Impaired Function of PD-1+ Follicular Regulatory T Cells in Systemic Lupus Erythematosus

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Abstract

Objective:

Aberrant autoantibody production is characteristic of systemic lupus erythematosus (SLE) but follicular regulatory T (TFR) cells potentially can suppress this abnormality. Here, we investigate functional changes in TFR cells from SLE patients.

Methods:

Circulating TFR cells were collected from 19 SLE patients and 14 healthy controls to compare molecular expression and *in vitro* suppressive capacity of follicular helper T (TFH) cell proliferation. We then tested IL-2 in SLE-TFR cells to check restoration of suppressor function.

Results:

Programmed death-1 (PD-1) expression in SLE-TFR cells was positively correlated with anti-DNA antibody levels and disease activity. These cells had impaired suppressive function for TFH cells with decreased expression of suppression mediators forkhead box p3 (Foxp3), cytotoxic T-lymphocyte antigen 4 (CTLA4), and IL-2 receptor alpha (IL2Rα). *In vitro* IL-2 stimulation restored expression of these molecules.

Conclusion:

SLE-TFR cells have functional TFH suppression defects but low-dose IL-2 therapy could be useful to restore this ability.

Background

Systemic lupus erythematosus (SLE) is a chronic, systemic, and progressive autoimmune disease that affects various organs and tissues (1). Although the pathophysiology of SLE is partly understood, pathogenic T-B cell interactions with autoantibody production are a hallmark of the disease. Of note, the anti-double stranded DNA (dsDNA) antibodies produced by these interactions have high disease specificity and are valuable in the prognostic role (2).

Several reports on the contribution of follicular helper T (TFH) cells for SLE autoantibodies exist. TFH cells themselves are a CD4 + helper T cell subset that facilitates B cell maturation and antibody secretion via cell surface molecules and cytokines such as programmed death-1 (PD-1) and interleukin (IL) -21 (3). These cells are reported to expand in the peripheral blood of SLE patients, promote autoantibody production, and associate with disease activity (4–6).

Follicular regulatory T (TFR) cells are a subset of regulatory T (Treg) cells that specifically regulate the function of TFH cells via expression of C-X-C chemokine receptor (CXCR) 5 and forkhead box P3 (Foxp3), allowing for localization to germinal centers (GCs) and suppression of TFH cell activation (7). In SLE, some reports have observed an altered balance of circulating TFH and TFR cells (8, 9); however, compared to the frequency change of TFR cells, much less is known regarding their function in SLE.

Here, we reveal an impaired suppressive function of TFR cells in SLE patients and the potential role of IL-2 for the restoration of this immunosuppressive function.

Methods

Patients

Peripheral blood mononuclear cells (PBMCs) and sera were collected from Japanese SLE patients (n = 19) or healthy volunteers free from autoimmune diseases, infections, or malignancies (n = 14). The patients met the revised criteria of the American College of Rheumatology for SLE (10) and/or the Systemic Lupus International Collaborating Clinics classification criteria for SLE (11) and had no comorbid autoimmune diseases. All clinical and biologically relevant patient information is shown in Supplementary Table 1. Disease activity was assessed with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) -2k (12). All patients were recruited at the University of Tsukuba Hospital from December 2019 to September 2020. All volunteers gave written consent to enroll in the study. Detailed information for all experimental methods is described in Supplementary Methods.

Statistical analysis

All data are expressed as mean ± standard deviation (SD) unless otherwise specified. Differences between groups were evaluated for statistical significance using the Mann-Whitney U test. Fisher's exact test was used for comparison of categorical value and correlations were evaluated using Spearman's correlation test. Friedman test was used to compare the molecular expression of the stimulated cells (Fig. 2F). *P* values less than 0.05 were considered statistically significant. Statistical analyses were performed using Prism version 9 (Graphpad Software, San Diego, CA).

Results

PD-1 expression of TFR cells was elevated and correlated with disease activity in SLE.

We analyzed circulating CD4 + CXCR5 + cells in 19 SLE patients (SLEs) and 14 age- and sex-matched healthy controls (HCs) (Fig. 1A). Comprehensive clinical features are shown in Supplementary Tables 1 and 2. Frequencies of TFH and TFR cells in these patients were comparable to HCs (Fig. 1B) although we found TFR cells expressed more PD-1 versus HCs. PD-1 in TFH cells trended higher in the SLEs group although this difference was not statistically significant (Fig. 1C). PD-1 in both cell types had positive correlations with anti-DNA antibody levels (Fig. 1D) and disease activity (Fig. 1E). TFH cells were also

correlated with observed clinical parameters; however, only TFR cells were positively correlated with serum IL-21 levels (Fig. 1F).

Although PD-1 in CD4 + CXCR5 + cells is regarded as an active disease marker, these data suggest that PD-1 expression in TFR cells also represents disease activity.

TFR cells from SLE patients had impaired suppressive function.

We subsequently explored the functional change of TFR cells within SLE patients. As expected, frequencies of Foxp3^{hi}CD45RA-activated TFR cells and Foxp3^{lo}CD45RA-non-suppressive TFR cells were altered in SLEs (Fig. 2A). An *in vitro* suppression assay with CD4 + CXCR5 + CD25^{hi} TFR cells (Supplementary Fig. 1) also revealed that suppression of proliferation and IL-21 production from TFH cells were impaired in SLEs-TFR cells compared to HCs-TFR cells (Figs. 2B and 2C). To clarify whether PD-1 expression in TFR cells is a loss-of-function marker, we tested PD-1 + TFR cells derived from HCs and observed a reduced suppressive capacity versus PD-1- TFR cells, though this was not statistically significant (Supplementary Fig. 2).

We further analyzed the decreased molecular expression of Foxp3, cytotoxic T-lymphocyte antigen 4 (CTLA4), and IL-2 receptor alpha (IL2Rα) expression in SLEs-TFR versus HC-TFR cells. Glucocorticoid-induced TNF receptor (GITR) expression tended to be higher in SLEs-TFR cells but this change was not statistically significant (Fig. 2D). Moreover, pyrosequencing of conserved non-coding sequence (CNS) 2 at the Foxp3 gene locus revealed hypomethylation in SLEs-TFR cells (Fig. 2E), meaning that epigenetic modulation could explain the suppressed Foxp3 transcription and functional declines observed in SLEs-TFR cells.

Having confirmed the altered function of SLEs-TFR cells, we next investigated the therapeutic potential for these cells by culturing them with various concentrations of recombinant IL-2/TCR stimulation *in vitro* for 96 hours. With 1.0 ng/mL of IL-2, PD-1 expression of TFR cells significantly decreased along with increased expression of Foxp3, IL2Rα, and CTLA4 (Fig. 2F).

These results indicate that PD-1 + SLEs-TFR cells have ablated suppressive function for TFH cell proliferation and activation but can be rescued by IL-2.

Discussion

Our study demonstrates the impaired suppressive function of PD-1 + TFR cells in SLE. PD-1 expression levels in these cells were positively correlated with both disease activity and disease-specific autoantibody production as well as decreased expression of CTLA4 and Foxp3. Moreover, *in vitro* IL-2 treatment was capable of restoring this lost effector function.

TFH and TFR cells have been proposed to play a critical role in SLE as defects within TFR cells could prevent suppression of TFH cells that stimulate production of possibly autoreactive antibodies. Studies

regarding circulating TFR cell numbers in SLE patients have generated controversial results (8, 9, 13) but we observed no quantitative differences within our cohort. Instead, our results identified impaired suppression of TFH cells in SLE-TFR cells.

PD-1 expression is difficult to interpret in TFR cells but, in TFH cells, PD-1 has been reported as an active state marker (14, 15). Although our study found no statistically significant difference in TFH cell PD-1 expression between SLEs and HCs, SLE patients are reported to have PD-1^{hi} active TFH cells (5). Moreover, since high PD-1 expression in Treg cells indicates a dysfunctional or 'exhausted' state (16, 17), the high expression of PD-1 in SLE-TFR cells we found was similarly related to impaired regulatory functions. This is in line with murine experiments in which Sage et al. reported that PD-1 signaling mediates the generation of TFR cells (18).

In SLE patients, Treg cell functions are reported to decline (19), with Foxp3 expression consistently decreased, possibly due to IL-2 deficiency or other mechanisms. As Hao et al. have reported the conversion of TFH cells to TFR cells by IL-2 (13), our study, which showed the recovery of Foxp3 and other regulatory molecules in TFR cells by *in vitro* IL-2 supplementation, was in line with these findings.

Certain limitations must be acknowledged. As we have described in the Supplementary Tables, almost all SLE patients were receiving glucocorticoids or immunosuppressants at the time of sample collection, possibly affecting TFH and TFR frequencies and phenotypes. Additionally, it has been recently reported that different subsets of immune cells exist in the inflammatory locus versus the peripheral blood (20). Analyses using peripheral blood, such as the present study, may thus not be able to fully explain the pathogenetic mechanisms of SLE.

Taken together, since we have confirmed the dysfunction of PD-1 + TFR cell suppression in SLE and found IL-2 to be restorative, low-dose IL-2 treatment could provide potential therapeutic benefits for SLE. Furthermore, assessing PD-1 expression molecularly rather than with only cell frequency is important for predicting TFR cell activation and humoral immune responses in SLE.

Conclusion

Quantitative abnormalities of TFR cells were reported in SLE. In the present study, we clarified that TFR cells are functionally impaired in SLE, with high expression of PD-1 which correlates the disease activity and anti-DNA antibody production. In addition, IL-2 could restore this impaired function. Assessing PD-1 expression molecularly rather than with only cell frequency is important for predicting TFR cell activation and humoral immune responses in SLE. Low-dose IL-2 treatment could provide potential therapeutic benefits for SLE in the perspective of TFR cell restoration.

List Of Abbreviations

CNS
conserved non-coding sequence

CTLA4
cytotoxic T-lymphocyte antigen 4
CXCR
C-X-C chemokine receptor
dsDNA
double stranded DNA
Foxp3
forkhead box protein 3
GC
germinal center
GITR
glucocorticoid-induced TNF receptor
HC
healthy control
IL
interleukin
IL2Rα
interleukin-2 receptor alpha
PBMC
peripheral blood mononuclear cell
PD-1
programmed death-1
SD
standard deviation
SLE
systemic lupus erythematosus
SLEDAI
systemic lupus erythematosus disease activity index
TFH
follicular helper T
TFR
follicular regulatory T
Treg
regulatory T

Declarations

Ethics approval and consent to participate

Written, informed consent was obtained from all patients and volunteers prior to their participation. The research protocol was approved by the clinical research ethics committee of the University of Tsukuba Hospital (#H24-164).

Consent for publication

Written, informed consent was obtained from all patients and volunteers prior to their participation. They all agreed to publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

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Authors’ contributions

IK, NM, and IM designed the study. IK and NM performed experiments and collected the data. IK, NM, AOh, AOs, YK, HT, and IM participated in critical discussions related to the study. IK and IM analyzed the data and wrote the manuscript. All authors have read and approved the manuscript for publication.

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Figures

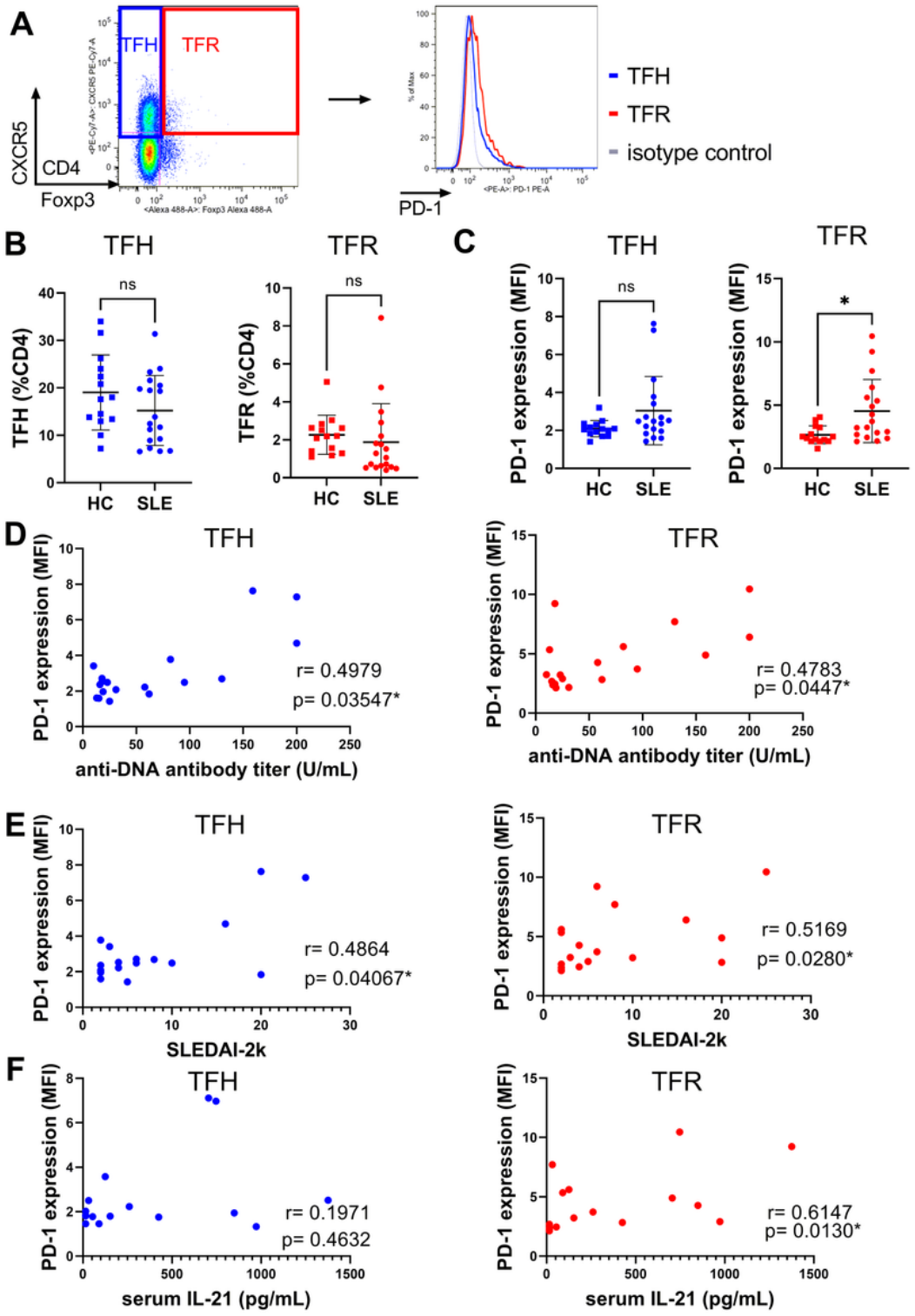

Figure 1

Elevated PD-1 expressions of TFR cells are associated with disease activity in SLE. A) Representative plots of flow cytometric analysis of peripheral CD4+ cells. B) and C) Comparison of follicular helper T (TFH) and follicular regulatory T (TFR) cell frequencies (B) and PD-1 expressions (C) in peripheral CD4+ cells between systemic lupus erythematosus (SLE) patients and HCs. PD-1 expression was compared using the mean fluorescence intensity ratio to isotype controls. D) and E) Correlation analysis of PD-1

expression in TFH and TFR cells with anti-DNA antibody titers (D) and disease activity (E) in SLE patients. These data were derived from medical records. F) Correlation analysis of PD-1 expression of TFH and TFR cells with serum IL-21 levels in SLE patients. Data are mean±SD. *: p< 0.05, ns: not significant. CXCR5: C-X-C chemokine receptor 5, Foxp3: forkhead box P3, TFH: follicular helper T, TFR: follicular regulatory T, PD-1: programmed death-1, SLEDAI: SLE disease activity index.

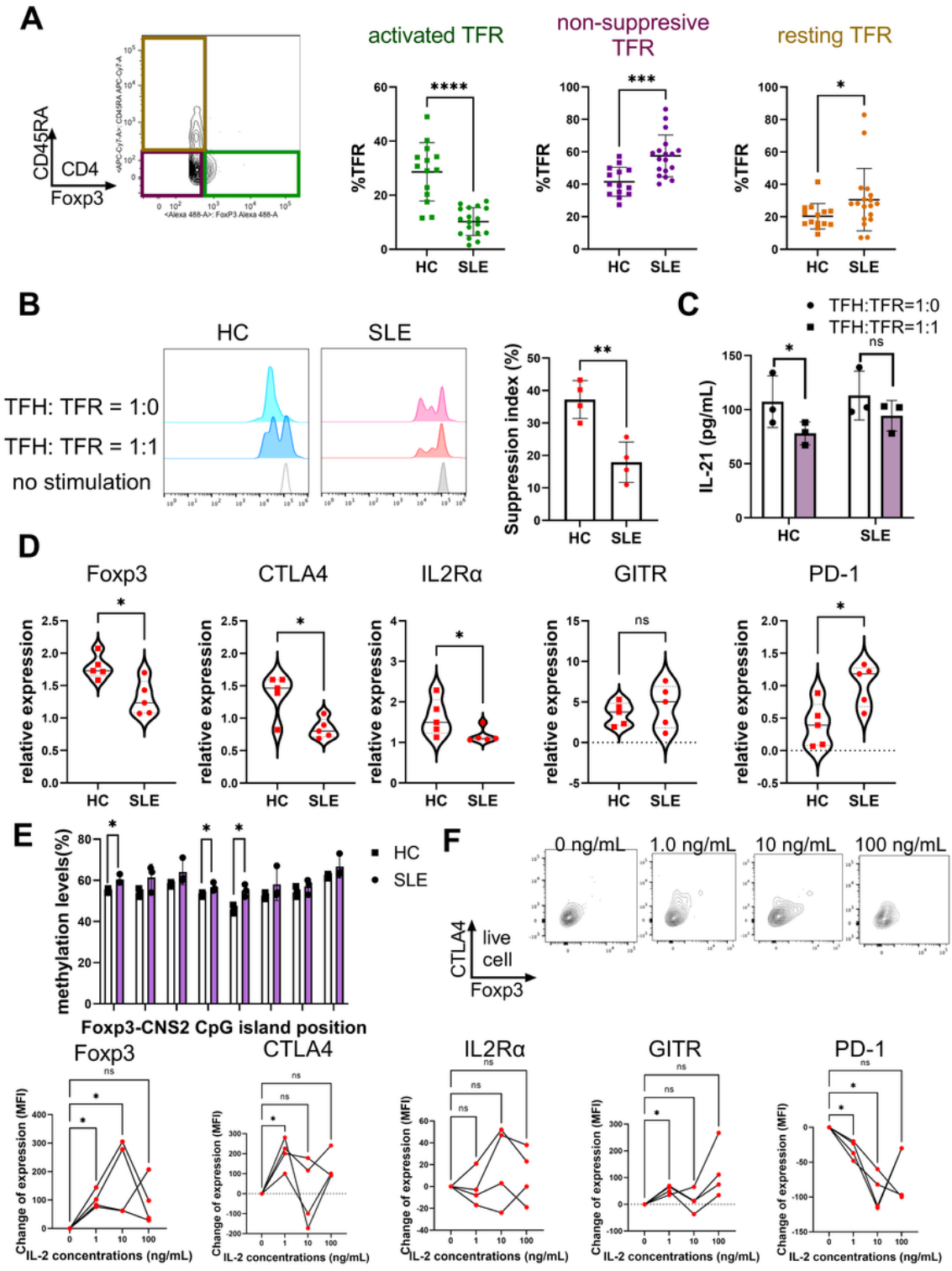

Figure 2

SLE-TFR cells are functionally impaired and IL-2 stimulation restores molecular expression-mediated suppressor function. A) Comparison of Foxp3 and CD45RA expression between HC- and SLE-TFR cells. Activated TFR cells are gated as CD45RA-Foxp3hi, non-suppressive TFR cells as CD45RA-Foxp3lo, and resting TFR cells as CD45RA+Foxp3lo. B) Comparison of suppressive capacity for TFH cell proliferation by TFR cells. CellTrace Violet-labelled TFH cells were cultured with or without presence of TFR cells under TCR stimulation for 96 hours. Suppressive capacity was calculated using a ratio of TFH cell proliferation under the concentration of TFR cells written in the figure. C) Comparison of IL-21 concentrations of TFH-TFR coculture supernatant. D) Comparison of molecular expressions of TFR cells as measured by qPCR. The gene expression values were normalized to those of the control gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). E) Methylation analysis of CNS2 in Foxp3 locus. Methylation levels of eight CpG islands were quantified by pyrosequencing. Methylation levels of TFH cells were analyzed for positive control of methylation. F) Change of molecular expression after IL-2 stimulation of SLE-TFR cells. TFR cells derived from SLE patients were cultured under TCR stimulation and various IL-2 concentrations for 96 hours. Molecular expression in cultured cells was analyzed with flow cytometry. Data are expressed as the mean fluorescent intensity changes from baseline (no IL-2 stimulation). Data are mean±SD. *: p< 0.05, **: p< 0.01, ***: p< 0.001, ****: p< 0.0001, ns: not significant. CTV: CellTrace Violet, CTLA4: cytotoxic T-lymphocyte antigen 4, GITR: glucocorticoid-induced TNF receptor.

Supplementary Files

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