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Pathogenic conversion of Foxp3+ T cells into $T_{H17}$ cells in autoimmune arthritis

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Autoimmune diseases often result from an imbalance between regulatory T ($T_{reg}$) cells and interleukin-17 (IL-17)-producing T helper ($T_{H17}$) cells; the origin of the latter cells remains largely unknown. Foxp3 is indispensable for the suppressive function of $T_{reg}$ cells, but the stability of Foxp3 has been under debate. Here we show that $T_{H17}$ cells originating from Foxp3+ T cells have a key role in the pathogenesis of autoimmune arthritis. Under arthritic conditions, CD25hiFoxp3+CD4+ T cells lose Foxp3 expression (herein called exFoxp3 cells) and undergo transdifferentiation into $T_{H17}$ cells. Fate mapping analysis showed that IL-17-expressing exFoxp3 T (exFoxp3 $T_{H17}$) cells accumulated in inflamed joints. The conversion of Foxp3+CD4+ $T_{reg}$ cells to $T_{H17}$ cells was mediated by synovial fibroblast-derived IL-6. These exFoxp3 $T_{H17}$ cells were more potent osteoclastogenic T cells than were naive CD4+ T cell–derived $T_{H17}$ cells. Notably, exFoxp3 $T_{H17}$ cells were characterized by the expression of Sox4, chemokine (C-C motif) ligand 20 (CCL20), IL-23 receptor (IL-23R) and receptor activator of NF-$kappa$B ligand (RANKL, also called TNFSF11). Adoptive transfer of autoreactive, antigen-experienced CD25hiFoxp3+CD4+ T cells into mice followed by secondary immunization with collagen accelerated the onset and increased the severity of arthritis and was associated with the loss of Foxp3 expression in the majority of transferred T cells. We observed IL-17+Foxp3+ T cells in the synovium of subjects with active rheumatoid arthritis (RA), which suggests that plastic Foxp3+ T cells contribute to the pathogenesis of RA. These findings establish the pathological importance of Foxp3 instability in the generation of pathogenic $T_{H17}$ cells in autoimmunity.

Foxp3-expressing $T_{reg}$ cells have an essential role in suppressing immune responses1–5. Mice deficient in Foxp3 develop fatal autoimmune diabetes6–8, and continuous expression of Foxp3 throughout life prevents autoimmunity6. Thus, the stability of Foxp3 expression influences the balance between tolerance and autoimmunity, as well as the efficacy of $T_{reg}$ cell–based therapies. The instability of Foxp3 may underlie the pathogenesis of autoimmune diabetes and lethal protozoa infection7–9, but this concept has been challenged by a report showing that Foxp3 expression is stable in in vivo disease models, including autoimmune arthritis10. A debate has arisen as to whether the plasticity of Foxp3-expressing $T_{reg}$ cells is pathologically relevant. $T_{reg}$ cell development and function are regulated by IL-2, which binds to the receptor complex containing IL-2Ralpha (also called CD25). It was recently shown that Foxp3+ cells are comprised of Foxp3-stable CD25hi and Foxp3-unstable CD25lo populations11–12, the former of which is composed of bona fide $T_{reg}$ cells with sustained Foxp3 expression11. However, the pathological importance of the latter Foxp3-unstable CD25lo population remains unclear.

RESULTS

$T_{H17}$ cells arise from CD25hiFoxp3+ T cells in arthritis

To evaluate the in vivo stability of Foxp3 in Foxp3+CD4+ T cells and its impact on collagen-induced arthritis (CIA), we adoptively transferred CD25hiFoxp3+CD4+ or CD25loFoxp3+CD4+ T cells into mice that we immunized with type II collagen 3 weeks before. One day after transfer, we subjected the mice to secondary immunization with collagen and monitored the production of collagen-specific antibodies (Supplementary Fig. 1). We found that the transfer of CD25hiFoxp3+CD4+, but not CD25loFoxp3+CD4+, T cells reduced joint swelling (Fig. 1a) and bone destruction (Fig. 1b) without affecting the production of collagen-specific antibodies (Supplementary Fig. 2). To examine the stability of Foxp3, we labeled donor CD25hiFoxp3+CD4+ or CD25loFoxp3+CD4+ T cells with carboxyfluorescein succinimidyl ester (CFSE) and monitored Foxp3 expression in donor-derived (CFSE+) CD4+ T cells 1 week after secondary immunization. The majority of CD25loFoxp3+CD4+ T cells...
Figure 1 CD25hiFoxp3+ T cells are unstable Foxp3+ T cells that convert to Th17 cells under arthritic conditions. (a,b) Clinical score (a) and microcomputed tomography analysis of calcaneus in the ankle joints (b) of immunized DBA/1 mice adoptively transferred with 5 × 10^5 CD25hi (a, n = 3; b, n = 6) or CD25lo (a, n = 5; b, n = 10) Foxp3+CD4+ T cells purified from untreated DBA/1 Foxp3hiCD2+ mice. (c) Frequency of Foxp3+ cells in CFSE+ donor-derived T cells. Representative data of five mice are shown. (d,e) Results from the immunized C57BL/6 Ly5.2 mice that were adoptively transferred with total hCD2+ (n = 12), CD25hi (n = 6) or CD25lo (n = 7) Foxp3+CD4+ T cells from untreated B6.Ly5.1 Foxp3hiCD2+ mice. (d) Frequency of hCD2+ cells in donor-derived CD4+ T cells. (e) Top, representative plots of Foxp3 and IL-17 expression in CD25hiFoxp3+ donor- or host-derived CD4+ T cells. Bottom, quantitative analysis of the frequency of IL-17+ cells in Foxp3−CD4+ T cells derived from CD25loFoxp3+ (Ly5.1+Ly5.2−) or naive CD4+ T (Ly5.1+Ly5.2+) donor cells or host cells (Ly5.1−Ly5.2+) (n = 10). All data are shown as the mean ± s.e.m. Statistical analyses were performed using unpaired two-tailed Student’s t test (*P < 0.05, **P < 0.01, ***P < 0.005; NS, not significant). Each dot indicates a single mouse.

>(95%) retained Foxp3 expression, but CD25loFoxp3+CD4+ T cells lost Foxp3 expression in the spleen (35%) and draining lymph nodes (dLNs) (75%) under arthritic conditions (Fig. 1c). These results suggest that the transferred CD25loFoxp3+CD4+ T cells failed to inhibit inflammation and bone destruction because of their loss of Foxp3.

To follow the transferred cells for a longer period of time, we used donor T cells from B6 Ly5.1+ Foxp3hiCD2+ congenic mice and analyzed Foxp3 and IL-17 expression 2 weeks after secondary immunization in donor-derived Ly5.1+CD4+ T cells in host mice with arthritis. The majority of transferred total Foxp3+ or CD25hiFoxp3+ T cells retained Foxp3 expression, which is consistent with a previous report10. In contrast, when we purified and transferred CD25loFoxp3+CD4+ T cells to the arthritic mice, these cells lost Foxp3 expression (30–50%) (Fig. 1d), and the percentage of IL-17+ expressing cells in the donor-derived Foxp3+CD4+ T cells (10–25%) was much greater than that in host-derived Foxp3+CD4+ T cells (<3%) (Fig. 1e and Supplementary Fig. 3). Thus CD25loFoxp3+CD4+ T cells preferentially lose Foxp3 and produce IL-17 in arthritic mice after adoptive transfer.

To determine the contribution of naive CD4+ and CD25loFoxp3+CD4+ T cells to Th17 cell development under arthritic...
Characterization of exFoxp3 T cells in arthritic mice

To monitor the localization of exFoxp3 T cells in vivo, we crossed Foxp3 bacterial artificial chromosome transgenic mice expressing the GFP-Cre recombinase fusion protein\(^7\) with ROSA26-YFP reporter mice\(^{19}\). GFP indicates cells that are currently expressing Foxp3, whereas YFP marks cells that are expressing or did express Foxp3.

Under arthritic conditions, the percentage of exFoxp3 (GFP−YFP−) cells (as a proportion of total CD4+ T cells) was higher in joints than in other lymphoid organs (Fig. 2a and Supplementary Fig. 4), suggesting a preferential accumulation of exFoxp3 T cells in the synovium. In addition, exFoxp3 (GFP−YFP+) T cells expressed higher levels of CCR6 and RANKL than did GFP−YFP− T cells in popliteal LNs (Fig. 2b). Notably, the percentage of IL-17+ cells among exFoxp3 T cells was highest in arthritic joints (Fig. 2c). These results collectively indicate that in vivo, exFoxp3 T cells acquire an activated T\(_{H17}\) phenotype (exFoxp3 T\(_{H17}\) cells) and accumulate in the inflamed synovium.

Instability of Foxp3+ T\(_{reg}\) cells under pathological conditions in vivo has been contentious, as the origin of this Foxp3-unstable population remains unclear. There are three possibilities for the origin of exFoxp3 T cells: thymus-derived T\(_{reg}\) (T\(_{reg}\)) cells, peripherally derived T\(_{reg}\) (pT\(_{reg}\)) cells and activated conventional T cells that transiently express Foxp3 (refs. 20,21). T\(_{reg}\) cells are by their suppressive function and are characterized by the expression and demethylated status of Foxp3 and other T\(_{reg}\) cell signature genes\(^{20,22}\). Flow cytometric analysis indicated that GITR (also called TNFRSF18), neuropilin 1 (Nrp1) and killer cell lectin-like receptor subfamily G member 1 (KLRG1) were similarly expressed by exFoxp3 T cells and GFP+YFP+ T cells (Fig. 2d). exFoxp3 T cells also expressed CD25, folate receptor 4 (FR4), OX40 (also called TNFRSF4), CD39 (also called ENTPD1), CD103 (also called ITGAE) and cytotoxic T lymphocyte–associated protein 4 (CTLA-4), albeit to a lesser extent compared to GFP+YFP+ T cells (Fig. 2d and Supplementary Fig. 5). Thus, although the expression level of a few T\(_{reg}\) marker genes, including CD25 and FR4, was lower in exFoxp3 T cells than in GFP+YFP+ T cells, exFoxp3 T cells expressed most of the phenotypic T\(_{reg}\) markers and were distinguishable from activated conventional T cells that transiently express Foxp3 (Fig. 2d).

Methylation analysis of the Foxp3, Il2ra and Cita4 loci\(^{22}\) in exFoxp3 T cells isolated from spleens and dLNs of arthritic mice revealed that the Foxp3 locus was largely methylated and the Il2ra locus was partially methylated in exFoxp3 (GFP+YFP+) T cells (Fig. 2e). As the Foxp3 locus in T\(_{reg}\) cells is known to be demethylated\(^{22}\), the data suggest that exFoxp3 T cells are not derived from T\(_{reg}\) cells. The Cita4 locus of exFoxp3 T cells was demethylated, making these cells distinct from effector memory T cells and in vitro–induced T\(_{reg}\) cells\(^{22}\).

Genome-wide expression analysis showed that exFoxp3 T\(_{H17}\) cells highly express Cxcr5, Ccr8, Rora and Rorc, which are preferentially expressed in pT\(_{reg}\) cells that are generated through antigen delivery or in T\(_{reg}\) cells in the gut lamina propria\(^{23}\) (Supplementary Figs. 5 and 6), but have lower expression of Il7r (encoding Helios) (Supplementary Figs. 5 and 6). These results suggest that exFoxp3 T\(_{H17}\) cells may...
belong to a subpopulation of pTreg cells rather than Treg cells. Taken together, it is possible that eFoxp3 T cells may comprise a previously unrecognized T cell population or pTreg cell–derived T cell subset that is distinct from Treg cells and activated conventional T cells. Future study of the methylation status of the Foxp3 TSDR region in pTreg cells under physiological and pathological conditions will be helpful to understand the origin of eFoxp3 T cells in more detail.

Synovial fibroblasts convert Foxp3+ T cells into Th17 cells

Accumulation of eFoxp3 Th17 cells in arthritic joints led us to hypothesize that cells that are resident in the joint interact with Foxp3+ cells, facilitating the conversion of Foxp3+ T cells to Th17 cells. To explore the role of synovial cells in the regulation of Foxp3 stability, we isolated Thy1+CD11b+ cells (synovial fibroblasts) and Thy1+CD11b+ cells (synovial macrophages) from the joints of arthritic mice and cocultured them with total Foxp3+CD4+ T cells purified from unmanipulated Foxp3hCD2 mice. Coculture of Thy1+CD11b+ cells, but not Thy1+CD11b+ cells, with Foxp3+CD4+ cells downregulated Foxp3 expression in Foxp3+CD4+ T cells (Fig. 3a). These eFoxp3 T cells upregulate IL-17 but do not express interferon-γ (IFN-γ) or IL-4 when cocultured with arthritic synovial fibroblasts (Fig. 3a,b), suggesting that arthritic synovial fibroblasts promote the conversion of Foxp3+ T cells to Th17 cells in the joints. In contrast, when we cocultured naive CD4+ T cells with Thy1+CD11b+ synovial fibroblasts, they did not differentiate into Th17 cells (Fig. 3b). We detected IL-17+Foxp3+ T cells, which may appear at the transition stage during the conversion of Foxp3+ T cells to Th17 cells, in the joints and lymph nodes of arthritic mice (Supplementary Fig. 7), as well as in the synovium of human subjects with RA (Supplementary Fig. 8).

We next investigated the mechanism by which synovial fibroblasts induce the conversion of Foxp3+CD4+ T cells into Th17 cells. The culture supernatant of synovial fibroblasts (Fig. 3c) or coculture of Foxp3+CD4+ T cells with transwell-separated synovial fibroblasts (Fig. 3d) induced conversion into Th17 cells, indicating that cell-cell contact is not essential for conversion. We explored the soluble factors mediating this conversion by analyzing the expression of various cytokines in the supernatant (Fig. 3e and data not shown) and found that IL-6 was highly produced by Thy1+CD11b+ cells (Fig. 3e). The expression of IL-6 by arthritic synovial fibroblasts was further enhanced by IL-17, suggesting a potential positive feedback loop (Fig. 3e). A neutralizing antibody against IL-6, but neither an antibody to TNF-α nor one to IL-1β, inhibited the generation of Th17 cells after the coculture of Foxp3+CD4+ T cells with synovial fibroblasts (Fig. 3f). These results indicate that synovial fibroblast–derived IL-6 has a crucial role in the conversion of Foxp3+CD4+ T cells to Th17 cells.

eFoxp3 Th17 cells are potent osteoclastogenic T cells

To evaluate the contribution of Th17 cells of a Foxp3+ T cell origin to the bone destruction that occurs in arthritis, we examined their ability to induce osteoclastogenesis by counting the number of osteoclasts defined by TRAP+ multinucleated cells (MNCs) and the expression of RANKL, a cytokine that is essential for osteoclast differentiation16, in comparison with both naive CD4+ T cell–derived Th17 cells and Foxp3+ T cells. We found that eFoxp3 Th17 cells had higher osteoclastogenic ability than naive CD4+ T cell–derived Th17 cells and Foxp3+ T cells. We detected Il-17+Foxp3+ T cells cultured in the presence of IL-2 did not have osteoclastogenic ability, which is consistent with a previous report24. We confirmed these findings further using Foxp3hCD2 mice crossed with IL–17–GFP knock-in mice, which enabled us to purify IL–17–expressing cells (Supplementary Fig. 9).
The osteoclastogenic ability of T\(_{H17}\) cells has been attributed mainly to their production of IL-17, which stimulates RANKL expression in fibroblasts, as T\(_{H17}\) cells alone cannot induce osteoclastogenesis despite their RANKL expression\(^2\). However, T\(_{H17}\) cells that were differentiated under T\(_{H17}\)-polarizing conditions still induced osteoclastogenesis, suggesting a potential contribution of T cell–derived RANKL or RANKL-inducing cytokines other than IL-17A (Fig. 4b). We observed that exFoxp3 T\(_{H17}\) cells expressed higher amounts of RANKL than naive CD4\(^+\) T cell–derived T\(_{H17}\) cells (Fig. 4c) and coculture with synovial fibroblasts further enhanced

**Figure 4**  exFoxp3 T\(_{H17}\) cells are osteoclastogenic T cells with distinct gene profiles. (a,b) Osteoclast differentiation in a coculture of BMMs, Thy1\(^+\) CD11b\(^-\) CIA synovial fibroblasts and the T cell subsets indicated. exFoxp3 T\(_{H17}\) indicates Foxp3\(^-\) T cells developed from Foxp3\(^+\) T cells under T\(_{H17}\)-polarizing conditions in this experiment. (a) Representative tartrate-resistant acid phosphatase (TRAP) staining. (b) Number of osteoclasts (TRAP\(^+\) MNCs) (n = 3 for exFoxp3 T\(_{H17}\) and Foxp3\(^+\) T cells, n = 4 for T17a\(^-\) T\(_{H17}\) cells, n = 6 for all other groups). WT, wild type. (c) Representative FACS profiles of RANKL and IL-17 expression in naive CD4\(^+\) T cell–derived T\(_{H17}\) cells (left) and exFoxp3 T\(_{H17}\) cells (middle and right) that differentiated under the conditions indicated. (d) Number of osteoclasts (TRAP\(^+\) MNCs) in a coculture of BMMs and the T cell subsets indicated (n = 3). (e) Number of osteoclasts (TRAP\(^+\) MNCs) in a coculture of BMMs, synovial fibroblasts and the T cell subsets indicated. (f) Analysis using exFoxp3 T\(_{H17}\) cells derived from Tnfsf11\(^+/\) Tmfn3\(^+/+\) or Lck-Cre Tnfsf11\(^+/\) Tmfn3\(^−/−\) mice. (g) Microarray analysis of selected T\(_{H17}\)-related genes in exFoxp3 T\(_{H17}\) cells and T\(_{H17}\) cells. (h) Quantitative RT-PCR analysis of differentially expressed genes in T\(_{H17}\) cells. (i) Mean fold change of three independent experiments is shown. All data are representative of three independent experiments with triplicate culture wells and are shown as the mean ± s.e.m. Statistical analyses were performed using unpaired two-tailed Student’s t test (*P < 0.05, ***P < 0.005).
Autoreactive CD25loFoxp3+ T cells promote arthritis

Self tolerance is maintained by thymic-derived stable Foxp3+CD4+ T cells, which have a higher affinity for self antigens.22,23. We hypothesized that unstable Foxp3+CD4+ T cells also contain self-reactive T cells and thus exert a potent arthritogenic effect after losing Foxp3 expression. To investigate the role of autoantigen-specific exFoxp3 T cells, we purified CD25loFoxp3+ CD25hiFoxp3+ cells, total Foxp3+ and effector memory CD44hiFoxp3+ CD44+ T cells from collagen-immunized DBA/1 Foxp3−CD2 mice, which harbor collagen-specific T cells. After CFSE labeling, we transferred these cells into immunized mice 1 d before secondary immunization. Notably, CD25loFoxp3+ T cells accelerated the onset and increased the severity of arthritic symptoms more than total Foxp3− or CD44hiFoxp3+ CD44+ T cells (Fig. 5a-c). In contrast, the transfer of CD25hiFoxp3+ CD4+ T cells markedly inhibited osteoclast formation. More than half (50–70%) of the CD25hiFoxp3+ T cells lost Foxp3 expression, whereas almost all (>96%) of the CD25loFoxp3+ cells retained Foxp3 expression (Fig. 5d). CD25loFoxp3+ T cells proliferated in response to type II collagen in vitro to a greater extent than did CD25hiFoxp3+ cells, suggesting that the CD25loFoxp3+ cells contained a higher number of autoreactive T cells (Fig. 5e).

To further examine whether the exacerbation of arthritic symptoms elicited by CD25loFoxp3+ CD4+ T cells is dependent on type II collagen-specific responses, we transferred CD25loFoxp3+ CD4+ T cells from ovalbumin (OVA)-immunized mice to collagen-immunized mice. OVA-specific CD25loFoxp3+ CD4+ T cells did not exacerbate arthritis scores (Fig. 5a). These results suggest that autoreactive pathogenic CD4+ T cells were generated mainly from CD25loFoxp3+ CD4+ T cells under arthritic conditions.

**DISCUSSION**

This study demonstrates that CD4+ T117 cells with arthritogenic and autoreactive properties arise from Foxp3+CD4+ T cells, thus establishing the in vivo pathological importance of Foxp3+CD4+ T cell conversion to T117 cells. The pathogenic function of IL-17+ exFoxp3 T cells may be enhanced by their higher affinity to self antigens, as well as their ability to accumulate and proliferate in inflamed tissues and stimulate...
osteoclastogenesis. Conversion of Foxp3+/CD4+ T cells into Th17 cells in the periphery was promoted by arthritic synovial fibroblasts, thereby uncovering a new interaction of immune and tissue-resident mesenchymal cells in the breakdown of self tolerance. We propose that the fate of plastic Foxp3+ T cells is a critical determinant of self tolerance versus autoimmunity. The balance between IL-2 and IL-6 regulates the development of Threg and Th17 cells from naive CD4+ T cells34, but the fate of plastic Foxp3+ T cells may also be determined by this cytokine balance35 (Supplementary Fig. 12). Blockade of IL-6 signalling increases the ratio of Foxp3+ to Th17 cells in the course of RA treatment36, suggesting that some of the beneficial effects of these therapies may derive from regulation of the plasticity of Foxp3+ T cell fate. Blockade of TNF-α also increases the ratio of Threg to Th17 cells37,38, and it was recently reported that this effect is attributable to the recovery of Threg cell function38. These reports suggest that regulating the balance of Threg to Th17 cells is important for the treatment of RA. Several RA-associated genes39, including Ptpn22, Ccr6 and Tnfifs14, are highly expressed in exFoxp3 Th17 cells (Supplementary Fig. 13), and we observed IL-17+Foxp3+ T cells in subjects with active RA (Supplementary Fig. 8), suggesting the potential role of exFoxp3 Th17 cells in the pathogenesis of RA. Notably, we found that exFoxp3 Th17 cells specifically express a set of surface molecules that may be useful in identifying arthritogenic T cells and may contribute to future diagnostic and therapeutic strategies for RA (Supplementary Fig. 10d). The presence of exFoxp3 Th17 cells may be used as a biomarker for RA and be useful for predicting responsiveness to anti–IL-6 therapy. Further characterization of the mechanisms underlying the conversion and plastic function of Foxp3+ T cells will provide new insights into the maintenance and restoration of self tolerance, and this will in turn lead to the development of new therapeutic strategies for autoimmune diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data have been deposited in the Gene Expression Omnibus database with accession code GSE48428. Note: Any Supplementary Information and Data Source files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

N.K. designed and performed experiments, interpreted the results and prepared the manuscript. K.O., S.S., T.N. and M.O. contributed to study design and manuscript preparation. T.K. contributed to microarray analysis. S.T. contributed to the analysis of human RA and osteoarthritis samples. I.A.B. generated Foxp3-GFP-Cre mice and contributed to study design and data interpretation. H.T. directed the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Mice were kept under specific pathogen–free conditions, and all animal experiments were performed with the approval of the Institutional Review Board at the University of Tokyo, B6.SJL (CD45.1+) and IL-17–GFP knock-in mice were obtained from the Jackson Laboratory and BIOCYTOGEN, respectively. Foxp3-Cre2ES2 knock-in mice,11 Foxp3-GFP-Cre mice,12 ROSA26-loxP-Stop-loxP-YFP reporter mice,13,14 Il17a−/− mice15 and Tnfsf11floxp mice41 were described previously. 8- to 12-week-old sex-matched mice were used for experiments unless otherwise mentioned.

Analysis of T cells in the synovium of subjects with RA or osteoarthritis. Human synovial tissue specimens were obtained from subjects undergoing joint replacement surgery or synovectomy at the Tokyo University Hospital. All the subjects with RA fulfilled the 2010 American College of Rheumatology–European League Against Rheumatism criteria for the classification of RA and provided written informed consent. This study was approved by the Institutional Review Board at The University of Tokyo. The tissue was digested with type II collagenase (1 mg ml−1; Worthington) for 2 h at 37 °C. After being filtered, cells were stimulated with phorbol myristate acetate and ionomycin for 5 h. After fixation, cells were examined for the expression of Foxp3, IL-17, CD3 and CD4. Synovitis activity was evaluated macroscopically by the redness and proliferative status of the synovium membrane.

Induction of CIA. We performed CIA in 8- to 12-week-old male C57BL/6J and DBA/1J mice. Mice were immunized with an emulsion consisting of 50 μl of chicken type II collagen (Sigma–Aldrich; 4 mg ml−1) and 50 μl of adjuvant given intradermally into the base of the tail at two sites. For the DBA/1 mice, we added heat-killed Mycobacterium tuberculosis H37Ra (Difco Laboratories; 5.0 mg ml−1) in incomplete Freund’s adjuvant (Difco Laboratories). For B6 mice, we added H37Ra (3.3 mg ml−1) in complete Freund’s adjuvant (DFA) (Difco Laboratories). Three weeks after the primary immunization, mice were challenged with the same collagen and CFA emulsion as the primary immunization. We judged the development of arthritis in the joint using the following criteria: 0, no joint swelling; 1, swelling of one finger joint; 2, mild swelling of the wrist or ankle; or 3, severe swelling of the wrist or ankle. The scores for all fingers of forepaws and hindpaws, wrists and ankles were totaled for each mouse (with a maximum possible score of 12 for each mouse).

T cell isolation and sorting. Single-cell suspensions were obtained from peripheral LNs and the spleen. Splenic erythrocytes were eliminated with red blood cell lysis buffer (Sigma–Aldrich). To purify the peripheral CD4+ T cell subpopulation obtained from Foxp3−/− mice, the pooled spleen and LN cells were subjected to depletion of any adherent cells by panning with goat anti-mouse IgG Fc receptor (Becton Dickinson) and subjected to FACS sorting using FACSAriaIII (with a maximum possible score of 12 for each mouse).

T cell differentiation in vitro. T cells were cultured in Iscove’s modified Dulbecco’s medium (Sigma–Aldrich) supplemented with 2 mM l-glutamine, 10% FBS, 50 μM 2-ME, 100 U ml−1 penicillin and 100 μg ml−1 streptomycin. The following reagents were used at the concentrations indicated: 5 μg ml−1 anti–IFN-γ (XMGL2, BD Biosciences), 5 μg ml−1 anti–IL-10 (11B11, BD Biosciences), 10 μg ml−1 anti–IL-6 (MOP-20F3, eBioscience), 10 μg ml−1 anti–TNF-α (1F11, BD Biosciences, 10 μg ml−1 anti–IL-1β (B122, eBioscience), 10 ng ml−1 recombinant mouse IL-1β (rmIL-1β, R&D Systems), 10 ng ml−1 rmIL-2 (R&D Systems), 100 ng ml−1 rmIL-6 (PeproTech), 10 ng ml−1 rmIL-12 (PeproTech), 10 ng ml−1 recombinant human transforming growth factor-β1 (rhTGFB-1) (R&D Systems) and 50 ng ml−1 IL-23 (R&D Systems). For T cell stimulation, the sorted T cells were stimulated with beads coated with monoclonal antibodies to CD3 and CD28 (Dynal; 25 μl per 1 × 10^6 cells) for 3 d. For T helper 1 cell polarization, naive CD4+CD62L+Foxp3−CD4+ T cell stimulation was performed in the presence of rmIL-12 and anti–IL-4. For T helper 17 cell polarization, naive CD4+ T cells were stimulated in the presence of rmIL-1β, rmIL-6, rmIL-23, rhTGFB-β1, anti–IFN-γ and anti–IL-4. Naive CD4+ T cells stimulated in the presence of anti–IFN-γ and anti–IL-4 were used as T0 cells. Foxp3−CD4+ T cell stimulation was performed in the presence of IL-2 were used as Foxp3+ T cells. For the coculture with synovial fibroblasts, synovial cells (1 × 10^6 cells per well) were cultured for 1 d before coculture using 96-well flat-bottom plates. The sorted T cells (1 × 10^6 cells per well) and beads coated with monoclonal antibodies to CD3 and CD28 were added to the culture of synovial fibroblasts. For transwell assays, synovial fibroblasts (6 × 10^4 cells per lower well) and T cells (1.5 × 10^6 cells per upper well) were cocultured using 0.4-μm pore 24-well transwell plates (Coster) in the presence of beads coated with monoclonal antibodies to CD3 and CD28.

Type II collagen–specific response. Titers of collagen-specific antibodies in the serum were measured by ELISA using the SBA Clonotyping System (SouthernBiotech). For the T cell proliferative response, CD25+Foxp3+CD4+ and CD25+Foxp3−CD4+ T cell populations were sorted from dLNs and spleens of collagen-immunized mice 1 d after immunization. Purified cells were labeled with CFSE and cultured (1 × 10^6 cells per well) in the presence of denatured type II collagen (100 μg ml−1), IL-2 (10 ng ml−1) and Thy1.2-depleted, mitomycin C (Sigma–Aldrich)–treated splenocytes (4 × 10^5 cells per well) using 96-well U-bottom plates for 3 d.

Preparation of arthritic synovial fibroblasts. Synovial tissues from the ankles of mice with CIA were minced and digested by type II collagenase (1 mg ml−1; Worthington) in DMEM (Sigma–Aldrich) for 2 h and then cultured in DMEM containing 20% FBS. To prepare Tnfsf11floxp arthritic synovial fibroblasts, Tnfsf11floxp mice were administered 2 mg of antibody to type II collagen (Chondrex) intravenously on day 0 and 50 μg of lipopolysaccharide intraperitoneally on day 3. Cultured fibroblasts during the fourth to seventh passages were used for the experiments. Thy1+CD11b+ synovial fibroblasts and Thy1−CD11b+ macrophages were sorted by FACSariaIII.

Flow cytometry. Antibodies conjugated with biotin, FITC, Alexa Fluor 488, PE, PerCP-Cy5.5, allophycocyanin (APC), Alexa Fluor 647, eFluor 450 or V500 were used at a 1:100 dilution unless otherwise mentioned. The following monoclonal antibodies were purchased from eBioscience: anti-human CD2 (RPA-2; 2,10), CD3 (OKT3), CD4 (OKT4), FOXP3 (362556), anti-mouse CD3ε (145-2C11), CD4 (RM4-5), CD11b (also called ITGAM) (M1/70), CD25 (PC61), CD49 (24DMS1), CD44 (IM7), CD45.1 (also called PTTPC) (A20), CD45.2 (104), CD26 (also called SELL) (MEL-14), CD90.2 (also called Thy1) (30G11), CD103 (2E7), OX40 (also called CD134) (OX-86), GITR (also called CD357) (DTA-1, 1:1600), Thy1 receptor-β (TGRB/β) (H57-597), CCR6 (140706), RANKL (IK22/5), KLRG1 (2F1), FR4 (eBio12A5; 1:400), Foxp3 (FJK-16), CTLA-4 (UC10-489), Ki-67 (B56), IFN-γ (XMGL2), IL-2 (11B11) and IL-17A (eBio1787). Anti-Helios (22F6) was purchased from BioLegend. Goat anti-mouse/rat NrP1 (FABS667, 1:40) was purchased from R&D Systems. For intracellular Foxp3 staining, the Foxp3 Staining Buffer Set (eBioscience) was used. For intracellular cytokine staining, cells were stimulated with 50 ng ml−1 phosphor myristate acetate (Sigma–Aldrich), 300 ng ml−1 ionomycin (Sigma–Aldrich) and GolgiPlug (BD Biosciences) for 5 h. After washing, cells were stained for surface antigens, fixed with 4% paraformaldehyde (Nacali Tesque) for 10 min at room temperature, permeabized and stained with monoclonal antibodies to cytokine diluted in Perm/Wash Buffer (BD Biosciences). For the measurement of cytokine concentration in the culture supernatants, BD Cytometric Bead Array was performed. Flow cytometric analysis was performed by FACSCanTo II with Diva software (BD Biosciences).

CpG methylation analysis by bisulfite sequencing. After sodium bisulfite treatment (MethylEasy Xced, Human Genetic Signatures) of genomic DNA, modified DNA was amplified by PCR and subcloned into pcUCl18 Hinc 2/BAP (Takara). PCR primers for the Foxp3 intron 1, Citad exon 2 and I2ra intron 1a regions were described previously.12
In vitro assay of osteoclast differentiation. Primary bone marrow cells were suspended in culture medium (α-MEM containing 10% FBS) supplemented with 10 ng ml$^{-1}$ macrophage colony-stimulating factor (M-CSF) (R&D Systems) for 2 d to obtain BMMs. For a coculture of BMMs and T cells, BMMs (5 × 10$^4$ cells per well) were cultured with sorted T cells (1 × 10$^5$ cells per well) in the presence of 10 ng ml$^{-1}$ M-CSF and beads coated with monoclonal antibodies to CD3 and CD28 for 7 d using a 96-well flat-bottom plate, and TRAP$^+$ MNCs (more than three nuclei) were counted. For a coculture of BMMs, synovial fibroblasts and T cells, BMMs (5 × 10$^4$ cells per well) and sorted T cells (1 × 10$^5$ or 5 × 10$^4$ cells per well) were cocultured with synovial fibroblasts (1 × 10$^3$ per well), which were isolated and cultured 1 d before coculture. Coculture was performed in the presence of beads coated with monoclonal antibodies to CD3 and CD28. After 5 d, TRAP$^+$ MNCs were counted.

Analysis of bone phenotype. The histomorphometric analysis has been described previously$^{41}$. The articular cartilage dysfunction score was calculated by the ratio of the toluidine blue-negative area to the total articular cartilage area. For the microcomputed tomography analysis, calcaneus in the ankle joints of arthritic mice 5 weeks after secondary immunization was subjected to three-dimensional microcomputed tomography. Computed tomography scanning was performed using a ScanXmate-A100S Scanner (Comscantechno). Three-dimensional microstructural image data were reconstructed, and structural indices were calculated using TRI/3D-BON software (RATOC).

GeneChip analysis. The GeneChip analysis was performed as described previously$^{42}$. IL-17GFP$^+$Foxp3$^{3CD2^+}$ cells differentiated from Foxp3$^{3CD2^+}$CD4$^+$ cells under Th17-polarizing conditions for 4 d were sorted and used as exFoxp3 Th17 cells. IL-17GFP$^+$Foxp3$^{3CD2^+}$ cells differentiated from naive CD4$^+$ cells under Th17-polarizing conditions were used as Th17 cells. IL-17GFP$^+$Foxp3$^{3CD2^+}$ cells after the culture of Foxp3$^{3CD2^+}$CD4$^+$ cells in the presence of IL-2 were used as Treg cells. Treg cells were also used for this analysis. 8–10 × 10$^5$ cells of each T cell subset after culture were subsequently subjected to RNA extraction. The total RNAs extracted from these cells were used for cDNA synthesis by reverse transcription, followed by synthesis of biotinylated cRNA through in vitro transcription. After cRNA fragmentation, hybridization with the Mouse Genome 430 2.0 Array (Affymetrix) was performed as described previously$^{42}$. The main part of the data set was deposited and can be obtained from the Genome Network Platform (http://genomenetwork.nig.ac.jp/). We performed microarray analysis using three sets of T cell samples that were independently prepared from 16–18 IL-17GFP$^+$Foxp3$^{3CD2^+}$ mice (51 mice in total). The microarray data have been deposited in the Gene Expression Omnibus database with accession code GSE48428.

Statistical analyses. Statistical analyses were performed using one-way analysis of variance with Newman-Keuls multiple comparison test and unpaired two-tailed Student's t test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$; NS, not significant; ND, not detected in all figures). All data are expressed as the mean ± s.e.m. The results are representative examples of more than three independent experiments. We estimated the sample size considering the variation and mean of the samples. We tried to reach the conclusion using as small a size of samples as possible. We usually excluded samples if we observed any abnormality in terms of size, weight or apparent disease symptoms in mice before performing experiments. However, we did not exclude animals here, as we did not observe any abnormalities in the present study. Neither randomization nor blinding was done in this study. Statistical tests are justified as appropriate for every figure, and the data meet the assumptions of the tests.