

## Basic Study

**Anti-tumor necrosis factor  $\alpha$  therapy associates to type 17 helper T lymphocytes immunological shift and significant microbial changes in dextran sodium sulphate colitis**

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## Abstract

### BACKGROUND

Anti-tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) represents the best therapeutic option to induce mucosal healing and clinical remission in patients with moderate-severe ulcerative colitis. On the other side gut microbiota plays a crucial role in pathogenesis of ulcerative colitis but few information exists on how microbiota changes following anti-TNF $\alpha$  therapy and on microbiota role in mucosal healing.

### AIM

To elucidate whether gut microbiota and immune system changes appear following anti TNF $\alpha$  therapy during dextran sulfate sodium (DSS) colitis.

### METHODS

Eighty C57BL/6 mice were divided into four groups: “No DSS”, “No DSS + anti-TNF $\alpha$ ”, “DSS” and “DSS + anti-TNF $\alpha$ ”. “DSS” and “DSS + anti-TNF $\alpha$ ” were treated for 5 d with 3% DSS. At day 3, mice within “No DSS+anti-TNF $\alpha$ ” and “DSS+anti-TNF $\alpha$ ” group received 5 mg/kg of an anti-TNF $\alpha$  agent. Forty mice were sacrificed at day 5, forty at day 12, after one week of recovery post DSS. The severity of colitis was assessed by a clinical score (Disease Activity Index), colon length and histology. Bacteria such as *Bacteroides*, *Clostridiaceae*, *Enterococcaceae* and *Fecalibacterium prausnitzii* (*F. prausnitzii*) were evaluated by quantitative PCR. Type 1 helper T lymphocytes (Th1), type 17 helper T lymphocytes (Th17) and CD4<sup>+</sup> regulatory T lymphocytes (Treg) distributions in the mesenteric lymph node (MLN) were studied by flow cytometry.

### RESULTS

Bacteria associated with a healthy state (*i.e.*, such as *Bacteroides*, *Clostridiaceae* and *F. prausnitzii*) decreased during colitis and increased in course of anti-TNF $\alpha$  treatment. Conversely, microorganisms belonging to *Enterococcaceae* genera, which are linked to inflammatory processes, showed an opposite trend. Furthermore, in colitic mice treated with anti-TNF $\alpha$  microbial changes were associated with an initial increase (day 5 of the colitis) in Treg cells and a consequent decrease (day 12 post DSS) in Th1 and Th17 frequency cells. Healthy mice treated with anti-TNF $\alpha$  showed the same histological, microbial and immune features of untreated colitic mice. “No DSS + anti-TNF $\alpha$ ” group showed a lymphomononuclear infiltrate both at 5<sup>th</sup> and 12<sup>th</sup> d at hematoxylin and eosin staining, an increase of in Th1 and Th17 frequency at day 12, an increase of *Enterococcaceae* at day 5, a decrease of *Bacteroides* and *Clostridiaceae* at day 12.

### CONCLUSION

Anti-TNF $\alpha$  treatment in experimental model of colitis improves disease activity but it is associated to an increase in Th17 pathway together with gut microbiota alteration.

**Key words:** Gut microbiota; Dextran sodium sulphate colitis; Immune system; T cells; Mesenchymal lymphnode; Tumor necrosis factor  $\alpha$

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**Core tip:** The effect of the gut microbiota on the immune responses is not limited to the gut. On the other hand, autoimmune diseases, such as inflammatory bowel disease, are associated to different degree of dysbiosis involving different bacterial genera. Using a colitic mouse model, we aimed to evaluate the impact of anti-tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) therapy on the intestinal immune system and gut microbiota concurrently. Healthy mice treated with anti-TNF $\alpha$  showed similar histological, microbial and immune features of untreated colitic mice, a lymphomononuclear infiltrate both at day 5 and 12 at hematoxylin and eosin staining, an increase of type 1 helper T lymphocytes and type 17 helper T lymphocytes at day 12, and finally increase of *Enterococcaceae* at day 5, a decrease of *Bacteroides* and *Clostridiaceae* at day 12.

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, relapsing, inflammatory disorder of the gastrointestinal tract due to a dysregulated immune response towards the microbiota<sup>[1]</sup>. Although many factors underlying IBD have been identified, the exact etiology is unknown, reflecting its complexity and the broad spectrum of its manifestations. IBD includes ulcerative colitis (UC) and Crohn's disease (CD), which show differences in the pathology and clinical characteristics<sup>[2]</sup> and with an increasing incidence, especially in adolescence<sup>[3-6]</sup>, becoming a global disease<sup>[7]</sup>.

IBD is a multifactor disease where environment, genetics, hygiene and microbiota interact with host immune system in a dynamic equilibrium<sup>[8,9]</sup>. This equilibrium between gut microbiota and immune system is stable throughout adulthood and in health condition, while it is perturbed in diseases like IBD and in predisposing risk conditions<sup>[10-12]</sup>. Recent studies indicate that a healthy commensal gut microbiota stimulates CD4<sup>+</sup> regulatory T lymphocytes (Tregs) that, in turn, are responsible for maintaining gut homeostasis<sup>[12]</sup>. Under homeostatic conditions, a balance exists between the production of pro-inflammatory cytokines by CD4<sup>+</sup> T lymphocytes and anti-inflammatory cytokines [e.g., interleukin (IL)-10] by Tregs<sup>[13]</sup>. The phyla *Bacteroidetes* and *Firmicutes* dominate the gut microbiota of healthy humans<sup>[14]</sup>.

In course of IBD a decrease of *Firmicutes* [and, among these, the species *Fecalibacterium prausnitzii* (*F. prausnitzii*)<sup>[15]</sup> and *Bacteroidetes* occurs with an increase in *Actinobacteria* and *Proteobacteria*<sup>[16,17]</sup>. Among genera a decrease of *Bacteroidaceae* and an increase of *Enterobacteriaceae* (specifically *Escherichia coli*) and *Clostridiaceae*<sup>[18-20]</sup> has been described, together with a reduced phylogenetic diversity associated to an increase of T lymphocyte differentiation toward type 1 helper T lymphocytes (Th1), type 17 helper T lymphocytes (Th17) subsets and a decrease of Treg<sup>[21-24]</sup>.

How these conditions are modified in course of medical treatment aimed to control active and chronic gastrointestinal inflammation, and whether this could have a role in non-response rates to biologic agents<sup>[24]</sup>, is not known. In a previous study we showed the importance of local immunity in course of anti-tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) therapy using animal model of IBD<sup>[25]</sup>. In this study, we aim to understand whether anti-TNF $\alpha$  therapy could affect the gut microbiota and intestinal mucosa immune activation in experimental model of this disease.

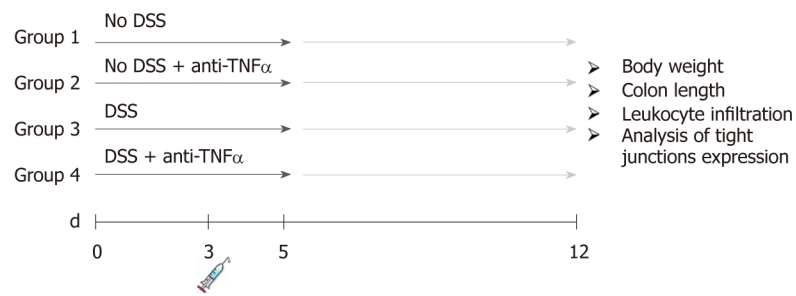
## MATERIAL AND METHODS

### Experimental acute colitis

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. C57BL/6 mice, 8 wk old, were fed for 5 d with 3% dextran sulfate sodium (DSS) polymers in drinking water (MP Biomedicals, Aurora, OH, United States) provided *ad libitum*. Mice were divided into four groups (Figure 1). Following three days from DSS treatment (day 3), two groups received an anti-TNF $\alpha$  drug iv [infliximab (IFX) 5 mg/kg]. Every day weight, fecal consistency, body weight of mice and the presence of occult fecal blood was monitored in mice as described<sup>[25,26]</sup>, in order to calculate disease activity index (DAI). Mice were sacrificed at 12<sup>th</sup> d after starting DSS, it means 7 d after DSS stop to evaluate the recovery post colitis. Some animals were sacrificed following 5 d from DSS (day 5), for intermediate measures in the moment of acute colitis. Parallel experiments were undertaken during same period also on healthy mice receiving drinking water instead of DSS.

### Biological samples collection and analysis

At the sacrifice from each animal was collected the colon, the mesenchymal lymph



**Figure 1** Experimental design of the dextran sulfate sodium colitis model. Acute colitis was induced by administration of 3% dextran sulfate sodium (DSS) to drinking water (DSS) for 5 d. At 3<sup>rd</sup> d, one singular injections of an anti-tumor necrosis factor  $\alpha$  was administrated. Each group counts 20 mice: 10 mice were sacrificed at day 5, 10 mice at day 12 after 1 wk of recovery post-DSS. DSS: Dextran sulfate sodium; TNF $\alpha$ : Tumor necrosis factor  $\alpha$ .

nodes (MLN) and stool and stored at  $-80^{\circ}\text{C}$ . The colonic length was evaluated. The last part of the small intestine was divided in three parts: two were stored at  $-80^{\circ}\text{C}$  for RNA and protein expression studies, one was fixed in 4% formalin and embedded in paraffin. The stools of animals were harvested at day 5 and 12, subsequently weighed, and stored at  $-80^{\circ}\text{C}$  for further analysis.

### General assessment of colitis

Colon length was measured as an indication of colonic inflammation. Briefly, the animals were anaesthetized and sacrificed by cervical dislocation. The colon was resected between the ileo-caecal junction and the proximal rectum, close to its passage under the pelvis. The colon was placed onto a non-absorbent surface and measured with a ruler, taking care not to stretch the tissue.

### Histological observation and scoring

Colon tissue was fixed in 4% formalin, embedded in paraffin and sliced into 3-mm-thick sections. Hematoxylin and eosin (HE)-stained sections were scored blindly on the basis of severity and extent of inflammation, as well as presence and extent of ulceration<sup>[27]</sup>. Scores ranged from 0 to 4 for each parameter; single values were summed up to obtain the overall histological score (maximum possible value equals 16). Photomicrographs were taken with  $40\times$  magnification on a Nikon E400 Eclipse microscope.

### RNA extraction and real time PCR

RNA was extracted from freeze colon using RNAqueous<sup>TM</sup> Total RNA Isolation Kit (Thermo-Fisher). cDNA was synthesized by High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed in duplicates using iQ SYBR Green Supermix (BioRad) on an iCycler iQ5 (BioRad) with the following scheme: an initial denaturation at  $95^{\circ}\text{C}$  for 3 min, 40 cycles of PCR amplification, each consisting of a denaturing step of  $95^{\circ}\text{C}$  for 10 s, annealing at  $55^{\circ}\text{C}$  for 30 s, and a final step at  $72^{\circ}\text{C}$  for 1 min. The primer pairs used were for CD3: forward 5'-ATGCGGTGGAACACTTTCTG-3' and reverse 5'-GCACGTCAACTCTACTGGT-3'; for  $\beta$ -actin: forward 5'-TGTTACCAACGTGGACGACA-3' and reverse 5'-CTGGGCATCTTTCA-3'.  $\beta$ -actin was used as an endogenous reference gene to normalize the expression of CD3.

### Western blot

Colon tissues were lysed in RIPA buffer [50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 0.05% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] supplemented with protease inhibitors (10  $\mu\text{g}/\text{mL}$  leupeptin, 20  $\mu\text{g}/\text{mL}$  aprotinin, 1 mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L  $\text{NaVO}_4$ , 100 mmol/L NaF). Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Protein extracts (50  $\mu\text{g}$ ) were resolved by 8% SDS-PAGE, transferred to PVDF membranes, and probed with rabbit polyclonal anti-ZO1 (1:500, Thermo Fisher Scientific) anti-Occludin (1:500, Thermo Fisher Scientific) anti-actin (1:1000, Sigma-Aldrich) primary antibodies. Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) were detected by use of the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and the ChemiDoc XRS system (Bio-Rad). Densitometric analysis was performed by using the ImageJ software (<http://imagej.nih.gov/ij/>).

### T lymphocyte activation and characterization

T lymphocytes were obtained from MLN of C57BL/6 mice, treated or not with DSS, using sterile strainers. MLN-derived T lymphocytes were then activated by plate-bound anti-CD3/anti-CD28 (10 and 4  $\mu\text{g}/\text{mL}$ , respectively) in fully supplemented RPMI 1640 medium containing 10% fetal calf serum, 2 mmol/L glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin for 48 h. At the end of incubation, T lymphocytes were stimulated with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A for 4 h, fixed in 4% formal saline and permeabilized with 0.1% saponin buffer prior to intracellular cytokine staining. Flow cytometry analysis was performed on an Epics XL Coulter instrument (Beckman Coulter). The following monoclonal antibodies (Mab) were used: PeCy5-labelled anti-CD4 Mab, PE-labelled anti-TNF $\alpha$  Mab, PE-labelled anti-IL6 Mab, PE-labelled anti-IL17A Mab, PE-labelled anti-FOXP3 Mab, PE CF594-labelled anti-interferon (IFN) $\gamma$  Mab, PE CF594-labelled anti-IL4 Mab, PE CF594-labelled anti-CD25 Mab (all from eBioscience, San Diego, CA, United States). Cell viability was assessed by Fixable Viability Dye eFluor 450 (eBioscience), according to the manufacturer's protocol.

### Fecal DNA extractions and qPCR

DNA from stool samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Inc., Valencia, CA, United States) according to manufacturer's instructions and as described previously<sup>[28]</sup>. qPCR was performed in duplicates by using iQ SYBR Green Supermix (BioRad) on an CFX96 cycler (BioRad) with the following scheme: an initial denaturation at 95 °C for 3 min, 40 cycles of PCR amplification, each consisting of a denaturing step of 95 °C for 10 s, annealing at 55 °C for 15 s, and a final step at 60 °C for 1 min. The primer pairs used were: *Fecalibacterium prausnitzii* Forward 5'-AGA TGG CCT CGC GTC CGA-3', Reverse 5'- CCG AAG ACC TTC TTC CTC -3', *Clostridiaceae* Forward 5'-CGG TAC CTG ACT AAG AAG-3', Reverse 5'-AGT TTY ATT CTT GCG AAC-3', *Enterococcaceae* Forward 5'-CAT TGA CGT TAC CCG CAG AAG AAG C-3', Reverse 5'-CTC TAC GAG ACT CAA GCT TGC-3', *Bacteroides* Forward 5'- GAA GGT CCC CCA CAT TG -3', Reverse 5'-CAA TCG GAG TTC TTC GTG-3.

As standards for qPCR was used DNA extracted from *Escherichia coli* ATCC 25922 (LGC STANDARD) suspension. DNA was extracted starting from a suspension of 0,5 MacFarland (approximately  $1.5 \times 10^8$  CFU/mL) using High pure PCR template preparation kit (Roche). Subsequently extracted DNA (approximately  $10^8$  copy/mL) was diluted two fold 1:100 to obtain  $10^6$  and  $10^4$  standards. The number of  $C_T$  was normalized in number of genome copy using a standard curve. The results were divided between T0 and T2, where T0 represented the conditions before any treatment, and T2 represented the changes that appeared after 5 or 12 d of administration of DSS and anti-TNF $\alpha$ , alone or in association. The exact number of days was specified in the figures.

### Bioinformatics analysis

Statistical analysis of bacterial genera and species were performed using R packages FactorMineR and factorextra<sup>[29]</sup>, while ggplot2 for graphical representations<sup>[30]</sup>. Principal component analysis (PCA) was computed in order to highlights differences in species relative abundances among samples. Finally, Mann-Whitney-Wilcoxon was performed for each bacterial species between T0 and T2 in every group considered in this study.

### Statistical analysis

ANOVA and adjusted Bonferroni post-hoc analysis were performed in order to assess differences in DAI, protein and RNA expression and T lymphocyte subsets among treatment groups. A *P* value < 0.05 was considered statistically significant. Statistical analysis was performed using IC STATA 12 for Mac.

## RESULTS

### Anti-TNF $\alpha$ ameliorates DAI and histological scores in a murine model of colitis

In the last 4 years, our group use a well-established model of colitis in mice, largely recognized in literature: the DSS model<sup>[26,31,32]</sup>. As shown in Figure 2A and B, during DSS treatment, particularly at the day 4 and 5, there was a significant increase of the DAI and a loss of body weight, that equates in an escalation of severity of the DSS-induced colitis. During the week of recovery, the values of body weight and DAI came back close to the controls, except in "No DSS + anti-TNF $\alpha$  group", that showed gain of DAI score and an unexpected loss of body weight. Colon length was measured

as an indication of colonic inflammation<sup>[33,34]</sup>. Bowels were resected from the 4 groups of mice and subjected to macroscopic and histopathological examination. The appearance of the organs from the DSS-treated mice showed obvious reddening and shortening of the colon, which are typical signs of acute intestinal inflammation (Figure 2C). Histological analysis, performed by HE staining, showed important modification on colonic mucosa, especially at day 5 not only in “DSS” group, but also in “No DSS + anti-TNF $\alpha$ ” (Figure 3A). After 5 d of DSS treatment, intestinal villi were flattened, the glandular epithelium disappeared, the goblet cells were no longer recognizable and there was an evident mononuclear cell infiltrate. In “DSS + anti-TNF $\alpha$ ” group, few glands were preserved, lymphomononuclear cell infiltrate was present. Mononuclear inflammatory cells were present also in colon of mice treated only with anti-TNF $\alpha$  (“No DSS + anti-TNF $\alpha$ ” group) at day 5 and 12. Following 7 d of recovery post-DSS, the “DSS” and “DSS + anti-TNF $\alpha$ ” groups still displayed atrophic goblet cells, but more preserved than day 5.

### **Anti-TNF $\alpha$ has effects on tight junction expression and lymphocyte infiltration**

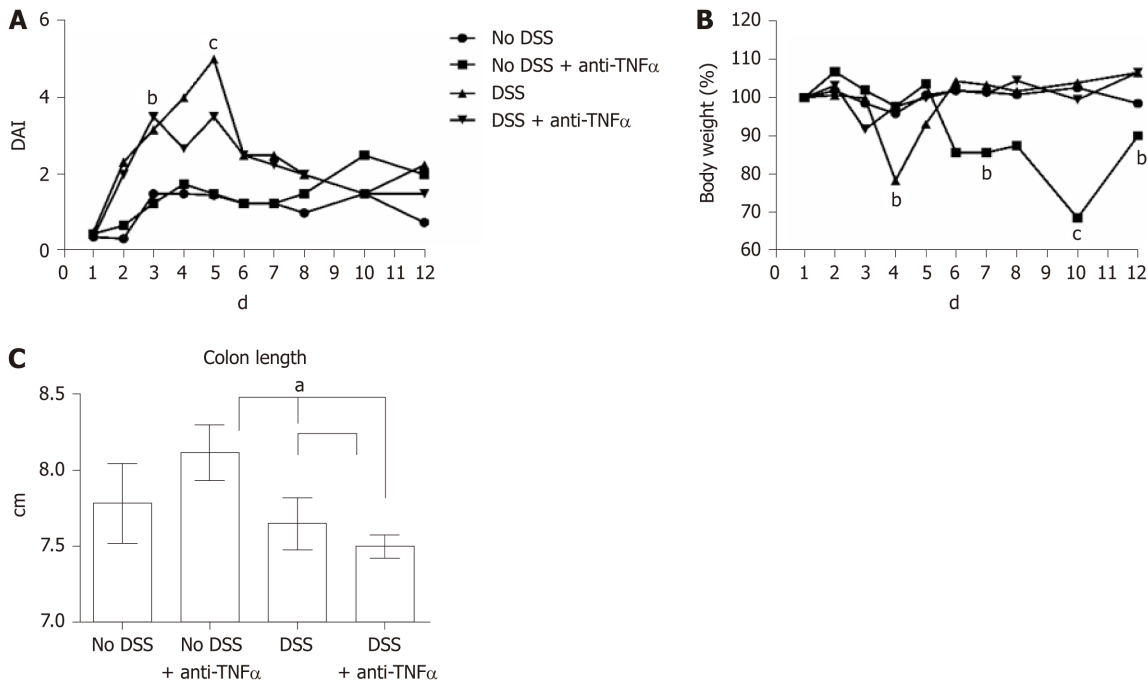
We analyzed the mononuclear cells infiltration in each condition (other than control group or “No DSS”) and the tight junction integrity of the colonic epithelium, and in particular occludin and ZO-1 (Figure 3B). Occludin expression decline was more evident at day 12 in “DSS” group (Figure 3B, right panel,  $P = 0.05$ ); ZO-1 decreased at day 5 (also in “No DSS + anti-TNF $\alpha$ ” group,  $P < 0.001$ ) and its levels remained lower than control mice also at day 12 (Figure 3B,  $P < 0.001$ ). CD3 expression was studied by qPCR in the last part of small intestine of mice from different groups. CD3 mRNA increased in “DSS group” during acute colitis at day 5 (Figure 3C, left panel). At day 12, after 7 d of recovery, CD3 mRNA level remained higher in samples from “No DSS + anti-TNF $\alpha$ ” group while decreased in other mice (Figure 3C, right panel,  $P < 0.001$ ); this finding correlates with the presence of lymphomononuclear cell infiltrate observed at day 5 and 12. The data in mucosa were confirmed by the data from MLN. In the presence of inflammatory processes, due in this case to DSS administration, Treg lymphocytes decreased early at day 5, except in “DSS + anti-TNF $\alpha$ ” group (Figure 4A and B); Th1 and Th17 lymphocytes increased especially later at day 12 (Figure 4A, C and D); these modifications are less prominent in “DSS + anti-TNF $\alpha$ ”. In “No DSS + anti-TNF $\alpha$ ” group, several MLN-derived T lymphocyte subsets were found modified at day 12. In particular, Th1 and Th17 lymphocytes were found to be more frequent than in the “DSS + anti-TNF $\alpha$ ” treatment group whereas they were found diminished as compared to the “DSS” treatment group.

### **The role of TNF $\alpha$ to maintain a healthy gut microbiota**

Increased epithelial tight junction permeability, with commensal bacteria, promotes intestinal CD4<sup>+</sup> T cell expansion<sup>[31]</sup>. The distribution of specific genera and species of bacteria in the different groups of mice was initially evaluated, by PCA (Figure 5A). As we expect, the biplot at T0 shows the samples clustered together, and reflects the homogeneity in the abundance of genera and species in the microbiota of inbred mice.

At T2, after 5<sup>th</sup> or 12<sup>th</sup> d of treatment, the samples from groups “DSS” or “DSS + anti-TNF $\alpha$ ” diverged from other T2’s samples and from their respective T0. Moreover, it is possible to observe a gradient of distance starting with “DSS” group. *Enterococcaceae* weighted on this gradient: they were main factor that causes this distance of T2 from T0 in “DSS” and “DSS + TNF $\alpha$ ” groups. We also compared, through Mann-Whitney-Wilcoxon, the distribution of genera *Bacteroides*, *Clostridiaceae*, *Enterococcaceae*, and of species *F. prausnitzii* in each group of mice. As we expected, in “No DSS” group no main changes occurred between T0 and T2, *i.e.*, between the beginning of the experiment and the sacrifice (Figure 5B, right panel). As described elsewhere<sup>[35]</sup>, animals with DSS induced acute colitis showed an increase of *Enterococcaceae*, and a decrease of *Bacteroides* and *Clostridiaceae*, that is also statistically significant ( $P < 0.05$ , Figure 5B, left panel). The level of *F. prausnitzii* is not affected. After 7 d of recovery in “DSS” group all bacteria modifications reached the T0 level again.

In “DSS + anti-TNF $\alpha$ ” group, the changes after 5 d were opposed to those seen in “DSS group”. In the “DSS + anti-TNF $\alpha$ ” group there is no evident effect of the inflammation on *Bacteroides*, *Clostridiaceae* and *F. prausnitzii* as their abundance increased while *Enterococcaceae* behaved as per “DSS” group. After 7 d of recovery the effects of anti-TNF $\alpha$  were maintained only by *F. prausnitzii* (Figure 5B, left panel). The increase of *F. prausnitzii* seems due the TNF $\alpha$  inhibition as in “No DSS + anti-TNF $\alpha$ ”: after 2 d from one singular administration of anti-TNF $\alpha$ , the abundance of *F. prausnitzii* increased and remained stable 10 d after drug administration. In “No DSS + anti-TNF $\alpha$ ”, the *Clostridiaceae* decreased at day 5 ( $P < 0.05$ ), and *Bacteroides* fell down at day 12 too ( $P < 0.05$ ). *Enterococacceae* show a moderate increase, but in this case the data were not significant (Figure 5B, left panel).



**Figure 2** Estimation of clinical and morphological changes of dextran sulfate sodium-induced colitis. A: The four-point disease activity index (DAI) was evaluated scoring daily body weight of mice, the percentage of body weight loss, the trait of the stool and the presence of occult blood in feces. B: Body weight, one of the DAI parameter, is also represented separately by percentage, where the mice weight at 1<sup>st</sup> d of experiment is equal to 100%. C: Colon length is another parameter to evaluate the healthy or disease state of the mice: the mean values in the 4 groups at day 5 are shown. <sup>a</sup>*P* < 0.05; <sup>b</sup>*P* < 0.01; <sup>c</sup>*P* < 0.0001. DSS: Dextran sulfate sodium; TNF $\alpha$ : Tumor necrosis factor  $\alpha$ ; DAI: Disease activity index.

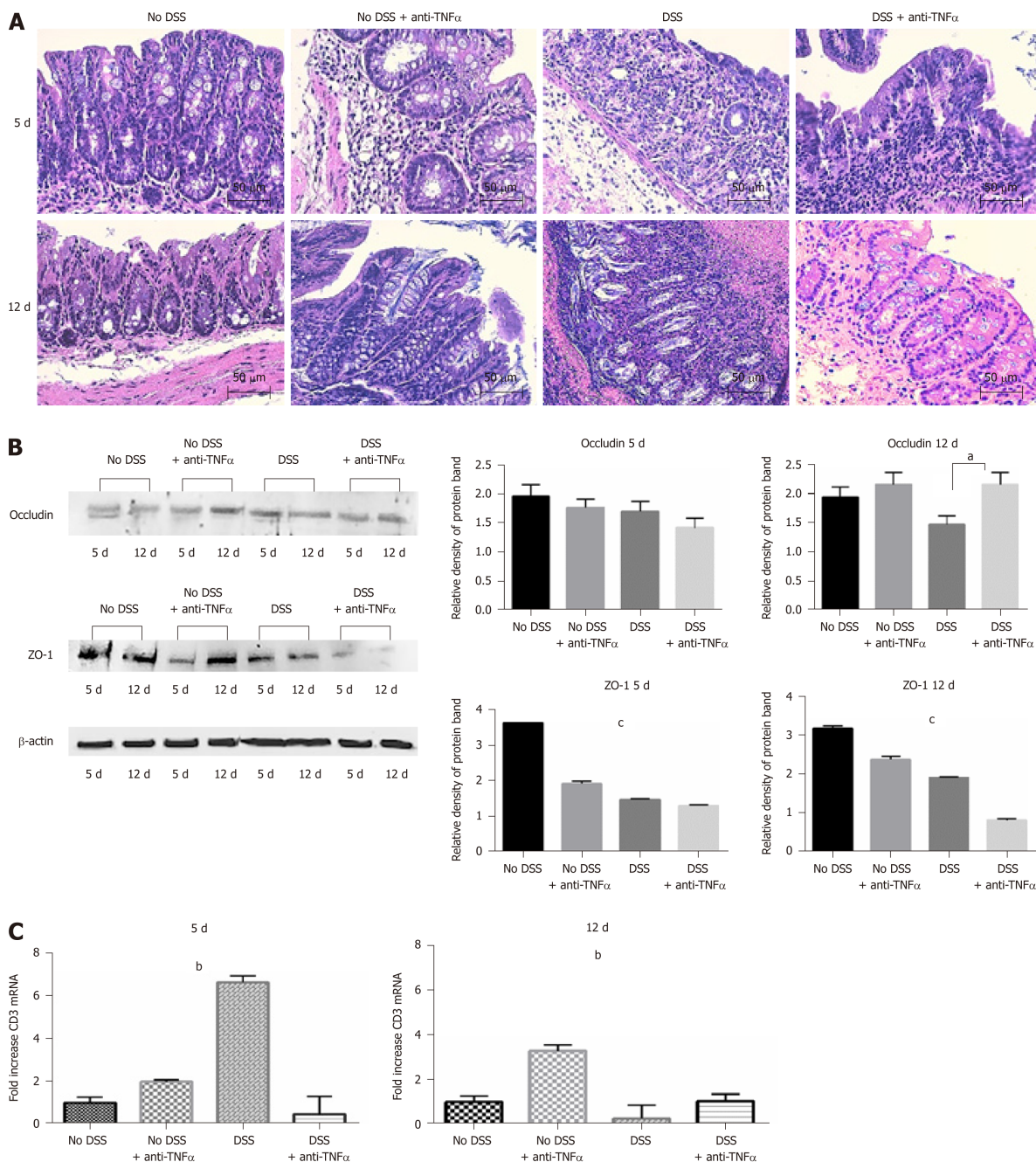
## DISCUSSION

An unfavorable alteration of the commensal structure of gut microbiota is referred to as “dysbiosis”; this includes a reduction in the number of tolerogenic bacteria and an overgrowth of potentially pathogenic bacteria (“pathobionts”) that can penetrate the intestinal epithelium and induce disease in certain genetic or environmental contexts<sup>[32,33]</sup>.

In the last decade a growing body of evidence suggests the crucial role of the gut microbiota in human health and studies are looking to define the link between “dysbiosis” and several pathologies<sup>[19,34,36]</sup>.

In this paper, we use an animal model of experimental colitis to study the behavior of few bacterial genera and species during inflammation and anti-TNF $\alpha$  therapy. A model of severe murine colitis, which most closely resembles human UC, results from administration of 40-50 kDa DSS in drinking water<sup>[37]</sup>. The mechanism by which DSS induces intestinal inflammation is due to the damage to the epithelial monolayer lining in the large intestine allowing the dissemination of pro inflammatory intestinal contents (*e.g.*, bacteria and their products) into underlying tissue. The DSS colitis model is very popular in IBD research due to its rapidity, simplicity, reproducibility and controllability<sup>[38,39]</sup>.

DSS treatment induces changes in the abundance of few bacteria, whose dynamics shift toward an unhealthy state, confirmed by clinical data in mice (DAI, changes in body weight and histological score) (Figure 2). We have previously described the efficacy of IFX in neutralizing also murine TNF $\alpha$ <sup>[25,40]</sup>. The aim of this study was to evaluate the effects of a selective immunosuppression on the gut microbiota, in particular on few genera and species involved in T lymphocytes expansion. To differentiate between the effects of DSS and of IFX, we used two control groups of mice: “No DSS” and “NO DSS + anti-TNF $\alpha$ ”. During the first evaluations, we noticed that the mice from “No DSS + anti-TNF $\alpha$ ” group were characterized by a raise of DAI (Figure 2A), due to the loss of body weight (Figure 2B). “No DSS + anti-TNF $\alpha$ ” group showed an increased mononuclear cell infiltrate compared to the control mice (Figure 3A), epithelial tight junction permeability (Figure 3B) and an increased expression of CD3 at qPCR (Figure 3C). In the first lymphoid station that drains the microbial products from intestine (MLN) Th1 and Th17 level increased at day 12 both in colitic mice and in control mice treated only with IFX (Figure 4A-D). Next, we looked at changes in *Bacteroides*, *Clostridiaceae*, *Enterococcaceae* and *F. prausnitzii* abundance. We have previously shown<sup>[15,16,18]</sup> that this mouse model mimics the changes that happens

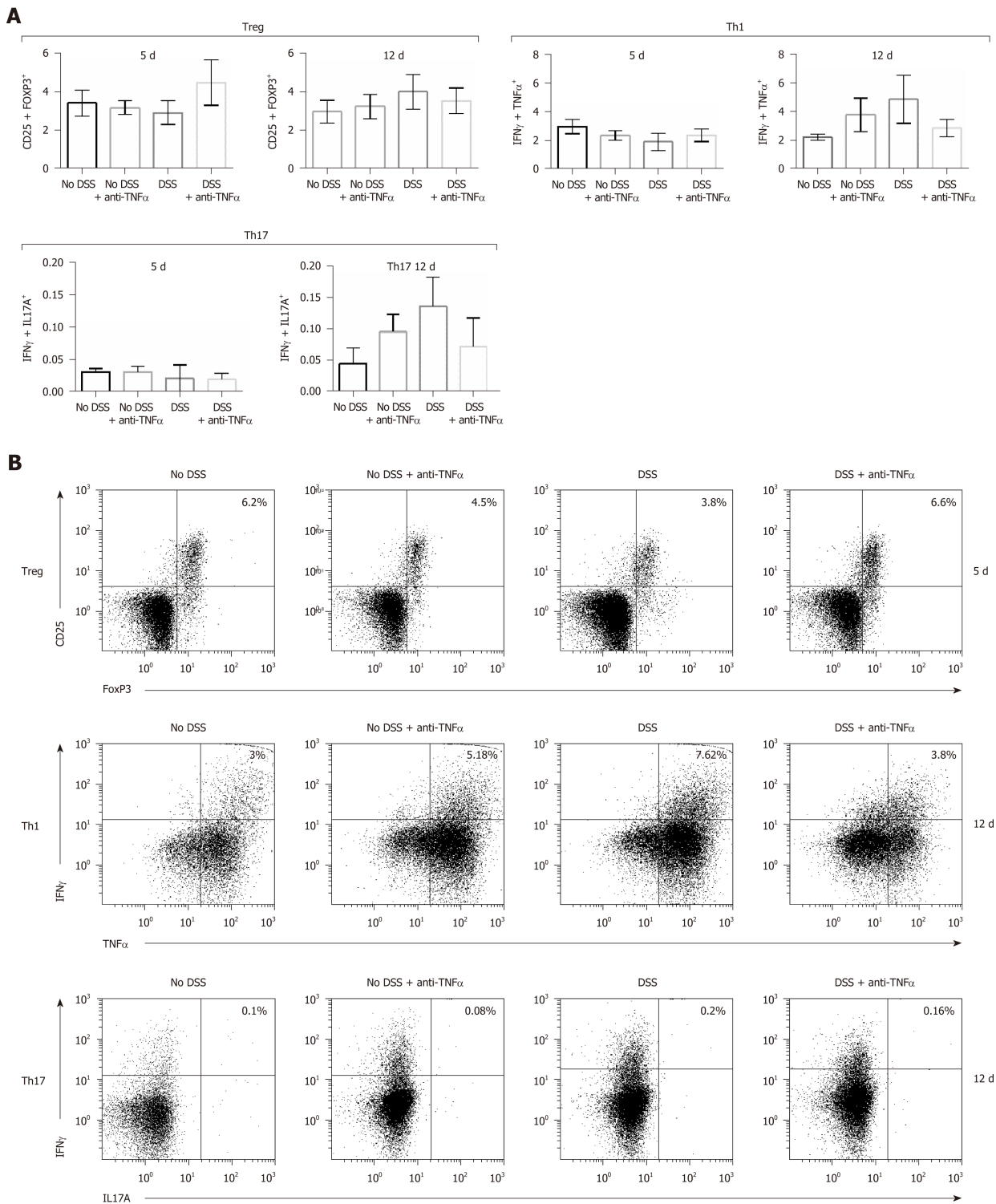


**Figure 3** Histological analysis confirms the strength of the dextran sulfate sodium model. A: Hematoxylin and eosin staining of colon sections of mice from 4 groups of treatment at day 5 and 12. Forty-fold magnification was used. B: Protein expression of tight junctions occludin and ZO-1 in colon tissue. Antibody anti- $\beta$ -actin was used as endogenous control. On the right, the histograms represent the mean of densitometry values from 3 different experiments. C: CD3 infiltration in colon mucosa was assessed by quantitative PCR of CD3 mRNA. One-way ANOVA (and non-parametric) was calculated; <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ ; <sup>c</sup> $P < 0.0001$ . DSS: Dextran sulfate sodium; TNF $\alpha$ : Tumor necrosis factor  $\alpha$ .

in the intestine of IBD patients. While in “No DSS” mice no main changes occurred between T0 and T2 (both at day 5 and at day 12, Figure 5B, right panel), in “No DSS + anti-TNF $\alpha$ ” “DSS + anti-TNF $\alpha$ ” and “DSS” groups there was an increase of *Enterococcaceae* abundance at day 5, a decrease of *Bacteroides* and *Clostridiaceae* at day 5 and day 12. This suggests that either DSS or anti-TNF $\alpha$  may cause a shift in the abundance of *Enterococcaceae*, *Bacteroides* and *Clostridiaceae* in the gut. The level of *Enterococcaceae*, *Bacteroides* and *Clostridiaceae* might have a role in the increase of CD3<sup>+</sup> cells in colon mucosa, in the loss of tight junction expression and the raise of Th1 and Th17 in MLN observed in mice treated only with IFX.

On the other hand, a positive effect due to IFX was the augmented abundance of *F. prausnitzii* at day 5 (after only 2 d from the i.v.) that remained also at day 12. The use of an anti-TNF $\alpha$  in healthy conditions resulted in dramatic changes in intestinal mucosa inflammation, modulation of tight junction permeability, and to an interesting

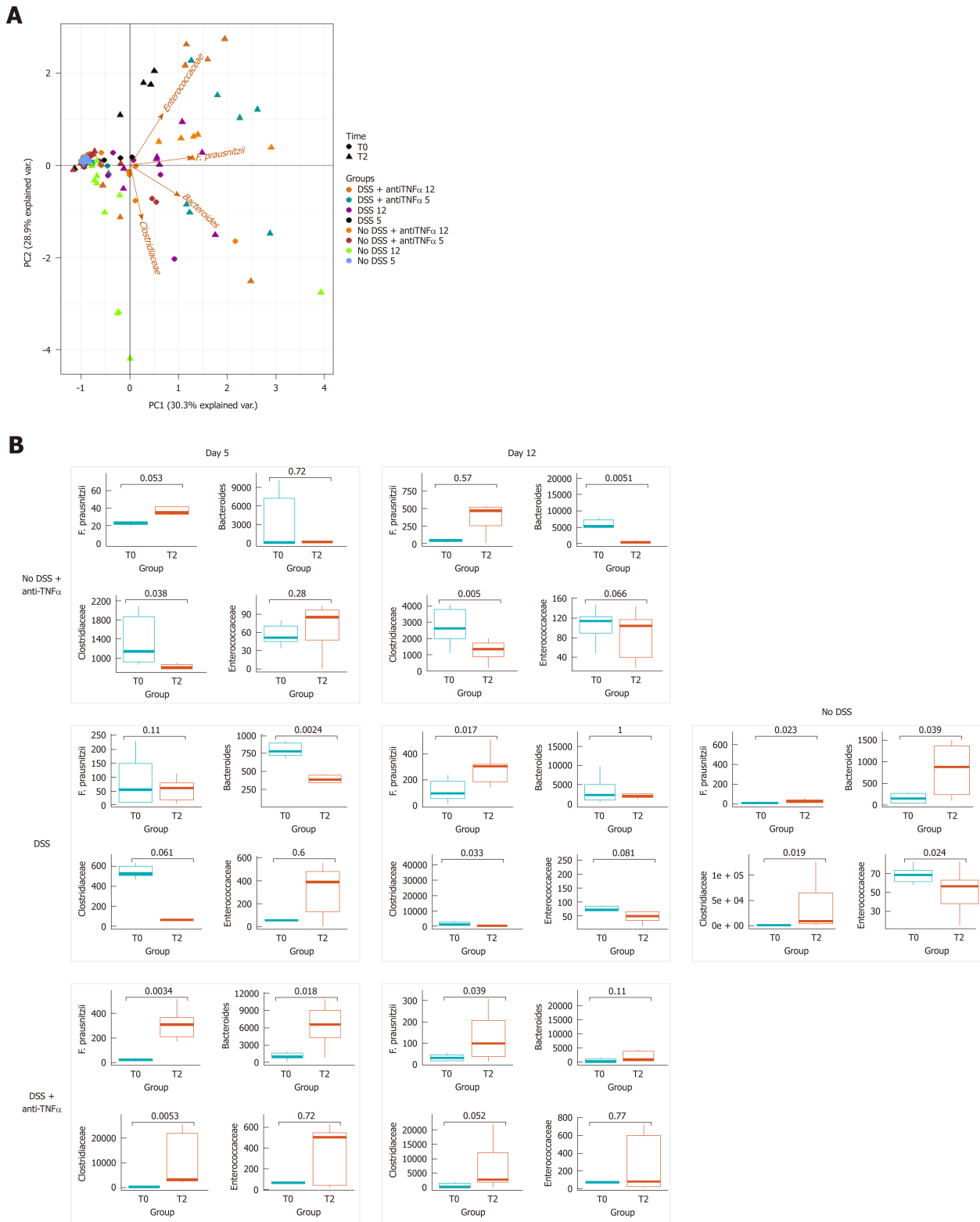




**Figure 4** T cells subsets characterization in presence of an anti-tumor necrosis factor  $\alpha$  agent. T cells were isolated from mesenteric lymphnode of mice and CD4<sup>+</sup> cells were studied by flow cytometry. In particular, CD4<sup>+</sup> regulatory T lymphocytes characterization was possible through the use of anti-CD25 and anti-FoxP3 antibodies (B); type 1 helper T lymphocytes expressed interferon (IFN) $\gamma$  and tumor necrosis factor  $\alpha$  (C); IFN $\gamma$ <sup>+</sup> and interleukin 17<sup>+</sup> cells identified the type 17 helper T lymphocytes cluster (D). In each panel (B), (C) and (D) the cells in the upper right quadrant were the double staining cells. Treg: CD4<sup>+</sup> regulatory T lymphocytes; Th1: Type 1 helper T lymphocytes; Th17: Type 17 helper T lymphocytes; DSS: Dextran sulfate sodium; TNF $\alpha$ : Tumor necrosis factor  $\alpha$ ; IFN $\gamma$ : Interferon  $\gamma$ ; IL: Interleukin.

shift in the overall intestinal mucosa T cell immune response at cytometry analysis, in parallel to variation of *Enterococcaceae*, *Bacteroides* and *Clostridiaceae* abundance. This observation, if confirmed in humans, has several potential implications. First, the dysbiotic effect of anti-TNF $\alpha$  must be considered in patients not responding to this drug. New and emerging data show potential positive effect of modulating gut microbiota composition in UC, including fecal microbiota transplantation<sup>[41,42]</sup>.

Furthermore, the shift toward Th17 pathway could be considered when deciding to



**Figure 5 Bacterial genera and species characterization in presence of an anti-tumor necrosis factor  $\alpha$  agent.** A: Principal component analysis was represented by a biplot, where the variability among samples and bacterial weight in the variability were expressed. B: Dot plot represents the distribution of bacteria at the beginning of the experiment and at the moment of sacrifice; in the case of "No DSS" mice, the samples from day 5 and 12 are collected together, since no significant changes occurred between day 5 and 12. In left panel, the results were distinguished based on the day of the end of experiment. DSS: Dextran sulfate sodium; TNF $\alpha$ : Tumor necrosis factor  $\alpha$ .

swap therapy in non-responder colitis patients: indeed emerging data suggest the positive effect of blocking the Th17/IL23 pathway also in UC, as shown by phase 3 induction trial of ustekinumab in UC patients<sup>[43]</sup>.

A direct translation to human disease cannot be done. Furthermore, no direct correlation has been shown between anti-TNF $\alpha$  use and change in microbiota

composition. It can be argued that microbial change could arise indirectly following the immunological changes due to IFX treatment. Finally, other factors, like the mucus layer, are also implied into the pathogenesis of DSS colitis, could exert an important role in these findings.

Overall our data suggest a deep knowledge of the interaction between drug treatment, immune system and microbiota is key to better understand IBD and its response to therapy.

## ARTICLE HIGHLIGHTS

### Research background

Anti-tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) represents the best therapeutic option to induce mucosal healing and clinical remission in patients with moderate-severe ulcerative colitis. On the other side gut microbiota plays a crucial role in pathogenesis of ulcerative colitis but few information exists on how microbiota changes following anti-TNF $\alpha$  therapy and on microbiota role in mucosal healing.

### Research motivation

The hypothesis behind this study is that anti-TNF $\alpha$  could induce a dysbiosis sustained by immunological changes: dysbiosis could represent one of the reasons for loss of response to anti-TNF $\alpha$  therapy or primary failure.

### Research objectives

With this manuscript we aimed to evaluate, in colitic mice as well as healthy mice, intestinal immune system status and gut microbiota modulation induced by anti-TNF $\alpha$  therapy. A more comprehensive approach including gut microbiota modulation, if clarified, could be assessed by dedicated studies on active gut microbiota modulation during biologic therapy in inflammatory bowel disease (IBD).

### Research methods

Healthy mice treated with anti-TNF $\alpha$  showed similar histological, microbial and immune features of untreated colitic mice, in particular a lymphomononuclear infiltrate both at 5<sup>th</sup> and 12<sup>th</sup> d at hematoxylin and eosin staining, an increase of type 1 helper T lymphocytes (Th1) and type 17 helper T lymphocytes (Th17) at day 12, and finally increase of *Enterococcaceae* at day 5, a decrease of *Bacteroides* and *Clostridiaceae* at day 12.

These findings are particularly relevant to understand the role that anti-TNF $\alpha$  modulation plays on gut microbiota (and vice-versa) also in humans: this finding could be of major interest in order to give more lights on mechanisms of loss of response to anti-TNF $\alpha$ , mechanisms of immunological shift with towards other pathways, like Th17 pathways, as well as novel potential therapeutic targets in IBD.

### Research results

Gut microbiota contributes to immune system priming, development and activation. In course of IBD a decrease of *Firmicutes* (and, among these, the species *Faecalibacterium prausnitzii*) together with an increase in *Proteobacteria* has been demonstrated, associated to an increase of T lymphocyte differentiation toward Th1, Th17 subsets and a decrease of CD4<sup>+</sup> regulatory T lymphocytes (Tregs). How these conditions are modified in course of medical treatment is not well clarified, particularly when a powerful modulator of the immune system, like anti-TNF $\alpha$ , is utilized.

### Research conclusions

Our study confirmed data from the literature. The same features were confirmed in the experimental model of colitis presented in this paper. Furthermore, it was shown that higher representation of *Bacteroides*, *Clostridiaceae* and *Faecalibacterium prausnitzii* and lower representation of *Enterococcaceae* are associated to a healthy state while the opposite happens during colitis. Anti-TNF $\alpha$  is able to induce changing in gut microbiota toward a health state in course of active colitis, but a more dysbiotic effect when utilized in healthy controls. Healthy related-microbial assessment is associated to higher Treg cells count and lower Th1 and Th17 frequency, condition modified in course of active colitis or use of anti-TNF $\alpha$  in course of healthy condition.

The study conclusion demonstrates a clear relationship between microbial changes and immunological changes in healthy as well as in controls. However, whether these findings correlate to human disease needs to be assessed in a dedicated study as well the need of checking and correction of dysbiosis in patients exposed to anti-TNF $\alpha$ .

### Research perspectives

This study strongly suggests a complete assessment of immune system and microbiota in course of powerful immunomodulatory therapy like anti-TNF $\alpha$  and opens new and important considerations. In order to personalize the therapeutic approach to an IBD patient, clinical studies assessing gut microbiota composition before starting an immunomodulatory therapy and in course of therapy are needed. Once an alteration has been demonstrated, the potential role of concomitant microbiota modulation could also be considered for newer and more personalized therapeutic approaches.

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