



# Induced regulatory T cells remain suppressive capability on effector T cells and synovial fibroblasts in collagen-induced arthritis

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## Abstract

Rheumatoid arthritis (RA) is a common autoimmune disorder initiated by inflammatory synovitis. Hyperproliferation of destructive synovial fibroblasts (SFs) is one of the pathogenic mechanisms of RA. Abnormalities in regulatory T cells (Tregs) may also play a critical role in this progression. To date, it is unclear whether both natural Tregs (nTregs) and induced Tregs (iTregs) share similar characteristics in RA progression and whether Tregs directly suppress the autoaggressive activities of SFs. In this study, we compared suppressive effects on effector T cells (Teffs) and inflamed SFs between nTregs and iTregs in a collagen-induced arthritis (CIA) model. Our results demonstrated that iTregs but not nTregs maintained a suppressive effect on Teffs after adoptive transfer into CIA mice. Additionally, we discovered that iTregs directly inhibited the destructive activities of CIA-SFs. Thus, this study suggests that administration of the iTreg subset has great potential for treatment of RA in the clinic in the future.

**Keywords** Induced regulatory T cells · Natural regulatory T cells · Synovial fibroblasts · Collagen-induced arthritis · Rheumatoid arthritis

## Introduction

Rheumatoid arthritis (RA), a classic autoimmune disease, is initiated with primary inflammatory synovitis, followed by articular cartilage destruction and bone erosion [1, 2]. Inflamed synovial fibroblasts (SFs) have a semitransformed, autoaggressive phenotype and have been demonstrated to be a main cell type that contributes to the pathogenesis of RA [2].

CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) act as a dominant regulator to maintain immune homeostasis and prevent autoimmune diseases [3, 4]. Abnormalities in the population or regulatory function of Tregs may be closely associated with the development of autoimmune disorders, including RA [5, 6]. Tregs generally consist of two types, thymus-derived natural Tregs (nTregs) and Tregs induced in the periphery *in vivo* or with IL-2 and TGF- $\beta$  *in vitro* (iTregs) [7, 8]. Our and

others' studies have provided much evidence that the nTreg and iTreg subsets have several differences [9–11].

Komatsu. et al. reported that when nTregs were adoptively transferred into a Collagen-Induced Arthritis (CIA) model, one typical animal model of RA, they preferentially lost Foxp3 expression, exhibited a pathogenic Th17 phenotype and infiltrated inflamed joints [12]. These Th17 cells destroyed the synovium, cartilage, and even bone and exacerbated arthritis progression. We wondered whether iTregs have a more stable biological phenotype after infiltration of the inflammatory microenvironment in a CIA model. To address this issue, we directly compared the suppressive characteristics of the nTreg and iTreg subsets on the activities of effector T cells (Teffs) in the inflammatory microenvironment in a CIA model. Furthermore, it is unclear whether Tregs directly target SFs in a CIA model. We also investigated this issue in this study.

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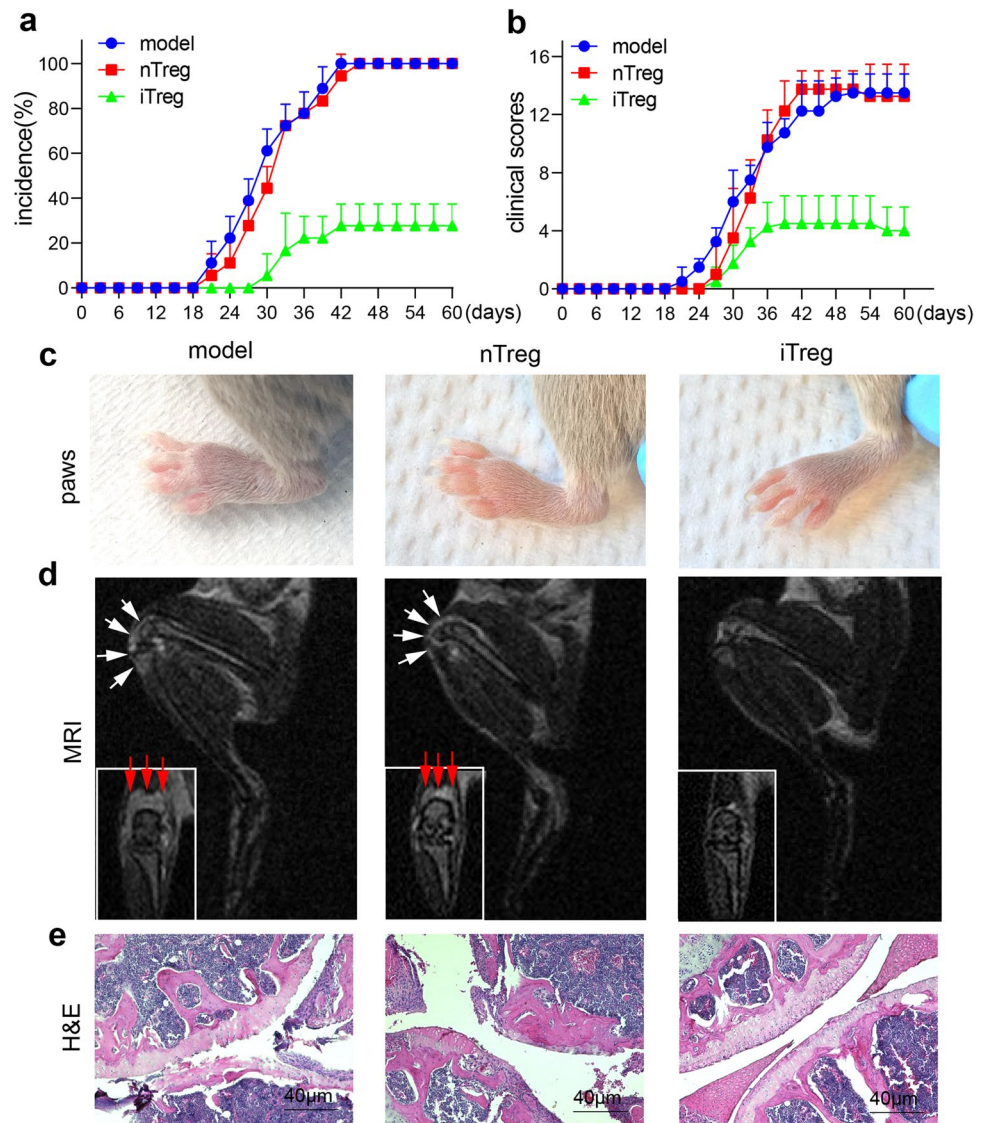
## Results

### The iTreg subset exhibits a protective effect limiting clinical progression in a CIA model

To identify whether the iTreg subset has a more stable regulatory effect on T effs than nTregs in a CIA model, we performed several experiments with DBA1/J mice. First, we established a CIA model with bovine type II collagen (CII) and Freund’s complete adjuvant (CFA) immunization. On day 14, we adoptively transferred 3 million nTregs or iTregs into the CIA model. Other CIA model mice were treated with the same amount of PBS as a control. Then, we recorded the clinical incidence and severity of CIA every day until day 60. Our results showed that the clinical incidence and severity of CIA failed to decrease following

infusion of nTregs, while infused iTregs significantly decreased the incidence and clinical scores and delayed the onset of CIA (Fig. 1 a, b). On day 45, the peak of the disease, we compared the four paws of each mouse. As we hypothesized, the extent of redness and swelling in the paws of the mice that received nTregs was obviously similar to the extent in model mice, but iTregs successfully prevented the development of joint swelling (Fig. 1c). We also evaluated the knee joints of the mice by MRI. Prominent synovial fluid effusion and swollen synovium were found in the knee joints in the model group and nTreg group, but in the iTreg group, fluid effusion was rarely detected (Fig. 1d). Then, the knee joints were subjected to H&E staining. The pathological results showed that in both CIA model mice and CIA model recipients treated with nTregs, the knee joints showed marked inflammatory changes, such as synovial

**Fig. 1** iTregs but not nTregs exhibit protective effects in a CIA model. DBA1/J mice were used for CIA induction. On day 14 after CII/CFA immunization,  $3 \times 10^6$  nTregs or iTregs were intravenously injected into CIA model mice. The incidence and clinical scores of CIA were recorded and compared every day (a, b). On day 44, images of hind paw swelling were taken for each mouse (c), the knee joints were subjected to MRI, and arthritis severity was evaluated blindly by two imaging specialists. The white arrows points swollen synovium and synovial effusion in sagittal imaging and the red arrows point to these lesions in coronal imaging. (d). Then, the mice were sacrificed, the knee joints were subjected to H&E staining ( $200\times$ , scale bars:  $40\ \mu\text{m}$ ), and the severity of arthritis was evaluated blindly by two pathologists (e).  $** P \leq 0.01$ ,  $*** P \leq 0.001$ ; error bars denote the SD. The experiments were repeated five times independently



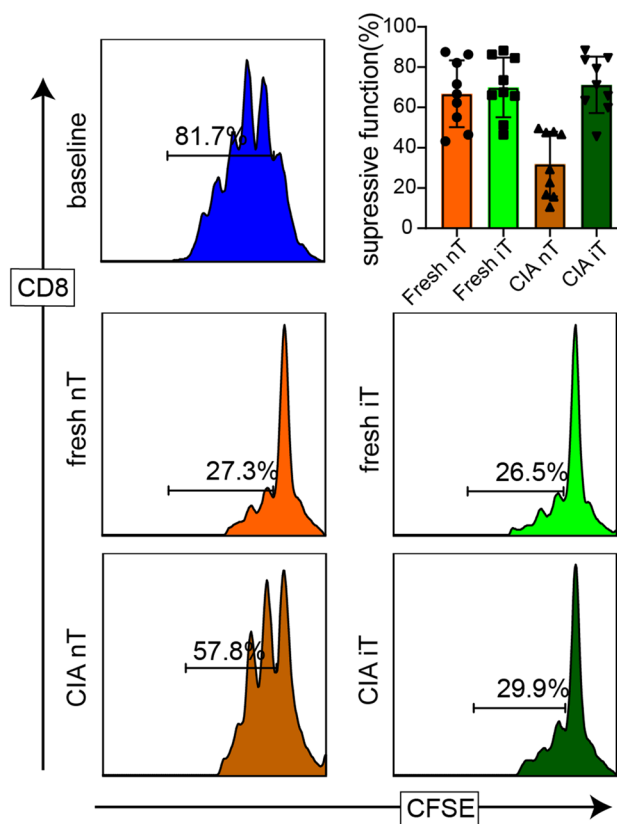
hyperplasia, inflammatory cell infiltration, severe pannus invasion and bone destruction. However, the iTreg treatment group showed an almost normal synovial membrane, less inflammatory cell infiltration and pannus invasion and no bone destruction (Fig. 1e).

### iTregs transferred into CIA model mice maintain the suppressive function that limits Teff activities

One previous study discovered that when adoptively transferred into a CIA model, nTregs preferentially lose both Foxp3 expression and regulatory function. To determine whether iTregs exhibit similar losses, we adoptively transferred 3 million Thy1.2<sup>+</sup> nTregs or iTregs into established Thy1.1<sup>+</sup> CIA model mice on day 45. After 3 days, the Thy1.2<sup>+</sup> Tregs were sorted from the axillary and inguinal lymph nodes by fluorescence-activated cell sorting (FACS). Then, we performed a standard functional assay *in vitro* [11], with fresh populations of both Treg subsets used as controls. The results showed that both fresh nTregs and fresh iTregs had similarly excellent regulatory functions limiting Teff proliferation (Fig. 2). After priming in CIA model mice, iTregs maintained their suppressive ability, with their suppressive function being equivalent to that of fresh iTregs. In contrast, nTregs dramatically lost their suppressive capacity against Teffs (Fig. 2).

### iTregs transferred into CIA model mice retain their suppressive effect on Teffs *in vivo*

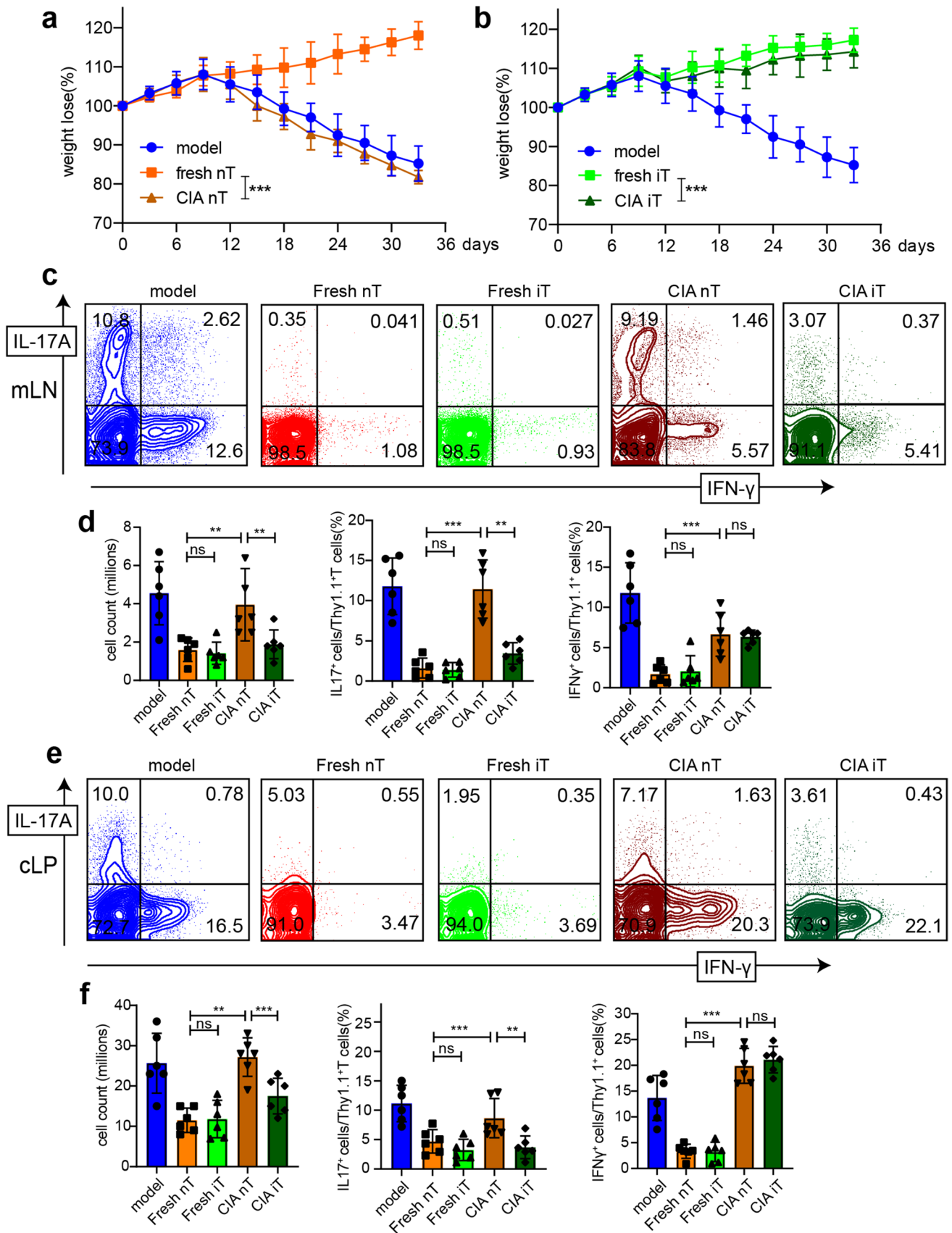
*In vivo*, the causes of disruption of the suppressive activities of Tregs can be more complicated than those *in vitro*. To exclude this concern, we established a colitis model with Rag1<sup>-/-</sup> mice since these mice naturally lack T cells [13, 14]. First, Thy1.1<sup>+</sup> DBA1/J mice were used to establish the CIA model. On day 45 after CII/CFA immunization, 3 × 10<sup>6</sup> fresh Thy1.2<sup>+</sup> nTregs or iTregs were adoptively transferred into the CIA model. After 3 days, these primed Tregs were sorted with FACS as mentioned above. Then, 1 million naive Thy1.1<sup>+</sup>CD4<sup>+</sup> T cells and the same number of primed nTregs or iTregs were injected into Rag1<sup>-/-</sup> mice simultaneously; some recipients received naive CD4<sup>+</sup> T cells alone as a control. We found that the recipients treated with naive CD4<sup>+</sup> T cells alone lost weight from day 9 onward and typically gradually developed severe colitis (Fig. 3a). Both fresh Treg subsets significantly protected the mice from colitis, and recipients gradually gained weight similar to naive Rag1<sup>-/-</sup> mice and exhibited only slight colitis, illustrating that both the nTreg and iTreg subsets have excellent suppressive function in the inflammatory microenvironment *in vivo*. However, after priming in the CIA model, the nTreg subset exhibited diminished effects on Teffs and failed to reduce the severity of colitis. Interestingly, the iTreg subset primed



**Fig. 2** The iTreg subset, after adoptive transfer into CIA model mice, retains its suppressive effects on Teffs *in vitro*. (a, b) Fresh nTregs or iTregs were prepared from naive Thy1.1<sup>+</sup> DBA1/J mice. CIA was induced in Thy1.2<sup>+</sup> DBA1/J mice with standard methods. Three million iTregs or nTregs were intravenously injected into CIA mice on day 44 after CII/CFA immunization. After 3 days, the two Treg subsets were harvested and sorted from the CIA model mice with FACS. The two Treg subsets were cocultured with the total effector T cells (Teffs) at a ratio of 1:2 for another 3 days. The proliferation of Teffs was detected and compared by flow cytometry. because CD4<sup>+</sup> and CD8<sup>+</sup> T cells shared similar tendency, but the proliferation rate of CD4<sup>+</sup> T cells was slower than CD8<sup>+</sup> T cells, So, we only exhibit the proliferation of CD8<sup>+</sup> T cells. \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ ; error bars denote the SD. The experiments were repeated nine times independently

in the CIA model retained its suppressive function and still effectively controlled the development of colitis (Fig. 3a, b).

Additionally, in the recipients treated with naive CD4<sup>+</sup> T cells alone, 10.8% of naive T cells differentiated into Th17 cells (producing only IL-17A) and 12.6% of those into Th1 cells (producing only IFN $\gamma$ ) in the mesenteric lymph nodes (mLNs) (Fig. 3c, d). A similar tendency was discovered in the colonic lamina propria (cLP) (Fig. 3e, f). Both fresh nTreg and iTreg subsets effectively suppressed the differentiation and proliferation of Teffs, such as Th17 and Th1 cells in the mLNs and cLP. After priming in the CIA model, in both lymph nodes and cLP, the suppressive activity on Th17 cells of





**Fig. 3** iTregs, after priming in CIA model mice, maintain the ability to regulate the activities of Tregs in a colitis model. (a, b) One million naive Thy1.1<sup>+</sup>CD4<sup>+</sup> T cells were intraperitoneally injected into Rag1<sup>-/-</sup> mice with  $1 \times 10^6$  iTregs or nTregs previously adoptively transferred into CIA model mice. We recorded the weights of every Rag1<sup>-/-</sup> mouse after cell transfer. (c-f) Thirty-three days later, the mesenteric lymph nodes (mLNs) and colonic lamina propria (cLP) were isolated, and the proportion of IL-17A<sup>+</sup> or IFN $\gamma$ <sup>+</sup> cells in Thy1.1<sup>+</sup>CD4<sup>+</sup> cells were detected with flow cytometry. \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ ; error bars denote the SD. The experiments were repeated six times independently

nTregs was almost significantly diminished, while iTregs maintained this capability similar to that observed with fresh iTregs. (Fig. 3c-f). In the cLP, a similar tendency on Th17 cells was discovered. Our results indicated that the iTreg subset has a more stable and superior ability to downregulate the differentiation and proliferation of Th17 cells, even following exposure to an inflammatory microenvironment *in vivo*.

While, after priming, both nTreg and iTreg subsets lost some suppressive function on Th1 cells in lymph nodes, even improved Th1 proliferation in cLP and exhibited no significance. It indicates that the suppressive function of Ag-experienced nTregs or iTregs on Th1 differentiation *in vivo* might be more complicated.

We also found that nTregs can inhibit the development of colitis, but cannot block CIA. We deduced that we use Rag1<sup>-/-</sup> mice to make colitis model with naive CD4<sup>+</sup> T cell and use DBA1/J mice to make CIA model with CII/CFA. These two models might have different activities to different autoantigen.

### The iTreg subset inhibits the inflammatory activities of CIA-SFs *in vivo*

Although Tregs exert their immunoregulatory function directly by targeting Tregs, B cells, macrophages, dendritic cells and osteoclasts [6, 11], it remains unclear whether Tregs directly affect the activities of CIA-SFs. To address this issue, we adoptively transferred 1 million fresh Thy1.2<sup>+</sup> nTreg or iTreg cells into CIA model mice on day 14 after CII/CFA immunization. On day 45, we isolated and passaged inflamed SFs and performed a series of evaluations. We found that iTregs but not nTregs markedly suppressed the migration and invasion of CIA-SFs (Fig. 4a, b). Additionally, iTregs significantly inhibited the proliferation of CIA-SFs, but nTregs failed to do so (Fig. 4c). Furthermore, ELISA results showed that the levels of TNF $\alpha$ , IL-6 and IL-1 $\beta$  produced by CIA-SFs from mice pretreated with iTregs were dramatically lower than those produced by CIA-SFs from model mice and mice pretreated with nTregs (Fig. 4d).

### iTregs suppress the inflammatory activities of CIA-SFs *in vitro*

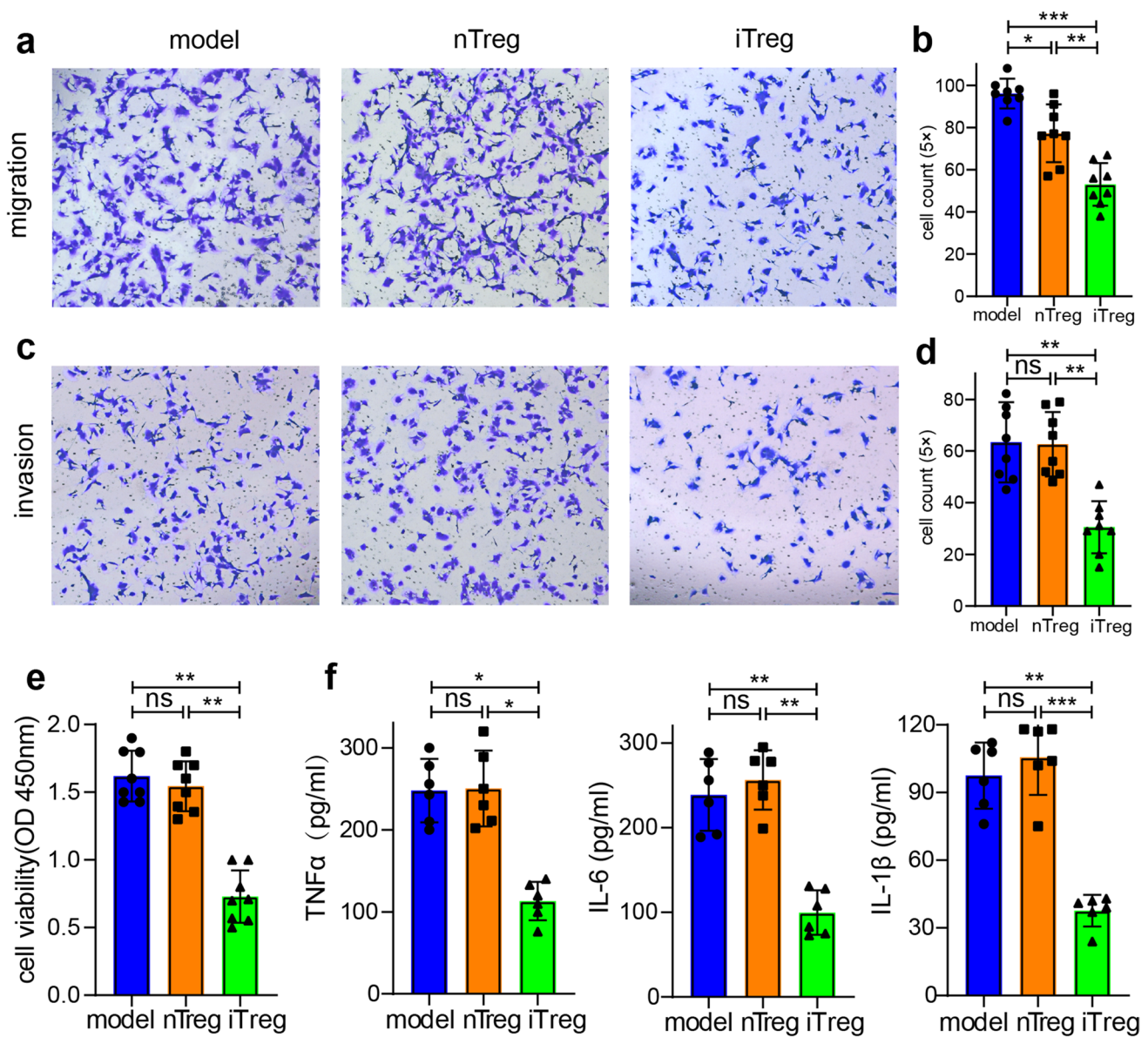
To exclude interference by other cells and cytokines in the CIA model, we also performed *in vitro* experiments. First, we prepared fresh nTregs or iTregs from naive wild type mice and inflamed SFs from CIA model mice 45 days after CII/CFA immunization. Then, we cocultured either the iTreg or nTreg subset with CIA-SFs for three days to determine whether the Treg subset had a suppressive function on the CIA-SFs. After three days, the CIA-SFs were harvested and subjected to a series of assays. Migration and invasion were measured in Transwell experiments as previously described, and the proliferation of CIA-SFs was measured with a CCK-8 assay. Consistent with our hypothesis, our results showed that the migration (Fig. 5a), invasion (Fig. 5b) and proliferation (Fig. 5f) of CIA-SFs were significantly decreased after iTreg treatment. Conversely, nTregs had poor suppressive effects on the inflammatory activities of CIA-SFs.

Given that VCAM-1 and NF- $\kappa$ B are critical proteins in the migration, invasion and proliferation of synovial fibroblasts [15, 16], we evaluated whether either the nTreg or iTreg subset suppresses the phosphorylation of NF- $\kappa$ B and expression of VCAM-1 in CIA-SFs by western blot analysis. Our results showed that iTregs but not nTregs markedly suppressed NF- $\kappa$ B phosphorylation and VCAM-1 expression in synovial fibroblasts, although the nTreg group showed a slight reduction in NF- $\kappa$ B phosphorylation compared to the CIA-SF only group (Fig. 5e). These results clearly provided evidence to confirm that iTregs have a superior suppressive function that alleviates CIA clinical severity by suppressing the inflammatory activities of CIA-SFs.

## Discussion

Tregs play a pivotal role in suppressing excessive autoimmune responses and maintaining immune self-tolerance [17, 18]. Dysfunction and/or aberrant proportions of Tregs have been demonstrated to be important mechanisms of RA pathogenesis [19, 20]. Furthermore, hyperproliferative pathogenic Th17 cells exacerbate the severity of RA by accelerating osteoclastogenesis, synovial neoangiogenesis and bone erosion. Hence, an aberrant ratio of Th17 cells to Tregs has been demonstrated to be one of the most important pathogenic mechanisms in the onset and progression of RA [21–23].

Tregs consist of two types: nTregs and Tregs induced in the periphery *in vivo* or iTregs [7, 8]. Interestingly, the two Treg subsets exhibit similar suppressive activities under some conditions, but they also show some distinctions when exposed to inflammatory microenvironments [4, 11].

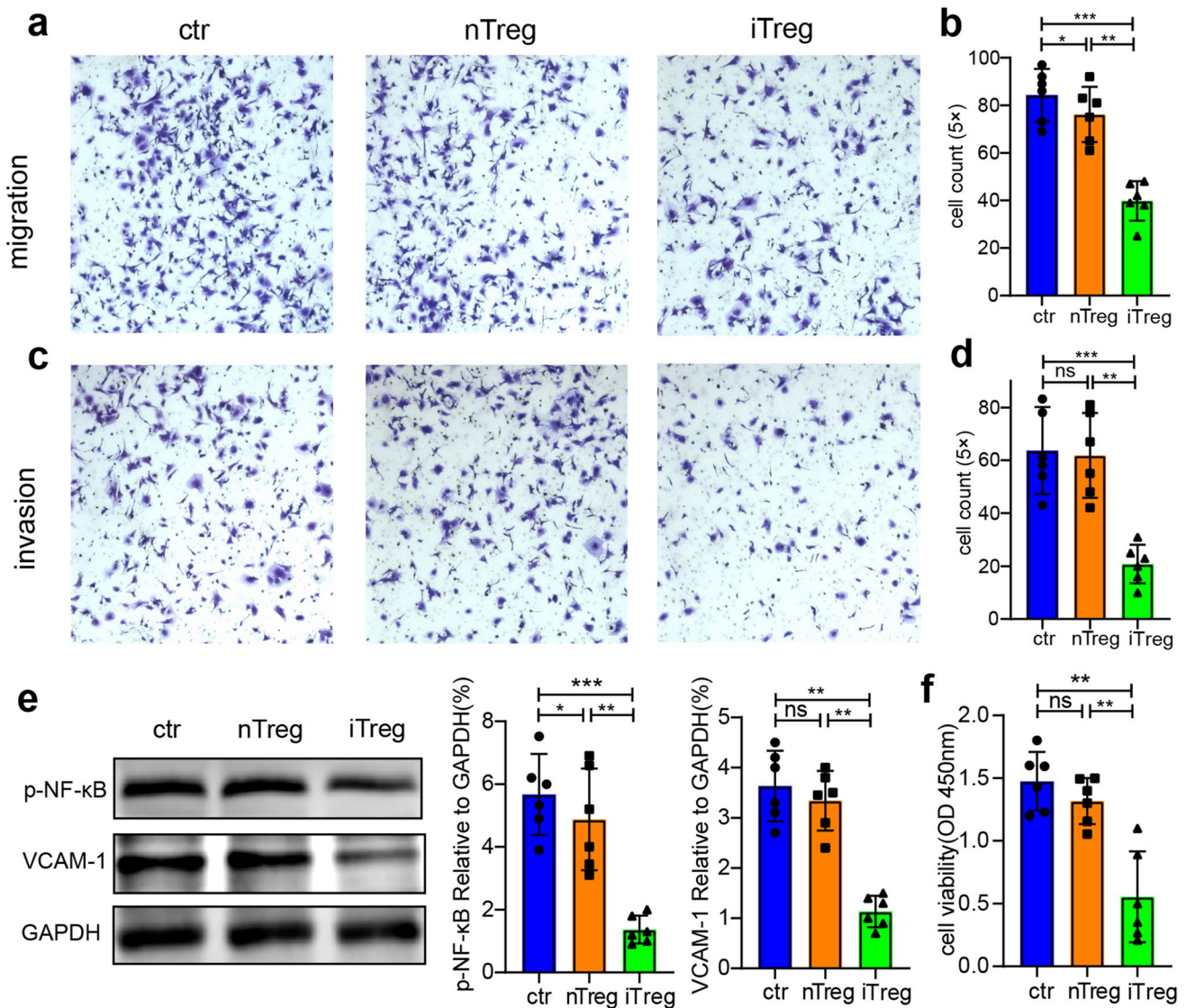


**Fig. 4** iTregs exhibit a protective effect in the CIA model by inhibiting the migration, invasion, proliferation, and proinflammatory cytokine expression of CIA-SFs. **(a, b)** DBA1/J mice were used for CIA induction. On day 14 after CII/CFA immunization,  $3 \times 10^6$  iTregs or nTregs were intravenously injected into the CIA model mice. On day 44, CIA-SFs were harvested and cultured in 24-well Transwell plates. The CIA-SFs were harvested after 12 h of culture in vitro. For the migration test, the population of CIA-SFs that passed through the membrane was determined by staining with crystal vio-

let (5x). **(c, d)** For the invasion test, the upper chambers were coated with Matrigel. After 24 h, the population of CIA-SFs that passed through the membrane was determined by staining with crystal violet. **(e)** We also cultured CIA-SFs in a 96-well plate. On day 3, the proliferation of CIA-SFs was detected with CCK-8. **(f)** On day 3, the production of proinflammatory cytokines was detected with ELISA kits. \*  $P \leq 0.05$ , \*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ ; error bars denote the SD. The experiments were repeated six times independently

Komatsu et al. reported that most nTregs lost Foxp3 expression and transdifferentiated into Th17 cells in a CIA model without cell–cell contact. Furthermore, blockade of IL-6 or TNFα signaling increased the ratio of Foxp3<sup>+</sup> to TH17 cells [12]. These pathogenic Th17 cells exhibited more aggressive capabilities for osteoclastogenesis and bone erosion than Th17 cells differentiated from naive T

cells [24]. This might explain why the nTreg subset loses regulatory activities and even exacerbates the progression when used to treat RA [10, 25]. We and other investigators also provided additional evidence that nTregs significantly prevent CIA but fail to control the disease at its peak because they are vulnerable to conversion into



**Fig. 5** The iTreg subset inhibits the migration, invasion, proliferation, and proinflammatory cytokine expression of CIA-SFs in vitro. **(a–d)** The CIA model was first induced with standard methods. On day 44 after CII/CFA immunization,  $3 \times 10^6$  iTregs or nTregs were intravenously injected into the CIA model mice. After 3 days, the two Treg subsets were harvested and sorted from the CIA model mice with FACS. Fresh SFs were prepared from naive DBA1/J mice and then cocultured with Treg subsets in 24-well Transwell plates for 3 days.

Then, after removal of the Treg subsets, the migration and invasion of CIA-SFs were tested (5 $\times$ ). **(e)** The phosphorylation of NF- $\kappa$ B and the expression of VCAM-1 in CIA-SFs were determined by western blot analysis, and GAPDH was used as an internal control. **(f)** The proliferation of CIA-SFs was measured with CCK-8 in 24-well plates. \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ ; error bars denote the SD. The experiments were repeated five times independently

T helper cells, such as Th17, Th1 and T follicular cells, under inflammatory conditions [26, 27].

Our recent study demonstrated that under autoimmune conditions, iTregs were resistant to Th17 cell transdifferentiation, retained Foxp3 expression and exerted effective regulatory functions in a CIA model [11]. The inflammatory microenvironment did not diminish the functional activities of iTregs [11], and the stability and function of nTregs were significantly compromised. We also found that CIA-SFs priming lowered the expression of CD137, ICOS, Helios,

CD39, CTLA-4, and IRF4 and increased the expression of GITR and Blimp-1 by nTregs. In contrast, CIA-SFs priming did not affect most of the marker expression on iTregs while increasing CD39 and CTLA-4 expression by iTreg [11]. These findings demonstrated that the nTreg and iTreg subsets have many biological differences in functional characteristics, especially under inflammatory conditions.

However, Th1 cell differentiation did not show a uniformed significant difference among mLNs, and cLP, especially in cLP. We deduced that nTregs lost Foxp3 expression,



and produced IL-17, or IFN $\gamma$ . While, iTregs resisted conversion to an IL-17-producing phenotype but an IFN $\gamma$ -producing phenotype, especially in the inflammatory site. But these iTregs retained an ability to suppress function and protected against the development of experimental autoimmune encephalomyelitis [28]. Hence, autoprotective versus autoaggressive functions in Tregs are not simply a binary relationship to be determined by their relative expression of Foxp3 versus IL-17, or IFN $\gamma$ .

In addition to targeting Teffs, especially Th17 cells, Tregs also regulate or suppress the activities of B cells, macrophages, mast cells, dendritic cells and osteoclasts [6, 11]. In rheumatoid arthritis, SFs act as the major cell type in inflamed synovial tissues and play a pivotal role in pathogenic mechanisms. Previous investigations have indicated that CIA-SFs can migrate to and invade the cartilage and bone, which contribute to pannus formation and synovial destruction [29].

In our study, we used two animal models, a colitis model and a CIA model, to determine whether the nTreg and iTreg subsets have functional differences after exposure to the CIA model. We found that nTregs adoptively transferred into the CIA model markedly lost their suppressive function against Teff activities both *in vitro* and *in vivo*. However, iTregs retained excellent regulatory functions limiting Teff proliferation.

We also investigated whether both Treg subsets directly regulate and affect the biological characteristics of inflamed SFs. Our results indicated that in both *in vitro* experiments and *in vivo* experiments, iTregs but not nTregs significantly inhibited the proinflammatory cytokine production, proliferation, migration and invasion of inflamed SFs. Furthermore, iTregs also suppressed the expression and phosphorylation of NF- $\kappa$ B and expression of VCAM-1 in SFs. Proinflammatory cytokines triggering the NF- $\kappa$ B pathway are known to be critical participants in the pathogenesis of autoimmune arthritis, and VCAM-1 facilitates autoimmune responses and facilitates the adherence of peripheral immune cells to endotheliocytes [16]. TNF $\alpha$ , IL-1 $\beta$ , and IL-6 play critical roles in the migration and invasion of SFs [30].

How to explain the different activities of Treg subsets under arthritic conditions? There are several possibilities listed as follows. First, relative to nTregs, iTregs exhibit a lower level of CD126 (IL-6 receptor) expression and show more passive responses to IL-6, which is an important cytokine in Th17 cell differentiation [11]. Second, iTregs derived with TGF- $\beta$  stimulation promote Bcl-2 expression, which exerts antiapoptotic effects [31]. Indeed, it has been demonstrated that iTregs have high expression of Bcl-2 [32]. Third, iTregs can secrete higher levels of IL-10 than nTregs to suppress the harmful self-directed activities of inflamed SFs [10].

**Conclusion:** In conclusion, this study demonstrates that iTregs may have potential for the prevention and treatment of patients with RA in the future.

## Materials and methods

### Mice

C57BL/6 (strain No.N000295), DBA1/J (strain No.N000219), Thy1.1<sup>+</sup> (strain No.T010974) and Rag1<sup>-/-</sup> (strain No.T004753) mice were purchased from P GemPharmtech Co, Ltd (Nanjing, China). The Thy1.1<sup>+</sup> mice and Rag1<sup>-/-</sup> mice are both C57BL/6 background. They were housed under specific pathogen-free conditions in the central laboratory of the First Affiliated Hospital of Guangdong Pharmaceutical College. All the mice used in our experiments were six to eight weeks old. The mice were treated according to the National Institutes of Health guidelines for the use of experimental animals, and the study received the approval of the Guangdong Pharmaceutical College Committees for the Use and Care of Animals.

### Antibodies and reagents

Naive CD4<sup>+</sup> T cell and nTreg isolation kits were purchased from Miltenyi Biotec (Cologne, Germany). Anti-CD3/CD28 antibody-coated beads from Gibco (New York, USA) and rhIL-2, rhTGF- $\beta$ , rmIL-6, and rmIL-23 from R&D Systems (Minneapolis, USA) were purchased for inducing or culturing Tregs *in vitro*. All of the anti-mouse immunofluorescence antibodies for FACS and flow cytometry were purchased from BioLegend (San Diego, USA). All of the primary and secondary antibodies for western blotting analysis were purchased from Cell Signaling Technology (Beverly, USA). All of the ELISA kits were purchased from eBioscience (San Diego, USA), and Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Incomplete Freund's adjuvant (IFA), a killed *Mycobacterium tuberculosis* strain and collagen II for CIA model induction were purchased from Sigma Aldrich (St. Louis, USA). Matrigel invasion chambers were purchased from BD Biosciences (Tokyo, Japan).

### Preparation of iTregs and nTregs

The preparation of iTregs and nTregs was performed as described in our previous study [11]. The proportion of cells expressing Foxp3 in iTregs was > 75%. We sorted CD4<sup>+</sup>CD25<sup>+</sup> cells from the lymph nodes as nTregs, achieving a purity of > 95%. In this CD4<sup>+</sup>CD25<sup>+</sup> cell population, no less than 75% of the CD4<sup>+</sup>CD25<sup>+</sup> cells expressed Foxp3.



## Treg suppressive function *in vitro*

The standard suppressive function of Tregs was performed as previously reported. The total effector T cells (Teffs) were enriched from lymphocytes and subsequently labeled with 1  $\mu$ M carboxyfluorescein succinimidyl ester (CFSE). Antigen-presenting cells (APCs) were irradiated with 30 Gy. The prepared Teffs and APCs were seeded in a 96-well plate (both at  $0.3 \times 10^6$ /well). Either iTregs or nTregs adoptively transferred into CIA model mice were added at a certain ratio relative to the Teffs. After 3 days, the proliferation of CD8<sup>+</sup> T cells was detected by flow cytometry (Because both CD4<sup>+</sup> and CD8<sup>+</sup> T cells shared similar tendency in these experiments. But the proliferation rate of CD4<sup>+</sup> T cells was slower than CD8<sup>+</sup> T cells. So, we always only exhibit the proliferation of CD8<sup>+</sup> T cells). the proliferation rate of CD8<sup>+</sup> T cells (PR) was be calculated as proliferating CD8<sup>+</sup> T cells (except for the primary CD8<sup>+</sup> T cells)/the total CD8<sup>+</sup> T cells \*100%, which was readed by flowjo v10.5.3 automatically [10]. the suppression function (%) = PR(baseline) -PR(treatment group) / PR(baseline) \*100%.

## Proliferation assay

For *in vivo* experiments, nTregs or iTregs were adoptively transferred into established CIA model mice. After 3 days, CIA-SFs were isolated. For *in vitro* experiments, CIA-SFs were isolated from CIA model mice and cocultured with either Treg subset sorted as described above (ratio 1:10) in a 48-well Transwell plate. The Treg subsets were seeded in the upper chamber, and the CIA-SFs were seeded in the lower chamber. After 3 days, the Tregs were removed by removing the upper chamber. To determine the proliferation of CIA-SFs, CCK-8 was added to each well, and the cells were incubated in a 37 °C incubator for 2 h. The absorbance at 450 nm was measured with a microplate reader.

## Migration and invasion assays

For *in vivo* experiments, nTregs or iTregs were adoptively transferred into established CIA model mice. After 3 days, CIA-SFs ( $2.5 \times 10^4$ ) were isolated and seeded in the upper chamber of a 24-well Transwell plate. For *in vitro* experiments, CIA-SFs were isolated from CIA model mice and cocultured with either Treg subset ( $2.5 \times 10^5$ ) sorted as described above in a 24-well Transwell plate. The Treg subsets were seeded in the lower chamber. The lower chamber was filled with DMEM containing 10% FBS, and the upper chamber was filled with DMEM without FBS. After 12 h, the CIA-SFs that passed through the membrane were stained with a crystal violet solution for 10 min. Micrographs of five random fields were captured and subjected to statistical analysis. The invasiveness of

CIA-SFs was determined using Matrigel invasion chambers for 24 h [11]. The upper side of the upper chamber was coated with fresh Matrigel. Tregs ( $1 \times 10^6$ ) were seeded in the lower chamber, and the culture medium was DMEM supplemented with 10% FBS. CIA-SFs ( $1 \times 10^5$ ) were seeded in the upper wells without FBS. The CIA-SFs that passed through the Matrigel membrane were identified with crystal violet staining and microscopy.

## Induction, assessment and treatment of arthritis

The induction and assessment of arthritis was performed as described in a previous study [11]. In brief, CIA was induced in DBA1/J mice through intradermal injection of 100  $\mu$ l of emulsion of bovine collagen II (2 mg/ml) and Complete Freund's Adjuvant containing inactivated *M. tuberculosis* (4 mg/ml) [at 1:1 (v/v) ratio]. A second immunization with 100  $\mu$ l of IFA was given on day 14 after the first CII/CFA immunization. For assessment of arthritis, clinical signs of the mice were assessed for every 3 days as follows: 0, normal joints; 0.5, swelling of one or more digits; 1, erythema and mild swelling of the ankle joint; 2, mild erythema and mild swelling involving the entire paw; 3, erythema and moderate swelling involving the entire paw; and 4, erythema and severe swelling involving the entire paw. The scores for four limbs were added together, and the maximal score for each mouse is 16.

For the Treg treatment CIA experiments,  $3 \times 10^6$  nTregs or iTregs were adoptively transferred into DBA/1 J mice on day 14 after CII/CFA immunization.

## Induction and assessment of colitis

The induction of colitis was performed as described in a previous study [33]. Naive Thy1.1<sup>+</sup>CD4<sup>+</sup> T cells (1 million) and Thy1.2<sup>+</sup> nTregs or iTregs (1 million) primed in CIA model mice were injected intraperitoneally into Rag1<sup>-/-</sup> mice simultaneously. The weight of the recipients was recorded every day. After 45 days, the cells of the MLNs and cLP were harvested, and the proportions of IL-17A<sup>+</sup> and IFN $\gamma$ <sup>+</sup> cells differentiated from naive CD4<sup>+</sup> T cells were detected with flow cytometry.

## MRI images

We acquired images by using 1.5 T micro-MRI from animal center of Sun Yet-San University. The mice were narcotized with diethyl ether firstly. An MRI was performed in the sagittal planes for all sequences. And the image sections were 2.0 mm thick.

## Isolation of synovial tissues from CIA model mice

After euthanasia, the four limbs of CIA mice were dissected at the joints. The auxiliary tissues, such as the skin, hair and nails, on the specimens were thoroughly removed under a microscope. The synovial tissues of the paws and ankles were exposed and digested in a digestive solution containing 1 mg/ml collagenase type IV on a 37 °C shaker for 1 h. The digestive solution including synovial fibroblasts was centrifuged at 1400 rpm for 4 min and washed with sterile PBS 3 times. The SFs were subcultured at 90% confluence prior to characterization at passage four. When CIA-SFs were cultured during passages 4–6, Thy1.2<sup>+</sup>CD11b<sup>-</sup> SFs were purified with an anti-Thy1.2-biotinylated antibody and anti-biotin microbeads by an autoMACS.

## Histopathology

The hind limbs of CIA mice were dissected, fixed with 4% paraformaldehyde and stained with H&E. Global histological changes were evaluated according to synovial thickness, SF proliferation, lymphoid cell infiltration and bone erosion.

## Enzyme-linked immunosorbent assay (ELISA)

As described for the migration and invasion assays, the supernatant of culture medium from CIA-SFs was collected for ELISA analysis of TNF $\alpha$ , IL-6, and IL-1 $\beta$ , after 3 days culture, according to the instruction of the manufacturers.

## Western blot analysis

CIA-SFs were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitor and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to standard methods. Membranes were incubated with antibodies against GAPDH (1:1,000), p-NF- $\kappa$ B (1:1,000), NF- $\kappa$ B (1:1,000), and VCAM-1 (1:1,000) overnight at 4 °C. Then, the membranes were washed and visualized with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1,000).

## Statistical analysis

GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) was used to calculate results, and data are presented as the mean  $\pm$  SEM. We assessed statistical differences between two groups with Student's *t* test and

among multiple groups with one-way ANOVA.  $P \leq 0.05$  was considered to indicate a significant difference.

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**Author's contribution** Aiqun Liu performed the majority of experiments, analyzed and interpreted the data. Qi Cui and Aiqun Liu drafted the manuscript. Sujuan Yang designed the experiments and revised the manuscript. All coauthors approved the final version.

**Data Availability** All data and materials as well as software application support the published claims and comply with field standards. All the data are available from the corresponding author on reasonable request.

## Declarations

**Conflict of Interest** The authors declare that they have no financial conflicts of interest.

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