International Immunopharmacology 68 (2019) 226–233

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Butyrate inhibit collagen-induced arthritis via Treg/IL-10/Th17 axis

Wenpeng Hui[a], Dapeng Yu[a], Zhong Cao[a], Xiwu Zhao[b,*]

[a] Department of Spinal Surgery, Shandong Provincial Western Hospital, No 4 Duanxing west Road, Jinan 250022, Shandong, China
[b] Department of Traumatic Orthopedics, Shandong Provincial Western Hospital, No 4 Duanxing west Road, Jinan 250022, Shandong, China

ARTICLE INFO

Keywords:
Rheumatoid arthritis
Butyrate
Treg cells
Th17 cells
IL-10

ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disorder demanding the development of novel therapeutic strategy. Butyrate is a functional short-chain fatty acid produced by the anaerobic intestinal microbiota. This study aimed to investigate the attenuation of butyrate on RA. The collagen-induced arthritis (CIA) mouse model was established and butyrate was administered in drinking water along with the collagen immunization. The histopathological features, clinical score, paw swelling, as well as the production of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6 and IL-17A were measured to determine the amelioration of butyrate on arthritis. The differentiation of Treg cells and Th17 cells in the splenic cells was assessed by flow cytometry. The expression of Foxp3, IL-10, Rorγt and IL-17A were detected by RT-PCR and FACS immunostaining. Anti-IL10R antibody was used in the CIA and $CD4^+$ cell cultures to mediate the effects of butyrate. Butyrate significantly inhibited expressions of IL-1β, IL-6 and IL-17A, but promoted the expression of IL-10. Butyrate also increased systematical Treg cells and reduced Th17 cells. Mechanism study revealed that butyrate directly enhanced the polarization of Treg cells but not Th17 cells. All effects of butyrate on RA were inversed by the co-administered anti-IL10R antibody. This study showed that butyrate administration inhibited arthritis in CIA mice model, suppressed the expression of inflammatory cytokines. The modulation may be mediated the differentiation of CD4 T cells towards Treg cells, which produce anti-inflammatory cytokine IL-10, and thus influenced the function of Th17 cells.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder, pathologically characterized by cartilage and bone destruction of synovial joints [1]. Although the pathologic mechanisms of RA have not been fully understood, studies investigating the etiology of RA have established the participation of regulatory T (Treg) cells, which were defective in suppression of IFN-γ and TNF-α production by conventional T cells in peripheral blood from active RA patients [2,3]. Given the pivotal role that proinflammatory cytokines in the induction and maintenance of RA, the regulation of these cytokines on Treg cells can be a target for protecting the host from autoimmune disease [4]. For instance, Treg cells depletion in mouse model leads to the onset of arthritis, while Treg cells replenishment ameliorates arthritic symptoms [5]. The importance of Treg cells in RA is also supported by the effectiveness of the cytotoxic T-lymphocyte-associated antigen 4-IgG1 (CTLA4-Ig) therapy, which is one of the most important biological disease-modifying antirheumatic drugs (DMARDs) by blocking T cell co-stimulation [6]. There are three classes of drugs generally used in the RA treatment: non-steroidal anti-inflammatory agents (NSAIDs), corticosteroids, and DMARDs. Although studies in patients with RA in the past decades have demonstrated that oral and biological DMARDs prevent joint pain, swelling, and erosion, these drugs could have drawbacks such as the lack of the eventual cure of the disease or even potentially life-threatening side effects [7]. Therefore, the development of new treatment strategies is required to meet clinical needs of RA. In view of the important role of Treg cells in RA, the regulation on Treg cells constitutes a useful target in the development of new treatment of RA.

Butyrate is the most extensively investigated short-chain fatty acid (SCFA) produced during the fermentation of dietary fiber by the anaerobic intestinal microbiota [8]. It has been reported that the concentration of butyrate was 3-fold in cancer cells when compared to the one in normal colonocytes [9]. In addition to serving as the primary energy supply in colonocytes, butyrate has been shown protection against colorectal cancer and inflammation, partially via inhibiting histone deacetylases (HDACs) [10]. Butyrate also regulates cytokine expression and differentiation into Tregs of T cells. Moreover, butyrate

Abbreviations: RA, rheumatoid arthritis; CIA, collagen-induced arthritis

* Corresponding author.
E-mail address: doctorzhaoxiwu@163.com (X. Zhao).

https://doi.org/10.1016/j.intimp.2019.01.018
Received 21 December 2018; Received in revised form 9 January 2019; Accepted 10 January 2019
Available online 16 January 2019

has been reported to ameliorate the development of colitis by modulating the differentiation of Treg cells [11]. Effector T cells, including Th1, Th2, and Th17 cells, are able to enhance aerobic glycolysis, which could inhibit Treg cells generation [12]. In general, the metabolic dynamics in activated T cells between regulatory or effect T cells is highly relevant to SCFA-mediated HDAC inhibition [11,13]. Overall, butyrate has been shown potent anti-inflammatory property by regulating the Treg cells in different models, and the present study was designed to explore the regulation of butyrate sodium in the rheumatoid arthritis mouse model, and to explore the underlying mechanism of action.

2. Materials and methods

2.1. Animals and induction of CIA

Female DBA/1J mice (8 weeks old) were purchased from Beijing VitalRiver Laboratory Animal Technology Co., Ltd. All animal experiments were carried out according to the protocol approved by the Animal Care and Use Committee of Shandong Provincial Western Hospital. After one week of acclimatization period, the mice were subcutaneously injected 100 μl of an emulsion containing a 1:1 ratio of bovine type II collagen (CII, Chondrex, Redmond, WA) with Freund's complete adjuvant (CFA, Chondrex) on day 0, followed by an immunization with 100 μl of an emulsion containing a 1:1 ratio of CII and Freund's incomplete adjuvant (IFA, Chondrex) on day 21. On the same day as the primary immunization, mice were provided with 100 mM sodium butyrate (Sigma Aldrich, St. Louis, MO) in drinking water for 5 weeks. The mice were examined three times per week for the presence of arthritis from day 21 to 35. The arthritis clinical score for the disease severity was determined according to a previously established report [14]. Briefly, it was a 0–16 scale which sums up the clinical score of four limbs, corresponding to the severity of erythema and swelling on different parts of limbs: 0, no sign of inflammation or redness; 1, slight erythema and mild swelling on the paw; 2, mild erythema and mild swelling across the entire paw; 3, severe swelling and redness from the ankle to digits; 4, maximal swelling and redness or obvious joint destruction. The scoring was executed by two independent observers.

2.2. Histopathological and paw swelling assessment of arthritis

On day 35 post the establishment of the CIA model, the mouse joint tissues were removed from hind legs, and fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. Five-micrometer tissue sections were prepared and stained with haemotoxylin and eosin (H&E, Sigma Aldrich), and observed under light microscope. Paw swelling of the mice at day 35 was determined by measuring the paw thickness using electronic digital caliper (Shandong Tools Manufacturing Co., Shandong, China) in a double blinded way.

2.3. Cytokine measurement by enzyme-linked immunosorbent assays (ELISAs)

At day 35, mice were euthanized and blood samples were collected from each group. The protein levels of cytokines, which include interleukin (IL)-1β, IL-6, IL-17A and IL-10 were quantified by the commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, serum samples were diluted and added into 96-well plates coated with purified primary polyclonal antibodies against IL-1β, IL-6, IL-17A and IL-10, followed by washing and incubation steps in the manual. The absorbance at 450 nm was recorded by microplate reader (Molecular Device, Sunnyvale, CA, USA), and the concentrations are expressed as pg/ml.

2.4. Staining and immunofluorescence analysis of cell populations by flow cytometry

The frequencies of Treg cells and Th17 cells were analyzed by FACS flow analysis according to the gating strategy described in previous research [15]. Briefly, at day 35 after immunization, $CD4^+$ T cells were isolated from the spleen, using the $CD4^+$ T cell isolation kit (Miltenyi Biotec, Shanghai, China). Approximately 1×10^6 cells collected from the spleen of arthritic mice with or without sodium butyrate administration, were incubated with CD4-APC antibodies (BD Biosciences, San Jose, CA, USA), washed and detected by flow cytometry. For surface and intracellular transcription factor staining, antibodies for FOXP3-APC, CD3-Percp and CD25-FITC were used for staining of Tregs. For intracellular staining of IL-17A and IFN-γ, $CD4^+$ T cells were fixed with 2% formaldehyde solution and stained with antibodies against these two cytokines (eBioscience, San Diego, CA, USA) in 0.3% saponin buffer. All flow cytometry analysis was conducted on FACSCalibur (BD Bioscience, Franklin Lakes, NJ, USA), and obtained data were analyzed with FlowJo software (Ashland, USA).

2.5. Cytokine analysis by real-time quantitative PCR

Total RNA was extracted from cultured $CD4^+$ cells or synovial tissues of CIA mice, and reverse transcribed to cDNA for quantitative PCR. The PCR reactions were performed with the BioRad CFX96 TouchTM System using iQTM SYBR1 Green Supermix (BioRad, Hercules, CA, USA) with the following primers: Foxp3 forward TTCCTTCCCAGAGT TCTTCCA, Foxp3 reverse CATTGAGT GTCCTCTGCCTCT; IL-10 forward CCCCAGGCAGAGAAGCATGGC, IL-10 reverse GGGGAGAAATCGATGA CAGCGCC; retinoic acid receptor-related orphan receptor gamma t (Rorγt) forward TCCTGCCACCTTGAGTATAGTCC, Rorγt reverse GGA CTATACTCAAGGTGGCAGGA; IL-17A forward ATCCCTCAAAGCTCAG CGTGTC, IL-17A reverse GGGTCTTCATTGCGGTGGAGAG; β-actin forward ATGTGGATCAGCAAGCAGGA, β-actin reverse AAGGGTGTAAAA CGC AGCTC.

2.6. In vitro Treg cells and TH17 cells differentiation

The in vitro $CD4^+$ T cells isolation and differentiation were performed according to previous description with slightly modification [16]. Briefly, $CD4^+$ T cells were enriched from splenocytes of mice with CD4-microbeads using MACS (Miltenyi Biotec). For naïve T cell enrichment, $CD4^+$ and $CD25^+$ T cells were depleted using beads with specific antibodies (Miltenyi Biotec). Naïve T cells were cultured at a density of 10^6 cells/ml for 5 days with plate-immobilized anti-CD3 and soluble anti-CD28 (both 2 μg/ml) in Click's medium supplemented with 10% FCS and ι-glutamine. Treg cell differentiation was achieved under TR1-inducing conditions by adding 0.5 ng/ml TGF-β1 and 30 ng/ml IL-27. For Th17 cell differentiation, naïve T cells were cultured in the same medium under Th17 polarizing condition, which contains 0.5 ng/ml TGF-β1, 10 ng/ml IL-6, 20 ng/ml IL-23 and 10 ng/ml IL-1β in the medium.

2.7. In vivo and in vitro IL-10 receptor blocking

For IL-10 receptor blocking in CIA mice model, anti-IL-10R antibodies were injected (i.v.) to the mice immunized with collagen from day 7 to day 28 (100 μg, every 3 days). Non-specific isotype IgG was injected as control. For the in vitro differentiated $CD4^+$ T cells, the supernatant from Treg cells culture (stimulated CD3/CD28 antibodies) was added to Th17 cells with anti- IL-10R Ab (50 μg/ml) or isotype Rat IgG for 3 days.

2.8. Statistical analysis

Data was presented as mean ± SEM. Statistical analysis was

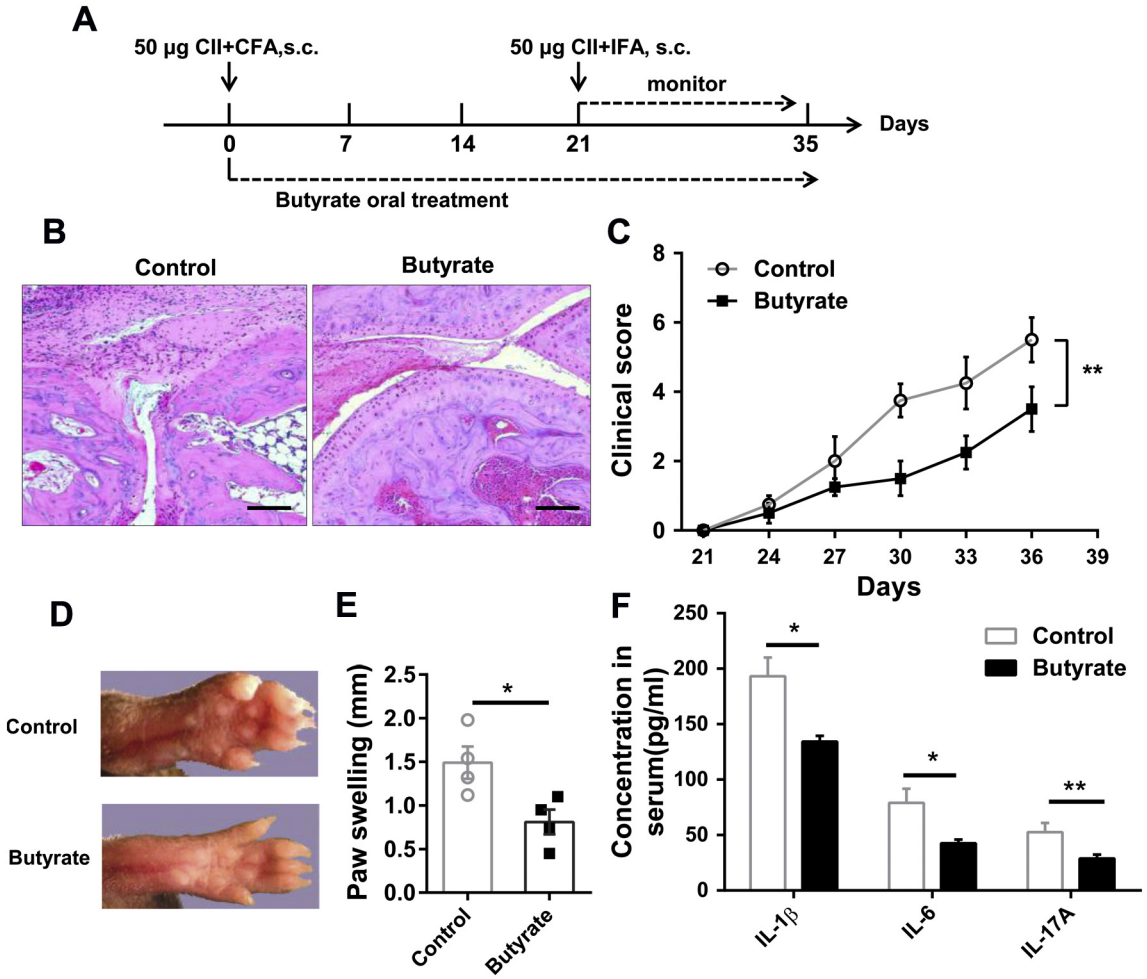

Fig. 1. Butyrate inhibited collagen-induced arthritis in mice. (**A**). Work flow of butyrate treatment for collagen-induced arthritis in mice. Briefly, mice were first immunized with injection (s.c.) of emulsion containing bovine type II collagen (CII) and CFA on day 0 and were subsequently immunized with emulsion containing CII and IFA on day 21. Mice were treated with sodium butyrate (100 mM) in drinking water from day 0 to day 35. (**B**). Histologic sections of joints of hind legs from each group were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy. Scale bar = 100 μm. (**C**). Clinical scores for assessment of joint injury from day 21 to day 35 were shown. (**D**). Hind legs from each group at day 35 were shown. (**E**). Paw swelling of the mice at day 35 was analyzed. (**F**). The concentrations of IL-1β, IL-6 and IL-17A in serum from each group at day 35 were evaluated by ELISA. Data are representative of three or more independent experiments with ≥4 mice per group. Data are the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ns = not significant.

performed with GraphPad Prism 5 (La Jolla, CA, USA). For the comparison between two groups, an unpaired Student's *t*-test was used. For the comparisons of the multiple groups, the one- or two-way analysis of variance was used. Statistical analysis was considered at $p < 0.05$.

3. Results

3.1. Butyrate inhibited collagen-induced arthritis in mice

As shown in the work flow (Fig. 1A), mice were first immunized with injection of emulsion containing bovine CII and CFA on day 0 and were subsequently immunized with emulsion containing CII and IFA on day 21. We found on day 24 after the first immunization, mice displayed the onset of arthritis (data not shown). The H&E staining results of the mouse joint tissues at day 35 post the establishment of the collagen-induced arthritis differs significantly in the control and butyrate groups. It is worth noting that no obvious side-effects could be found in the mice treated by butyrate during the period of experiments. Briefly, in the vehicle control group, we can see severe infiltration of inflammation and apparent synovial hyperplasia, both of which were significantly attenuated in the butyrate treatment group (Fig. 1B). Notably, the average clinical score in butyrate group was significantly lower than the one in the vehicle group across the progression of the RA in mice, and the largest difference was observed at day 35 (Fig. 1C, $p < 0.01$). As seen in Fig. 1D and E, the hind paw of the control group is swelling with approximately 1.5 mm thickness in the control group, whereas the thickness in butyrate group was decreased to 0.7 mm ($p < 0.05$). We also measured the concentrations of pro-inflammatory cytokines including IL-1β, IL-6 and IL-17A in the mice serum, the results showed that the levels of all three cytokines were significantly downregulated in the butyrate group (Fig. 1F, $p < 0.05$ for IL-1β and IL-6, $p < 0.01$ for IL-17A, respectively). All of these measurements demonstrated that the butyrate treatment alleviated collagen-induced arthritis compared with that of vehicle control group.

3.2. Butyrate treatment increased systematical Treg cells and reduced Th17 cells in arthritis

To determine the anti-inflammatory mechanism of butyrate treatment in collagen-induced arthritis model, we further assessed the concentration of anti-inflammatory cytokine IL-10 in the mice serum at day 35. The result showed that the IL-10 concentration in butyrate group is significantly elevated than the control group (Fig. 2A). Interestingly, the frequency of colonic Tregs increased from 6.0% in the

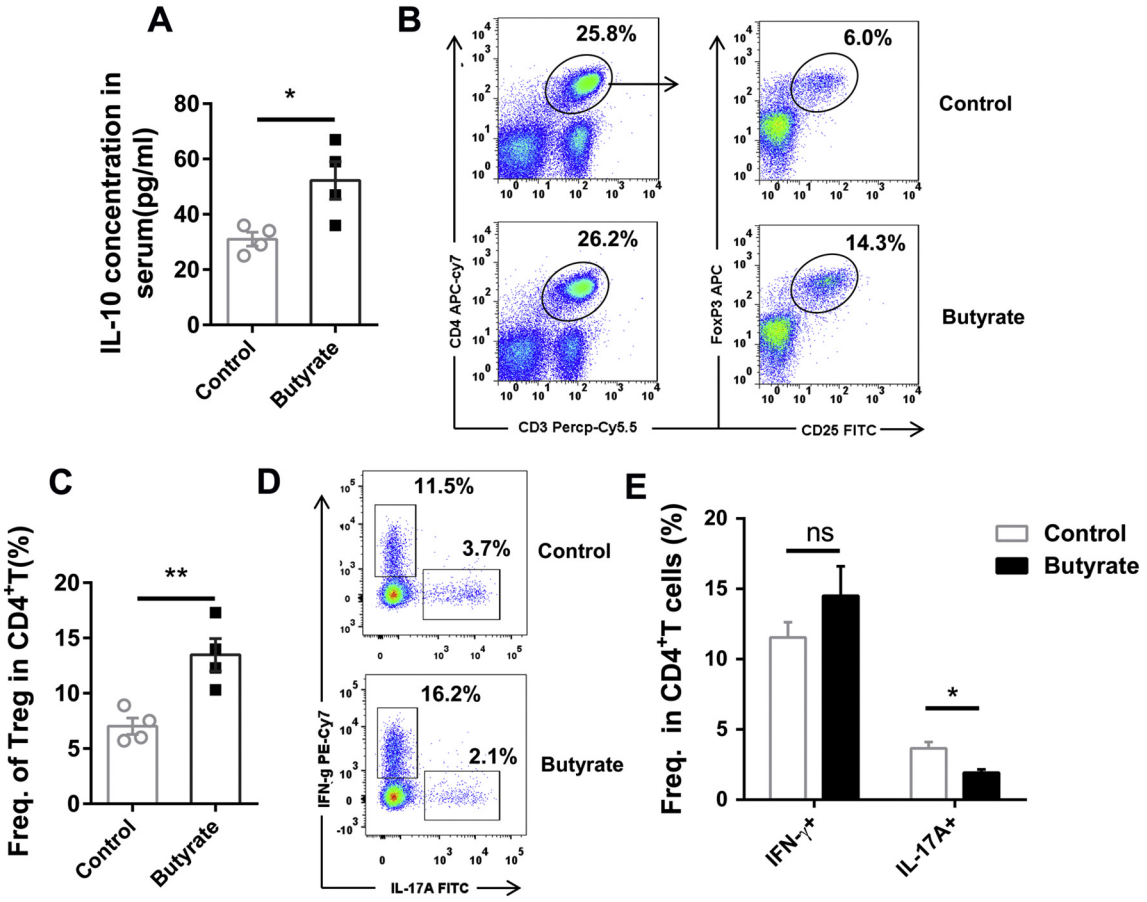

Fig. 2. Butyrate treatment increased systematical Treg cells and reduced Th17 cells in arthritis. (A). IL-10 concentration in serum at day 35 was evaluated by ELISA. (**B**). Treg cells in spleen at day 35 were analyzed by FACS. Gating strategy was shown. (**C**). Statistical analysis of Treg cells in B was shown. (**D**). Th17 and Th1 cells in spleen at day 35 were analyzed by FACS. Gating strategy was shown. (**E**). Statistical analysis of Th17 and Th1 cells in D were shown. Data are representative of three or more independent experiments with ≥4 mice per group. Data are the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ns = not significant.

control group to 14.3% in the mice received sodium butyrate in the drinking water, whereas the frequency of Th17 cells changed from 3.7% to 2.1% (Fig. 2B–D), suggesting cell regulation on Th17 by butyrate was at a lesser extent. Previous reports have shown that SCFAs impact the expansion of Tregs, as well as the differentiation of Th1 and Th17 cells (21). Correspondingly we evaluated the effects of SCFAs on the production of pro-inflammatory cytokines and found butyrate was able to selectively inhibit expression of IL-17A but not of IFN-γ (Fig. 2E).

3.3. Butyrate directly promoted the polarization of Treg but not Th17 cells

To understand the regulation difference between Treg and Th17 cells of butyrate, in vitro cell cultures and differentiation were performed. Specifically, $CD4^+$ T cells were cultured under Treg-inducing conditions in the presence of 0.2 mM of sodium butyrate for 4 days. As demonstrated in Fig. 3A, the mRNA levels of FoxP3 and IL-10 in $CD4^+$ T cells were significantly higher in the cells administrated with butyrate ($p < 0.05$). Moreover, FACS analysis of FoxP3 expression in $CD4^+$ T cells showed that the frequency of $FoxP^+$ cells was increased from 29.8% in the control group to 35.6% in the butyrate group (Fig. 3B and C, $p < 0.05$). In contrast, the mRNA expression levels of RORγt and IL-17A in $CD4^+$ cells did not show significant difference (Fig. 3D). Consistently, the FACS results showed similar frequency of $IL\text{-}17A^+$ $CD4^+$ T cells (Fig. 3E and F).

3.4. Butyrate inhibited collagen-induced arthritis through IL-10 signal pathway

Consistent with the results in the cultured $CD4^+$ cells, butyrate significantly upregulated the mRNA expression level of IL-10 in synovial tissues of mice (Fig. 4A, $p < 0.05$). To anchor the modulation role of butyrate on IL-10, anti-IL-10R Ab were injected from day 7 to day 28 (see workflow shown in Fig. 4B). The histological staining results of the mouse joint tissues at day 35 showed that the co-administration of anti-IL-10R Ab forfeited the amelioration of butyrate to the arthritis, as severe infiltration of inflammation was observed (Fig. 4C). In addition, the average clinical scores in the anti-IL-10R Ab group are similar to the ones in the control group, which are significantly higher than the ones in the butyrate group (Fig. 4D, $p < 0.01$). As shown in Fig. 4E, the paw swelling in anti-IL-10R Ab group is close to the control group, which is significantly higher than the one in butyrate group. Moreover, Fig. 4F shows that the co-administration of anti-IL-10R Ab also inversed the inhibition of butyrate on the expression of pro-inflammatory cytokines including IL-1β, IL-6 and IL-17A. Taken together, anti-IL-10R Ab forfeited all of these parameters altered by butyrate treatment in collagen-induced arthritis model compared with that of vehicle control group.

3.5. Butyrate inhibited Th17 function in a Treg-IL-10-dependent manner

To further explore the mechanisms underline the inhibition on Th17, we established in vitro experiment to test the participation of IL10 in this process. Firstly, flow cytometry results showed that the Th17 cells isolated from the synovial tissues presented expression of

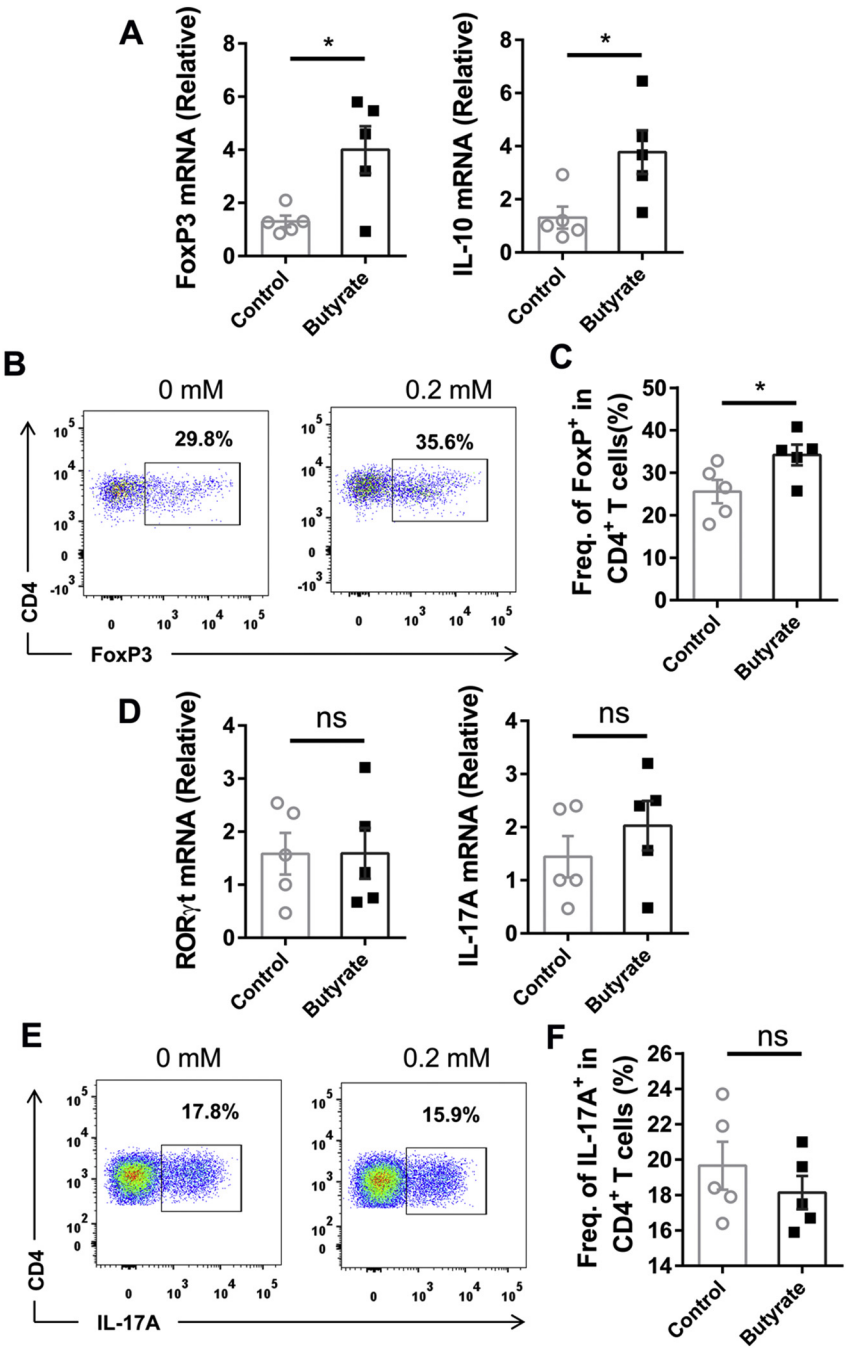

Fig. 3. Butyrate directly promoted the polarization of Treg but not Th17 cells. (**A–C**). $CD4^+$ T cells were cultured under Treg-inducing conditions in the presence of 0.2 mM of sodium butyrate for 4 days. The mRNA levels of FoxP3 and IL-10 in $CD4^+$ T cells were determined by Real-time PCR (**A**). FACS analysis of FoxP3 expression in $CD4^+$ T cells was shown (**B**). The frequency of $FoxP^+$ cells was shown from FACS **(C)**. (**D–F**). $CD4^+$ T cells were cultured under Th17-inducing conditions in the presence of 0.2 mM of sodium butyrate for 4 days. The mRNA levels of RORγt and IL-17A in $CD4^+$ T cells were determined by Real-time PCR (**D**). FACS analysis of IL-17A expression in $CD4^+$ T cells was shown (**E**). The frequency of $IL\text{-}17A^+$ cells was shown from FACS (**F**). Data are representative of three or more independent experiments. Data are the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, ns = not significant.

receptor towards IL-10 (Fig. 5A). Further, Th17 and Treg cells were induced separately, and then the supernatant of cultured Treg cells were added to the induced Th17 cells, followed by the addition of anti-IL-10R or control isotype (Fig. 5B). As shown in Fig. 5C and D, the resulting data confirmed that anti-IL-10R Ab significantly promoted the mRNA and protein expression levels of IL-17A and IL-1β, which were downregulated by butyrate. In contrast, the expression of nuclear hormone receptor RORγt remained the same across different groups, suggesting the specific regulation of butyrate on IL-10.

4. Discussion

Intestinal commensal microbes play important roles in gut immune homeostasis, through balancing between pro- and anti-inflammatory mechanisms, and regulating the differentiation of various T cell types [13]. An obvious example is that antibiotics have been reported to alleviate symptoms in several different autoimmune diseases including RA [17]. Potential therapeutic effects of SCFAs, the bowel microbial fermentation products, have been recently emphasized in a great variety of experimental models of autoimmunity or inflammatory diseases mediated by T cells [18–20]. Among them, a four-carbon SCFA butyrate has been shown to present anti-inflammatory properties by inducing Treg cells differentiation in mice [11]. Controversially, higher concentrations of butyrate could result in the pro-inflammatory development reflected by the IFN-γ-production in T cells [15]. A more comprehensive understanding of how the bacterial products influence host immune system may play a part in new therapies by reinforcing immunity and reducing inflammatory conditions in different autoimmune disorders. In this study we investigated the regulation role of butyrate in rheumatoid arthritis, which is a chronic autoimmune disease. In order to determine if butyrate is capable of alleviating the symptoms of RA, we administered butyrate in drinking water to

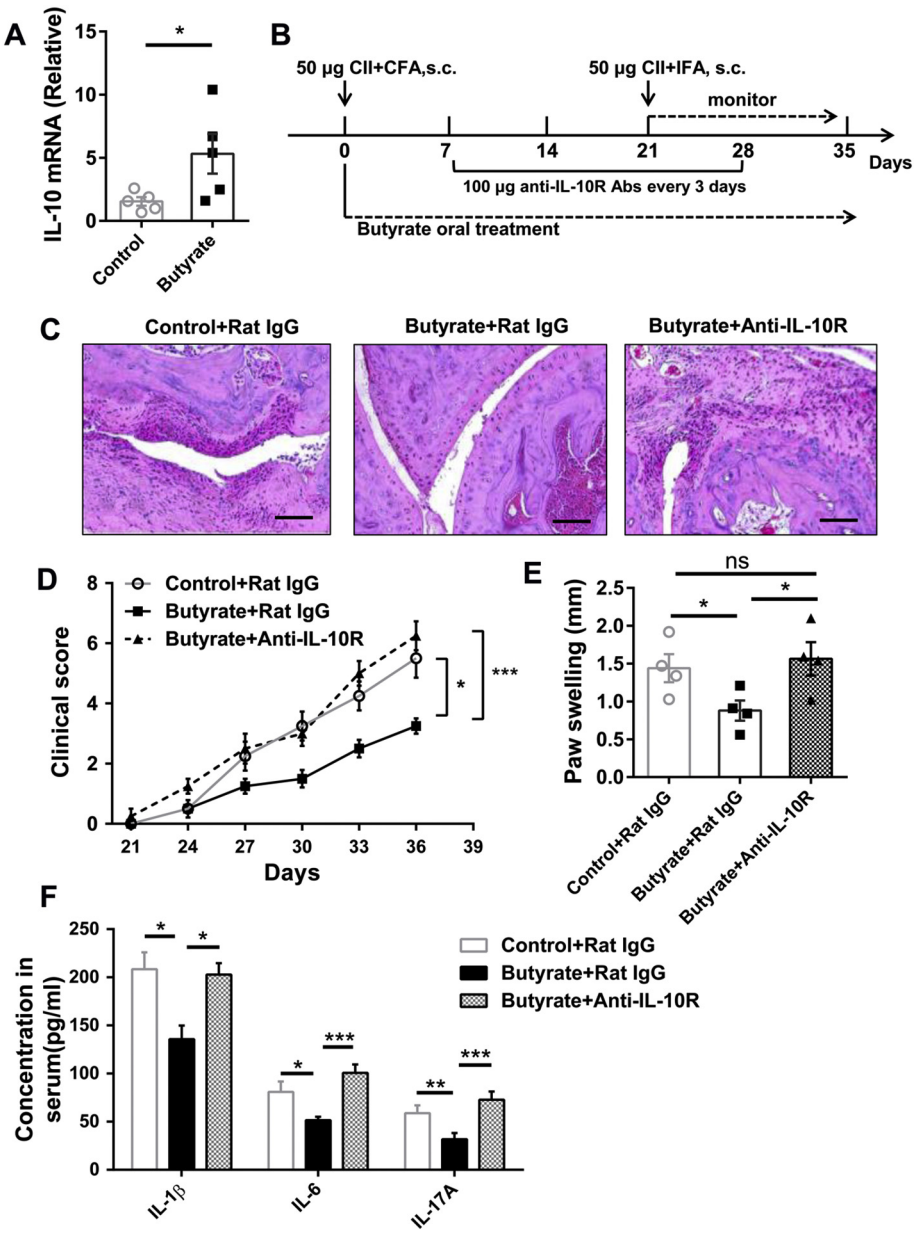

Fig. 4. Butyrate inhibited collagen-induced arthritis through IL-10 signal pathway. (**A**). The level of IL-10 mRNA in synovial tissues from mice at day 35 was evaluated by Real-time PCR. (**B–F**). Work flow of butyrate and anti-IL-10R treatment in collagen-induced arthritis. Briefly, mice were first immunized with injection (s.c.) of emulsion containing CII and CFA on day 0 and were subsequently immunized with emulsion containing CII and IFA on day 21. Mice were treated with sodium butyrate (100 mM) in drinking water from day 0 to day 35. Anti-IL-10R antibodies were injected (i.v.) from day 7 to day 28 (100 μg, every 3 days). The mice were injected with Rat IgG as control (B). (**C**). Histologic sections of joints of hind legs from each group were stained with H&E and analyzed by light microscopy. Scale bar = 100 μm. (**D**). Clinical scores for assessment of joint injury from day 21 to day 35 were shown. (**E**). Paw swelling of the mice at day 35 was analyzed. (**F**). The concentration of IL-1β, IL-6 and IL-17A in serum from each group at day 35 were evaluated by ELISA. Data are representative of three or more independent experiments with ≥4 mice per group. Data are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, ns = not significant.

collagen-induced arthritis mice for 5 weeks. Our data suggested very strong inhibition of butyrate on the histopathological parameters, clinical score, paw swelling, as well as the production of pro-inflammatory cytokines including IL-1β, IL-6 and IL-17A in the mice serum, when compared to the untreated control CIA mice.

Given the key roles of CD4 T cells in mediating autoimmune responses, we further investigated the regulation of butyrate on the different population of CD4 T cells. Naive CD4 T cells may differentiate into T helper (Th) cells and Treg cells [21]. Th17 cells are unique pro-inflammatory cells characterized by RORγt and IL-17 [22]. and an inflammatory environment controls the balance between Treg and Th17 cell differentiation, as defined by their patterns of cytokine production and function towards pro- or anti-inflammation. That is to say, the differentiation into Th17 effector cells, which provoke a highly inflammatory immune response, or into Treg cells, which suppress immune system, is the mechanism behind T-cell mediated inflammation in auto-immune diseases [23]. In our case, to further explore the inhibition mechanism of butyrate on RA, we analyzed the splenic Treg cell subsets in butyrate and control mice by FACS analysis. In RA, the $CD4^+CD25^+$ regulatory T cell subset represents approximately 5–10% of the $CD4^+$ T cell population [24], and our data fell in this range, indicating the successful establishment of the CIA moel. Our results also showed that butyrate administration significantly increased the frequency of Treg cells, which is in agreement with the synergic effects of $Foxp3^+$ regulatory T cells with natural Treg cells to promote immune tolerance and inhibit autoimmunity [12]. Since naive T cells can differentiate towards other effector populations including Th1, Th2, and Th17 cells, which are characterized by the signature cytokines of IFN-γ, IL-4, and IL-17, respectively [12], we also used different gating strategy to evaluate the frequency of IFN-γ^+ and IL $17A^+$ CD4+ cells. The results clearly showed that butyrate increased the splenic Th17 cells frequency but not the Th1 cells. Notably, serum from CIA mice treated with butyrate also demonstrated significantly higher concentration of the anti-inflammatory cytokine IL10, indicating that butyrate promotes the T cell receptor activation towards anti-inflammatory cytokine milieu practically.

The phenomena that butyrate promotes Treg cells differentiation triggered our curiosity to know whether butyrate directly affects T cells by enhancing the expression of Foxp3, the lineage specific transcription factor of Treg cells. We evaluated the effects of butyrate in vitro, and

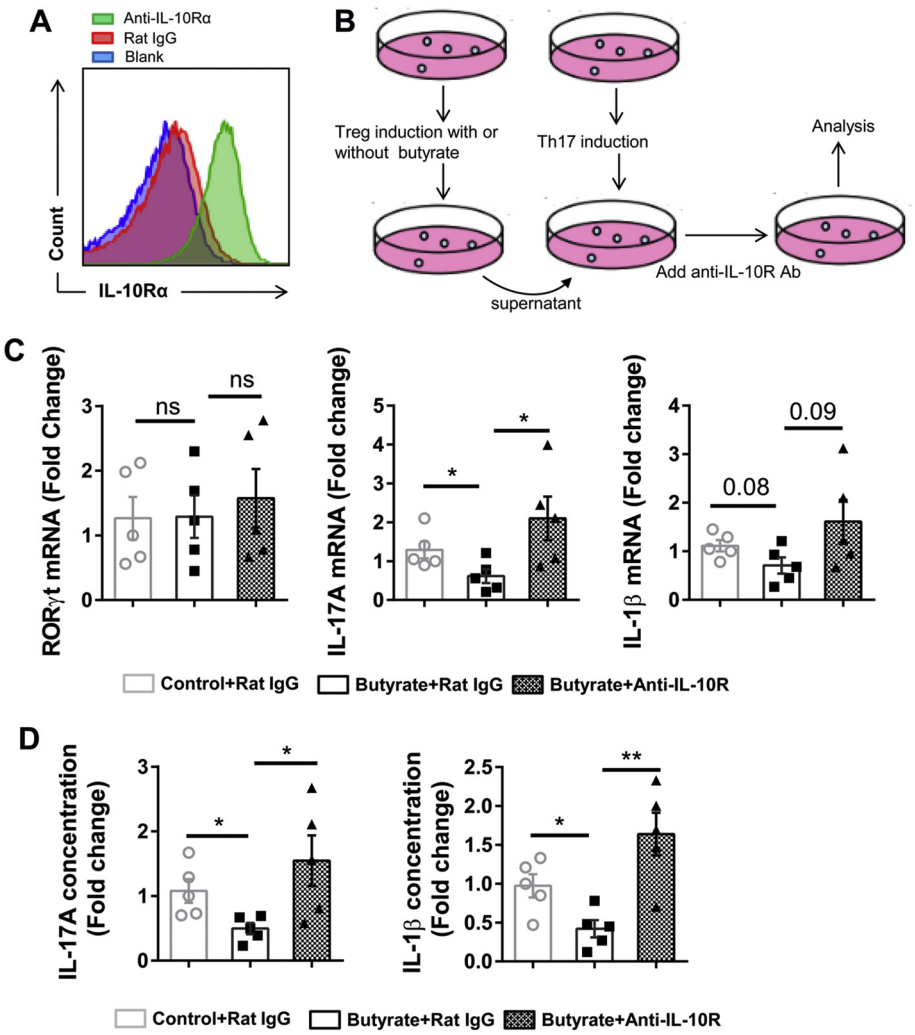

Fig. 5. Butyrate inhibited Th17 function in a Treg-IL-10-dependent manner. (**A**). IL-10Ra expression on Th17 cells (gating on $CD45^+CD4^+IL\text{-}17A^+$ cells) from synovial tissues of mice after collagen-induced arthritis were analyzed by FACS. (**B–D**). Th17 and Treg were induced in vitro respectively. Butyrate (0.2 mM) was added when Treg induction. The supernatant from Treg cells culture was added to Th17 cells with anti- IL-10R Ab (50 mg/mL) or isotype Rat IgG1,k for 3 days (**B**). The mRNA levels of RORγt, IL-17A and IL-1β in $CD4^+$ T cells were determined by Real-time PCR (**C**). The concentrations (relative to group of "Control + Rat IgG") of IL-17A and IL-1β in supernatant from each group were evaluated by ELISA **(D)**. Data are representative of three or more independent experiments. Data are the mean ± SEM. $*p < 0.05$, $**p < 0.01$, ns = not significant.

found that butyrate at 0.2 mM upregulated the mRNA expression levels of FoxP3 and IL-10 in cultures of stimulated $CD4^+$ T cells. Treg cells treated with butyrate presented higher expression of Foxp3 protein on cells than those from butyrate-free cultures, which is consistent with the Treg cells isolated from the spleen of butyrate-treated mice. We also detected the expression of IL17A and the specific transcription factor ROR- γ t for TH17, but did not see significant alteration between the butyrate treated and control cells. Consistently, FACS results showed no significant change of the protein expression of IL17A on $CD4^+$ T cells. These results indicated that butyrate directly promoted the polarization of Treg but not Th17 cells.

To this end, we observed that IL-10 concentration was significantly elevated in the serum of the butyrate treated CIA mice, as well as the upregulated mRNA level of IL-10 in the butyrate induced $CD4^+$ T cells. It has been known that $CD4^+$ T regulatory type 1 cells (TR1) secrete high levels of IL-10 and are known to play a major role in maintaining immune tolerance via their potent immune-regulatory activity [16]. Intriguingly, we also saw the upregulation of IL-10 mRNA in the synovial tissues of butyrate treated mice, thus we are fascinated to further explore the role of IL10 regulated by butyrate. As expected, we found anti-IL-10R Ab inversed the protection of butyrate on all of the detected indices of arthritis including histopathological characteristics, clinical score, paw swelling, as well as the concentrations of IL-1β, IL-6 and IL-17A in the mice serum. These results suggested butyrate inhibited collagen-induced arthritis through the regulation of IL-10.

We also saw the expression of IL-10 receptor on the surface of Th17 cells, and butyrate induced Treg cells significantly decreased the expression of IL-17A and IL-1β expression in Th17 cells, but did not change the expression of ROR-γt, indicating that could only regulate the function of Th17 cells, rather than regulate the polarization of Th17 cells. Notably, these effects of butyrate were forfeited by the addition of anti-IL-10R Ab. Taken together, we concluded the treatment of butyrate on naive T cells cultured under the Treg-cell-polarizing conditions promoted the IL-10 expression of Treg cells, and further inhibited the pro-inflammatory cytokines secreted by Th17 cells. Various studies have suggested that the IL-10 production by Treg cells could be of potential value in pharmaceutical intervention during inflammatory conditions [25]. However, this strategy has been hampered by the obstacles against obtaining homogeneous IL-10-producing T cells populations reproducibly. We believe that the direct promotion of butyrate on the IL-10-producing regulatory cells provides the foundation of the clinical usage of butyrate in the autoimmune inflammatory disease RA.

5. Conclusions

In conclusion, the present study showed that butyrate administration inhibited arthritis in CIA mice model, suppressed the expression of inflammatory cytokines. The modulation may be mediated the differentiation of CD4 T cells towards Treg cells, which produce anti-inflammatory cytokine IL-10, and thus influenced the function of Th17 cells.

Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

None.

References

[1] J.J. Cush, P.E. Lipsky, Phenotypic analysis of synovial tissue and peripheral blood lymphocytes isolated from patients with rheumatoid arthritis, Arthritis Rheum. 31 (1988) 1230–1238.
[2] M.R. Ehrenstein, J.G. Evans, A. Singh, S. Moore, G. Warnes, D.A. Isenberg, et al., Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF alpha therapy, J. Exp. Med. 200 (2004) 277–285.
[3] F. Flores-Borja, E.C. Jury, C. Mauri, M.R. Ehrenstein, Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 19396–19401.
[4] S. Sakaguchi, Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self, Nat. Immunol. 6 (2005) 345–352.
[5] N. Komatsu, H. Takayanagi, Chapter nine - regulatory T cells in arthritis, in: A. Liston (Ed.), Progress in Molecular Biology and Translational Science, Academic Press, 2015, pp. 207–215.
[6] J.J. Miner, L.E. Cook, J.P. Hong, A.M. Smith, J.M. Richner, R.M. Shimak, et al., Therapy with CTLA4-Ig and an antiviral monoclonal antibody controls chikungunya virus arthritis, Sci. Transl. Med. 9 (2017).
[7] A. Chopra, M. Saluja, A. Venugopalan, Effectiveness of chloroquine and inflammatory cytokine response in patients with early persistent musculoskeletal pain and arthritis following chikungunya virus infection, Arthritis Rheumatol. 66 (2014) 319–326.
[8] G. den Besten, K. van Eunen, A.K. Groen, K. Venema, D.J. Reijngoud, B.M. Bakker, The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism, J. Lipid Res. 54 (2013) 2325–2340.
[9] D.R. Donohoe, L.B. Collins, A. Wali, R. Bigler, W. Sun, S.J. Bultman, The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation, Mol. Cell 48 (2012) 612–626.
[10] H.J. Flint, K.P. Scott, P. Louis, S.H. Duncan, The role of the gut microbiota in nutrition and health, Nat. Rev. Gastroenterol. Hepatol. 9 (2012) 577–589.
[11] Y. Furusawa, Y. Obata, S. Fukuda, T.A. Endo, G. Nakato, D. Takahashi, et al., Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells, Nature 504 (2013) 446–450.
[12] L.Z. Shi, R. Wang, G. Huang, P. Vogel, G. Neale, D.R. Green, et al., HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells, J. Exp. Med. 208 (2011) 1367–1376.
[13] N. Arpaia, C. Campbell, X. Fan, S. Dikiy, J. van der Veeken, P. deRoos, et al., Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation, Nature 504 (2013) 451–455.
[14] N. Kong, Q. Lan, M. Chen, J. Wang, W. Shi, D.A. Horwitz, et al., Antigen-specific transforming growth factor beta-induced Treg cells, but not natural Treg cells, ameliorate autoimmune arthritis in mice by shifting the Th17/Treg cell balance from Th17 predominance to Treg cell predominance, Arthritis Rheum. 64 (2012) 2548–2558.
[15] M. Kespohl, N. Vachharajani, M. Luu, H. Harb, S. Pautz, S. Wolff, et al., The microbial metabolite butyrate induces expression of Th1-associated factors in CD4(+) T cells, Front. Immunol. 8 (2017) 1036.
[16] L. Brockmann, N. Gagliani, B. Steglich, A.D. Giannou, J. Kempski, P. Pelczar, et al., IL-10 receptor signaling is essential for TR1 cell function in vivo, J. Immunol. 198 (2017) 1130–1141.
[17] P. Toivanen, Normal intestinal microbiota in the aetiopathogenesis of rheumatoid arthritis, Ann. Rheum. Dis. 62 (2003) 807–811.
[18] R.S. Longman, D.R. Littman, The functional impact of the intestinal microbiome on mucosal immunity and systemic autoimmunity, Curr. Opin. Rheumatol. 27 (2015) 381–387.
[19] S.M. Vieira, O.E. Pagovich, M.A. Kriegel, Diet, microbiota and autoimmune diseases, Lupus 23 (2014) 518–526.
[20] A. Haghikia, S. Jorg, A. Duscha, J. Berg, A. Manzel, A. Waschbisch, et al., Dietary fatty acids directly impact central nervous system autoimmunity via the small intestine, Immunity 43 (2015) 817–829.
[21] J. Zhu, H. Yamane, W.E. Paul, Differentiation of effector CD4 T cell populations, Annu. Rev. Immunol. 28 (2010) 445–489.
[22] P.R. Mangan, L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, et al., Transforming growth factor-beta induces development of the T(H)17 lineage, Nature 441 (2006) 231–234.
[23] D.R. Littman, A.Y. Rudensky, Th17 and regulatory T cells in mediating and restraining inflammation, Cell 140 (2010) 845–858.
[24] D. Cao, V. Malmstrom, C. Baecher-Allan, D. Hafler, L. Klareskog, C. Trollmo, Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis, Eur. J. Immunol. 33 (2003) 215–223.
[25] K.W. Moore, R. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.