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Ph.D Thesis

Elucidation of the therapeutic effect of HDAC-selective inhibitors on collagen-induced arthritis

(Abridged Version)

コラーゲン誘導性関節炎における HDAC アイソザイム選択的阻害剤の治療効果の解明

〈要約版〉

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Abbreviation List

RA	Rheumatoid arthritis	IL	interleukin
Treg	regulatory T cells	H&E	ematoxylin-eosin
Tfr	follicular regulatory T	BSA	bovine serum albumin
Tfh	follicular helper T	DMSO	dimethyl sulfoxide
Bcl-6	B-cell lymphoma 6	LPS	lipopolysaccharide
CXCR5	C-X-C chemokine receptor type 5	JAK	Janus kinases
GC	germinal center	Foxp3	forkhead box P3
PBS	Phosphate-buffered saline		
mAb	monoclonal antibody		
PPs	Peyer's patches		
mLNs	mesenteric lymph nodes		
AxLNs	axillary lymph nodes		
InLNs	inguinal lymph nodes		
PD-1	programmed death receptor-1		
ICOS	inducible costimulatory molecule		
CTLA4	cytotoxic T-lymphocyte antigen-4		
GITR	glucocorticoid-induced TNF receptor		
Blimp-1	B-lymphocyte-induced maturation protein 1		
Gata3	GATA Binding Protein 3		
SCFAs	short-chain fatty acids		
mLNs	mesenteric lymph nodes		
TNF-α	tumor necrosis factor-α		
OPD	o-phenylenediamine dihydrochloride		
CIA	collagen-induced arthritis		
CAIA	collagen antibody-induced arthritis		
TRAP	Tartrate-resistant acid phosphatase		
M-CSF	macrophage colony-stimulating factor		
RANKL	receptor activator of nuclear factor kappa-B ligand		
BMDMs	bone marrow-derived macrophages		
DMARDs	disease-modifying antirheumatic drugs		
HDACs	Histone deacetylases		

Part I

Screening and studying of compounds promoting Tfr-cell differentiation

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that affects about 1% of the global population and can occur at any age. Its cause is unknown, but it is thought to be triggered by an infectious disease or environmental exposure. RA is characterized by inflammation of the synovium, neovascularization, and massive local T-cell infiltration, which leads to joint deformity and loss of function, as well as damage to cartilage structures and breakdown of the joint capsule [1]. This results in a high disability rate. The pathogenesis of RA and its treatment is still being researched. In recent decades, there have been significant advances in the understanding of the pathogenesis of RA, leading to the identification of new therapeutic targets [2].

Recently, a subset of regulatory T (Treg) cells called follicular regulatory T (Tfr) cells has been identified in lymphoid tissues [3]. Tfr cells differentiated from Treg cells and have similar characteristics to both Treg cells and follicular helper T (Tfh) cells, including high expression of a transcription factor, B-cell lymphoma 6 (Bcl-6) and C-X-C chemokine receptor type 5 (CXCR5) [4]. Tfr cells play a crucial role in preventing autoimmune diseases caused by the production of self-reactive antibodies (autoantibodies). Tfr cells have been found to regulate the differentiation of Tfh and B cells in the germinal center (GC) of secondary lymphoid organs, regulating in the selection of high-affinity antibodies, and preventing autoantibody production [5-8]. Because of the antiautoimmue property, we considered that understanding the mechanisms of Tfr-cell differentiation and increasing the number of this cell population would lead to a new approach to the prevention and treatment of various autoimmune diseases caused by autoantibody production [9].

However, even after a decade of Tfr-cell discovery, the process of their differentiation is still not fully understood. Clarifying the details of Tfr-cell differentiation is crucial for (1) understanding the regulatory mechanisms of autoantibody production and (2) developing therapeutic agents targeting Tfr cells for the treatment of autoimmune diseases. In this study, I have used a newly developed *in vitro* Tfr-cell differentiation culture to evaluate a library of hundreds of compounds. By using a chemical biology approach, I aimed to identify the molecules involved in Tfr-cell differentiation and function. A list of compounds that can induce Tfr cells has been compiled, and their therapeutic effects have been tested in mouse models of RA.

2. Material and Methods

2.1 Mice

Six- to seven-week-old C57BL/6J Jcl mice were purchased from Clea Japan (Tokyo, Japan). Foxp3^{hCD2}Bcl-6-tdTomato mice were bred at the animal facility of Keio University Faculty of Pharmacy. All mice were treated under protocols approved by the Animal Studies Committees of at Keio University Faculty of Pharmacy (Animal Experiment Approval Number A2022-201). All mice were fed *ad libitum* a purified diet based on the composition of CE-2 (Clea Japan).

2.2 Compound library sources

- Intestinal microbiota metabolites: Kindly provided by Kyodo Milk Industry
- SCADS kits: Kindly provided by SCADS Inhibitor Kit, Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

2.3 Induction of Tfr-cell differentiation

2.3.1 Naïve CD4$^+$ T cells isolation

Naïve CD4$^+$ T cells were isolated using the MojoSort mouse CD4 T cell isolation kit (BioLegend, San Diego, CA, USA) with the following protocol. Spleen tissue was collected from the Foxp3^{hCD2} Bcl-6-tdTomato mouse, and the adipose tissue attached to the spleen was removed. The spleen was placed on a 100 μm cell filter (Greiner Bio-One, Tokyo, Japan) and ground with a syringe plunger. The cells were suspended in a 2E2P buffer. The cells were then centrifuged at 4°C for 10 min at 300 × *g*, resuspended in 4 ml MojoSort buffer, and filtered through a 100 μm cell filter into new tubes. The cells

were counted, and adjusted cell concentration to 1 x 10^8 cells/ml. After adding the biotin antibody cocktail according to a cell suspension: cocktail = 10:1 ratio, the liquid was mixed well and incubated on ice for 15 min. Streptavidin nanobeads were added to cell suspension: nanobeads = 10:1 ratio; the liquid was mixed well and incubated on ice for 15 minutes. The cell suspension was adjusted to 3 ml/tube by adding MojoSort buffer, and the tube was placed in a magnet for 5 minutes. The liquid was poured out and collated, and repeated the magnet collection step with labeled cells for a total of 2 separations. All the collected liquid was then centrifuged at 4°C for 10 min at 300 × *g* and used for cell culture.

2.3.2 Cell culture

Naïve $CD4^+$ T cells from normal $Foxp3^{hCD2}$ Bcl-6-tdTomato mice spleen were isolated according to the previous description. They were seeded on a high-binding 96-well plate (Corning, Inc., NY, USA) at a concentration of 1 × 10^5 cells/well, which was incubated with 5 μg/ml anti-TCRβ mAb overnight in the 4°C refrigerators in advance. The anti-CD28 antibody, IL-2, and TGF-β1 were also added by dilution to RPMI 1640 medium containing 5% FBS (MP Bio, New York, NY, USA) at final concentrations of 2 μg/ml, 10 ng/ml, and 0.1 ng/ml, respectively. Cells were incubated in a CO_2 incubator at 37°C for 2 days. Afterward, cells were washed, and 1/4 volume of each well was resuspended in a mixture of rmIL-2, rmIL-21, rmIL-6, anti-IL-4, anti-IFN-γ and anti-ICOS antibodies that were diluted in high-level RPMI 1640 medium containing 5% FBS at final concentrations of 2 ng/ml, 25 ng/ml, 25 ng/ml, 10 μg/ ml, 10 μg/ml, and 5 μg/ml. The cell suspension is transferred to a new 96-well flat-bottom plate with the test compounds to be assayed and incubated in a CO_2 incubator at 37°C for 3 days.

Antibody Name	Clone	Manufacturer
*InVivo*MAb anti-mouse TCRβ	H57-597	Bio X Cell, Lebanon, NH, USA
*InVivo*MAb anti-mouse CD28	37.51	Bio X Cell
*InVivo*MAb anti-mouse IL-4	11B11	Bio X Cell
*InVivo*MAb anti-mouse IFNγ	R4-6A2	Bio X Cell
*InVivo*MAb anti-mouse IL-2	11B11	Bio X Cell
LEAF Purified anti-human/mouse/rat CD278 (ICOS) antibody	C398.4A	BioLegend
recombinant human (rh) TGF-β1	-	BioLegend
recombinant mouse (rm) IL-2	-	BioLegend
recombinant mouse (rm) IL-6	-	BioLegend
recombinant mouse (rm) IL-21	-	BioLegend

2.3.3 Flow cytometry

Cells obtained from 2.3.1 were transferred to 96-well V-bottom plates, centrifuged at 300 × *g* for 5 min at 4°C, resuspended in a monoclonal antibody (mAb) against CD16/32 (93; BioLegend) dilution in Phosphate-buffered saline (PBS) containing 2% FBS and 0.1% NaN_3, and blocked on ice for 15 min. After centrifugation and washing twice, 50 μl of the extracellular antibody mixture from the table below (1) is added and allowed to react for 30 min at 4°C in the refrigerator. After two centrifugations and washes, cells were resuspended in PBS containing 2% FBS and 0.1% NaN_3 and analyzed with a FACSAria III (BD Biosciences, San Jose, CA, USA). The data obtained were analyzed using FlowJo 10 software (BD Biosciences).

(1) Antibody mixture for extracellular use

Reagent	Clone	Manufacturer	Dilution
BV786 Rat Anti-Mouse CD4	RM4-5	BD Biosciences	1:200
redFluor™ 710 Anti-Mouse CD45	30-F11	Tonbo Biosciences	1:200

Brilliant Violet 711™ anti-mouse TCR β	H57-597	BioLegend	1:200
Brilliant Violet 650 anti-mouse CD25	PC61	BioLegend	1:200
Brilliant Violet 421 anti-human CD2	TS1/8	BioLegend	1:200
FITC anti-mouse CD279 (PD-1)	29F.1A12	BioLegend	1:50
APC anti-mouse CD185 (CXCR5)	L138D7	BioLegend	1:50
tdTomato	-	-	-
7-aminoactinomycin D (7-AAD) Viability Staining Solution	-	BioLegend	1:200

2.3.4 Cell culture media and buffers

・5R medium

Name	Manufacturer	Final Conc.
Fetal Bovine Serum (Heat inactivated)	MP Bio	5%
1 mol/L hydroxyethyl piperazine ethanesulfonic acid (HEPES) buffer solution	nacalai tesque, Kyoto, Japan	0.01 mol/L
Penicillin-Streptomycin mixed solution	nacalai tesque	100 μg/ml penicillin 100 μg/ml streptomycin
Roswell Park Memorial Institute 1640	Sigma-Aldrich	93%

・2E2P buffer

Name	Manufacturer	Final Conc.
D-PBS (-)	nacalai tesque	96%
Fetal Bovine Serum (Heat inactivated)	MP Bio	2%
1 mol/L HEPES Buffer Solution	nacalai tesque	0.01 mol/L
0.5 mol/L ethylenediaminetetraacetic acid (EDTA)	nacalai tesque	2 mmol/L
Penicillin-Streptomycin mixed solution	nacalai tesque	100 μg/ml penicillin 100 μg/ml streptomycin

・N2P buffer

Name	Manufacturer	Final Conc.
D-PBS (-)	nacalai tesque	96%
Fetal Bovine Serum (Heat inactivated)	MP Bio	2%
1 mol/L HEPES Buffer Solution	nacalai tesque	0.01 mol/L
10% NaN3	FUJIFILM Wako Pure Chemicals	0.1%

・2R medium

Name	Manufacturer	Final Conc.
Roswell Park Memorial Institute 1640 (RPMI)	nacalai tesque	96%
Fetal Bovine Serum (Heat inactivated)	MP Bio	2%
1 mol/L HEPES Buffer Solution	nacalai tesque	0.01 mol/L
Penicillin-Streptomycin mixed solution	nacalai tesque	100 µg/ml penicillin 100 µg/ml streptomycin

2.4 Autoantigen Immunization model

2.4.1 Immunization protocol

Human insulin powder (FUJIFILM Wako Pure Chemicals, potency 27.5 U/mg or higher) was dissolved to 45 U/ml in D-PBS (nacalai tesque) and insulin was inactivated by standing in a warm bath at 60°C for 1 hour. The dissolved insulin was stored at -20°C. An equal volume of aliquots of human insulin and 2% Alhydrogel adjuvant (Aluminium hydroxide gel, InvivoGen, San Diego, CA, USA) were mixed by pipetting for 5 minutes on inc. The emulsion was injected subcutaneously into the right dorsal surface of male C57BL/6J Jcl mice for immunization at an insulin dose of 4.5 U per mouse.

2.4.2 Administration method

Six- to seven-week-old male C57BL/6J Jcl mice were randomly divided into 4 groups, each consisting of six mice. The groups were as follows: control PBS group, putrescine

group (Tokyo Chemical Industry, Tokyo, Japan), and cadaverine group (Tokyo Chemical Industry) on day 0. Putrescine and cadaverine were dissolved in sterile PBS at a concentration of 5 mg/ml. The polyamines were orally administered via gavage at a dose of 10 ml/kg for 28 days. All mice were immunized with insulin as the previous method on day 18 and dissected on day 28.

2.4.3 Cell isolation

Spleen was obtained from Foxp3hCD2 Bcl-6-tdTomato mice, placed on a 100 μm cell filter (Greiner Bio-One), ground with a syringe plunger, and suspended in RPMI 1640 medium containing 2% FBS, then centrifuged at 280 × *g* for 10 min at 4°C in 1× erythrocyte lysis buffer (BioLegend). The cells were then centrifuged at 300 × *g* for 10 min at 4°C, resuspended in 1× Red Blood Cell Lysis Buffer (BioLegend), placed on ice for 5 min to lyse red blood cells, added PBS containing 2% FBS and 2 mM EDTA, and passed through a 100 μm cell filter to remove cell clumps. The cells were resuspended in PBS containing 2% FBS and 2 mM EDTA by centrifugation at 280 × *g* for 10 min at 4°C.

Peyer's patches (PPs), mesenteric lymph nodes (mLNs), axillary lymph nodes (AxLNs), and inguinal lymph nodes (InLNs) were removed from mice, placed on 100 μm cell filters (Sysmex, Kobe, Japan), ground with a syringe plunger, and suspended in RPMI 1640 medium containing 2% FBS. The obtained cells were then resuspended in PBS containing 2% FBS and 2 mM EDTA by centrifugation at 280 × *g* at 4°C for 10 min.

2.4.4 Blood collection

Blood was collected from the heart with a heparinized syringe under anesthesia with isoflurane (Pfizer, New York, NY, USA) during the dissection of mice. Blood samples were centrifuged at 1000 × *g* for 10 min at room temperature, and the supernatant was

collected to obtain a plasma sample. Serum samples were collected by this procedure. Prepared samples were stored in a freezer at -80°C prior to measurement.

2.4.5 Flow cytometry

Cells obtained from 2.4.3 and 2.4.4 were transferred to 96-well V-bottom plates and centrifuged at 300 × *g* for 5 min at 4°C. For cell counting, 200 µl of adjusted cell suspension was mixed with 200 µl of the cell counting mixture in the table below (3), and the number of $CD45^+$ 7-AAD^- cells was calculated by the ratio of the number of $CD45^+$ 7-AAD^- cells to the number of precision counting beads. For cell staining, cells were resuspended in a monoclonal antibody (mAb) against CD16/32 (93; BioLegend) dilution in PBS containing 2% FBS and 0.1% NaN_3, and blocked on ice for 15 min. After centrifugation and washing twice, 50 µl fluorescently labeled surface staining antibodies were added according to the table below and incubated for 30 min at 4°C in the refrigerator. After centrifugation and washing twice, the cells were fixed. For fixation, the cells were incubated for 45 min at room temperature using a transcription factor buffer set (BD Biosciences) according to the instructions provided. After three centrifugation washes with permeabilization buffer (eBioscience, Waltham, MA, USA), intracellular transcription factors were stained by adding fluorescently labeled staining antibodies according to the table below and incubating for 45 minutes at room temperature protected from light. After centrifugations and washes twice, cells were resuspended in PBS containing 2% FBS and 0.1% NaN_3 and analyzed with FACS LSR II or FACS Celesta (BD Biosciences). The data obtained were analyzed using FlowJo 10 software (BD Biosciences).

(1) Antibody mixture for extracellular use

Antibody and Reagent	Clone	Manufacturer	Dilution

BV786 Rat Anti-Mouse CD4	RM4-5	BD Biosciences	1:200
BV650 Rat Anti-Mouse CD25	PC61	BioLegend	1:100
BV605 Hamster Anti-Mouse TCR β chain	H57-597	BioLegend	1:200
BV510 Rat Anti-Mouse CD45	30-F11	BioLegend	1:200
PB Rat Anti-Mouse GL7	GL7	BioLegend	1:200
Brilliant Violet 421 anti-human CD2	TS1/8	BioLegend	1:200
PerCP-eF710 Hamster Anti-Mouse CD279 (PD-1)	J43	BioLegend	1:100
APC Rat Anti-Mouse CD185 (CXCR5)	L138D7	BioLegend	1:25
APC-R700 Rat Anti-Mouse CD19	1D3	BioLegend	1:200
APC-Cy7-conjugated anti- Fixable Viability Stain (FVS) 780	-	Tonbo Biosciences	1:200

(2) Antibody mixture for intracellular use

Reagent	Clone	Manufacturer	Dilution
PE-CF594 Hamster Anti-Mouse Bcl-6	K112-91	eBioscience	1:25
PE Rat Anti-Mouse Foxp3	MF-14	BioLegend	1:50

(3) Mixture for cell counting

Reagent	Clone	Manufacturer	/sample
Precision Count Beads	-	Biolegend	50 µl
eFluor 450 anti-mouse CD45	30-F11	eBioscience	0.4 µl
TruStain Fcx™ (anti-mouse CD16/32) antibody	93	Biolegend	2.0 µl
7-AAD Viability Staining Solution	-	Biolegend	2.0 µl
D-PBS containing 2% NBCS	-	-	145.6 µl

(4) Cell fixation

Fix/Perm working solution: Fixation/Permeabilization concentrate: Diluent buffer= 1: 3

Perm/Wash working solution: Perm/Wash buffer: Distilled water = 1: 4

2.5 Statistical analyses

Statistical analysis was performed using GraphPad Prism (version 19.0). All data were expressed as mean ± standard error of the mean (SEM). Repeated-measures two-way ANOVA or one-way ANOVA was used for comparison between multiple means. The statistical significance of differences between groups was determined using an unpaired two-tailed Student's t-test or Welch's t-test. The data were considered statistically significant when $p < 0.05$.

3. Results

3.1 Screening results on the effect of intestinal microbiota metabolites in Tfr-cell differentiation

Tfr cells are a newly identified subpopulation of Treg cells that are characterized by the expression of markers such as CXCR5, Bcl6, programmed death receptor-1 (PD-1), and inducible costimulatory molecule (ICOS), as well as the Treg cell markers cytotoxic T-lymphocyte antigen-4 (CTLA4), glucocorticoid-induced TNF receptor (GITR), and forkhead box P3 (Foxp3) [4]. $CD25^+$ Tregs can upregulate CXCR5 and PD-1 during an immune response by decreasing CD25 expression and increasing B-lymphocyte-induced maturation protein 1 (Blimp-1) expression. This process is transcriptionally regulated by Bcl-6, which eventually leads to the differentiation and development of Tfr cells [10]. Bcl-6, a transcriptional repressor, plays a central role in immune regulation and is essential for the GC response by controlling the differentiation of Tfr cells, Tfh cells, and GC B cells. In the absence of Bcl-6, the increased expression of transcription factors such as GATA Binding Protein 3 (Gata3) and Blimp1 in Tregs can promote the development of Th2 cells and ultimately lead to increased immune/hyperimmune responses in the organism [11]. Therefore, I have defined $Foxp3^+$ $CD25^-$ $PD\text{-}1^+$ $CXCR5^+$ $Bcl6^+$ cells as Tfr cells and based on changes in CXCR5 and Bcl6 as indicators of the effect of compounds on Tfr-cell differentiation.

Numerous studies have demonstrated a relationship between the number of intestinal microbiota and the development of the intestinal immune system, with intestinal microorganisms influencing the development of inflammatory responses, including autoimmune diseases and allergies. For example, short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate are by-products of dietary fiber fermentation and are among the most representative metabolites of the microbiota [12]. Previous research has shown that SCFAs can modulate host intestinal immunity and reduce the colonic

inflammatory response by increasing intestinal epithelial cell integrity, specific antibody responses, and colonic Treg numbers [13, 14]. Through the G protein-coupled receptor GPR43, SCFAs upregulate Foxp3 and IL-10 gene expression in Treg cells, thereby suppressing effector $CD4^+$ T (Teff) cells and alleviating autoimmune disease [15]. Based on these findings, I speculate that intestinal microbiota metabolites may have a similar effect on Tfr cells.

In the *in vitro* screening system using mouse splenocytes described in Material and Methods 2.3, I tested the effects of 47 compounds from the intestinal content and a positive control drug on Tfr-cell differentiation. The results showed that γ-Aminobutyric acid, putrescine, cadaverine, and 3-phenylpropionic acid all had significant effects in promoting Tfr-cell differentiation.

I then further screened the four compounds that showed the greatest potential. Following the previous *in vitro* screening system, different concentrations of compounds were added. Putrescine and cadaverine not only exhibited the best effect in promoting cell differentiation, but also had a clear dose-dependent relationship. Based on these results, two polyamines, putrescine and cadaverine were selected for further *in vitro* and *in vivo* experiments to determine their potential for promoting Tfr-cell differentiation.

3.2 Screening results of SCADS inhibitor kits compounds on Tfr-cell differentiation

The SCADS inhibitor suite consists of a small molecule compound that has a clear target molecule, is well established as an inhibitor in current science. When a small compound inhibitor affects a specific phenotype in an experiment, it is a sign that the target is related to that phenotype. Using such small compound inhibitors can reveal the mechanisms behind unknown phenotypes and may also lead to the development of new targeted drugs for phenotype-related diseases. I tested the effect of the inhibitors in the SCADS kit on the Tfr-cell differentiation process by screening approximately 400 compounds contained in SCADS.

Due to limited experimental capacity, it was not possible to test all 400 compounds at once. Therefore, the screening experiments were conducted in batches. However, the results of different batches could not be directly compared due to the influence of cell status. To address this issue, the index of $Bcl6^{+}CXCR5^{+}$ cells as Tfr cells was calculated as a relative value of compounds promoting Tfr-cell differentiation using each Tfr control group as a baseline. The results of the experiments showed that three compounds - the telomerase inhibitor TMPyP4, the IGF-1R inhibitor OSI-906, and the HDAC inhibitor vorinostat - had a positive effect on Tfr-cell differentiation. Among these compounds, the HDAC inhibitor vorinostat had the best results.

Previous research, not just in our laboratory, has demonstrated that butyrate, a pan-HDAC inhibitor, has a positive therapeutic effect in models of rheumatoid arthritis [9, 16]. However, its adverse effects are significant, limiting its clinical use [17-19]. As a result, specific and selective HDAC inhibitors, which can be administered at lower concentrations, may produce more favorable outcomes and reduce side effects for the treatment of chronic conditions. Thus, I have chosen to utilize an HDAC1 selective inhibitor, TTA03-107, synthesized and provided by Prof. Suzuki at Osaka University in my next *in vivo* experiment series in part II.

3.3 Efficacy of polyamines in mouse insulin immunization model

The aim of this experiment was to investigate the effects of putrescine and cadaverine on Tfr cells in mice through oral administration. Previous research in our laboratory indicated that Tfr cell numbers in mice peak 10 days after immunization with insulin as an autoantigen. Therefore, in this study, mice were immunized with a subcutaneous injection of insulin after 18 days of continuous administration. 10 days after immunization, the mice were dissected to analyze the number and percentage of Tfr and Tfh cells in the spleen and various lymph nodes.

The results showed that mice treated with 50 mg/kg polyamines for 4 weeks had a lower body weight gain rate compared to the control group treated with PBS. This result implies that long-term and high doses of polyamines may have a negative impact on mouse health. To analyze the effects of cadaverine and putrescine on Tfr-cell differentiation *in vivo*, spleen, Peyer's patches, mesenteric lymph nodes, axillary lymph nodes, and inguinal lymph nodes of mice were collected. The cells in the axillary and inguinal lymph nodes were grouped together due to their low cell numbers. The results showed that the two polyamines did not significantly affect the number of $CD45^+$ cells in the lymphoid tissues compared to the control group. Only the putrescine-treated mice showed a significant increase in $CD45^+$ cells in Peyer's patches. These data suggest that putrescine might only have an effect on the intestine.

The frequency and number of Tfr cells were then observed. The results showed that the frequency and number of Tfr cells in the polyamine-treated mice were comparable among the three groups. However, there was a significant increase in the number of Tfr cells in the Peyer's patches of the putrescine group, due to the higher total number of $CD45^+$ cells. The changes in Bcl-6 expression in Tfr cells were also examined, with similar results. Putrescine and cadaverine did not increase the frequency and number of $Bcl\text{-}6^+$ Tfr cells, except in Peyer's patches. These results suggest that polyamines have no effect on Tfr-cell differentiation *in vivo* despite their promoting effect on Tfr-cell differentiation *in vitro*.

4. Discussion

In this study, I screened compounds for the Tfr cell-inducing effect of microbiota-derived metabolites and SCADS kits inhibitors. Among the microbiota-derived metabolites, cadaverine and putrescine, both polyamines, demonstrated the most pronounced effect. Previous research has shown that spermidine, another polyamine, can enhance the differentiation of naive T cells to Treg cells in mice and humans, leading to reduced pathology in a mouse model of colitis [20-22]. However, there has been no previous research on the effects of polyamines on Tfr cells. These results show that polyamines can also promote the differentiation of Treg cells to Tfr cells *in vitro*. Contrary to the *in vitro* results, further *in vivo* experiments in mice showed that cadaverine and putrescine have no ability to promote Tfr-cell differentiation. This may be due to the lower plasma concentration of polyamines, as *in vitro* experiments showed that cadaverine and putrescine only promote Tfr-cell differentiation at concentrations higher than 1 mM, which is not achievable in the plasma.

The SCADS inhibitor kits contain a range of inhibitors for small molecule compounds. By examining the effects of these inhibitors on Tfr-cell differentiation, it is possible to infer the molecular mechanisms involved in this process. In my experiments, three targets were found to be particularly effective in promoting Tfr differentiation: telomerase, IGF-1R, and HDAC. Telomerase is an enzyme that lengthens telomeres, the protective caps on the ends of eukaryotic chromosomes, through the addition of telomeric DNA. By maintaining telomere length, telomerase allows cells to divide more times before telomeres become depleted, thereby increasing the lifespan of cells [23]. Telomerase has been shown to have a strong positive correlation with activation-induced cell proliferation and survival [24]. However, T-cell differentiation has a negative correlation with proliferative capacity, with T-cell differentiation resulting in a decrease in proliferative capacity [25-27]. This suggests that telomerase inhibitors may enhance T-cell differentiation by inhibiting T cell proliferation, although more research is needed to

confirm this. IGF-1R is a cell surface receptor for the hormone IGF-1, which is a growth-promoting factor that is very close in structure to insulin and plays a role in regulating cell proliferation, transformation, and differentiation. Previous studies have shown that IGF-1 or IGF-1R inhibitors can protect against certain conditions, such as cirrhosis and diabetic nephropathy, by blocking the HMGB1-induced TLR4/MyD88/NF-κB pathway [28, 29]. The expression of IGF-1R in the peripheral blood of patients with rheumatoid arthritis is correlated with increased systemic inflammation. As a result, a study has demonstrated the ability to alleviate experimental arthritis by inhibiting IGF-1R signaling and modulating the balance of IL-6-dependent Th17/Treg cell formation [30, 31]. My experiments indicate that Tfr cells, a subpopulation of Treg cells, can also be regulated by IGF-1R inhibitors, with the level of Tfr-cell differentiation significantly increased in the presence of an IGF-1R inhibitor. This study also found that HDAC inhibitors significantly enhance Tfr-cell differentiation, which is consistent with previous findings in the laboratory. However, my unpublished data from the laboratory showed that oral administration of HDAC inhibitors to mice did not significantly increase Tfr cell frequency, Tfr cell number, or Tfr/Tfh cell ratio in lymph nodes compared to control groups. On the other hand, other studies have demonstrated that HDAC inhibitors, such as pan-HDAC inhibitors VPA, SAHA, and short-chain fatty acids, have a significant therapeutic effect on autoimmune diseases [32, 33]. These findings suggest that HDAC inhibitors may ameliorate autoimmune diseases through alternative pathways aside from promoting Tfr-cell differentiation. As such, the focus of my next study will be to identify the targets and mechanisms of HDAC inhibitors in improving rheumatoid arthritis.

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Part II

Study on the mechanism of attenuates autoimmune arthritis by HDAC1-selective inhibitor TTA03-107

1. Introduction

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune arthritis characterized by synovitis and the progressive destruction of cartilage and bone, leading to irreversible joint destruction. Macrophages and osteoclasts have been demonstrated as major cellular components in the pathogenesis of RA. Indeed, the infiltration of macrophages into synovial tissues is a hallmark of RA in the early phase. Activated macrophages in synovial tissues produce pro-inflammatory chemokines and cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, which recruit other immune cells and promote joint inflammation. In addition, enhanced differentiation and activation of osteoclasts are observed in RA, resulting in the destruction of cartilage and bone [1,2]. Both macrophages and osteoclasts in joint tissue originate from circulating monocytes. Thus, current antirheumatic therapy mainly targets the activation and differentiation of macrophages and osteoclasts to reduce disease severity.

Histone deacetylases (HDACs) play a critical role in the regulation of transcription by controlling reversible histone lysine acylation. On the basis of the similarity of nucleic acid sequence and function, HDACs are classified into four classes, with a total of 18 members in humans and mice [5]. The therapeutic effects of pan-HDAC inhibitors (HDACi) have been examined in rodent models of autoimmune diseases as well as in tissue samples from patients. Their anti-inflammatory effects were reported to be prominent in both cases [3-5]. However, their adverse effects, such as fatigue, anorexia, diarrhea, vomiting, weight loss, and alterations in serum biochemical markers, have greatly limited their clinical use in RA [6]. Against this background, it has been proposed that HDAC isozyme-selective inhibitors may have fewer adverse effects than pan-HDAC inhibitors

for the treatment of chronic disease. The current emerging trend in drug development is to identify HDAC isozyme-selective inhibitors with both immunomodulatory activity and improved safety.

The class I HDAC family consists of HDAC1, HDAC2, HDAC3, and HDAC8, the first two of which are ubiquitously expressed. HDAC1 and HDAC2 form homo- and heterodimers, thereby working together as catalytic components of the co-repressor complex at some gene loci [7]. Interestingly, recent observations in RA patients have identified elevated transcript levels of HDAC1 in synovial fibroblasts and tissues [8-9]. Similarly, lipopolysaccharide (LPS) stimulation upregulates HDAC2 expression in macrophages; this is essential for the subsequent production of pro-inflammatory cytokines, such as IL-12p70 and TNF-α [10]. Strikingly, T-cell-specific deletion of HDAC1 completely prevents the development of CIA, suggesting that HDAC1 activity in T cells plays an indispensable role in the pathogenesis of RA [9]. Furthermore, the injection of HDAC1-targeting siRNA into CIA mice attenuates arthritis, probably by increasing IL-10 production [8]. These observations shed light on the high therapeutic potential of chemical inhibitors against class I HDAC isozymes, especially HDAC1 and HDAC2, in RA. Indeed, studies in the past two decades have shown that synthesized class I HDAC inhibitors targeting mostly HDAC1 and HDAC2 have anti-rheumatoid effects in collagen-induced arthritis (CIA) and collagen antibody-induced arthritis (CAIA) mouse models of autoimmune arthritis. The inhibition of HDAC1 and HDAC2 suppresses osteoclast differentiation and activity and the production of inflammatory mediators from monocytes and macrophages [4, 11-13]. In addition to the chemical compounds, a commensal bacteria-derived natural class I HDAC inhibitor, butyrate, attenuates autoimmune arthritis in osteoclast-dependent and -independent manners [14-18]. Nonetheless, until recently, no HDAC1- and HDAC2-selective inhibitors have been available.

In this study, I investigated the therapeutic efficacy of a novel HDAC1 isozyme-specific inhibitor, TTA03-107, in the CIA and CAIA models. I found that HDAC1 inhibition

attenuated the severity of arthritis in both of these models. HDAC1 inhibition by TTA03-107 decreased IL-17A production *in vivo* and inhibited RORγt^{+} Th17-cell differentiation *in vitro* while barely affecting osteoclast differentiation. TTA03-107 also prevented macrophage differentiation and inflammatory cytokine production. Together, my data suggest that TTA03-107 is a potential candidate for tsDMARD targeting macrophages.

2. Materials and Methods

2.1 Mice

C57BL/6J Jcl mice and DBA/1JJmSlc mice were purchased from CLEA Japan and Sankyo Labo Service (Tokyo, Japan), respectively. They were fed CE-2 pellet chow (CLEA Japan). All mice were housed and bred at the Keio University Faculty of Pharmacy under protocols approved by Keio University Institutional Animal Care and Use Committee (Animal Experiment Approval Number A2022-201).

2.2 Compounds

- Sodium valproate: VPA, FUJIFILM Wako Pure Chemicals
- Prednisolone: Predni, Tokyo Chemical Industry
- TTA03-107: Kindly provided by Prof. Takayoshi Suzuki (Graduate School of Medical Science, Kyoto Prefectural University of Medicine)

All compounds were dissolved in dimethyl sulfoxide (DMSO) as a stock solution.

2.3 Mouse rheumatoid arthritis model

2.3.1 Collagen-induced arthritis model

Six- to seven-week-old male DBA/1JJmsSlc (Sankyo Labo Service, Japan) mice were injected intracutaneously with 50 μl (2 mg/ml) of immunization-grade bovine type II collagen (CII; Chondrex, Woodinville, WA, USA) emulsified in 50 μl (1 mg/ml) of complete Freund's adjuvant (M. Tuberculosis: 2 mg/ml, Chondrex) at the base of the tail on day 1. On day 21, the mice were injected with bovine type II collagen emulsified in incomplete Freund's adjuvant as a booster.

On day 21, they were randomly divided into three groups of 12 mice each, namely, control DMSO, VPA, and TTA03-107 groups. TTA03-107 and VPA were suspended in

MediDrip Sucralose (Clear H_2O; Westbrook, ME, USA) at a concentration of 0.03 or 8 mg/ml, respectively. Vehicle DMSO was diluted in MediDrip Sucralose at 0.003%. The reagents were orally administered as drinking water starting on the day of booster immunization. All mice were dissected on day 43. In terms of mouse dosage, for the HDACi suspension, a 0.03 mg/ml suspension would be 6 mg/kg/day, and for the VPA suspension, an 8 mg/ml suspension would be 1200 mg/kg/day.

2.3.2 Purification of anti-collagen IgG

To purify collagen-recognizing IgG, Amicon Ultra-15 Centrifugal Filter Units (100 kDa, Millipore, Burlington, MA, USA) were used for serum collected from CIA model mice in accordance with the manufacturer's instructions. Briefly, after pre-rinsing the filter with MilliQ water, serum was added up to a volume of 15 ml and centrifuged at 5,000 × *g* for 30 min. After centrifugation, the concentrated sample was collected and the protein concentration was assayed to adjust the concentration to 50 mg/ml. The samples were stored at −80°C until later use.

2.3.3 Collagen antibody-induced arthritis model

Six- to seven-week-old male DBA/1JJmsSlc mice were randomly divided into five groups consisting of 10 mice each, namely, control DMSO, VPA (reference group), prednisolone (reference group), and TTA03-107 groups, on day 0. To produce collagen antibody-induced arthritis, 5 mg of IgG purified from CIA mice was administered intraperitoneally on day 0, followed by 50 μg of LPS (*E. coli* O111; FUJIFILM Wako Pure Chemicals) on day 3. TTA03-107, VPA, and prednisolone were suspended in MediDrip Sucralose and orally administered as drinking water at concentrations of 0.03, 0.03, 8, and 0.015 mg/ml, respectively, starting on day 1. All mice were dissected on day 22.

In terms of mouse dosage, for the TTA03-107 suspension, a 0.03 mg/ml suspension would be 6 mg/kg/day, for the VPA suspension, an 8 mg/ml suspension would be 1200

mg/kg/day, for prednisolone suspension, a 0.005 mg/ml suspension would be 1 mg/kg/day.

2.3.4 Clinical evaluation of arthritis

Clinical parameters were measured using an articular index score. The articular index scoring was performed using a scale of 0–4: 0, normal; 1, red and swollen toe joint; 2, swollen toe joint and ankle; 3, swelling of the paw; and 4, swelling of all of the feet, including the ankle joint. The sum of the scores of the four limbs was expressed as the clinical score, with a maximum of 16 points.

2.3.5 Blood collection

Blood was collected from the heart with a heparinized syringe under anesthesia with isoflurane (Pfizer) during dissection of mice. Blood samples were centrifuged at 1000 × *g* for 10 min at room temperature and the supernatant was collected to obtain a plasma sample. Serum samples were collected by this procedure. Prepared samples were stored in a freezer at -80°C prior to measurement.

2.3.6 Histological analysis

For histological analysis, paws were skinned and fixed in Mildform 10N (FUJIFILM Wako Pure Chemicals), decalcified in EDTA buffer for 15 days, and then embedded in paraffin. The tissue cross-sections were stained with hematoxylin-eosin (H&E; SKK Organization Science Research Institute, Yokohama, Japan). Briefly described, tissues were washed with tap water, soaked in 70%, 80%, 90%, and 100% ethanol and 100% xylene for 1 hour each for dehydration, then embedded in paraffin, sectioned with a microtome using the coronal section of the ankle joint as the dividing surface, and attached to a glass slide. The slides were placed twice in 100% xylene for 10 minutes each and in 100%, 90%, 80% and 70% EtOH for 5 minutes each, degreased and washed

under running water until free of alcohol odor. After rinsing, the slides were stained with hematoxylin stain for approximately 3 minutes, washed with warm water at approximately 40°C to remove excess stain and soaked in eosin for 2 min. Eosin was washed with 95% EtOH and 100% EtOH for two 10 minutes washes to completely remove excess stain. After natural drying, they were permeabilized with xylene for 3 min and then sealed with neutral resin. The histological changes were examined under a microscope (All-in-One Fluorescence Microscope, KEYENCE) by blinded observers. Synovial inflammation, bone erosion, cartilage damage, and leukocyte infiltration were scored using a three-parameter system in which individual scores were summed: joint inflammatory cell infiltration, synovial hyperplasia, and cartilage and bone destruction (0 = normal, 1 = mild, 2 = moderate, 3 = marked, 4 = severe). The histological score was calculated by summing the scores.

2.3.7 Serum and joint cytokine measurements (ELISA)

The serum and joint were collected from CIA and CAIA model mice for cytokine measurements. For joint cytokine measurements, frozen tissues (whole joints, including synovium, adjacent tissues, and bone) were pulverized with a mortar and pestle filled with liquid nitrogen. Approximately 80 mg of tissue was transferred to a 5 ml tube, resuspended in total 2 ml of RIPA of tissue, and frozen overnight at −80°C. This was followed by centrifugation at 500 × *g* for 10 min at 4°C. The supernatant was transferred to a 1.5 ml tube and collected for cytokine analysis. The levels of IL-1β, TNF-α, and IL-17A were measured using ELISA kits (all from BioLegend). Joint cytokine data are expressed as pg cytokine/g tissue (data were multiplied by a dilution factor for conversion from pg/ml to pg/g).

The ELISA assay steps are briefly described below. Specific primary antibodies were diluted 200-fold in coating buffer, added to 96-well ELISA plates (Thermo Scientific, Waltham, MA, USA) at 100 μl/well and incubated overnight at 4°C for encapsulation.

After encapsulation, 0.05% Polyoxyethylenesorbitan monolaurate (Tween 20) (nacalai tesque)/D-PBS (nacalai tesque) (hereafter referred to as Wash Buffer) is added at 200 µl/well and washed three times. Standards and samples diluted with 1% bovine serum albumin (BSA) (nacalai tesque)/D-PBS (hereafter referred to as Dilution Buffer) are added at 100 µl/well to a 96-well plate and incubated for 1-2 hours at room temperature, followed by three washes with Wash Buffer. Specific secondary antibodies diluted 200-fold with Dilution Buffer are added to a 96-well plate at 100 µl/well and incubated for 1 hour at room temperature, followed by 3 washes with Wash Buffer. Avidin-HRP diluted 1000x with dilution buffer at 100 µl/well is added to a 96-well plate and incubated for 1 hour at room temperature, followed by 3 washes with wash buffer. Then 1-Step ultra TMB-ELISA substrate solution (Thermo Scientific) was added at 100 µl/well and incubated for 20 min~1 h at room temperature for color development reaction. After confirming the color development, 1.2 M H_2SO_4 was added to each well to stop the color development reaction, and the absorbance at 450 nm was measured with a plate reader.

2.3.8 Determination of serum IgG concentration using ELISA

Serum total IgG antibody titers were determined by using the Mouse IgG ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). Affinity purified Goat anti-Mouse IgG-Fc Coating Antibody was diluted 200-fold in D-PBS, added at 100 µl/well to a 96-well ELISA plate (Thermo Scientific), and incubated overnight at 4°C for encapsulation. After coating, 0.05% Tween 20/D-PBS (hereafter referred to as Wash Buffer) was added at 200 µl/well and washed three times. Blocking was performed by adding 2% BSA/D-PBS (hereafter referred to as Dilution Buffer) at 200 µl/well and incubating for 2 hours at room temperature. Then wash 3 times with Wash Buffer. Standards and serum samples diluted 10,000-fold using Dilution Buffer are added to 96-well plates at 100 µl/well and incubated for 1-2 hours at room temperature and then washed 3 times with Wash Buffer. HRP-conjugated Goat Anti-Mouse IgG-Fc Detection Antibody (A90-131P-40) is diluted

8,000-fold in Dilution Buffer, added to 96-well plates at 100 μl/well, incubated for 1 hour at room temperature and washed 3 times with Wash Buffer. To a solution prepared in the ratio of 0.1 M citric acid: 0.2 M Na_2HPO_4: d-water = 1: 1: 2, add 0.016% by volume of 30% H_2O_2 (nacalai tesque) and a final concentration of 0.4 mg/ml of o-phenylenediamine dihydrochloride (OPD) and mix (hereinafter referred to as OPD solution). After confirming color development, 1.2 M H_2SO_4 was added to each well to stop the color development reaction, and the absorbance at 492 nm was measured with a plate reader.

Serum CII-specific IgG antibody titers were measured by using ELISA grade type II collagen (Chondrex) measurements. ELISA grade type II collagen was diluted to 5 μg/ml in 10 mM acetic acid solution, added to a 96-well ELISA plate at 100 μl/well, and incubated overnight at 4°C for encapsulation. After encapsulation, wash buffer is added at 200 μl/well and washed three times. Blocking is performed by adding dilution buffer at 200 μl/well and incubating at room temperature for 2 hours. Then wash 3 times with Wash Buffer. Standards (using control group mouse serum as relative standards) and 10,000-fold dilutions of serum samples are added to 96-well plates at 100 μl/well and incubated for 1-2 hours at room temperature and washed three times with Wash Buffer. The capture antibody, which is Rabbit Anti-Mouse IgG (H+L)-HRP (Southern Biotech), Goat Anti-Mouse IgG_1-HRP (Southern Biotech), HRP-rabbit anti-mouse IgG_{2a} (Southern Biotech) and HRP-rabbit anti-mouse IgG_{2b} (Southern Biotech), respectively, is diluted 500-fold in dilution buffer, added to the 96-well plate at 100 μl/well, incubated for 1 hour at room temperature and washed 3 times with wash buffer. Add OPD solution at 100 μl/well and incubate the color development at room temperature for 30 min to 1hr. After confirming the color development, add 1.2 M H_2SO_4 to each well to stop the color development reaction and measure the absorbance at 450 nm with a plate reader.

2.4 Osteoclast differentiation assay

2.4.1 Cell isolation and culture method

Bone marrow (BM) was isolated from mice in accordance with the following protocol. In brief, erythrocyte lysis was performed on bone marrow cells from the femur and tibia from male C57BL/6J Jcl mice (6-8 weeks old, Clea Japan), the femur and tibia of C57BL/6J Jcl mice were collected, the surrounding muscle tissue was removed and soaked in medical alcohol for 5 minutes. The leg bones were removed in a clean bench and transferred to a Petri dish placed on ice, the bone ends were cut with scissors and the bone marrow was rinsed several times with a 10 ml syringe containing 2R solution. The bone ends were also cut into small pieces. The cell suspension and the bone pieces after multiple rinses were collected through a 100 μm cell filter, and the BM cells were collected by centrifugation at 500 × *g* for 5 min at 4°C. The precipitated cells were suspended in red blood cell (RBC) lysis solution (eBioscience) and placed on ice for 1 min to lyse erythrocytes. Add 10 times the volume of 2R solution, collect the cell suspension through a 100 μm cell filter, and centrifuge at 500 × *g* for 5 min at 4°C to collect BM cells. The precipitated cells were suspended in 2R solution, stained with 0.5% trypan blue stain and counted.

To induce osteoclast differentiation, BM cells were cultured in alpha minimum essential medium (α-MEM) with 10% FBS (MP Biomedicals, Irvine, CA, USA) containing 20 ng/ml recombinant mouse macrophage colony-stimulating factor (rmM-CSF; BioLegend) for 3 days. Cells were then seeded into 96-well plates and cultured with complete α-MEM containing 20 ng/ml M-CSF and 50 ng/ml recombinant mouse receptor activator of nuclear factor kappa-B ligand (RANKL; BioLegend) with or without TTA03-107 at 37°C in a 5% CO_2 incubator. The culture medium was replaced every 2 days.

2.4.2 TRAP staining

After 6 days, cells were fixed and stained for Tartrate-resistant acid phosphatase (TRAP) activity using a TRAP staining kit according to the manufacturer's protocol

(Cosmo Bio Co., LTD., Tokyo, Japan). TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. The staining assay steps are briefly described below. The medium is removed and washed once with 100 μl/well of PBS. Add 50 μl of 10% formalin neutral buffer solution to each well, fix for 5 minutes at room temperature, and then wash 3 times with 250 μl/well of deionized water. The color development substrate was dissolved in 5 ml of buffer containing tartrate and left to stand for at least half an hour. Add 50 μl of chromogenic substrate to each well. Incubate at 37°C for 20-60 minutes and wash with deionized water to stop the reaction.

2.5 Th17 differentiation assay

2.5.1 culture method

To establish the effect of TTA03-107 on Th17 cells differentiation, naïve $CD4^+$ T cells from normal C57BL/6 Jcl mouse spleen were isolated using MojoSort™ mouse CD4 T cell isolation kit (BioLegend) as previous description. They were seeded on a high-binding 96-well plate (Corning) at a concentration of 1×10^5 cells/well, which had been incubated with 5 μg/ml anti-TCRβ mAb overnight in the 4℃ refrigerators in advance. The cells were cultured in complete RPMI-1640 (nacalai tesque) with 10% FBS for 2 days under Th17 cell-polarizing conditions. The cells were cultured in complete media supplemented with CD28 (at final concentration of 2 μg/ml), IL-2 (10 μg/ml), IL-4 (10 μg/ml), IFN-γ (10 μg/ml), rmIL-6 (25 ng/ml), rmIL-1β (10 ng/ml), and rmIL-23 (10 ng/ml). Then, the medium was replaced with a new one and the cells continued to be cultured under these conditions for 3 days with or without different concentrations of TTA03-107 at 37°C in a 5% CO_2 incubator.

Antibody Name (Clone Name)	Manufacturing Company
*InVivo*MAb anti-mouse TCRβ (H57-597)	Bio X cell

*InVivo*MAb anti-mouse CD28 (37.51)	Bio X cell
Purified anti-mouse IL-2 (JES6-1A12)	BioLegend
*InVivo*MAb anti-mouse IL-4 (11B11)	Bio X cell
*InVivo*MAb anti-mouse IFNγ (R4-6A2)	Bio X cell
recombinant mouse (rm) IL-6	BioLegend
recombinant mouse (rm) IL-1β	BioLegend
recombinant mouse (rm) IL-23	BioLegend

2.5.2 Flow cytometry

Th17 cells were collected after being cultured under Th17 polarized conditions. For intracellular cytokine staining, Th17 cells were stimulated with a cell activation cocktail (500×; BioLegend) with final concentrations of phorbol 12-myristate 13-acetate (PMA) of 50 ng/ml and of ionomycin of 1000 ng/ml for 4 h. A protein transport inhibitor cocktail (500×; eBioscience) was added after 1 h. Then cells were transferred to 96-well V-bottom plates, centrifuged at 300 × *g* for 5 min at 4°C. For cell staining, cells were resuspended in a monoclonal antibody (mAb) against CD16/32 (93; BioLegend) dilution in PBS containing 2% FBS and 0.1% NaN_3, and blocked on ice for 15 min. After centrifugation and washing twice, add 50 μl fluorescently labeled surface staining antibodies according to the table below and incubate for 30 min at 4°C in the refrigerator. After centrifugation and washing twice, the cells were vortexed and fixed. For fixation, the cells were incubated for 45 min at room temperature using a transcription factor buffer set (BD Biosciences) according to the instructions provided. After three centrifugation washes with permeabilization buffer (eBioscience), intracellular transcription factors were stained by adding fluorescently labeled staining antibody according to the table below and incubating for 45 minutes at room temperature protected from light. After centrifugations and washes twice, cells were resuspended in PBS containing 2% FBS and 0.1% NaN_3

and analyzed with FACS LSR II or FACS Celesta (BD Biosciences). The data obtained were analyzed using FlowJo 10 software (BD Biosciences).

(1) Antibody mixture for extracellular use

Reagent	Clone	Manufacturer	Dilution
BV605-conjugated anti-CD4	RM4-5	BD Biosciences	1:200
BV786-conjugated anti-CD25	2A3	BD Biosciences	1:200
PCP-Cy5.5-conjugated anti-CD45	30-F11	BioLegend	1:200
APC-Cy7-conjugated anti-FVS780	-	Tonbo Biosciences	1:200

(2) Antibody mixture for intracellular use

Reagent	Clone	Manufacturer	Dilution
PE-CF594-conjugated anti-RORγt	Q21-559	BD Biosciences	1:25
PE-conjugated anti-Foxp3	FJK-16s	eBioscience	1:100

(3) Cell fixation

Fix/Perm working solution: Fixation/Permeabilization concentrate: Diluent buffer= 1: 3

Perm/Wash working solution: Perm/Wash buffer: Distilled water = 1: 4

2.6 BMDMs isolation and macrophage assay

2.6.1 Cell isolation and culture method

Bone marrow-derived macrophages (BMDMs) were isolated as previously described. After isolation, BM cells were cultured in a differentiation medium for 3 days, then the medium was changed to a new one for 3 days with or without different concentrations of TTA03-107. The differentiation medium consisted of high-glucose Dulbecco's Modified Eagle Medium (nacalai tesque) with 10% FBS, and a final concentration of 20 ng/ml rm M-CSF (BioLegend) was also added. BMDMs were polarized to M1 macrophages by 10

hrs stimulation with 100 ng/ml LPS (E. coli O111125-05181; FUJIFILM Wako Pure Chemicals), followed by 6-h stimulation with 20 ng/ml nigericin (InvivoGen) to activate the NLRP3 inflammasome. After culture under the polarized conditions, cell supernatants and cells were collected for ELISA and flow cytometry assays, respectively.

2.6.2 Flow cytometry

Macrophages obtained from the study after treated with M1 polarized culture system were transferred to 96-well V-bottom plates, centrifuged at 300 × *g* for 5 min at 4°C, resuspended in a monoclonal antibody (mAb) against CD16/32 (93; BioLegend) dilution in PBS containing 2% FBS and 0.1% NaN_3, and blocked on ice for 15 min. After centrifugation and washing twice, 50 μl of the extracellular antibody mixture from the table below (1) is added and allowed to react for 30 min at 4°C in the refrigerator. After two centrifugations and washes, cells were resuspended in PBS containing 2% FBS and 0.1% NaN_3. Flow cytometry was performed on a BD LSR II, and analyzed with FlowJo software (BD Biosciences, v10.8).

(1) Antibody mixture for extracellular use

Reagent	Clone	Manufacturer	Dilution
BUV 395-conjugated anti-CD45	30-F11	BD Biosciences	1:200
BUV 737-conjugated anti-CD11b	M1/70	BD Biosciences	1:200
PE-conjugated anti-F4/80	QA17A29	BioLegend	1:200
PE-Cy7-conjugated anti- CD163	S15049I	BioLegend	1:200
PE-CF594-conjugated anti-CD40	3/23	BioLegend	1:200
AF700-conjugated anti-CD206	C068C2	BioLegend	1:200
APC-Cy7-conjugated anti- FVS780		Tonbo Biosciences	1:200

(2) Antibody mixture for intracellular use

Reagent	Clone	Manufacturer	Dilution
APC-conjugated anti-Arg1	A1exF5	Invitrogen	1:100
and PCP-Cy5.5-conjugated anti-iNOS	CXNFT	Invitrogen	1:100

(3) Cell fixation

Fix/Perm working solution: Fixation/Permeabilization concentrate: Diluent buffer= 1: 3

Perm/Wash working solution: Perm/Wash buffer: Distilled water = 1: 4

2.6.3 Cell supernatant cytokine measurements (ELISA)

For the *in vitro* experiment cytokine measurements, the cell supernatant was collected at the end of the M1 polarized culture conditions. IL-1β, TNF-α and IL-6 were measured using ELISA kits (all from BioLegend) according to the manufacturer's instructions. The ELISA assay steps are briefly described as 2.3.7.

2.7 Cultivations, buffering solutions used of cell culture

• Culture medium

・Osteoclasts cell culture medium

Name	Manufacturer	Final Conc.
Fetal Bovine Serum (Heat inactivated)	MP Biomedicals	10%
1 mol/L HEPES Buffer Solution	nacalai tesque	0.01 mol/L
β-mercaptoethanol	Thermo Scientific	55 μM
Penicillin-Streptomycin mixed solution	nacalai tesque	100 μg/ml penicillin 100 μg/ml streptomycin
alpha minimum essential medium (α-MEM)	Gibco	88 %

・Th17 cell culture medium

Name	Manufacturer	Final Conc.
Fetal Bovine Serum (Heat inactivated)	MP Biomedicals	10%
1 mol/L HEPES Buffer Solution	nacalai tesque	0.01 mol/L
β-mercaptoethanol	Thermo Scientific	55 μM
Penicillin-Streptomycin mixed solution	nacalai tesque	100 μg/ml penicillin 100 μg/ml streptomycin
complete RPMI-1640	nacalai tesque	88 %

・Macrophage culture medium

Name	Manufacturer	Final Conc.
Fetal Bovine Serum (Heat inactivated)	MP Biomedicals	10%
1 mol/L HEPES Buffer Solution	nacalai tesque	0.01 mol/L
β-mercaptoethanol	Thermo Scientific	55 μM
Penicillin-Streptomycin mixed solution	nacalai tesque	100 μg/ml penicillin 100 μg/ml streptomycin
High-glucose Dulbecco's Modified Eagle Medium	nacalai tesque	88 %

2.8 Statistical analyses

Statistical analysis was performed using GraphPad Prism (version 19.0). All data were expressed as mean ± standard error of the mean (SEM). Repeated-measures two-way ANOVA or one-way ANOVA was used for comparison between multiple means. The statistical significance of differences between groups was determined using an unpaired Student's t-test or Welch's t-test. The data were considered statistically significant when $p < 0.05$.

3. Results

3.1 Antiarthritic Efficacy of TTA03-107 in Mouse CIA Model

To examine the therapeutic effect of TTA03-107 in the RA mouse model, I administered TTA03-107 in drinking water (approximately 5 mg/kg/day) to CIA-induced DBA/1J mice at the booster immunization onward. I also administered a class I HDAC inhibitor, VPA (approximately 1,200 mg/kg/day), which was shown to prevent CIA development [17] as a positive reference. The mice administered TTA03-107 had a higher rate of body weight gain than the mice in the control group, while the VPA-treated group displayed lower body weight gain with a sharp reduction at the initial phase. Water consumption was also significantly lower in the VPA-treated group than in the other groups. According to the clinical score data obtained based on the scoring criteria, I found that TTA03-107 treatment alleviated the severity of arthritis. In contrast, there was no change in clinical scores between the VPA-treated and control groups. Upon histological examination, a broken joint structure with a large amount of inflammatory cell infiltration and cartilage destruction was evident in the control group, whereas the treatment with TTA03-107 significantly ameliorated these changes with few signs of inflammation. I also evaluated the serum levels of CII-specific IgG, a hallmark of CIA, 3 weeks after the booster immunization. The administration of TTA03-107 reduced CII-specific IgG_{2b} but not IgG_1 and IgG_{2a}, nor CII-specific total IgG. Because CII-specific IgG_1 and IgG_{2a} play primary roles in the pathogenesis of CIA, TTA03-107 may attenuate arthritis through a mechanism other than preventing autoantibody production. Therefore, I further explored the effect of TTA03-107 on the production of inflammatory cytokines, TNF-α and IL-17A, which make critical contributions to the pathogenesis of CIA [19]. The serum levels of these inflammatory cytokines were moderately but significantly reduced in the TTA03-107-treated groups. These observations suggest that TTA03-107 mitigates

the development of arthritis symptoms in CIA mice by suppressing inflammatory cytokine production rather than CII-specific IgG immune responses.

3.2 TTA03-107 exerts an antiarthritic effect in the later phase

To test whether TTA03-107 is engaged in arthritis at the later phase of pathogenesis after autoantibody generation, I treated CAIA model mice with TTA03-107 via drinking water. I also administered the conventional first-line DMARD prednisolone as a positive reference. Similar to the CIA experiment, the TTA03-107-treated group did not show a change in body weight gain. By contrast, treatment with VPA or prednisolone decreased body weight, especially until day 13 after disease onset. The VPA treatment group consumed less water at the beginning of the experiment, whereas water consumption was comparable among the other three groups. TTA03-107 treatment ameliorated arthritic symptoms such as swelling of the ankle joint and paw, skin congestion, and stiffness. The therapeutic effect of TTA03-107 was comparable to that of prednisolone. Meanwhile, VPA did not significantly alter the severity of arthritis. I further evaluated inflammatory cytokines in the serum and joint tissue extract in the CAIA model and observed that TTA03-107 markedly suppressed the levels of TNF-α, IL-1β, and IL- 17A in the serum. A similar tendency was also observed in the joint tissue extract. These findings indicate that TTA03-107 exerts a therapeutic effect in the later phase of arthritis development.

3.3 TTA03-107 treatment suppresses osteoclast differentiation

TTA03-107 treatment almost completely reduced the damage of cartilage and bone in the CIA model, and therefore I hypothesized that it might reduce arthritis severity by affecting osteoclast differentiation. To test this hypothesis, I assessed the osteoclast differentiation of BM cells in the presence or absence of TTA03-107 by TRAP staining. I observed that TTA03-107 moderately suppressed osteoclast formation only at higher

concentrations, suggesting that the direct effect of HDAC1 inhibition on osteoclast differentiation may be minimal and is unlikely to contribute to its antiarthritic activity.

3.4 TTA03-107 treatment reduces RORγt^{+} Th17-cell differentiation *in vitro*

Our previous study showed that commensal bacteria-derived butyrate, as a class I HDAC inhibitor, ameliorates arthritis by inhibiting autoantibody production through enhancing the differentiation of follicular regulatory T (Tfr) cells [15]. However, TTA03-107 showed the no effects on the production of CII specific IgG in CIA model except IgG_{2b} which suggest that TTA03-107 affect arthritis not through Tfr cell induction.

Th17 cells serve as potent osteoclastogenic T cells in the RA model; RANKL derived from Th17 cells supports osteoclastogenesis to enhance bone destruction. Considering that pan-HDAC inhibitors inhibit Th17-cell differentiation and the T-cell-specific deletion of HDAC1 completely suppresses IL-17A production, I assumed that TTA03-107 might prevent the destruction of cartilage and bone in the CIA model by inhibiting Th17-cell differentiation. To test this assumption, I adopted an *in vitro* Th17 culture and analyzed the expression of RORγt, a characteristic transcription factor of Th17 cells. The frequency of RORγt^{+} Th17 cells and RORγt mean fluorescent intensity were reduced upon TTA03-107 treatment, but there was no significant dose-response relationship.

3.5 TTA03-107 treatment downregulates inflammatory cytokine production by macrophages

Class I HDAC inhibitors have been recognized to suppress the expression of pro-inflammatory cytokines in response to LPS stimulation in macrophages. Although the mechanism of HDAC1 involvement remained unclear, it is possible that HDAC1 inhibition also affects macrophage hyperresponsiveness. To test this, I prepared BM-derived macrophages under M1 polarizing conditions in the presence of TTA03-107 before stimulating them with LPS. The levels of IL-1β, TNF-α, and IL-6 were significantly

reduced in M1 macrophages treated with TTA03-107 in a dose-dependent manner. To examine whether TTA03-107 treatment changes the M1 macrophage phenotype toward the M2 phenotype, I analyzed the expression of an M1 macrophage marker, iNOS, and an M2 macrophage marker, arginase 1 (Arg1), by flow cytometry. I found that TTA03-107 treatment downregulated iNOS expression while upregulating Arg1 expression. These results suggest that TTA03-107 downregulated the transcriptional status of genes involved in the differentiation and production of inflammatory cytokines of M1 macrophages, probably by promoting the conversion of M1 to M2 phenotype.

4. Discussion

Immunosuppressive and immunomodulatory agents for treating RA are generally called disease-modifying antirheumatic drugs (DMARDs). DMARDs are classified as either synthetic DMARDs (sDMARDs) or biologic DMARDs (bDMARDs). The most widely used DMARDs for RA therapy are conventional sDMARDs (csDMARDs), such as methotrexate, which target the entire immune system and slow down disease progression, although they take approximately a month to start working. bDMARDs and targeted sDMARDs (tsDMARDs) tend to work faster than csDMARDs [20, 21]. However, bDMARDs are expensive and approximately 40% of RA patients do not respond to therapy using anti-TNF-α antibodies, a representative bDMARD. In addition, currently available tsDMARDs are limited to inhibitors of Janus kinases (JAK), which are associated with concerns about increased susceptibility to viral infection [22]. Moreover, some individuals who do not respond to JAK inhibitor treatment have been reported. There is thus a need for a rational therapeutic approach with novel tsDMARDs that target non-JAK molecules for RA patients, especially bDMARD non-responders.

Herein, I addressed the therapeutic potential of a novel HDAC1-selective inhibitor, TTA03-107, for mouse models of RA. TTA03-107 ameliorated disease severity and the degree of inflammation in the joints of both CIA and CAIA models without affecting their body weight. In comparison, a class I HDAC inhibitor, VPA, reduced the body weight and had little if any effect on the development of arthritis in both models. Given that other class I HDAC inhibitors have a strong DMARD effect, the lower therapeutic effect of VPA in the current study is an anomaly [11-13], which may be due to the different administration routes used. A previous study demonstrated that the intraperitoneal administration of VPA significantly improved the incidence and disease severity of CIA [17]. Meanwhile, I administered VPA orally, which may be inappropriate with regard to achieving efficacy due to the associated low bioavailability. Moreover, the VPA treatment group consumed less water presumably due to its high concentration. Therefore, the dosage of VPA was

calculated based on the reduced volume of water consumption, and thus the reduction of water consumption is unlikely to affect the results.

In line with previous findings from mice with T-cell-specific deletion of HDAC1 [9], the current study shows that HDAC1 inhibition by TTA03-107 treatment significantly decreased IL-17A levels in both serum and joint tissue in the CIA model. The reduction of IL-17A is probably attributable to T-cell-intrinsic HDAC1 inhibition to arrest Th17 differentiation and also to the downregulation of IL-6 and IL-1β by macrophages in the joint tissues. Indeed, my *in vitro* BMDM experiment confirmed that TTA03-107 reduces the production of TNF-α, IL-1β, and IL-6. Moreover, TTA03-107 upregulated the M2 macrophage marker Arg1 while downregulating the M1 marker iNOS. These phenomena imply that HDAC1 inhibition is related to the M1 to M2 macrophage transition, although there is a need for further work aimed at developing a deeper understanding of the molecular mechanisms involved.

CII-specific IgG_{2b} production was slightly abated by TTA03-107 treatment in the CIA model, although the other IgG subclasses remained unchanged. Our lab previously reported that a class I HDAC inhibitor, butyrate, suppresses germinal center responses by inducing follicular regulatory T-cell differentiation in the CIA model [15, 18]. Therefore, the abatement of CII-specific IgG_{2b} by TTA03-007 may also be attributable to the attenuation of germinal center responses to CII. Additionally, class I HDAC inhibition by butyrate also regulates osteoclast differentiation and bone homeostasis by inducing metabolic reprogramming of osteoclasts, reducing joint inflammation and bone destruction in mice in the CIA and CAIA models [14, 16]. Meanwhile, TTA03-107 showed only a minor effect on osteoclast differentiation even at a high concentration. Thus, it is plausible that osteoclast regulation by class I HDAC inhibition is mediated by its effect on HDAC2, HDAC3, or HDAC8, but not HDAC1. Taken together, my observations illustrate that TTA03-107 alleviates the development of inflammatory arthritis by inhibiting

inflammatory cytokine production from inflammatory macrophages and subsequent Th17 responses in the autoimmune arthritis models.

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