Use of laser microdissection in the analysis of renal-infiltrating T cells in murine lupus

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Abstract
Objective: To clarify the role of T cells in kidney pathology of three widely used murine lupus models.
Material and methods: Cells infiltrating the glomeruli and perivascular areas in MRL/lpr (n = 10 female), NZB × NZW F1 (B/W F1) (n = 9 female), and BXSB (n = 10 male) mice were captured by laser microdissection (LMD). Samples were subjected to nested reverse transcription polymerase chain reaction (RT-PCR) with primers specific to β-actin, T-cell receptor β chain (TCR-β), interleukin (IL)-10, IL-13, IL-17, and interferon-γ (IFN-γ). Frozen sections of lesions were also stained immunohistochemically for tissue and cellular characterization.
Results: T cells infiltrating the glomeruli and perivascular areas predominantly produced IFN-γ, IL-13, and IL-17 in MRL/lpr, B/W F1, and BXSB mice, with IL-17 expression in glomeruli of BXSB mice being significantly lower than that of MRL/lpr and B/W F1 mice. IL-10 was detected only in the perivascular areas of MRL/lpr and B/W F1 mice and not in glomeruli isolates. Immunohistochemical staining revealed positive for the expression of Thy-1, CD4, CD8, and B220 in glomeruli and perivascular areas from all three strains of mice.
Conclusions: Cytokine balance in murine SLE is complex and cannot be attributed simply to the balance between Th1 and Th2 cells. Th17 cells may play a critical role in disease pathology, possibly with greater contribution toward disease progression in MRL/lpr and B/W F1 mice than in BXSB mice. Furthermore, these findings lend support to the concept that different molecular mechanisms underlie glomerulonephritis as compared to vasculitis.

Key words: murine lupus, glomerulonephritis, vasculitis, laser microdissection, IL-17.

Introduction
Systemic lupus erythematosus (SLE) is an autoimmune disease for which the mechanism of pathogenesis remains unclear. Murine lupus models such as MRL/MpJ-lpr/lpr (MRL/lpr), NZB × NZW F1 (B/W F1), and male BXSB mice have provided much insight into the pathogenesis of lupus. As each of these models was generated to mimic SLE by a unique genetic modification, similarities and divergence amongst these models could provide crucial clues to the fundamental mechanisms of disease. MRL/lpr mice are homozygous for the lymphoproliferative (lpr) mutation and spontaneously develop glomerulonephritis, vasculitis, and arthritis, with characteristic production of rheumatoid factor, immune-complexes, and anti-double-stranded DNA autoantibody [1, 2]. Male BXSB mice develop SLE faster than females, which is opposite of that observed in humans [1-3]. Female B/W F1 mice spontaneously produce auto-antibodies starting at 5-6 months of age and eventually die from kidney failure [4, 5]. Among these mouse models, the symptoms of SLE-prone B/W F1 mice bear the most resemblance to those of human SLE patients [4, 5].

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 diseases in these models [6-9], and the imbalance of T-cell-derived cytokines have been implicated to contribute to disease progression. Indeed, some investigators propose that a predominance toward Th1 generation could play a significant role in driving lupus-like autoimmune disease in MRL/lpr mice [10, 11]. Cytokine profiles of spleen cells in BXSB mice showed mainly increases in the Th1 cy-
tokine IFN-γ (interferon γ) [12]. IFN-γ accelerated, while anti-IFN-γ antibody (Ab) or soluble IFN-γ receptor prevented (onset or progression of) SLE in NZB/W F1 mice [13, 14]. Interestingly, Th2-type responses have also been observed with the development of SLE, where the numbers of IL-4-producing cells have been found to be higher in some lupus-susceptible strains [15, 16]. Similarly, treatment with blocking anti-IL-4 Ab or soluble IL-4R reduced autoantibody production and nephritis in NZB/W F1 and MRL-Fas mice [17, 18]. Additionally, IL-13, also a Th2 cytokine, has been found to play a critical role in immunoglobulin production and the induction of antibody class switch in response to rheumatoid factors in human SLE patients [19, 20]. Finally, IL-17, an inflammatory cytokine expressed by a recently identified lineage of effector CD4+ T cells (Th17) [21], has also been implicated in the pathogenesis of autoimmune and inflammatory diseases including SLE [22]. We previously reported that both IL-13 and IL-17 were produced in MRL/lpr mice [23], however little is known about these two cytokines in NZB/W F1 and BXSB mice. We have also reported that renal T cell infiltrates may consist of a mixture of Th1, Th2 and Th17 cells in lupus patients [24]. As of present, the roles of individual Th1, Th2, and Th17 cell subsets in SLE remain unclear.

Laser microdissection (LMD) is a well-established method for isolating individual cells or subcellular structures from a heterogeneous cell population [25]. It could be used to precisely harvest cells of interest from a tissue specimen in a rapid and practical manner. Cell-, DNA-, RNA-, and protein-based techniques have been used in combination with LMD to gather important information regarding the genome, transcriptome, and more recently, proteome of individual microdissected cells. 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 [26]. In the present study, we employed RT-PCR following LMD to analyze cytokine mRNA profiles as well as protein expression by immunohistochemistry to further define the role of T cells in the kidneys of murine lupus models. We found that individual T cell subsets may have differential contributions to SLE progression depending on the murine model.

**Material and methods**

**Mice**

Female MRL/MpJ-lpr (MRL/lpr) mice (6-10 weeks) were purchased from Charles River Japan (Yokohama, Japan) and bred at the animal facility of the University of Tsukuba, Japan. At 20-24 weeks of age, ten MRL/lpr mice were sacrificed and spontaneous development of glomeru-

![Fig. 1. Renal histological characterization in murine lupus models. Kidney sections from MRL/lpr mice (female), B/W F1 mice (female), and BXSB mice (male) were stained with hematoxylin and eosin. MRL/lpr and B/W F1 mice with developing glomerulonephritis and vasculitis, and BXSB with developing glomerulonephritis were used. Almost no infiltrating cells were detected at the perivascular areas of BXSB mice (original magnification 200× in A-C, and 100× in E, F).](image)
Histopathologic and immunohistologic examinations

Kidney samples were fixed with 10% formalin in 0.1M phosphate buffer, pH 7.2, and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin (HE) for histologic examination by light microscopy. For immunohistochemical staining, kidneys samples were frozen in OCT compound (Sakura Finetek USA, Torrance, CA) and stored at –80°C until use. Sections (5 μm thick) were cut on a Cryostat (Leica Microsystems, Wetzlar, Germany). Immunohistochemical staining was performed by avidin-biotin complex. Primary antibodies (Becton Dickinson, Mountain View, CA) were as follows: rat anti-mouse L3T4 for CD4 T cells; rat anti-mouse Ly-2 for CD8 T cells; rat anti-mouse B220 (CD45R) and anti-mouse Thy-1 for T cells. Immunoreactivity was visualized with diaminobenzidine (DakoCytomation, Carpinteria, CA) for 10 min, and sections were counterstained with hematoxylin. In negative controls, the 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 seconds. 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 the computer mouse, glomeruli and perivascular areas of infiltrating cells of similar sizes were selected for RNA extraction in thin-walled PCR tubes (0.5 ml), and subsequently dissected with a laser microbeam.

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), and 1 μl of each first-strand reaction was amplified with primers specific to β-actin, T-cell receptor β chain (TCR-Cβ), 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 seconds 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). The number of samples positive for a given cytokine was divided by the number of samples positive for TCR-Cβ to obtain the percentage of T cells positive for the cytokine of interest.

Statistical analysis

Statistical significance was determined with the χ² test. Statistical significance was set at p < 0.05.

Results

Histopathologic and immunohistologic examinations

MRL/lpr mice (10 females) and B/W F1 mice (9 females) with developing glomerulonephritis and vasculitis, as well as BXSB mice (10 males) with developing glomerulonephritis were used. Unlike MRL/lpr and B/W F1, almost no infiltrating cells were detected at the perivascular areas of BXSB mice (Fig. 1).

To verify the tissue localization of T cells associated with glomerulonephritis and vasculitis, presence of surface protein markers was examined immunohistochromically. Thy-1, B220, CD4, and CD8 staining was observed in glomeruli from all three strains, especially in the glomerular circumferential areas of MRL/lpr mice (Fig. 2). These results indicated that the major site of T cell infiltration was at the glomerular circumferential areas. Thy-1, B220, CD4, and CD8 staining was also observed in perivascular areas of MRL/lpr and B/W F1 mice (Fig. 3). Areas positive for Thy-1 were larger than those positive for B220, CD4, and CD8 in both the glomeruli and perivascular areas.

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

Single glomeruli and perivascular areas of similar sizes as the glomeruli were captured by LMD (Fig. 4). We then analyzed gene expression of laser microdissected sections by nested RT-PCR (Fig. 5). Samples from ten MRL/lpr mice (292 pooled samples positive for both β-actin-and TCR-Cβ- by RT-PCR), nine B/W F1 mice (315 pooled...
samples positive for both β-actin-and TCR-Cβ- by RT-PCR), and ten BXSB mice (188 pooled samples positive for both β-actin-and TCR-Cβ- by RT-PCR) were used. The number of samples positive for a given cytokine was divided by the number of samples positive for TCR-Cβ to obtain the percentage of T cells positive for the cytokine of interest. T cells infiltrating the glomeruli and perivascular areas prominently produced IFN-γ, IL-13, and IL-17 in MRL/lpr, B/W F1, and BXSB mice. However, the amount of IL-17 expressing cells in the glomeruli of BXSB mice was lower than that of MRL/lpr and B/W F1 mice.

In the glomerular lesions, the percentage of positive IFN-γ samples was 48.6% in T cells of the B/W F1 mice, which is significantly lower than in the BXSB mice (66.4%, p < 0.05). IFN-γ expression of almost the same percentage (63.1%) was found in the MRL/lpr mice. The percentages of positive IL-13 samples were 68.0%, 63.3%, and 63.5% in MRL/lpr mice, B/W F1 mice, and BXSB mice, respectively (Fig. 5A). The percentages of positive IL-17 samples were 50.7% and 60.8% in the MRL/lpr and B/W F1 mice, both of which were significantly greater than that from BXSB mice (29.7%) (Fig. 5A). The expression level

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**Table 1. Oligonucleotide primer sequences**

| PCR products | Oligonucleotide sequence | Product size (bp) | RT-PCR cycles |
|--------------|--------------------------|------------------|---------------|
| **Mouse β-actin** | | | |
| First PCR | 5’ sense | TGGTACCACTGGGACGACA | 415 | 25 |
| | 3’ antisense | TTGTAGTCAAGCAGCTTTT | | |
| Nested PCR | 5’ sense | GATCTGGCAACCACACCTTCT | 366 | 25 |
| | 3’ antisense | CTTCAGCTGTGTTGTTGAA | | |
| **Mouse TCR-Cβ** | | | |
| First PCR | 5’ sense | CCCAAGGTCTCTTGGTTGGA | 339 | 25 |
| | 3’ antisense | GCCTCTGACATGTGAGCTTCA | | |
| Nested PCR | 5’ sense | AAGGCTACCCCTGTTGCTC | 193 | 25 |
| | 3’ antisense | AGTGGTTCGAGAATTGTCG | | |
| **Mouse IFN-γ** | | | |
| First PCR | 5’ sense | GCCTCATTGAATCACACCTG | 468 | 25 |
| | 3’ antisense | CGATTACAGTCTTGGCTA | | |
| Nested PCR | 5’ sense | TTTGAGGCTCAACACCCACA | 343 | 30 |
| | 3’ antisense | TGGTCAAGGAAATAGTTG | | |
| **Mouse IL-10** | | | |
| First PCR | 5’ sense | CTTGCACTACGCAAAGCCACA | 451 | 30 |
| | 3’ antisense | TTTCACAGGGGAGAAATCG | | |
| Nested PCR | 5’ sense | AGCAAGCCTACGAGAAAGAG | 372 | 30 |
| | 3’ antisense | TCTCACCCAGGAAATTCAAA | | |
| **Mouse IL-13** | | | |
| First PCR | 5’ sense | CTGTGAGCCTGTCTCTCTC | 233 | 30 |
| | 3’ antisense | TTGGTGAGCGAGTAGAGACG | | |
| Nested PCR | 5’ sense | AAATGCTACCTGAGCTTGTGCT | 198 | 25 |
| | 3’ antisense | CTTCCAGTCCTGATGACTG | | |
| **Mouse IL-17** | | | |
| First PCR | 5’ sense | CATGCGAGAGGGTGTACCTT | 212 | 25 |
| | 3’ antisense | AGCCTCTCTCGCTCAGACG | | |
| Nested PCR | 5’ sense | CGTGTTAATGCATGACATATC | 166 | 25 |
| | 3’ antisense | ACATAAACAGGCCAGTCAGT | | |

RT-PCR – reverse transcription polymerase chain reaction; TCR-Cβ – T-cell receptor β chain; IL – interleukin; IFN-γ – interferon γ
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Fig. 2. Detection of T cells in glomerulonephritis. Frozen sections of glomeruli were immunostained with mouse antibodies against Thy-1, B220, CD4, and CD8. Thy-1, B220, CD4, and CD8 staining were observed in glomeruli of MRL/lpr, B/W F1, and BXSB mice, especially in the glomerular circumference areas of MRL/lpr mice (original magnification 200×).

Fig. 3. Detection of T cells in vasculitis frozen sections of perivascular lesions were immunostained with mouse antibodies against Thy-1, B220, CD4, and CD8. Thy-1, B220, CD4, and CD8 staining was observed in the perivascular areas of MRL/lpr and B/W F1 mice (original magnification 100×).
of IL-10 was not detectable in the glomeruli of all three mouse models (Fig. 5).

Similar to glomerular infiltrating T cells, the perivascular infiltrating T cells also expressed high levels of IFN-γ, IL-13, and IL-17 in MRL/lpr and B/W F1 mice (Fig. 5B). Additionally, IL-10 expression was detected at the same strength in the perivascular infiltrates from these two strains (Fig 2B-b). The percentages of positive IFN-γ and IL-13 samples were higher than 60%, with IFN-γ at 66.9% and 60.2%, and IL-13 present at 72.5% and 61.6% in the MRL/lpr and B/W F1 mice, respectively (Fig. 5B). The percentages of samples positive for IL-17 were 41.7%, and 56.6% in MRL/lpr and B/W F1 mice, respectively. The percentages samples positive for IL-10 were 64.4% in the MRL/lpr mice and 49.7% in the B/W F1 mice (Fig. 5B).

Discussion

Systemic lupus erythematosus (SLE) is an autoimmune disease for which the pathogenesis remains unclear. Although the etiology of SLE is unknown, a number of key factors have been identified. T cells play an important role

Fig. 4. Targeted infiltrating T cells selected and cut by laser microdissection (LMD). Glomeruli and perivascular areas of similar sizes containing infiltrating cells (black arrows) were selected (left panels) and then captured (right panels) by LMD.
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In the development of autoimmune disease in the three most used mouse models: MRL/lpr mice, B/W F1 mice, and BXSB [6-9]. Imbalance of CD4+ T-cell subsets has been suggested to play a role in the pathophysiology of SLE. CD4+ T-helper cells are crucial in the regulation of immune response, and their deregulation has been associated with the pathogenesis of various autoimmune diseases. In addition to the classical Th1 and Th2 CD4+ T cell subtypes, another helper T cell subset, Th17, was recently discovered, with characteristic high production of IL-17 [27]. IL-17 is a proinflammatory cytokine, the presence of which has been observed in many inflammatory conditions [27]. Indeed, IL-17 has been found to contribute to the pathogenesis of many autoimmune and inflammatory diseases including renal diseases such as SLE [28, 29].

To define the role of T cells in the kidneys of lupus model mice, we analyzed cytokine mRNA expression by LMD and RT-PCR and surface protein marker expression by immunohistochemistry. Our data indicated that T cells infiltrating the glomeruli and perivascular areas predominantly produced IFN-γ, IL-13, and IL-17 in lupus model such as MRL/lpr, B/W F1, and BXSB mice. However, the level of IL-17 in the glomeruli of BXSB mice was found to be lower than that in MRL/lpr and B/W F1 mice. IL-10 was detected only in the perivascular areas of MRL/lpr and B/W F1 mice but not in glomeruli. Immunohistochemical staining revealed that the expression of Th1, CD4, CD8, and B220 was observed in both the glomeruli and perivascular areas from all three strains. The areas of Thy-1 and B220 staining were larger than the areas of CD4 and CD8 staining in glomerular and perivascular areas of MRL/lpr mice, but not in B/W F1 and BXSB mice. Similar findings in which the areas of Thy-1 and B220 staining containing large numbers of CD4-CD8-B220+Thy-1+T cell have been reported in the lymph nodes and spleens of MRL/lpr mice [2]. Our previous data have also shown an abundance of CD4-CD8- B220+Thy-1+ T cells in MRL/lpr mouse kidney by RT-PCR [23].

The high levels of IFN-γ expression in the glomeruli and perivascular areas indicated that IFN-γ may play a crucial role in the development of glomerulonephritis and vasculitis in these models of lupus. Moreover, gene knockout of IFN-γ or IFN-γ receptor eliminated disease in spontaneous and xenobiotic-induced lupus models [10, 13, 30]. However, Th2-type responses have also been associated with the development of SLE. IL-4 and IL-10 play prominent roles in the pathogenesis of lupus-associated tissue injury in mice [18, 31]. We presently found high expression of IL-13 in the glomerular and perivascular lesions of these lupus models (Fig. 5). Thus, IL-13 may also be an important factor in the pathogenesis of glomerulonephritis and vasculitis. We speculate that Th2 cells play an active role in the development 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 both antigen-presenting cells and T cells [32]. IL-10 gene-deficient mice develop severe lupus with an earlier appearance of skin lesions, increased lymphadenopathy, more severe glomerulonephritis, and higher mortality than littermate controls [33]. In addition, IL-10 down regulates murine lupus by inhibiting pathogenic Th1 cytokine responses [33]. In the present study, IL-10 was observed only in perivascular lesions by RT-PCR (Fig. 5). Therefore, we speculate that the molecular mechanism for glomerulonephritis differs from that of vasculitis in murine SLE. IL-10 is also produced by other CD4+ regulatory T cells (Tregs) such as Type 1 Tregs and CD4+CD25+ Tregs [34]. Recently, the role of regulatory T cells in the pathogenesis of human autoimmune diseases, including rheumatoid arthritis and SLE, was reported [35, 36]. In MRL/lpr and B/W F1 mice, it is possible that IL-10 produced by Type 1 Tregs and CD4+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 reported by Andreas et al. in rats [37]. Our results support the hypothesis that vasculitis and glomerulonephritis are under the control of different genes and involve different pathologic processes [38]. In the current study, we observed IL-17 mRNA expression in both glomerular and perivascular lesions, suggesting that IL-17 is a key factor in the development of glomerulus nephritis and vasculitis in murine lupus models. However, the percentage of positive IL-17 samples in BXSB mice was significantly lower than that in MRL/lpr and B/W F1 mice (Fig. 5A). We conjecture that IL-17 might play a minor role in BXSB mice as compared to the other two strains. BXSB males are affected much earlier than females due to the presence of an as yet unidentified mutant gene located on the Y chromosome, designated as Yaa (Y-linked autoimmune acceleration). The Yaa gene has been shown to be responsible for the acceleration of lupus-like autoimmune syndrome in inbred BXSB mice. Consequently, the pathologic processes of BXSB mice may be different from those of MRL/lpr and B/W F1 mice. Our results showed that in the glomeruli of B/W F1 mice, the level of IFN-γ was lower than that from BXSB mice, while IL-17 was higher. Due to the successful characterization of the Th17 lineage, it has been recognized that IFN-γ exhibits inhibitory action toward the differentiation of Th17 cells [21, 39]. Nakae et al. [40] also found that IL-17 can suppress Th1 cell differentiation in the presence of exogenous IL-12 in vitro, and likewise, IFN-γ can down-regulate Th17 cell differentiation. Therefore, in B/W F1 mice, the possibility that Th17 is suppressing Th1 cell differentiation via IL-17 is consistent with the finding that IFN-γ levels are low.

Although SLE has been classically considered as an autoantibody- and immune complex-driven disease, recent work indicates that IL-17 is involved in different aspects of its pathogenesis. Its potent proinflammatory capacity, along with the effects it exerts on a variety of
cells, suggests that its unregulated production has indeed widespread consequences in lupus. Th17 cells serve as an independent Th helper effector cell subset promoting inflammation through cytokine secretion. These cytokines have stimulatory effects on B cells, and instigate local inflammation and tissue damage leading subsequently to the pathogenesis of SLE. In conclusion, our results suggest that the cytokine balance is complex and not due simply to the Th1 and Th2 balance, but that Th17 cells might critically contribute to the pathogenesis of murine lupus models. These findings also support the concept that different molecular mechanisms underlie glomerulonephritis and vasculitis, and that IL-17 might play a more important role in MRL/lpr and B/W F1 mice than in male BXSB mice.

We would like to thank the Summus Biological Chemical Institute for their kindly help with the experiments.

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