Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Altered homeostasis of CD4$^+$ FoxP3$^+$ regulatory T-cell subpopulations in systemic lupus erythematosus

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doi:10.1111/j.1365-2567.2008.02937.x
Received 5 June 2008; revised 3 July 2008; accepted 25 July 2008.
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Summary

The role of naturally occurring regulatory T cells (Treg), known to be phenotypically heterogeneous, in controlling the expression of systemic lupus erythematosus (SLE) is incompletely defined. Therefore, different subpopulations of CD4$^+$ FoxP3$^+$ Tregs in patients with active or inactive SLE were investigated and compared with those of healthy subjects and patients with ankylosing spondylitis (AS). Characterization of different subsets of circulating CD4$^+$ FoxP3$^+$ Tregs was examined using flow cytometry. CD4$^+$ CD25^{high} T cells were sorted and examined for suppressive activity *in vitro*. The results showed first that a significant decrease in the frequency of CD4$^+$ CD25^{high} FoxP3$^+$ T cells was present in patients with active SLE (n = 58), compared with healthy controls (n = 36) and AS patients (n = 23). In contrast, the frequencies of CD25^{low} FoxP3$^+$ and CD25$^-$ FoxP3$^+$ CD4$^+$ T cells were significantly increased in patients with active SLE by comparison with the control subjects. The elevation of these two putative Treg subpopulations was associated with lower plasma levels of complement C3 and C4 in patients with SLE. In addition, the ratios of the three subsets of CD4$^+$ FoxP3$^+$ Tregs versus effector T cells (CD4$^+$ CD25$^+$ FoxP3$^-$) were inversely correlated with the titer of anti-double-stranded DNA IgG in patients with inactive, but not active, SLE. These results suggest that the pathogenesis of SLE may be associated with a defect in the homeostatic control of different Treg subsets.

Keywords: anti-dsDNA IgG; FoxP3; regulatory T cells; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE), a chronic and often debilitating autoimmune disease, is characterized by a loss of immune tolerance to self-antigens (Ags) and by the persistent production of pathogenic autoantibodies.[1] While the exact pathogenic mechanisms remain to be elucidated, recent studies have suggested dysregulation of the regulatory T cells (Tregs), particularly the naturally occurring Tregs, as one of the major factors conferring risk for the expression of human autoimmune diseases, including SLE. For example, defective suppressor function in CD4$^+$ Treg cells with relatively high levels of CD25 (CD25^{high}) has been demonstrated in patients with multiple sclerosis and autoimmune polyglandular syndrome Type II.[2,3] Also, while some studies have suggested that the frequency of CD4$^+$ CD25$^{+/high}$ T cells is decreased in both adult[4–6] and paediatric[7] patients with SLE, the contribution of various subsets of CD4$^+$ FoxP3$^+$ T cells has not been investigated. Moreover, there are conflicting

Abbreviations: Ag, antigen; APC, antigen-presenting cell; AS, ankylosing spondylitis; c.p.m., counts per minute; CTLA-4, cytotoxic T-lymphocyte antigen 4; dsDNA, double-stranded DNA; FACS, fluorescence-activated cell sorter; FoxP3, forkhead box P3; IgG, immunoglobulin G; GITR, glucocorticoid-induced tumour necrosis factor receptor-related protein; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; Teff, effector T cell; Treg, regulatory T cell.

data regarding the function of $CD4^+$ $CD25^{high}$ T cells in patients with SLE,[6,8] which may be a result of the heterogeneity of the Treg population.

Two subsets of $CD4^+$ Tregs have been classified as natural and adaptive Tregs.[9,10] Naturally occurring Tregs develop during normal T-cell maturation in the thymus and represent 1–2% of $CD4^+$ T cells in the peripheral blood.[11,12] They typically express high levels of CD25, as well as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR).[13] Most importantly, natural Tregs express a specific transcription factor, forkhead box P3 (FoxP3), which is critical in the generation and development of the regulatory function of $CD4^+$ $CD25^+$ Tregs.[14]

Similarly to natural Tregs, adaptive Tregs, including transforming growth factor- β (TGF-β)-expressing T-helper 3 (Th3) cells and interleukin (IL)-10-producing T-regulatory 1 (Tr1) cells, originate from the thymus, but they are developed throughout the course of the immune response *in vivo*. Of interest is the finding that naïve $CD4^+$ T cells can be converted, *de novo,* into $CD25^+$ $FoxP3^+$ and $CD25^-$ $FoxP3^+$ suppressor T cells upon subimmunogenic stimulation.[15] Also, it has been shown that TGF-β can induce FoxP3 expression, blurring the distinctions between the different adaptive Treg subsets.[16] Therefore, alteration of the tissue and cytokine milieu may tip the balance in the composition of Treg subpopulations, which may, in turn, contribute to the pathogenesis of autoimmune diseases.

While the detailed mechanisms are under active investigation, natural and adaptive Treg-cell subsets may differ functionally in their mechanism of suppression. Natural Tregs primarily mediate suppression by CTLA-4, whereas adaptive Tregs initiate the suppressive cascade in a cytokine-dependent manner.[9,17] In addition, the degree to which T-effector cells (Teffs) are resistant to Treg suppression is also important in immune regulation. For example, not only is GITR constitutively expressed by $CD4^+$ $CD25^+$ Tregs but it is also expressed by activated Teffs.[18] Previous studies showed that ligation of GITR leads to an Ag-non-specific proliferation and activation of $CD4^+$ $CD25^+$ Tregs,[13,19] while the engagement of GITR renders Teffs resistant to Treg suppression.[20]

In this study, we used intracellular FoxP3, together with CD25 staining, to distinguish different subsets of $CD4^+$ $FoxP3^+$ T cells and Teffs ($CD4^+$ $CD25^+$ $FoxP3^-$), clearly, in human peripheral blood mononuclear cells (PBMCs). We found a decrease in $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells and a dramatic increase of $CD25^{low}$ $FoxP3^+$ and $CD25^-$ $FoxP3^+$ T cells in patients with active SLE. We also demonstrated that $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells from SLE patients exhibit a potent ability to inhibit activated naïve $CD4^+$ T cells *in vitro*. Interestingly, the ratio of $CD4^+$ $CD25^{low/-}$ $FoxP3^+$ T cells to Teffs was significantly associated with disease activity and autoantibody levels in SLE patients. This study shows a comprehensive quantification of FoxP3-expressing $CD4^+$ Treg subsets in SLE patients and examines their relevance to disease progression. This study will help us to understand, in greater detail, the role of different subsets of Tregs, and potentially to optimize novel therapies based on the expansion of Tregs in SLE.

Materials and methods

Subjects

We enrolled individuals who were 18 years of age or older, and used a protocol approved by the institutional review board at the Kaohsiung Medical University. Informed consent was provided according to the Declaration of Helsinki. The study subjects included patients with inactive SLE [SLE disease activity index (SLEDAI) score ≤ 3], patients with active SLE (SLEDAI score > 3), healthy controls and subjects with ankylosing spondylitis (AS) as patient controls. Healthy volunteers with no history of autoimmune diseases were enrolled in the study. Any normal donors or patients with signs of infection were excluded before the study start. Patients with SLE fulfilled the American Rheumatism Association revised criteria for SLE.[21] Treatment regimens in the patients with inactive and active SLE were as follows: prednisolone alone ($n = 10$ and 14, respectively); prednisolone + hydroxychloroquine ($n = 14$ and 33, respectively); and prednisolone + hydroxychloroquine + azathioprine ($n = 2$ and 5, respectively).

The clinical information of the SLE patients is shown in Table 1. The following were measured in the SLE patients in the clinical immunology laboratory of the Kaohsiung Medical University Hospital: differential white cell count; the titer of anti-double-stranded DNA (dsDNA) immunoglobulin G (IgG) (Pharmacia & Upjohn, Freiburg, Germany); and the level of plasma complement C3 and C4 (Beckman Coulter, Fullerton, CA). These were all performed in parallel with the analysis of Treg-cell subpopulations.

Cell isolation and flow cytometry

To diminish non-specific staining by monocytes, total T cells were negatively selected from the peripheral blood from study subjects (StemCell Tech., Vancouver, BC, Canada). For fluorescence-activated cell sorter (FACS) analysis (BD Biosciences, Mountain View, CA), the following conjugated antibodies were used: CD4 (RPA-T4), human FoxP3 (PCH101), HLA-DR (LN3), CD25 (B1.49.9), CD127 (hIL-7R-M21), CD45RO (UCHL1), CD45RA (HI100), CTLA-4 (BNI3), GITR (110416) and

Table 1. Clinical information for control subjects and patients with systemic lupus erythematosus (SLE) included in this study

	Normal control	Inactive SLE	Active SLE	AS
Patient no.	36	29	58	23
Age[1] (range)	37 ± 12 (22–70)	40 ± 11 (21–70)	36 ± 11 (21–61)	43 ± 12* (21–70)
Gender (female/male)	34/2	27/2	52/6	4/19**
SLEDAI score[1]	NA	1·5 ± 1·0	7·5 ± 5·3	NA
Prednisolone (users/non-users)	NA	26/3	54/4	NA
Hydroxychloroquine (users/non-users)	NA	16/13	40/18	NA
Azathioprine (users/non-users)	NA	2/27	5/53	NA
Drug (users/non-users)	NA	26/3	54/4	NA

AS, ankylosing spondylitis; NA, not applicable; SLEDAI, systemic lupus erythematosus disease activity index.
*$P < 0{\cdot}05$ compared with active SLE, results analyzed using the Mann–Whitney *U*-test; **$P < 0{\cdot}05$ compared with normal control, results analyzed using Fisher's exact test.
[1]Mean ± standard deviation.

isotype controls. All antibodies were used at concentrations titrated for optimal staining. The samples were run on a FACScan or an LSRII flow cytometer, collecting data on 10^5 lymphocytes (gated by forward-scatter and side-scatter properties), and were analyzed using FCS EXPRESS software (De Novo Software, Thornhill, ON, Canada) and CELLQUEST PRO software (BD Biosciences).

For cell sorting, PBMCs were purified by Ficoll–Hypaque gradient centrifugation and this was followed by $CD4^+$ T-cell isolation. $CD4^+$ $CD25^{high}$ T cells, $CD4^+$ $CD45RA^{high}$ naïve T cells and $CD4^+$ $CD25^-$ T cells were separated on a FACSVantage^{TM} SE cell sorter to a purity of > 97%. $CD4^+$ $CD25^{high}$ T cells contained only 1–2% of $CD4^+$ T cells with the highest CD25 expression, as previously reported.[12] The purity and phenotype of samples were further tested by intracellular FoxP3 staining that revealed at least > 94% $FoxP3^+$ cells in the sorted $CD4^+$ $CD25^{high}$ T-cell population and < 1% $FoxP3^+$ cells in sorted naïve T cells.

Proliferation assays

For the assessment of $CD4^+$ $CD25^{high}$ T-cell suppressive activity, different numbers of sorted $CD4^+$ $CD25^{high}$ T cells were cocultured with 2500 autologous naïve responder T cells ($CD4^+$ $CD45RA^{high}$) and 50 000 autologous mitomycin C-treated, $CD4^+$ T-cell-depleted PBMCs in 96-well U-bottom plates coated with 0·5 μg/ml of anti-CD3 (UCHT1). Cells were cultured in RPMI-1640 supplemented with 5% human AB serum. After 5 days of culture, 1 μCi of [^{3}H]thymidine was added and the cells were cultured for a further 16 hr. [^{3}H]Thymidine incorporation was measured in counts per minute (c.p.m.) using a scintillation counter. To compare independent assays, proliferation of responder T cells was set at 100%. Thus, per cent suppression of Tregs was defined as 1 − (c.p.m. incorporated in the coculture of $CD4^+$ $CD25^{high}$ T cells with responder T cells/c.p.m. of responder cells alone) × 100%.

Statistical analysis

To adjust for the gender and age effect on Treg percentages in different groups, the general linear model was adopted to compare the group differences. The frequencies of different Treg subsets were considered as response variables, individually. The variables of group, gender and age were included in each general linear model. The significant level of group variables was then considered as significantly different after adjusting for gender and age effects. Statistical comparisons of data among groups of normal and SLE subjects (without the AS group) were performed using the non-parametric Mann–Whitney *U*-test. Correlations were determined by non-parametric Spearman's correlation. Probability (*P*) values of $< 0{\cdot}05$ were considered significant. All statistical tests were performed using SPSS for Windows, version 13.0. (SPSS Inc., Chicago, IL).

Results

Demographic and clinical information of study subjects

The healthy volunteers were matched with the inactive and active SLE patients for both age and gender. In addition, we chose AS patients as patient controls because a previous study had mentioned that the function of $CD4^+$ $CD25^{high}$ T cells from AS patients was normal.[2] Within the study group, the mean age of patients with active SLE was significantly younger than that of AS patients. Patients with AS were predominantly male, while patients with SLE were primarily female (Table 1). Therefore, statistical comparisons of data among groups of study subjects were performed using a general linear

model to adjust for age and gender effects, as described in the *Materials and methods*.

Definition and phenotypic characterization of Treg subsets and Teff in patients and controls

To clarify the proportions of different populations of Tregs in the peripheral blood of study subjects, including SLE patients, the expression of CD25 and FoxP3 on $CD4^+$ T cells was examined to define the $CD25^{high}$ $FoxP3^+$, $CD25^{low}$ $FoxP3^+$ and $CD25^-$ $FoxP3^+$ subsets. As shown in Fig. 1a, most $CD4^+$ $CD25^{high}$ T cells are $FoxP3^+$ cells (termed $CD25^{high}$ Tregs).[12] In the phenotypic analysis of the cells from SLE patients (Fig. 1a lower panel), the population of $CD25^{low}$ and $CD25^-$ cells among $CD4^+$ T cells contained a substantial number of the cells co-expressing $FoxP3^+$ (termed $CD25^{low}$ Tregs and $CD25^-$ Tregs, respectively). $CD4^+$ $CD25^+$ $FoxP3^-$ T cells may represent recently activated $CD4^+$ T cells (termed Teffs).[22]

We further characterized these putative Treg subsets and Teffs using CD127 and other surface markers associated with activation and Ag presentation. Using multicolor analysis (Fig. 1b), most $CD4^+$ $FoxP3^+$ T cells, irrespective of their CD25 expression, were $CD127^{low/-}$, whereas $CD4^+$ $CD25^+$ $FoxP3^-$ T cells contained around 50% of $CD127^+$ cells and at least 80% of $CD45RO^+$ cells, both in the controls and patients. As CD127 could be used to discriminate between Tregs and activated T cells,[23,24] our data support that $CD4^+$ $FoxP3^+$ T cells, irrespective of their CD25 expression, represent Treg subsets and that $CD4^+$ $CD25^+$ $FoxP3^-$ T cells represent Teffs.

As to other markers, $CD25^{high}$ Tregs are mainly $HLA\text{-}DR^{low}$, $CD45RO^+$ and $CD45RA^{low/-}$, both in the controls (black line) and in patients (color line). In addition,

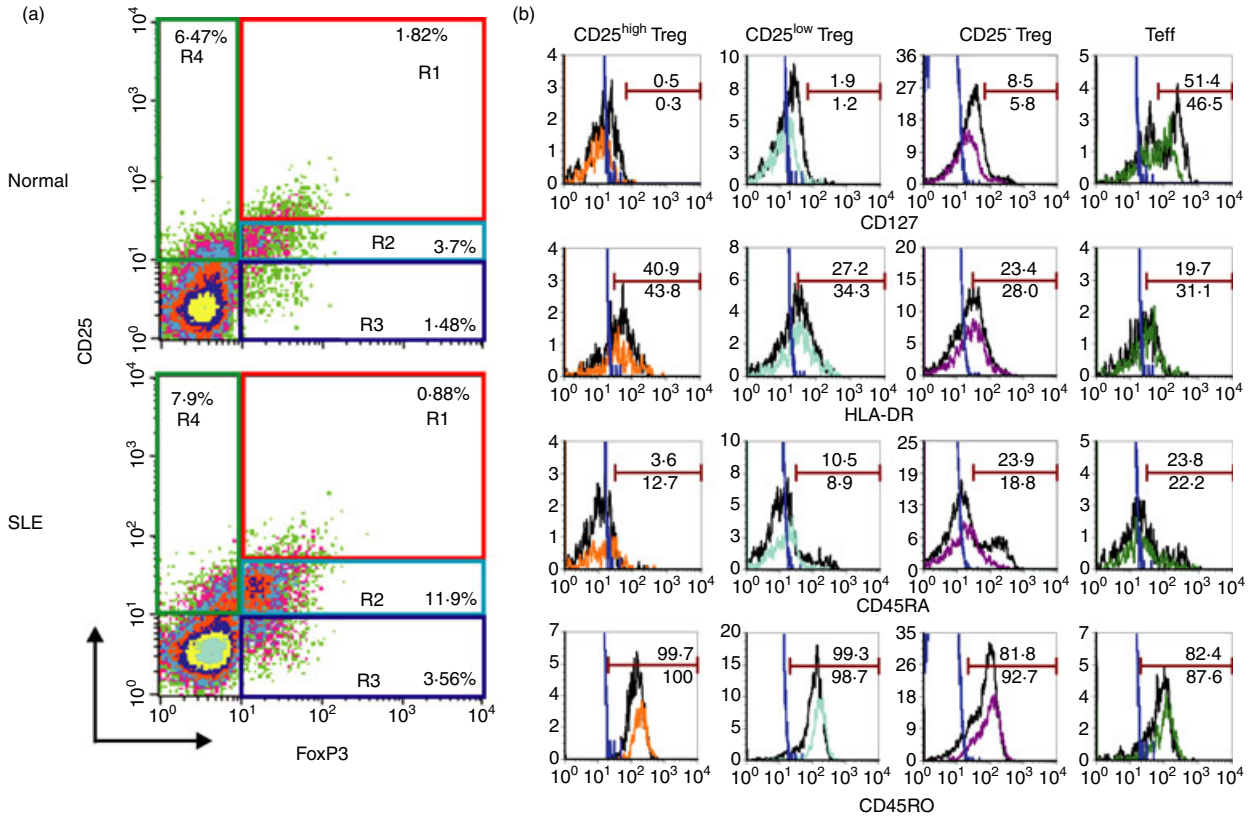

Figure 1. Characterization of different subsets of regulatory T cells (Tregs) and effector T cells (Teffs) in patients with systemic lupus erythematosus (SLE) and in controls. (a) Purified T cells from peripheral blood mononuclear cells (PBMCs) were stained with monoclonal antibodies specific for CD4, CD25 and intracellular forkhead box P3 (FoxP3) and analyzed using a FACScan. Representative dot-plots show the expression of CD25 and FoxP3 on gated $CD4^+$ T cells from one healthy donor (upper panel) and from one patient with SLE (lower panel). Quadrants were established using appropriate isotype controls. $CD4^+$ $CD25^{high}$ $FoxP3^+$ (R1), $CD4^+$ $CD25^{low}$ $FoxP3^+$ (R2) and $CD4^+$ $CD25^-$ $FoxP3^+$ (R3) cells were termed as $CD25^{high}$ Tregs, $CD25^{low}$ Tregs and $CD25^-$ Tregs, respectively. $CD4^+$ $CD25^+$ $FoxP3^-$ (R4) cells were termed as Teffs. (b) PBMCs were stained with monoclonal antibodies specific for CD4, CD25, CD127, HLA-DR, CD45RO, CD45RA and intracellular FoxP3 and analyzed using LSRII. Histograms show surface marker expression on different Treg subsets and Teffs of one healthy donor (black line) and one patient with SLE (color line). The blue lines represent the isotype control. Data are representative of five independent controls and four independent patients.

$CD25^-$ Tregs in the controls may consist of memory and naïve subsets, based on their CD45RO and CD45RA expression, as shown in Fig. 1b. However, $CD25^-$ Tregs in patients with SLE were mainly $CD45RO^+$ and $CD45RA^{low/-}$, possibly reflecting the recent activation of patient's $CD25^-$ Tregs by self-Ags *in vivo*.

Frequencies of circulating $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells ($CD25^{high}$ Tregs) were significantly decreased in patients with active SLE

As shown in Fig. 2, the percentage of circulating $CD25^{high}$ Tregs was significantly lower in patients with active SLE (0·61 ± 0·41%) that in healthy donors (0·86 ± 0·39%). Although no significant difference in Treg preponderance was observed between subjects with inactive SLE (0·69 ± 0·33%) and healthy controls, the percentage of $CD25^{high}$ Tregs in patients with inactive SLE tended to be lower than that in healthy controls. We also found that the absolute numbers of $CD25^{high}$ Tregs in patients with inactive ($3{\cdot}64 \pm 3{\cdot}39 \times 10^3$/ml) and active ($2{\cdot}33 \pm 2{\cdot}06 \times 10^3$/ml) SLE were significantly lower than those found in healthy controls ($5{\cdot}58 \pm 2{\cdot}11 \times 10^3$/ml).

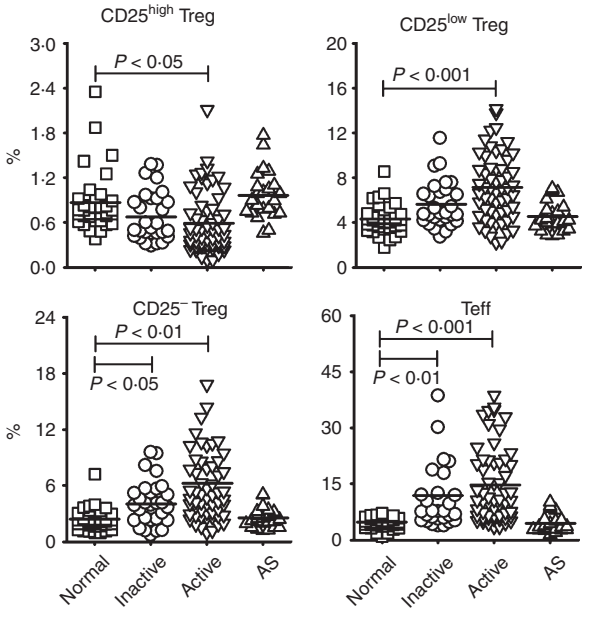

Figure 2. The percentages of different regulatory T-cell (Treg) subsets and effector T cells (Teffs) in patients with systemic lupus erythematosus (SLE) and in healthy controls. The results are shown as percentages of $CD25^{high}$ Tregs, $CD25^{low}$ Tregs, $CD25^-$ Tregs and Teffs in normal controls ($n = 36$), in patients with inactive SLE ($n = 29$), in patients with active SLE ($n = 58$) and in patients with ankylosing spondylitis (AS) ($n = 23$). Symbols represent the frequencies of Treg subsets and Teffs in individual samples. The line within the vertical points marks the mean for each group. A *P*-value of $< 0{\cdot}05$ was considered as significant following analysis of the results using a general linear model adjusted for gender and age. See Fig. 1 for other definitions.

The percentages of $CD25^{low}$ $FoxP3^+$, $CD25^-$ $FoxP3^+$ and $CD25^+$ $FoxP3^-$ populations were increased among $CD4^+$ T cells in SLE patients

In contrast to the reduction of $CD25^{high}$ Tregs, the proportions of $CD25^{low}$ Tregs, $CD25^-$ Tregs and Teffs were significantly elevated in SLE patients (Fig. 2). The proportions of circulating $CD25^{low}$ Tregs, as well as $CD25^-$ Tregs, were significantly increased in patients with inactive SLE (5·62 ± 2·02%, 4·14 ± 2·38%, respectively) and active SLE (7·1 ± 2·92%, 6·26 ± 4·87%, respectively) compared with the proportions in healthy donors (4·31 ± 1·29%, 2·37 ± 1·18%, respectively). However, the absolute counts of $CD25^{low}$ Tregs and $CD25^-$ Tregs were similar among these three subject groups ($CD25^{low}$ Tregs: normal, $2{\cdot}8 \pm 1{\cdot}12 \times 10^4$/ml, inactive, $2{\cdot}62 \pm 2{\cdot}01 \times 10^4$/ml, active, $2{\cdot}57 \pm 1{\cdot}68 \times 10^4$/ml; $CD25^-$ Tregs: normal, $1{\cdot}49 \pm 0{\cdot}7 \times 10^4$/ml, inactive, $1{\cdot}85 \pm 1{\cdot}77 \times 10^4$/ml, active, $2{\cdot}19 \pm 2{\cdot}19 \times 10^4$/ml). The apparently normal absolute Treg numbers found in patients with SLE may be partly the result of lymphopenia associated with the disease, which led to a decrease in the number of total $CD4^+$ T cells in patients with both inactive ($4{\cdot}93 \pm 3{\cdot}62 \times 10^5$/ml) and active ($4{\cdot}24 \pm 3{\cdot}78 \times 10^5$/ml) SLE, compared with that seen in normal controls ($6{\cdot}69 \pm 1{\cdot}93 \times 10^5$/ml). As expected, when compared with healthy controls (4·68 ± 1·54%), patients with SLE showed a significantly increased percentage of Teffs (inactive, 11·97 ± 10·1%; active, 14·71 ± 9·8%) (Fig. 2). Absolute counts of Teffs were also significantly elevated in patients with active ($5{\cdot}44 \pm 5{\cdot}24 \times 10^4$/ml) but not inactive ($4{\cdot}5 \pm 3{\cdot}07 \times 10^4$/ml) SLE, compared with those in healthy controls ($3{\cdot}15 \pm 1{\cdot}44 \times 10^4$/ml).

Regarding patients with AS, the percentages of $CD25^{high}$ Tregs, $CD25^{low}$ Tregs, $CD25^-$ Tregs and Teffs (0·97 ± 0·33%, 4·56 ± 1·36%, 2·51 ± 1·06%, 4·43 ± 2·39%, respectively) did not significantly differ from those found in normal controls (Fig. 2).

Altered relative ratios of $CD25^{high}$ Tregs and $CD25^{low}$ Tregs versus Teffs in patients with SLE

Owing to the importance of the delicate balance between Tregs and Teffs, the ratios of these two populations were further analyzed and the results are shown in Fig. 3. There was a significantly lower ratio of both $CD25^{high}$ Tregs and $CD25^{low}$ Tregs versus Teffs in patients with inactive and active SLE than in healthy individuals ($CD25^{high}$ Tregs/Teffs: inactive, 0·1 ± 0·08, active, 0·07 ± 0·06, normal, 0·23 ± 0·24; $CD25^{low}$ Tregs/Teffs: inactive, 0·67 ± 0·37, active, 0·65 ± 0·38, normal, 1·13 ± 0·86). However, there was no significant difference

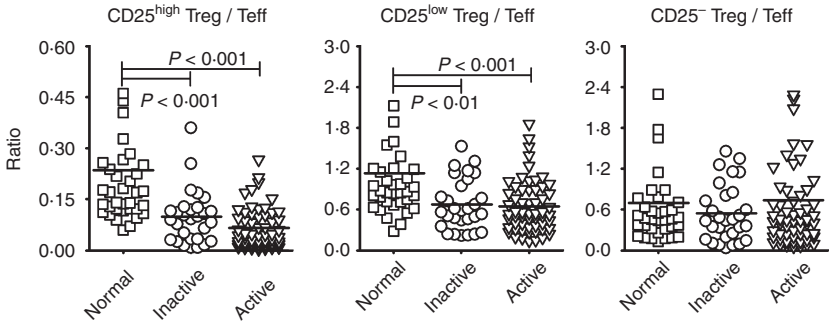

Figure 3. The comparisons of ratios of regulatory T-cell (Treg) subsets versus effector T cells (Teffs) in patients with SLE and in healthy controls. The ratio was defined as the percentage of the indicated Treg subset divided by the percentage of Teffs from the same individual. The ratio was calculated from individual data shown in Fig. 2. A *P*-value of $< 0{\cdot}05$ was considered significant following analysis of the results using the Mann–Whitney *U*-test. See Fig. 1 for other definitions.

in the ratio of CD25$^-$ Tregs to Teffs between patients with inactive or active SLE and healthy controls (inactive, $0{\cdot}54 \pm 0{\cdot}42$, active, $0{\cdot}74 \pm 1{\cdot}26$, normal, $0{\cdot}7 \pm 0{\cdot}78$).

The imbalance between Tregs and Teffs correlated with levels of anti-dsDNA IgG in patients with inactive SLE

We next examined whether the change in the distribution of the Treg subsets is correlated with the clinical and serologic features of SLE. First, the titer of anti-dsDNA IgG was, as expected, positively correlated with the percentage of Teffs in patients with inactive SLE (Fig. 4a), but this correlation was not seen in active SLE (data not shown). Second, it was found that the ratios of CD25^{high} Tregs, CD25^{low} Tregs, or CD25$^-$ Tregs versus Teffs were inversely correlated with the titer of anti-dsDNA IgGs in patients with inactive SLE (Fig. 4a), but not in those with active disease (data not shown). In addition, we compared the percentages of different Treg subsets in SLE patients exhibiting normal complement levels or low complement levels. The percentages of CD25^{low} Tregs and

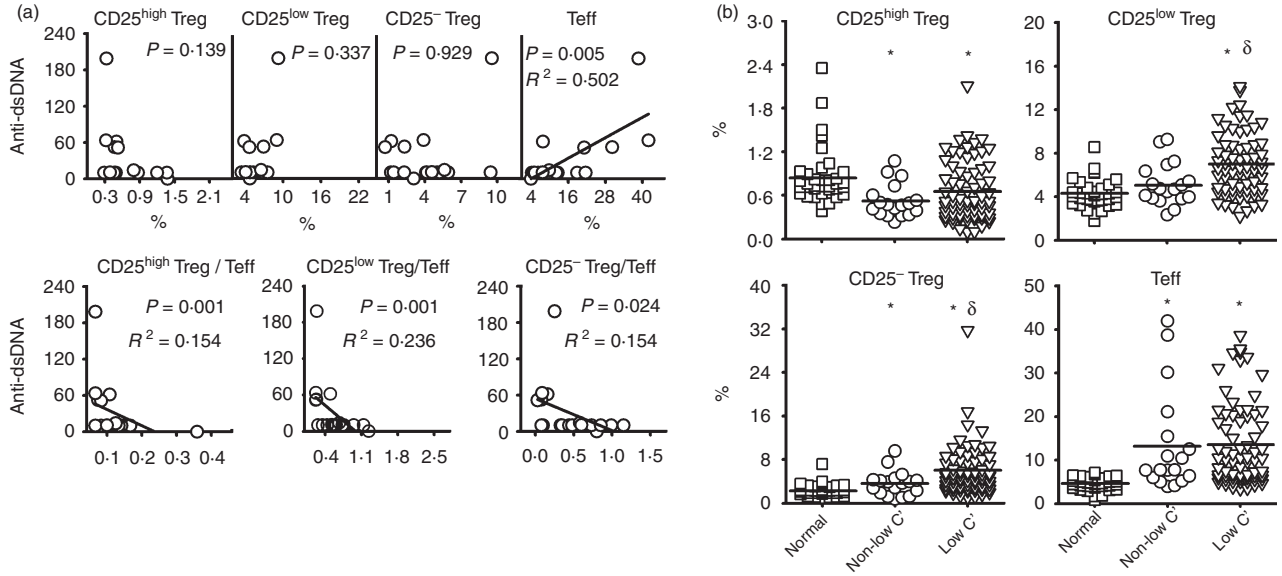

Figure 4. The relationships between disease activity and the frequencies or relative ratios of regulatory T-cell (Treg) subsets and effector T cells (Teffs) in patients with systemic lupus erythematosus (SLE). (a) The ratios of different Treg subsets versus Teffs were negatively correlated with the titre of anti-double-stranded DNA immunoglobulin G (anti-dsDNA IgG) in patients with inactive SLE ($n = 18$). (b) The results are shown as the percentage of CD25^{high} Tregs, CD25^{low} Tregs, CD25$^-$ Tregs and Teffs in healthy controls ($n = 36$); SLE patients exhibiting normal complement levels (Non-low C') ($n = 18$); and SLE patients exhibiting low complement levels (Low C') ($n = 69$). Lines within the vertical points mark the mean for each group. A *P*-value of $< 0{\cdot}05$ was considered as significant following analysis of the results using the Mann–Whitney *U*-test. $^*P < 0{\cdot}05$ compared with normal subjects; $\delta P < 0{\cdot}05$ compared to SLE subjects with normal complement levels. See Fig. 1 for other definitions.

$CD25^-$ Tregs in patients with low complement levels (6·9 ± 2·8%, 6·06 ± 4·58%, respectively) were significantly higher than those in patients with normal complement levels (5·16 ± 1·94%, 3·61 ± 2·28%, respectively). However, no significant differences in the percentages of $CD25^{high}$ Tregs and Teffs were found in these two groups (Fig. 4b). We also compared the percentages of different Treg subsets and the ratios of Tregs versus Teffs in SLE patients with or without nephritis; however, we did not find any statistically significant differences between these two groups (data not shown).

$CD25^{high}$ Tregs displayed normal function in patients with SLE

Previous studies examined SLE patient Treg function using $CD4^+$ $CD25^-$ T cells as responding T cells.[6,8] However, our data showed that $CD4^+$ $CD25^-$ T cells contained significantly higher levels of $FoxP3^+$ cells in patients with inactive (4·14 ± 2·38%) and active (6·26 ± 4·87%) SLE compared with control donors (2·37 ± 1·18%) (Fig. 2). To avoid the contamination of $FoxP3^+$ cells in responder T cells, we sorted only highly pure naïve T cells ($CD4^+$ $CD45RA^{high}$) as responder cells. When activated with a plate-bound anti-CD3, these naïve T cells responded with robust proliferation, although the magnitude of the proliferative response was lower than that induced by $CD4^+$ $CD25^-$ cells in the same culture conditions.

We cocultured sorted $CD4^+$ $CD25^{high}$ T cells and autologous naïve T responder cells in the presence of autologous antigen-presenting cells (APCs) at different ratios. As shown in Fig. 5, $CD4^+$ $CD25^{high}$ T cells from both healthy individuals and patients with SLE significantly inhibited T-cell proliferation in a dose-dependent manner. In fact, $CD4^+$ $CD25^{high}$ T cells from normal individuals and SLE patients demonstrated similar capacities for suppressing the proliferative responses of naïve T cells at varying ratios of Treg cells/naïve T cells.

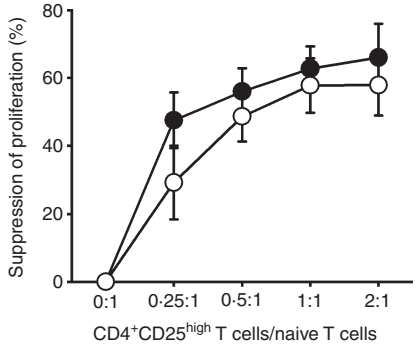

Figure 5. The $CD25^{high}$ regulatory T-cell (Treg) suppressive function in patients with systemic lupus erythematosus (SLE) appears normal *in vitro*. Naïve T cells ($CD4^+$ $CD45RA^{high}$) were stimulated alone or cocultured with autologous $CD4^+$ $CD25^{high}$ T cells in the presence of mitomycin C-treated autologous antigen-presenting cells (APCs) at the indicated ratios. SLE patients (○, $n = 7$, three inactive and four active) and age- and gender-matched healthy individuals (●, $n = 6$) were tested for $CD25^{high}$ Treg activity. The per cent suppression of proliferation was calculated as described in the '*Materials and methods*'. The average proliferative response of APCs was 583 counts per minute (c.p.m.) in the control group and 1224 c.p.m. in the patient group. The average stimulation index of responder T cells was 8·3 in the control group and 9·9 in the patient group. Sorted $CD4^+$ $CD25^{high}$ T cells proliferated poorly after activation with monoclonal anti-CD3, while the average stimulation index of $CD25^{high}$ Tregs in both groups was < 2·0. See Fig. 1 for other definitions.

Discussion

The role of Tregs in the pathogenesis of SLE has been suggested, but the exact mechanism remains to be defined. This study provides, to our knowledge, the first evidence for an increased prevalence of circulating $CD25^{low}$ and $CD25^-$ of $CD4^+$ $FoxP3^+$ T cells in SLE patients. The results also showed that quantitative imbalances between different $CD4^+$ $FoxP3^+$ subsets and Teffs were associated with disease activity and autoantibody production in patients with SLE. While the causal relationship remains to be established, this study suggests that the delicate balance between Tregs and Teffs may be important in the pathogenesis of human lupus.

Several studies demonstrated that $CD25^{low}$ $FoxP3^+$ cells are Tregs with a naive phenotype, which is reflected in their low expression of CD45RO and CD95, as well as their resistance to CD95 ligand (CD95L)-mediated apoptosis. In contrast, $CD25^{high}$ $FoxP3^+$ Tregs exhibited a predominantly memory phenotype with high expression of CD45RO and CD95, and displayed sensitivity to CD95L-induced apoptosis.[22,25,26] As a corollary, Miyara *et al.*[6] suggested that enhanced CD95-mediated apoptosis of $CD4^+$ $CD25^{high}$ Tregs could result in reduced frequencies of Treg in active SLE. The sensitivity to apoptosis may thus determine, in part, the relative frequency of the Treg subpopulations. This possibility would be consistent with our finding of a decreased frequency in the $CD25^{high}$ Tregs in patients with active SLE. Furthermore, the increased frequency of $CD25^{low}$ Tregs, as seen in our study patients, may compensate for the loss of $CD25^{high}$ Tregs in active SLE. However, this compensation may not be enough to regulate the autoimmune response because the ratio of $CD25^{low}$ Tregs versus Teffs in both inactive and active SLE still remains significantly lower than that in normal controls (Fig. 3). Thus, our data suggest that the pathogenesis of SLE might be associated with not only a reduction in $CD25^{high}$ Tregs, but also the quantitative imbalance between $CD25^{low}$, and $CD25^-$ Tregs, and Teffs.

Active systemic inflammation against self-Ags, as observed in patients with SLE, may induce T-cell

activation, resulting in increased CD25 expression. Our data indeed showed a significantly higher frequency of $CD4^+$ $CD25^+$ $FoxP3^-$ T cells in SLE patients compared with normal controls (Fig. 2). In addition, systemic inflammation may induce the differentiation of naïve $CD4^+$ T cells into $CD25^+$ $FoxP3^+$ and $CD25^-$ $FoxP3^+$ Tregs, or so-called adaptive Tregs.[15,16,27] Liu *et al.*[23] also found that that FoxP3 was expressed in 8–10% of the human $CD4^+$ T cells, independently of CD25 expression in normal individuals. Although it is currently unclear whether such differentiation of adaptive Tregs exists in human inflammatory diseases *in vivo*, it is tempting to speculate that $CD4^+$ $CD25^-$ $FoxP3^+$ T cells in SLE patients might represent adaptive Tregs induced by a systemic autoimmune response. It is also possible that $CD25^{low}$ Tregs in SLE patients may contain not only thymically derived Tregs with a naïve phenotype,[22,25] but also peripherally converted Tregs.

In addition to the disproportionate numbers of Tregs and Teffs, the functions of different Treg subsets in SLE still need further clarification. The *in vitro* suppressive data of $CD4^+$ $CD25^{high}$ T cells (Fig. 5) support the presence of normally functioning, suppressive $CD4^+$ $CD25^{high}$ T cells in SLE patients. The data are consistent with those of Miyara *et al.*,[6] but not with those of Valencia *et al.*[8] This discrepancy might be a result of the different monoclonal antibodies and types of APCs used for *in vitro* stimulation. Regarding the functions of $CD25^{low}$ Tregs and $CD25^-$ Tregs in normal individuals, Liu *et al.* demonstrated that $CD4^+$ $CD127^{low/-}$ T cells, which include both $CD25^{low}$ $FoxP3^+$ and $CD25^-$ $FoxP3^+$ subsets, could suppress the allogeneic mixed-lymphocyte response as well as classical '$CD4^+$ $CD25^{high}$' Tregs.[23] However, our phenotypic analysis of different Treg subsets showed a reduced frequency of CTLA-4-expressing, $CD25^{low/-}$ Tregs in SLE patients compared with those in normal controls (data not shown). On the other hand, it should be taken into account that activation-induced expression of FoxP3 mRNA has been reported for *in vitro*-stimulated $CD4^+$ T cells.[28–30] Furthermore, acetylation of several lysines in the forkhead domain of the FoxP3 protein determines the optimal Treg function.[31] These observations raise questions about whether $CD25^{low}$ Tregs and $CD25^-$ Tregs in patients with SLE exhibit suppressive activity.

Immune regulation is not only controlled by Tregs but also by the status of responder T cells. A previous study demonstrated that activated responder T cells from the synovial fluid of patients with rheumatoid arthritis were more resistant to suppression mediated by $CD4^+$ $CD25^+$ T cells than responder cells from the peripheral blood of these patients.[32] This study also found an increased percentage of $CD4^+$ $CD25^+$ T cells in synovial fluid, but not in the peripheral blood, of patients with rheumatoid arthritis.[32] Therefore, the degree to which Teffs are resistant to Treg suppression in SLE patients requires further investigation.

One important characteristic of SLE is the production of anti-dsDNA IgG. Although the role of Tregs on T-cell-mediated immunity has been widely explored, the effects of Tregs on humoral immunity are less clear. Seo *et al.*[33] have shown that the tolerance breakdown of autoreactive B cells in *lpr/lpr* mice requires the help of $CD4^+$ T cells and the overcoming of suppression by $CD4^+$ $CD25^+$ Tregs. In agreement with the previous study,[33] our data showed that the ratios of $CD4^+$ $FoxP3^+$ subsets versus Teffs were inversely correlated with the titer of anti-dsDNA IgG in patients with inactive SLE (Fig. 4a). This suggests that as the number and/or function of Tregs decline relative to those of Teffs, tolerance begins to be breached in SLE patients. Thus, for autoimmune diseases with altered frequencies of Tregs, an analysis of Teffs should be carried out in parallel.

On the other hand, the imbalance between Treg subsets and Teffs did not correlate with the level of anti-dsDNA IgG in patients with active SLE (data not shown). We speculate that the high dose of drug treatment may affect the levels of autoantibodies in patients with active SLE. The main treatment approach for lupus patients in this study was the combination of prenisolone, hydroxychloroquine and azathioprine. Among these drugs, corticosteroids, such as prednisolone, can control inflammation through the suppression of multiple inflammatory genes that are activated in chronic inflammatory diseases. Prednisolone also suppresses T-lymphocyte proliferation by inhibiting nuclear factor-κB, which eventually down-regulates IL-2 expression.[34,35] With a lack of T-cell help, IgG autoantibody production would significantly decrease. Thus, the higher dose of prenisolone used in active SLE may interfere with the titre of anti-dsDNA IgG, and therefore no correlation was found between Tregs versus Teffs ratios and the anti-dsDNA IgG titer in those study patients. This possibility clearly needs to be further investigated. As to the effect of treatment on the distribution of Tregs, the percentage of those three Treg subsets in the patients did not correlate with daily prednisolone dose (data not shown).

In summary, our study examined the frequencies of different subpopulations of $CD4^+$ $FoxP3^+$ T cells, which may represent a comprehensive Treg pool in human PBMCs from normal individuals and patients with SLE. Our study also found that altered homeostasis, as evidenced by an imbalance between different $CD4^+$ $FoxP3^+$ subsets and Teffs, were associated with disease activity, complement consumption and the production of anti-dsDNA IgG in SLE patients. Expanding the frequencies of Tregs, especially in an Ag-specific manner, may be a potential therapeutic strategy for treating autoimmune diseases such as SLE.

Acknowledgements

This work was supported by grants from the National Science Council (NSC 94-2320-B-037-030 and NSC 95-2320-B-037-009) and by the funds KMU-EM-97-1.2b. We sincerely thank Dr Shau Ku Huang for critical review of the manuscript. We also thank the Center for Resources, Research and Development of KMU and the Department of Medical Research, KMUH for providing LSRII and FACSVantage, respectively.

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