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Title

CD4+ CD52lo T-cell expression contributes to the development of systemic lupus erythematosus

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CD4⁺ CD52lo T-cell expression contributes to the development of systemic lupus erythematosus☆☆☆

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1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease caused by a reduction of tolerance to nuclear antigens [1]. In SLE, multiple organs such as the kidneys, brain, and skin are injured by inappropriate immune responses, including auto-antibody production, auto-reactive T cells and B cells, auto-antigen presenting cells, and a local activation of the complement system [1]. If the treatment of SLE is inadequate, fatal complications can eventually occur.

CD52, also known as Campath-1, is a glycosylphosphatidylinositol (GPI)-anchored cell-surface glycoprotein that is widely expressed in lymphocytes, monocytes and eosinophils [2]. Changes in the quantity and/or quality of CD4⁺ CD52hi T cells are suggested to be involved in autoimmune disease [3]. CD4⁺ CD52lo T cells play a suppressor role, whereas CD4⁺ CD52lo T cells have an activator function. It has been reported that CD4⁺ CD52lo T cells are induced by glutamic acid decarboxylase (GAD) 65 or tetanus toxoid from healthy human peripheral blood mononuclear cells (PBMCs), and that the activated CD4⁺ CD52lo T cells can produce interferon-gamma (IFN-γ) [4].

Importantly, the production of inflammatory cytokines, including IFN-γ, was inhibited under a co-culture with CD4⁺ CD52lo and CD4⁺ CD52hi T cells through the soluble fusion protein of CD52 cleaved by cell-surface CD52 and the immunoglobulin crystallizable fragment (CD52-Fc) [4]. This soluble CD52 is cleaved by phospholipase C, which functions as a ligand of siglec-10 on CD4⁺ CD52lo T cells [4]. The signal of siglec-10 inhibits the phosphorylation of the T cell receptor-associated kinases Lck and Zap70 and T-cell activation [4].

Keywords: CD52, T cells, Systemic lupus erythematosus

⁎ The authors declare no conflict of interest.
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These findings indicate that an imbalance between CD4+ CD52hi T cells and CD4+ CD52lo T cells is involved in the pathogenesis of autoimmune diseases. However, the expression and function of these cells in SLE have not been clarified. In this study, we examined the expressions of CD4+ CD52hi and CD4+ CD52lo T cells in SLE patients, and we evaluated their underlying mechanism of action in the pathogenesis of SLE.

2. Materials and methods

2.1. Study design and patients

We analyzed blood samples from individuals treated at Nagasaki University Hospital (Nagasaki, Japan) and Sasebo Chuo Hospital (Sasebo, Japan) during a 4-year period from 2013 through 2016: 64 patients with SLE (51 female and 13 male; mean age 41.4 years, standard deviation [SD] 15.4 years), 23 patients with rheumatoid arthritis (RA) (18 female and 5 male; mean age 65.3 years, SD 10.1 years), and 33 healthy controls (HCs) (22 female and 11 male; mean age 29.2 years, SD 6.5 years). The demographic characteristics of the subjects are summarized in Table 1. The subjects included in the analysis between CD4+ CD52hi T cells, circulating follicular helper-like T cells (Tfh-like cells) and plasmablasts are shown separately in Suppl. Table S1. These subjects were included in the group of all subjects in Table 1.

All of the SLE patients fulfilled at least 4 of the 11 revised criteria of the American College of Rheumatology (ACR) [5]. Disease activity was assessed with the Safety of Estrogens in Lupus Erythematosus National Assessment-SLE Disease Activity Index (SELENA-SLEDAI) [6]. All of the RA patients fulfilled the 2010 RA classification criteria issued by the ACR/European League Against Rheumatism (EULAR) [7]. All of the patients and HCs gave their informed consent to participate in the study and have their data published, and the study was approved by the Institutional Review Board of Nagasaki University (approval no. 12012397-5).

2.2. Mice

Female MRL/Mp-Tnfrsf6fl/fl (MRL/lpr) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and sacriﬁced at the end of their 6th or 16th week of age. Prior to sacriﬁce, the mice were maintained in an SPF animal facility (Nagasaki University), and the experiments were approved by the Institutional Animal Care Committee of Nagasaki University (approval no. 1412261190-6).

Table 1

<table>
<thead>
<tr>
<th>Demographics and characteristics of the subjects.</th>
<th>SLE (n = 64)</th>
<th>RA (n = 23)</th>
<th>HC (n = 33)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Gender (female:male)</td>
<td>51:13</td>
<td>18:5</td>
<td>22:11</td>
<td>0.52</td>
</tr>
<tr>
<td>Age (yrs)</td>
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<td>65.3 ± 15.4</td>
<td>29.2 ± 15.4</td>
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<td>SELENA-SLEDAI</td>
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<td>14 (21.9%)</td>
<td>11.0 ± 10.6</td>
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<tr>
<td>untreated</td>
<td>14 (21.9%)</td>
<td>11.0 ± 10.6</td>
<td>6 (9.4%)</td>
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<tr>
<td>Prednisolone (mg/day)</td>
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<td>14 (21.9%)</td>
<td>11.0 ± 10.6</td>
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<tr>
<td>Immunosuppressive drugs</td>
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<td>11.0 ± 10.6</td>
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<td>Cyclosporine</td>
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<td>Lymphocyte counts (×109/L)</td>
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<td>Anti-ds DNA antibody (U/ml)</td>
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<td>C3 (mg/dl)</td>
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<td>C4 (mg/dl)</td>
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<tr>
<td>CH50 (U/ml)</td>
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<tr>
<td>IgG (mg/dl)</td>
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</tbody>
</table>

**SLE**: systemic lupus erythematosus; **RA**: rheumatoid arthritis; **HC**: healthy controls; **SELENA-SLEDAI**: Safety of Estrogen in Lupus Erythematosus National Assessment-SLE Disease Activity Index; **WBC**: white blood cells. Values are the number (%) of patients or the mean ± SD. Analyses were conducted by using Kruskal-Wallis test and Fisher’s exact test.

2.3. Flow cytometry

We isolated peripheral blood mononuclear cells (PBMCs) from the blood samples of patients and HCs using density-gradient centrifugation with a RosetteSep™ system (Stemcell Technologies, Grenoble, France). Isolated PBMCs were stained for flow cytometry with antibodies (Abs) against CD3 (SK7; BD Biosciences, San Diego, CA), CD4 (SK3; BD Biosciences), CD8a (HI38; Biologend, San Diego, CA), CD45 (H100; Biologend), CXCR5 (J252D4; Biologend), ICO5 (C98H4A; Biologend), PD-1 (EH12.2H7; Biologend), CD19 (HB19; Biologend), CD27 (O323; Biologend), CD38 (HIT2; Biologend), or CR7 (G043H7; Biologend) for 30 min at 4°C.

Spleens were excised from the mice by teasing the organs through a nylon mesh. Isolated cells were stained for flow cytometry with Abs against CD3 (17A2A; Biologend), CD4 (1GK1.5; Biologend), CD8a (53–67; Biologend) and CD52 (B-2G; MBL, Nagoya, Japan). Stained cells were analyzed by multiparameter flow cytometry using a FACS CANTO II (BD Biosciences) or FlowSight Imaging Flow Cytometer (Amnis Corp., Seattle, WA) and FlowJo ver. 10 software (Tree Star, Ashland, OR).

2.4. Cell sorting

Cell sorting was performed in a FACS Aria II cell sorter (BD Biosciences). PBMCs from SLE patients (n = 5) were isolated and then incubated with antibodies against CD3, CD4, CD8, CD45 and CD52 for 30 min at 4°C. Cells were sorted into two different populations: (1) CD3+ CD45+ CD4+ CD8− CD52hi cells and (2) CD3+ CD45+ CD4− CD8− CD52lo cells. The purity of the sorted cell populations ranged from 94% to 99%.

2.5. ELISA

Soluble CD52 in serum from the human blood samples was measured by a Human CAMPATH-1 Antigen (CD52) Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Cusabio, College Park, MD).

2.6. Gene microarray analysis

To reveal the genetic characteristics of CD4+ CD52lo T cells in SLE, we performed cDNA microarrays. CD4+ CD52lo T cells and CD4+ CD52hi T cells from five SLE patients were sorted by flow cytometry. The RNA was obtained from CD3+ CD45+ CD4+ CD8− CD52hi cells and CD3+ CD45+ CD4+ CD8− CD52lo T cells. Total cellular RNA was extracted using an RNasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations. RNA was quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). We performed the cDNA microarray using a SurePrint G3 Human GE 8x60K Microarray (Agilent Technologies). The in vitro transcription, oligonucleotide array hybridization, and scanning were conducted with Takara Bio protocols (TaKaRa Bio, Shiga, Japan). We conducted a scatter plot analysis by using GeneSpring GX ver. 7.3.1 software (Agilent Technologies). The data have been deposited in the Gene Expression Omnibus repository (GSE94815).

2.7. Analysis of the effects of ligands

CD4 T cells were isolated using CD4 RosetteSep™. The isolated CD4 T cells (1 × 106) in RPMI 1640 medium with 10% fetal bovine serum (HyClone, Logan, UT) plus penicillin/streptomycin were incubated for up to 24 h at 37°C (in 5% CO2) in 96-well round-bottomed plates with or without the following ligands: growth-related oncogene (GRO) (50 ng/ml; Peprotech, Rocky Hill, NJ), monocyte chemoattractant protein-1 (MCP-1) (20 ng/ml; Prospeck, East Brunswick, NJ), regulated
upon activation normal T-cell expressed and secreted (RANTES) (10 ng/ml; Peprotech), thymus and activation-regulated chemokine (TARC) (10 ng/ml; Novus Biologicals, Littleton, CO), and interleukin-8 (IL-8) (100 ng/ml; Prospec).

2.8. Statistical analyses

One-way analysis of variance (ANOVA), unpaired t-test, Wilcoxon signed rank test, paired t-test and Fisher’s exact test were used. Spearman’s correlation coefficient with two-tailed p-values was determined in the analysis of correlations. Statistical analyses were performed with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA) or JMP® 13 (SAS Institute Inc., Cary, NC). A p-value < 0.05 was considered significant.

3. Results

3.1. The proportion of CD4+CD52lo T cells was increased in the SLE patients

We analyzed single CD3+ CD45+ CD4+ cells, dividing them into CD52hi and CD52lo cell subsets, which have been reported as the suppressor phenotype and effector-like phenotype, respectively [4]. We found that the percentage of CD4+CD52lo T cells in the SLE patients was significantly higher than those in the HC (p < 0.01) and RA (p < 0.05) groups (Fig. 1A, B). The expansion of CD8+ CD52lo T cells was not seen in SLE patients (Suppl. Fig. S1A).

No significant difference was found in the percentage of conventional Tregs (identified as CD3+ CD4+ CD25+ CD127− cells) among the three groups (Suppl. Fig. S1B, C). There was no correlation between the percentage of Tregs and CD4+CD52hi T cells in SLE patients (Suppl. Fig. S1D). The serum soluble CD52 was also analyzed by ELISA. The titer of serum soluble CD52 was decreased in the SLE group compared to the RA and HC groups (Fig. 1C).

3.2. The expansion of CD4+ CD52lo T cells was correlated with disease activity and autoantibody production in the SLE patients

To clarify whether the proportion of CD4+ CD52lo T cells can be a biomarker of SLE, we next analyzed the correlations between the percentage of CD4+ CD52lo T cells in the PBMCs and clinical parameters including the SLEDAI value, the serum IgG concentration, serum anti-double strand-DNA antibody titer, and serum complement titer in the SLE patients. We found that the percentage of CD4+CD52lo T cells in the SLE patients showed a positive correlation with the SLEDAI, the anti-double strand-DNA antibody titer, and the serum IgG concentration (Fig. 2).

Though the age differed among the three groups, there was no correlation between the percentage of CD4+ CD52lo T cells and age in either SLE patients or all subjects (Suppl. Fig. S2A, B). On the basis of these results, we think that the age of subjects does not affect the proportion of CD4+ CD52lo T cells. There was no difference in gender among the three groups, while the proportion of CD4+ CD52lo T cells was higher in females than males in both SLE patients and all subjects (Suppl. Fig. S2C, D).

There was no correlation between the titer of serum soluble CD52 and the SLEDAI score in SLE patients (Suppl. Fig. S3).

3.3. The CD4+ CD52lo T cells increased with the disease development in MRL/lpr mice

To clarify the relationship between SLE progression and the expression of CD4+ CD52lo T cells, we used MRL/lpr mice, which develop immune complex-mediated nephritis and exhibit progressive lymphadenopathy by age 8–10 weeks [8]. We compared the percentage of CD4+ CD52lo T cells from splenocytes between pre-onset (6 weeks old) and established disease (16 weeks old). As shown in Fig. 3, the percentage of CD4+ CD52lo T cells was significantly increased in the 16-week-old mice compared to the 6-week-old mice (Fig. 3).

Fig. 1. Analysis of CD4+ CD52+ T cells. A, B: The percentage of CD4+ CD52lo T cells in the SLE patients (n = 64) was significantly higher than those of the HC (n = 33) and RA (n = 23) groups. C: Soluble CD52 was decreased in the SLE group compared to the other groups. Bars: mean ± SD. *p < 0.05, **p < 0.01 by one-way ANOVA. SLE: systemic lupus erythematosus; HC: healthy control; RA: rheumatoid arthritis.
3.4 The correlation of circulating follicular helper-like T cells and CD4+ CD52lo T cells

Since our data showed that the percentage of CD4+ CD52lo T cells was correlated with both the anti-ds-DNA antibody titer and the serum concentration of IgG, we determined the correlation between the percentage of CD4+ CD52lo T cells, Tfh-like cells, and plasmablasts (CD3− CD19+ CD38+ CD27+) from the SLE patients (n = 27), the HC (n = 9) and the RA patients (n = 9). The demographic characteristics of these subjects are summarized in Suppl. Table S1. The percentages of Tfh-like cells (ICOShigh PD-1high in CD4+ CD45RA− CXCR5high) and plasmablasts (CD38+ CD27+ in CD3− CD19+) in the SLE patients were significantly higher than those of the other groups (Fig. 4A, B, D, E), which is consistent with a previous report [9]. We found that the percentage of Tfh-like cells in the SLE group was positively correlated with that of CD4+ CD52lo T cells (Fig. 4C), but there was no correlation between the plasmablasts and CD4+ CD52lo T cells (Fig. 4F). To clarify that Tfh-like cells and CD4+ CD52lo cells are not overlapped populations, we next analyzed CD45RA− CXCR5+ in both CD4+ CD52hi cells and CD4+ CD52lo T cells in patients with SLE. We found that the percentage of CD45RA− CXCR5+ cells was lower in the CD4+ CD52lo T cells than the CD4+ CD52hi T cells (Suppl. Fig. S4). From this result, we concluded that there was little overlap between the Tfh-like cells and CD4+ CD52lo cells.

3.5 TARC induced the development of CD4+ CD52lo T cells into CD4+ CD52hi T cells in the SLE patients

The expression of messenger RNA from CD4+ CD52lo T cells and CD4+ CD52hi T cells was analyzed by microarray analysis. To identify
factors that affect the induction of CD4+ CD52lo T cells, we compared the genes expressing the chemokine receptors between CD4+ CD52lo T cells and CD4+ CD52hi T cells. Of 35,324 genes, the expression of 3158 genes was >1.5-fold higher in CD4+ CD52lo T cells (Fig. 5A). Increased expression levels of several chemokine ligands and their cognate receptors have been found in lupus-prone mice and SLE patients [10]. We focused on the chemokine receptors that were upregulated by >1.5-fold in CD4+ CD52lo T cells, i.e., chemokine receptor 2 (CCR2), CCR5, CCR8 and C-X-C motif chemokine receptor 2 (CXCR2).
To examine the function of these chemokine receptors in CD4+ CD52lo T cells, we conducted in vitro experiments using CD4+ T cells from SLE patients. The isolated CD4+ T cells were incubated with or without ligands related to these chemokine receptors for 24 h: GRO as a ligand of CXCR2, MCP-1 as a ligand of CCR2, RANTES as a ligand of CCR5, TARC as a ligand of CCR8, and IL-8 as a ligand of CXCR2. We used flow cytometry to analyze the percentage of CD4+ CD52+ T cells after the stimulation of these ligands, and we found that only TARC induced the development of CD4+ CD52hi T cells into CD4+ CD52lo T cells (Fig. 5B, C).

4. Discussion

Our findings revealed that the percentage of CD4+ CD52lo T cells in the peripheral blood of the SLE patients was increased, and the expansion of CD4+ CD52lo T cells was positively correlated with the SLEDAI score, the anti-ds-DNA antibody titer, and the serum IgG concentration. In addition, the expansion of circulating Tfh-like cells, which is responsible for B-cell differentiation in the germinal center, was positively correlated with the percentage of CD4+ CD52lo T cells. These results suggested that increased CD4+ CD52lo T cells along with decreased serum soluble CD52 are involved in the pathogenic autoantibody production.

We also found that the gene expression of CCR8 was increased in CD4+ CD52lo T cells. The in vitro experiments showed that TARC, which is known as one of the ligands for CCR8, induced the development of CD4+ CD52hi T cells into CD4+ CD52lo T cells.

Humans with type 1 diabetes have been reported to have a lower frequency and diminished function of CD4+ CD52hi T cells responsive to the autoantigen GAD65 [4]. CD4+ CD52hi T cells exert a suppressor function by releasing soluble CD52 [4]. Soluble CD52 binds to siglec-10 on activated T cells, including CD4+ CD52lo T cells, which leads to the inhibition of these cells by impairing the phosphorylation of the T-cell receptor-associated kinases Lck and Zap70 [4]. An anti-CD52 antibody (alemtuzumab) has been used to treat multiple diseases: RA [13–15], non-Hodgkin’s lymphoma [16,17], T-cell lymphoma [18,19], and relapsing-remitting multiple sclerosis [20,21].
The efficacy of alemtuzumab is brought about by anti-CD52 antibody-mediated lymphocytic depletion as immune ablation. However, multiple sclerosis patients treated with alemtuzumab have been reported to develop autoimmune diseases with autoantibodies, such as Grave's disease, immune thrombocytopenic purpura, and anti-glomerular basement membrane disease [22,23]. It has been suggested that anti-CD52 antibody treatment may induce autoimmune diseases by a preferential removal of CD4+ CD52lo regulatory T cells and soluble CD52 [4,24]. In light of our present findings, a decreased percentage of CD4+ CD52lo T cells along with decreased soluble CD52 in the serum of patients with SLE may contribute to the impairment of the inhibitory signal of activated T cells.

Our present investigation revealed that the expansion of CD4+ CD52lo T cells correlates with disease activity and autoantibody production in both SLE patients and a murine lupus-prone model. It was proposed that follicular helper T cells are necessary for B-cell differentiation in the germinal center [25] and that circulating Tfh-like cells, identified as CD4+ CD45RA−CXCR5hi ICOShi PD-1hi, were increased in the PBMCs from individuals with SLE [9]. In the present study, the expansion of Tfh-like cells was positively correlated with CD4+CD52lo T cells in the SLE patients, suggesting that CD4+ CD52lo T cells are involved in the abnormal production of antibodies through the differentiation of Tfh-like cell-mediated plasma cells. However, our investigation could not clarify the mechanisms by which the CD4+CD52lo T cells induce Tfh-like cells. We speculate that CD4+ CD52lo T cells from SLE patients may produce IL-21, which skews toward Tfh-like cells. Further research is required to elucidate the precise mechanisms underlying the induction of Tfh-like cells by CD4+CD52lo T cells.

To determine whether the proportion of CD4+ CD52lo T cells could be a candidate marker for the clinical response of SLE patients, we evaluated the percentage of CD4+ CD52lo T cells before and after intravenous cyclophosphamide (IVCY) treatment, and as shown in Suppl. Fig. S5, we observed that the percentage of CD4+ CD52lo T cells was reduced after the induction of IVCY therapy. Previous studies showed that CD52 expression is downregulated as lymphocytes divide and upregulated after the induction of cell quiescence through the expression of the transcription factor LKLF [Jung Krüppel-like factor] [26,27].

Cyclophosphamide is an alkylating agent that interferes with the duplication of DNA, resulting in an impairment of cell division [28]. Cell quiescence induced by IVCY may contribute to the decrease of CD4+ CD52lo T cells in SLE patients. In addition, our present experiments demonstrated that in the murine lupus-prone model, the percentage of CD4+ CD52lo T cells was greater in the 16-week-old mice compared to the 6-week-old mice (Fig. 3). In light of these results, we propose that CD4+ CD52lo T cells could serve as a useful biomarker for evaluating the disease activity and therapeutic responses of individuals with SLE.

Interestingly, even though the expansion of CD8+ CD52lo T cells was not seen in SLE patients, the CD8+ CD52lo T cells were expanded in the patients with RA (Suppl. Fig. S1A). Abundant CD8+ T cells have been shown to infiltrate the rheumatoid synovium [29] and are also detected in preclinical stages [30]. CD8+ CD52lo T cells may play some role in the pathogenesis in RA. Further research is needed on this point.

In a present study, there was no correlation between the percentage of CD4+ CD25−CD127− regulatory T cells and CD4+ CD52lo T cells in the SLE patients. In addition, the microarray analysis in the present study showed that the mRNA expression level of FOXp3, a master transcription gene in Tregs, was nearly identical between CD4+ CD52lo T cells and CD4+ CD52lo T cells (1.16-fold difference). In the previous studies, the generation of human CD4+ regulatory T-cell clones against the pancreatic islet autoantigen GAD65 was indicated to be a function of regulatory T cells [4,31]. These regulatory T-cell clones did not show CD25 expression or inhibitory mechanisms with typical Tregs. The clones also showed that a depletion of CD25+ T cells from PBMCs did not affect the generation of CD4+ CD52lo suppressive T cells [4]. These results support our present findings and suggest that CD4+ CD52lo T cells with suppressor functions are not derived from CD4+ CD25+ conventional regulatory T cells.

We also analyzed the differentiation of T cells such as effector memory, central memory and naive cells in both CD4+ CD52lo T cells and CD4+ CD52hi T cells (shown in Suppl. Fig. S6). We found that CD4+ CD52lo T cells were polarized into effector T cells. The microarray analysis revealed that the mRNA expression of IFNG, the gene encoding IFN-γ, was upregulated in CD4+ CD52lo T cells compared to CD4+ CD52hi T cells (2.66-fold difference). These results suggest that CD4+ CD52lo T cells in SLE patients have the activated effector functions.

TARC, also known as chemokine ligand 17 (CCR17), is expressed in the thymus and is produced by dendritic cells, endothelial cells, keratinocytes and fibroblasts [32]. TARC has affinity as a ligand for CCR4 and CCR8, which are predominantly expressed by Th2 cells [12,33]. In the present study, we observed that (1) the expression of CCR8 but not CCR4 mRNA was increased in CD4+ CD52lo T cells, and (2) a recombinant TARC protein induced the development of CD4+ CD52lo T cells into CD4+ CD52lo T cells in SLE patients. A previous study implicated TARC in T-cell activations and the development of CCR8-transfected Jurkat cells [12]. It was also reported that the concentration of TARC is elevated in serum of SLE patients [34] and from New Zealand mice, a lupus-prone murine model [35]. Collectively, these findings suggest that TARC, by interacting with CCR8, may induce the development of CD4+ CD52lo T cells into CD4+ CD52lo T cells via direct or indirect mechanisms.

In conclusion, our data suggest that an increased percentage of CD4+ CD52lo T cells, in conjunction with an increased percentage of Tfh-like cells, is involved in the pathogenic autoantibody production and that TARC may contribute to the development of SLE via an aberrant induction of CD4+ CD52lo cells.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2017.10.004.

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Author contributions

Drs. Ichinose and Koga had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study design: K. Ichinose, T. Koga.
Statistical analysis: M. Umeda.

References


