ORIGINAL ARTICLE

Abatacept suppresses the telomerase activity of lymphocytes in patients with rheumatoid arthritis

SHORT RUNNING TITLE: Telomerase activity in RA

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AUTHOR CONTRIBUTIONS

Daitaro Kurosaka designed the study and administered abatacept. Kazuhiro Otani acquired and analyzed the data. Kazuhiro Otani wrote the initial draft of the manuscript. Both authors interpreted the data, reviewed the manuscript, and approved the final version.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Abstract

Aim: Telomere is a component of chromosomes that protects their ends from various stresses. The telomeres shorten during cell division, and their length is maintained by telomerase. The telomerase activity of lymphocytes was shown to be upregulated on lymphocyte activation, and abatacept was found to suppress the activation of T lymphocytes involved in pathogenesis of rheumatoid arthritis. Therefore, we investigated the effect of abatacept on lymphocyte telomerase activity in patients with rheumatoid arthritis.

Method: This study included 11 patients diagnosed with rheumatoid arthritis based on American College of Rheumatology 2010 criteria, who received abatacept treatment from August 2012 to August 2013. We collected their clinical data and obtained peripheral blood samples before starting abatacept, and 1, 3, 6, and 12 months after treatment. Peripheral blood mononuclear cells were extracted using Ficoll density gradient centrifugation, and T and B lymphocytes were sorted by magnetic beads. The telomerase activity of lymphocytes was determined using the telomeric repeat amplification protocol.

Results: The telomerase activity of T lymphocytes declined from 0.357 to 0.161 ($P<0.01$) at 12 months after abatacept treatment, and that of B lymphocytes declined from 0.554

to 0.202 (P<0.01). The telomerase activity of B lymphocytes, but not that of T lymphocytes, was also significantly downregulated 1 month after treatment.

Conclusion: Abatacept suppressed the telomerase activity of both T and B lymphocytes, although that of B lymphocytes was downregulated before T lymphocytes. These findings imply that the clinical efficacy of abatacept during the early phase depends on the suppression of B lymphocytes.

Keywords: Abatacept, rheumatoid arthritis, telomerase, T lymphocytes, B lymphocytes

INTRODUCTION

The telomere complex is composed of DNA and protein, and protects the ends of chromosomes,[1] with telomere DNA shortening when cells divide.[1,2] Telomerase is a reverse transcriptase that contains template RNA and replicates the telomere DNA sequence.[2] The telomerase activity of cancer cells is upregulated,[3] so their telomere length is maintained.[3] In lymphocytes, telomerase activation occurs when the cells are stimulated *in vitro*.[4]

Abnormal activity of telomerase is reported in some autoimmune diseases.[5] For example, the telomerase activity of peripheral blood mononuclear cells (PBMCs) is upregulated in mixed connective tissue disease and Sjögren's syndrome.[5] We previously investigated the telomerase activity of peripheral blood cells in patients with systemic lupus erythematosus,[6–8] and found that it was upregulated in T lymphocytes at both the active and inactive phases. However, telomerase activation of B lymphocytes only occurred during the active phase.[8] Patients with rheumatoid arthritis (RA) were also found to have high telomerase activity in lymphocytes of the peripheral blood and in the synovium.[9]

Abatacept is a fusion protein consisting of an extracellular domain of human cytotoxic T lymphocyte-associated antigen 4 and a modified Fc portion of human

immunoglobulin G1.[10,11] It inhibits the co-stimulation signal of T lymphocytes mediated by CD80/86 in antigen-presenting cells and CD28 in T lymphocytes, and was shown to be effective against RA.[10–12] In this study, we examined the changes in telomerase activity of peripheral T and B lymphocytes in RA patients treated with abatacept.

MATERIALS AND METHODS

Patients and healthy controls

We recruited 49 patients with RA who received abatacept treatment at Jikei University from August 2012 to August 2013. We followed them up for 1 year, and analyzed the data of 11 patients from whom we could obtain blood samples at all observation points. We also used the data of six healthy age- and gender-matched volunteers from our database. Abatacept was administered according to the package insert. This study was approved by the Ethics Committee of Jikei University School of Medicine (approval number 25-133(7268)), and we obtained the prior written consent of patients for their participation.

Blood samples and clinical data collection

We collected clinical data and blood samples before abatacept treatment, and 1, 3, 6, and 12 months after treatment. Clinical data included sex, age, Disease Activity Score

in 28 joints based on C-reactive protein (DAS28-CRP), anti-citrullinated peptide antibody (ACPA) and rheumatoid factor (RF) titers, immunoglobulin G (IgG) and matrix metalloproteinase 3 (MMP-3) concentrations, and treatment before abatacept.

Isolation of T and B lymphocytes

We isolated PBMCs from peripheral blood samples by Ficoll density gradient centrifugation using Histopaque®-1077 (Sigma Aldrich, St. Louis, MO) according to the manufacturer's guidelines. PBMCs were purified into CD19-positive cells (B cells) and CD3-positive cells (T cells) by a magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany).

Measurement of telomerase activity

We measured telomerase activity using the telomeric repeat amplification protocol (TRAP) assay with the TRAPEZE® telomerase detection kit (Millipore, Burlington, MA), according to the manufacturer's guidelines with a slight modification. Each sample of cells (1.0×10^5) was suspended and lysed in 100 μl of 1 × CHAPS lysis buffer, homogenized, and incubated on ice for 30 min. The cell lysate was centrifuged at 20400 × *g* for 20 min at 4°C, then 10 μl of the supernatant was mixed with 2.5 μl 10 ×TRAP buffer, 0.5 μl 50 × dNTPs, 0.5 μl TS primer, 0.5 μl primer mixture, 10.8 μl distilled water, and 0.2 μl Taq polymerase. The mixture was incubated at 30°C for 10 min, and

subjected to PCR in a thermal cycler (GeneAmp 9700; Applied Biosystems, Foster City, CA) for 30 cycles of 30 s at 94°C and 30 s at 60°C. The PCR product was electrophoresed on a 12% polyacrylamide gel, stained with SYBR Gold (Molecular Probes, Eugene, OR), and photographed with a UV transilluminator equipped with a ChemiDoc CCD camera (Bio-Rad, Hercules, CA). Images were analyzed with Image J software (National Institutes of Health, Bethesda, MD). All bands > 50 bp were measured with a densitometer, and the sum total of absorbance values was regarded as the amount of TRAP products. The internal control band was also measured by densitometry, and the absorbance was used as the internal control value. Telomerase activity was defined as the value for the TRAP product divided by that of the internal control. All experiments were performed in triplicate, and the mean value was used for analysis.

Statistical analysis

Statistical analysis was performed by GraphPad Prism 4 software (Graph Pad, La Jolla, CA). Differences between the means of two groups were analyzed by the unpaired t-test. Correlations between the telomerase activity of T and B lymphocytes, DAS28-CRP, ACPA, RF, IgG, and MMP-3 were analyzed by the Pearson correlation coefficient. Changes in telomerase activity and DAS28-CRP were analyzed by repeated measures analysis of variance and Bonferroni's multiple comparison test as a post hoc test. We

defined $P<0.05$ as significant.

RESULTS

Patient background

The mean age of the patients was 62.0±11.7 years (Table 1), and three males and eight females were included in the study. Before abatacept treatment, the average DAS28-CRP score was 3.57±0.95, the mean dosage of prednisolone was 4.65±4.37 mg/day, and that of methotrexate was 2.55±2.31 mg/week. One patient was treated with tocilizumab.

Comparison of telomerase activity between RA patients and healthy controls

We measured the telomerase activity of T lymphocytes and B lymphocytes, and compared values from RA patients with those of healthy controls (Fig. 1). In T lymphocytes, the telomerase activity of RA patients (0.357±0.177) was significantly higher than that of healthy controls (0.043±0.018). Similarly, in B lymphocytes, the telomerase activity of RA patients (0.554±0.274) was significantly higher than that of healthy controls (0.086±0.051).

Correlation between telomerase activity and clinical data

The telomerase activities of T lymphocytes and B lymphocytes were not correlated (Fig.

2). Moreover, there was no significant correlation between telomerase activity and DAS28-CRP, ACPA, RF, IgG, or MMP-3 clinical data (Fig. 2).

Time course of DAS28-CRP and lymphocyte telomerase activity following abatacept treatment

We measured DAS28-CRP and telomerase activity of lymphocytes before and after abatacept treatment (Fig. 3). The mean DAS28-CRP decreased 1 month after the administration of abatacept. Similarly, the telomerase activity of B lymphocytes decreased significantly 1 month after treatment, while that of T lymphocytes did not decrease significantly 1 month after the administration of abatacept.

Comparison of telomerase activity between patients who achieved remission within 1 month after abatacept treatment and other patients

We divided patients into two groups based on their DAS28-CRP scores. The first group included those who achieved remission (DAS28-CRP <2.3) within 1 month after abatacept treatment, and the second included the remaining patients. The telomerase activities of these two groups before abatacept treatment are shown in Figure 4. The T lymphocyte telomerase activity of the remission group tended to be lower than that of the non-remission group. However, this was not observed in B lymphocytes. Following

the administration of abatacept, the telomerase activity of both groups tended to decrease as shown in Figure 5.

DISCUSSION

The telomerase activity of lymphocytes infiltrating synovial tissue, and that of peripheral blood lymphocytes was previously shown to be upregulated in RA patients compared with healthy controls.[9] Moreover, Figen reported that the expression of human telomerase reverse transcriptase mRNA, which is associated with telomerase activity, was correlated with RA disease activity.[13] However, the telomerase activity of peripheral T and B lymphocytes had not been compared previously between RA patients and healthy controls.

We herein isolated T and B lymphocytes from PBMCs, and showed that telomerase activity of both types of lymphocyte was higher in RA patients than in healthy controls. However, there was no correlation between the telomerase activity of T and B lymphocytes, even though the activation of B lymphocytes is considered to be dependent on T lymphocytes.[10] Therefore, we speculated that the mechanism of activation of B lymphocytes is not completely dependent on T lymphocytes in RA patients. Additionally, we found that the telomerase activity of lymphocytes did not

correlate with DAS28-CRP, ACPA, RF, IgG, or MMP-3, although this should be verified in a larger study because the current sample size was small.

Abatacept inhibits the co-stimulation signal of T lymphocytes mediated by CD80/86 in antigen-presenting cells and CD28 in T lymphocytes.[12] As expected, the telomerase activity of T lymphocytes in the present study decreased after the administration of abatacept. T lymphocytes producing interleukin-17 or interferon-gamma were reported to be decreased in RA patients after the administration of abatacept.[14] Moreover, CD4-positive and CD28-negative T lymphocytes also decreased and the repertoire of T lymphocytes was increased in RA patients after the administration of abatacept.[15] We are interested in the telomerase activity of these fractions of T lymphocytes, so intend to investigate them in future work.

Conversely, Fujii et al. previously reported that the telomerase activity of T lymphocytes in RA patients could not be upregulated by stimulation with anti-CD3/CD28 beads compared with healthy controls, and that the telomerase activity of CD71-positive activated T lymphocytes was lower in RA patients than in healthy controls[16]. This indicated that the ability to upregulate telomerase activity in T lymphocytes under certain conditions may be insufficient in RA patients. However,

various factors are known to activate telomerase in T lymphocytes[17], and different cytokines were reported to stimulate T lymphocytes in RA patients[18]. Therefore, the upregulation of telomerase activity in T lymphocytes observed in the present study may reflect that of already activated lymphocytes. Imberti et al. reported that abatacept did not affect the ability to upregulate telomerase activity in lymphocytes when they were stimulated with anti-CD3 monoclonal antibody[15]; therefore, abatacept may not restore the ability to upregulate lymphocyte telomerase activity under certain conditions. However, our data show that abatacept may have an effect on telomerase activity of already activated lymphocytes in RA patients. It is unclear whether decreasing telomerase activity is beneficial in the treatment of RA, so we also intend to investigate this in future work

The present study investigated the telomerase activity of B lymphocytes, which was found to decrease after the administration of abatacept. This is in agreement with previous studies that showed abatacept affected not only T but also B lymphocytes in RA patients.[19, 20] Iwata et al. demonstrated the phosphorylation of spleen tyrosine kinase in the peripheral B lymphocytes of RA patients 24 weeks after the administration of abatacept,[19] while Gazuau et al. reported a decrease in peripheral B lymphocytes 6 months after the administration of abatacept in RA patients with a good

drug response.[20]

We were particularly interested in our finding that the telomerase activity of B lymphocytes decreased 1 month after the administration of abatacept because this has not previously been reported. Additionally, we detected a significant decrease in the telomerase activity of T lymphocytes 3 months after the administration of abatacept, which was later than that seen in B lymphocytes. Although it is generally considered that abatacept suppresses B lymphocytes through the suppression of T lymphocytes, we hypothesize that abatacept has a direct effect on B lymphocytes.

Postmarketing surveillance in Japan revealed a change in DAS28-CRP of 0.73 ±1.03 from baseline to 1 month after the administration of abatacept.[21] This compares with a change of 1.06±1.14 in the present study, suggesting that our current group of patients were good responders to abatacept. Because the telomerase activity of B lymphocytes decreased 1 month after the administration of abatacept, we expect that the change in B lymphocytes is important for the clinical efficacy of abatacept at this time point.

We divided the patients into two groups based on whether they achieved remission (DAS28-CRP <2.3) within 1 month after the administration of abatacept. Although the change was not significant, the telomerase activity of T lymphocytes

tended to be lower in the remission group than in the non-remission group. There was little difference in the telomerase activity of B lymphocytes. However, after the administration of abatacept, the telomerase activity of T and B lymphocytes tended to decrease in both groups. Therefore, we speculated that one of the differences between these groups is the T lymphocyte telomerase activity before abatacept treatment, which appeared to be low in patients who achieved remission within 1 month. This tendency implied that the clinical efficacy of abatacept in the early phase depended on the suppression of B lymphocytes. However, it is unclear whether the downregulation of telomerase activity itself is related to the effectiveness of abatacept in the early phase. Therefore, these speculations should be confirmed in future work because the number of patients in the present study was small.

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Table 1 Patient background

Sex	Age (years)	DAS28 CRP	ACPA (U/ml)	RF (IU/ml)	IgG (mg/dl)	MMP3 (ng/ml)	DMARDs (mg)				Biologics
							PSL	MTX	SASP	TAC	
F	68	3.71	99	102	1351	335	10		1000		tocilizumab
F	55	3.12	84.4	143	1563	84.3		8			
F	57	4.71	322	106.4	2982	132.4					
F	68	2.71	n.d.	64.1	992	73		8			
F	52	3.13	n.d.	n.d.	1847	67.5	2	12			
F	50	3.95	21.5	179	1521	86.8					
M	45	4.11	203	n.d.	1454	255.3	8		1000		
F	78	5.13	444	2805	1994	165.6					
F	78	3.64	5.4	623	n.d.	39.6	10		1000		
M	74	3.41	681	262.3	1012	611.4	15			0.5	
M	57	1.67	18.7	n.d.	1498	239	6				
average	62.0 ±11.7	3.57 ±0.95	208.8 ±232.2	535.6 ±934.1	1621 ±571	190.0 ±168.0	4.64 ±4.37	2.55 ±2.31			

Average values are the means ± standard deviation. M: male; F: female; ACPA: anti-citrullinated peptide antibody; RF: rheumatoid factor; IgG: immunoglobulin G; MMP3: matrix metalloproteinase 3; DMARDs: disease-modifying anti-rheumatic drugs; PSL: prednisolone; MTX: methotrexate; SASP: salazosulfapyridine; TAC: tacrolimus; n.d.: no data

Figure legends

Figure 1 Telomerase activity of peripheral blood lymphocytes in patients with rheumatoid arthritis and healthy controls. Triangles represent the telomerase activity of patients; squares represent controls. Horizontal bars represent the average telomerase activity. * $P<0.01$.

Figure 2 Correlation between the telomerase activity of T and B lymphocytes, DAS28-CRP, ACPA, RF, IgG, and MMP-3. Squares represent patients before the administration of abatacept. Regression lines are shown, and the correlation coefficient is r.

Figure 3 Change of DAS28CRP and telomerase activity of lymphocytes after the administration of abatacept. Each symbol represents the average of each time point. Bars show standard deviations. *$P<0.01$, **$P<0.05$, n.s. not significant.

Figure 4 Telomerase activity in the groups demonstrating remission or not before the administration of abatacept. Squares represent patients in the non-remission group; triangles represent patients in the remission group. Horizontal bars represent the average telomerase activity.

Figure 5 Change of telomerase activity in the remission and non-remission groups.

Each symbol represents the average of each time point. Bars show standard deviations.

Figure 1

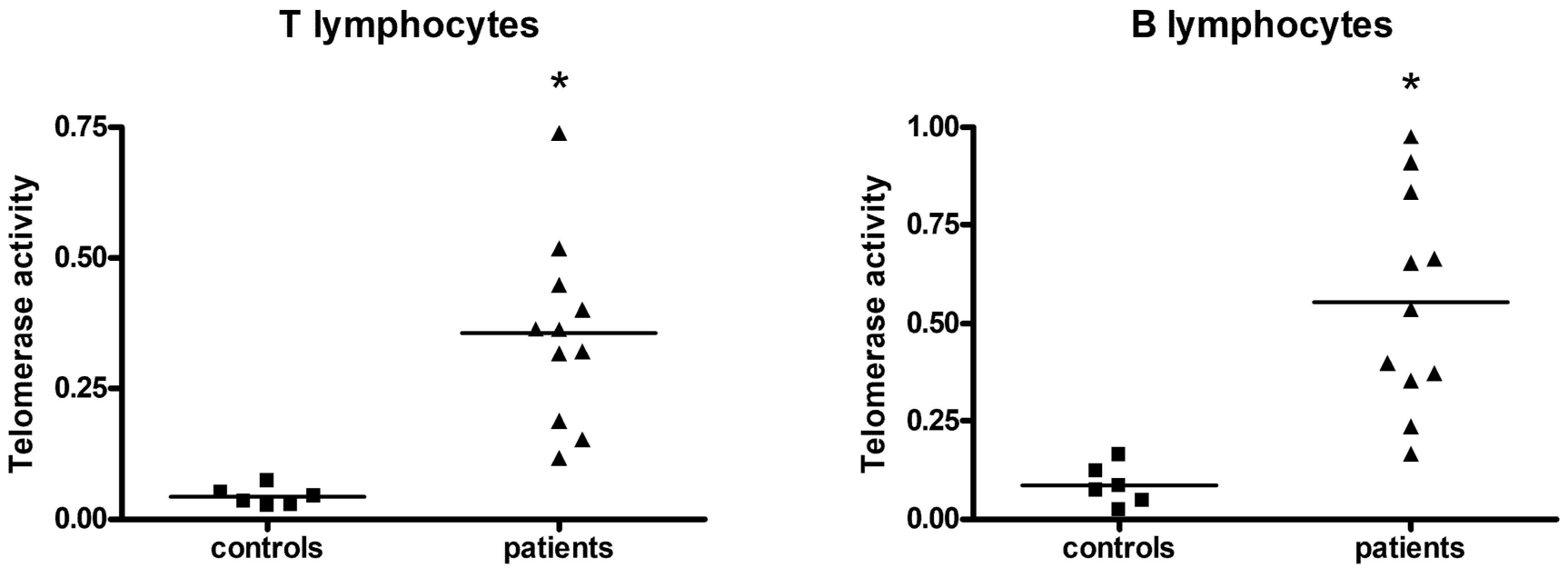

T lymphocytes
B lymphocytes
Telomerase activity
0.75
0.50
0.25
0.00
1.00
0.75
0.50
0.25
0.00
*
*
controls
patients
controls
patients

Figure 2

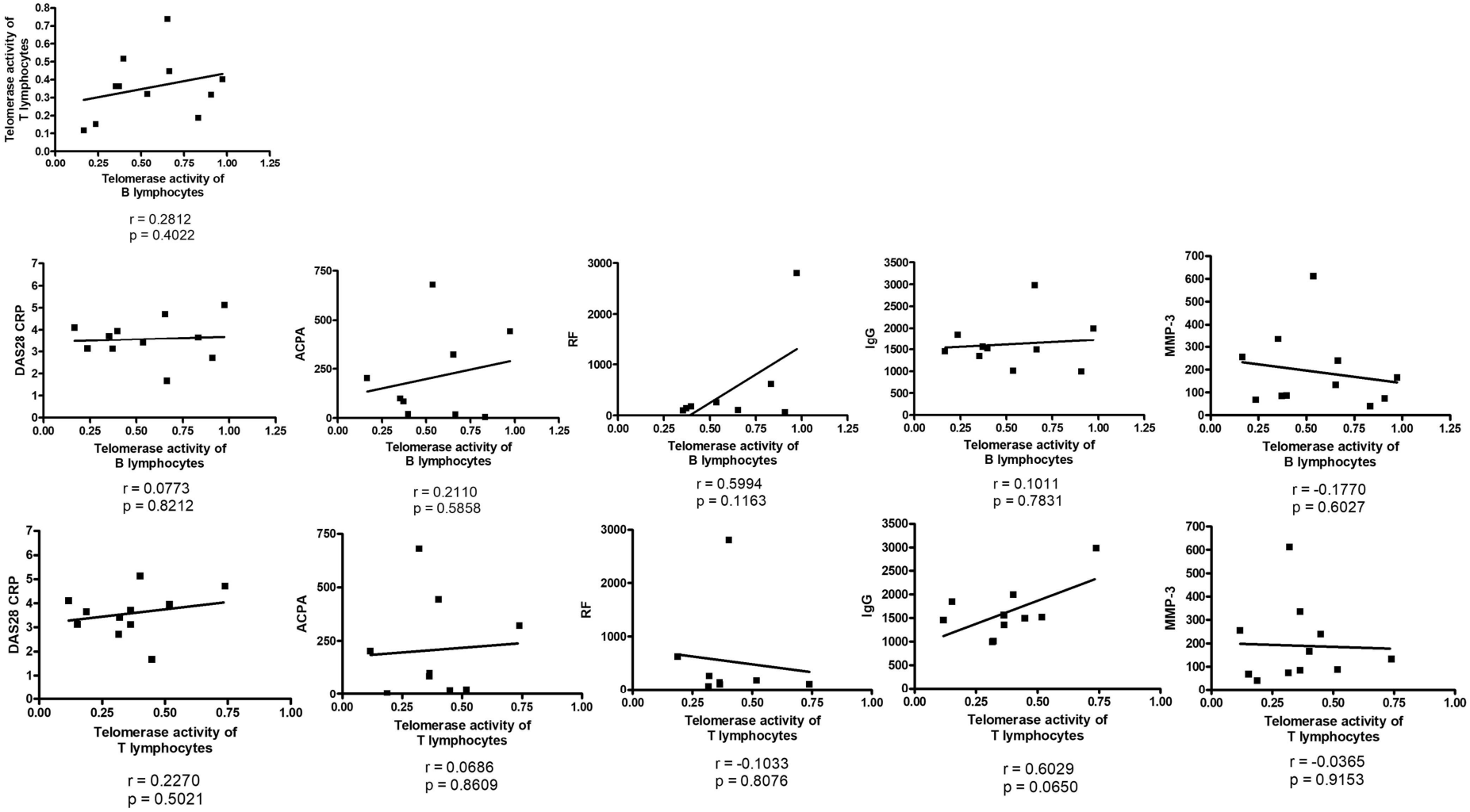

Telomerase activity of T lymphocytes
Telomerase activity of B lymphocytes
r = 0.2812
p = 0.4022
DAS28 CRP
Telomerase activity of B lymphocytes
r = 0.0773
p = 0.8212
ACPA
Telomerase activity of B lymphocytes
r = 0.2110
p = 0.5858
RF
Telomerase activity of B lymphocytes
r = 0.5994
p = 0.1163
IgG
Telomerase activity of B lymphocytes
r = 0.1011
p = 0.7831
MMP-3
Telomerase activity of B lymphocytes
r = -0.1770
p = 0.6027
DAS28 CRP
Telomerase activity of T lymphocytes
r = 0.2270
p = 0.5021
ACPA
Telomerase activity of T lymphocytes
r = 0.0686
p = 0.8609
RF
Telomerase activity of T lymphocytes
r = -0.1033
p = 0.8076
IgG
Telomerase activity of T lymphocytes
r = 0.6029
p = 0.0650
MMP-3
Telomerase activity of T lymphocytes
r = -0.0365
p = 0.9153

Figure 3

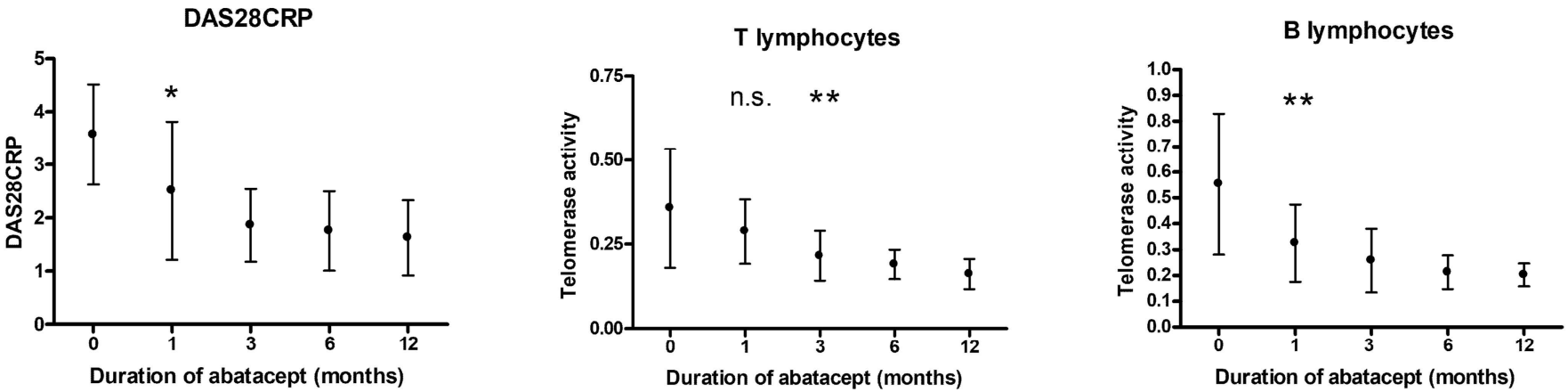

DAS28CRP
DAS28CRP
5
4
3
2
1
0
*
0
1
3
6
12
Duration of abatacept (months)
T lymphocytes
Telomerase activity
0.75
0.50
0.25
0.00
n.s.
**
0
1
3
6
12
Duration of abatacept (months)
B lymphocytes
Telomerase activity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
0
1
3
6
12
Duration of abatacept (months)

Figure 4

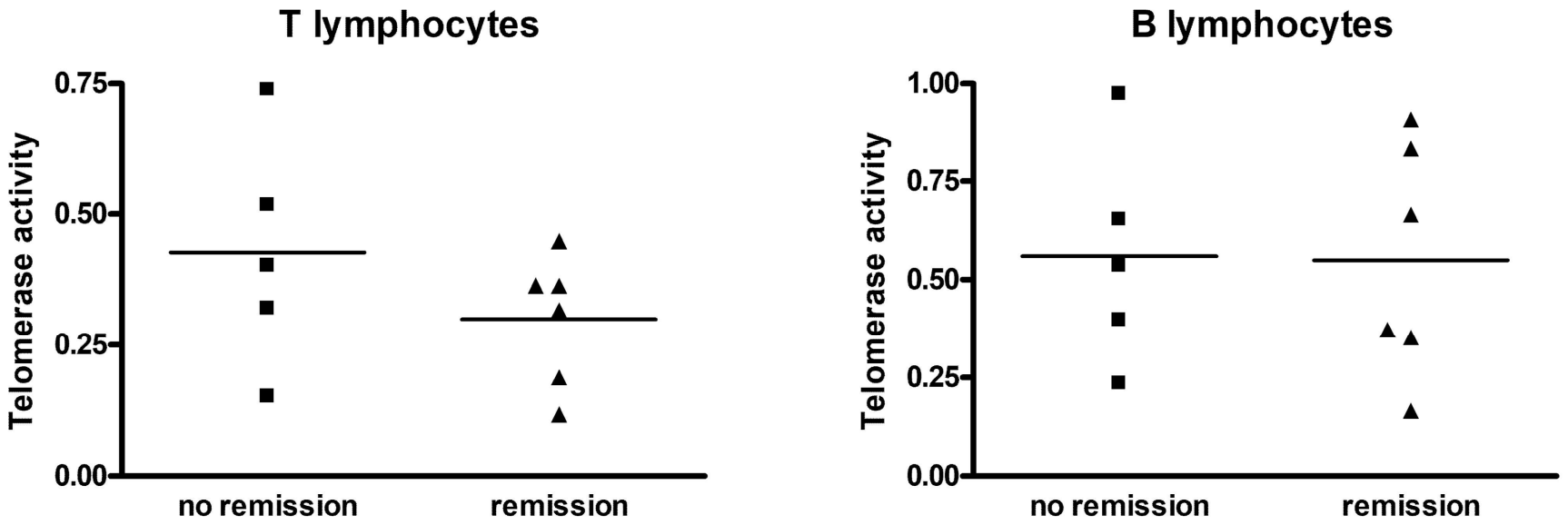
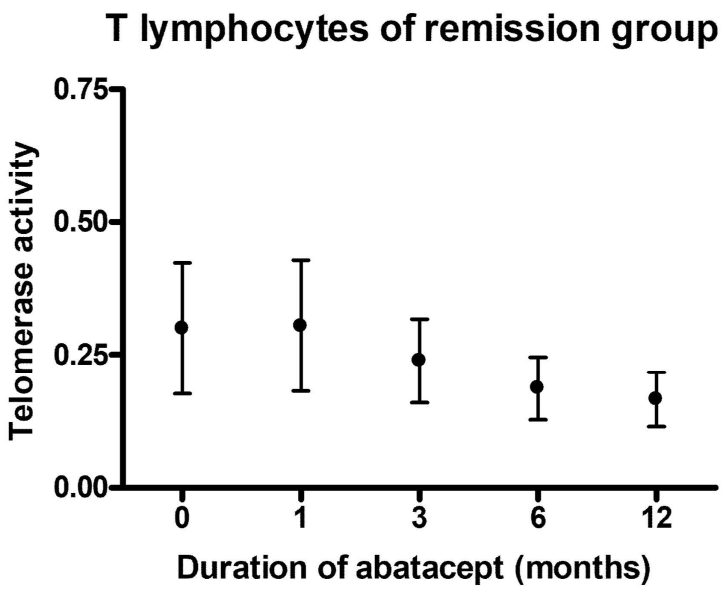
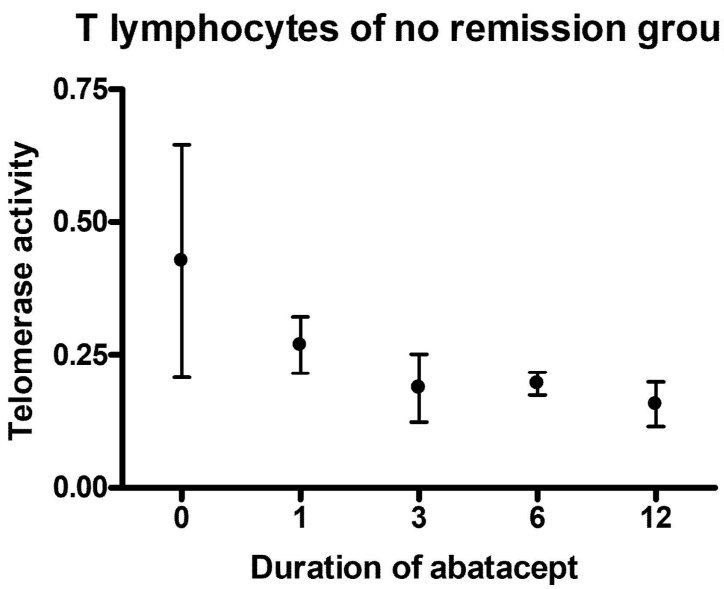

T lymphocytes
Telomerase activity
0.75
0.50
0.25
0.00
no remission
remission
B lymphocytes
Telomerase activity
1.00
0.75
0.50
0.25
0.00
no remission
remission

Figure 5


T lymphocytes of remission group
Telomerase activity
0.75
0.50
0.25
0.00
0
1
3
6
12
Duration of abatacept (months)


T lymphocytes of no remission group
Telomerase activity
0.75
0.50
0.25
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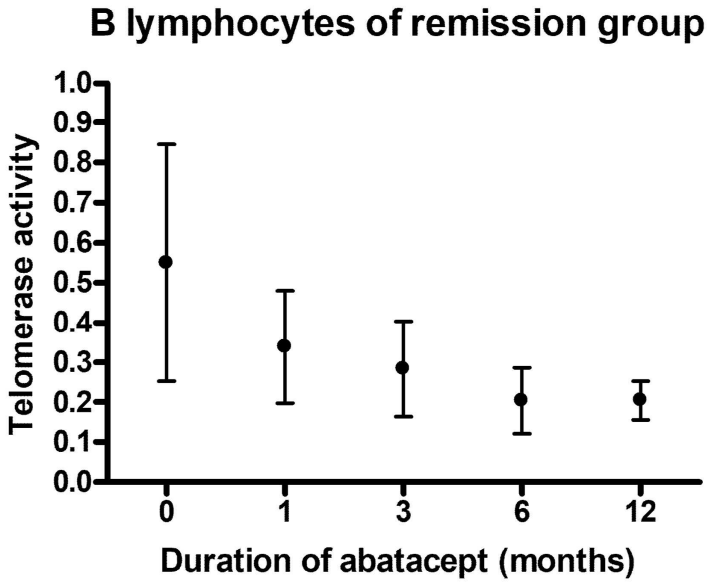
12
Duration of abatacept


B lymphocytes of remission group
Telomerase activity
1.0
0.9
0.8
0.7
0.6
0.5
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0.3
0.2
0.1
0.0
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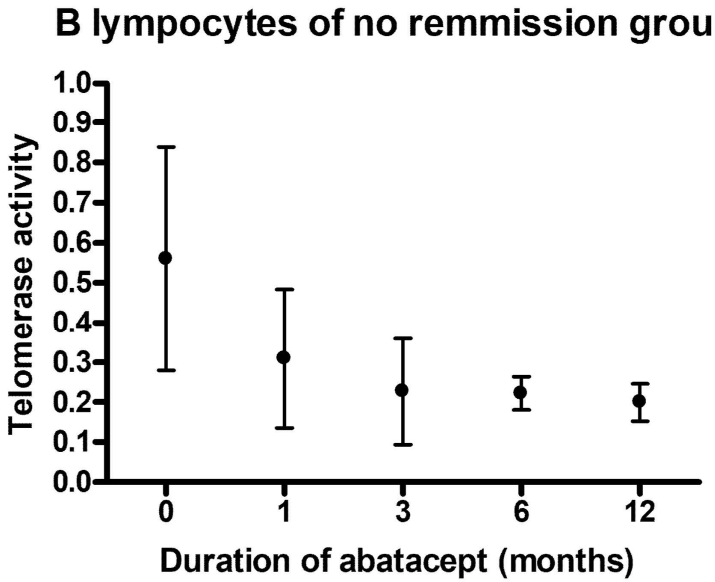
1
3
6
12
Duration of abatacept (months)


B lympocytes of no remmission group
Telomerase activity
1.0
0.9
0.8
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0.4
0.3
0.2
0.1
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0
1
3
6
12
Duration of abatacept (months)