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Original Article

Novel benzenediamine derivative FC99 ameliorates zymosan-induced arthritis by inhibiting RORγt expression and Th17 cell differentiation

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Increased IL-17-producing helper T (Th17) cells have been observed in patients with rheumatoid arthritis (RA). The retinoic-acid-related orphan nuclear receptor (RORγt) is the master regulator of Th17 cells. Our previous research showed that FC99 possesses anti-inflammation activity. However, to date the effects of FC99 on RORγt expression in Th17 cell differentiation have not been investigated yet. In the present study, we found that FC99 significantly attenuated arthritis-like symptoms, i.e., suppressing the development of paw edema in zymosan-induced arthritis (ZIA) mice. H&E staining showed that the infiltration of inflammatory cells in ankle synovial tissues was significantly suppressed. FC99 also reduced the mRNA levels of pro-inflammatory cytokines in ankle synovial tissues as shown by Q-PCR analysis. The protein levels of the pro-inflammatory cytokines in sera were also suppressed after FC99 treatment. Moreover, FC99 decreased the RORγt mRNA level in spleen tissues. Th17 cell percentage was significantly decreased in spleens and draining lymph nodes (dLNs). The mRNA and protein levels of IL-17A and IL-23 were reduced after FC99 treatment in ZIA mice. Furthermore, *in vitro* experiments showed that FC99 inhibited the expression of IL-6 in LPS-induced RAW264.7 cells and BMDCs. Moreover, FC99 significantly inhibited the RORγt expression in PMA-induced CD4$^+$ T cells and LPS-induced RAW264.7 cells. These data indicate that FC99 improves arthritis-like pathological symptoms *in vivo* and *in vitro*, which might be related to the inhibition of RORγt expression in Th17 cells. Our findings suggest that FC99 may be a potential therapeutic candidate for the treatment of RA and other inflammatory disorders.

Keywords FC99; Th17 cells; IL-17; RORγt; rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA), the most frequent autoimmune chronic inflammatory disease of the joints, is characterized by the inflammation of synovial membrane, the infiltration of inflammatory cells, and the subsequent destruction of the joints [1–5]. Clinical data have shown that methotrexate, sulfasalazine, and glucocorticoids alleviate the symptoms of RA. The availability of medications targeted toward tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 has revolutionized the treatment of RA [6,7]. However, chronic administration of these agents is thought to directly associate with an increased risk of urinary tract and respiratory infections and pneumonia [6,7].

Accumulating evidence has shown that IL-17-producing helper T (Th17) cells play a pivotal role in the pathogenesis of RA [8]. Increased levels of IL-17 have been observed in patients with RA and IL-17 synergizes with other cytokines such as TNF-α and IL-6 to enhance the inflammatory response [9–11]. Moreover, the receptor for IL-17 was also abnormally over-expressed in peripheral blood and synovium of RA patients [12,13]. As it is well known, Th17 cells develop upon the influence of a unique cocktail of cytokines such as IL-6 and IL-23 as well as the coordinated activation of a complex network of transcription factors. IL-6 is an indispensable factor for generating Th17 cells from naïve T cells [14], while IL-23 facilitates the maintenance, expansion, and further differentiation of Th17 cells [15–18]. Recently, the therapeutic potential of anti-IL-17 therapy was evaluated in a Phase I study, and it was found that LY2439821, a potent anti-IL-17 antibody, indeed reduced the joint inflammation and erosion of RA patients [19].

Of note, retinoic-acid-related orphan nuclear receptor (RORγt) is the master regulator of Th17 cells [14]. Pharmacological inhibition of RORγt activity could offer a

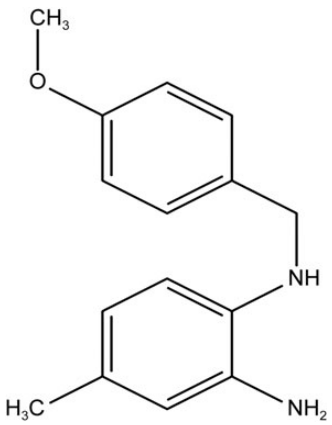

Figure 1. The chemical structure of FC99

potential therapeutic approach for the treatment of immune disorders including RA [19]. Our previous studies showed that Fumigaclavine C (FC), which was isolated from an endophytic fungus of a salinity-tolerant medicinal plant *Cynodon dactylon* (Gramineae), protected against liver injury, experimental colitis, and vasorelaxant effect [20,21]. Moreover, based on FC bioactivity backbone, a series of small-molecule FC derivatives were designed and synthesized. Strikingly, we found that FC99 (*N*1-[(4-methoxy)-methyl]-4-methyl-1,2-benzenediamine, **Fig. 1**), a novel benzenediamine derivative, possessed anti-inflammation activity and inhibited the activation of macrophage to attenuate experimental sepsis [22]. However, to date the effect of FC99 on RORγt expression in Th17 cell differentiation has not been investigated.

In the present study, we used zymosan-induced arthritis (ZIA) model mice and found that FC99 significantly attenuated arthritis-like symptoms, i.e., suppressing the development of paw edema, the infiltration of inflammatory cells, and the expression of inflammatory cytokines. Moreover, FC99 decreased the RORγt expression, Th17 cell percentage, and IL-17 level in ZIA mice. Furthermore, *in vitro* experiments showed that FC99 significantly inhibited the RORγt expression in $CD4^+$ T cells exposed to phorbol-12-myristate-13-acetate (PMA) stimulation. These data indicate that FC99 improves arthritis-like pathological symptoms in ZIA mice, which is related to the inhibition of RORγt expression in Th17 cells. Our findings suggest that FC99 may be a potential therapeutic candidate for the treatment of RA.

Materials and Methods

Animals

Male C57BL/6 mice (20−25 g) were obtained from Model Animal Research Center of Nanjing University (Nanjing, China) and kept under pathogen-free housing conditions in a 12-h light and 12-h dark cycle in accordance with institutional guidelines for animal care and use based on the Guide for the Animal Care Committee at Nanjing University. Mice were acclimated for a minimum of 2 weeks before experiments. All experiments were performed according to Chinese animal protection laws and had been approved by the local government authorities.

Induction of joint inflammation by zymosan

The zymosan-induced arthritis mouse model was set up as previously described [23]. In brief, 15 mg of zymosan (Sigma-Aldrich, St. Louis, USA) was dissolved in 1 ml of sterile phosphate buffer saline (PBS) and the solution was boiled twice before sonication. C57BL/6 mice were anesthetized by intra-peritoneal (i.p.) injection of 20 μl of 4% chloral hydrate (Sigma-Aldrich) diluted in sterile water and then injected with 10 μl zymosan solution into the right footpad (day 0).

FC99 treatment of the ZIA mice

FC99 was dissolved in dimethyl sulfoxide (Sigma-Aldrich) and diluted with saline to the indicated concentrations prior to use. C57BL/6 mice were randomly divided into the following three groups (eight mice per group): (i) normal mice (control group); (ii) ZIA model mice (vehicle-treated group); and (iii) ZIA mice with FC99 treatment (FC99-treated group). On day 0, mice in FC99-treated group started to receive FC99 treatment (100 mg/kg body weight) by i.p. injection twice every day. Mice in the vehicle-treated group received 10 μl PBS by i.p. injection daily. Mice in the control group were not treated. All mice were sacrificed by cervical dislocation on day 7.

Morphological and histopathological evaluation

Inflammation severity in mice was assessed daily by measuring paw thickness using a dial calliper (Shanghai Measuring and Cutting Tools Company Ltd., Shanghai, China). Development of arthritis was assessed on day 7 by blinded observers. The clinical severity of arthritis in each paw was quantified according to a graded scale from 0 to 4 as follows: 0, no swelling; 1, swelling in one digit or mild edema; 2, moderate swelling affecting several digits; 3, severe swelling affecting most digits; and 4, the most severe swelling.

For histopathological analysis, ankle segments were fixed in 10% buffered formalin phosphate, and paraffin-embedded sections (4 μm) were prepared for hematoxylin and eosin (H&E) staining (Beyotime, Shanghai, China). Histological scores were blindly determined and histopathological changes were scored using the standard described previously [24]. Inflammation was graded based on the index of inflammatory cells into synovium (infiltrate) and the joint cavity (exudate) with an arbitrary scale from 0 (no inflammation) to 3 (severe inflammation) [23].

Reverse transcription and real time quantitative PCR (Q-PCR)

Total RNAs were extracted from spleen and ankle synovial tissues using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Then, 1 μg of RNA was reverse-transcribed in a 20 μl system using a Revert Aid First-Strand cDNA Synthesis kit (Fermentas, Waltham, USA). Subsequently, 1 μl of complementary DNA was used as the template for the Q-PCR using SybrGreen PCR Master Mix (with Rox) (Invitrogen) and 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA). Each Q-PCR contained 0.1 mM of each primer, 5 μl of the SYBR Green PCR master mix (including AmpliTaq Gold DNA polymerase with buffer, dNTPs mix, SYBR Green I dye, ROX dye, and 10 mM $MgCl_2$), and 1 μl of the template cDNA in a 10-μl total reaction volume. The typical amplification program included activation of the enzyme at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, then annealing and extension at 60°C for 30 s. The C_t (cycle threshold) value for each gene was determined using the automated threshold analysis function of the ABI instrument. The relative expression levels of TNF-α, IL-6, IL-1β, IL-17A, IL-23, and RORγt against GAPDH were calculated as $2^{-\Delta\Delta Ct}$ according to the manufacturer's instructions. The primers for Q-PCR are listed in **Table 1**.

ELISA of serum cytokines

At the end of therapy, the blood sample was obtained from each mouse by tail vein, and the serum was collected and stored at −20°C. To quantify the cytokine levels in the serum of treated and control mice, enzyme-linked immunosorbent assay (ELISA) analyses for IL-6, IL-23, IL-17A, and TNF-α were performed using mouse IL-6 kit and TNF-α kit (Biolegend, San Diego, USA) as well as IL-23 kit and IL-17A kit (Dakewei, Shenzhen, China) according to the manufacturer's instructions. Absorbance was determined using an ELx-800 Universal Microplate Reader (BIO-TEK, Vermont, USA).

Table 1. Primers used for quantitative PCR in this study

Gene	Primer sequence
TNF-α	F primer: 5-CCACCACGCTCTTCTGTCTAC-3
	R primer: 5-AGGGTCTGGGCCATAGAACT-3
IL-6	F primer: 5-TGATGCACTTGCAGAAAACA-3
	R primer: 5-ACCAGAGGAAATTTTCAATAGGC-3
IL-1β	F primer: 5-AGACCCTTGCTTCCCTTCAT-3
	R primer: 5-AGGCGTGTCGCTCTCATACT-3
IL-17A	F primer: 5-TCAGCGTGTCCAAACACTGAG-3
	R primer: 5-CGCCAAGGGAGTTAAAGACTT-3
IL-23	F primer: 5-CAGCAGCTCTCTCGGAATCTC-3
	R primer: 5-TGGATACGGGGCACATTATTTTT-3
RORγt	F primer: 5-ACCTCCACTGCCAGCTGTGTGCTGTC-3
	R primer: 5-TCATTTCTGCACTTCTGCATGTAGACT GTCCC-3

Flow cytometry

Briefly, mouse spleen and draining lymph node (dLN) cells were isolated from mice and then passed through a 70-μm cell strainer, centrifuged, and resuspended in a red blood cell lysing buffer at 37°C for 2 min to lyse the red blood cells. For intracellular IL-17A staining [25], cells were incubated with 5 ng/ml PMA (Invitrogen) and 1 ng/ml ionomycin (Enzo Life Sciences, Inc.) for 5 h. After 30 min, brefeldin A (Enzo Life Sciences, Inc.) was added at a final concentration of 10 ng/ml. After washing, cells were stained with CD4-FITC (eBiosciences, San Diego, USA). After permeabilization of the cells with Cytofix/Cytoperm (BD Biosciences, San Diego, USA), cells were stained with IL-17A-PE (eBiosciences). For intracellular Foxp3-PE staining [26], cells were permeabilized with Cytofix/Cytoperm, and stained with Foxp3-PE (eBiosciences). After washing, cells were stained with CD4-FITC and CD25-APC (eBiosciences). After staining, cells were analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson, San Diego, USA) with the Flow Jo software.

Preparation of $CD4^+$ T cells

Spleen cells were isolated as described in the above section. $CD4^+$ T cells were further isolated at high purity from the spleen cells by a cell isolation kit (Miltenyi Biotec Technology & Trading, Shanghai, China), and resuspended in RPMI 1640 medium (Gibco Invitrogen, Paisley, UK) supplemented with 10% FBS (Gibco Invitrogen) (4×10^4 cells/100 μl). Cells were pretreated with 25, 50, or 100 μM FC99 for 2 h, and then stimulated with 1 μM PMA. The expression of RORγt in $CD4^+$ T cells was measured by Q-PCR.

Cell culture and treatment

The mouse macrophage cell lines RAW264.7 and EL-4 purchased from ATCC (American Type Culture Collection, Manassas, USA) were cultured in DMEM (Gibco Invitrogen) containing 10% heat-inactivated FBS. Primary mouse bone marrow-derived dendritic cells (BMDCs) were prepared as described before [27–29] and maintained in RPMI 1640 medium supplemented with 10% FBS.

Freshly isolated mouse BMDCs and RAW264.7 cells were suspended in RPMI 1640 medium. Cells were pretreated with 25, 50, or 100 μM FC99 for 2 h, and then stimulated with 100 ng/ml LPS (Sigma-Aldrich). The expression of IL-6 in both of them was examined by Q-PCR as described above. The expression of RORγt in RAW264.7 cells was also measured by Q-PCR.

The EL-4 cells were suspended in DMEM medium. Cells were pretreated with 25, 50, or 100 μM FC99 for 2 h, and then stimulated with 1 μM PMA. The expression of IL-17A was examined by Q-PCR.

Molecular docking modeling

The crystal structures of the protein complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The three-dimensional structure of FC99 was constructed using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2010)], and then was energetically minimized by using MMFF94 with 5000 iterations and minimum RMS gradient of 0.10. Molecular docking of FC99 into the RORγt protein complex structure (4NIE.pdb, downloaded from the PDB) was carried out using the Discovery Studio (version 3.5) as implemented through the graphical-user interface DS-CDOCKER protocol. All bound water molecules and ligands were eliminated from the protein and the polar hydrogen was added to the proteins.

Statistical analysis

Data were expressed as mean ± SEM. The statistical differences between two groups were performed using Student's *t*-test, and comparison among three groups was performed using one-way analysis of variance. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using commercially available statistical software GraphPad Prism (GraphPad, San Diego, USA).

Results

Effects of FC99 on arthritis onset in ZIA mice

To determine whether FC99 was efficacious in RA, ZIA mice were treated by i.p. injection with FC99 at a dose of 100 mg/kg body weight on day 0. During the experiment, the first sign of joint inflammation was typically evaluated by paw swelling on day 1. The results showed that the paws swelled and rapidly reached maximal diameter in vehicle-treated mice, while FC99 significantly diminished the paw swelling (**Fig. 2A**). Moreover, a marked presence of cell infiltration histopathologically lasted till day 7 in the paws of vehicle-treated mice, while the infiltration of lymphoid cells and cellular exudates was significantly suppressed in FC99-treated mice ($P < 0.05$) (**Fig. 2B**). These data suggest that FC99 may reduce the zymosan-induced articular inflammatory response.

Effects of FC99 on the expression of pro-inflammatory cytokines in ZIA mice

Inflammatory cytokines are thought to be high in the synovium of RA patients and experimental arthritis models. Herein, the expressions of TNF-α, IL-6, IL-17A, and IL-1β in ankle synovial tissues of treated mice were measured by Q-PCR. The results showed that FC99 significantly reduced the mRNA expressions of the pro-inflammatory cytokines, except IL-1β (**Fig. 3A**). Moreover, FC99 also significantly inhibited the protein levels of TNF-α, IL-6, and IL-17A in mouse sera (**Fig. 3B**). As it is well known, most of IL-17A is secreted by Th17 cells.

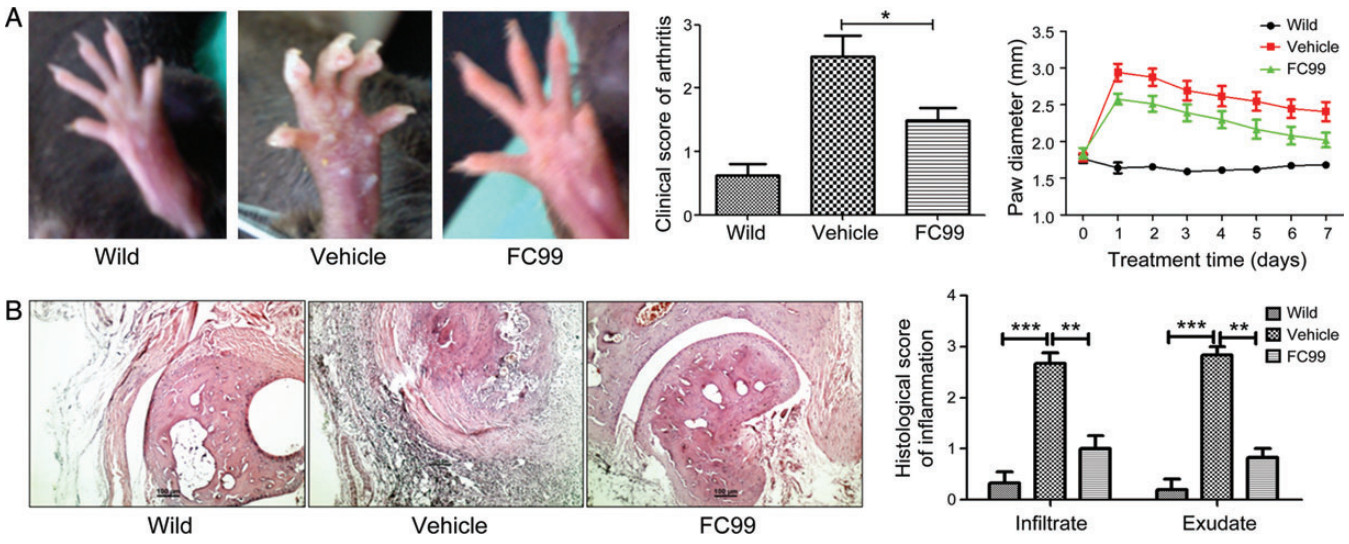

Figure 2. FC99 reduces zymosan-induced articular inflammatory response (A) Paw swelling in vehicle-treated mice and FC99-treated mice over time after injection. Paw diameters were averages of eight mice in each group. (B) Representative sections of the ankle joints from all mice groups and FC99 on day 7, stained with H&E (×4). Quantification of joint inflammation from mice of all groups. Data are expressed as mean ± SEM ($n = 8$ mice per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

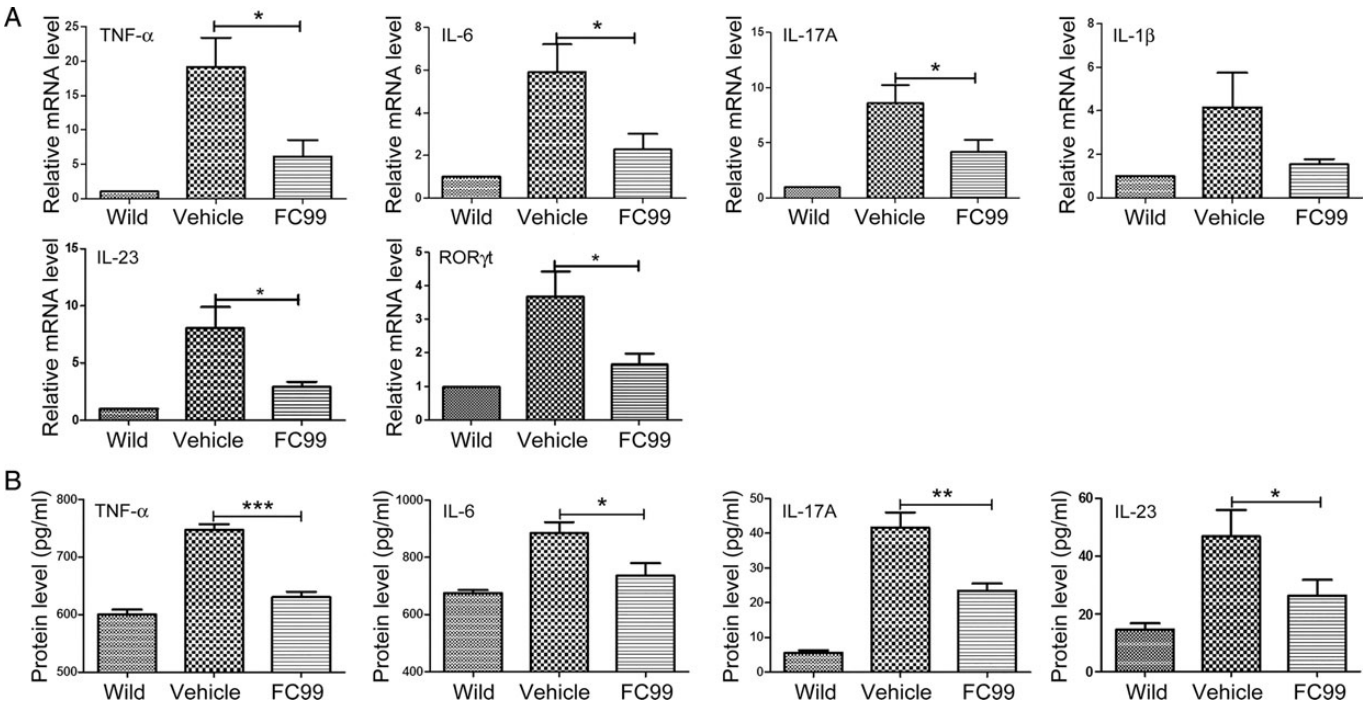

Figure 3. Effects of FC99 on the expression pro-inflammatory cytokines in ZIA mice (A) FC99 suppressed the cytokines generation in the ankle synovial extracts and spleen. Ankle synovial extracts mRNA expressions of IL-6, TNF-α, IL-17A and IL-1β in all mice groups were determined by Q-PCR. Spleen extracts mRNA expressions of IL-23 and RORγt in all mice groups were determined by Q-PCR. (B) The protein levels of IL-6, TNF-α, IL-17A, and IL-23 in the serum were also measured by ELISA. Data were expressed as mean ± SEM ($n = 8$ mice per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

IL-23 facilitates the maintenance, expansion, and further differentiation of Th17 cells. The expression of RORγt is required for survival of Th17 cells. Interestingly, FC99 suppressed the expression of IL-23 in the spleens (**Fig. 3A**) as well as the protein level of IL-23 in mouse sera (**Fig. 3B**). Moreover, FC99 also inhibited the mRNA expression of RORγt by Q-PCR in the mouse spleens (**Fig. 3A**).

FC99 modulates the balance of Th17 and Treg cells in ZIA mice

To analyze the effects of FC99 on T cells in ZIA model mice, the spleen and dLN cells of all mice were measured by flow cytometry. The results showed that FC99 decreased the percentage of $CD4^+IL\text{-}17^+$ T (Th17) cells compared with that from spleens ($P < 0.05$) and dLNs ($P < 0.01$) of vehicle-treated mice (**Fig. 4A**). Meanwhile, the $CD25^+$ $FOXP3^+$ regulatory $CD4^+$ T (Treg) cells were significantly increased by FC99 (**Fig. 4B**). These data indicate that FC99 may modulate the balance of Th17 and Treg cells.

FC99 inhibits the expression of pro-inflammatory cytokines in LPS-induced RAW264.7 cells and BMDCs

Macrophages and BMDCs can produce pro-inflammatory cytokines. When exposed to bacterial LPS (100 ng/ml) for 6 h, the expression of pro-inflammatory cytokine IL-6 was significantly increased in both RAW264.7 cells and BMDCs, while FC99 treatment significantly suppressed the IL-6 expression in a dose-dependent manner ($P < 0.001$) (**Fig. 5A,B**). This demonstrated the inhibitory effect of FC99 on inflammatory response. Given that IL-6 is elevated in RA patients and indispensable for the Th17 cell differentiation, we suppose that FC99 may relieve symptoms of RA.

FC99 inhibits IL-23 and RORγt expression related to Th17 cell differentiation

Furthermore, to verify the impact of FC99 on the expression of RORγt, the $CD4^+$ T cells were isolated and pretreated with different concentrations of FC99 for 2 h, and then incubated with PMA for 6 h. The expression of RORγt was examined by Q-PCR and the results showed that FC99 significantly reduced the PMA-induced RORγt expression ($P < 0.001$) in a dose-dependent manner (**Fig. 6A**, left). In addition, LPS can induce RORγt mRNA expression in macrophages [30]. We confirmed that FC99 also significantly down-regulated the RORγt mRNA expression in LPS-induced RAW264.7 cells (**Fig. 6A**, right). IL-17A mRNA expression was also suppressed in PMA-stimulated EL-4 cells in a dose-dependent manner (**Fig. 6B**).

Interaction between FC99 and RORγt

Now that FC99 was found to significantly decrease RORγt expression *in vivo* and *in vitro*, we deduce that FC99 should bind to the active center of RORγt. A docking study was performed by computer calculation, and the data were

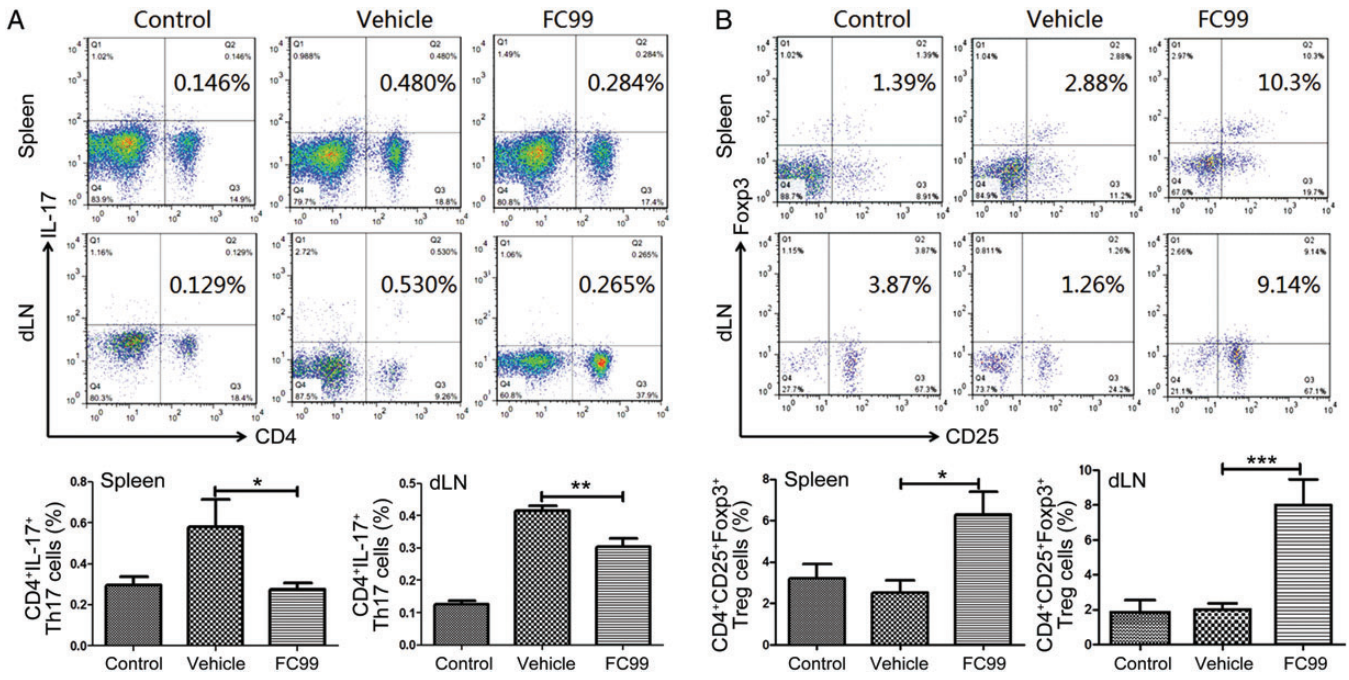

Figure 4. FC99 modulates the balance of Th17 and Treg cells (A,B) The percentage of Th17 and Treg cells in spleens and dLNs of mice was measured by flow cytometry. Data were expressed as mean ± SEM ($n = 8$ mice per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

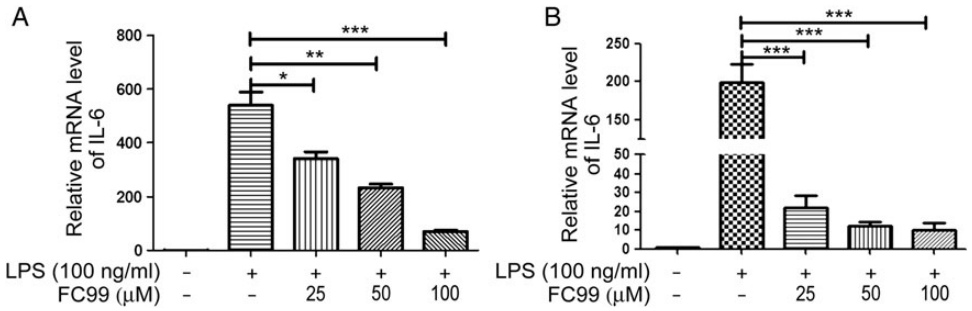

Figure 5. FC99 possesses anti-inflammation activity on RAW264.7 cells and BMDCs (A) RAW264.7 cells and (B) BMDCs were pretreated with 25, 50, and 100 μM FC99 for 2 h, and then stimulated with 100 ng/ml LPS for 6 h, the expression of pro-inflammatory cytokine IL-6 was measured by Q-PCR. Data were expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

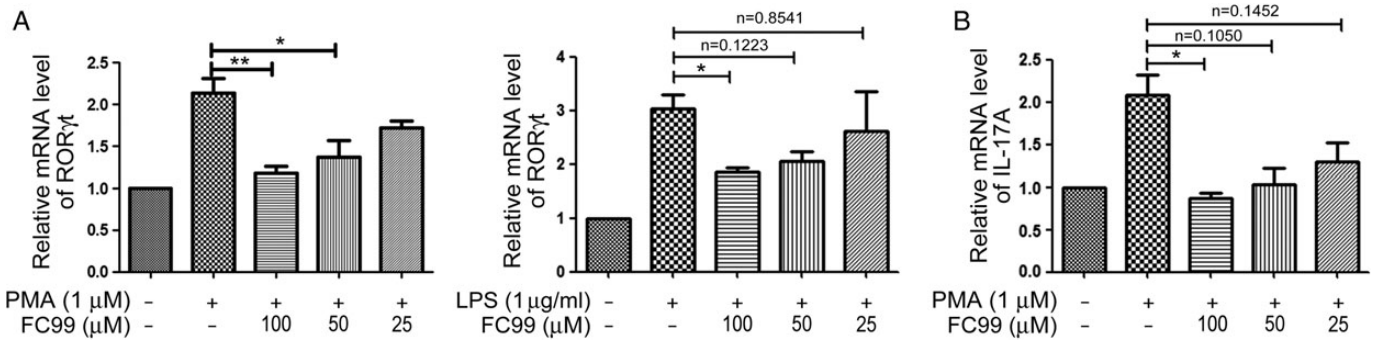

Figure 6. FC99 inhibits IL-23 and RORγt expression related to Th17 cell differentiation (A) The influence of FC99 treatment on the expression of RORγt in PMA activated $CD4^+$ T cells and LPS activated RAW264.7 cells. (B) The influence of FC99 treatment on the expression of IL-17A in PMA activated EL-4 cells. Data were expressed as mean ± SEM ($n = 8$ mice per group). *$P < 0.05$, **$P < 0.01$.

presented as supplemental data (**Supplementary Fig. S1**). The results demonstrated that FC99 could indeed bind to the active center of RORγt through certain chemical bonds.

Discussion

Pharmacologically targeting to RORγt for therapeutic intervention of an autoimmune disease has been reported before [31]. RORγt was also reported to be essential for the survival and differentiation of Th17 cells [32,33]. Th17 cells were shown to play a pivotal role in autoimmune diseases, including psoriasis, RA, multiple sclerosis, and inflammatory bowel disease [34,35]. Th17 cells secret the inflammatory cytokine IL-17, a cytokine that has been detected in the synovial fluid of RA patients. IL-17A acts in synergy with IL-1β and TNF-α to drive the inflammatory environment in joints of RA patients [36,37]. In the present study, we demonstrated that the novel small-molecule compound FC99 showed therapeutic efficacy in ZIA mice through inhibiting the expression of RORγt. FC99 strongly suppressed the development of paw swelling, inflammatory cell infiltration, and the expression of inflammatory cytokines, suggesting that FC99 can also significantly reduce joint inflammation and improve clinical scores.

Ordinarily, the differentiation of Th17 cells depends on a unique cocktail of cytokines such as IL-6 and IL-23 [14]. IL-6 is a necessary cytokine that generates Th17 cells from naïve T cells through the activation of RORγt [14]. IL-23 facilitates maintenance, expansion and further differentiation of Th17 cells [15]. In this study, FC99 was found to reduce the expression of pre-inflammatory cytokines IL-6 and IL-23. Moreover, FC99 could also significantly inhibit RORγt expression both *in vivo* and *in vitro* (**Fig. 6A**). RORγt is the master regulator of Th17 cells. When expression of RORγt is inhibited, Th17 cell differentiation will be suppressed.

In addition, the docking study demonstrated that there was mild action between FC99 and RORγt. This indicated that the differentiation of Th17 cells in FC99-treated mice was related to RORγt expression and FC99 appeared to drive an immunosuppressive environment in the ZIA mice. As we all know, the small-molecule compound may have many targets and the signaling of RORγt is very complex. So it is necessary for us to explore how FC99 inhibits the expression of RORγt in future studies.

In summary, in the present study we found that FC99 significantly attenuated arthritis-like symptoms. FC99 decreased the RORγt expression, Th17 cell percentage, and IL-17 level in ZIA mice. FC99 significantly inhibited the RORγt expression in $CD4^+$ T cells exposed to PMA stimulation. These data indicate that FC99 improves arthritis-like pathological symptoms in ZIA mice, which is related to the inhibition of RORγt expression in Th17 cells. Our findings suggest that FC99 may be a potential therapeutic candidate for the treatment of RA.

Supplementary Data

Supplementary data is available at *ABBS* online.

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