

Expression of intracellular transforming growth factor-beta1 in CD4⁺CD25⁺ cells in patients with systemic lupus erythematosus

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Background and Purpose: The CD4⁺CD25⁺ regulatory T (Treg) cells exert immunoregulatory functions in various autoimmune diseases, in part through transforming growth factor-beta1 (TGF-β1), and can be expanded by TGF-β1 stimulation in normal subjects. This study aimed to examine intrinsic TGF-β1 expression and the response to TGF-β1 stimulation of this CD4⁺CD25⁺ subset in patients with systemic lupus erythematosus (SLE).

Methods: Flow cytometry with multicolor staining of CD4⁺, CD25⁺, and TGF-β1 was used to quantify the percentage of CD4⁺CD25⁺ T cells in fresh peripheral blood and TGF-β1-stimulated peripheral blood mononuclear cell (PBMC) cultures, and their corresponding intracellular TGF-β1 expression.

Results: In fresh peripheral blood, we found that decreased percentages of CD4⁺CD25⁺/CD4⁺ in SLE patients were associated with disease activity and renal involvement. Intracellular TGF-β1 expression of CD4⁺CD25⁺ cells was significantly elevated in SLE compared with matched controls ($p < 0.001$). In addition, there was significant negative correlation between TGF-β1 expression and percentage of CD4⁺CD25⁺ cells present ($r = -0.432$, $p = 0.004$). Nevertheless, in ex vivo unstimulated PBMC cultures, the percentage and intracellular TGF-β1 expression of CD4⁺CD25⁺ cells of SLE were normalized to the levels of the control group. In TGF-β1-stimulated PBMC cultures, CD4⁺CD25⁺ cells and their intracellular TGF-β1 expression were significantly increased ($p < 0.001$), both in SLE and controls. Moreover, the increments in the percentage of CD4⁺CD25⁺ cells and intracellular TGF-β1 expression by TGF-β1 stimulation were comparable in SLE and controls, and were not significantly influenced by disease activity or renal involvement in SLE.

Conclusions: CD4⁺CD25⁺ cells were deficient in peripheral blood but not impaired either in intrinsic TGF-β1 expression or in response to TGF-β1 stimulation in patients with SLE. This study suggests that TGF-β1, by inducing CD4⁺CD25⁺ cells, has potential clinical application in treating SLE.

Key words: CD4-positive T-lymphocytes; Lupus erythematosus, systemic; T-lymphocytes, regulatory; Transforming growth factor-beta1

Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterized by a breakdown of self-tolerance, resulting in pathogenic autoantibodies, immune complexes, and multiorgan injury. Overactivation of B

lymphocytes is one of the hallmarks of SLE [1]. It has been demonstrated that sustained hyperactivity of autoreactive B lymphocytes and failure of feedback regulation to maintain tolerance to self-antigens is T-cell-dependent and leads to perpetuation of SLE [2].

Considerable progress has been made in identifying regulatory T (Treg) cells that were believed to suppress harmful autoimmunity [3-5]. Defined by the distinct origins and cytokine profiles, Treg cells can be classified into at least three categories:

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CD4⁺CD25⁺ cells [3], type 1 T regulatory (Tr1) cells [6] and T-helper type 3 (Th3) cells [7]. Among them, the CD4⁺CD25⁺ Treg cells have been demonstrated to play a crucial role in maintaining immune tolerance [8], namely in sustaining self-tolerance and control of a variety of immune responses to non-self (such as microbial antigens of allogeneic transplantation antigens) or quasi-self (such as tumor-associated antigens) [9].

Recent studies showed that mice with depletion of CD4⁺CD25⁺ T cells were susceptible to the development of spontaneous lupus-like disease [10], and had higher titres of anti-double-strand/single-strand DNA antibodies [11]. In addition, the adoptive transfer of CD4⁺CD25⁺ Treg cells generated *ex vivo* can protect mice from developing chronic graft-versus-host disease with a lupus-like syndrome and alter the course of established disease [12], evidence that CD4⁺CD25⁺ T cells play an important role in the pathogenesis of SLE.

CD4⁺CD25⁺ Treg cells can be classified into thymus-derived natural Treg cells [13,14], and those induced in the periphery [15]. Naturally occurring CD4⁺CD25⁺ Treg cells express a high level of CD25, the forkhead/winged-helix family transcription factor forkhead box P3, cytotoxic T lymphocyte antigen-4, and glucocorticoid-induced tumor necrosis factor receptor family-related receptor (GITR), to exert immunosuppressive function [16]. CD4⁺CD25⁺ Treg cells can also be adaptively induced by specific tolerogenic stimulation *in vivo* [17] or expanded from naïve CD4⁺ T cells in the peripheral blood upon transforming growth factor-beta (TGF- β) or interleukin-10 stimulation [18-20]. The acquired CD4⁺CD25⁺ Treg cells then suppress naïve T-cell responses, prevent autoimmunity, and delay allograft rejection by producing TGF- β 1 or interleukin-10 [18,19].

TGF- β 1 is one of the most important cytokines in immune regulation. TGF- β 1 can suppress T- or B-cell functions, and enable CD8⁺ cells to develop suppressor activity [21,22]. Most importantly, TGF- β 1 serves as not only a costimulatory factor in the development of CD4⁺CD25⁺ Treg cells [18,19], but also an important mediator for those cells to exert their immunoregulatory function in various autoimmune diseases [23]. Ohtsuka and colleagues have demonstrated that production of both the total and biologically active form of TGF- β 1 is reduced in subjects with SLE [24], and decreased production of the total TGF- β 1 inversely correlates with disease activity of SLE [25].

We have studied SLE patients in Taiwan, but found no association of TGF- β 1 activity with SLE [26]. In the present study, we endeavored to study the important correlation between TGF- β 1, CD4⁺CD25⁺ Treg cells and SLE.

Methods

Patients and controls

Thirty patients fulfilling the 1997 revised classification criteria of American Rheumatism Association for SLE [27] and 20 healthy blood donors were evaluated for this study. Informed consent was obtained from each participant. The clinical disease activity for each patient with SLE was scored using the SLE disease activity index (SLEDAI) [28]. Patients were designated to be disease-active when their major clinical manifestations were present or SLEDAI was >3 . Patients were enrolled if they were free of infection, had white blood cell count $>4 \times 10^9/L$, and had not received methylprednisolone or cyclophosphamide pulse therapy within 3 months.

Preparations of fresh peripheral blood

Blood samples were obtained by venipuncture. One mL of sterile heparinized whole blood was stimulated with 0.01 ng/mL of staphylococcal enterotoxin B (Sigma, St. Louis, MO, USA) and 10 ng/mL of recombinant human interleukin-2 (BD Biosciences, San Jose, CA, USA) in the presence of 2 μ M monensin (protein transporter inhibitor, BD GolgiStop; BD Biosciences) for 4 h at 37°C in a humidified 5% carbon dioxide incubator. Cells were harvested and erythrocytes were lysed by adding 2 mL of 1 \times ammonium chloride lysing reagent (BD Pharm Lyse™; BD Biosciences) for 10 min. Samples were washed with 0.2% bovine serum albuminphosphate buffered saline (Pharmingen-Stain Buffer, BD Biosciences) and underwent multi-color staining for cell surface markers and intracellular cytokines.

Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated from 30 mL heparinized venous blood by centrifugation over Ficoll-Hypaque density gradient (Amersham Biosciences, Uppsala, Sweden) at 400 g for 25 min. The cells (1×10^7 cells/mL) were added to 24-well microtiter plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) in AIM-V serum-free lymphocyte medium (Gibco,

Grand Islands, NY, USA). Cells were cultured in the presence or absence of 1 ng/mL of TGF- β 1 (R&D Systems Inc, Minneapolis, MN, USA) for 5 days. After harvest, cells were further stimulated with 0.01 ng/mL of staphylococcal enterotoxin B and 10 ng/mL of recombinant human interleukin-2 in the presence of 2 μ M monensin for 4 h at 37°C in a humidified 5% carbon dioxide incubator. Cells were then harvested and underwent multicolor staining for cell surface markers and intracellular cytokines.

Flow cytometry analysis

PBMCs were washed twice in bovine serum albumin-phosphate-buffered saline staining buffer and incubated with normal human serum 20 min at 4°C to block Fc gamma receptors, washed twice again, and were stained with Cy-Chrome™ (PE-Cy5)-conjugated CD4 and fluorescein isothiocyanate-conjugated CD25 monoclonal antibody 30 min at 4°C.

After cell surface staining, the cells were fixed and permeabilized in Cytotofin/Cytoperm solution for 20 min at 4°C and washed twice in Perm/Wash solution. Cells were then incubated with mouse anti-human TGF- β 1 immunoglobulin G1 (IgG1) monoclonal antibody for 30 min at 4°C, washed twice in Perm/Wash solution, and then incubated with phycoerythrin-conjugated rat anti-mouse IgG1 mAb for 30 min at 4°C. After staining, cells were washed twice in Perm/Wash solution, re-suspended in fluorescence-activated cell sorter (FACS) staining buffer, and flow cytometry was performed on a FACSCalibur™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Isotype controls for each sample were run in conjunction with surface markers and TGF- β 1 staining, respectively.

The analysis was performed using CellQuest software (BD Biosciences). 1×10^5 cells were requested. Viable lymphocytes were gated by plotting forward scatter versus side scatter. Cells were then gated for CD4 versus CD25. Results were expressed as percentage of cells. Intracellular TGF- β 1 was assessed on histogram by gated CD4⁺CD25⁺ cells and presented as log geometric mean fluorescence intensities (GeoMFIs).

Conjugated monoclonal antibodies, isotype-matched controls of mouse IgG1, and reagents were obtained from Becton Dickinson Immunocytometry Systems (BD Biosciences); mouse anti-human TGF- β 1 IgG1 monoclonal antibody was purchased from R&D Systems Inc.

Statistical analysis

Comparisons of percentages of CD4⁺CD25⁺ T cells and GeoMFI of intracellular TGF- β 1 expression for freshly prepared and cultured PBMC between groups were analyzed by Student's *t* test for the independent samples. Differences were considered to be significant at $p < 0.05$. All the calculations were performed using Statistical Package for the Social Sciences for Windows (Version 10.1; SPSS Taiwan Corp., Taipei, Taiwan) software package.

Results

Clinical characteristics of patients and controls

Table 1 lists the clinical characteristics of patients with SLE in this study (28 females, 2 males; age range, 23–63 years; mean age, 32.9 years). Among them, 12 patients (40.0%) were disease-active, 16 (53.3%)

Table 1. Clinical characteristics of systemic lupus erythematosus (SLE) patients

Characteristic ^a	SLE			<i>p</i> ^b
	Total (n = 30)	Active (n = 12)	Inactive (n = 18)	
Gender (F:M)	28:2	12:0	16:2	NS
Age (years; mean \pm SD)	32.9 \pm 8.8	31.1 \pm 5.9	34.2 \pm 10.3	NS
Nephritis (no.) [%]	18 (60.0)	6 (50.0)	12 (66.7)	NS
C3 (mg/dL; mean \pm SD)	82.4 \pm 27.4	61.7 \pm 11.2	96.2 \pm 26.3	0.001
C4 (mg/dL; mean \pm SD)	17.2 \pm 9.3	10.9 \pm 7.2	21.3 \pm 8.4	0.002
Anti-double-stranded DNA (IU/mL; mean \pm SD)	31.3 \pm 51.8	59 \pm 69.5	12.9 \pm 23.5	0.014
SLEDAI (mean \pm SD)	4.5 \pm 6.2	10.2 \pm 6.4	0.8 \pm 1.0	0.001
Prednisolone (mg/day; mean \pm SD)	9.9 \pm 5.1	14.2 \pm 4.0	7.1 \pm 3.7	<0.001

Abbreviations: F = female; M = male; SD = standard deviation; C = complement; SLEDAI = SLE disease activity index; NS = not significant

^aNormal ranges: C3, 79–152 mg/dL; C4, 16–38 mg/dL; anti-double-stranded DNA, <40 IU/mL.

^bStudent's *t* test.

were well-controlled and inactive, and 2 were (6.7%) in remission and not taking any medication. Eighteen patients (60.0%) had biopsy-proven renal involvement with at least 5 years' follow-up, 2 (6.7%) had central nervous system disease, and 12 (40.0%) had mild disease mainly affecting the skin and joints. The control group (19 females, 1 male; age range, 19-52 years; mean age, 31.5 years) comprised unrelated healthy

blood donors and college students who were age- and gender-matched with the patient group.

Peripheral CD4⁺CD25⁺ cells and intracellular TGF- β 1 expression in SLE

The relative amount of CD4⁺CD25⁺ T cells was analyzed and presented as percentage of CD4⁺ T cells. As shown in Fig. 1, the flow cytometric results

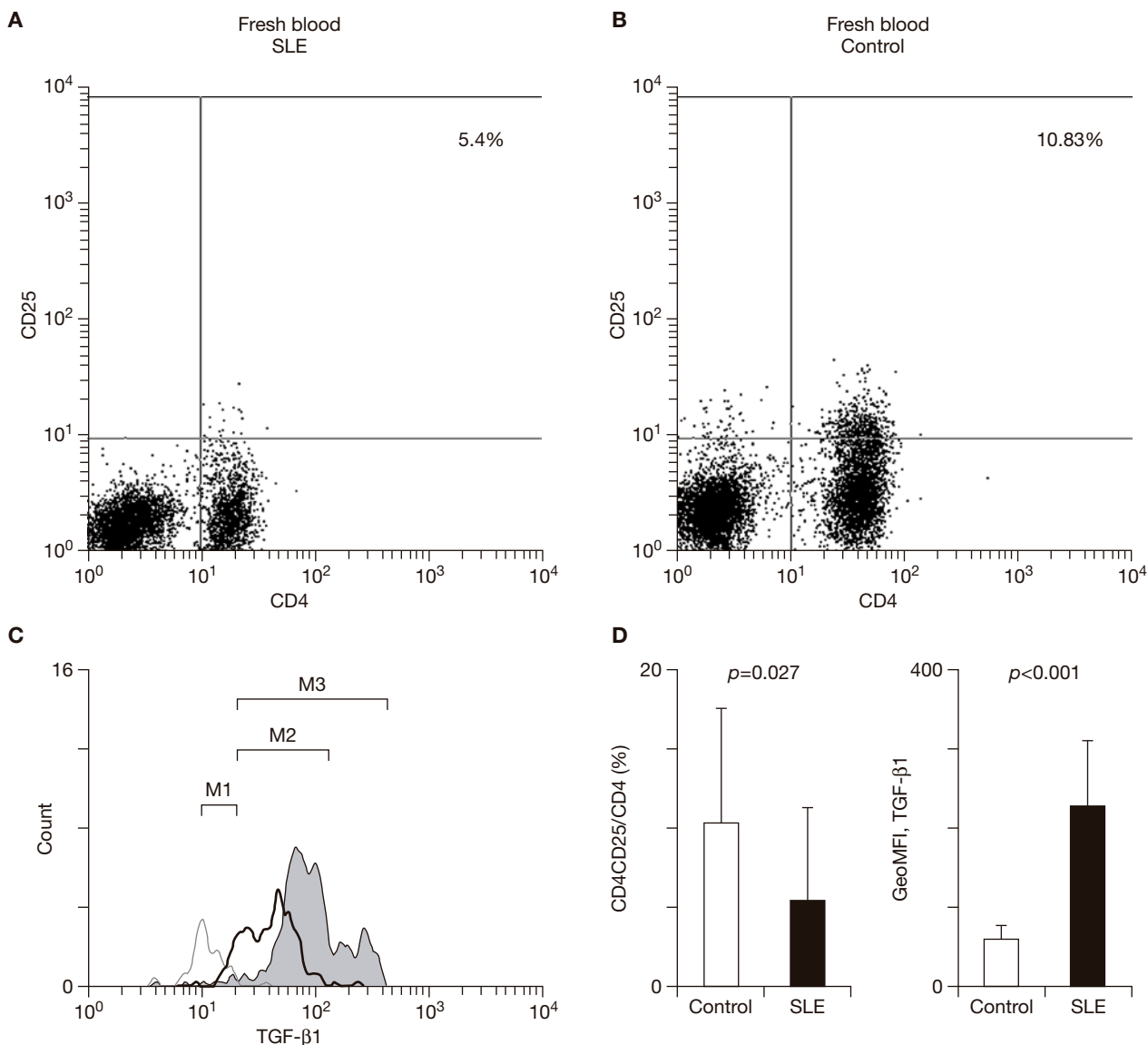


Fig. 1. CD25 and transforming growth factor-beta1 (TGF- β 1) expression in CD4⁺ cells in freshly prepared peripheral blood. The percentage of CD4⁺CD25⁺ T cells was gated by plotting CD4⁺ versus CD25⁺ T cells. (A) Percentage of CD25⁺ in CD4⁺ T cells of a representative systemic lupus erythematosus (SLE) patient (no.12). (B) Percentage of CD25⁺ in CD4⁺ T cells of a representative normal control donor (no. 10). (C) TGF- β 1 expression was plotted by histogram by gating CD4⁺CD25⁺ T cells. Filled histogram represents the location of the TGF- β 1-producing CD4⁺CD25⁺ T cells of the representative SLE patient (no. 12), the black solid line the normal control donor (no. 10), and the gray solid line the isotype control. The log geometric mean fluorescence intensities (GeoMFIs) were determined (M1, isotype control; M2, normal control donor; M3, SLE patient). (D) Mean percentage of CD4⁺CD25⁺/CD4⁺ and mean GeoMFI of intracellular TGF- β 1 expression in control (white bar) and SLE (black bar) groups.

revealed that CD4⁺CD25⁺ T cells (right upper quadrant) accounted for 5.4% of total CD4⁺ T cells in one representative SLE patient (Fig. 1A) and 10.83% of total CD4⁺ T cells in one representative normal control (Fig. 1B). To evaluate the expression capability of TGF- β 1 of the CD4⁺CD25⁺ Treg cells, the subset of CD4⁺CD25⁺ T cells was gated and the GeoMFI of intracellular TGF- β 1 was determined (Fig. 1C). The GeoMFI of intracellular TGF- β 1 of the SLE patient (M3) was apparently higher than that of the control (M2). Fig. 1D demonstrates that the mean (\pm standard deviation) percentage of CD4⁺CD25⁺/CD4⁺ of SLE patients was lower ($5.44 \pm 5.83\%$) than in controls ($10.22 \pm 7.42\%$, $p=0.027$), but the mean intracellular TGF- β 1 expression by the CD4⁺CD25⁺ cells of SLE patients (mean GeoMFI, 224.72 ± 83.72) was higher than that of the normal controls (60.53 ± 15.46 , $p<0.001$). Thus, there was an inverse correlation between the intracellular TGF- β 1 expressions and the frequencies of CD4⁺CD25⁺ T cells ($r = -0.432$, $p=0.004$) in freshly prepared peripheral blood from SLE and control subjects ($n = 50$, Fig. 2).

Effects of TGF- β 1 stimulation

It has been reported that TGF- β 1 can upregulate CD25 expression in naïve CD4⁺ T cells and can induce suppressive activity of CD4⁺CD25⁺ T cells in normal human peripheral blood [18,19]. In order to study the expandability of CD4⁺CD25⁺ Treg cells upon TGF- β 1 stimulation in SLE compared with controls, 3-color flow cytometry analysis was performed to

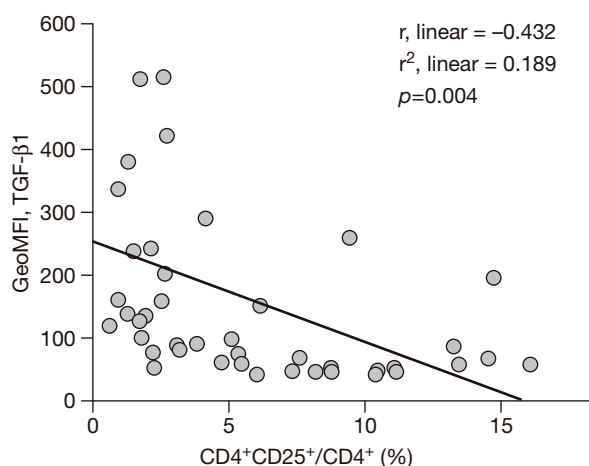


Fig. 2. Correlation between transforming growth factor-beta1 (TGF- β 1) expression and the frequency of CD4⁺CD25⁺ regulatory T cells in freshly prepared peripheral blood from systemic lupus erythematosus patients and controls ($n = 50$). GeoMFI = geometric mean fluorescence intensity.

examine the relative number and TGF- β 1 expression of CD4⁺CD25⁺ T cells in the absence and presence of TGF- β 1-stimulated PBMC cultures. In the absence or presence of TGF- β 1 at the final concentration of 1 ng/mL for 5 days, CD4⁺CD25⁺ cells increased from 10.2% (Fig. 3A) to 29.7% (Fig. 3B) of total CD4⁺ cells in a representative SLE patient. Intracellular TGF- β 1 expression of the TGF- β 1-stimulated CD4⁺CD25⁺ cells (M3) in this patient increased about 1.8-fold compared with that of the unstimulated CD4⁺CD25⁺ cells (M2) [Fig. 3C]. The mean percentages of CD4⁺CD25⁺ cells and their TGF- β 1 expressions in TGF- β 1-stimulated PBMC cultures are shown in Fig. 3D. SLE patients had a similar percentages of CD4⁺CD25⁺/CD4⁺ in unstimulated PBMC cultures (mean, $17.96 \pm 7.74\%$) compared with normal controls (mean, $15.11 \pm 5.56\%$). After TGF- β 1 stimulation, the mean percentage of CD4⁺CD25⁺/CD4⁺ increased similarly in SLE patients (from 17.96% to 31.07%) and controls (15.11% to 31.88%). The increments in the percentages of CD4⁺CD25⁺ T cells on TGF- β 1 stimulation were prominent ($p<0.001$), and were similar in SLE and control groups.

Unlike freshly prepared CD4⁺CD25⁺ T cells, the intracellular TGF- β 1 expression (GeoMFI) of CD4⁺CD25⁺ T cells in unstimulated PBMC cultures of SLE patients was not significantly different from that of controls (mean, 52.00 ± 21.93 vs 48.17 ± 23.60). There was a significant increase in intracellular TGF- β 1 expression of CD4⁺CD25⁺ T cells in response to TGF- β 1 stimulation, of about 5.5-fold in the control group (mean, 265.24 ± 62.95 , $p<0.001$) and 4.1-fold in SLE patients (mean, 240.62 ± 127.77 , $p<0.001$), with no statistically significant difference between the SLE and control groups.

Associations of CD4⁺CD25⁺ T cells with disease activity and lupus nephritis

In freshly prepared PBMC, the percentage of CD4⁺CD25⁺/CD4⁺ cells was decreased significantly in SLE patients with active disease ($p=0.058$) and lupus nephritis ($p=0.036$) [Table 2]. However, there was no statistically significant difference in intracellular TGF- β 1 expression of CD4⁺CD25⁺ cells between these groups.

In PBMC cultures, the relative increment in the percentages of CD4⁺CD25⁺ T cells in response to TGF- β 1 stimulation was slightly lower in patients with active SLE compared to those with inactive SLE (mean, 87.98% vs 121.31%), but higher in patients with nephritis compared with patients without nephritis (mean,

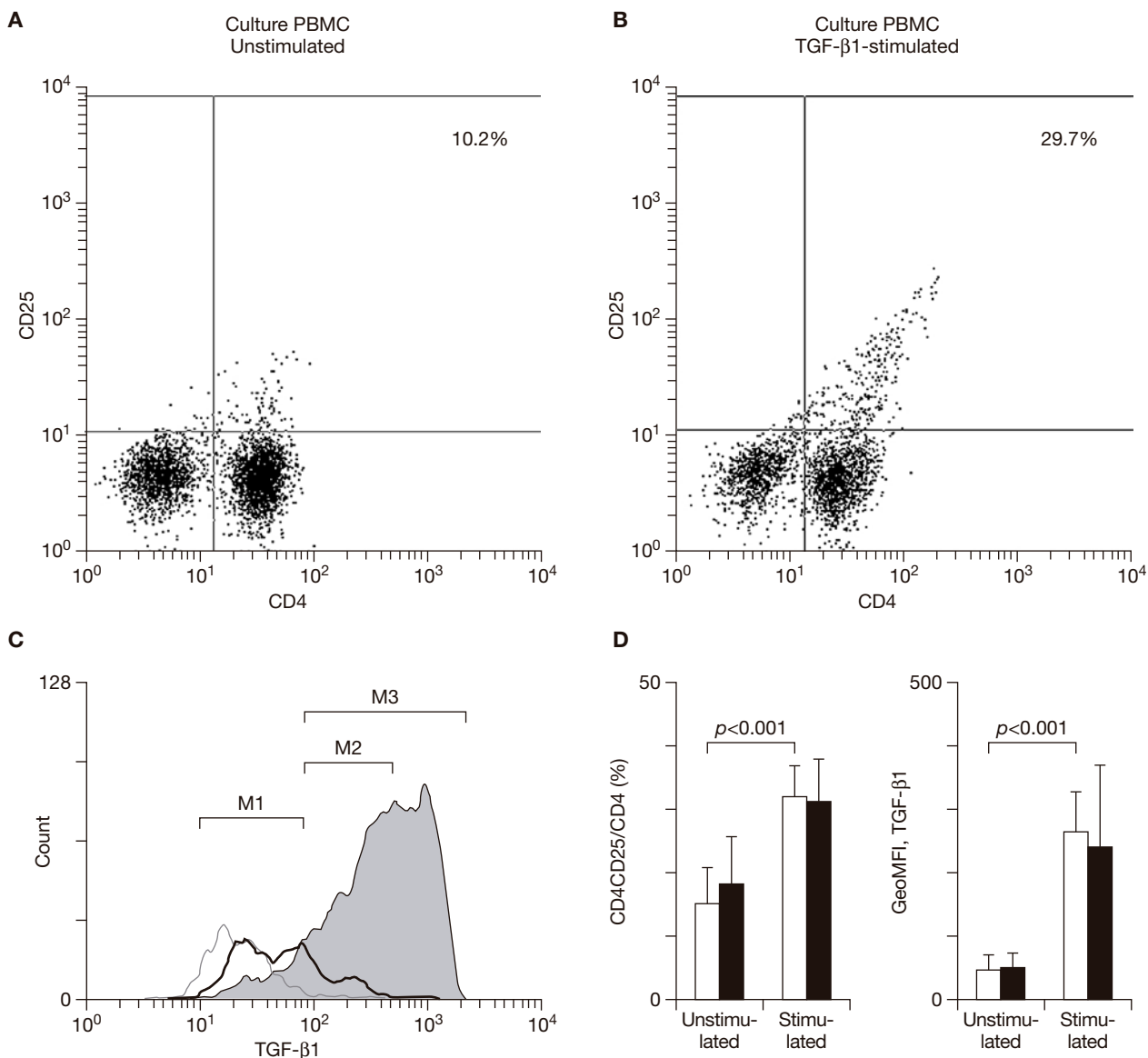


Fig. 3. CD25 and transforming growth factor-beta1 (TGF- β 1) expression in CD4⁺ cells after culture for 5 days in unstimulated and TGF- β 1 (1 ng/mL)-stimulated peripheral blood mononuclear cell (PBMC) cultures. (A) Percentage of CD25⁺CD4⁺ in CD4⁺ T cells in unstimulated PBMC culture of a representative systemic lupus erythematosus (SLE) patient (patient no.10). (B) Percentage of CD25⁺CD4⁺ in CD4⁺ T cells in TGF- β 1-stimulated PBMC culture of the representative SLE patient. (C) TGF- β 1 expression was plotted by histogram gating CD4⁺CD25⁺ T cells. Filled histogram represents the location of the TGF- β 1-stimulated CD4⁺CD25⁺ T cells, the black solid line the unstimulated CD4⁺CD25⁺ T cells, and the gray solid line the isotype control. The geometric mean fluorescence intensities (GeoMFIs) were determined (M1, isotype control; M2, unstimulated CD4⁺CD25⁺ cells; M3, TGF- β 1-stimulated CD4⁺CD25⁺ cells, of the representative SLE patient). (D) Mean percentage of CD4⁺CD25⁺/CD4⁺ cells and mean GeoMFI of intracellular TGF- β 1 expression in unstimulated and TGF- β 1-stimulated PBMC of the control (white bar) and SLE (black bar) groups.

131.08% vs 85.48%). Nevertheless, there were no statistically significant differences between these groups. The increment in TGF- β 1 expression of CD4⁺CD25⁺ T cells by TGF- β 1 stimulation was similar between patients with or without disease activity (mean, 346.75% vs 357.53%), but higher in patients with nephritis compared to those without nephritis (mean, 393.17% vs

295.13%), the latter difference being of only marginal statistical significance ($p=0.059$).

Discussion

A number of animal studies have shown that CD4⁺CD25⁺ Treg cells play an important role in

Table 2. Association of the relative amounts (percentage) and intracellular transforming growth factor-beta1 (TGF- β 1) expression of CD4⁺CD25⁺ cells in fresh blood and TGF- β 1-stimulated peripheral blood mononuclear cell (PBMC) cultures in patients with systemic lupus erythematosus according to disease activity and presence of nephritis

Variable	Fresh blood		PBMC cultures	
	CD4 ⁺ CD25 ⁺ / total CD4 ⁺ cells (%)	GeoMFI TGF- β 1	Percent stimulation, CD4 ⁺ CD25 ⁺ / total CD4 ⁺ cells ^a	Percent stimulation, GeoMFI TGF- β 1 ^b
Disease activity				
Active (n = 12) [mean \pm SD]	3.68 \pm 1.89	197.66 \pm 157.53	87.98 \pm 85.27	346.75 \pm 277.00
Inactive (n = 18) [mean \pm SD]	6.73 \pm 7.45	187.09 \pm 176.44	121.31 \pm 61.51	357.53 \pm 283.39
<i>p</i> ^c	0.058	NS	NS	NS
Nephritis				
Positive (n = 18) [mean \pm SD]	3.78 \pm 2.82	176.24 \pm 137.79	131.08 \pm 67.02	393.17 \pm 217.17
Negative (n = 12) [mean \pm SD]	7.73 \pm 8.20	212.14 \pm 226.24	85.48 \pm 66.31	295.13 \pm 196.86
<i>p</i>	0.036	NS	NS	0.059

Abbreviations: GeoMFI = geometric mean fluorescence intensity; SD = standard deviation; NS = not significant

^aPercent stimulation (relative increment) = ((percentage of TGF- β 1 stimulated CD4⁺CD25⁺ T cells/percentage of unstimulated CD4⁺CD25⁺ T cells - 1) \times 100%).

^bPercent stimulation (relative increment) = ((GeoMFI of TGF- β 1 expression in TGF- β 1 stimulated CD4⁺CD25⁺ T cells/GeoMFI of TGF- β 1 expression in unstimulated CD4⁺CD25⁺ T cells - 1) \times 100%).

^cStudent's *t* test.

maintaining self-tolerance and preventing autoimmunity [14]. TGF- β 1 is one of the most important mediators of the function of this subset of T cells in the periphery [29,30]. Co-administration of neutralizing antibodies to TGF- β 1 abrogated the *in vivo* autoimmune-preventive or tolerance-inducing activity of CD4⁺CD25⁺ Treg cells [31]. It has been reported that both New Zealand black/white (NZB/NZW) F1 (BWF1) and Swiss Webster \times New Zealand black F1 (SNF1) lupus-prone mice [10] and NZB/NZW lupus mice [11] have a lower percentage of CD4⁺CD25⁺ T cells in their CD4 repertoire compared to their gender- and age-matched non-autoimmune counterparts, while the percentage of CD4⁺CD25⁺ T cells is decreased in the peripheral blood of patients with SLE [32-34]. Nevertheless, the examination of CD4⁺CD25⁺ T cells has remained limited in SLE.

In the present study, we first confirmed the previous findings that the natural CD4⁺CD25⁺ Treg cells in fresh PBMC were reduced in number in SLE [10,11,32-34]. Furthermore, our results showed that the intracellular TGF- β 1 expressions of these Treg cells were significantly increased compared to normal controls. Together with significantly negative correlations between TGF- β 1 expression and the level of CD4⁺CD25⁺ cells, these results suggested that the decreased CD4⁺CD25⁺ subset functioned normally and even compensatorily. Most importantly, the decrease in CD4⁺CD25⁺ cells in PBMC of SLE patients was presumed not to be caused by the

immunosuppressive therapy that they received, because the expression of TGF- β 1 in these cells was not suppressed. This finding of increased TGF- β 1 expression in CD4⁺CD25⁺ cells was coincident with the recent report by Lee et al [34] that the frequency of CD4⁺CD25⁺ cells was significantly decreased in pediatric patients with active SLE, whereas the surface expression of GITR, which is expressed preferentially at high level on CD4⁺CD25⁺ cells and involved in regulating the functions of CD4⁺CD25⁺ cells, is significantly increased in CD4⁺CD25⁺ cells.

Consistent with previous reports using human peripheral blood [18,19], we found that TGF- β 1 could enhance the expression of CD25 phenotype from naïve CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells and stimulate suppressive activity via TGF- β 1 expression *in vitro*. We also found that the costimulatory effects of TGF- β 1 on cell numbers and TGF- β 1 expression in CD4⁺CD25⁺ T cells were comparable between patients with SLE and gender- and age-matched healthy controls. Together with the study in lupus-prone mice showing that the number of CD4⁺CD25⁺ T cells restores to the level seen in normal mice following tolerance induction by intranasal administration of autoantigenic peptides [10], these results suggested that SLE patients could have normal acquired CD4⁺CD25⁺ T cell responses in the periphery.

Contrary to a study finding no clinical correlation between levels of CD4⁺CD25⁺ T cells and SLEDAI

scores [33], but consistent with data showing that CD4⁺CD25⁺ T cells are decreased in patients with clinically active SLE [32,34], we noted that our SLE patients tended to have lower levels of natural CD4⁺CD25⁺ Treg cells when the diseases were active (SLEDAI above 3) or when they had renal involvement. Nevertheless, the intracellular TGF- β 1 expression of natural CD4⁺CD25⁺ Treg cells was not statistically influenced by disease activity or the presence of nephritis. The inducibility of acquired CD4⁺CD25⁺ Treg cells and TGF- β 1 expression by TGF- β 1 stimulation were not affected by disease activity, although patients with lupus nephritis had slightly increased increments of TGF- β 1 expression after TGF- β 1 stimulation. These patients with disease activity and nephritis usually received a larger dose of steroid and immunosuppressive therapy, but still had relatively normal TGF- β 1 expression and stimulation response, again suggesting that the deficiency in natural CD4⁺CD25⁺ Treg cell population was not related to the immunosuppressive therapy, but rather, might contribute to a susceptibility to major manifestations of SLE, nephritis in particular.

Wu and Staines reported that natural CD4⁺CD25⁺ Treg cells in lupus-prone mice are deficient even at the ages of 4-6 weeks (immature mice) and well before the disease develops [10], suggesting that the deficiency of natural CD4⁺CD25⁺ Treg cells may be spontaneous or inherent. Nevertheless, the data presented in this study showed that the deficiency of fresh peripheral CD4⁺CD25⁺ Treg cells in patients with SLE could be normalized to the levels of controls even in unstimulated PBMC cultures, without putatively inherent defect. Additional research examining whether the deficiency of natural CD4⁺CD25⁺ Treg cells is spontaneous or secondary to other serum or cellular factors in SLE would be of great value in understanding the role of CD4⁺CD25⁺ Treg cells in the pathogenesis of SLE.

In summary, natural CD4⁺CD25⁺ Treg cells were deficient in SLE patients and the deficiency correlated with disease activity and the presence of nephritis. Natural CD4⁺CD25⁺ Treg cells in SLE had higher TGF- β 1 expression to compensate for the reduced numbers. Control of cell numbers and regulatory function was effectively induced through TGF- β 1 expression by CD4⁺CD25⁺ Treg cells in TGF- β 1-stimulated PBMC cultures in patients with SLE and in healthy controls. These results help to clarify the importance of TGF- β 1 expressing CD4⁺CD25⁺ Treg cells in the

pathogenesis of SLE and verify that TGF- β 1 could be a potential target for clinical application in treating SLE.

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