Sirolimus selectively increases circulating Treg cell numbers and restores the Th17/Treg balance in rheumatoid arthritis patients with low disease activity or in DAS28 remission who previously received conventional disease-modifying anti-rheumatic drugs

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Abstract

Objective

Regulatory T (Treg) cells are crucial players in the prevention of autoimmunity. Mechanistic target of rapamycin (mTOR) signalling negatively controls the development and function of Treg cells. The aim of the present study was to evaluate the effects of rapamycin, under the generic name sirolimus, on $CD4^+CD25^+FoxP3^+$ Treg cells in rheumatoid arthritis (RA) patients with low disease activity or in DAS28 remission.

Methods

Fifty-five RA patients and 60 healthy controls were enrolled in this study. All patients had previously received conventional disease-modifying anti-rheumatic drugs (DMARDs) and were considered to have a low DAS28 score (≤3.2). Peripheral blood samples and clinical information were obtained at baseline and following 6 and 12 weeks of sirolimus treatment, or after 12 weeks of conventional treatment. Peripheral blood samples were also obtained from the healthy controls. The circulating levels of lymphocyte subpopulations were assessed by flow cytometry.

Results

Thirty-five patients received sirolimus and 20 patients continued treatment with conventional DMARDs. The absolute counts and proportions of $CD4^+CD25^+FoxP3^+$ Treg cells were significantly lower in all RA patients with DAS28 ≤ 3.2 as compared with those in healthy controls. By contrast, the difference in circulating Th17 cell numbers was not significant. Sirolimus administration resulted in elevations in circulating Treg cell numbers and significant reductions in the Th17/Treg cell ratio, whereas the circulating level of Treg cells and the Th17/Treg cell ratio in patients under conventional treatment both showed a tendency of reduction. Furthermore, a greater proportion of patients under sirolimus treatment achieved DAS28-based remission at 12 weeks.

Conclusion

Sirolimus can favourably expand Treg cells in RA patients with DAS28 ≤3.2, consequently restoring a healthy balance of Th17/Treg cells, which might improve the likelihood of long-term and sustained clinical remission and reduce the probability of disease flare-ups in RA.

Key words

rheumatoid arthritis, sirolimus, regulatory T cells, Th17 cells, low disease activity, remission, DAS28

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by persistent polyarticular synovitis, systemic inflammation, and autoantibody production, eventually resulting in joint destruction and disability (1). Long-lasting clinical remission or at least low disease activity (LDA) has been established as a treatment target, which can prevent RA-related disability (1, 2). In practice, if a state of major responses has been achieved by 3 months after therapy initiation, the likelihood of reaching the target at 6 months is very high (3, 4). However, many patients lose drug responsiveness and experience a flare-up in disease activity over time after reaching LDA or Disease Activity Score in 28 joints (DAS28) remission under therapy with conventional disease-modifying anti-rheumatic drugs (DMARDs). Although the precise reasons for this regression are not known, an imbalance between Th17 and regulatory T (Treg) cells might play an important role.

As recently identified $CD4^+$ T cell subsets, the roles of Th17 and Treg cells have been extensively investigated in rheumatic diseases (5). Th17 cells are leading actors in RA-related autoimmunity, whereas Treg cells suppress the autoreactive activities of effector $CD4^+$ T cells and thus maintain immune tolerance. Patients with inactive RA show a reduced frequency of peripheral Treg cells (6). Moreover, we previously found a decreased number and frequency of Treg ($CD4^+CD25^+FoxP3^+$) cells and an increased ratio of Th17/Treg cells in the peripheral blood of RA patients with LDA ($2.6 \leq$ DAS28 ≤ 3.2) and/or in DAS28 remission (DAS28 <2.6), while the levels of circulating Th17 cells ($CD4^+IL\text{-}17^+$) were similar to those of healthy subjects (unpublished data), suggesting that a reduction in Treg cells might be the leading cause of the Th17/Treg imbalance in RA patients with LDA or in DAS28 remission.

Treg cells are essential for immune homeostasis and are crucial players in the prevention of autoimmunity. The phosphatidylinositol 3-kinase (PI3K)-Akt-mechanistic target of rapamycin (mTOR) signaling axis negatively controls the development, homeostasis, and function of $CD4^+CD25^+FoxP3^+$ Treg cells (7, 8). Rapamycin, an mTOR inhibitor, exhibits immunosuppressive abilities by inhibiting the serine/threonine kinase mTOR downstream of PI3K and Akt (9), and has therefore been developed as a medication to prevent organ transplant rejection under the generic designation of sirolimus (10). In addition, sirolimus may be efficacious in patients with several autoimmune diseases (8), and was shown to effectively expand the subpopulation of $CD4^+CD25^+FoxP3^+$ Treg cells in patients with clinically active systemic lupus erythematosus (SLE) (11). We also demonstrated an increase of Treg cells in patients with active RA after receiving sirolimus treatment (12); however, the response of Treg cells to sirolimus treatment in RA patients with DAS28 ≤ 3.2 is still unclear. Therefore, the purpose of this study was to investigate the influence of sirolimus treatment on $CD4^+CD25^+FoxP3^+$ Treg cells in RA patients with DAS28 ≤ 3.2 who previously received conventional DMARDs.

Materials and methods

Subjects and study design

A total of 55 RA patients (mean age ± SD, 48.7±14.0 years, 41 females) who met the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 criteria (13), and 60 healthy controls (mean age ± SD, 46.9±10.2 years, 45 females) were enrolled in the study. All patients previously received DMARDs treatment and were considered to have LDA ($2.6 \leq$ DAS28 ≤ 3.2) or were in DAS28 remission (DAS28 <2.6). Disease activity in patients was assessed using the DAS28 (14) during clinic visits, and a flare-up was defined as DAS28 >3.2 (15). Cut-off points of the DAS28 for remission (<2.6), low (2.6 to 3.2), moderate (>3.2 to ≤ 5.1), and high (>5.1) disease activity were used (16). Healthy volunteers had no evidence of inflammatory syndromes, no autoimmune or inflammatory diseases, no recent acute or chronic infectious diseases, and no history of cancer. They also had not recently received steroids or immunosuppressive drugs. The clinical characteristics of all RA patients were retrospectively

collected from their medical records. This study was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University, and written informed consent was obtained from all patients and controls.

Thirty-five RA patients with DAS28 ≤3.2 received sirolimus at a dose of 0.5 mg every 2 days. Concomitant treatment with methotrexate or leflunomide, non-steroidal anti-inflammatory drugs, glucocorticoids (≤10 mg of prednisone or the equivalent per day), or a combination of these drugs was permitted. Clinical disease activity [as measured by the DAS28 and erythrocyte sedimentation rate (ESR)] and immunological assessments were determined at baseline (0W), and at 6 weeks (6W) and 12 weeks (12W) post-sirolimus treatment. Twenty RA patients with DAS28 ≤3.2 continued their present conventional treatment and were also evaluated for clinical disease activity and an immunological assay at 0W and 12W. The decision for sirolimus treatment or conventional treatment was made by a physician. During follow-up visits, the category and dosage of DMARDs and glucocorticoids prescribed to all RA patients could be reduced according to changes in disease activity at the physician's discretion.

Peripheral blood samples were taken from the patients undergoing sirolimus treatment at baseline and after 6 and 12 weeks, and were taken from the patients under conventional treatment at baseline and after 12 weeks. Peripheral blood samples were also obtained from the healthy subjects, which were used in parallel as controls for immunological analyses.

Analysis of lymphocyte subpopulations by flow cytometry

Whole-blood samples were collected in EDTA anticoagulant tubes according to manufacturer recommendations (BD Biosciences, San Jose, CA, USA) and were analysed to determine percentages and absolute counts of lymphocyte subpopulations by flow cytometry. The following monoclonal antibodies conjugated with a fluorochrome were used for $CD3^+/CD4^+/CD8^+$ T lymphocyte populations: fluorescein isothiocyanate (FITC)-CD3, phycoerythrin (PE)-CD8, peridinin chlorophyll protein (PerpCP)-CD45, and allophycocyanin (APC)-CD4. The following monoclonal antibodies were used for B lymphocytes and natural killer (NK) cell populations: FITC-CD3, PE-CD16+CD56, PerpCP-CD45, and APC-CD19. The percentages and absolute counts of $CD3^+CD19^-$ T cells, $CD3^-CD19^+$ B cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and NK cells ($CD3^-CD16^+CD56^+$) were automatically calculated using BD Multitest software (BD Biosciences). $CD4^+$ T cell

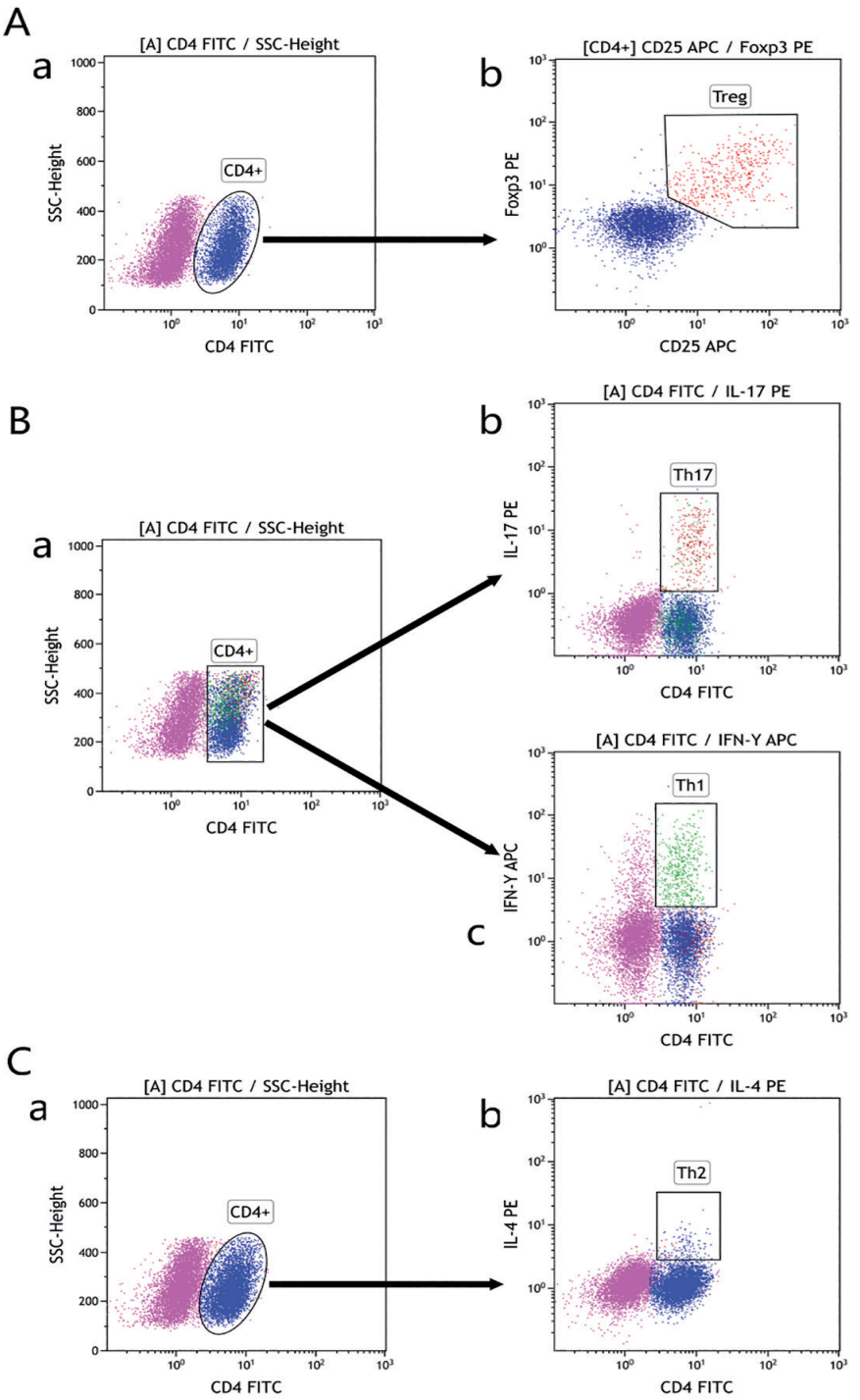

Fig. 1. Analysis of lymphocyte subpopulations by flow cytometry.
A: Gating strategy for Treg cells: **a** expressed as a single parameter of CD4 and scatter; **b** Treg ($CD4^+CD25^+FoxP3^+$). **B**: Gating strategy for Th17 and Th1 cells: **a** expressed as a single parameter of CD4 and scatter; **b** Th17 ($CD4^+IL\text{-}17^+$); **c** Th1 ($CD4^+IFN\text{-}\gamma^+$). **C**: Gating strategy for Th2 cells: **a** expressed as a single parameter of CD4 and scatter; **b** Th2 ($CD4^+IL\text{-}4^+$). Treg: regulatory T cells, Th: T helper cells.

subsets were stained using the standard protocol with FITC-conjugated anti-CD4 and APC-conjugated anti-IFN-γ (intracellular staining) for Th1 cell analysis; FITC-conjugated anti-CD4 and PE-conjugated anti-IL-4 (intracellular staining) for Th2 cell analysis; FITC-conjugated anti-CD4 and PE-conjugated anti-IL-17A (intracellular staining) for Th17 cell analysis; and FITC-conjugated anti-CD4, APC-conjugated anti-CD25, and PE-conjugated anti-FoxP3 (intracellular staining) for Treg cell analysis. The percentages and absolute counts of $CD4^+$ T lymphocyte subsets were automatically calculated using BD Multitest software (BD Biosciences). All immunofluorescence antibodies were purchased from BD Biosciences, and the results were expressed as percentages of the parental lineage gate and absolute counts (cells/μl). Gating strategies are described in Fig. 1.

Statistical analysis

Data are expressed as the number of cases for category-based variables, mean ± stanard deviation for continuous variables with normal distributions (age), and median and 25th and 75th percentiles (IQR) for continuous variables with non-normal distributions. The differences between groups were statistically analysed with SPSS 20.0 statistical software (SPSS Inc., Cary, NC, USA) using the χ^2 test, parametric *t* test, or non-parametric Wilcoxon rank sum test as appropriate. A mixed linear model with an unstructured covariance matrix was used for the repeated-measures analysis, and the parameters of the model were assessed by restricted maximum likelihood (REML) estimation. The repeated-measures analysis was performed with SAS 8.0 statistical software (SAS Institute Inc., Cary, NC, USA). A *p*-value <0.05 (2-sided) was considered statistically significant.

Results

Clinical characteristics of the RA patients

Baseline clinical characteristics of the 55 patients with RA, including age, sex, duration, autoantibodies, ESR, DAS, prednisone dose, and medication use, are shown in Table I. None of the parameters differed significantly between RA patients that received sirolimus treatment and conventional treatment. Note that data collected between 10 and 14 weeks of treatment were included in the "12 weeks" assessment period.

Table I. Characteristics of RA patients with DAS28 ≤3.2 under sirolimus treatment and conventional treatment at baseline.

	RA patients under sirolimus treatment	RA patients under conventional treatment
Number of patient	35	20
LDA	18	12
DAS28 remission	17	8
Age (years)[a]	48.6 ± 14.8	52.0 ± 12.7
female, no. (%)	25 (71.4)	16 (80.1)
Duration of RA (months)[b]	60 (24–124)	56 (26–128)
Antibodies		
RF positive, no. (%)	25 (71.4)	14 (70.0)
ACPA positive, no. (%)	27 (77.1)	15 (75.0)
ESR (mm/1h)[b]	14 (6-25)	11 (7-29)
DAS28[b]	2.53 (2.15-2.73)	2.64 (2.21-2.96)
Prednisone		
number of patient	23	12
dose (mg/day)[b]	5 (3.3–10)	5 (2.5–9.5)
Use of concomitant agents		
methotrexate	10	7
leflunomide	8	3
hydroxychloroquine	25	15
salazosulfapyridine	21	12

[a] Results expressed as mean ± standard deviation (*M* ± *SD*). [b] Results expressed as median and 25th and 75th percentiles. The independent-samples *t*-test was performed for quantitative variables with normal distributions; the chi-squared test was performed for category-based variables. The Wilcoxon rank sum test test was performed for quantitative variables with non-normal distributions. RA: rheumatoid arthritis; LDA: low disease activity; DAS28: Disease Activity Score in 28 joints; RF: rheumatoid factor; ACPA: anti-citrullinated peptides antibody; ESR: erythrocyte sedimentation rate.

Decreased absolute counts and proportions of Treg cells, and increased ratio of Th17/Treg cells in the peripheral blood of RA patients with DAS28 ≤3.2

As shown in Table II, the absolute counts and proportions of Treg cells ($CD4^+CD25^+FoxP3^+$) were both significantly lower in RA patients with DAS28 ≤ 3.2 as compared with those in healthy volunteers (p=0.003, p=0.000). Additionally, the median Th17/Treg cell ratio was significantly elevated in RA patients (p=0.000). The frequencies and counts of circulating Th17 cells ($CD4^+IL\text{-}17^+$) were higher in RA patients than those of healthy subjects, but this difference was not statistically significant (p=0.059, p=0.233). These results indicated that a reduction in Treg cells might be the leading cause of the Th17/Treg imbalance in RA patients with DAS28 ≤3.2.

We also compared the levels of Th17 cells and Treg cells between the patients under sirolimus treatment or conventional treatment and healthy controls. Both the absolute counts and proportions of Treg cells were significantly decreased in patients under sirolimus treatment at baseline compared to those of healthy controls (p=0.035, p=0.000), and a similar result was observed for patients under conventional treatment. Compared with that in healthy subjects, the Th17/Treg cell ratio was significantly increased in RA patients at 0W under sirolimus or conventional treatment (p=0.001, p=0.002), although the differences in Th17 cells were not significant between patients with sirolimus or conventional treatment and healthy subjects (Table II, Fig. 2). There were no significant differences in the numbers of Treg cells, Th17 cells, and the Th17/Treg ratio between RA patients that received sirolimus treatment and conventional treatment.

The frequencies or absolute counts of Th1 ($CD4^+IFN\text{-}\gamma^+$) and Th2 ($CD4^+IL\text{-}4^+$) cells were not significantly different between RA patients and healthy subjects.

Table II. Absolute counts and proportions of $CD4^+$ T lymphocyte subpopulations in the peripheral blood of all study participants.

	Healthy controls (n=60)	All RA patients (n=55)	RA patients under sirolimus treatment (n=35)	RA patients under conventional treatment (n=20)
$CD4^+$T subsets (cells/μl)				
Th17	7.03 (4.43-10.30)	7.76 (4.59-12.65)	8.13 (4.59-13.92)	7.25 (4.54-10.40)
Treg	34.25 (22.66-45.35)	22.04 (16.49-37.24)*	22.27 (16.49-42.34)#	20.36 (16.26-31.71)*
Th1	81.70 (18.63-146.67)	71.97 (33.03-121.83)	69.81 (38.46-127.13)	73.16 (14.10-105.91)
Th2	9.87 (5.94-15.07)	9.89 (4.63-15.59)	10.51 (6.43-17.04)	8.47 (3.90-10.93)
Th17/Treg	0.20 (0.15-0.33)	0.35 (0.23-0.51)$	0.33 (0.23-0.53)*	0.38 (0.20-0.47)*
Th1/Th2	8.13 (1.79-14.43)	7.23 (4.35-13.79)	7.13 (4.58-12.23)	7.96 (1.73-18.39)
$CD4^+$T subsets %				
Th17 %	1.01 (0.66-1.41)	1.29 (0.82-1.72)	1.09 (0.77-1.69)	1.20 (0.74-1.74)
Treg %	4.72 (3.89-6.19)	3.42 (2.90-4.74)$	3.28 (2.70-4.27)$	3.47 (2.07-4.59)#
Th1 %	11.30 (3.32-19.97)	10.12 (5.14-18.49)	10.12 (5.19-18.02)	10.36 (4.33-20.68)
Th2 %	1.48 (0.93-2.10)	1.45 (0.78-2.37)	1.34 (0.78-2.37)	1.60 (0.78-2.60)

Results expressed as median and 25th and 75th percentiles. Statistics: Wilcoxon rank sum test. RA: rheumatoid arthritis; Th: T helper cell; Treg: regulatory T cells. *$p<0.01$, #$p<0.05$, $$p<0.001$ *vs*. healthy controls.

Table III. Absolute counts and proportions of lymphocytes in the peripheral blood of all study participants.

	Healthy controls (n=60)	All RA patients (n=55)	RA patients under sirolimus treatment (n=35)	RA patients under routine treatment (n=20)
Lymphocyte (cells/μl)				
T	1291 (1072-1473)	1268 (918-1909)	1385 (1086-2115)#	1247 (812-1430)
B	179 (137-264)	148 (76-229)#	157 (83-252)	143 (64-177)*
$CD4^+$ T	639 (550-879)	656 (467-846)	656 (523-1274)	658 (419-762)
$CD8^+$ T	422 (334-587)	497 (324-826)	491 (426-910)*	483 (278-738)
NK	282 (189-402)	203 (145-345)#	237 (146-363)	190 (134-298)#
Lymphocyte %				
T %	72.5 (64.2-76.0)	76.0 (71.0-82.0)*	77.0 (71.0-83.0)*	74.5 (68.0-76.0)
B %	10.5 (8.0-13.8)	8.0 (5.0-12.0)*	8.0 (5.0-12.0)#	9.0 (6.0-11.0)#
$CD4^+$ T%	39.0 (32.2-44.7)	41.0 (35.0-48.0)	41.0 (36.0-48.0)	42.0 (33.0-47.5)
$CD8^+$ T %	23.0 (20.0-32.2)	28.0 (22.0-39.0)*	29.0 (22.0-36.0)#	26.5 (20.2-42.0)
NK %	16.0 (11.2-20.0)	13.0 (8.0-17.0)#	12.0 (7.0-16.0)*	16.5 (8.5-20.0)

Results expressed as median and 25th and 75th percentiles. Statistics: Wilcoxon rank sum test. RA: rheumatoid arthritis; NK: natural killer cell; *$p<0.01$, #$p<0.05$ *vs*. healthy controls.

Although the median Th1/Th2 cell ratio was reduced in patients with RA, this did not differ significantly from that observed in healthy subjects (Table II). Moreover, the frequencies of T cells and $CD8^+$ T cells were higher in RA patients with DAS28 ≤3.2 than those in healthy volunteers (p=0.004, p=0.008), whereas the percentages and absolute counts of B cells and NK cells were lower in RA patients relative to those in healthy subjects (p=0.004, p=0.011 and p=0.044, p=0.025, respectively). Furthermore, there were no significant differences in $CD4^+$ T cell numbers between RA patients and healthy volunteers (p=0.251, p=0.951). No significant differences in Th1, Th2 cells, or other lymphocyte subpopulations were observed between RA patients under sirolimus treatment and conventional treatment (Tables II and III).

We also analysed the data according seropositive or seronegative status for rheumatoid factor (RF) and anti-citrullinated peptides antibody (ACPA) in both groups of patients. There were no significant differences in the levels of Th17 cells, Treg cells, and the Th17/Treg ratio between patients with RF seropositivity and seronegativity, or patients with ACPA seropositivity and seronegativity in both groups at baseline. However, the proportions of Treg cells were lower in patients with RF seropositivity than in patients with RF seronegativity (3.04% and 4.53%, p=0.039) under conventional treatment at 12W.

Changes in lymphocyte subpopulations of RA patients after treatment

Among the 35 RA patients with LDA or in DAS28 remission who received sirolimus therapy, immunological assessments could not be performed for three patients at 6W for non-medical reasons. Changes in the lymphocyte subpopulations in all RA patients were compared among 0W (n=35), 6W (n=32), and 12W (n=35). As shown in Fig. 2A, sirolimus administration for 12 weeks increased the absolute counts and percentages of Treg cells in RA patients (p=0.013, p=0.002). The Th17/Treg cell ratio was reduced from a median of 0.33 at baseline to 0.16 at 12W (p=0.005), and there was no difference from that of healthy volunteers, indicating that sirolimus treatment restored the immunological balance of Th17/Treg cells. By contrast, no significant differences were observed in the percentages or absolute counts of Th17, Th1, or Th2 cells before and after sirolimus treatment. Additionally, there were no significant differences in the percentages

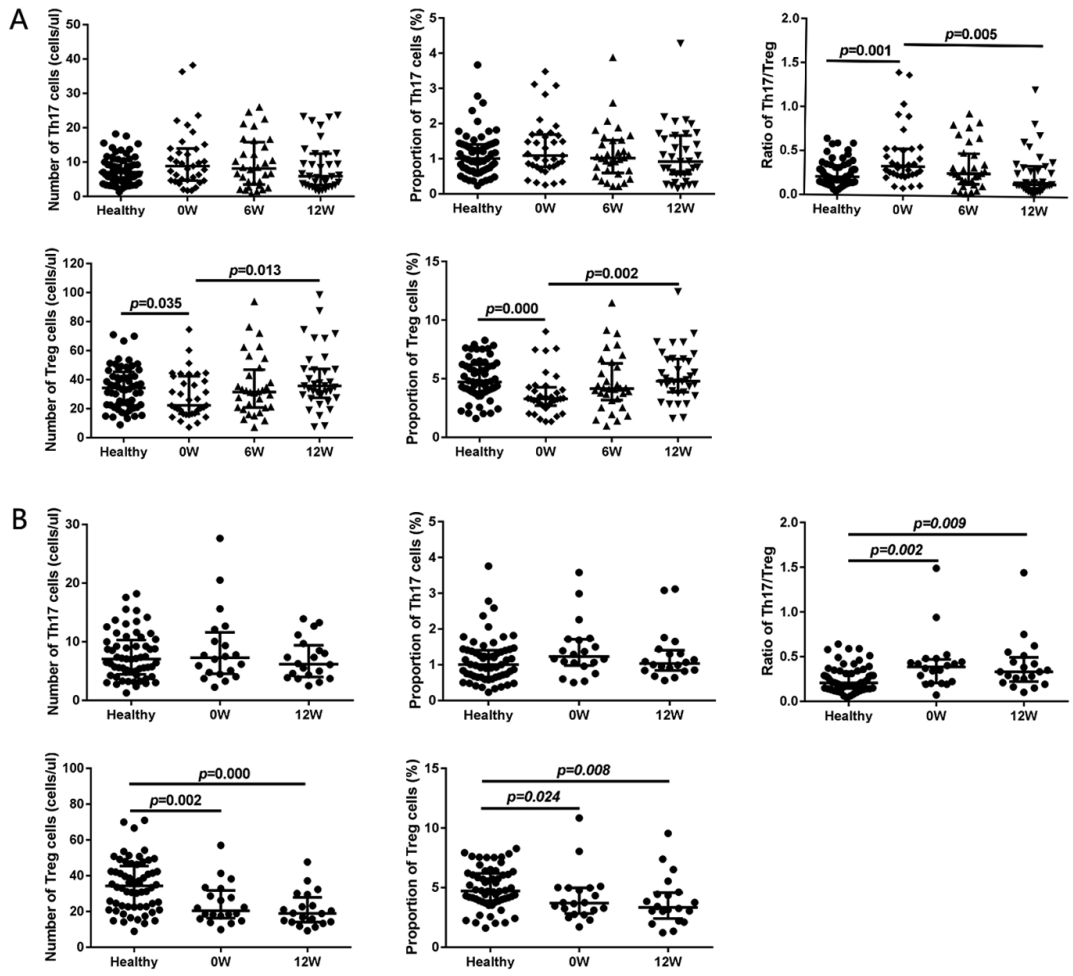

Fig. 2. The absolute counts and proportions of Th17 cells ($CD4^+IL\text{-}17^+$), Treg cells ($CD4^+CD25^+FoxP3^+$), and the ratio of Th17/Treg in healthy controls and RA patients before and after treatment.
A: Healthy controls and RA patients before and after sirolimus treatment.
B: Healthy controls and RA patients before and after conventional treatment.
Th17: T helper 17, Treg: T regulatory cells, RA: rheumatoid arthritis. Black bars: median with interquartile range. Statistics: Wilcoxon rank sum test, Mixed linear model with unstructured covariance.

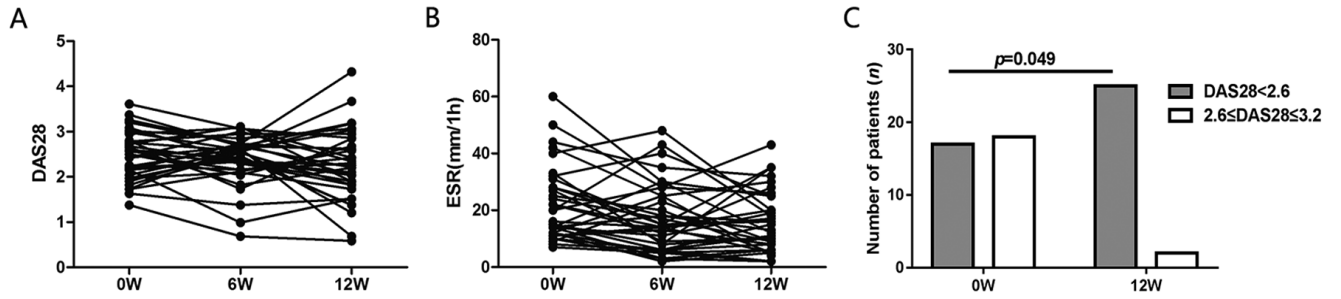

Fig. 3. Clinical response of RA patients to sirolimus treatment. **(A)** DAS28 score and **(B)** ESR in RA patients at baseline, and at 6 and 12 weeks of sirolimus treatment. (**C**) The percentage of RA patients achieving DAS28 remission at 12 weeks post-sirolimus treatment was significantly higher than that at baseline. RA: rheumatoid arthritis; DAS28: Disease Activity Score in 28 joints; ESR: erythrocyte sedimentation rate. Statistics: Wilcoxon rank sum test (A, B) or χ^2 test (C).

or absolute counts of the other lymphocyte subsets or NK cells before or after sirolimus treatment.

For RA patients that continued the conventional treatment for 12 weeks, both the percentages and absolute counts of Th17 cells declined, but the changes were not statistically significant (p=0.117, p=0.552). Additionally, the percentage and absolute counts of Treg cells were lower at 12W than at 0W, although the differences were not significant (p=0.279, p=0.317). The Th17/Treg cell ratio was also reduced at 12W compared to that at baseline (0.38 *vs.* 0.33), but the change was not significant (p=0.655). Furthermore, the median Th17/Treg ratio at 12W was significantly higher than that of the healthy volunteers (0.33 *vs.* 0.20, p=0.009; Fig. 2B), indicating that the abnormal immune balance between Th17 and Treg cells was sustained in patients under 12-week conventional treatment. There were no significant differences in the percentages or absolute counts of the other lymphocyte subsets in the RA patients under conventional treatment between 0W and 12W.

Clinical response to sirolimus

Overall, the RA patients who received sirolimus treatment showed a tendency of reduction in disease activity at the end of the study [DAS28: 2.25 *vs.* 2.53; ESR: 13 *vs.* 20 mm/1h] as compared

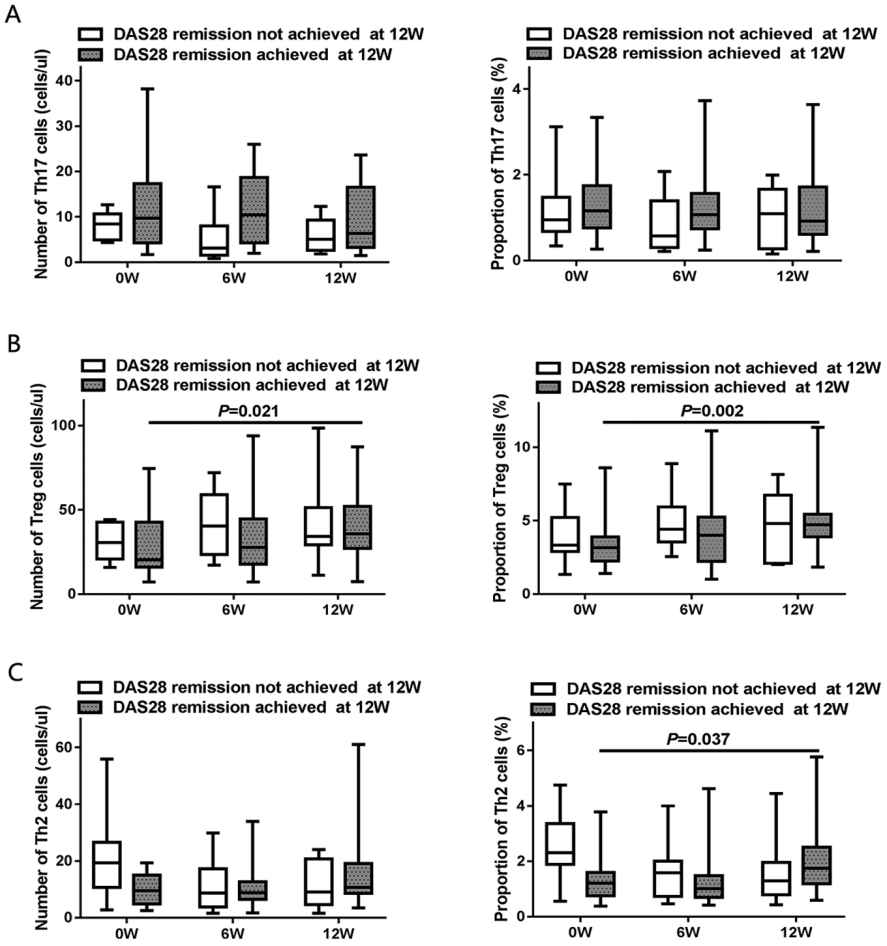

Fig. 4. Changes of lymphocyte subpopulations in RA patients under sirolimus treatment according to DAS28 remission at 12 weeks. Baseline levels and changes in the absolute counts and percentages of **(A)** Th17 cells, **(B)** Treg cells, and **(C)** Th2 cells between 0W and 12W in patients who achieved (n=25) or did not achieve (n=10) DAS28 remission. RA: rheumatoid arthritis; DAS28: Disease Activity Score in 28 joints; Th17: T helper 17; Treg: regulatory T cells. Black bars: medians. Statistics: Wilcoxon rank sum test.

with that at baseline, but the difference was not significant (Fig. 3A, Fig. 3B). By 12W, 25 (71.4%) patients reached DAS28 remission (DAS28 <2.6), and 8 patients (22.9%) exhibited LDA (DAS28: 2.6 to 3.2). The proportion of patients achieving DAS28 remission at 12W was higher than that at 0W (χ^2=3.880, p=0.049; Fig. 3C); however, two patients showed DAS28-defined disease flare-up (DAS28: 3.66 and 4.32) at 12W, and their Treg cell percentages were 2.01% and 2.08%, respectively, which were lower than the medians of healthy volunteers (4.72%) and RA patients post-sirolimus treatment (4.78%).

Reductions in glucocorticoid use during the study were permitted at the physician's discretion, and at 12W, 14 of the 35 glucocorticoid-treated patients (40.0%) were receiving prednisone at a >30% reduced dose as compared with that administered at baseline [the median prednisone dose at baseline was 5 mg (IQR: 3.5–7.5 mg) *vs*. 3.5 mg (IQR: 1.0–5.0 mg) at 12W].

During the 12-week study period, no serious adverse events occurred. Some slight adverse events, including rash, oral ulcers, and alopecia, occurred in three patients with RA. All symptoms and signs were minor and did not require special medical care.

Baseline levels and changes in lymphocyte subpopulations of RA patients in DAS28 remission at 12W

All RA patients who received sirolimus treatment were classified into two groups: those who achieved DAS28 remission (DAS28 <2.6) at 12W (n=25) and those who did not (n=10) (Fig. 4). We then evaluated the changes in different lymphocyte subpopulations at 0W, 6W, and 12W. After sirolimus treatment, the Th17 cell number declined in patients achieving DAS28 remission, but this change was not significant (p=0.157; Fig. 4A). The absolute number and proportion of Treg cells increased in patients achieving DAS28 remission at 12W (p=0.021 and p=0.002, respectively; Fig. 4B). Moreover, the Th2 cell percentage significantly increased during the 12-week period in patients achieving DAS28 remission at 12W (p=0.037; Fig. 4C). Changes in other lymphocyte subpopulations were not significant in RA patients achieving DAS28 remission at 12W, and there were no significant changes in any lymphocyte subpopulations in the RA patients not achieving DAS28 remission at 12W.

Discussion

In this study, we demonstrated that circulating $CD4^+CD25^+FoxP3^+$ Treg cells are decreased in RA patients with LDA or in DAS28 remission who previously received conventional DMARDs. Moreover, the reduced Treg cells increased after sirolimus treatment, which restored a healthy balance between Th17 and Treg cells. There were also more patients that achieved DAS28 remission (DAS28 <2.6) and showed a tendency of reduction in DAS28 score after 12-week sirolimus treatment.

Many immunosuppressants used to treat RA were designed to broadly suppress T cell function, including that of Treg cells (17), which may be the primary reason for the decreased circulating Treg cell numbers and imbalance in Th17/Treg cells detected in RA patients with LDA and/or in DAS28 remission who previously received conventional DMARDs. The imbalance between Th17 and Treg cells has been identified as a crucial event in RA pathogenesis (7, 18). Treg cells are essential for maintaining effective immune tolerance and a homeostatic balance of Th17/Treg, and thus could be targeted to treat and prevent systemic autoimmune diseases, including RA (7, 19). Therefore, there has been increasing interest in promoting immune tolerance via immunoregulation and

developing Treg-friendly regimens to minimise or eliminate the immunosuppression of conventional medications on RA.

The transcription factor forkhead box P3 (FoxP3) is indispensable for Treg cell development and function. The PI3K-Akt-mTOR-signaling axis negatively regulates FoxP3 expression, and mTOR acts as a critical negative regulator of Treg differentiation and expansion (20). Previous studies have demonstrated that rapamycin could promote expansion of functional $CD4^+CD25^+FoxP3^+$ Treg cells *in vitro* (21, 22); however, $CD4^+CD25^+FoxP3^+$ Treg cells induced and proliferated *ex vivo* are unstable in terms of lineage specialisation and suppressive function, as demonstrated by the loss of FoxP3 expression and acquisition of Th cell-like functions upon transfer into the host (23). In this setting, the administration of sirolimus to RA patients offers the opportunity to favorably shift the balance toward Treg expansion at the expense of effector T cells, consequently restoring a healthy balance between Th17 and Treg cells through a qualitative and/or quantitative increase in Treg cells. As previously described (11, 12), sirolimus can expand $CD4^+CD25^+FoxP3^+$ Treg cells in patients with active SLE and active RA *in vivo*. Consistently, our study also demonstrated that sirolimus can promote the expansion of circulating Treg cells and restore a healthy balance between Th17 and Treg cells in RA patients with DAS28 ≤3.2. This might be partly explained by the removal of the suppression exerted by mTOR signaling on Treg cells.

Moreover, we observed a significant increase in Treg cell number during sirolimus treatment in patients that reached or maintained DAS28 remission at 12W, with a higher proportion of patients achieving DAS28 remission at 12W compared to that measured at baseline. This was accompanied by a reduction in corticosteroid usage. These results further support that the restoration of Treg cells promotes remission in RA patients.

In this study, the levels of pro-inflammatory T-cell lineage, including Th17 ($CD4^+IL\text{-}17^+$) cells, Th1 ($CD4^+IFN\text{-}\gamma^+$) cells, and Th2 ($CD4^+IL\text{-}4^+$) cells, were not significantly different between RA patients with LDA or in DAS28 remission and healthy controls, or before and after 12-week sirolimus treatment. However, the proportion of Th2 cells was significantly higher in sirolimus-treated patients achieving DAS28 remission at 12W than that at baseline. This phenomenon requires further investigation.

It is important to note that we were not able to evaluate the changes of proinflammatory and anti-inflammatory cytokines in the patients during the course of sirolimus treatment. Thus, the function of Treg cells in RA with LDA or in DAS28 remission during sirolimus treatment will need to be clarified in future studies. In addition, as this is a preliminary study, our results will need to be confirmed on a wider scale, and the molecular mechanisms responsible for controlling sirolimus-mediated Treg cell proliferation also require further investigation.

In conclusion, this study provides reference data for evaluating changes in various lymphocyte subpopulations in RA patients with LDA/DAS28 remission under sirolimus treatment. The abnormally reduced levels of circulating Treg cells in RA patients with LDA/DAS28 remission could be corrected by the sirolimus-mediated blockade of mTOR signaling, which reestablished the immune balance of Th17 and Treg cells. These results emphasise the benefit of therapeutic approaches based on the promotion of Treg cells in RA. By blocking the mTOR-signaling pathway, sirolimus might facilitate Treg cell differentiation and expansion, explaining the increased number of patients that achieved DAS28 remission under this treatment. The quantitative expansion of Treg cells and a restored immune balance between Th17 and Treg cells in patients might improve the likelihood of long-term and sustained drug-free remission, and reduce the probability of disease flare-ups (24). These findings provide valuable insights that might potentially lead to changes in clinical practice for the routine treatment of RA.

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