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Oral treatment with enrofloxacin creates anti-inflammatory environment that supports induction of tolerogenic dendritic cells

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ABSTRACT

Background: Oral enrofloxacin treatment altered the gut microbiome promoting anti-inflammatory bacteria. The dysbiosis promotes regulatory cell induction in the intestines and in the periphery, which suppresses contact sensitivity. Bacterial-derived signals promote regulatory cell induction both directly and indirectly by influencing the phenotype of dendritic cells (DC).

Methods: Oral treatment with broad-spectrum antibiotic enrofloxacin was used to evaluate how gut flora perturbation shapes the immune response in the gut and the periphery.

Results: Enrofloxacin-induced dysbiosis creates an anti-inflammatory environment characterized by increased IL-10 concentration in the gut lumen and tissues. The production of IFN-γ and IL-17A did not change. Oral enrofloxacin treatment skewed the profile of the immune response towards an anti-inflammatory phenotype locally in small intestinal Peyer's Patches (PP) and systematically in the spleen (SPL). Enrofloxacin administration changed immune response in PP by increasing TGF-β secretion from an increased percentage of TGF-β-producing. In the SPL, enrofloxacin treatment increased the secretion of TGF-β and IL-10 and decreased the secretion of IL-17A and IFN-γ. The shift in cytokine profile correlated with a higher percentage of latency-associated peptide and IL-10-producing cells and a decreased percentage of IFN-γ-producing T cells. This anti-inflammatory immune response in the PP and SPL promoted a higher frequency of tolerogenic DC.

Conclusion: Our data indicate that two-week enrofloxacin treatment induces dysbiosis, skews immune response towards an anti-inflammatory phenotype, and elevates secretion of TGF-β and IL-10 in the intestines and periphery. Additionally, we observed higher frequencies of tolerogenic DC, characterized by CD11b and IL-10 expression, which are known inducers of Treg cells.

1. Introduction

Evidence suggests that gut microbes, which represent an enormous source of interactions between microbes and immunity, play a very important role in various health problems like autoimmune and allergic diseases [1]. Natural gut flora is dominated by phyla *Firmicutes* and *Bacteroidetes* but also contains the less abundant *Proteobacteria* and *Actinobacteria*. Some bacterial species such as *Bacteroides fragilis* [2] and bacteria belonging to *Clostridium* cluster XIVa [3] have shown strong immunoregulatory properties. The shift in microbiota composition is known as dysbiosis. Altered microbiota composition is observed in many autoimmune and inflammatory diseases [4], however, it is not known if these shifts precede or are consequences of the disease. Directed modification of intestinal bacteria could prevent or ameliorate the development of numerous diseases in both scenarios [4].

Antibiotics commonly cause dysbiosis. We have shown previously that treatment with the broad-spectrum antibiotic enrofloxacin before induction of collagen-induced arthritis (CIA) aggravates disease [5]. On the other hand, broad-spectrum antibiotics and mixtures of antibiotics have also been shown to reduce the severity of experimental autoimmune encephalomyelitis (EAE) [6], dextran sodium sulfate (DSS)-induced colitis [7], spontaneous colitis in IL-10$^{-/-}$ mice [8], and

Abbreviations: CIA, collagen-induced arthritis; CS, contact sensitivity; DC, dendritic cell; DSS, dextran sodium sulfate; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; FCS, fetal calf serum; ICC, intracellular cytokines; IEL, intraepithelial lymphocytes; IFN-γ, interferon-gamma; IL, interleukin; LAP, latency-associated peptide; mAb, monoclonal antibody; PA, pyruvate; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PP, Peyer patch; PSA, polysaccharide A; ROS, reactive oxygen species; SCFA, short-chain fatty acids; SFB, segmented filamentous bacteria; SPL, spleen; SPLC, splenocytes; TGF-β, transforming growth factor β; Th, helper T lymphocytes; TLR2, Toll-like receptor 2; TNP-Ig, TNP-conjugated mouse immunoglobulins; Tr1, type 1 regulatory T cells; Treg, Regulatory T cells

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allergic contact dermatitis in mice [9]. Observed alleviation of such inflammatory responses was associated with the induction of T and B cells with regulatory potential [6,9–10].

Our previous study in a mouse model of contact sensitivity (CS) showed that oral treatment with enrofloxacin induced gut microbiota dysbiosis characterized by increased proportion of short-chain fatty acids (SCFA) -producing *Clostridium coccoides* (cluster XIVa), *Clostridium coccoides – Eubacterium rectale* (cluster XIVab), *Bacteroidetes* and *Bifidobacterium* spp., but decreased segmented filamentous bacteria (SFB) [9]. The enrofloxacin-induced dysbiosis reduced the severity of CS by supporting the induction of a variety of regulatory cells in periphery such as $TCR\alpha\beta^+CD4^+CD25^+FoxP3^+$ T regulatory cells (Treg), $CD19^+B220^+CD5^+IL\text{-}10^+$, $IL\text{-}10^+$ type 1 regulatory T (Tr1) cells, and $IL\text{-}10^+TCR\gamma\delta^+$ cells [9]. However, we didn't observe changes in these cells until immunization, which may suggest that enrofloxacin-induced dysbiosis creates an immune environment supporting the induction of regulatory cells.

Two anti-inflammatory cytokines, interleukin 10 (IL-10) and transforming growth factor β (TGF-β), are involved in the induction of regulatory cells directly and indirectly *via* induction of tolerogenic dendritic cells (DC). The production of both cytokines is influenced by gut microbes [3,11]. The major sources of these cytokines in the gastrointestinal tract are epithelial cells [12] and leukocytes. Anti-inflammatory cytokine TGF-β supports the induction of Treg cells [3,13] and promotes the induction of $IL\text{-}10^+$ Tr1 lymphocytes [14], whereas IL-10 is involved in induction of Tr1 cells [15]. Epithelia-derived IL-10 supports the generation of tolerogenic $CD11b^+$ DCs that produce IL-10 [16]. Interestingly, probiotics administration supports the induction of tolerogenic DCs, which promotes inducible Treg cell development and activates naturally occurring natural Treg lymphocytes [17].

In this study, we further explored the mechanism of how enrofloxacin-induced dysbiosis supports the induction of regulatory cells by modulating the immune environment in the gut and the periphery.

2. Materials and methods

2.1. Mice

C57BL/6 mice (6 weeks old) were obtained from the breeding unit of the Department of Medical Biology, Jagiellonian University, College of Medicine. Mice were kept under specific pathogen-free conditions in individually ventilated cages using the Aero-Mouse IVC Green Line system (Tecniplast S.p.A., Buguggiate, Italy) and fed autoclaved food and water ad libitum. All experiments were conducted according to guidelines of the Jagiellonian University Medical College.

2.2. Reagents

Baytril (latin: Enrofloxacinum) was purchased from Bayer Animal Health GmbH (Leverkusen, Germany). RPMI 1640 medium, penicillin, streptomycin and non-essential amino acids (NEAA) were purchased from Gibco (Grand Island, NY, USA). Pyruvate (PA) and L-glutamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). And fetal calf serum (FCS) was purchased from PAA Laboratories (Pasching, Austria).

2.3. Treatment with enrofloxacin

Mice received drinking water alone or water with enrofloxacin (0.27 mg/ml) for two weeks before each sample collection. Analyzed biological material originated from mice with previously documented dysbiosis [9].

2.4. Intestinal lavage collection and tissue culture of intestinal biopsies

Two weeks post enrofloxacin treatment, small and large intestines were collected and the intestinal content was flushed out using the same amount of sterile phosphate-buffered saline (PBS). 1 cm long fragments of distal ileum were cut off, weighed, and placed into PBS with 1% penicillin and streptomycin. The fragments were excised longitudinally, washed extensively and transferred into fresh PBS with 1% penicillin and streptomycin. Following the wash, the tissue fragments were placed into 600 μl RPMI 1640 culture medium supplemented with penicillin, streptomycin, non-essential amino acids (NEAA) and 5% FCS. Supernatants were collected and then tested for cytokine concentration using ELISA kits after 5 days of culture.

2.5. Cell culture of lymphoid organs

To evaluate influence of enrofloxacin treatment on immune cells in the intestines, Peyer's patches (PP) were collected into homogenization buffer (10 mM Tris-base, 150 mM NaCl, pH = 8.0) and disrupted using a mechanical homogenizer at 50 Hz for 3 min (Tissue homogenizer TissueLyser LT, Qiagen GmbH, Hilden, Germany). The tissue homogenates were centrifuged (4000 rpm, 30 min, 4 °C), supernatants were collected, and cytokine concentration was evaluated by ELISA.

To evaluate the impact of enrofloxacin treatment on the immune profile in the periphery, we measured cytokine production in spleen (SPL) cells isolated from mice receiving water with or without enrofloxacin. After two weeks of treatment, mice were sacrificed. SPL were collected, ground and tissue fragments were removed using cell strainers. The cells were washed 3x with PBS + 1%FCS, centrifuged (1200 rpm, 10 min, 4 °C), and cell pellets were re-suspended in RPMI 1640 culture medium supplemented with penicillin, streptomycin, NEAA, and 5% FCS. The cells were counted and seeded at a density of 3×10^6/ml in flat-bottom 24 well Falcon plates with 100 μg of TNP-Ig antigen. After 48 h, culture supernatants were collected and tested for cytokine concentration by ELISA.

2.6. Measurement of cytokines concentrations

The concentration of IL-10 and interferon-gamma (IFN-γ) was measured in culture supernatants using mouse ELISA kits purchased from BD Pharmingen (San Diego, CA, USA) according to the product instructions. The concentration of IL-17A was measured using a mouse ELISA Ready-SET-Go kit from eBioscience (San Diego, CA, USA) according to the product instructions. To measure the concentration of TGF-β, anti-TGF-β1 capture monoclonal antibodies (mAb) and anti-TGF-β biotinylated detection mAb were used; both of these mAb were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). TGF-β1 standard was purchased from R&D Systems Inc., (McKinley Place, NE, USA). The TMB reagent set obtained from BD Pharmingen (San Diego, CA, USA) was used as the substrate.

The ELISA plates were measured using a Microplate Absorbance Reader Synergy HT reader from BioTek Instruments Inc. (Winooski, VE, USA).

2.7. FACS analysis

For flow cytometry analysis, SPL and PP were isolated and single-cell suspensions were prepared, followed by staining with fluorochrome-conjugated mAbs.

For T cell phenotyping, anti-CD4-PerCP-Cy5.5, anti-TCRβ-APC-Cy7 (BioLegend, San Diego, CA) and anti-LAP-Pe-Cy7 mAbs were used. For DC analysis anti-CD11b-APC-Cy7, anti-CD11c-Per-Cy5.5 (BioLegend, San Diego, CA, USA) and anti-B220-PE-Cy7 (eBioscience Inc. San Diego, CA, USA) mAbs were used.

To detect intracellular IL-10 and IFN-γ, ICC (intracellular cytokines) staining was performed. Single-cell suspensions of SPL and PP were cultured for 4 h with phorbol 12-myristate 13-acetate (PMA), ionomycin (Sigma Chemical Co., St Louis, MO) and Golgi Plug (eBioscience, San Diego, CA) before staining for surface markers of T cells and DCs [9]. Finally, the cells were fixed and permeabilized with an intracellular

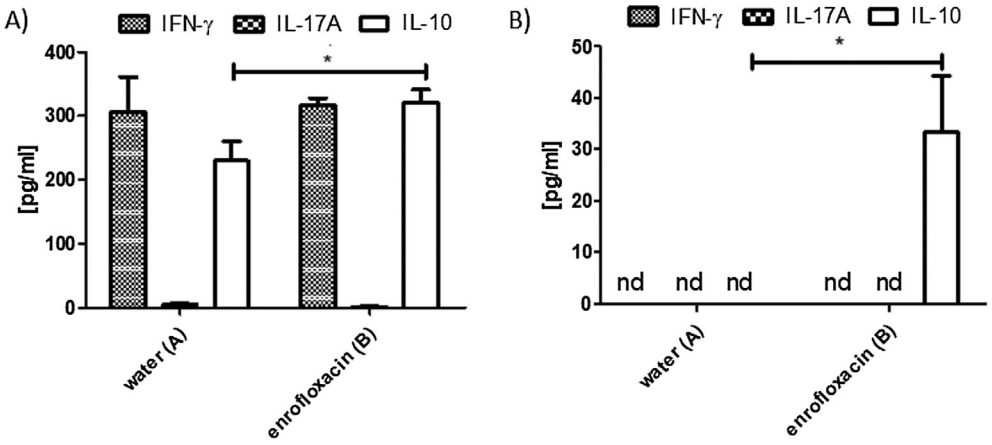

Fig. 1. Oral treatment with enrofloxacin increases IL-10 concentration in (A) small intestine and (B) large intestine lavages. Adult mice received water (group A) or water with enrofloxacin (group B) for two weeks. Then, intestinal lavages from small and large intestines were collected and tested for IL-10, IL-17A, and IFN-γ. Results are shown as means ± SE. n = 5–6 *P < 0.05. Significance was determined using a *t*-test (A), and a one-sample *t*-test comparing the mean of group B to the mean value of group A, which is zero (B). nd- not detected – below ELISA kit sensitivity.

staining buffer kit (eBioscience Inc., San Diego, CA, USA) before staining with anti-IFN-γ-APC, anti-IL-10-FITC or anti-IL-10-PE mAbs, (BD Biosciences, San Jose, CA, USA). The cells were analyzed with a Flow cytometer FACS Canto II (Becton Dickinson & Co., San Jose, CA, USA) and data were analyzed using FACSDiva software.

3. Results

3.1. Antibiotic induced-dysbiosis creates an anti-inflammatory environment in the gut

Epithelial and immune cells create the immune environment in the gut. To evaluate the impact of enrofloxacin-induced dysbiosis on intestinal immune status, cytokine concentration was tested in intestinal lavages from mice receiving water with or without enrofloxacin.

Data present in Fig. 1 show that enrofloxacin-induced dysbiosis significantly increased concentration of anti-inflammatory IL-10 in small (Fig. 1A) and large intestine (Fig. 1B) lavages. The concentration of pro-inflammatory IL-17A and IFN-γ did not change in small intestine lavages after antibiotic treatment and was not detected in large intestine lavages of both water and enrofloxacin treated mice.

To confirm that treatment with enrofloxacin for two weeks creates an anti-inflammatory environment, we evaluated cytokine production by small intestine biopsies. Tissue culture was made only from small intestine biopsies as the cytokine concentration in large intestine lavages was below the threshold level of detection.

Data presented in Fig. 2 show that enrofloxacin-induced dysbiosis significantly increased IL-10 production by small intestine biopsies (Fig. 2A). The concentration of IFN-γ (Fig. 2B) and IL-17A (Fig. 2C) did not change significantly after enrofloxacin treatment, although the production of IFN-γ was decreased when compared to the water treated group.

3.2. Antibiotic-induced dysbiosis skews immune response in PP towards an anti-inflammatory cytokine profile

Data presented above show that treatment with enrofloxacin promotes an anti-inflammatory cytokine milieu in the small and large intestines. Since both epithelial and immune cells could be the source of IL-10, we evaluated the impact of enrofloxacin-induced dysbiosis on immune cells, specifically. To achieve this, we measured cytokine production in PP, which are organized lymphoid tissues across the small intestines. Data presented in Fig. 3 shows a two-fold higher concentration of anti-inflammatory IL-10 (Fig. 3A) and TGF-β (Fig. 3B) in PP homogenates collected from mice receiving water with enrofloxacin (group B) compared to the water treated group (group A). Interestingly, the production of pro-inflammatory IL-17A (Fig. 3D) decreased significantly after enrofloxacin treatment, while the level of IFN-γ (Fig. 3C) did not change.

To evaluate the cellular source of anti-inflammatory cytokines in PP, we determined the phenotype of local T cells by flow cytometry. To detect TGF-β^+ cells, we stained for latency-associated peptide (LAP), which interacts with TGF-β. Fig. 4 shows an increased percentage of TCRβ^+CD4$^+$LAP$^+$ cells (Fig. 4B) in enrofloxacin-treated mice (group B) compared to the water-receiving group (group A). Antibiotic treatment did not significantly change the percentages of TCRβ^+CD4$^+$IL-

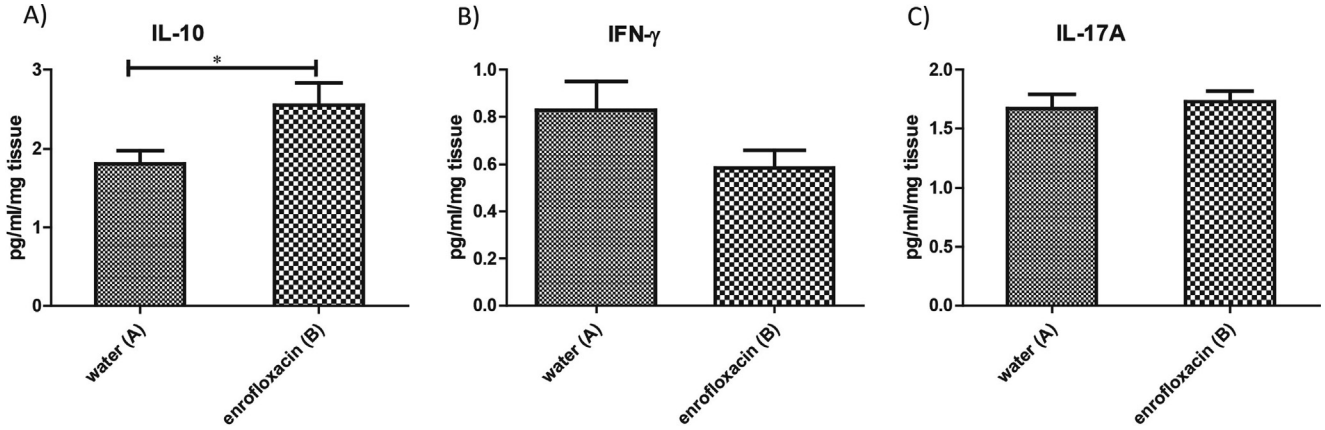

Fig. 2. Oral treatment with enrofloxacin creates an anti-inflammatory environment in the gut tissues. Adult mice received water (group A) or water with enrofloxacin (group B) for two weeks. Mice were sacrificed and small intestine biopsies were collected and cultured for 5 days. Tissue culture supernatants were tested for IL-10 (A), IFN-γ (B), and IL-17A (C). Results are shown as means ± SE. n = 7–8 *P < 0.05.

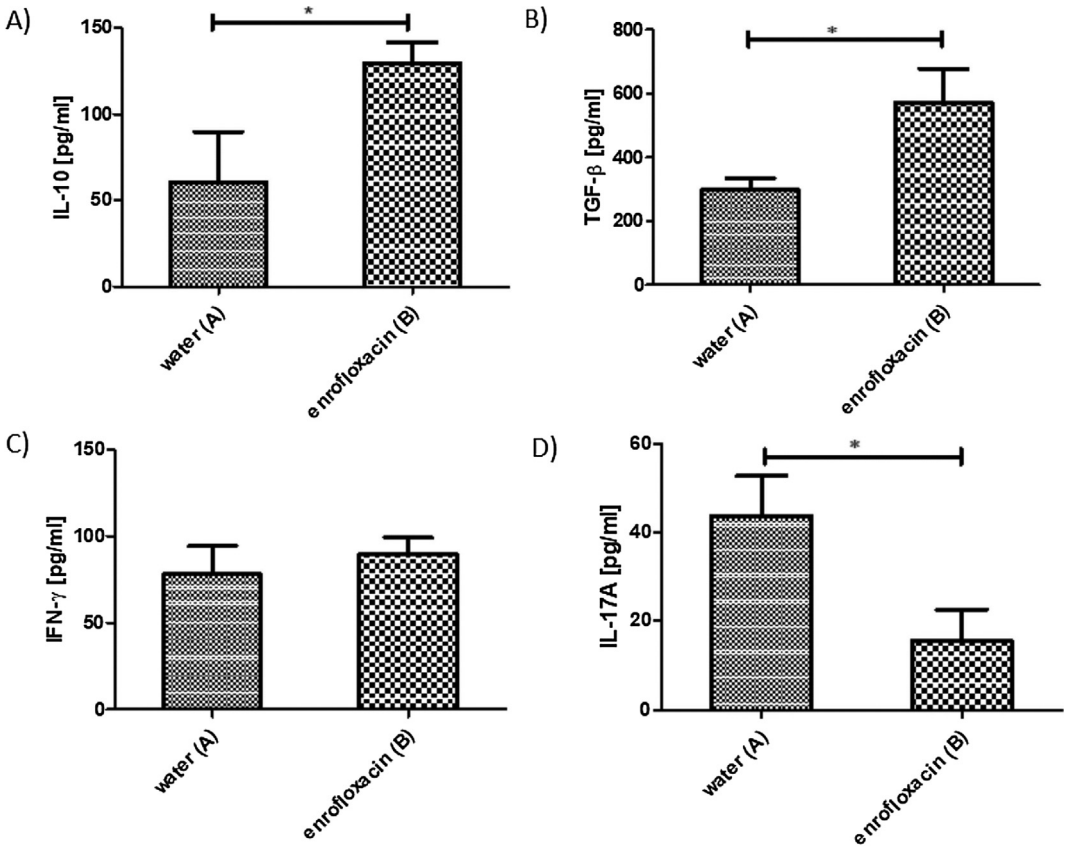

Fig. 3. Oral treatment with enrofloxacin skews immune response in the PP towards an anti-inflammatory cytokine profile. Adult mice received water (group A) or water with enrofloxacin (group B) for two weeks. Mice were sacrificed, and PPs were collected and homogenized. The homogenates were tested for IL-10 (A), TGF-β (B), IFN-γ (C), and IL-17A (D). Results are shown as means ± SE. n = 4–6 *P < 0 0.05.

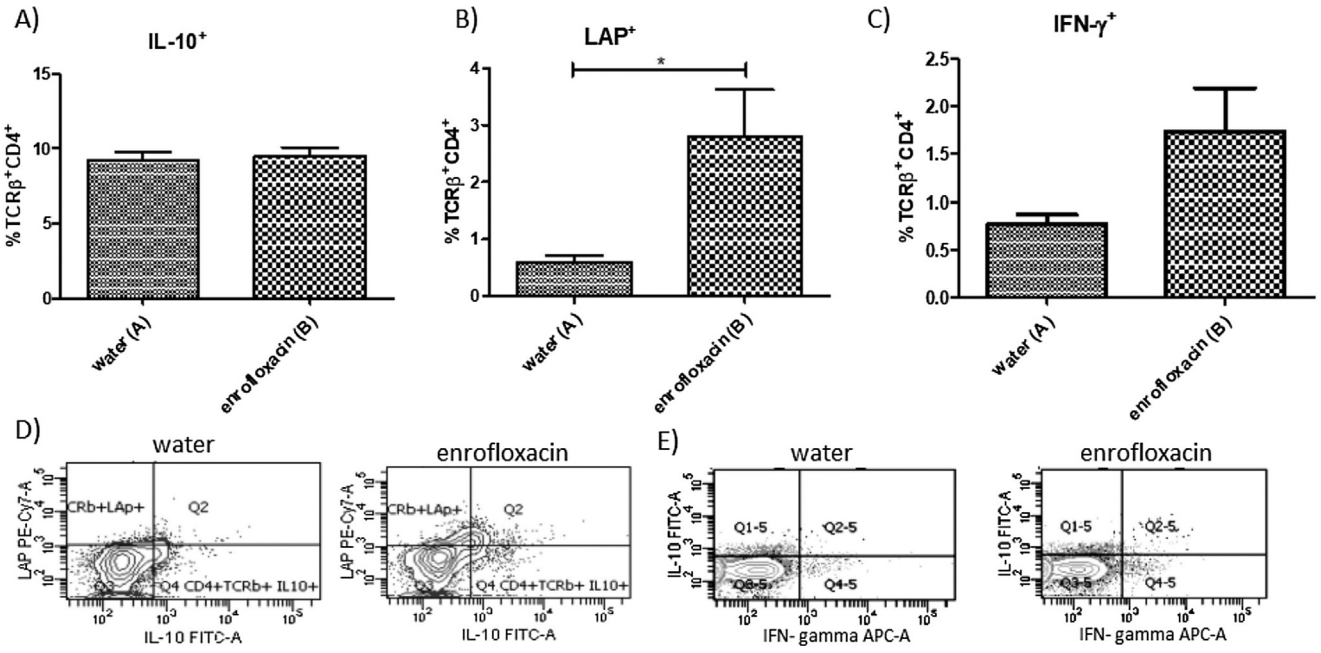

Fig. 4. Oral treatment with enrofloxacin affects cytokine production by T cells. Adult mice received water (group A) or water with enrofloxacin (group B) for two weeks. Mice were sacrificed, and PPs were collected. Single-cell suspensions stained with fluorochrome-conjugated antibodies for TCRβ$^+$ and CD4$^+$ and IL10$^+$ (A), LAP$^+$ (B), and INF-γ$^+$ (C), and analyzed by flow cytometry. The flow cytometry plots of IL10$^+$ and LAP$^+$ (D) positive cells and IFN-γ+ (E) positive cells from water and enrofloxacin-treated mice. Results are shown as means ± SE. n = 4–5 *P < 0.05.

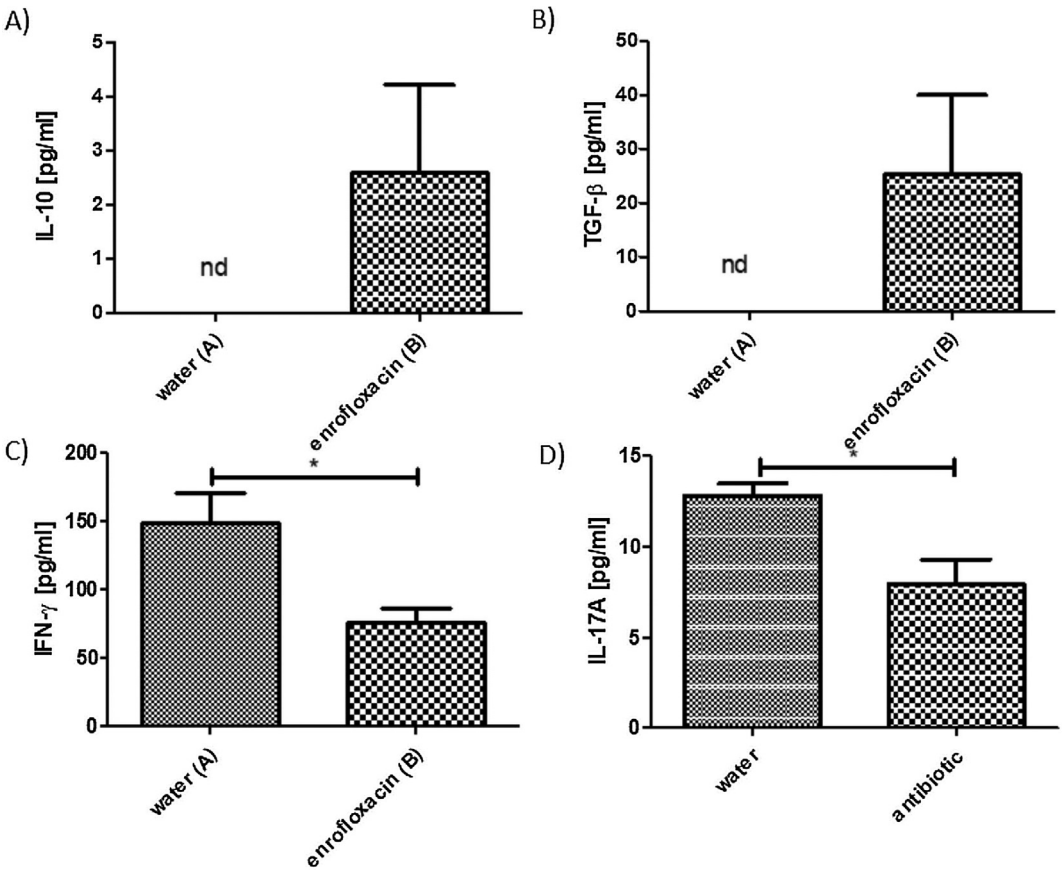

Fig. 5. Oral treatment with enrofloxacin creates an anti-inflammatory environment in the periphery. Adult mice received water (group A) or water with enrofloxacin (group B) for two weeks. Mice were sacrificed, and SPLs were collected. Single-cell suspensions were cultured for 48 h with 100 μg TNP-Ig, and culture supernatants were evaluated for IL-10 (A), TGF-β (B), IFN-γ (C), IL-17A (D). Results are shown as means ± SE. n = 4 *P < 0.05. Significance was determined using a *t*-test. nd-not detected – below ELISA kit sensitivity.

10^+ cells (Fig. 4A) or TCRβ$^+$ CD4$^+$ IFN-γ$^+$ (Fig. 4C).

3.3. Antibiotic-induced dysbiosis creates an anti-inflammatory environment in the periphery

To determine if enrofloxacin-induced dysbiosis affects the immune status in the periphery, mice received drinking water or water with enrofloxacin for two weeks before SPL collection and cell culture with TNP-Ig antigen.

Data presented in Fig. 5 show a trend towards increased level of anti-inflammatory IL-10 (Fig. 5A) and TGF-β (Fig. 5B) in SPL cells from enrofloxacin-treated (group B) mice compared to water-receiving mice (group A). On the contrary, treatment with enrofloxacin (group B) significantly decreased the production of INF-γ (Fig. 5C) and IL-17A (Fig. 5D) by SPL cells compared with water-treated controls (group A).

Additionally, we investigated the skewing of immune responses in the SPL on a cellular basis by flow cytometry. Fig. 6 shows that enrofloxacin-receiving mice (group B) have higher percentages of anti-inflammatory TCRβ$^+$CD4$^+$IL-10$^+$ (Fig. 6A) and TCRβ + CD4$^+$LAP$^+$ (Fig. 6B) cells compared to water-treated control mice (group A). Simultaneously, Simultaneously, the antibiotic-treated group has decreased the percentage of pro-inflammatory TCRβ$^+$ CD4$^+$ IFN-γ$^+$ (Fig. 6C) cells compared to the control.

3.4. Treatment with enrolfloxacin induces tolerogenic DC in the intestines and in the periphery

Our data indicate that the oral application of enrofloxacin creates an anti-inflammatory environment in the gut and the periphery characterized by higher levels of IL-10 and TGF-β and lower levels of IL-17A in PP and SPL. Anti-inflammatory cytokines are known inducers of tolerogenic DC, which drive the development of regulatory cells.

To test if the anti-inflammatory environment in the gut and the periphery created by treatment with enrofloxacin supports the induction of tolerogenic DC, we performed flow cytometry analysis of PP (Fig. 7A) and SPL (Fig. 7B). Fig. 7 shows that oral treatment with enrofloxacin increased the percentage of IL-10-producing B220$^-$CD11c$^+$CD11b$^+$ DC in PP and SPL.

4. Discussion

Various inflammatory diseases present altered microbial communities in the intestines. Alteration of microbial communities in the intestines is observed in various inflammatory diseases. Reports are showing that treatment with antibiotics modify microbiota composition and subsequently affect immunity [4]. Our previous work has shown that enrofloxacin-induced intestinal dysbiosis characterized by increased levels of SCFA- producing *Clostridium coccoides* (cluster XIVa), *Clostridium coccoides – Eubacterium rectale* (cluster XIVab), *Bacteroidetes* and *Bifidobacterium* spp., but decreased SFB, effectively suppress induction of the CS response in mice. Suppression of CS was due to induction of numerous regulatory cells: TCRαβ$^+$CD4$^+$CD25$^+$FoxP3$^+$ Treg, CD19$^+$B220$^+$CD5$^+$IL-10$^+$, IL-10$^+$ Tr1, and IL-10$^+$TCRγδ$^+$ cells [9]. Suppression of unwanted immune responses by induction of regulatory cells may have valuable therapeutic potential for the treatment of various inflammatory diseases. Our present study, aimed to evaluate how enrofloxacin-induced shifts in the intestinal microbiota promote the induction of cells with regulatory potential by characterization of

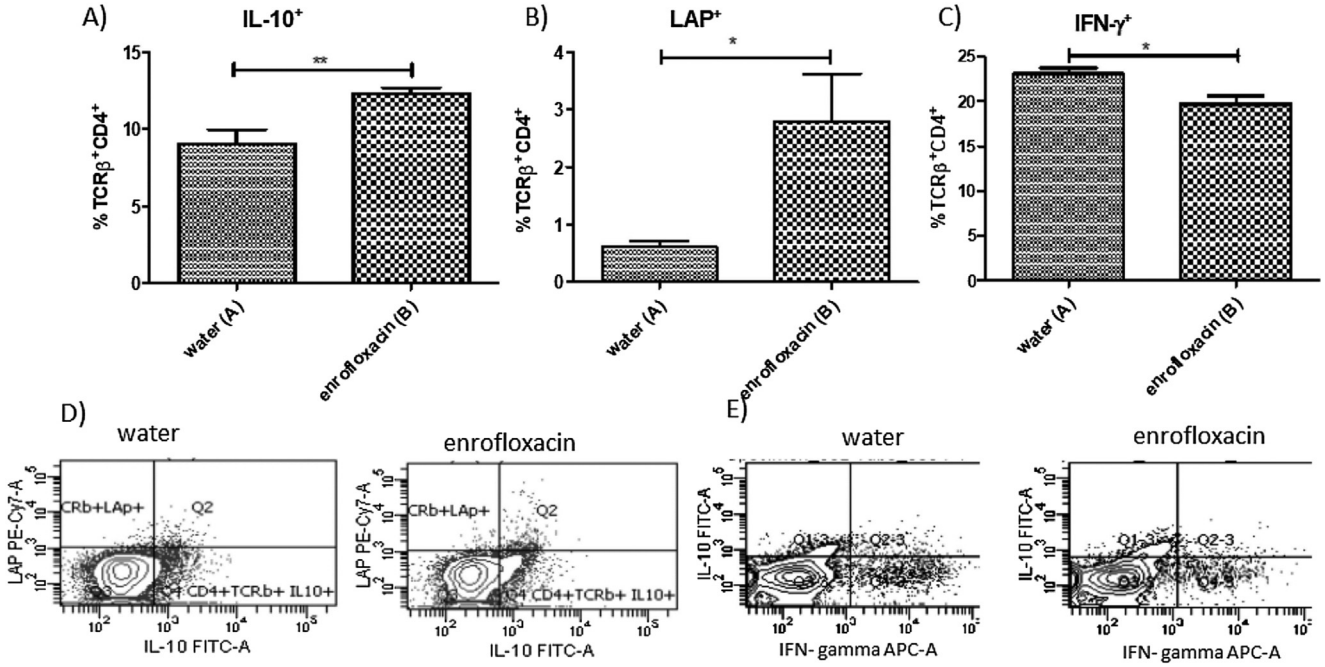

Fig. 6. Oral treatment with enrofloxacin modulates immune status in the SPL. Adult mice received water (group A) or water with enrofloxacin (group B) for two weeks. Mice were sacrificed, and SPLs were collected. Single-cell suspensions were stained with fluorochrome-conjugated mAb for TCRβ$^+$ and CD4$^+$ and (A) IL10$^+$ (B) LAP$^+$ cells and (C) INF-γ$^+$ and analyzed by flow cytometry. The flow cytometry plots of IL10$^+$ and LAP$^+$ (D) cells and IFN-γ + positive cells (E) from water and enrofloxacin-treated mice. Results are shown as means ± SE. n = 4–5 *P < 0.005 and **P < 0.01.

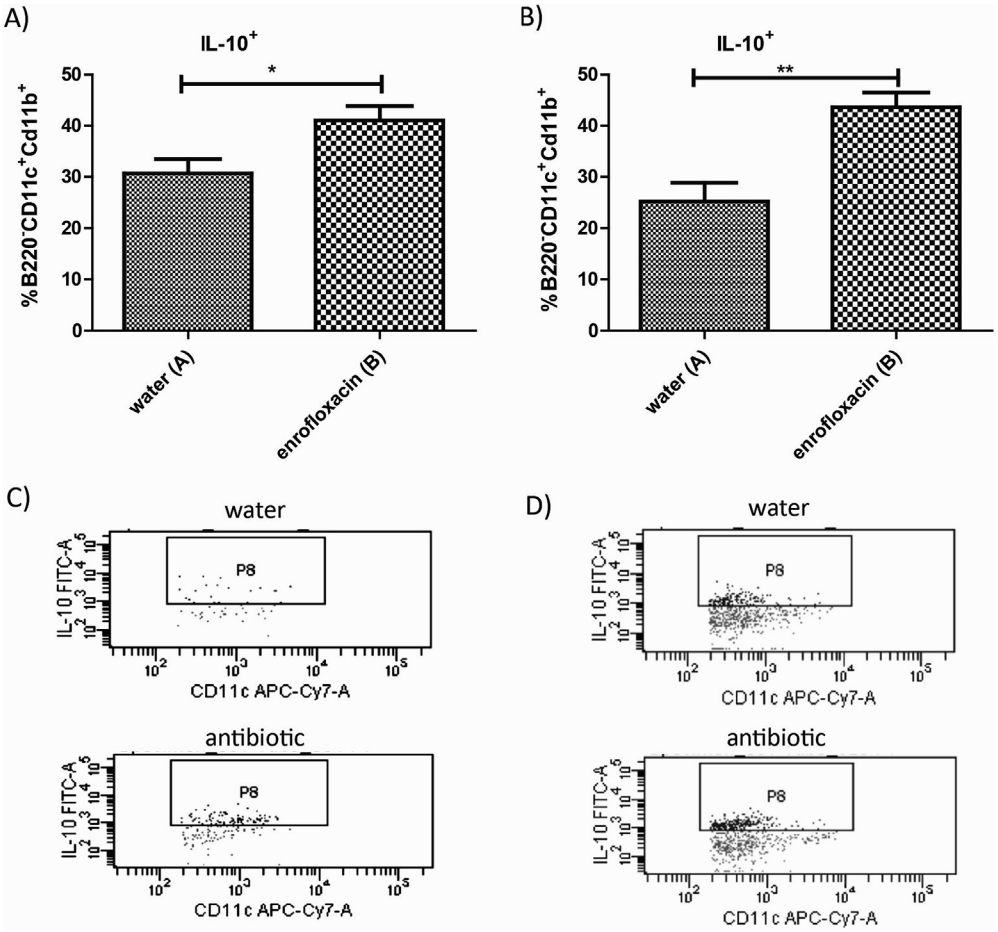

Fig. 7. Oral treatment with enrofloxacin induces tolerogenic DC. Adult mice received water (group A) or water with enrofloxacin (group B) for two weeks. Mice were sacrificed, and PPs and SPLs were collected. Single-cell suspensions of PP (A) and SPL (B) were stained with fluorochrome-conjugated mAb for B220, CD11c, CD11b, and intracellular IL-10 and evaluated by flow cytometry. The flow cytometry plots of IL10$^+$ producing cells from PP (C) and SPL (D) from water and enrofloxacin-treated mice. Results are shown as means ± SE. n = 4–5 *P < 0.05 and **P < 0.01.

the immune environment in the intestines and the periphery and this could guide the development of the adaptive immune response.

The adaptive immune response is induced after antigen endocytosis, processing, and presentation by DC to naïve T lymphocytes. Skewing of naïve T cells to lymphocytes with regulatory potential requires an anti-inflammatory cytokine environment generated by tissue-resident cells and antigen-presenting cells. Two cytokines, IL-10 and TGF-β, with strong anti-inflammatory potential, are implicated in the induction of regulatory cells directly and indirectly through the induction of DC. To evaluate the impact of intestinal dysbiosis induced by two-week oral treatment with enrofloxacin on the immune environment in the intestinal mucosae, we measured cytokine production *in vivo* by testing intestinal lavages and *in vitro* and by measuring cytokine production by intestine biopsies. Our data show that enrofloxacin-induced dysbiosis increases the concentration of anti-inflammatory IL-10 in small and large intestinal lavages (Fig. 1) and supernatants from the small intestine biopsies cultures (Fig. 2). Simultaneously, no change in IL-17A and IFN-γ production was observed in intestinal lavages and biopsies culture.

IFN-γ and IL-17A are T cell cytokines, which are the signatures of T helper lymphocytes type 1 (Th1) and type 17 (Th17) lineages, respectively. These T cell subsets differentiate in response to antigen in the context of pro-inflammatory cytokines produced as a response to pathogens. These effector populations mount adequate immune responses by orchestrating innate immune cells to clear infections. Th1-derived IFN-γ activates macrophages to fight intracellular pathogens and supports immunoglobulin production while Th17-derived IL-17A recruits and activates neutrophils to eliminate extracellular bacteria and fungi [18]. Increased production of both cytokines is implicated in autoimmunity and other inflammatory diseases such as CS. Their induction could be inhibited by IL-10 directly or indirectly by the potential of IL-10 to induce regulatory cells. IL-10 is a potent anti-inflammatory cytokine secreted by T and B lymphocytes, a wide range of innate immune cells such as monocytes, macrophages, DC, mast cells, as well non-immune cells such as keratinocytes and stromal cells. IL-10 is a potent anti-inflammatory cytokine as it inhibits the function of innate immune cells by inhibiting reactive oxygen production (ROS) production, expression of adhesion molecules, and production of pro-inflammatory IL-1β, TNF-α, IL-6, IL-8, and MIP-1α. IL-10 also suppresses induction of the adaptive immune response by inhibiting antigen presentation through reducing MHC II and B7-1 and B7-2 costimulatory molecule expression. DCs exposed to IL-10 and those that secrete IL-10 favor the development of regulatory T cells that suppress adaptive immunity. Additionally, IL-10 has been shown to downregulate adaptive immunity directly by reducing the production of Th1 and Th2 cytokines and suppressing T cell proliferation by reducing IL-2 production.

No change of pro-inflammatory IFN-γ and IL-17A production after enrofloxacin treatment suggests that gut dysbiosis does not impair cytokine production by Th1 and Th17 cells, however higher levels of anti-inflammatory IL-10 implies the potential to skew immune response towards anti-inflammatory. This data correlates well with other studies showing that oral treatment with broad-spectrum antibiotic mixtures modulates immune responses toward an anti-inflammatory state. However, the mechanisms of skewing the immune response vary between studies. The production of IFN-γ and IL-17A could be decreased [19] or unchanged with a simultaneous increase in the level of anti-inflammatory IL-10 [20]. The expression level of anti-inflammatory IL-10 cytokines in the intestinal mucosa has been the most variable between studies as some investigators observed reduced IL-10 expression [19], others have pointed out that expression is unchanged [21], while still, some found increases in IL-10 levels [20,22]. These discrepancies may be due to experimental differences such as the type, dose, and length of antibiotic treatment used. The maturity of the immune system is another factor affecting the immune response in mice [23]. Early-life antibiotic treatment increases the risk of developing allergic diseases [4]. Two-week-old pups breastfed with a streptomycin sulfate, ampicillin, metronidazole, and vancomycin mixture had increased expression of IL-10 in colon mucosae while no difference in IL-4, IL-17, or IFN-γ were observed [20]. Interestingly, this type of skewing is similar to one described in the present study.

Immune and non-immune cells: mostly epithelial and, to a lesser extent, stromal cells, mount the immune environment in gut mucosa. Between epithelial cells of the intestinal wall are intestinal immune cells (IEL: intraepithelial lymphocytes). They are also immersed in the lamina propria between stromal cells and create organized structures such as PP.

To evaluate the impact of enrofloxacin-induced dysbiosis on immune cells within the gut mucosae, we measured the immune status of cells present in PP. Our study shows that enrofloxacin treatment has no influence on INF-γ production (Fig. 3C) and the percentage of INF-γ-producing TCRαβ + CD4 + lymphocytes (Fig. 4C), implying that dysbiosis does not impact Th1 cell differentiation. These results are in line with other studies [24]. Interestingly, decreased production of IL-17A (Fig. 3D), may imply reduced T cell differentiation towards a Th17 population. However, it does not exclude the possibility that the production of IL-17A is reduced not because of reduced differentiation to Th17 cells but inhibited production by innate immune cells such as lymphoid tissue inducer cells, NK, NKT cells, macrophages, and Paneth cells [25].

Simultaneously, enrofloxacin treatment increased the production of IL-10 (Fig. 3A) and TGF-β (Fig. 3B) in PP. These data are in line with flow cytometry data showing an increased frequency of LAP-positive $TCR\beta^+CD4^+$ in PP (Fig. 4B). We did not observe an increase in IL-10 production by $TCR\alpha\beta^+CD4^+$ cells; however, this could be explained by increased IL-10 production by cells other than T lymphocytes such as DC (Fig. 7A).

Flow cytometry data also confirmed an increase in the percentage of $TCR\beta^+CD4^+LAP^+$ cells (Fig. 4B), which corresponds to a membrane-bound form of TGF-β. TGF-β is a pleiotropic cytokine with non-immune and immune activities. It supports tissue remodeling by inducing fibroblast proliferation, increasing extracellular matrix (ECM) deposition, and supporting angiogenesis. Its immunoregulatory activity plays an important role in adaptive immunity. It was shown that TGF-β-deficient mice present an elevated autoimmune response characterized by circulating antibodies to nuclear antigens and immune complex deposition, but that these animals could be protected from autoimmune manifestations when crossed with MHC II-deficient animals [26]. TGF-β was shown to be a negative regulator of the immune response, however, its precise function depends on the immune context. Also, mice deficient in TGF-β receptors develop multi-organ inflammation, which is the most pronounced in the lungs and intestines [27]. Additionally, TGF-β is a crucial factor inducing immunoglobulin isotype class switching to IgA [28]. Predominantly, TGF-β inhibits Th1 and Th2 differentiation, cytokine production, and stimulates formation of iTregs.

We then evaluated the role of enrofloxacin-induced dysbiosis in the development of systemic immune responses by measuring cytokine production by splenocytes. We observed increased levels of IL-10 (Fig. 5A) and TGF-β (Fig. 5B) and significantly decreased levels of proinflammatory INF-γ (Fig. 5D) and IL-17 (Fig. 5D), indicating skewing of the immune response towards an anti-inflammatory phenotype. These data correspond well with the flow cytometry data where we observed an increase in the frequency of $TCR\beta^+CD4^+LAP^+$ (Fig. 6B) and $TCR\alpha\beta^+CD4^+$ $IL10^+$ (Fig. 6A) lymphocytes and decrease in the percentage of pro-inflammatory $TCR\alpha\beta^+CD4^+$ $IFN\text{-}\gamma^+$ (Fig. 6C) cells in the SPL. Our studies are in line with Dimmitt et al., where a mixture of streptomycin, ampicillin, metronidazole, and vancomycin provided during breastfeeding resulted in significantly lower production of IFN-γ and IL-17 in the SPLs of two-week-old pups. Simultaneously they observed a higher production of IL-10 [20].

Our data indicate that the immune environment created by two-week enrofloxacin treatment promotes induction of cells with an anti-

inflammatory phenotype due to high levels of IL-10 and TGF-β secreted. These cytokines were implicated in the induction of regulatory cells. TGF-β enhances conversion of naïve T cells to FoxP3$^+$ expressing inducible Tregs (iTregs) [3,13] and supports induction of IL-10$^+$ Tr1 lymphocytes [14]. Production of both IL-10 and TGF-β cytokines is regulated by gut microorganisms. SCFA-producing bacteria like some *Clostridium* species induce production of TGF-β by intestinal tissues [3], while *Bacteroides fragilis*-derived bacterial wall constituent, polysaccharide A (PSA), increases IL-10 production by FoxP3$^+$ Tregs after Toll-like receptor 2 (TLR2) binding [29]. Data showing that gut microorganisms regulate IL-10, TGF-β production, and are implicated in regulatory cell induction are in accordance with our data showing that enrofloxacin-induced dysbiosis promotes induction of regulatory cells in the CS model [9].

Additionally, both IL-10 and TGF-β support the induction of regulatory cells indirectly by induction of tolerogenic DC. The development of tolerogenic DC is under the influence of microbial signals acting directly on DC [17] or through intestinal epithelial cells (IEC) and stromal cells [30]. Flow cytometry analysis of immune cells from PP (Fig. 7A) and SPL (Fig. 7B) showed that two weeks of antibiotic treatment increases the number of B220$^-$CD11c$^+$CD11b$^+$ DC that secrete IL-10. The tolerogenic DC support regulatory cell induction, which is in line with our previous studies, showing an increase in the frequency of regulatory cells after enrofloxacin treatment and immunization [9].

These data are in line with studies showing that modification of intestinal bacteria through probiotic bacteria supplementation, including the use of different *Lactobacilli* species and *Bifidobacterium bifidum*, induces IL-10-producing tolerogenic DC which subsequently increase conversion of naïve T cells to iTreg and activate naturally occurring Tregs [17].

Our study shows that two-week enrofloxacin treatment generates an anti-inflammatory environment in the gut and the periphery, and supports the induction of IL-10-producing tolerogenic DC. An increase in the frequency of tolerogenic DC may promote the generation of regulatory cells seen after but not before immunization.

This data implies that directed modification of gut microbiota to a state similar to what we observed after two-week enrofloxacin treatment could have therapeutic potential due to its immunomodulatory activity.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105966.

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