Discussion

IL-18 and IL-2 are important cytokines that can induce IFN-γ production by NK cells [11]. IL-18 and IL-2 administered daily acted synergistically to induce ILD. ILD mice show severe infiltration of NK cells in the lungs and have high levels of IFN-γ in both serum and lung [19]. Depletion of NK cells by anti-NK1·1 monoclonal antibody or anti-asialo GM1 antibody treatment prevented this effect. Furthermore, the morbid effects of IL-18 and IL-2 were reduced in IFN-γ-deficient mice. These findings suggest that the increase of NK cells and elevation of IFN-γ seem to play a role in the pathogenesis of IL-18/IL-2-induced ILD in mice.

In other mouse models of ILD, BLM induces pulmonary fibrosis and the TGF-β pathway plays an important role in the pathogenesis of ILD [24,27–29,36]. The present study also showed increased expression of TGF-β mRNA in the lung in the early stage of ILD after injection of IL-18/IL-2. Thus, TGF-β seems to be involved in the pathogenesis of IL-18/IL-2-induced ILD and BLM-induced pulmonary fibrosis. Surprisingly, mice treated with SB-431542 delayed mortality in IL-18/IL-2-induced ILD. In addition, IL-18/IL-2-induced NK cell infiltration in the lung was decreased significantly following treatment with SB-431542 and also in $Smad3^{-/-}$ mice. Histological analysis demonstrated that SB-431542 reduced cell infiltration significantly in ILD mice. It was reported that injection of IL-18/IL-2 induced the expression of IFN-γ and TNF-α in sera, and IFN-γ and IL-6 in the lung [19]. In the lung, treatment with SB-431542 reduced the expression of IFN-γ and IL-6 from IL-18/IL-2-induced ILD mice, but in sera the expression of IFN-γ and TNF-α was not changed. Furthermore, IL-18/IL-2-induced ILD was improved in $Smad3^{-/-}$ mice. These findings indicate the involvement of Smad-mediated TGF-β signalling in the pathogenesis of murine ILD.

Bellone *et al.* [37] reported that TGF-β inhibited activated NK cells *in vitro*. Our preliminary study confirmed that exogenously added TGF-β suppressed NK cells *in vitro* (data not shown). However, in the present study, we demonstrated that shutdown of TGF-β signalling by SB-431542 or Smad3 knock-out down-regulated NK cells migration into the lung *in vivo*, indicating that TGF-β enhanced the infiltration of NK cells into the lung. The discrepancy might be due to the difference between the effects of TGF-β on NK cells *in vitro* and those *in vivo*. We proposed that TGF-β could enhance the migration of NK cells into the lung *in vivo*, whereas TGF-β suppressed the proliferation of NK cells *in vitro*.

Okamoto *et al.* [19] showed that certain chemokines, such as CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1 and CXCL10, were up-regulated in the lungs of IL-18/IL-2-induced ILD mice. In contrast, in the lungs of B6 mice treated with SB-431542 and in $Smad3^{-/-}$ mice, chemokine mRNAs were down-regulated. Interestingly, larger proportions of NK cells were noted in the spleens of SB-431542-treated mice with ILD, although their proportion in the lungs was reduced. The latter finding may be due to redistribution and accumulation of NK cells in the spleen. Thus, inhibition of TGF-β signalling could, potentially, be a useful therapeutic strategy in ILD through regulation of NK cells infiltration in the lung.

In conclusion, the present study showed that inhibition of TGF-β signalling regulated IL-18/IL-2-induced ILD through inhibition of NK cells and down-regulation of certain chemokines in the lung. These findings support the notion that TGF-β signalling plays an important role in the pathogenesis of ILD.

Acknowledgements

We thank Dr Chuxla Deng (National Institute of Diabetes and Digestive and Kidney Diseases, MD) for kindly providing Smad3 deficient ($Smad3^{-/-}$) mice, and Dr F. G. Issa, for the critical reading of the manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research by Japan Society for the Promotion of Science and the Japanese Ministry of Health, Labour and Welfare.

Disclosure

None of the authors has any conflict of interest with the subject matter or materials discussed in the manuscript.

References

1 King TE Jr. Clinical advances in the diagnosis and therapy of the interstitial lung diseases. Am J Respir Crit Care Med 2005; **172**:268–79.

2 Luna MA, Bedrossian CW, Lichtiger B, Salem PA. Interstitial pneumonitis associated with bleomycin therapy. Am J Clin Pathol 1972; **58**:501–10.

3 Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. J Exp Med 1989; **170**:655–63.

4 Nakao A, Fujii M, Matsumura R *et al.* Transient gene transfer and expression of smad7 prevents bleomycin-induced lung fibrosis in mice. J Clin Invest 1999; **104**:5–11.

5 Scheule RK, Perkins RC, Hamilton R, Holian A. Bleomycin stimulation of cytokine secretion by the human alveolar macrophage. Am J Physiol 1992; **262**:386–91.

6 Smith RE, Strieter RE, Phan SH *et al.* Production and function of murine macrophage inflammatory protein-1 alpha in bleomycin-induced lung injury. J Immunol 1994; **153**:4704–12.

7 Zhang K, Gharaee-Kermani M, Jones ML, Warren JS, Phan SH. Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. J Immunol 1994; **153**:4733–41.

8 Hoshino T, Nakamura H, Okamoto M *et al.* Redox-active protein thioredoxin prevents proinflammatory cytokine- or bleomycin-induced lung injury. Am J Respir Crit Care Med 2003; **168**:1075–83.

9 Kuwano K, Hagimoto N, Kawasaki M *et al.* Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. J Clin Invest 1999; **104**:13–19.

© 2010 British Society for Immunology, *Clinical and Experimental Immunology*

10 Nakamishi K, Yoshimoto T, Tsustumi H, Okamura H. Interleukin-18 regulates both th1 and th2 response. Annu Rev Immunol 2001; **19**:423–74.
11 Hoshino T, Robert H, Wiltrout RH, Young HA. IL-18 is a potent coinducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. J Immunol 1999; **162**:5070–7.
12 Hoshino T, Yagita H, Ortaldo JR, Wiltrout RH, Young HA. *In vivo* administration of IL-18 can induce IgE production through TH2 cytokine induction and up-regulation of CD40 ligand (CD154) expression on CD4+ T cells. Eur J Immunol 2000; **30**:1998–2006.
13 Hoshino T, Kawase Y, Okamoto M *et al.* IL-18- transgenic mice: *in vivo* evidence of a broad role for IL-18 in modulating immune function. J Immunol 2001; **166**:7014–18.
14 Wild JS, Sigounas A, Sur N *et al.* IFN-gamma-inducing factor (IL-18) increases allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma. J Immunol 2000; **164**:2701–10.
15 Yoshimoto T, Min B, Sugimoto T *et al.* Nonredundant roles for CD1d-restricted natural killer T cells and conventional CD4+ T cells in the induction of immunoglobulin E antibodies in response to interleukin 18 treatment of mice. J Exp Med 2003; **197**:997–1005.
16 Sims JE. IL-1 and IL-18 receptors, and their extended family. Curr Opin Immunol 2002; **14**:117–22.
17 Nakatani-Okuda A, Ueda H, Kashiwamura S *et al.* Protection against bleomycin-induced lung injury by IL-18 in mice. Am J Physiol Lung Cell Mol Physiol 2005; **289**:280–7.
18 Hoshino T, Okamoto M, Sakazaki Y, Kato S, Young HA, Aizawa H. Role of proinflammatory cytokines IL-18 and IL-1β in bleomycin-induced lung injury in humans and mice. Am J Respir Cell Mol Biol 2009; **41**:661–70.
19 Okamoto M, Kato S, Oizumi K *et al.* Interleukin18 (IL-18) in synergy with IL-2 induced lethal lung injury in mice: a potential role for cytokines, chemokines, and natural killer cells in the pathogenesis of interstitial pneumonia. Blood 2002; **99**:1289–98.
20 Chen ES, Greenlee BM, Wills-Karp M, Moller DR. Attenuation of lung inflammation and fibrosis in interferon-gamma-deficient mice after intratracheal bleomycin. Am J Respir Cell Mol Biol 2001; **24**:545–55.
21 Yang HZ, Cui B, Liu HZ *et al.* Targeting TLR2 attenuates pulmonary inflammation and fibrosis by reversion of suppressive immune microenvironment. J Immunol 2009; **182**:692–702.
22 Suzuki T, Chow CW, Downey GP. Role of innate cells and their products in lung immunopathology. Int J Biochem Cell Biol 2008; **40**:1348–61.
23 Kakizaki T, Kohno M, Watanabe M *et al.* Exacerbation of bleomycin-induced injury and fibrosis by pneumonectomy in the residual lung of mice. J Surg Res 2009; **154**:336–44.
24 Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-β regulation of immune responses. Ann Rev Immunol 2006; **24**:99–146.
25 Bonniaud P, Kolb M, Galt T *et al.* Smad3 null mice develop airspace enlargement and are resistant to TGF-β-mediated pulmonary fibrosis. J Immunol 2004; **173**:2099–108.
26 Higashiyama H, Yoshimoto D, Okamoto Y, Kikkawa H, Asano S, Kinoshita S. Receptor-activated Smad localization in bleomycin-induced pulmonary fibrosis. J Clin Pathol 2007; **60**:283–9.
27 Venlatesan N, Pini L, Ludwig MS. Changes in Smad expression and subcellular localization in bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 2004; **287**:L1342–7.
28 Zhao Y, Geverd DA. Regulation of Smad3 expression in bleomycin-induced pulmonary fibrosis: a negative feedback loop of TGF-β signaling. Biochem Biophys Res Commun 2002; **294**:319–23.
29 Zhao J, Shi W, Wang YL *et al.* Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. Am J Physiol Lung Cell Mol Physiol 2002; **282**:L585–93.
30 Laping NJ, Grygielko E, Mathur A *et al.* Inhibition of transforming growth factor (TGF)-β1-induced extracellular matrix with a novel inhibitor of the TGF-β type I receptor kinase activity: SB-431542. Mol Pharmacol 2002; **62**:58–64.
31 Inman GJ, Nicolás FJ, Callahan JF *et al.* SB-431542 is a potent and specific inhibitor of transforming growth factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol 2002; **62**:65–74.
32 Matsuyama S, Iwadate M, Kondo M *et al.* SB-431542 and Gleevec inhibit transforming growth factor-β-induced proliferation of human osteosarcoma cells. Cancer Res 2003; **63**:7791–8.
33 Higashiyama H, Yoshimoto D, Kaise T *et al.* Inhibition of activin receptor-like kinase 5 attenuates bleomycin-induced pulmonary fibrosis. Exp Mol Pathol 2007; **83**:39–46.
34 Yang X, Letterio JJ, Lechleider RJ *et al.* Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO J 1999; **18**:1280–91.
35 Li S, Kishihara K, Akashi N *et al.* Vδ1^{+} T cells are crucial for repertoire formation of γδ T cells in the lung. Biochem Biophys Res Commun 2008; **365**:246–51.
36 Batram U, Speer CP. The role of transforming growth factor beta in lung development and disease. Chest 2004; **125**:754–65.
37 Bellone G, Aste-Amezage M, Trinchieri G, Rodeck U. Regulation of NK cell functions by TGF-β1. J Immunol 1995; **155**:1066–73.

© 2010 British Society for Immunology, *Clinical and Experimental Immunology*

Clinical & Experimental Immunology
The Journal of Translational Immunology
immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2009.04031.x

Laser microdissection-based analysis of cytokine balance in the kidneys of patients with lupus nephritis

Y. Wang,* S. Ito,* Y. Chino,* D. Goto,* I. Matsumoto,* H. Murata,* A. Tsutsumi,* T. Hayashi,* K. Uchida,† J. Usui,‡ K. Yamagata‡ and T. Sumida*
*Division of Clinical Immunology, Doctoral Program in Clinical Sciences, †Molecular and Biological Oncology, and ‡Division of Pathophysiology of Renal Diseases, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tennodai, Tsukuba, Japan

Accepted for publication 28 August 2009
Correspondence: S. Ito, Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan.
E-mail: s-ito@md.tsukuba.ac.jp

Summary

To determine the cytokine balance in patients with lupus nephritis (LN), we analysed kidney-infiltrating T cells. Renal biopsy samples from 15 systemic lupus erythematosus (SLE) patients were used. In accordance with the classification of International Society of Nephrology/Renal Pathology Society, they were categorized into Class III, Class III+V (Class III-predominant group, $n = 4$), Class IV, Class IV+V (Class IV-predominant group, $n = 7$) and Class V ($n = 4$) groups. The single-cell samples of both the glomelular and interstitial infiltrating cells were captured by laser-microdissection. The glomerular and interstitial infiltrating T cells produced interleukin (IL)-2, IL-4, IL-10, IL-13 and IL-17 cytokines in the Class III-predominant, Class IV-predominant and Class V groups. Interferon-gamma was detected only in the glomeruli of the Class III-predominant and Class V group samples. The expression level of IL-17 was correlated closely with clinical parameters such as haematuria, blood urea nitrogen level, SLE Disease Activity Index scores in both glomeruli and interstitium, urine protein level in glomeruli and serum creatinine and creatinine clearance levels in interstitium. This suggests that the glomerular infiltrating T cells might act as T helper type 1 (Th1), Th2 and Th17 cells while the interstitial infiltrating T cells, act as Th2 and Th17 cells in the Class III-predominant and Class V groups. In contrast, both the glomerular and interstitial infiltrating T cells might act as Th2 and Th17 cells in the Class IV-predominant group. The cytokine balances may be dependent upon the classification of renal pathology, and IL-17 might play a critical role in SLE development.

Keywords: laser-microdissection, lupus nephritis, SLEDAI, Th17

Introduction

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease characterized by various clinical manifestations. T cell-derived cytokine production plays a determinant role in SLE development. Previous studies have reported that an imbalance in cytokine production between T helper type 1 (Th1) and Th2 T cells (predominance of Th2 cytokine) in the peripheral blood of SLE patients is associated with the pathogenesis of the disease [1–3]. In contrast, Akahoshi *et al.* [4] demonstrated that a substantial predominance of Th1-type response took place in the peripheral blood samples of lupus nephritis (LN) patients categorized in WHO Class IV. Not only T cells in the peripheral blood, but also the balance in cytokine production between Th1 and Th2 cells in the kidney has drawn a great deal of attention. Masutani *et al.* [5] analysed the expression levels of interferon (IFN)-γ and interleukin (IL)-4 on intrarenal T cells as well as those in the peripheral blood samples from SLE patients with diffuse proliferative LN by immunohistochemistry, demonstrating the predominance of Th1 type response. They suggested that the Th1 : Th2 ratio in the peripheral blood might directly reflect the local histopathological findings. However, Murata *et al.* [6] indicated that the kidney-infiltrating T cells could produce Th2 type cytokines such as IL-4 and IL-10 through reverse transcription–polymerase chain reaction (RT–PCR), and made an assumption that this discrepancy might arise from a difference in sensitivity between the methods used in detection of cytokines. The expression level of IL-13, one of the Th2 type cytokines, was reported to be higher in the serum from the rheumatoid arthritis (RA), SLE, Sjögren's syndrome and

systemic sclerosis patient groups than that in the normal healthy control group [7]. Morimoto *et al.* [8] also showed elevated expression level of IL-13 in SLE patients. Recently, it has been reported that naive murine CD4$^+$ T helper cells can be induced to differentiate into Th1, Th2, Th17 and regulatory phenotypes [9]. IL-17 is a proinflammatory cytokine, as possibly known from the pathological conditions of various inflammatory diseases in both humans and mice [9]. We have reported previously that both IL-13 and IL-17 were produced in the murine LN (MRL/lpr mice) cells; however, we did not analyse them at a single-cell level [10]. The laser microdissection (LMD) technique has been adopted recently to obtain tissue samples exclusively from specific regions of interest. This new technique has been used successfully in various fields, including oncology [11], endocrinology [12], gastroenterology [13], rheumatology [14–16] and nephrology [10,17–19]. With this technique, attempts to analyse single-cell gene expression were made [13,16,20]. In our study, we analysed the single-cell expression levels of cytokines, including IL-13 and IL-17, by infiltrating T cells in the kidneys of LN patients.

Patients and methods

Renal biopsy samples were obtained from 15 SLE patients, two minor glomerular abnormalities (MGA) patients (female, 16 years old; male, 14 years old) and one minimal change nephrotic syndrome (MCNS) patient (male, 14 years old), and used in our experiments. In accordance with the classification criteria defined by International Society of Nephrology/Renal Pathology Society (ISN/RPS) [21,22], renal pathologies were diagnosed as: Class III, three cases; Class III+V, one case; Class IV, two cases; Class IV+V, five cases; and Class V, four cases. To ensure consistency with the World Health Organization (WHO) classification criteria, a further membranous lesion (Class V) may be added to Class III or Class IV in ISN/RPS. They were categorized as Class III-predominant group (Class III-predominant group included patients with both Class III and Class III+V, $n = 4$) and Class IV-predominant group (including patients with both Class IV and Class IV+V, $n = 7$). The patients, who had underwent renal biopsy before 2004, had already been classified in accordance with the WHO classification criteria [23] at the time of biopsy, but in this study were re-evaluated by nephrologists in accordance with the ISN/RPS classification criteria. The SLE Disease Activity Index (SLEDAI) scores [24], histological activity index (AI) and chronicity index (CI) scores [25] at renal biopsy are shown as Table 1. This study was approved by the ethical committee of Tsukuba University Hospital (no. 392). Prior written consent was given by the patients.

Immunohistological examinations

Five-μm-thick sections were obtained from the renal biopsy specimens of the SLE patients. Immunohistochemical

Table 1. Clinical characteristics of patient and positivity of dissected T cells.

No.	Age	Sex	Classification	Pre-s	UP (g/day)	Haematuria (RBC/HPF)	Urinary cast	Pyuria	BUN (mg/dl)	Cr (mg/dl)	Ccr (ml/min)	ADNA (U/ml)	CH50 (U/ml)	SLEDAI	TCR-cβ/ β-actin (%)	AI	CI
1	45	F	III (A)	No	5·0	10–19	+	–	10·1	0·54	92·1	> 300	4·8	17	18/28 (64·3%)	5	2
2	52	F	III (C)	Yes	0·46	1–4	–	–	20·7	1·01	38·9	< 2	40·2	0	15/34 (44·1%)	1	8
3	52	F	III (A) + V	No	0·3	1–4	–	–	10·0	0·57	84·0	64·7	18·2	9	18/26 (69·2%)	0	3
4	29	F	IV-G (A/C)	No	8·0	56	+	+	31·1	1·10	48·1	100·0	12·0	21	28/36 (77·8%)	16	3
5	25	F	IV-G (A/C)	Yes	5·0	5–9	+	–	15·0	0·60	93·0	> 300	2·1	19	18/25 (72·0%)	11	3
6	58	F	IV-S(A/C)+V	No	6·1	1–4	+	–	10·0	0·56	159·0	45	24·5	16	53/67 (79·1%)	12	4
7	59	F	IV-G(A/C)+V	No	2·3	1–4	–	+	26·1	0·95	51·5	64·7	18·2	9	19/28 (67·9%)	5	2
8	55	F	IV-G (A) + V	No	0·8	1–4	–	–	13·1	0·49	100·0	94·6	7·4	13	14/27 (51·9%)	10	0
9	18	F	IV-S (A) + V	Yes	0·46	1–4	–	–	12·3	0·48	108·5	58·1	9·5	18	22/31 (70·1%)	6	3
10	28	F	V	No	7·7	0–1	–	–	13·8	0·70	96·7	16·7	41·6	5	18/26 (69·2%)	1	2
11	39	F	V	Yes	5·3	0–1	–	–	12·4	0·51	138·1	5·4	50·7	4	22/32 (68·8%)	0	2
12	26	F	V	Yes	2·1	5–9	–	–	13·5	0·60	95·4	23·1	42·9	19	46/62 (74·2%)	0	0
13	22	M	IV-G(A/C)+V	Yes	4·15	30–50	+	–	16·0	0·90	110·8	< 5·0	20·5	10	7/20 (35·0%)	8	6
14	38	F	III (A/C)	No	0·85	1–4	+	–	18·7	0·62	141·7	> 300	23·2	13	18/31 (58·1%)	6	4
15	60	F	V	No	0·49	20–99	–	–	16·6	0·73	99·6	20·3	54·0	22	27/40 (67·5%)	0	3

S: segmental; G: global; A: active; C: chronic; Pre-s: pretreatment with steroid; UP: urine protein; RBC/HPF: red blood cell/high power field; BUN: blood urea nitrogen; Cr: serum creatinine; Ccr: creatinine clearance; Anti-ds: anti-double-stranded DNA; CH50: 50% haemolytic unit of complement serum; SLEDAI: systemic lupus erythematosus Disease Activity Index; AI: activity index score; CI: chronicity index score.

© 2009 British Society for Immunology,

staining was performed by the avidin–biotin complex technique. Primary antibodies used included murine anti-human IFN-γ (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-IL-4, 10 (Research & Diagnostics Systems, Minneapolis, MN, USA); and polyclonal rabbit anti-human IL-17 and IL-13 (Santa Cruz Biotechnology). Staining was performed on the sections using normal murine IgG or rabbit immunoglobulin (Ig)G, a primary antibody, as a negative control. We also performed staining on sections of the renal biopsy samples of MGA and MCNS patients using anti-human IL-17 as the control.

Tissue sampling by laser microdissection

Frozen sections (10 μm thick) from the renal biopsy specimens of the SLE patients were stained with 0·05% toluidine blue solution (pH 7·0) (Wako Pure Chemical Industries, Osaka, Japan) and the individual single cells infiltrating into glomeruli and interstitiums were selected and dissected with laser-microdissection system (AS-LMD; Leica Microsystems Japan, Tokyo, Japan) (Fig. 2A).

RNA extraction and nested RT–PCR

Total RNA was extracted from the LMD samples by the Isogen method (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA using the ThermoScript RT–PCR System (Invitrogen Life Technologies, Carlsbad, CA, USA) and amplified with primers specific to β-actin, T cell receptor β chain (TCR-Cβ), IL-2, IL-4, IL-10, IL-13, IL-17 and IFN-γ for nested RT–PCR (Table 2).

Statistical analysis

All data were expressed as mean ± standard error of the mean. Statistically significant differences between groups were determined using the Mann–Whitney *U*-test. A simple linear regression analysis was used to evaluate the correlation between the two parameters. The statistical significance was defined as $P < 0{\cdot}05$.

Results

Detection of T cells in glomeruli and interstitium

Stained IL-4, IL-10 and IL-13 were observed in the glomerular and interstitial areas of the specimens from the SLE patients of the Class III-predominant, IV-predominant and Class V groups, especially in the latter area of the Class IV-predominant group (Fig. 1A) (the immunohistochemical data for the Class III-predominant and Class V groups are not shown). Many IL-4 cells were observed predominantly, mainly in the glomerular and interstitial cells, especially in intraglomerular infiltrating cells, in the Class IV-predominant group, while there were only a few IL-4-positive cells in the tubular epithelial cells (TEC) (Fig. 1Aa, b). IL-10- and IL-13-positive cells were observed prominently in the glomerular and interstitial infiltrating cells (Fig. 1Ac–f). Some stained IL-10-positive cells were observed in TEC (Fig. 1Ac, d). IL-17-positive cells were observed mainly in the glomerular and interstitial infiltrating cells and TECs, especially in intraglomerular cells of the Class IV-predominant group (Fig. 1Ag, h). Almost no IL-17-positive cells were observed in the glomeruli of the Class III-predominant (Fig. 1Ba) and Class V group (not shown) samples. However, IFN-γ cells were not observed in all the specimens (Fig. 1Bb) (the immunohistochemical data for the Class III-predominant groups are shown). Normal rabbit IgG was used as a negative control (Fig. 1Bc). IL-17-positive cells were not observed in all the specimens from the MGA and MCNS patients (Fig. 1C). This demonstrates that IL-17 may be produced preferentially in SLE patients.

Analysis of gene expression by laser microdissection and nested RT–PCR

Of 622 glomerular and interstitial infiltrating cells, 513 (82·5%) were β-actin-positive, among which 343 (66·7%) were TCR-Cβ-positive; these 343 cells were deemed to be T cells and used for cytokine analysis (Table 1). The number of positive samples for each cytokine/ TCR-$C\beta^{+}$ cells was expressed as a percentage.

The glomerular and interstitial infiltrating T cells produced IL-2, IL-4, IL-10, IL-13 and IL-17 cytokines in the Class III-predominant, Class IV-predominant and Class V groups. The positivity of cytokines is shown in Table 3 and Fig. 2B. The percentages of positive IL-4, IL-10 and IL-13 samples were more than 70%, 67% and 41%, respectively, in all the groups. The expression levels of IL-2 were low in each of the predominant groups. IFN-γ was detected only in the glomeruli of the Class III-predominant and Class V groups (32·3 ± 12·9% and 24·0 ± 10·0%, $P < 0{\cdot}05$) (Table 3 and Fig. 2B). In the glomerular lesions, the percentage of positive IL-17 samples was 64·7 ± 10·1% and 70·7 ± 6·0% in the Class IV-predominant and V groups, while it was significantly greater than in the Class III-predominant group (44·7 ± 5·9%, $P < 0{\cdot}05$) (Fig. 2Bb). In the interstitial lesions, the positivity of IL-17 (48·0 ± 4·2%) was also significantly lower in the Class III-predominant groups than that in the Class IV-predominant group (69·1 ± 8·9%, $P < 0{\cdot}05$) (Fig. 2Bc).

Correlation between the expression levels of cytokines and clinical parameters in SLE patients

We analysed the correlation between the expression levels of Th1 (IL-2), Th2 (IL-4, IL-10, and IL-13) and Th17 (IL-17)

Table 2. Oligonucleotide primer sequences.

PCR products		Oligonucleotide sequence	Product size (bp)	RT–PCR cycles
β-actin				
First PCR	5′ sense	GGCATCCTCACCCTGAAGTA	496	25
	3′ anti-sense	CCATCTCTTGCTCGAAGTCC		
Nested PCR	5′ sense	AAATCTGGCACCACACCTTC	262	25
	3′ anti-sense	AGGGCATACCCCTCGTAGAT		
TCR-Cβ				
First PCR	5′ sense	ACATAAGGAAGGCTGCATGG	249	30
	3′ anti-sense	CGTTTTGATCATGGTGTGTGG		
Nested PCR	5′ sense	ATCAGGTGTGTGGGACTTTG	217	30
	3′ anti-sense	GACTCAGGACAGTGACATCA		
IFN-γ				
First PCR	5′ sense	TCTGCATCGTTTTGGGTTCTC	346	25
	3′ anti-sense	TCAGCTTTTCGAAGTCATCTC		
Nested PCR	5′ sense	TGTTACTGCCAGGACCCATAT	242	30
	3′ anti-sense	ACTCTTTTGGATGCTCTGGTC		
IL-2				
First PCR	5′ sense	ACTACCAGGATGCTCACATT	267	25
	3′ anti-sense	AAGGTAATCCATCTGTTCAGA		
Nested PCR	5′ sense	GCCACAGAACTGAAACATCTT	201	30
	3′ anti-sense	TTCTACAATGGTTGCTGTCTC		
IL-4				
First PCR	5′ sense	CTTCCCCCTCTGTTCTTCCT	318	25
	3′ anti-sense	TTCCTGTCGAGCCGTTTCAG		
Nested PCR	5′ sense	CTAGCATGTGCCGGCAACTTT	273	25
	3′ anti-sense	TCGGATCAGCTGCTTGTGCCT		
IL-10				
First PCR	5′ sense	ACAGCTCAGCACTGCTCTGT	327	30
	3′ anti-sense	AGTTCACATGCGCCTTGATG		
Nested PCR	5′ sense	CCCAGTCTGAGAACAGCTGCAA	210	30
	3′ anti-sense	CTGGGTCTTGGTTCTCAGCTT		
IL-13				
First PCR	5′ sense	CTATGCATCCGCTCCTCAAT	391	30
	3′ anti-sense	TTTACAAACTGGGCCACCTC		
Nested PCR	5′ sense	ATTGCTCTCACTTGCCTTGG	229	25
	3′ anti-sense	TCCTGTGGGTCTTCTCGATC		
IL-17				
First PCR	5′ sense	CTTCACCCTGTGGAACGAAT	262	30
	3′ anti-sense	CGGAATTGGTTCTGGAGTGT		
Nested PCR	5′ sense	GAGCACATGCACCACATACC	170	25
	3′ anti-sense	AGGAAACAGTCGCGGAGTGT		

RT–PCR: reverse transcription polymerase chain reaction; TCR-Cβ: T cell receptor β chain; IL: interleukin; IFN-γ: interferon-gamma; bp: base pairs.

Table 3. Positivity of cytokines in glomeruli and interstitiums (%).

	Glomeruli			Interstitiums		
	Class III predominant	Class IV predominant	Class V	Class III predominant	Class IV predominant	Class V
IL-2	19·7 ± 10·3	23·7 ± 20·3	44·6 ± 12·8	25·6 ± 10·5	2·8 ± 21·7	27·9 ± 12·3
IFN-γ	32·3 ± 12·9	n.d.	24·0 ± 10·0	3·1 ± 3·8	n.d.	1·3 ± 1·6
IL-4	88·6 ± 9·1	80·4 ± 13·5	85·7 ± 7·1	90·6 ± 7·5	84·6 ± 13·5	70·9 ± 16·7
IL-10	67·2 ± 5·1	67·7 ± 14·3	70·0 ± 10·3	70·5 ± 6·4	84·3 ± 6·5	79·8 ± 8·1
IL-13	60·6 ± 15·7	47·6 ± 20·5	62·3 ± 9·3	52·0 ± 9·2	41·5 ± 13·4	62·3 ± 7·6
IL-17	44·7 ± 5·9	64·7 ± 10·1*	70·7 ± 6·0*	48·0 ± 4·2	69·1 ± 8·9*	62·6 ± 12·3

*$P < 0{\cdot}05$ *versus* Class III-predominant groups. Results are expressed as mean ± standard error of the mean. Statistical significance was determined using the Mann–Whitney *U*-test. IL; interleukin; IFN-γ: interferon-gamma; n.d.: not determined.

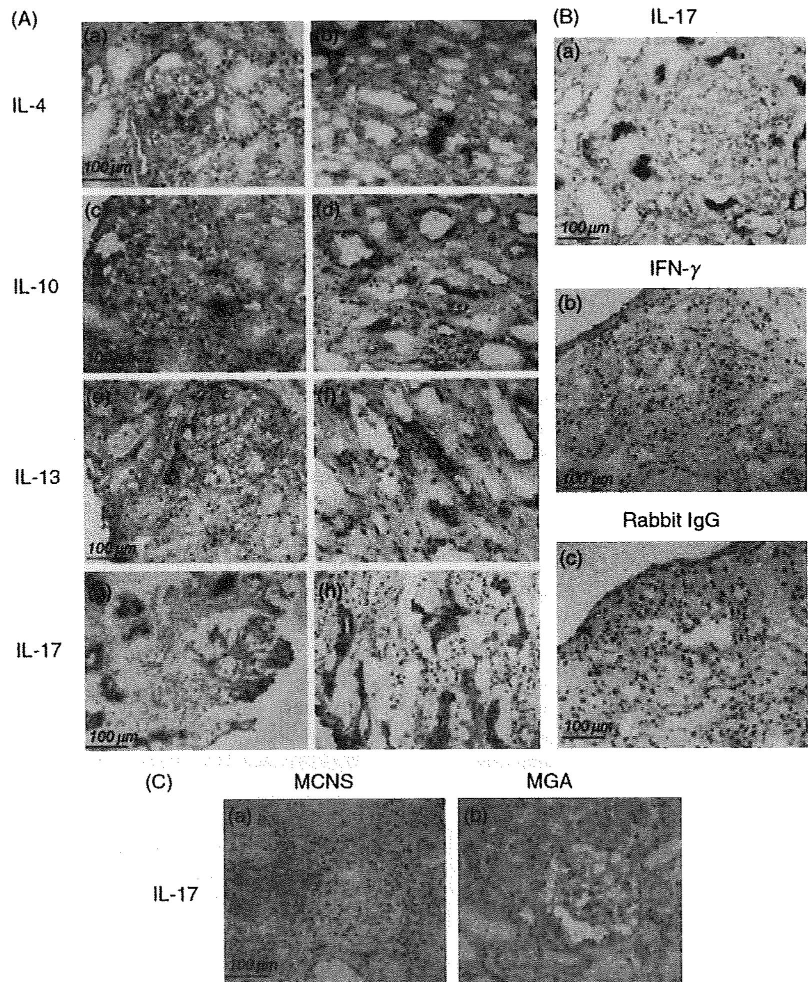


Fig. 1. Detection of T cells in glomeruli and interstitium. (A) Stained interleukin (IL)-4, IL-10, IL-13 and IL-17 were observed in glomeruli and interstitium areas of the Class IV-predominant groups. Many IL-4 cells are observed prominently, mainly in the cells infiltrating into the glomeruli and interstitium areas, especially in intraglomerular infiltrating cells. There are only a few IL-4 cells in the tubular epithelial cells (TEC) (a, b). IL-10 and IL-13 cells are observed prominently in some of the cells infiltrating into the glomeruli and interstitium areas (c–f). Some stained IL-10 cells were observed in TEC (d). Many IL-17 cells are observed prominently, mainly in the cells infiltrating into the glomeruli, interstitium areas and TEC (g, h), especially in intraglomerular cells and TEC (g) (original magnification × 100 in a–h). (B) In the Class III-predominant group, some stained IL-17 cells were observed in TEC but almost no IL-17-positive cells were observed in glomeruli (a). Interferon (IFN)-γ cells were not observed in all the specimens (b). Normal rabbit immunoglobulin (Ig)G was stained as negative control (c) (original magnification × 100). (C) IL-17-positive cells were not observed in all the specimens of minor glomerular abnormalities (MGA) and minimal change nephrotic syndrome (MCNS) (a, b) (original magnification × 100).

cytokines and clinical parameters in SLE patients, such as the urine protein (UP) level, haematuria, blood urea nitrogen (BUN) level, serum creatinine (Cr) level, creatinine clearance (Ccr), 50% haemolytic unit of complement serum (CH50), anti-double-strand DNA (anti-ds DNA) antibodies, SLEDAI scores, histological AI and CI (Table 4). Good and significant correlation data are shown in Fig. 3.

Correlation between Th1 cytokine and clinical parameters. In glomeruli, as known from the tendency of the point distribution on the charts, the parameters, BUN ($r = 0{\cdot}27$), Ccr ($r = 0{\cdot}31$), AI ($r = -0{\cdot}28$), CI ($r = 0{\cdot}39$) and SLEDAI ($r = -0{\cdot}21$) ($P < 0{\cdot}05$) showed a weak correlation with the expression level of IL-2 (Table 4). The expression level of IL-2 showed a good correlation with anti-ds DNA antibodies ($r = -0{\cdot}53$, Fig. 3Aa) and a significant correlation with CH50 ($r = 0{\cdot}80$, $P < 0{\cdot}001$, Fig. 3Ab). In the interstitium, haematuria ($r = -0{\cdot}36$), BUN ($r = -0{\cdot}24$), Cr ($r = -0{\cdot}35$) and CH50 ($r = 0{\cdot}37$) showed a weak correlation with the expression level of IL-2 (Table 4); Ccr ($r = 0{\cdot}63$) and CI ($r = 0{\cdot}404$) showed a good correlation with the expression level of IL-2 (Fig. 3Ac, d).

Correlation between Th2 and clinical parameters. In the glomeruli, haematuria ($r = 0{\cdot}44$), BUN ($r = -0{\cdot}44$), Cr ($r = -0{\cdot}41$) and CI ($r = -0{\cdot}59$) showed a good correlation with the expression level of IL-4 (Fig. 3Ba–d); SLEDAI ($r = -0{\cdot}36$) and AI ($r = -26$) showed a weak correlation with IL-4 (Table 4). The expression level of IL-10 showed a weak correlation with haematuria ($r = -0{\cdot}23$), BUN ($r = -0{\cdot}39$), Ccr ($r = 0{\cdot}27$), CI ($r = 0{\cdot}28$) and CH50 ($r = 0{\cdot}31$). However, there was almost no finding that showed any correlation with the expression level of IL-13 except for BUN ($r = -0{\cdot}21$), AI ($r = -0{\cdot}32$) and CH50 (Table 4).

In the interstitiums, there was a weak correlation in the expression level of IL-4 with haematuria ($r = 0{\cdot}24$), CH50 ($r = -0{\cdot}34$), AI ($r = 0{\cdot}22$), CI ($r = -0{\cdot}33$) and anti-ds DNA antibodies ($r = 0{\cdot}28$) (Table 4). IL-10 showed a good correlation with UP ($r = 0{\cdot}59$) (Fig. 3Be) and a weak correlation with SLEDAI ($r = -0{\cdot}26$) (Table 4). The percentage of IL-13

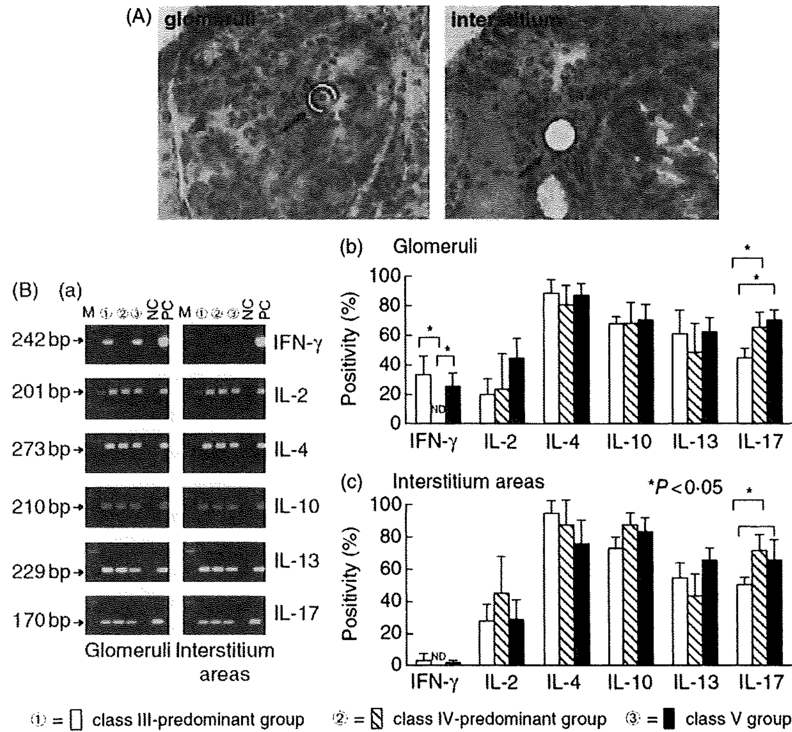


Fig. 2. (A) Targeted infiltrating cells selected and cut by laser microdissection (LMD). The glomeruli and interstitium areas of a single infiltrating cell (black arrows) were selected and dissected with a laser microbeam one by one. (B) Analysis of cytokine gene expression in lesions. (a) Detection of cytokines in the lesions of the renal biopsy specimens from the patients by nested polymerase chain reaction (PCR). Specific expression of interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10, IL-13 and IL-17 were identified in the lesions of the glomeruli and interstitium areas from the renal biopsy specimens of the patients in the Class III-predominant groups ($n = 4$), Class IV-predominant groups ($n = 7$) and Class V groups ($n = 4$). M: molecular size marker; NC: negative control; PC: positive control cDNA clone. (b) Expression of IFN-γ, IL-2, IL-4, IL-10, IL-13 and IL-17 mRNAs in the glomeruli areas of the Class III-predominant (white bars), Class IV-predominant (hatched bars) and Class V (black bars) groups was analysed by nested reverse transcription–polymerase chain reaction (RT–PCR). (c) Expression of IFN-γ, IL-2, IL-4, IL-10, IL-13 and IL-17 mRNAs in the interstitium areas of the Class III-predominant (white bars), Class IV-predominant (hatched bars) and Class V (black bars) groups was analysed by nested RT–PCR (n.d. = not determined). The number of positive samples is shown as a percentage. Error bars represent ± standard error. $P < 0{\cdot}05$, by Mann–Whitney U-test.

samples showed a weak correlation with UP ($r = -0{\cdot}35$), haematuria ($r = -0{\cdot}31$) and Ccr ($r = 0{\cdot}37$) (Table 4), and a good correlation with BUN ($r = -0{\cdot}68$), Cr ($r = -0{\cdot}49$), CH50 ($r = 0{\cdot}48$), AI ($r = -0{\cdot}54$) and anti-ds DNA antibodies ($r = -0{\cdot}43$) (Fig. 3C).

Correlation between Th17 and clinical parameters. In the glomeruli, UP ($r = 0{\cdot}33$), AI ($r = 0{\cdot}26$), CI ($r = -0{\cdot}34$) and BUN ($r = 0{\cdot}26$) showed a weak correlation with the expression level of IL-17 (Table 4). Haematuria ($r = 0{\cdot}54$) and SLEDAI ($r = 0{\cdot}54$) showed a significantly positive correlation with the expression level of IL-17 (Fig. 3Da, b). In the interstitiums, the positive IL-17 samples showed a weak correlation with BUN ($r = 0{\cdot}37$), Cr ($r = 0{\cdot}38$), AI ($r = 0{\cdot}29$), CI ($r = -0{\cdot}27$) and Ccr ($r = -0{\cdot}36$) (Table 4), and a good correlation with haematuria ($r = 0{\cdot}47$) and SLEDAI ($r = 0{\cdot}54$) (Fig. 3Da, b). In particular, focusing upon patients whose SLEDAI scores are more than 10, there is a highly significant correlation between SLEDAI scores and the expression levels of IL-17 both in the glomeruli ($r = 0{\cdot}81$, $P < 0{\cdot}05$) and the interstitiums ($r = 0{\cdot}87$, $P < 0{\cdot}001$) (Fig. 3Dc).

Discussion

A cytokine balance of T helper cells in the kidneys of LN patients has drawn a great deal of attention [5,6]. We analysed the single-cell cytokine profile of the samples from the LN patients, including IL-13 and IL-17, by LMD. We observed the predominance of the Th2 cytokine both in the glomeruli and the interstitiums; this corresponds to the results of the study using whole kidneys by Murata *et al.* [6]. However, IFN-γ was observed only in the glomeruli of the ISN/RPS Class III-predominant and Class V groups. Chan *et al.* [19] reported that up-regulation of IFN-γ, IL-2 and T-bet (the Th1 transcription factor) was observed and no difference was observed in glomerular expression level of any

Table 4. Correlation between the levels of cytokines and clinical parameters.

	Glomeruli									
	SLEDAI	CH50	ADNA	haematuria	Cr	BUN	AI	CI	UP	Ccr
IL-2	−0.214*	0.795***	−0.53**	0.363*	0.114	−0.27*	−0.279*	0.387*	0.045	0.31*
	0.049	0.002	0.018	0.046	0.242	0.047	0.047	0.045	0.435	0.047
IL-4	−0.361*	−0.065	0.016	−0.437**	−0.405*	−0.441**	−0.262*	−0.591**	−0.115	−0.095
	0.046	0.592	0.47	0.042	0.041	0.044	−0.048	0.01	0.34	0.367
IL-10	−0.156	0.308*	0.194	−0.231*	0.019	−0.391*	−0.091	0.282*	0.187	0.265*
	0.268	0.047	0.143	0.048	0.472	0.045	0.626	0.046	0.049	0.047
IL-13	0.001	0.342*	−0.192	0.162	0.023	−0.213*	−0.319*	0.022	0.038	0.127
	0.499	0.047	0.045	0.146	0.467	0.049	0.047	0.531	0.445	0.325
IL-17	0.541**	0.123	−0.157	0.543**	0.029	0.264*	0.227*	−0.341*	0.333*	0.007
	0.017	0.33	0.278	0.018	0.458	0.049	0.049	0.046	0.047	0.488
	Interstitium									
	SLEDAI	CH50	ADNA	haematuria	Cr	BUN	AI	CI	UP	Ccr
IL-2	−0.125	0.37*	−0.175	−0.362*	−0.348*	−0.24*	0.173	0.404**	0.154	0.63**
	0.327	0.045	0.265	0.046	0.046	0.048	0.268	0.037	0.29	0.016
IL-4	0.178	−0.34*	0.279*	−0.24*	−0.168	0.07	0.221*	−0.333*	−0.062	−0.084
	0.262	0.046	0.047	0.044	0.273	0.401	0.048	0.046	0.413	0.381
IL-10	−0.26*	−0.116	−0.02	−0.094	0.207	0.037	0.195	0.061	0.586**	−0.058
	0.047	0.339	0.471	0.369	0.059	0.447	0.091	0.414	0.012	0.418
IL-13	0.058	0.483**	−0.436**	0.31*	−0.486**	−0.675**	−0.541**	−0.117	−0.35*	0.371*
	0.418	0.033	0.025	0.047	0.039	0.002	0.018	0.338	0.047	0.046
IL-17	0.544**	−0.134	0.476*	0.471*	0.379*	0.374*	0.294*	−0.273*	−0.028	−0.364*
	0.018	0.316	0.036	0.038	0.045	0.042	0.047	0.048	0.459	0.047

Correlation between clinical parameters and cytokines was assessed by using the Pearson correlation coefficient test (r-value showed in up, *$r = 0{\cdot}2–0{\cdot}4$, weak correlation; **$r = 0{\cdot}4–0{\cdot}7$, good correlation; ***$r = 0{\cdot}7–0{\cdot}9$, significant correlation. P-value showed in down). UP: urine protein; RBC/HPF: red blood cell/high power field; BUN: blood urea nitrogen; Cr: serum creatinine; Ccr: creatinine clearance; ADNA: anti-double-stranded DNA; CH50: 50% haemolytic unit of complement serum; SLEDAI: systemic lupus erythematosus Disease Activity Index; AI: activity index score; CI: chronicity index; IL: interleukin.

target genes between the WHO Classes. However, as they reported, they did not analyse at a single-cell level; therefore, they could not identify the cellular origin of the detected mRNA, which is likely to be the reason for the discrepancy between their results and our results. Morimoto *et al.* [8] reported that Th2 predominance in the peripheral blood might induce renal lesions, and the co-existence of Th1 and Th2 might cause haemolytic anaemia or pulmonary lesions in SLE patients. Our result demonstrates that Th1 has a role in protecting the kidneys of LN patients; this corresponds to the results of the experiments on the peripheral blood of the SLE patients reported by Morimoto *et al.* Although, conventionally, it was believed that enhanced Th1 cell activation and IFN-γ production might contribute to the development of autoimmune diseases [26,27], certain findings have exploded this general hypothesis. For example, experimental autoimmune nephritis and collagen-induced arthritis (CIA) was exacerbated in mice treated with anti-IFN-γ-neutralizing antibodies and in IFN-γ-deficient or IFN-γ receptor-deficient mice [28]. Haas *et al.* [29] reported that IFN-γ might play a key role in suppressing the development of nephritis in MRL/lpr mice (SLE models).

In addition to the helper T cells classified into Th1 and Th2 types, another helper T cell subset, Th17, has been discovered recently [9]. It has been observed that IL-17 has a proinflammatory role in many inflammatory conditions [9], contributing to the pathogenesis of autoimmune and inflammatory diseases, including SLE [30].

Elevated concentrations of proinflammatory cytokines (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) in the SLE patients were reported [31]. Dong *et al.* [32] reported that the cultured peripheral blood mononuclear cells (PBMC) of LN patients stimulated by IL-17 produced significantly high levels of IL-6, IgG and anti-ds DNA antibodies. However, IL-17 did not increase them in cultured PBMC of normal controls [32]. Crispin *et al.* [33] have demonstrated that $CD3^+$ $CD4^-CD8^-$ double-negative (DN) T cells from SLE patients produce significant amounts of IL-17 and IFN-γ. Furthermore, $IL\text{-}17^+$ and DN T cells are found in renal biopsy specimens from LN patients. In our study, we have confirmed successfully the production of IL-17 in infiltrating T cells in the kidneys (glomeruli and interstitiums) of the LN patients at a single-cell level. This suggests that IL-17 may play an important role in the LN patients. It was reported

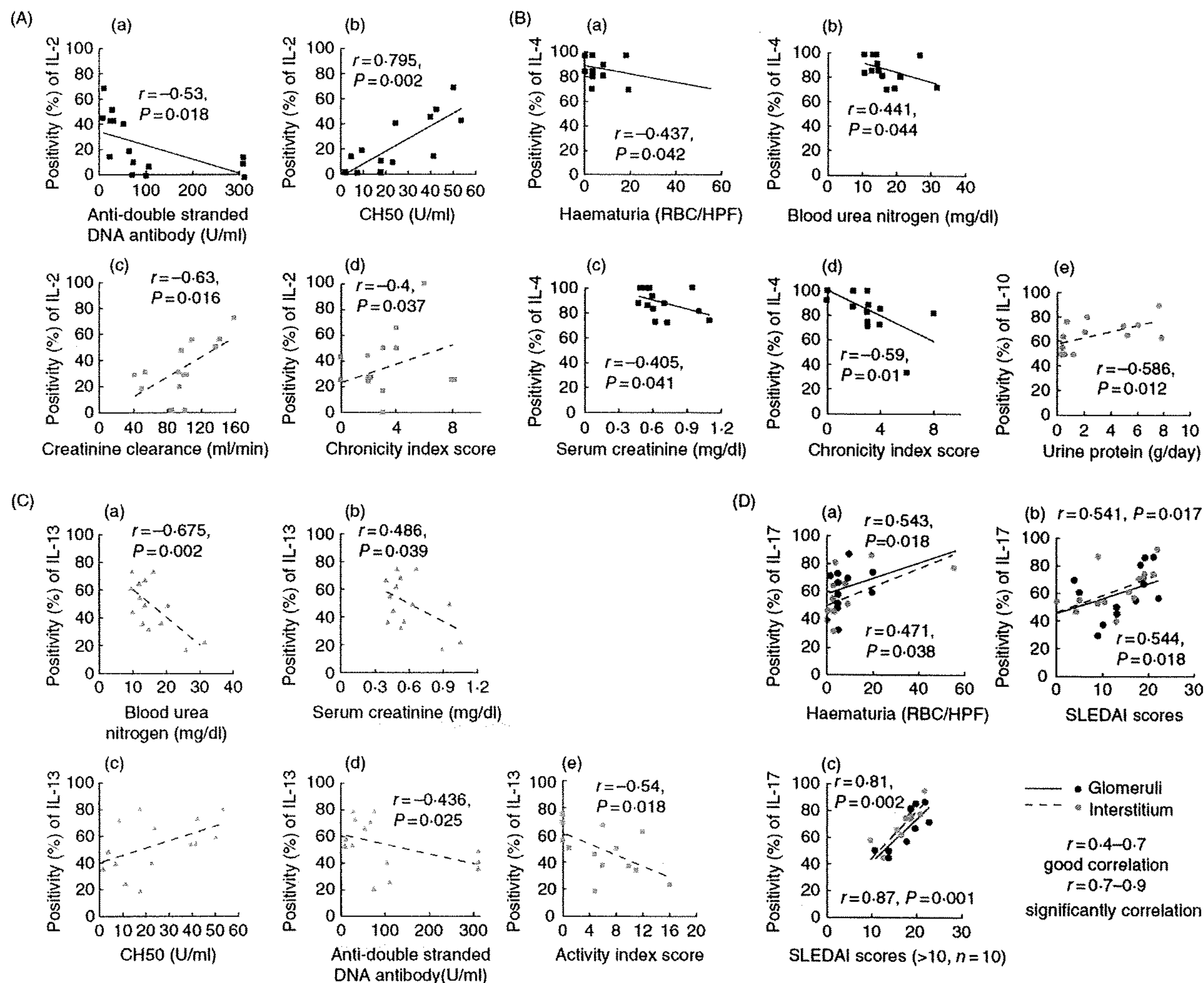


Fig. 3. Correlation between T helper type 1 (Th1), Th2 and Th17 cytokines and clinical and laboratory parameters in systemic lupus erythematosus (SLE). (A) Correlation between the levels of Th1 cytokine interleukin (IL)-2 and anti-double-strand (ds) DNA antibodies (a), 50% haemolytic unit of complement serum (CH50) (b), creatinine clearance (c) and chronicity index score (d) in glomeruli (black full line and points) and interstitium (black dashed line and grey points). (B) Correlation between the levels of Th2 cytokines–IL-4 and haematuria (a), blood urea nitrogen (b), serum creatinine (c) and chronicity index score (d) in glomeruli (black full line and points). Correlation between the levels of IL-10 and urine protein in interstitium (black dashed line and grey points). (C) Correlation between the levels of IL-13 and blood urea nitrogen (a), serum creatinine (b), 50% haemolytic unit of complement serum (CH50) (c), anti-ds DNA antibodies (d) and Activity Index scores (e) in interstitium. (D) Correlation between the levels of Th17 cytokine (IL-17), haematuria (a) and SLE Disease Activity Index (SLEDAI) scores (b) in glomeruli (black full line and points) and interstitium (black dashed line and grey points). Correlation between the level of IL-17 and SLEDAI scores (> 10) in glomeruli and interstitium (c). A simple linear regression analysis was used to evaluate the correlation between the two parameters, $P < 0{\cdot}05$.

that cyclosporine A might inhibit the production of IL-17 in the healthy control and RA patient groups [34]. Cyclosporine A also inhibits IL-15-induced IL-17 production in the CD4⁺ T cells through down-regulation of PI3K/Akt and nuclear factor-kappa B (NF-κB) [35]. Inhibition of IL-15-induced IL-17 production by tacrolimus was also observed in CD4⁺ T cells [35]. It may be considered that the inhibition of IL-17 is an important mechanism of the efficacy of these two kinds of calcineurin inhibitors in the steroid-resistant LN patients.

To confirm cytokine production in the kidney by RT–PCR, we conducted immunohistochemical experiments. The production of IL-13 and IL-17 were also observed by immunohistochemistry. Stained IL-17-positive cells were observed not only in the glomeruli or interstitiums, but also in the tubular epitheliums of LN patients (Fig. 1). Crispin *et al.* [33] reported that IL-17-positive cells were found by immunofluorescence mainly in the tubule-interstitial zone, the area where cellular infiltration is mainly found. We made stains for IL-17-positive cells with anti-human IL-17 in

 © 2009 British Society for Immunology,

the specimens from MGA and MCNS patients; no IL-17-positive cells were observed (Fig. 1Ca, b). This has demonstrated that IL-17 may be produced preferentially in SLE patients. Matsumura *et al.* also found stained IL-17 in the tubular epitheliums of LN patients by immunohistochemistry (personal communication). Thus, production of IL-17 in the tubules was confirmed by the RT–PCR and LMD methods. We believe that the RT–PCR technique is more sensitive than immunohistochemistry and can be used for quantification of the production of each cytokine.

We analysed the correlation between the expression levels of Th1, Th2 and Th17 cytokines and clinical parameters. We found that the levels of IL-2, IL-4, IL-10, IL-13 and IL-17 have a correlation with some clinical and laboratory parameters (Fig. 3). A negative correlation was found between the level of IL-2 and haematuria, BUN, Cr, anti-ds DNA antibody and SLEDAI, except for Ccr, CH50 and CI. However, the IL-17 level was correlated positively with UP, haematuria, BUN, Cr, AI and SLEDAI, while correlating negatively with CI and Ccr (Fig. 3). These findings indicate that IL-2 and IL-17 play opposite roles in SLE development. It is suggested that IL-2 may play a role in protecting against SLE development, while IL-17 might have a reverse effect. Wong *et al.* [36] showed significant and positive correlations of plasma IL-17 concentrations with SLEDAI scores in the patients without renal disease. Yang *et al.* [37] showed that patients with active SLE (SLEDAI > 6) exhibit an increased proportion of Th17 cells in $CD3^-CD8^-$ T cells from PBMC compared with healthy individuals by flow cytometric analysis, and a significant positive correlation between the percentage of Th17 cells and the SLEDAI score. Doreau *et al.* [38] also found that the serum of patients with SLE had higher concentrations of IL-17 than did the serum of healthy people, and that IL-17 abundance correlated with the disease severity of SLE. In our study, the level of IL-17 correlated positively and significantly with SLEDAI scores both in the glomeruli and the interstitiums. A highly significant correlation was observed between SLEDAI scores and the level of IL-17 in both the glomeruli and the interstitiums of active SLE patients (SLEDAI > 10) (Fig. 3D). We also found that the level of IL-17 has positive correlations with AI and negative correlations with CI in both glomerulus and interstitium, although correlations were weak (Table 4). This suggests that IL-17 may play an important role in the inflammatory process of a renal disease during the acute phase of SLE patients. With few IFN-γ-positive samples, we did not analyse the correlation between IFN-γ and the clinical and laboratory parameters. IFN-γ was observed only in the glomeruli of ISN/RPS Class III-predominant and Class V groups; accordingly, IFN-γ might play a role in protecting against the inflammatory process in LN patients, as with IL-2. The IL-2 level correlatates good positively with CI, suggesting that IL-2 might act during the chronic stage of glomerulonephritis (Fig. 3A and Table 4). Nakae *et al.* [39] found that IL-17 can suppress Th1 cell differentiation in the presence of exogenous IL-12 *in vitro*, and IFN-γ can down-regulate Th17 cell differentiation. Not only IFN-γ but also IL-4 can suppress IL-17 production *in vitro* [40,41]. Chu *et al.* [42] demonstrated further that IFN-γ might regulate susceptibility to CIA through suppression of IL-17, and IFN-γ and IL-4 together had a synergistic effect on suppression of type II collagen (CII)-specific IL-17 production during CII restimulation *in vitro*. This might be the reason why the expression levels of IFN-γ and IL-4 were higher in the ISN/RPS Class III-predominant group than those of other classes, whereas that of IL-17 was lower. Th2 cytokine showed inconsistent results, but it seems likely that IL-13 plays a protective role in lupus nephritis (Fig. 3C, Table 4).

In conclusion, we have shown that the glomerular infiltrating T cells might act as Th1, Th2 and Th17 cells, while the interstitial infiltrating T cells, as Th2 and Th17 cells in the Class III-predominant and Class V groups. In contrast, both the glomerular and interstitial infiltrating T cells might act as Th2 and Th17 cells in the Class IV-predominant group. The cytokine balances may be dependent on the classification of renal pathology and IL-17 might play a critical role in SLE development.

Acknowledgements

This study was supported by the Health and Labour Sciences Research Grants for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan. We thank medical scientists of Chiba-East National Hospital (Department of Rheumatology, Allergy and Clinical Immunology National Hospital Organization Chiba-East National Hospital) for their helpful suggestions for this study.

Disclosure

None of the authors have any conflict of interest with the subject matter or materials discussed in the manuscript.

References

1 Funauchi M, Ikoma S, Enomoto H, Horiuchi A. Decreased Th-1 like and increased Th-2 like cells in systemic lupus erythematosus. Scand J Rheumatol 1998; **27**:219–24.
2 Viallard JF, Pellegrin JL, Ranchin V *et al.* Th1 [IL-2, interferon-gammma (IFN-γ)] and Th2 (IL-10, IL-4) cytokine production by peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE). Clin Exp Immunol 1999; **115**:189–95.
3 Richaud-Patin Y, Alcocer-Verela J, Llorente L. High levels of TH2 cytokine gene expression in systemic lupus erythematosus. Rev Invest Clin 1995; **47**:267–72.
4 Akahoshi M, Nakashima H, Tanaka Y *et al.* Th1/Th2 balance of peripheral T helper cells in systemic lupus erytematosus. Arthritis Rheum 1999; **42**:1644–8.
5 Masutani K, Akahoshi M, Tsuruya K *et al.* Predominance of Th1

immune response in diffuse proliferative lupus nephritis. Arthritis Rheum 2001; **44**:2097–106.
6 Murata H, Matsumura R, Koyama A *et al.* T cell receptor repertoire of T cells in the kidneys of patients with lupus nephritis. Arthritis Rheum 2002; **46**:2141–7.
7 Spadaro A, Rinaldi T, Riccieri V, Taccari E, Valesini G. Interleukin-13 in autoimmune rheumatic diseases: relationship with the autoantibody profile. Clin Exp Rheumatol 2002; **20**:213–6.
8 Morimoto S, Tokano Y, Kaneko H, Nozawa K, Amano H, Hashimoto H. The increased interleukin-13 in patients with systemic lupus erythematosus: relations to other Th1-, Th2-related cytokines and clinical findings. Autoimmunity 2001; **34**:19–25.
9 Afzari B, Lombardi G, Lechler R, Lord GM. The role of T helper (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. Clin Exp Immunol 2007; **148**:32–46.
10 Wang Y, Ito S, Sumida T *et al.* Use of laser microdissection in the analysis of renal-infiltrating T cells in MRL/lpr mice. Mod Rheumatol 2008; **18**:385–93.
11 Lechner S, Muller-Landner U, Renke B, Scholmerich J, Ruschoff J, Kullmann F. Gene expression pattern of laser microdissected colonic crypts of adenomas with low grade dysplasia. Gut 2003; **52**:1148–53.
12 Hong SH, Nah HY, Lee JY, Gye MC, Kim CH, Kim MK. Analysis of estrogen-regulated genes in mouse uterus using cDNA microarray and laser capture microdissection. J Endocrinol 2004; **181**:157–67.
13 Shi X, Kleeff J, Zhu ZW *et al.* Gene-expression analysis of single cells-nested polymerase chain reaction after laser microdissection. World J Gastroenterol 2003; **9**:1337–41.
14 Judex M, Neuman E, Gay S, Muller-Lander U. Laser-mediated microdissection as a tool for molecular analysis in arthritis. Methods Mol Med 2004; **101**:93–105.
15 Hashimoto A, Tarner IH, Bohle RM *et al.* Analysis of vascular gene expression in arthritis synovium by laser-mediated microdissection. Arthritis Rheum 2007; **56**:1094–105.
16 Hofbauer M, Wiesener S, Babbe H *et al.* Clonal tracking of autoaggressive T cells in polymyositis by combining laser microdissection, single-cell PCR, and CDR3-spectratype analysis. Proc Natl Acad Sci USA 2003; **100**:4090–5.
17 Peterson KS, Huang JF, Zhu J *et al.* Characterization of heterogeneity in the molecular pathogenesis of lupus nephritis from transcriptional profiles of laser-captured glomeruli. J Clin Invest 2004; **113**:1722–33.
18 Fries JW, Roth T, Dienes HP, Weber M, Odenthal M. A novel evaluation method for paraffinized human renal biopsies using quantitative analysis of microdissected glomeruli and VCAM-1 as marker of inflammatory mesangial cell activation. Nephrol Dial Transplant 2003; **18**:710–6.
19 Chan RW, Lai FM, Li EK *et al.* Intrarenal cytokine gene expression in lupus nephritis. Ann Rheum Dis 2007; **66**:886–92.
20 Todd R, Margolin DH. Challenge of single-cell diagnostics: analysis of gene expression. Trends Mol Med 2002; **8**:254–7.
21 Weening JJ, D'Agati VD, Schwartz MM *et al.* The classification of glomerulonephritis in systemic lupus erythematosus revised. J Am Soc Nephrol 2004; **15**:241–50.
22 Weening JJ, D'Agati VD, Schwartz MM *et al.* The classification of glomerulonephritis in systemic lupus erythematosus revised. Kidney Int 2004; **65**:521–30.
23 Churg J, Bernstein J, Glassock RJ. Renal disease: classification and atlas of glomerular disease, 2nd edn. Tokyo: Igaku-Shoin, 1995.
24 Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. Arthritis Rheum 1992; **35**:630–40.
25 Austin HA 3rd, Muenz LR, Joyce KM, Antonovych TT, Balow JE. Diffuse proliferative lupus nephritis: identification of specific pathologic features affecting renal outcome. Kidney Int 1984; **25**:689–95.
26 Wang B, Esche C, Mamelak A, Freed I, Watanabe H, Sauder DH. Cytokine knockouts in contact hypersensitivity research. Cytokine Growth Factor Rev 2003; **14**:381–9.
27 Crane IJ, Forrester JV. Th1 and Th2 lymphocytes in autoimmune disease. Crit Rev Immunol 2005; **25**:75–102.
28 Matthys P, Vermeire K, Heremans H, Billiau A. The protective effect of IFN-gamma in experimental autoimmune diseases: a central role of mycobacterial adjuvant-induced myelopoiesis. J Leukoc Biol 2000; **68**:447–54.
29 Haas C, Ryffel B, Le Hir M. IFN-gamma is essential for the development of autoimmune glomerulonephritis in MRL/Ipr mice. J Immunol 1997; **158**:5484–91.
30 Bi Y, Liu G, Yang R. Th17 cell induction and immune regulatory effects. J Cell Physiol 2007; **211**:273–8.
31 Wong CK, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus 2000; **9**:589–93.
32 Dong GF, Ye R, Shi W *et al.* IL-17 induces autoantibody overproduction and peripheral blood mononuclear cell overexpression of IL-6 in lupus nephritis patients. Chin Med J 2003; **116**:543–8.
33 Crispin JC, Oukka M, Bayliss G *et al.* Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. J Immunol 2008; **181**:8761–6.
34 Zhang C, Zhang J, Yang B, Wu C. Cyclosporine A inhibits the production of IL-17 by healthy individuals and patients with rheumatoid arthritis. Cytokine 2008; **42**:345–52.
35 Cho ML, Ju JH, Kim KW *et al.* Cyclosporine A inhibits IL-15-induced IL-17 production in CD4+ T cells via down-regulation of PI3K/Akt and NF-κB. Immunol Lett 2007; **108**:88–96.
36 Wong CK, Lit LC, Tam LS, Li EK, Wong PT, Lam CW. Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity. Clin Immunol 2008; **127**:385–93.
37 Yang J, Chu Y, Yang X *et al.* Th17 and natural Treg cell population dynamics in systemic lupus erythematosus. Arthritis Rheum 2009; **60**:1472–83.
38 Doreau A, Belot A, Bastid J *et al.* Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus. Nat Immunol 2009; **10**:778–85.
39 Nakae S, Iwakura Y, Suto H, Galli SJ. Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. J Leukoc Biol 2007; **81**:1258–68.
40 Harrington LE, Hatton RD, Mangan PR *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005; **6**:1123–32.
41 Park H, Li Z, Yang XO *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005; **6**:1133–41.
42 Chu CQ, Swart D, Alcorn D, Tocker J, Elkon KB. Interferon-gamma regulates susceptibility to collagen-induced arthritis through suppression of interleukin-17. Arthritis Rheum 2007; **56**:1145–51.

Research article

Altered peptide ligands inhibit arthritis induced by glucose-6-phosphate isomerase peptide

Keiichi Iwanami[1], Isao Matsumoto[1,2], Yohei Yoshiga[1], Asuka Inoue[1], Yuya Kondo[1], Kayo Yamamoto[1], Yoko Tanaka[1], Reiko Minami[1], Taichi Hayashi[1], Daisuke Goto[1], Satoshi Ito[1], Yasuharu Nishimura[3] and Takayuki Sumida[1]

[1]Department of Clinical Immunology, Doctoral Program in Clinical Science, Graduate School of Comprehensive Human Science, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8575, Japan
[2]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan
[3]Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8556, Japan

Corresponding author: Isao Matsumoto, ismatsu@md.tsukuba.ac.jp

Received: 4 May 2009 Revisions requested: 2 Jul 2009 Revisions received: 23 Sep 2009 Accepted: 9 Nov 2009 Published: 9 Nov 2009

Arthritis Research & Therapy 2009, 11:R167 (doi:10.1186/ar2854)
This article is online at: http://arthritis-research.com/content/11/6/R167
© 2009 Iwanami *et al.*; licensee BioMed Central Ltd.
This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Introduction Immunosuppressants, including anti-TNFα antibodies, have remarkable effects in rheumatoid arthritis; however, they increase infectious events. The present study was designed to examine the effects and immunological change of action of altered peptide ligands (APLs) on glucose-6-phosphate isomerase (GPI) peptide-induced arthritis.

Methods DBA/1 mice were immunized with $hGPI_{325-339}$, and cells of draining lymph node (DLN) were stimulated with $hGPI_{325-339}$ to investigate the T-cell receptor (TCR) repertoire of antigen-specific CD4+ T cells by flow cytometry. Twenty types of APLs with one amino acid substitution at a TCR contact site of $hGPI_{325-339}$ were synthesized. CD4+ T cells primed with human GPI and antigen-presenting cells were co-cultured with each APL and cytokine production was measured by ELISA to identify antagonistic APLs. Antagonistic APLs were co-immunized with $hGPI_{325-339}$ to investigate whether arthritis could be antigen-specifically inhibited by APL. After co-immunization, DLN cells were stimulated with $hGPI_{325-339}$ or APL to investigate Th17 and regulatory T-cell population by flow cytometry, and anti-mouse GPI antibodies were measured by ELISA.

Results Human $GPI_{325-339}$-specific Th17 cells showed predominant usage of TCRVβ8.1 8.2. Among the 20 synthesized APLs, four (APL 6; N329S, APL 7; N329T, APL 12; G332A, APL 13; G332V) significantly reduced IL-17 production by CD4+ T cells in the presence of $hGPI_{325-339}$. Co-immunization with each antagonistic APL markedly prevented the development of arthritis, especially APL 13 (G332V). Although co-immunization with APL did not affect the population of Th17 and regulatory T cells, the titers of anti-mouse GPI antibodies in mice co-immunized with APL were significantly lower than in those without APL.

Conclusions We prepared antagonistic APLs that antigen-specifically inhibited the development of experimental arthritis. Understanding the inhibitory mechanisms of APLs may pave the way for the development of novel therapies for arthritis induced by autoimmune responses to ubiquitous antigens.

Introduction

Rheumatoid arthritis (RA) is characterized by symmetrical polyarthritis and joint destruction. Although the etiology is considered autoimmune reactivity to some antigens, the exact mechanisms are not fully understood. Pathological examinations show that most of the lymphocytes infiltrating the synovium in RA are CD4+ T cells, which can recognize some antigens and expand oligoclonally intraarticularly [1]. These findings imply the possible role of CD4+ T cells in the pathogenesis of RA. Previous studies showed that cytotoxic T-lymphocyte antigen-4 immunoglobulin and tacrolimus have remarkable effects on RA, and stressed the importance of CD4+ T cells in the pathogenesis of RA [2-4].

Ab: antibody; APC: antigen-presenting cell; APL: altered peptide ligand; CII: collagen type II; DLN: draining lymph node; ELISA: enzyme-linked immunosorbent assay; GPI: glucose-6-phosphate isomerase; IFN: interferon; IL: interleukin; MBP: myelin basic protein; MHC: major histocompatibility complex; PBS: phosphate-buffered saline; PLP: proteolipid protein; RA: rheumatoid arthritis; rhGPI: recombinant human glucose-6-phosphate isomerase; TCR: T-cell receptor; Th: T-helper; TNF: tumor necrosis factor.

Although the exact helper T-cell lineage critical in RA remains elusive, previous animal studies reported that Th17 cells play a crucial role and that Th1 cells may have a protective role against the progress of arthritis in most mouse models with the exception of proteoglycan-induced arthritis in Balb/c mice [5]. Collagen-induced arthritis in the C57BL/6 background is markedly suppressed in IL-17-deficient mice [6], and glucose-6-phosphate isomerase (GPI)-induced arthritis in the DBA/1 background and antigen-induced arthritis in the C57BL/6 background are also suppressed by the administration of anti-IL-17 antibodies (Abs) [7,8]. In these models, complete Freund's adjuvant is used for the induction of arthritis; therefore it is possible that the components of *Mycobacterium tuberculosis* affect the cytokine dependency. The arthritis seen in IL-1 receptor antagonist-deficient mice in the Balb/c background and SKG mice in the Balb/c background, however, is completely suppressed in IL-17-deficient mice [9,10]. These findings indicate that Th17 cells play a central role in murine models independent of mouse strains and target antigens.

IL-17 is also considered to play a crucial role in host defense. IL-17 signaling seems essential for the recruitment of neutrophils to the alveolar space in pneumonia caused by *Klebsiella pneumoniae*, *Mycoplasma pneumoniae* and *Pneumocystis jiroveci* [11-13]. IL-17 is also involved in mucosal host defense against oropharyngeal candidasis via salivary antimicrobial factors, in addition to neutrophil recruitment [14]. Furthermore, IL-17 production by $\gamma\delta$ T cells is essential against peritonitis caused by *Escherichia coli* [15]. In this regard, anti-cytokine therapies such as infliximab and tocilizumab have been applied to clinical treatment and have shown striking effects on RA [16-19]; anti-IL-17 therapy could therefore be useful in the treatment of RA. Blockade of IL-17 could increase the likelihood of infections, however, and the use of such a strategy would be limited just like the case of infliximab and tocilizumab.

Altered peptide ligands (APLs) are peptides with substitutions in amino acid residues at T-cell receptor (TCR) contact sites, and can be either agonistic, antagonistic with partial activation or antagonistic [20]. These three different actions seem to depend on the site and residue of the peptide substitution [21]. The antagonistic APLs can inhibit the function of limited T-cell populations, and thus they could be potentially useful as antigen-specific therapy for autoimmune diseases in which T cells play a pathogenic role. Indeed, APLs have been proven effective in the suppression of several autoimmune models. In an arthritis model, previous studies identified type II collagen $CII_{245-270}$ as a prominent T-cell epitope in collagen-induced arthritis in DBA/1 mice, and found that co-immunization with the analog peptide (I260A, A261B(hydroxyproline), F263N), also known as A9, significantly suppressed the disease [22,23]. As reported previously, however, the type II collagen residues CII 260 (I) and CII 263 (F) are I-Aq (MHC class II of DBA/1 mice) binding sites, and A9 was confirmed not to bind I-Aq molecules [24,25]. The analog peptide A9 therefore seems to differ from conventional APLs, and the inhibitory effect and the mechanisms of conventional APLs on arthritis remain to be defined.

Several models of arthritis have so far been described and analyzed to understand the etiological mechanisms of RA. GPI-induced arthritis, a murine model of RA, is induced by immunization of DBA/1 mice with recombinant human GPI (rhGPI) [26]. We demonstrated previously that the Th17 subset of $CD4^+$ T cells played a central role in the pathogenesis of GPI-induced arthritis; GPI-specific $CD4^+$ T cells were skewed to T_H-17 at the time of onset, and blockade of IL-17 resulted in a significant amelioration of arthritis [7]. We have also demonstrated that the major epitope of $CD4^+$ T cells in GPI-induced arthritis was $hGPI_{325-339}$, and immunization with the peptide induced severe polyarthritis (GPI peptide-induced arthritis) [27].

The present work is an extension to the above studies. Specifically, we explored the antigen-specific Th17 inhibition, explored the effects of APLs on arthritis, and investigated the inhibitory mechanisms of APLs, using the T-cell-dependent model of GPI peptide-induced arthritis. The results showed that many $hGPI_{325-339}$-specific $CD4^+$ T cells employed Vβ8.1 8.2 as the TCR repertoire, and co-immunization with APL (N329S, N329T, G332A, or G332V) significantly inhibited the development of arthritis. Our analysis of the inhibitory mechanisms of APLs indicates that our APLs can function as TCR antagonists; however, they can differentiate naïve $CD4^+$ T cells to Th17 cells, but not Th2 cells or regulatory T cells. Based on these findings, we define a new aspect for APLs, and propose that they may provide the basis for the invention of new antigen-specific therapy.

Materials and methods

Mice

DBA/1 mice were purchased from Charles River Laboratories, Yokohama, Japan. All mice were kept under specific pathogen-free conditions, and all experiments were conducted in accordance with the institutional ethical guidelines.

Glucose-6-phosphate isomerase and synthetic peptides

The rhGPI and recombinant mouse GPI were prepared as described previously [28,29]. Briefly, human or mouse GPI cDNA was inserted into the plasmid pGEX-4T3 (Pharmacia, Uppsala, Sweden) for expression of glutathione-*S*-transferase-tagged proteins. *E. coli* harboring pGEX-hGPI plasmid was allowed to proliferate at 37°C, before the addition of 0.1 mM isopropyl-β-d-thiogalactopyranoside to the medium, followed by further culture overnight at 30°C. The bacteria were lysed with a sonicator and the supernatant was purified with a glutathione-sepharose column (Pharmacia). The purity was estimated by SDS-PAGE.

Peptides for screening were synthesized with 70% purity by Wako Pure Chemical Industries, Ltd (Osaka, Japan), and peptides of a major peptide and antagonistic altered peptide ligands were synthesized with 90% purity by Invitrogen (Carlsbad, CA, USA). $OVA_{323-339}$ peptide was purchased from AnaSpec (San Jose, CA, USA).

Induction of arthritis

DBA/1 mice were immunized with 10 μg synthetic peptides for GPI peptide-induced arthritis in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA), and in the indicated experiments 50 μg altered peptide ligands were used with 10 μg $GPI_{325-339}$ for co-immunization. The synthetic peptides were emulsified with complete Freund's adjuvant at a 1:1 ratio (v/v). For induction of arthritis, 150 μl emulsion was injected intradermally at the base of the tail, and 200 ng pertussis toxin was injected intraperitoneally on days 0 and 2 after immunization.

The arthritis score was evaluated visually using a score of 0 to 3 for each paw. A score of 0 represented no evidence of inflammation, 1 represented subtle inflammation or localized edema, 2 represented easily identified swelling that was localized to either the dorsal or ventral surface of the paws, and 3 represented swelling in all areas of the paws.

Screening of antagonistic altered peptide ligands

Mice were sacrificed on the indicated day. Spleens and draining lymph nodes (DLNs) were harvested, and splenocytes were hemolyzed with a solution of 0.83% NH_4Cl, 0.12% $NaHCO_3$ and 0.004% $EDTA_2Na$ in PBS. Single-cell suspensions were prepared in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. $CD4^+$ T cells from DLNs and $CD11c^+$ dendritic cells from spleens were isolated by magenetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the collected cells (>97%) was confirmed by flow cytometry. $CD11c^+$ dendritic cells treated with 50 μg/ml mitomycin C were used as antigen-presenting cells (APCs). The purified $CD4^+$ T cells and APCs were co-cultured with 10 μM synthetic peptide at a ratio of 1:3 in 96-well round-bottom plates (Nunc, Roskilde, Denmark) at 37°C under 5% carbon dioxide for 72 hours. The supernatants were assayed for IL-10 and IL-17 by the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

Pre-pulse assay

The pre-pulse assay was conducted as described previously [30]. Briefly, $CD11c^+$ APCs from spleens (4×10^4/well) were cultured with a suboptimal concentration of $GPI_{325-339}$ (3 μM) for 2 hours. In the meantime, native peptides were loaded onto APCs and presented on MHC. After 2 hours of incubation, APCs were washed twice to remove unbound peptides, and 30 μM each antagonistic APL was added. After 18 hours of culture, $CD4^+$ T cells (2×10^4/well) from DLNs were added, and after an additional 72 hours of culture the supernatants were assayed for IL-17 and IL-10 by ELISA. The inhibition ratio was calculated as follows:

1 – (IL-17 concentration in the presence of native peptides and APLs / IL-17 concentration in the presence of native peptides only) x 100 (%)

Flow cytometry

Mice were sacrificed on the indicated day. The popliteal lymph nodes were harvested and single-cell suspensions were prepared as described above. Cells (1×10^6/ml) were stimulated with 100 μg/ml rhGPI in 96-well round-bottom plates (Nunc) for 24 hours and GoldiStop (BD PharMingen, San Diego, CA, USA) was added for the last 2 hours of each culture. Cells were first stained extracellularly, fixed and permeabilized with Cytofix/Cytoperm solution (BD PharMingen) and then stained intracellularly. Regulatory T cells were stained with the Mouse Regulatory T cell Staining kit (eBioscience, San Diego, CA, USA) according to the protocol supplied by the manufacturer. For TCR repertoire screening, the Mouse TCR Screening Panel (BD PharMingen) was used. Samples were acquired on FACSCalibur (BD PharMingen) and data were analyzed with FlowJo (Tree Star, Ashland, OR, USA).

Analysis of anti-glucose-6-phosphate isomerase antibody

Sera were taken from immunized mice on day 28 and were diluted 1:500 (for IgG, IgG_{2a}, IgG_{2b} and IgG_3) or 1:8,000 (for IgG_1) in blocking solution (25% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) in PBS) for antibody analysis. We also prepared 96-well plates (Sumitomo Bakelite, Tokyo, Japan) coated with 5 μg/ml recombinant mouse GPI for 12 hours at 4°C. After washing twice with a washing buffer (0.05% Tween20 in PBS), the blocking solution was used for blocking nonspecific binding for 2 hours at room temperature. After two washes, 150 μl diluted serum was added and incubated for 2 hours at room temperature. After three washes, alkaline phosphatase-conjugated anti-mouse IgG, horseradish peroxidase-conjugated anti-mouse IgG_1, IgG_{2a}, IgG_{2b} (Zymed Laboratories, San Francisco, CA, USA) or IgG_3 (Invitrogen) was added at a final dilution of 1:5,000 for 1 hour at room temperature. After three washes, color was developed with substrate solution (1 alkaline phosphatase tablet (Sigma-Aldrich) per 5 ml alkaline phosphatase reaction solution (containing 9.6% diethanolamine and 0.25 mM $MgCl_2$, pH 9.8)) or tetramethylbenzidine (KPL, Gaithersburg, MD, USA). Plates were incubated for 20 to 60 minutes at room temperature and the optical density was measured by a microplate reader at 405 nm (for IgG) or at 450 nm (for IgG_1, IgG_{2a}, IgG_{2b} and IgG_3).

Statistical analysis

All data are expressed as the mean ± standard error of the mean or standard deviation. Differences between groups were

examined for statistical significance using the Mann-Whitney U test. Differences of arthritis incidence between groups were examined using Fisher's exact test. $P < 0.05$ denotes the presence of a statistically significant difference.

Results

Designing and screening antagonistic altered peptide ligands

We reported previously that the major T-cell epitope in GPI-induced arthritis is $hGPI_{325-339}$, and immunization with the peptide provokes symmetrical polyarthritis (GPI peptide-induced arthritis) [28]. To investigate the effects of APLs in GPI peptide-induced arthritis, we first designed APLs of $hGPI_{325-339}$. Since the MHC binding sites of $hGPI_{325-339}$ exist at P1 (I328), P4 (F331), and P7 (E334) (IWYINCF-GCETHAML) [25,28], the amino acid residues of the TCR contact sites at P0 (Y327), P2 (N329), P3 (C330), P5 (G332), P6 (C333), and P8 (T335) were substituted for another peptide to design 20 types of APLs (Table 1).

To select antagonistic APLs, CD4+ T cells primed with rhGPI and APCs were co-cultured with each APL. The IL-17 production was markedly lower with APL 2, APL 5, APL 6, APL 7, APL 9, APL 10, APL 11, APL 12, APL 13, and APL 18 than with $hGPI_{325-339}$, and therefore these APLs were considered candidates of antagonistic APLs (Figure 1a). None of the APLs induced IL-4 and IL-10 production (data not shown). We next explored the potency of the APLs in inhibiting IL-17 production in the presence of $hGPI_{325-339}$. In the pre-pulse assay, APL 6 (N329S), APL 7 (N329T), APL 12 (G332A), and APL 13 (G332V) significantly reduced IL-17 production by CD4+ T cells primed with rhGPI in the presence of $hGPI_{325-339}$ ($P < 0.01$) (Figure 1b). We therefore considered these four APLs as antagonistic APLs.

Inhibition of arthritis by antagonistic altered peptideligands

Since GPI peptide-induced arthritis is mediated by Th17 and antagonistic APLs can suppress IL-17 production, we explored the efficacy of the prepared APLs on the inhibition of

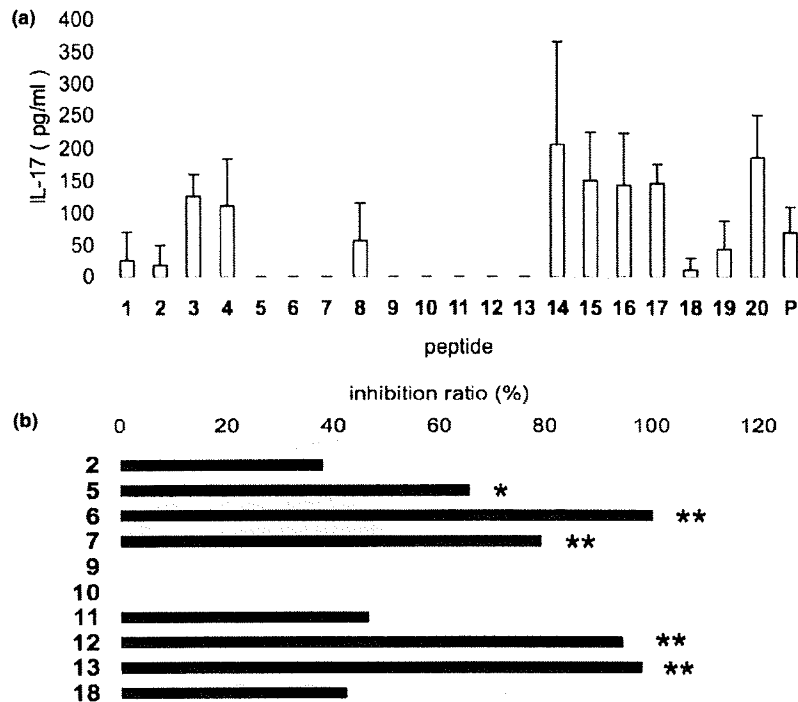
Table 1

$hGPI_{325-339}$-derived altered peptide ligands used in the present study

325 to 339	I	W	Y	I	N	C	F	G	C	E	T	H	A	M	L
APL 1	--	--	N		--	--	--	--	--	--	--	--	--	--	--
APL 2	--	--	Q		--	--	--	--	--	--	--	--	--	--	--
APL 3	--	--	S		--	--	--	--	--	--	--	--	--	--	--
APL 4	--	--	T		--	--	--	--	--	--	--	--	--	--	--
APL 5	--	--	--		Q	--		--	--		--	--	--	--	--
APL 6	--	--	--		S	--		--	--		--	--	--	--	--
APL 7	--	--	--		T	--		--	--		--	--	--	--	--
APL 8	--	--	--		--	N		--	--		--	--	--	--	--
APL 9	--	--	--		--	Q		--	--		--	--	--	--	--
APL 10	--	--	--		--	S		--	--		--	--	--	--	--
APL 11	--	--	--		--	T		--	--		--	--	--	--	--
APL 12	--	--	--		--	--		A	--		--	--	--	--	--
APL 13	--	--	--		--	--		V	--		--	--	--	--	--
APL 14	--	--	--		--	--		--	N		--	--	--	--	--
APL 15	--	--	--		--	--		--	Q		--	--	--	--	--
APL 16	--	--	--		--	--		--	S		--	--	--	--	--
APL 17	--	--	--		--	--		--	T		--	--	--	--	--
APL 18	--	--	--		--	--		--	--		N	--	--	--	--
APL 19	--	--	--		--	--		--	--		Q	--	--	--	--
APL 20	--	--	--		--	--		--	--		S	--	--	--	--

The MHC binding sites exist at glucose-6-phosphate isomerase (GPI) 328 (I), GPI 331 (F) and GPI 334 (E) as indicated (underlined). The amino acid residues of the T-cell receptor contact sites at P0 (Y327), P2 (N329), P3 (C330), P5 (G332), P6 (C333), and P8 (T335) were substituted for the indicated peptides to design 20 types of altered peptide ligands (APLs).

Figure 1



Altered peptide ligands markedly suppress IL-17 production by glucose-6-phosphate isomerase-primed CD4+ T cells. Altered peptide ligand (APL) 6, APL 7, APL 9, APL 12 and APL 13 markedly suppress IL-17 production by glucose-6-phosphate isomerase (GPI)-primed CD4+ T cells. Mice were sacrificed on day 8 after immunization. CD4+ T cells were purified from draining lymph node cells of recombinant human GPI (hGPI)-immunized DBA/1 mice, and CD11c+ antigen-presenting cells (APCs) were purified from spleen cells. **(a)** CD4+ T cells primed with hGPI and APCs were co-cultured with 10 μM synthetic peptide for 72 hours. The supernatants were assayed for IL-17 by ELISA. P, positive control ($hGPI_{325-339}$). **(b)** CD11c+ APCs were cultured with a suboptimal concentration $GPI_{325-339}$ (3 μM) for 2 hours, washed twice to remove unbound peptides, and 30 μM each antagonistic APL was added. After 18 hours of culture, CD4+ T cells (2 × 10^4/well) were added, and after an additional 72 hours of culture, the supernatants were assayed for IL-17 by ELISA. The inhibition ratio was calculated as stated in Pre-pulse assay. Data presented as average ± standard deviation of three culture wells. **P* < 0.05, ***P* < 0.01 (Mann--Whitney U test). Representative data of two independent experiments.

arthritis. First, we immunized DBA/1 mice with each APL alone, and confirmed that no overt arthritis developed (data not shown). DBA/1 mice were then co-immunized with $hGPI_{325-339}$ and each APL to explore the development of arthritis. Mice co-immunized with APL 6, APL 12 and APL 13 developed significantly attenuated arthritis after day 12, and those co-immunized with APL 7 after day 16, compared with mice immunized with $hGPI_{325-339}$ alone ($P < 0.05$) (Figure 2, upper panel). Co-immunization with APL 13 also significantly suppressed the incidence of arthritis ($P < 0.05$) (Table 2). Co-immunization with $hGPI_{325-339}$ and APL 15, an agonistic APL, however, did not affect the severity or course of arthritis (Figure 2, middle panel). Moreover, mice co-immunized with $hGPI_{325-339}$ and $OVA_{323-339}$ also had a similar course of arthritis to $hGPI_{325-339}$ alone (Figure 2, lower panel).

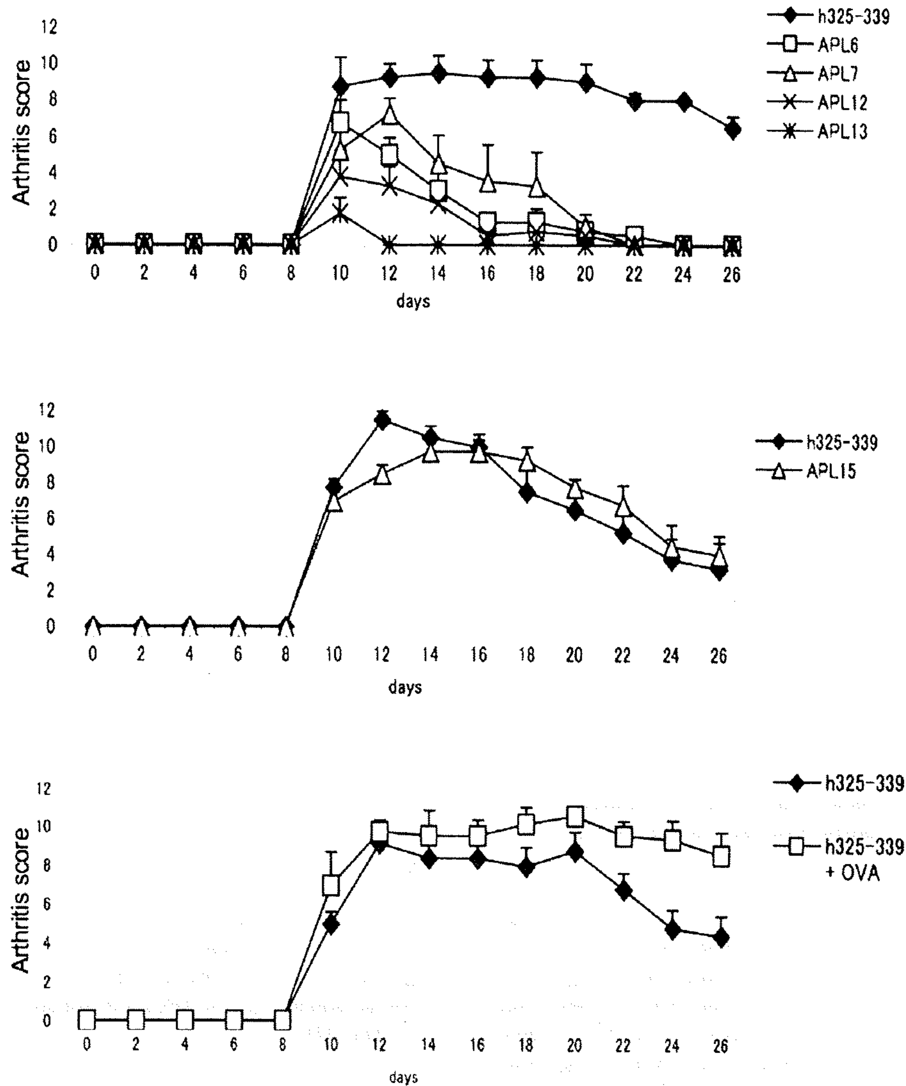
Identification of TCRVβ usage of $hGPI_{325-339}$-specificTh17 cells

To investigate the inhibitory mechanisms of the antagonistic APLs, we explored TCRVβ usage of $hGPI_{325-339}$-specific CD4+ T cells. The CD4+ T cells primed with $hGPI_{325-339}$ were stimulated with $hGPI_{325-339}$ *in vitro* and the TCRVβ repertoire was analyzed by flow cytometry and compared with that before stimulation. Stimulation with $hGPI_{325-339}$ expanded the population of CD4+ T cells with TCRVβ8.1 8.2 (Figure 3a). We also found that much of IL-17 was produced by CD4+ T cells with TCRVβ8.1 8.2 following stimulation with $hGPI_{325-339}$ (Figure 3b). These data indicate that many $hGPI_{325-339}$-specific Th17 cells use TCRVβ8.1 8.2.

Effect of antagonistic altered peptide ligands on differentiation of Th17 and regulatory T cells

In vitro analysis showed that the antagonistic APLs suppressed IL-17 production, and that co-immunization with the APLs inhibited the development of arthritis. We therefore explored the effect of APLs on Th17 differentiation. Our previous report suggested that cross-reactivity of CD4+ T cells primed with $hGPI_{325-339}$ to $mGPI_{325-339}$ was critical for the development of arthritis. We therefore assessed the population of $mGPI_{325-339}$ reactive Th17 cells in the DLNs of mice co-

Figure 2



Co-immunization with antagonistic altered peptide ligands suppresses the development of arthritis. Mice were co-immunized with antagonistic altered peptide ligand (APL) 6, APL 7, APL 12, APL 13 (upper panel), the agonistic APL 15 (middle panel) or OVA peptide (lower panel). Progression of arthritis was significantly suppressed in mice co-immunized with APL 6, APL 12 and APL 13 after day 12, and in mice co-immunized with APL 7 after day 16 ($P < 0.05$, Mann--Whitney U test). Data presented as mean arthritis score (± standard error of the mean) of four mice in one representative experiment of at least two independent experiments.

immunized with each APL. IL-17 production by CD4+ T cells with TCRVβ8.1 8.2 or other TCRVβ with stimulation of $mGPI_{325-339}$was not affected by co-immunization with APLs (Figure 4a), and neither was affected IL-17 production with $hGPI_{325-339}$ (data not shown). Unexpectedly, IL-17 production was considerable with stimulation of the corresponding APLs (Figure 4b). ELISA showed undetectable levels of IL-4, and the IL-10 production, and IFNγ production was not affected (data not shown). Co-immunization with APLs did neither affect the population of regulatory T cells nor the expression of CD25 (Figure 5), and stimulation of DLN cells of co-immunized mice with APLs *in vitro* did not induce the expansion of regulatory T cells (data not shown).

Identification of TCRVβ usage of altered peptide ligand-specific CD4+ T cells

The unexpected data mentioned above suggested that APL-specific CD4+ T cells were developed by co-immunization. We

Table 2

Effects of co-immunization with altered peptide ligands on the development of arthritis

Co-immunization	Incidence	Day of onset	Maximum severity
None	8/8	10 ± 0.0	10.9 ± 1.4
APL 6	8/8	10 ± 0.0	7.5 ± 2.0*
APL 7	6/8	10 ± 0.0	7.8 ± 1.7*
APL 12	7/8	10.3 ± 0.8	5.0 ± 1.2**
APL 13	3/8†	10 ± 0.0	4.0 ± 0.0**

Mice were co-immunized with 10 μg glucose-6-phosphate isomerase $hGPI_{325-339}$ and 50 μg indicated antigen. Data presented as incidence or mean ± standard deviation. †$P < 0.05$ (Fisher's exact test). *$P < 0.005$, **$P < 0.001$ (Mann-Whitney U test).

therefore investigated TCRVβ usage of APL-specific CD4+ T cells. The CD4+ T cells primed with each APL were stimulated with the corresponding APL *in vitro* and the TCRVβ repertoire was analyzed by flow cytometry and compared with that before stimulation. Stimulation with APL 6, APL 7 and APL 12 induced expansion of the population of CD4+ T cells with TCRVβ8.1 8.2; however, this expansion was not so remarkable as that of $hGPI_{325-339}$-specific CD4+ T cells (Figures 3a and 6). Interestingly, stimulation with APL 13 hardly induced the expansion of the population of CD4+ T cells with TCRVβ8.1 8.2 (Figure 6) or any other specific Vβ chain, although each APL stimulation could proliferate CD4+ T cells primed with the corresponding APL as efficiently as $hGPI_{325-339}$ (data not shown).

Effects of antagonistic altered peptide ligands on anti-mouse glucose-6-phosphate isomerase antibody production

Since administration of anti-CD4 monoclonal Abs with immunization prevents the development of arthritis and completely inhibits the production of anti-mGPI Abs in GPI-induced arthritis, mGPI is considered a thymus-dependent antigen to the humoral immune response [26]. We therefore next investigated the effects of APLs on antibody production. Co-immunization with APL 6, APL 7, APL 12 and APL 13 significantly suppressed the titers of anti-mGPI Abs ($P < 0.01$, $P < 0.005$, $P < 0.001$ and $P < 0.001$, respectively) (Figure 7). We also investigated the anti-mGPI IgG isotype. Co-immunization with APL 7, APL 12 and APL 13 significantly suppressed the titer of anti-mGPI IgG_1 isotype ($P < 0.005$, $P < 0.001$ and $P < 0.01$, respectively). Any other anti-mGPI IgG isotype was hardly detected, however, and any bias to specific isotype was not found as an effect of APL.

Discussion

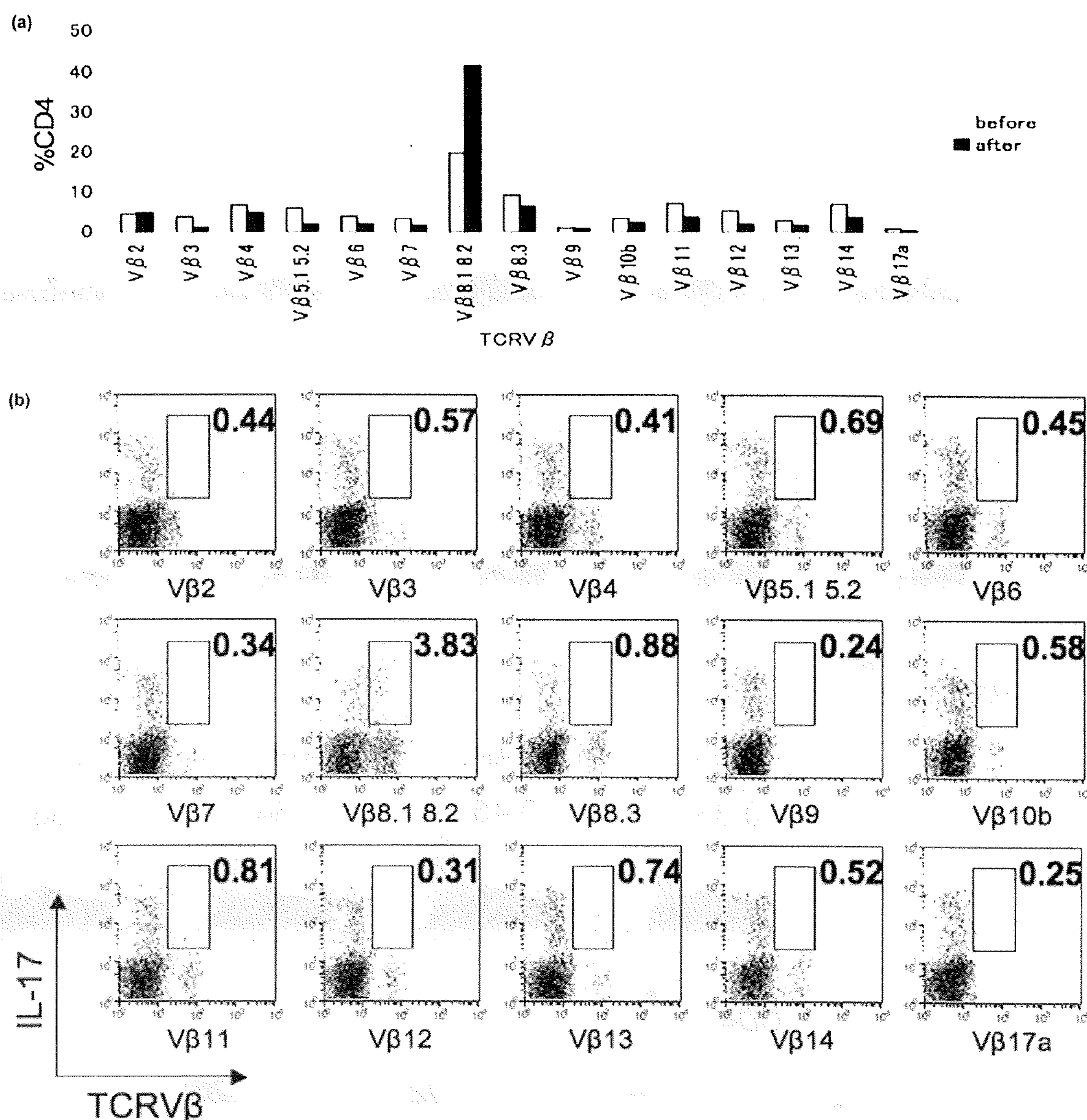
APLs are considered useful for antigen-specific therapy of autoimmune diseases and allergy. Treatments with APLs have so far been tested in several autoimmune models, and especially experimental autoimmune encephalitis has been enthusiastically examined for APLs designed as a single amino acid substitution of a TCR contact site. In experimental autoimmune encephalitis in Lewis rats, co-immunization with the APL (K91A) of myelin basic protein MBP_{87-99} strongly inhibited the development of the disease by suppression of IFNγ and TNFα, not T-cell proliferation [31]. Furthermore, another study of experimental autoimmune encephalitis in SJL mice showed that co-immunization with the APL (W144Q) of myelin proteolipid protein $PLP_{139-151}$ also inhibited the disease, and that the T-cell clone specific for the APL (W144Q) possessed the Th0 or Th2 phenotype [32].

Although one study used conventional APLs in collagen-induced arthritis [33], unconventional APLs with substitutions at MHC binding sites were mainly tested in arthritis models. Myers and colleagues reported that the analog peptide (I260A, A261B(hydroxyproline), F263N) significantly suppressed collagen-induced arthritis by inducing Th2 cells in DBA/1 mice [34]. They also reported the suppression of collagen-induced arthritis in HLA-DR1 and HLA-DR4 transgenic mice using other analog peptides with substitutions at MHC binding sites [35,36]. Another group reported also that the antigen-specific proinflammatory response to the human cartilage glycoprotein-39 (263 to 275) epitope was suppressed in DR4 transgenic mice by APLs with substitution at MHC binding sites [37].

In our study, we designed various APLs (N329S, N329T, G332A, or G332V) of $hGPI_{325-339}$ with substitutions at TCR contact sites, and showed that co-immunization with the individual APL significantly inhibited the development of arthritis. Although the APLs had antagonism to Th17 primed with hGPI cells *in vitro* (Figure 1), analysis of the mechanisms of the effect of co-immunizing APLs showed normal development of $hGPI_{-325-339}$-specific Th17 cells and APL-specific Th17 cells *in vivo* (Figure 4). Co-immunization with $hGPI_{325-339}$ and the APL might have induced both $hGPI_{-325-339}$-specific Th17 clones and APL-specific Th17 clones by the adjuvant effects of complete Freund's adjuvant and pertussis toxin.

Since both the TCR signal and the co-stimulatory signal are essential for priming of naïve T cells, our data suggested the potency of the APLs as agonists to some TCRs. It is likely that

Figure 3



Human glucose-6-phosphate isomerase-specific Th17 cells use TCRVβ8.1 8.2. Many glucose-6-phosphate isomerase $hGPI_{325-339}$-specific Th17 cells use TCRVβ8.1 8.2. Mice were immunized with 10 μg $hGPI_{325-339}$, and draining lymph node cells on day 6 were stimulated with 20 μM $hGPI_{325-339}$ *in vitro*. **(a)** Ratios of TCRVβ repertoire to CD4+ T cells. The TCRVβ repertoire of CD4+ T cells was analyzed by flow cytometry: before stimulation with $hGPI_{325-339}$ *in vitro* for 72 hours, and after stimulation. **(b)** GoldiStop was added in the last 4 hours of the 24-hour culture. Flow cytometry analysis for IL-17 was gated in $CD4^{high}$ cells. Representative data of two independent experiments.

an antigen acts as an agonist to one T-cell clone and as an antagonist to another T-cell clone because any TCR can cross-react with various antigens. Although the antigen specificity is mainly determined by the complementary determining regions, the different ratio of TCRVβ usage between $hGPI_{325-339}$-specific CD4+ T cells and APL-specific CD4+ T cells (especially APL 13) indicates that each CD4+ T cell is a different clone that leads to different antigen specificity, and does not cross-react to the APLs and $hGPI_{325-339}$ to conduct positive TCR signals, respectively. Our previous paper showed that T cells primed with $hGPI_{325-339}$ could cross-react to $mGPI_{325-339}$ and that the cross-reactivity to $mGPI_{325-339}$ was crucial for induction arthritis [27]. The findings that immunization with the APLs (APL 6, APL 7, APL 12, APL 13) alone