RORγt+Foxp3+ regulatory T cells in the regulation of autoimmune arthritis

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Abstract

RORγt+Foxp3+ regulatory T (Treg) cells, known as T regulatory 17 cells (Tr17 cells), are a novel subset of Treg cells, which have the potential to regulate the development of experimental autoimmune encephalomyelitis (EAE) thorough a specific repression of T helper 17 (Th17) cell mediated inflammation. However, the function of Tr17 cells the development of other autoimmune diseases such as autoimmune arthritis remains unclear. Collagen induced arthritis (CIA) was found to be prolonged in Foxp3creRORγtfl/fl mice, in which Tr17 cells were deleted, compared with Foxp3wtRORγtfl/fl mice. Tr17 cells were significantly increased in ankle joints compared with draining lymph nodes after the onset of arthritis. CC chemokine receptor 6 (CCR6) was up-regulated on Tr17 cells compared to RORγt negative Treg cells. CD25, cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF-receptor (GITR), and inducible T-cell co-stimulator (ICOS) expression was also up-regulated on Tr17 cells compared to RORγt negative Treg cells. IL-10-producing cells and Blimp-1+ cells were increased in Tr17 cells compared to RORγt-Treg cells. Tr17-enriched Treg cells significantly suppressed proliferation of conventional T cells compared with CCR6-Treg cells. Tr17 cells increased during the clinical course of CIA and accumulated in inflamed joints. These cells expressed CD25, CTLA4, GITR, and ICOS molecules and up-regulated Blimp-1 and over-produced IL-10. Moreover, CCR6+ Treg cells significantly suppressed cell proliferation. Taken together, it appears that Tr17 cells play a crucial role in the regulation of autoimmune arthritis.

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Summary

RORγt^{+}Foxp3^{+} regulatory T (Treg) cells, known as T regulatory 17 cells (Tr17 cells), are a novel subset of Treg cells, which have the potential to regulate the development of experimental autoimmune encephalomyelitis (EAE) thorough a specific repression of T helper 17 (Th17) cell mediated inflammation. However, the function of Tr17 cells the development of other autoimmune diseases such as autoimmune arthritis remains unclear. Collagen induced arthritis (CIA) was found to be prolonged in Foxp3$^{\mathrm{cre}}$RORγt$^{\mathrm{fl/fl}}$ mice, in which Tr17 cells were deleted, compared with Foxp3$^{\mathrm{wt}}$RORγt$^{\mathrm{fl/fl}}$ mice. Tr17 cells were significantly increased in ankle joints compared with draining lymph nodes after the onset of arthritis. CC chemokine receptor 6 (CCR6)

was up-regulated on Tr17 cells compared to RORγt negative Treg cells. CD25, cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF-receptor (GITR), and inducible T-cell co-stimulator (ICOS) expression was also up-regulated on Tr17 cells compared to RORγt negative Treg cells. IL-10-producing cells and Blimp-1$^+$ cells were increased in Tr17 cells compared to RORγt$^-$Treg cells. Tr17-enriched Treg cells significantly suppressed proliferation of conventional T cells compared with CCR6$^-$Treg cells. Tr17 cells increased during the clinical course of CIA and accumulated in inflamed joints. These cells expressed CD25, CTLA4, GITR, and ICOS molecules and up-regulated Blimp-1 and over-produced IL-10. Moreover, CCR6$^+$ Treg cells significantly suppressed cell proliferation. Taken together, it appears that Tr17 cells play a crucial role in the regulation of autoimmune arthritis.

Key words

Autoimmune arthritis, CD4$^+$ T cells, regulatory T cells, RORγt, Foxp3, T regulatory 17 cells Tr17 cells

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune arthritis leading to severe inflammation, and destruction of articular joints. Susceptibility of RA is associated with the major histocompatibility complex (MHC) class. Drugs that regulate CD4$^+$T cell activation, such as calcineurin inhibitors and abatacept, have been shown to be effective for RA treatment; thus, CD4$^+$T cells are a necessary component of the pathogenesis and continuity of RA[1,2,3].

Differentiation of CD4$^+$T cells into various T helper (Th) cell subsets is regulated by the expression of specific transcriptional factors. Th17 cells are a subset of Th cells that are related with the pathology of RA[4,5]. Retinoic acid receptor-related orphan receptor-γt (RORγt), a transcriptional factor induced in the presence of IL-6 and TGFβ, regulates IL-17 production and CC chemokine receptor 6 (CCR6) expression in CD4$^+$ T cells, resulting in the differentiation of Th17 cells[6,7,8,9]. Because IL-17 produced from joint-infiltrated Th17 cells amplifies the joint inflammation through the facilitation of neutrophil migration into inflamed joints, and the expression of receptor activator of nuclear factor-ϰB ligand (RANKL) on Th17 cells induces bone destruction via the promotion of osteoclast differentiation, RORγt expression was suggested to play an important role in RA development[10,11].

Regulatory T cells are one of the T cell subsets that regulate T cell-induced inflammation. Forkhead box P3 is transcriptional factor (Foxp3) regulates Treg cell differentiation and function through the induction of IL-10 production and cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression[12]. Treg cells and their specific transcription factor, Foxp3, is assumed to play an important role in suppression of RA development[13,14]. Recently,Th17-specific effector Treg cells, known as T regulatory 17 (Tr17) cells, have been reported to express both RORγt and Foxp3 and regulate experimental autoimmune encephalomyelitis (EAE)[15]. In our previous study of Collagen induced arthritis (CIA), we suggested that RORγt$^+$Foxp3$^+$Tr17-like cells might suppress the development of arthritis through the preferential infiltration into inflamed joints and high production of IL-10 in RORγt Tg mice[16]. On the other hand, RORγt$^+$Treg cells include a subpopulation called CD25^{lo}RORγt$^+$Treg cells, which express low levels of CD25 (unstable Foxp3 expression) and can convert to highly arthritogenic Th17 cells[17]. Therefore, we investigated the precise role of Tr17 cells in autoimmune arthritis. In this study, Foxp3^{cre}RORγt$^{fl/fl}$ mice with Tr17 cell deletion showed prolongation of more severe arthritis. Moreover, after the onset of arthritis, Tr17 cells were significantly increased in ankle joints compared with draining lymph nodes, and showed high IL-10 production. Tr17-enriched Treg cells also significantly suppressed proliferation of conventional T cells. Collectively, our study indicates the critical role of Tr17 cells in the inhibition of autoimmune arthritis aggravation thorough high expression of suppressive molecules such as IL-10 and preferential infiltration to inflamed joints.

Methods

Mice

Age- and sex-matched C57BL/6 mice, C57BL/6-Foxp3GFP reporter mice, RORγt$^{fl/fl}$ mice, and Foxp3^{cre}RORγt$^{fl/fl}$ mice (age: 6-12 weeks) were used in our experiments. RORγt$^{fl/fl}$ mice were purchased

from The Jackson Laboratory (USA). C57BL/6-Foxp3GFP reporter mice and Foxp3^{cre} mice were provided by B. Malissen (Université de la Méditerranée, Marseille, France) and A. Yoshimura (Keio University, Japan), respectively. All mice were maintained under specific pathogen-free conditions. All experiments described in this report were performed according to the *Guide for the Care and Use of Laboratory Animals* at the University of Tsukuba and were approved by the Animal Ethics Review Committee of the University of Tsukuba.

Induction of collagen-induced arthritis

Native chicken CII was obtained from Sigma-Aldrich (St. Louis, MO). CII was dissolved in 0.01 M acetic acid and emulsified in Complete Freund's Adjuvant (CFA). CFA was prepared by mixing 5 mg heat-killed Mycobacterium tuberculosis (H37Ra; Difco Laboratories, Detroit, MI) and 1 mL Incomplete Freund's Adjuvant (Sigma-Aldrich). Mice were immunized intradermally at the base of the tail with 200 mg of CII in CFA on days 0 and 21. Arthritis was evaluated visually, and changes in each paw were scored on a scale of 0 to 3 as follows; 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, 3 = ankylosis. The score was summed-up for each limb (maximum score = 12).

Histological assessment

Mice were sacrificed on day 56 post first CII immunization, and both hind limbs were removed. After fixation and decalcification, the joints were cut into sections and stained with hematoxylin and eosin. Quantification of histological changes was carried out by two independent and blinded observers and a histological score was assigned to each joint based on the degree of inflammation and erosion, as described previously[18,19].

Measurement of collagen-specific IgG titers

At 49 days post first CII immunization, serum was collected, then diluted at a ratio of 1:3,000 in blocking solution containing 1% bovine serum albumin in PBS. Collagen-specific total IgG titers were measured by coated 10 mg/mL of CII in PBS on 96-well plates. The optical density was read at 450 nm using a microplate reader.

Cell isolation

Inguinal lymph nodes were collected (draining lymph nodes [LNs]) and used for the experiments. CD4$^+$ cells from draining LNs were isolated by positive selection using the magnetic activated cell sorting (MACS) system with anti-CD4 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4$^+$GFP$^+$Treg (Foxp3$^+$Treg) or CD4$^+$GFP$^-$T cells were isolated with MoFlo cell sorter (DakoCytomation, Glostrup, Denmark) from MACS-isolated CD4$^+$ T cells of C57BL/6-Foxp3GFP mice. The isolated cells were then used forcell culture and flow cytometry.

Cell culture

Sorted CD4$^+$GFP$^+$ Treg cells or CD4$^+$GFP$^-$ T cells (2×10^5 cells/well on 96-well flat-bottom plate) were cultured in RPMI 1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 50 μM 2-mercaptoethanol. The sorted cells were cultured in the presence PMA 50 ng/mL, ionomycin 1 μL/mL, and Golgistop 1 μL/mL in a flat-well for 8 h and analyzed using cytokine staining.

Joint infiltrating cell isolation after the induction of CIA

Ankle joints were collected after CIA induction. The skin was removed from the ankle joints and shaken in 5 mL- RPMI 1640 medium (Sigma-Aldrich) containing 10% FBS, 100 units/mL of penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, and 0.385 mg Liberase. The ankle joints were dismantled and single cells were extracted and suspended in culture medium, and isolated with OptiPrep (60 %v/w iodixanol, Takara Bio) according to the manufacturer's instructions.

Flow cytometry

For flow cytometry, the cell surface was stained with the following antibodies specific for mouse proteins: anti-CD4, anti-CCR6, anti-CD25, anti-CTLA-4, anti-GITR, and anti-ICOS (BioLegend). Mouse Regulatory T Cell Staining Kit (eBioscience; San Diego, CA) was used to stain the transcription factors with anti-Foxp3 (Biolegend) and anti-RORγt antibodies (BD), anti- Blimp-1 (Biolegend) and anti-Helios (Biolegend), or intracellular cytokine staining with anti-IL-17A and anti-IL-10 (BioLegend). Data were acquired using the FACSVerseFlow Cytometer (Becton Dickinson; Mountain View, CA) or the SH800 Cell Sorter (Sony, Tokyo, Japan) and analyzed with FlowJo software (Tree Star; Ashland, OR).

In vitro suppression assay and Treg culture

Responder cells were labelled with 6 μM Cell trace violet (Thermo Fisher), then cultured with or without unlabeled Treg cells at a 1:1 ratio for 72 h in the presence of Dynabeads Mouse T-activator CD3/CD28 (1 bead/cells) in round-bottomed 96-well dishes. The proliferation inhibition rate on the responder was calculated as: (1-(Cell trace violet percentage Treg plus responder cells co-culture/responder cells alone)) x 100%.

Statistical analysis

Data are expressed at mean ± standard error of the mean (SEM). Differences between groups were examined for statistical significance using the Student's t-test, Tukey's test

or Chi-square test. P values less than 0.05 were considered significant.

Results

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To confirm the functional role of Tr17 cells on the development of arthritis, we generated Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice in which the gene encoding RORγt specifically in Foxp3-expressing cells and was induced CIA in these mice. We first checked RORγt expression in Foxp3^{+} Treg cells after the immunization of CII in Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice, and compared it with Foxp3$^{\text{wt}}$RORγt$^{\text{fl/fl}}$control mice. Frequency of RORγt-expressing Foxp3^{+}Tr17 cells was significantly decreased in Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice compared with Foxp3$^{\text{wt}}$RORγt$^{\text{fl/fl}}$ mice (Figure 1a). While the incidence and severity of arthritis was almost unchanged in Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice compared with Foxp3$^{\text{wt}}$RORγt$^{\text{fl/fl}}$ mice, severe arthritis was significantly prolonged in Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice than in Foxp3$^{\text{wt}}$RORγt$^{\text{fl/fl}}$ mice from 56 days after 1st-CII immunization (Figure 1b, c). Joint inflammation and erosion scores tended to be increased in Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice compared with Foxp3$^{\text{wt}}$RORγt$^{\text{fl/fl}}$ mice (Figure 1d). We examined serum CII-specific IgG in Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice using ELISA, because CII-specific IgG level is known to correlate with the development of CIA. There was no difference in CII-specific total IgG between Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ mice and Foxp3$^{\text{wt}}$RORγt$^{\text{fl/fl}}$ mice at day 75 post first CII immunization (Figure 1e). These results proved significant prolongation of arthritis in CIA with Tr17-deficient mice, and it was not associated with anti-CII antibody.

Infiltration of Tr17 cells into ankle joints after onset of CIA through high expression of CCR6

To evaluate the characteristics of Tr17 cells, we analyzed draining LN cells harvested from C57BL/6 mice at days 10, 42, and 56 after 1st-CII immunization, and ankle joint (AJ) cells also harvested after arthritis development. Tr17 cells appeared in LN following CII immunization and increased in the ankle joints compared to LN (Figure 2a, b). Next, we wanted to investigate how Tr17 cells accumulate in inflamed joints after the onset of CIA. A previous study reported that CC chemokine receptor 6 (CCR6) expression contributed to the recruitment of arthritogenic Th17 cells to the inflamed joints[9]; therefore, we examined the expression of CCR6 in Tr17 cells. FACS analysis revealed that CCR6 expression cells was significantly up-regulated in Tr17 cells compared to not only RORγt-negative Treg cells but also RORγt-positive Th17 cells (Figure 2c). Interestingly, when compared to Foxp3$^{\text{wt}}$RORγt$^{\text{fl/fl}}$ control mice, expression of CCR6 was significantly down-regulated in the Foxp3-positive Treg cells but not the Foxp3-netgative T cells of Foxp3$^{\text{cre}}$RORγt$^{\text{fl/fl}}$ cKO mice (Supplementary Figure 1), suggesting that infiltration of Treg cells to inflamed

joints might be impaired in Foxp3^{cre}RORγt$^{fl/fl}$ cKO mice. These findings indicate that joint infiltration of Tr17 cells might contribute to the regulation of CIA prolongation through increased expression of CCR6.

Immunological characteristics of Tr17 cells after CII immunization

We analyzed the expression of cell surface markers related to Treg cell stability and function in Tr17 cells or RORγt-negative Treg cells. Treg-specific molecules such as CD25, CTLA-4, and GITR were significantly increased in Tr17 cells compared to RORγt\sout^{-}-negative Treg cells both of which were isolated from draining LNs on 10 days after 1st-CII-immunization (Figure 3a, b, c). Compared to RORγt-negative Treg cells, ICOS expression was also upregulated in Tr17 cells, which was previously reported as a characteristic marker molecule of Tr17 cells[15](Figure 3d). Moreover, most of the Tr17 cells expressed Helios, indicating that Tr17 cells in CIA might be differentiated from thymus-derived Treg cells, as in a previous report on the EAE model[15](Figure 3e). In Foxp3^{cre}RORγt$^{fl/fl}$cKO mice, although CD25 expression on Treg cells was comparable to Foxp3^{wt}RORγt$^{fl/fl}$ control mice, CTLA-4 and GITR expression on Treg cells was significantly decreased compared to Foxp3^{wt}RORγt$^{fl/fl}$control mice at 10 days after 1st CII-immunization (Supplemental Figure 2); thus, deletion of RORγt in Treg cells results in down-regulation of characteristic Treg cell molecules. These results suggested that Tr17 cells detected in the course of CIA retained a Treg cell nature with characteristic features of Tr17 cells as reported in the EAE model, and that deletion of RORγt might attenuate Treg cell function.

Cytokine production and transcription factor expression in Tr17 cells

To determine the cytokine production with T cell subsets in CIA, we analyzed draining LN cells harvested from C57BL/6-Foxp3GFP mice at day 10 post-1st-CII-immunization. CD4^{+}GFP^{+}Treg cells and CD4^{+}GFP^{-} T cells were isolated and cultured with PMA/ionomycin for 8 h. IL-10 and IL-17 production from these cells were analyzed using flow cytometry, and its relationship to the expression of Foxp3 and RORγt was assessed. IL-10 produced was much higher in Tr17 cells compared to RORγt-negative Treg cells (Figure 4a), whereas IL-17 production was lower in Tr17 cells than RORγt-positive non-Treg cells (Figure 4a). To clarify why IL-10 was increased in Tr17 cells compared to RORγt-negative Treg cells, we checked the expression of Blimp1 and Foxp3. Blimp-1 is essential for the production of IL-10 by Foxp3^{+}Treg cells[20] and preserves Treg cell stability at sites of inflammation[21]. The frequency of Blimp-1 positive cells was increased in Tr17 cells compared to RORγt-negative Treg cells (Figure 4b). Moreover, expression level of Foxp3 was significantly higher in Tr17 cells compared to RORγt-negative Treg cells in spite of the findings that RORγt expression was significantly enhanced in Tr17 cells (Figure 4b). These results indicated that enhanced expression of Blimp-1 might induce increased IL-10 production and maintain expression of Foxp3 in Tr17 cells under inflammatory conditions.

Suppression activity of Tr17 cells

To provide an answer to the question of whether Tr17 cells can suppress effector CD4^{+} T cells, we tried to compare the ability of RORγt-positive and -negative Treg cells to inhibit the proliferation of effecter CD4^{+} T cells in vitro. Because it is difficult to isolate RORγt-positive cells by their expression, we tried to enrich them using CCR6 expression. FACS analysis revealed that about 30% of CCR6^{+} Foxp3-positive Treg cells expressed RORγt (Supplementary Figure 3) and, thus, we used CCR6^{+} Treg cells as Tr17-enriched Treg cells (Figure 5a). To check the equivalence between Tr17 cells and CCR6^{+}Treg cells, we examined the expression of the cell surface molecules typically highly expressed in Tr17 cells (as mentioned above; Figure 2). FACS analysis revealed nearly identical expression levels of CD25, CTLA-4, and GITR between Tr17 cells and CCR6^{+}Treg cells (Supplementary Figure 4) and, thus, it was thought to be reasonable to use CCR6^{+}Treg cell for evaluation of suppressive function as enriched-Tr17 cells.

CD4^{+}GFP^{+} Treg cells and CD4^{+}GFP-conventional T cells were isolated from LN in C57BL/6-Foxp3GFP reporter mice 10 days after 1st CII-immunization, and cocultured with stimulation of T cell receptor in vitro. CCR6^{+}Tr17-enriched Treg cells suppressed the proliferation of conventional T cells (Figure 5b, c). Interestingly, compared to CCR6^{-}Treg cells, the suppressive capacity of CCR6^{+}Tr17-enriched Treg cells was significantly enhanced (Figure 5b, c). These results showed the possibility that Tr17 cells have enhanced

suppressive capability compared with RORγt-negative Treg cells.

Discussion

Foxp3$^+$ regulatory T cell are required to prevent autoimmunity against self-antigens and prevent tissue destruction resulting from excessive immune response. Treg cells differentiate into distinct subsets to inhibit the immune response caused by distinct T helper cell subsets. RORγt$^+$Foxp3$^+$Treg cells have been identified as Tr17 cells, because they regulate Th17 cell-mediated immune response in EAE[15]. By analyzing T cell-specific RORγt transgenic mice, we also discovered the potential of RORγt$^+$Foxp3$^+$Tr17-like cells to suppress the development of CIA[16]. In this study, we observed significant exacerbation of CIA in Foxp3^{cre}RORγt$^{fl/fl}$ mice accompanied by the disappearance of Tr17 cells, and thus it was suggested that Tr17 cells might be a specific regulatory cell subset in the development of autoimmune arthritis.

How do Tr17 cells regulate the pathogenesis of autoimmune arthritis? We propose the following two possibilities: 1) enhancement of suppressive function as Treg cells; and 2) preferential migration of Tr17 cells into inflamed joints.

First, we examined the regulatory function of Tr17 cells and found that suppressor molecules such as CTLA-4, GITR, and IL-10 were significantly up-regulated in Tr17 cells compared to RORγt negative Treg cells after CIA induction. CTLA-4 expression on Treg cells is required to suppress CD4$^+$T and CD8$^+$T cells via the blockade between CD28 on T cells and CD80/86 on dendritic cells. It is also required to prevent the differentiation from naive T cells to Th-subsets and induce T cells anergy[22,23,24]. It was also reported that CIA was exacerbated in CTLA-4 KO mice[25]. GITR, which is a potential Treg cell marker, controls their suppressive phenotype[26], and regulates autoreactive CD4$^+$T cell activation in EAE[27]. IL-10 is an anti-inflammatory cytokine and plays a crucial role in preventing inflammatory and autoimmune pathologies[28]. A previous study reported that IL-10$^{-/-}$ mice showed CIA deterioration[29] and another study found that Th17 cells express IL-10 receptor and are directly regulated by IL-10[30]. It was also pointed out that immune response of Th17 cells were specifically suppressed by Tr17 cells via IL-10. Consequently, enhanced expression of suppressor molecules in Tr17 cells might play an essential role in regulation against immune response especially by arthritogenic T cells in autoimmune arthritis.

We analyzed the second possibility according to the preferential migration of Tr17 cells into inflamed joints. Treg cells can undergo stimulus-specific differentiation, which is regulated by transcription factors associated with the differentiation of conventional CD4$^+$ T cells[31]. Moreover, differentiated Treg cells have unique migratory properties matched to the stimulus that elicited the initial response. CCR6 is one of the chemokine receptors which is expressed on various immune cells and is required for their trafficking via chemokine ligand CCL20[9]. Past studies have revealed that CCR6 expressed in RORγt-positive Treg cells regulates their recruitment and inhibition of Th17 cell-mediating inflammation[15,32]. Interestingly, CCR6 expression was regulated by RORγt, and also contributed to the recruitment of arthritogenic Th17 cells to the inflamed joints[9]. Our study showed that Tr17 cells accumulated in inflamed joints after the onset of CIA, and that CCR6 was highly expressed in Tr17 cells compared to RORγt-negative Treg cells. Moreover, Treg cell-specific deletion of RORγt Foxp3^{cre}RORγt$^{fl/fl}$ cKO mice resulted in reduction of CCR6-expressing Treg cells. Collectively, we speculated that Tr17 cells might preferentially infiltrate into inflamed joints and regulate arthritogenic Th17-mediated inflammation resulting in suppression of the prolongation of autoimmune arthritis.

Why do Tr17 cells maintain their suppressive capacity despite the expression of RORγt, the master transcription factor in the differentiation of Th17 cells? Past reports have pointed out that RORγt-positive Treg cells had almost the same pattern of methylations status of the Treg-specific demethylated region (TSDR) and other Treg-associated epigenetic loci indicates as RORγt-negative Treg cells[33] and that Tr17 cells highly expressed Blimp-1, which is master transcriptional factor of IL-10 and regulates IL-17 expression, and that Tr17 cells have a role on preservation of stability of Treg cells at inflammation sites[20,21,34,35]. In the current study, Foxp3 expression in Tr17 cells was comparable to RORγt-negative Treg cells. In addition, Tr17 cells highly expressed Blimp-1 compared to RORγt-negative Treg cells. Accordingly, we speculated that RORγt-expressing Tr17 cells maintained their suppressive function through high expression of Blimp-1 in

inflammation.

This study has some limitations. First, we could not clarify the exact mechanism of how Tr17 cells regulate Th17 cell-mediated inflammatory response in vivo and in vitro. To investigate this, it is essential that RORγt-positive cells are isolated with a high degree of purity, and experiments such as adoptive cell transfer of RORγt-positive Tr17 cells into RORγt Foxp3^{cre}RORγt$^{fl/fl}$ cKO mice and in vitro suppression assay against RORγt-positive Th17 cells are performed. The second limitation is that little is known concerning Tr17 cells in the patients with RA. Further analysis should be performed to elucidate the precise role of Treg cells in the development of RA.

In conclusion, our experiments show that Tr17 cells increase during the clinical course of CIA and accumulate in inflamed joints. Tr17 cells express CD25, CTLA4, and GITR molecules and over-produced IL-10 via Blimp-1 up-regulation Moreover, Tr17-enriched CCR6^{+}Treg cells significantly suppress cell proliferation. Taken together, our study demonstrates that Tr17 cells play a crucial role in the regulation of autoimmune arthritis.

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Disclosures

The authors declare that they have no competing interests.

Data Availability Statement

Data are available from the corresponding author on reasonable request.

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Author contributions

K. F., Y. K., I. M., and T. S. designed the project. K. F., Y. K. M. S. and M. Y. performed the experiments. K. F., Y. K., M. S., M. Y., S. S. A. I. T. R. and H. T. analyzed and interpreted the data. K. F., Y. K., I. M. and T. S. contributed to draft the manuscript. T. S. assumed the final responsibility to submit the manuscript for publication. All authors read and approved the final manuscript.

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Figure Legends

Φιγυρε 1. Συππρεσσιον οφ ΄IA ιν Φοξπ3$^{\varsigma\rho\varepsilon}$POPγτ$^{\varphi\lambda/\varphi\lambda}$ μιχε

a) Lymphocytes from the draining lymph nodes were harvested from Foxp3^{wt}RORγt$^{fl/fl}$ mice (n = 5) and Foxp3^{cre}RORγt$^{fl/fl}$ mice (n = 5) at 10 days after immunization of type II collagen (CII). The expression of RORγt in Foxp3^{+}Treg cells was analyzed using flow cytometry. b-c) Foxp3^{wt}RORγt$^{fl/fl}$mice (n =13) and Foxp3^{cre}RORγt$^{fl/fl}$mice (n = 17) were immunized intradermally with CII emulsified with complete Freund's adjuvant (CFA) on days 0 and 21. Incidence (b) and severity (c) of CIA were analyzed from 0 to 75 days after the first immunization of CII. Data were obtained from three independent experiments. d) At 56 days post first CII immunization, joint pathology was evaluated on decalcified hematoxylin and eosin-stained sections. Inflammation and bone erosion scores were assessed in both groups. e) At 75 days post first CII immunization, serum samples were collected from Foxp3^{wt}RORγt$^{fl/fl}$ mice (n = 7) and Foxp3^{cre}RORγt$^{fl/fl}$ mice (n = 6) for measurement of CII-specific total IgG levels.

Figure 2. Infiltration of Tr17 cells into ankle joints after onset of CIA through high expression of CCR6

a) Lymphocytes were harvested from draining lymph nodes on 10, 42, and 56 days and from ankle joints on 42 and 56 days after immunization of CII emulsified with complete Freund's adjuvant in C57BL/6 mice. a) The expression of RORγt in Foxp3^{+}Treg cells was chronologically analyzed using flow cytometry. b) Bar graph shows percentage of Tr17 cells in LN and AJ on day 10, 42, and 56. c) At 10 days after CII immunization CCR6 expression on Tr17 cells, RORγt^{-}Treg cells, and RORγt^{+}non-Treg cells in lymph node were analyzed using flow cytometry.

Figure3. Immunological characteristics of Tr17 cells after CII immunization

a-e) At 10 days after CII immunization, CD25, cytotoxic T-lymphocyte antigen 4 (CTLA-4), Glucocorticoid-induced TNF-receptor (GITR), inducible T-cell co-stimulator (ICOS), and Helios expression on Tr17 cells and RORγt^{-}Treg cells in lymph node were analyzed using flow cytometry (n = 4-6).

Figure 4. Cytokine production and transcription factor expression in Tr17 cells

a) Lymphocytes in draining lymph node were harvested from C57BL/6-Foxp3 GFP reporter mice 10 days after first CII immunization. CD4^{+}GFP^{+} Treg cells and CD4^{+}GFP^{-} T cells were isolated and stimulated with TCR stimulation *in vitro* . The expression of IL-10 and IL-17 in RORγt^{+}GFP^{+} Tr17 cells was analyzed using flow cytometry and compared with that in RORγt^{-}GFP^{+} cells and RORγt^{+}GFP^{-} cells. b) At 10 days after CII immunization, Blimp-1, Foxp3, and RORγt expression in Tr17 cells, RORγt^{-}Treg cells, and RORγt^{+}non-Treg cells in lymph node were analyzed using flow cytometry.

Figure5. Suppression activity of Tr17 cells

a) CD4^{+}T cells isolated from C57BL/6-Foxp3GFP reporter mice. b) Cell trace violet-labeled CD4^{+}GFP^{-} T cells from C57BL/6-Foxp3GFP reporter mice cultured with or without CD4^{+}CCR6^{+}GFP^{+}Treg cells or CD4^{+}CCR6^{-}GFP^{+}Treg cells from C57BL/6-Foxp3GFP reporter mice and stimulated with anti-CD3/28 beads for 72 h. c) Bar graphs show percentage of inhibition rate of conventional T cells.

Figure 6. Schematic diagram of potential

Tr17 cells increased in LNs before induction of CIA and Tr17 cells increased during the clinical course of CIA and accumulated in inflamed joints. Tr17 cells highly expressed CD25, CTLA4, GITR, and ICOS molecules and over-produced IL-10. Tr17 cells suppressed proliferation of conventional T cells. Tr17 cells played a crucial role in the regulation of autoimmune arthritis trough infiltration into ankle joints.

Supplemental Figure 1. CCR6 expression of cKO Treg cells

Lymphocytes in draining lymph node were harvested from Foxp3^{wt}RORγt$^{fl/fl}$ mice (n =5) and Foxp3^{cre}RORγt$^{fl/fl}$ mice (n = 5) 10 days after immunization of CII. The expression of CCR6 on Foxp3^{+}Treg cells or Foxp3^{-}T cells was analyzed using flow cytometry. Bar graphs show percentage of CCR6^{+}T cells (left), MFI of CCR6 (right).

Supplemental figure 2. Functional molecule expression of cKO Treg cells

Lymphocytes in draining lymph node were harvested from Foxp3^{wt}RORγt$^{fl/fl}$ mice (n = 5) and Foxp3^{cre}RORγt$^{fl/fl}$ mice (n = 5-6) on 10 days after immunization of CII. a-c) The expression of CD25, CTLA-4, and GITR on Foxp3^{+}Treg cells was analyzed using flow cytometry. Bar graphs show percentage of Treg cells expressing each molecule (left) and MFI of each molecule (right).

Supplemental figure 3. Profile of CCR6-positiveTreg cells

a) Lymphocytes in draining lymph node were harvested from C57BL/6 mice (n = 6). CCR6 expression on CD4^{+} T cells (left). RORγt expression on Foxp3^{+}CCR6^{+}Treg cells or Foxp3^{+}CCR6^{-}Treg cells (right). b) Bar graphs show expression of RORγt^{+}Treg cells in Foxp3^{+}CCR6^{+}Treg cells and Foxp3^{+}CCR6^{-}Treg cells.

Supplemental Figure 4. Comparison of phenotype between Tr17 cells and CCR6^{+}Treg cells

At 10 days after CII immunization, CD25, CTLA-4, and GITR expression on Tr17 cells and CCR6^{+}Treg cells in lymph node were analyzed using flow cytometry (n = 4-6).

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