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Title	CD4+CD25(high)CD127(low/-) Treg cell frequency from peripheral blood correlates with disease activity in patients with rheumatoid arthritis.
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CD4$^+$ CD25^{high} CD127$^{low/-}$ T$_{reg}$ cell frequency from peripheral blood correlates with disease activity in patients with rheumatoid arthritis

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Running title: CD4$^+$CD25^{high}CD127$^{low/-}$ T cells in RA

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Abstract

Objectives. To investigate whether the frequency of peripheral blood (PB) T_{reg} cells correlates with the clinical disease activity of rheumatoid arthritis (RA).

Methods. PB T_{reg} cells, defined as the $CD4^+ CD25^{high} CD127^{low/-}$ population, were examined by flow cytometry in 48 RA patients, including 13 who had never received disease-modifying anti-rheumatic drugs (DMARDs), 19 with active disease who were receiving (N = 14) or had received (N = 5) DMARDs and 16 in remission receiving DMARDs.   The clinical disease activity of the patients was defined by disease activity score 28 (DAS28).   The association of DAS28, C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) with the frequency of PB T_{reg} cells was examined.

Results. The frequency of PB T_{reg} cells in patients with RA was significantly low compared with that of healthy controls (N = 14).   Among the 3 populations of RA patients, T_{reg} cell frequency was lowest in active RA patients.   In contrast, the T_{reg} cell frequency of RA patients in remission was similar to that of healthy controls.   Accordingly, the frequency of $CD4^+ CD25^{high} CD127^{low/-}$ T_{reg} cells negatively correlated with DAS28, CRP and ESR in patients with RA.

Conclusions. The present data suggest that T_{reg} cells, defined as the $CD4^+ CD25^{high} CD127^{low/-}$ population, may contribute to the pathogenesis of RA and be an indicator of disease activity.

Key Indexing Terms: regulatory T cell (T_{reg} cell), CD127 (IL-7Rα), rheumatoid arthritis, disease activity

List of Abbreviations used

ACR: American College of Rheumatology

CRP: C-reactive protein

DAS: disease activity score

DMARDs: disease-modifying anti-rheumatic drugs

ESR: erythrocyte sedimentation rate

FCM: flow cytometry

FOXP3: forkhead box P3

IL: interleukin

PB: peripheral blood

PBMCs: peripheral blood mononuclear cells

RA: rheumatoid arthritis

T_{reg}: regulatory T

Introduction

CD4$^+$ regulatory T cell (T$_{reg}$)-cell deficiency or absence is known to correlate with the development or exacerbation of autoimmune diseases, implying a crucial role for T$_{reg}$ cells in maintaining immunological self-tolerance [1, 2].   In recent years, T$_{reg}$ cell counts and function have also been examined in rheumatoid arthritis (RA) patients [3-8].   T$_{reg}$ cell function in patients with active RA is supposed to be impaired, a trend that seems to be reversed by TNF antagonist therapy [6-7]; however, T$_{reg}$ cell counts in peripheral blood (PB) have varied across studies [3, 4].   These discrepancies can probably be ascribed to differences in the labeling and definition of CD4$^+$CD25$^+$ T cells [9].   Among CD4$^+$CD25$^+$ T cells, only those expressing large amounts of CD25, e.g., CD4$^+$CD25^{high} T cells, which highly express forkhead box P3 (FOXP-3), exert suppressive effects [9, 10].   The intracellular staining process for FOXP-3 is somewhat time-consuming as compared with cell surface staining in clinical practice; thus, a more convenient marker on the cell surface closely correlating with FOXP-3 expression is awaited. In this regard, Saleem et al. recently revealed that the frequency of CD62L$^+$ T$_{reg}$ cells in PB from RA is associated with sustained remission during TNF antagonist therapy [11].

Another candidate cell surface molecule for the identification of T$_{reg}$ cells is CD127.   Two recent studies have demonstrated that down-regulation of the interleukin (IL)-7 receptor α chain, CD127, distinguishes T$_{reg}$ cells from activated T cells demonstrating a significant correlation between the FOXP3 and CD127$^{low/-}$ phenotype at the same time that it functionally suppresses the CD127$^{low/-}$ population [12, 13].

For these reasons, we undertook the present study examining whether the frequency of T$_{reg}$ cells correlates with the clinical disease activity of RA by staining cells with CD4, CD25 and CD127.   The frequency of CD4$^+$CD25^{high}CD127$^{low/-}$ T$_{reg}$ cells negatively correlated with disease activity score 28 (DAS28), C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR).   This is a more convenient method of detecting T$_{reg}$ cells in clinical practice and may follow the data of Saleem et al. [11] suggesting that T$_{reg}$ cells contribute to the pathogenesis of RA.

Materials and Methods

Study population

RA patients (n=48) and healthy controls (n=14) were included in this study.　All of the patients fulfilled the 1987 criteria of the American College of Rheumatology (ACR) for RA [14]. All patients were seropositive for rheumatoid factor and/or anti-cyclic citrullinated peptide (CCP) antibodies.　Informed consent was obtained from all patients and healthy controls. Each patient provided a signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University.　Clinical response to the therapy was evaluated by DAS28 (high disease activity > 5.1, moderate disease activity < 5.1 and > 3.2, low disease activity < 3.2, remission < 2.6).　RA patients were divided into 3 groups: (1) patients naïve to disease-modifying anti-rheumatic drugs (DMARDs) (N=13); (2) active-RA patients (N=19) whose disease activity were over moderate (DAS28>3.2), including both those who were receiving (N = 14) or had received (N = 5) DMARDs; (3) RA patients achieving clinical remission (DAS28<2.6) with concomitant use of DMARDs (n=16).　All patients in the active-RA group and remission group were on stable therapy.　Patient characteristics are shown in Table 1.　The median (range) of age and the ratio of gender of healthy controls were 34.5 (27-50) years and 4: 10 (male: female), thus they were statistically younger than patients with RA.　We examined a correlation of age and the each population of T cell frequency among 14 healthy controls by Spearman's rank correlation, and did not find any association (data not shown).

Cell isolation and Analysis by FCM

Peripheral blood samples were collected in heparin.　Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Hypaque density centrifugation and used for flow cytometry (FCM).　Freshly isolated PBMCs were stained with 3 colors: FITC-labeled CD4, PE-Cy5-labeled CD25 and PE-labeled CD127, or PE-Cy5-labeled CD4, PE-labeled CD25 and FITC-labeled FOXP3 (clone: PCH101, intracellular staining) by the standard protocol.　All

antibodies were products of eBioscience.　For flow cytometric analysis, lymphocytes were
gated morphologically.

Statistical analyses

　　Within-group comparisons were made using Mann-Whitney's U test.　Changes from the
baseline were compared using Wilcoxon's signed rank test.　Correlations were assessed with
Spearman's correlation coefficient test.　The overall significance level for statistical analysis
was 5% (two-sided).　*P* values less than 0.05 were considered statistically significant.

Results

Patient characteristics are shown in Table 1.   CRP, ESR and DAS28 in the remission group were significantly lower than in the other two groups.   CRP and ESR in the active-RA patients who were receiving or had received DMARDs were significantly higher than those of DMARD-naïve RA patients, but DAS28 was not significantly different between the two groups.

Although most $CD4^+ CD25^+ CD127^{low/-}$ T cells were positive for FOXP3, a portion of this population was negative for FOXP3 (Figure 1A, middle).   On the other hand, almost all $CD4^+ CD25^{high} CD127^{low/-}$ T cells were positive for FOXP3 (Figure 1A, right side).

Phenotypes of peripheral blood $CD4^+$ T cells of RA patients and healthy controls were compared (Table 2).   There were no significant differences in the frequencies of $CD4^+ CD25^-$ T cells and $CD4^+ CD25^+$ T cells between DMARD-naïve RA patients and healthy volunteers. The frequency of $CD4^+ CD25^+ CD127^{low/-}$ T cells (Figure1A box-a) was significantly lower in the active-RA patients who were receiving or had received DMARDs than in healthy controls (p< 0.05).

We counted the frequency of $CD4^+ CD25^{high} CD127^{low/-}$T cells, for which we used the cut-off value of less than 5% CD127 expression among $CD4^+$ T cells (Figure 1B).   We have adopted this method to identify the $CD4^+ CD25^{high} CD127^{low/-}$T cells accurately in individual.   The frequency of this population was lower in the DMARD-naïve RA patient group than in the healthy controls (p<0.01), and was lower in the active-RA group with DMARDs than in the DMARD-naïve RA group (p<0.01).   Further, the frequency of this population was higher in the remission group than in the active-RA group with DMARDs (p<0.0001).

We investigated the correlation between the phenotype of peripheral blood T_{reg} cells and the markers of disease activity such as CRP, ESR and DAS28, in the 48 RA patients (Table 3). The frequencies of $CD4^+ CD25^+$ T cells and $CD4^+ CD25^+ CD127^{low/-}$ T cells were not correlated with disease activity.   However, the frequency of $CD4^+ CD25^{high} CD127^{low/-}$ T cells was negatively correlated with CRP, ESR and DAS28, respectively (p<0.0001).

As mentioned above, $CD4^+$ T cells were likely almost all positive for CD127 (Figure 2A);

however, a large CD127$^{low/-}$ population was detected among CD4^{+}CD25^{+} T cells in healthy

individuals (Figure 2B).   In patients with RA, the expression of this population was lower than

that in healthy individuals (Figure 2C), but recovered after the patient achieved clinical

remission (Figure 2D).

Discussion

Recent data obtained from patients with RA during TNF antagonist therapy have suggested that TNF down-modulates the function of human $CD4^+ CD25^+$ T_{reg} cells [6-8]. Therefore, T_{reg} cells may dynamically fluctuate, depending on the disease status of RA, and reflect the disease activity of RA. We have focused on a convenient cell surface staining method to identify T_{reg} cells and tried to investigate the association of T_{reg} cell frequency with the disease activity of RA.

CD25 and CD127 were used to identify the T_{reg} cell population in the present study. Since FOXP-3 is strongly expressed in $CD4^+ CD25^{high} CD127^{low/-}$ population, $CD4^+ CD25^{high} CD127^{low/-}$ cells can be estimated as T_{reg} cells [12, 13]. Additionally, the clinical differences between RA patients and healthy controls as well as the clinical parameters among RA patients were most predominantly found in the $CD4^+ CD25^{high} CD127^{low/-}$ population. We have set the cut-off of CD127 expression as less than 5% in the individual case; thus, our definition may correctly identify the frequency of naturally arising T_{reg} cells. The healthy controls were younger than the patients with RA in the present study. Although there was no correlation between T_{reg} cell frequency and age of healthy control, the previous study [4] demonstrated a weak negative correlation between age and T_{reg} cell frequency. The use of glucocorticoid was more frequent in active RA group as compared with DMARDs-naïve RA group as well as remission group. Influence of glucocorticoid regarding to the function or number of T_{reg} cells might be controversial [15, 16]. Therefore, further age-matched experiment of glucocorticoid-naïve patients is necessary to confirm our results.

DMARDs may alter the function of T cells in patients with RA. However, we have found in the present study that T_{reg} cell frequency may depend not on the use of DMARDs but on the disease activity of RA since the T_{reg} cell frequency was lowest in active RA patients whereas that of RA patients in remission was similar with that in healthy controls despite the administration of DMARDs in both groups. In addition, the T_{reg} cell frequency was not statistically different between DMARD-naïve RA patients and RA patients in remission

receiving DMARDs, and an inverse correlation was found between the disease activity of RA and the T_{reg} cell frequency, also supporting the above speculation.   Fluctuation of T_{reg} cell frequency depending on the disease activity, in fact, is found in our representative case. Difference of the present study as compared with previous reports is to estimate T_{reg} cells as FOXP-3^{bright} cells.   As shown in the previous report [3, 4], the difference of CD4$^+$CD25$^+$ T cell frequency between the patients with RA and healthy controls was not significant.   In addition to CD127, similar result is obtained as to estimate T_{reg} cells as CD62 ligand$^+$ FOXP-3^{bright} cells [11].   Since we have not performed follow-up analysis of T_{reg} cell frequency in each case, a prospective follow-up study should be performed to establish that the CD4$^+$CD25^{high}CD127$^{low/-}$ T_{reg} cell population does in fact reflect changes in disease activity of RA.   Further examinations, including studies with a larger number and with follow-up observation, are needed to confirm the present observation.

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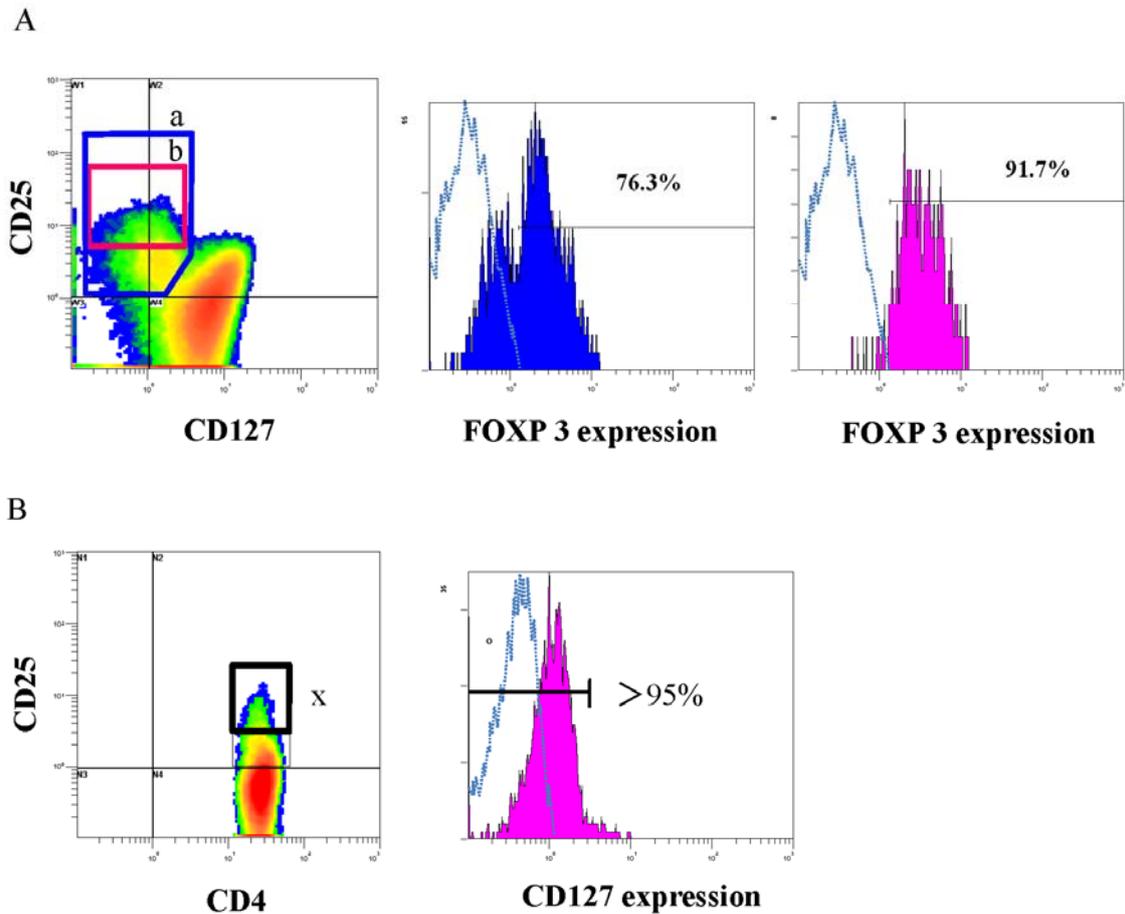
Figure Legends

A

B

Figure 1.

CD 4^+ CD25^{high} CD127$^{low/-}$ population as the phenotype of T$_{reg}$ cells.

We showed a representative data of several healthy samples.   **A**, Plots are gated for CD4$^+$ T

cells.   CD25$^+$ CD127$^{low/-}$ cells and CD25^{high} CD127$^{low/-}$ cells are boxed in box-a and box-b

(left side).   Expressions of FOXP3 in box-a and box-b are shown on the middle and right-hand

side.   **B**, Mononuclear cells were stained for CD4, CD25, and CD127.   Plots are gated for

CD4$^+$ T cells.   CD4$^+$ CD25^{high} population, with the cut-off of CD127 expression as less than

5% among CD4$^+$ T cells (right side), is boxed as CD4$^+$ CD25^{high} CD127$^{low/-}$ T cells (left side,

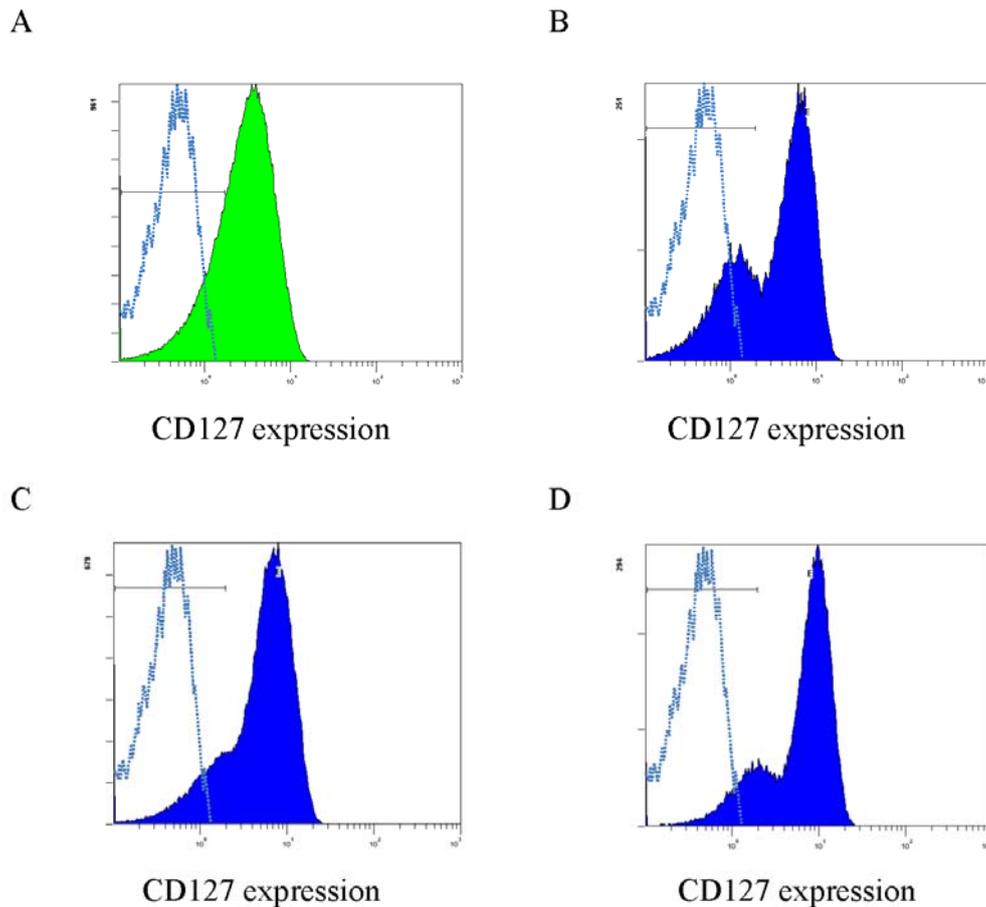box-x).   Box-x is individually adjusted.

Figure 2.

Changes in the proportion of the CD127$^{low/-}$ population among CD4$^+$ CD25$^+$ T cells.

We showed a representative data of several samples.   Expression of CD127 among CD4$^+$ T cells (A) and CD4$^+$ CD25$^+$ T cells (B-D) are shown.   **A and B**, PBMC samples were collected from healthy individuals. Although CD4$^+$ T cells are likely almost all positive for CD127 (A), the expression of CD127 among CD4$^+$ CD25$^+$ T cells is bipolar, with both CD127$^{low/-}$ cells and CD127$^+$ cells in healthy controls (B).   **C and D**, PBMC samples were collected from an early RA patient (male, disease duration; 3 months).   The proportion of CD127$^{low/-}$ cells among CD4$^+$ CD25$^+$ T cells decreased before this patient was treated (DAS28; 4.27) (C).   After this patient achieved clinical remission by treatment with bucillamine, the expression of CD127 among CD4$^+$ CD25$^+$ T cells recovered to nearly the same level as that in healthy controls (D). The frequencies of CD4$^+$ CD25$^+$ CD127$^{low/-}$ cells (Figure 1A box-a) were 5.73 % before therapy and 7.21 % after achieving clinical remission.
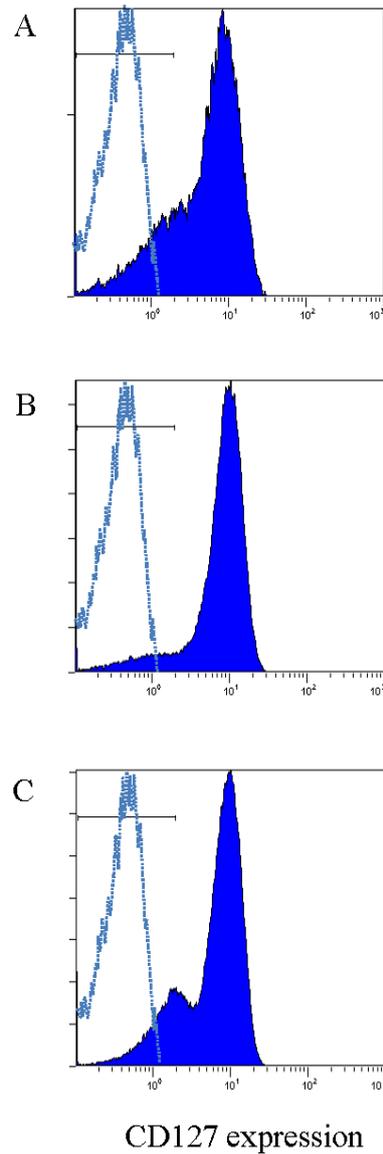
A

B

C

CD127 expression

Figure 3

The proportion of the CD127 $^{low/-}$ population among CD4$^+$ CD25$^+$ T cells in the three RA groups.

We showed a representative data from each 3 RA groups (**A**, DMARDs-naïve RA group; **B**,

active RA group; **C**, remission group).   Expressions of CD127 among CD4+ CD25+ T cells

are shown.   The frequencies of CD4$^+$ CD25$^+$ CD127 $^{low/-}$ cells (Figure 1A box-a) were 3.28 %,

2.24%, and 5.77 % in A, B, and C, respectively.

Table 1. Patient characteristics

	Healthy controls	DMARDs-naïve RA group	Active RA group	Remission group
Patient numbers	14	13	19	16
Age (years [a])	34.5 (27-50)	59 (39-81)	57 (19-79)	58 (26-76)
Gender (male/female)	4/10	3/10	1/18	4/12
Duration of disease (years [a])	-	0.25 (0.15-1.5)	3.5* (0.5-28)	2.5* (0.33-22)
CRP (mg/dl [a])	-	0.54** (0.04-5.35)	2.59**# (0.16-10.08)	0.07 (0.01-0.12)
ESR (mm/hr [a])	-	48** (12-120)	57.4**# (1-127)	13.5 (5-26)
DAS28 [a]	-	4.68** (3.54-7.75)	5.70** (3.21-8.16)	1.83 (1.13-2.54)
Therapy	-	-	MTX:13, SASP:1	MTX:11, SASP:2, BU:1, ETN:1, IFX:3
	-			
Concomitant glucocorticoid(n)[$]	-	2	10***#	0

[a] Median (range)

*$p<0.0001$, vs. DMARD-naïve RA group, ** $p<0.0001$, vs. Remission group, ** $p<0.001$, vs. Remission group

# $p<0.05$, vs. DMARD-naïve RA group

Within-group comparisons were made using Mann-Whitney's U test and χ^2 test (Fisher's exact probability test when appropriate).

[$]Doses of glucocorticoid were less than 7.5mg daily.

BU: bucillamine, ETN: etanercept, IFX: infliximab, MTX: methotrexate, SASP: salazosulfapyridine

Table 2

Phenotype of peripheral blood CD4$^+$ T cells in RA patients and healthy controls

	CD4$^+$CD25$^-$	CD4$^+$CD25$^+$	CD4$^+$CD25$^+$CD127$^{low/-}$	CD4$^+$CD25^{high}CD127$^{low/-}$
Healthy controls (n=14)	84.7	15.3	3.63	3.76
	(72.4-87.2)	(12.8-27.6)	(2.34-7.54)	(2.11-9.80)
DMARDs-naïve RA group (n=13)	78.3	21.7	3.12	2.23**
	(63.9-88.3)	(11.7-36.1)	(1.72-5.73)	(0.57-5.18)
Active RA group (n=19)	79.9	20.1	2.76*	1.35***[#]
	(63.9-87.2)	(12.8-36.1)	(1.25-5.31)	(0.41-2.21)
Remission group (n=16)	80.6	19.4	3.35	2.98[$]
	(66.6-90.1)	(9.9-33.4)	(2.13-7.21)	(1.34-4.89)

% CD4$^+$T cells; Median (range)

* $p<0.05$, ** $p<0.01$, *** $p<0.0001$, vs. HC, [#] $p<0.01$, vs. DMARDs-naïve RA group, [$] $p<0.0001$, vs. Active RA group

Within-group comparisons were made using Mann-Whitney's U test

Table 3

The correlations between T_{reg} and RA disease activity in 48 RA patients

	CRP		ESR		DAS28	
	r	*p*	r	*p*	r	*p*
CD4$^+$CD25$^+$	0.03	*NS*	0.12	*NS*	0.04	*NS*
CD4$^+$CD25$^+$CD127$^{low/-}$	-0.21	*NS*	-0.20	*NS*	-0.17	*NS*
CD4$^+$CD25^{high}CD127$^{low/-}$	-0.65	<0.0001	-0.58	<0.0001	-0.61	<0.0001

The correlations were assessed with Spearman's correlation coefficient test.