Adiponectin promotes fibroblast-like synoviocytes producing interleukin-6 to enhance T follicular helper cells response in rheumatoid arthritis

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Abstract

Objective

Rheumatoid arthritis (RA) is characterised by the overproduction of autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibody. T follicular helper (Tfh) cells are a specialised Th subset that provides signals to B cells, promoting the secretion of antibodies. Our previous studies showed that the frequency of circulating Tfh cells were markedly increased in RA patients and positively correlated with disease activity and the levels of anti-CCP autoantibody. Adiponectin (AD) is an adipokine secreted mainly by adipocytes. Our previous work has demonstrated that AD is highly expressed in the inflamed synovial joint tissue and correlates closely with progressive bone erosion in RA patients. However, it remains unknown whether AD aggravates the severity of RA via modulating Tfh cells. This study aims to investigate whether AD exerts effect on Tfh cells in RA.

Methods

$CD4^+$ T cells were purified from peripheral blood mononuclear cells (PBMCs) of healthy controls (HC), and adiponectin receptor 1 (AdipoR1) expression on the surface of $CD4^+CXCR5^+PD\text{-}1^+$ (Tfh) cells was detected by flow cytometry. Purified HC $CD4^+$ T cells were cultured with different concentration fetal bovine serun (FBS) in the presence or absence of AD. The percentages of Tfh cells were analysed by flow cytometry. RA or osteoarthritis (OA) fibroblast-like synoviocytes (FLSs) were stimulated with AD for 72h and then co-cultured with HC $CD4^+$ T cells through cell-to-cell contact or in a transwell system. The percentages of Tfh cells were analysed by flow cytometry and the levels of soluble factors such as interleukin-(IL)-6, IL-21, IL-12 and IFNγ in the supernatants were determined by Human Magnetic Bead Panel or Enzyme linked immunosorbent assay (ELISA). Then anti-IL-6 antibody and/or anti-IL-21 antibody was added to the co-culture system, and the percentages of Tfh cells were analysed by flow cytometry. The frequency of Tfh cells in the joint tissue of collagen-induced arthritis (CIA) mice was examined by flow cytometry. The mRNA expression of Tfh cell transcription factors and functional molecules such as B-cell lymphoma 6 (Bcl-6), B lymphocyte maturation protein 1 (Blimp-1), IL-6, IL-21, IL-12 and IFNγ in the joints of CIA mice were detected by real time PCR (RT-PCR).

Results

Adiponectin receptor 1 (AdipoR1) expression was detected on the surface of Tfh cells. However, in the present study, we did not find that AD has a direct effect on Tfh cell generation in vitro. Nonetheless, AD-stimulated RA FLSs could promote Tfh cell generation, predominantly via IL-6 production and this upregulated effect was partially abolished upon neutralising IL-6. Finally, intraarticular injection of AD aggravated synovial inflammation with increased frequency of Tfh cells in the joints of AD-treated CIA mice.

Conclusion

Our study demonstrated that AD-stimulated RA FLSs promote Tfh cell generation, which is mainly mediated by the secretion of soluble factor IL-6. This finding reveals a novel mechanism for AD in RA pathogenesis.

Key words

rheumatoid arthritis, T follicular helper cell, adiponectin, IL-6

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Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease which is characterised by synovial inflammation and hyperplasia, autoantibody production, cartilage and bone destruction, and systemic complication (1, 2). However, to date, the exact mechanism of RA remains unknown. Therefore, exploring the pathogenesis of RA is still a challenge.

T follicular helper (Tfh) cells are a specialised Th subset that could promote the differentiation of B cells into memory B cells or plasma cells (PCs), and are crucial for the processes of class switch recombination. Tfh cells are characterised by high expression of CXC chemokine receptor 5 (CXCR5), which facilitates Tfh cell migration to B cells follicles (3, 4). Moreover, Tfh cells express high levels of costimulatory molecules such as programmed death 1 (PD-1), inducible T cell costimulator (ICOS), interleukin-(IL)-21, cytoplasmic adaptor protein signalling lymphocytic activation molecule (SLAM)-associated protein (SAP) and B-cell lymphoma 6 (Bcl-6) (5, 6). Nurieva *et al*. found that IL-6, IL-21 and Bcl-6 were critical for Tfh cell differentiation (7), and that Tfh cell differentiation in humans required IL-12 and activin A signalling (8, 9). Moreover, IL-27, a heterodimeric cytokine was critical for the survival of Tfh cells as well as for the marker expression, such as CXCR5, PD-1 and ICOS (10). Interferon γ (IFNγ), a cytokine that associated with Tfh cell production, could regulate the T-bet expression to promoting pathogenic autoreactive antibodies production (11, 12). Our previous studies showed that the frequency of circulating Tfh cells were markedly increased in RA patients and positively correlated with disease activity and the levels of anti-CCP autoantibody (13, 14), suggesting that Tfh cells might participate in the pathogenesis of RA.

Adiponectin (AD), also called Acrp30, adipoQ, ApM1, and GBP28, is a 28-30 kDa collagen-like protein mainly secreted by adipocytes. The growing evidence suggest that AD plays an important role in regulating immune and inflammatory processes (15). Our previous and other studies demonstrated that the level of AD was highly expressed both in the serum, synovial fluids (SF) and inflamed synovial joint and correlated closely with progressive bone erosion in RA patients (16-19). Moreover, local level of AD was positively correlated with IL-6 level in RA synovial fluids. *In vitro*, stimulation of RA synoviocytes with AD could promote a high expression of IL-6 (20), which was necessary for Tfh cell differention. Therefore, in this study, we attempt to reveal that whether AD participates in RA through inducing Tfh cell response.

Methods

Cell isolation and cultures

$CD4^+$ T cells were separated from peripheral blood mononuclear cells (PBMCs) of healthy control (HC), and then sorted by positive selection with magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions and the purity was >97% (Supplementary Fig. 1).

RA fibroblast-like synoviocytes (FLSs) and osteoarthritis (OA) FLSs were obtained from the synovial tissue of RA patients or OA patients who underwent therapeutic synovectomy or arthroplasty, as described previously (21). And the disease 28-joint count disease activity score (DAS28) of RA patients was >3.2. The study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (2013-SR-178). All study subjects signed written informed consent before participating in the study. The characteristics of RA and OA patients are shown in Supplementary Fig 2. The tissues were minced into small pieces and incubated with 4 mg/ml type I collagenase in Dulbecco's modified eagle medium (DMEM) (Gibco) for 4 h. The cells were collected by filtering the suspension through nylon mesh (70μm), followed by extensive washing with phosphatebuffered saline (PBS). The cells were then centrifuged at 400 g for 6 min and collected in plastic cell culture dishes, cultured in DMEM with 10% fetal bovine serum (FBS) (Gibco) and 100 U/ml 1% penicillin-streptomycin (Gibco). A homogeneous population (<1% $CD3^+$, <1% $CD19^+$, <1% $CD68^+$, <1% $CD11b^+$,

and <1% $CD14^+$) was identified as RA FLSs (Suppl. Fig. 3) (22). Only passage 3 to passage 8 cells were used for the culture experiments. $CD4^+$ T cells (4×10^5) that obtain from 10 different HC respectively were cultured in 96-well plates in 1640 medium (Gibco) with serum-free, 2% FBS, 5% FBS and 10% FBS respectively. $CD4^+$ T cells were stimulated with anti-CD3 mAb (5μg/ml, eBioscience) and anti-CD28 mAb (3μg/ml, eBioscience) in different concentrations of AD (5μg/ml and 25μg/ml, Peprotech) for 72h and this experiments were repeated three times.

Co-culture of FLSs and $CD4^+$ T cells

RA FLSs or OA FLSs (4×10^4) that each from 3 different patients were allowed to adhere to 24-well plates in DMEM medium (Gibco) containing 10% FBS (Sigma) in the presence of AD (25μg/ml). After 72h, the supernatants were discarded and the FLSs were washed with PBS twice. Then $CD4^+$ T cells were added into the plate in the presence of anti-CD3 mAb (5μg/ml, eBioscience) and anti-CD28 mAb (3μg/ml, eBioscience) for 72h in a cell-to-cell contact or transwell system. RA/OA FLSs from one patient were co-cultured with $CD4^+$ T cells from different HC. This experiments were repeated three times. Anti-IL-6 antibody (0.5μg/ml, eBioscience) or/and anti-IL-21 antibody (0.2μg/ml, eBioscience) were added to the above co-culture system. The frequency of Tfh cells was detected by flow cytometry.

RNA interference

siRNA specifically targeting human AdipoR1 (sense 5'-GCUCUUUCACACCGUCUAUTT-3', antisense 5'-AUAGACGGUGUGAAAGAGCTT-3') were purchased from Invitrogen Biotechnology. 20 pM siRNA oligonucleotides were transfected into RA FLSs with Lipofectamine® 2000 Reagent (invitroger) in accordance with the manufacturer's instructions. Control siRNA was used as a negative control. Knockdown efficiencies were monitored by quantitative real time PCR (RT-PCR) and by western blot after transfection (Suppl. Fig. 4). RA FLSs transfected with AdipoR1 siRNA (4×10^4) were allowed to adhere to 24-well plates in DMEM medium (Gibco) containing 10% FBS (Sigma) in the presence of AD (25μg/ml) for 72h, then co-cultured with $CD4^+$ T cells for 72h. Levels of IL-6 in the culture supernatants were measured by ELISA.

Human Magnetic Bead Panel

Levels of IL-6, IFNγ, IL-27 and IL-12 in the culture supernatants were measured by Human Magnetic Bead Panel (MILLIPLEX® MAP, Merck KGaA) according to the manufacturer's recommendations. Each sample was prepared in triplicate. 1. Add 200 μl of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20–25°C). 2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. 3. Add 25μl of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 standard (Background). 4. Add 25 μl of Assay Buffer to the sample wells. 5. Add 25 μl of control culture medium to the background, standards, and control wells. 6. Add 25 μl of neat Samples into the appropriate wells. 7. Vortex Mixing Bottle and add 25 μl of the Mixed or Premixed Beads to each well. 8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16–18 hours) at 4°C. 9. Gently remove well contents and wash plate two times following instructions listed in the PLATE WASHING section. Each sample was prepared in triplicate. An Axon scanner 4000B with GenePix software was used to collect fluorescence intensities.

Enzyme linked immunosorbent assay (ELISA)

Levels of IL-21 and IL-6 in the culture supernatants were measured by ELISA (R&D Systems) according to the manufacturer's recommendations.

Flow cytometric analysis

The following antibodies were used for surface staining: anti-human CD4-FITC, CXCR5-APC, PD1-PE (eBioscience), Anti-Adiponectin Receptor 1 (AdipoR1, abcam), Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG (H+L) (FMS). Data were acquired using a Fluorescence Activated Cell Sorter (FACS) calibur system (Backman Biosciences) and analysed by CytExpert software and Flowjo software. All samples were treated according to the manufacturer's recommendations.

Experimental animals

Six- to eight-week-old DBA/1J mice were purchased from the Shanghai Laboratory Animal Center, China. Mice were fed under pathogen-free conditions at experimental animal center of Nanjing Medical University. All experiments were conducted according to the animal care and use committee guidelines. CIA mice were induced as previously described (23). Firstly, 100μg of bovine type II collagen (CII) (Chondrex) was dissolved in 0.05M acetic acid with an equal volume of Freund's complete adjuvant (Difco). Then DBA/1J mice were intradermally administered at the base of tail. On Day 21, booster injections were administered with 75μg of type II collagen and Freund's incomplete adjuvant (Difco) near the primary injection site. CIA mice were intraarticularly injected with AD (10μg AD in 10μl PBS) into knee joints on day 17, day 20 and day 23 post first CII-immunisation. Other CIA mice were treated with same volume of PBS as controls.

RNA extraction and RT-PCR analysis

Joint tissues were collected for RT-PCR analysis. RNA samples were extracted by Trizol reagent (Invitrogen) and then RNA was converted to complementary deoxyribonucleic acid (cDNA) using Prime Script TM RT regent Kit according to the manufacturer's instructions (Takara). PCR primers used for RT-PCR were as follows: for GAPDH, forward 5'-AGGTCGGTGTGAACGGATTTG-3', reverse 5'-TGTAGACCATGTTGAGGTCA-3; for Bcl6, forward 5'- CACACCCGTCCATCATTGAA, reverse 5'-TGTCCTC-ACGGTGCCTTTTT-3'; for Blimp-1, forward 5'-TTCTCTTGGAAAAACGTGTGGG-3', reverse

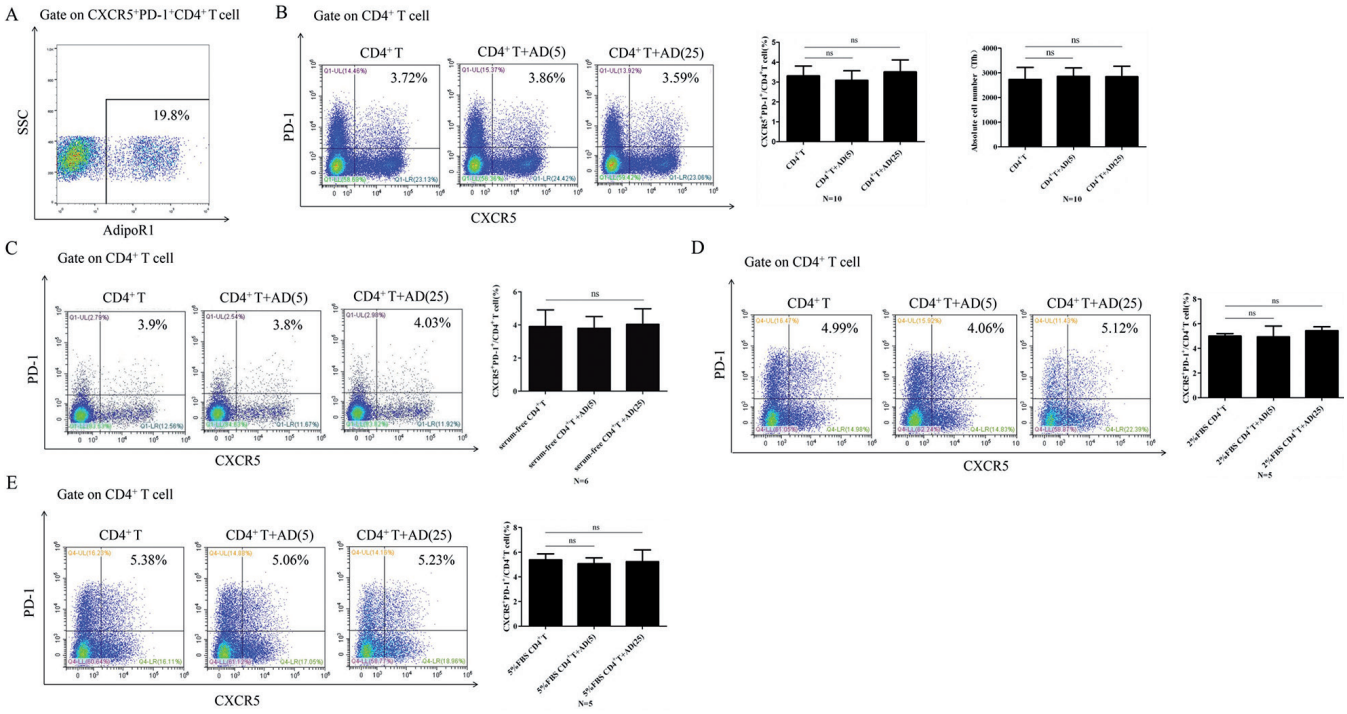

Fig. 1. AD had no direct role in Tfh cells development.
A: The expression of AD receptor1 (AdipoR1) on Tfh cells were detected by flow cytometry (n=3).
B: HC CD4+ T cells were stimulated with anti-CD3 mAb (5μg/ml, eBioscience) and anti-CD28 mAb (3μg/ml, eBioscience) in different concentrations of AD (5μg/ml and 25μg/ml, Peprotech) for 72h. The frequency or absolute number of Tfh cells (CD4+CXCR5+PD-1+T cells) were detected by flow cytometry.
C-**D**-**E**: HC CD4+ T cells were cultured in 1640 medium (Gibco) with serum-free (C), 2% FBS (D), 5% FBS (E) with different concentrations of AD (5μg/ml and 25μg/ml, Peprotech) for 72h respectively, then the frequency of Tfh cells were detected by flow cytometry.

5'-GGAGCCGGAGCTAGACTTG-3'; for IL-6, forward 5'-CTG CAA GAG ACT TCC ATC CAG TT-3', reverse 5'-GAA GTA GGG AAG GCC GTG G-3'; for IL-21, forward 5'- TGCTAGCTC-CAGCCTCAGTCT-3'; for IL-12, forward 5'- ATGACCCTGTGCCTTG-GTG-3' reverse 5'-GAAGCAGGAT-GCAGAGCTTC-3'; for IFNγ, forward 5'-GGAATGAGGTTGATCCGTTT-3' reverse 5'-TGCCAGATTCGTATGGT-GTAA-3'; The RT-PCR analysis was detected by an ABI 7900 system (Applied BioSystems Inc) and the cycling parameters were as follows: 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. Relative expression of target genes were calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) tests and Student's *t*-test. The values of $p<0.05$ were considered significant (*$p<0.05$, ** $p<0.01$ and *** $p<0.001$).

Results

AD showed indirect effect on Tfh cell differentiation

In order to determine the role of AD in the development of Tfh cells, we first examined whether AdipoR1 was expressed on the surface of Tfh cells. Flow cytometry result showed that AdipoR1 was expressed on Tfh cells (17.7%±3.19%, Fig. 1A). Then we tested whether AD had regulation on Tfh cells. However, no change of Tfh cells was observed after stimulation with AD either in the frequency or absolute number of Tfh cells (Fig. 1B). To exclude the influence of AD in fetal bovine serum (FBS), we repeated the above experiment in serum-free, 2% and 5% FBS DMEM medium respectively. Similar results were observed among the three groups (Fig. 1C-D-E). Previous study showed that synovial fibroblasts (SFs) protected T cells from apoptosis, which might had profound effects on the persistence of inflammation in RA (24). Frommer *et al*. revealed that AD could upregulate the migration of SFs and lymphocytes in RA (25). Therefore, we next assessed whether AD could induce Tfh cell production via stimulating RA FLSs. RA FLSs were firstly stimulated by AD for 72h, and then co-cultured with HC CD4+ T cells for 72h in a cell-to-cell contact system. We found that the frequency of Tfh cells was remarkedly upregulated in AD-stimulated RA FLSs and CD4+ T cell co-culture system, but this phenomenon was independent of the concentration of AD (Fig. 2A). However, there was no difference in Tfh cell frequency in the AD-stimulated OA FLSs and CD4+ T cell co-culture system (Fig. 2B). These results confirmed that AD had an indirect role in Tfh cell development.

IL-6 produced by AD-stimulated RA FLSs promoted Tfh cell generation

To further explore how AD-stimulated RA FLSs enhanced the frequency of Tfh cells, AD-stimulated RA FLSs were co-cultured with HC CD4+ T cells in a transwell system. The result showed that there was no difference in Tfh cell frequency between cell-to-cell contact and transwell system (Fig. 3A),

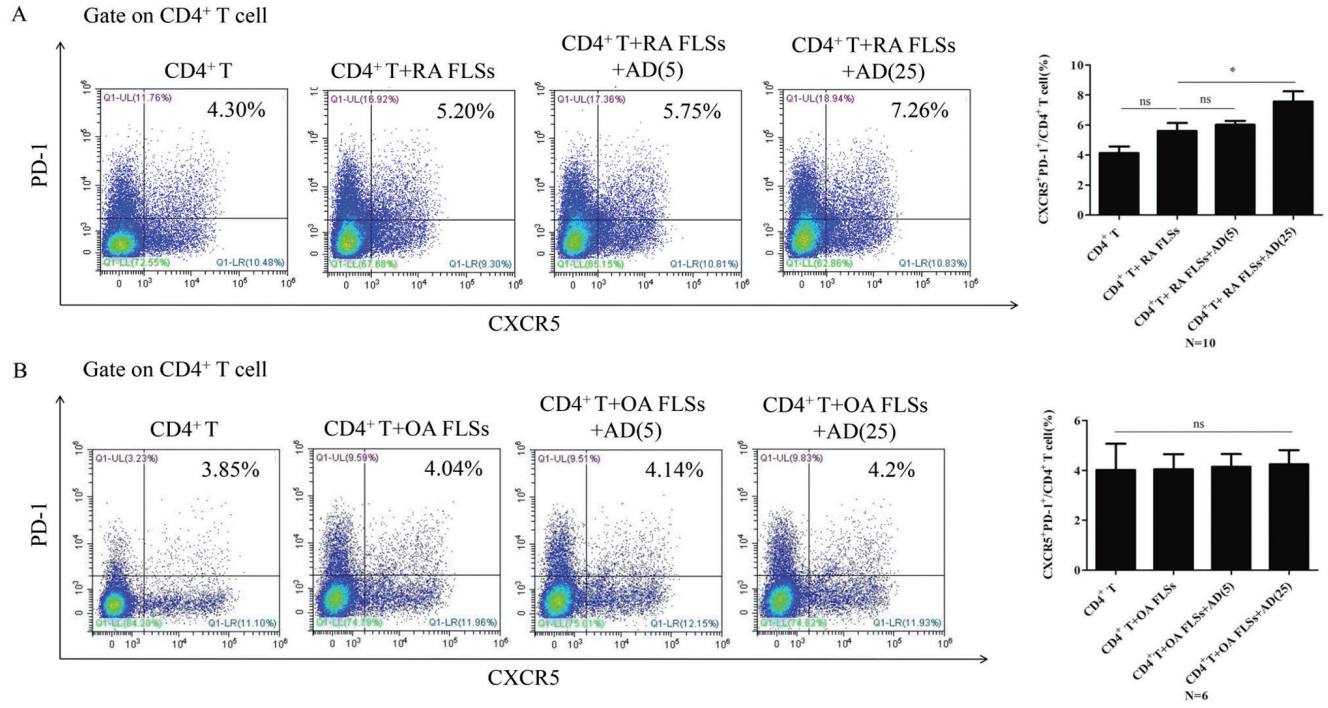

Fig. 2. AD had an indirect role in Tfh cells development.
A-B: RA FLSs and OA FLSs were firstly stimulated by AD (25μg/ml, Peprotech) for 72h, and then co-cultured with HC CD4+ T cells through cell-to-cell contact for 72h. The frequency of Tfh cells were detected by flow cytometry.

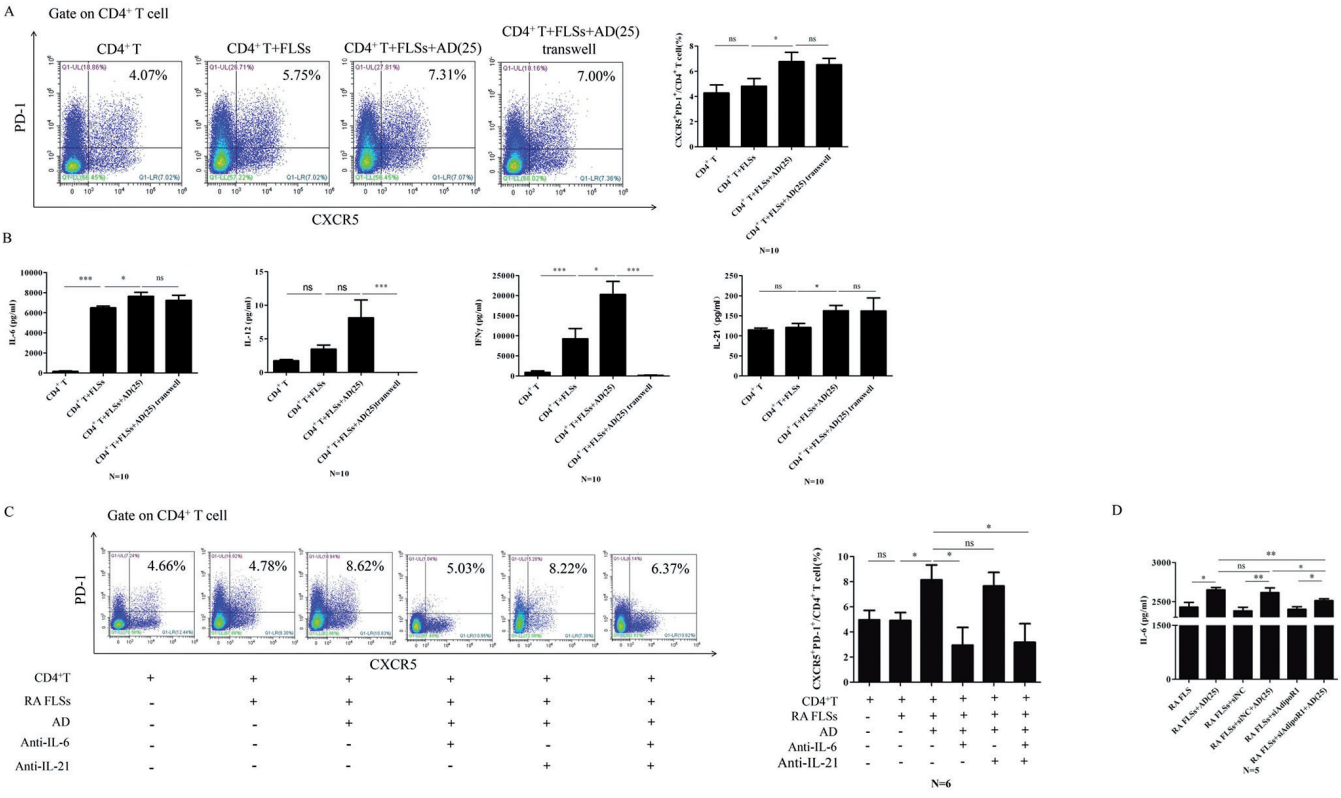

Fig. 3. IL-6 produced by AD-stimulated RA FLSs promoted Tfh cell generation.
A: RA FLSs were firstly stimulated by AD (25μg/ml, Peprotech) for 72h. The supernatant was discarded and the FLSs were washed with PBS twice and then co-cultured with HC CD4+ T cells through transwell system for 72h. The frequency of Tfh cells were detected by flow cytometry.
B: The levels of IL-6, IL-21, IL-27, IFNγ and IL-12 in the supernatants of each group were examined by microarray or ELISA.
C: Anti-IL-6 antibody (0.5 ug/ml, eBioscience) or/and anti-IL-21 antibody (0.2 ug/ml, eBioscience) were added to the above co-culture system. The frequency of Tfh cells was detected by flow cytometry.
D: RA FLSs transfected with AdipoR1 siRNA were stimulated with AD for 72h. The level of IL-6 in the supernatants were detected by ELISA.

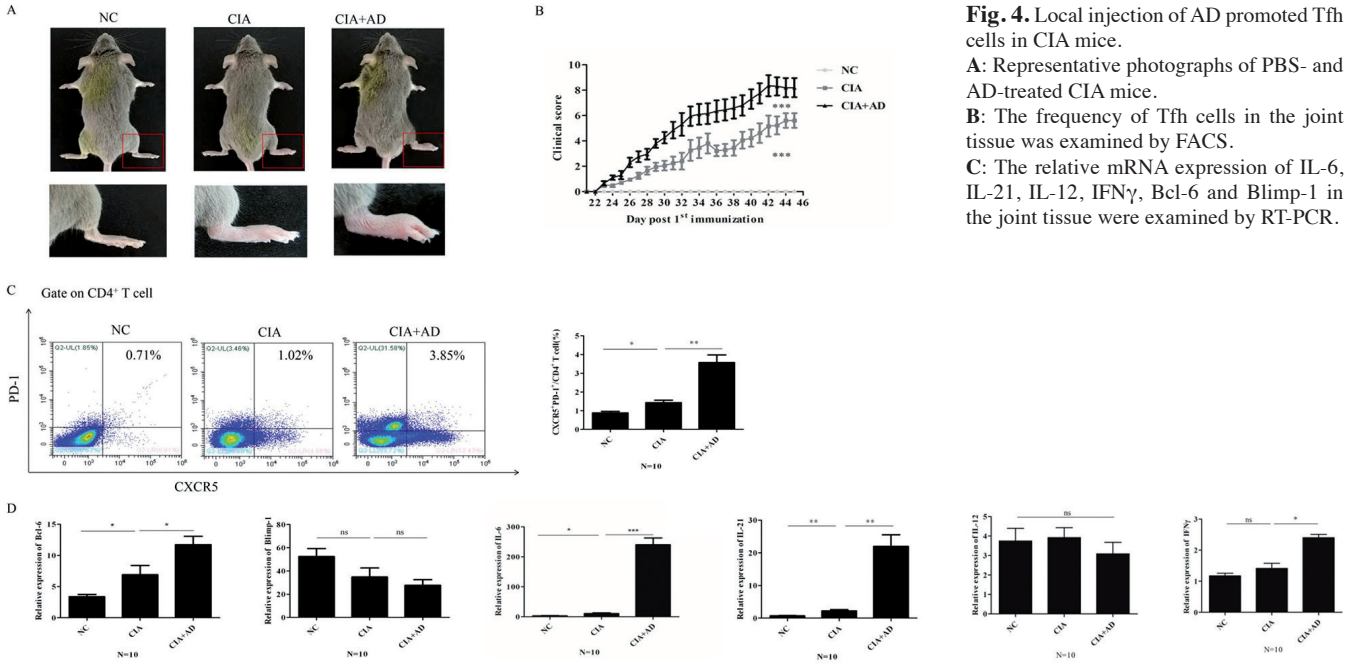

Fig. 4. Local injection of AD promoted Tfh cells in CIA mice.
A: Representative photographs of PBS- and AD-treated CIA mice.
B: The frequency of Tfh cells in the joint tissue was examined by FACS.
C: The relative mRNA expression of IL-6, IL-21, IL-12, IFNγ, Bcl-6 and Blimp-1 in the joint tissue were examined by RT-PCR.

indicating that AD-stimulated RA FLSs promoted Tfh cell production mainly by the secretion of soluble factors.

Given that no differences in Tfh cell frequency were detected through cell-to-cell contact or transwell system, we focused on the soluble factors in AD-stimulated RA FLSs. Previous study showed that the positive regulators such as IL-6, IL-21, IFNγ, IL-27 and IL-12 were critical for Tfh cell differentiation (26). Thus, the supernatants of each experiment group were collected and the levels of above soluble factors were detected by protein microarray or ELISA. Consistent with increased frequency of Tfh cells, IL-6 and IL-21 levels were significantly enhanced in the supernatant of co-culture system (Fig. 3B). IL-12 and IFNγ levels were only increased in cell-to-cell contact system but not in the transwell system (Fig. 3B). IL-27 was below standard in all groups. These results suggested that IL-6 and IL-21 might be involved in RA FLSs-mediated promotion on Tfh cells. To further confirm that, anti-IL-6 antibody or/and anti-IL-21 antibody were added to the co-culture system respectively. We found that neutralising IL-6 but not IL-21 significantly downregulated the frequency of Tfh cells (Fig. 3C). To find the source of IL-6, RA FLSs were stimulated by AD. The IL-6 protein expression were markedly elevated in the supernatant of RA FLSs after stimulation with AD and downregulated after interfering with AdipoR1 expression on RA FLSs (Fig. 3D). This result indicated that IL-6 was the mainly soluble factors by AD-stimulated RA FLSs that mediated Tfh cell generation.

Local injection of AD promoted Tfh cell expansion in CIA mice

Our previous work had confirmed that AD could accelerate bone erosion by prompting Th17 cell differentiation and enhancing receptor activator of nuclear factor-κB ligand (RANKL) expression (27). As shown in Fig. 4A-B, local AD-treated CIA mice exhibited earlier onset of arthritis and higher arthritis scores. Then we sought to determine whether AD could affect Tfh cell generation in the joint of CIA mice. Flow cytometry showed that the $CD4^+CXCR5^+PD\text{-}1^+$ T cells were significantly enhanced in the joint of local AD-treated CIA mice compared to untreated CIA mice (Fig. 4C). We also found that the mRNA expression of Tfh cell transcription factors and functional factors such as Bcl-6, IL-6, IL-21 and IFNγ in the joint were significantly upregulated. However, the expression of Blimp-1 and IL-12 were not changed (Fig. 4D). These results suggested that AD was the triggering factors responsible for enhanced Tfh cell production during CIA development.

Discussion

In this study, we first discovered that AD had indirect regulation on the development of Tfh cells. This process was mediated by stimulating RA FLSs to secret IL-6. The finding *in vitro* was consistent to the animal results that Tfh cell frequency was highly expressed in the joint of local AD-treated CIA mice.

It is reported that the serum concentrations of AD were significantly higher in RA patients and positively associated with radiographic progression (28, 29). But the opposite result showed that in RA patients with severe disease, the levels of C-reactive protein (CRP) inversely correlated with circulating AD concentrations (30). This result indicated that high-grade inflammation was independently and negatively correlated with circulating AD concentrations in RA. Although there was controversy in the role of AD at the systemic level of RA, it is regarded that high concentrations of AD in the joint and the synovial fluid were correlated closely with progressive bone erosion of RA patients (16-19). This suggested that a high level of AD in the joint might contribute to synovitis and joint destruction.

AD exerts its inflammatory effect mainly by acting on cartilage, synovium, bone, and various immune cells (31, 32). Moreover, Frommer *et al*. found that AD had pathophysiological modu-

lation in RA effector cells, including FLSs, lymphocytes, endothelial cells and chondrocytes (33). AD exerts its function via three receptors: AdipoR1 which was predominantly expressed in skeletal muscle, AdipoR2 expressed more abundantly in the liver, and T-cadherin, mainly expressed in the cardiovascular system (31). In RA, AD plays its proinflammatory function mainly depending on AdipoR1 (32). In our experiment, the expression of AdipoR1 was detected on the surface of Tfh cells. These data prompted us to test whether high concentration of AD could induce Tfh cell development. Unexpectedly, we did not observe direct effect of AD on Tfh cells. These results implied that AD might regulate the production of Tfh cells through other ways.

Substantial evidence suggested that FLSs carried a variety of innate immune receptors and were able to present antigen. In RA, FLSs promoted joint destruction via their attachment to cartilage and the interaction between FLSs and T cells could facilitate T cell recruitment (34, 35). It has been reported that AD induced IL-6 production from FLSs by binding to the AdipoR1 through activating the AMP activated protein kinase (AMPK)-p38-nuclear factor-κB signalling pathway (36, 37). Our previous study demonstrated that AD was highly expressed in the inflamed synovial joint tissue and correlated closely with progressive bone erosion in RA patients (19). Thus, we sought to explore whether AD could induce Tfh cell production through changing the local joint microenvironment. As expected, we found that AD-stimulated FLSs could promote Tfh cell generation. This increase in Tfh cells would make B cells receive excessive survival signal and produce self-reactive antibodies (38-41), such as RF and anti-CCP, which are related to disease activity (42). Therefore, our results provided new evidence for a previously unrecognised role of AD on Tfh cells in RA, although this was an indirect effect. Moreover, we found AD upregulated Tfh cell production mainly due to the secretion of soluble factors IL-6. IL-6 was a major early inducer of Tfh cell differentiation program, which through upregulated Bcl-6 protein expression (43, 44). Although we also observed that supernatant IL-21 levels were mildly enhanced and correspond to an increase in the proportion of Tfh cells, blockade of IL-21 did not change the Tfh cell frequency. According to the literature, both IL-12 and IFNγ were important soluble factors for Tfh cell differentiation (8, 11). In our experiment, we observed that supernatant levels of IL-12 and IFNγ, especially IFNγ, were only increased in cell-to-cell contact system but not in the transwell system (Fig. 3B). Furthermore, the low levels of IL-12 and IFNγ in the supernatant of transwell system were not in accord with high Tfh cell proportion in the transwell group (Fig. 3A), suggesting that both IL-12 and IFNγ could only be induced by cellular interaction and were not the major soluble factors involved in the process of Tfh cell generation in RA.

Taken together, it is possible that the high level of AD changed the local microenvironment of the joint that favours Tfh cell development in RA, suggesting that AD might serve as a potential therapeutic target for the treatment of RA.

References

1. FIRESTEIN GS: Evolving concepts of rheumatoid arthritis. *Nature* 2003; 423: 356-61.
2. MCINNES IB, SCHETT G: The pathogenesis of rheumatoid arthritis. *N Engl J Med* 2011; 365: 2205-19.
3. BREITFELD D, OHL L, KREMMER E *et al*.: Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 2000; 192: 1545-52.
4. CHEVALIER N, JARROSSAY D, HO E *et al*.: CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol* 2011; 186: 5556-68.
5. KING C, TANGYE SG, MACKAY CR: T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* 2008; 26: 741-66.
6. VINUESA CG, TANGYE SG, MOSER B, MACKAY CR: Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* 2005; 5: 853-65.
7. NURIEVA RI, CHUNG Y, HWANG D *et al*.: Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 2008; 29: 138-49.
8. SCHMITT N, MORITA R, BOURDERY L *et al*.: Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity* 2009; 31: 158-69.
9. LOCCI M, WU JE, ARUMEMI F *et al*.: Activin A programs the differentiation of human TFH cells. *Nat Immunol* 2016; 17: 976-84.
10. BATTEN M, RAMAMOORTHI N, KLJAVIN NM *et al*.: IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells. *J Exp Med* 2010; 207: 2895-906.
11. NAKAYAMADA S, POHOLEK AC, LU KT *et al*.: Type I IFN induces binding of STAT1 to Bcl6: divergent roles of STAT family transcription factors in the T follicular helper cell genetic program. *J Immunol* 2014; 192: 2156-66.
12. MYLES A, GEARHART PJ, CANCRO MP: Signals that drive T-bet expression in B cells. *Cell Immunol* 2017; 321: 3-7.
13. LIU R, LI X, ZHANG Z *et al*.: Allogeneic mesenchymal stem cells inhibited T follicular helper cell generation in rheumatoid arthritis. *Sci Rep* 2015; 5: 12777.
14. LIU R, WU Q, SU D *et al*.: A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. *Arthritis Res Ther* 2012; 14: R255.
15. MAO X, HONG JY, DONG LQ: The adiponectin signaling pathway as a novel pharmacological target. *Mini Rev Med Chem* 2006; 6: 1331-40.
16. SCHAFFLER A, EHLING A, NEUMANN E *et al*.: Adipocytokines in synovial fluid. *JAMA* 2003; 290: 1709-10.
17. PENATTI A, FACCIOTTI F: Differences in serum and synovial $CD4^+$ T cells and cytokine profiles to stratify patients with inflammatory osteoarthritis and rheumatoid arthritis. *Arthritis Res Ther* 2017; 19: 103.
18. PRESLE N, POTTIE P, DUMOND H *et al*.: Differential distribution of adipokines between serum and synovial fluid in patients with osteoarthritis. Contribution of joint tissues to their articular production. *Osteoarthritis Cartilage* 2006; 14: 690-95.
19. TAN W, WANG F, ZHANG M, GUO D, ZHANG Q, HE S: High adiponectin and adiponectin receptor 1 expression in synovial fluids and synovial tissues of patients with rheumatoid arthritis. *Semin Arthritis Rheum* 2009; 38: 420-27.
20. MANGGE H, ALMER G, TRUSCHNIG-WILDERS M, SCHMIDT A, GASSER R, FUCHS D: Inflammation, adiponectin, obesity and cardiovascular risk. *Curr Med Chem* 2010; 17: 4511-20.
21. KIM H, CHO M, KIM K, JUHN J, HWANG S, YOON C: Up-regulation of IL-23p19 expression in rheumatoid arthritis synovial fibroblasts by IL-17 through PI3-kinase-, NF-jB and p38 MAPK-dependent signalling pathways. *Rheumatology* (Oxford) 2007; 46: 57-64.
22. REVU S, NEREGÅRD P, AF KLINT E, KOROTKOVA M, CATRINA AI: Synovial membrane immunohistology in early-untreated rheumatoid arthritis reveals high expression of catabolic bone markers that is modulated by methotrexate. *Arthritis Res Ther* 2013; 15: R205.
23. LO CK, LAM QL, SUN L *et al*.: Natural killer cell degeneration exacerbates experimental arthritis in mice via enhanced interleukin-17

production. *Arthritis Rheum* 2008; 58: 2700-11.
24. OSPELT C: Synovial fibroblasts in 2017. *RMD Open* 2017; 3: e000471.
25. FROMMER KW, SCHAFFLER A, BUCHLER C *et al*.: Adiponectin isoforms: a potential therapeutic target in rheumatoid arthritis? *Ann Rheum Dis* 2012; 71: 1724-32.
26. JOGDAND GM, MOHANTY S, DEVADAS S: Regulators of Tfh cell differentiation. *Front Immunol* 2016; 7: 520.
27. SUN X, FENG X, TAN W *et al*.: Adiponectin exacerbates collagen-induced arthritis via enhancing Th17 response and prompting RANKL expression. *Sci Rep*. 2015; 5:11296.
28. RHO YH, SOLUS J, SOKKA T *et al*.: Adipocytokines are associated with radiographic joint damage in rheumatoid arthritis. *Arthritis Rheum* 2009; 60: 1906-14.
29. KLEIN-WIERINGA IR, VAN DER LINDEN MP, KNEVEL R *et al*.: Baseline serum adipokine levels predict radiographic progression in early rheumatoid arthritis. *Arthritis Rheum* 2011; 63: 2567-74
30. GONZALEZ-GAY MA, LLORCA J, GARCIA-UNZUETA MT *et al*.: High-grade inflammation, circulating adiponectin concentrations and cardiovascular risk factors in severe rheumato id arthritis. *Clin Exp Rheumatol* 2008; 26: 596-603
31. VERSINI M, JEANDEL PY, ROSENTHAL E, SHOENFELD Y: Obesity in autoimmune diseases: not a passive bystander. *Autoimmun Rev* 2014; 13: 981-1000.
32. GOMEZ R, CONDE J, SCOTECE M, GOMEZ-REINO JJ, LAGO F, GUALILLO O: What's new in our understanding of the role of adipokines in rheumatic diseases? *Nat Rev Rheumatol* 2011; 7: 528-36.
33. FROMMER KW, ZIMMERMANN B, MEIER FM *et al*.: Adiponectin-mediated changes in effector cells involved in the pathophysiology of rheumatoid arthritis. *Arthritis Rheum* 2010; 62: 2886-99.
34. NEUMANN E, LEFEVRE S, ZIMMERMANN B, GAY S, MULLER-LADNER U: Rheumatoid arthritis progression mediated by activated synovial fibroblasts. *Trends Mol Med* 2010; 16: 458-68.
35. JUAREZ M, FILER A, BUCKLEY CD: Fibroblasts as therapeutic targets in rheumatoid arthritis and cancer. *Swiss Med Wkly* 2012; 142: w13529.
36. TANG CH, CHIU YC, TAN TW, YANG RS, FU WM: Adiponectin enhances IL-6 production in human synovial fibroblast via an AdipoR1 receptor, AMPK, p38, and NF-kappa B pathway. *J Immunol* 2007; 179: 5483-92.
37. EHLING A, SCHAFFLER A, HERFARTH H *et al*.: The potential of adiponectin in driving arthritis. *J Immunol* 2006; 176: 4468-78.
38. SCHAERLI P, WILLIMANN K, LANG AB, LIPP M, LOETSCHER P, MOSER B: CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* 2000; 192: 1553-62.
39. WALKER LS, GULBRANSON-JUDGE A, FLYNN S *et al*.: Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. *J Exp Med* 1999; 190: 1115-22.
40. KIM CH, ROTT LS, CLARK-LEWIS I, CAMPBELL DJ, WU L, BUTCHER EC: Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* 2001; 193: 1373-81.
41. MA J, ZHU C, MA B *et al*.: Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* 2012; 2012: 827480.
42. HU YL, METZ DP, CHUNG J, SIU G, ZHANG M: B7RP-1 blockade ameliorates autoimmunity through regulation of follicular helper T cells. *J Immunol* 2009; 182: 1421-28.
43. ETO D, LAO C, DITORO D *et al*.: IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PloS One* 2011; 6: e17739.
44. CHOI YS, ETO D, YANG JA, LAO C, CROTTY S: Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol* 2013; 190: 3049-53.