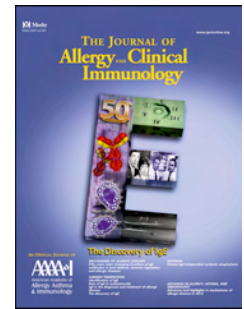


Accepted Manuscript

Broad spectrum antibiotic enrofloxacin modulates contact sensitivity through gut microbiota in a murine model

Anna Strzępa, PhD, Monika Majewska-Szczepanik, PhD, Francis M. Lobo, MD, Li Wen, MD, PhD, Marian Szczepanik, PhD



PII: S0091-6749(17)30056-8

DOI: [10.1016/j.jaci.2016.11.052](https://doi.org/10.1016/j.jaci.2016.11.052)

Reference: YMAI 12598

To appear in: *Journal of Allergy and Clinical Immunology*

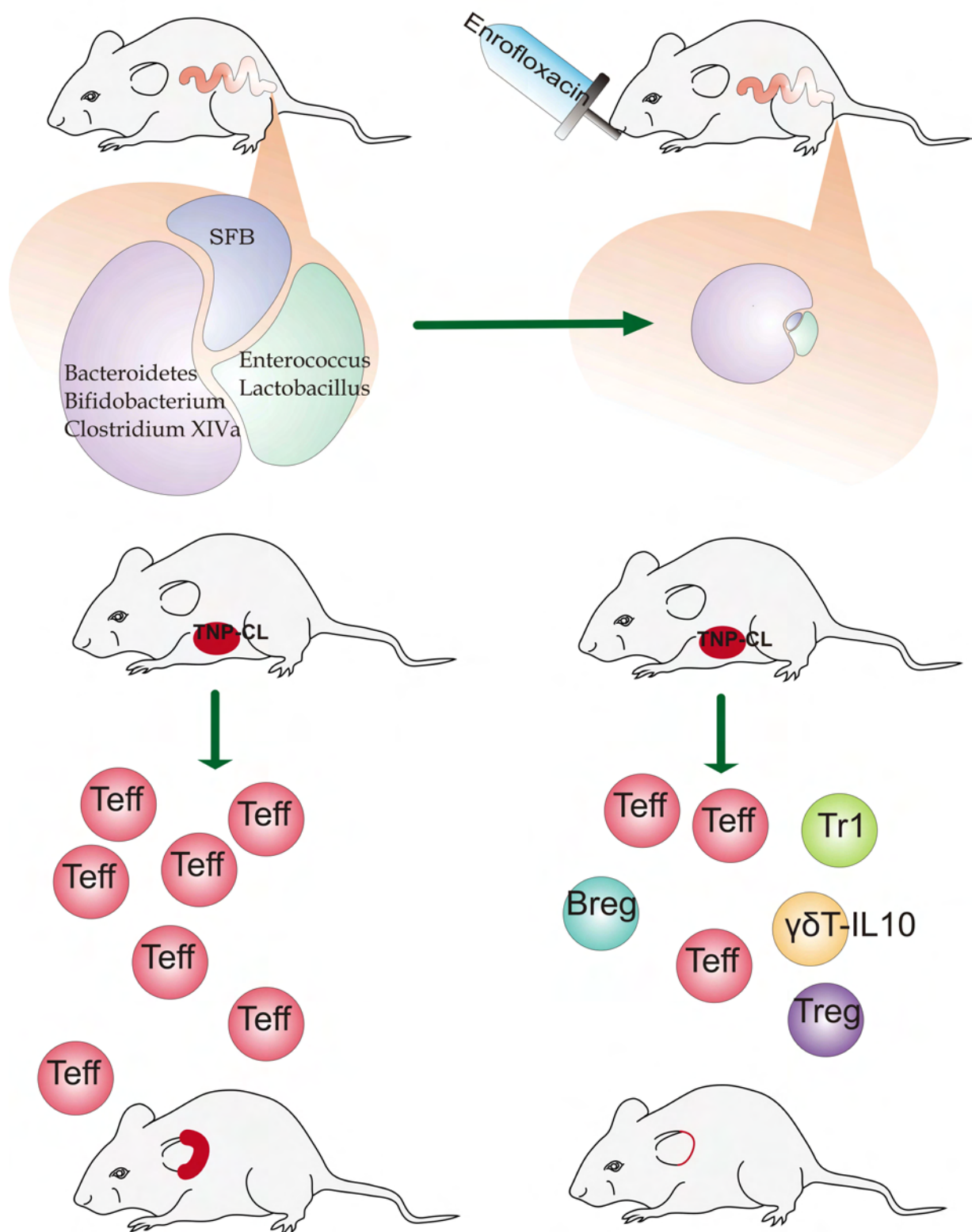
Received Date: 10 January 2016

Revised Date: 10 November 2016

Accepted Date: 23 November 2016

Please cite this article as: Strzępa A, Majewska-Szczepanik M, Lobo FM, Wen L, Szczepanik M, Broad spectrum antibiotic enrofloxacin modulates contact sensitivity through gut microbiota in a murine model, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2016.11.052.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Broad spectrum antibiotic enrofloxacin modulates contact sensitivity through gut microbiota in a murine model

Anna Strzępa PhD^a, Monika Majewska-Szczepanik PhD^a, Francis M. Lobo MD^b,

Li Wen MD, PhD^c and Marian Szczepanik PhD^a

^a Department of Medical Biology, Faculty of Health Sciences, Jagiellonian University Medical College, ul. Kopernika 7a, 31-034 Krakow, Poland

^b Section of Allergy and Clinical Immunology, Department of Internal Medicine, Yale University School of Medicine, New Haven CT., USA

^c Department of Internal Medicine, Section of Endocrinology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520-8013 USA

Corresponding author: Marian Szczepanik, PhD; Department of Medical Biology, Jagiellonian University Medical College, ul. Kopernika 7a, 31-034 Krakow, Poland. E-mail: mmszczep@cyf-kr.edu.pl. Tel/Fax: +48 12 422 99 49

Co-corresponding author: Li Wen, li.wen@yale.edu

Supported by grant from National Science Center 2011/01/B/NZ6/00300 in Poland and K/ZDS/004650 to MS and NIH (DK088181 and DK092882) to LW and K/DSC/002879 to AS.

Abstract

Background: Medical advances in the field of infection therapy has led to an increasing use of antibiotics, which, apart from eliminating pathogens, also partially eliminate naturally existing commensal bacteria. It has become increasingly clear that less exposure to microbiota early in life may contribute to the observed rise in “immune-mediated” diseases including autoimmunity and allergy.

Objective: We sought to test whether the change of gut microbiota with the broad spectrum antibiotic enrofloxacin will modulate contact sensitivity (CS) in mice.

Methods: Natural gut microbiota were modified by oral treatment with enrofloxacin prior to sensitization with TNP-CI followed by CS testing. Finally, adoptive cell transfers were performed to characterize the regulatory cells that are induced by microbiota modification.

Results: Oral treatment with enrofloxacin suppresses CS and production of anti-TNP IgG1 antibodies. Adoptive transfer experiments show that antibiotic administration favors induction of regulatory cells that suppress CS. Flow cytometry and adoptive transfer of purified cells show that antibiotic-induced suppression of CS is mediated by $\text{TCR}\alpha\beta^+\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ Treg, $\text{CD19}^+\text{B220}^+\text{CD5}^+\text{IL-10}^+$, IL-10^+ Tr1, and $\text{IL-10}^+\text{TCR}\gamma\delta^+$ cells. Treatment with the antibiotic induces dysbiosis characterised by increased proportion of *Clostridium coccoides* (cluster XIVa), *Clostridium coccoides* – *E. rectale* (cluster XIVab), *Bacteroidetes* and *Bifidobacterium* spp., but decreased segmented filamentous bacteria. Transfer of antibiotic-modified gut microbiota inhibits CS, but this response can be restored through oral transfer of control gut bacteria to antibiotic-treated animals.

Conclusion: Oral treatment with a broad spectrum antibiotic modifies gut microbiota composition and promotes anti-inflammatory response, suggesting that manipulation of gut microbiota can be a powerful tool to modulate the course of CS.

Key messages:

- Oral treatment with broad spectrum antibiotic enrofloxacin leads to gut dysbiosis associated with increased levels of anti-inflammatory bacteria.
- An alteration in gut microbiota profile favors induction of a variety of regulatory cells that inhibit CS in mice.
- Transfer of antibiotic-modified gut microbiota inhibits CS, indicating that targeted modulation of gut microbiota could have therapeutic potential.

Capsule summary:

Antibiotic-induced dysbiosis, characterized by increased levels of *Bacteroidetes*, *Bifidobacterium* spp., *Clostridium* cluster XIVa and XIVab, but decreased proportions of segmented filamentous bacteria, effectively inhibits contact sensitivity by inducing a variety of regulatory cells.

Key words: contact sensitivity, gut microbiota, immunoregulation, dysbiosis, regulatory cells

Abbreviations:

AD: atopic dermatitis

ALNC: axillary and inguinal lymph node cells

CIA: collagen-induced arthritis

CS: contact sensitivity

FACS: Fluorescence-activated cell sorting

EAE: experimental autoimmune encephalomyelitis

i.p.: intraperitoneal

MLNC: mesenteric lymph node cells

MPO: myeloperoxidase

NOD: non-obese-diabetic

PP: Peyer patch

SFB: segmented filamentous bacteria

SPLC: splenocytes

T1D: type 1 diabetes

Th: Helper T lymphocytes

Treg: Regulatory T cells

TNP-Cl: trinitrophenyl chloride

Introduction

Advances in medicine in the field of infection therapy over the last several decades has led to a drastically increasing application of antibiotics, which, apart from eliminating pathogens, also partially eliminate naturally existing commensal bacteria. In addition,

unlabeled broad-spectrum antibiotics are often present in the food supply, which also can change the composition of gut microbiota. A study in a group of healthy human volunteers showed that treatment with ciprofloxacin for 5 days decreases diversity, richness, and evenness of fecal microbiota during antibiotic treatment [1]. Although the microbiota mostly returned to pretreatment composition following ciprofloxacin treatment, several bacterial taxa failed to recover, indicating that changes to the microbiota can persist following a short course of oral antibiotics [2]. Similar to human studies, mouse models have revealed that treatment with ampicillin, cefoperazone, vancomycin or clindamycin causes long-lasting changes in the gut microbiota that persist after cessation of antibiotic treatment [3, 4, 5].

It has become increasingly clear that less exposure to microbiota early in life may contribute to the observed rise in the diseases characterized by immune dysregulation, including allergy, autoimmunity, metabolic disorders, and even neoplastic diseases [6]. A number of studies have demonstrated the increased risk of developing asthma and food allergy in children who have been exposed to antibiotics in the first year of life [7, 8, 9]. Similar findings are also observed in animal studies, such as in the non-obese-diabetic (NOD) mouse, which is known to be very sensitive to the change of the environment, such that a cleaner environment enhances the rate of onset and incidence of type 1 diabetes (T1D) [10]. This is also the case in animal models of allergic asthma [11, 12]. Studies carried out in recent years have confirmed a strong connection between the natural gut microbiota and the immune response. However, this interaction may differ in various experimental models. In an animal model of multiple sclerosis, for instance, a strong inhibition of inflammation has been observed in mice treated orally with an antibiotic [13]. Similar observations have been reported in an animal model of human rheumatoid arthritis [14].

Atopic dermatitis (AD) is a prominent clinical example of an immune-mediated disease that is affected by environmental factors. AD has become a considerable clinical problem, having nearly tripled in prevalence over the last 30 years in developed countries. The disease afflicts 15-30% of children and about 2-10% of adults [15]. Yet another form of skin disease with an underlying immune-mediated hypersensitivity reaction is contact sensitivity (CS) to haptens. Allergic contact dermatitis, as opposed to AD, is a typical CS response in humans and has become a significant cause of morbidity. Indeed, contact dermatitis linked to exposure to chemical substances in the workplace constitutes about 30% of all occupational diseases and represents a severe social and economic problem [16].

The hygiene hypothesis has been advanced as an explanation for the dramatic rise in the prevalence of allergic diseases in industrialized societies. Supporters of this hypothesis note that decreasing microbial exposure of many types, including bacteria, parasites, and viruses, has been associated with the rise in incidence of allergic diseases and autoimmune disorders [17]. Increasing evidence suggests that gut microbes, which represent an enormous source of interaction between microbes and immunity, play a very important role in various health problems [6]. However, it is not clear what effects are exerted by natural gut microbiota on the course of CS responses. Given the common use of antibiotics in industrialized societies, in the setting of ever increasing exposure to potential CS-generating haptens in the ‘chemicalized’ modern environment, we see that it is important to study the effect of the gut microbiota on the CS response. In the current work, therefore, we test our hypothesis that change of gut microbiota with a broad spectrum antibiotic will modulate CS response in mice.

METHODS

Mice

C57BL/6 mice, at 6 weeks old, were taken from the breeding unit of the Department of Medical Biology, Jagiellonian University, College of Medicine. Mice were kept under pathogen-free conditions in individual cages using Aero-Mouse IVC Green Line system (Tecniplast S.p.A., Buguggiate, Italy). All experiments were conducted in compliance with the guidelines of the 1st Local Ethics Committee of the Jagiellonian University College of Medicine.

Reagents

Trinitrophenyl chloride (TNP-Cl) (Chemica Alta, Edmonton, Canada); Evans blue, formamid, hexadecyltrimethylammonium bromide, lysozyme, magnesium chloride, JumpStart™ Taq ReadyMix™ for qPCR, o-dianisidine dihydrochloride, sodium dodecyl sulfate, Tris-EDTA, and hydrogen peroxide were obtained from Sigma (St. Louis, MO, USA). Silica beads were from BioSpec Products (USA); proteinase K was from Roche Diagnostics (Germany). Baytril (enrofloxacin) was purchased from Bayer Animal Healthcare GmbH (Leverkusen, Germany).

Sensitization and elicitation of contact sensitivity (CS) *in vivo*

Contact hypersensitivity to TNP-Cl was induced as described previously [18]. Vascular permeability, MPO assay and concentration of IFN- γ was measured as previously described [18].

Treatment with enrofloxacin

Mice received drinking water containing the broad spectrum antibiotic enrofloxacin (0.27 mg/ml) or water alone for two weeks prior to sensitization with TNP-Cl. In some experiments mice received enrofloxacin *i.p.* (5 mg/kg) or PBS for two weeks prior to further tests.

Evaluation of gut flora depletion

Serial dilutions of colon contents from enrofloxacin-treated or untreated mice were cultured on general agar plates (blood agar, Bioshop Inc., Burlington, Canada) at 37°C for 48 h or 72 h in aerobic and anaerobic conditions respectively. Total bacteria per gram of sample was calculated using CFU.

Adoptive cell transfer of CS and cell mixing assay to evaluate suppression (transfer out protocol).

Donors of CS-immune effector cells were contact sensitized with 5% TNP-Cl. Axillary and inguinal lymph nodes (ALN) were harvested from TNP-Cl-immune mice on day “+4,” and 7×10^7 immune cells were incubated for 30 min. at 37°C in RPMI 1640 medium alone, washed, and then injected *i.v.* into normal syngeneic recipients (positive transfer). For the cell mixing assay, 7×10^7 of the CS-effector immune cells from TNP-Cl contact sensitized donors were incubated for 30 min. at 37°C with 5×10^7 ALN or SPL, or 1×10^7 Peyer patch (PP) or mesenteric lymph node (MLN) cells, from mice treated orally with enrofloxacin for 2 wks prior to TNP-Cl immunization. After incubation the cell mixture was transferred *i.v.* into naïve recipients. Mice were challenged with the hapten within 30 min. after cell transfer and tested for CS 24 h later.

Transfer into tolerized mice to confirm active tolerance (transfer in protocol).

To confirm data found in the “transfer out” cell mixing assay, 5×10^7 ALN or SPLC, or 1×10^7 PP or MLNC, isolated from donors orally treated with enrofloxacin for 2 wks prior to TNP-Cl, were transferred *i.v.* into syngeneic recipients 2 h prior to CS induction, or one day prior to CS elicitation (day “+3”). Mice then were challenged on the ears and tested for CS. In

some experiments MACS purified cell populations were transferred as indicated to characterize their regulatory activity.

Extraction of bacterial DNA from the gut content

Gut content was collected by lavage with sterile PBS and centrifuged. The pellet was resuspended in sterile PBS and mixed vigorously. After centrifugation, the supernatant without the solid fraction was collected and centrifuged. The pellet was resuspended in 300 μ l of Tris-EDTA. Samples were frozen and thawed several times followed by chemical disruption with lysozyme and SDS, followed by protein removal using silica beads. DNA extraction with phenol:chloroform:isoamyl alcohol (25:24:1) solution was performed as described elsewhere.

PCR conditions

To evaluate dysbiosis after oral antibiotic administration, RT-PCR was performed with 10.5 ng of DNA using CFX96 Touch. Detection of selected gut bacteria species and group was based on amplification of conserved 16S rDNA fragments. The primer sequences are summarized in table I found in Online Respository. PCR conditions were as described previously [19, 20]. 16S rDNA detected by universal primers and probes, detecting all bacteria, was used as reference gene [21]. The expression level was determined by the values received from mixed DNA samples extracted from control mice and presented as $\Delta\Delta$ ct [22]. Values for all bacteria were subtracted from values obtained for selected bacteria [23].

Transfer of gut microbiota

Cohousing was performed by housing mice given oral enrofloxacin with untreated animals for two weeks prior to TNP-Cl sensitization and CS test. The experiment of “adoptive microbiota

transfer” (FMT- fecal microbiota transplantation) was performed by oral gavage with the fecal material from the donors that were or were not treated *per os* (*p.o.*) with enrofloxacin. 24 h after stopping enrofloxacin, the lumen contents of large intestine were harvested in 4 ml of sterile PBS, mixed vigorously, and centrifuged. The fecal supernatant (300 μ l) was orally inoculated twice a week for two weeks [24] prior to CS induction.

Statistics

Data in graphs are shown as mean $\pm$ SE. *t*-Student test or ANOVA followed by Tukey’s multiple comparison test was used. Statistical significance was set at $P < 0.05$.

Description of all of the materials and methods used in this study can be found in the Methods section in this article’s Online Repository at www.jacionline.org.

RESULTS

Oral application of broad spectrum antibiotic enrofloxacin alleviates CS in mice

To determine the influence of gut microbiota on CS, mice were treated orally or intraperitoneally (*i.p.*) with the broad spectrum antibiotic enrofloxacin prior to TNP-Cl sensitization. Oral treatment with enrofloxacin reduced CS, as measured by ear swelling, in actively immunized mice (Fig 1A, Group D vs. B), whereas *i.p.* injection of enrofloxacin does not affect ear swelling (Group F vs. B). Both oral or *i.p.* enrofloxacin treatment did not affect the body weight of mice, regardless of TNP-Cl immunized, when compared to animals not treated with enrofloxacin (Fig 1B, Groups C and E vs. A and Groups D and F vs. B). Oral antibiotic application significantly reduced the number of both aerobic (Fig 1C) and anaerobic (Fig 1D) bacteria in colon contents (Groups C and D vs. A and B). Intraperitoneal injection of

antibiotic did not affect bacterial counts of aerobes (Fig 1C) and anaerobes (Fig 1D, Groups E and F vs. A and B). A decrease of CS in actively immunized mice due to antibiotic treatment was confirmed by adoptive lymphocyte transfer experiments. Transfer of axillary and inguinal lymph node cells (ALNC) and splenocytes (SPLC) isolated from TNP-Cl sensitized donors that were orally treated with enrofloxacin to naïve mice significantly reduced CS when compared to positive control (ALNC and SPLC from hapten-sensitized mice not treated with antibiotic) (Fig 1E, Group C vs. B). Additionally, oral treatment with antibiotic prior to CS induction and elicitation increased production of TNP-specific IgG1 antibodies but reduced synthesis of anti-TNP IgG2a antibodies (Fig 1F, Group C vs. B). The ear swelling data were confirmed in further experiments showing that oral treatment with enrofloxacin prior to TNP-Cl sensitization results in decreased vascular permeability (Fig 2A) and lower ear weight (Fig 2B). Additionally, oral application of enrofloxacin before induction of CS resulted in reduced myeloperoxidase (MPO) activity, as well as decreased IFN- γ concentration in ear extracts (Fig 2C and Fig 2D).

Oral treatment with antibiotic induces cells that inhibit CS *in vivo*.

To test if decreased CS responses after antibiotic treatment can be transferred into naïve recipients, we performed adoptive transfer (“transfer out”) experiments, in which naïve recipient mice were given TNP-Cl immune cells (7×10^7 cells) that were pre-incubated with ALNC or SPLC (5×10^7), or mesenteric lymph node (MLNC) (1×10^7 cells), or Peyer patch (PP)-derived lymphocytes (1×10^7 cells) isolated from syngeneic animals that were orally treated with antibiotic and sensitized with TNP-Cl (Fig 3A, Groups C-F). The recipient mice were tested for CS immune response after cell transfer. Mice in the positive control group received immune cells only (Group B). ALNC, SPLC, MLNC and PP from donors treated with antibiotic prior to TNP-Cl immunization inhibit the effector function of TNP-Cl-

sensitized CS-effector cells (Fig 3A, Groups C-F vs. B). Additionally, employing a “transfer in” protocol, we showed that injection of ALNC, SPLC, MLNC or PP cells isolated from donors treated with antibiotic and sensitized with TNP-Cl into actively sensitized recipients one day before ear challenge (day “+3”) suppressed CS response (Fig 3B; Groups C-F vs. B). Our results suggest that cells from antibiotic-treated mice prior TNP-Cl sensitization suppress the effector phase of the CS response. To determine if the antibiotic-induced cells also play a role during the CS induction phase, ALNC, SPLC, MLNC and PP cells isolated from mice receiving antibiotic prior to TNP-Cl sensitization were transferred into syngeneic mice on the day of TNP-Cl contact immunization (day “0”). These transferred cells interfere with the induction phase of the CS response (Fig 3C, Groups C-F vs. B).

Oral antibiotic treatment prior to TNP-Cl sensitization induces distinct populations of regulatory cells.

As shown above, pretreatment with antibiotic prior to hapten sensitization results in the induction of regulatory cells in ALN, SPL, MLN and PP. To determine which type of the regulatory cells is involved in the suppression of CS in our model, we phenotyped ALN, SPL, MLN and PP cells by flow cytometry after staining with different fluorochrome-conjugated antibodies. We found that the percentage of both $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ Treg and $\text{CD19}^+ \text{B220}^+ \text{CD5}^+ \text{IL-10}^+$ Breg cells in PP were increased in mice orally treated with antibiotic followed by TNP-Cl sensitization when compared to hapten-sensitized mice that had not been treated with antibiotic (Fig 4A, Group D vs. B). Moreover, we observed nonsignificant increase of $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ Treg cells in PP in mice treated with antibiotic without hapten sensitization, compared to the antibiotic-untreated mice (Fig 4A, Group C vs. A). FACS analysis of MLN showed that antibiotic treatment and hapten sensitization increased the frequency of both $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ Treg and

TCR $\alpha\beta$ ⁺ CD4⁺ IL-10⁺ Tr1 cells when compared to antibiotic-untreated, hapten-sensitized mice (Fig 4B, Group D vs. B). Interestingly, we also found an increased frequency of TCR $\gamma\delta$ ⁺ IL-10⁺ lymphocytes in ALN and SPL of antibiotic-treated, hapten-sensitized mice compared to the animals with hapten sensitization in the absence of antibiotic treatment (Figs 4C and 4D, Group D vs. B).

Adoptive transfer of purified regulatory cell populations inhibits CS *in vivo*.

To test the suppressive function of the regulatory cells induced by antibiotic treatment, we purified CD4⁺CD25⁺ cells or CD19⁺ cells from PP of antibiotic-treated and hapten-sensitized donors and then transferred CD4⁺CD25⁺ (3.9×10^5), CD19⁺ (3.3×10^5), or whole PP cells (1×10^7) into naïve mice prior to TNP-Cl immunization. Each of these populations suppressed CS, although the suppression by CD4⁺CD25⁺ cells was greater (Fig 5A, Groups D and E vs. B). Our results suggest that Treg and Breg cells may be involved in the observed immunoregulation. We also tested the regulatory cells from MLN using the same approach. Total MLN cells (1×10^7) and purified CD4⁺CD25⁺ cells (6×10^5) from MLN of antibiotic-treated and hapten-sensitized mice equally inhibited CS reactions in the recipients (Fig 5B, Group D vs. B). Finally, we tested the immune regulatory function of the TCR $\gamma\delta$ cells that were observed to be increased after antibiotic treatment. We found that transfer of 5×10^7 unpurified ALN or SPL of antibiotic-treated and hapten-sensitized donors down-regulated CS response in the recipient mice (Fig. 5C and 5D, Group C vs. B). Similar to the unpurified ALN and SPL, purified TCR $\gamma\delta$ from ALN (1.5×10^6) or SPL (1×10^6) of antibiotic-treated and hapten-sensitized donors down-regulated CS response in the recipient mice (Fig. 5C and 5D, Group D vs. B).

Treatment with antibiotic shifts the bacterial population toward an anti-inflammatory profile.

Oral antibiotic treatment significantly reduced both aerobic and anaerobic bacteria number in the gut. Recent studies indicate that some gut bacteria can promote anti-inflammatory responses, whereas others can induce inflammatory reactions [25]. To test if the observed down-regulated CS response in antibiotic-treated mice is the result of dysbiosis, we evaluated the composition of some common gut microbiota using RT-PCR. We found that the some species of gut bacteria changed significantly between treated and untreated mice, whereas other bacterial species remained unaffected (Fig 6). Among the tested species, the relative abundance of *Enterococcus spp.* and *Lactobacillus* were similar between untreated and antibiotic-treated mice (Fig 6A, Group B vs. A), whereas antibiotic treatment significantly increased the relative abundance of *Clostridium coccoides* (cluster XIVa) and *Clostridium coccoides* – *E. rectale* (cluster XIVab) compared to untreated mice (Fig 6B, Group B vs. A). Antibiotic treatment also increased the relative abundance of *Bacteroidetes* and *Bifidobacterium spp.* compared to untreated mice (Fig 6C, Group B vs. A). In contrast, antibiotic treatment significantly decreased the relative abundance of segmented filamentous bacteria (SFB) compared to the control mice (Fig 6D, Group B vs. A).

Direct transfer of gut flora modulates CS response *in vivo*.

To determine the direct role of gut microbiota in down-regulation of CS response associated to antibiotic treatment, we took two approaches to foster microbiota exchange between mice. First we performed a cohousing experiment by keeping mice that were previously antibiotic-treated or untreated for two weeks in the same housing, followed by sensitization with TNP-Cl and testing for CS immune response. We found that naïve mice co-housed with antibiotic-treated animals show decreased CS compared to control mice (Fig 7A, Group E vs. B). The

suppression of CS response in this group was similar to the suppression seen in the antibiotic-treated mice with or without cohousing (Fig 7A, Group E vs. C, D and F). Next, we transferred gut microbiota from the mice with or without antibiotic treatment directly to naïve mice through oral gavage, followed by induction of the CS response. The mice that received gut bacteria from antibiotic treated donors showed decreased CS after TNP-CI sensitization (Fig 7B, Group C vs. B), whereas there was no effect on CS response when the naïve recipients were given gut microbes from non-antibiotic-treated donors (Fig 7B, Group D vs. B). Furthermore, the suppressed CS response seen in antibiotic-treated mice was reversed by oral transfer of gut microbes from non-antibiotic treated mice (Fig 7C, Group E vs. C and D), while gavage of naïve mice with gut flora from non-antibiotic treated control animals did not affect CS (Fig 7C, Group F vs. B).

DISCUSSION

There is mounting evidence that the homeostasis of the gut microbiome is important for the balance and regulation of the immune system in both animals and humans. Recent studies have shown that microbiota play a crucial role in fostering the development of numerous T cell subsets including Th17 and Treg lymphocytes [26]. Germ-free mice have imbalanced Th1/Th17 responses that are beneficial in some autoimmune disorders, including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) [27, 28] but appear to be detrimental for others, such as T1D. The involvement of microbiota in the development of autoimmunity and other immune-mediated diseases has also been revealed by observing the effect of removing some gut bacteria with antibiotics [13, 14]. It has been shown that adult mice treated with broad spectrum antibiotics develop ameliorated forms of animal models of Th17-mediated psoriasis [29] and Th1 mediated EAE [13] but

show no effects in Th2-mediated allergic responses [30]. On the other hand, broad spectrum antibiotics administrated early in life increase susceptibility to Th17 mediated psoriasis [29] and to Th2-mediated atopic dermatitis [30]. Increasing evidence suggests that imbalance in homeostasis of gut bacterial species is associated with atopic disorders [31, 32, 33]. Metagenome sequence analyses of the gut microbiome from patients with atopic dermatitis showed that enrichment of a subspecies of the major species of gut bacteria *Faecalibacterium prausnitzii* is strongly associated with atopic dermatitis [34]. It has been revealed that operational taxonomic units belonging to *F. prausnitzii* F06 are significantly more abundant in the atopic dermatitis-associated microbiota. The authors postulate that interactions between dysbiosis in *F. prausnitzii* and dysregulation of gut epithelial inflammation might underlie the chronic progression of atopic dermatitis [34].

The influence of microbiota on CS response is not well explored. A few studies suggest that the proliferation of *Bifidobacterium pseudolongum* in the intestinal tract could be partially responsible for the reduction of DNFB-induced CD8 Tc1-mediated CS response in mice [35, 36], and oral administration of the probiotic *Lactobacillus casei* alleviates CD8 Tc1-mediated CS to DNFB [37]. Currently there are no studies, however, investigating the role of microbiota in CD4⁺ Th1-mediated CS responses. The novel findings of our study are, first, that the same antibiotic, enrofloxacin, administrated via different routes leads to different effects on CS *in vivo*. Second, we show that oral administration of enrofloxacin disturbs the natural composition of gut microbiota and induces different regulatory immune cells which suppress CS inflammatory response. Third, we show that the suppression of CS inflammatory response can be transferred to a new host by each population of regulatory cells. Fourth, we show that co-housing the mice with altered gut microbiota or fecal matter transfer can confer the protection to the new hosts. Finally, we show that the anti-inflammatory effect mediated by altered gut microbiota can be reversed by introducing normal gut microbiota. It is

interesting that systemic administration of the same antibiotic, through *i.p.* injection, did not affect CS response *in vivo*, suggesting the importance of antibiotic activity within the intestinal lumen. Our study provides strong evidence that gut microbiota play an important role in skin allergic inflammatory response. Concordantly, we find decreased CS responses after treatment with enrofloxacin prior to TNP-Cl sensitization in mice with highly divergent MHC haplotypes, namely NOD and BALB/c mice, suggesting that the observed phenomenon does not depend on the MHC background (unpublished observation).

The treatment with the antibiotic either orally or *i.p.* did not affect animal body weight, suggesting that the observed modulation of the CS response is not a result of side effects related to antibiotic treatment. The culture of gut content showed that oral but not *i.p.* administration of the antibiotic significantly reduced number of both aerobic and anaerobic gut bacteria. Our findings are in line with recent data showing that reduction of gut commensal bacteria after oral, but not *i.p.*, treatment with antibiotic impairs the development of EAE [13]. Analysing the composition of gut microbiota, we find an increase of *Bifidobacterium spp.*, in agreement with the study by other investigators showing that consumption of prebiotic fructo-oligosaccharide, which is associated with proliferation of *Bifidobacterium pseudolongum* in the intestinal tract of mice, reduces CS [38].

The CS seen after transfer of ALNC and SPLC from antibiotic-treated and TNP-Cl-sensitized mice is weaker compared with CS after transfer of ALNC and SPLC isolated from non-antibiotic treated, TNP-Cl-sensitized mice. However, this experiment did not answer the question of whether antibiotic treatment prior to hapten sensitization affects the induction of CS-effector cells themselves, or is involved in the induction of regulatory cells. To answer this question, two approaches have been taken in our study. Using a “transfer out” protocol, we show that cotransfer of CS-effector cells with immune cells (ALNC or SPLC or MLNC or

PP) isolated from antibiotic-treated donors results in decreased CS response when compared to the control mice that received CS-effector cells alone. This strongly suggests that immune cells from antibiotic-treated mice contain regulatory cells. Our results further demonstrate that the regulatory cells can act at both the induction and the effector phase of the CS response. Phenotyping the immune cells revealed an increased percentage of $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ Treg cells in both MLN and PP of antibiotic-treated mice compared with non-treated mice. This is also in line with a study by Ochoa-Reparaz and colleagues showing an increase of Foxp3^+ Treg cells in MLN and cervical LN of antibiotic-treated mice [13]. In addition to Foxp3^+ Treg cells, we observe an increase of $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{IL-10}^+$ lymphocytes in MLN of antibiotic treated mice compared with non-treated mice. Our results suggest that alteration of gut microbiota can induce both $\text{TCR}\alpha\beta^+ \text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ Treg cells in MLN and IL-10^+ Tr1 cells [39]. Interestingly, phenotypic analysis in our study also reveals an increase of B regulatory cells that are $\text{CD19}^+ \text{B220}^+ \text{CD5}^+ \text{IL-10}^+$ in PP of antibiotic treated mice. A study in an EAE model system has shown that antibiotic-treated mice have an increased percentage of $\text{CD19}^+ \text{B220}^+$, IL-10-producing, CD5^+ Breg cells in peripheral lymph organs [40]. It has been reported that CD19^+ B cells also can negatively regulate CS responses [41]. Our results further indicate that IL-10 producing $\text{TCR}\gamma\delta^+$ cells are among the immune regulatory cells from antibiotic-treated mice. Our findings add another population of microbiota-associated regulatory cells that has not been appreciated to date. We have reported previously the involvement of $\text{TCR}\gamma\delta$ cells in negative regulation of CS response [42]. Our current study indicates that oral administration of antibiotic prior to hapten sensitization induces four different subsets of regulatory cells. In this study, we show the ability of these four populations to suppress the CS response *in vivo* by transferring each purified cell subset individually. These experiments prove that each cell population indeed has the ability to suppress the CS response *in vivo* after adoptive transfer. Our study also provides evidence that

alteration of gut microbiota due to the oral administration of antibiotics may affect the development of regulatory cells.

It is known that antibiotic treatment decreases not only the number of bacteria but also the diversity of the microbiota. This effect can last from days to weeks after the cessation of antibiotic administration, and it is possible that some bacterial species could be depleted from the community permanently [43]. Moreover, antibiotics can affect the immune system, favorably or harmfully, through the change of bacterial composition. Recent studies have shown that *Bacteroides fragilis* increases the suppressive activity of Treg cells, whereas certain *Clostridium* strains increase the frequency of IL-10-producing Tregs [44, 45]. Other studies reported that gavage of commensal bacteria of the *Lactobacillus* or *Bifidobacterium* genera can induce Treg cells [46, 47]. However, the studies of SFB in mice have demonstrated that SFB is closely associated with the induction of Th17 lymphocytes [48]. In this study, we demonstrate that oral treatment with the antibiotic increases the relative abundance of *Clostridium coccoides* (cluster XIVa), *Clostridium coccoides* – *E. rectale* (cluster XIVab), *Bacteroidetes* and *Bifidobacterium spp.*, all of which have been shown to promote anti-inflammatory responses [25]. In contrast, oral antibiotic treatment decreases the relative abundance of SFB, which are known to promote IL-17 mediated inflammatory response [48]. Our results suggest that antibiotic-induced dysbiosis of gut microbiota favors an anti-inflammatory status and ameliorates contact sensitivity of skin inflammatory response. This is further confirmed by co-housing naïve mice with antibiotic-treated mice and by gut microbiota transfer from antibiotic-treated mice to naïve mice, both of which suppress CS responses in naïve mice. More importantly, we show that the CS response can be restored through oral transfer of control gut bacteria to antibiotic-treated animals, suggesting that changes in the gut flora can revert to a natural homeostatic state through oral transfer. It is noteworthy that the route of antibiotic administration is essential for our findings presented in

this study, as systemic antibiotic administration via the *i.p.* route had little effect on the immune regulation of CS.

In summary, our work shows that oral treatment with the broad spectrum antibiotic enrofloxacin modifies gut microbiota composition to promote an anti-inflammatory response as seen in reduced CS responses. The observed changes in microbiota promote the induction of different populations of regulatory cells that inhibit CS *in vivo*. The suppression of the CS response induced by antibiotic effects on the microbiota is reversible, as gavage of antibiotic treated mice with control gut flora restores CS reactions. Thus, targeted manipulation of gut microbiota, leading to increased level of anti-inflammatory bacteria could be a therapeutic strategy to ameliorate allergic contact dermatitis.

Acknowledgements: We would like to thank Dr. Philip W. Askenase for his help.

REFERENCES

1. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16s rRNA sequencing. *PLoS Biol* 2008;6:e280.
2. Ubeda C, Pamer EG. Antibiotics, microbiota and immune defense. *Trends Immunol* 2012;33:459-66.
3. Ubeda C, Taur Y, Jeng RR, Equinda MJ, Son T, Samstein M, et al. Vancomycin-resistant *Enterococcus* domination of intestinal microbiota is enabled by antibiotic

- treatment in mice and precedes bloodstream invasion in humans. *J Clin Invest* 2010; 120:4332-41.
4. Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML, Young VB. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infect Immun* 2009;77:2367-75.
 5. Buffie CG, Jarchum I, Equinda M, Lipuma L, Goborne A, Viale A, et al. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infect Immun* 2012; 80:62-7.
 6. Zeissig S, Blumberg SR. Life at the beginning: perturbation of the microbiota by antibiotics in early life and its role in health and disease. *Nat Immunol* 2014;15:307-10.
 7. Marra F, Marra CA, Richardson K, Lynd LD, Kozyrskyj A, Patrick DM et al. Antibiotic use in children is associated with increased risk of asthma. *Pediatrics* 2009; 123:1003–10.
 8. Kozyrskyj AL, Ernst P, Becker AB. Increased risk of childhood asthma from antibiotic use in early life. *Chest* 2007;131:1753–59.
 9. Metsälä J, Lundqvist A, Virta LJ, Kaila M, Gissler M, Virtanen SM. Mother's and offspring's use of antibiotics and infant allergy to cow's milk. *Epidemiology* 2013; 24:303–9.
 10. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, et al. Innate immunity and intestinal microbiota in the development of type 1 diabetes. *Nature* 2008;455:1109-13.
 11. Hill DA, Artis D. Intestinal bacteria and the regulation of immune cell homeostasis. *Annu Rev Immunol* 2010;28:623-67.

12. Penders J, Stobberingh EE, Van Den Brandt PA, Thijs C. The role of the intestinal microbiota in the development of atopic disorders. *Allergy* 2007;62:1223–36.
13. Ochoa-Repáraz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, et al. Role of gut commensal microflora in development of experimental autoimmune encephalomyelitis. *J Immunol* 2009;183:6041-50.
14. Ebringer A. Therapy: Gut-mediated autoimmune arthritis treated with antibiotics. *Nat Rev Rheumatol* 2010;6:622-23.
15. Bieber T. Atopic dermatitis. *N Engl J Med* 2008;358:1483–94.
16. Diepgen TL, Weisshaar E. Contact dermatitis: epidemiology and frequent sensitizers to cosmetic. *J Eur Acad Dermatol Venereol* 2007;21: suppl 2:9-13.
17. Simpson CR, Anderson WJ, Helms PJ, Taylor MW, Watson L, Prescott GJ, et al. Coincidence of immune-mediated diseases driven by Th1 and Th2 subsets suggests a common aetiology. A population-based study using computerized general practice data. *Clin Exp Allergy* 2002;32:37-42.
18. Majewska-Szczepanik M, Strzępa A, Marcińska K, Wen L, Szczepanik M: Epicutaneous immunization with TNP-Ig and zymosan induces TCR $\alpha\beta^+$ CD4 $^+$ contrasuppressor cells that reverse skin-induced suppression via IL-17A. *Int Arch Allergy Immunol* 2014;164:122-36.
19. Rinttilä T, Kassinen A, Malinen E, Krogus L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004;97:1166-77.
20. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, et al. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl Environ Microbiol* 2002; 68:5445-51.

21. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002;148:257-66.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001; 25:402-8.
23. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Dore J, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiology* 2009;9:123.
24. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006;444:1027-31.
25. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012;336:1268-73.
26. Alexander KL, Targan SR, Elson CO 3rd. Microbiota activation and regulation of innate and adaptive immunity. *Immunol Rev* 2014;260:206-20.
27. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 2011;108:4615-22.
28. Wu HJ, Ivanov II, Darce J, Hattori K, Shima T, Umesaki Y, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 2010; 32:815-27.
29. Zhanvit P, Konkel JE, Jiao X, Kasagi S, Zhang D, Wu R, et al. Antibiotics in neonatal life increase murine susceptibility to experimental psoriasis. *Nat Commun* 2015; 6:8424.

30. Strzępa A, Majewska-Szczepanik M, Kowalczyk P, Woźniak D, Motyl S, Szczepanik M. Oral treatment with enrofloxacin early in life promotes Th2-mediated immune response in mice. *Pharmacol Rep* 2016; 68:44–50.
31. Candela M, Rampelli S, Turroni S, Severgnini M, Consolandi C, De Bellis G, et al. Unbalance of intestinal microbiota in atopic children. *BMC Microbiol* 2012;12:95.
32. Penders J, Stobberingh EE, van den Brandt PA, Thijs C. The role of the intestinal microbiota in the development of atopic disorders. *Allergy* 2007; 62:1223-36.
33. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 2012; 129:434-40.
34. Song H, Yoo Y, Hwang J, Na YC, Kim HS. *Faecalibacterium prausnitzii* subspecies-level dysbiosis in the human gut microbiome underlying atopic dermatitis. *J Allergy Clin Immunol* 2016; 137:852-60.
35. Fujiwara R, Sasajima N, Takemura N, Ozawa K, Nagasaka Y, Okubo T, et al. 2,4-Dinitrofluorobenzene-induced contact hypersensitivity response in NC/Nga mice fed fructo-oligosaccharide. *J Nutr Sci Vitaminol (Tokyo)* 2010; 56:260-5.
36. Fujiwara R, Takemura N, Watanabe J, Sonoyama K. Maternal consumption of fructo-oligosaccharide diminishes the severity of skin inflammation in offspring of NC/Nga mice. *Br J Nutr* 2010; 103:530-8.
37. Hacini-Rachinel F, Gheit H, Le Luduec JB, Dif F, Nancey S, Kaiserlian D. Oral probiotic control skin inflammation by acting on both effector and regulatory T cells. *PLoS ONE* 2009; 4:e4903.
38. Watanabe J, Sasajima N, Aramaki A, Sonoyama K. Consumption of fructo-oligosaccharide reduces 2,4-dinitrofluorobenzene-induced contact hypersensitivity in mice. *Br J Nutr* 2008; 100:339-46.

39. Pot C, Apetoh L, Kuchroo VK. Type 1 regulatory T cells (Tr1) in autoimmunity. *Semin Immunol* 2011;23:202-8.
40. Ochoa-Repáraz J, Mielcarz DW, Haque-Begum S, Kasper LH. Induction of a regulatory B cell population in experimental allergic encephalomyelitis by alteration of the gut commensal microflora. *Gut Microbes* 2010;1:103-8.
41. Watanabe R, Fujimoto M, Ishiura N, Kuwano Y, Nakashima H, Yazawa N, et al. CD19 expression in B cells is important for suppression of contact sensitivity. *Am J Pathol* 2007;171:560-70.
42. Szczepanik M, Anderson LR, Ushio H, Ptak W, Owen MJ, Hayday AC, et al. $\gamma\delta$ T cells from tolerized $\alpha\beta$ T cell receptor (TCR)-deficient mice inhibit contact sensitivity-effector T cells in vivo, and their IFN- γ production in vitro. *J Exp Med* 1996;184:2129-40.
43. Jakobsson HE, Jemberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PloSOne* 2010;5:e9836.
44. Kosiewicz MM, Zirnheld AL, Alard P. Gut microbiota, immunity, and disease: complex relationship. *Front Microbiol* 2011;2:180.
45. Ochoa-Repáraz J, Mielcarz DW, Wang Y, Begum-Haque S, Dasgupta S, Kasper DL, et al. A polysaccharide from the human commensal *Bacteroides fragilis* protects against CNS demyelinating disease. *Mucosal Immunol* 2010;3:487-95.
46. Karimi K, Inman MD, Bienenstock J, Forsythe P. *Lactobacillus reuteri*-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med* 2009;179:186-93.

47. Zhang LL, Chen X, Zheng PY, Lou Y, Lu GF, Liu ZQ, et al. Oral Bifidobacterium modulates intestinal immune inflammation in mice with food allergy. *J Gastroenterol Hepatol* 2010;25:928-34.
48. Gaboriau-Routhiau V, Rakotobe S, Lécuyer E, Mulder I, Lan A, Bridonneau C, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 2009;31: 677-89.

Figure legends

FIG 1.

Oral application of broad spectrum antibiotic alleviates CS in mice. Mice received plain water (Groups A and B), or water containing enrofloxacin (0.27 mg/ml) (Groups C and D), or were *i.p.* injected with enrofloxacin (5 mg/kg) (Groups E and F) for two weeks prior to TNP-Cl or sham immunization and test. **A**, Ear swelling. **B**, Body weight. **C** and **D**, Aerobic and anaerobic bacteria in content of large intestine. **E**, Adoptive transfer of CS. **F**, Production of TNP-specific IgG1 and IgG2a. Results shown as mean $\pm$ SE. $n = 8-16$ (A), $n = 5-6$ (B, C and D), $n = 14$ (E) and $n = 5-7$ (F). ns = non-significant. * $P < 0.05$ and ** $P < 0.01$ and *** $P < 0.001$.

FIG 2.

Oral treatment with antibiotic prior to CS induction inhibits inflammatory response in the ears. **A**, Vascular permeability. **B**, Ear weight. **C**, MPO activity in ear extracts. **D**, IFN- γ concentration in ear extracts. Results shown as mean $\pm$ SE. $n = 10-14$ (A, B and D), $n = 12$ (C). * $P < 0.05$ and *** $P < 0.001$.

FIG 3.

Oral treatment with antibiotic induces cells that suppress CS *in vivo*. Transfer of ALNC or SPLC or MLNC or PP cells from donors treated orally with enrofloxacin and then sensitized. **A**, “Transfer out“. **B**, Transfer in” prior to challenge. **C**, “Transfer in” prior to immunization. Results shown as mean $\pm$ SE. $n = 10-14$ (A and B), $n = 8-12$ (C). *** $P < 0.001$ and * $P < 0.05$.

FIG 4.

Oral treatment with antibiotic prior to TNP-Cl sensitization induces various populations of regulatory cells. Water (Groups A and B) or water with enrofloxacin (Groups C and D) receiving mice were (Groups B and D) or were not (Groups A and C) sensitized with TNP-Cl prior to FACS analysis of lymphoid organs. **A**, Percentage of TCR $\alpha\beta^+$ CD4 $^+$ CD25 $^+$ FoxP3 $^+$ Treg and CD19 $^+$ B220 $^+$ CD5 $^+$ IL-10 $^+$ Breg cells in PP. **B**, Percentage of TCR $\alpha\beta^+$ CD4 $^+$ CD25 $^+$ FoxP3 $^+$ Treg and TCR $\alpha\beta^+$ CD4 $^+$ IL-10 $^+$ Tr1 cells in MLN. **C**, Percentage of TCR $\gamma\delta^+$ IL-10 $^+$ lymphocytes in ALN. **D**, Percentage of TCR $\gamma\delta^+$ IL-10 $^+$ lymphocytes in SPL. Results shown as mean $\pm$ SE. $n = 4-5$. ** $P < 0.01$ and * $P < 0.05$.

FIG 5.

Transfer of purified regulatory cells prior to TNP-Cl immunization inhibits CS *in vivo*. Whole immune cells from PP, MLN, ALN or SPL or adequate MACS-purified cells from each organ were transferred to naïve recipients prior to hapten immunization and CS test. **A**, Transfer of purified CD4 $^+$ CD25 $^+$ or CD19 $^+$ cells from PP. **B**, Transfer of purified CD4 $^+$ CD25 $^+$ cells from MLN. **C**, Transfer of purified TCR $\gamma\delta$ from ALN. **D**, Transfer of purified TCR $\gamma\delta$ from SPL. Results shown as mean $\pm$ SE. $n = 8$. *** $P < 0.001$ and ** $P < 0.01$ and * $P < 0.05$.

FIG 6.

Treatment with antibiotic shifts the bacteria profile towards anti-inflammatory flora in mice treated with water containing enrofloxacin. Relative abundance of bacterial conservative 16S rDNA fragments in gut: **A**, *Lactobacillus* and *Enterococcus* spp.; **B**, *Clostridium coccoides* (cluster XIVa) and *Clostridium coccoides* – *E. rectale* (cluster XIVab); **C**, *Bacteroidetes* and *Bifidobacterium* spp; **D**, SFB. Results shown as mean $\pm$ SE. n = 11. ** $P < 0.01$ and * $P < 0.05$.

FIG 7.

Transfer of gut flora modulates CS response *in vivo*. **A**, Cohousing experiment. **B**, Transfer of gut flora from enrofloxacin treated donor mice. **C**, Transfer of gut flora from naive mice. Results shown as mean $\pm$ SE. n = 10-12 (A), n = 10-14 (B), n = 8 (C). *** $P < 0.001$ and ** $P < 0.01$ and * $P < 0.05$.

	Primers
Clostridium coccoides – Eubacterium rectale subgroup (Clostridium XIVab)	F: 5'-AAATGACGGTACCTGACTAA-3' R: 5'-CTTTGAGTTTCATTCTTGCGAA-3'
Clostridium coccoides - Clostridium XIVa	F: 5'-AAATGACGGTACCTGACTAA-3' R: 5'-CTTTGAGTTTCATTCTTGCGAA-3'
Enterococcus spp.	F: 5'-CCCTTATTGTTAGTTGCCATCATT-3' R: 5'-ACTCGTTGTACTTCCCATTGT-3'
Lactobacillus	F: 5'-AGCAGTAGGGAATCTTCCA-3' R: 5'-CACCGCTACACATGGAG-3'
Bacteroidetes	F: 5'-GGTGTCTGGCTTAAGTGCCAT-3' R: 5'-CGGA(C/T)GTAAGGGCCGTGC-3'
Bifidobacterium spp.	F: 5'-TCGCGTC(C/T)GGTGTGAAAG-3' R: 5'-CCACATCCAGC(A/G)TCCAC-3'
SFB	F: 5'-GACGCTGAGGCATGAGAGCAT R: 5'-GACGGCACGGATTGTTATTCA
Bacteria	F: 5'-TCCTACGGGAGGCAGCAGT-3' R: 5'-GGACTACCAGGGTATCTAATCCTGTT-3' (6-FAM)-5'-CGTATTACGCGGCTGCTGGCAC-3'-(TAMRA)

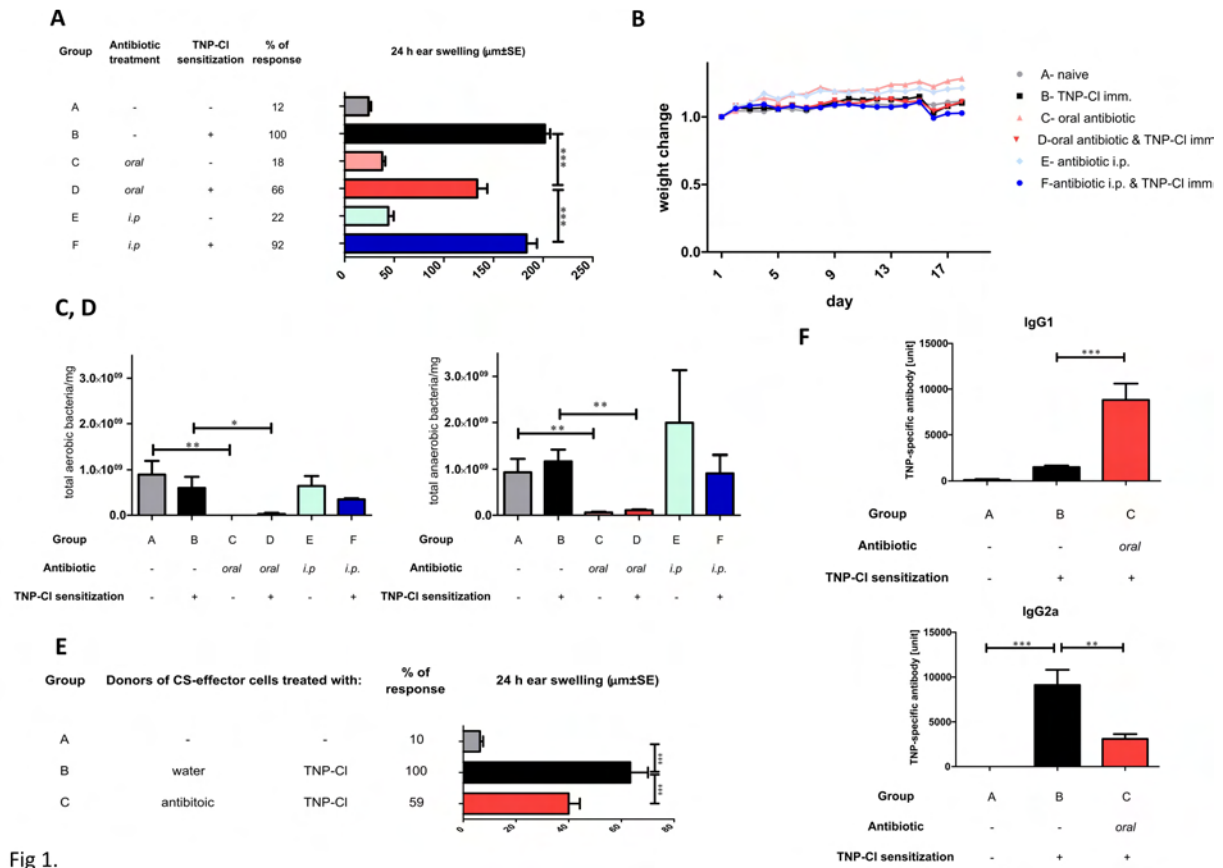
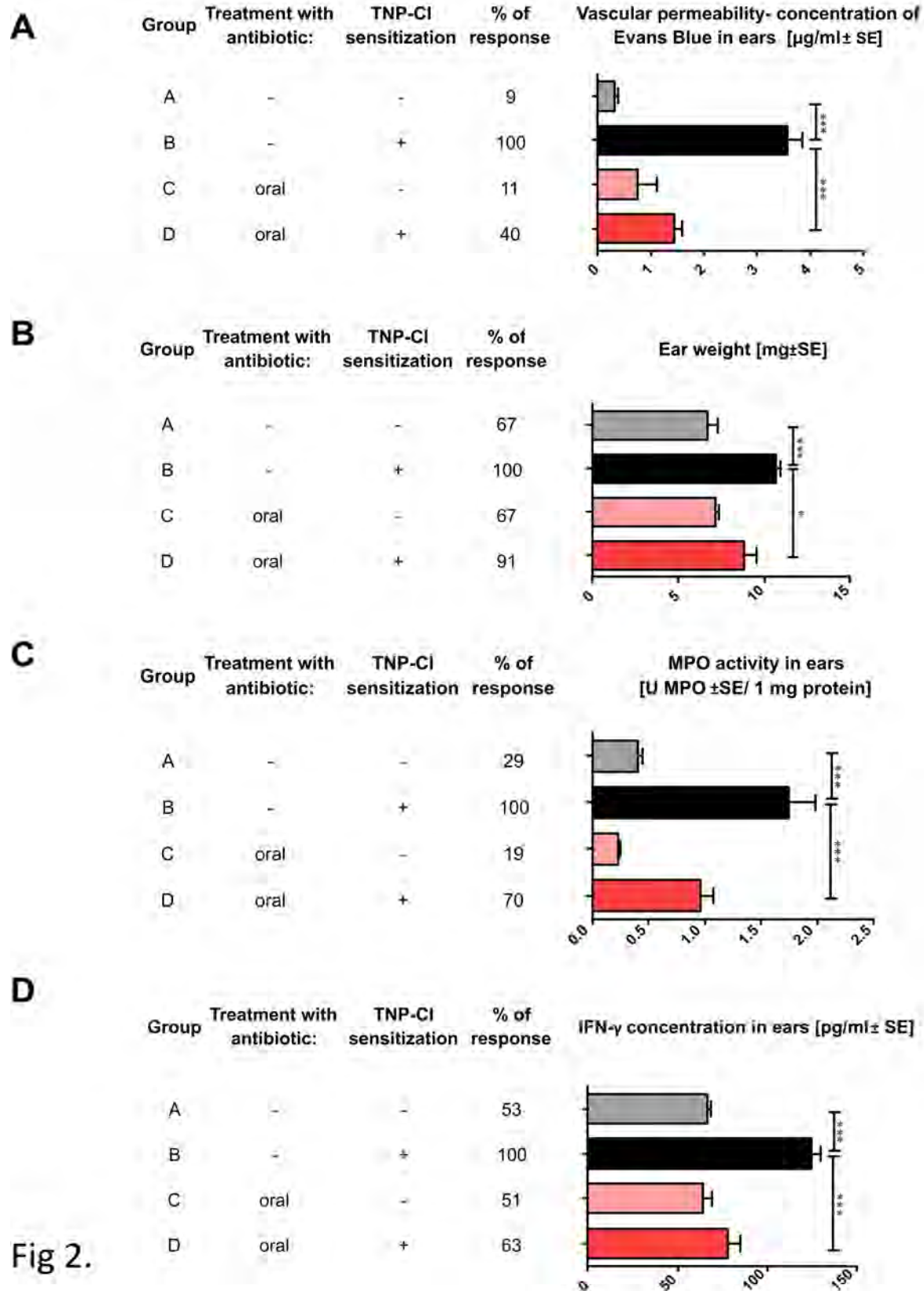


Fig 1.



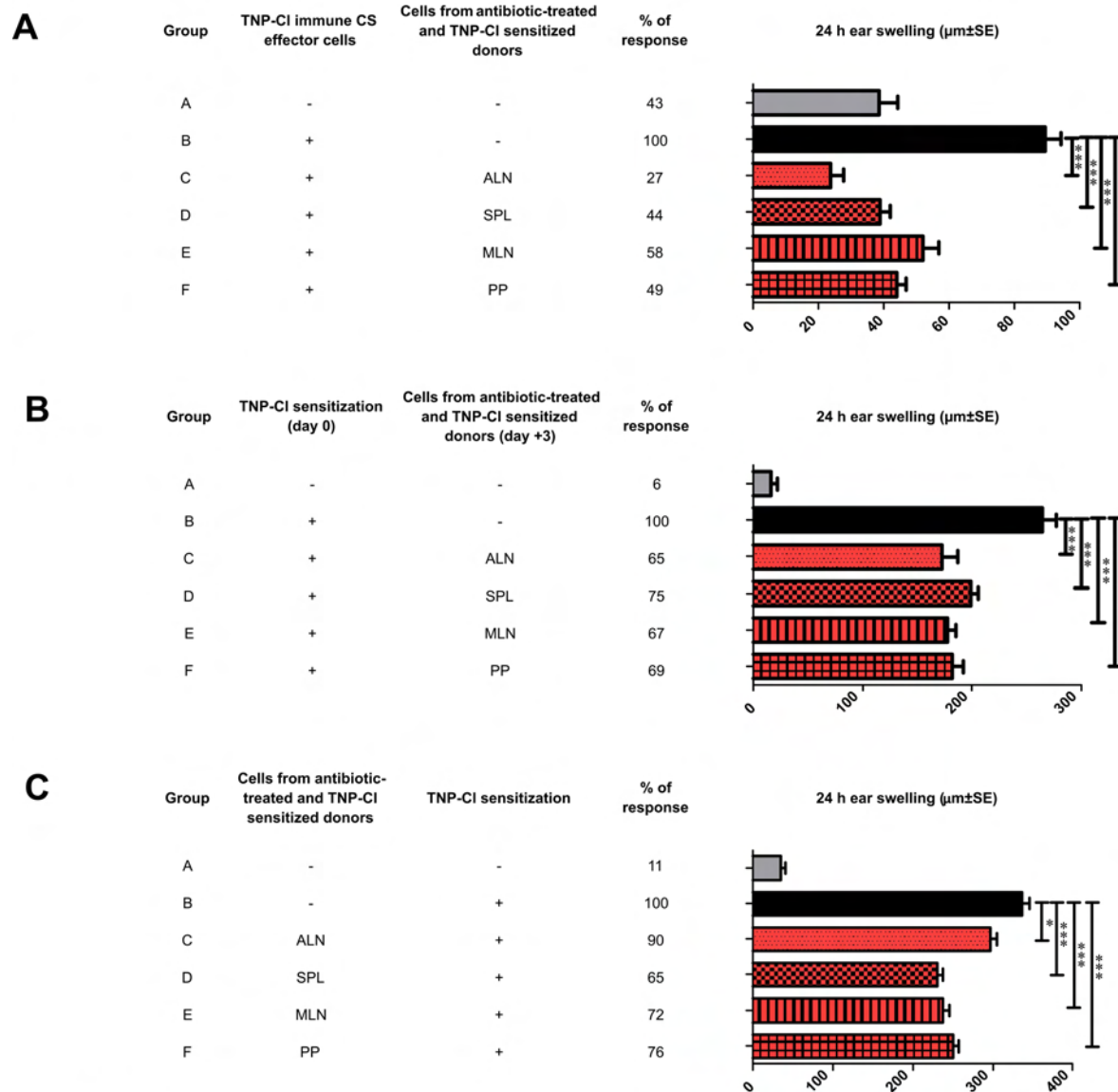


Fig 3.

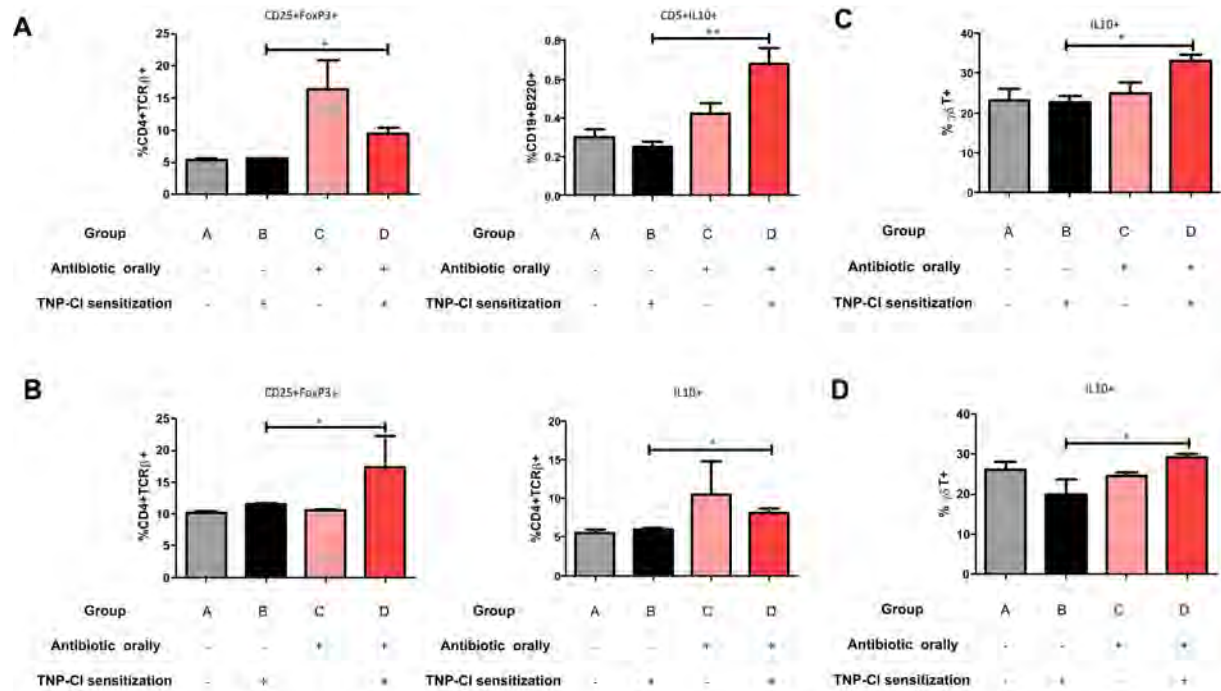


Fig 4.

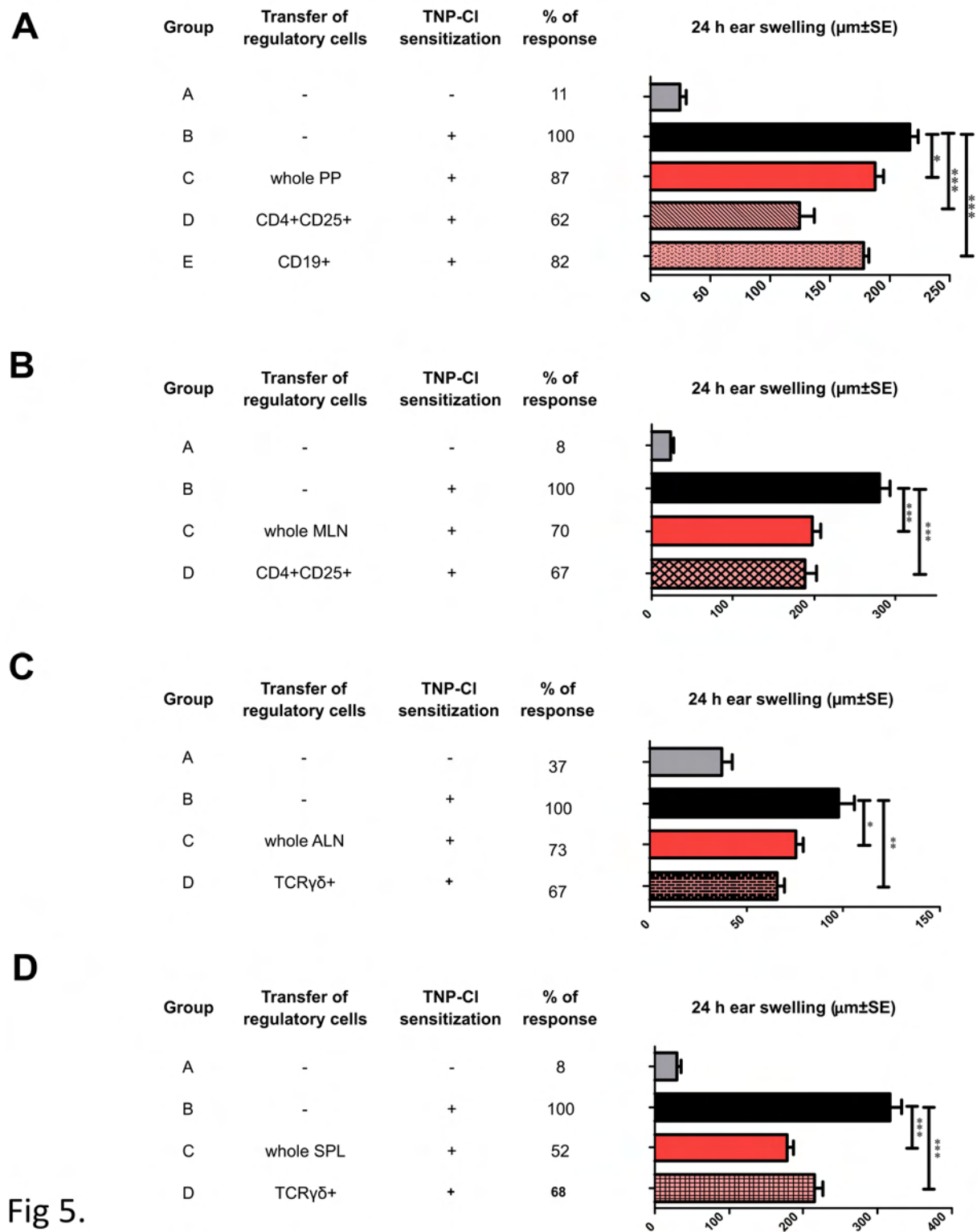


Fig 5.

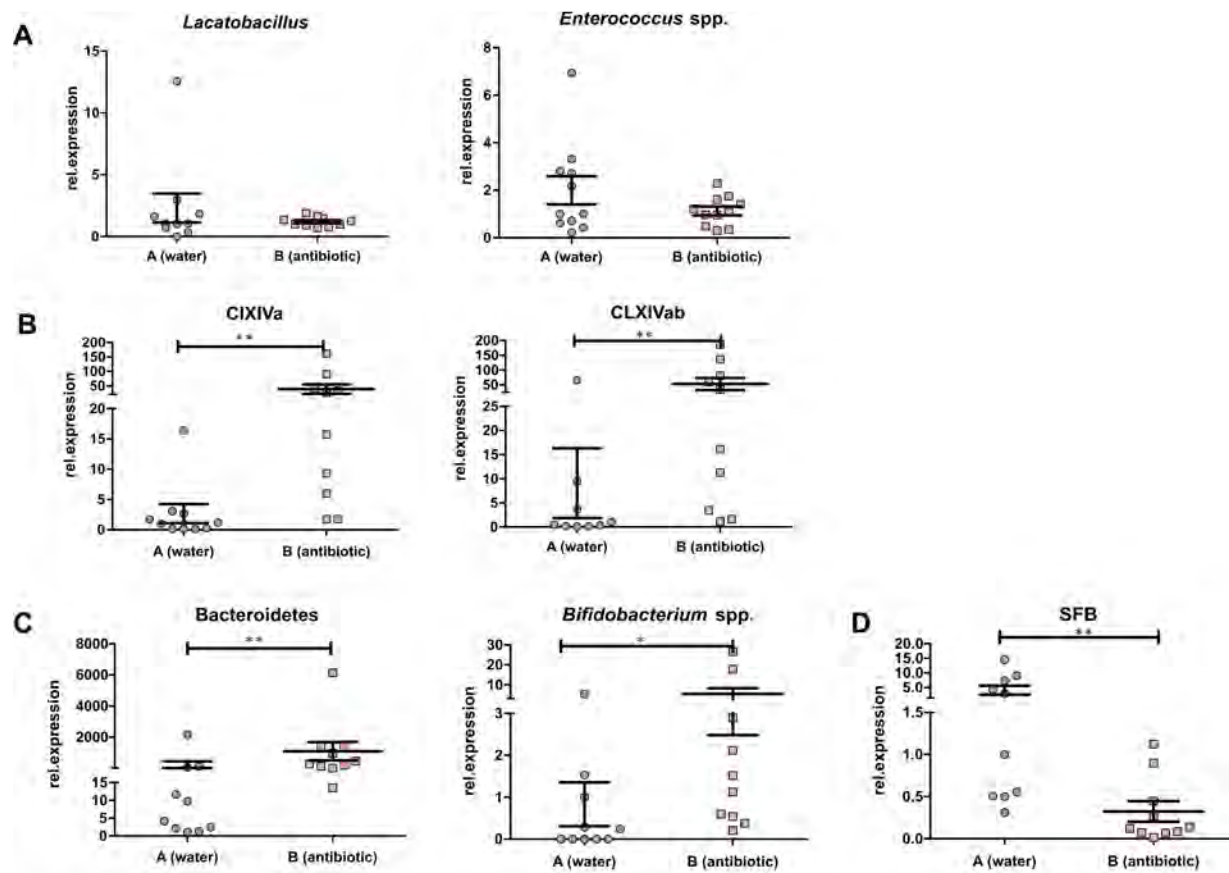


Fig 6.

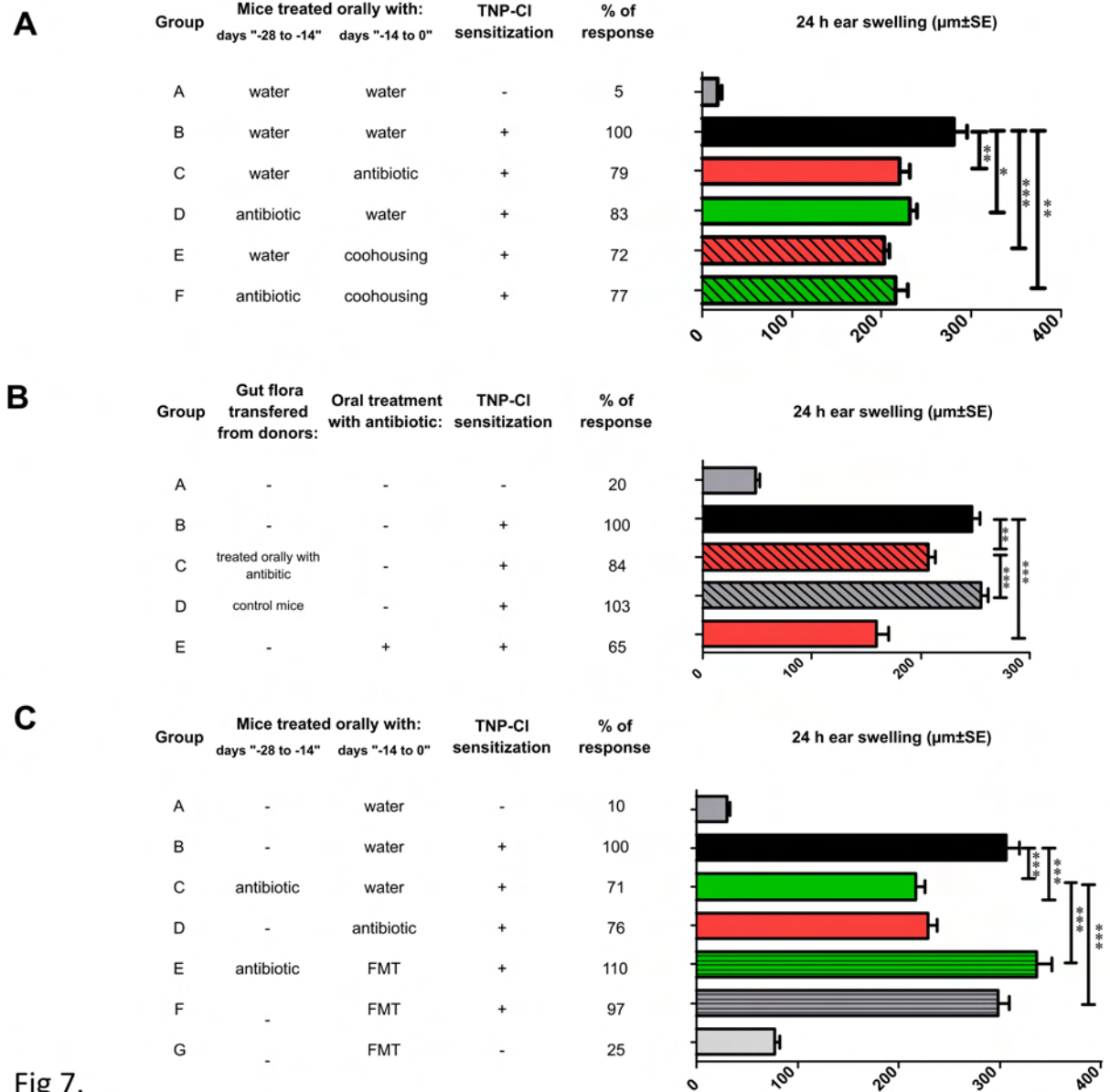


Fig 7.

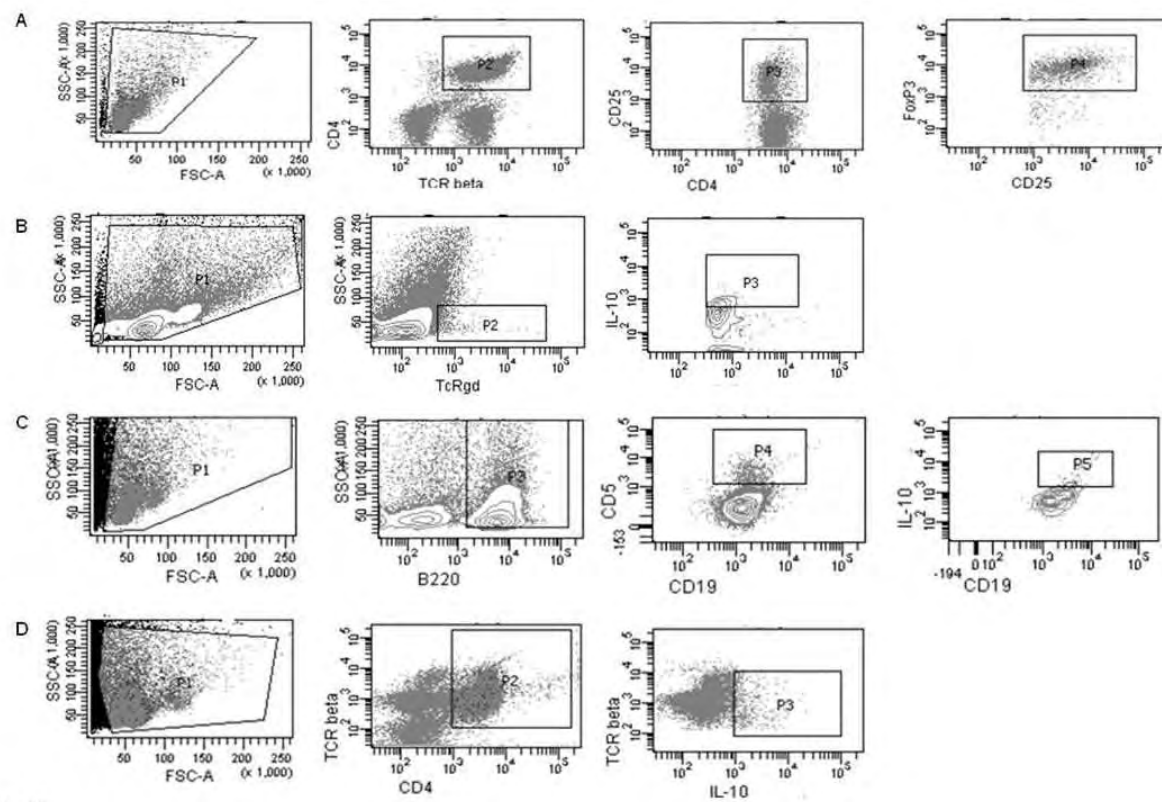


Fig E1.

SUPPLEMENTAL METHODS**Sensitization and elicitation of contact sensitivity (CS) *in vivo***

Mice were sensitized by topical application of 0.15 ml of 5% TNP-Cl in acetone-ethanol mixture (1:3) to the shaved abdomen and chest. Control mice were shaved and painted with the acetone-ethanol mixture alone as a sham sensitization. Four days later, animals were challenged on both sides of the ears with 10 μ l of 0.4% TNP-Cl in olive oil-acetone mixture (1:1). Resulting ear thickness was measured prior to testing with a micrometer (Mitutoyo, Tokyo, Japan) by an observer unaware of the experimental groups and then again at 24 h after challenge. Negative control consisted of littermate sham sensitized animals that were similarly challenged. The ear swelling was expressed in μ m $\pm$ SE [18]. Ear swelling was further confirmed by the measurements of ear weight, vascular permeability, MPO activity and IFN- γ concentration in ear extracts. Additionally, concentration of anti-TNP IgG1 and IgG2a was measured in mouse sera by ELISA.

Vascular permeability test

To assess very early changes in vascular permeability, TNP-Cl immunized or naïve mice were challenged with 10 μ l of 0.4% TNP-Cl and injected with 1% Evans blue dye (83 μ g/g body weight) 23 h later. 1 hour after Evans blue injection mice were anesthetized and sacrificed. Ears were removed and 6 mm diameter punch of the ear was made with biopsy punch (Frey Products Corp., cat# BP60). Ear punches were transferred to tubes containing 1 ml of formamid. After 18 h incubation at 37°C the samples were centrifuged and the optical density (OD) of Evans blue in the supernatant was read at 565 nm against blank containing formamid [18].

Myeloperoxidase (MPO) assay

Neutrophil infiltration to the inflamed ears was indirectly quantitated using a MPO assay, as described previously [18]. Ears were removed 24 h post challenge and 6 mm diameter punch of the ear was made. Biopsies were collected from the distal site of CHS responses and were homogenized in 0.5% hexadecyltrimethylammonium bromide pH = 6.0 (50 mg of tissue/ml). The homogenates were freeze – thawed 3 times, centrifuged at 40,000 g. 0.1 ml aliquots were mixed with 2.9 ml phosphate buffer (pH = 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 5×10^{-4} % H_2O_2 and incubated at 25°C for 20 min. The absorbance was measured at 460 nm in 96-well flat bottom plates. MPO activity was expressed in units per protein concentration (U/mg of protein).

In vitro measurement of IFN- γ in CS ear extracts

To determine local production of IFN- γ in elicited TNP-Cl CS, mice were immunized with 5% TNP-Cl or sham sensitized and challenged with 10 μ l of 0.4% TNP-Cl four days later. Ears were removed 24 h post challenge and 6 mm diameter punch of the ear was made. Biopsies were collected from the distal site of CS ear responses. The biopsies were frozen rapidly in liquid N_2 and were subsequently thawed and extracted in 300 μ l cold PBS containing a mixture of proteinase inhibitors on ice with a tissue microhomogenizer [18]. Concentration of IFN- γ was measured by ELISA with the use of BD OptEIA Set (BD Biosciences, San Diego, CA).

FACS analysis

Axillary lymph nodes, SPL, MLN and PP were isolated and single cell suspensions prepared and stained prior to analysis. Cell subpopulations were analyzed using

fluorochrome-conjugated mAbs. For the analysis of T cell subpopulations CD4-PerCP-Cy5.5 and TCR β -APC-Cy7 (BioLegend, San Diego, CA), CD25-APC and TCR $\gamma\delta$ -PE (BD Biosciences, San Jose, CA) and CD4-FITC (Life Technologies Corporation, Carlsbad, CA) were used. For B cell identification B220-PE-Cy7 and CD19-PerCP-Cy5.5 (eBioscience Inc, San Diego, CA) and CD5-FITC (BD Biosciences, San Jose, CA) were used.

Treg cells were determined by staining with anti-FoxP3-PE mAb (BD Biosciences, San Jose, CA) using the mouse Treg staining kit (eBioscience) according to manufacturers instructions. To detect intracellular IL-10, ICC (intracellular cytokines) staining was performed. Single cell suspension from lymphoid tissues were cultured for 4 h with PMA, ionomycin (Sigma Chemical Co., St Louis, MO) and Golgi Plug (eBioscience, San Diego, CA), followed by surface markers staining of T and B cells. After washing, the cells were fixed and permeabilized followed by staining with IL-10-PE-Cy7 or IL-10-PE. The cells were analyzed with a FACS Canto II (Becton Dickinson) and data were analyzed using FACSDiva software.

Supplementary Figure Legend

FIG E1.

Representative flow cytometry gating strategies for: **A**, TCR $\alpha\beta$ ⁺CD4⁺CD25⁺FoxP3⁺ T-regulatory cells (Treg); **B**, IL-10⁺TCR $\gamma\delta$ ⁺ cells; **C**, CD19⁺B220⁺CD5⁺IL-10⁺ B-regulatory cells (Breg); **D**, TCR $\alpha\beta$ ⁺ CD4⁺ IL-10⁺ Tr1.

Table I

The primers sequences used to detect selected gut bacteria species