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論文内容の要旨

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder with chronic inflammation of the joints characterized by the proliferation of synovial cells, angiogenesis, and infiltration of T cells. The causative factors of RA still remain to be elucidated; however, a combination of genetic and environmental factors is likely to contribute to the development of RA. Without appropriate treatment, RA causes joint damage, disability, and a decrease in quality of life. RA is typically managed with a combination of non-pharmacological (e.g., exercise and physical therapies) and pharmacological treatments. The latter includes nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and biologics. In this study, we sought to identify a potential drug candidate for RA, mainly focusing on the expansion of follicular regulatory T (Tfr) cells. The study will consist of two sections; in the first part evaluated the compounds' ability to promote Tfr-cell differentiation, and in the second part, I assessed their efficacies against synovial inflammation and joint damage in RA animal models. I also analyzed the mode of action of the selected compound.

Part I. Screening of Compounds with Tfr Cell Differentiation Promoting Activity

1-1. Background

Tfr cells are a subtype of regulatory T (Treg) cells that share molecular characteristics with both Treg cells and follicular helper (Tfh) cells. For instance, Tfr cells express Foxp3, a hallmark

of Treg cells, in addition to Bcl-6 and CXCR5, which are functional markers of Tfh cells. Tfr cells play a vital role in inhibiting the production of autoantibodies, which attack the body's own tissues. Therefore, this cell subset has been considered as a significant target for the prevention and treatment of autoimmune disorders. However, despite the discovery of Tfr cells over a decade ago, their differentiation process has yet to be fully understood.

Furthermore, technical difficulty in inducing Tfr cells *in vitro* has hampered the cell differentiation mechanism, as well as the establishment of an *in vitro* screening system targeting Tfr-cell differentiation. Our laboratory previously found culture conditions to induce the differentiation of Tfr cells. By taking advantage of this culture system, I attempted to establish an *in vitro* screening system to identify bioactive compounds that facilitate Tfr-cell differentiation.

1-2. Methods

① Compound screening using the Tfr cell differentiation system

Naive T cells were isolated from the spleens of Bcl-6-tdTomato/Foxp3^{hCD2} reporter mice and cultured in the conditions to induce the differentiation of Tfr cells. Compounds and intestinal metabolites were added to the culture, and the expression of the Bcl-6 was evaluated by flow cytometry after 5 days to determine its ability to induce the differentiation of Tfr cells.

② Autoantigen Immunization model

An immunization model was established by subcutaneous injection of emulsified denatured insulin following 18 days of polyamine administration via drinking water. Ten days later, the mouse lymphoid tissues were collected to analyze the changes in the frequency and number of Tfr cells.

1-3. Results

① Screening results in the effect of intestinal microbiota metabolites in promoting Tfr cell differentiation

Tfr cells were defined as Foxp3^{+} CD25^{-} PD-1^{+} CXCR5^{+} Bcl6^{+} in this culture condition. The effects of 47 intestinal microbiota metabolites on Tfr cell differentiation were evaluated by changes in CXCR5 and Bcl6 expression (Fig. 1). γ-Aminobutyric acid, putrescine, cadaverine, and 3-phenylpropionic acid significantly promoted Tfr cell differentiation, with

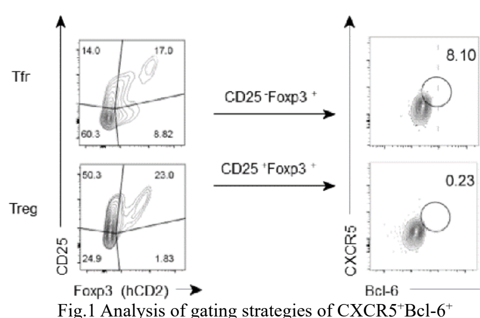

Fig.1 Analysis of gating strategies of CXCR5^{+}Bcl-6^{+}

putrescine and cadaverine showing the most potent effects in a dose-dependent relationship.

② Screening results on the effect of SCADs kits compounds in promoting Tfr cell differentiation

The SCADs inhibitor suite (SCADs kits) is a systematic collection of small molecule inhibitors. The experimental results showed that the telomerase inhibitor TMPyP4, IGF-1R inhibitor OSI-906, and HDAC inhibitor vorinostat all had beneficial effects, with HDAC inhibitor being the most effective.

③ Efficacy of polyamines in insulin immunization model

Treatment with polyamines resulted in a decrease in body weight gain rate in mice compared to the control group treated with PBS. While there was no significant difference in the number of $CD45^+$ cells in lymphoid tissues between the polyamine-treated group and the control group, treatment with putrescine resulted in a significant increase in Peyer's patches. The frequency and number of Tfr cells in polyamine-treated mice were not significantly different from the control group, except for an increase in Tfr cells in Peyer's patches in the putrescine group. Similar results were also observed in the changes in Bcl-6 expression.

1-4. Discussion

The ability of various compounds to induce Tfr cells was evaluated using microbiota-derived metabolites and SCADS inhibitor kits. Of the microbiota-derived metabolites, polyamines cadaverine and putrescine displayed the strongest effect *in vitro*, but they did not promote Tfr cell differentiation in mice. The SCAD inhibitor kits contained several small molecule compounds, and three targets that were effective in promoting Tfr differentiation were identified: telomerase, IGF-1R, and HDAC. Previous research has shown that butyrate, a pan-HDAC inhibitor, has a therapeutic effect on rheumatoid arthritis models, but it also has significant adverse effects. Therefore, selective HDAC inhibitors, which can be used at lower concentrations, were explored as a potential means of improving therapeutic outcomes and reducing side effects in the treatment of chronic diseases. The selective HDAC1 inhibitor TTA03-107 was subjected to further *in vivo* experiments to evaluate pharmacological efficacy in the RA model, as described in Part II.

Part II. Study on the Mechanism of HDAC1 Selective Inhibitor TTA03-107 to Suppress Autoimmune Arthritis

2-1. Background

Histone deacetylases (HDACs) regulate gene expression by controlling reversible histone lysine acetylation. Pan-HDAC inhibitors (HDACi) have been investigated as potential therapeutics for

autoimmune diseases in rodent models and patient tissue samples, but their adverse effects have limited their use in the treatment of rheumatoid arthritis (RA). HDAC isozyme-selective inhibitors may have fewer negative effects and be more effective in the treatment of chronic diseases. The class I HDAC family includes HDAC1, HDAC2, HDAC3, and HDAC8, and HDAC1 transcript levels are elevated in synovial fibroblasts and tissues in RA. In mouse models of autoimmune arthritis, class I HDAC inhibitors targeting HDAC1 and HDAC2 have shown anti-rheumatoid effects. Inhibition of them also suppresses osteoclast differentiation and activity and reduces inflammatory mediator production from monocytes and macrophages. However, until recently, HDAC1- and HDAC2-selective inhibitors were not available.

2-2. Methods

① Collagen-induced arthritis model

The DBA/1 mice were used for a mouse arthritis model through the use of two percutaneous injections of emulsified collagen at the base of the tail. On day 21, the mice were divided into control, VPA, and TTA03-107 groups and administered orally via drinking water. On day 43, all mice were dissected.

② Collagen antibody-induced arthritis model

The DBA/1 mice were established as a mouse arthritis model through the intraperitoneal injection of purified IgG and LPS on day 0 and day 3, respectively. From the outset, the mice were divided into control, VPA, prednisolone, and TTA03-107 groups and administered orally via drinking water. All mice were dissected on day 22.

③ Osteoclast differentiation assay

Bone marrow was isolated from C57B6/J mice to induce osteoblast differentiation. Cells were cultured in α-MEM medium containing rmM-CSF for 3 days, followed by a medium containing rmM-CSF and RANKL for an additional 6 days. The medium was replaced with a fresh one every 2 days. Cells were then fixed and stained using a TRAP staining kit. TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts.

④ Th17 cell differentiation assay

Naïve T cell was isolated from C57B6/J mice and cultured in 1640 medium containing differentiation stimulants for 3 days to induce Th17 cell differentiation. The cells were then continued to be cultured for 2 days after changing to a fresh medium and were analyzed using flow cytometry after fixation and staining.

⑤ Macrophage differentiation assay

To induce macrophage differentiation, bone marrow was isolated from C57B6/J mice, and the cells were cultured in a high-glucose DMEM medium containing rmM-CSF for 3 days. The cells were then continued to be cultured for 2 days after changing to a fresh medium, followed by a medium containing LPS and nigericin for 10 hours. The cells were analyzed using flow cytometry after fixation and staining.

2-3. Results

① Antiarthritic Efficacy of TTA03-107 in Mouse CIA Model

In a study involving CIA model mice, TTA03-107 was administered via drinking water. Mice treated with TTA03-107 had higher body weight gain compared to the control group, while mice treated with valproic acid (VPA) had lower body weight gain and lower water consumption. TTA03-107 treatment reduced the severity of arthritis, as evidenced by a reduction in inflammatory cell infiltration and cartilage destruction (Fig. 2), as well as a decrease in serum levels of TNF-α, IL-1β, and IL-17A. However, no changes in clinical scores were observed between the VPA-treated and control groups. TTA03-107 also reduced CII-specific IgG2b but had no impact on other IgG levels. These suggest TTA03-107 may have a beneficial effect on arthritis-related inflammation.

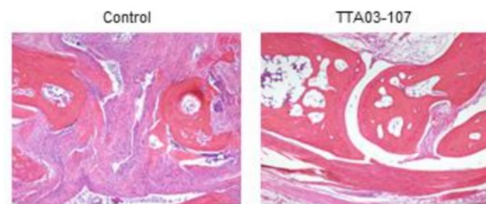

Fig.2 H&E staining graphs for histological changes

② TTA03-107 exerts an antiarthritic effect in the later phase

TTA03-107 was administered to CAIA model mice via drinking water and did not affect body weight gain. It effectively relieved arthritic symptoms and suppressed TNF-α, IL-1β, and IL-17A levels in serum and joint tissue, similar to prednisolone treatment. VPA had no significant effect on arthritis severity and decreased water consumption at the start of the experiment.

③ TTA03-107 treatment suppresses osteoclast differentiation

The osteoclast differentiation of BM cells was evaluated in the presence or absence of TTA03-107 using TRAP staining. It was observed that TTA03-107 only moderately suppressed osteoclast formation at higher concentrations, suggesting a minimal direct effect of HDAC1 inhibition on osteoclast differentiation and an unlikely contribution to its antiarthritic activity.

④ TTA03-107 treatment reduces RORγt^{+} Th17-cell differentiation *in vitro*

An *in vitro* Th17 culture was established, and the expression of RORγt was analyzed. TTA03-107 treatment resulted in a reduction of both the frequency and the mean fluorescent intensity of

RORγt⁺ Th17 cells, but there was no significant dose-response relationship.

⑤ TTA03-107 treatment downregulates inflammatory cytokine production by macrophages

The levels of IL-1β, TNF-α, and IL-6 were significantly reduced in TTA03-107-treated M1 macrophages in a dose-dependent manner. Flow cytometric analysis revealed that TTA03-107 downregulated iNOS expression and upregulated arginase 1 (Arg1) expression (Fig. 3). These results suggest that TTA03-107 may downregulate the transcription of genes involved in the production of inflammatory cytokines in M1 macrophages, potentially through promoting M1 to M2 phenotype conversion.

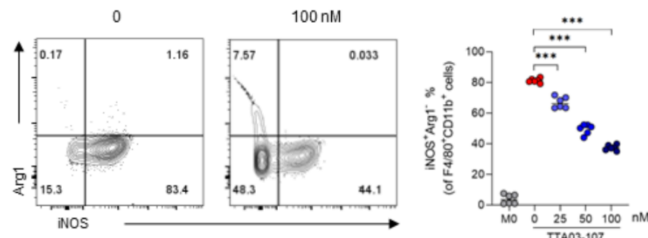

Fig.3 iNOS⁺Arg1⁻ cells (M1) cells within macrophage cell population

2-4. Discussion

The HDAC1-selective inhibitor TTA03-107 was investigated for its therapeutic potential in mouse models of rheumatoid arthritis (RA). TTA03-107 was effective in reducing disease severity and inflammation in the joints of CIA and CAIA models without affecting body weight. HDAC1 inhibition by TTA03-107 treatment significantly decreased IL-17A levels in both serum and joint tissue in the CIA model. This effect was likely due to T-cell-intrinsic HDAC1 inhibition, which arrests Th17 differentiation, as well as the downregulation of IL-6 and IL-1β by macrophages in the joint tissues. An *in vitro* BMDM experiment showed that TTA03-107 reduced the production of TNF-α, IL-1β, and IL-6 and induced M2 macrophage marker Arg1 while downregulating M1 marker iNOS. TTA03-107 slightly decreased CII-specific IgG2b production in the CIA model, while other IgG subclasses remained unchanged. This may be due to the attenuation of germinal center responses to CII. TTA03-107 had only a minor effect on osteoclast differentiation at high concentration, suggesting that regulation of osteoclasts by class I HDAC inhibition may be mediated by HDAC2, HDAC3, or HDAC8, but not HDAC1. Overall, TTA03-107 demonstrates the potential for the treatment of inflammatory arthritis by inhibiting inflammatory cytokine production from inflammatory macrophages and subsequent Th17 responses in autoimmune arthritis models.

論文審査結果の要旨

2023 年 2 月 9 日に実施された副査審査会において、最初に学位申請者が英語にて約 30 分のプレゼンテーションを行い、その後、約 45 分間で英語にて質疑応答を行った。最後に、申請者が退出して論文審査委員による協議を行った。

審査委員からは、腸内細菌代謝物ライブラリを用いた Tfr 細胞分化誘導効果スクリーニングの目的と方法の妥当性に対して質問があった。この質問に対し、過去の講座内の研究例を引き合いに、代謝物ライブラリには Tfr 細胞分化誘導効果を持つ代謝物が含まれる可能性が高いので、実施するに当たって十分な妥当性がある旨を回答した。また実際に幾つかの代謝物を同定して博士論文に記載している点を、合わせて回答した。審査会では 1 次スクリーニングのデータしか提示しなかったために、審査委員からは、どのようにスクリーニング系を構築したのかを示して議論すべきである旨提案があった。この提案に従って博士論文の記述を見直し、議論すると回答した。関連して、スクリーニングで同定した Tfr 細胞分化誘導効果を持つポリアミンをマウスに投与した際には、Tfr 細胞分化誘導効果を示さないことについて審査委員からいくつか質問があがった。まずポリアミンが広範な機能を持つことから、Tfr 細胞分化誘導メカニズムを解析するには不適なのでは無いかとの質問に対しては、指摘の通りにメカニズム解析は難しく、あくまでも *in vitro* での効果であると回答した。さらに、関節リウマチ患者におけるポリアミン濃度についての質問があり、これまでに報告がない旨回答した。スクリーニングに関連した質問として、阻害剤ライブラリの中で Tfr 細胞分化抑制活性を持つ化合物があるという指摘があった。これに対して、指摘の通り Tfr 細胞分化を解明する上では極めて有用な情報であるため、今後の研究課題になると回答した。スクリーニング結果から数百種類のヒット化合物があるのにも関わらず、HDAC 阻害剤に着目するに至った理由について質問があった。これに対して HDAC 阻害剤である SAHA が最も高い Tfr 細胞分化誘導活性を持つ点を説明すると同時に、実際には数個の化合物のみが Tfr 細胞分化誘導活性を持つ点を説明した。誤解を与えやすいグラフであったため、博士論文と公聴会では修正したグラフを提示すると回答した。Tfr 細胞分化誘導活性能において次点にあったテロメラーゼ阻害薬に関して検討したのか質問を受けたが、時間的制約より HDAC 阻害剤に標的を絞って研究を行った旨を回答した。

次に、審査委員より HDAC 阻害剤による関節リウマチ治療薬の新規性やインパクトについて質問が出された。この質問に対して、これまで汎用されてきた HDAC 阻害剤が、14 種類存在する HDAC の広く阻害してしまう事実を述べ、本研究による HDAC1 と HDAC2 のそれぞれのアイソザイムを選択的に阻害することによる利点と説明した。その利点としては、選択的な阻害による副作用の軽減や、選択的阻害剤を利用することによる関節リウマチの症状抑制の背後にある分子メカニズムの解明に資するため、十分な新規性とインパクトがあると回答した。また研究開始時に想定していた、

Tfr を標的とした関節リウマチ治療薬というアイデアは申請者に独自のものであることも説明して理解を得た。

今回用いた HDAC1 の選択的阻害剤と、HDAC2 選択的阻害剤の構造的特徴、特異性、HDAC に対する選択性、生物学的利用能等、標的細胞について、審査委員から多くの質問と指摘が述べられた。まず両者の構造を示して議論を深めることが重要であるという指摘に対しては、HDAC2 選択的阻害剤が特許出願の観点から博士論文に構造は載せることが難しいことを説明し理解を得た上で、公聴会では両者の構造を示して議論する意向だと回答した。次に HDAC1 の選択的阻害剤と、HDAC2 選択的阻害剤の IC_{50} と生物学的利用能を事前に解析した上で、マウスへの投与実験に用いたのかという質問に対しては、IC_{50} は化合物提供者の大阪大学の鈴木孝禎教授のグループが解析していること、また生物学的利用能については自身で LC-MS/MS を用いて予備実験を行った上で投与濃度を決定した旨を回答した。各阻害剤の特異性についての質問に対しては、鈴木教授のグループが解析を実施した旨を回答し、Kd 値等のデータを公聴会と博士論文で示して丁寧に議論すると回答した。さらに、関節リウマチマウスモデルに対して、幾つか濃度を設定した上で予備検討した上で本試験を実施したことを回答した。また実際に関節リウマチの阻害剤として考えた場合に、どちらの阻害剤が有望なのかという質問に対しては、HDAC1 阻害による強い副作用を説明して HDAC2 選択的阻害剤がより有望である旨回答した。*in vitro* では Tfr 細胞分化誘導効果がある HDAC1 選択的阻害剤と HDAC2 選択的阻害剤が、関節リウマチモデルでは Tfr 細胞の分化を誘導しない点を指摘されたことに対しては、生体内では阻害対象となる細胞が多く存在し、関節リウマチモデルではマクロファージや破骨細胞に対する効果がより顕著出たためではないかと意見を述べた。HDAC1 および HDAC2 選択的阻害剤が関節リウマチモデルに対して示すのが予防効果なのか治療効果なのかという質問に対しては、発症後に投与する実験でも症状の増悪を抑制することから予防・治療両面の効果があると回答した。HDAC1 および HDAC2 選択的阻害剤がマクロファージと破骨細胞分化をそれぞれ抑制するメカニズムについて考察を求められたが、現状のデータでは考察が困難であるので、今後トランスクリプトーム解析等を実施して明らかにしていく旨回答した。

関節リウマチモデルの評価方法について、審査委員からは理解が難しいという指摘があった。この点については、口頭で説明し理解を得るとともに、スコアリングの詳細を公聴会で簡潔に平易に説明すると回答した。また、審査委員からは博士論文の不備について指摘があった。審査会の経緯も踏また上で博士論文の適切な修正について指導があり、対応する旨を回答した。学位審査会において、申請者は審査委員からの質問に対して丁寧に回答しており、本研究およびその関連領域に対する知識と理解は十分であると評価された。

公聴会では、HDAC に共通する亜鉛結合モチーフに起因する阻害剤との相互作用について考慮すべきであるとのとの指摘があり、申請者はその通りである旨回答した。

HDAC1 選択的阻害剤と HDAC2 選択的阻害剤のこれまでの HDAC 阻害剤に対するアドバンテージに対する質問に対しては、副作用の低さが見込まれることを回答した。また、*in vitro* で HDAC1 選択的阻害剤と HDAC2 選択的阻害剤が Tfr 細胞の分化誘導能を持つ理由を問われ、その分子メカニズムは今後の課題であるという回答を行った。Treg 細胞ではなく、Tfr 細胞に着目して研究を実施した理由について問われたが、過去の報告で HDAC 阻害剤が Tfr 細胞の分化を促進することが判明していることが理由であると答えた。HDAC1 選択的阻害剤と HDAC2 選択的阻害剤の体内での安定性についての質問に対しては、血中濃度を測定しているので問題ないと回答した。ステロイド製剤によるヒトでの体重減少と食欲についての報告と本研究の結果の差異を指摘されたが、炎症を抑制しているので問題ないと回答した。

全体として申請者の博士論文、学位審査会および公聴会での発表・試問に対する回答は、概ね満足出来るものであった。

論文目録

【主論文に関する原著論文】

Wei Z., Hoshina N., Itoh Y., Tojo T., Suzuki T., Hase K., Takahashi D. A Novel HDAC1-Selective Inhibitor Attenuates Autoimmune Arthritis by Inhibiting Inflammatory Cytokine Production. *Biological and Pharmaceutical Bulletin*, 2022, 45(9): 1364-1372.