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CD45RA–Foxp3^{high} activated/effector regulatory T cells in the CCR7 + CD45RA–CD27 + CD28 + central memory subset are decreased in peripheral blood from patients with rheumatoid arthritis

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ABSTRACT

Human CD4+ T cells can be classified as either naïve, central memory (T_{CM}), or effector memory (T_{EM}) cells. To identify the CD4+ T cell subsets most important in the pathogenesis of rheumatoid arthritis (RA), we phenotypically defined human CD4+ T cells as functionally distinct subsets, and analyzed the distribution and characteristics of each subset in the peripheral blood. We classified CD4+ T cells into six novel subsets based on the expression of CD45RA, CCR7, CD27, and CD28. The CCR7 + CD45RA– CD27 + CD28+ T_{CM} subset comprised a significantly smaller proportion of CD4+ T cells in RA patients compared to healthy controls. The frequency of TNF-α-producing cells in the CCR7–CD45RA– CD27 + CD28+ T_{EM} subset was significantly increased in RA. Furthermore, within the CCR7 + CD45RA– CD27 + CD28+ T_{CM} subset, which was decreased in peripheral blood from RA, the proportions of total Foxp3+ Treg cells and CD45RA–Foxp3^{high} activated/effector Treg cells were significantly lower in RA patients. Our findings suggest that the increased proportion of TNF-α-producing cells and the decreased proportion of CD45RA–Foxp3^{high} activated/effector Treg cells in particular subsets may have critical roles in the pathogenesis of RA.

1. Introduction

Rheumatoid arthritis (RA) is characterized by synovial membrane hyperplasia and infiltration by inflammatory cells, including activated T cells. T cells contribute to the initiation and perpetuation of the inflammation underlying RA, leading to joint destruction and disability. Activated T cells proliferate and induce monocytes, macrophages, and synovial fibroblasts to produce pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, and stimulate osteoclastogenesis, matrix metalloproteinase secretion, and immunoglobulin production by B cells.

Human T cells can be divided into functionally distinct subsets. Two primary categories are T cells that have not been exposed to antigen (naïve) and those that are antigen-experienced (memory). The memory subpopulation is a heterogeneous pool, and previous studies using two surface markers, CCR7 and CD45RA, led to the following proposed phenotypic classification of human T cells: naïve T cells as CCR7 + CD45RA+, central memory T cells (T_{CM}) as CCR7 + CD45RA– and effector memory T cells (T_{EM}) as CCR7–CD45RA– [1–3]. Recent reports suggest that this phenotypic classification of T cells is useful for investigating human autoimmune diseases such as RA [4], systemic lupus erythematosus (SLE) [5], and granulomatosis with polyangiitis (Wegener's granulomatosis) [6].

The phenotypic subdivisions of human CD4+ T cell subsets are less studied than those of CD8+ T cells. CD4 + CCR7 + CD45RA– T_{CM} cells have been shown to produce IL-2, and CD4 + CCR7–CD45RA– T_{EM} cells to predominantly produce IFN-γ, IL-4, and TNF-α, although a small population of CD4 + CCR7–CD45RA– T_{EM} cells produces IL-2. In addition to CD45RA and CCR7, CD27 and CD28 have been used as surface markers to characterize CD4+ T cells. Appay et al. established a model of CD4+ T cell differentiation characterized by a sequential down-regulation of CCR7, CD27 and then CD28, accompanied by changes of their functional characteristics [7–9]. CD27 and CD28 are the main costimulatory molecules required to promote T cell activation, although memory T cells

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seem to be less dependent on CD27 and CD28 for their reactivation than naive T cells [10,11]. CD4+ T cells gain cytotoxic potential with the acquisition of lytic granules with granzymes, as they lose CD27 expression, and acquisition of perforin at the CD28 negative stage, so that highly differentiated CD4+ T cells become cytotoxic. With the down-regulation of CD27 and CD28, CD4+ T cells exhibit higher IFN-γ-producing ability and shorter telomere length [12–14]. Although most studies have used either CD27 or CD28 in conjunction with CD45RA and CCR7 to define the CD4+ T cell subsets, we recently discriminated CD4+ T cells into five major subsets using all four markers, CD45RA, CCR7, CD27, and CD28. We defined the function of each population based on its ability to produce IFN-γ, IL-4, and IL-2, and proposed that this phenotypic characterization would be useful in the study of immunological diseases [15,16].

Human regulatory T cells (Treg cells) play an indispensable role for the maintenance of self tolerance and immune homeostasis [17]. Quantitative and/or qualitative deficiencies in Treg cells could lead to the development of autoimmune diseases [18–20]. Foxp3 is a key transcription factor for the development and function of natural CD4+ Treg cells. However, recent studies have shown that human Foxp3 + CD4+ T cells are not homogeneous in gene expression, phenotype and suppressive function. Miyara et al. revealed that human Foxp3 + CD4+ T cells can be separated into three functionally and phenotypically unique subpopulations, based on the expression of Foxp3 and their cell surface phenotype. The three distinct subpopulations are as follows: (fraction (Fr.) I) CD45RA + Foxp3^{low} naïve Treg cells; (Fr. II) CD45RA–Foxp3^{high} activated/effector Treg cells, both of which are suppressive *in vitro*; and (Fr. III) non-suppressive cytokine-secreting CD45RA–Foxp3^{low} non-Treg cells. Terminally differentiated CD45RA–Foxp3^{high} activated/effector Treg cells rapidly died whereas CD45RA + Foxp3^{low} naïve Treg cells proliferated and converted into activated/effector Treg cells *in vitro* and *in vivo*. Recent studies report the proportion of the three subpopulations differed among patients with immunological diseases such as SLE and sarcoidosis [21–23].

In the present study, we phenotypically classified human peripheral blood CD4+ T cells into six novel functionally distinct major subsets using the four cell-surface markers CD45RA, CCR7, CD27 and CD28, and characterized the pro-inflammatory and regulatory characteristics of each subset. In addition, we classified Foxp3 + CD4+ T cells into three functionally subpopulations and studied their distribution in peripheral blood from RA patients.

2. Materials and methods

2.1. Sample collection

This study was approved by the ethics committee at the Kobe University Graduate School of Medicine. The human samples were used in accordance with the guidelines of Kobe University Hospital, and written informed consent was obtained from all subjects. The blood samples were obtained from patients with RA (n = 32) and age- and sex-matched healthy volunteers (n = 19) at Kobe University Hospital. The clinical characteristics of the RA patients are summarized in Table 1. All the patients met the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 classification criteria for RA, and none of them had any complicating diseases.

2.2. Five-color flow cytometric analysis of human CD4+ T cell subsets

The peripheral blood mononuclear cells (PBMCs) were purified from the peripheral blood of RA patients and healthy subjects by using Ficoll-Paque solution (GE Healthcare, Uppsala, Sweden). After the cells were washed with Flow Cytometry Staining Buffer (eBioscience), they were stained with anti-CD27 (BioLegend), anti-CD28 (Beckman Coulter), anti-CD45RA, anti-CCR7 (BD Biosciences), and anti-CD4 mAbs for 30 min at 4 °C. The percentage of each subset was determined using a Moflo™ cell sorter (Beckman Coulter).

Table 1
Characteristics of the study population.

RA patients	
N (male/female)	32 (6/26)
Median age (range)	46 ± 6.7 (37 – 60)
Years with disease (range)	7.1 ± 7.6 (0.5 – 38)
DAS28 average score (range)	3.1 ± 1.3 (1.3 – 6.2)
Medication	
Prednisolone *N* (dose; average ± SD/range)	7 (6.2 ± 3.5 mg/0.5 mg – 10 mg)
Methotrexate *N* (dose; average ± SD/range)	16 (7.8 ± 1.5 mg/4 mg – 10 mg)
Other DMARDs	22
Anti-TNF drug	11
Abatacept	1
Healthy volunteers	
N (male/female)	19 (3/16)
Median age (range)	43 ± 8.8 (29 – 60)

N; number.

2.3. Eight-color flow cytometric analysis for cytokine production by human CD4+ T cell subsets stimulated with PMA/ionomycin

For cytokines analysis, PBMCs were cultured with PMA/ionomycin (final concentrations: PMA 10 μg/ml, ionomycin 1 μg/ml), and then breferdin A (10 μg/ml) was added for the subsequent 4 h. Thereafter, the cells were stained with mAbs described above. The cells were fixed and permeabilized with Fixation and Permeabilization Buffer (eBioscience), stained with anti-IFN-γ (BioLegend), anti-IL-17 (eBioscience) and anti-TNF-α (BD Biosciences) mAbs, and analyzed using the Moflo™ cell sorter.

2.4. Six-color flow cytometric analysis for Foxp3 expression by human CD4+ T cell subsets

For analysis of Foxp3 expression, PBMCs were stained with the five mAbs described above. The surface-stained cells were fixed and permeabilized with FOXP3 Fix/Perm Buffer (Biolegend), and stained with anti-Foxp3 mAbs (BioLegend). The cells were analyzed using the Moflo™ cell sorter.

2.5. Statistical analysis

Statistical analysis was accomplished by Mann–Whitney *U*-test using the GraphPad Prism ver 5 software. Values of $P < 0.05$ were considered significant.In the boxplots, the box represents the interquartile range, the line across the box is the median and the whiskers represent the 5th and 95th percentiles.

3. Results

3.1. The CCR7+CD45RA–CD27+CD28+ T_{CM} subset is significantly decreased in the peripheral blood CD4+ T cells from RA patients

We classified the peripheral blood CD4+ T cells from healthy subjects and RA patients into the six subsets by four cell-surface markers, CD45RA, CCR7, CD27, and CD28. Flow cytometry analysis revealed six major populations of human CD4+ T cells (Fig. 1A). The CCR7 + CD45RA–CD27–CD28+ T_{CM} subset was not studied previously, because it was considered as a minor population containing few cells [15]. However, we found that 5% of the CD4+ T cells belonged to this subset (Fig. 1A). We therefore considered the

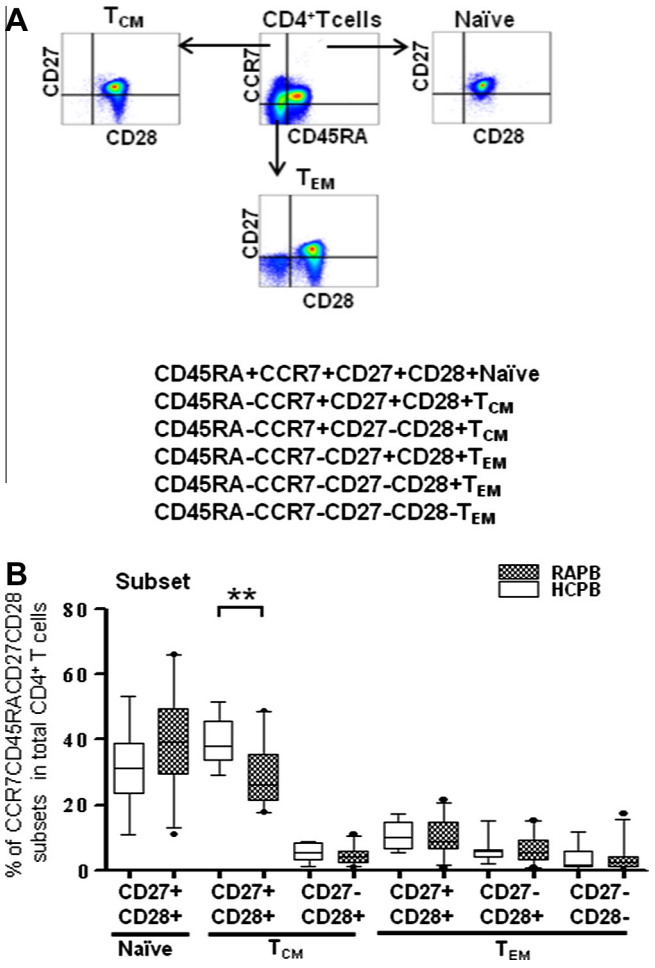

Fig. 1. The CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset is significantly decreased in peripheral blood from RA patients. (A) Peripheral blood CD4+ T cells from healthy subjects were classified into six major populations using CD45RA, CCR7, CD27, and CD28. Representative flow cytometry results are shown. (B) The distribution of the CD4+ T cell subsets was studied in RA patients (RA) and healthy controls (HC) (n = 23 in RA, n = 11 in HC). $^{*}P < 0.05$; $^{**}P < 0.01$.

CCR7 + CD45RA–CD27–CD28+ T_{CM} subset as a major population, and carried out the functional analysis for the six subsets. On the other hand, we did not perform the functional analysis of the CCR7–CD45RA– T_{EM} subset in this study because we have shown that this subset was rarely found in the Japanese population in our previous report [15].

We then compared the distribution of the six subsets between healthy subjects and RA patients (HCPB and RAPB). We found that the proportion of cells belonging to the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset was significantly decreased in RA patients compared to healthy controls (Fig. 1B); CD45RA + CCR7 + CD27 + CD28+ Naïve (Mean ± SD, RA; 39.6 ± 14.5%, HC; 32.2 ± 12.8%), CCR7 + CD45RA–CD27 + CD28+ T_{CM} (RA; 29.3 ± 10.9%, HC; 38.9 ± 7.5%), CCR7 + CD45RA–CD27–CD28+ T_{CM} (RA; 4.4 ± 2.9%, HC; 5.4 ± 2.6%), CCR7–CD45RA–CD27 + CD28+ T_{EM} (RA; 10.2 ± 3.7%, HC; 10.9 ± 4.1%), CCR7–CD45RA–CD27–CD28+ T_{EM} (RA; 6.4 ± 3%, HC; 5.8 ± 3.6%), and CCR7–CD45RA–CD27–CD28– T_{EM} (RA; 3.2 ± 1.7%, HC; 3.2 ± 3.7%).

3.2. Phenotypic characterization of the six subsets classified by four surface markers

To clarify the pro-inflammatory and regulatory characteristics of each subset, we next investigated the percentages of pro-inflammatory cytokine (IFN-γ, IL-17, and TNF-α)-producing cells and of Foxp3+ cells using eight-color flow cytometry. We found that the CCR7 + CD45RA–CD27–CD28+ T_{CM} and CCR7–CD45RA–CD27–CD28+ T_{EM} populations included more cells positive for pro-inflammatory cytokines (IFN-γ, IL-17, and TNF-α) than the other subsets. The CCR7–CD45RA–CD27–CD28– T_{EM} subset had few IL-17-producing cells, but contained moderate numbers of IFN-γ- and TNF-α-positive cells (Fig. 2A and Additional file 1). Consistent with a previous report [24], only a very small number of Foxp3+ cells were observed in the CD27-negative subsets (CCR7 + CD45RA–CD27–CD28+ T_{CM}, CCR7–CD45RA–CD27–CD28+ T_{EM}, and CCR7–CD45RA–CD27–CD28– T_{EM}). These results suggest that particular phenotypically defined CD4+ T cell subsets play an important role in the inflammatory cytokine production or regulatory function of CD4+ T cells.

3.3. The frequency of Foxp3+ cells in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset was decreased, while that of TNF-α-producing cells in the CCR7–CD45RA–CD27 + CD28+ T_{EM} subset was increased in peripheral blood from RA patients.

Then we compared the distribution of cytokine-producing and Foxp3+ cells in the six subsets among all the CD4+ T cells between RA patients and healthy subjects. Since naïve and the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset comprise the largest populations in peripheral blood (Fig. 1A and B), the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset contained the most inflammatory cytokine-producing cells in total CD4+ T cells (Fig. 2B). Although CCR7–CD45RA– T_{EM} cells have a tendency to produce more inflammatory cytokines (Fig. 2A and Additional file 1), they are not the major cytokine-producing subsets in total CD4+ T cells. We demonstrated that the proportion of TNF-α-producing cells in the CCR7–CD45RA–CD27 + CD28+ T_{EM} subset was significantly higher in RA patients. Moreover, the frequency of Foxp3+ cells in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} population was significantly decreased in peripheral blood from RA patients (Fig. 2B). These results suggest that the increased proportion of inflammatory cytokine-positive CD4+ T cells and the decreased proportion of regulatory T cells in particular subsets may have critical roles in the pathogenesis of RA.

3.4. The frequency of activated/effector Treg cells in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset was decreased in the peripheral blood CD4+ T cell subsets from RA patients.

Miyara et al. have recently shown that human Foxp3 + CD4+ Treg cells can be separated into three functionally and phenotypically unique subpopulations based on the expression of Foxp3 and CD45RA; CD45RA + $Foxp3^{low}$, naïve Treg cells (Fr. I), CD45RA–$Foxp3^{high}$, activated/effector Treg cells (Fr. II), and CD45RA–$Foxp3^{low}$, non-Treg cells (Fr. III) [21].

We classified human peripheral blood CD4+ T cells from RA patients and healthy controls using CD45RA and Foxp3 (Fig. 3A). The frequency of CD45RA–$Foxp3^{high}$ activated/effector Treg cells (Fr. II) was significantly decreased in the peripheral blood CD4+ T cells from RA patients (Mean ± SD, RA; 1 ± 0.9%, HC; 2.1 ± 1.8%) (Fig. 3B). As a result of the decrease of Fr. II, more than half of the Foxp3+ Treg cells were the CD45RA–$Foxp3^{low}$, non-Treg cells (Fr. III) in peripheral blood from RA patients. Furthermore, the frequency of CD45RA−$Foxp3^{high}$ activated/effector Treg cells (Fr. II) in the CCR7 + CD45RA–CD27 + CD28 + T_{CM} subset was significantly decreased in RA patients (Mean ± SD, RA; 0.4 ± 0.4%, HC; 1.3 ± 1.1%) (Fig. 3B).

We next investigated the correlation of the frequency of each Foxp3+ T cell fraction (Fr. I, II, and III) in peripheral blood CD4+ T cells with the clinical disease activity score for 28 joints (DAS28) in RA patients. We found that the percentage of CD45RA + $Foxp3^{low}$ naïve Treg cells (Fr. I) was negatively correlated with DAS28-CRP (Fig. 3C).

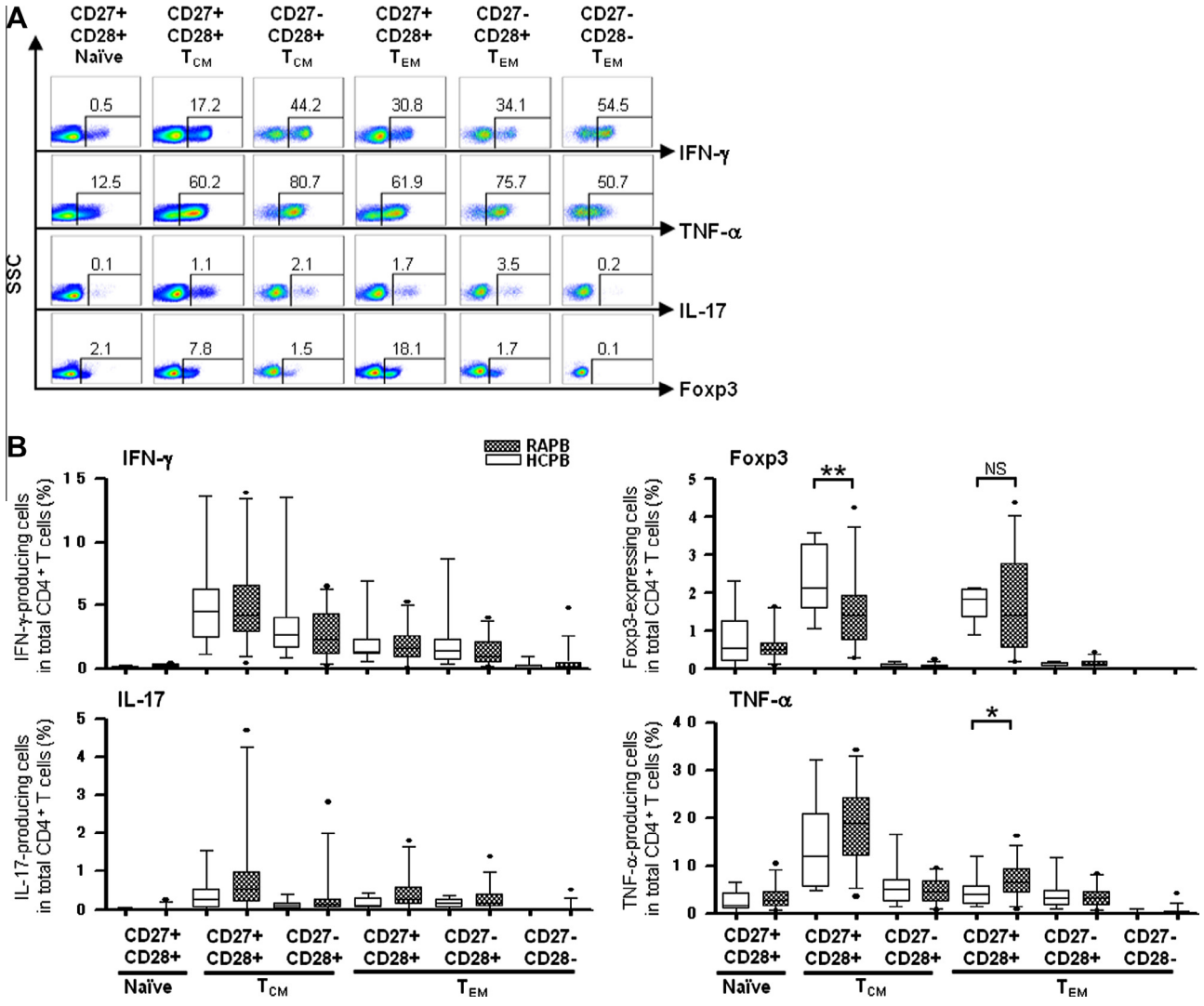

Fig. 2. Pro-inflammatory and regulatory characteristics of the six subsets. (A) The frequency of IFN-γ-, IL-17-, or TNF-α-producing cells, and of Foxp3+ cells in each subset was analyzed by flow cytometry. Representative flow cytometry results are shown. (B) The frequency of pro-inflammatory cytokine-producing cells, and of Foxp3+ cells among all the CD4+ T cells in individuals was analyzed in RA and HC ($n = 27$ in RA, $n = 15$ in HC). $^{*}P < 0.05$; $^{**}P < 0.01$; NS, not significant.

4. Discussion

The impaired function of Treg cells has been implicated in the development of autoimmune diseases, including RA [25]. Numerous studies dealt with Treg population in RA with various conclusions regarding the frequency of circulating Treg cells. Most studies reported decreased [26,27] or normal proportions [28,29] of Treg cells, whereas some other groups reported an increase [30,31]. Maldonado et al. reported that the CD45RA–CD62L + CD4+ T_{CM} subset was significantly increased in RA patients [4]. These conflicting results may be due to differences in race, disease activity, detection markers, gating strategies or parameters [32,33]. Heterogeneity of Treg cells could be an another cause. Although most previous reports defined Treg cells as Foxp3+ or CD4+/CD25+ cells, substantial evidence revealed that Foxp3+ and CD4+/CD25+ Treg cells represent a heterogeneous population, which can be divided into several distinct subsets with unique functional properties [34].

We found that, among peripheral blood CD4+ T cells, Foxp3 was almost exclusively expressed on the CCR7 + CD45RA–CD27 + CD28+ T_{CM} and CCR7–CD45RA–CD27 + CD28+ T_{EM} subsets, while the other subsets contained only a small percentage of Foxp3+ cells. Treg cells use multiple mechanisms to inhibit dendritic cell (DC) function and block the initiation of autoimmunity. The selective deletion of IL-10 in Treg cells in mice results in the development of spontaneous colitis and exaggerated immune responses at other environmental interfaces, such as the skin and lungs; however, these animals do not develop systemic autoimmunity [35]. In contrast, the loss of CTLA4 expression in Treg cells in mice results in severe systemic autoimmune disease characterized by massive lymphadenopathy and splenomegaly [36]. Therefore, Treg-derived IL-10 is considered to be essential for immunoregulation in non-lymphoid, mucosal tissues, such as intestines, lungs, and skin. On the other hand, in lymph nodes, Treg cells can inhibit the priming of effector T cells by preventing DC maturation, and by blocking the initiation of systemic autoimmunity through CTLA4-dependent mechanisms [35]. Notably, we showed in this study that most of the Foxp3+ Treg cells could be classified into two groups, the CCR7 + CD45RA–CD27 + CD28+ T_{CM} and CCR7–CD45RA–CD27 + CD28+ T_{EM}, and that the number and frequency of Foxp3+ Treg cells in the CCR7 + CD45RA– T_{CM} population were significantly decreased in RA patients; in contrast the Foxp3+ Treg cells in the CCR7–CD45RA– T_{EM} population were similar between RA patients and healthy controls. Therefore, the

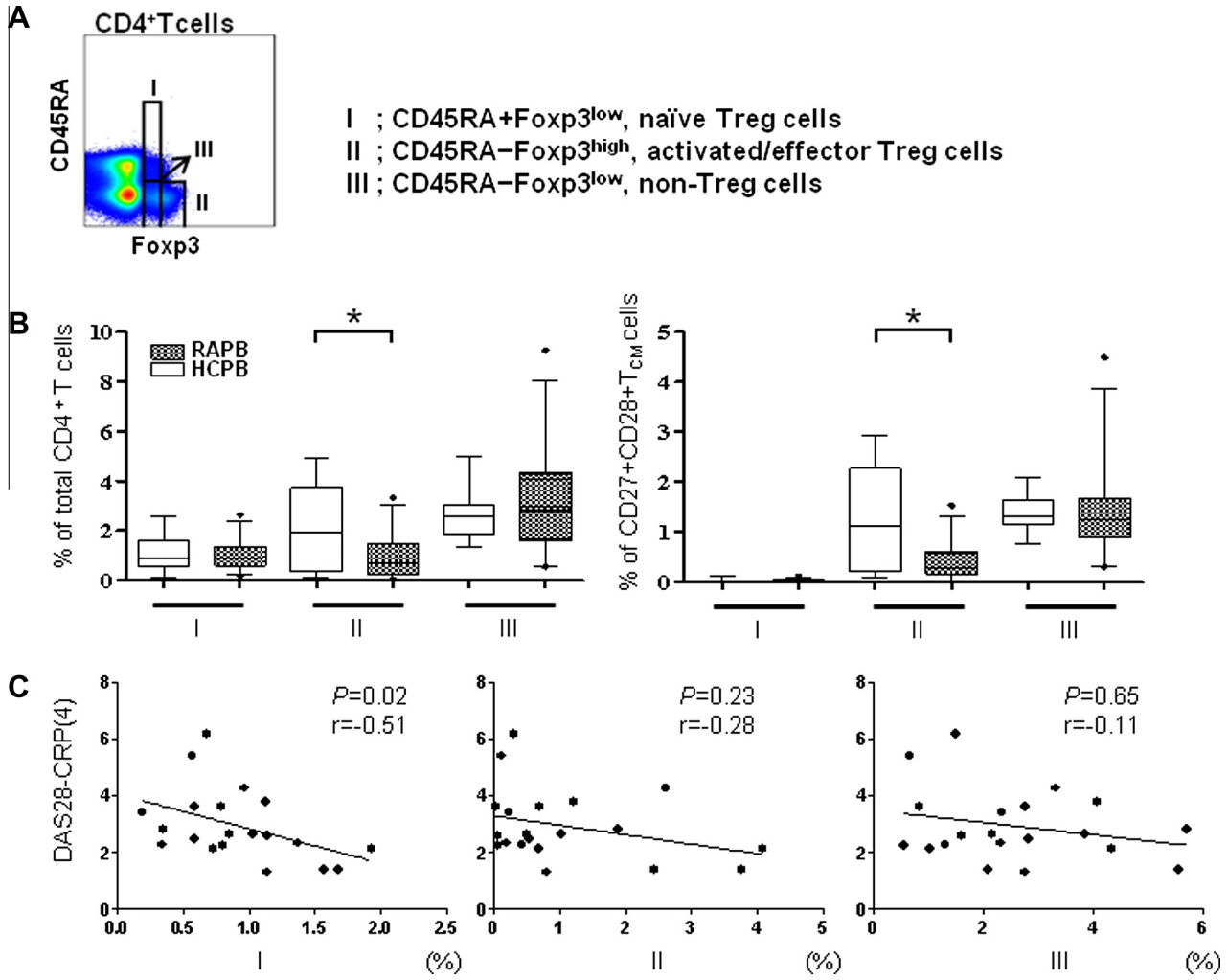

Fig. 3. The frequency of CD45RA–Foxp3^{high} effector/activated Treg cells in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset was decreased in peripheral blood from RA patients. (A) Peripheral blood (PB) CD4+ T cells from RA patients and HC were classified into three fractions using CD45RA and Foxp3. A representative flow cytometry result is shown. (B) The frequency of each Foxp3+ T cell fraction among all CD4+ T cells (left panel) and in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset (right panel) were analyzed in PB from RA patients and HC (n = 27 in RA, n = 15 in HC). (C) Correlation of the frequency of each Foxp3+ T cell fraction (Fr. I, II, and III) in PB CD4+ T cells with the disease activity score (DAS28) in RA patients. Correlation was established by calculating the Spearman's rank correlation coefficients. $^{*}P < 0.05$; $^{**}P < 0.01$.

reduced number of Foxp3+ Treg cells in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset, which resides mainly in the lymphoid tissues, may break down the immune tolerance and cause systemic autoimmunity in RA.

Miyara et al. recently proposed a novel classification approach for Foxp3+ Treg cells, and demonstrated that the proportion of the three subpopulations differed among patients with immunological diseases. They showed that patients with active sarcoidosis showed a considerable increase in the proportion of CD45RA–Foxp3^{high} activated/effector Treg cells among CD4+ T cells and a decrease in the proportions of CD45RA + Foxp3^{low} naïve Treg cells [21]. They also showed that SLE patients with active disease had a decreased proportion of CD45RA–Foxp3^{high} activated/effector Treg cells, and an increased proportion of CD45RA + Foxp3^{low} naïve Treg cells. A recent paper reported that the proportion of CD45RA–Foxp3^{high} activated/effector Treg among peripheral blood CD4+ T cells was decreased in patients with RA and Behçet's disease [23]. In this study, we demonstrated that the frequency of CD45RA–Foxp3^{high} activated/effector Treg cells was significantly decreased among CD4+ T cells and in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset in peripheral blood from RA pateints. Previous studies have revealed the capacity of TNF-α which plays critical roles in the pathogenesis of RA, to abrogate the suppressive function of Treg cells *in vitro* or to alter Foxp3 expression through their binding to TNF-receptor 2 expressed on Treg cells [37]. Thus, the increased production of TNF-α may contribute the decrease of CD45RA–Foxp3^{high} activated/effector Treg cells in RA patients.

We found the tendency that RA patients with high disease activity showed a decrease in CD45RA + Foxp3^{low} naïve Treg cells. A significant negative correlation was observed between the ratio of naïve Treg cells and DAS28. This result suggests that naïve Treg cells may have a protective role in the pathophysiology of RA.

In summary, we phenotypically classified human CD4+ T cells into six novel functionally distinct subsets and demonstrated that the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset was significantly decreased in the peripheral blood from RA patients compared with healthy controls. In addition, the proportion of TNF-α-producing cells in the CCR7–CD45RA–CD27 + CD28+ T_{EM} subset was significantly increased, and the proportion of Foxp3-positive cells in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset was significantly decreased in RA patients. Furthermore, the frequency of activated/effector Treg cells in the CCR7 + CD45RA–CD27 + CD28+ T_{CM} subset was significantly decreased in the peripheral blood CD4+ T cells. Our results suggest that the increased proportion of

TNF-α-producing cells and the decreased proportion of CD45RA–Foxp3^{high} activated/effector Treg cells in particular subsets may have critical roles in the pathogenesis of RA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.120.

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