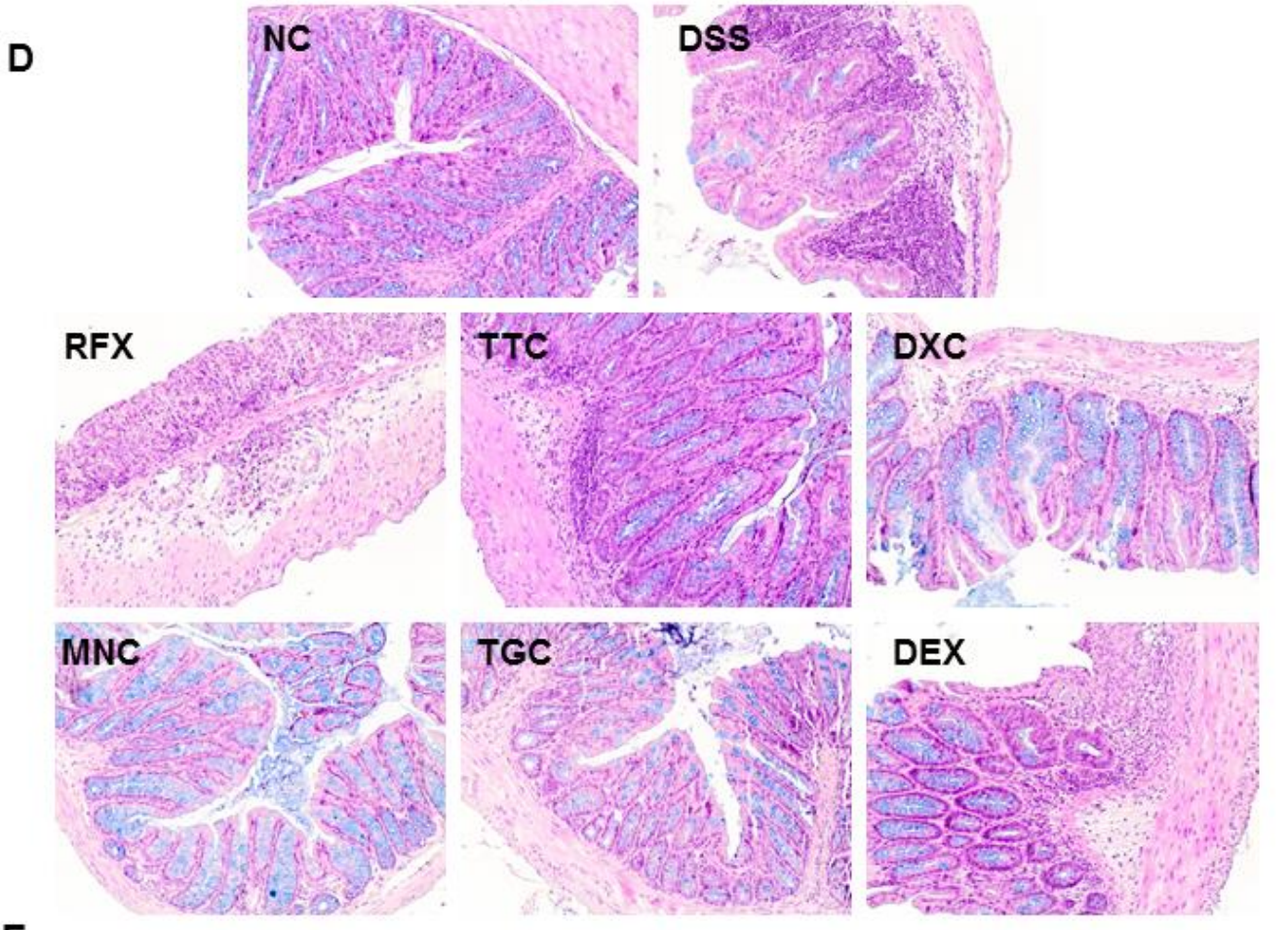
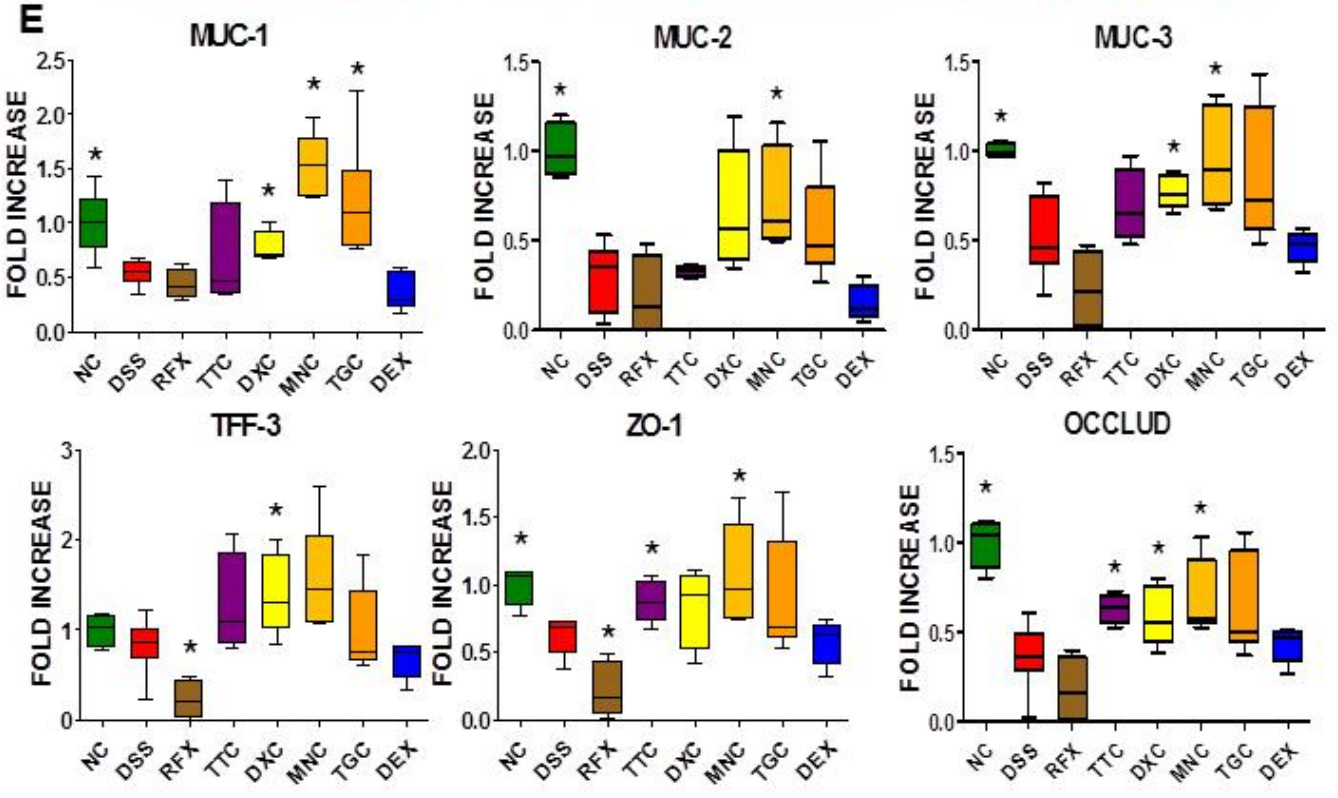


Figure 18: Comparative study in DSS colitis.





The histological evaluation of the colonic samples confirmed the intestinal anti-inflammatory effects exerted by the immunomodulatory tetracyclines (Fig.18C & D). DSS-induced colitis mainly affected the mucosa, with more than 70% of the colonic surface affected by the epithelial ulceration. The presence of oedema between the mucosa and muscularis layers was accompanied by an intense inflammatory cell infiltration that sometimes also affected inner intestinal layers. Moreover, an important alteration of the crypt structure was observed, with high mitotic activity and intense goblet cell mucin depletion. Confirming previous results, the administration of the immunomodulatory tetracyclines doxycycline, minocycline and tigecycline, to colitic mice, resulted in a clear improvement in the colonic histological score (Fig. 18C & D). A preservation of the mucosal layer was observed in these groups, with the presence of restored goblet cells replenished with their mucin content. As a result, a significant reduction in the microscopic score values of colitic mice treated with DXC, MNC or TGC was observed in comparison with the colitic control group (Fig. 18C & D). However, when the groups of colitic mice treated with TTC, rifaximin or dexamethasone were considered, no beneficial effect was noted in the histologically, thus confirming the lack of any intestinal anti-inflammatory effect of these treatments in this experimental model.

The biochemical evaluation of the colonic segments provided additional information about the effects of the different treatments. In this sense, the mucin depletion observed after the administration of DSS in the histological analysis was associated to a reduced gene expression or the Mucins MUC-1, MUC-2 and MUC-3. Similarly, the expression of other makers of epithelial barrier integrity, like ZO-1 and occludin, was also reduced in untreated colitic mice (Fig 18E). In agreement with the results commented above, the three immunomodulatory tetracyclines restored the expression of these mucins as well as the expression of tight junction proteins. Tetracycline improved the expression of ZO-1 and occludin, but showed no significant effects when the expression of the mucins was assayed. Additionally, although the expression of TFF-3 was not reduced in the DSS control group, all the tetracyclines increased its expression, while rifaximin substantially reduced it levels. On the other side, the effects of dexamethasone were not only not associated with a restoration of the expression of these protective markers, but instead, dexamethasone caused a further reduction in their expression when compared with untreated colitic mice.

In addition, the expression of the different inflammatory markers evaluated, including TNFα, IL-1β, IL-6, MMP-9, CCL2 and CXCL2, was significantly increased in the DSS control group, in comparison with non colitic mice (Fig.19) This increased expression was also observed in the group of colitic mice treated with rifaximin, in agreement with the higher DAI values and microscopic score presented by these mice in the intestinal inflammatory process in this experimental model of colitis.(Fig. 19). On the other hand, the administration of immunomodulatory tetracyclines significantly reduced the expression of IL-1β, IL-6, MMP-9 and CXCL2 in the colonic tissue; however, they did not reduce TNFα expression significantly, which was the only cytokine ameliorated by dexamethasone (Fig. 19). Curiously, in comparison with the control colitic group, a strong and consistent increase in CCL2 expression was observed in the groups treated with doxycycline, minocycline and tigecycline; and to a lesser extent, with tetracycline.

This suggests that, although these compounds exert a general anti-inflammatory effect, it is not necessary that all inflammatory pathways are reduced; in fact, an increased monocyte recruitment could be involved in the mechanisms involved in their beneficial effects in these conditions. Similarly, the improvement on epithelial barrier function does not seem to be just a consequence of the amelioration of the inflammatory process, but a direct protective action of these drugs promoting the different barrier funtion mechanisms could also be involved in the effect observed.

The evaluation of the TLR expression profile in the DSS model revealed an important reduction in TLR4 expression upon the induction of colitis (Fig 19). The immunomodulatory tetracyclines partially restored the expression of this receptor, showing statistically significant differences with the control group. In addition, the expression of TLR2 was also reduced in DSS colitis, although to a lesser extent, and all antibiotics increased its expression, while dexamethasone further reduced it.

Figure 19. Gene expression analysis in the comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DSS model of mouse colitis. NC: Non-colitic group, DSS: DSS-colitic group. mRNA expression of the indicated genes quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DSS control group.

Figure 19: Comparative study in DSS colitis.

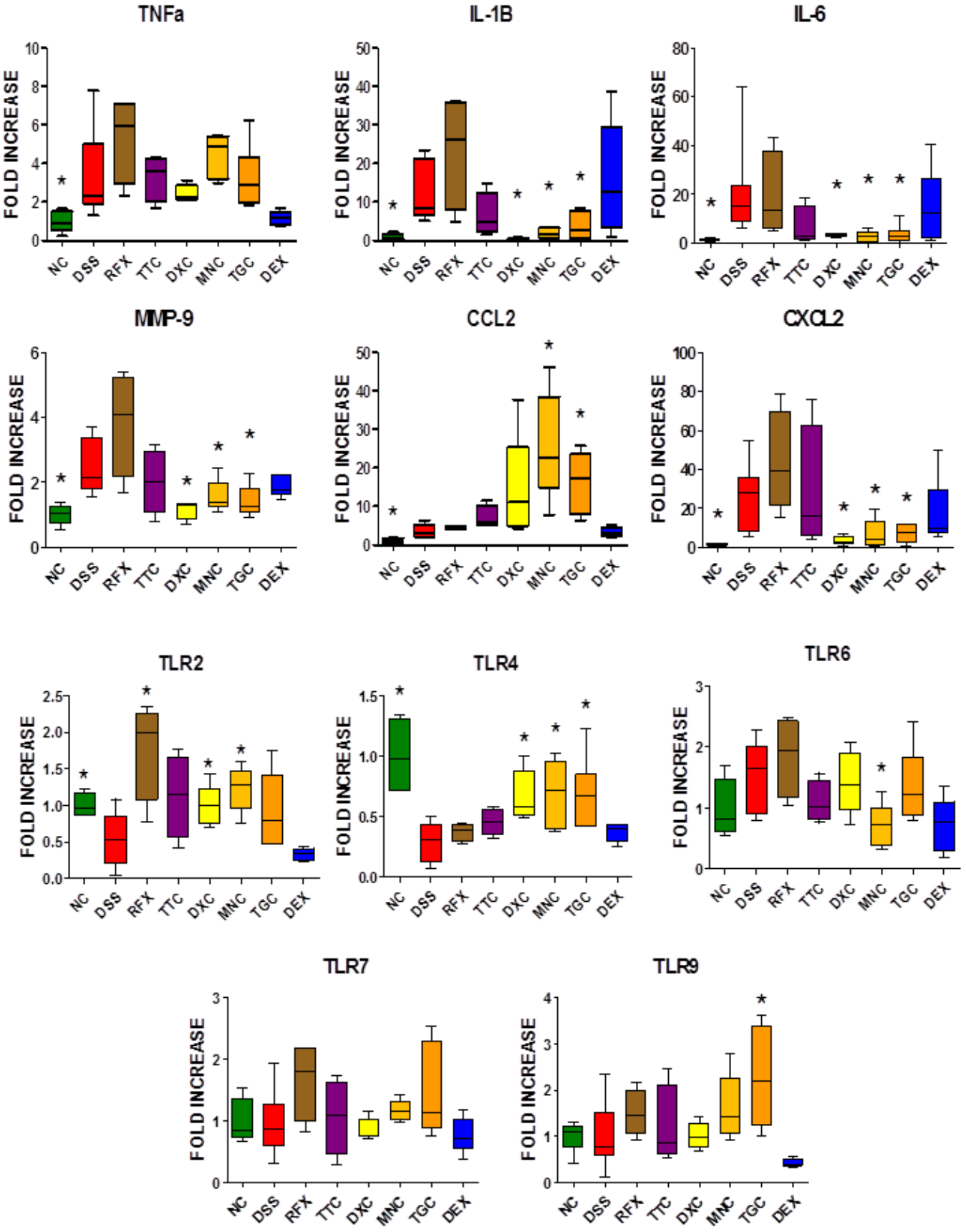


Figure 20: Comparative study in DSS colitis.

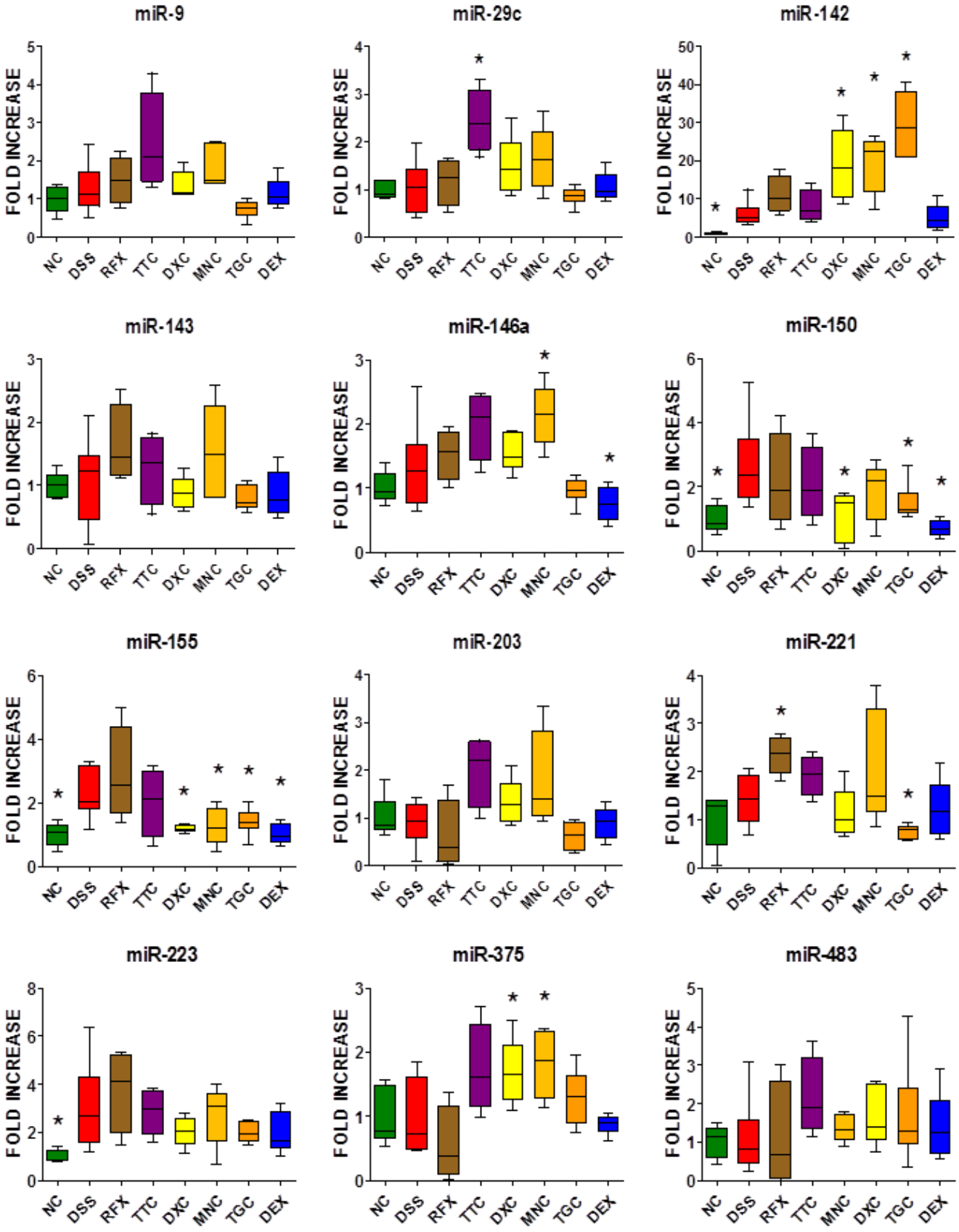


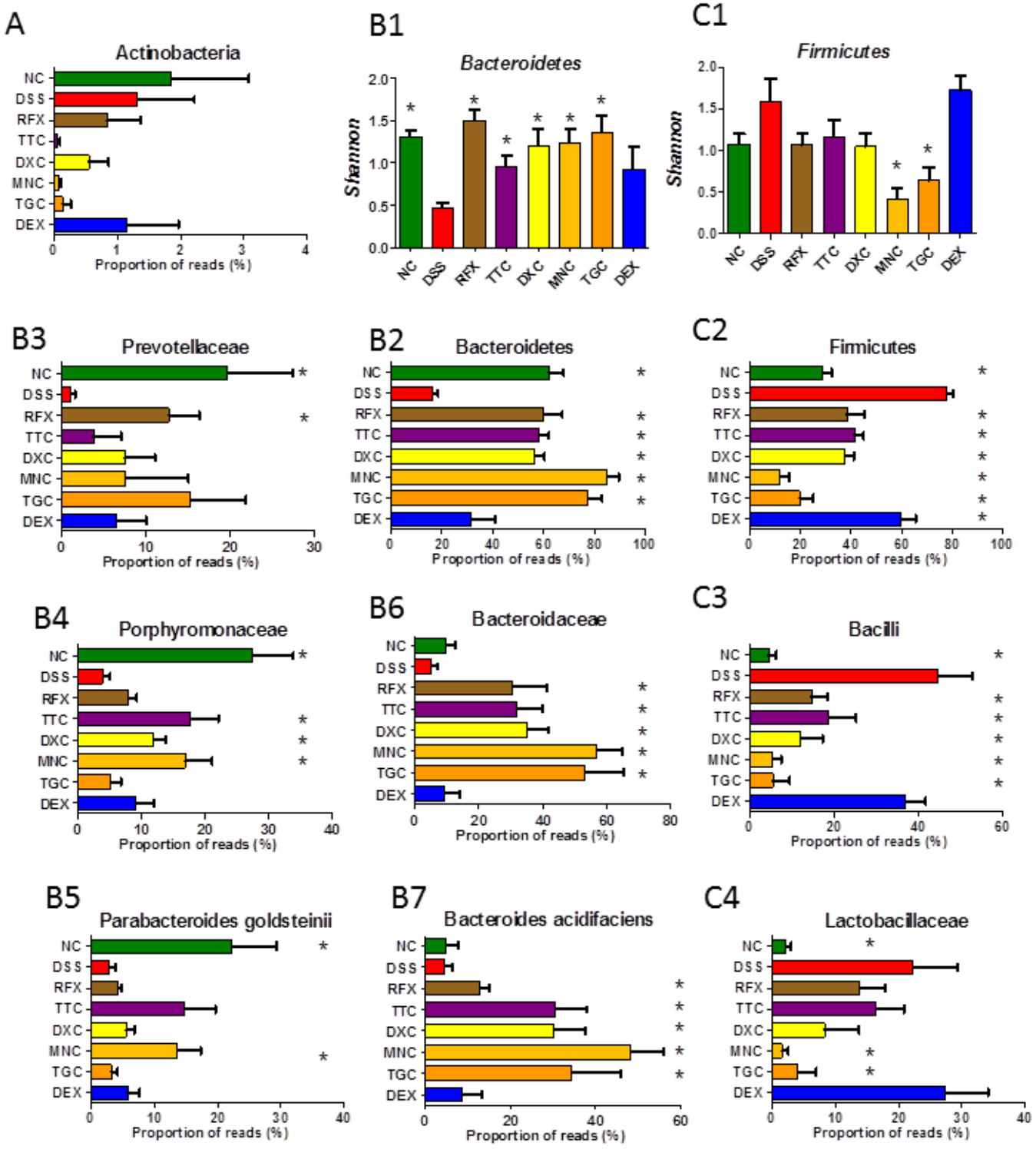
Figure 20. micro-RNA expression analysis in the comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in the DSS model of mouse colitis. NC: Non-colitic group, DSS: DSS-colitic group. Expression of the indicated micro-RNAs quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DSS control group.

When considering the colonic expression of the different miRNAs evaluated, the inflammatory process initiated by the DSS induced a statistically significant up-regulation of miR-142, miR-150, miR-155 and miR-223 (Fig. 20). No significant modifications were observed for the other miRNAs evaluated. In general, the immunomodulatory properties of tetracyclines and dexamethasone were associated with a significant reduction on miR-155 and miR-150 expression (Fig 20). Of note, the administration of TTC, DXC or MNC to colitic mice resulted in an increased expression of some of the miRNAs studied, including miR-29c, miR-146a, miR-203 and miR-375, that were not modified in the control colitic group in comparison with non colitic mice (Fig 20). However, the most consistent and surprising effect observed in this regard was the significantly higher expression of miR-142 found in mice treated with one of the three immunomodulatory tetracyclines, in comparison with the levels detected in the control colitic group, which were already increased from the basal expression(Fig. 20). Interestingly, the effect caused by tetracyclines on miRNA142 expression follows a similar pattern than that observed when CCL2 expression was analysed (Fig 20).

Figure 21. Estimation of the phylogenetic diversity of the gut microbiota in Non-colitic group (NC), DSS-colitic group (DSS), and rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) treated groups in the DNBS model of mouse colitis. Total community α-diversity values are included in the table. Phyla and lower taxa specific measures are included in graphics: abundance of *Actinobacteria* phylum **(A);** diversity **(B1)** and abundance **(B2-B7)** within *Bacteroidetes* phylum; diversity **(C1)** and abundance **(C2-C4)** within *Firmicutes* phylum. Data expressed as means ± SEM. *P<0.05 *vs.* DSS control group.

Figure 21: Comparative study in DSS colitis.

INDEX	Margalef	Chao1	1-Simpson	Shannon	Pielou
NC	10,1 ±2,02	115,5 ±20,2	0,85 ±0,04	2,70 ±0,25	0,61 ±0,04
DNBS	8,5 ±2,50	104,8 ±33,2	0,77 ±0,08	2,27 ±0,32	0,55 ±0,06
RFX	6,1 ±0,31	59,1 ±3,2	0,89 ±0,01	2,64 ±0,11	0,67 ±0,03
TTC	5,6 ±1,48	67,2 ±15,6	0,81 ±0,07	2,16 ±0,34	0,55 ±0,04
DXC	7,1 ±2,01	83,5 ±21,9	0,83 ±0,07	2,43 ±0,35	0,61 ±0,06
MNC	5,4 ±1,21	73,9 ±23,0	0,69 ±0,06	1,77 ±0,26	0,46 ±0,04
TGC	6,0 ±1,36	81,8 ±18,2	0,76 ±0,11	2,11 ±0,40	0,53 ±0,07
DEX	10,0 ±2,79	117,4 ±31,3	0,92 ±0,02	2,97 ±0,22	0,67 ±0,04



As in the DNBS model, the microbial composition in the intestinal content of these mice was determined by pyrosequencing, and changes in microbial communities were evaluated by α-diversity indexes, relative abundance of the taxonomic groups and overall differences among samples, determined by beta-diversity analysis. In general, a reduction in richness (Margalef and Chao1 indices) and diversity (Shanon index) was observed in the DSS group compared to the NC one, although statistically significant differences were not reached at this time point (Fig 21, Table). Antibiotic treatments did not restore the indices of richness to values similar to those of the NC group, but instead they even reduced them more. (Fig 21, Table). However, as observed in the DNBS model, significant differences where obtained when the Shannon index, related to bacterial diversity, was determined in two of the most predominant phyla, *Bacteroidetes* and *Firmicutes*, independently. In fact, statistically significant differences were found when considering *Bacteroidetes* diversity, which was reduced in control DSS-colitic mice when compared to healthy mice (Fig. 21 B2). Of note, the treatment of colitic mice with the different antibiotics significantly counteracted the decrease this index, obtaining similar values in these animals to those obtained in NC mice (Fig 21 B1). When the Shannon index was calculated in *Firmicutes*, control and DEX-treated colitic mice showed a higher diversity than NC mice (Fig. 21 C1). Antibiotic treatment to colitic mice was typically associated with decreased Shannon index values, showing antibiotic treated mice a similar diversity to that found in NC group; of note, the highest reductions in this index were achieved after MNC and TGC treatments, being the values corresponding to these two groups significantly different to those from the DSS control group (Fig 21 C1). The differences in the relative abundance in these phyla followed the same pattern as observed for the diversity: The proportion of reads in the phylum Bacteroidetes was significantly reduced in the DSS-control and DEX treated groups, whereas colitic mice treated with the antibiotics showed similar proportions to NC mice (Fig 21 B2). Similarly, Firmicutes were increased in DSS-control and DEX treated mice, and antibiotic treatments, especially MNC and TGC, reduced their abundance, (Fig 21 C2). In comparison with the results obtained for CD1 mice used for the DNBS model of colitis, the phylum Actinobacteria was not highly represented in these animals, which highlights the existence of important differences in terms of microbiota composition among different strains of mice and/or colonies, and experimental models of disease. However, the impact that antibiotic treatment had on this phylum in DSS-colitis was similar to that previously described in the DNBS model, and it was also associated with a reduction in the abundance of Actinobacteria (Fig 21 A).

Most of the changes found on the phylum Firmicutes are due to marked differences in the proportion of sequences of the class *Bacilli*, which was increased in colitic mice up to 8 fold. The treatment with antibiotics significantly counteracted this increase (Fig 21 C3). In turn, variations observed in the class *Bacilli* were determined by the misbalance of the family *Lactobacillaceae* (Fig 21 C4), being *Lactobacillus murinus* the most abundant species identified. DSS control mice also showed increased abundance of Clostridiaceae, although no further differences were observed within this family.

The modifications in Bacteroidetes were analysed down to family level. Differences between NC and DSS groups were mainly associated with *Prevotellaceae* (Fig 21 B3) and *Porphyromonaceae* (Fig 21 B4) families, which were significantly reduced in colitic animals. In general, antibiotic treatment increased their abundance, an increased that was statistically significant with RFX in the case of Prevotellacae and with TTC, DXC and MNC for the *Porphyromonaceae* family. Withinthe latter, *Parabacteroides goldsteinii* was the most dominant species (Fig 21 B5). Finally, despite this model of colitis was not associated to differences on *Bacteroidaceae* (Fig 21 B6), antibiotic administration to colitic mice caused a dramatic increase in the reads of this family, mainly associated with the species *Bacteroides acidifaciens,* which was statistically significant for all groups treated with antibiotics (Fig 21 B7). Mean bacterial composition of each bacterial group down to family level is represented on multi-layered pie charts on Figure 22.

Figure 22. Microbiota composition of Non-colitic group (NC), DSS-colitic group (DSS), and rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) treated groups in the DSS model of mouse colitis. Pie-charts collectively represent mean abundance of the different taxa included in *Bacteria* domain down to family level.

Figure 23. Comparison of microbiota composition between Non-colitic group (NC), DSS-colitic group (DSS), and rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) treated groups in the DSS model of mouse colitis (β-diversity analysis). **A)** Heatmap with relative abundance of the 10 most abundant orders, include hierarchical clustering of samples with the method of minimum variance of Ward Phylum. **B)** PCA plot representation based on the ordination of the distance matrix build with a dissimilarity analysis at genus level using the taxon-based Bray-Curtis complementary algorithm. Green ellipse includes NC samples red ellipse includes DSS samples and purple ellipse includes sample from antibiotic-treated groups (RFX, TTC, DXC, MNC & TGC).

Figure 22: Comparative study in DSS colitis.

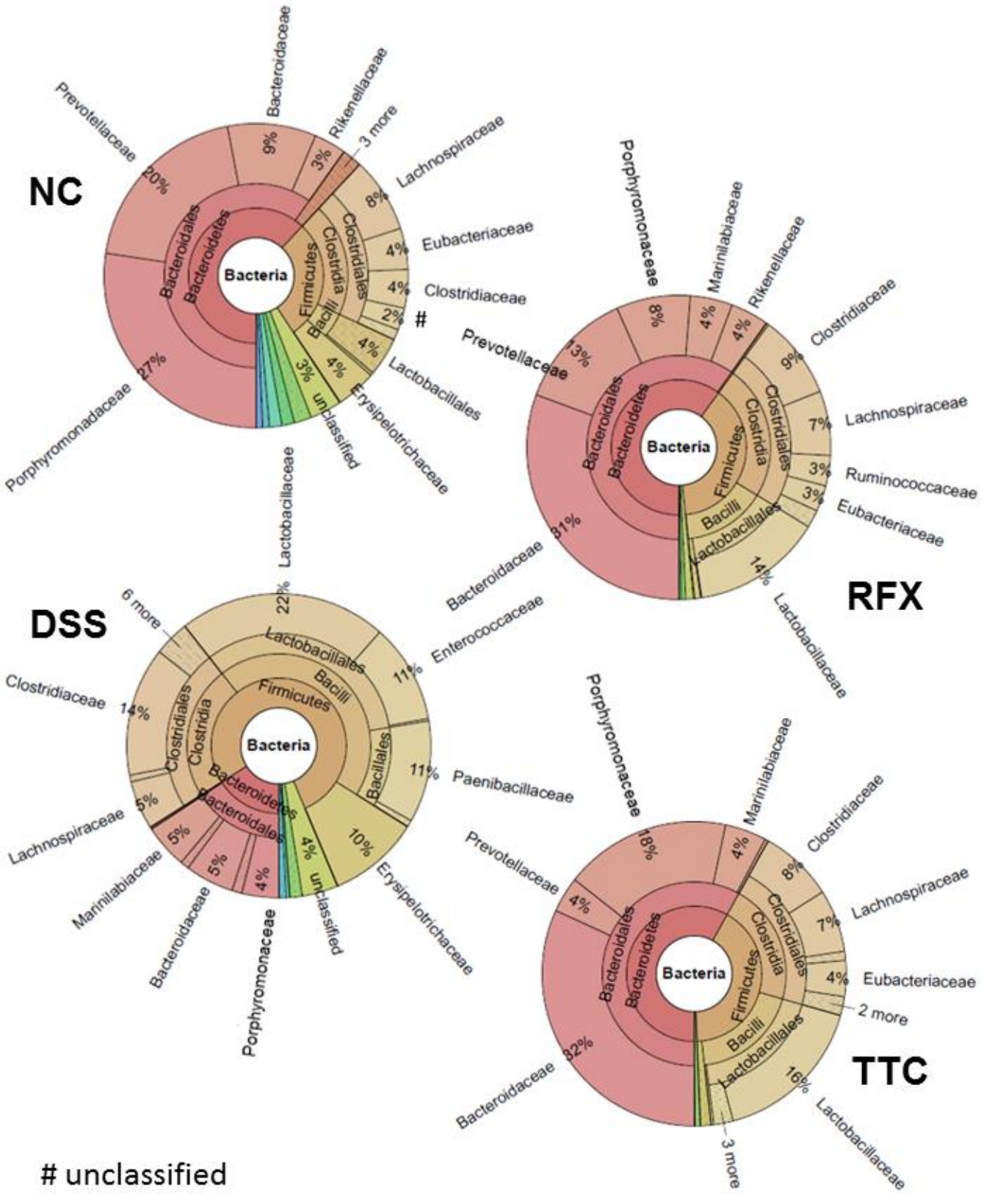


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Figure 22: Comparative study in DSS colitis.

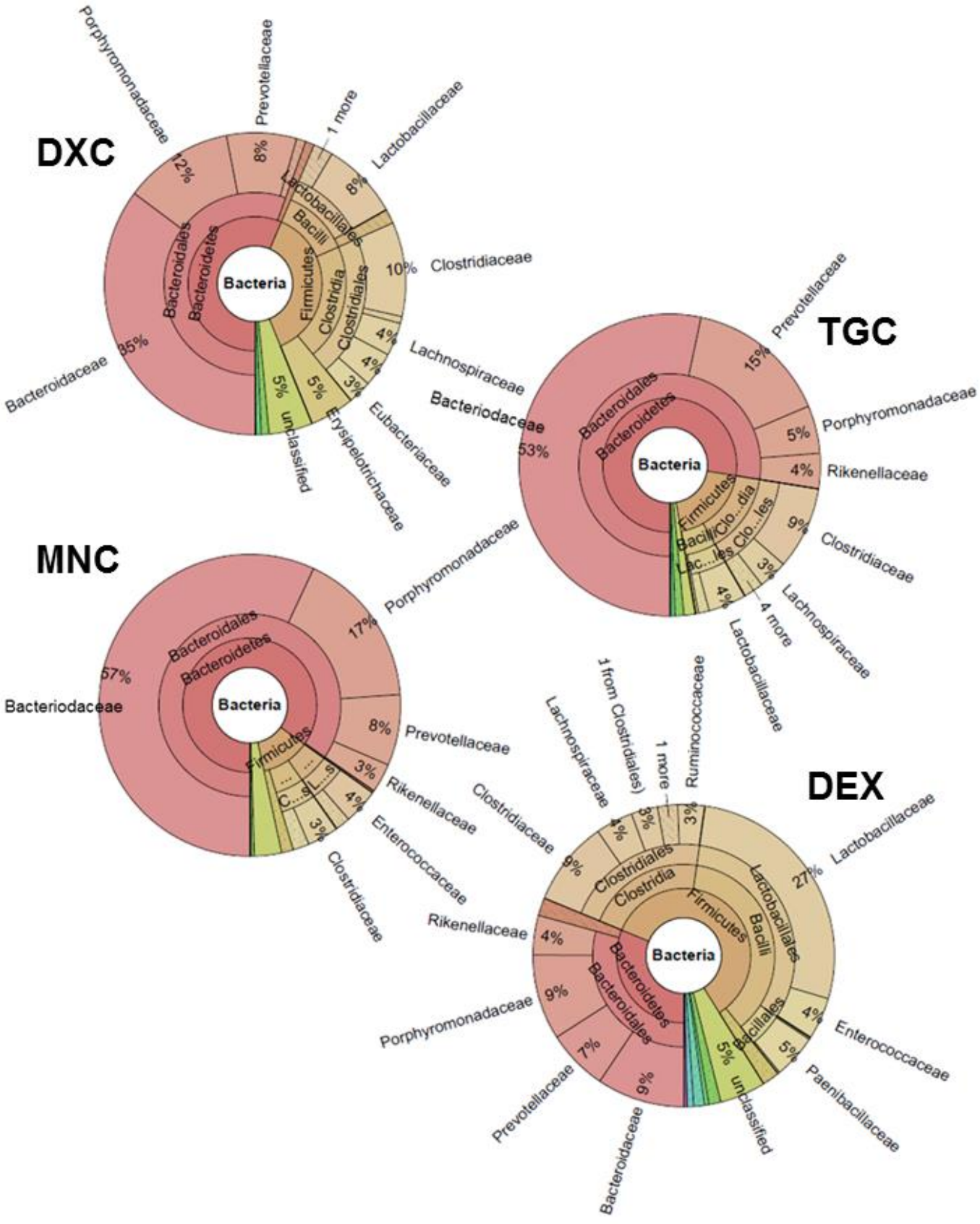
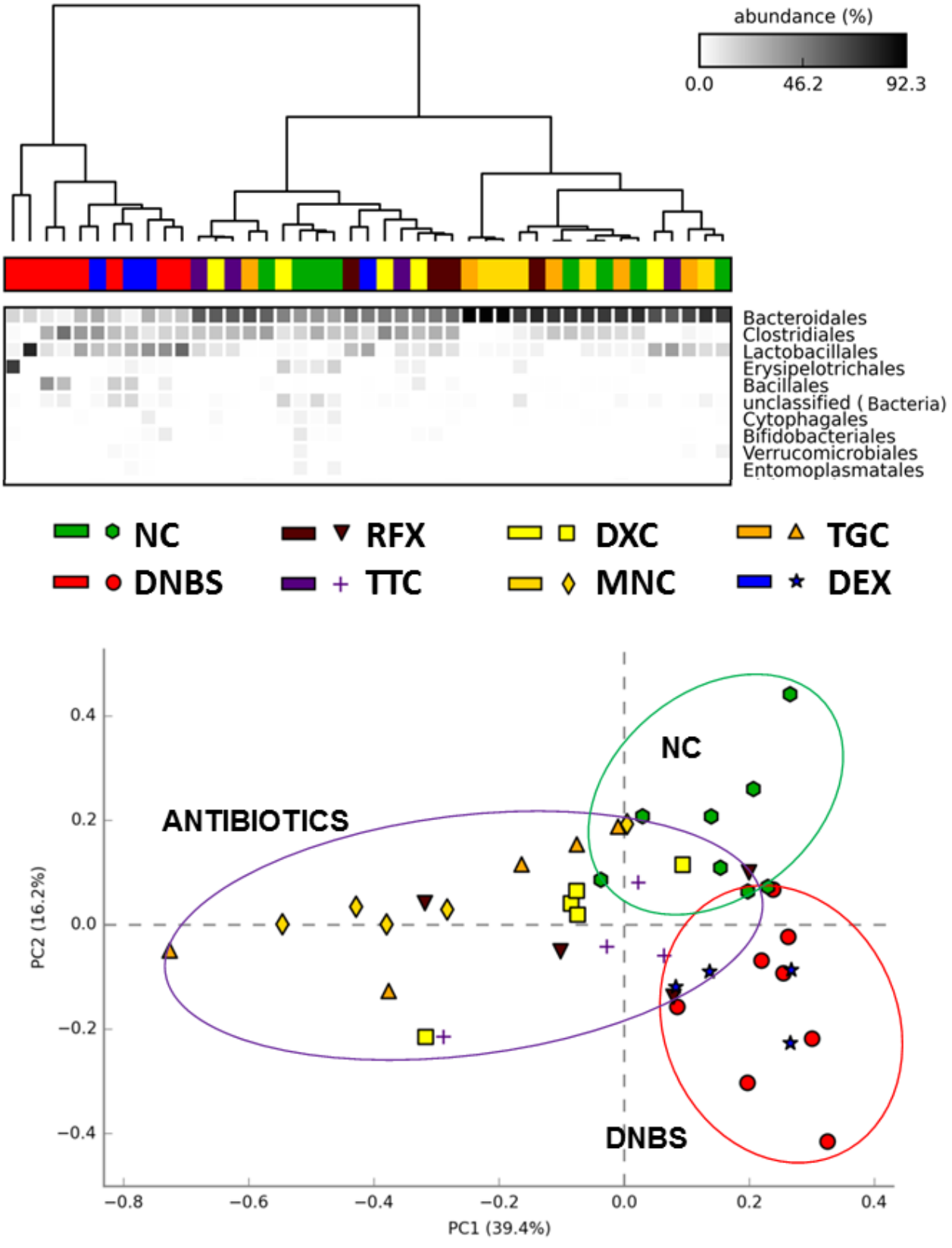


Figure 23: Comparative study in DSS colitis.



Beta-diversity assessment based on hierarchical clustering and principal component analysis (PCA) represent the main differences observed between the different groups. Order level heatmap showing the 10 most abundant taxons illustrates that DSS control and DEX-treated individuals cluster together based on their different composition with the other groups, mainly of Bacteroidales. Antibiotic treated and healthy mice fall into a different branch with no specific pattern of distribution observed at this level (Fig 23A). Based on the ordination of the distance matrix, a clear demarcation of antibiotic treated mice along PC1 axis 1 is apparent (which explains 39.4% of the variance). Based on PC2 (16.2%), bacterial assemblages of healthy mice differ from those of colitic mice without antibiotic treatment (DSS and DEX), which cluster on the same region, while no differences among antibiotic action is explained by this component (Fig 23B).

2.1. FUNCTIONAL EVALUATION OF MICROBIOTA

The relevance of microbial communities on intestinal homeostasis is determined by its functions, and specific metabolic pathways are not restrained to determined evolutionary related groups[737]. After studying the microbial variations and the effects associated to each pharmacological intervention, we concluded that the antibiotic action could be beneficial in DNBS colitis, while its impact on DSS induced intestinal inflammation was not sustained by the results obtained. Therefore, in order to collectively evaluate the functional role of DSS induced dysbiosis and the influence of tetracycline treatments, we proposed an additional experiment: to assess the ability of the intestinal microbiota to confer protection against DSS-induced colitis in a model of fecal microbiota transference (Fig.24).

The depletion of endogenous microbiota in recipient mice was performed as detailed on the M&M section. Four groups underwent this procedure(Fig.24): one was given the vehicle, PBS, while the other three received the microbiota of NC, untreated DSS-colitis and MNC-treated DSS colitis animals respectively. These last three groups had previously been subject to the same experimental protocol used on the previous comparative study. After the fecal transfer, the susceptibility of these groups to develop DSS-induced intestinal inflammation was evaluated at the peak of the disease(Fig.24).

The administration of the initial antibiotic cocktail correlated with a slow but progressive weight loss, which was quickly recovered upon antibiotic interruption and microbial transference (Fig.24). This is in agreement with the metabolic function of the intestinal microbiota, which contributes to the recovery of energy from non-digested diet components, and the observation that germ-free mice require a 30% additional food intake to maintain their body weight. It is also indicative of the successful establishment of the exogenous microbiota, while the subtle weight recovery observed in the vehicle treated control group might be indicative of the reconstitution of the endogenous microbiota, which is never completely eliminated, and occurs at a slower rate (Fig.24).

Figure 24: Functional assessment of MNC-treated DSS-colitis microbiota

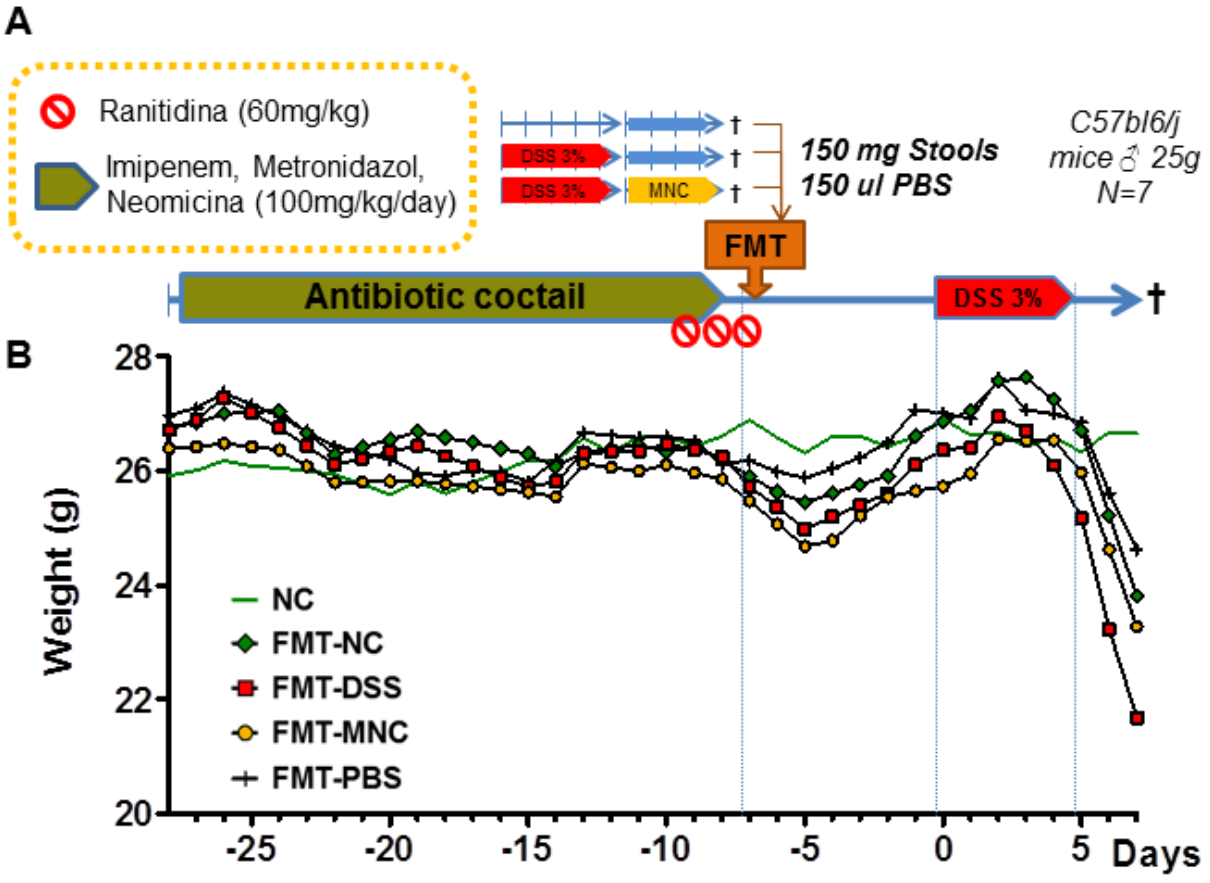


Figure 24. Functional assessment of the role of MNC-treated DSS-colitis microbiota in the development of intestinal inflammation. **A)** Schematic illustration of the experimental design followed. The microbiota of recipient mice was depleted by administering an antibiotic cocktail prior to the transfer. Stools from non-colitic (NC), DSS-colitic (DSS) and minocycline-treated (MNC) DSS-colitic mice were collected and transferred into the microbiota-depleted recipients. A week later, colitis was induced in faecal microbiota transferred mice (FMT) by administering 3%DSS for a period of 5 days. Mice were sacrificed 2 days later and the colonic inflammatory process was evaluated. NC: non-colitic (NC), FMT-NC: FMT from NC animals, FMT -DSS: FMT from DSS-colitic animals, FMT-MNC: FMT from MNC-treated DSS-colitic animals, and FMT-PBS: microbiota depleted group administered the FMT vehicle (PBS). **B)** Mean body weight evolution (g) of the different groups throughout the experimental period.

The results show that the evolution of colitis was characterized by a stronger weight loss and elevated DAI values in the group of mice recipient of microbiota from untreated colitic animals compared with all other groups (Fig. 25A & B). These differences were statistically significant at days 5, 6 and 7 for the weight evolution, and at days 5, 6 and 7 for the DAI, with the exception of PBS group, that did not show statistical differences in DAI evaluation(Fig. 25 B). This might be indicative of an increased susceptibility conferred by the intestinal microbiota resulting from a previous colitic process. Macroscopic parameters showed minor changes when the FMT-DSS group was compared with the FMT-NC and FMT-MNC groups. A more pronounced reduction in the colonic length was observed in the FMT-DSS group, while similar colonic weight values were found in all colitic groups (Fig 25 C & D).

The results from the microscopic evaluation were more representative of the disease evolution observed on the different groups, evidencing a more severe histological damage in the FMT-DSS group than in the others (Fig.25 E). The histological features were mainly associated with the area affected by ulceration, while dense inflammatory infiltrate was present in all groups although at similar degrees (Fig. 25 F).

No major differences were observed in the expression of barrier function markers. In general, improved levels of these markers were observed in the vehicle control group and a controversial increase was found in ZO-1 expression in this group, compared to basal expression levels of non-colitis mice. In general, this might represent a preventive effect of the antibiotic cocktail used on this model.

Figure 25: Functional assessment of MNC-treated DSS-colitis microbiota

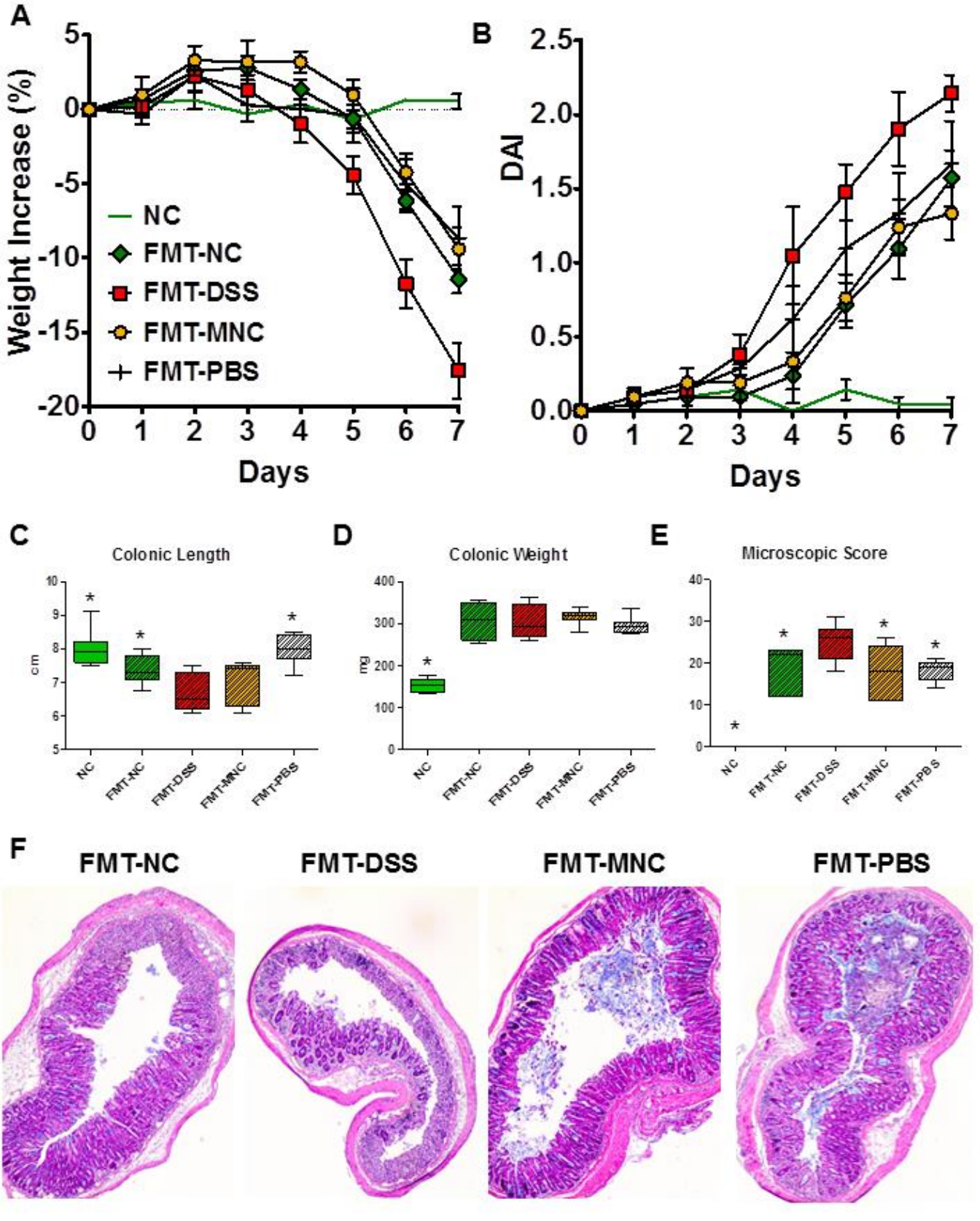


Figure 25: Functional assessment of MNC-treated DSS-colitis microbiota (continuation)

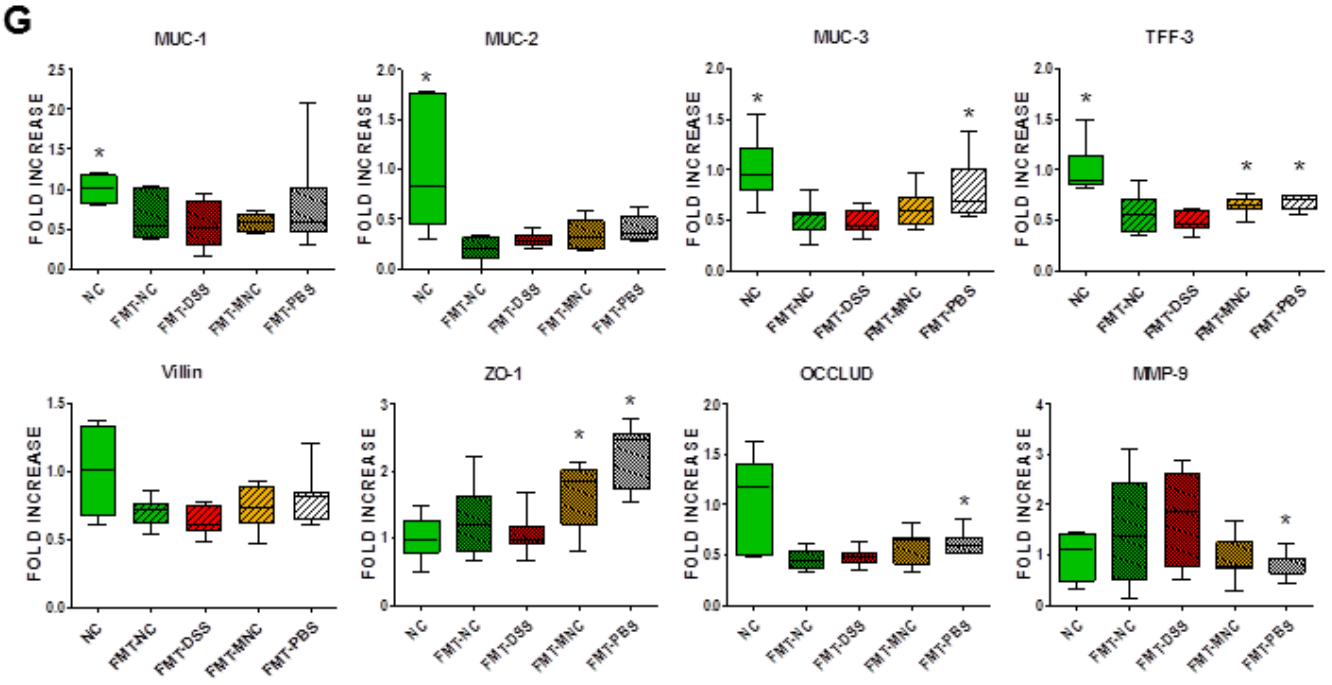


Figure 25. Functional assessment of the role of MNC-treated DSS-colitis microbiota in the development of intestinal inflammation. **A)** Body weight evolution (%weight increase)(means ± SEM) of the different groups during DSS-colitis post FMT. **B)** Disease Activity Index (DAI) values (means ± SEM) assigned based on the criteria described in (Cooper et al., 1993), during DSS-colitis post FMT. **C)** Colonic length and **D)** weight of the different groups after DSS-colitis post FMT. **E)** Microscopic damage score assigned according the criteria described in (Camuesco et al., 2004). Data are expressed as median (range). **F)** Representative histological sections of colonic mucosa of the different experimental groups stained with haematoxylin and eosin (10x magnification). **G)** mRNA expression in colonic tissue quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* FMT-DSS group.

Minor differences were observed between the microbiota transferred groups. An increased in the expression of TFF-3, tight junction proteins and a reduction in MMP-9 expression was evidenced in the FMT-MNC. Additionally, no differences were observed in the expression of the cytokines TNFα, IL-6 and IL-1b between the different groups, which suggests that acute activation of the immune response may not be yet affected at this early time point despite the amelioration observed histologically.

AIM 3

Our previous studies have highlighted the huge therapeutic potential of immunomodulatory tetracyclines on intestinal inflammation. Tigecycline did also exert anti-inflammatory activity and, together with doxycycline and minocycline, prevented mortality associated with acute intestinal inflammation. The antibiotic action seems to be relevant for the effect of these tetracyclines in DNBS-induced colitis, to which other properties, such as inhibition of cell death, fibrosis, oxidative stress, and their immunomodulatory activity also contribute. In DSS induced colitis, however, the antibiotic properties may not play a predominant role in the general anti-inflammatory effect observed with tetracyclines. In this model, a marked improvement in the intestinal barrier protection and their inherent anti-inflammatory activity are the factors that seem to lead their rapid beneficial effect. Intriguingly, the latter may not follow conventional mechanisms. We found indeed, that these tetracyclines potentiated some inflammatory pathways. An increased expression of miR-142 and recruitment of monocytes and dendritic cells may underlay the mechanisms responsible for their early and effective improvement of acute intestinal inflammation. Even more, their dual effect on macrophage activation *in vitro* seems quite controversial with the beneficial effects they displayed *in vivo*, especially when considering the important contribution of this immune cell population to intestinal homeostasis and its key role in the pathogenesis of the DSS model of colitis[294]. These findings suggest that minocycline might induce a certain degree of immune activation which may underlay the mechanisms by which it controls acute intestinal inflammation. Considering all the above, we thought that giving an answer to the following question is a crucial step to uncover the mechanism of action behind the intestinal anti-inflammatory activity of these tetracyclines:

Do tetracyclines potentiate the immune response to control intestinal inflammation?

To provide an answer to this question, we focused on the evaluation of the changes in the immune response during the distinct stages of the inflammatory process, paying special attention to the immune compartment of the colonic lamina propria (cLP). Being minocycline the most studied tetracycline regarding its immunomodulatory properties, we focused on evaluating its effects in the immune response that takes place during DSS colitis. Minocycline early effects were studied after 2 of treatment, in order to capture the initial events that may be triggering later changes in the immune response, and the further development of the immune response in the presence of this antibiotic was determined after 4 days of treatment.

For this, intestinal inflammation was induced following the same experimental design of previous experiment, in which mice were administered 3%DSS in the drinking water for a period of 5 days. Mice were then treated with MNC (50 mg/kg) for either 2 or 4 days (Fig.26 A). After this, the animal were sacrificed and the immune cells populations were isolated from the blood, colon LP, mesenteric lymph nodes and spleen and analysed by FACS as detailed on the protocols section.

Data from preliminary experiments in which a panel of antibodies to identify B cells, T cells, neutrophils and other $CD11b^+$ and $CD11c^+$ populations was used, showed that after 4 days of treatment, minocycline reduced the population of neutrophils present in the colon LP while increasing the number of monocytic myeloid cells, in line with the effects previously observed in the expression on their chemoattractants CXCL2 and CCL2, respectively. Strikingly, elevated numbers of the rest of granulocytes (excluding neutrophils) as well as T cells were also detected on treated mice.

Considering these observations, we developed and optimised three different panels to further characterise the nature of the immunomodulatory action exerted by minocycline. As described in the material and methods section (Table XX), a first panel was used to identify the presence of B cells ($B220^+$), T cells ($CD3^+$), eosinophils ($SiglecF^+$), neutrophils ($Ly6G^+$) and other $CD11b^+$ and $CD11c^+$ populations. Macrophages and dendritic cells, were further characterised in a second panel designed to evaluate the phenotype of antigen presenting cells, including specific markers such as. And finally, a third panel was developed to identify the different T cell subsets, cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells,

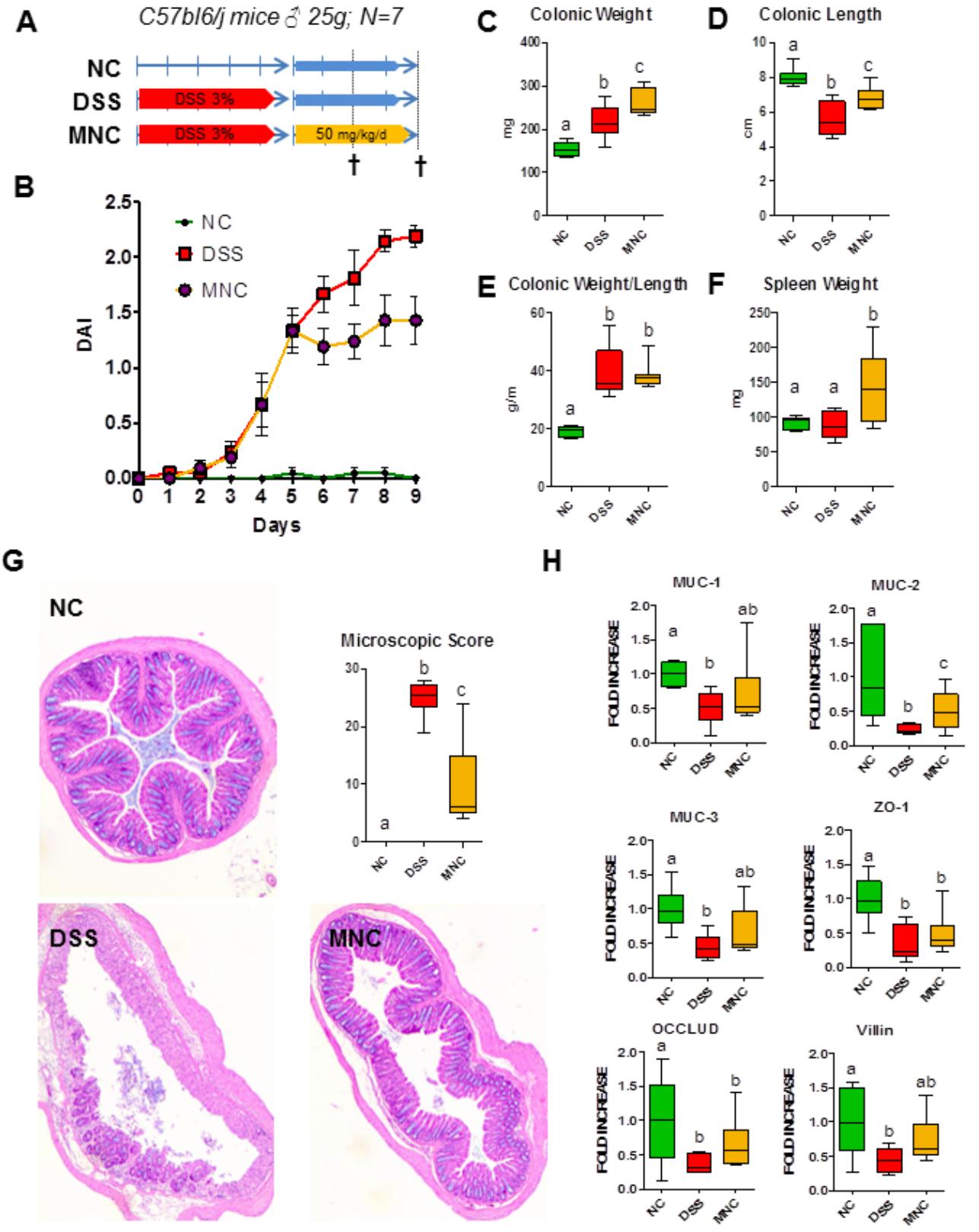
and specific markers to identify Th1, Th2, Th17 and Tregs within T helper cells were also included

Confirming our previous data, minocycline treatment to DSS colitic mice significantly ameliorated the evolution of the colitic process, and according to the length of the treatment, this effect was more pronounced after 4 days of minocycline administration(Fig.26 B). Reduced DAI values were observed for the MNC-treated group, which also showed a marked improvement histologically with a lower histological damage score(Fig.26 G). This effect was confirmed biochemically. A restored expression of mucins and tight junction proteins was observed in the MNC -treated group together with a reduction in IL-1β and IL-6 expression, and a slighter decrease in the expression of TNFα (Fig.26 H). However, colonic weight/length ration was not modified, but separate analysis of these two measures show how an amelioration of colonic shortening is compensated by an increase on weight(Fig.26 C-E). This might be representative of an increase oedema associated with the inflammatory process, while tissue remodelling is reduced. An elevated immune response is also suggested by increased spleen weight (Fig.26 F).

At the two different time points, immune cells were isolated from the lamina propria, cell numbers were determined, and the different populations were analysed by FACS. Percentages of the different populations were referred to life cells and multiplied by the total count of cells obtained after digestion of lamina propria in order to provide the total number of each population. . The percentage and/or total cell numbers for each population are provided.

Figure 26: Evaluation of the effects of minocycline on the immune response during DSS colitis. **A)** Schematic illustration of the experimental design followed. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group (50 mg/kg/d) (n=7). **B)** Disease Activity Index (DAI) values (means ± SEM) in DSS mice colitis over the 9-day experimental period, based on the criteria described in (Cooper et al., 1993). **C)** Colonic weight, **D)** Colonic length, **E)** Colonic weight/length ratio, and **F)** Spleen weight of the different groups of colitic mice. **G)** Histological sections of colonic mucosa stained with haematoxylin and eosin and microscopic damage score assigned according the criteria described in (Camuesco et al., 2004). **H)** mRNA expression of MUC-1, MUC-2, MUC-3, ZO-1, Occludin, and Villin quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DSS group.

Figure 26: Evaluation of the effects of MNC on the immune response



3.1. EFFECTS IN THE IMMUNE RESPONSE DURING DSS COLITIS: 2 DAYS OF MNC TREATMENT.

Two days after the colitis induction, no changes in the different immune cell populations were observed in the blood of colitic control mice when compared with healthy animals (Fig. 27A). Surprisingly, a strong increase in circulating myeloid cells ($CD45^+CD11b^+$) was observed in the MNC-treated group, compared to the levels of these cells found in NC and DSS-colitic animals (Fig 27 A1). This included an increase in neutrophils ($Ly6G^+$), eosinophils ($SiglecF^+$) and monocytic cells ($Ly6G^-SiglecF^-SSC^{lo}$), and is indicative of the systemic impact on the inflammatory process and suggests that systemic immune changes were accelerated by minocycline treatment.

When immune changes were analysed on the lamina propria, differences between healthy and colitic mice were observed, which included the increase of neutrophils ($Ly6G^+$), monocytic myeloid cells ($CD11b^+Ly6G^-SiglecF^-SSC^{lo}$), B cells ($B220^+$) and T cells ($CD3^+$). Particularly, at this time point, dendritic cells with a tolerogenic phenotype ($CD11b^-CD103^+$) were reduced while inflammatory macrophages ($Ly6C^+MHCII^-$) and $CD4^+$ T cells, mainly $FoxP3^+$ Tregs, accumulated in the colon of colitic mice. The effect of minocycline was not evidenced at this time point when the lamina propria leukocytes were analysed in this group, which showed no statistical differences with colitic control animals (Fig 27 B).

Figure 27: Evaluation of the effects of 2 days of minocycline treatment on the immune response during DSS colitis. **A)** Analysis of immune cell populations in the blood of the different experimental groups. The top panel (A1) shows the total percentage of $CD45^+$ $CD11b^+$ myeloid cells, and the percentages of neutrophils ($Ly6G^+$), eosinophils ($SiglecF^+$) and monocytic cells ($Ly6G^-$ $SiglecF^-$ SSC^{lo}) within the $CD45^+CD11b^+$ cells. The bottom panel (A2) shows the total percentage of $CD45^+$ $CD11b^-$ cells and the percentages of $CD11c^+$, T cells ($CD3^+$) and B cells ($B220^+$) within the $CD11b^-$ cells. **B)** Analysis of immune cell populations in the colonic lamina propria of the different experimental groups. B1) Absolute cell numbers of $CD45^+$ $CD11b^+$ myeloid cells, and the number neutrophils ($Ly6G^+$), eosinophils ($SiglecF^+$) and monocytic cells ($Ly6G^-$ $SiglecF^-$ SSC^{lo}). B2) Absolute cell numbers of $CD45^+$ $CD11b^-$ cells and the number of $CD11c^+$, T cells ($CD3^+$) and B cells ($B220^+$). B3-6) Absolute cell numbers of: B3) Inflammatory MΦ, B4) tolerogenic DCs, B5) $CD4^+$ T cells, B6)T regs. Data are expressed as mean ± SEM (n=5). NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group.

Figure 27: Evaluation of the effects of MNC on the immune response: 2-days of treatment

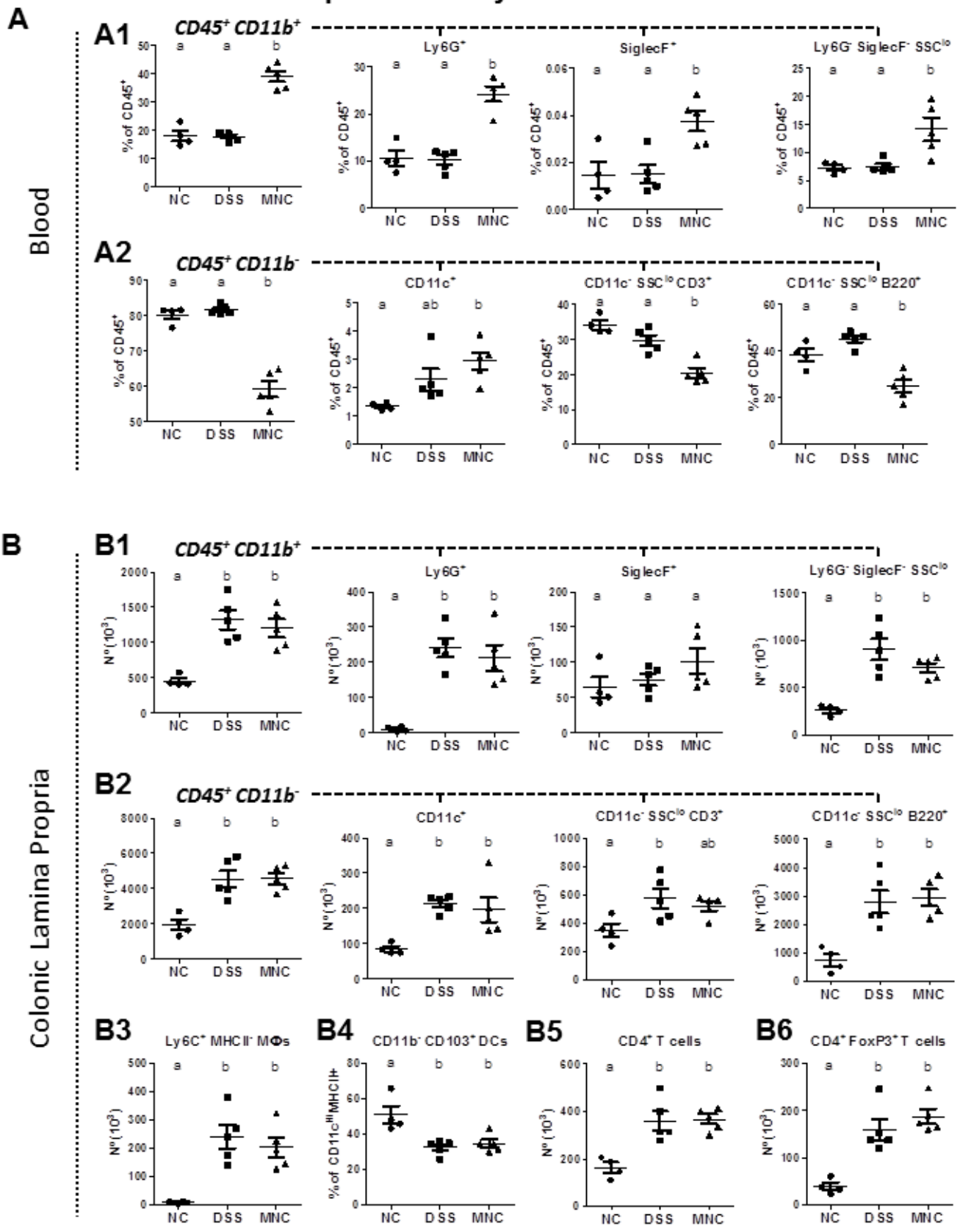


Figure 28: Evaluation of the effects of MNC on the immune response: 2-days of treatment

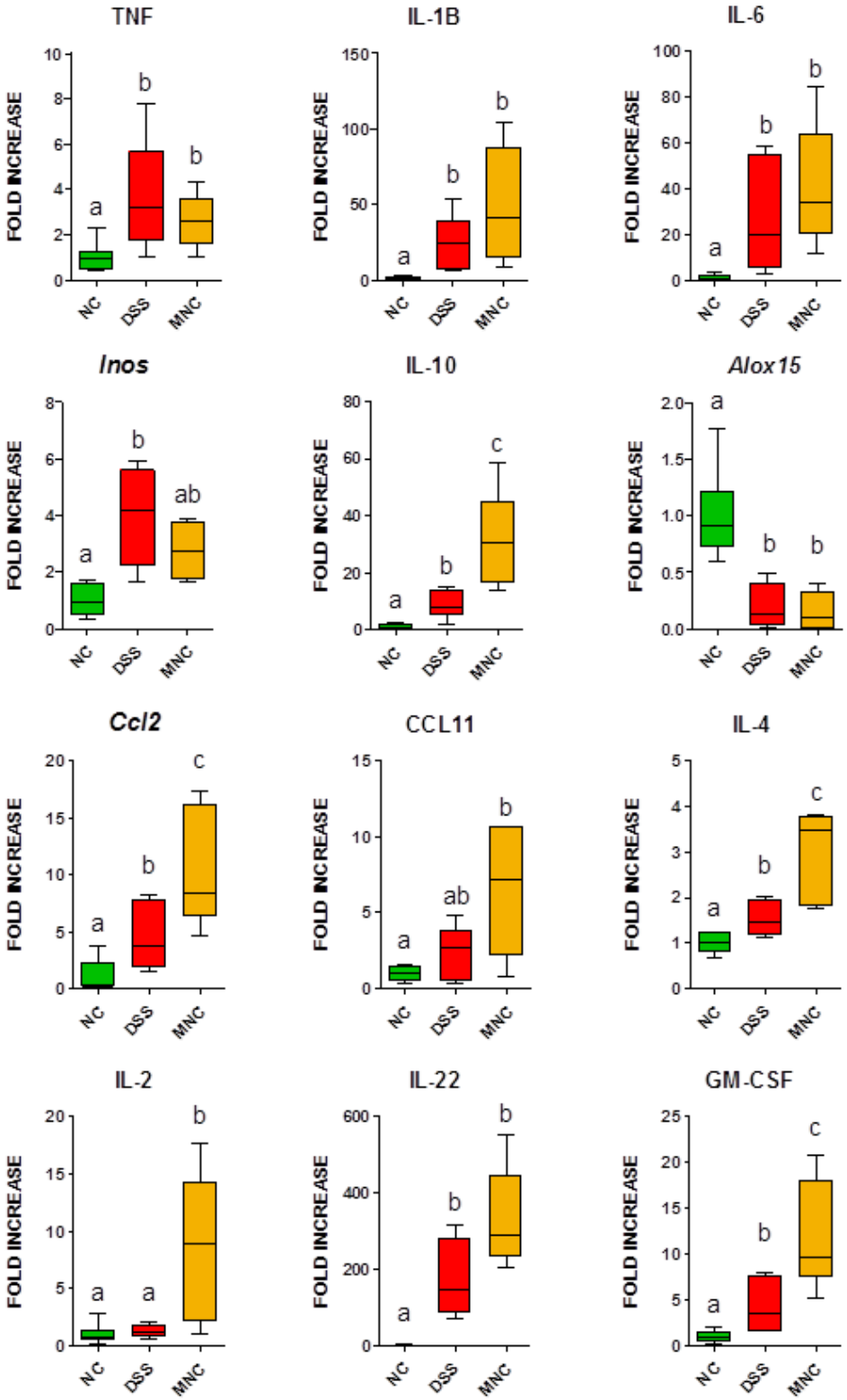


Figure 28: Evaluation of the effects of 2 days of minocycline treatment on the immune response during DSS colitis. mRNA expression of the indicated genes quantified by real-time PCR. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *P<0.05 *vs.* DSS group (n=5).

Despite local changes in immune population were not observed, gene expression analysis in the colonic tissue did show important differences between treated and untreated mice (Fig 28). Increased expression of the monokines IL-1β and IL-6 was observed in colitic animals, and this was further potentiated in minocycline treated mice, which may be representative of the effect observed with minocycline in macrophage function *in vitro*. Additionally, IL-10 was also expressed at higher levels in the mice treated with minocycline, while iNOS was ameliorated. When the expression of *Alox15* was evaluated, the gene encoding for the 12/15-lipooxigenases, critical enzymes involved in the synthetic pathway of anti-inflammatory lipid mediators, all colitic mice showed reduced levels compared to NC group. Minocycline treatment induced the expression of the chemokines CCL2 and CCL11, involved in the recruitment of monocyte and dendritic cell, the first, and eosinophils, the latter (Fig. 28). Accordingly with a potentiation of type-2 immune response and eosinophil recruitment, IL-4 expression was also up-regulated by minocycline treatment.

IL-2 expression, which promotes T cell expansion and stabilises Treg polarization, was strongly increase in minocycline treated mice, while no difference was observed between NC and DSS groups. A remarkable 200 fold increase in IL-22 expression was observed in the DSS control group, and this level was doubled in the MNC-treated group (Fig.28). Colitic control mice also showed an increased expression of GM-CSF, levels that where almost triplicated by the treatment with minocycline (Fig. 28), and may contribute to the potentiation of the immune response both at locally and systemically. The changes observed on these markers two days after the colitic induction may explain the immune variations appreciated at latter stage.

Enhanced expression of IL-4, IL-10 and GM-CSF could promote the progression of the development of inflammatory macrophages into the homeostatic phenotype. Additionally, IL-4 is linked with type 2 immune responses and eosinophils recruitment, which was also suggested by the higher

levels of Ccl11, produced by macrophages observed in minocycline treated mice. Such a strong increase of IL-22 may not be originated on Th22 cells but rather ILC3. This population represents a more reliable source of this cytokine, as well as GM-CSF. Increased IL-1β production on macrophages could potential ILC3 function to accelerate and improve the protective immune response at the mucosa.

Although no striking differences in the immune cell populations were observed at this time point between minocycline and DSS control mice, the enhanced levels of the aforementioned cytokines suggest a potentiation of the immune response in minocycline treated mice.

3.1. IMMUNE RESPONSE ON DSS COLITIS: 4 DAYS OF MNC TREATMENT.

On a first analysis of the immune compartment of the colon LP we found an increased immune cell infiltration, measured as the number of CD45+ cells, in all colitic animals, including in the MNC-treated group, in which the number of CD45+ cells was indeed slightly increased compared to the untreated colitic group (fig. 29). A particular increase in the myeloid lineage ($CD11b^{+}$) in the cLP was observed in MNC treated animals in comparison with untreated colitic control mice, while no differences in the total numbers of $CD11b^{-}$ cells were found in between treated and untreated mice(Fig x). Similarly, no changes were found in the number of $CD11b^{-}$ $CD11c^{+}$ cells after minocycline treatment (Fig. 29).

Within the lymphocyte compartment, gated as $CD11c^{-}$ SSC^{lo} cells and further segregated based on CD3 and B220 expression to identify B cells and T cells populations respectively, an intense recruitment of B cells was observed in colitic mice, as well as increased number of T cells (Fig 29). Minocycline treatment did not modify the number of B cells, although at this time point it did increase the total number of T cells in comparison with both healthy and DSS-colitc mice.

Figure 29: Evaluation of the effects of MNC on the immune response: 4-days of treatment

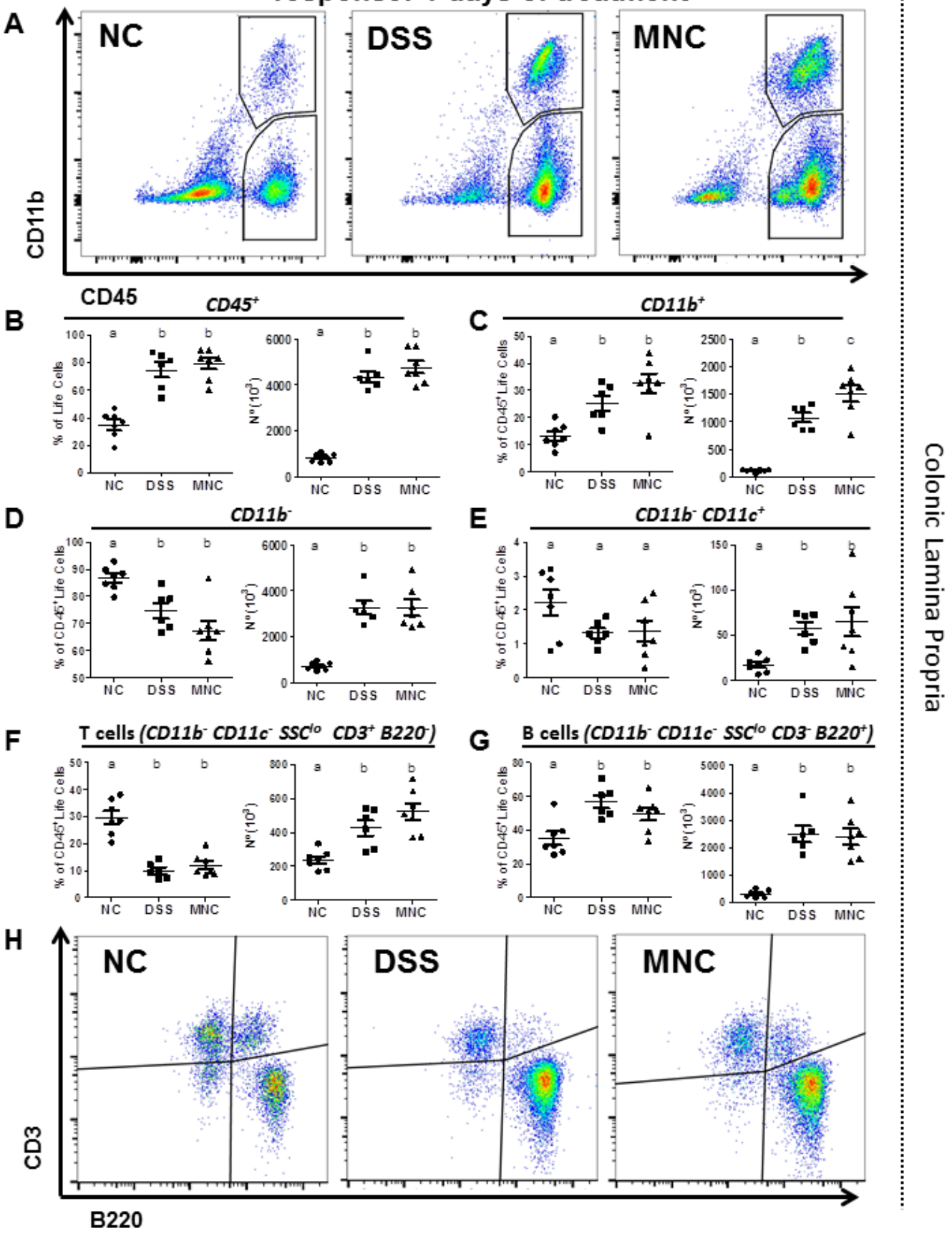
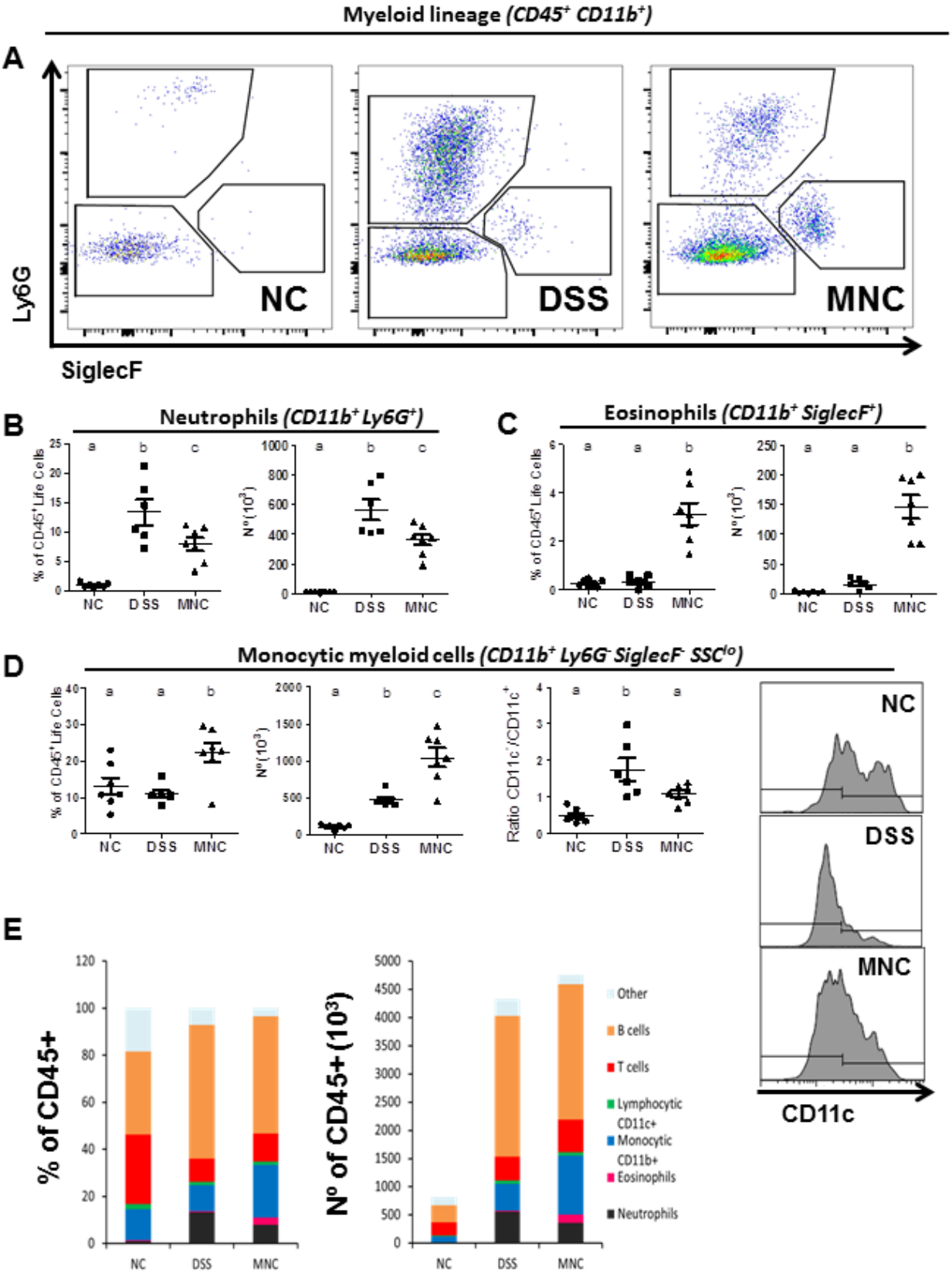


Figure 29: Evaluation of the effects of 4 days of minocycline treatment on the immune response in the colonic lamina propria during DSS colitis. **A)** Representative flow cytometry analysis of live cells from the cLP showing the $CD11b^+$ and $CD45^+$ populations of cells. **B-G)**) Percentage (left) and absolute cell numbers (right) of: B) $CD45^+$ cells, C) $CD11b^+$ cells, D) $CD11b^-$ cells, E) CD11b- CD11c+ cells, F) CD11b- CD11c- SSClo CD3+ B220- T cells, and G) CD11b- CD11c- SSClo CD3- B220+ B cells. **H)** Representative flow cytometry analysis of $CD11b^-CD11c^-SSC^{lo}$ cells from the cLP showing the $CD3^+$ and $B220^+$ cell populations. Data are expressed as mean ± SEM. NC: Non-colitic group(n=7), DSS: DSS-colitic group(n=6), MNC: minocycline-treated colitic group(n=7).

Among the myeloid compartment (CD11b) (Fig 29), neutrophil ($Ly6G^+SiglecF^-$) infiltration was significantly increased in colitic animals, but the recruitment of this population to the colon LP was significantly reduced by minocycline. No changes were observed in the number of eosinophils (Ly6G-SiglecF+) upon colitis induction. However, confirming the data from our preliminary experiments and consequently with the higher expression levels of CCL11, IL-4 and GM-CSF observed in the colonic tissue at day 2, a strong increase in the number of eosinophils was observed in the MNC treated group. Within the $CD11b^+Ly6G^-SiglecF^-$ fraction, SSC^{hi} (granulocytes) and SSC^{lo} populations (monocytic myeloid cells) were discriminated, and the expression of CD11c was evaluated among the latter (Fig 30). An increase in myeloid monocytic cells, which mainly represent macrophages and a subset of dendritic cells $CD11b^+$, was observed in colitic animals, which was potentiated by minocycline treatment (Fig. 30). This correlates with the increased Ccl2 mRNA expression previously found in this group (Fig. 30).

Figure 30: Evaluation of the effects of 4 days of minocycline treatment on the innate immune response in the colonic lamina propria during DSS colitis. A) Representative flow cytometry analysis of xxx cells from the cLP showing the Ly6G+ and SiglecF+ populations of cells. B-C) Percentage (left) and absolute cell numbers (right) of: B) neutrophils (Ly6G+ cells) and C) Eosinophils (SiglecF+ cells). D) Percentage and absolute cell numbers, ratio of CD11c+/CD11c- cells and representative flow cytometry analysis of CD11c expression in monocytic myeloid cells (CD11b+ Ly6G- SiglecF- SSClo). Data are expressed as mean ± SEM. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group.

Figure 30: Evaluation of the effects of MNC on the innate immune response: 4-days of treatment



Additionally, increased CD11c staining was observed in this population in NC group (Fig 30), indicative of a mature phenotype, while the DSS control group was dominated by $CD11c^-$ cells, suggesting they are cells derived from recently recruited precursor monocytes. An intermediate profile between the DSS and NC groups was observed in minocycline treated mice, and the ratio of CD11c +/- cells in this group was statistically significant reduced in comparison with the colitic control group.

An accurate identification of macrophages and dendritic cells and the characterization of their phenotypes requires the concomitant use of different antibodies raised against their multiple surface markers. Using this strategy, our results confirmed the presence of elevated number of macrophages ($CD11b^+Ly6G^-SSC^{lo}F4/80^+$) and dendritic cells ($SSC^{lo}F4/80^-CD11c^{hi}MHC^+$) in the colonic lamina propria of colitic animals, numbers that where even higher in the MNC treated group (Fig. 31). The CCL2-CCR2 axis mediates the recruitment of dendritic cells and $Ly6C^{hi}$ monocytes to intestine, both in the steady state and during inflammation. In the context of inflammation, once in the lamina propria, the inflammatory milieu arrests the maturation process of recently recruited monocytes at the $Ly6C^+MHCII^-$ stage, preventing their development into resident macrophages ($Ly6C^-MHCII^+$). Therefore, they accumulate in the inflamed intestine as $Ly6C^+MHCII^-$ inflammatory MΦs with a M1 activation phenotype that sustains inflammation[294]. This monocyte-macrophage differentiation waterfall can be observed in Fig 31, which also illustrates the accumulation of the initial $Ly6C^{hi}$ population in colitic animals. However, despite the enhanced monocyte recruitment, MNC-treated group showed similar numbers of inflammatory MΦs to the DSS control group, while the intermediate and resident macrophage populations (Fig 31) were significantly increased. The latter are considered as M2-like activated macrophages, and they are involved in the regulation of intestinal homeostasis, mucosal healing and epithelial regeneration, contributing to the resolution of the inflammatory response. Therefore, minocycline treatment accomplished to restore the balance between inflammatory and resident macrophage populations (Fig 31), which followed a similar but accentuated profile than previous $CD11c^{+/-}$ monocytic cells ratio.

Figure 31: Evaluation of the effects of MNC on the innate immune response: 4-days of treatment.

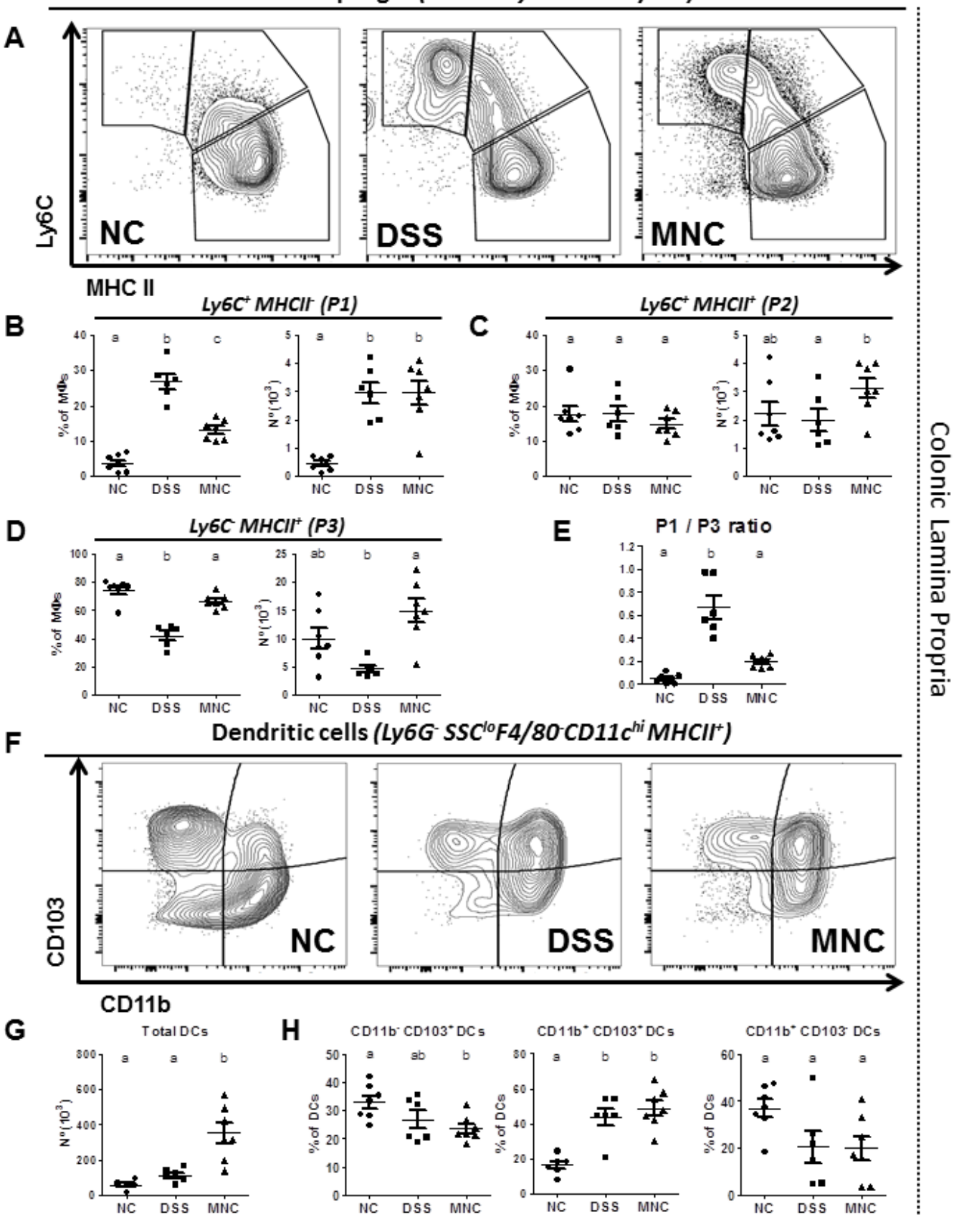


Figure 31: Evaluation of the effects of 4 days of minocycline treatment on the innate immune response in the colonic lamina propria during DSS colitis. **A-E)** Analysis of the macrophage population. A) Representative flow cytometry analysis of macrophages ($CD11b^+Ly6G^-SSC^{lo}F4/80^+$ cells) from the cLP showing the expression of Ly6G and MHCII and illustrating the monocyte-macrophage waterfall. B-D) Percentage (left) and absolute cell numbers (right) of: B) Inflammatory macrophages (P1)($Ly6C^+MHCII^-$ cells), C) Intermediate macrophage population (P2) ($Ly6C^+MHCII^+$ cells) and D) Resident intestinal macrophages (P3)($Ly6C^-MHCII^+$ cells). **F-H)** Analysis of the dendritic cell population. F) Representative flow cytometry analysis of ($Ly6G^-SSC^{lo}F4/80^-CD11c^{hi}MHCII^+$)cells from the cLP showing the expression of CD103 and CD11b. G) Absolute cell numbers of DCs, H) Percentage of $CD11b^-CD103^+$, $CD11b^+CD103^+$ and $CD11b^+CD103^-$ DCs. Data are expressed as mean ± SEM. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group.

Based on their expression of CD11b and CD103, dendritic cells can be divided in four subsets. As represented in Fig 31, $CD11b^-CD103^+$ and $CD11b^+CD103^-$ DCs are the predominant populations found in the healthy intestine but upon activation of the inflammatory response, DCs polarize into the $CD11b^+CD103^+$ phenotype, as observed in the DSS-colitic group (Fig. 31). This has been identified as the main migratory population during intestinal inflammation. They are involved in the priming of the adaptive immune response in MLNs, and in particular, they drive the differentiation of Th17 mucosal immune responses. The total number of dendritic cells was increased in minocycline treated mice compared to untreated controls, however, minocycline treatment also promoted the polarization towards the migratory phenotype.

Figure 32: Evaluation of the effects of 4 days of minocycline treatment on the T cell immune response in the colonic lamina propria during DSS colitis. Absolute cell numbers of T cell ($CD3^+$) populations: A) $CD8^+$, B) $CD4^+$, C) $CD4^+$ $IFN\gamma^+$, D) $CD4^+$ $IL\text{-}17A^+$, E) $CD4^+$ $IL\text{-}4^+$ and F) $CD4^+$ $FoxP3^+$. Percentage of $CD4^+$ T cells: G) $IL\text{-}4^+$ $IL\text{-}17A^+$ and H) $IL\text{-}4^+FoxP3^+$. Data are expressed as mean ± SEM. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group.

Figure 33: Evaluation of the effects of 4 days of minocycline treatment on mRNA expression of the indicated genes quantified by real-time PCR. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group. Fold increase calculated *vs.* NC group. Boxes graphs represent ± SEM range, with middle line indicating the median and the whiskers, extreme values. *$P<0.05$ *vs.* DSS group.

Figure 32: Evaluation of the effects of MNC on the T cell immune response 4-days of treatment.

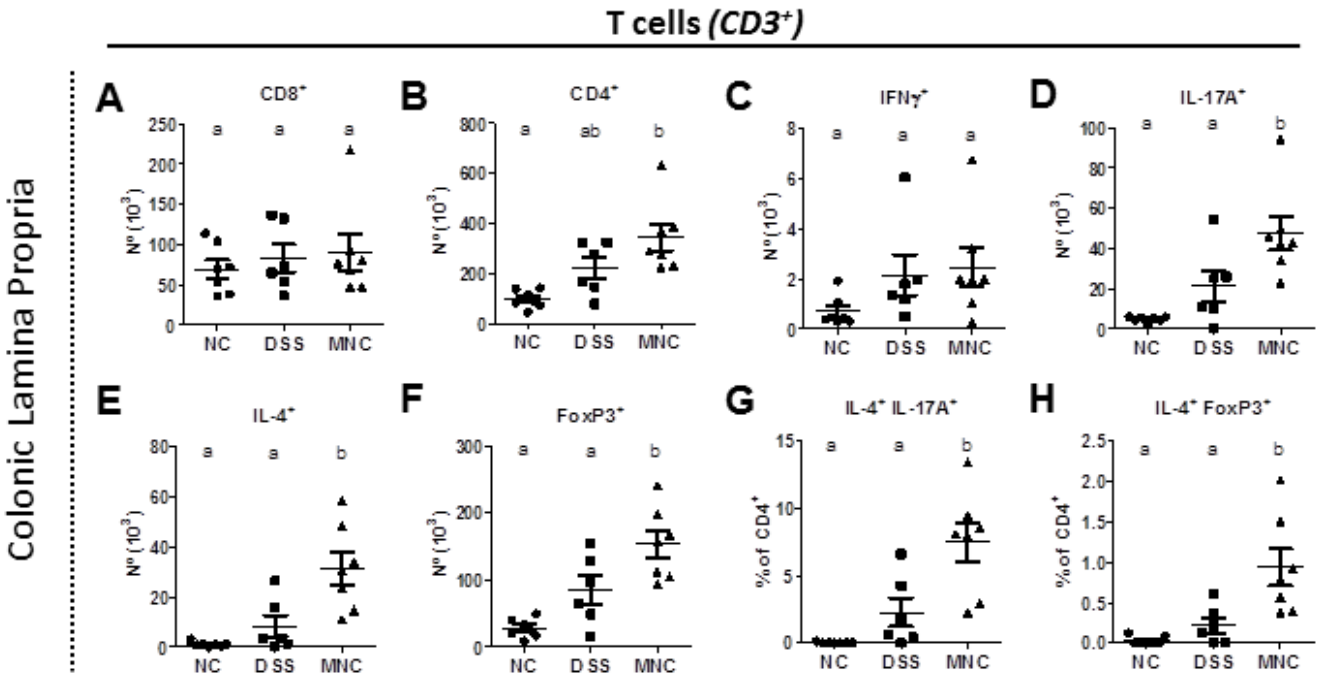
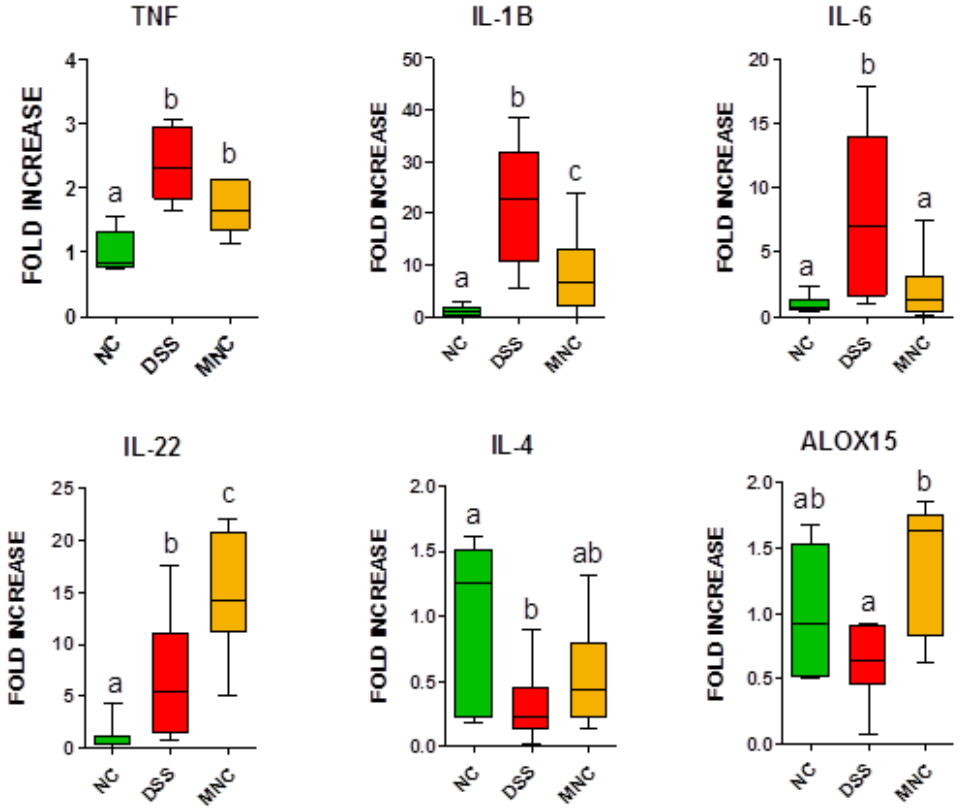


Figure 33: Evaluation of the effects of MNC on colonic immune gene expression: 4-days of treatment.

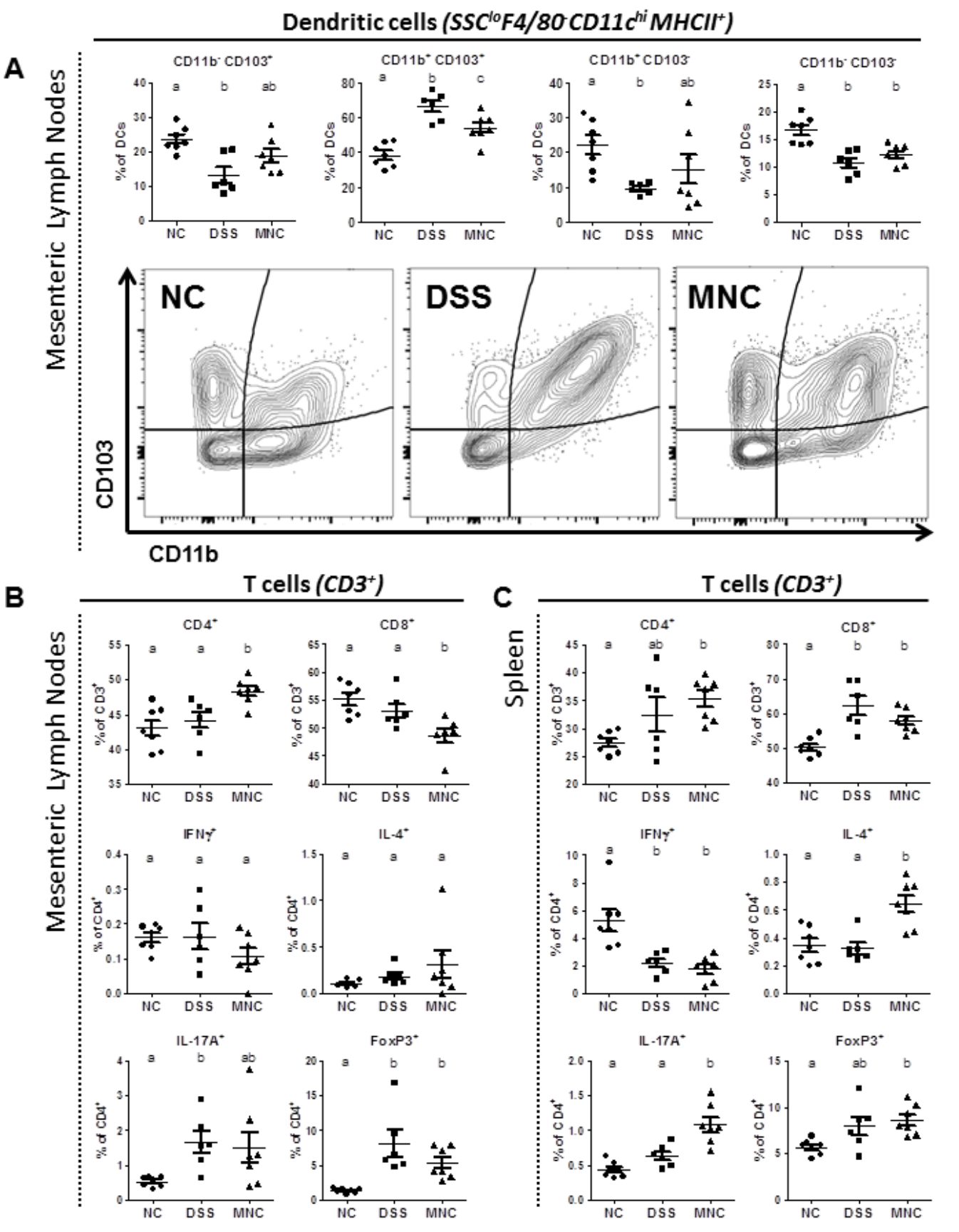


CD3+ T cells were found increased in all colitic animals compared to NC mice (Fig 32), which was mainly due to an increase in $CD4^+$ helper T cells, while no differences were found in the numbers of $CD8^+$ cytotoxic T cells (Fig 32). We then analysed the different T helper cell subsets and found that, although no statistically significant differences were obtained between the NC and DSS control group, DSS-induced inflammation was related to an increase in the IL-17 producing Th17 and Foxp3+ regulatory T cell populations in the colon LP. Tregs dominated the T cell compartment, both in healthy and colitic animals, representing around 30% of the $CD4^+$ T cells (Fig. 32), while the lowest numbers were found for the IFNγ-producing Th1 subset (Fig. 32). Minocycline treatment to colitic mice induced a significant increase in the numbers of Treg and Th17 cells when compared with healthy mice. In line with the higher number of IL-17 producing cells, the mRNA expression of IL-22, another Th17 cytokine, was also increased in colitic animals, and its expression was further potentiated by minocycline treatment (Fig. 32). Furthermore, an increase in Th2 cells was also observed in the MNC-treated group, as shown by the presence of higher numbers of IL-4 producing T cells and an increased IL-4 mRNA expression in the colon LP of these mice (Fig 32). Increased percentages of $IL\text{-}4^+$ $IL\text{-}17A^+$ double positive cells and IL-4 producing cells within the $FoxP3^+$ populations were also observed in MNC treated mice (Fig. 32), which may suggest a higher degree of plasticity between these T cell subsets after minocycline treatment..

Finally, as observed in Fig 33, Alox15 expression was found significantly increased on colonic tissue of MNC group compared to DSS control, which showed reduced levels than healthy mice. This finding correlates with increased presence of eosinophils and Th2 cells, as well as alternatively activated macrophages, which have been described to be actively involved in the resolution phase of acute inflammation.

Figure 34: Evaluation of the effects on the immune response in secondary lymphoid organs. **A)** Analysis of the dendritic cell population in the mesenteric lymph nodes. The bottom panel shows a representative flow cytometry analysis of DCs cells ($SSC^{lo}F4/80^-CD11c^{hi}MHCII^+$) showing the expression of CD103 and CD11b. **B)** Analysis of the $CD3^+$ T cell populations in the mesenteric lymph nodes. Percentages of $CD4^+$ and $CD8^+$ within $CD3^+$ T cells, and percentages of $IFN\gamma^+$, $IL\text{-}4^+$, $IL\text{-}17A^+$ and $FoxP3^+$ cells within the CD4+ T helper cells. **C)** Analysis of the CD3+ T cell populations in the spleen. Percentages of $CD4^+$ and $CD8^+$ within $CD3^+$ T cells, and percentages of $IFN\gamma^+$, $IL\text{-}4^+$, $IL\text{-}17A^+$ and $FoxP3^+$ cells within the CD4+ T helper cells. Data are expressed as mean ± SEM. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group.

Figure 34: Evaluation of the effects of MNC on the immune response: 4-days of treatment.

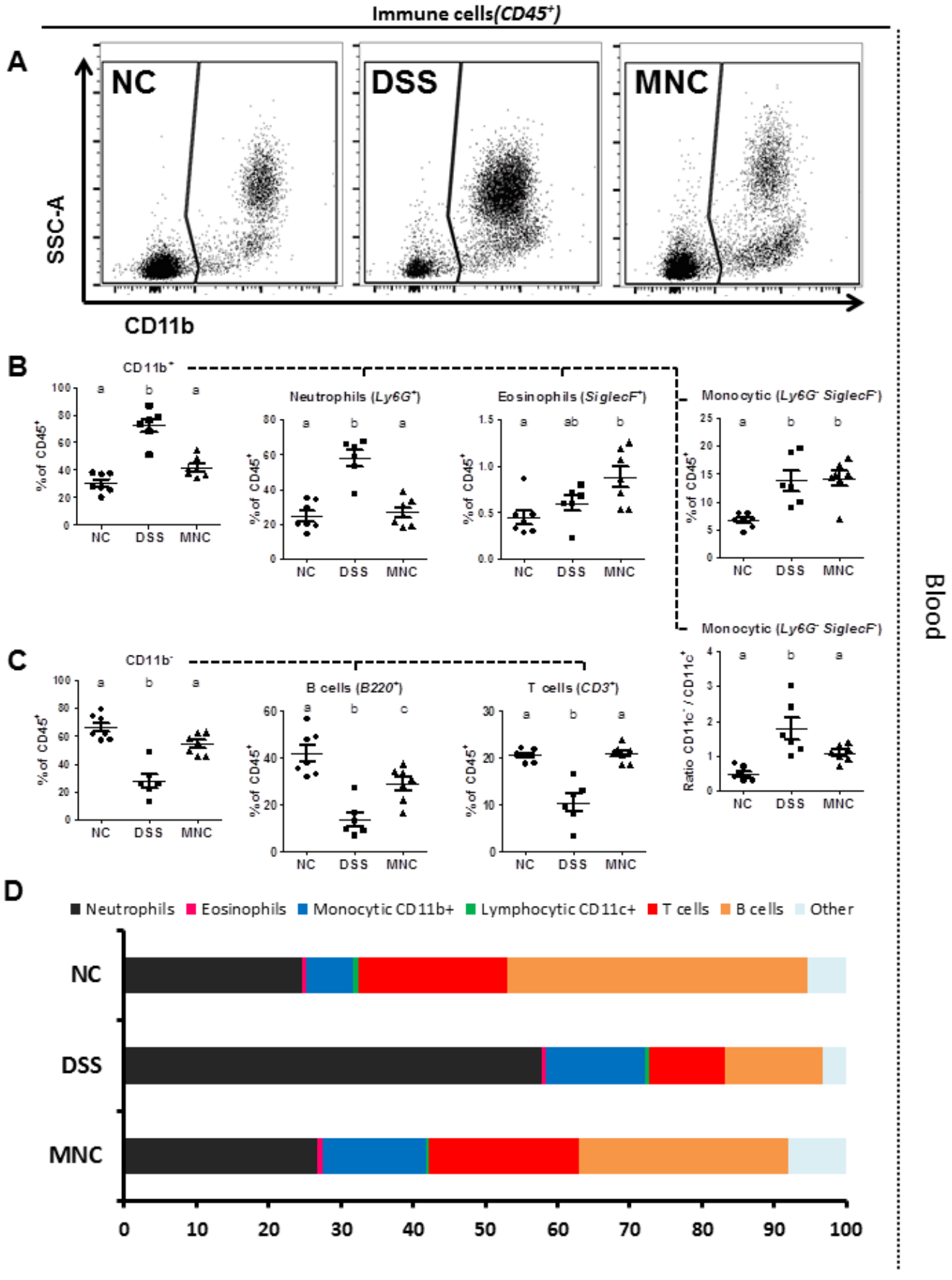


In view of these findings, we aimed at characterising the immune cell population at the local (mLN) and systemic (spleen) secondary lymphoid organs. In the analysis of the dendritic cells populations in the mesenteric lymph nodes we found an increase in the $CD11b^{+}CD103^{+}$ DC population in colitic mice, similar to that observed in the colon LP (Fig. xx) Minocycline treatment partially reversed this change, reducing the $CD11b^{+}CD103^{+}$ DC population while increasing the numbers of $CD103^{+}CD11b^{-}$ and $CD103^{-}CD11b^{+}$ cells (Fig. 34). Among the T cells, the proportion of $CD4^{+}$ T cells was increased in minocycline treated colitic mice, while the CD8+ T cells were reduced. Within the T helper subset, as observed in the colon LP, IL-17+ and FoxP3+ T cells we present at higher percentage in colitic than in non-colitic mice, but no differences were observed between DSS control and minocycline treated mice.

Consistent with the increased spleen weight of MNC treated animals, increased total cell numbers were also observed in this group. Similar to that found in the colon LP and the mLN, Treg and Th17 populations were slightly elevated in DSS control mice, while statistically significant higher percentages of Th2, Th17 and Treg subsets were found in minocycline treated mice (Fig 34) . Curiously, the IFNγ-producing population, which was not modified in the locations previously studied was found significantly reduced in the spleen on both colitic groups (Fig 34). An important fact that should be taken into account is the relative contribution of each subsets. While Tregs where the most abundant Th cells in the three locations evaluated, higher numbers of Th17 cells were observed in the colonic LP and MLN while on the spleen the Th1 subset was are more abundant. Th2 cells were found at similar numbers than Th17 cells in the cLP of MNC treated mice, but they were reduced at other sites. This profile suggests that T cell responses follow the pattern dictated by the immune signals at the focus of inflammation, hence the more pronounce chances are observed locally, while systemically the general well-been of mice might exert a stronger influence.

Figure 35: Evaluation of the effects of 4 days of minocycline treatment on the immune cell populations in the blood during DSS colitis. **A)** Representative flow cytometry analysis of CD45+ cells showing the CD11b and SSC signals. **B)** Percentage of CD45+ CD11b+ myeloid cells, and percentages of neutrophils ($Ly6G^{+}$ cells), Eosinophils ($SiglecF^{+}$ cells) and monocyctic cells ($Ly6G^{-}SiglecF^{-}SSC^{lo}$), and ratio of $CD11c^{+}/CD11c^{-}$ monocytic cells. **C)** Percentage of CD45+ CD11b- cells, and percentages of B ($B220^{+}$) and T ($CD3^{+}$ cells), within the CD45+ CD11b- cells. Data are expressed as mean ± SEM. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group.

Figure 35: Evaluation of MNC-immunomodulation: 4-days treatment.



Finally, a strong increase in circulating myeloid lineage cells, and in particular neutrophils, was observed in the blood of colitic mice, compared to the levels of these cells found in NC and MNC-treated animals. This intense neutrophilia, as represented on Fig 35, is indicative of the systemic impact of the inflammatory process. Consequently the percentage of cells from the other lineages appeared reduced in DSS-colitic mice. Minocycline treatment significantly reduced the presence of neutrophils in blood, an effect that generally re-established the percentages of the different subsets to baseline (Fig. 35). This effect might follow the general improvement of the inflammatory response, which could probably be associated a the reduced translocation of bacterial antigens and the reduced release of systemic inflammatory mediations, such as IL-6 and TNFα, previously observed in the MNC treated group (Fig 35). However, myeloid monocytic cells were still elevated in minocycline treated mice as they were in untreated colitic mice, and an increase in eosinophils was also observed in this group. This finding leads to conclude that, despite treated mice showed reduced neutrophilia, pathways that specifically increase monocytes and eosinophils might still be activated, supporting the increased recruitment of these populations observed at the inflamed tissue.

The differences in expression observed at these two different time points highlight the different kinetics followed by different inflammatory mediators, which may be of great relevance for an adequate control of the inflammatory process.

DISCUSSION

Tetracyclines have been on the brink of disappearance from the therapeutic arsenal due to the antibiotics abuse made in the past decades. The chemical modifications introduced in these compounds not only rescued their utility as antibiotics, but also improved their pharmacological profile. The discovery of these novel and interesting properties has propelled that, nowadays, minocycline and doxycycline are being tested in more than 200 clinical trials each all over the world. Their therapeutic potential goes far beyond their antibiotic properties and the good pharmacokinetic profile makes them valuable drugs. Extensive pre-clinical research has unveiled the potential of minocycline on numerous complex diseases such as skin, bone and joint disorders, ischemia and tissue damage, neuro-immune diseases and cancer[589]. Of note, the combination of their antibiotic activity and many non-antibiotic properties results of great interest for the treatment of complex diseases where inflammation and tissue damage is associated with microbial misbalance. This particular crossroad is found in inflammatory bowel disease.

The complexity of IBD, both in the aetiology and clinical features, hinder the therapeutic success. Main strategies are aimed, mainly by supressing the immune system and the production of pro-proinflammatory mediators, rather than restoring the reported altered bacterial composition. This opened a therapeutic gap for compounds like tetracyclines. Minocycline has already proved its benefit on experimental models of IBD[629], [630], [738]. Two different studies showed that minocycline reduced intestinal inflammation in the models of DSS and TNBS induced colitis and another report showed improved intestinal function after 5-FU induced intestinal mucositis. The beneficial effects have been linked with many of the properties that characterize minocycline, such as antibiotic activity, antioxidant and immunomodulatory properties, and the inhibition of enzymes involved in the process. However, the administration of antibiotics, including

minocycline, during a prolonged period of time is not recommended due to adverse events that this approach can induce in the IBD patient. Furthermore, a combination of minocycline treatment with a probiotic has been proposed to fill that therapeutic gap on the management of IBD[628]. This study showed that an initial administration with minocycline induced remission on acute intestinal inflammation and proposed that the antibiotic activity would favour the establishment of the probiotic, used as a chronic maintenance therapy. In view of the positive results obtained with minocycline, we proposed to increase the knowledge on the therapeutic potential of this family of tetracyclines on intestinal inflammation and elucidate their mechanism of action.

Doxycycline: Does it exert anti-inflammatory activity on experimental colitis?

Doxycycline is a second generation tetracycline with improved serum half-life[527], [545], [546], [548], [551] and stronger ability to inhibit MMPs than other tetracyclines[652]. It is the most widely used tetracycline and one of the OMS chosen essential drugs, being therefore well known and safe. Despite doxycycline has not been studied as deeply as minocycline, it retains most of its non-antibiotic actions[568] and therefore the potential to ameliorate intestinal inflammation[673], [739]. Therefore, we initiated this general aim with the evaluation of the therapeutic potential of doxycycline in experimental models of colitis.

The results of the first aim of this thesis have evidenced the immunomodulatory properties of doxycycline, and proved that its administration to colitic animals results in an amelioration of the inflammatory process, being its efficacy comparable to the one previously showed by minocycline[629]. The immunomodulatory properties previously ascribed to doxycycline[485] have been confirmed in vitro, and it has been evidenced that doxycycline has an equivalent potential to that of minocycline in reducing the production of NO and the chemokine IL-8. NO production by macrophages, one of the main sources of NO in inflammatory conditions, and IL-8 secretion by intestinal epithelial cells are both implicated in the migration of neutrophils to the inflammatory focus[733]. The capability of doxycycline to inhibit the production of these mediators would prevent the deleterious effects of their overproduction in the colonic tissue, therefore contributing to the amelioration of the inflammatory process evidenced in the *in vivo* studies that confirm previous reports[740].

Dose-response in TNBS and DSS colitis

As shown in the TNBS model of rat colitis, both minocycline and doxycycline reduced the tissue damage caused by the unspecific oxidative response of leucocytes, as reflected in the decreased MPO activity and the higher levels of the antioxidant glutathione found in the colonic specimens from rats treated with the antibiotics Moreover, the increased production of IL-1β in the inflamed intestine, one of the main inflammatory cytokines involved in this model of colitis, was also counteracted by the antibiotics. These observations were corroborated by histological analysis, which showed a reduced leukocyte infiltration, together with milder tissue damage in tetracycline-treated colitic rats.

The anti-inflammatory effect displayed by a given compound in a particular experimental model could be conditioned by differences between models and the

type of damage involved. However, in our study doxycycline activity was manifested in two different models of colitis and in two different animals, confirming its intestinal anti-inflammatory effects. Of note, its capacity to influence not only the expression of pro-inflammatory cytokines, but also makers of mucosal barrier function and enzymes involved in the inflammatory response and tissue remodeling, highlights the ability of this compound to target multiple factors involved in the intestinal inflammatory response.

Combination of doxycycline and *S. boulardii*

It is evident that the impact of the immunomodulatory properties of these tetracyclines in ameliorating the intestinal inflammatory conditions can be reinforced by its antibiotic activity[629]. The crucial role that the microbiota plays in the development of IBD[741] has justified the therapeutic use of antibiotics through many years, in order to reduce the bacterial load of the intestinal mucosa. However, the long term administration of antibiotics that would be required in chronic conditions like IBD is restricted due to the appearance of drug side effects[183].Therefore, to assist in the management of this complex disease, it would be of need the development of novel therapeutic strategies that combine an immunomodulatory activity and the ability to restore the luminal microbial balance in the intestine, and that are also safe in the long-term. This could be the case of a therapy based on the combination of immunomodulatory antibiotics and probiotics[628]. Probiotics have also been shown to modulate the immune response and decrease mucosal permeability, positively affecting the intestinal barrier function[502], [504]. Following this hypothesis and the design of previous studies, we propose the therapeutic association of doxycycline, to induce remission, with *Saccharomyces bourlardii,* to prevent the relapses.

The mouse model of DSS reactivated colitis mimics the relapsing nature of human IBD. In this model, doxycycline was only administered for a short period,

to target acute inflammation and induce remission. Meanwhile, S. *boulardii* was given during all the experimental protocol, as a basal therapy to improve colonic homeostasis and prevent the reactivation of the colitis (induced by the second cycle of DSS). Both the combination of doxycycline and *S. boulardii* and either treatment separately were able to promote the recovery of the mice at the different time points evaluated, although the effect of the association was stronger and more prolonged. This strategy exerted its effect by simultaneously modifying different key players in the pathogenesis of IBD[45], including chemoattractants (ICAM-1) and cytokines (IL-12, TNFα, IL-1β and IL-17), markers of the barrier function (MUC-1, MUC-3) and inflammatory and proteolytic enzymes (MMP-2, MMP-9, iNOS). However, the mechanisms behind these multiple effects are not completely understood. Different pathways are involved in the inflammatory response of the intestine. Th1 lymphocytes have been classically associated with IBD[131], while Th17 cells have lately received an increasing interest[742]. The role of each pathway should not be taken individually, since their associated cytokines can synergize with each other, thus generating the vicious cycle that maintains the chronic inflammatory response in the intestine. In this regard, a treatment able to target a greater number of these factors would more efficiently facilitate the recovery of the inflamed colonic tissue, as shown when colitic mice received the association of doxycycline and *S. boulardii*. This reasoning could also be applied to the defensive mechanisms of the intestinal epithelial barrier. The combined treatment increased the expression of MUC-1 and MUC-3, constituents of the mucus layer in the colon, and therefore prevented the access of antigens from the intestinal lumen, one of the main factors that promote the exacerbated immune response[743].

All these actions contribute to the progression of the colonic inflammatory process. As shown in the histological analysis, there is a higher mucosal damage and inflammatory infiltrate, which may be associated with an increased bacterial

translocation. The ability of the treatments to reduce the alteration of the intestinal homeostasis is manifested in the evolution of the DAI. This index reveals in a simple visual way how the antibiotic and the probiotic display different actions to achieve a synergic beneficial effect. Doxycycline treatment showed higher effectiveness in recovering the animal status after colitis induction. Since the antibiotic administration was stopped after five days, it was less effective than the *S. boulardii* in maintaining the recovery or preventing the relapse of colitis, while the probiotic alone showed a fewer acute effect. The combination of both treatments, however, resulted in a mayor beneficial effect, improving the animal status in all the phases of the process, ameliorating tissue damage and modulating the expression of most of the inflammatory markers evaluated. This combination better controls the relapsing inflammatory process although we have not been able to show the mechanism behind it since we did not observed any significant additive effect when they were assayed in vitro. Further studies on this field are required to determine if the immunomodulatory pathways of the antibiotic and the probiotic converge.

This knowledge will definitely improve to join combinatory strategies like the one proposed here.

Immunomodulatory tetracyclines: How do their different properties contribute to control intestinal inflammation?

Doxycycline and minocycline have proven their potential to control intestinal inflammation. No significant differences have been observed in their effects, which support the idea that they share similar mechanisms of action. The combination of their biological properties can? contribute to their beneficial effect[611], being this the reason of their high effectiveness. However, the degree of contribution of each activity is not completely clear, since it is difficult to differentiate cause and consequence, and an improvement of a given maker may result from the amelioration of the inflammatory process, rather than a direct mechanism. Also, the effects of the antibiotic could be both positive and detrimental in these conditions. A comparative study of minocycline and tetracycline was previously performed in the TNBS model of colitis[629], and it was shown that although tetracycline exerted some therapeutic effect, this was achieved to a lesser extent than with minocycline. However, no information is available regarding other models of intestinal inflammation and the presence of some beneficial non-antibiotic properties on the tetracycline compound may also contribute to this protection. Therefore, in order to further investigate the mechanisms of action of this family of compounds, we tested different tetracyclines in two different experimental models of colitis and compared their effects with those showed by compounds without with segregated antibiotic and immunomodulatory activities.

With this aim, the most studied tetracyclines, doxycycline and minocycline, were assayed together with tigecycline and tetracycline. Tigecycline is a third generation minocycline derivate that, although it has never been assayed on intestinal inflammation, it retains many of the non-antibiotic properties of minocycline, and thus we hypothesised that similar intestinal anti-inflammatory effect could be expected (cita: Dunston 2011). Tetracycline was considered as the reference compound of their antibiotic activity despite, as pointed above, additional beneficial properties should not be excluded. To overcome this limitation, we also included an antibiotic of a different family: Rifaximin is a non-systemic rifamycin-derived antibiotic that exhibits low gastrointestinal absorption while retaining potent antibacterial activity[744], thus providing a local antibiotic action. Additionally, rifaximin has been reported to act as a specific agonist for the human pregnane X receptor (PXR), but not in rodents[745]. This is important to consider since this mechanism confers specific protection from intestinal inflammation, and it has been widely studied on IBS and IBD in pre-clinical and clinical trials, providing positive results[746]–[751]. However, since rifaximin does not activate PXR in mice, the effects observed in the experimental models of mouse colitis could be theoretically ascribed only to its antibiotic properties. In addition, dexamethasone, one of the most potent and widely used corticoids, was selected as an immunomodulatory drug without antibiotic activity.

In vitro evaluation of these compounds on macrophages showed their ability to inhibit iNOS activity and NO production, thus confirming the spectrum of immunomodulatory activity previously proposed. According to this activity, we presented the six drugs in a graded spectrum from their antibiotic action to the immunomodulatory activity, in increasing order of potency: RFX < TTC < DXC < MNC < TGC < DEX. However, it is interesting to note that some previous studies have highlighted that the immunomodulatory activity of tetracyclines may

involve the release of pro-inflammatory mediators in specific immune cell populations, such as monocytes and alveolar macrophages[701], [713], although they typically displayed an anti-inflammatory effect. We confirmed this dual activity in these assays performed with BMDM, since they reduced iNOS expression, a classical marker of LPS-induced M1 activation, while increasing TNFα, IL-1β and IL-6, even at basal conditions. This controversial effect, compared to the classical immunomodulatory one exerted by dexamethasone, suggests that different and specific mechanisms may govern the action of tetracyclines on macrophages[677]. Considering the important regulatory role of macrophages in the immune response and inflammation, it is surprising how this activity has not attracted more attention. This might be of special relevance in intestinal inflammation[294]. In fact, it could weaken the potential benefit of tetracyclines or even potentiate the established inflammatory response. However, other possibility is that this activity may not be present in intestinal macrophages, since an evident intestinal antiinflammatory effect was observed so far, and this dual activity has not been observed in other specific macrophage populations, such as peritoneal macrophages and microglia[713], [714]. Further discussion on this concern will be addressed later.

The *in vivo* comparative studies were performed in two different models, in mice. The DNBS model of colitis represents many features of intestinal inflammation observed in human CD, with transmural intestinal inflammation that leads to fibrotic lesions, strictures and obstruction[34], [35], [145]. DSS-induced colitis resembles many features of human UC, typically associated to a disruption of the epithelial barrier that facilitates the mucosal injury through the exposure of the lamina propria to luminal contents. [34], [151], [152].

The damage induced by intracolonic DNBS administration is quite similar to that usually observed in the TNBS model in rats, which has been used in the aim 1 of this thesis as well as in a previous study describing the intestinal

antiinflammatory effects of minocycline[629], [630]. The ethanol eases/facilitates the access of DNBS-haptenized luminal? proteins to the mucosa, where they activate the immune system. Additionally, DNBS initiates the generation of free radicals and other reactive species, thus promoting a situation of local oxidative stress in the intestine, which in combination with neutrophil induced damage, are important features of this model[145], [147]. Therefore, the well-known/previously reported antioxidant properties of tetracyclines[597]–[599] could be especially beneficial against the course of the inflammatory process in this experimental model of colitis. In addition, this model is characterized by intense tissue remodelling and fibrosis, as illustrated by the increased colonic weight/length ratio as well as the upregulated MMP expression in comparison with non colitic mice. The well-known ability of tetracyclines to inhibit MMP expression and activity[652] can probably contribute to its intestinal anti-inflammatory activity in this model, thus limiting the progression of tissue damage.

The most relevant data obtained in this study was the ability of tetracyclines to reduce the elevated mortality rate to less by half. This also implies that, due to the variability of the disease, subsequent evaluation in control group relies on less affected members while tetracyclines treated groups also include severely affected individuals, rescued from dying. Colonic evaluation at day 6 showed a marked colonic shortening and tissue damage. Tetracyclines achieved an impressive reduction of these parameters on such a severe colitis, while rifaximin and dexamethasone did not. Therefore, due to the low number of remaining individuals in these groups, subsequent evaluation did not always provide reliable results. The recovery of mucosal architecture and the presence of mucus-filled goblet cells in mice treated with tetracyclines can be considered of great relevance when considering the protective role of the mucus layer to keep off/away bacterial triggers from the mucosa. All antibiotics improved the

evolution of animal weight, but the effects observed with tetracycline or rifaximin were delayed in time, ~~with~~ the latter displaying minor activity. This might indicate that the antibiotic action itself could indirectly protect from disease progression. The advantage of tetracycline over rifaximin, in addition to their non-antibiotic properties, could also relay on its absorption, providing an antibiotic protection against disseminated infection in the host.

The beneficial effects exerted by tetracyclines on intestinal barrier function were confirmed after evaluation of gene expression of tight junction proteins (ZO-1)? and TFF-3, which are important factors/elements affected in IBD[252], [752]. However, and despite increased mucin staining was observed in the histological sections from colitic animals treated with the different tetracyclines, the expression of the mucin genes (MUC-1, MUC-2 and MUC-3) was not significantly modified in these experimental groups. The effects observed in the histochemical evaluation might be underappreciated on whole colonic gene expression since histological sample was taken localized on the distal site, where DNBS damage was mainly located.

The protection exerted by tetracyclines also affected pathways involved in the expression of inflammatory cytokines, chemokines involved in the recruitment of neutrophils (CXCL2) and tissue remodelling proteins (MMP-9), which contribute to reduce tissue damage associated to inflammation. Surprisingly, tetracyclines increased the expression of the monocyte chemoattractant protein CCL2, which was reduced in this model. This could indicate that newly arrived macrophages may not play a predominant role in the colonic inflammatory process induced by DNBS. Therefore, an increased presence of macrophage activity in the colonic tissue from tetracycline-treated animals could contribute to the immunomodulatory activity exerted by these antibiotics. Of note, most studies with this model found opposed results to those reported here, since increased CCL2 expression has been reported to occur in the

affected tissue[753]. One possible explanation for these discrepancies is that in all these studies the evaluation of the colonic inflammatory status was performed 72 hours after DNBS instillation, when the inflammatory reaction peaks. However, the established inflammatory process cannot control the severe damage induced, and the affected tissue quickly progresses to develop fibrosis, obstruction and necrosis. Therefore, when the colonic tissue was evaluated 6 days after initiation of colitis, this may not accurately represent the inflammatory reaction but rather its detrimental impact. By contrast, tetracyclines managed to block/ameliorate the progression of the inflammatory reaction into loss of function, which may be the reason that justifies their ability to increase the colonic expression of CCL2 in the inflamed tissue. Additionally, several studies have reported that CCL2 elicits polarising effects of/on? T cell response, and when there are increased levels, it may promote a Th2-mediated response in detriment of the Th1 subset[754]–[756]. Curiously, various studies have also shown that alternatively activated macrophages are necessary to control inflammation in this model of colitis without inducing fibrosis[757], [758]. This type of immune response, and the communication with B cells, has been observed to mediate the therapeutic effects of helminths in experimental colitis[759], an action that has also been observed in the absence of T cells[760]. Considering all the above, the observed increase of CCL2 expression in colitic mice treated with tetracyclines cannot be considered as a secondary detrimental effect, but indeed a possible mechanism of their protective action in this model.

Numerous studies have reported the key role that PRR display in intestinal homeostasis, by preserving its barrier function and promoting its repair when disrupted[267], [761]. In this task, the interplay of TLRs between the microbiota and the host can be crucial to accurately interprete the signals mediated by commensal bacteria. For this reason, we included their evaluation in our study.

Despite in DNBS-control colitic mice none of the TLRs evaluated was significantly modified in comparison with non colitic mice, colitic mice treated with tetracyclines showed elevated levels of some of them, such as TLR2, TLR9 and TLR6, although the latter was only upregulated with the immunomodulatory tetracyclines. TLR9 has shown to be important for protection against experimental colitis[762, p. 9]. In fact, TLR9$^{-/-}$ mice has delayed wound repair in experimental colitis?, and therefore, increased TLR9 expression in colitic mice treated with tetracyclines can potentiate the protective effect of these microbial derived signals. TLR2/TLR6 pair is involved in recognition of diacylated lipopeptides[763], [764]. The signals mediated by TLR6 are essential for the immune-regulatory properties of lactic acid bacteria, widely known for their health benefits[765]. Additionally, genetic variants in TL2 and TLR6 have been associated with IBD, and deficient innate immune response to bacteria caused by these variants results in more extensive disease localization in UC and in colonic disease in CD[766]. Expression of TLR6 has been found on the cell surface in monocytes, monocyte-derived iDCs, and neutrophils, but not on B, T, or NK cells[767], although another study showned higher expression of TLR6 in B1 cells compared with conventional B cells and DCs[768]. Identification of the effects mediated by this receptor has been controversial: some studies have observed an activation of Th17 responses, which has been identified as both protective[769]–[771] and detrimental[772], while others reported suppressive action on the same pathway[773]. Therefore, the context of TLR6 mediated effects probably determines the results its activation.

MiRNAs are important epigenetic regulators involved in many pathological conditions, such as IBD[736], [774], [775]. Altered miRNA signature observed in IBD patients mostly coincide with changes observed in the DNBS model of experimental colitis in the present study, with an increase in miR-9, miR-155 and miR-223 while miR-143 and miR-375 were significantly reduced[736], [776]–[779].

Activation of the NFkB pathway regulate the expression of miR-9[780] and miR-155 [781]–[783]. The latter has been already observed to be increased in other experimental models of colitis, like the TNBS-model of colitis in mice, being correlated its expression with TNFα release by activated $CD4^+$ T cells[784]. The immunomodulatory properties exerted by tetracyclines and dexamethasone were associated to a reduced expression of miR-9; however, miR-155 was not significantly modified, being TNFα expression unaltered??. Interestingly, miR-223 expression was the most up-regulated among the different miRNAs assayed in the DNBS model. This is specifically expressed in the granulocytic lineage and increase as granulocytes mature[785], [786]. The increase of its expression may represent the crucial role that neutrophils play in DNBS-induced inflammation. Of note, tetracyclines reduced its expression, as well as that of the neutrophil chemoattractant CXCL2. In a previous study, it was reported that miR-142-3p and miR-223 were the most up-regulated miRNAs in the mucosa of UC patients[787]. By contrast, miR-142-3p was downregulated in this model. A plausible explanation for this discrepancy may be that DNBS inflammation resembles human CD, and therefore, miR-142 expression could be useful to differentiate distinct features of intestinal inflammation. In this regard, the up-regulation achieved by tetracyclines in miR-142 may indicate specific actions on this type of inflammation. Strikingly, miR-223 mediates the up-regulation of miR-142[788], while miR-155 can reduce its expression[789]. Neither changes observed in this model, nor the action of tetracyclines indicate that miR-142 variations could follow the effect of these other microRNAs.

In DNBS colitis, the expressions of miRNA-143 and miR-375 were downregulated. MiR-143 has shown to reduce epithelial-mesenchymal transition through targeting ERK5, which has been reported to be involved in tumor growth[790]. In the present study, the reduced expression of this miRNA correlated with the increased epithelial proliferation that is? required to restore

the epithelial wall. However, none of the treatments significantly modified its expression in colitic mice. Finally, it has been reported that miR-375 inhibits KLF5, an antagonist of KLF4, which is a goblet cell differentiation factor. IL-13, a Th2 cytokine, stimulate miR-375 expression and a down-stream production of TSLP, an epithelial cytokine involved in the Th2 pathway/type 2 immune response/type 2 inflammatory pathway[734], [791]. This highlights the relevance of this miRNA in the protection of mucosal homeostasis and supports the effect observed in this model, prone of a Th1-mediated inflammation. The up-regulation achieved by tetracyclines correlates with the increased presence of goblet cells, and reinforce the notion previously exposed for CCL2, thus inducing mucosal protection by favouring a Th2 response and improvement of barrier function.

The evaluation of the changes in the microbial community associated with this model of colitis showed major abundance variations, specially a lower taxonomic levels, as it has also been observed in other models of acute colitis[792]–[794]. Unfortunately, almost no information regarding microbial characterization in DNBS or the similar TNBS model of colitis can be obtained in the scientific literature up to date. Additionally, the few studies available show different results, such as decreased diversity within Firmicutes phylum in TNBS mice colitis[457], [460], while a recent study found no differences, similarly to our results[795]. Therefore, we have tried to extract conclusions based on dysbiotic changes observed and the therapeutic effect of the associated treatments.

Surprisingly, although no statistical differences were observed, microbial diversity increased in this model of colitis, which was associated with the increase in the Bacteroidetes abundance and diversity. Although IBD has generally been associated with reduced diversity, other extreme conditions, such as fasting, has been reported shown to increase colonic microbial diversity in various animal species, including mice[796]. Of note, the severe impact of this

model on mice wellbeing extremely reduced food intake, and induced colonic obstruction. This effect could create the condition for the bacterial overgrowth and increased diversity observed in the present study.

Additionally, the impact of antibiotics on microbial composition follows a similar pattern than DNBS, but further accentuated. Considering the beneficial therapeutic effect that antibiotics achieved in DNBS colitis, we can propose two possible hypotheses to explain it?. The first, and most likely, the antibiotic administration to colitic mice promoted a reduction in the bacterial load, therefore reducing exacerbated antigenic stimulation[466], [476], [477]. Our second hypothesis explores the possibility that microbial alterations represent the adaptation of the microbial ecosystem to the changing environment, a dynamism that may not be detrimental. Therefore, the administration of antibiotics in this model can promote these changes, thus providing an increased adaptability of the microbiota to the colonic insult induced by colitis. A clear example is the reduction in *Bifidobacterium pseulongum*, from a 50% abudance to 20% in DNBS and DEX groups and less than 10% in all groups treated with antibiotics. A recent study suggests that probiotics of the genus *Bifidobacterium* should be used more cautiously since increased *Bifidobacterium* has been associated with active IBD[797].

Other interesting finding was the increase in Blautia, which belongs to the family *Lachnospiraceae*, specifically in the groups treated with immunomodulatory tetracyclines and in the DNBS control. A beneficial anti-inflammatory association of *Blautia* has been observed in colorectal cancer and inflammatory pouchitis following ileal pouch-anal anastomosis[798], which can be linked to the metabolitc ability to degrade non-digestible fiber and produce SCFA[799]. Increased information regarding the collective function of the microbiome would be of great value to determinate whether variations in the

composition associate with protective functionality or these are just a secondary consequence.

The DSS model allows to better control the grade of intestinal inflammation induced, which provides a useful tool considering that success of a therapeutic agent might variate with the severity of the process. Having observed the protection of tetracyclines from the elevated mortality induced by DNBS colitis, we decided to also evaluate these compounds on a fatal colitis, which has not been specifically assessed before. The three immunomodulatory tetracyclines ameliorated the evolution of the intestinal inflammation and clearly improved the survival rate. Rifaximin lacked any therapeutic effect. Conversely, tetracycline progressively improved the evolution of colitic mice and reduced mortality down to 50%. This narrow effect might indicate that, despite local antibiotic action do not induce protection in this model, systemic effect of tetracycline, either due to its antibiotic or other properties, contribute to ameliorate this acute inflammatory process. Interestingly, the activity exerted by dexamethasone, with an initial reduction of DAI, could not rescue mice from disease progression and death. Despite corticoids are widely used in IBD to treat the symptomatology of the inflammation, it has been evidenced that they inhibit wound repair[800]. This action is mediated by their anti-inflammatory effects, inhibition of HIF-1, a key transcriptional factor in healing wounds[801] and suppression of cellular wound responses, including fibroblast proliferation and collagen synthesis[802]. Additionally, systemic corticosteroids may also increase the risk of wound infection[803]. Therefore, interfering with inflammation, they also impair the protection conferred by this response. In consequence, their use in acute flares of intestinal inflammation should be reviewed, because while targeting symptoms we may be delaying the course of the disease by interfering with naturally protective mechanisms.

We then evaluated the effects of the treatments on a second experiment with increased survival. The influence of immunomodulatory tetracyclines is manifest from the first day and previous studies with minocycline and doxycycline has already evaluated their effect at advanced time points, which might represent the overall benefit obtained with the treatment. Therefore, in order to investigate/elucidate their mechanism of action, we decided to evaluate the effect of these compounds on DSS colitis after 4 days of treatment. Similar evolution was observed than in the previous experiment and final DAI correlated with the microscopic evaluation, which supports the great benefit that immunomodulatory tetracyclines accomplish in such a short time.

The improvement of the epithelial barrier function achieved by immunomodulatory tetracyclines is supported by restored expression of mucins, tight junction proteins and TFF-3. The immunomodulatory action of dexamethasone did not improved mucosal healing, which is supported by the findings of other studies, exposed above[800]. Tetracycline improved some of these markers, but due to the lack of protection of a local antibiotic effect of rifaximin on this model, tetracycline treated mice likely profit/benefit from the other non-antibiotic actions. Another plausible explanation is that the systemic antibiotic action achieved by tetracycline limits bacterial translocation and infection, reducing exacerbated intestinal inflammation and mucosal damage.

The study of inflammatory markers correlated with previous observations, and only immunomodulatory tetracyclines achieved to ameliorate most of the mediators evaluated. It confirmed that rifaximin exacerbated intestinal inflammation. Compared with previous results on DNBS colitis, therapeutic success of local antibiotic action may strongly variate with the characteristics of the inflammatory process. Of note, rifaximin has not shown clinical benefit in patients with moderate to severe active UC, although it resulted in a lower incidence of recurrence in CD[804]. On the one hand, this could be the effect

observed in our study, since DSS colitis closer UC while DNBS model resembles CD. On the other hand, preventive use of antibiotics will likely benefit any type of intestinal inflammation by reducing initial bacterial load, while microbial changes induce once the inflammation is stablished may not always have a positive impact.

CCL2 represented an interesting example of the differences between these two models of colitis: CCL2 was reduced in DNBS colitis but up-regulated in DSS. Surprisingly, immunomodulatory tetracyclines increased the expression of this gene in both models, a controversial result of enough consistency for being ignored. It indicates that this is not a consequence of ameliorated intestinal inflammation, but rather a direct immunomodulatory mechanism. This chemokine is involved in monocyte and dendritic cell recruitment to inflammatory sites. Dendritic cells are important regulators between innate and adaptive immunity, and macrophages key players on the regulation of the inflammatory response, being one of the main immune populations involved in DSS induced colitis (Bain). Additionally, we confirmed before that tetracyclines display dual pro-/anti-inflammatory actions on this cell type in vitro. Therefore, this result suggests that a potentiation of macrophages inflammatory response could be taking place in this context. At first sight, this hypothesis confront the anti-inflammatory activity observed with tetracyclines on this and many other inflammatory disorders. But some arguments already support that this effect might contribute to their protective effect on the mucosa, considering the role of macrophages in mucosa healing and resolution and the Th2-promoting activity of CCL2, exposed above[754], [756]–[758].

This was not the only contradictory result. When we evaluated the expression of microRNAs on DSS colitis, the same patter of CCL2 was observed on miR-142-3p, both in DSS and DNBS colitis: Immunomodulatory tetracyclines raised the expression level of miR-142, indistinctly of the regulation of this gene

in each different model. Additionally, the fold change observed on this microRNA is unusual for microRNA expression, where variations are sutile despite being biologically relevant considering their wide regulatory properties. In view of this result, the preference expression of this microRNA on immune cells and the correlation with CCL2 regulation, the effect mediated by tetracyclines may relay on the potentiation of an unidentified immune parthway, opposed to the immune suppressive effect of classical anti-inflammatories drugs, such as dexamethasone. Loss of miR-142 leads serious immunodeficiency with very low immune response toward soluble antigens and viruses[805], [806]. It also attenuate the immune response: miR-142 was first identified for inhibiting IL-6 production by DCs in response to LPS[807], [807]; miR-142 targets IRAK-1, involved in the inflammatory response to LPS-TLR4[808]; PPARγ directly induce the expression of miR-142, which target HMGB1, supporting the mechanism of the anti-inflammatory effects of PPARγ [809]; IL-4 and IL-13 induce miR-142 expression, which targets SOCS1 and consequently prolongs STAT6 phosporilation, involved in the response to these cytokines[810]; Finally, the effect of this miRNA in CD4+ DCs also favour the generation of type-2 immunity. MiR-142 is highly expressed in this population under the control of PU.1, Runx1, and IRF4[811], [812] and it has been observed that loss of miR-142 induced a phenotype on DCs similar to that observed in mice deficient for IRF4 [813], [814] with loss of CD4+ DCs and elevated Irf8 which directs the expression of IL-12[815] and IL- 18[816], thereby promoting Th1 responses in contrast to the Th2 bias induced by Irf4. In conclusion, although the source of elevated miR-142 still needs to be determined, available information support that up-regulation of miR-142 may account for a protective mechanism to improve control of the inflammatory response associated to intestinal mucosa.

Regarding other microRNAs, DSS colitis was also associated with elevated expression of miR150, related to effector T helper[735], [817] and humoral

response[817], miR-155, involved in NFkB pathway[781]–[783], and miR-223, associated wih the granulocytic lineage[785], [786]. Their expression was downregulated by dexamethasone as well as immunomodulatory tetracyclines, which might represent a direct effect of the first while it could also be a consequence secondary to ameliorated intestinal inflammation in the groups DXC, MNC, and TGC . Strikingly, up-regulation of miR-375 by tetracyclines on this experiment as well support an active protection provided by goblet cells[734], [791], which correlates with mucin expression and histological observations.

Reduced expression of TLR2 and TLR4 was observed in DSS colitis, with the antibiotic effect associated with increased TLR2 and restored expression of TLR4 in mice treated with immunomodulatory tetracyclines. The need of TLRs for sensing the microbial environment and repair of intestinal barrier function has already been exposed[267], [761]. Both TLR2$^{-/-}$ mice and TLR4$^{-/-}$ mice develop more severe colitis in response to DSS[761], [818], and therefore increased expression of these PRRs might account for increased protection and response to threatening environment. While restored levels of TLR4 achieved with immunomodulatory tetracyclines might represent the improvement of the epithelial barrier, the fact that all antibiotics consistently increased TLR2 expression above levels of healthy mice suggest that microbial changes induced may direct specific up-regulation of TLR2 on determinate cells. Such association with specific group of microorganism is difficult to stablish, taking into account the vast complexity of microbiota, and no information regarding this possible mechanism has been found.

Modifications in microbial compositions have been widely study on DSS colitis[792]–[794]. On this model, mucosal damage precedes the modification of microbiota, which alters a consequence of imbalanced homeostasis. This has been observed in a longitudinal study on DSS colitis, in which initial modifications

were initially observed at lower taxonomic levels, with inner group dynamics, while dysbiosis at higher taxonomic levels became more pronounced and evident at later time points[794]. One specific example is a transient increase in *Bacteroides acidifaciens* as inflammation progress. We found higher abundance in all colitic groups in the DNBS model but not in DSS colitis. However, an increase of this single bacterium was observed in all mice treated with antibiotics. This may be responsible for the increase observed in TLR2. Additionally, it could also explain a negative impact of the antibiotic action by predisposing to the dysbiosis that characterises later stages of the inflammatory process.

The overgrowth of Bacteroidaceae was the major contribution to restore the composition of the Phyla Bacteroidetes, while in healthy mice it was dominates by other families, such as Prevotellaceae and Porphyromonaceae. Although antibiotic treatment increased the abundance of these groups as well, it highlights the relevance of perform an evaluation at deeper taxonomic levels. The effect observed here with antibiotics, which partially restored microbiota at phylum level, can readily guide to the wrong conclusion that antibiotic action correct the dysbiosis generated in intestinal inflammation. However, no benefit was observed with rifaximin, whose impact on microbiota outline the general effect of other antibiotics. This, together with inner group changes, as represented on the PCoA analysis at genera level, indicate that antibiotic may not benefit this model of intestinal inflammation. Even more, it could be deleterious/detrimental, as observed with rifaximin, that lack any other relevant activity as far as we know.

Of note, some differences between antibiotics were also observed. Reduced actinobacteria in mice treated with tetracyclines and, more interesting, decreased abundance of Lactobacillaceae in the groups receiving immunomodulatory tetracyclines, while it drastically increased in all other colitic groups. As previously exposed with Bifidobacterium, Lactobacillus has also been found

increased in IBD[797]. Then, low abundance of Lactobacillus could be regarded as a homeostatic indicative, since it was only observed in healthy mice and those receiving an effective treatment.

These studies provided some insights into the possible contribution of the antibiotic effect. Due to the complexity of the microbiome and the contribution of different bacteria to similar pathways, metagenomics studies would provide direct and valuable information about the collective functionality of alterations induced by inflammation and the different treatments. Despite that, it would still be difficult to answer the question of whether these functions could provide a real benefit. Therefore, in order to directly assess that issue, we performed a transference of faecal microbiota into recipient mice, depleted of their original microbiota by a long term administration of antibiotics, a model widely used in this type of studies [819], [820]. Since our biggest concerned about the antibiotic effect was on DSS colitis, this was the model used for the evaluation. We hypothesize, from the negative effect of rifaximin, that the antibiotic impact could be detrimental. However, mice transferred with stools from colitic mice treated with minocycline were less susceptible to intestinal inflammation than those receiving stools from colitic controls, a similar effect to transfer healthy microbiota. This protection was observed on the evolution of colitis and histological study, but none of the inflammatory markers were modified and minor changes were observed in markers of barrier functions. It may highlight that minor differences are not reflected in the peak of acute inflammation. Additionally, the complexity of the experimental setting itself introduces many confounding factors. Using antibiotics to deplete the microbiota have a strong impact on intestinal homeostasis, such as alterations in the pattern of TLR expression, intestinal motility and even inducing a mild inflammation[821]. It has been observed that, for a successful treatment with faecal transference, the previous administration of a short antibiotics does provide any benefit[822], but

the aim of this procedure is to improve an existing microbiota by increasing the diversity and proportion of beneficial bacteria, not the evaluation of an existing community. The use of germ free do not provide a more natural recipient: their defensive barriers are underdeveloped, being raised in a completely different environment, and show increased susceptibility to disease[823]–[825]. Finally, limitless conditionings influence the settle of transferred microbiota, such as the route of inoculation and the adaptation of the ecosystem to this new environment. However, differences in bacterial load, which could be another factor behind differential susceptibilities, do not seem likely here, since one week is enough time for the microbiota to growth and stabilize. Therefore, despite many limitations of this method, we cannot find an experimental design that will improve this type of study, which is an area of great interest though.

Having said that, although the basis of the protection observed remains obscure, at least certain degree of protection could be transferred by this procedure. This indicates that the impact of a short treatment with tetracyclines might benefit dysbiotic changes by reducing the predisposition to future colitis. The use of antibiotics has been generally associated with increased risk of opportunistic infections by disruption of the ecological barrier. However, this adverse effect was greatest with clindamycin, fluoroquinolones and cephalosporins, whereas tetracyclines have not been associated with an increased risk[826]. This support that the protection provided in various types of intestinal inflammation could be synergistically combined with therapeutic strategies as we proposed in aim 1: tetracyclines induce an accelerated mucosal healing and inflammatory resolution that can be maintained in remission with a probiotic that boost intestinal protective barriers. This therapeutic strategy exploits natural defensive mechanisms to effectively control intestinal inflammation. Therefore, it is a promising strategy to use in combination with current treatments, when

needed, or in substitution of those of expensive cost, numerous adverse reactions and a negative impact on defensive barriers of the intestine.

The mechanism of action: Do tetracyclines potentiate the immune response mediated by macrophages to control intestinal inflammation?

On the previous aims of this thesis we showed that tetracyclines, by means of their many properties, can reduce intestinal inflammation. But whether the immunomodulatory activity has a major contribution was still open to discussion when considering the potentiation of some inflammatory mediators observed in vivo and on macrophages. Therefore, an immune boost mediated by this population could be expected, which may either dampen or contribute to the benefit achieved by their other protective properties. In order to assess/answer this query, on the aim 3 of this thesis we specifically focused on the study of immune populations involved in DSS induced colitis. Minocycline is the most studied tetracycline and thus we selected it to conduct this study. The evolution of the immune populations on the intestine and blood were studies after 2 and 4 days of treatment after DSS removal.

DSS intake progressively damage the intestinal epithelium, losing the protective barrier that keep away luminal microbes from the immune compartment at the lamina propria. The release of danger signals upon tissue damage and the access of bacterial antigens trigger the beginning of the inflammatory reaction, aimed at restoring intestinal homeostasis. This was

characterized by an increased recruitment of neutrophils, monocytes and dendritic cells and B cells and T cells. All this populations progressively increased in colitic animals at days 2 and 4 of treatment, and disease score generally reach peak values between these time points. As intestinal inflammation progress, inflammatory signals reach systemic level and stimulated hematopoietic stem cells to sustain the immune response. Consequently, 4 days after DSS removal, colitic mice displayed increase myeloid cells on blood, supporting the ongoing innate immune response at the intestine. Neutrophils are the main primary responders that attempt to control the infection by releasing proteases and generation of an oxidative burst, that indiscriminately attack bacteria and host tissue. The inflammatory response is coordinated by recently arrived macrophages, that conditioned by the inflammatory milieu accumulates with an inflammatory phenotype. Finally, dendritic cells migrate to mesenteric lymph nodes and initiate the adaptive immune response associated to mucosal damage, being the increase in Tregs and Th17 subsets the most important changes observed. This was the evolution of the immune response observed on this model of colitis at the time points assayed, and published data agree with these observations. As long as this inflammatory reaction perpetuates, pro-inflammatory cytokines will sustain the recruitment and activation of inflammatory cells and their associated tissue damage, which impede the recovery of a functional protective barrier and lead to chronic inflammation.

What was the effect of the treatment? Minocycline first potentiated a pro-inflammatory immune response. After two days of treatment, it increased the expression of the chemokines CCL11 and CCL2, involved the recruitment of eosinophil, the first, and monocyte and dendritic, the second. Up-regulation of the cytokines IL-1β, IL-6, GM-CSF, IL-22, IL-2, IL-4 and IL-10 was also observed. No significant immune changes were observed at this time on the lamina propria, but leukocyte pool in blood already displayed the inflammatory

phenotype observed on the control group after 4 days of treatment, with an increase on the myeloid lineage, stimulated by GM-CSF as well as inflammatory cytokines.

Two days later, the composition of immune cells on blood was already recovering basal levels, with reduced neutrophilia although monocytes and eosinophils were still increased. Lamina propria leukocytes showed reduced numbers of neutrophils, while eosinophils, macrophages, dendritic cells and T cells were increased as compared to untreated mice. However, the macrophage pool showed a significant shift towards the alternatively activated phenotype, associated with mucosal healing and homeostatic functions. The phenotype of dendritic cells was not modified, and increased Tregs and Th17 cells were also found on the colon as well as in lymph nodes and spleen. Last, Th2 subset appeared increased as well, which correlates with increase IL-4 expression observed at this and previous time point. Despite this general immune boost, histological damage was toughly reduced and epithelial barrier markers were significantly improved.

Supported by these observations, we hypothesize that the pro-inflammatory actions of immunomodulatory tetracyclines on macrophages do not dampen the beneficial effect ascribed to their other non-antibiotic properties, but rather reinforce and explain the potent activity of this compounds to resolve mucosal inflammation.

CONCLUSSIONS

☑ Doxycycline exerts intestinal anti-inflammatory activity, similar to that previously observed with minocycline. The combination of doxycycline with *S. boulardii* effectively control relapsing inflammation, which supports the potential use of this therapeutic association for the treatment of inflammatory bowel diseases, in which doxycycline is used to induce remission and long term probiotic administration, helps to prevent the relapses.

☑ Doxycycline, minocycline and tigecycline, thanks to their combination of immunomodulatory and antibiotic properties, confer a superior protection against acute intestinal inflammation compared to conventional antibiotics and corticosteroids. The nature of the inflammatory process might determine whether the impact of their antibiotic activity on intestinal microbiota contribute to this protection, while a negative impact of their short-term administration has not being observed. However, the immunomodulatory properties of these drugs conferred protection from colitis-induced tissue damage and mortality, regardless the features of the inflammatory reaction involved in the experimental settings evaluated. This immunomodulation involve specific pathways associated with innate immune sensing and epigenetic regulation of mucosal epithelial and immune barriers.

☑ The mechanism of action of immunomodulatory tetracyclines, as shown for minocycline, includes the activation of natural pathways leading to mucosal protection and healing and inflammatory resolution. Minocycline shape the inflammatory and innate immune response controlled by macrophages, which first potentiate the release of inflammatory mediators and the recruitment of monocytes, dendritic cells and eosinophils. This action, which may involve innate lymphoid cells activation, accelerates the recovery of barrier function and generates anti-inflammatory macrophages and regulatory T cells, thus reducing neutrophil recruitment and inflammation-associated tissue damage. The potential of this mechanism strongly support the use of immunomodulatory tetracyclines to control the complexity of intestinal inflammation.

CONCLUSIONES

☑ La doxiciclina muestra actividad anti-inflamatoria intestinal, similar a la observada previamente con minociclina. La combinación de doxiciclina con *S. bourlardii* controla efectivamente una inflamación recurrente, lo cual apoya el uso de esta asociación terapéutica en el tratamiento de la enfermedad inflamatoria intestinal, donde la doxiciclina se utilice para inducir remisión y el tratamiento prolongado con probióticos ayude a prevenir su reactivación.

☑ La doxiciclina, minociclina y tigeciclina, gracias a la combinación de propiedades inmunomoduladoras y antibióticas, confieren una protección superior contra la inflamación intestinal aguda, en comparación con antibióticos y corticoides convencionales. La naturaleza del proceso inflamatorio puede determinar si el impacto de la actividad antibiótica contribuirá positivamente a su efecto protector, mientras no se han observado perjuicios de su uso a corto plazo. Sin embargo, las propiedades inmunomoduladoras de estos fármacos contribuyen a la protección frente al daño tisular y mortalidad inducidos por colitis, sin importar la naturaleza de la reacción inflamatoria desencadenada en los diferentes modelos experimentales en los que se han sido evaluadas. Este efecto inmunomodulador implica la activación de vías asociadas a la activación de la inmunidad innata y la regulación epigenética de la barreras epitelial e inmune de la mucosa intestinal.

- ☑ El mecanismo de acción de las tetraciclinas inmunomoduladoras, como se ha observado con minociclina, incluye la activación de vías naturales destinadas a proteger y reparar la mucosa y resolver la inflamación. La minociclina refuerza la función de los macrófagos en la inflamación y respuesta inmune innata, en primer lugar potenciado la liberación de mediadores inflamatorios y el reclutamiento de monocitos, células dendríticas y eosinófilos. Esta acción, que probablemente implica a las células linfoides innatas, acelerata la recuperación de la barrera protectora y genera macrófagos antiinflamatorios y células T reguladoras, consiguiendo frenar la llegada de neutrófilos y el daño tisular asociado a la inflamación. El potencial de este mecanismo respalda fuertemente el uso de tetraciclinas inmunomoduladoras en el control de la inflamación intestinal.

MATERIALS AND METHODS

REAGENTS

All chemicals were obtained from Sigma (Madrid, Spain), unless otherwise stated. Most relevant reagents used in the experimental protocols described below are included in the following table, as well as the list with primer sequences used in real-time qPCR. Tigecycline was provided by the pharmaceutical service of the hospital Virgen de las Nieves (Granada, Spain). The doses of antibiotics used in the animal models were chosen according to previous results of our group and were equivalent to the therapeutic dose in humans (calculated as described elsewhere [1]). *Saccharomyces bourlardii* CNCMI-745 was provided by Biocodex (Beauvais, France).

Table 2. Primer sequences used in real-time PCR assays in colonic tissue

Gene		Sequence5´-3´	Annealing T (ºC)
GAPDH	FW	5'-CCATCACCATCTTCCAGGAG	60
	RV	5'-CCTGCTTCACCACCTTCTTG	
MUC-1	FW	5'-GCAGTCCTCAGTGGCACCTC	60
	RV	5'-CACCGTGGGGCTACTGGAGAG	
MUC-3	FW	5'-CGTGGTCAACTGCGAGAATGG	60
	RV	5'-CGGCTCTATCTCTACGCTCTC	
MMP-2	FW	5'-TGCCGGCACCACTGAGGACTAC	56
	RV	5'-GGGCTGCCACGAGGAACA	
MMP-9	FW	5'-TGGGGGGCAACTCGGC	60
	RV	5'-GGAATGATCTAAGCCCAG	
iNOS	FW	5'-GTTGAAGACTGAGACTCTGG	56
	RV	5'-GACTAGGCTACTCCGTGGA	
IL-1β	FW	5'-TGATGAGAATGACCTCTTCT	55
	RV	5'-CTTCTTCAAAGATGAAGGAAA	
TNFa	FW	5'-AACTAGTGGTGCCAGCCGAT	56
	RV	5'-CTTCACAGAGCAATGACTCC	
IL-6	FW	TAGTCCTTCCTACCCCAATTTCC	60
	RV	TTGGTCCTTAGCCACTCCTTC	
occludin	FW	ACGGACCCTGACCACTATGA	56
	RV	TCAGCAGCAGCCATGTACTC	
ZO-1	FW	GGGGCCTACACTGATCAAGA	56
	RV	TGGAGATGAGGCTTCTGCTT	
MCP-1	FW	CAGCTGGGGACAGAATGGGG	62
	RV	GAGCTCTCTGGTACTCTTTTG	

IN VITRO STUDIES

Caco-2 (human colon adenocarcinoma cells), RAW 264.7 (mouse macrophages) and L929 (mouse fibroblasts) cell lines were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain). Bone marrow-derivated macrophages where obtained from C57b6l/j mice. Unless otherwise stated, cells were 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. Cell viability of tested conditions was ensured in advance by the MTT-test following the manufacturer's instructions[2].

CACO-2 CELL LINE

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-treated for 24 h with minocycline (MNC) (50 μM) or different concentrations of doxycycline ranging from 1 to 50 μM. To study the effects of *Saccharomyces boulardii*, cells were pre-incubated for 2 h with the probiotic at a concentration of 10^8 UFC/ml, and they were washed three times afterwards. Probiotic conditioned medium was obtained by incubating the probiotic in medium at 10^8 UFC/ml for 2 h, followed by centrifugation and collection of the supernatant. Cells were incubated with the conditioned medium for 2 h. In the association studies, cells were incubated with doxycycline (25 μM) for 24 h and *S.boulardii* or its conditioned medium were added during the last 2 h. Following the pre-treatments, the cells were stimulated with IL-1β (1 ng/ml) for 20 h. Untreated unstimulated cells and untreated cells were used as negative and positive controls. Then the supernatants were collected, centrifuged at 10000×g for 5min and stored at −80 °C until IL-8 determination was performed by ELISA (Biosource, Invitrogen™), according to manufacturer's instructions.

RAW 264.7 CELL LINE

RAW264.7 cells were seeded onto 24-well plates at a density of 5×10^5 cells per well and grown until confluence. They were cultured with antibiotics and/or *S.boulardii* or conditioned medium as described above and then stimulated with LPS (100 ng/ml) for 24 h; similarly, positive and negative controls were included. Supernatants were collected and centrifuged at 10000×g for 5min, and nitrite levels were measured using the Griess assay[3].

BONE MARROW-DERIVATED MACROPHAGES

BMDM were generated as described previously[4] with some modifications. Bone marrow was isolated from femurs of 6-8 week-old C57Bl6/j (Janvier, St Berthevin Cedex, France). Mice were killed by cervical dislocation, the adherent tissue was removed and both femurs were dissected. The bone ends were cut off, and the marrow tissue was flushed by irrigation with DMEM. The marrow plugs were dispersed by passing them through a 25-gauge needle, and the cells were suspended by vigorous pipetting and washed. Cells were cultured in 150 mm Petri dishes with 40 ml of DMEM containing 20% FBS and 30% Lcell- conditioned medium as a source of macrophage colony-stimulating factor (M-CSF)[5]. Cells were incubated at 37ºC in a humidified 5% CO2 atmosphere. After 6 days of culture, a homogeneous population of adherent macrophages was obtained. Cells were scraped and seeded at 10^6 cells/ml. After one day, cells were incubated with the drugs for 24h and then stimulated with LPS at 10 ng/ml. RNA extraction and gene expression were assessed after 3h of stimulation, while 24h time was used for evaluation of cytokine production by ELISA. Untreated unstimulated cells and untreated cells were used as negative and positive controls.

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. *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.

TNBS MODEL OF RAT COLITIS

Female Wistar rats (180–200 g) were obtained from Janvier (St BerthevinCedex, France). Colonic inflammation was induced as previously described[6], 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. The treatment with antibiotics started the day of the colitis induction, to avoid their possible preventive effect, as it has been widely described[7]. Four colitic groups were treated with antibiotics, as illustrated on the experimental design included with the results of this experiment. The antibiotics were dissolved in 2 ml of distilled water and administered daily by oral gavage. TNBS control and a non-colitic group, which did not undertake colitis induction protocol, were included for reference and received the vehicle. Treatment continued for 7 days until the death of the rats with an overdose of halothane. Animal body weights, occurrence of diarrhea and water and food intake were recorded daily throughout all the experiment.

Once the animals were sacrificed, the colon was removed aseptically and placed on an ice-cold plate and longitudinally opened. 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. [8].

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).

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.

DNBS MODEL OF MOUSE COLITIS

Male CD1 mice, weighing 25–30 g, were obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain). DNBS colitis, a variant of the TNBS method first described in rats[6], was induced as previously reported[9] with minor modifications. Briefly, mice anaesthetized with 2% enflurane were injected in the distal 4 cm of the colon with 100 µl of a EtOH:H_2O 1:1 solution containing 4 mg of DNBS, using a 1 ml syringe and a polyethylene catheter. Mice were maintained in a downright position inside the anaesthetic chamber to avoid the loose of the haptenizing dose. After 15 minutes, mice were taking out for recovery. Control mice injected with EtOH:H_2O vehicle solution were included on preliminary experiments while setting up this models. These also develop minor inflammation that resolves within 3 days, since the EtOH do not just vehicularize the DNBS, but also contributes to the mechanism of colitis induction. Therefore, non-colitic controls used on the evaluation of the treatments did not undertake this procedure. Six hours after colitis inductions, to avoid a possible preventive effect, mice started their respective treatments (as illustrated on the experimental design included at the beginning of results), by oral gavage in a total volume of 200µl of sterile water, while non-treated mice received the vehicle only. Body weight was measured daily to follow the evolution of colitis and, after 6 days of treatment, mice were sacrifice by cervical dislocation.

The colon was then resected and stools were collected aseptically. Adherent tissue was removed and the colon was rinsed with ice-cold saline. Afterwards, the colonic segment was weighed and its length measured under a constant load (2 g). 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 colic tissue was subsequently minced, frozen in liquid nitrogen and stored for subsequent evaluations.

DSS MODELS OF MOUSE COLITIS

On the experiments included in aim 1, we used female C57BL/6J mice (7-9 weeks old; approximately 20 g), while on aims 2 and 3, male C57BL/6J mice (7-9 weeks old; approximately 25 g) were used, both obtained from Janvier (St Berthevin Cedex, France). The colitis was induced in all groups except the non-colitic control by adding DSS (3% w/v) (36-50KDa, MP Biomedicals, Ontario, USA) in the drinking water. Colitis induction vary according to many factors, such as the percentage of DSS used and the batch, as well as mouse strain, gender and colony[10], [11] and therefore, period of induction varied among different experiments, being also adjusted to the severity of intestinal inflammation required for each study. Generally, DSS intake was interrupted when colitic mice reached a mean DAI of 1.4 - 1.8. Food and water consumption were measured daily to discard differences in the colitis induction process among groups. For the reactivated model of colitis, the mice were subjected to a second cycle of DSS, ten days after the first (at day 17), inducing a relapse of intestinal inflammation to resemble human IBD course. Treatments were administered in 200µl of sterile water, while control groups received the vehicle only. To assist in the interpretation of results, detailed information regarding colitic induction and treatment periods, as well as dosage, has been illustrated on the experimental design included at the beginning of each results section.

Animal body weight, the presence of gross blood in the feces and stool consistency were evaluated daily by the same observer. These parameters were assigned a score according to the criteria proposed previously by Cooper et al.[12] and used to calculate an average daily disease activity index (DAI).

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

Score	Weight loss	Stool consistency	Rectal bleeding
0	None	Normal	Normal

1	1 - 5 %	Mucous traces	Perianal blood traces
2	5- 10 %	Loose stools	Blood traces on stools
3	10 – 20 %	Diarrhoea	Bleeding
4	> 20 %	Gross 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).

Mice were sacrificed by cervical dislocation. For flow cytometric analysis performed in aim 3, mice were anesthetized with ketamine/xylazine (100 and 7.5 mg/kg respectively) and blood was collected by cardiac puncture. Then, mice were sacrificed by cervical dislocation and mesenteric lymph nodes and spleen were also collected for flow cytometric analysis, as well as the colon, used in all the studies. Colonic stools were collected aseptically and the colon was then washed with saline solution, weighed, and its length was measured under a constant load (2 g). Representative whole gut specimens (0.5 cm length) were taken at 1cm from the distal region and were fixed in 4% buffered formaldehyde for histological studies. The remaining colonic tissue was subsequently divided for RNA isolation, biochemical determinations and flow cytometric analysis, when required.

FAECAL MICROBIOTA TRANSFERENCE MODEL.

The functional evaluation of changes induced on intestinal microbial communities was performed by transference of microbiota to recipient mice, previously depleted of their original microbiota by applying an experimental protocol based on previously described methodologies[13], [14]. Depletion of intestinal microbiota was induced by daily administration of an antibiotic

cocktail of metronidazole, neomycin and imipenem, all three at the dose of 100mg/kg, for 3 weeks. Antibiotic administration was interrupted one day before transference to avoid its effect on exogenous microbiota. Additionally, three doses of ranitidine (60mg/kg) were given 2 days before and 2 hours before the transference to reduce acid secretion in order to improve the establishment of exogenous microbiota. Four groups of animals followed this protocol, which was coordinated with experimental design of the groups providing of the microbial communities to evaluate: a non-colitic group and two colitic groups, DSS control without treatment and one treated with minocycline for 4 days, following the previous experimental design. Microbial contents from cecum and colon of these experimental groups were collected and quickly submerged on PBS to reduce contact with oxygen. Pooled content of mice at each group were filtered and adjusted at the proportion stools:PBS of 150mg:150µl, of which recipient mice received 300µl by oral gavage. Of the four groups depleted of endogenous microbiota, one was given PBS alone to control the impact of the experimental procedure alone, while the other three received microbiota of each group to analyse. After inoculation, mice were maintained one week under standard conditions to allow exogenous microbiota to settle and stabilize.

After that, the susceptibility of the different groups to a challenge of acute intestinal inflammation was evaluated as described above. Colitis was induced by administration of DSS (3%) for 5 days followed by 2 days of normal water, killing the animals at the peak of intestinal damage.

PROTOCOLS

GRIESS ASSAY

Nitrite accumulated in cell media was convert into a deep purple azolic compound by Griess reagents (0.1 % N-(1-naphthy) ethylenediamine solution and 1% sulphanilamide in 5% (v/v) phosphoric acid solution). Photometric measurement of the absorbance at 550 nm due to this azo chromophore accurately determines nitrite concentration[3].

MICROSCOPIC EVALUATION

Colonic cross-sections were embedded in paraffin and full-thickness sections of 4 μm were obtained and stained with haematoxylin and eosin, combined with histochemical staining of mucins with alcian blue[15], when indicated. The microscopic damage was evaluated by a pathologist observer, who was blinded to the experimental groups, according to the following criteria[16].

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).

MIELOPEROXIDASE ACTIVITY

Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al.[17]. 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 (460 nm) changes that resulted from decomposition of H_2O_2 in the presence of O-dianisidine.The results were obtained by comparison with a standard curve of MPO and expressed as mili-units of MPO per gram of wet tissue; one unit of MPO activity was defined as that degrading 1µmol of hydrogen peroxide/min at 25^{o}C.

GLUTATHIONE CONTENT

Total glutathione (GSH) content was quantified with the recycling assay described by Anderson et al.[18] and in which GSH 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.

COLONIC PROTEIN EXTRACTION FOR IL-1B DETERMINATION BY ELISA

Colonic samples for IL-1β determinations were immediately weighed, minced on an ice-cold plate and suspended (1:5 w/v) in a lysis buffer containing 20mM 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, 500 μM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml iodoacetamide and 2 mM sodium orthovanadate. The tubes were placed in an orbital rotor (4ºC) for 20 min and centrifuged at 9000×g for 10 min at 4ºC; the supernatants were frozen at −80ºC until assay. The cytokine was quantified by enzyme-linked immunoabsorbent assay (R&D Systems Europe, Abingdom, UK) and the results were expressed as pg/g wet tissue.

RNA EXTRACTION AND RT-QPCR EVALUATION OF GENE EXPRESSION

For the analysis of gene expression on the experiments included on the aim 1, total RNA from colonic samples was isolated using Trizol® (Ambion, Austin, TX, USA) following the manufacturer's protocol. On the comparative studied on DSS and DNBS models, both microRNA and mRNA expression were evaluated. The conservation of small RNAs was ensured by isolation of total RNA from colonic samples using miRNeasy mini Kit (Qiagen, Hilden, Germany) according manufacturer's instructions, with tissue homogenization performed with QIAzol reagent on a PrecellysR24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). On subsequent experiments on aims 2 and 3, total RNA from colonic samples was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

For the evaluation of mRNA expression, 3μg of RNA were reverse transcribed using oligo(dT) primers (Promega, Madison, WI, USA). Real time quantitative PCR amplification and detection was performed on optical-grade 48 well plates in an EcoTM Real-Time PCR System (Illumina, CA, USA). Each reaction was

composed of 10 ng of cDNA, 5 µL of the KAPA SYBR® FAST qPCR Master Mix (KapaBiosystems, Inc., Wilmington, MA, USA) and specific primers and PCR-grade water up to a final volume of 10 µL. The thermal cycling program consisted of an initial activation step of 2 min at 95 °C, followed by 40 cycles with 3-step clycing: 15 s at 94 °C for denaturation, 30s at the annealing temperature and 30 s at 70°C for extension step. To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was measured. Then RNA relative quantitation was calculated using the ΔΔCt method.

For simultaneous evaluation of miRNAs and mRNA, 500 ng of RNA were reverse transcribed using the miScript II RT kit from Qiagen (Qiagen, Hilden, Germany). Then, RT-qPCR evaluation of mRNA expression was performed as described above while microRNA determination followed a different protocol. For microRNA expression each reaction was composed of 5 µL QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 1 µL miScript Universal Primer, 1 µL miScript Primer Assay, 20 ng of cDNA from the RT reaction and PCR-grade water up to a final volume of 10 µL. The thermal cycling program consisted of an initial activation step of 15 min at 95 °C, followed by 40 cycles with 3-step clycing: 15 s at 94 °C for denaturation, the annealing step at 55°C s for 30 s and 30 s at 72°C for extension step. To normalize microRNA expression, the expression of the housekeeping gene, small nucleolar RNA, C/D box 95 (SNORD95) was measured. The miRNA relative quantitation was done using the ΔΔCt method.

DNA EXTRACTION AND PYROSEQUENCING

DNA from faecal content was isolated using phenol:chloroform, modified from (Sambrook J, 2001)[20]. To compare how 16S rRNA gene sequence recovery was affected by storage and purification methods, total DNA from stool samples was PCR amplified using primers targeting regions flanking the variable regions 1 through 3 of the bacterial 16S rRNA gene (V1-3), gel purified, and analyzed using the 454/Roche GS FLX technology (Branford, CT, USA).
The amplification of a 600-bp sequence in the variable region V1-V3 of the 16S rRNA gene was performed using barcoded primers. PCR was performed in a total volume of 15 µL for each sample containing the universal 27F and Bif16S-F

forward primers (10 µmol/L) at a 9:1 ratio, respectively, and the barcoded universal reverse primer 534R (10 µmol/L) in addition to dNTP mix (10 mmol/L), FastStart 10× buffer with 18 mmol/L of MgCl2, FastStart HiFi polymerase (5 U in 1 mL), and 2 µL of genomic DNA. The dNTP mix, FastStart 10× buffer with MgCl2, and FastStart HiFi polymerase were included in a FastStart High Fidelity PCR System, dNTP Pack (Roche Applied Science). The PCR conditions were as follows: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 5 min, and final step at 4 °C. After PCR, amplicons were further purified using AMPure XP beads (Beckman-Coulter) to remove smaller fragments. DNA concentration and quality were measured using a Quant-iT™ PicoGreen® dsDNA Assay Kit. Finally, the PCR amplicons were combined in equimolar ratios to create a DNA pool (109 DNA molecules) that was used for clonal amplification (emPCR) and pyrosequencing according to the manufacturer's instructions.

CELL ISOLATION AND FACS STUDIES

Protocol X includes detailed optimized procedure used for cell isolation from the different organs. Colon LP cells isolation protocol followed as previously described methods[21], [22]. Once cell suspension was obtained, cells were stained and analysed by FACS. Surface-staining antibodies were added together with a viability stain (Invitrogen) for 20 minutes at 4°C. For intracellular cytokine expression, cells were previously stimulated PMA (50 ng/ml,) and ionomycin (1 µg/ml, both Sigma-Aldrich) with GolgiPlug™ for 4,5 hours, at 37°C. Prior to intracellular staining, when required, cells were fixed in Fixation/Permeabilization buffer (eBioscience). Antibodies for intracellular stains were used together with the FoxP3 staining kit (eBioscience) following the manufacturer's instructions. Antibodies were from Miltenyi unless otherwise stated. Samples were acquired using a FACSVerse™, FACSCanto II™ or FACScalivur™ cytometers (Becton Dickinson, USA).

ANALYSIS & STATISTICS

ANALYSIS OF PYROSEQUENCING DATA

Obtained reads from 16S ribosomal DNA sequencing were scored for quality, and any poor quality and short reads were removed. Sequences were selected to estimate the total bacterial diversity of the DNA samples in a comparable manner and were trimmed to remove barcodes, primers, chimeras, plasmids, mitochondrial DNA and any non-16S bacterial reads and sequences <150 bp. MG-RAST (metagenomics analysis server)[23] with the Ribosomal Database Project (RDP) were used for analyses of all sequences. The pipeline takes in bar coded sequence reads, separates them into individual communities by bar code, and utilizes a suite of external programs to make taxonomic assignments with RDP database[24] and estimate phylogenetic diversity. Operational taxonomic units (OTUs) were obtained with minimum e-value of 1e-5, minimum alignment length of 15 base pairs and minimum identity threshold was set at 95% in order to reach genus and species level.

The output file included the abundance profile of OTUs of each sample down to species level, which was used for subsequent analysis[25], [26]. Each sample was normaliced by calculation of relative abudance and used for evaluation of different ecological parameters at species level. Parameters indicative of α-diversity (within each sample) of the gut bacterial communities included: Margalef (species richness relative to community size), Chao1 (an estimate of a total community richness based on rare species), Simpson (similarity, probability that 2 chosen invividual will fall into the same group), Shannon (diversity of the community) and Pielou (evenness, proportion of the diversity observed against the maximal diversity expected). Pie-charts of each group mean relative abundance were obtained with Krona tool[27]. The output file was further used for β-diversity analysis using Statistical Analysis of Metagenomic Profiles (STAMP) software package version 2.1.3 [28].

ANALYSIS OF FACS DATA

Flow cytometry panels and gating strategy were defined according to previous set up experiments and FMO controls. Data was analysed using FlowJo software (Tree Star, USA). Gating strategies of the three different panels are showed with results. Total count of each population was obtained an results were expressed as total number of life cells, percentage of $CD45^+$ life cells or percentage of the

parent populations. For total number (used on colonic lamina propria leukocytes), percentage of life cells from the analysis was multiplied by the cell count obtained after tissue digestion and divided by the weight ration of the tissue fraction used for digestion/total organ.

STATISTICS

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 $P<0.05$.

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