

# Elevated Gene Expression of Th1/Th2 Associated Transcription Factors Is Correlated with Disease Activity in Patients with Systemic Lupus Erythematosus

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**ABSTRACT.** *Objective.* T-box expressed in T cells (T-bet) and GATA-binding protein 3 (GATA-3) are transcriptional factors that play a crucial role in Th1 and Th2 development. We investigated the immunomodulatory roles of T-bet and GATA-3 and Th1/Th2 related cytokines in the pathogenesis of systemic lupus erythematosus (SLE) and their association with disease activity.

*Methods.* Gene expressions of T-bet, GATA-3, interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin 4 (IL-4) in peripheral blood mononuclear cells, and plasma concentrations of the Th1/Th2 cytokines IFN- $\gamma$ , IL-18, and IL-4, were assayed in 80 patients with SLE and 40 sex and age matched healthy subjects by real-time quantitative polymerase chain reaction and ELISA.

*Results.* The mRNA levels of T-bet and IFN- $\gamma$  and the relative expression levels of T-bet/GATA-3 and IFN- $\gamma$ /IL-4 were significantly higher, in contrast to the lower expressions of GATA-3 and IL-4, in SLE patients than controls (all  $p < 0.05$ ). In all SLE patients, there were significant correlations in mRNA expression of T-bet with IFN- $\gamma$  ( $r = 0.590$ ,  $p < 0.0001$ ), and of GATA-3 with IL-4 ( $r = 0.245$ ,  $p = 0.029$ ). The relative expressions of T-bet/GATA-3 and IFN- $\gamma$ /IL-4 correlated with lupus disease activity ( $r = 0.229$ ,  $p = 0.042$ ;  $r = 0.231$ ,  $p = 0.040$ , respectively). Plasma IL-18 concentration was increased significantly in all SLE patients ( $p < 0.05$ ). The elevated plasma Th1/Th2 cytokine ratio IL-18/IL-4 correlated positively with disease activity in all SLE patients ( $r = 0.250$ ,  $p = 0.025$ ).

*Conclusion.* There is an association between expression of Th1/Th2 transcription factors and cytokines in SLE. The elevated gene expressions of Th1/Th2 transcription factors and cytokines should provide a useful tool for assessing the functional status of T-helper lymphocytes in SLE disease development. (First Release Nov 15 2006; J Rheumatol 2007;34:89–96)

## Key Indexing Terms:

SYSTEMIC LUPUS ERYTHEMATOSUS

CYTOKINES

SYSTEMIC LUPUS ERYTHEMATOSUS DISEASE ACTIVITY INDEX

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by various immunological abnormalities, including dysregulated activation of both T and B lymphocytes, and subsequent polyclonal activation of circulating B cells that produces a large quantity of autoreactive antibodies<sup>1,2</sup>. The etiology and pathogenic mechanism of this immunological disorder have not been clearly elucidated. Aberrant production and imbalance of T helper (Th) lympho-

cyte cytokines have been shown to be involved in the pathogenesis of autoimmune diseases<sup>3</sup>. It has been suggested that SLE is a Th2-polarized disease because of its production of autoantibodies specific for self-antigens<sup>4</sup> and increases in plasma concentration of the Th2 cytokines IL-6 and IL-10 in active SLE<sup>5</sup>. However, other studies have demonstrated that plasma cytokines for Th1 response including IL-12, tumor necrosis factor- $\alpha$ , and interferon- $\gamma$  (IFN- $\gamma$ ) were also significantly elevated in patients with SLE<sup>6-8</sup>. The ratios of Th1 and Th2 cytokines have been investigated<sup>3</sup> to determine the cytokine homeostasis, and indicate Th1 or Th2 predominance during the development of disease. Other work showed a negative correlation of the ratio between IFN- $\gamma$ /IL-10-secreting cells and SLE Disease Activity Index (SLEDAI) by enzyme-linked immunospot analysis of freshly isolated stimulated peripheral blood mononuclear cells (PBMC)<sup>9</sup>, while another study reported a positive and significant correlation of ratios with disease activity using *in vitro* stimulated PBMC<sup>3</sup>. From the observed high ratio of IFN- $\gamma$ /IL-4, Akahoshi, *et al* sug-

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gested a significant predominance of Th1 among SLE patients with lupus nephritis and proliferative glomerulonephritis<sup>10</sup>. Our previous findings also showed that plasma concentrations of the proinflammatory cytokine IL-18 were significantly elevated in SLE patients<sup>11,12</sup>, and there was a positive correlation between the IL-18/IL-4 ratio and the SLEDAI, suggesting an unbalanced cytokine profile with Th1 predominance<sup>11</sup>. These phenomena imply that the Th cytokine response in SLE is very complex, and the issue of polarized Th1 and/or Th2 cytokine expression in SLE remains unsettled.

Recent studies have focused on the molecular mechanisms of Th1 and Th2 cell development. Many intracellular molecules appear to control Th1/Th2 development and the related production of cytokines. Significant progress has also been made in identifying factors that control the transcription of Th precursor cells. T-box expressed in T cells (T-bet) and GATA-binding protein 3 (GATA-3) are the 2 major transcription factors that regulate the expression of Th1 or Th2 cytokine genes and play important roles in differentiation of Th cells<sup>12</sup>. T-bet, a Th1-specific transcription factor, has been postulated to initiate Th1 development while inhibiting Th2 cell differentiation<sup>13</sup>. GATA-3 is a member of the GATA zinc finger protein family, and enhances the development of the Th2 phenotype while inhibiting Th1 cells<sup>14-16</sup>. Studies of patients with asthma and atopic dermatitis have shown increased gene expression of the GATA-3 transcription factor<sup>17,18</sup>. Recent publications also reported an alteration of T-bet levels in patients with inflammatory diseases of the gastrointestinal tract<sup>19,20</sup>. Upregulation of T-bet expression with induction of Th1-mediated immunopathology was evident in Crohn's disease<sup>19,20</sup>. Transcriptional regulation with parallel suppression of Th1 and Th2 cytokine production was similarly reported in Kawasaki disease<sup>21</sup>. Although the importance of these transcription factors has been demonstrated in these immunological disorders, little information is available about the expression profile and interactions of these transcription factors in SLE. To investigate their immunopathological roles and influence on Th1/Th2 status in SLE pathogenesis and disease activity, we analyzed the mRNA expression of T-bet, GATA-3, IFN- $\gamma$ , and IL-4 in PBMC and plasma cytokine IFN- $\gamma$ , IL-18, IL-4, and IL-10 protein concentrations from SLE patients with or without renal disease, and compared the results with those of normal healthy subjects.

## MATERIALS AND METHODS

**Patients, controls, and blood samples.** Eighty Chinese patients with SLE (78 women, 2 men) treated at the Rheumatology Outpatient Clinic of Prince of Wales Hospital, Hong Kong, were recruited with informed consent. Ethical approval for the study was obtained from the Clinical Research Ethics Committee of The Chinese University of Hong Kong and New Territories East Cluster Hospitals. Diagnosis of SLE was established according to the 1982 revised American Rheumatism Association criteria<sup>22</sup>, and disease activity was evaluated by SLEDAI score<sup>23</sup>. Active lupus disease was defined as a SLEDAI score  $\geq 6$ <sup>23</sup>. Patients were classified as having active or inactive disease, and were divided further into 2 groups: 40 with renal disease (RSLE group) and 40 without renal disease (SLE group). The rationale for this was

that SLE patients with advanced or severe disease will develop renal impairment, thus representing most of the active disease group. RSLE patients were defined by persistence of proteinuria ( $> 0.5$  g/24 h) or the presence of cellular casts, persistent hematuria, or renal biopsy showing mesangial, focal proliferative, diffuse proliferative or membranous glomerulonephritis. Forty sex and age matched healthy Chinese subjects were recruited as controls. Fifteen milliliters of EDTA venous peripheral blood were collected from each patient and control.

**Isolation of RNA from PBMC.** Mononuclear cells were isolated from EDTA blood by gradient centrifugation on Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden). PBMC were then applied to an RNeasy mini-column (Qiagen GmbH, Hilden, Germany) and was processed according to the manufacturer's recommendations. "On-column DNase digestion" was performed using an RNase-free DNase preparation (Qiagen) as described by the manufacturer. Total RNA was eluted with 15  $\mu$ l of RNase-free water and stored at  $-80^{\circ}\text{C}$ . The amount of RNA was determined using the Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY, USA) and normalized to 1  $\mu\text{g}/\text{ml}$  for each subsequent real-time quantitative polymerase chain reaction (RT-PCR) process.

**RT reverse transcription-PCR.** One step real-time quantitative reverse transcription-PCR was used for all mRNA quantifications. In this method, the *rTth* polymerase functions as both a reverse transcriptase and a DNA polymerase. The primers for all mRNA assays were intron-spanning. The sequences of the amplification primers and TaqMan minor groove binding probes for T-bet, GATA-3, IFN- $\gamma$ , and IL-4 are listed in Table 1. Calibration curves for mRNA quantification were prepared by serial dilutions of HPLC-purified single-strand synthetic DNA oligonucleotides for the 4 transcripts (Proligo Pty Ltd., Singapore) with concentrations ranging from  $2.5 \times 10^1$  to  $2.5 \times 10^6$  copies/ $\mu$ l. The RT-PCR reactions were set up according to the manufacturer's instructions (EZ *rTth* RNA PCR reagent set; Applied Biosystems, Foster City, CA, USA) in a reaction volume of 25  $\mu$ l. Each reaction contained 1  $\times$  EZ buffer; 200 nM of each primer; 100 nM fluorescent probe; 3 mM Mn(OAc)<sub>2</sub>; 300  $\mu$ M each of dATP, dCTP, and dGTP; 600  $\mu$ M dUTP; 5 U of *rTth* polymerase; and 0.5 U of uracil N-glycosylase. Extracted RNA (3 ng) was used for amplification.

Two thermal profiles were used for the 4 RT-PCR assays. The reaction was initiated at  $50^{\circ}\text{C}$  for 2 min in the presence of uracil N-glycosylase, followed by reverse transcription at  $60^{\circ}\text{C}$  for 30 min. After 5 min denaturation at  $95^{\circ}\text{C}$ , PCR was carried out for 40 cycles with a denaturation step of  $94^{\circ}\text{C}$  for 20 s and annealing/extension for 1 min at  $56^{\circ}\text{C}$  (T-bet, IFN- $\gamma$ , and IL-4) and  $60^{\circ}\text{C}$  (GATA-3). The real-time RT-QPCR was performed in duplicate in an Applied Biosystems 7700 Sequence Detector and amplification data were collected and analyzed by the sequence detection system software (ver. 1.9; Applied Biosystems). Multiple negative water blanks were included in every analysis. Results were expressed as copies/ng RNA. The sensitivities of the

Table 1. Primers and probe sequences used for real-time RT-QPCR.

Target	Oligonucleotide	Sequence
T-bet	Sense	5'-CTT TCC AAG AAA CCC AGT TCA-3'
	Antisense	5'-CAG CTG AGT AAT CTC GGC ATT-3'
	Probe	5'-(FAM) TGC CGT GAC TGC CTA-3'
GATA-3	Sense	5'-CAC AAT ATT AAC AGA CCC CTG ACT-3'
	Antisense	5'-TTG CTA GAC ATT TTT CGG TTT CT-3'
	Probe	5'-(FAM) TGA AGA AGG AAG GCA TCC A-3'
IFN $\gamma$	Sense	5'-TGC CAG GAC CCA TAT GTA AAA-3'
	Antisense	5'-GCT ACA TCT GAA TGA CCT GCA T-3'
	Probe	5'-(FAM) AAG CAG AAA ACC TTA AGA AA-3'
IL-4	Sense	5'-GCT TGA ATT CCT GTC CTG TGA AG-3'
	Antisense	5'-TTA GCC TTT CCA AGA AGT TTT CCA-3'
	Probe	5'-(FAM) AAG CCA ACC AGA GTA C-3'

FAM: 6-carboxyfluorescein.

amplification steps of these assays were sufficient to detect T-bet, GATA-3, IFN- $\gamma$ , and IL-4 calibrator targets at 75, 37.5, 18, and 18 copies, respectively. Replicate RNA extraction and RT-PCR analysis showed that the coefficients of variation of threshold cycle (Ct) values of the analytical systems for T-bet, GATA-3, IFN- $\gamma$ , and IL-4 mRNA were 2.19%, 0.8%, 1.16%, and 1.33%, respectively.

**Cytokine assays.** Plasma concentrations of IFN- $\gamma$  and IL-4 were measured by ELISA using specific reagent kits (R&D Systems, Minneapolis, MN, USA) and also for IL-18 (Biosource International, Camarillo, CA, USA).

**Statistical analysis.** Numerical data were expressed as median (interquartile range; IQR) if they were not in Gaussian distribution. Differences among groups were compared by Kruskal-Wallis ANOVA, followed by Dunn's multiple comparisons post-test. Dunn's post-test compares the difference and calculates a p value for each pair of columns. Calculation of the p values takes into account the number of comparisons made. Nonparametric Spearman rank correlation test was used to assess correlations among mRNA transcripts and cytokine protein concentrations with SLEDAI scores. All analyses were performed using SPSS for Windows, version 9.0 (SPSS Inc., Chicago, IL, USA). Probability value < 0.05 was considered significant.

## RESULTS

Forty patients with SLE with renal disease (RSLE group), 40 patients without renal disease (SLE group), and 40 sex and age matched controls were studied. Details of duration of diagnosis, SLEDAI score, plasma urea and creatinine concentrations, and drug treatment are summarized in Table 2. Using a SLEDAI cutoff score  $\geq 6$ , there were 29 patients with active lupus disease and 52 with inactive disease. The proportions of the SLE group and RSLE group with active disease were 7.5% and 65%, respectively. Active kidney disease was manifested in 67.5% of patients with RSLE according to the presence of urinary casts, hematuria, proteinuria, or pyuria. The mean duration of glomerulonephritis in RSLE patients was 9.6 years.

**Quantitative analysis of T-bet, GATA-3, IFN- $\gamma$ , and IL-4 mRNA expression.** As shown in Table 3, T-bet and IFN- $\gamma$

mRNA expression was significantly higher in all SLE patients including RSLE and SLE groups than in controls (all  $p < 0.05$ ). There was also a decrease in IL-4 and GATA-3 expression in all SLE patients and in patients with renal impairment (all  $p < 0.05$ ). Consistent with these observations, when the patients were stratified into active disease (SLEDAI  $\geq 6$ ) and inactive disease (SLEDAI < 6) groups for further evaluation, we found a parallel increase in T-bet and IFN- $\gamma$  expression in patients with active disease [median (IQR) 5050 (3420–6450) vs 3600 (2380–5000) and 326 (199–500) vs 208 (133–321) copies/ml, respectively; all  $p < 0.05$ ] and a concurrent decrease in IL-4 and GATA-3 expression [55 (42–80) vs 79 (55–121) and 6280 (4140–8100) vs 8600 (5000–12,700) copies/ml, respectively; all  $p < 0.05$ ]. Further, assessment of the relative expression of the 2 transcription factors and cytokine genes showed the expression ratios of T-bet/GATA-3 and IFN- $\gamma$ /IL-4 were significantly elevated in all SLE patients including renal and non-renal subgroups (all  $p < 0.05$ ). Parallel to this observation, even more significant increases in the T-bet/GATA-3 and IFN- $\gamma$ /IL-4 expression ratios were noted in patients with active lupus disease (all  $p < 0.05$ ; Table 4).

To evaluate the significance of T-bet and GATA-3 in the differentiation of Th1 and Th2 cells *in vivo*, we also investigated for correlation of mRNA levels of T-bet and IFN- $\gamma$ , GATA-3 and IL-4. There was a significant correlation between the mRNA expression of T-bet and that of IFN- $\gamma$  ( $r = 0.590$ ,  $p < 0.0001$ ), and GATA-3 with that of IL-4 ( $r = 0.245$ ,  $p = 0.029$ ). In addition, the relative expression of T-bet/GATA-3 showed a positive correlation with that of IFN- $\gamma$  ( $r = 0.320$ ,  $p = 0.003$ ), and IFN- $\gamma$ /IL-4 ( $r = 0.229$ ,  $p = 0.040$ ).

We then studied the relationship between expression of T-bet, GATA-3, IFN- $\gamma$ , and IL-4 mRNA and lupus disease activity. The mRNA expression of neither the 2 transcription fac-

Table 2. Demographics and clinical histories of SLE patients with renal involvement (RSLE) and without renal disease (SLE), and controls.

	RSLE	SLE	Controls
No.	40	40	40
Female/male	39/1	39/1	39/1
Age, yrs*	36 $\pm$ 8 (22–55)	38 $\pm$ 9 (19–51)	40 $\pm$ 9 (19–55)
Duration of diagnosis, yrs*	12.1 $\pm$ 6.4 (0.3–29.2)	12.8 $\pm$ 6.1 (1.3–28.2)	NA
SLEDAI score*	8 $\pm$ 4 (2–18)	3 $\pm$ 2 (0–8)	NA
Treatment with prednisolone			
Patients, no. (%)	36 (90.0)	23 (57.5)	NA
Daily dose, mg	6.1 $\pm$ 5.4	3.2 $\pm$ 4.4	
Treatment with hydroxychloroquine			
Patients, no. (%)	16 (40.0)	24 (60.0)	NA
Daily dose, mg	85.0 $\pm$ 112.2	130.0 $\pm$ 111.4	
Treatment with azathioprine			
Patients, no. (%)	22 (55.0)	3 (7.5)	NA
Daily dose, mg	35.6 $\pm$ 36.6	7.5 $\pm$ 28.4	
Plasma urea, mmol/l*	8.2 $\pm$ 6.2 (2.8–36.5)	4.9 $\pm$ 1.4 (2.6–8.2)	NA
Plasma creatinine, $\mu$ mol/l*	108.7 $\pm$ 79.6 (57.0–472.0)	76.0 $\pm$ 10.5 (57.0–106.0)	NA

\* Values are mean  $\pm$  SD (range); NA: not applicable.

Table 3. Gene expression levels of T-bet, GATA-3, IFN- $\gamma$ , and IL-4 in PBMC of SLE patients with non-renal disease (SLE), renal disease (RSLE), and controls.

mRNA transcript		Median (interquartile range), Copies/ng RNA	p
T-bet	Control	3600 (2380–5000)	
	SLE	5165 (3458–8913)	0.001**
	RSLE	4884 (2999–6163)	0.049*
	SLE + RSLE	6350 (4800–8370)	0.022*
GATA-3	Control	8370 (5000–12040)	
	SLE	6418 (4964–9852)	0.316
	RSLE	6278 (4487–7994)	0.042*
	SLE + RSLE	6350 (4800–8370)	0.022*
IFN- $\gamma$	Control	208 (133–321)	
	SLE	340 (190–546)	0.029*
	RSLE	331 (211–547)	0.045*
	SLE + RSLE	330 (188–532)	0.015*
IL-4	Control	79 (55–121)	
	SLE	66 (42–104)	0.224
	RSLE	60 (42–82)	0.029*
	SLE + RSLE	62 (42–88)	0.042*
T-bet/GATA-3	Control	0.47 (0.35–0.58)	
	SLE	0.69 (0.43–1.31)	0.001**
	RSLE	0.63 (0.37–0.86)	0.041*
	SLE + RSLE	0.66 (0.38–1.16)	0.002**
IFN- $\gamma$ /IL-4	Control	3.08 (1.64–5.07)	
	SLE	4.27 (2.54–9.34)	0.032*
	RSLE	6.19 (2.97–9.29)	0.006**
	SLE + RSLE	5.06 (2.88–9.61)	0.005**

\*  $p < 0.05$ ; \*\*  $p < 0.01$  compared to controls.

Table 4. Gene expression levels of T-bet, GATA-3, IFN- $\gamma$ , and IL-4 in PBMC of SLE patients with active disease (ASLE), inactive disease (ISLE), and controls. Active lupus disease was defined as a SLEDAI score  $\geq 6$ .

mRNA transcript		Median (interquartile range), Copies/ng RNA	p
T-bet	Control	3600 (2380–5000)	
	ISLE	4980 (3170–8300)	0.005**
	ASLE	5050 (3240–6450)	0.015*
	ISLE + ASLE	4920 (3070–6830)	0.002**
GATA-3	Control	8370 (5000–12040)	
	ISLE	6420 (5000–8740)	0.104
	ASLE	6280 (4140–8100)	0.011*
	ISLE + ASLE	6350 (4800–8370)	0.022*
IFN- $\gamma$	Control	208 (133–321)	
	ISLE	351 (184–534)	0.008**
	ASLE	326 (199–500)	0.046*
	ISLE + ASLE	330 (188–532)	0.015*
IL-4	Control	79 (55–121)	
	ISLE	66 (45–95)	0.186
	ASLE	55 (42–80)	0.020*
	ISLE + ASLE	62 (42–88)	0.042*
T-bet/GATA-3	Control	0.47 (0.35–0.58)	
	ISLE	0.66 (0.44–1.22)	0.002**
	ASLE	0.80 (0.37–1.12)	0.009**
	ISLE + ASLE	0.66 (0.38–1.16)	0.002**
IFN- $\gamma$ /IL-4	Control	3.08 (1.64–5.07)	
	ISLE	4.27 (2.63–9.02)	0.032*
	ASLE	7.78 (4.19–9.89)	< 0.001***
	ISLE + ASLE	5.06 (2.88–9.61)	0.005**

\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared to controls.

tors nor the 2 cytokines correlated with disease activity. In contrast, we found that the ratios of expressions of T-bet and GATA-3 correlated with the SLEDAI scores in all SLE patients and the RSLE group ( $r = 0.229$ ,  $p = 0.042$ ;  $r = 0.321$ ,  $p = 0.04$ , respectively; Figures 1A, 1C). A similar correlation was also seen between the relative expression of IFN- $\gamma$ /IL-4 and disease activity in the SLE patients and the RSLE group ( $r = 0.231$ ,  $p = 0.04$ ;  $r = 0.337$ ,  $p = 0.030$ , respectively; Figures 1B, 1D). It is noteworthy that we also observed more significant positive correlations between T-bet/GATA-3 and IFN- $\gamma$ /IL-4 and disease activity in patients with active SLE disease ( $r = 0.430$ ,  $p = 0.023$ ;  $r = 0.394$ ,  $p = 0.038$ , respectively; Figures 1E, 1F), suggesting that relative expression of the transcription factors played a significant role in modulating the activation of T cells in SLE patients, leading to disease exacerbation.

**Plasma concentration of Th1 and Th2 cytokines.** Plasma IL-18 concentration was significantly elevated in all SLE patients compared to controls [median 248 (IQR 180–335) vs 158 (137–219) pg/ml, respectively;  $p < 0.001$ ]. IL-4 was elevated only in the RSLE group [0.09 (0.03–0.130) pg/ml; all  $p < 0.05$ ]. The plasma IFN- $\gamma$  concentrations of patients and controls were too low and close to the assay's detection limit, they therefore were not taken into consideration. Weak correlation of disease activity with IL-4 concentration was also noted in the SLE group, although it did not achieve statistical significance ( $p = 0.058$ ). The Th1/Th2 cytokine ratio of IL-18/IL-4 was also significantly higher in all SLE patients than the control group [676 (112–4602) vs 486 (34–2056);  $p = 0.009$ ], while the IL-18/IL-4 ratio correlated positively with SLE disease activity ( $r = 0.250$ ,  $p = 0.025$ ).

In contrast to the positive correlation of mRNA expression levels between the transcription factors, as described, the concentrations of cytokine proteins circulating in plasma did not correlate with the corresponding cytokine mRNA expression, or that of transcription factors, except the level of plasma IL-4 protein, which showed a modest correlation with the relative expression of T-bet/GATA-3 mRNA ( $r = 0.237$ ,  $p = 0.034$ ).

## DISCUSSION

SLE is an idiopathic disease characterized by variable autoimmune inflammatory tissue destruction. Studies have attempted to determine the cellular pathways that contribute to autoimmune responses and the subsequent inflammatory disease<sup>24</sup>. Nevertheless, the mechanisms that lead to the aberrant autoinflammatory syndrome are not clearly understood. Defective T cell censorship has been postulated to play a crucial role in pathogenesis and disease manifestation. An imbalance in the Th1/Th2 cytokine ratio is thought to be pathogenic<sup>3,9</sup>. However, experimental findings have been inconsistent and the issue of polarized Th1 and/or Th2 cytokine expression in SLE remains unsettled. Recent studies have focused on the molecular mechanisms of Th1 and Th2 cell development. The transcription factors T-bet and GATA-3 are thought to be key

regulators of Th1/Th2 differentiation. Although alteration of T-bet or GATA-3 mRNA expression is evident in patients with various inflammatory diseases of the gastrointestinal tract, Crohn's disease, autoimmune glomerulonephritis, allergic asthma, atopic dermatitis, and rheumatoid arthritis<sup>17-20,25-27</sup>, little information is available about the interactions of these transcription factors in SLE.

We focused on mRNA expression levels of the Th1 and Th2 cytokines and transcription factors that regulate the differentiations of Th1 and Th2 cells. We found a reciprocal pattern in mRNA expression of Th1/Th2-associated transcription factors and cytokines in SLE: the mRNA levels of T-bet and IFN- $\gamma$  were significantly higher in SLE patients than in controls, in contrast to the lower expressions of GATA-3 and IL-4. There was a significant correlation between the mRNA expression of T-bet and that of IFN- $\gamma$ , and GATA-3 with IL-4. Previous studies have suggested that T-bet is the master regulator of Th1 lineage commitment that strongly promotes IFN- $\gamma$  expression<sup>13,28</sup>, and GATA-3 is expressed during the course of Th2 differentiation in response to IL-4<sup>14,15</sup>. In this regard, our results confirmed that T-bet and GATA-3 are involved in regulating potential production of IFN- $\gamma$  and IL-4 in SLE. In addition, the reciprocal upregulation in mRNA expression of T-bet and IFN- $\gamma$  and suppressed expression of GATA-3 and IL-4 in patients with SLE suggested that peripheral blood T cells are functionally activated in terms of Th1 cell lineage. As we also observed, the transcription factors and cytokine expressions showed no correlation with the dosages of prednisolone, hydroxychloroquine, and azathioprine in patients with renal and non-renal SLE (all  $p > 0.05$ ).

It is clear that several factors determine the fate of activated T cells, including antigen form, dose, type of antigen-presenting cells, costimulatory molecules, chromatin structure, and most important, cytokines present in the local environment of the cells at the time of stimulation. GATA-3 transcription is activated via pathways that involve IL-4-dependent activation of the signal transducer and activator of transcription (STAT-6)<sup>14-16</sup>. While GATA-3 expression is critical for Th2 development, GATA-3 induction also inhibits Th1 differentiation by increasing IL-4 production and by inhibiting the crucial transcription factor T-bet<sup>29</sup>. T-bet activates the IFN- $\gamma$  gene by chromatin remodeling, leading to secretion of IFN- $\gamma$ , and increases both IFN- $\gamma$  and IL12R $\beta$ 2 chain, further enhancing both IFN- $\gamma$  and IL-12 signals<sup>30,31</sup>. Retroviral expression has shown that T-bet may also inhibit GATA-3 expression and IL-4 and IL-5 production in developing and developed Th2 lymphocytes<sup>13</sup>. The interaction and coexpression between transcription factors and the cytokines thus provide positive and negative feedback regulation for Th1 and Th2 development. Recent *in vitro* studies have shown that changes in the ratio of expression of T-bet and GATA-3 reflect parallel changes in the IFN- $\gamma$  and IL-4, thereby influencing the Th1/Th2 status<sup>32,33</sup>. Together, these reports suggest that the fate of T cell differentiation depends crucially on the dynam-

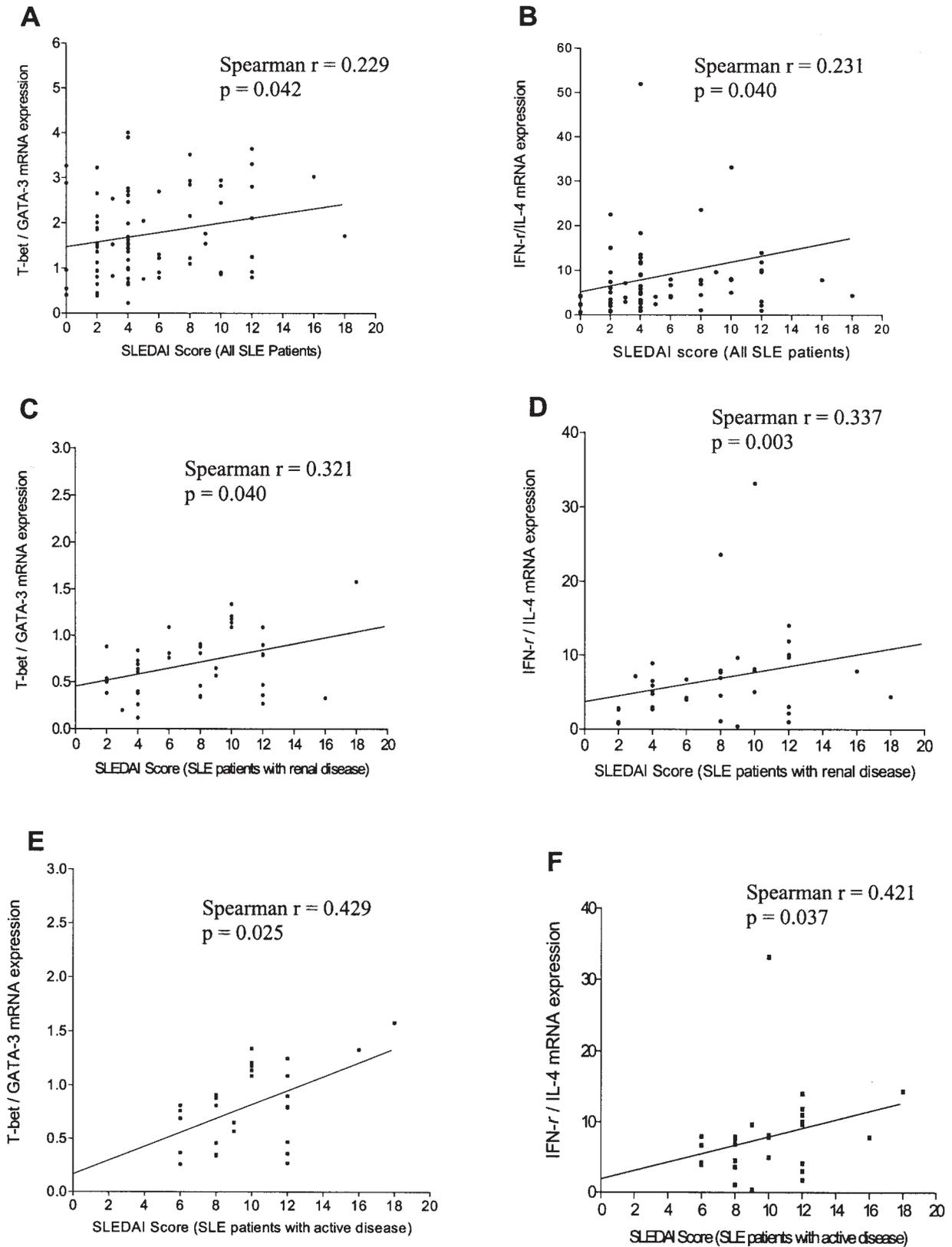


Figure 1. Correlations of T-bet/GATA-3 and IFN- $\gamma$ /IL-4 mRNA expression ratios with SLEDAI score in all SLE patients (A, B), SLE patients with renal disease (C, D), and SLE patients with active lupus disease (E, F). Active disease was defined as a SLEDAI score  $\geq 6$ . Data were analyzed by Spearman's rank correlation test.

ics of GATA-3 and T-bet expression within individual T cells, and that a balance between T-bet and GATA-3 is crucial to control Th1/Th2 polarization. We calculated the relative mRNA ratio to explore the dynamic phenomenon of T-bet and GATA-3 expression in patients with SLE. The relative gene expression levels of T-bet/GATA-3 and IFN- $\gamma$ /IL-4 were significantly elevated in SLE patients. Most importantly, there was a strong correlation between T-bet/GATA-3 and IFN- $\gamma$ /IL-4 or IFN- $\gamma$  expressions. These results further support the notion that there was a tendency of Th1 polarization in our SLE patient cohort. Further, we observed positive correlations between mRNA expression of T-bet/GATA-3, IFN- $\gamma$ /IL-4, and lupus disease activity, suggesting that the relative predominance of T-bet and GATA-3 may determine Th1/Th2 differentiation and subsequent involvement in the pathogenesis and development of SLE disease.

We also compared plasma Th1 and Th2 cytokine profiles in SLE patients and controls. In accord with other findings, we observed elevated plasma Th1 cytokine IL-18 concentrations in SLE patients, and the increase in IL-18/IL-4 ratios correlated positively with lupus activity scores. Recent evidence has shown that IL-18, along with IL-12, is a potent inducer of inflammatory mediators by T cells, causing severe inflammatory disorders in autoimmune diseases such as rheumatoid arthritis<sup>34,35</sup>. Along this line, we also performed a separate *in vitro* study to investigate responsiveness of PBMC to synergistic activation by IL-12 and IL-18 in the *ex vivo* production of inflammatory chemokines. Indeed, baseline evaluation of the plasma cytokines showed that SLE patients exhibited a parallel increase in plasma circulating IL-12 and IL-12/IL-4 cytokine ratios compared to controls. Notably, the ratio of IL-12/IL-4 also correlated positively with SLE disease activity. Considered together with findings in the present study, the Th1/Th2 cytokine ratios could reflect the development and progression of Th1-mediated inflammatory disease in our patients with SLE.

The cytokine network is complex and directed by multiple factors. Th1 and Th2 immune responses have traditionally been studied by measuring intrinsic or extrinsic cytokine production by immunocompetent cells harvested directly from the peripheral blood, or using multistep procedures to isolate the blood cells<sup>36-38</sup>. Cell processing or purification procedures could artificially change the metabolism and environment of the target cells, leading to uncontrolled cell activation, elimination of essential cellular and molecular components, and stimulatory and inhibitory mediators present in the whole blood *in vivo*. Thus, the levels of cytokines measured in these cells may not reflect *in vivo* conditions, leading to discrepancies in interpretations among many studies<sup>3,9,10</sup>. Compared to methods that require lengthy isolation procedures for cell preparation, RT-QPCR measurement of T-bet and GATA-3 in PBMC may provide an immediate "snapshot" of cytokine status reflecting the *in vivo* physiological condition. Since T-bet and GATA-3 may antagonize each other's expression through

direct negative feedback, the dynamics of T-bet and GATA-3 expression and thus the relative predominance of their expression could better determine the Th1/Th2 fate of a T-helper cell.

Our study suggests that the ratio of T-bet/GATA-3 expression is more informative than the level of either transcription factor alone, which may be disproportionately affected by changes in their coexpression in cell populations. The T-bet/GATA-3 expression ratio not only enhances our understanding of Th1/Th2 polarization, it may also serve as a supplementary tool for further assessment of Th1/Th2 status and development of SLE disease activity.

## REFERENCES

1. Mills JA. Systemic lupus erythematosus. *N Engl J Med* 1994;330:871-9.
2. Kotzin BL. Systemic lupus erythematosus. *Cell* 1996;85:303-6.
3. Viallard JF, Pellegrin JL, Ranchin V, et al. Th1 (IL-2, IFN- $\gamma$ ) and Th2 (IL-10, IL-4) cytokine production by peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Clin Exp Immunol* 1999;115:189-95.
4. Mohan C, Adams S, Stanik V, Datta SK. Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. *J Exp Med* 1993;177:1367-81.
5. Houssiau FA, Lefebvre C, Vanden Berghe M, Lambert M, Devogelaer JP, Renaud JC. Serum interleukin 10 titers in systemic lupus erythematosus reflect disease activity. *Lupus* 1995;4:393-5.
6. Tokano Y, Morimoto S, Kaneko H, et al. Levels of IL-12 in the sera of patients with systemic lupus erythematosus — relation to Th1- and Th2-derived cytokines. *Clin Exp Immunol* 1999;116:169-73.
7. Davas EM, Tsirogianni A, Kappou I, Karamitsos D, Economidou I, Dantis PC. Serum IL-6, TNF alpha, p55 srTNF alpha, p75 srTNF alpha, srIL-2 alpha levels and disease activity in systemic lupus erythematosus. *Clin Rheumatol* 1999;18:17-22.
8. Al-Janadi M, al-Balla S, al-Dalaan A, Raziuddin S. Cytokine profile in systemic lupus erythematosus, rheumatoid arthritis, and other rheumatic diseases. *J Clin Immunol* 1993;113:58-67.
9. Hagiwara E, Gourley MF, Lee S, Klinman DK. Disease severity in patients with systemic lupus erythematosus correlates with an increased ratio of interleukin-10: interferon-gamma-secreting cells in the peripheral blood. *Arthritis Rheum* 1996;39:379-85.
10. Akahoshi M, Nakashima H, Tanaka Y, et al. Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. *Arthritis Rheum* 1999;42:1644-8.
11. Wong CK, Li EK, Ho CY, Lam CW. Elevation of plasma interleukin-18 concentration is correlated with disease activity in systemic lupus erythematosus. *Rheumatology Oxford* 2000;39:1078-81.
12. Wong CK, Ho CY, Li EK, Tam LS, Lam CW. Elevated production of interleukin-18 is associated with renal disease in patients with systemic lupus erythematosus. *Clin Exp Immunol* 2002;130:345-51.
13. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000;100:655-9.
14. Zhang DH, Yang L, Ray A. Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. *J Immunol* 1998;161:3817-21.
15. Ouyang W, Lohning M, Gao Z, et al. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity* 2000;12:27-37.

16. Lee GR, Fields PE, Flavell RA. Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. *Immunity* 2001;14:447-59.
17. Macaubas C, Lee PT, Smallacombe TB, et al. Reciprocal patterns of allergen-induced GATA-3 expression in peripheral blood mononuclear cells from atopics vs. non-atopics. *Clin Exp Allergy* 2002;32:97-106.
18. Arakawa S, Hatano Y, Katagiri K. Differential expression of mRNA for Th1 and Th2 cytokine-associated transcription factors and suppressors of cytokine signalling in peripheral blood mononuclear cells of patients with atopic dermatitis. *Clin Exp Immunol* 2004;135:505-10.
19. Neurath MF, Weigmann B, Finotto S, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* 2002;195:1129-43.
20. Matsuoka K, Inoue N, Sato T, et al. T-bet upregulation and subsequent interleukin 12 stimulation are essential for induction of Th1 mediated immunopathology in Crohn's disease. *Gut* 2004;53:1303-8.
21. Kimura J, Takada H, Nomura A, et al. Th1 and Th2 cytokine production is suppressed at the level of transcriptional regulation in Kawasaki disease. *Clin Exp Immunol* 2004;137:444-9.
22. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271-7.
23. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH, the Committee on Prognosis studies in SLE. Derivation of the SLEDAI: a disease activity index for lupus patients. *Arthritis Rheum* 1992;35:630-40.
24. Davidson A, Diamond B. Autoimmune disease. *N Engl J Med* 2001;345:340-50.
25. Nakamura Y, Ghaffar O, Olivenstein R, et al. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. *J Allergy Clin Immunol* 1999;103(2 Pt 1):215-22.
26. Kawashima M, Miossec P. mRNA quantification of T-bet, GATA-3, IFN-gamma, and IL-4 shows a defective Th1 immune response in the peripheral blood from rheumatoid arthritis patients: link with disease activity. *Clin Immunol* 2005;25:209-14.
27. Kawashima M, Miossec P. Effect of treatment of rheumatoid arthritis with infliximab on IFN gamma, IL4, T-bet, and GATA-3 expression: link with improvement of systemic inflammation and disease activity. *Ann Rheum Dis* 2005;64:415-8. Epub 2004 Jul 29.
28. Szabo SJ, Sullivan BM, Stemmann C, Sato AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 2002;295:338-42.
29. Ho IC, Glimcher LH. Transcription: tantalizing times for T cells. *Cell* 2002;109 Suppl:S109-20.
30. Mullen AC, High FA, Hutchins AS, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 2001;292:1907-10.
31. Afkarian M, Sedy JR, Yang J, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 2002;3:549-57.
32. Chakir H, Wang H, Lefebvre DE, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. *J Immunol Methods* 2003;278:157-69.
33. Yates A, Callard R, Stark J. Combining cytokine signalling with T-bet and GATA-3 regulation in Th1 and Th2 differentiation: a model for cellular decision-making. *J Theor Biol* 2004;231:181-96.
34. Dinarello CA. IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J Allergy Clin Immunol* 1999;103:11-24.
35. Fehniger TA, Shah MH, Turner MJ. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol* 1999;162:4511-20.
36. Zangerle PF, De Groote D, Lopez M, et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood: II. Application to rheumatoid arthritis and osteoarthritis. *Cytokine* 1992;4:568-75.
37. Narumi S, Takeuchi T, Kobayashi Y, Konishi K. Serum levels of IFN-inducible protein-10 relating to the activity of systemic lupus erythematosus. *Cytokine* 2000;12:1561-5.
38. Eriksson C, Eneslatt K, Ivanoff J, Rantapaa-Dahlqvist S, Sundqvist KG. Abnormal expression of chemokine receptors on T-cells from patients with systemic lupus erythematosus. *Lupus* 2003;12:766-74.