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Cytokines and regulatory T cells in ankylosing spondylitis

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Aims

To investigate the correlations among cytokines and regulatory T cells (T-regs) in ankylosing spondylitis (AS) patients, and their changes after anti-tumour necrosis factor- α (TNF- α) treatment.

Methods

We included 72 AS patients with detailed medical records, disease activity score (Bath Ankylosing Spondylitis Disease Activity Index), functional index (Bath Ankylosing Spondylitis Functional Index), and laboratory data (interleukin (IL)-2, IL-4, IL-10, TNF- α , interferon (IFN)- γ , transforming growth factor (TGF)- β , ESR, and CRP). Their peripheral blood mononuclear cells (PBMCs) were marked with anti-CD4, anti-CD25, and anti-FoxP3 antibodies, and triple positive T cells were gated by flow cytometry as T-regs. Their correlations were calculated and the changes after anti-TNF- α therapy were compared.

Results

The frequency of T-regs in PBMCs was positively correlated to ESR and CRP in AS ($r = 0.35$ and 0.43 ; $p = 0.032$ and 0.027 , respectively), and there was also a significant correlation between serum level of TNF- α and CRP ($p = 0.041$). The frequency of T-regs in PBMCs positively correlated to serum levels of TNF- α , IL-10, and TGF- β , while IL-2, IL-4, and IFN- γ showed opposite results. After anti-TNF- α treatment, there were significantly lower serum levels of TNF- α , IL-10, TGF- β , and frequency of T-regs in PBMCs among these AS patients ($p = 0.026$, 0.032 , 0.029 , and 0.037 , respectively).

Conclusion

In AS patients, proinflammatory cytokine may give positive feedback to induce more T-reg production and anti-inflammatory cytokine secretion to suppress this inflammatory status, and they can be reversed by anti-TNF- α therapy. However, the detailed interactions among T-regs and complex cytokine networks in autoinflammatory diseases still need more studies and further functional assay.

Cite this article: *Bone Joint Res* 2023;12(2):133–137.

Keywords: Cytokine, Regulatory T cell, Ankylosing spondylitis, Anti-tumour necrosis factor- α

Article focus

■ To examine the changes of cytokines and regulatory T cells (T-regs) in ankylosing spondylitis (AS) with anti-tumour necrosis factor- α (TNF- α) treatment.

Key messages

- Tregs positively correlated to anti-inflammatory cytokines, but negatively to target effector T cell (T-eff) cytokines in AS patients.
- Inflammation may give feedback to induce T-regs and anti-inflammatory cytokines to alleviate this vicious circle.

■ Expression of T-regs in peripheral blood mononuclear cells (PBMCs) could be reversed by anti-TNF- α therapy in patients with AS.

Strengths and limitations

- We demonstrated the changes and relationships between cytokines and T-regs in AS patients under biological (anti-TNF- α) treatment.
- These AS patients were recruited from one medical centre in Taiwan, and the sample size of this study was relatively limited.

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doi: 10.1302/2046-3758.122.BJR-2022-0195.R1

Bone Joint Res 2023;12(2):133–137.

Table I. Demographic and clinical characteristics of the total ankylosing spondylitis patients (n = 72).

Characteristic	Data
Mean age, yrs (SD)	48.63 (12.75)
Male/female, n	59/13
Mean disease duration, yrs (SD)	18.62 (12.53)
Axial with peripheral arthritis, n	49
Axial disease only, n	23
Uveitis, n	15
Mean BASDAI (SD)	5.72 (2.42)
Mean BASFI (SD)	4.81 (2.96)
Mean CRP, mg/dl (SD)	4.53 (1.86)
Mean ESR, mm/h (SD)	48.26 (19.72)

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; SD, standard deviation.

Introduction

Seronegative spondyloarthritis (SpA) is a family of chronic autoinflammatory rheumatic diseases, characterized by inflammatory back pain and peripheral arthritis.¹ It contains subtypes, such as ankylosing spondylitis (AS), reactive arthritis, psoriatic arthritis, inflammatory bowel disease related arthritis, and non-radiological axial SpA. AS is the prototype of SpA, and it predominantly involves the axial joints as well as sacroiliitis. AS occurs predominantly in young adult males and has a strong association with human leucocyte antigen-B27.² New bone formation results in ossification and progressive ankylosing of adjacent vertebral bodies with bamboo change ('bamboo spine'), which leads to limitation of spinal mobility and reduced quality of life. There were also extra-articular manifestations, such as uveitis, enthesitis, osteoporosis, and inflammatory colitis.^{1,3} Measurement of CRP is the most widely used method to assess the inflammation status in AS, but it could be interfered with by various confounding factors and is not a specific biomarker for monitoring disease activity. Cytokines are involved in many steps in the pathogenesis of AS,² and could promote the autoinflammatory process; we had already applied biologics with anti-tumour necrosis factor- α (TNF- α) and anti-interleukin (IL)-17A to clinically treat advanced AS patients.

Regulatory T cells (T-regs) belong to a T cell subpopulation to possess the suppressive effect in autoinflammatory status or autoimmune diseases.⁴ T-regs have a crucial housekeeping role in maintaining the balance and homeostasis between inflammation and anti-inflammation. Target effector T cells (T-effs) are suppressed by blocking IL-2 expression, which is necessary for T-reg cell expansion and survival,⁵ physically eliminating T-effs by apoptosis, or inhibiting T-eff function by transforming growth factor (TGF)- β and IL-10.⁶ Herein, we designed a prospective study to investigate the characteristics among T-regs, pro-/counter-inflammatory cytokines (including proinflammatory cytokine: TNF- α ; anti-inflammatory cytokines: IL-10 and TGF- β ; Th1-type

Table II. Correlations (r) among disease activity scores, inflammatory reactants, regulatory T cells, and cytokines in 72 ankylosing spondylitis patients before anti-tumour necrosis factor- α therapy.

Variable	BASDAI	BASFI	ESR	CRP
CD4 ⁺ CD25 ⁺ FoxP3 ⁺	0.16	0.10	0.35*	0.43*
IL-2	-0.18	-1.38	-0.08	-0.19
IL-4	0.17	0.11	0.18	0.19
IFN- γ	-0.17	0.02	-0.04	-0.08
TNF- α	0.22	0.23	0.16	0.27*
IL-10	0.11	0.10	0.08	0.07
TGF- β	0.12	-0.09	0.16	0.16

Values are shown as r, determined with Spearman's rank correlation test.

*p < 0.05 (significant difference).

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF- α , tumour necrosis factor- α .

cytokine: interferon (IFN); Th2-type cytokine: IL-4; and T cell differentiated related cytokine: IL-2), disease activity/functional index in AS patients, and those who received biological treatments with anti-TNF- α agents.

Methods

Patient recruitment and study design. This research was approved by the ethical committee of Taipei Veterans General Hospital, and informed and written consent was obtained from all participants. Blood samples were obtained from 72 AS patients who fulfilled the 1984 modified New York criteria⁷ and visited one tertiary rheumatology centre. All AS patients received anti-TNF- α biological agents (44 patients received adalimumab and 28 patients received golimumab) for three months. Clinical and laboratory assessments were performed on the same day. We evaluated disease activity using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), and assessed functional ability using the Bath Ankylosing Spondylitis Functional Index (BASFI).^{8,9}

Clinical demographic and laboratory characteristics. The clinical characteristics of 72 AS patients (59 male and 13 female) are shown in Table I. The mean age and disease duration of these patients were 48.63 years (standard deviation (SD) 12.75) and 18.62 years (SD 12.53), respectively. A total of 49 AS patients had both axial and peripheral joint involvement, while 23 suffered from axial disease only. Overall 15 patients had uveitis. Mean ESR and CRP levels were 48.26 mm/hr (SD 19.72) and 4.53 mg/dl (SD 1.86), respectively. In addition, BASDAI and BASFI data were recorded.

Gating strategy of CD4, CD25, and FOXP3 expression cells as T-regs by flow cytometric analysis. Whole blood samples were collected (before and after anti-TNF- α biological agent treatments for three months) in heparin-containing tubes, and peripheral blood mononuclear cells (PBMCs) were isolated immediately by the Ficoll-Hypaque gradient centrifugation for the subsequent flow cytometric analysis. PBMCs were incubated with

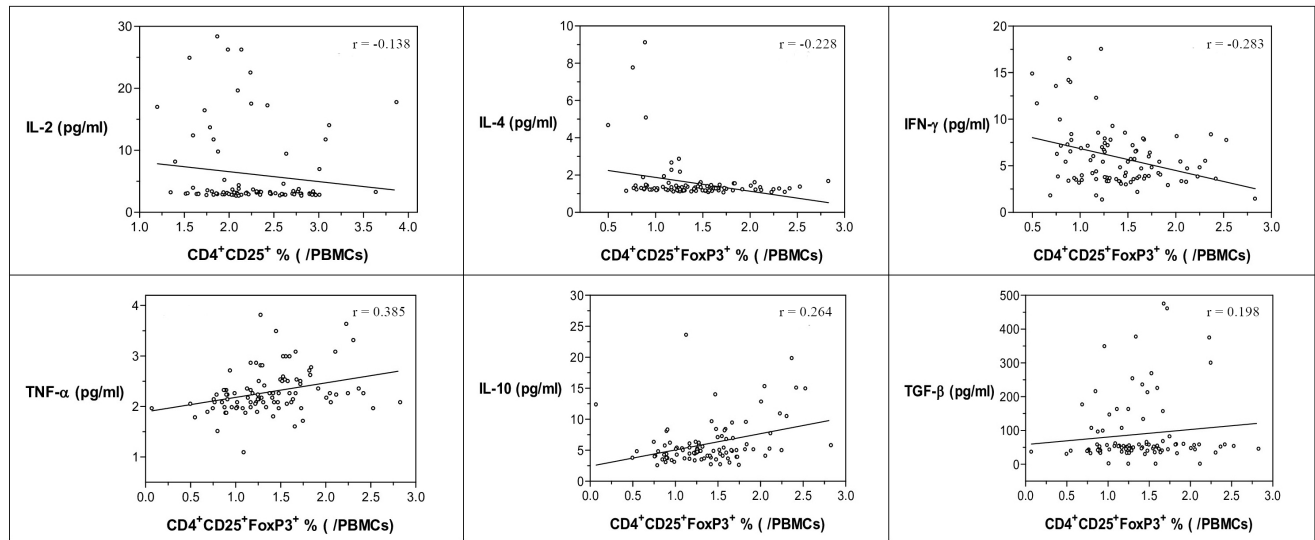


Fig. 1

Correlations among CD4⁺CD25⁺FoxP3⁺-Tregs and cytokines (interleukin (IL)-2, IL-4, interferon (IFN)- γ , tumour necrosis factor- α (TNF- α), IL-10, and transforming growth factor (TGF)- β) in total ankylosing spondylitis (AS) patients. Values are shown as r , determined with Spearman's rank correlation test. PBMCs, peripheral blood mononuclear cells; T-regs, regulatory T cells.

FITC-conjugated anti-CD4 antibody (clone: RPA-T4; BD Biosciences, USA) and PE-conjugated anti-CD25 antibody (clone: M-A251; BD Biosciences). Anti-FOXP3 antibody (PE-Cy5 clone: PCH101; eBioscience, Thermo Fisher Scientific, USA) was used in the intracellular immunostaining for flow cytometric analysis. After being washed with phosphate-buffered saline twice, cells were fixed and permeabilized by using the fix/permeabilization buffer purchased from eBioscience. Cells were then stained with PE-conjugated anti-Foxp3 antibody in the presence of the fix/permeabilization buffer for 30 minutes. Stained cells were washed with the fix/permeabilization buffer twice and immediately analyzed by a Becton Dickinson FACScan analyser (BD Biosciences). While performing the flow cytometric analysis, CD4-positive or CD4-negative lymphocyte population was gated for further analyzing expression of CD25 and Foxp3. Triple positive with CD4⁺CD25⁺FoxP3⁺ lymphocytes in PBMCs were gated as T-regs. For measurement of ESR and CRP, ESR was checked by Westergren method and CRP by nephelometry (Behring Nephelometer Analyzer-II; CSL Behring, Germany). After anti-TNF- α treatment was given for three months, we re-checked the expression of CD4/CD25/Foxp3 T cells in PBMCs, as well as serum cytokines. **Cytokine assay.** Serum levels of proinflammatory cytokine (TNF- α), counter-inflammatory cytokines (IL-10, TGF- β), Th1-type cytokine (IFN- γ), Th2-type cytokine (IL-4), and T cell-differentiated cytokine (IL-2) were tested (before and after anti-TNF- α biological agent treatment for three months). These cytokines were measured with a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) including IL-2/IL-4/IL-10/IFN- γ /TGF- β (eBioscience) and TNF- α (Cayman Chemical Company, USA)

according to the manufacturer's instructions, and carried out in duplicate.

Statistical analysis. We used SPSS software version 17 (SPSS, USA). All statistical tests were two-tailed and statistical significance was defined as p -value below 0.05. Data were summarized as means (SDs) for continuous variables, and frequency and percentage for categorical variables. The association between continuous variables was evaluated using Spearman's rank correlation test, and the association between categorical variables was evaluated using chi-squared test. Also, continuous variables were compared using Kruskal-Wallis test or Mann-Whitney U test for independent samples, and Wilcoxon signed-rank test for paired data. Data were evaluated by non-parametric method, to analyze group differences and associations.

Results

Correlations among clinical parameters, T-regs, and cytokines in AS patients. Correlations (r) among different parameters in 72 AS patients were calculated (Table II). The highest correlation coefficient observed was between CD4⁺CD25⁺FoxP3⁺-Tregs and CRP ($r = 0.43$; $p = 0.027$, Spearman's rank correlation test), and there were also significant positive correlations between T-regs and ESR as well as CRP. In contrast, the correlation of BASDAI and BASFI with all other parameters was poor and non-significant. However, in AS patients with higher disease activity (BASDAI > 4), BASDAI had a significantly positive correlation with serum TNF- α level ($p = 0.043$, Spearman's rank correlation test), a trend positive correlation with CD4⁺CD25⁺Foxp3⁺ T-regs ($p = 0.082$, Spearman's rank correlation test), but also a negative

Table III. Regulatory T cells and cytokines in 72 ankylosing spondylitis patients before and after anti-tumour necrosis factor- α therapy for three months.

Treg markers (Tregs/PBMCs) % or cytokines	Before anti-TNF- α therapy	After anti-TNF- α therapy	p-value*
Mean CD4 ⁺ CD25 ⁺ FoxP3 ⁺ (SD)	1.49 (0.36)	1.12 (0.27)	0.037
Mean IL-2, pg/ml (SD)	7.14 (2.11)	14.40 (8.21)	0.007
Mean IL-4, pg/ml (SD)	1.31 (0.24)	5.66 (4.43)	< 0.001
Mean IFN- γ , pg/ml (SD)	4.72 (1.91)	11.70 (6.65)	< 0.001
Mean TNF- α , pg/ml (SD)	2.33 (0.60)	1.12 (0.40)	0.026
Mean IL-10, pg/ml (SD)	5.02 (2.35)	2.94 (1.90)	0.032
Mean TGF- β , pg/ml (SD)	55.52 (23.81)	27.93 (13.17)	0.029

Statistical significance defined as p-value below 0.05.

*Wilcoxon signed-rank test.

IFN, interferon; IL, interleukin; PBMCs, peripheral blood mononuclear cells; SD, standard deviation; TGF, transforming growth factor; TNF- α , tumour necrosis factor- α ; T-regs, regulatory T cells.

correlation with IL-4 ($p = 0.117$, Spearman's rank correlation test). The baseline relationships between T-regs and serum cytokine levels are displayed in Figure 1, and the results show that the frequencies of T-regs in PBMCs were in an inverse correlation with IL-2 ($r = -0.138$; $p = 0.073$, Spearman's rank correlation test), as well as with IL-4 and IFN- γ ($r = -0.228$ and -0.283 ; $p = 0.046$ and 0.033 , respectively, Spearman's rank correlation test); while they positively correlated with TNF- α and IL-10 ($r = 0.385$ and 0.264 ; $p = 0.029$ and 0.042 , respectively, Spearman's rank correlation test), as well as with TGF- β ($r = 0.198$; $p = 0.062$, Spearman's rank correlation test).

Frequencies of T-regs in PBMCs and serum level of cytokines in AS patients before and after anti-TNF- α treatment. The frequencies of cell numbers of T-regs in PBMCs in AS patients were significantly higher before than after anti-TNF- α treatment for three months ($p = 0.037$, Wilcoxon signed-rank test) (Table III). In cytokine assay, serum levels of TNF- α , IL-10, and TGF- β in AS patients were significantly higher before anti-TNF- α treatment than after, while IL-2, IL-4, and IFN- γ revealed opposite results ($p = 0.026$, 0.032 , 0.029 , 0.007 , < 0.001 , and < 0.001 , respectively, Wilcoxon signed-rank test).

Discussion

A role of immunological tolerance is to enable the immune system to discriminate self- from non-self-antigens, and the suppressive function of T-regs on autoimmunity has prompted us to hypothesize that the dysregulation of T-regs is associated with AS. The interaction between T-regs and autoimmune rheumatic diseases has often been investigated, however there were still discrepant results in numerous studies.¹⁰⁻¹² In this study, compared with after anti-TNF- α treatment, AS patients have increased T-regs frequency in PBMCs in higher disease activity status, and the results are compatible with previous T-reg studies in other autoimmune diseases.¹¹ Previous studies have shown that there are more CD4⁺ CD25⁺ T cells with Foxp3⁺ in inflammatory arthritis patients than in normal individuals.¹² The increased percentage of the T-regs in PBMCs before anti-TNF- α treatment suggests that an augmented immunosuppressive process is present in the inflammatory status. This may result from the existence

of a passive feedback reaction that T-regs are induced and activated by underlying inflammatory status.

We also found that TNF- α , IL-10, and TGF- β serum levels were significantly higher while IL-2, IL-4, and IFN- γ were lower in AS patients before than after anti-TNF- α treatment. Previous reports have demonstrated that the increased susceptibility of patients to infection/inflammation was possibly related to the reduced T helper 1 (Th1)-type immune (IFN- γ) responses and a skewing toward increased counter-inflammatory (IL-10 and TGF- β) immune reactivity.^{6,13,14} Th2-type cytokine IL-4 could also inhibit T-regs and induce T-effs.¹³ These observations underlie the idea that inflammation triggers the development of a counter-inflammatory adaptive immune response that may help to control excessive innate immune inflammatory reactivity.^{12,15} As we already knew, CD25 is the α -chain of the IL-2 receptor on T cells. Hence, while high expression of CD4⁺CD25⁺Foxp3⁺T-regs performed the 'suppressor role' in T-effs, IL-2 was deemed to be absorbed by T-regs with decreasing serum concentration.⁴ Therefore, in this study the positive correlation between TNF- α and T-regs suggests that the proinflammatory cytokine TNF- α in AS patients induced more inflammation processes, then it recruited more T-regs which secrete counter-inflammatory cytokines such as IL-10 and TGF- β to suppress this inflammation status,⁴ consume IL-2, and inhibit IL-4 and INF- γ production (Figure 1).

In spite of the above interesting findings, the present investigation still has some limitations. First, it was an investigation involving a single centre which could include only a relatively small number of AS patients, and further studies with larger sample sizes are required. Second, due to cross-sectional design of the study, data regarding the expression of T-regs in PBMCs and cytokines throughout the whole clinical course of AS were not all available. Finally, we only measured the frequencies of T-regs and serum levels of cytokines; we will design functional studies in the future.

In conclusion, we have demonstrated the correlations among cytokines, T-regs, disease activity scores, and inflammatory reactants in patients with AS and their changes after anti-TNF- α therapy. Inflammatory status

may induce more T-reg production and anti-inflammatory cytokine secretion to suppress it. After anti-TNF- α therapy, the inflammation reaction was ameliorated and T-reg production or anti-inflammatory cytokines secretion were reversed. However, in order to further elucidate the complex network between cytokines and Tregs in AS, functional studies will be needed in the future.

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Funding statement:

- The authors received no financial or material support for the research, authorship, and/or publication of this article.

ICMJE COI statement:

- The authors have declared no conflict of interest.

Acknowledgements:

- We thank the patients and their caregivers as well as the investigators who contributed to this study.

Ethical review statement:

- This research was approved by the ethical committee of Taipei Veterans General Hospital.

Open access funding

- The authors report that the open access funding for their manuscript was self-funded.

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