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Oral treatment with enrofloxacin early in life promotes Th2-mediated immune response in mice

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ABSTRACT

Background: Th2 lymphocytes play a crucial role in the development of allergy. These pathologies are caused by coordinated production of the cytokines IL-4, IL-5 and IL-13 that regulate the activity of eosinophils, basophils and B cells. According to the 'hygiene hypothesis', the reduced exposure to microorganisms favors allergy occurrence. The advances in medicine in the field of infection therapy promoted an increasing application of antibiotics which, apart from eliminating pathogens, also partially eliminate the microbiota.

Methods: Epicutaneous (EC) immunization with ovalbumin (OVA) followed by OVA challenge was used to study the influence of partial gut flora depletion by oral treatment with enrofloxacin on type-2 immune response.

Results: Current work describes the influence of enrofloxacin application on anti-OVA antibody production and cytokine synthesis in young and adult mice. Immune response in adult mice is less sensitive to modification of natural gut flora. We observed that enrofloxacin treatment of adult mice leads to significant decrease of anti-OVA IgG2a production while synthesis of anti-OVA IgE was not changed. The production of type-1 (IFN- γ), type-2 (IL-4, IL-5, IL-10, IL-13) and Th17-associated (IL-17A) cytokines was inhibited. On the other hand, treatment of young mice with enrofloxacin significantly upregulates the production of anti-OVA IgE and inhibits the secretion of anti-OVA IgG2a antibodies. Additionally, treatment with enrofloxacin early in life prior to OVA immunization results in increased production of type-2 (IL-4, IL-10 and IL-13) cytokines.

Conclusion: Our results clearly indicate that the immune system is more vulnerable to decreased bacterial exposure early in life that may promote development of allergy.

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Introduction

Q4 Allergic disorders develop as a consequence of immune responses to normally innocuous environmental antigens and now afflict roughly 25% of people in the developed countries [1]. These diseases include asthma, food allergy, atopic dermatitis,

rhinoconjunctivitis, sinusitis, urticaria, anaphylaxis, and insect and drug allergy, all of which can occur either alone or in combination [2]. It is believed that Th2 lymphocytes play a crucial role in this type of diseases. The phenotype of Th2 cell is characterized by the coordinated production of the cytokines IL-4, IL-5 and IL-13 which, in conjunction with other inflammatory mediators, direct the activation and tissue accumulation of eosinophils, basophils and B cells switching to pathogenic IgE [3].

Along with the development of our civilization and the increasing awareness of hygiene, there has been a permanent rise in the prevalence of autoimmune and allergic diseases. The observation collected in human is also directly reflected in animal studies. Hence, for example in non-obese diabetic mice (NOD), a change in the environment that reduces the number of microorganisms increases

Abbreviations: ALNC, axillary lymph node cells; CFU, colony forming unit; CIA, collagen-induced arthritis; EC, epicutaneous; FCS, fecal calf serum; IFN- γ , interferon gamma; Ig, immunoglobulin; IL, interleukin; NOD, non-obese diabetic mice; OVA, ovalbumin; PBS, phosphate-buffered saline; T1D, type 1 diabetes; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; TMB, 3,3',5,5'-tetramethylbenzidine.

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the prevalence of type 1 diabetes (T1D), whereas the increase in the number of microorganisms in the surroundings decreases this prevalence [4]. Similar observations were made in an animal model of asthma which reflects the conditions observed in asthma patients [5].

Moreover, the advances in medicine in the field of infection therapy promoted an increasing application of antibiotics which, apart from eliminating pathogens, also partially eliminate the natural human bacterial flora. Studies conducted over many years have shown that the bacteria living in the alimentary tract have an essential role in the processes of food digestion, maintaining homeostasis, modulating lipid metabolism, promoting angiogenesis, supporting the immunity to infections, and maintaining the immune cell homeostasis [6].

The studies carried out in recent years have confirmed a strong connection between the natural gut flora and immune response. This interaction may differ in various experimental models. A strong inhibition of inflammatory reaction was observed, for instance, in the animal model of multiple sclerosis in mice treated orally with an antibiotic [7]. Similar observations were made in an animal model imitating rheumatoid arthritis [8]. Our previous study employing broad-spectrum antibiotic enrofloxacin showed that oral application of this therapeutic prior to the initiation of collagen-induced arthritis (CIA) aggravates disease symptoms in DBA1 mice [9].

In the current work, we determined the influence of partial gut flora depletion with broad spectrum enrofloxacin on type-2 immune response in mice.

Materials and methods

Mice

6–8 weeks old female BALB/c mice were 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) and fed autoclaved food and water *ad libitum*. All experiments were conducted to the guidelines of Jagiellonian University College of Medicine. All experiments were repeated 2–3 times, and a representative experiment is shown in the figures.

Reagents

Ovalbumin (Grade V) and phosphate-buffered saline (PBS) were from Sigma–Aldrich (St. Louis, MO, USA). Enrofloxacin was purchased from Bayer Animal Healthcare GmbH (Leverkusen, Germany). DNA analysis was performed using sodium dodecyl sulfate (SDS), JumpStart™ Taq ReadyMix™ for qPCR, lysozyme, Tris–EDTA (Sigma–Aldrich, St. Louis, MO, USA), silica beads (BioSpec Products, Bartlesville, OK, USA) and proteinase K (Roche Diagnostics, Mannheim, Germany). RPMI 1640 was from Gibco (Grand Island, NY, USA), whereas fetal calf serum (FCS) was from PAA Laboratories (Pasching, Austria). The levels of OVA-specific IgE and IgG2a antibodies in sera were tested using reagents from mouse ELISA sets for IgE (BD Pharmingen, San Diego, CA, 555248) and for IgG2a (BD Pharmingen, San Diego, CA, 552576). The units of antibodies were calculated according to an internal standard. The concentration of IL-4, IL-5, IL-10 and IFN- γ in culture supernatants was measured with the use of mouse ELISA sets purchased from BD Pharmingen, San Diego, CA. Additionally, the concentration of IL-17A was evaluated using a mouse ELISA Ready-SET-Go kit (eBioscience, San Diego, CA). 3,3',5,5'-Tetramethylbenzidine (TMB) (BD Biosciences, San Jose, CA) reagent set was used as the substrate in our ELISAs (BD Pharmingen, San Diego, CA). The

level of IL-13 was measured by using a mouse IL-13 immunoassay (R&D Systems, Inc., Minneapolis, MN).

Treatment with enrofloxacin

6–8 weeks old BALB/c mice received drinking water containing enrofloxacin (0.27 mg/ml) or water alone for 2 weeks prior to tests. When enrofloxacin treated animals were immunized with OVA, enrofloxacin application was continued for additional 7 days. To study the influence of early exposure to enrofloxacin on antibody production, nursing pups were exposed to enrofloxacin during breastfeeding. Antibiotic treatment of mothers began immediately after delivery and was continued for 2 weeks. After the weaning, 6 weeks old mice were additionally treated with enrofloxacin for 3 weeks prior to immunization with OVA, as described above.

Evaluation of gut flora depletion

Mice received drinking water containing enrofloxacin (0.27 mg/ml) or water alone for 3 weeks. Then, animals were sacrificed and colon content was collected and serial dilutions were cultured in general agar plates (blood agar, Bioshop Inc., Burlington, Canada) for 48 h or 72 h at 37 °C in aerobic and anaerobic conditions respectively. Total bacteria per gram of sample were calculated based on the Colony Forming Units (CFU) counted in each serial dilution [7,9]. Additionally, gut flora depletion was evaluated by quantitative PCR analysis of 16S rDNA genes by real-time RT-PCR. Briefly, stool pellets were collected and total DNA was extracted with *phenol:chloroform:isoamyl alcohol* (25:24:1) method. Quantification of rDNA was performed with universal primers (forward; 5'-TCCTACGGGAGGCAGCAGT-3'), (reverse; 5'-GGACTACCAAGGGTATCTAATCCTGT-3') and probe (6-FAM)-5'-CGTATTACCGCGGCTGCTGGCAC-3'-(TAMRA) using CFX96 Touch (Bio-Rad Laboratories Inc., Hercules, CA, USA).

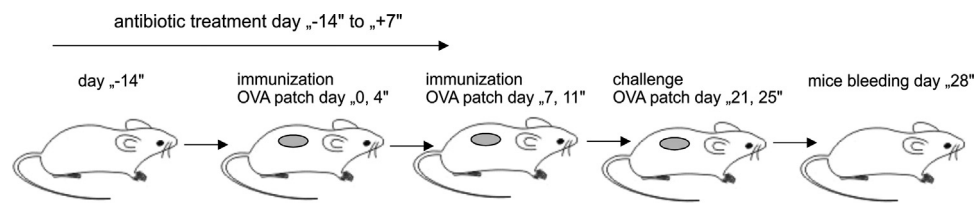
Epicutaneous immunization with OVA

Epicutaneous immunization of mice was performed according to the method described by Spergel et al. [10] and Wang et al. [11] with our modifications. Briefly, immunization was performed by applying to the shaved skin of the mouse dorsum a 1 cm² gauze patch soaked with a solution containing 100 μ g OVA in a volume of 100 μ l PBS on day "0". Mice in control group were patched with PBS alone. The patches were secured by adhesive tape wrapped around the midsection. The patches were left in place from day "0" until day "+4" when they were replaced with fresh patches and kept to day "+7".

The refreshed patches were then replaced on day "+7" and kept until day "+11" and replaced once again with fresh patches that were kept until day "+14". On day "+14", the patches were removed and the mice were rested for 1 week. The animals were then EC challenged by patching with 100 μ g OVA in sterile PBS on day "+21". Four days later (day "+25") patches were replaced by fresh ones and left until day "+28". After that, patches were removed, mice were bled and sera were used to test level of OVA-specific IgE and IgG2a antibodies. The scheme of immunization and challenge is described in Scheme 1.

Measurement of OVA-specific IgE and IgG2a antibodies

OVA-specific antibodies were measured according to the method described by Spergel et al. [10] and Wang et al. [11] with our modifications. Briefly, 96-well microtiter plates were coated with 50 μ g/ml of OVA in 0.1 M NaHCO₃ at 4 °C overnight. The plates were washed with PBS–Tween 20 (0.05%), then blocked



Scheme 1.

with 10% PBS–FCS for 1 h at room temp. Serial dilutions of sera in 10% PBS–FCS (50 μ l volume) were then added to the wells and incubated for 2 h at room temp. After washing with PBS–Tween 20, a mixture of biotinylated anti-mouse isotype-specific antibody and streptavidin-horseradish peroxidase (diluted 1:250 in 10% PBS–FCS) was added and plates were incubated for 1 h at room temp. The plates were then washed with PBS–Tween and then developed with TMB substrate for 30 min. The reaction was then stopped and absorption was measured at 450 nm with a wavelength correction at 570 nm subtracted from these values. Results were expressed as IgE-20 and IgG2a-40 arbitrary units.

In vitro cell culture and cytokine immunoassay

Axillary and inguinal lymph nodes were dissected from experimental mice. The cell suspensions were prepared by gentle teasing and then washed two times with PBS. 3×10^6 of axillary and inguinal lymph node cells (ALNC) from mice that were or were not treated with enrofloxacin prior to EC exposure to PBS or OVA were cultured in 1 ml RPMI 1640 medium supplemented with 5% FCS in the presence of 100 μ g/ml OVA in flat 24 well Falcon plates [12]. After 48 h, culture supernatants were collected and samples were tested for IL-4, IL-5, IL-10, IL-13, IL-17A and IFN- γ concentration by ELISA.

Statistics

Data in graphs are shown as mean $\pm$ SE. ANOVA followed by Tukey's multiple comparison test was used where indicated. Statistical significance was set at $p < 0.05$.

Results

Partial depletion of natural gut flora in adult mice inhibits the production of OVA-specific IgG2a antibodies

6–8 weeks old mice received drinking water alone or water containing enrofloxacin (0.27 mg/ml) for 2 weeks prior to EC immunization with OVA (Groups C and D). Treatment with enrofloxacin or water was continued during the first week of OVA immunization and then all the mice were switched to drinking water without enrofloxacin till the challenge (Scheme 1). The control mice received drinking water alone or water with the enrofloxacin but were not immunized with OVA prior to the challenge (Groups A and B).

Data presented in Fig. 1 show that partial depletion of natural gut flora evaluated by CFU counts (Fig. 1A and B; Group B vs. A) and quantitative PCR analysis (Fig. 1F; Group B vs. A) non-significantly decreased anti-OVA IgE antibody production when compared to control mice (Fig. 1C; Group D vs. C). However, oral treatment with enrofloxacin significantly decreased synthesis of anti-OVA IgG2a antibodies (Fig. 1D; Group D vs. C). Treatment with enrofloxacin did not affect the body weight of experimental mice (Fig. 1E; Groups B and D vs. A and C).

Oral treatment with enrofloxacin prior to OVA immunization inhibits type-1 and type-2 cytokines and also suppresses the production of IL-17A

To determine if partial depletion of natural gut flora with a broad spectrum antibiotic enrofloxacin affects cytokine production, ALNC were collected from mice receiving water alone or enrofloxacin in drinking water prior to OVA immunization (Groups C and D). In control groups, mice received water alone or water with enrofloxacin without subsequent OVA immunization. Data presented in Fig. 2 show that treatment with enrofloxacin prior to OVA immunization results in decreased production of IL-4 (Fig. 2A; Group D vs. C), IL-5 (Fig. 2B; Group D vs. C), IL-10 (Fig. 2C; Group D vs. C), IL-13 (Fig. 2D; Group D vs. C), IL-17A (Fig. 2E; Group D vs. C) and IFN- γ (Fig. 2F; Group D vs. C) by ALNC.

Treatment with enrofloxacin early in life shifts the immune response from type-1 toward type-2 inflammation

To test the influence of early exposure to enrofloxacin on antibody production, the newborn mice were exposed to enrofloxacin during breastfeeding for 2 weeks and then again received enrofloxacin at 6 weeks of age prior to EC immunization with OVA. Data presented in Fig. 3 show that treatment with enrofloxacin early in life significantly upregulates the production of anti-OVA IgE antibodies (Fig. 3A; Group D vs. C) and inhibits the secretion of anti-OVA IgG2a antibodies (Fig. 3B; Group D vs. C).

Enrofloxacin application to young mice promotes the production of type-2 cytokines

To determine whether treatment with enrofloxacin early in life modulates cytokine production, ALNC from offspring of female mice receiving water alone or water with enrofloxacin prior to OVA immunization (Groups C and D) were cultured in the presence of the antigen. In control groups, offspring of mice receiving water alone or water with enrofloxacin were not immunized with OVA. Data presented in Fig. 4 show that treatment with enrofloxacin early in life prior to OVA immunization results in an increased production of IL-4 (Fig. 4A; Group D vs. C), IL-10 (Fig. 4B; Group D vs. C) and IL-13 (Fig. 4C; Group D vs. C). There was no difference in IL-17A (Fig. 4D) and IFN- γ (Fig. 4E) production between all the groups, whereas IL-5 was undetectable (data not shown).

Discussion

The development of balanced and adequate immune response in the intestines and periphery is governed by fine-tuned signals from gut bacteria. The immune system in neonates resembles one found in germ-free mice, hallmarked by enhanced Th2 response and elevated IgE levels [13]. Bacterial colonization at birth not only primes the proper development of the immune system but also skews cytokine profile toward Th1-polarised, which provides protection against infections. Decreased exposure to bacteria in Western countries, so called “hygiene

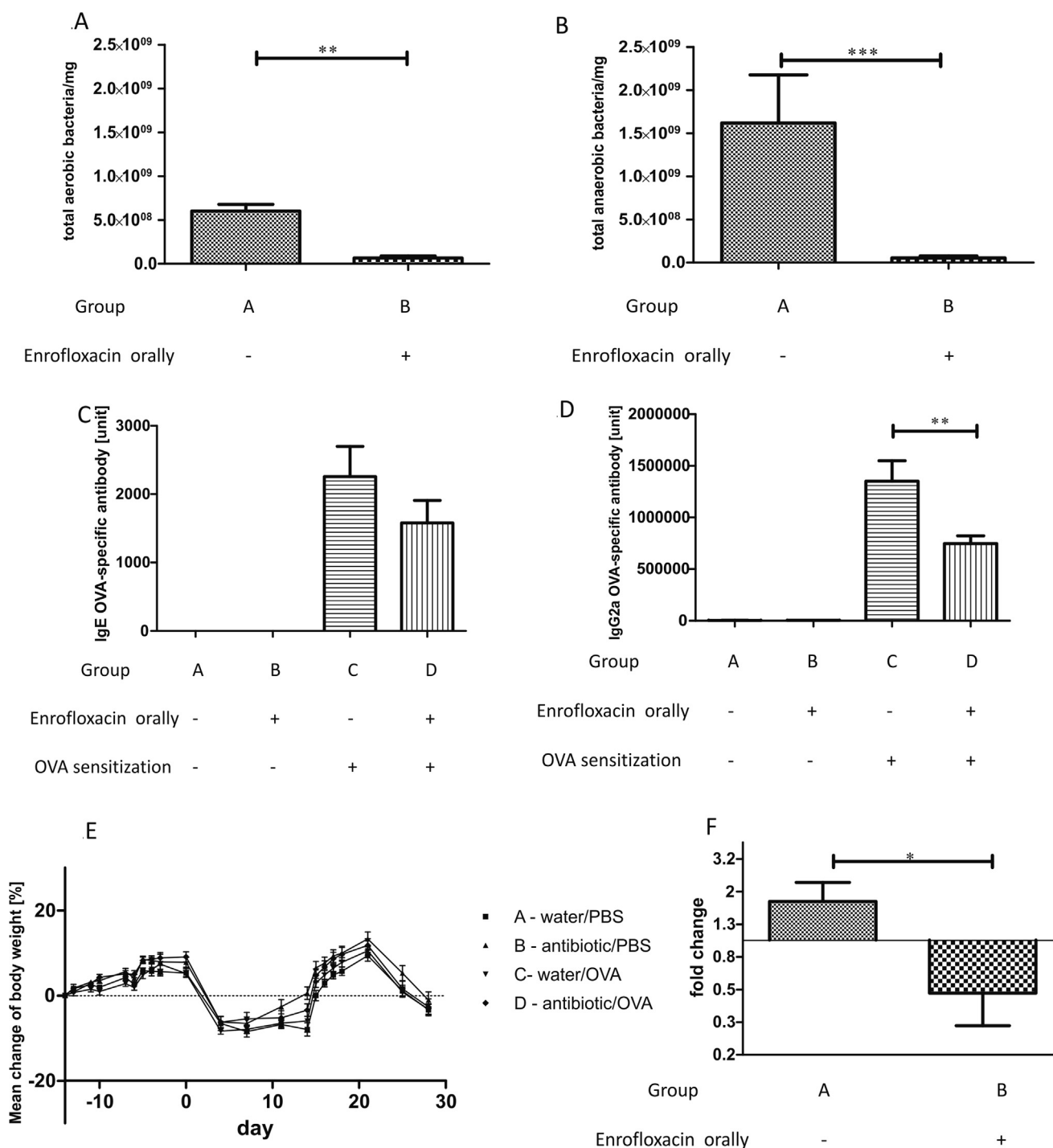


Fig. 1. Partial depletion of natural gut flora in adult mice inhibits the production of OVA-specific IgG2a antibodies. (A and B) Oral treatment with enrofloxacin partially depletes aerobic and anaerobic bacteria in the gut. BALB/c mice received water alone (Group A) or water with enrofloxacin (0.27 mg/ml) (Group B) for 3 weeks. Then, mice were sacrificed and colon content was collected and serial dilutions were cultured in general agar plates for 48 h or 72 h at 37 °C in aerobic and anaerobic conditions respectively. Total bacteria per gram of sample were calculated based on the CFU counted in each serial dilution. Data expressed as mean $\pm$ SE. $n = 4-6$; ** $p \leq 0.01$; *** $p \leq 0.001$. (C and D) Depletion of gut flora does not affect anti-OVA IgE antibody production (C) but inhibits synthesis of anti-OVA IgG2a antibodies (D). BALB/c mice were treated with enrofloxacin before EC immunization with OVA. After the challenge with OVA, sera were collected and concentration of OVA-specific antibodies was measured. Data expressed as mean $\pm$ SE. $n = 6-12$; ** $p \leq 0.01$. (E) Treatment with enrofloxacin does not affect body weight. To rule out detrimental influence of oral treatment with enrofloxacin on animal health, the body weight of mice treated with enrofloxacin or water was monitored daily. Data expressed as mean $\pm$ SE. $n = 6-12$; $p = ns$. (F) Oral treatment with enrofloxacin results in 10 folds reduction of gut bacteria number. BALB/c mice received water alone (Group A) or water with enrofloxacin (0.27 mg/ml) (Group B) for 3 weeks. Stool pellets were collected and 16S rDNA copy number was quantified by real-time PCR. Data expressed as mean $\pm$ SE. $n = 9$; * $p < 0.05$.

hypothesis", is regarded as a factor leading to the increased prevalence of Th2-mediated diseases [14]. It is known that treatment with antibiotics, apart from eliminating pathogenic bacteria, can also affect microbiota that may result in dramatic rise in atopic diseases [15–17].

This study was aimed to determine the influence of partial gut flora depletion with broad spectrum antibiotic enrofloxacin on type-2 immune response in mice. Additionally, we tested if the influence of enrofloxacin application on type 2 immune response depends on the maturity of the immune system.

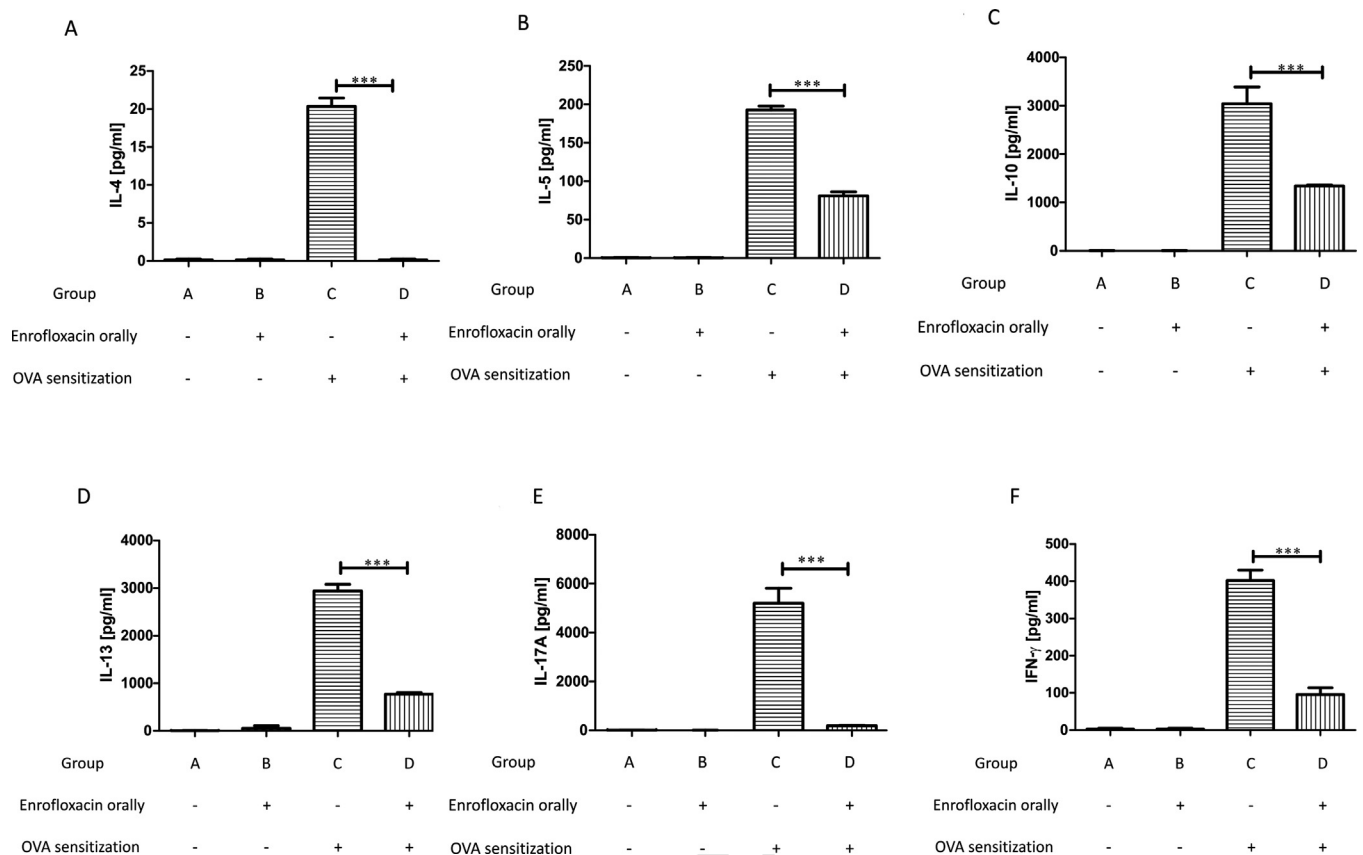


Fig. 2. Oral treatment with enrofloxacin prior to OVA immunization inhibits type-1, type-2 and Th17 associated cytokines (A–F). BALB/c mice were treated orally with water alone or water with enrofloxacin prior to OVA-sensitization (Groups C and D). Control mice were treated orally with water or enrofloxacin without subsequent OVA sensitization (Groups A and B). After 2 weeks of immunization with OVA, ALNC were collected and 3×10^6 cells were cultured in 1 ml RPMI 1640 medium supplemented with 5% FCS in the presence of 100 μ g/ml OVA. After 48 h culture, supernatants were collected and tested for: IL-4 (A), IL-5 (B), IL-10 (C), IL-13 (D), IL-17A (E) and IFN- γ (F). Results are presented as mean $\pm$ SE. $n = 4$; $***p \leq 0.001$.

To this end, we performed two sets of experiments with young and adult mice treated with enrofloxacin prior to EC immunization with OVA. Our data show that enrofloxacin application in adult mice decreases aerobic and anaerobic bacteria load as determined by CFU (Fig. 1A and 1B). Additionally, PCR analysis of conservative 16S rDNA fragments revealed almost 10 fold reduction in 16S rDNA copies number. Observed limited exposure to bacteria in adult mice has no impact on the production of OVA-specific IgE

antibodies (Fig. 1C). However, reduced exposure to gut bacteria signals is associated with diminished synthesis of OVA-specific IgG2a antibodies (Fig. 1D). Treatment with enrofloxacin does not affect the body weight of mice, suggesting that observed changes in antibody production were not caused by a toxic effect of the used therapeutic (Fig. 1E). It is well known that antibody production is regulated by cytokines [1,18,19]. To determine if the decreased production of IgG2a in adult mice treated with

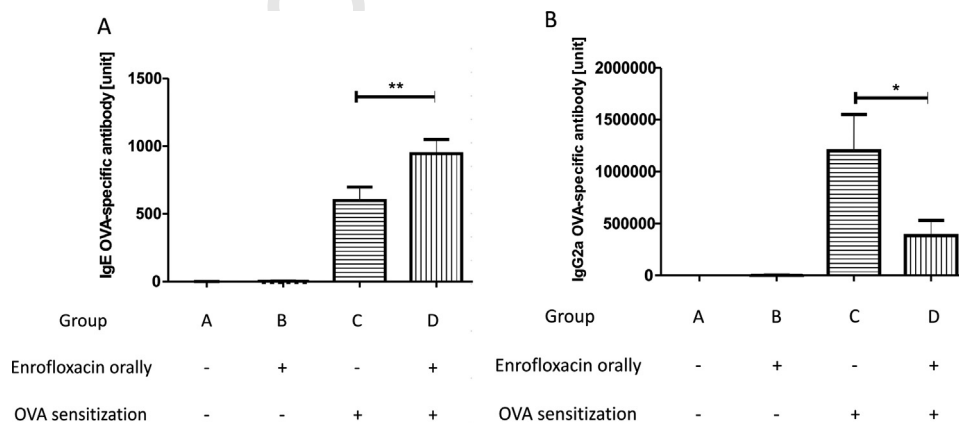


Fig. 3. Treatment with enrofloxacin early in life shifts the immune response from type-1 toward type-2 inflammation (A and B). Newborn mice were exposed to enrofloxacin for 2 weeks during breastfeeding. At 6 weeks of age they were treated again with water alone or water with enrofloxacin prior to EC immunization with OVA. After OVA-challenge sera were collected and concentration of anti-OVA IgE (A) and anti-OVA IgG2a antibodies (B) was measured. Data expressed as mean $\pm$ SE. $n = 18-24$; $**p \leq 0.01$; $*p \leq 0.05$.

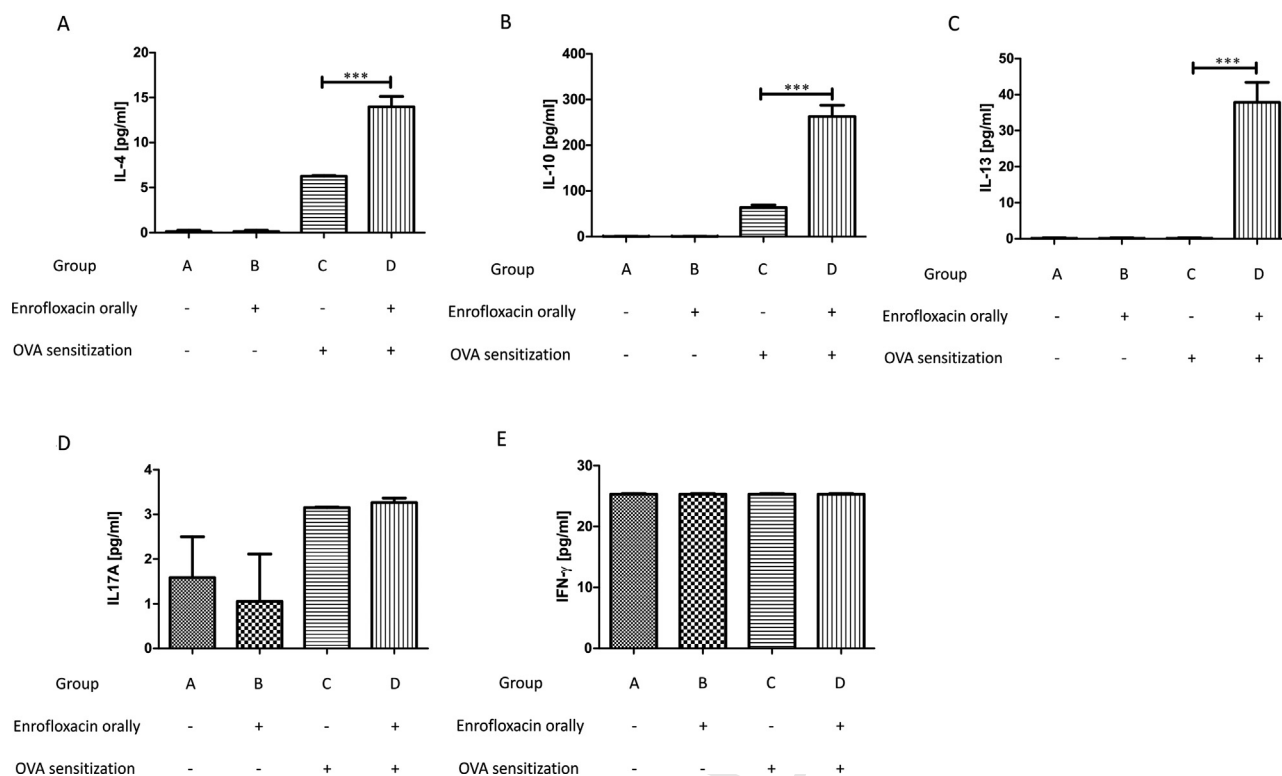


Fig. 4. Enrofloxacin application to young mice promotes the production of type-2 cytokines (A–D). Young mice were exposed to enrofloxacin during breastfeeding. At 6 weeks of age they received again water or enrofloxacin prior to immunization with OVA (Groups C and D). Control mice were not sensitized with OVA (Groups A and B). ALNCs were collected and 3×10^6 ALNC were cultured in 1 ml RPMI 1640 medium supplemented with 5% FCS in the presence of 100 $\mu\text{g/ml}$ OVA. After 48 h cultures, supernatants were collected and tested for: IL-4 (A); IL-10 (B); IL-13 (C); IL-17A (D) and IFN- γ (E). Data expressed as mean $\pm$ SE. $n = 4$; $p = \text{ns}$; *** $p \leq 0.001$.

enrofloxacin is associated with changes in cytokine production, ALNC were cultured and supernatants were tested for type-1, type-2 and Th17 associated cytokines.

We found that the production of type-2 cytokines IL-4 (Fig. 2A), IL-5 (Fig. 2B), IL-10 (Fig. 2C), IL-13 (Fig. 2D), Th17-associated cytokine IL-17A (Fig. 2E) and type-1 cytokine IFN- γ (Fig. 2F) is significantly decreased in mice treated with enrofloxacin prior to OVA immunization. Reduced release of type-1 cytokine IFN- γ and Th17-associated cytokine IL-17A corresponds with suppressed IgG2a production, as both cytokines play a crucial role in IgG production [18,19]. On the other hand, reduced production of type-2 cytokines has no significant effect on IgE synthesis in adult mice. As Th1 and Th2 immune responses are interconnected, one could speculate that diminished type-1 immune response could potentiate type-2 inflammation skewing and facilitate the development of atopic diseases accompanied by elevated IgE production. Our results show that after full maturation of the immune system, Th2 skewing of immune response is not under direct control of gut flora, although intestinal microbiota are able to influence type-2 cytokine production. The observed suppression of cytokine production could be an effect of inadequate priming of the immune system by reduced gut bacteria exposure or could be associated with the induction of regulatory cells due to antibiotic-induced dysbiosis. However, we cannot rule out any direct influence of enrofloxacin on the immune system or mouse physiology. It has been already shown by others that some antibiotics, e.g. vancomycin, have potential to down-regulate TNF- α production [20].

Further experiments aimed to evaluate the influence of enrofloxacin on immune response in young mice. Our data show that treatment with enrofloxacin early in life has profound effect on the immune system as it shifts the response from type-1 to type-2. The production of anti-OVA IgE antibodies (Fig. 3A) is

elevated while the secretion of anti-OVA IgG2a antibodies is inhibited (Fig. 3B). Cytokine production by ALNC from enrofloxacin treated mice indicates that modification of gut flora signals early in life favors the production of type-2 cytokines: IL-4 (Fig. 4A) and IL-13 (Fig. 4C) that support IgE synthesis and the development of allergic reactions. Our finding is in line with the data presented by Bashir et al. showing that gut flora modification by treatment with a mixture of antibiotics beginning at 2 weeks of age can modify susceptibility to allergy [21].

Many epidemiological studies suggest that proper bacterial signaling early in life is crucial to prevent atopic diseases later in life. Signals received from bacteria should be diverse, for proper shaping of the immune response. Children living on farms exposed to a wide variety of microbial antigens develop less atopic diseases later in life [22]. On the other hand, decreased gut bacteria diversity during first years of life correlates with higher incidence of asthma or allergies in children [23]. Pattern of gut colonization in atopic children was also altered and showed less *Enterococci* and *Bifidobacteria* but more *Clostridia* [24,25]. Antibiotics are a known cause of decreased bacteria load as well as microbial diversity in the gut. The observed changes in gut bacterial communities are long-lasting [26], but their impact seems to be more profound in young children where proper bacterial signaling is essential for the immune system maturation and tolerance induction. Some epidemiological studies suggest association between the usages of broad spectrum antibiotics early in life and an increased risk of atopic diseases later in life [15–17]. On the contrary, children exposed to bacterial compounds in the form of probiotic in post-natal period develop less atopic diseases [27]. Thus, our results from young mice correspond well with epidemiological studies. The data from young mice combined with the results from mature mice indicate the presence of time window, where the

mice are especially prone to modified signals received from bacterial flora.

Our observation is in line with results presented by Russell et al. showing that treatment of young but not adult mice with vancomycin predisposes to asthma [28]. On the other hand, other groups showed that antibiotic application to adult mice before intranasal challenge leads to enhanced IgE response [29,30]. However, we cannot directly compare these results with our findings. Both aforementioned experimental models evaluated the influence of antibiotic application on the development of allergic response after intranasal but not epicutaneous challenge. Moreover, different antibiotic treatment regime was used in these studies comparing application of a mixture of antibiotics [29] to cefoperazone alone [30]. Additionally, our work evaluated the level of antigen specific IgE, whereas in aforementioned papers total IgE was measured. This differential impact of bacterial signal deprivation on IgE immune response in old mice is in contrast with model-independent elevation of IgE response in young mice. Thus, our results support the hypothesis that the immune system is more vulnerable to decreased bacterial exposure early in life, while later in life its net effect could be influenced by other yet unidentified factors.

During early life enrofloxacin application prevents proper development of type 1 response and promotes uninterrupted development of type 2 immune response. Proper priming of the immune system by bacteria early in life results in resilience of the immune system to changes imposed by enrofloxacin application later in life.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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