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The role of stem cell-like memory T cells in systemic lupus erythematosus

전신홍반루푸스에서 기억 줄기 T 세포의 역할

2017 년 02 월

서울대학교 대학원

의학과 중개의학(면역학)전공

이 예 지

A thesis of the Degree of Master of Science

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February 2017

The Department of Medicine,

Seoul National University

College of Medicine

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by

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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Translational Medicine) at Seoul National University College of Medicine

February, 2017

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이 논문을 의학석사 학위논문으로 제출함

2017년 2월

서울대학교 대학원

의학과 중개의학(면역학)

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이예지의 의학석사 학위논문을 인준함

2017년 2월

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위 원 _____ (인)

ABSTRACT

A novel memory T subset, stem cell-like memory T (Tscm) cell, was recently discovered [1]. Previous studies have shown that Tscm cells can produce their own precursor cells and differentiate into other T cell subsets. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by characteristic systemic inflammatory features caused by immune complex-mediated complement activation. The perpetuation of the autoimmune reaction in this disease suggests the presence of abnormal immune stem cells. In this thesis, I investigated the presence and function of Tscm cells in SLE. The Tscm cells were defined by the presence of the following surface markers: $CD3^+$, $CD4^+/CD8^+$, $CD45RO^-$, $CCR7^+$, $CD62L^+$, $CD45RA^+$, $CD27^+$, $CD28^+$, $CD127^+$, $CD122^+$, and $CD95^{hi}$. The proportions of $CD4^+$ and $CD8^+$ Tscm cells among naïve $CD4^+$ and $CD8^+$ T cells or the total $CD4^+$ and $CD8^+$ T cells were significantly increased in SLE patients compared to those of the healthy controls. Tscm cells from SLE patients can proliferate and renew themselves and differentiate into naïve-like ($CD3^+$, $CD4^+/CD8^+$, $CD45RO^-$, $CCR7^+$ and $CD45RA^+$), central memory ($CD3^+$, $CD4^+/CD8^+$, $CD45RO^+$, $CCR7^+$, and $CD45RA^-$) and effector memory ($CD3^+$, $CD4^+/CD8^+$, $CD45RO^+$, $CCR7^-$, and $CD45RA^-$) T lymphocytes. The stimulated Tscm cells of SLE patients secreted more IFN-γ, TNF-α and IL-2 compared with those from the HCs. Tscm cells from SLE patients can differentiate into Tfh cells, and the differentiated Tfh cells can make B cells to produce antibodies. Tfh cells are

induced by Tscm cells through upregulation of IL-21 and downregulation of Blimp-1 which are controlled by the transcription levels of TCF-1. The proportion of Tscm cells showed no association with the SLE disease activity suggesting that Tscm cells determine the development of disease but not the disease activity.

Taken together, this study shows that the proportion of Tscm cells is increased in SLE patients and that Tfh cells differentiated from Tscm cells play a role in maintaining SLE by helping B cells produce autoantibodies.

Key words: Stem cell-like memory T cells, Systemic lupus erythematosus, Follicular helper T cells

Student number: 2014-22225

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LIST OF ABBREVIATIONS

SLE: Systemic Lupus Erythematosus

HC: Healthy controls

Tnaive: Naive T cells

Tcm: Central Memory T cells

Tem: Effector Memory T cells

PMA: Phorbol 12-myristate 13-acetate

IFN: Interferon

IL: Interleukin

TCR: T cell receptor

SEB: Staphylococcal Enterotoxin B

MHC: Major Histocompatibility Complex

GC: Germinal Center

cDNA: complementary DNA

ELISA: Enzyme-linked Immunosorbent Assay

qPCR: quantitative Polymerase Chain Reaction

TNF-α: Tumor Necrosis Factor-alpha

INTRODUCTION

Human T lymphocytes are composed of naive T lymphocytes and memory T lymphocytes. T cells, which experience the maturation process in thymus, remain “naive” until they encounter antigens. Naive T lymphocytes can proliferate and differentiate into memory subsets after reencountering their specific antigens. Memory T cells are long-lived cells and express high levels of CD45RO in humans and CD44 in mice [1]. Memory T cells are composed of central memory T lymphocytes and effector memory T lymphocytes. Central memory T lymphocytes can home to secondary lymphoid organs such as lymph nodes with their surface chemokine receptor CCR7 and L-selectin. In contrast, effector memory T lymphocytes home to peripheral tissues [2].

Recently, a new subset of memory T cells, labeled as stem cell-like memory T lymphocytes (Tscm), was found [3]. The Tscm cells, like stem cells, can proliferate and renew themselves and differentiate into mature lymphocytes when stimulated through the T cell receptor. Human Tscm cells can be distinguished by their characteristic surface phenotypes, $CD45RO^-$ $CCR7^+$ $CD45RA^+$ $CD62L^+$ $CD27^+$ $CD28^+$ IL-$7R^+$ IL-$2R^+$ $CD95^{hi}$. The $CD45RO^-$, $CCR7^+$, $CD45RA^+$, and $CD62L^+$ markers represent the naivety of the cells, and the $CD95^+$ marker is used to discriminate Tscm cells from pure naïve T cells. Tscm cells can be $CD4^+$ Tscm or $CD8^+$ Tscm cells depending on the presence of the CD4 or CD8 surface marker.

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease characterized by the increased production of autoantibodies that recognize self-antigens such as double-stranded DNA and U-rich ribonucleoproteins (RNPs, a collection of small single-stranded RNAs associated with nuclear proteins) [4]. These anti-nuclear antibodies (ANA) are complexed with their cognate autoantigens and are deposited in the skin or other internal organs such as the kidneys or nervous system inducing immune cell activation and the secretion of pro-inflammatory cytokines such as IFN-α and TNF-α and finally damaging the affected organs [5]. Persistent pro-inflammatory immune cells are considered to contribute to the pathogenesis of SLE.

Recently, follicular helper T (Tfh) cells were suggested to have a role in the pathogenesis of SLE. Tfh is a key immuno-regulator of germinal center formation and humoral immunity [6-8]. As a specialized subset of helper T cells, it activates B cells in the germinal centers causing plasma cells to produce autoantibodies. It is known that Tfh cells can be identified by chemokine receptor 5 (CXCR5), inducible co-stimulator (ICOS), programmed cell death 1 (PD-1), and transcription factor B cell lymphoma 6 (Bcl-6) [9]. CXCR5 and its ligand CXCL13 are required for T cell migration into B follicles and for their co-localization. ICOS mediates the association between Tfh cells and B cells through the delivery of activation signals to $CD4^+$ T cells. PD-1, which is known to repress TCR signaling, acts as a negative regulator of Tfh differentiation. Bcl-6 behaves like a key regulator of Tfh differentiation [4]. By the action of Tfh cells on B cells, in secondary lymphoid organs such

as lymph nodes, B cell maturation processes such as immunoglobulin isotype switching and affinity maturation proceeds. As a result, memory B cells and long-lived plasma cells are generated [10]. In SLE, many autoreactive IgG^+ memory B cells are somatically mutated [11]. Moreover, the ratio of somatically mutated antibody-producing plasmablasts is increased in SLE peripheral blood [12]. Thus, it can be inferred that autoantibody-producing B cells are generated from the GCs of secondary lymphoid organs by the help of Tfh cells in SLE patients. In fact, several studies have suggested that Tfh has a role in human SLE [13, 14]. One study showed an increased frequency of circulating Tfh (cTfh) cells in SLE patients compared with healthy controls. Among the different subsets of Tfh cells ($CXCR3^+$ $CCR6^-$ (cTfh1), $CXCR3^-$ $CCR6^-$ (cTfh2) and $CXCR3^-$ $CCR6^+$ (cTfh17)), activated cTfh cells [15] were increased in SLE patients producing higher levels of autoantibodies [16] or circulating plasmablasts [17].

Therefore, I hypothesized that the proportion of pathogenic Tscm cells is increased in SLE patients and that Tfh cells derived from Tscm cells can contribute to the development of SLE. In this study, I investigated 1) the levels of Tscm cells in SLE patients, 2) the functional characteristics of Tscm cells from SLE patients, 3) the mechanism of Tfh cells from Tscm cells in SLE patients, and finally 4) the functional ability of Tfh cells to make B cells produce immunoglobulins.

MATERIALS AND METHODS

1. Sample collection and clinical information

A total of 52 SLE patients were enrolled in this study. Fifty-seven healthy persons were also included as controls. After obtaining peripheral blood, peripheral blood mononuclear lymphocytes were isolated using the Ficoll-Hypaque (GE healthcare, NJ, USA) gradient.

All of the SLE patients met the revised classification criteria of SLE by the American College of Rheumatology [18]. Disease activity was assessed using the SLE disease activity index, SLEDAI [19], and laboratory investigations included white blood cell count, ESR, and complement levels (C3 and C4). The autoantibodies of the SLE patients were measured including the antinuclear antibodies, anti-dsDNA, anti-SSA/Ro, and anti-SSB/La antibodies.

2. Antibodies and reagents, flow cytometry and cell sorting

IL-15 was obtained from Peprotech (Rocky Hill, NJ, USA). Dynabeads Human T-cell activator CD3/CD28 was obtained from ThermoFisher (Waltham, MA, USA). Anti-CD127 antibody emitting the Qdot655 color was obtained from Biolegend (San Diego, CA, USA), and anti-CCR7 antibody emitting the PE color was obtained from R&D (Minneapolis, MN, USA). All

of the other antibodies including CD3 (clone SK7), CD4 (SK3), CD8 (SK1), CD45RO (UCHL1), CD62L (DREG-56), CD45RA (HI100), CD28 (CD28.2), CD27 (M-T271), CD127 (A019D5), CD95 (DX2), CD122 (Mik-β3), CXCR5 (RF8B2), ICOS (DX29), Bcl-6 (K112-91) and PD-1 (EH12.1) were obtained from BD Biosciences (San Jose, CA, USA).

B cell isolation kit was purchased from Miltenyi Biotec (Germany). To exclude the possibility of pre-stimulation, the negative isolation kit was used.

After blocking the Fc receptors of PBMCs ($1x10^7$ cells/mL) with purified mouse anti-human IgG (BD Biosciences, San Jose, CA, USA), the PBMCs were stained with the specific fluorescent antibodies to detect Tscm, central memory T lymphocyte (Tcm), naïve T lymphocyte (Tnaive), effector memory T lymphocyte (Tem), or follicular helper T lymphocyte (Tfh) cells.

Tscm was designated as $CD3^+$ $CD4^+/CD8^+$ $CD45RO^-$ $CCR7^+$ $CD62L^+$ $CD45RA^+$ $CD27^+$ $CD28^+$ $CD127^+$ $CD122^+$ $CD95^{hi}$. Detailed gating is explained in Figure 1. Tcm was designated as $CD3^+$ $CD4^+/CD8^+$ $CD45RO^+$ $CCR7^+$ $CD45RA^-$; Tnaive was designated as $CD3^+$ $CD4^+/CD8^+$ $CD45RO^-$ $CCR7^+$ $CD45RA^+$ $CD62L^+$ $CD27^+$ $CD95^-$, and Tem was designated as $CD3^+$ $CD4^+/CD8^+$ $CD45RO^+$ $CCR7^-$ $CD45RA^-$. Tfh was designated as $CD3^+$ $CD4^+$ $CXCR5^+$ $ICOS^+$ $PD\text{-}1^+$ $Bcl\text{-}6^+$.

All fluorescence-stained cells were detected with LSR Fortessa (BD Biosciences, San Jose, CA, USA) and sorted using the FACSAria instrument (BD Biosciences, San Jose, CA, USA). All data were analyzed with the FlowJo software (Treestar, Ashland, OR, USA).

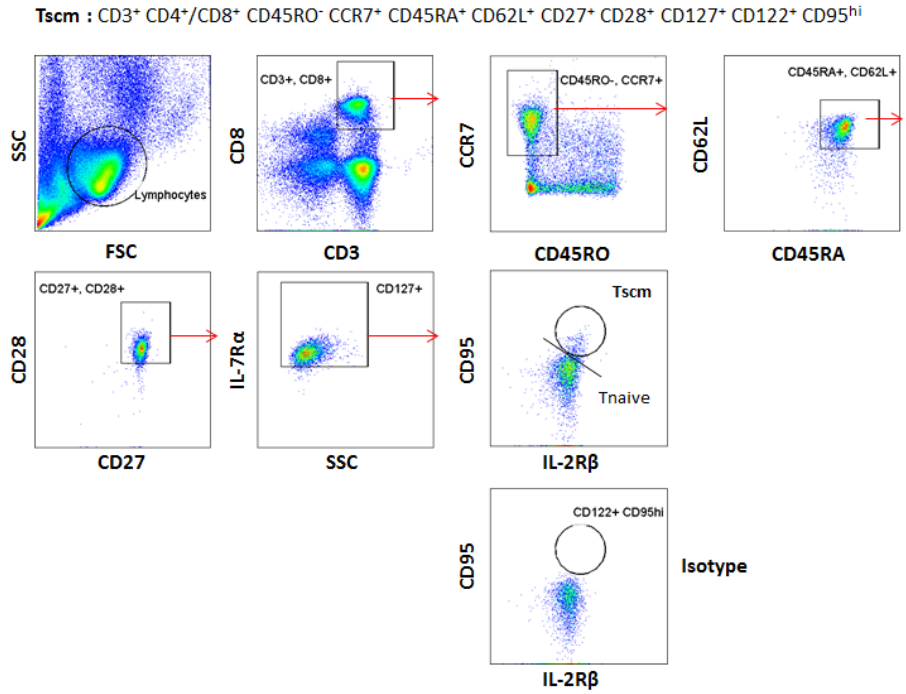

Figure 1. Flow cytometry panel for the detection of Tscm cells

Tscm cells were designated as $CD3^+$, $CD4^+$ or $CD8^+$, $CD45RO^-$, $CCR7^+$, $CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$, $CD127^+$, $CD122^+$ and $CD95^{hi}$ cells. The attached graph shows the precise flow cytometry gating strategy. The isotype antibody emitting the same color as CD95 was used to discriminate $CD95^{hi}$ cells easily.

3. Self-renewal ability of the Tscm cells

Before cytokine treatment, cell proliferation was determined by 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; 5uM; 10min/ Invitrogen, Waltham, MA, USA) staining. 30 ng/mL of IL-15 were added to sorted Tnaive or Tscm or Tcm or Tem to evaluate the response to the treatment with IL-15 for 8 days. $CFSE^{low}$ cells were identified as the parental phenotype or differentiated other cell type using flow cytometry markers.

4. Multipotency of Tscm cells

Cells were stimulated with anti-CD3/CD28-coated beads (Dynabeads, Thermo Fisher, Waltham, MA, USA) for 6 days. Anti-CD3/CD28-coated beads were added to the cells at a 1:1 ratio. Differentiated cells were analyzed using flow cytometry markers after staining with CD3 (clone SK7), CD4 (SK3), CD8 (SK1), CD45RO (UCHL1), CCR7 (150503), and CD45RA (HI100).

5. Cytokine analysis

Sorted Tnaive, Tscm, Tcm, and Tem cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ug/mL) and Ionomycin (1 mM) (both from Sigma Aldrich, Darmstadt, Germany) for 5 hours in the presence of monensin (Golgistop, BD Biosciences, San Jose, CA, USA). After the treatment, the Fc

receptor was blocked, and surface markers (CD3, CD8, CD45RA, CCR7, and CD45RO) were stained. Next, the cells were permeabilized with a fix/perm solution (eBioscience, San Diego, CA, USA) and then stained for intracellular cytokines: IFN-γ (clone B27), IFN-α (7N4-1) (BD bioscience, San Diego, CA, USA), TNF-α (MAb11) and IL-2 (MQ1-17H12) (ebioscience, San Diego, CA, USA).

6. Quantitative real-time PCR

The transcription levels of the Tfh cells was assessed by real-time qPCR. After extracting RNA from the sorted Tscm cells using RNeasy Mini kit (Qiagen, Hilden, Germany), cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The transcribed levels of the Tfh-associated molecules (Bcl-6, CXCR5, ICOS, PD-1, IL-21, Blimp-1, LEF-1, TCF-1) were detected with Bio-rad CFX 96 (Hercules, CA, USA) using SYBR Green (Qiagen, Hilden, Germany) and the fold changes in expression were measured with the $2^{-\Delta\Delta Ct}$ method with the results normalized to β-actin for gene expression. Image data were obtained from the cDNA on the gel electrophoresis using 1% agarose. Gel images were visualized with GelDoc (Bio-rad Laboratories, Hercules, CA, USA).

7. Immunoglobulin production with the co-culture assay

Tscm cells were stimulated with anti-CD3/CD28 beads for 6 days and then placed in a co-culture with an equal number of autologous B cells in the presence of 200 ng/mL of staphylococcal enterotoxin B (SEB) (Toxin technology, Sarasota, FL, USA) in RPMI 1640 (Welgene, Gyeongsan, Korea) with 10% fetal bovine serum (Biowest, Nuaillé, France) and 1% penicillin/streptomycin (Gibco, Waltham, MA, USA). The supernatant and cells were harvested at day 6 to analyze the total IgG.

The total IgG assay consisted of an enzyme-linked immunosorbent assay (ELISA) using the culture supernatant according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). All samples were measured in triplicate.

8. Statistical analysis

The data were expressed as the mean ± SEM. For continuous variables, Student's t-test was used if the sample number was enough while the Mann Whitney U test was used when the sample number was not enough. All of the graphs in the manuscript were made with the Prism software (GraphPad software, Inc., La Jolla, CA, USA). All statistical analyses were done with SPSS (IBM, Armonk, NY, USA) using the Student's T-test and Mann Whitney U test.

RESULTS

Increased levels of $CD4^+$ or $CD8^+$ Tscm cells in patients with SLE

The proportion of Tscm cells among naive-like T or total T lymphocytes for PBMCs was measured in 52 SLE patients and 57 healthy controls (HCs) (Fig 2). Naive-like T cells were designated as $CD4^+$ or $CD8^+$, $CCR7^+$ $CD45RO^-$ $CD45RA^+$ $CD62L^+$ T cells, and Tscm cells were designated as $CD4^+$ or $CD8^+$, $CCR7^+$ $CD45RO^-$ $CD45RA^+$ $CD62L^+$ $CD27^+$ $CD28^+$ $CD127^+$ $CD122^+$ $CD95^{hi}$ cells.

Among the naive-like T cells, the proportion of $CD4^+$ and $CD8^+$ Tscm cells (Tscm cells/naive-like T cells) was significantly higher in the SLE patients than in the HCs (for $CD4^+$ T cells, 2.2875±0.2205 (SLE) vs 1.1118±0.1310 (HC), p-value≦0.001 ; for $CD8^+$ T cells, 5.3574±0.6274 (SLE) vs 3.4053±0.4449 (HC), p-value=0.013) (Fig 2. A). Among the total $CD4^+$ T cells, the ratio of Tscm to $CD4^+$ T cells (Tscm cells/$CD4^+$ T cells) was higher in the SLE patients than in the healthy controls (0.6276±0.0972 (SLE) vs 0.4010±0.0410 (HC), p-value=0.035) (Fig 2. B, left). However, among the total $CD8^+$ T cells, the proportion of Tscm cells was comparable between the SLE patients and HCs (1.0363±0.1446 (SLE) vs 0.7895±0.0615 (HC), p-value=0.121) (Fig 2. B, right). To measure the absolute number of $CD4^+$/$CD8^+$ Tscm cells, Accucheck counting beads (Thermo Fisher (Waltham, MA, USA))

were used. The absolute number of $CD4^+$/$CD8^+$ Tscm cells was not different between the SLE patients and the HCs (for $CD4^+$ T cells, 3199.2973±650.9093 (SLE) vs 4930.1373±615.8670 (HC), p-value=0.061; for $CD8^+$ T cells, 6076.5526± 1113.0701 (SLE) vs 4074.6471± 375.2328 (HC), p-value=0.095) (Fig 2. C).

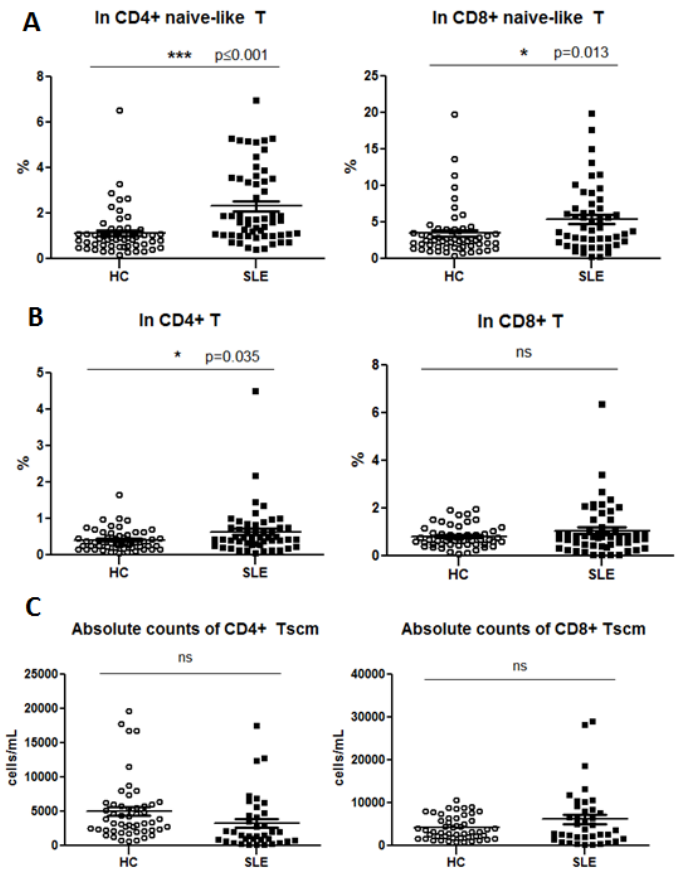

Figure 2. The proportion of $CD4^+/CD8^+$ Tscm cells in naive-like T cells and the proportion of $CD4^+/CD8^+$ Tscm cells in total $CD4^+/CD8^+$ T cells.

PBMCs from 57 healthy controls and 52 SLE patients were investigated. (A) The ratio of Tscm cells per naive-like T cells ($CD4^+$ or $CD8^+$ $CD45RO^-$ $CCR7^+$ $CD45RA^+$ $CD62L^+$) and (B) the ratio of Tscm cells per total $CD4^+$ or $CD8^+$ T cells were measured. (C) The absolute counts of Tscm cells were also calculated. Open circles are the healthy control group, and black squares are the SLE group. The bars indicate the means ± SEM.

ns, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***,$p \leq 0.001$;

Tscm cells of the SLE patients can differentiate into naïve-like T, central memory, and effector memory T cells.

It has been reported that Tscm cells can differentiate into Tcm and Tem cells as well as Tscm cells themselves when adequate stimuli is applied. To determine whether SLE Tscm cells can differentiate into daughter subsets, Tscm cells sorted from 8 SLE patients were stimulated with anti-CD3/CD28 beads for 6 days at 37℃ with Tscm cells from the HCs as the control cells. After stimulation, $CD4^+$ Tscm cells from the SLE patients could differentiate into naive-like T ($CD3^+$ $CD4^+$ $CCR7^+$ $CD45RA^+$), central memory T ($CCR7^+$ $CD45RA^-$), effector memory T ($CCR7^-$ $CD45RA^-$), and terminal effector memory T ($CCR7^-$ $CD45RA^+$) cells (Fig 3. A). The Tscm cells that showed only naïve-like T cell markers ($CCR7^+$ $CD45RA^+$) expressed memory, effector surface markers in addition to their original naïve-like T markers when stimulated while the central memory T cells could differentiate only into the central memory T and effector memory T phenotypes. Additionally, effector memory T cells could differentiate only into the effector memory T cell phenotype. The differentiation patterns were similar between the SLE and HC Tscm cells. However, the SLE $CD4^+$ Tscm cells tended to differentiate more toward the effector subsets, effector memory and terminal effector memory T cells (Fig 3. B) (In HCs, Naïve-like T : Tcm : Tem : Temra = 26.58 : 26.35 : 37.70 : 9.39 (%) ; In SLE, Naïve-like T : Tcm : Tem : Temra = 20.65 : 17.2 : 51.68 : 10.47 (%)). Moreover, $CD4^+$ Tscm cells replicated themselves.

Additionally, Tscm cells existed in CFSE-diluted $CD4^+$ T cells.

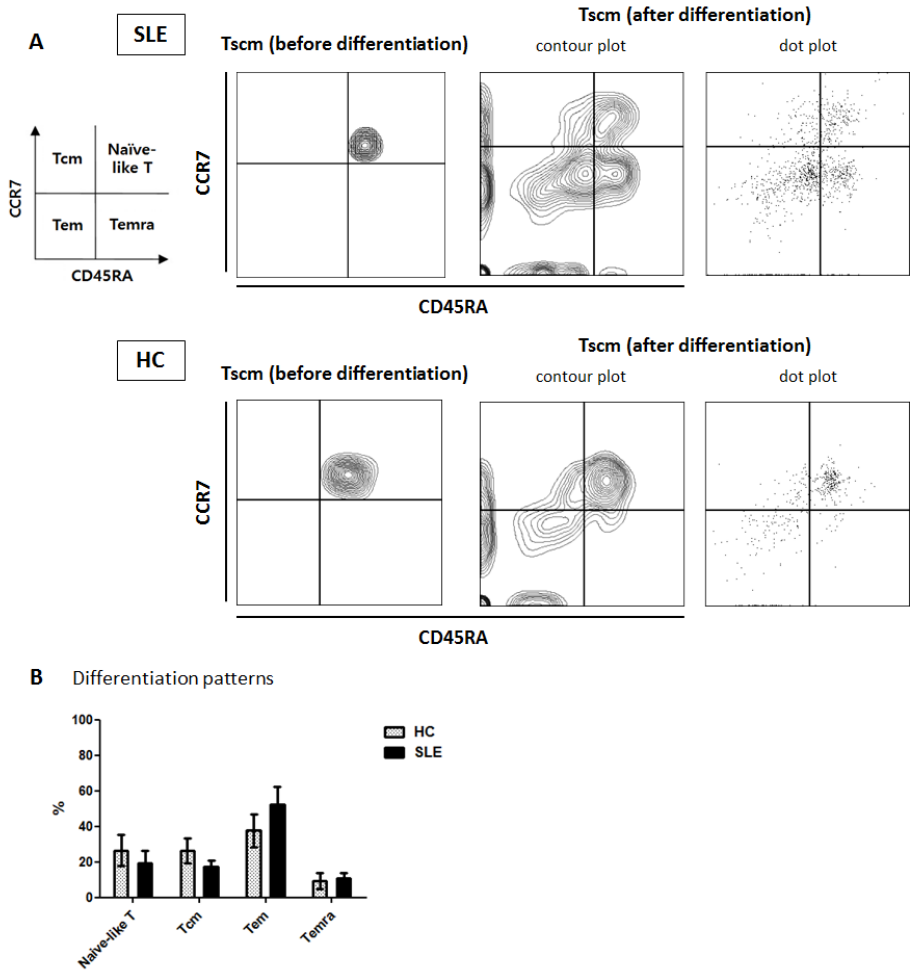

Figure 3. Tscm cells could differentiate toward other T cell subsets via T cell stimulation.

(A) Sorted Tscm cells from 8 SLE patients and 7 healthy controls were stimulated with anti-CD3/CD28 beads for 6 days. Before and after TCR stimulation, differentiated Tscm cells expressed the phenotypes of naïve-like T, central memory T, effector memory T, and terminal effector memory T cells discriminated by the expression of CCR7 and CD45RA ($CD3^+$ $CD4^+$ gated).

(B) $CD4^+$ Tscm differentiation patterns are depicted above. The dotted white bars denote the Tscm distribution after the TCR stimulation for HC, and the black bars denote the Tscm distribution after the TCR stimulation for SLE.

Differentiated T cell subsets from SLE patients secrete more cytokines.

To understand the immunologic characteristics of the differentiated cells from the Tscm cells, the cytokine secretion pattern was analyzed. Each subset of T cells from 9 SLE patients or 7 HCs were stimulated with anti-CD3/CD28 beads for 6 days at 37℃. After activating each T cell subset with PMA/Ionomycin for 5 hours at 37℃, intracellular IFN-γ, IL-2, TNF-α, and IFN-α were measured using intracellular flow cytometry. The activated Tscm cells from the SLE patients showed higher levels of IFN-γ, IL-2, and TNF-α compared with those from the HC Tscm cells, while the level of IFN-α was lower in the SLE patients. Cytokines were detected more profoundly in the memory and effector cells, as expected (Fig 4).

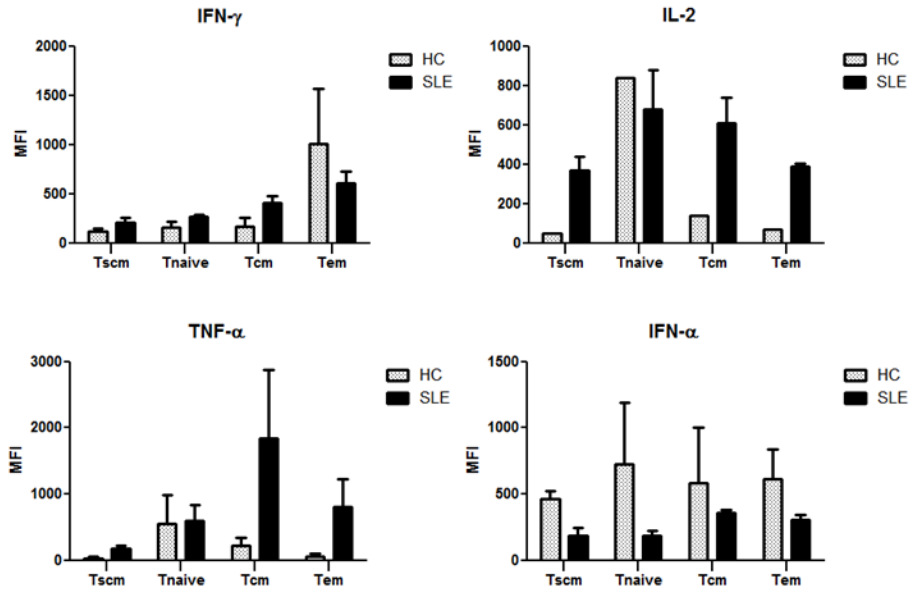

Figure 4. Differentiated T cell subsets from the SLE patients secrete pro-inflammatory cytokines.

To identify cytokines secreted by the activated Tscm cells, sorted Tscm cells were TCR-stimulated. For comparison, naïve ($CD3^+$ $CD4^+$ $CD45RO^-$ $CCR7^+$ $CD45RA^+$ $CD62L^+$ $CD27^+$ $CD95^-$), central memory and effector memory T cells were also stimulated. Each activated subset of T cells was stimulated with 50 ug/mL of PMA and 1 mM of Ionomycin for 5 hours with GolgiStop. The dotted white bars represent the healthy controls, and the black bars represent the SLE patients.

MFI, Mean Fluorescence Intensity

Tscm cells can differentiate into follicular helper T cells.

Sorted Tscm cells from 7 SLE patients and 7 healthy controls (HCs) were examined for their potential to differentiation into Tfh cells after stimulation with anti-CD3/CD28 beads for 6 days at 37℃ by staining the Tfh surface markers.

The results show that the Tscm cells can differentiate into Tfh cells evident by the $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ $ICOS^+$ surface markers (Fig 5. A). Compared with the HCs, the Tscm cells from the SLE patients differentiated more into Tfh cells (Fig 5. B). The level of transcription factor Bcl-6 was also high in the SLE Tscm cells than in the HC Tscm cells. For the $CD4^+$ T cells, the average expression of $CXCR5^+$ $ICOS^+$ $PD\text{-}1^+$ cells in the SLE patients was 9.3 (%). For the HC $CD4^+$ T cells, the average expression was 5.41 (%). Moreover, for the $CD4^+$ T cells, the average expression of $Bcl\text{-}6^+$ cells in the SLE patients and HCs was 34.294 (%) and 19.2 (%), respectively.

Production of Tfh by Tscm was reconfirmed at the RNA level. RNA extracted from sorted Tscm cells was reverse-transcribed into cDNA, and the RNA levels of Bcl-6, CXCR5, PD-1, ICOS, and β-actin were detected by real-time quantitative PCR (Fig 5. C). The primers used are listed in Table 2, and the fold changes in expression were measured by the $2^{-\Delta\Delta Ct}$ method. The results were normalized to β-actin for gene expression. The transcription levels of Tscm after stimulation were also calculated based on the levels of Tscm before stimulation. In the HC Tscm cDNA, except for CXCR5, the

transcription levels of Bcl-6, PD-1, and ICOS were down-regulated when compared with the transcription levels of Tscm before stimulation. In contrast, in the SLE Tscm cells, the transcription levels of the Tfh-associated markers were up-regulated compared with the gene levels of Tscm before stimulation.

When the gel image of the replicated cDNA was analyzed, the same results were observed (Fig 5. D).

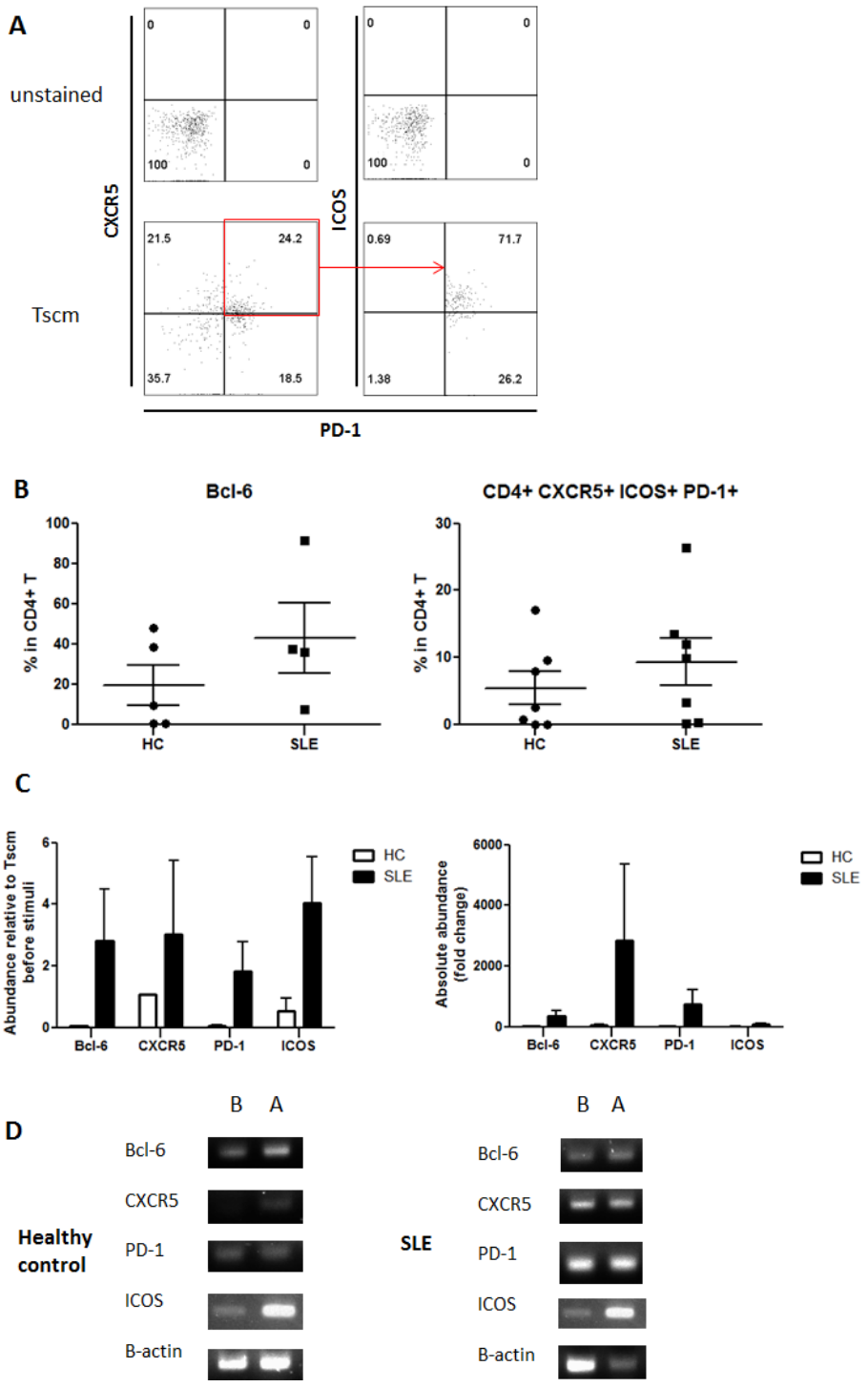
A
unstained
Tscm
CXCR5
ICOS
PD-1
0
100
21.5
24.2
35.7
18.5
0.69
71.7
1.38
26.2
B
Bcl-6
CD4+ CXCR5+ ICOS+ PD-1+
% in CD4+ T
HC
SLE
C
Abundance relative to Tscm before stimuli
Absolute abundance (fold change)
Bcl-6
CXCR5
PD-1
ICOS
D
B A
Healthy control
SLE
Bcl-6
CXCR5
PD-1
ICOS
B-actin

Figure 5. Tscm cells from SLE patients can differentiate into follicular helper T cells.

To examine whether Tscm cells can differentiate into Tfh cells, sorted Tscm cells were stimulated with anti-CD3/CD28 beads for 6 days at 37℃. (A) Tfh cells were designated as $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ $ICOS^+$ cells. Positive cells were divided by comparing unstained cells. (B) Expression of Bcl-6, the transcription factor for Tfh cells, was assessed. (C) Real-time quantitative PCR data. RNA was extracted from sorted Tscm cells, and cDNA was extracted using reverse transcriptase. The real-time qPCR primers are listed below (Table 2). In the right graph, the cDNA of the TCR-stimulated Tscm cells was measured, and the fold changes were calculated based on the highest ΔC_T value. (D) The images show hybridized cDNA with primers associated with the Tfh markers on electrophoresis gel. Left lane was the hybridized cDNA of Tscm before stimuli (marked 'B') and right lane was the hybridized cDNA of Tscm after stimuli (marked 'A').

Numbers in the plots indicate the percentage of cells in each quadrant.

Increased IL-21, down-regulation of Blimp-1, and up-regulation of TCF-1 lead to differentiation into follicular helper T cells.

Bcl-6 is a classical transcription factor of follicular helper T cells [9]. IL-21 is known to be a prerequisite for maintaining Bcl-6 [20]. Therefore, the IL-21 RNA levels were measured by real-time qPCR (Fig 6. A). Compared with the baseline IL-21 transcription levels of the Tscm cells before stimuli, the transcription levels of the IL-21 genes by the Tscm cells were up-regulated in SLE after differentiation. The levels of IL-21 RNA before and after differentiation from the HC Tscm cells were comparable. Blimp-1 is a negative regulator of Bcl-6 [21]. Bcl-6 and Blimp-1 are central, opposing master regulators of Tfh differentiation. The transcription level of Blimp-1 (PRDM-1) was examined by real-time qPCR (Fig 6. B). Additionally, the repressor of Blimp-1, LEF-1, and the TCF-1 gene levels were examined too (Fig 6. C). In accordance with the fact that TCF-1 promotes Tfh differentiation [22, 23], the transcription levels of TCF-1(*Tcf-7*) were up-regulated in SLE. However, the transcription levels of LEF-1(*Lef-1*) were down-regulated in the SLE patients and healthy controls. Interestingly, the transcription level of Blimp-1 was down-regulated in the SLE Tscm cells suggesting that Tfh differentiation was promoted by the increased IL-21 levels, up-regulated TCF-1, and down-regulated Blimp-1.

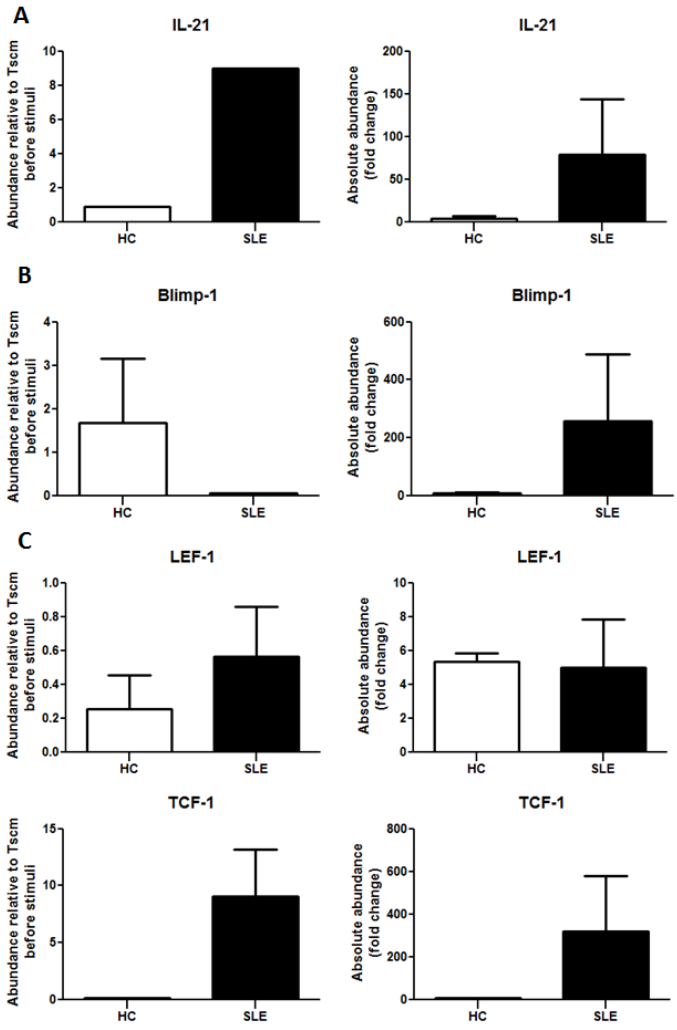

Figure 6. Increased IL-21, up-regulation of TCF-1 and down-regulation of Blimp-1 promoted the generation of Tfh cells.

The transcription levels of IL-21, LEF-1, TCF-1, and Blimp-1 were measured to investigate the mechanisms of Tfh differentiation. Tscm cells isolated from PBMCs were stimulated with anti-CD3/CD28 beads for 6 days. The Tscm cells directly isolated from the PBMCs and the TCR-stimulated Tscm cells were compared. (A) Transcription level of IL-21 by real-time qPCR. (B)

Transcription levels of the Blimp-1 gene measured by real-time qPCR. (C) Transcription level of the LEF-1 and TCF-1 genes measured by real-time qPCR. In the graphs on the left side, the levels were calculated and compared with the levels of Tscm before stimuli. In the graphs on the right side, the cDNA of the TCR-stimulated Tscm cells was measured, and the fold changes were calculated and compared with the highest ΔC_T value. White bars represent the healthy controls, and the black bars represent the SLE patients.

Generated follicular helper T cells have their own trait: Tscm-derived Tfh cells help B cells in antibody production *in vitro*.

A T-dependent antigen forms a germinal center (GC) when it infiltrates into secondary lymphoid organs. The GC comprised of B cells undergoing somatic hyper-mutation of the B cell receptor. In this process, B cells with high-affinity receptors are selected and differentiated into plasma cells and memory B cells with the aid of T cells. The T cells, which provide B cells with help, are called follicular helper T (Tfh) cells. Tfh cells are essential because of their roles in offering survival signals to GC B cells and promoting differentiation [24].

To investigate the functional role of Tscm-derived Tfh cells, antibody production by B cells was measured in the presence of Tfh cells. Sorted Tscm cells were stimulated with anti-CD3/CD28 beads for 6 days at 37℃. To measure the ability of the Tfh cells in helping B cells to produce antibodies, the same numbers of Tscm cells after stimulation were placed with autologous B cells (Fig 7. A). Then, 200 ng/mL of SEB was used to crosslink the T/B cells [25]. The T-B co-culture was maintained for 6 days, and the secreted antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (Fig 7. B). Consistent with a previous study [17], the amount of secreted IgG antibodies was increased in SLE when the Tfh cells and autologous B cells were co-cultured (12007.8075±4457.5425 (SLE) vs 2082.1780±517.822 (HC).

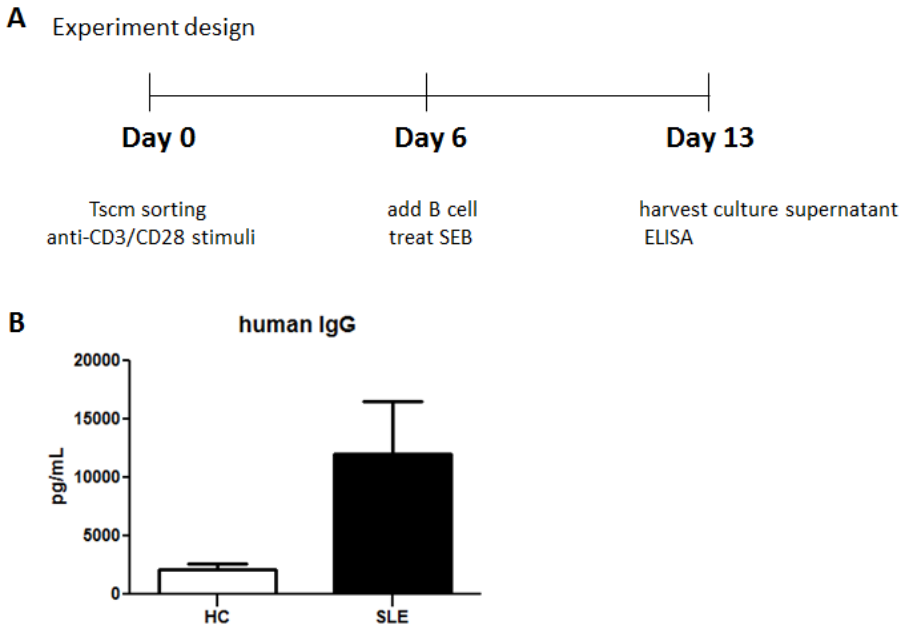

Figure 7. Follicular helper T cells – B cells co-culture and IgG assay

(A) Experiment design. Day 0, sorted Tscm cells were stimulated with anti-CD3/CD28 beads for 6 days. On day 6, after removing the TCR stimulant, proliferated cells were counted. During the 6 days, the Tscm cells proliferated; thus, their cell numbers usually increased. Autologous B cells were added at a ratio of 1:1. In addition, SEB was added at a concentration of 200 ng/mL to the experimental group and 0 ng/mL to the control group. On day 13, secreted IgG levels were detected with ELISA. (B) Secretion of human immunoglobulin. Human immunoglobulins from activated B cells were measured with ELISA. The white bar represents the HCs, and the black bar represents the SLE group.

Correlation between Tscm cells and disease activity in SLE

To evaluate the clinical significance of Tscm cells in SLE, the association between the level of Tscm and the clinical manifestations of SLE were evaluated.

The proportion of Tscm in naive T cells was not associated with the disease activity of SLE (SLEDAI) (Fig 8. A). The level of Tscm cells was not different depending on the presence of anti-dsDNA, anti-cardiolipin antibody, and serum C3/C4 (Fig 8. B and C ; Table 3).

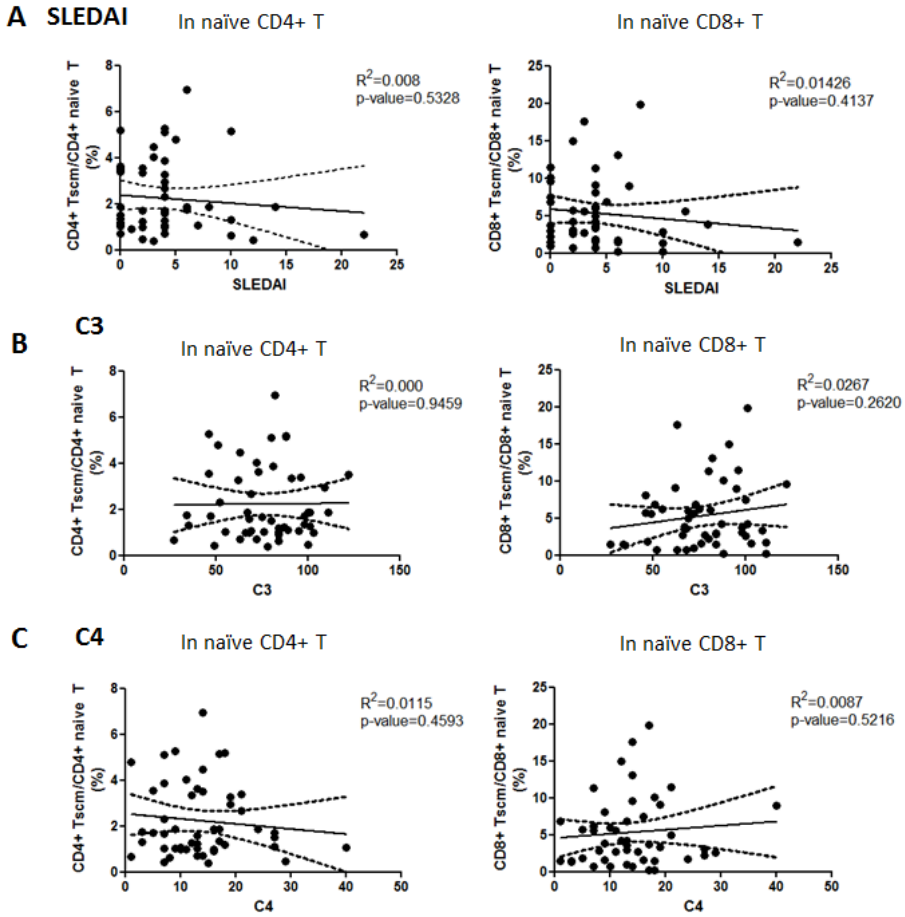

Figure 8. No association between the proportion of Tscm cells and lupus disease activity.

(A) Association between SLEDAI and the ratio of Tscm cells ($CD4^+$ Tscm/naive $CD4^+$ T, $CD8^+$ Tscm/naive $CD8^+$ T). Relationship between the ratio of Tscm cells ($CD4^+$ Tscm/naive $CD4^+$ T ; $CD8^+$ Tscm/naive $CD8^+$ T) and the (B) C3 or (C) C4 level. R-square and p-value are described in each graph.

Table 1. Clinical characteristics of the patients

	SLE patients (n = 52)
Age at diagnosis, years, mean ± SD	41.2 ± 12.9
Female, n (%)	48 (92.3)
SLE duration, years, median ± IQR	8.83 [5.67-14.75]
SLEDAI-2k score at experiment, median ± IQR	4 [2-6]
Laboratory and serological feature	
White blood cell count (x 10^3/ul), median ± IQR	5.13 [4.28-7.25]
Lymphoocyte (%), median ± IQR	22 [14.1-34.6]
Hemoglobin (mg/dl), median ± IQR	12.5 [11.4-14]
Platelet (x 10^3/uL), median ± IQR	229 [169-283]
ESR (mm/hour), median ± IQR	21 [12-33]
Anti-dsDNA titer (IU/ml), median ± IQR	13.85 [5.33-44.80]
C3 (mg/dl), median ± IQR	80 [65 – 96]
C4 (mg/dl), median ± IQR	13 [8 – 18]
Treatment, n(%)	
Corticosteroids	46 (88.5)
Corticosteroid dose (prednisolone equivalent), mg/day	5 [2.5 – 10]
Hydroxychloroquine	25 (48.1)
Mycophenolate mofetil	10 (19.2)
Azathioprine	10 (19.2)
Methotrexate	3 (5.8)
NSAIDs	12 (23.1)

ESR, erythrocyte sedimentation rate; IQR, interquartile range; NSAIDs, Nonsteroidal anti-inflammatory drugs; SLE, systemic lupus erythematosus; SLEDAI-2K, SLE disease activity index 2000. z

Table 2. Real-time qPCR primer sequences

5’ to 3’	**Forward**	**Reverse**
Bcl-6	CTGCAGATGGAGCATGTTGT	TCTTCACGAGGAGGCTTGAT
CXCR5	GCTAACGCTGGAAATGGA	GCAGGGCAGAGATGATTT
PD-1	ATGCAGATCCCACAGGCGCCCTGGCC	TCAGAGGGGCCAAGAGCAGTGTCCATC
ICOS	GTGCTCACTGGGAGTGGAAT	GTCAACTGGGTTCAGCAAT
β-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
IL-21	CCACAAATCAAGCTCCCAAG	CAGGGACCAAGTCATTCACA
Blimp-1 (*PRDM-1*)	TCCAGCACTGTGAGGTTTCA	TCAAACTCAGCCTCTGTCCA
LEF-1	AATGAGAGCGAATGTCGTTG	GCTGTCTTTCTTTCCGTGCTA
TCF-1 (*TCF-7*)	TCCAGAGCCCCTGGAGGACG	GGGCTGATTGGCCTTGTGCA

Table 3. Ratio and absolute counts of the Tscm cells according to the presence of autoantibodies in SLE.

Autoantibodies		CD4+ Tscm (in CD4+ naïve T)	CD4+ Tscm absolute counts	CD8+ Tscm (in CD8+ naïve T)	CD8+ Tscm absolute counts
Anti-dsDNA	High, mean (SD)	2.4004 (1.8898)	2834.8421 (4233.9928)	4.4512 (3.3146)	5933.85 (7536.0848)
	Low, mean (SD)	2.0733 (1.3514)	4063.0667 (3923.5285)	5.6548 (4.4223)	6861.1333 (6632.7116)
	P-value	0.797	0.128	0.396	0.202
Anti-Sm	Positive, mean (SD)	1.7079 (1.22)	1947.8462 (1722.3891)	4.51 (4.4292)	7703.786 (7151.238)
	Negative, mean (SD)	2.7038 (1.5896)	5402.125 (5048.5045)	7.098 (4.8223)	6917 (7487.4168)
	P-value	0.074	**0.045***	0.07	0.637
Anti-SSA/Ro	Positive, mean (SD)	2.5405 (1.5958)	3259.1538 (3396.887)	5.9626 (5.6923)	4856.9167 (5727.9709)
	Negative, mean (SD)	1.8782 (1.5575)	3163.5455 (4051.9900)	5.3482 (4.4575)	7347 (7233.7795)
	P-value	0.123	0.569	0.832	0.225
Anti-SSB/La	Positive, mean (SD)	2.5763 (1.5488)	3400 (2829.2233)	7.3138 (7.8984)	7948.6 (11943.65)
	Negative, mean (SD)	2.3579 (1.8445)	3881.65 (4945.4483)	5.7004 (4.1765)	5702.55 (4794.4366)
	P-value	0.380	0.921	0.983	0.717
Anti-cardiolipine	Positive, mean (SD)	2.7114 (1.6914)	3078.1818 (4001.0112)	4.6593 (3.4066)	4501.8182 (5333.8473)
	Negative, mean (SD)	2.1305 (1.3814)	4723.5385 (5193.0229)	5.9741 (5.2172)	5554.4615 (4612.8624)
	P-value	0.454	0.252	0.68	0.424
Serum C3	High, mean (SD)	2.3756 (1.7416)	5500.8 (5092.0983)	6.1317 (5.1842)	7881.533 (7354.946)
	Low, mean (SD)	2.0304 (1.4221)	1631.4545 (1794.3983)	4.4607 (3.6103)	4899.3913 (6409.7044)
	P-value	0.436	**0.012***	0.194	0.194
Serum C4	High, mean (SD)	2.3674 (1.7428)	4393.9231 (4644.4179)	6.1714 (5.6331)	7397.4615 (8200.907)
	Low, mean (SD)	2.0607 (1.4504)	2553.4583 (3468.815)	4.5459 (3.2353)	5389.68 (6125.0607)
	P-value	0.492	0.181	0.235	0.4

Mann Whitney U test; Tscm, stem cell-like memory T; ANA, antinuclear antibody; dsDNA, double-strand DNA ; *, p≤ 0.05; **, p≤ 0.01; ***,p≤ 0.001

DISCUSSION

This study shows that the proportions of $CD4^+$ Tscm cells are increased in patients with SLE compared with healthy controls and that isolated Tscm cells can replicate themselves and can differentiate into different subsets of T lymphocytes, including naïve-like T, central memory and effector memory T lymphocytes. Tscm cells can also differentiate into Tfh cells, which are regarded as a main driver of SLE pathogenesis by helping B cell activation in the germinal center of secondary lymphoid organs. Differentiation of Tfh cells is made possible by the regulation of IL-21 and Blimp-1 which are in turn regulated by TCF-1. Tfh differentiated from Tscm cells can make autologous B cells secrete immunoglobulins.

In this study, Tscm cells were designated as $CD3^+$ $CD4^+$/$CD8^+$ $CD45RO^-$ $CCR7^+$ $CD45RA^+$ $CD62L^+$ $CD27^+$ $CD28^+$ CD127 $(IL\text{-}7R\alpha)^+$ CD122 $(IL\text{-}2R\beta)^+$ $CD95^{hi}$ cells. Among the Tscm surface markers, $CCR7^+$, $CD45RO^-$, $CD45RA^+$, and $CD62L^+$ represent the naive characteristics of T cells. CD28 is a costimulatory molecule, which interacts with CD80 (B7-1) and CD86 (B7-2) of antigen presenting cells. Optimal stimulation of T cells requires stimulatory signals through CD28 in addition to TCR stimulation. Binding of CD27 and its ligand CD70 promotes TCR-induced expansion of both $CD4^+$ and $CD8^+$ T cells [26]. CD27 and CD28 are more highly expressed by central memory T cells and naive T cells. CD95 is a pro-apoptotic molecule. Gattinoni et al.

suggest that CD95 might have a functional and crucial role in the self-renewal and persistence of memory T cells by inducing T cell factor (TCF)-β-catenin signaling and suggested that T cell factor (TCF)-β-catenin signaling has a role in the maintenance of memory T cells [3]. Y. Amasaki et al. reported increased expression of CD95 molecules on the T cells of SLE patients and their role in leukopenia and lymphopenia [27]. Increased CD95 in the PBMCs of SLE patients can partly be explained by the increased Tscm cells in the SLE patients.

The proportions of $CD4^+$ Tscm or $CD8^+$ Tscm were increased in the SLE patients. The pathogenesis of SLE is characterized by the presence of various autoantibodies produced by activated B cells [13]. Because the proper activation of B cells requires the help of $CD4^+$ T cells, increased levels of $CD4^+$ Tscm, as a source of helper T cell, is important. In contrast, the numbers of $CD4^+$ Tscm cells were comparable between the SLE patients and HCs when the absolute numbers of Tscm cells were counted with the Accucheck counting beads (Fig 2. C). Contradictory results are due to the fact that SLE patients generally suffer from leukopenia or lymphopenia.

Stem cells are characterized by their self-renewal nature and their ability to differentiate into different cell subsets [3]. Tscm, as stem cell-like cells, should show the essential characteristics of stem cells. As shown in this study, the Tscm cells, regardless of whether they were from the SLE patients or HCs, could replicate themselves and effectively differentiate into naïve-like T,

memory, and effector T cell subsets when the appropriate stimulus was administered (Fig 3), fulfilling the requirement as a stem cell.

Even though the Tscm cells from the SLE patients show similar characteristics to those from the HCs, the cytokine patterns secreted from these cells after activation were different from those of the HCs.

Inflammatory cytokines, type I interferon (IFN-α), type II interferon (IFN-γ), IL-6, IL-1, and TNF-α have been suggested as critical cytokines in SLE patients. Furthermore, immunomodulatory cytokines IL-10 and TGF-β, as well as IL-21, IL-17, and IL-2, were recently identified as crucial in SLE [28]. In this study, the secreted levels of IFN-γ, TNF-α, and IL-2 from the activated Tscm cells of the SLE patients were higher than those of the HCs (Fig 4). This result suggests that the Tscm cells of the SLE patients have inherent abnormalities toward pro-inflammatory cytokine patterns. It also suggests that simply targeting differentiated cells may not be enough to eradicate the SLE completely. Interestingly, secretion of IFN-α, which is regarded as a key cytokine in SLE, was not increased in the SLE patients compared with the HCs. As previously known, IFN-α secreted by plasmacytoid dendritic cells is more influential than IFN-α secreted by T cells [29-31].

In this study, Tfh cells were differentiated from Tscm cells. Tfh cells have important roles in the pathogenesis of SLE by helping B cells make autoantibodies against self-tissue. To reveal the underlying mechanism of the production of Tfh cells from Tscm cells, IL-21 and Blimp-1 were analyzed

from activated Tscm cells. Maintenance of Bcl-6, which is the critical transcription molecule of Tfh cells [9], is balanced by IL-21, which maintains Bcl-6, and Blimp-1, which down-regulates Bcl-6 [20]. The increased level of IL-21 and decreased level of Blimp-1 in the activated Tscm cells in this study suggest that a favorable balance toward Tfh cells is achieved in the SLE Tscm cells when they are appropriately stimulated.

During the differentiation process of Tfh cells from Tscm cells, the expression levels of TCF-1 significantly increased after stimulation of the Tscm cells from the SLE patients compared with that of the HCs (Fig 5). According to Choi et al., Wnt-β-catenin signaling transcription factors LEF-1 and TCF-1 can differentiate activated $CD4^+$ T cells into Tfh cells in a mouse animal model [32]. In their study, Tcf7(encoding TCF-1)$^{-/-}$ or Lef1(encoding LEF-1)$^{-/-}$Tcf7$^{-/-}$ mice had significantly lower numbers and frequencies of GC Tfh cells (Bcl-6^+ $CXCR5^+$ PD-1^{hi}) and $GL7^+$ Fas^+ GC B cells. In a human cell line, *in vitro* data showed the binding of TCF-1 to Bcl-6 using 293 T human embryonic kidney cells [22]. On the other hand, the association between LEF-1 and Tfh cells has not been identified. TCF-1 represses the function of Blimp-1 (encoded by prdm-1) by binding them. Blimp-1 is known as a repressor of Bcl-6 which is essential in the differentiation of Tfh cells. Thus, the up-regulation of TCF-1 promotes differentiation toward Tfh cells. Therefore, it can be inferred that increased expression of TCF-1 caused by the activation of Wnt-β-catenin signaling suppresses the Blimp-1 transcription

factor and increases the level of Bcl-6 which subsequently leads to Tfh cells.

The serum levels of IL-6 were significantly elevated in the SLE patients compared with the healthy controls [32, 33]. Moreover, for the development of early Bcl-6^+ $CXCR5^+$ Tfh cells, IL-6 signals are known to be required [34]. In another study, activation of STAT-3 by pre-treated IL-6 increased the TCF-1 and β-catenin levels [35]. In the case in which IL-6 levels are high in SLE patients, the development of early Tfh cells may be fulfilled by the increased TCF-1.

The proportion of $CD4^+$ Tscm cells among naive T cells showed no association with the disease activity of SLE, which was measured by SLEDAI (Figure 8). The level of Tscm was not different depending on the presence of specific autoantibodies. These results suggest that Tscm cells contribute to the development and maintenance of the disease while disease activity is determined by other factors.

There are several limitations in this study. First, the number of SLE patients may not be enough to reveal the fine role of Tscm in SLE pathogenesis. Second, all the patients were Koreans. Therefore, further studies may be needed to confirm the generalizability of our study to other ethnic groups.

In conclusion, the proportion of Tscm cells was increased in the SLE patients compared with the HCs, and the Tscm cells can maintain SLE by its ability to differentiate into as well as maintain follicular helper T cells.

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국문 초록

새로 발견된 기억 T 세포인 기억 줄기 T 세포(Tscm)가 최근에 발견되었다. 이전의 연구에서 기억 줄기 T 세포는 그들 자신의 전구 세포를 만들 수 있고 다른 T 세포들로 분화할 수 있다고 밝힌 바 있다. 전신홍반루푸스는 자가면역질환으로, 면역 복합체-매개성 보체 활성화로 인한 전신 염증성 소견이라는 특징을 가진다. 이 질병에서 자가면역적 반응이 지속됨은 비정상적인 면역 줄기 세포가 있다고 예상할 수 있다. 이 학위논문에서 나는 전신홍반루푸스의 Tscm 세포의 존재와 기능을 조사하였다. Tscm 세포는 $CD3^+$ $CD4^+/CD8^+$ $CD45RO^-$ $CCR7^+$ $CD62L^+$ $CD45RA^+$ $CD27^+$ $CD28^+$ $CD127^+$ $CD122^+$ $CD95^+$ 세포로 정의하였다. 미접촉 $CD4^+/CD8^+$ T 세포 중 $CD4^+$ 그리고 $CD8^+$ Tscm 세포의 비율이 건강 대조군에 비해 SLE 환자에서 매우 증가하였다. SLE 환자의 Tscm 세포는 그들 자신을 복제할 수 있었고, 미접촉 유사 T 세포 ($CD3^+$ $CD4^+/CD8^+$ $CD45RO^-$ $CCR7^+$ $CD45RA^+$), 중심 기억 T ($CD3^+$ $CD4^+/CD8^+$ $CD45RO^+$ $CCR7^+$ $CD45RA^-$), 효과 기억 T ($CD3^+$ $CD4^+/CD8^+$ $CD45RO^+$ $CCR7^-$ $CD45RA^-$) 림프구로 분화할 수 있었다. 전신홍반루푸스 환자의 Tscm 세포에 자극을 가한 후 건강 대조군과 비교했을 때, 인터페론-γ, 종양과다인자-α 그리고 인터류킨-2 를 더 많이 분비하였다. 전신홍반루푸

스 환자의 Tscm 세포는 Tfh 세포로 분화할 수 있었고, 분화된 Tfh 세포는 B 세포가 항체를 생성할 수 있도록 만들 수 있었다. Tfh 세포는 IL-21 의 증가와 TCF-1 조절 인자들의 발현 수준을 통해 통제되는 Blimp-1 의 억제를 통해 유도되었다. Tscm 세포의 비율은 SLEDAI 로 측정되는 전신홍반루푸스 질병 활성도와 아무 연관이 없었다는 것으로 보아, Tscm 세포는 질병의 활성도가 아닌 질병의 발달을 결정한다고 할 수 있다.

종합적으로, 이 연구는 Tscm 세포의 비율이 SLE 환자에서 증가되어 있고, Tscm 세포로부터 분화된 Tfh 세포는 B 세포의 항체 생성을 도움으로서 전신홍반루푸스를 유지하는 데에 역할을 한다는 것을 설명하고 있다.

주요어: 기억 줄기 T 세포, 전신홍반루푸스, follicular 도움 T 세포
학 번: 2014-22225