Immunomodulatory tetracyclines shape the intestinal inflammatory response inducing mucosal healing and resolution.

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ABSTRACT:

Background and Purpose: Immunomodulatory tetracyclines are well-characterised drugs with a pharmacological potential beyond their antibiotic properties. Particularly, minocycline and doxycycicline have shown beneficial effects in experimental colitis, although pro-inflammatory actions have also been described in macrophages. Therefore, we aimed to characterise the mechanism behind their effect in acute intestinal inflammation.

Experimental Approach: A comparative pharmacological study was first used to elucidate the most relevant actions of immunomodulatory tetracyclines: doxycycline, minocycline, tigecycline and other antibiotic or immunomodulatory drugs were assessed in bone-marrow derived macrophages and in DSS-induced mouse colitis, where different barrier markers, inflammatory mediators, microRNAs, TLRs, and the gut microbiota composition were evaluated. Then, the sequential immune events that mediate the intestinal anti-inflammatory effect of minocycline in DSS-colitis were characterised.

Key Results: We have identified a novel immunomodulatory activity of tetracyclines, potentiating the innate immune response and leading to an enhanced resolution of inflammation. This is also the first report describing the intestinal anti-inflammatory effect of tigecycline. A minor therapeutic benefit seems to derive from their antibiotic properties. Conversely, immunomodulatory tetracyclines potentiate macrophage cytokine release *in vitro* and, while improving mucosal recovery in colitic mice, they up-regulate *Ccl2, miR-142, miR-375* and *Tlr4*. In particular, minocycline initially enhances IL-1β, IL-6, IL-22, GM-CSF and IL-4 colonic production and monocyte recruitment to the intestine, subsequently increasing $Ly6C^{-}MHCII^{+}$ macrophages, Tregs and type-2 intestinal immune responses.

Conclusion and Implications: Immunomodulatory tetracyclines potentiate protective immune pathways leading to mucosal healing and resolution, representing a promising drug reposition strategy for the treatment of intestinal inflammation.

Key words: Immunomodulatory tetracyclines | intestinal inflammation | macrophages | mucosal healing | resolution.

Abbreviations: DSS – dextran sodium sulfate; DAI – Disease Activity Index; NC – Non-colitic; RFX – rifaximin; TTC – tetracycline; DXC – doxycycline; MNC – minocycline; TGC – tigecycline; DEX – dexamethasone; BMDM – Bone marrow-derived macrophages; cLP – colonic lamina propria; DC – Dendritic Cell; Mφ – Macrophage; Th – T helper cell; Treg – Regulatory T cell; ILC – Innate Lymphoid Cell; IBD – Inflammatory Bowel Disease.

INTRODUCTION

Immunomodulatory antibiotics are an interesting therapeutic strategy for intestinal inflammation, targeting both the altered microbiota and the exacerbated inflammatory response. In particular, minocycline and doxycycline have shown promising results in experimental colitis (Huang et al., 2009b; Garrido-Mesa et al., 2011a, 2011b, 2015). These are well known tetracyclines with proven benefits in many inflammatory conditions (Garrido-Mesa et al., 2013a). Their intestinal anti-inflammatory effect has been mainly associated with the reduction of inducible nitric oxide synthase (iNOS) and matrix metalloproteinase (MMP) activity (Huang et al., 2009b) and direct immunomodulatory and antibiotic properties (Garrido-Mesa et al., 2011a, 2011b, 2015). However, the relevance of these activities to the overall anti-inflammatory effect has not been specificaly assessed. Of note, although their actions within the immune system are generally anti-inflammatory, a certain degree of controversy has been observed in monocytes and macrophages (Mφ): while immunomodulatory tetracyclines inhibit the inflammatory activity of microglia and peritoneal Mφs, increased activation and cytokine production has been observed in monocytes (Kloppenburg et al., 1996), alveolar Mφs (Bonjoch et al., 2015) and RAW264.7 colonic Mφ cell line (Dunston et al., 2011).

In this regard, although the inflammatory reaction may cause harm and tissue damage, a powerful intestinal mucosal immune system is also needed to protect and restore intestinal homeostasis (Mowat and Agace, 2014), where Mφs play a key role (Gross et al., 2015). Indeed, unlike other locations, the intestinal Mφ pool is continuously replenished from $CCR2^{+}Ly6C^{hi}$ blood monocytes, which then differentiate into $Ly6C^{-}MHCII^{+}$ resident Mφs in the steady state. In inflammatory conditions however, their differentiation is arrested and $Ly6C^{+}MHCII^{-}$ Mφs accumulate (Bain et al., 2013), which display an M1/pro-inflammatory phenotype and produce cytokines that drive the inflammatory reaction. By contrast, intestinal resident $Ly6C^{-}MHCII^{+}$ Mφs are tolerogenic and display an M2-like phenotype, contributing to mucosal healing, resolution of inflammation and maintenance of intestinal homeostasis (Sherman and Kalman, 2004; Pull et al., 2005). Hence, a differential activity of tetracyclines on intestinal Mφs might be of special relevance and a full understanding of their mechanisms is required to expand their therapeutic application to intestinal inflammatory conditions.

The present study aims to characterise the mechanisms of action of immunomodulatory tetracyclines in acute intestinal inflammation, by comparing their effects with other antibiotics or immumomodulatory drugs and studying their impact in the course of the immune response developed in DSS-induced colitis in mice. Our results confirm the relevance of their differential immunomodulatory activity for their anti-inflammatory effect, and allow establishing a link between the initial up-regulation of innate immunity and an improved mucosal healing and resolution. Thus, we have demonstrated that the enhancement of mucosal-protective immune pathways is a key immunomodulatory mechanism of tetracyclines in acute colitis, which is of great interest to prevent the chronification of intestinal inflammation.

METHODS

In vitro studies

RAW264 murine macrophage and L929 murine fibroblast cell lines were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in standard conditions. Bone marrow-derived macrophages (BMDM) were obtained from the bone-marrow of C57BL/6J mice cultured for 6 days in DMEM supplemented with 20% FBS and 30% L929-supernatant containing M-CSF factor. Cells were plated at $1x10^6$ cells/ml and the drugs were added for 24h before stimulation with LPS (100 ng/ml for RAW cells or 10 ng/ml for BMDM) for either 3h for RNA isolation, or 24h for cytokine determination by ELISA (PeproTech EC Ltd, London, UK) or nitrite determination by Griess assay (Green et al., 1982). Briefly, for nitrite determination, 100µl of Griess reagent (0.1 % N-(1-naphthy) ethylenediamine solution and 1% sulphanilamide in 5% (v/v) phosphoric acid solution, mixed in a proportion 1:1) was added to 100µl of cell supernatant and incubated for 10 minutes. The concentration of the product of the reaction, a coloured azolic compound, can be determined by photometric measurement of the absorbance at 550 nm. Cell viability of tested conditions was measured by the MTS-based assay (Promega, Madison, WI, USA).

In vivo studies

All animal 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. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015). Male C57BL/6J mice (6-8 weeks, 25 g) were obtained from Janvier (St Berthevin Cedex, France) and kept in specific pathogen–free facilities at University of Granada Biological Services Unit at 23 ± 1°C, with a relative humidity of 50–70% andon a regular 12 h dark/light cycle. Mice were housed in Makrolom cages (Ehret, Emmerdingen, Germany), with a maximum of 8 mice per cage, with dust □free laboratory bedding and enrichment. They were fed standard chow diet (Panlab A04, Panlab, Barcelona, Spain) and provided drinking water ad libitum.

To investigate the mechanism behind the beneficial activity previously reported for tetracyclines (Garrido-Mesa et al., 2011a, 2011b, 2015) and, in particular, to characterize their impact on the pathways involved in the initiation and resolution of acute intestinal inflammation, we focused on the experimental model of colitis triggered by DSS-induced mucosal injury, the most widely used model of acute colitis (Wirtz et al., 2007). A curative treatment protocol was used considering the lack of preventive effect observed in previous studies (Garrido-Mesa et al., 2011a) and taking in consideration the limitations on antibiotic usage in the clinical practise. Colitis was induced by adding DSS (3% w/v) (36-50KDa, MP

Biomedicals, Ontario, USA) in the drinking water for a period of 5 or 6 days as indicated. Mice were then randomised and treated with the different drugs for either 2, 4 or 6 days depending on the study. Disease evolution was monitored by a daily determination of the disease activity index (DAI), calculated as described in table 1. Mice were anesthetised with ketamine/xylazine (100 and 7.5 mg/kg respectively) for blood collection by cardiac puncture when required and sacrified by cervical dislocation and the whole colon length was resected. Stools were collected aseptically for pyrosequencing. The colonic tissue was washed in PBS and samples were collected for subsequent histological, biochemical and immunological evaluations.

Histology

Representative colonic specimens were taken at 1cm from the distal region, fixed in 4% paraformaldehyde and embedded in paraffin. Histochemical staining of mucins was performed by incubation of 4 μm re-hydratated sections in Alcian Blue 1% in Acetic acid 3% for 30 minutes prior to conventional haematoxylin and eosin staining. Colonic microscopic damage was evaluated by a pathologist observer blinded to the experimental groups according to the criteria described in table 2.

Colonic explant culture and cytokine determination by ELISA

Whole thick colonic punch biopsies (3 per specimen) (Miltex, York, PA, USA) were obtained from distal, medial and proximal regions, and incubated in 0.5ml of medium supplemented with gentamycin 50μg/ml for 24h. Cytokine concentration in culture supernatant was measured by ELISA (PeproTech EC Ltd, London, UK).

RNA extraction and gene expression analysis

Representative colonic samples were taken for RNA extraction and gene expression studies. In the comparative pharmacological study on DSS colitis where both microRNAs and mRNAs were evaluated, total RNA was isolated with miRNeasy mini Kit (Qiagen, Hilden, Germany) and 500 ng of RNA was reverse transcribed using the miScript II RT kit from Qiagen (Qiagen, Hilden, Germany). For other studies, RNA was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany) and 3μg of RNA was reverse transcribed using oligo(dT) primers (Promega, Madison, WI, USA). RT-qPCR of microRNAs was performed using the QuantiTect SYBR Green PCR Master Mix with miScript Universal Primers and the specific miRNA primer sequences (Qiagen, Hilden, Germany). For mRNA expression, RT-qPCR was performed using KAPA SYBR® FAST qPCR Master Mix (KapaBiosystems, Inc., Wilmington, MA, USA). Detection was performed on optical-grade 48 well plates in an EcoTM Real-Time PCR System (Illumina, CA, USA). The small nucleolar RNA C/D box 95 (SNORD95) and GAPDH were measured to normalise microRNA and mRNA expression (ΔCt), respectively. Fold increase values for gene expression analysis were calculated using normalised expression levels ($2^{-\Delta Ct}$) referred to the mean of NC control group (Fold Increase = $2^{-\Delta Ct}/2^{-\Delta Ct}{}_{NC}$). SNORD95, miRNA and reverse universal primer for miRNA (Qiagen) and IL-22 (PrimerDesign) were sourced commercially. The remaining specific primer sequences (Sigma) are detailed in table 3.

Bacterial DNA pyrosequencing and analysis

DNA from faecal content was isolated using phenol:chloroform extraction and ethanol purification (Sambrook and Russell, 2006). 16S rRNA gene sequence recovery and integrity 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 Titanium technology (Roche Diagnostics, 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 $MgCl_2$, and FastStart HiFi polymerase were included in a FastStart High Fidelity PCR System, dNTP Pack (Roche Applied Science, Penzberg, Germany). 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, Inc., Indianapolis, IN, USA) to remove smaller fragments. DNA concentration and quality were measured using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the PCR amplicons were combined in equimolar ratios to create a DNA pool (10^9 DNA molecules) that was used for clonal amplification (emPCR) and pyrosequencing according to the manufacturer's instructions.

Obtained reads from 16S ribosomal DNA sequencing were scored for quality, and any poor quality and short reads were removed. Sequences 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) was used to analyse the sequences and make taxonomic assignments with Ribosomal Database Project (RDP). Operational taxonomic units (OTUs) were obtained with minimum e-value of 1e-5, minimum alignment length of 15bp and minimum identity threshold was set at 95%. The relative abundance of OTUs of each sample was calculated on the output file and used for subsequent analysis, including the determination of ecological parameters indicative of α- and β-diversity, determined using Statistical Analysis of Metagenomic Profiles (STAMP) software package version 2.1.3.

Cell isolation and flow cytometry analysis

Cell from the colonic lamina propria (cLP) were isolated as described (Scott et al., 2017) using a digestion media composed of HBSS without Mg^{2+} or Ca^{2+}, 10% of FBS, and 0.5mg/ml collagenase V (Sigma), 0.65mg/ml collagenase D, 30µg/ml DNase I and 1mg/ml dispase II (all Roche). Blood (300µl) was collected and red blood lysis was performed as needed. Surface-staining antibodies were added to the cell suspension together with a viability stain (Invitrogen) and FcR blocking reagent (Miltenyi) for 20 minutes at 4°C. For intracellular cytokine expression, cells were previously stimulated PMA (50 ng/ml) and ionomycin (1 µg/ml) (Sigma-Aldrich) in the presence of GolgiPlug™ (eBioscience) for 4.5 hours, at 37°C. For intracellular staining of cytokines and transcription factors, cells were fixed in Fixation/Permeabilization buffer (FoxP3 staining kit, eBioscience) and antibodies were added following the manufacturer's instructions. Antibodies were from Miltenyi unless otherwise stated: α-mouse CD45 (30F11), α-human/α-mouse CD11b (M1/70.15.11.5), α-mouse Ly6G (REA526), α-mouse SiglecF (REA798), α-mouse MHCII (M5/114.15.2), α-mouse Ly6C (1G7.G10), α-mouse CD103 (2E7), α-mouse CD11c (N418), α-mouse F4/80 (REA126), α-mouse B220 (RA3-6B2, BD Bioscience), α-mouse CD3 (17A2), α-mouse CD8 (53-6.7), α-mouse CD4 (RM4-5, BD Bioscience), α-mouse IL-4 (BVD4-1D11), α-mouse IFNγ (XMG1.2, BD Pharmigen), α-mouse IL-17A (eBio17B7, eBioscience), α-mouse FoxP3 (FJK-16s, eBioscience). Samples were acquired using a FACSVerse™ or FACSCanto II™ cytometers (Becton Dickinson, USA) and data was analysed using FlowJo software (Tree Star, USA). Percentages of the different populations referring to live cells were multiplied by the total count to provide the total number of each population.

Data and Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in Pharmacology (Curtis et al., 2015). Statistical significance was only evaluated in data sets with n≥5 with one-way analysis of variance (ANOVA) and *post hoc* Tukey's Multiple Comparison tests. Survival curves were analysed with the Gehan-Breslow-Wilcoxon test. Non-parametric data 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 $P<0.05$.

Materials

All chemicals were obtained from Sigma (Madrid, Spain), unless otherwise stated. Drug doses used in mice were equivalent to the therapeutic dose in humans.

RESULTS

Immunomodulatory tetracyclines have a dual effect on macrophages *in vitro*

The immunomodulatory activity of different tetracyclines was initially compared in LPS-activated RAW246 macrophages. Tigecycline (TGC) was the most potent inhibitor of NO production, followed by minocycline (MNC) and doxycycline (DXC), although the activity of dexamethasone (DEX) was stronger. On the other hand, no significant inhibitory effect was observed for tetracycline (TTC) or rifaximin (RFX) at the viable concentrations assayed (Fig1A). Then, the immunomodulatory tetracyclines were evaluated in bone-marrow derived Mφs (BMDM), to characterise the dual anti-/pro-inflammatory activity described in this cell type (Kloppenburg et al., 1996; Dunston et al., 2011; Bonjoch et al., 2015). LPS activation of BMDM induced the expression of *Inos* and the release of IL-1β, IL-6, and TNFα. DEX reduced all these markers, whereas the immunomodulatory tetracyclines reduced *Inos* mRNA levels but potentiated cytokine release (Fig1B-E).

Immunomodulatory tetracyclines ameliorate DSS-colitis, showing a better profile than rifaximin, tetracycline and dexamethasone

Initially, the compounds were assayed following a fatal colitis protocol in mice induced by administering 3% DSS for 6 days. Then, mice were treated for 6 days with: 1) RFX (200 mg/kg/day), a non-absorbable antibiotic; 2) TTC (250 mg/kg/day), included as reference of tetracyclines' antibiotic activity; 3-5) immunomodulatory tetracyclines: DXC (25 mg/kg/day), MNC (50 mg/kg/day) and TGC (25 mg/kg/day); and 6) DEX (2.4 mg/kg/day), included as reference of an anti-inflammatory drug without antibiotic activity. The administration of DXC, MNC and TGC significantly reduced disease activity index (DAI) (Fig2A) from the first day of treatment and throughout the study; however, DEX-treated mice experienced an increase in DAI values after the third day of treatment, in contrast with the initial reduction observed. TTC-treated mice showed a slight improvement, although no statistical differences were observed in comparison with DSS-control, and RFX did not show any beneficial effect. Moreover, a high mortality rate was experienced from day 8 in colitic mice, with only 30% of the animals surviving until the end of the study, and only those colitic groups treated with immunomodulatory tetracyclines showed a significantly increased survival (Fig2B).

Subsequently, the effect of these drugs was tested in a less aggressive colitis protocol, in which DSS intake (3%) was maintained for 5 days, followed by a 4-day period of treatment. The DAI evolution followed a similar pattern: only the immunomodulatory tetracyclines significantly ameliorated DAI values (Fig3A). Histological analysis showed that DSS-colitis mainly affected the mucosa, with epithelial ulceration in more than 70% of the colonic surface.

Major histological alterations affected the crypt structure, with high mitotic activity, mucin depletion in goblet cells, presence of oedema and intense inflammatory cell infiltration. Immunomodulatory tetracyclines significantly reduced the microscopic damage score, preserving the mucosal layer and restoring the presence of mucus filled goblet cells. However, no histological improvement was observed in colitic mice treated with RFX, TTC or DEX (Fig3B-C). The mucin depletion observed in DSS-control mice was associated with reduced expression of *Muc-1, Muc-2* and *Muc-3,* and of the epithelial barrier integrity makers *Zo-1* and *Occludin*. Importantly, their expression was improved in animals treated with immunomodulatory tetracyclines, which also showed an upregulation of *Tff-3* expression. In constrast, RFX and DEX treatments did not restore the expression of these protective markers, which appeared even further reduced in RFX-treated mice (Fig3D).

When different inflammatory mediators were considered, DSS-induced colitis was linked to an increased expression of *Tnfα, Il-1β, Il-6, Mmp-9, Ccl2* and *Cxcl2* (Fig4). The treatment with immunomodulatory tetracyclines significantly reduced *Il-1β, Il-6, Mmp-9* and *Cxcl2* expression, while the other drugs showed no effect. Strikingly, *Ccl2* expression was strongly potentiated in mice treated with DXC, MNC and TGC, and to a lesser extent with TTC. Recent studies have highlighted the role of microRNAs in the regulation of intestinal inflammation (Biton et al., 2011; Pekow and Kwon, 2012). In our study, DSS-colitis induced a significant up-regulation of *miR-142, miR-150* and *miR-155* (Fig4). The tetracyclines and DEX reduced *miR-150* and *miR-155* expression, which have been associated with T helper cell and humoral responses (Monticelli et al., 2005) and the NFκB pathway (Tili et al., 2007), respectively. The administration of tetracyclines to colitic mice resulted in an up-regulation of *miR-375*, which aligns with increased *Tff-3* expression, both related to goblet cell function (Biton et al., 2011). *MiR-142*, preferentially expressed in immune cells (Kramer et al., 2015), was the most upregulated miRNA upon colitis induction, and, strikingly, its expression was further increased in mice treated with the immunomodulatory tetracyclines, an effect similar to the one observed in *Ccl2* expression. In addition, the colonic inflammatory process has been associated with changes in microbial sensing through TLRs (Franchimont et al., 2004). We observed a significant reduction in *Tlr2* and *Tlr4* expression in DSS-colitic mice; while *Tlr2* levels were restored by all antibiotics, *Tlr4*, highly expressed by enterocytes and required to preserve barrier function and promote its repair (Franchimont et al., 2004; Fukata et al., 2005), was significantly up-regulated by immunomodulatory tetracyclines (Fig4).

When considering the impact on colonic microbiota composition, while no statistical differences were observed in α-diversity at this time point (table 4), inner taxonomic groups showed a higher degree of variation, as it has been described at early stages of intestinal inflammation (Schwab et al., 2014). *Bacteroidetes* abundance was reduced in DSS-control compared to healthy mice, while *Firmicutes* abundance increased. Antibiotic administration to colitic mice counteracted these changes, while DEX treatment showed minor effect (Fig5A). Order level heatmap and clustering analysis ilustrates these results: based on their different composition, mainly of *Bacteroidales,* DSS and DEX animals cluster in a separated branch from antibiotic-treated and healthy mice (Fig5B). However, analysis at lower taxonomic levels revealed that antibiotic treatment showed a divergent impact. PCA at genus level delimitate three different clusters with different microbiota composition: NC control, DSS and DEX-treated colitic mice, and antibiotic-treated groups. No major differences were observed among the antibiotics, which exerted a higher impact in the microbiota composition (distributed along PC1 axis, which explains 39.4% of the variance) than the colitis itself (separated in PC2, accounting for 16.2% of variability) (Fig5C). As an example of this divergence, colitis-associated reduction of *Bacteroidetes* included families such as *Porphyromonaceae* and *Prevotellaceae,* while the impact of antibiotics was greater within *Bacteroidaceae*, mainly due to an increase in the abundance of *Bacteroides acidifaciens*. Within the Phylum *Firmicutes,* antibiotics counteracted the increase in *Bacilli* class observed in colitis and, within it, only MNC and TGC significantly reduced *Lactobacillaceae* family (Fig5A).

Minocycline potentiates the early inflammatory response and promotes mucosal healing and resolution in DSS colitis

Considering their effects on Mφs *in vitro* and the differential immunomodulatory activity observed for tetracyclines *in vivo*, particularly the upregulation of *Ccl2* and *miR-142* associated with the generation of type-2 immunity (Gu et al., 2000; Belz, 2013), we analysed the effects of MNC on the initial immunological events of the intestinal inflammatory process. Once colitis was established after 5 days of 3% DSS treatment, mice were treated with MNC (50 mg/kg) for 2 days; at this time point, the colonic inflammatory status was evaluated biochemically and circulating and colonic Lamina Propria (cLP) immune populations were isolated and analysed by flow cytometry. No major differences were observed in blood leukocytes between NC and DSS-colitic mice; however, a strong increase in circulating neutrophils, eosinophils and monocytic myeloid cells was observed in MNC-treated animals (Fig6A). The analysis of cLP immune cells showed clear differences between healthy and colitic mice, with B cells, $CD4^+$T cells and neutrophils, being raised in the latter. In particular, inflammatory Mφs ($Ly6C^+MHCII^-$) and $FoxP3^+$Tregs accumulated in the colon of colitic mice. We did not detect major changes in cLP immune populations at this time point. However, gene expression and cytokine production analysis in colonic tissue showed important changes related to MNC treatment. As opposed to what was found at later time points (Fig4), the characteristic higher production of Il-1β and Il-6 in DSS-control mice was further increased in MNC-treated mice. Additionally, other inflammatory mediators were also up-regulated in the MNC-treated group in comparison with

DSS-controls, such as *Il-10, Il-2, Ccl2* and *Ccl11* expression, and IL-4, GM-CSF and IL-22 concentration in the supernatant of colonic explants from MNC-treated mice were similarly increased (Fig5C-D).

Then, the effects of MNC were characterised after 4 days of treatment, when MNC intestinal anti-inflammatory activity was fully displayed, with lower DAI values and marked histological improvement (Fig7A-B). At this time point, the systemic immune response in colitic mice was characterised by an increase in circulating myeloid cells, particularly neutrophils. Interestingly, MNC treatment significantly reduced the neutrophilia, while myeloid monocytic cells and eosinophils were still elevated in this group (Fig7C-D).

Flow cytometry analysis of cLP leukocytes showed that the CD45$^+$ cell number was slightly higher in MNC-treated group than in DSS-control, mainly associated with increased presence of CD11b$^+$ myeloid cells (Fig8A-B). Among them, MNC treatment significantly reduced neutrophils while it increased the numbers of eosinophils and monocytic myeloid cells (Fig8C-D). These findings align respectively with increased *Ccl11* and *Ccl2* transcripts detected in the colon of mice after 2 days of MNC treatment (Fig6C). The phenotype of cLP Mφs (CD11b$^+$Ly6G$^-$SSC^{lo}F4/80$^+$) and DCs (SSC^{lo}F4/80$^-$CD11c^{hi}MHC$^+$) was further characterised, confirming that MNC-treated mice presented an increased number of Mφs and DCs in the cLP (Fig8E and H). The monocyte-Mφ differentiation waterfall (Fig8F) illustrates the accumulation of the initial Ly6C^{hi} population in the inflamed intestine. Despite that MNC-treated mice had higher numbers of Mφs in the cLP than the DSS-controls, both groups had similar numbers of inflammatory Mφs, while the intermediate and tissue-resident Mφ populations were significantly increased in MNC-treated mice (Fig8G). Among intestinal DCs, DSS-colitis induced their polarization towards the CD11b$^+$CD103$^+$ phenotype (Fig8I), the main migratory population. MNC treatment resulted in an increase of the total number of DCs in the cLP without altering the polarization of DCs (Fig8H). When considering the lymphoid compartment, a strong B cell infiltrate was observed in colitic mice, which was not modified by MNC treatment. Within the CD3$^+$lymphocyte compartment, no differences were observed in CD8$^+$T cells numbers amongst the different groups (Fig9A). However, the number of cLP CD4$^+$T cells, and particularly of IL-17$^+$ and Foxp3$^+$CD4$^+$T helper cells, was higher in colitic mice than in healthy controls, and these appeared further increased in MNC-treated mice (Fig9A). As observed before, the production of IL-22, a Th17-related cytokine, was increased in colonic explants from colitic animals, being even higher in MNC-treated colitic mice (Fig9B). Higher numbers of IL-4-producing Th2 lymphocytes were found in the cLP of MNC-treated mice, while no differences were observed amongst NC and DSS groups (Fig9A). Additionally, colonic explants from MNC-treated mice produced higher IL-4 than NC and DSS groups (Fig9B). Interestingly, increased numbers and percentages of IL-4$^+$IL-17A$^+$ and IL-17A$^+$FoxP3$^+$

double positive $CD4^+$T cells were also found in the cLP of MNC-treated mice when compared to control groups (Fig9A), which may suggest a higher degree of plasticity between these T cell subsets after MNC treatment (Gagliani et al., 2015). In addition, and in contrast to what was observed after 2 days of treatment, IL-1β and IL-6 cytokine release by colonic explant cultures from the MNC-treated group was now reduced in comparison to the DSS-control (Fig9B). Since eosinophils, Th2 cells and alternatively activated Mφs are actively associated with the resolution phase of acute inflammation, and considering the higher numbers of these cells found in the cLP of MNC-treated mice, we evaluated the expression of *Alox15*, which encodes for the enzyme 12/15-lipoxygenase, involved in the synthesis of pro-resolving lipid mediators (Wang and Colgan, 2017). Interestingly, *Alox15* expression was significantly up-regulated in the colonic tissue of the MNC-treated group compared to the DSS-control (Fig9C).

DISCUSSION

Following previous reports describing a beneficial anti-inflammatory activity obtained with minocycline and doxycycline in pre-clinical models of intestinal inflammation (Huang et al., 2009b, 2009a; Garrido-Mesa et al., 2011a, 2011b, 2015), we aimed to further investigate the potential of this interesting therapeutic family of immunomodulatory antibiotics. We have identified an important link between the effect of immunomodulatory tetracyclines and the activation of specific inflammatory pathways leading to the resolution of inflammation, which supports the potential of these molecules as organ protective agents (Griffin et al., 2010, 2011). Moreover, this study also constitutes the first description of the intestinal anti-inflammatory activity of tigecycline. Our results suggest that the antibiotic activity per se does not exert a significant contribution to the anti-inflammatory effect of tetracyclines in this model of colitis, since all antibiotics had a similar impact in microbiota but no beneficial effect was observed with RFX or TTC. However, immunomodulatory tetracyclines have demonstrated a prompt effect, driving a strong improvement of the epithelial barrier integrity and reducing colitis-associated mortality rate. These findings support the idea that the activation of innate immune protection, as opposed to the immune inhibition caused by dexamethasone, could in fact constitute an advantage in the treatment of intestinal inflammation. Particularly, considering the effects displayed by tetracyclines on macrophages *in vitro*, and the up-regulation of *Ccl2* in the colonic tissue of tetracycline-treated colitic mice, we proposed that a potentiation of the MΦ response might underlay their anti-inflammatory effect. Although sustainably elevated cytokine levels may perpetuate the inflammatory process, an adequate initial inflammatory response is required for an effective recovery. In fact, GWAS have shown that an immune deficit underlies the pathogenesis of Inflammatory Bowel Disease (IBD)(Lees et al., 2011), characterised by diminished cytokine production by monocytes and an impaired ability of the inflammatory response to restore intestinal homeostasis, as reported in Crohn's Disease patients (Marks, 2011). Of note, immunomodulatory tetracyclines potentiated innate cytokine release by LPS-activated BMDM, a mechanism that contributes to clear bacterial infection and promote epithelial barrier protection (Wittkopf et al., 2015). This hypothesis was confirmed *in vivo* when we observed increased innate cytokine release after 2 days of MNC treatment.

MΦ production of IL-1β induces cytokine release by innate lymphoid cells (ILC)-3 (Mortha et al., 2014), which are the initial source of IL-22 upon mucosal damage (Sanos et al., 2009) and the major source of GM-CSF in the gut (Mortha et al., 2014). Considering this, and in view of the enhanced levels of the aforementioned cytokines in MNC-treated mice, the initial events mediating its anti-inflammatory effects may include the promotion of MΦ response and their crosstalk with ILCs. Systemically, we observed higher numbers of circulating myeloid cells after 2 days of MNC treatment, linking with higher levels of GM-CSF, IL-6 and IL-1β, important players in emergency myelopoiesis (Root and Dale, 1999; Hsu et al., 2011). Locally, GM-CSF increases *Ccl2* expression and contributes to the maintenance of the MΦ, DC and Treg populations (Tanimoto et al., 2008; Mortha et al., 2014). IL-22 also has a crucial role in host defence and tissue recovery inducing epithelial proliferation, repair and production of protective molecules, such as mucins, IL-10 and the “alarmins” IL-25, IL-33 and TSLP (Nagalakshmi et al., 2004; Zheng et al., 2008; Lindemans et al., 2015). Additionally, we detected increased IL-2 expression in MNC-treated animals, which may be related to Treg expansion and IL-10 production (Barthlott et al., 2005), as well as to the potentiation of ILC function, inducing IL-22 expression in ILC3s (Crellin et al., 2010) and, together with “alarmins”, driving ILC2 activation and type-2 immune pathways (Roediger et al., 2015; Halim et al., 2016). These favour mucosal protection and oppose to the detrimental immune response observed in chronic inflammatory disorders.

The protective consequences of these immune changes are reflected in the effects evidenced for tetracyclines at later time points, such as the increase in *miR-375*, *Tff-3* and *Tlr4* expression, recovered goblet cell function and improved epitelial barrier integrity (Biton et al., 2011). Subsequent to *Ccl11* and *Ccl2* up-regulation, eosinophils, macrophages and DCs accumulated in the cLP of MNC-treated mice. Eosinophil activity attenuates experimental colitis (Masterson et al., 2015) and increased eosinophils have been found during the remission phase of ulcerative colitis (Lampinen et al., 2005). MNC treatment promoted monocyte recruitment but also their differentiation into $Ly6C^-MHCII^+$ Mφs despite the surrounding inflammatory conditions. GM-CSF, IL-10 and IL-4 have been described to promote the polarization of inflammatory MΦs towards the homeostatic and alternatively activated Mφ phenotypes, implicated in bacterial and apoptotic cell clearance and supporting local regulatory responses and mucosal healing (Hunter et al., 2010; Bain et al., 2013). Similarly, MNC promoted DCs recruitment and the increase in migratory DCs ($CD11b^+CD103^+$) correlated with an increase in $CD4^+$T cell priming in this group. Particularly, higher numbers of Tregs, Th17 and Th2 subsets were present in cLP of MNC-treated mice. Although Th17 cells have initial protective functions in intestinal inflammation, exacerbated Th17 responses can lead to perpetuated inflammation and tissue damage. Of note, Th17 cells can differentiate into Treg cells during the resolution of inflammation (Gagliani et al., 2015), as well as into the Th2 subset in response to IL-4 (Lee et al., 2009), and MNC treatment promoted a higher degree of plasticity among these T cell subsets, particularly between Th17 and Th2. Additionally, CCL2 enhances IL-4 secretion by T cells and elicits Th2 polarising effects, and *miR-142* has an important role in DC priming of Th2 responses (Gu et al., 2000; Belz, 2013), both up-regulated in MNC-treated mice.

Alternative therapeutic strategies that exploit counterregulatory pathways, such as parasites used to skew mucosal immune responses and favour barrier protection, involve alternatively activated macrophages, eosinophils, Th2 cells and Tregs (Smith et al., 2007; Hunter et al., 2010; Gause et al., 2013; Driss et al., 2016). These cells play a key role in the resolution of intestinal inflammation, for example, by producing anti-inflammatory lipid mediators that activate this process (Sherman and Kalman, 2004; Wang and Colgan, 2017). Correlating with these immune changes, *Alox15* was up-regulated in the MNC- treated group, suggesting the initiation of the resolution phase. In fact, at this time point, colonic IL-6 and IL-1β levels dropped, the number of cLP neutrophils was reduced and the efficacy of the treatment was evident both macroscopically and histologically.

All together, these results indicate that the pro-inflammatory actions of immunomodulatory tetracyclines in MΦs, rather than being detrimental, strongly contribute to mucosal protection. The benefits of DXC and MNC, previously reported in experimental colitis (Huang et al., 2009b; Garrido-Mesa et al., 2011a, 2011b, 2015) and in a model of 5-FU induced intestinal mucositis (Huang et al., 2009a), were attributed to their antibiotic activity and other mechanisms such as MMPs inhibition and antioxidant effects (Garrido-Mesa et al., 2013b). We have now demonstrated that immunomodulatory tetracyclines, by promoting the innate immune response, actively induce mucosal healing and lead to an accelerated resolution of the process. This mechanism represents the success of the inflammatory response, aimed at restoring tissue homeostasis (Sherman and Kalman, 2004; Rutgeerts et al., 2007). Even though therapies aimed at a specific target have a special interest in Pharmacology due to the rationale to avoid side effects, there is increasing awareness of their lack of efficacy in complex pathologies such as IBD, due to counter-regulatory pathways that sustain inflammation (Biancheri et al., 2013). By contrast, the high therapeutic benefit observed with tetracycines in preclinical models of IBD and other multifactorial diseases might be precisely related to their pleiotropic properties, influencing different factors involved in the inflammatory response (Griffin et al., 2010; Garrido-Mesa et al., 2013a). The benefit of their non-antibiotic properties has already proved clinical relevance, so it is reasonable to believe that tetracyclines can be repurposed for other non-infectious pathologies in the future, and we hope this report will contribute to draw the attention over these interesting drugs. This, together with their well known and safe profile, makes them very promising candidates for future translational studies into human disease. Similarly, further research into multi-target drugs and ways of exploiting pro-resolving pathways warrants interesting results in complex chronic pathologies (Medina-Franco et al., 2013).

Considering the epidemiological association of antibiotic intake and IBD development (Ungaro et al., 2014), the use of antibiotics seems to be discouraged. However, the disruption caused by antibiotics is particularly relevant earlier in life (Miyoshi et al., 2017; Örtqvist et al., 2018) and, when used to treat IBD patients, it has not been reported that antibiotics worsen or perpetuate the disease. We previously reported that, when taken in a preventative manner, no significant differences were observed, suggesting that the modification of the microbiota in this case had no deleterious effects, neither beneficial (Garrido-Mesa et al., 2011a). On this ground, here we proposed a curative treatment (once inflammation is established). But considering our new findings, indicating that the microbiota composition was not restored in antibiotic-treated groups, and the risks of dysbiosis associated with antibiotic intake, e.g. leading to *C. difficile* infection (Owens et al., 2008), the long-term administration of tetracyclines in this context might seem discouraged as well. Of note, even though their use has not been associated with increased *C. difficile* infection when compared to other antibiotics (Deshpande et al., 2013). With this in mind, the recently developed chemically-modified tetracyclines, devoid of antibiotic activity, offer a promising tool to explore the potential of the immunomodulatory properties of tetracyclines, allowing for long-term therapy if needed (Lokeshwar, 2011). However, the mechanism that we describe here suggests that tetracyclines would be best indicated as short-term treatment to induce remission of acute episodes. Acute intestinal inflammation is the second leading cause of death worldwide, given the high mortality rates found in developing countries (Liu et al., 2012) and it also implies significant costs to developed societies (Glass et al., 2014). Thus, by potentiating host-protective pathways, immunomodulatory tetracyclines become a promising strategy for the treatment of acute intestinal inflammation. Whether tetracyclines can induce remission on a chronified pathology, where the protective effect of innate immunity is overridden, or whether a long-term therapy could be applied are questions that require further investigation in a chronic setting. Nonetheless, considering the defects on innate protective mechanisms that underlie IBD (Marks, 2011), and that the majority of IBD patients experience relapsing acute inflammatory flares (Baumgart and Carding, 2007), therapeutic approaches that aim at restoring these protective mechanisms are the next logical approach in IBD and our findings aling with the current understanding of the disease. The effects described for tetracyclines in this study could benefit the course of the pathology, used as short-term treatment to promote the resolution of the inflammatory flares. Once induced, remission could be maintained by additional therapies with fewer side effects, such as probiotics. Indeed, we have already evaluated the potential of this strategy in previous studies, where minocycline or doxycycline administration was followed by long-term maintenance treatment with probiotics, showing very promising results (Garrido-Mesa et al., 2011b, 2015).

In conclusion, available pharmacological treatments for IBD are mainly aimed at reducing the symptoms of inflammation under an "acceptable" threshold, but they have not succeeded at modifying the course of the disease, and IBD patients without a robust mucosal healing have worse outcomes (Baert et al., 2010). Thus, by striking at the immune system, current pharmacological treatments may interfere with the natural protective pathways activated by the inflammatory response. We have now generated solid evidence of the benefit of strengthening these defensive mechanisms with the administration of immunomodulatory tetracyclines. This adds to the broad range of promising properties exerted by this safe and well-known family of compounds, offering an appealing drug-reposition strategy to manage intestinal inflammatory conditions.

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TABLES

Table 1: 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.

Table 2: 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.

Table 3: RT-qPCR primer sequences

Gene	Gene ID		Sequence5´-3´	Annealing T (ºC)
Gapdh	14433	FW	5'-CCATCACCATCTTCCAGGAG	**60**
		RV	5'-CCTGCTTCACCACCTTCTTG	
Muc-1	17829	FW	5'-GCAGTCCTCAGTGGCACCTC	**60**
		RV	5'-CACCGTGGGGCTACTGGAGAG	
Mic-2	***17831***	FW	5'-GATAGGTGGCAGACAGGAGA	**60**
		RV	5'-GCTGACGAGTGGTTGGTGAATG	
Muc-3	***666339***	FW	5'-CGTGGTCAACTGCGAGAATGG	**60**
		RV	5'-CGGCTCTATCTCTACGCTCTC	
Ttf-3	***21786***	FW	5'-CCTGGTTGCTGGGTCCTCTG	**60**
		RV	5'-GCCACGGTTGTTACACTGCTC	
Zo-1	***21872***	FW	5'-GGGGCCTACACTGATCAAGA	**56**
		RV	5'-TGGAGATGAGGCTTCTGCTT	
Occludin	***18260***	FW	5'-ACGGACCCTGACCACTATGA	**56**
		RV	5'-TCAGCAGCAGCCATGTACTC	
Mmmp-9	***17395***	FW	5'-TGGGGGGCAACTCGGC	**60**
		RV	5'-GGAATGATCTAAGCCCAG	
Inos	***18126***	FW	5'-GTTGAAGACTGAGACTCTGG	**56**
		RV	5'-GACTAGGCTACTCCGTGGA	
Alox15	***11687***	FW	5'-TTTTTGACAAGGAGGTGATGAGC	**57**
		RV	5'-GAAGCAAGTGTCAATATCCAG	
Tlr2	***24088***	FW	5'-CCAGACACTGGGGGTAACATG	**60**
		RV	5'CGGATCGACTTTAGACTTTGGG	
Tlr4	***21898***	FW	5'-GCCTTTCAGGGAATTAAGCTCC	**60**
		RV	5'-AGATCAACCGATGGACGTGTAA	
Cxcl2	***20310***	FW	5'-CAGTTAGCCTTGCCTTTGTTCAG	**62**
		RV	5'-CAGTGAGCTGCGCTGTCCAATG	
Ccl2	***20296***	FW	5'-CAGCTGGGGACAGAATGGGG	**62**
		RV	5'-GAGCTCTCTGGTACTCTTTTG	
Ccl11	***20292***	FW	5'-AGTAACTTCCATCTGTCTCC	**51**
		RV	5'-TGGTGATTCTTTTGTAGCTC	
Tnfα	***21926***	FW	5'-AACTAGTGGTGCCAGCCGAT	**56**
		RV	5'-CTTCACAGAGCAATGACTCC	
Il-1β	***16176***	FW	5'-TGATGAGAATGACCTCTTCT	**55**
		RV	5'-CTTCTTCAAAGATGAAGGAAA	

Il-6	***16193***	FW	5'-TAGTCCTTCCTACCCCAATTTCC	**60**
		RV	5'-TTGGTCCTTAGCCACTCCTTC	
Il-2	***16183***	FW	5'-TGATGGACCTACAGGAGCTCCTGA	**60**
		RV	5'-GAGTCAAATCCACAACATGCC	
Il-10	***16153***	FW	5'-TCCTTAATGCAGGACTTTAAGGG	**56**
		RV	5'-GGTCTTGGAGCTTATTAAAAT	
Il-4	***16189***	FW	5'-AGCTAGTTGTCATCCTGCTC	**53**
		RV	5'-AGTGATGTGGACTTGGACTC	
Gm-csf	***16981***	FW	5'-CTACTACCAGACATACTGCC	**51**
		RV	5'-GCATTCAAAGGGATATCAG	
miR-142-3p		FW	5'-UGUAGUGUUUCCUACUUUAUGGA	**55**
miR-150-5p		FW	5'-UCUCCCAACCCUUGUACCAGUG	**55**
miR-155-5p		FW	5'-UUAAUGCUAAUUGUGAUAGGGGU	**55**
miR-375-3p		FW	5'-UUUGUUCGUUCGGCUCGCGUGA	**55**

Table 4: α-diversity measures of intestinal microbiota

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
DSS	**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

Table 4: Comparison of α-diversity measures of intestinal microbiota between non-colitic group (NC) (n=8), DSS-colitic group (DSS) (n=8), and rifaximin (RFX) (n=4), tetracycline (TTC) (n=4), doxycycline (DXC) (n=5), minocycline (MNC) (n=5), tigecycline (TGC) (n=5) and dexamethasone (DEX) (n=4) treated groups in the DSS model of mouse colitis. Data expressed as means ± SEM.

FIGURE LEGENDS

Figure 1: Comparative study of the effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) on MΦ activity *in vitro*. **A)** Nitrite production by LPS-stimulated RAW 264 MΦs. 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. Data is expressed as mean ± SEM (n=6). **B)** *Inos* mRNA expression quantified by real-time PCR and **C-E)** Cytokine concentration in the culture supernatant quantified by ELISA, in LPS-stimulated (10 ng/ml) BMDM after 24h of pre-incubation with the different treatments (25µM). Data expressed as mean ± SEM (n=6). Fold increase is calculated *vs.* unstimulated untreated cells. *P<0.05 *vs*. stimulated untreated cells.

Figure 2: Comparative study of the intestinal anti-inflammatory effects of rifaximin (RFX), tetracycline (TTC), doxycycline (DXC), minocycline (MNC), tigecycline (TGC) and dexamethasone (DEX) in DSS-fatal colitis. NC: Non-colitic group, DSS: DSS-colitic group. (n=10, DSS n=20) **A)** Schematic illustration of the experimental design and Disease Activity Index (DAI) values (means) assigned based on the criteria described in table 1. **B)** Survival curves (%) of the different groups during the 6-day treatment period and their P values *vs.* DSS control group.

Figure 3: 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. **A)** Schematic illustration of the experimental design followed and Disease Activity Index (DAI) mean values assigned based on the criteria described in table 1, during the 9-days experimental period (n=8, DSS n=14). **B)** Microscopic damage score assigned according the criteria described table 2. **C)** Representative histological sections of colonic mucosa of the different experimental groups stained with haematoxylin, eosin and alcian blue (40x magnification). **D)** Colon mRNA expression quantified by real-time PCR. Fold increase calculated *vs.* NC group. Boxes graph represents ±SEM range, median (middle line) and extreme values (whiskers) (NC, DSS, DXC, MNC and TGC, n=6; RFX, TTC and DEX, n=5). *P<0.05 *vs.* DSS control group.

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(whiskers) (NC, DSS, DXC, MNC and TGC, n=6; RFX, TTC and DEX, n=5). *P<0.05 *vs.* DSS control group.

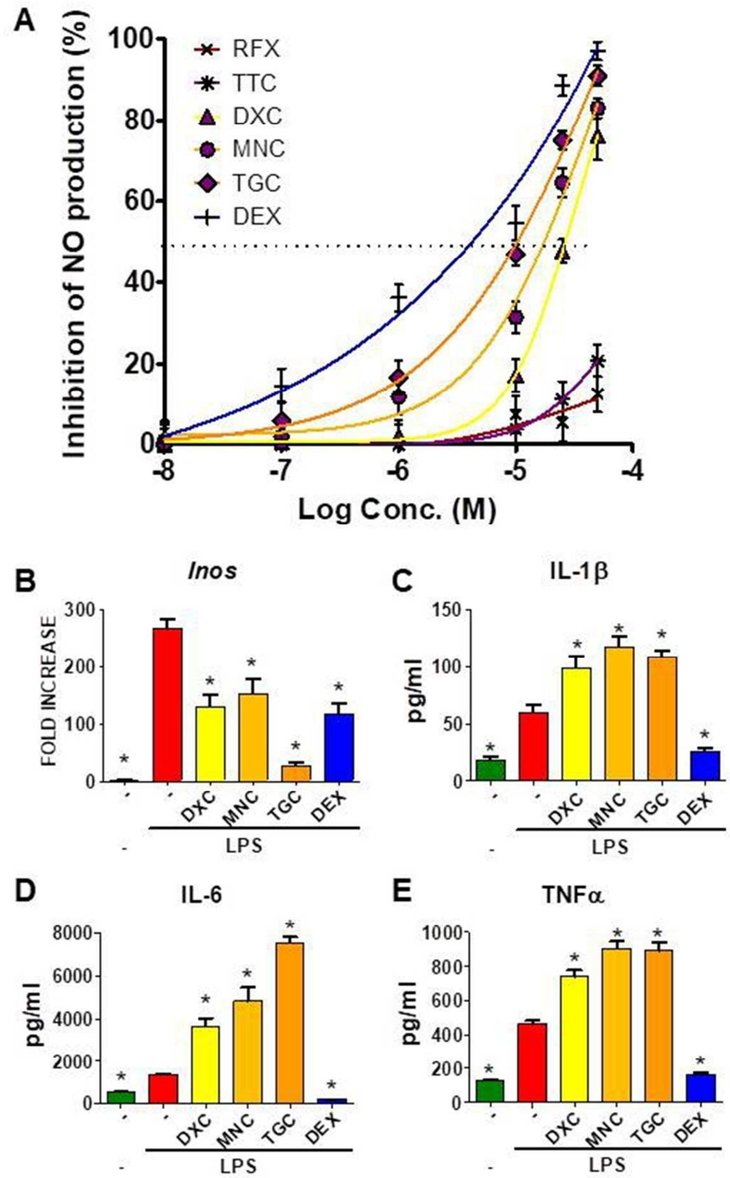
Figure 5: Comparison of microbiota composition between Non-colitic group (NC) (n=8), DSS-colitic group (DSS) (n=8), and rifaximin (RFX) (n=4), tetracycline (TTC) (n=4), doxycycline (DXC) (n=5), minocycline (MNC) (n=5), tigecycline (TGC) (n=5) and dexamethasone (DEX) (n=4) treated groups in the DSS model of mouse colitis. **A)** Relative abundance of various taxonomic groups. Data expressed as means ± SEM. **B)** Heatmap with relative abundance of the 10 most abundant orders, include hierarchical clustering of samples based on order level composition analysed with the method of minimum variance of Ward **C)** 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.

Figure 6: Evaluation of the effects of 2 days of minocycline treatment on the immune response in DSS-colitic mice. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group. **A)** % of the indicated immune cell populations in the blood of the different experimental groups. **B)** Analysis of immune cell populations in the cLP of the different experimental groups. Absolute cell numbers of B cells (B220$^+$), CD4$^+$T cells (CD3$^+$), Tregs (CD3$^+$CD4$^+$FoxP3$^+$), neutrophils (Ly6G$^+$) and Ly6C$^+$MHCII$^-$ Mφs. Scatter plots represent individual values (dots) and mean ± SEM. **C)** colon mRNA expression quantified by real-time PCR. Fold increase calculated *vs.* NC group. **D)** Cytokine concentration in the supernatant of colonic explant cultures quantified by ELISA. Boxes graph represents ±SEM range, median (middle line) and extreme values (whiskers) (n=5). *P<0.05 *vs.* DSS control group, #P>0.05 vs NC control group.

Figure 7: Evaluation of the effects of 4 days of minocycline treatment on the immune response in DSS-colitic mice. **A)** Schematic illustration of the experimental design followed and Disease Activity Index (DAI) values (means ± SEM) over the 9-day experimental period, calculated based on the criteria described in table 1. NC: Non-colitic group DSS: DSS-colitic group, MNC: minocycline-treated colitic group (50 mg/kg/d) (n=7). **B)** Histological sections of colonic mucosa stained with haematoxylin, eosin and alcian blue (40x magnification) and microscopic damage score assigned according the criteria described in table 2. Boxes graph represents ±SEM range, median (middle line) and extreme values (whiskers). **C)** Representative flow cytometry analysis of circulating CD45$^+$ cells. **D)** Percentage of the indicated cell populations within the CD45$^+$ cells present in the blood of NC, DSS and MNC mice. Scatter plots represent individual values (dots) and mean ± SEM. (n=7, DSS n=6) *P<0.05 *vs.* DSS control group, #P>0.05 vs NC control group.

Figure 8: Evaluation of the effects of 4 days of minocycline treatment on the cLP immune response during DSS colitis. NC: Non-colitic group, DSS: DSS-colitic group, MNC: minocycline-treated colitic group (50 mg/kg/d) (n=7, DSS n=6). **A)** Representative flow cytometry analysis of live cells from the cLP showing the CD11b$^+$ and CD45$^+$ cell populations. **B)** Absolute cell numbers of immune cells (CD45$^+$) and CD45$^+$CD11b$^+$ myeloid cells. **C)** Representative flow cytometry analysis of CD11b$^+$ cells from the cLP showing the Ly6G$^+$ and SiglecF$^+$ populations. **D**) Absolute cell numbers of: neutrophils (Ly6G$^+$), eosinophils (SiglecF$^+$) and myeloid monocytic cells (CD11b$^+$ Ly6G$^-$ SiglecF$^-$ SSC^{lo}). **E)** Total number of MΦs (CD11b$^+$Ly6G$^-$SSC^{lo}F4/80$^+$ cells) **F)** Representative flow cytometry plots showing showing the expression of Ly6G and MHCII by MΦs from the cLP and illustrating the monocyte-MΦ waterfall. G**)** Percentage (left) and absolute cell numbers (right) of: Inflammatory MΦs (Ly6C$^+$MHCII$^-$ cells), Intermediate MΦ population (Ly6C$^+$MHCII$^+$ cells) and Resident intestinal MΦs (Ly6C$^-$MHCII$^+$ cells). **H)** Absolute cell numbers of DCs (Ly6G$^-$SSC^{lo}F4/80$^-$ CD11c^{hi}MHCII$^+$) and percentage of CD11b$^+$CD103$^+$ DCs. **I)** Representative flow cytometry analysis of DCs from the cLP showing the expression of CD103 and CD11b. Scatter plots represent individual values (dots) and mean ± SEM. *P<0.05 *vs.* DSS control group, #P>0.05 vs NC control group.

Figure 9: Evaluation of the effects of 4 days of minocycline treatment in the immune response in the cLP during DSS colitis. NC: Non-colitic group; DSS: DSS-colitic group; MNC: minocycline-treated colitic group (50 mg/kg/d) (n=7, DSS n=6). **A)** Absolute cell numbers of lymphocyte populations. Scatter plots represent individual values (dots) and mean ± SEM. **B)** Cytokine concentration in the culture supernatant of colonic explants culture quantified by ELISA. **C)** Colonic mRNA expression of Alox15 quantified by real-time PCR. Boxes graph represents ±SEM range, median (middle line) and extreme values (whiskers). *P<0.05 *vs.* DSS control group, #P>0.05 vs NC control group.



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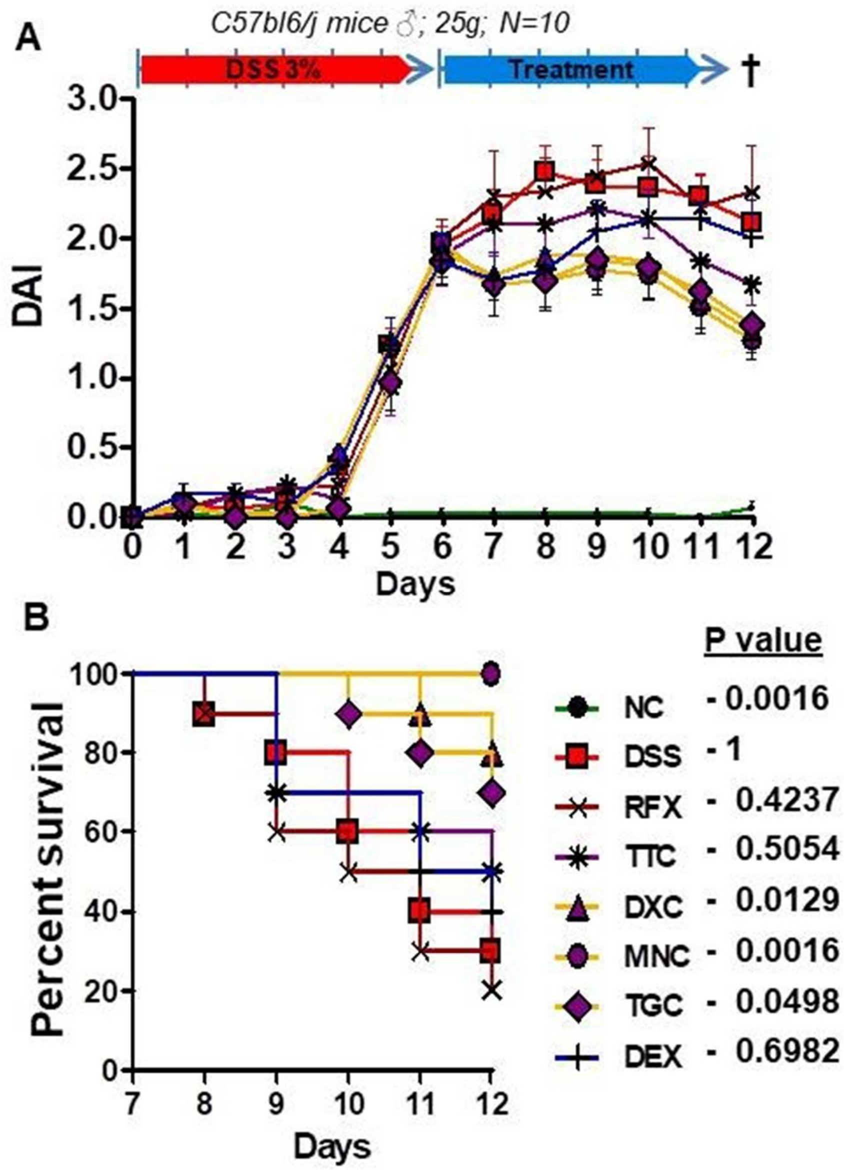

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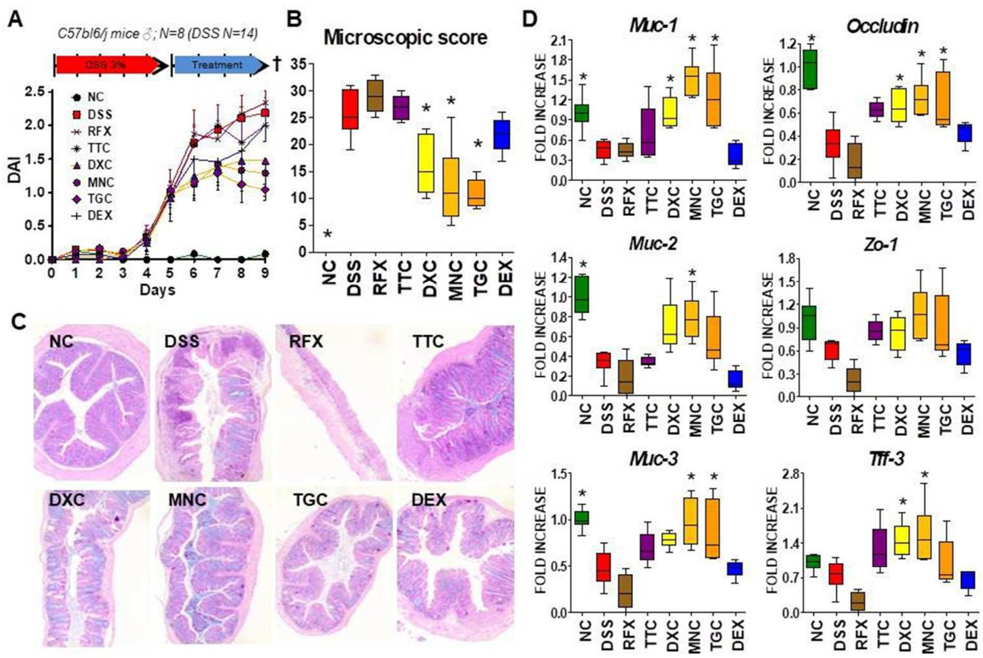

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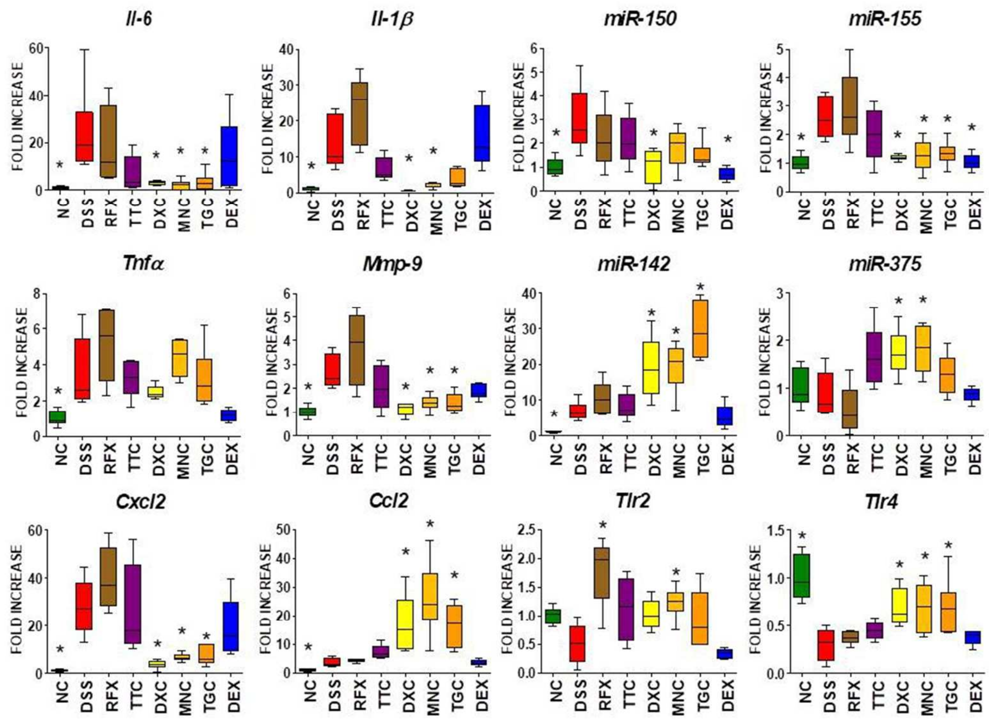

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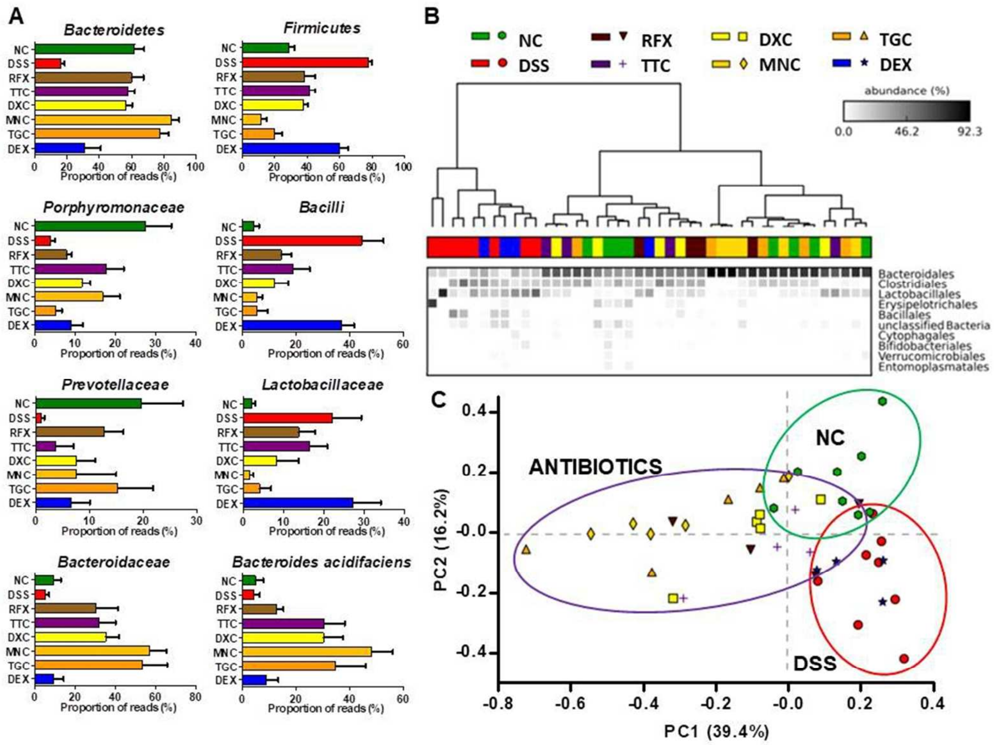

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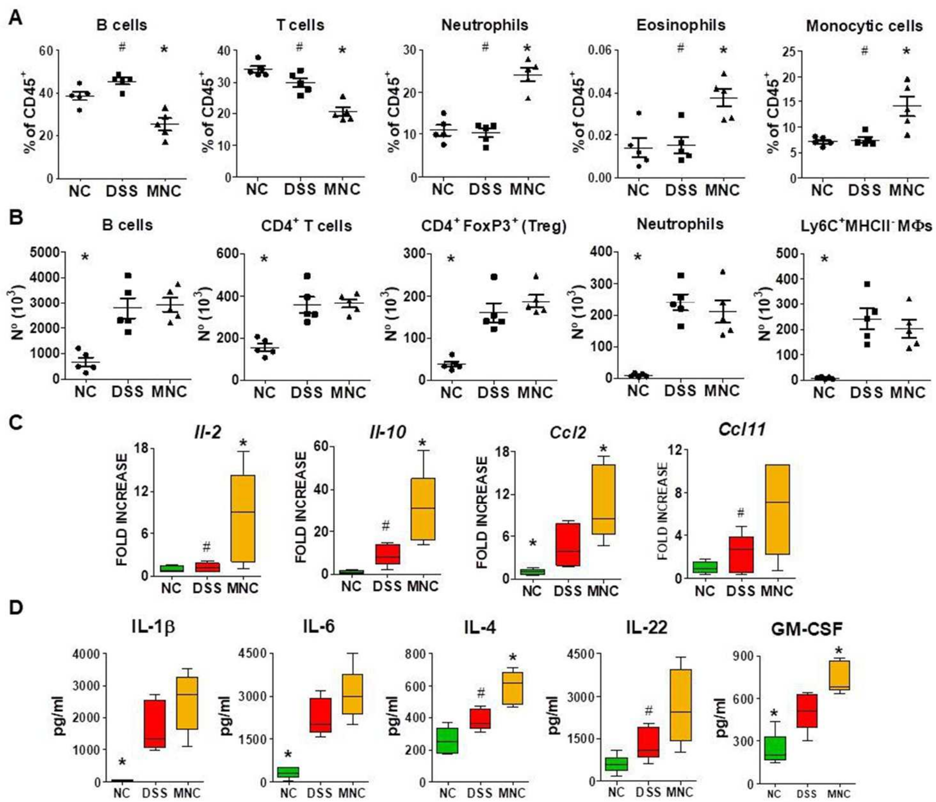

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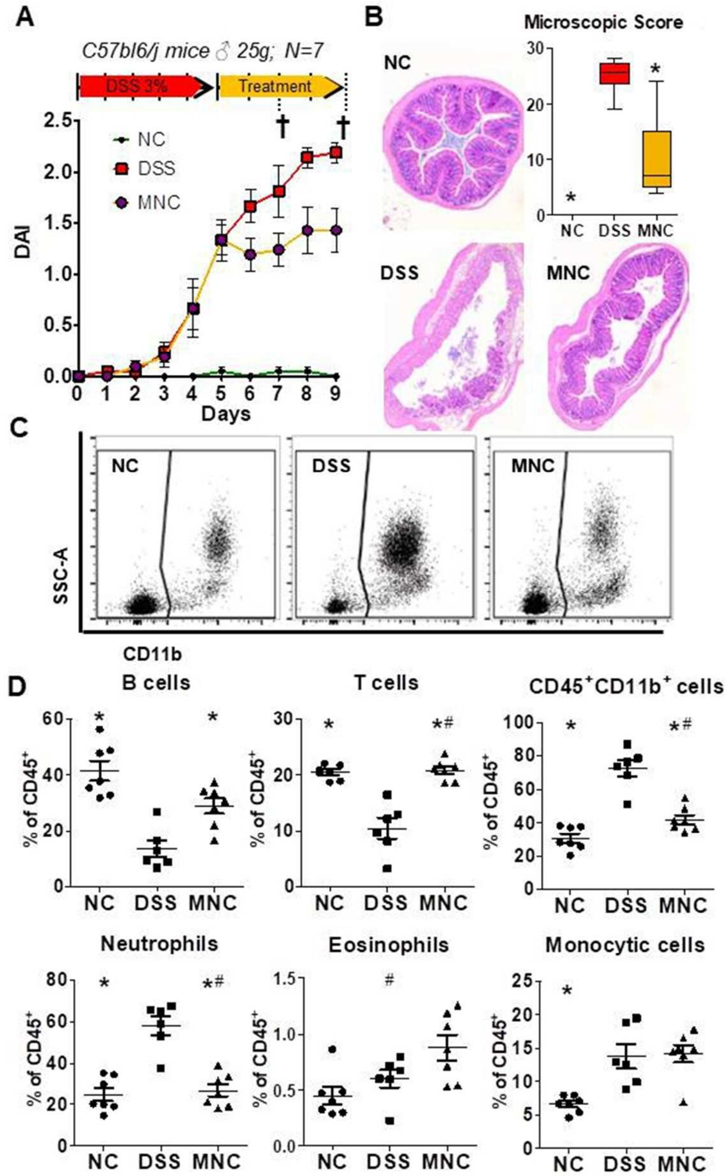

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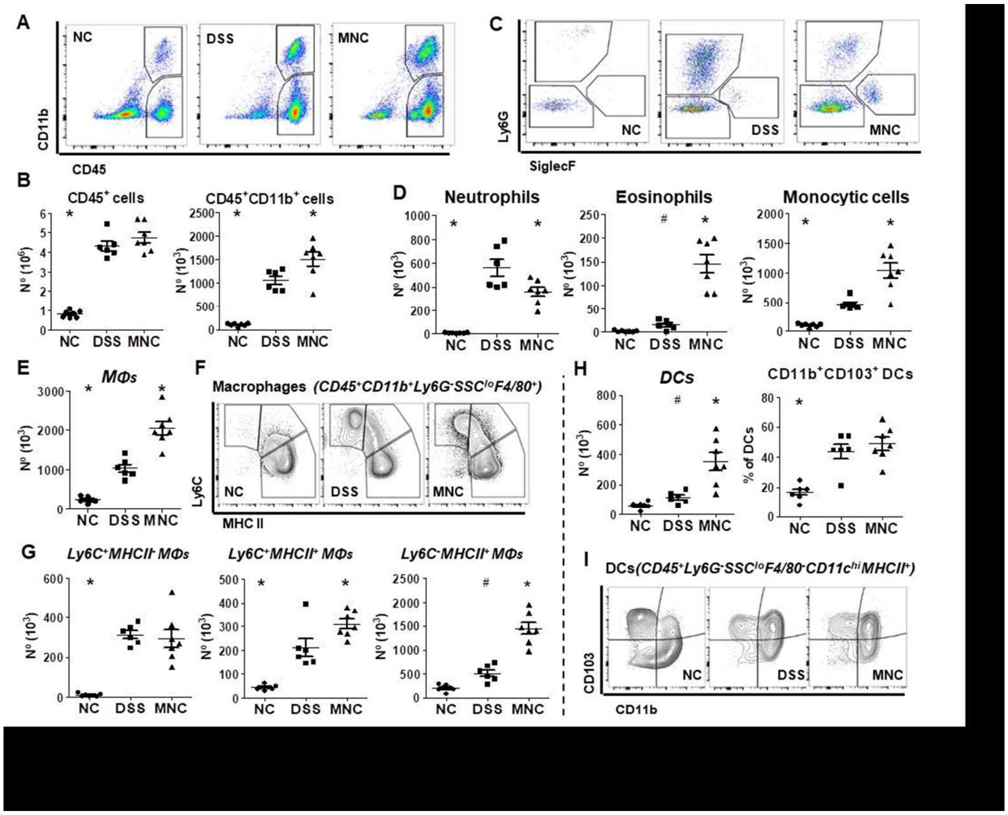

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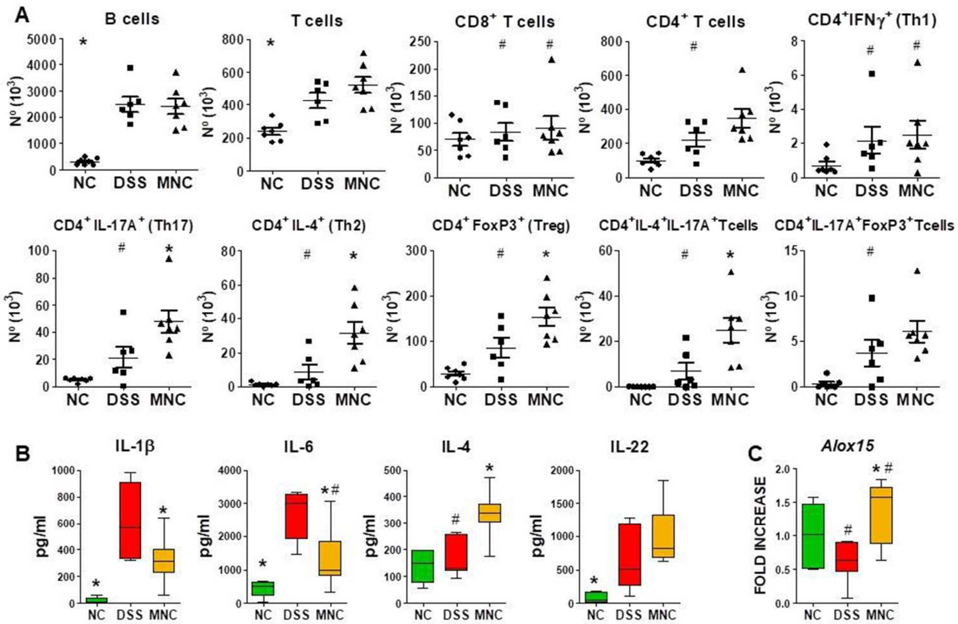

Figure 9: Evaluation of the effects of 4 days of minocycline treatment in the immune response in the cLP during DSS colitis. NC: Non-colitic group; DSS: DSS-colitic group; MNC: minocycline-treated colitic group (50 mg/kg/d) (n=7, DSS n=6). A) Absolute cell numbers of lymphocyte populations. Scatter plots represent individual values (dots) and mean ± SEM. B) Cytokine concentration in the culture supernatant of colonic explants culture quantified by ELISA. C) Colonic mRNA expression of Alox15 quantified by real-time PCR. Boxes graph represents ±SEM range, median (middle line) and extreme values (whiskers). *P<0.05 vs. DSS control group, #P>0.05 vs NC control group.

171x109mm (150 x 150 DPI)

Dear Dr Garrido Mesa,

Re 2018-BJP-0110-RP.R1: "Immunomodulatory tetracyclines shape the intestinal inflammatory response inducing mucosal healing and resolution."

Your revised paper has been seen by an editor and expert referees. I enclose below the comments received that set out a number of additional points which will need your attention before we can consider the submission further. I would urge you to give these points your careful attention.

I hope that you will be prepared to make the necessary amendments and submit a revised manuscript within three months. This should be accompanied by a statement of how you have responded to the criticisms raised, preferably numbered point by point. Should you decide that you do not wish to submit a revised manuscript to BJP, please contact the Editorial Office so we may withdraw your manuscript from the system.

Please highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text. Please read the author instructions carefully prior to re-submission.

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Thank you for submitting your work to the British Journal of Pharmacology.

Yours sincerely,

Dr Mark Giembycz

Senior Editor, British Journal of Pharmacology
giembycz@ucalgary.ca

Senior Editor

The sample sizes in Table 4 must be at least N = 5. Please revise.
The authors must also provide clinical significance for their findings as suggested by reviewer 3.
The role of gut microbiota that is also raised by reviewer 3 must be addressed.

Comments to the Author
One of the referees has serious doubts about the clinical significance of the study. Please address this issue in any revision.

Dear Dr. Giembycz

The data presented in table 4 was obtained in the same pyrosequencing analysis as the results showed in Figure 5. As we mentioned in more detail in our previous response to reviewers comments concerning sample size: Due to technical problems (no amplification in some samples) and loss of sample recovery, particularly in the groups with lower or no therapeutic effect (owing to the diarrheic process), we could only reach n=4. Therefore, beside the most relevant groups of the study have an n≥5 (untreated controls and the tree groups treated with immunomodulatory tetracyclines), to comply with the guidance of BJP, the statistical analysis is not shown for the microbial data (figure 4 and 5). This was mentioned in the text (lines 232-235). We apologise for the reduced N in these groups, but even though we tried to sequence again some of the samples that did not amplify, we got the same lack of result. Please also consider the fact that many researchers find the same difficulties and have to report reduced sample size in these studies (Yuksel et al., 2015; Kim et al., 2017; Shin et al., 2017).

Please find bellow our response to the concerns raised by reviewer 3 concerning the clinical relevance of the study and the role of intestinal microbiota. Some of these questions were already answered in the response to reviewers 1 and 2, and modifications were already included in the previous version of the manuscript regarding these topics (red coloured text). We have now included additional changes in the manuscript in order to emphasize the clinical relevance of our findings and set the microbial studies in the context of the role of the microbiota in these conditions (new modifications highlighted in yellow). However, despite the interesting role of dysbiosis and antibiotics in the aethiology of IBD (well known), antibiotics have been long used, and they are still used, to treat IBD without published record of a negative impact on the course of the disease. On this ground, here we proposed a curative treatment (once disease is established) and our results indicate that the impact of tetracyclines on the microbiota does not have a major contribution to their effect, whereas the novel immunomodulatory mechanism described here does it. Therefore, while we have extensively answered below to reviewer 3, we believe that these novel findings should be the focus of the discussion and the manuscript. May you consider appropriate to include any additional comment, please let us know and we will undertake the suggested amendments.

Reviewers' Comments to Author:

Reviewer: 2

Comments to the Author

Dear Authors,

The revised manuscript represents the contents and the discussion points well. The point-to-point response to the reviewers' comments are concise and clear.

Reviewer: 3

Comments to the Author

This manuscript is of good quality, well written and I have no specific complaints on the methodology. However, one limitation of the scientific proposal is the lack of a clinical view of this approach. There is no mention about the impact of tetracycline treatment on gut microbiota. The microbiota is one of the most important regulators of gut homeostasis, and its alteration has been linked to IBD triggering and perpetuation in humans. The immunomodulatory effect of tetracycline (already known) appears very limited without a look at what happens on microbiota today. It is a good manuscript, but lacking of a more modern view of IBD treatments, and in this form remains a an end in itself.

We completely agree with the reviewer about the relevance of the microbiota in intestinal inflammatory conditions. This was indeed one of the reasons why we performed a comparative study including different antibiotics (figures 2-5). We did not only evaluate their intestinal anti-inflammatory effect here, but we specifically assessed the impact of the treatments in the microbiota composition (shown in table 4 and figure 5). These studies showed that the impact exerted on the microbiota composition by all the antibiotics tested was very similar, while only those with immunomodulatory properties ameliorated the inflammatory response, suggesting that the immunomodulatory effect is significantly more relevant than the antibiotic one. Therefore, this study encourages and provides the scientific support for the development and evaluation of tetracycline-based molecules, devoid of antibiotic properties but retaining the immunomodulatory potential leading to the activity described here for the first time. This was discussed in lines 404-407, 478-481, 508-517 and 529-535 of the manuscript (new modifications highlighted in yellow, previous modifications in red text)

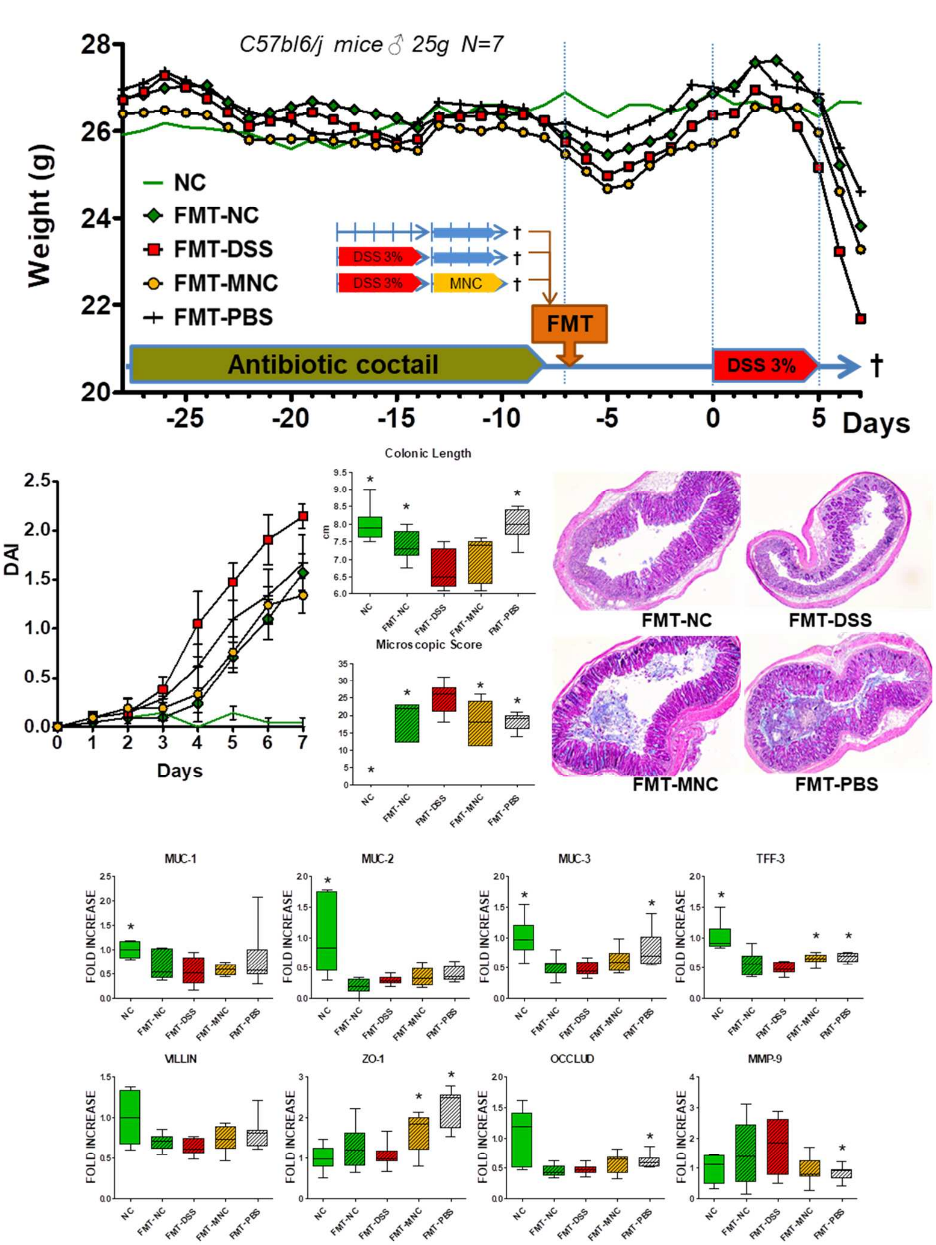
We are well aware of the epidemiological reports that associated antibiotic intake and IBD development, i.e. (Shaw et al., 2011; Ungaro et al., 2014). But as the most recent studies have determined, the disruption cause by antibiotics leading to IBD is particularly relevant earlier in life (Miyoshi et al., 2017; Aniwan et al., 2018; Örtqvist et al., 2018), not as an immediate trigger. This indicates that the cause-effect relationship is not only the microbial dysbiosis, but also the impact on the development of the immune system (Schulfer et al., 2018). But it is obvious that antibiotics play a role, we are not denying that, and we do find this field interesting

and relevant for consideration. However, this association does not apply to our study and the proposed clinical applications for several reasons:

1) **We are not suggesting the use of antibiotics in patients without IBD or even as a preventive treatment in IBD patients before the development of an inflammatory flare, but as a curative short-term treatment. This is also based on a previous report describing that, when taken in a preventative manner, no significant differences were observed in between untreated and minocycline-treated mice, suggesting that the modification of the microbiota in this case had no deleterious effects, neither beneficial** (Garrido-Mesa et al., 2011a)**.**
2) **For the application suggested here, it is interesting to note the lack of reports describing that antibiotic intake by IBD patients is linked to a perpetuation of the disease (we couldn't find any at least). Considering the availability of studies describing the role of antibiotics in the aetiology of IBD (some referenced above) but the lack of the others, it seems that once the pathology/susceptibility has been developed, microbial disruption by antibiotics may not have such an important impact. Indeed, antibiotics have been long used in IBD, so it is likely that a deterioration of the pathology would have already been described. So clinicians still consider antibiotics among the therapeutic arsenal for IBD and there are even clinical trials including antibiotics due to their antibiotic properties, and tetracyclines in particular: https://clinicaltrials.gov/ct2/show/NCT02606032?term=doxycycline&cond=IBD&rank=4;https://clinicaltrials.gov/ct2/show/NCT02033408?term=doxycycline&cond=IBD&rank=1;https://clinicaltrials.gov/ct2/show/NCT01783106?term=doxycycline+AND+Antibiotics&rank=3. Therefore, antibiotics are used by clinitians despite the aetiological association and, on this ground, immunomodulatory tetracyclines could provide a therapeutic advantage over other antibiotics. As a short-term treatment (3-7 days), these drugs could push the on-going inflammation down a resolution pathway that could be maintained with "microbiota-friendly" drugs such as probiotics, a therapeutic combination that we previously reported to be effective in relapsing experimental colitis** (Garrido-Mesa et al., 2011b, 2015)**. Also take in consideration that the potential benefit of the activity described here should not only be limited to IBD, but also to other intestinal inflammatory conditions.**
3) **Having said that, we do share the reviewers concern about the adverse effects of the antibiotic action of tetracyclines, even when given therapeutically and not for a long-period. But no drug is devoid of adverse effects, so this should not be a limitation for its evaluation, but further encourage studies in order to improve it. Especially when the therapeutic option has proved added interest, as this and previous reports do. Following on from the results obtained here (it is not our intention for this report to remain as an end in itself), we have initiated the evaluation of one chemically-modified tetracycline provided by Galderma, which we hope will retain the anti-inflammatory mechanisms described here without the disadvantage of the antibiotic activity. These compounds are currently being**

evaluated in clinical trials for their potential to inhibit metastasis. Additionally, we have also initiated studies to determine the functional role of the microbiota changes observed here. We have started with faecal transference experiments, giving microbiota derived from NC, DSS and MNC groups (similar setting used in this study) to recipient mice and evaluating the differential susceptibility to DSS colitis. We observed that mice receiving microbiota derived from NC and MNC groups showed slightly reduced susceptibility than the mice receiving DSS faecal contents. These results are too preliminary and we need to further characterised and confirm these results, but it is at least encouraging since we did not observed a negative impact.

Functional assessment of MNC-treated DSS-colitis microbiota



We have further discussed the impact of the microbiota and the possible implications in the revised version of the manuscript (lines 136-139 and 500-507, in addition to previously related comments in lines 404-407, 478-481, 508-517 and 529-535), but please note that these are not the most interesting results of the study and, therefore, should not be the focus of the manuscript.

Regarding the clinical relevance, it is evident that our studies are preclinical, and the relevance and clinical significance of these findings will have to be assessed in the appropriate clinical studies. However, from a pharmacological point of view, we believe that the results of our study, and the potential of the novel mechanism described here, encourage to continue with this line of research. Although the presence of immunomodulatory effects on tetracyclines has previously been reported, their ability to potentiate a protective innate response has never been described before until now. Indeed, our findings align with the more modern view of IBD pathology: considering how our understanding has evolved in recent years, and the key role that we now know defects in innate immunity and mucosal barrier play in IBD pathogenesis, the development and study of new therapeutic approaches that aim at restoring these protective mechanisms is the next logical approach in IBD.

As commented in the manuscript, the fact that these drugs are already approved and have been safely used in clinical practice for over 40 years is of key relevance for any human translational studies. It is also relevant to mention their evaluation and used in non-infectious pathologies (Garrido-Mesa et al., 2013) **and, in fact, there are numerous clinical trials testing these drugs (255 for minocycline, 295 for doxycycline and 68 for tigecycline), not only in infectious conditions (https://clinicaltrials.gov/). Having these antibiotics proved clinical relevance besides their antibiotic properties previously, it is reasonable to believe they can also be repurposed for other pathologies in the future, and we hope this report will contribute to draw the attention of clinicians and pharmaceutical companies into the potential of the mechanism described here. Unfortunately, it is not in our hands to initiate the required clinical trials, but to provide scientific base for others to carry on with this therapeutic opportunity.**

We have modified the manuscript to include some the comments mentioned above to emphasise the clinical relevance of our findings and to show how this therapeutic option could impact IBD therapy (new modifications included in lines 492-495 and 527-529, in addition to previously related comments in lines 35-36, 71-77, 399-404, 414-422, 481-492, 495-499, 513-524 and 536-545).

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29th July 2018

Dear Dr Mark Giembycz

Re: Immunomodulatory tetracyclines shape the intestinal inflammatory response inducing mucosal healing and resolution. Garrido-Mesa *et al*.

Please find attached a detailed response to the concerns raised by the reviewer, where we have addressed the issues regarding the implications of the antibiotic impact on the microbiota as well as we have explained the reasons that support the clinical relevance of the novel activity described in here. The manuscript has being revised accordingly to highlight these points.

We hope you find the amendments made appropriated and you consider the revised manuscript suitable for publication.

With best wishes

Natividad Garrido-Mesa (on behalf of the authors)

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