Abatacept therapy reduces $CD28^+CXCR5^+$ follicular helper-like T cells in patients with rheumatoid arthritis

S. Fukuyo, S. Nakayamada, S. Iwata, S. Kubo, K. Saito, Y. Tanaka

The First Department of Internal Medicine, University of Occupational and Environmental Health, Fukuoka, Japan.

Abstract

Objective

The characteristics of T cells targeted by abatacept (ABT) in cases of rheumatoid arthritis (RA) are still unknown. The goal of the study was to determine the pathogenicity of T cells and the predictors of therapeutic effects of ABT.

Methods

We analysed the peripheral T cell phenotype of 34 RA patients via flow cytometry. The correlation of the phenotypes of $CD4^+$ T cells with clinical disease activity and change in $CD4^+$ T cell subsets at baseline and 24 weeks after ABT treatment were evaluated.

Results

RA patients showed an increase in the proportion of $CD28^-$ cells among $CD4^+$ cells, which was significantly high in patients who had not achieved remission after ABT therapy. The proportions of $CD4^+CXCR5^+$ T follicular helper-like (Tfh-like) cells increased in RA patients compared to healthy donors. The proportions of Tfh-like cells among $CD4^+CD28^+$ cells were significantly higher than those among $CD4^+CD28^-$ cells. The proportion of Tfh-like cells was higher in anti-cyclic citrullinated peptide antibody (ACPA)-positive patients. By contrast, the proportions of $CD4^+CXCR3^+$ T helper 1-like (Th1-like) cells and effector memory phase T cells among $CD4^+CD28^-$ cells were significantly higher than those among $CD4^+CD28^+$ cells, and the proportion of these cells did not correlate with disease activity. After ABT therapy, the proportion of Tfh-like cells among $CD4^+CD28^+$ cells was significantly reduced.

Conclusion

These results imply that $CD4^+$ $CD28^+$ Tfh-like cells could possibly be the targets of ABT. Conversely, $CD4^+$ $CD28^-$ cells may be a potential predictor of treatment resistance.

Key words

T follicular helper cells, CD28, abatacept, rheumatoid arthritis

Shunsuke Fukuyo, MD, PhD
Shingo Nakayamada, MD, PhD
Shigeru Iwata, MD, PhD
Satoshi Kubo, MD, PhD
Kazuyoshi Saito, MD, PhD
Yoshiya Tanaka, MD, PhD

Please address correspondence and reprint requests to:
Prof. Yoshiya Tanaka,
The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Japan,
1-1 Iseigaoka, Yahata-nishi-ku, Kitakyushu, Fukuoka, 807-8555, Japan
E-mail: tanaka@med.uoeh-u.ac.jp

Received on August 14, 2016; accepted in revised form on November 10, 2016.

Funding: this study was supported in part by a Research Grant-In-Aid for Scientific Research by the Ministry of Health, Labor and Welfare of Japan; the Ministry of Education, Culture, Sports, Science and Technology of Japan; and the University of Occupational and Environmental Health, Japan.

Competing interests: Y. Tanaka has received consulting fees, speaking fees, and/or honoraria from Abbvie, Chugai, Astellas, Takeda, Santen, Mitsubishi-Tanabe, Pfizer, Janssen, Eisai, Daiichi-Sankyo, UCB, GlaxoSmithKline, and Bristol-Myers and has received research grants from Mitsubishi-Tanabe, Chugai, MSD, Astellas, and Novartis; the other co-authors have declared no competing interests.

Introduction

Rheumatoid arthritis (RA), which occurs as a result of an immune abnormality associated with the hyperactivation of autoreactive T cells, is a systemic inflammatory disease. Activated $CD4^+$ T helper (Th) cells, which are divided into various subsets, play an important role in the pathogenesis of RA. Th1 and Th2 cells have long been known to be associated with the pathology of RA (1). Th17 cells are responsible for inducing and maintaining inflammation by producing IL-17 (2, 3) and increasing its levels in the synovial fluids of RA patients (4, 5). T follicular helper (Tfh) cells, which is to promote and then maintain the germinal centre response, necessary for B cell memory and long-lived high affinity plasma cell formation, constitute a new subset that is garnering attention (6, 7). These cells are reported to exist in greater quantities in the peripheral blood of RA patients and it is hypothesised that the interaction between these $CD4^+$ T cells is the underlying basis for the complex pathology of RA (8).

Abatacept (ABT) is a fusion protein composed of the extracellular domain of CTLA-4 and the Fc region of human IgG_1. ABT exerts a therapeutic effect by blocking the co-stimulatory signal transmitted via the binding of CD80/CD86 on APCs and of CD28 on T cells, thereby inhibiting T cell activation (9). However, in actuality, few reports have proven the effects of ABT on human T cells. An *In vivo* study conducted to date reported that ABT inhibits Tfh cell activation and B cell-mediated antibody production in a murine model of arthritis (10). We recently reported that abatacept treatment reduces the levels of phosphorylated Syk in peripheral B cells in RA patients (11). While ABT exerts a marked effect in RA patients, treatment resistance has been noted in some cases (12, 13). These findings highlight the diversity of RA pathology. Therefore, identifying the target for ABT therapy may not only yield a greater understanding of RA pathology but also allow for customised therapy.

The objective of this study was to assess the link between ABT and phenotype and phenotypic changes of peripheral $CD4^+$ T cells in the context of disease activity, antibody production, and treatment response in RA patients.

Materials and methods

Patients

We included RA patients who met the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 diagnosis criteria for RA, and received DMARD therapy at the University Hospital of the University of Occupational and Environmental Health, Japan, from 2012 to 2014. Patients resistant to treatment included those who had a DAS28 erythrocyte sedimentation rate (ESR) score >3.1 for RA activity, despite treatment with adequate doses of antirheumatic drugs (mainly methotrexate) for a minimum of 3 months, and those who showed no or moderate response, according to the European League Against Rheumatism (EULAR) improvement criteria. This group was selected for treatment with ABT.

Fifteen patients were treated with 10 mg/kg of ABT at baseline, week 2, and every 4 weeks thereafter. Heparinised whole blood samples were taken at baseline and at week 24 during treatment with ABT. In addition, heparinised whole blood samples were taken from healthy volunteers. Detailed demographic and clinical characteristics of the RA patients are provided in Table 1. The mean age of the healthy donors differed from that of RA patients (control: 36.0±7.5, 5 men and 9 females, *vs*. RA: mean 62.2±14.2, 6 men and 28 females). We assessed clinical disease activity using the Simplified Disease Activity Index (SDAI) and the disease activity score (DAS) based on 28 joints and ESR. The Human Ethics Review Committee of the university reviewed and approved our study, including the collection of peripheral blood samples from healthy adults and RA patients. Each subject provided a signed participation consent form.

Flow cytometric analysis

After obtaining informed consent, blood was withdrawn during routine laboratory screening. Peripheral blood mononuclear cells (PBMCs) were isolated from

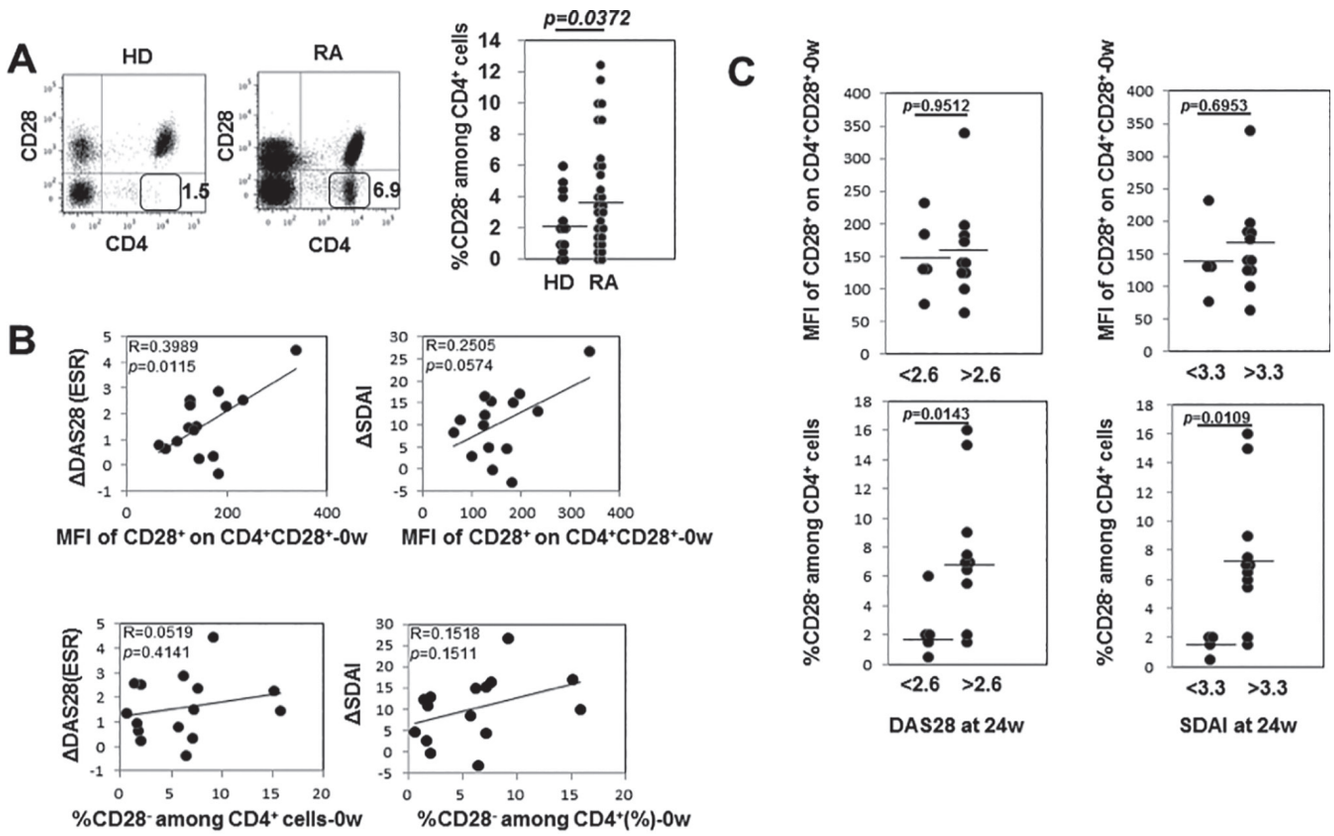

Fig. 1. Contribution of CD28 expression on $CD4^+$ T cells in response to abatacept therapy.
(A) The proportion of $CD28^-$ cells among $CD4^+$ cells in RA patients (n=34) compared with healthy donors (HD) (n=14). The statistical difference was determined by two-sided Student's *t*-test. Difference with $p<0.05$ was considered significant.
(B) (upper) The correlation between CD28 expression levels on $CD4^+CD28^+$ cells at baseline and the changes in disease activities assessed by ΔDAS28 and ΔSDAI at 24 weeks after abatacept (ABT) therapy (n=15). MFI : Mean Fluorescence Intensity. (bottom) The correlation between the proportion of $CD28^-$ cells among $CD4^+$ cells at baseline and ΔDAS28 or ΔSDAI at 24 weeks after ABT therapy. The Spearman rank correlation was calculated to assess the correlation between the data.
(C) (top) The levels of CD28 expression on $CD4^+CD28^+$ cells at baseline in RA patients who failed to achieve remission at 24 weeks after ABT therapy (DAS28 >2.6, SDAI >3.3) compared with RA patients who achieved remission. (bottom) The proportion of $CD28^-$ cells among $CD4^+$ cells at baseline in RA patients who failed to achieve remission at 24 weeks after ABT therapy (DAS28 >2.6, SDAI >3.3) compared with RA patients who achieved remission. The statistical difference was determined by two-sided Student's *t*-test. Difference with $p<0.05$ was considered significant.

heparinised blood by lymphocyte separation medium (LSM) (PAA Laboratories GmbH, Pasching, Austria) density gradient centrifugation. PBMCs were resuspended in PBS/3% human IgG (Baxter International Inc., Vienna, Austria) in order to block Fc receptors and prevent non-specific antibody binding, and then incubated for 15 min at 4°C in the dark with combinations of fluorescein isothiocyanate (FITC)-labelled antibodies against CXCR5, CXCR3, and CCR7 (R&D Systems, Minneapolis, MN, USA), and CD38, phycoerythrin (PE) against CD28, phycoerythrin-cyanin 7 (PE-Cy7) against CD4, and APC-Cy-7 against CD45RA (BD Bioscience, San Jose, CA, USA). The cells were then washed with PBS containing 1% BSA. Background fluorescence was assessed using appropriate isotype- and fluorochrome-matched control mAbs. We had processed and stained in exactly the same procedures in both healthy donors and RA patients. After staining with the indicated antibodies, cells were analysed by flow cytometry (FACSCalibur™ , BD Bioscience). The cells were collected and analysed with FlowJo software (Tree Star). Absolute cell numbers were calculated from whole blood counts obtained via routine laboratory testing.

Statistical analysis

All values are presented as mean values ± standard deviation (SD). Proportions of lymphocyte subpopulations were compared using the Student's *t*-test for normally distributed values, and the Mann-Whitney test for values without Gaussian distribution. Relationships between the various outcomes were examined using Pearson's correlation coefficient, Spearman's rank correlation tests, and two-sided paired *t*-test, as appropriate. A *p*-value of less than 0.05 was considered significant in all statistical tests. All statistical analyses were performed using GraphPad Prism® (GraphPad Prism 4.0 by Graph Pad software Inc.) and JMP (JMP 12.0 by JMP software Inc.) software.

Results

Contribution of CD28 expression on $CD4^+$ T cells in response to abatacept therapy

First, we examined the properties of T cells in the peripheral blood of RA

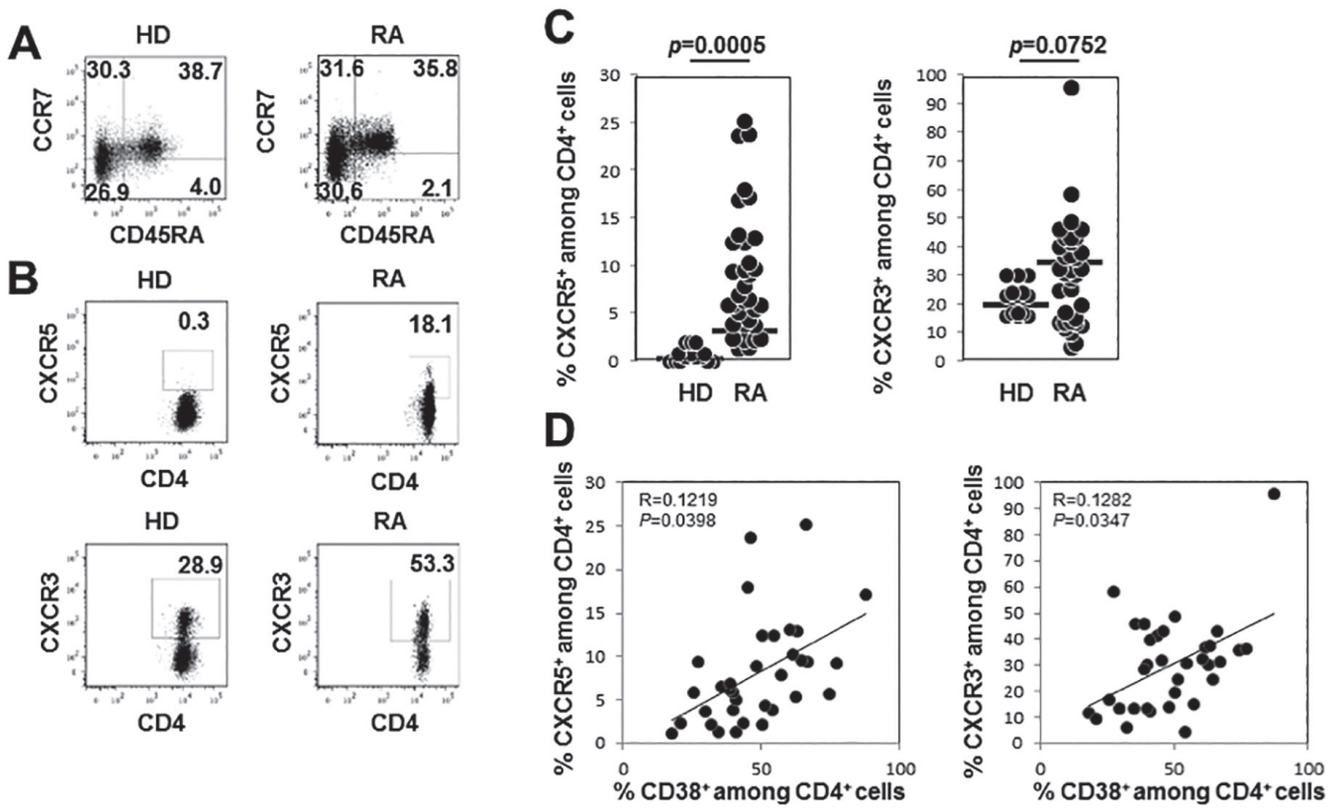

Fig. 2. The proportion of Th1-like and Tfh-like cells in the peripheral blood of RA patients.
(A) Representative expression of CCR7 *vs*. CD45RA expression on $CD4^+$ cells in RA patients and healthy donors (HD). **(B)** Representative expression of CXCR5 or CXCR3 on $CD4^+$ cells in RA patients and HD. **(C)** The proportion of $CXCR5^+$ (Tfh-like) or $CXCR3^+$ (Th1-like) cells among $CD4^+$ cells in RA patients compared with HD. The statistical difference was determined by two-sided Student's *t*-test. Difference with $p<0.05$ was considered significant. **(D)** The correlation between the proportion of $CD38^+$ among $CD4^+$ cells and the proportion of $CXCR5^+$ (Tfh-like) or $CXCR3^+$ (Th1-like) among $CD4^+$ cells. Each data point represents an individual subject, and horizontal lines show the median. The Spearman rank correlation was calculated to assess the correlation between the data.

patients. A significant difference in the expression of the co-stimulatory molecule CD28 was observed between RA patients with high disease activity (n=34) and healthy controls (n=14). The proportion of $CD28^-$ cells among $CD4^+$ cells was significantly increased in the peripheral blood of RA patients (p=0.0372) (Fig. 1A). ABT therapy improved disease activity (SDAI/DAS28) (Supplementary Fig. 1A). Baseline expression levels of CD28 on $CD4^+CD28^+$ cells, and changes in disease activities at 24 weeks after ABT therapy, were compared in patients with bio-naïve RA and high disease activity (n=15). A correlation was observed between expression levels of CD28 on peripheral $CD4^+CD28^+$ cells and changes in disease activities, as determined by ΔDAS28 (p=0.0115) and ΔSDAI (p=0.0574). In contrast, the proportion of $CD28^-$ cells among $CD4^+$ cells did not reveal a correlation with ΔDAS28 or ΔSDAI 24 weeks after ABT therapy (Fig. 1B). Furthermore, patients who failed to achieve remission at 24 weeks after ABT therapy (DAS28 >2.6, SDAI >3.3) showed a higher baseline proportion of $CD28^-$ cells among $CD4^+$ cells than observed in patients who achieved remission (DAS28 remission: p=0.0143; SDAI remission: p=0.0109) (Fig. 1C). No significant difference was observed in the levels of CD28 expression on $CD4^+CD28^+$ cells in patients who responded to ABT therapy (Fig. 1C). The baseline DAS28 and SDAI of responder group was lower than those of non-responder (3.8±1.2 *vs*. 5.5±1.3, 13.1±4.6 *vs*. 23.9±10.4, respectively) (Supplementary Table I). This may reflect that ABT is more effective in patients with lower inflammatory markers such as CRP, as previously reported (12). Thus, the expression levels of CD28 on $CD4^+CD28^+$ cells were associated with the therapeutic effects of ABT. However, RA patients who did not respond to ABT characteristically possessed $CD28^-$ cells among peripheral $CD4^+$ cells.

Activated Th1-like and Tfh-like cells are increased in the peripheral blood of RA patients

Next, as T helper cells are composed of various subsets, we analysed the subsets of $CD4^+$ T cells in the peripheral blood of RA patients. When classified by helper cell subset among $CD4^+CD45RA^-$ memory T cells (Fig. 2A), RA patients demonstrated a significant increase in $CD4^+CXCR5^+$ T (Tfh-like) cells in the peripheral blood compared to healthy controls (p=0.0005) (Fig. 2B-C). A tendency towards an increased proportion of $CD4^+CXCR3^+$ T cells (Th1-like) compared to healthy controls was also observed (p=0.0752) (Fig. 2B-C). Furthermore, when examining $CD38^+$ cells (which are activation

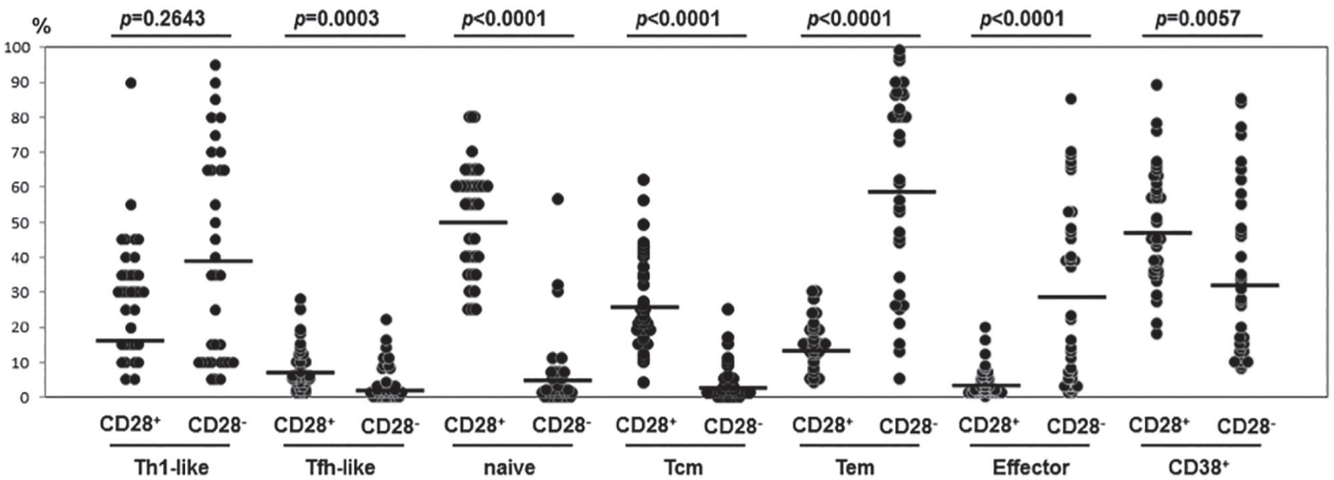

Fig. 3. The phenotypic difference of $CD4^+CD28^+$ cells and $CD4^+CD28^-$ cells. The proportion of $CD4^+$ $CXCR3^+$ (Th1-like), $CD4^+$ $CXCR5^+$ (Tfh-like), $CD4^+$ $CCR7^+$ $CD45RA^+$ (naive), $CD4^+$ $CCR7^+$ $CD45RA^-$ (Tcm), $CD4^+$ $CCR7^-$ $CD45RA^-$ (Tem), $CD4^+$ $CCR7^-$ $CD45RA^+$ (effector), and $CD38^+$ cells among $CD4^+CD28^+$ cells or $CD4^+CD28^-$ cells. Each data point represents an individual subject, and horizontal lines show the median. The statistical difference was determined by two-sided Student's *t*-test. Difference with $p<0.05$ was considered significant.

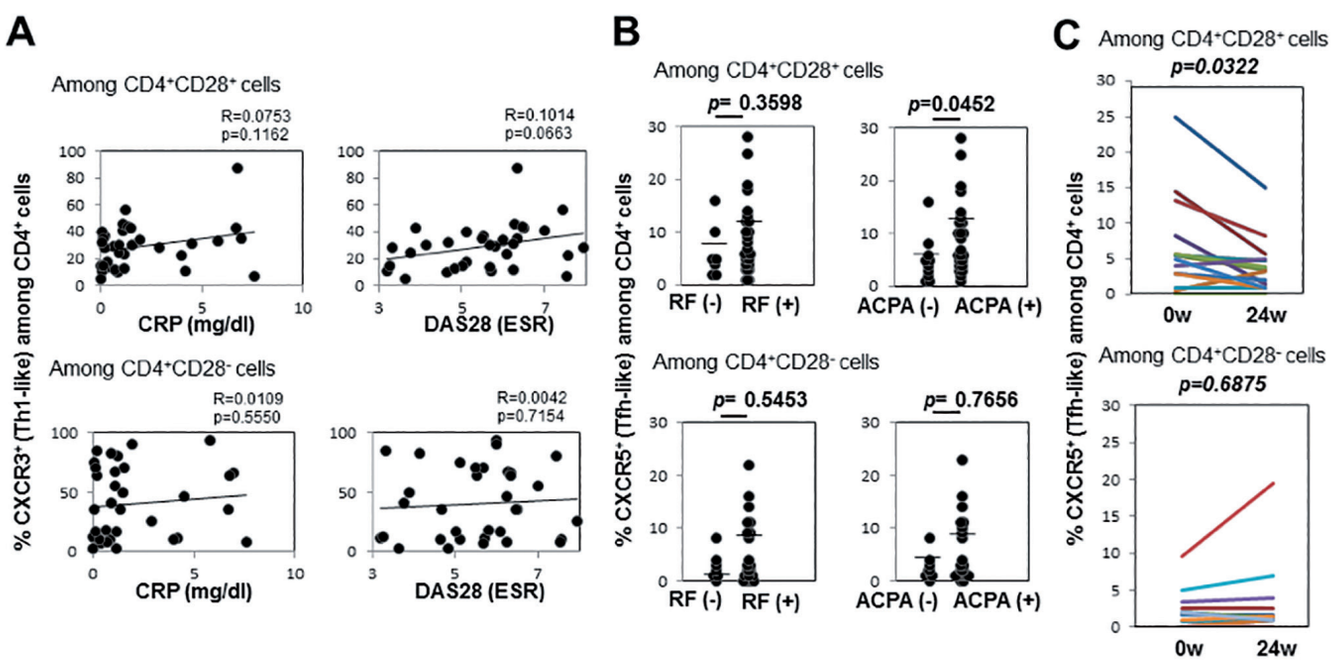

Fig. 4. The correlation between the phenotype of $CD4^+CD28^+$ cells and $CD4^+CD28^-$ cells and the disease activity and antibody production. **(A)** The correlation between the proportion of $CXCR3^+$ (Th1-like) cells in $CD4^+CD28^+$ cells and $CD4^+CD28^-$ cells and CRP and DAS28ESR was assessed. The Spearman rank correlation was calculated to assess the correlation between the data. **(B)** The correlation between the proportion of $CXCR5^+$ (Tfh-like) cells in $CD4^+CD28^+$ cells and $CD4^+CD28^-$ cells and positive or negative for RF (normal limit at our hospital; <15 IU/ml) and ACPA (normal limit at our hospital; <4.5 IU/ml) was assessed. Each data point represents an individual subject, and horizontal lines show the median. The statistical difference was determined by two-sided Student's *t*-test. Difference with $p<0.05$ was considered significant. (C) The change of the proportion of $CXCR5^+$ (Tfh-like) cells in $CD4^+CD28^+$ cells and $CD4^+CD28^-$ cells between at baseline and 24 weeks after ABT treatment.

targets), the percentage of both Tfh-like cells and Th1-like cells tended to correlate with the percentage of $CD38^+$ cells among $CD4^+$ cells (Tfh-like cells: R=0.1219, p=0.0398; Th1-like cells: R=0.1282, p=0.0347) (Fig. 2D), indicating that there was an increase in activated Th1-like and Tfh-like cells in the peripheral blood of RA patients.

$CD4^+CD28^+CXCR5^+$ (Tfh-like) cells are the possible therapeutic targets of ABT

In the differentiation stages classified as the naïve or memory phase, no difference was observed between RA patients and healthy controls (a representative case is shown in Fig. 2A). The proportions of Tfh-like cells, $CD45RA^+CCR7^+$ naïve and $CD45RA^-CCR7^+$ central memory (Tcm) phase T cells among $CD4^+CD28^+$ cells were significantly higher than those of $CD4^+CD28^-$ cells (p=0.0003, $p<0.0001$, $p<0.0001$ respectively) (Fig. 3).

In addition, we analysed the correlation of their respective phenotypes with disease activity and autoantibod-

Table I. Patient baseline demographics and clinical characteristics.

	n=34
Sex (M/F)	6/28
Age (years)	62.2 ± 14.2
Disease duration (months)	138 ± 167
Prednisolone dose (mg/day) (% of use)	4.3 ± 1.0 (9%)
Methotrexate dose (mg/week) (% of use)	13.9 ± 3.0 (79%)
Tender joint count (28)	12.4 ± 8.7
Swollen joint count (28)	6.8 ± 4.9
CRP (mg/dl)	2.0 ± 2.3
ESR (mm/hr)	38.3 ± 30.8
RF (IU/ml) (% of positivity)	201 ± 511 (79%)
Anti-CCP antibody (% of positivity)	(71%)
MMP-3	177 ± 138
IgG (mg/dl)	1518 ± 382
DAS28 (CRP)	4.9 ± 1.3
DAS28 (ESR)	5.5 ± 1.3
CDAI	25.7 ± 7.2
SDAI	27.1 ± 14.2
HAQ	1.6 ± 0.9

Table II. Changes in the subsets of $CD4^+$ T cell at 24 weeks after ABT therapy.

		0w	24w	*p*-value
$CD4^+$ (%)	$CD28^+$	96.2 ± 2.9	94.2 ± 7.3	0.2290
	$CD28^-$	3.8 ± 2.9	5.7 ± 7.3	0.2345
Tem (%)	$CD28^+$	21.4 ± 7.7	23.8 ± 14.7	0.6189
	$CD28^-$	72.7 ± 22.2	80.0 ± 9.5	0.3236
Tcm (%)	$CD28^+$	27.6 ± 14.0	27.5 ± 13.5	0.7623
	$CD28^-$	1.4 ± 1.7	1.4 ± 2.0	0.4151
Effector (%)	$CD28^+$	4.9 ± 5.5	7.6 ± 12.1	0.6885
	$CD28^-$	24.2 ± 21.8	15.4 ± 7.9	0.2824
Naïve (%)	$CD28^+$	45.3 ± 12.4	40.1 ± 17.1	0.6998
	$CD28^-$	1.8 ± 1.9	4.5 ± 8.5	0.2989
$CD4^+CXCR3^+$Th1-like (%)	$CD28^+$	21.4 ± 13.4	27.9 ± 12.9	0.2646
	$CD28^-$	25.6 ± 29.5	30.4 ± 24.4	0.6510
$CD4^+CXCR5^+$Tfh-like (%)	$CD28^+$	6.2 ± 6.1	3.7 ± 4.9	0.0322
	$CD28^-$	2.2 ± 2.8	2.9 ± 6.0	0.6875
$CD38^+$ (%)	$CD28^+$	45.0 ± 15.7	36.4 ± 15.3	0.2475
	$CD28^-$	38.2 ± 25.3	34.1 ± 21.8	0.9292

ies. Among $CD4^+CD28^+$ cells, Th1-like cells tended to correlate with the DAS28 (p=0.0663), and a significantly higher proportion of Tfh-like cells was observed in the ACPA-positive group compared to the ACPA-negative group (p=0.0452) (Fig. 4A-B). Importantly, while no change was observed in the overall proportion of $CD28^+$ cells among $CD4^+$ cells at 24 weeks after ABT therapy, the proportion and the number of Tfh-like cells among $CD4^+CD28^+$ cells was significantly reduced (Fig. 4C, Table II and Supplementary Fig. 1B). No significant changes were observed in any other type of cells (Table II). We also compared the percentages of $CD28^+$, $CD28^-$, $CXCR5^+$ cells and CD28 MFI with disease activity at baseline and after therapy (Supplementary Fig. 2). The association between expression levels of CD28 on $CD4^+$ T cells and SDAI was only detected before ABT therapy but not after therapy. Therefore, Tfh-like cells among $CD4^+CD28^+$ cells were considered to be targets for ABT therapy.

$CD4^+CD28^-$ cells are associated with treatment resistance

The proportions of effector and effector memory (Tem) cells among the $CD4^+CD28^-$ cells were significantly higher than among $CD4^+CD28^+$ cells (p<0.0001, p=0.0001, respectively) (Fig. 3). Although not significant, a tendency towards a higher proportion of Th1 was observed among $CD4^+CD28^-$ cells (Fig. 3). However, among $CD4^+CD28^-$ cells, no correlation whatsoever was observed between Th1-like and disease activity, or between Tfh-like cells and autoantibodies (Fig. 4A-B). At 24 weeks after ABT therapy, no change was observed in the proportion of $CD28^-$ cells among $CD4^+$ cells or the proportion of Th1-like cells (Table II). Thus, the $CD4^+CD28^-$ cells demonstrated no qualitative improvement following ABT therapy, indicating potential involvement in resistance to ABT therapy.

Discussion

In the present study, we demonstrated that the expression pattern of the co-stimulatory molecule CD28 on $CD4^+$ T cells contributes to T cell subset differences and pathogenicity, as well as to the response to ABT therapy. Namely, $CD4^+CD28^+$ cells are composed of Th1-like cells and of Tfh-like cells. We found that the expression levels of CD28 on $CD4^+CD28^+$ T cells correlated with the ABT-based disease activity improvement rate, and that Tfh-like cells among $CD4^+CD28^+$ cells may thus be targeted by ABT.

Th1 cells are known to be increased in the synovial fluid and peripheral blood of RA patients. Historically, an imbalance between Th1 and Th2 cells has been considered an important factor in the pathology of RA (14). However, after Th17, Treg, and Tfh cells were identified, the pathology of RA is now considered to be the result of a more complex mechanism than previously proposed (8). Recent clinical data-based analyses of peripheral blood in RA patients have focused on the importance of Tfh cells (15). Furthermore, we recently discovered the plasticity of Th1 and Tfh cells in the process of T helper cell differentiation and activation (16). We therefore conducted this study with a focus on Th1 cells as the classic subset and Tfh cells as the novel subset.

Th1 cells produce large quantities of interferon gamma (IFN-γ), activate macrophages and other immune-mediated cells, and are involved in the pathology of inflammation (17). The induc-

tion of Th1 cells is strongly linked to the upregulation of the chemokine receptor CXCR3. CXCR3 binds three chemokines chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11 to induce migration of activated T cells. CXCR3 and its ligands are an inflammatory chemokine system, capable of coordinating T cell responses in the inflamed periphery. Th1 cell-mediated inflammation is characterised by the recruitment of IFN-γ producing CD4 T cells. T-bet is the master transcription factor of Th1 cells. T-bet directly activates transcription of a set of genes important for Th1 cell function (18). However, $CXCR3^+$ T cells may be consist of $CXCR5^+$ (Tfh-like) and $CXCR5^-$ cells (Th1) (19). Since we did not measure IFN-γ, T-bet and double-staining of CXCR3 and CXCR5, the expression of CXCR3 alone may not be sufficient for the definition of Th1 cells. Therefore, we referred $CD4^+CXCR3^+$ T cells as "Th1-like" cells.

Tfh cells are T cells specialised for action on B cells. These cells express surface markers such as IL-21, the transcription factor B cell lymphoma 6 (Bcl-6), the chemokine (C-X-C motif) receptor 5 (CXCR5) and the inducible co-stimulator (ICOS) and programmed death-1 (PD-1) (6, 20). CXCR5 and its ligand CXCL13 are important for B cell follicle formation in secondary lymphoid organs. Furthermore, expression of CXCR5 is a feature that T cell has been localised to B cell follicles and germinal centres in secondary lymphoid organs (21). Tfh cells express large amounts of Bcl-6, which is necessary and sufficient for the development of Tfh cells *in vivo* (22). But, it was previously reported that $CD4^+CXCR5^+$ cells expressing Bcl-6 were absent in blood (23), alongside lacking other features of the central population. The circulating $CD4^+CXCR5^+CCR7^{lo}PD\text{-}1^{hi}$ subset was indicative of active Tfh differentiation in lymphoid organs and correlated with clinical indices in autoimmune diseases. It is suggested that circulating $CXCR5^+$ helper T cells are primarily generated before germinal centres (23). Thus, we referred $CD4^+CXCR5^+$ "Tfh-like" cells to the circulating pool as cells with a Tfh phenotype.

The migration of Tfh cells to B-cell follicles multiplies B cells by producing IL-4, IL-21, and IFN-γ, and induces both class switching and differentiation into antibody production cells and memory cells. The functional differentiation of B cells occurs in the germinal centre; however, in an environment lacking Tfh cells, the formation of the germinal centre is inhibited (24, 25). Although Tfh cells are important in antibody reactions *in vivo*, an excessive increase of Tfh cells induces autoimmune disorders through the production of autoantibodies (26). In fact, we demonstrated an association between increased Tfh-like cells and ACPA production in the peripheral blood of RA patients. Recently, we have described that levels of the phosphorylation of Syk (pSyk) in peripheral blood B cells were higher in RA patients compared to healthy donors, and patients with higher pSyk levels were strongly positive for ACPA. Abatacept treatment reduced the levels of pSyk and the proportion of Tfh cells (11). These data suggest that B and T cell interactions as a potential target for abatacept therapy for RA. Engagement of CD80/86 by abatacept inhibited the second signaling for T cell activation and induced activation of the enzyme indoleamine 2,3-dioxygenase, (IDO), in the expression of CD80/86 cells, which degraded tryptophan and promoted apoptosis (27). These data suggest that abatacept might affect the function of APCs.

In the present study, we demonstrated that the expression levels of CD28 on $CD4^+CD28^+$ T cells were correlated with the rate of improvement (ΔDAS28ESR/ΔSDAI) following 24 weeks after ABT therapy. This indicated that, in the case of RA, expression levels of CD28 on $CD4^+CD28^+$ T cells were not only important for the activation of T cells, but may also be a target for ABT therapy. We recently reported that abatacept treatment reduces the proportion of circulating Tfh cells in RA patients (11). Moreover, this study showed that a significant reduction in the proportion of Tfh-like cells in $CD4^+CD28^+$ cells, but not in $CD4^+CD28^-$ cells, was observed following 24 weeks after ABT therapy. We further performed extensive analysis for markers of therapy response *i.e.* correlation of reduction of Tfh-like cells and ABT therapy response. However, we could not find any association. Our preliminary data showed that the proportion of $CD4^+CD28^+CXCR5^+$ Tfh-like cells was significantly high in ACPA positive patients; however that did not correlate with disease activity score (Supplementary Fig. 2). As reported previously, ABT significantly decreased RF and ACPA levels (28). Although we propose that ABT appears to preferentially inhibit the differentiation or proliferation of Tfh-like cells in $CD4^+CD28^+$ cells, further studies need to confirm whether ABT targets Tfh cells accompanied by a concomitant reduction in levels of ACPA. On the other hand, CD28-dependent signaling is required for optimal T follicular helper cell maturation and expansion, and its inhibition prevents loss of self-tolerance in a mouse model (10). Although Tfh cells express other surface markers such as PD-1 and ICOS, our findings suggested that the expression of CD28 and the differentiation stage may be a predictive factor for directing therapy. Although ABT therapy improved disease activity, there might be the possibility of type 2 errors regarding the possible predictive factors, because the number of patients in remission after ABT therapy was very small (SDAI: 4, DAS: 5). We may need to increase the number of patients to prove the significance of our findings in future in-depth studies. Despite the limited number of samples, it should be noted that ABT therapy decreased $CD4^+CXCR5^+$ Tfh-like cells.

In this study, we learned that there was a significantly higher amount of $CD4^+CD28^-$ cells in patients who failed to achieve remission following 24 weeks after ABT therapy than in patients who did achieve remission. Scarsi *et al*. have previous reported that patients having low baseline numbers of $CD8^+CD28^-$ T cells had a more than 4-fold higher probability of achieving remission by ABT within 6 months than patients with higher levels of these cells (29). In addition to that, we demonstrated for the first time that $CD4^+CD28^-$ cells associated with response to ABT therapy. On the basis of previous reports, the

synovial fluid and peripheral blood of RA patients contains an increased proportion of T cells not expressing the co-stimulatory molecule CD28 (31, 32). $CD4^+CD28^-$ cells infiltrate muscle tissue in dermatomyositis patients and brain cells in multiple sclerosis patients (33, 34). Thus, $CD4^+CD28^-$ T cells in peripheral blood might also reflect the pathogenesis of RA, because the expansion of $CD4^+CD28^-$ T cells in peripheral blood positively correlates with the presence of extra-articular manifestations (35). We speculated that the loss of CD28 expression could not be affected by ABT therapy. $CD4^+CD28^-$ T cells are less susceptible to suppression by $CD4^+CD25^{high}$ regulatory T cells than conventional $CD4^+$ T cells (36).

We further confirmed that $CD4^+CD28^-$ cells were composed of effector memory or effector cells, and that no change was observed in their proportion at 24 weeks after ABT therapy. This indicated that $CD4^+CD28^-$ cells were not associated with disease activity or autoantibody production, and therefore, they were not targets for ABT therapy. $CD4^+CD28^-$ cells contain Tem cells, which lack of CCR7 expression, and are located in circulating peripheral tissue or blood (37). Tem cells show an immediate effector function of maintaining preformed cytotoxic granules for rapid cytolysis of infected host cells (38). They play a pathogenic role in the immune disorders of RA. Costimulatory molecules such as CD28-CD80/CD86 and CD40-CD40L are essential for T cell activation, and lack of costimulatory signaling leads to T cell dysfunction (39, 40). Yet, even if CD28 is absent on T cells, they are not anergic and receive costimulatory signals via APCs (41). We and other research demonstrated that crosslinking of CD3 and β1 integrin not only mediates adhesion, but also provides costimulatory signals to T cells (42, 43). Furthermore, previous studies have shown that $CD4^+CD28^-$ cells are resistant to apoptosis after IL-2 deprivation and activation which is associated with the increased expression of Bcl-2 (44) and Fas-associated death domain-like IL-1-converting enzyme inhibitory protein (45). The functional role of $CD4^+CD28^-$ cells is still a topic for future research.

This study has a number of limitations. First, the ages of the RA patients and those of the healthy subjects did not match. Because the $CD28^-CD4^+$ T cell count in the peripheral blood has previously been reported to be higher in the elderly (30), our findings showing an increased $CD28^-CD4^+$ T cell count in patients with RA may have indirectly reflected the age difference between HC and RA. However, among both RA patients and healthy subjects who were included in our analyses (as well as among all participants included in both groups), the age did not correlate with the proportion of $CD28^-CD4^+$ T cells (Supplementary Fig. 3). Similarly, the age did not correlate with the proportion of $CXCR5^+CD4^+$ T cells (Supplementary Fig. 3). Therefore, future studies will need to be carried out in age-matched groups in order to determine whether the number of $CD28^-CD4^+$ T cells and $CXCR5^+CD4^+$ T cells increase in a specific manner in RA. However, the finding of the influence of differences in CD28 expression on the responsiveness to abatacept therapy is the most important finding in this study. The low proportion of $CXCR5^+CD4^+$ T cells was another limitation. Compared to the proportion of $CXCR5^+CD4^+$ T cells in healthy subjects (mean value: approximately 8%) in a study of juvenile dermatomyositis previously reported by Morita *et al*. (19), the proportion of $CXCR5^+CD4^+$ T cells found in the healthy subjects examined in our study was extremely low (mean value: approximately 0.8%). Meanwhile, similar to our findings, the proportions of $CXCR5^+CD4^+$ T cells were also extremely low (mean value: approximately 1%) in a study previously reported by Ma *et al*. (46). The differences between each of the aforementioned reports may have been associated with technical issues (differences in reagents and experimental conditions), age differences, and racial differences.

Taken together, $CD4^+CD28^+CXCR5^+$ Tfh-like cells in the peripheral blood of RA patients are considered to be targets for ABT therapy. Conversely, $CD4^+CD28^-$ cells, which do not contribute to autoantibody production or disease activity, are primarily composed of terminally differentiated effector Th1-like cells and are potentially predictive of treatment resistance. Thus, both quantitative and qualitative analyses of T helper cells may be useful in understanding the pathology of RA and predicting the therapeutic effects of ABT.

Acknowledgements

The author thanks all the medical staff in all the institutions for providing the study data. The authors also thank Ms. T. Adachi and Ms. N. Sakaguchi for the excellent technical assistance.

References

1. FIRESTEIN GS: Evolving concepts of rheumatoid arthritis. *Nature* 2003; 423: 356-61.
2. BETTELLI E, OUKKA M, KUCHROO VK: T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 2007; 8: 345-50.
3. MCGEACHY MJ, CUA DJ: Th17 cell differentiation: the long and winding road. *Immunity* 2008; 28: 445-53.
4. CHABAUD M, DURAND JM, BUCHS N *et al*.: Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum* 1999; 42: 963-70.
5. KOTAKE S, UDAGAWA N, TAKAHASHI N *et al*.: IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* 1999; 103: 1345-52.
6. CROTTY S: Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011; 29: 621-63.
7. FAZILLEAU N, MARK L, MCHEYZER-WILLIAMS LJ, MCHEYZER-WILLIAMS MG: Follicular helper T cells: lineage and location. *Immunity* 2009; 30: 324-35.
8. ROBERTS CA, DICKINSON AK, TAAMS LS: The interplay between monocytes/macrophages and CD4 (+) T cell subsets in rheumatoid arthritis. *Front Immunol* 2015; 6: 571.
9. MARTIN MOLA E, BALSA A, MARTINEZ TABOADA V *et al*.: Abatacept use in rheumatoid arthritis: evidence review and recommendations. *Reumatol Clin* 2013; 9: 5-17.
10. PLATT AM, GIBSON VB, PATAKAS A *et al*.: Abatacept limits breach of self-tolerance in a murine model of arthritis via effects on the generation of T follicular helper cells. *J Immunol* 2010; 185: 1558-67.
11. IWATA S, NAKAYAMADA S, FUKUYO S *et al*.: Activation of Syk in peripheral blood B cells in patients with rheumatoid arthritis: a potential target for abatacept therapy. *Arthritis Rheumatol* 2015; 67: 63-73.
12. KUBO S, NAKANO K, NAKAYAMADA S *et al*.: Clinical, radiographic and functional efficacy of abatacept in routine care for rheumatoid arthritis patients: Abatacept Leading Trial for RA on Imaging Remission (ALTAIR) study. *Clin Exp Rheumatol* 2016; 34: 834-41.

13. NÜSSLEIN HG, ALTEN R, GALEAZZI M *et al*.: Efficacy and prognostic factors of treatment retention with intravenous abatacept for rheumatoid arthritis: 24-month results from an international, prospective, real-world study. *Clin Exp Rheumatol* 2016; 34: 489-99.
14. MIOSSEC P, VAN DEN BERG W: Th1/Th2 cytokine balance in arthritis. *Arthritis Rheum* 1997; 40: 2105-15.
15. LIU R, WU Q, SU D *et al*.: A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. *Arthritis Res Ther* 2012; 14: R255.
16. NAKAYAMADA S, KANNO Y, TAKAHASHI H *et al*.: Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity* 2011; 35: 919-31.
17. MURPHY KM, REINER SL: The lineage decisions of helper T cells. *Nat Rev Immunol* 2002; 2: 933-44.
18. GROOM JR, LUSTER AD: CXCR3 in T cell function. *Exp Cell Res* 2011; 317: 620-31.
19. MORITA R, SCHMITT N, BENTEBIBEL SE *et al*.: Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011; 34: 108-21.
20. KING C: New insights into the differentiation and function of T follicular helper cells. *Nat Rev Immunol* 2009; 9: 757-66.
21. KIM CH, ROTT LS, CLARK-LEWIS I, CAMPBELL DJ, WU L, BUTCHER EC: Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* 2001; 193: 1373-81.
22. YU D, RAO S, TSAI LM *et al*.: The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 2009; 31: 457-68.
23. HE J, TSAI LM, LEONG YA *et al*.: Circulating precursor CCR7(lo) PD-1(hi) CXCR5$^+$ CD4$^+$ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity* 2013; 39: 770-81.
24. NURIEVA RI, CHUNG Y, MARTINEZ GJ *et al*.: Bcl6 mediates the development of T follicular helper cells. *Science* 2009; 325: 1001-5.
25. JOHNSTON RJ, POHOLEK AC, DITORO D *et al*.: Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 2009; 325: 1006-10.
26. LINTERMAN MA, RIGBY RJ, WONG RK *et al*.: Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* 2009; 206: 561-76.
27. BOZEC A, ZAISS MM, KAGWIRIA R *et al*.: T cell costimulation molecules CD80/86 inhibit osteoclast differentiation by inducing the IDO/tryptophan pathway. *Sci Transl Med* 2014; 6: 235.
28. SCARSI M, PAOLINI L, RICOTTA D *et al*.: Abatacept reduces levels of switched memory B cells, autoantibodies, and immunoglobulins in patients with rheumatoid arthritis. *J Rheumatol* 2014; 41: 666-72.
29. SCARSI M, ZIQLIOLI T, ARIO' P: Baseline numbers of circulating CD28-negative T cells may predict clinical response to abatacept in patients with rheumatoid arthritis. *J Rheumatol* 2011; 38: 2105-11.
30. VALLEJO AN, NESTEL AR, SCHIRMER M, WEYAND CM, GORONZY JJ: Aging-related deficiency of CD28 expression in CD4$^+$ T cells is associated with the loss of gene-specific nuclear factor binding activity. *J Biol Chem* 1998; 273: 8119-29.
31. WARRINGTON KJ, TAKEMURA S, GORONZY JJ, WEYAND CM: CD4$^+$,CD28- T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. *Arthritis Rheum* 2001; 44: 13-20.
32. PAWLIK A, OSTANEK L, BRZOSKO I *et al*.: The expansion of CD4$^+$CD28$^-$ T cells in patients with rheumatoid arthritis. *Arthritis Res Ther* 2003; 5: R210-3.
33. FASTH AE, DASTMALCHI M, RAHBAR A *et al*.: T cell infiltrates in the muscles of patients with dermatomyositis and polymyositis are dominated by CD28 null T cells. *J Immunol* 2009; 183: 4792-9.
34. BROUX B, PANNEMANS K, ZHANG X *et al*.: CX(3)CR1 drives cytotoxic CD4$^{(+)}$CD28$^{(-)}$ T cells into the brain of multiple sclerosis patients. *J Autoimmun* 2012; 38: 10-9.
35. MARTENS PB, GORONZY JJ, SCHAID D, WEYAND CM: Expansion of unusual CD4$^+$ T cells in severe rheumatoid arthritis. *Arthritis Rheum* 1997; 40: 1106-14.
36. THEWISSEN M, SOMERS V, HELLINGS N, FRAUSSEN J, DAMOISEAUX J, STINISSEN P: CD4$^+$CD28null T cells in autoimmune disease: pathogenic features and decreased susceptibility to immunoregulation. *J Immunol* 2007; 179: 6514-23.
37. WOODLAND DL, KOHLMEIER JE: Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat Rev Immunol* 2009; 9: 153-61.
38. SALLUSTO F, LANZAVECCHIA A, ARAKI K, AHMED R: From vaccines to memory and back. *Immunity* 2010; 33: 451-63.
39. LENSCHOW DJ, WALUNAS TL, BLUESTONE JA: CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 1996; 14: 233-58.
40. POLLARD KM, ARNUSH M, HULTMAN P, KONO DH: Costimulation requirements of induced murine systemic autoimmune disease. *J Immunol* 2004; 173: 5880-7.
41. SCHMIDT D, GORONZY JJ, WEYAND CM: CD4$^+$ CD7$^-$ CD28$^-$ T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* 1996; 97: 2027-37.
42. KAMIGUCHI K, TACHIBANA K, IWATA S, OHASHI Y, MORIMOTO C: Cas-L is required for β_1 integrin-mediated costimulation in human T cells. *J Immunol* 1999; 163: 563-8.
43. NAKAYAMADA S, SAITO K, NAKANO K, TANAKA Y: Activation signal transduction by β1 integrin in T cells from patients with systemic lupus erythematosus. *Arthritis Rheum* 2007; 56: 1559-68.
44. SCHIRMER M, VALLEJO AN, WEYAND CM, GORONZY JJ: Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4$^+$CD28$^-$ T cells from rheumatoid arthritis patients. *J Immunol* 1998; 161: 1018-25.
45. VALLEJO AN, SCHIRMER M, WEYAND CM, GORONZY JJ: Clonality and longevity of CD4$^+$ CD28null T cells are associated with defects in apoptotic pathways. *J Immunol* 2000; 165: 6301-7.
46. MA J, ZHU C, MA B *et al*.: Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. *Clin Dev Immunol* 2012; 2012: 827480.