Mod Rheumatol (2008) 18:385–393
DOI 10.1007/s10165-008-0074-8

ORIGINAL ARTICLE

Use of laser microdissection in the analysis of renal-infiltrating T cells in MRL/*lpr* mice

Yingge Wang · Satoshi Ito · Yusuke Chino · Keiichi Iwanami · Takanori Yasukochi · Daisuke Goto · Isao Matsumoto · Taichi Hayashi · Kazuhiko Uchida · Takayuki Sumida

Received: 25 January 2008 / Accepted: 12 March 2008 / Published online: 2 May 2008

Abstract To clarify the role of T cells in the kidneys of MRL/MpJ-*lpr* (MRL/*lpr*) mice, cytokine mRNA expression was analyzed, and tissue localization of T cells was examined by immunohistochemistry. Cells infiltrating the glomeruli, glomerular circumference, and perivascular areas in ten female MRL/*lpr* mice were captured by laser microdissection (LMD). Nested reverse transcription polymerase chain reaction (RT-PCR) of samples was performed with primers specific for β-actin, T-cell receptor β chain (TCR-Cβ), Thy-1, B220, CD4, CD8, interleukin (IL)-2, IL-4, IL-10, IL-13, IL-17, and interferon (IFN)-γ. Frozen sections of lesions were also stained immunohistochemically. B220, MAC-1, Thy-1, CD4, and CD8 staining was observed in glomeruli and perivascular areas, especially in glomerular circumference areas. T cells infiltrating the glomeruli, glomerular circumference areas, and perivascular areas produce INF-γ, IL-13, and IL-17 predominately. IL-10 positivity was identified in 60% of perivascular T cells but not in a substantial number of glomerular or periglomerular T cells. The results of our study suggest that the pathogenesis of renal lesions in MRL/*lpr* mice is complex and not due simply to the Th1 and Th2 balance. These findings also support the concept of different molecular mechanisms for glomerulonephritis and vasculitis in these mice.

Keywords Glomerulonephritis · Laser microdissection (LMD) · MRL/*lpr* mice · T cells · Vasculitis

Y. Wang · S. Ito (✉) · Y. Chino · K. Iwanami · T. Yasukochi · D. Goto · I. Matsumoto · T. Hayashi · T. Sumida
Division of Clinical Immunology,
Major of Advanced Biomedical Applications,
Graduate School of Comprehensive Human Science,
University of Tsukuba, 1-1-1 Tennodai,
Tsukuba, Ibaraki 305-8575, Japan
e-mail: s-ito@md.tsukuba.ac.jp

K. Uchida
Molecular and Biological Oncology,
Graduate School of Comprehensive Human Science,
University of Tsukuba, Tsukuba, Japan

Introduction

MRL/MpJ-*lpr/lpr* (MRL/*lpr)* mice comprise an animal model for systemic lupus erythematosus (SLE). They are homozygous for the lymphoproliferative (lpr) mutation and spontaneously develop glomerulonephritis, vasculitis, and arthritis, characterized by the production of rheumatoid factor, immune complex, and anti-double-stranded DNA autoantibody [1, 2]. The lpr mutation, a nearly disabling insertion of a retrotransposon in the Fas apoptosis gene, results in defective activation-induced cell death of peripheral T cells and CD4-CD8- and B220 + Thy-1 + T cells in the lymph nodes and spleen [2, 3]. The lpr cell is able to produce various cytokines, such as IFN-γ, TNF-α, TGF-β and IL-6 [4].

Although the etiology of SLE is unknown, a number of key factors have been identified. T cells play an important role in the development of autoimmune disease in MRL/*lpr* mice [5, 6]. The respective roles of the two T-helper cell subsets Th1 and Th2, which possess differential capacities with respect to cytokine secretion, in the development and progression of SLE have not been well defined. Some investigators suggest that an imbalance toward Th1 predominance plays a significant role in the progression of lupus-like autoimmune disease in MRL/*lpr* mice [7, 8]. However, others have shown that IL-4 and IL-10

(Th2 cytokines) play prominent roles in the pathogenesis of lupus-associated tissue injury in mice [9, 10]. IL-13 is a Th2 cytokine that plays significant roles in immunoglobulin production and in the switching of antibody isotypes in relation to rheumatoid factors in SLE in humans [11, 12]. Little is known about IL-13 in MRL/*lpr* mice. IL-17 is an inflammatory cytokine that is expressed in a recently identified lineage of effector CD4 + T cells (TH-17) [13], which contribute to the pathogenesis of autoimmune and inflammatory diseases including SLE [14]. The roles of individual Th1, Th2, and Th-17 cell subsets in SLE remain unclear. Vasculitis and glomerulonephritis, both manifestations of murine lupus, are thought to be associated with an increase in circulating immune complexes and autoantibodies but under the control of different genes [14]. Anti-DNA and anti-myeloperoxidase autoantibodies appear to have a limited pathogenic role in glomerulonephritis and vasculitis in MRL/*lpr* mice [15].

To identify differences in the etiology of glomerulonephritis and vasculitis, we analyzed cytokine mRNA expression in cells infiltrating glomerular and perivascular areas. Laser microdissection (LMD) is a well-established method for isolating individual cells or subcellular structures from a heterogeneous cell population [16]. In recent years, cell-, DNA-, RNA-, and protein-based techniques have been used with LMD to gather important information regarding the genome, transcriptome, and more recently, proteome of individual microdissected cells. LMD is used to precisely harvest cells of interest from a tissue specimen in a rapid and practical manner. Together with reverse transcription polymerase chain reaction (RT-PCR) techniques, LMD can be used to study genetic alterations, gene expression, and protein expression in defined cell populations from complex normal and diseased tissues [17]. In this study, to clarify the role of T cells in the kidneys of MRL/*lpr* mice, we analyzed cytokine mRNA expression by LMD and RT-PCR and protein expression by immunohistochemistry.

Materials and methods

Mice

Female MRL/MpJ-*lpr* (MRL/*lpr*) mice, 6–10 weeks of age, were purchased from Charles River Japan (Yokohama, Japan) and bred at the animal facility of the University of Tsukuba. Tsukuba, Japan. At the age of 20–24 weeks, ten mice were killed, and glomerulonephritis and vasculitis in the kidneys that had developed spontaneously were confirmed by histologic examination (data not shown). All animal experiments were performed with the approval of the Animal Research Committee of the University of Tsukuba.

Histopathologic and immunohistologic examinations

Kidney samples were fixed with 10% formalin in 0.01 M phosphate buffer, ph 7.2, and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin (H&E) for histologic examination by light microscopy. For immunohistochemical staining, kidneys samples were frozen in OCT compound (Sakura Finetek USA, Torrance, CA, USA) and stored at −80 °C until use. Sections (5 μm thick) were cut on a Cryostat (Leica Microsystems, Wetzlar, Germany). Immunohistochemical staining was performed with the avidin–biotin complex technique. Primary antibodies (Becton Dickinson, Mountain View, CA, USA) were as follows: rat anti-mouse L3T4 for CD4 T cells; rat anti-mouse Ly-2 for CD8 T cells; rat anti-mouse MAC-1 (CD11b) for activated macrophages; and rat anti-mouse B220 (CD45R) and anti-mouse Thy-1 for T cells. Immunoreactivity was visualized with diaminobenzidine (DakoCytomation, Carpinteria, CA, USA) for 10 min, and sections were counterstained with hematoxylin. In negative controls, primary antibody was substituted with antibody diluent.

Tissue sampling by laser microdissection

Kidney specimens were frozen in OCT compound, and 10-μm-thick cryostat sections were prepared and mounted onto polyethylene membrane. Sections were fixed immediately in 70% ethanol for 3 min and washed for 1 min in diethylpyrocarbonate (DEPC)-treated water and then stained rapidly in 0.05% toluidine blue solution (pH 7.0) (Wako Pure Chemical Industries, Osaka, Japan) for 30 s. After two 1-min washes in DEPC-treated water, samples were dehydrated in a dryer for 10 min. Dried samples were set on the computer-controlled microscope stage of the laser-microdissection system (AS-LMD; Leica Microsystems Japan, Tokyo, Japan) and observed under a charge-coupled device camera from above. With a computer mouse, glomeruli and perivascular areas of infiltrating cells of similar size were selected and dissected with a laser microbeam. We dissected 5–10 glomeruli and 5–10 perivascular samples, and the samples were pooled for RNA extraction in thin-walled PCR tubes (0.5 ml). Ten mice (80 pools of both β-actin- and TCR-Cβ-positive samples by RT-PCR) were used for analysis. Since periglomerular cell infiltration is observed in the kidney of MRL/lpr mice, four mice were also examined in glomerular circumference areas (16 pools of both β-actin- and TCR-Cβ-positive samples by RT-PCR).

RNA extraction and nested reverse transcription polymerase chain reaction

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 with a ThermoScript RT-PCR System (Invitrogen Life Technologies, Carlsbad, CA, USA), and 1 μl of each first-strand reaction was amplified with primers specific for β-actin, T-cell receptor β chain (TCR-Cβ), Thy-1, B220, CD4, CD8, IL-2, IL-4, IL-10, IL-13, IL-17, and IFN-γ for RT-PCR (Table 1). Cycle conditions were as follows: 1 min at 94 °C for denaturation, 30 s at 58–63 °C for annealing, 1 min at 72 °C for elongation, and then 7 min at 72 °C after the last cycle. Samples were amplified twice for 20–30 cycles with a Thermal Cycler Dice (TaKaRa, Kyoto, Japan). PCR products were visualized by electrophoresis on 2% agarose gels and ethidium bromide staining. Gels were analyzed with an electronic ultraviolet transilluminator (Ultra-Lum, Claremont, CA, USA).

Statistical analysis

Statistical significance was determined with the χ^2 test. Statistical significance was set at $P < 0.05$.

Results

Detection of T cells in glomerulonephritis and vasculitis

To verify the tissue localization of T cells associated with glomerulonephritis and vasculitis, protein expression was examined immunohistochemically. B220, MAC-1, Thy-1, CD4, and CD8 staining was observed in both glomeruli and perivascular areas, especially in glomerular circumference areas (Fig. 1). These results indicated that the major infiltration of T cells and macrophages was in the glomerular circumference areas. Areas positive for Thy-1, B220, and MAC-1 were larger than those positive for CD4 and CD8 in both glomeruli and perivascular areas, corresponding to the RT-PCR results (described below).

Analysis of gene expression by laser microdissection and nested reverse transcription polymerase chain reaction

Ten female MRL/*lpr* mice with developing glomerulonephritis and vasculitis were killed at 20–24 weeks of age. Glomeruli and perivascular areas of a similar size were captured by LMD. Four mice were also used to examine areas of glomerular circumference infiltrating cells (Fig. 2). We then analyzed gene expression by nested RT-PCR. Thy-1 and B220 mRNA expression was observed in glomerular and perivascular areas (Fig. 3). Thy-1 mRNA was observed in 93.8% of glomerular circumference areas, and B220 mRNA was observed in 31.3%. Thy-1 positivity in glomeruli, glomerular circumference areas, and perivascular areas did not differ significantly, but B220 positivity in glomerular circumference areas did differ from that in glomeruli and perivascular areas ($P < 0.0001$). CD4 positivity was 22.5% in glomeruli and 12.5% in glomerular circumference areas, and CD8 positivity was 35% in glomeruli and 25% in glomerular circumference areas. In perivascular areas, CD4 positivity was increased to 65%, and CD8 positivity was increased to 42.5%. CD4 positivity in perivascular areas differed significantly from that in glomeruli and glomerular circumference areas ($P < 0.0001$) (Fig. 3). Thy-1, CD4, and CD8 positivity were similar between glomeruli and glomerular circumference areas. Immunohistochemical results also supported a major infiltration of T cells in the glomerular circumference areas (Fig. 1). INF-γ and IL-13 sample positivity were 75 and 87.5% in glomeruli T cells, 68.8 and 81.3% in glomerular circumference areas T cells, with similarly high positivity (62.5 and 70%, respectively) in perivascular T-cell samples (Fig. 4a, b). IL-10 was positive in 60% of perivascular T-cell samples, a percentage that was significantly greater than in glomeruli and glomerular circumference areas ($P < 0.0001$). IL-17 mRNA (Th-17 type) was also detected in glomerular and perivascular lesions. Positivity was 35, 62.5, and 30% in glomeruli, glomeruli circumference areas, and perivascular areas, respectively (Fig. 4a, b). Detection statuses of cytokines in each pooled sample (both β-actin- and TCR-Cβ-positive samples) are shown in Table 2.

Discussion

To clarify the role of T cells in the kidneys of MRL/*lpr* mice, we analyzed cytokine mRNA expression by LMD and RT-PCR and the localization of specific proteins by immunohistochemistry. Our data indicated that T cells infiltrating glomeruli, glomerular circumference areas, and perivascular areas produce INF-γ, IL-13, and IL-17. IL-10 was detected only in perivascular lesions. Large numbers of CD4-CD8- and B220 + Thy-1 + T cell have been reported in the lymph nodes and spleen of MRL/*lpr* mice [2]. Our results also showed an abundance of CD4-CD8- and B220 + Thy-1 + T cells in MRL/*lpr* mouse kidney. Areas of Thy-1 and B220 staining were larger than areas of CD4 and CD8 staining in glomerular and perivascular areas, corresponding to the RT-PCR results. Our results probably included T cells and B cells; we did not analyze single cells with the LMD method.

The high levels of INF-γ expression in glomeruli, glomerular circumference areas, and perivascular areas indicated that INF-γ plays critical roles in the development

Table 1 Oligonucleotide primer sequences

PCR products	Oligonucleotide sequence		Product size (bp)	RT-PCR cycles
Mouse β-actin				
First PCR	5′ sense	TGTTACCAACTGGGACGACA	415	25
	3′ antisense	TTTGATGTCACGCACGATTT		
Nested PCR	5′ sense	GATCTGGCACCACACCTTCT	366	25
	3′ antisense	CTCTCAGCTGTGGTGGTGAA		
Mouse TCR-Cβ				
First PCR	5′ sense	CCCAAGGTCTCCTTGTTTGA	339	25
	3′ antisense	GGCCTCTGCACTGATGTTCT		
Nested PCR	5′ sense	AAGGCTACCCTCGTGTGCT	193	25
	3′ antisense	AGTGGTTTCGAGGATTGTGC		
Mouse Thy-1				
First PCR	5′ sense	CCAATGAGGATGAGGGCTTA	424	25
	3′ antisense	GAAGAGGCAGGTTGCAAGAC		
Nested PCR	5′ sense	CATTCTCAGCCACCACACAC	302	25
	3′ antisense	GGCTCTCCCTTGGTAAGCTG		
Mouse B220				
First PCR	5′ sense	GGGTTGTTCTGTGCCTTGTT	425	25
	3′ antisense	TGTGAGAGTCTGCGTTGTCC		
Nested PCR	5′ sense	TCTCTTGGAAAGTGCAGAAA	323	25
	3′ antisense	CATGTTCTGGTTCCTCAGCA		
Mouse CD4				
First PCR	5′ sense	TGGAGTTGTGGGTGTTCAAA	464	25
	3′ antisense	CTGGAGCTTGAGGTCTTTGG		
Nested PCR	5′ sense	GACCCTGACCTTGGATAGCA	314	30
	3′ antisense	CCTTCTCTGCCTTCCACATC		
Mouse CD8				
First PCR	5′ sense	TCAGTTCTGTCGTGCCAGTC	423	25
	3′ antisense	TACAAGGGGCTCTGATGTCC		
Nested PCR	5′ sense	GACATCTCAGCCCCAGAGAC	310	25
	3′ antisense	CACCGCTAAAGGCAGTTCTC		
Mouse INF-γ				
First PCR	5′ sense	GCGTCATTGAATCACACCTG	468	25
	3′ antisense	CGCATTCACAGTCTTGGCTA		
Nested PCR	5′ sense	TTTGAGGTCAACAACCCACA	343	30
	3′ antisense	TGGTCAAAGAGAAATAGTTG		
Mouse IL-2				
First PCR	5′ sense	CCCACTTCAAGCTCCACTTC	441	25
	3′ antisense	AGGGCTTGTTGAGATGATGC		
Nested PCR	5′ sense	AAGCTCTACAGCGGAAGCAC	369	30
	3′ antisense	TCCACCACAGTTGCTGACTC		
Mouse IL-4				
First PCR	5′ sense	TCAACCCCCAGCTAGTTGTC	406	25
	3′ antisense	TCCATTTGCATGATGCTCTT		
Nested PCR	5′ sense	TGTACCAGGAGCCATATCCA	310	25
	3′ antisense	TGGACTCATTCATGGTGCAG		
Mouse IL-10				
First PCR	5′ sense	CTTGCTCTACCAAAGCCACA	451	30
	3′ antisense	TTTTCACAGGGGAGAAATCG		

Table 1 continued

PCR products	Oligonucleotide sequence		Product size (bp)	RT-PCR cycles
Nested PCR	5′ sense	AGCAGCCTTGCAGAAAAGAG	372	30
	3′ antisense	TCTCACCCAGGGAATTCAAA		
Mouse IL-13				
First PCR	5′ sense	CTGTGAGCCTTGTCCTCCTC	233	30
	3′ antisense	TTGGTGAGCCAGTGAGACG		
Nested PCR	5′ sense	AAAGTGCCCTGAGCCTTGTC	198	25
	3′ antisense	CTTCCCAGTCCTGATGACTG		
Mouse IL-17				
First PCR	5′ sense	CATGCAGGAGGTGGTACCTT	212	25
	3′ antisense	AGCTTCTTCTCGCTCAGACG		
Nested PCR	5′ sense	CCTGACCAAACTCAGCAATC	166	25
	3′ antisense	ACATAAACAGCAGGTCCAGT		

RT-PCR reverse transcription polymerase chain reaction, *TCR-Cβ* T-cell receptor β chain, *IL* interleukin, *IFN-γ* interferon-gamma

of glomerulonephritis and vasculitis in this model of SLE. INF-γ is a Th1 cytokine that has been shown to induce Th1-dependent autoantibody isotypes [18, 19], produce inflammatory cytokines and nitric oxide [8], induce macrophage activation, and induce apoptosis of tubular epithelial cells [20] in MRL/*lpr* mice. Many studies have shown that IFN-γ gene deficiency in these mice dramatically reduces glomerulonephritis, evidently through the reduced production of IgG2a (an Ig isotype associated with the Th1 phenotype) anti-dsDNA antibody [10, 19, 20]. In contrast, transgenic expression of IFN-γ in the epidermis of mice without autoimmune issues leads to the development of inflammatory skin disease resembling lupus erythematosus, with 30% of female mice also developing severe immune complex-mediated glomerulonephritis [21, 22].

Our present study showed high expression of IL-13 in glomerular and perivascular lesions and of IL-10 only in perivascular lesions. We speculate that Th2 cells play an active role in the development of glomerulonephritis and vasculitis. IL-13 is a Th2 cytokine that plays a significant role in immunoglobulin production and in the switching of antibody isotypes in relation to rheumatoid factors in patients with SLE [11, 12]. An increased IL-13 level indicates that this cytokine is involved in the pathogenesis of SLE [12, 23]. Shimizu et al. [24] reported the presence of IL-13 in CD4 + T cells from WSX-1−/− MRL/*lpr* mice (MRL/*lpr* mice deficient for the IL-27 receptor and that develop a disease resembling human membranous glomerulonephritis) and WSX-1+/− mice. However, there are few reports of MRL/lpr mice. Our present study showed that IL-13 is highly expressed in the kidneys of MRL/*lpr* mice. Thus, IL-13 may be an important factor in the pathogenesis of glomerulonephritis and vasculitis.

IL-10 is a regulatory cytokine that inhibits Th1 cytokine production and the proliferation of CD4 + T cells via indirect effects on antigen-presenting cells and direct effects on T cells [25]. The precise role of IL-10 in the pathogenesis of lupus remains uncertain; however, studies have suggested that this cytokine is pathogenic in both humans and mice with SLE [26]. IL-10 is presumed to be an important modulator of disease activity in humans with SLE. Patients with lupus produce large amounts of IL-10, with enhanced gene expression in peripheral blood mononuclear cells [27], and the serum level of IL-10 correlates with disease activity [28]. IL-10 gene-deficient mice develop severe lupus, with an earlier appearance of skin lesions, increased lymphadenopathy, more severe glomerulonephritis, and higher mortality than in control littermates [29]. In addition, IL-10 down-regulates murine lupus by inhibiting pathogenic Th1 cytokine responses [29]. In the present study, IL-10 was observed only in perivascular lesions. Therefore, we speculate that the molecular mechanism for glomerulonephritis differs from that of vasculitis in this SLE mouse model. IL-10 is also produced by other CD4 + regulatory T cells (Tregs), such as Type 1 Tregs and CD + CD25 + Tregs [30]. Recently, the role of regulatory T cells in the pathogenesis of human autoimmune diseases, including rheumatoid arthritis and SLE, was reported [31, 32]. In MRL/*lpr* mouse, it is possible that IL-10 produced by Type 1 Tregs and CD + CD25 + Tregs plays an important role in the pathogenesis of perivascular lesions. Another possibility is that endothelial cells in perivascular lesions produce IL-10, as Andreas et al. [33] reported in rats. Our present results support the hypothesis that vasculitis and glomerulonephritis are under the control of different genes and involve different pathologic processes [15].

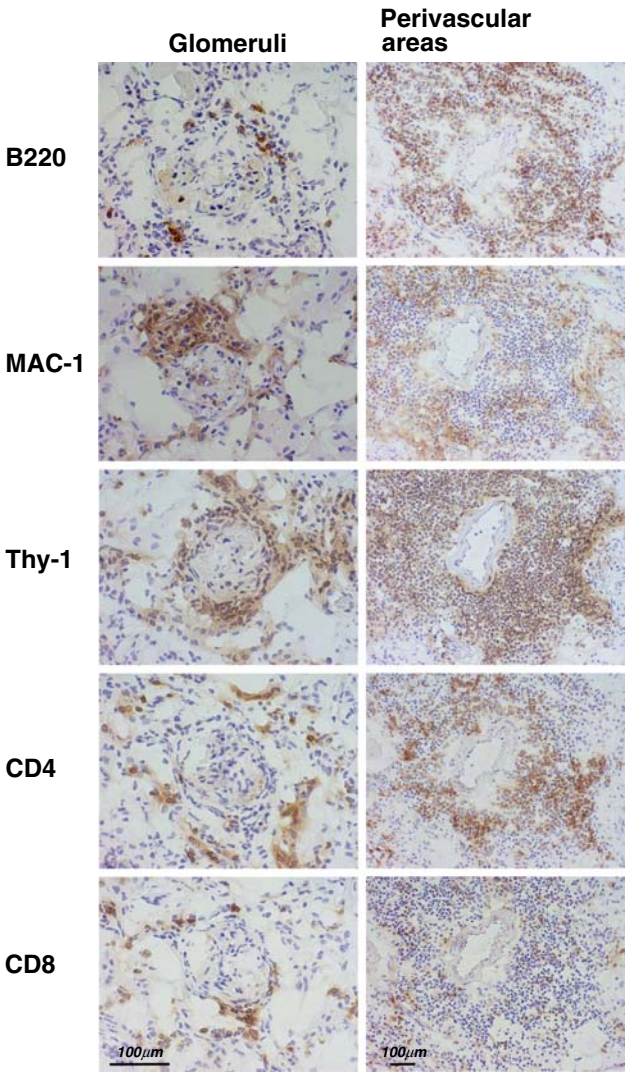

Fig. 1 Detection of T cells in glomerulonephritis and vasculitis. Serial frozen sections of glomeruli and perivascular lesions were immunostained with mouse antibodies against B220, MAC-1 (corresponding to activated macrophages), Thy-1, CD4, and CD8 (100×)

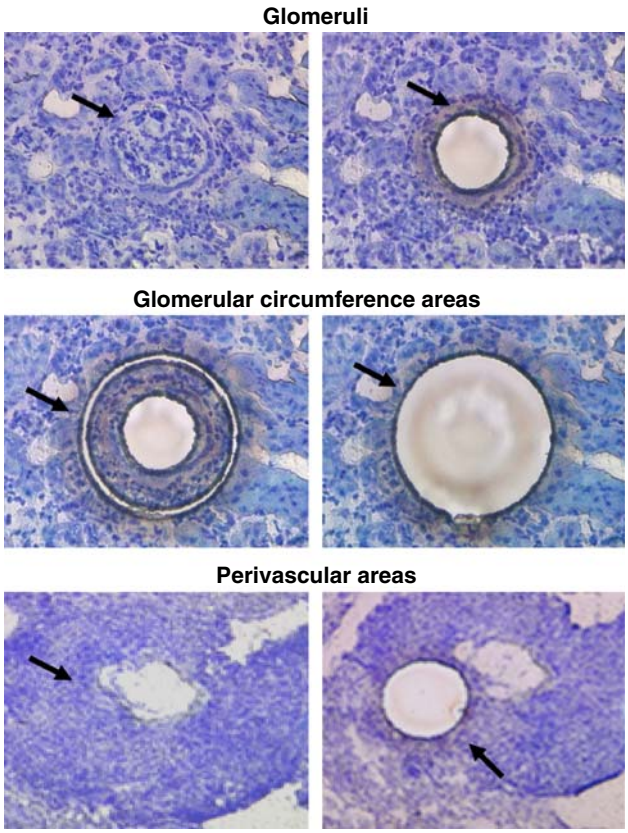

Fig. 2 Targeted infiltrating T cells selected and cut by laser microdissection (LMD). Glomeruli and areas of perivascular infiltrating cells of a similar size (*black arrows*) were selected (*left panels*) and were captured (*right panels*) by LMD. In four mice, cells infiltrating glomerular circumference areas were also examined

IL-17 (also called IL-17A) is a cytokine produced by TH17 cells, a recently described lineage of effector CD4 + T cells that contribute to the pathogenesis of autoimmune and inflammatory diseases such as rheumatoid arthritis, asthma, lupus, and allograft rejection [34–36]. IL-17 has been associated with the etiology of human SLE [37]. Wong et al. [37] reported increased IL-17 levels in patients with SLE. Dong et al. [38] reported that IL-17 induces autoantibody (anti-dsDNA IgG) overproduction and peripheral blood mononuclear cell overexpression of IL-6 in patients with lupus nephritis. There have been few studies on the role of IL-17 in mouse models of lupus. Cai et al. [39] reported the presence of IL-17 in cultures of stimulated splenocytes from MRL+/+ mice. In the present study, we observed IL-17 mRNA expression in both glomerular and perivascular lesions (35 and 30% positivity, respectively), suggesting that IL-17 is a key factor in the development of glomerulus nephritis and vasculitis in MRL/lpr mice. We used LMD in combination with RT-PCR for the analysis of cytokine expression. This method may be useful when examining the role of cytokine balance in murine lupus nephritis.

We could not demonstrate that IFN-γ, IL-2, IL-10, IL-13 and IL-17 are produced by CD4–CD8-and B220 + Thy-1 + T cell in the kidneys of MRL/*lpr* mice with the LMD method. Murray et al. [4] clarified that in lymph nodes, fluorescence-activated cell sorter (FACS)-purified B220 + Thy-1 + T cells (lpr cells) and B220-Thy-1 + T cells produce IFN-γ, TNF-α, TGF-β and IL-6. However, mRNA for IL-1, IL-4, or IL-5 was not detected in either T cell population. They did not analyze IL-2, IL-10, IL-13, and IL-17.

Nested RT-PCR is not a quantitative analysis when performed as described here, but is usually used as a method of judging whether target mRNA expression is positive or not, such as in the study by Chen et al. [40] of immunoglobulin G via LMD. There are some

reports which use quantitative real-time PCR (qRT-PCR). Maeda et al. [41] prepared 12–16 μm-thick sections and Khodosevich et al. [42] dissected 3,000–5,000 cells by LMD for a qRT-PCR experiment. However, in the present study, we were afraid that the amount of RNA necessary for qRT-PCR would not be obtained.

In conclusion, T cells infiltrating the glomeruli, glomerular circumference areas, and perivascular areas produce INF-γ, IL-13, and IL-17 predominately. IL-10 was detected only in perivascular lesions. These results suggest that the pathogenesis of nephritis in MRL/*lpr* mice is complex and not due simply to the Th1 and Th2 balance. These findings also support the concept that the molecular mechanism underlying glomerulonephritis differs from that

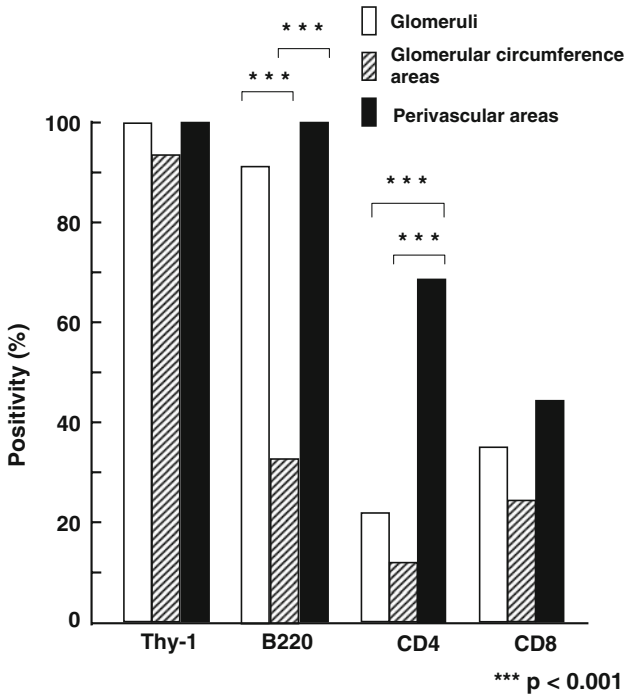

Fig. 3 Thy-1, B220, CD4 and CD8 mRNA expression in lesions. Expressions of mouse Thy-1, B220, CD4, and CD8 mRNAs in the lesions of glomeruli (*white bars*), glomerular circumference areas (*hatched bars*), and perivascular areas (*black bars*) were analyzed by nested reverse transcription polymerase chain reaction (RT-PCR). The number of positive samples is shown as a percentage. Differences between two groups were analyzed with the χ^2 test ($n = 16$ for glomerular circumference samples and 40 for glomeruli and perivascular samples in each group)

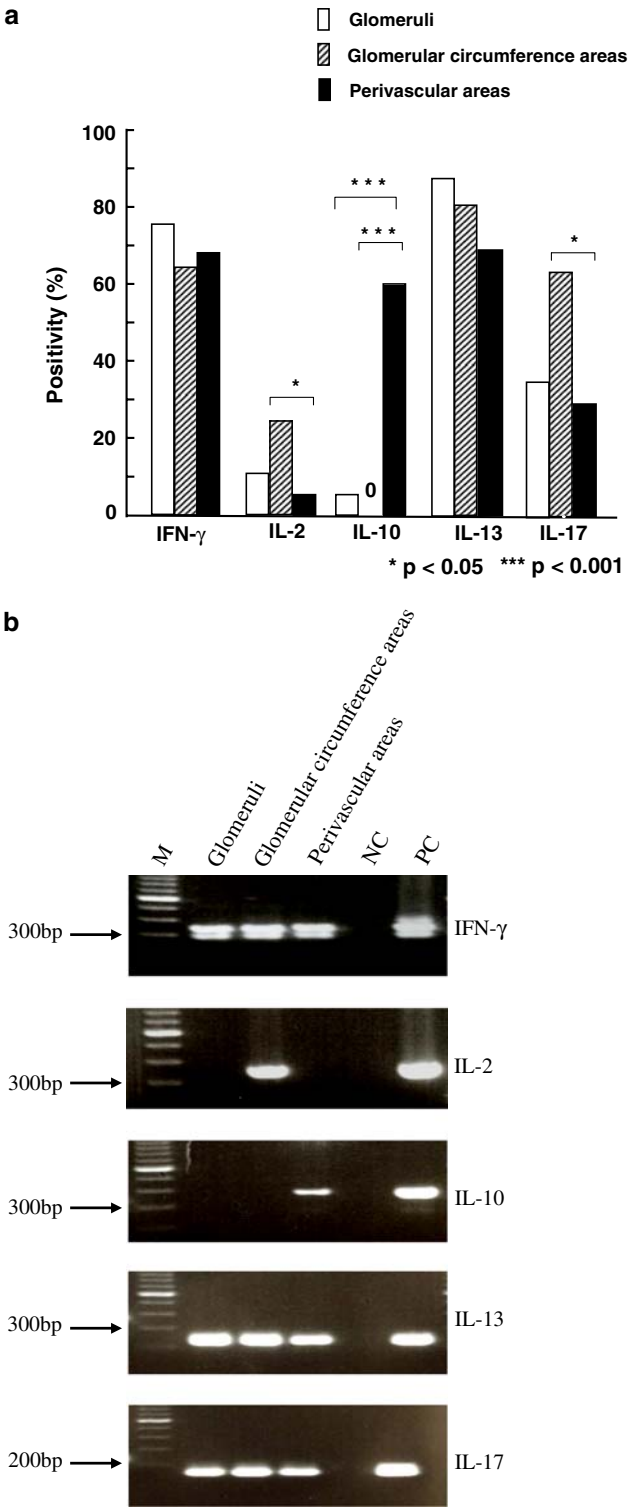

Fig. 4 Analysis of cytokine gene expression in lesions. **a** Expressions of interferon-gamma (IFN-γ), interleukin (IL)-2, IL-10, IL-13, and IL-17 mRNAs in the lesions of glomeruli (*white bars*), glomerular circumference areas (*hatched bars*), and perivascular areas (*black bars*) were analyzed by nested reverse transcription polymerase chain reaction (RT-PCR). The number of positive samples is shown as a percentage. Differences between two groups were tested with the χ^2 test ($n = 16$ for glomerular circumference samples and 40 for glomeruli and perivascular samples in each group). **b** Detection of cytokines in lesions of MRL/*lpr* mice by PCR. Specific expressions of interferon-gamma (IFN-γ), interleukin (IL)-2, IL-10, IL-13, and IL-17 were demonstrated in the lesions of glomeruli, glomerular circumference areas and perivascular areas in MRL/*lpr* mice by RT-PCR. *M* molecular size marker, *NC* negative control, *PC* positive control cDNA clone

Table 2 Expression statuses of cytokine mRNAs in pooled samples of glomeruli, glomerular circumference areas and perivascular areas

Pools	IFN-γ	IL-2	IL-10	IL-13	IL-17	Pools	IFN-γ	IL-2	IL-10	IL-13	IL-17
G1	+	+	−	+	−	P1	−	−	+	+	−
G2	+	−	−	−	+	P2	−	−	+	+	+
G3	+	−	−	+	−	P3	+	−	+	+	+
G4	+	−	−	+	−	P4	+	−	+	+	−
G5	+	+	−	+	+	P5	−	−	+	−	−
G6	+	−	−	+	−	P6	−	−	+	−	+
G7	+	−	−	+	−	P7	+	−	+	+	−
G8	+	−	−	+	+	P8	+	−	+	+	−
G9	+	−	−	+	−	P9	+	+	−	−	−
G10	−	−	−	+	−	P10	+	−	−	+	−
G11	+	−	−	+	+	P11	+	−	+	−	−
G12	+	+	−	+	+	P12	+	−	+	+	−
G13	+	−	−	+	−	P13	+	−	+	−	−
G14	+	−	−	+	−	P14	+	−	−	−	−
G15	−	+	−	+	−	P15	+	−	−	−	+
G16	−	−	−	+	−	P16	−	−	−	+	+
G17	−	−	−	+	−	P17	+	−	+	−	−
G18	−	−	−	+	−	P18	−	−	+	−	−
G19	−	−	−	−	+	P19	−	−	−	+	+
G20	−	−	−	−	−	P20	+	−	−	+	−
G21	−	−	+	+	−	P21	+	−	−	+	−
G22	+	−	+	+	−	P22	−	−	−	−	+
G23	+	−	−	+	+	P23	−	−	+	+	+
G24	+	−	+	+	−	P24	+	−	−	+	−
G25	+	−	−	+	−	P25	+	−	−	+	−
G26	+	−	−	+	−	P26	+	−	−	+	+
G27	+	−	−	−	−	P27	−	−	−	+	−
G28	−	−	−	+	−	P28	+	−	+	+	−
G29	+	−	−	−	−	P29	−	−	+	+	+
G30	+	−	−	+	−	P30	+	−	+	+	−
G31	+	−	−	+	−	P31	−	−	+	+	−
G32	+	−	−	+	+	P32	+	−	+	+	−
G33	+	−	−	+	−	P33	−	+	+	+	−
G34	+	−	−	+	+	P34	+	−	+	+	−
G35	+	+	−	+	+	P35	+	−	−	−	−
G36	+	−	−	+	−	P36	−	−	+	+	−
G37	+	−	−	+	+	P37	+	−	−	+	+
G38	+	−	−	+	+	P38	+	−	+	−	−
G39	−	−	−	+	+	P39	+	−	+	+	+
G40	+	−	−	+	+	P40	−	−	+	+	−
GC1	−	+	−	−	−						
GC2	+	+	−	+	+						
GC3	+	−	−	+	+						
GC4	−	−	−	+	−						
GC5	−	+	−	+	+						
GC6	−	−	−	+	−						
GC7	+	+	−	+	+						
GC8	+	−	−	+	+						
GC9	+	−	−	+	+						
GC10	+	−	−	+	−						
GC11	−	−	−	−	−						
GC12	+	−	−	+	+						
GC13	+	−	−	+	+						
GC14	+	−	−	−	−						
GC15	+	−	−	+	+						
GC16	−	−	−	+	+						

IL interleukin, *IFN-γ* interferon-gamma, *G* glomeruli, *GC* glomerular circumference areas, *P* perivascular areas

underlying vasculitis in MRL/*lpr* mice. LMD combined with RT-PCR may be useful when examining the pathogenesis of murine lupus nephritis.

Acknowledgments 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 declare that there is no conflict of interest in this article.

References

1. Andrews BS, Eisenberg RA, Theofilopoulos AN, Izui S, Wilson CB, McConahey PJ, et al. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. J Exp Med. 1978;148:1198–215.
2. Furukawa F, Yoshimasu T. Animal models of spontaneous and drug-induced cutaneous lupus erythematosus. Autoimmun Rev. 2005;4:345–50.
3. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature. 1992;356:314–7.
4. Murray L, Martens C. Abnormal T cells from lpr mice down-regulate transcription of interferon-gamma and tumor necrosis factor-alpha in vitro. Cell Immunol. 1990;126:367–76.
5. Koh DR, Ho A, Rahemtulla A, Fung-Leung WP, Griesser H, Mak TW. Murine lupus in MRL/lpr mice lacking CD4 or CD8 T cells. Eur J Immunol. 1995;25:2558–62.
6. Peng SL, Madaio MP, Hughes DP, Crispe IN, Owen MJ, Wen L, et al. Murine lupus in the absence of alpha beta T cells. J Immunol. 1996;156:4041–9.
7. Takahashi S, Fossati L, Iwamoto M, Merino R, Motta R, Kobayakawa T, et al. Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. J Clin Invest. 1996;97:1597–604.
8. Huang FP, Feng GJ, Lindop G, Stott DI, Liew FY. The role of interleukin 12 and nitric oxide in the development of spontaneous autoimmune disease in MRL/MP-lpr/lpr mice. J Exp Med. 1996;183:1447–59.
9. Schorlemmer HU, Dickneite G, Kanzy EJ, Enssle KH. Modulation of the immunoglobulin dysregulation in GvH- and SLE-like diseases by the murine IL-4 receptor (IL-4-R). Inflamm Res. 1995;44:s194–6.

10. Peng SL, Moslehi J, Craft J. Roles of interferon-gamma and interleukin-4 in murine lupus. J Clin Invest. 1997;99:1936–46.
11. Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc Natl Acad Sci USA. 1993;90:3730–4.
12. Spadaro A, Rinaldi T, Riccieri V, Taccari E, Valesini G. Interleukin-13 in autoimmune rheumatic diseases: relationship with autoantibody profile. Clin Exp Rheumatol. 2002;20:213–6.
13. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005;6:1133–41.
14. Bi Y, Liu G, Yang R. Th17 cell induction and immune regulatory effects. J Cell Physiol. 2007;211:273–8.
15. Nose M, Nishimura M, Ito MR, Toh J, Shibata T, Sugisaki T. Arteritis in a novel congenic strain of mice derived from MRL/Lpr lupus mice: genetic dissociation from glomerulonephritis and limited autoantibody production. Am J Pathol. 1996;149:1763–9.
16. Pinzani P, Orlando C, Pazzagli M. Laser-assisted microdissection for real-time PCR sample preparation. Mol Aspects Med. 2006;27:140–59.
17. Ladanyi A, Sipos F, Szoke D, Galamb O, Molnar B, Tulassay Z. Laser microdissection in translational and clinical research. Cytometry A. 2006;69:947–60.
18. Schwarting A, Wada T, Kinoshita K, Tesch G, Kelley VR. IFN-gamma receptor signaling is essential for the initiation, acceleration, and destruction of autoimmune kidney disease in MRL-Fas(lpr) mice. J Immunol. 1998;161:494–503.
19. Haas C, Ryffel B, Le Hir M. IFN-gamma is essential for the development of autoimmune glomerulonephritis in MRL/lpr mice. J Immunol. 1997;158:5484–91.
20. Balomenos D, Rumold R, Theofilopoulos AN. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. J Clin Invest. 1998;101:364–71.
21. Seery JP, Carroll JM, Cattell V, Watt FM. Antinuclear autoantibodies and lupus nephritis in transgenic mice expressing interferon-gamma in the epidermis. J Exp Med. 1997;186:1451–9.
22. Seery JP. IFN-gamma transgenic mice: clues to the pathogenesis of systemic lupus erythematosus? Arthritis Res. 2000;2:437–40.
23. Morimoto S, Tokano Y, Kaneko H, Nozawa K, Amano H, Hashimota 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.
24. Shimizu S, Sugiyama N, Masutani K, Sadanaga A, Miyazaki Y, Inoue Y, et al. Membranous glomerulonephritis development with Th2-type immune deviations in MRL/lpr mice deficient for IL-27 receptor (WSX-1). J Immunol. 2005;175:7185–92.
25. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683–765.
26. Beebe AM, Cua DJ, de Waal Malefyt R. The role of interleukin-10 in autoimmune disease: systemic lupus erythematosus (SLE) and multiple sclerosis (MS). Cytokine Growth Factor Rev. 2002;13:403–12.
27. Csiszar A, Nagy G, Gergely P, Pozsonyi T, Pocsik E. Increased interferon-gamma (IFN-γ), IL-10 and decreased IL-4 mRNA expression in peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE). Clin Exp Immunol. 2000;122:464–70.
28. Houssiau FA, Lefebvre C, Vanden Berghe M, Lambert M, Devogelaer JP, Renauld JC. Serum interleukin 10 titers in systemic lupus erythematosus reflect disease activity. Lupus. 1995;4:393–5.
29. Yin Z, Bahtiyar G, Zhang N, Liu L, Zhu P, Robert ME, et al. IL-10 regulates murine lupus. J Immunol. 2002;169:2148–55.
30. Beissert S, Schwarz A, Schwarz T. Regulatory T cells. J Invest Dermatol. 2006;126:15–24.
31. van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeder FP, Taams LS. CD4 + CD25 + regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum. 2004;50:2775–85.
32. Crispin JC, Martınez A, Alcocer-Varela J. Quantification of regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun. 2003;21:273–6.
33. Hocke AC, Ermert M, Althoff A, Brell B, N'Guessan PD, Suttorp N, et al. Regulation of interleukin IL-4, IL-13, IL-10, and their downstream components in lipopolysaccharide-exposed rat lungs. Comparison of the constitutive expression between rats and humans. Cytokine. 2006;33:199–211.
34. Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. J Leukoc Biol. 2002;71:1–8.
35. Moseley TA, Haudenschild DR, Rose L, Reddi AH. Interleukin-17 family and IL-17 receptors. Cytokine Growth Factor Rev. 2003;14:155–74.
36. Kolls JK, Linden A. Interleukin-17 family members and inflammation. Immunity. 2004;21:467–76.
37. Wong CK, Ho CY, Li EK, Lam CW. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus. 2000;9:589–93.
38. Dong G, Ye R, Shi W, Liu S, Wang T, Yang X, 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.
39. Cai P, König R, Khan MF, Qiu S, Kaphalia BS, Ansari GA. Autoimmune response in MRL+/+ mice following treatment with dichloroacetyl chloride or dichloroacetic anhydride. Toxicol Appl Pharmacol. 2006;216:248–55.
40. Chen Z, Gu J. Immunoglobulin G expression in carcinomas and cancer cell lines. FASEB J. 2007;21:2931–8.
41. Maeda K, Lee DS, Yanagimoto Ueta Y, Suzuki H. Expression of uterine sensitization-associated gene-1 (USAG-1) in the mouse uterus during the peri-implantation period. J Reprod Dev. 2007;53:931–6.
42. Khodosevich K, Inta D, Seeburg PH, Monyer H. Gene expression analysis of in vivo fluorescent cells. PLoS ONE. 2007;2:e1151.