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Microarray Analysis of Glomerular Gene Expression in Murine Lupus Nephritis

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Abstract. To elucidate the molecular mechanism of glomerular events in lupus nephritis, we performed genome-wide mRNA expression analysis of glomeruli microdissected from lupus mice. MRL/lpr mice (12-week-old) were orally given vehicle or prednisolone (10 mg/kg per day) for 4 weeks. Renal histology of MRL/lpr mice revealed mesangial proliferative glomerulonephritis with cellular infiltration of macrophages, T cells, and neutrophils. We identified 567 up-regulated genes in MRL/lpr glomeruli compared to control congenic mice. Those included complement components, adhesion molecules, chemokines and their receptors, and molecules related to antigen presentation. Over 130 genes were considered preferentially or exclusively expressed in hematopoietic cell lineages possibly reflecting leukocytes accumulation. Of note is the finding that chemokines and chemokine receptors (CCL3, CCL4, CCL5, CXCL9, CXCL10, CXCL11, CXCL16, CCR5, CXCR3, and CXCR6) that are related to T helper 1 (Th1) cells accumulation were up-regulated concomitantly with increased expression of Ebi3, a subunit of IL-27 that plays a role in Th1 predominance. These changes were accompanied by increased mRNA expression of many genes that were inducible by Th1 cytokine interferon-γ. Prednisolone markedly attenuated glomerular lesion and leukocyte influx parallel with the reduction of enhanced gene expression. The present study shows additional evidence supporting glomerular Th1 cells accumulation and their role. Our data also provide an important resource in seeking new therapeutic targets to lupus nephritis.

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Keywords: lupus nephritis, chemokine, Th1, interferon-γ, IL-27

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by widespread loss of immune tolerance to self-antigen. Studies on pathogenesis of SLE have been facilitated by the availability of murine lupus models. MRL/lpr mice represent a prototypic murine model of SLE, which spontaneously develop lethal renal disease that is characterized mainly by diffuse proliferative glomerulonephritis and interstitial nephritis. This strain has an autoimmune MRL background and bears the *lpr* mutation (1), the result of a retroviral insertion in Fas gene, leading to nearly complete absence of the proapoptotic Fas protein and interfering with Fas-induced lymphocyte apoptosis. As a consequence of this mutation, autoreactive lymphocytes escape thymic selection (2), leading to their proliferation and increased circulating immune complexes, which are deposited in the glomerular microvasculature and triggers glomerular inflammation.

Although various mediators from various cell types have been implicated in this model, the molecular mechanism of lupus glomerular lesion is still poorly understood. Microarray technology enables simultaneous monitoring of genome-wide mRNA expression analysis, providing an opportunity to study complex

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molecular interactions in disease processes (3). In fact, this technology was successfully applied to kidney tissue of MRL/lpr mice (4). In their study, however, whole kidney samples were used for the microarray analysis. The architecture of the kidney is complex and different structural regions of the kidney exhibit highly distinctive patterns of gene expression (5). Immune complex deposits are detected not only within the glomeruli and vessels but also tubular basement membranes. The pathologic features of lupus nephritis are diverse, variably affecting the different renal compartments, including glomeruli, tubules, interstitium, and vasculature in different ways. Therefore, to elucidate the molecular events in lupus glomeruli, it is advantageous to study the glomerular gene expression profile separately from that of the interstitium and tubules. Microdissection has been applied as a method to acquire morphologically selected regions of a large variety of tissues using laser irradiation under the light-microscopy (6). Therefore, we combined microdissection and microarray technologies and examined glomerular genome–wide mRNA expression profiles of lupus-prone mice and therapeutic effects of glucocorticoid. We then tried to correlate expression changes with glomerular pathologic alterations to gain further insight into the molecular mechanisms of glomerular lesion of MRL/lpr mice.

Materials and Methods

Animals and drugs

All experiments were conducted in accord with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

MRL/lpr mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama) and MRL+/+ mice, from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed under a condition free of specific pathogens in our animal facility on standard laboratory chow.

Prednisolone (PSL; Wako Pure Chemical Industries, Ltd., Osaka) was suspended in 800 μg/ml of 0.5% methylcellulose solution (vehicle solution).

Experimental protocol

Twelve-week-old female MRL/lpr and MRL+/+ mice were used. MRL+/+ mice are congenic to MRL/lpr but have normal Fas protein and develop autoimmunity only later in life. These mice were given orally vehicle solution (12.5 ml/kg body weight) everyday and served as the control (+/+ group, n = 10). MRL/lpr mice were orally given either PSL at a dose of 10 mg/kg (lpr + PSL group, n = 8) or vehicle solution (lpr group, n = 10) everyday. After 4 weeks, mice were housed in metabolic cages for collection of 24-h urine samples. After urine collection, all mice were anesthetized with ether and a blood sample was collected from abdominal vein. Both kidneys were then perfused with ice-cold heparinized saline through the left ventricle and were immediately removed. For microdissection, a kidney was split into two halves and one half was embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo), snap-frozen in liquid nitrogen, and stored at −80°C until use. Another half of the renal tissue was fixed overnight with cold 4% paraformaldehyde in 0.01 M phosphate-buffered saline, pH 7.2 for histology and immunohistochemistry. Serum and urine creatinine concentration, blood urea nitrogen (BUN), and protein concentration of urine were determined by a standard laboratory method with auto-analyzers.

Histopathological examinations

Paraffin sections were stained with hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS) for the light microscopic histological examination. Glomerular injury was evaluated by the glomerular score, which is was based on the activity index (7), with exception of the tubulointerstitial abnormalities. Briefly, cellular proliferation, fibrinoid necrosis/karyorrhexis, cellular crescent, hyaline thrombi/wire loop, and glomerular leukocyte infiltration were graded from 0 to 3 for about 50 glomeruli for each sample and the summation was expressed as the glomerular score in each case.

Immunohistochemical examinations

The populations of infiltrating cells in the glomeruli were examined by immunohistochemical examinations. Immunohistochemical examinations were performed for CD3ε (T cell, M-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD68 (macrophage; Serotec, Oxford, UK), neutrophil (7/4, Serotec), and Ki67 (proliferating cell, TEC-3; Dako Cytomation, Ltd., Kyoto). Four-micron-thick sections of paraffin embedded specimens were used in all examinations. After deparaffinization and rehydration, endogenous peroxidase activity was quenched by 3% H_2O_2 for 10 min at room temperature. For antigen retrieval, sections were treated either by autoclaving (0.01 M citric acid buffer, solution, 121°C, 20 min) to stain CD3ε, neutrophil, and Ki-67 or incubated with 0.05% proteinase (proteinase XXIV; Sigma-Aldrich, Tokyo) for 20 min at room temperature for CD68. Then the sections were incubated with 5% skim milk for 30 min at room temperature. Next, the sections were incubated with the first antibody for 1 h (CD3ε, Ki67) at room temperature or overnight at 4°C (CD68,

neutrophil). Each first antibody was diluted with 1% bovine serum albumin in 0.01 M phosphate saline buffer solution to 1:150 (CD3ε) and 1:50 (CD68, Ki67 and neutrophil). Histofine Simple Stain Mouse MAX-PO (rat) for CD68, neutrophil, and Ki67 and (G; goat) for CD3ε (Nichirei Corp., Tokyo) were used as secondary antibodies according to the manufacturer's instructions. Immuno-reaction products were developed using 3,3'-diaminobenzine as the chromogen. Proliferating cells and infiltrating cells such as T cells, macrophages, and neutrophils were counted in 50 glomeruli for each sample.

Laser-capture microdissection and RNA extraction

The methods of preparation of frozen fixed tissue, microdissection, and RNA extraction were essentially the same as those reported by Irie et al. (8). The frozen blocks were sliced by a cryomicrotome (HM560; Micron, Walldorf, Germany) at a thickness of 8 μm, and each tissue section was affixed to a slide to which an original thin film (Meiwa fosis, Tokyo) had been attached by silicone adhesive (GE Toshiba Silicone, Tokyo). The sliced sample was returned to room temperature (20 s) and was fixed in 99.8% methanol for 3 min and then stained with 1% toluidine blue solution with 1% sodium tetraborate. A Laser Microbeam System (P.A.L.M., Bernried, Germany) was used for laser microdissection. Ten glomeruli excluding crescentic lesion or Bowman's capsules were individually microdissected and collected into a 1.5-ml tube from sliced samples that were randomly chosen from each group (n = 5). Total RNA was then extracted from each sample of the laser-microdissected glomeruli according to the procedure based on the acid guanidinine-phenol-chloroform method and was finally resuspended in 20 μl RNase free water. In a preliminary experiment, the quality and quantity of the extracted RNA were checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA synthesis, amplification, and array hybridization

A 10-μl aliquot of extracted RNA was amplified using T7 RNA polymerase promoter-attached, adaptor ligation-mediated, and PCR amplification followed by the in vitro T7-transcription (TALPAT) method described by Aoyagi et al. (9). Biotinylated cRNA was then synthesized using GeneChip® Expression 3'-Amplification Reagents for IVT Labeling Kits (Affymetrix, Santa Clara, CA, USA) and fragmented. Hybridization cocktails contained 15 μg cRNA and GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix) were hybridized according to the standard Affymetrix protocols. GeneChip® Mouse Genome 430 2.0 Arrays, which comprise about 45,000 probe-sets, included approximately 34,000 well-substantiated mouse genes. Chips were washed and stained using the GeneChip Fluidics Station 450 according to the EukGE-WS2v5 fluidics protocol (Affymetrix) and scanned with the GeneChip Scanner 3000 (Affymetrix).

Array analysis

We normalized all intensity data by scaling the average signal intensity of 100 maintenance genes on each array and determined whether transcript was detected ("present"), undetected ("absent"), or at the limit of detection ("marginal") using GeneChip Operating Software (GCOS) 1.0 (Affymetrix). Microarray signal data were subsequently analyzed with GeneSpring 7.3 software (Silicon Genetics, Santa Clara, CA, USA). To identify the genes whose expression levels were up-regulated in MRL/lpr glomeruli, normalized gene expression signals were subjected to further analysis if they fulfilled all of the following criteria: 1) its expression was "present" on more than 3 out of 5 lpr group samples, 2) its expression was statistically different among groups by Kruskal-Wallis test (P<0.05), 3) its expression was up-regulated in lpr group compared to the +/+ group by post hoc testing (Student-Newman-Keuls by using rank sum, P<0.05), and 4) its expression in the lpr group was up-regulated more than two-fold compared to those in the +/+ group.

Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

The remaining 10 μl RNA extracted above was reverse transcribed using TaqMan Reverse Transcription Reagent (Applied Biosystems, NJ, USA) according to the manufacturer's instructions. Real-time RT-PCR was performed for complement component 1, q subcomponent, C chain (C1qc), Histocompatibility 2, D region locus 1 (H2-D1), intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), chemokine (C-C motif) ligand 2 (Ccl2), chemokine (C-X-C motif) ligand 10 (CXCL10), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). All primers were obtained from Takara Bio Inc. (Shiga). Real-time RT-PCR was performed using the SYBR® Premix Ex Taq™ (Takara Bio Inc.) and the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Data were analyzed using the comparative cycle threshold (Ct) method as a means of relative quantitation of gene expression, normalized to the endogenous reference (GAPDH) and relative to a calibrator (normalized Ct values obtained from renal tissue of MRL/lpr mice), and expressed as 2-$\Delta\Delta$Ct, as described by the manufacturer (Applied Biosystems).

Statistical analysis

Values were presented as the mean ± S.E.M. All values other than expression data were analyzed using analysis of variance combined with Student-Newman-Keuls test. *P* values at <0.05 were considered significant.

Results

Characteristics of mice

Characteristics of mice were listed in Table 1. Body weight was not different among the groups. Serum creatinine and BUN were elevated in the lpr group compared with those in +/+ group and were decreased by prednisolone treatment. Creatinine clearance of the lpr group was lower than those of the +/+ group. Prednisolone treatment for 4 weeks slightly improved this reduction but not significantly. The average value of urinary protein excretion was increased in lpr mice but not statistically significant due to scattering of data.

Histopathology and immunohistochemistry

At the age of 16 weeks, the glomeruli of the lpr group showed mesangial cell proliferation and leukocyte infiltration with occasional formation of cellular crescent (Fig. 1). The severity of glomerular lesions was

Table 1. Animal characteristics and glomerular lesion

	+/+	lpr	lpr + PSL
Body weight (g)	35.2 ± 0.6	36.1 ± 1.3	32.9 ± 0.7
Serum-creatinine (mg/dl)	0.12 ± 0.00	0.19 ± 0.02*	0.14 ± 0.01†
Blood urea nitrogen (mg/dl)	15.0 ± 0.8	46.8 ± 6.2*	25.4 ± 2.0†
Creatinine clearance (ml/min)	0.57 ± 0.10	0.27 ± 0.04*	0.35 ± 0.03*
Proteinuria (mg/day)	2.05 ± 0.39	9.41 ± 4.93	1.68 ± 0.32
Ki67 (proliferation)	3.6 ± 0.6	38.1 ± 6.6*	7.3 ± 1.7†
Glomerular score	0.2 ± 0.1	9.1 ± 0.7*	3.3 ± 0.8*†

Values are means ± S.E.M. of eight to ten animals for each group at the age of 16 weeks. +/+: MRL+/+ mouse (control), lpr: MRL/lpr mouse, lpr + PSL: MRL/lpr mouse treated with prednisolone. *P<0.05 versus +/+; $^{\dagger}P$<0.05 versus lpr.

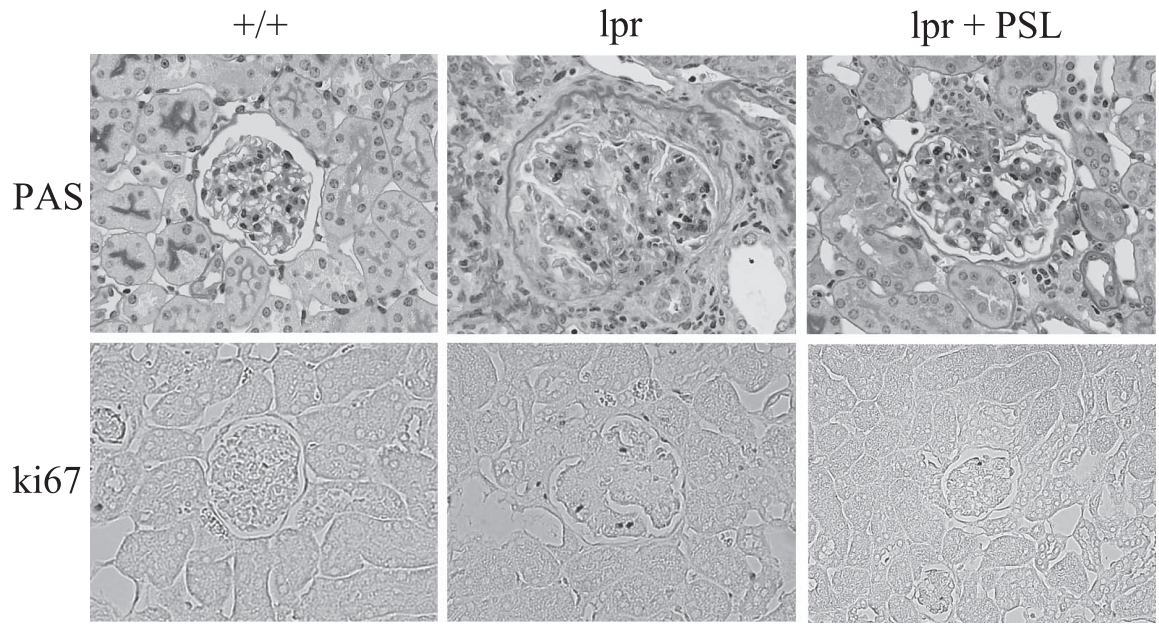

Fig. 1. Representative glomerular histopathology and proliferating cells from each group of mice. Upper panel: Periodic acid-Shiff (PAS) staining. At the age of 16 weeks, MRL/lpr mice showed mesangial proliferative glomerulonephritis with cellular infiltration and formation of cellular crescent. Glomeruli of prednisolone-treated MRL/lpr mice showed slight mesangial cell proliferation but less cellular infiltration and negligible cellular crescent. Lower panel: Ki67-positive cells indicate proliferating cells that were increased in the glomeruli of MRL/lpr mice. +/+: MRL+/+ mice, lpr: MRL/lpr mice, lpr + PSL: MRL/lpr mice treated with prednisolone. Magnification: ×400.

evaluated by the glomerular score (Table 1). Glomerular scores were higher in the lpr group than those in the +/+ group. Prednisolone treatment significantly attenuated the increased glomerular score observed in the lpr group. Ki67-positive cells, which show the presence of proliferating cells such as mesangial cells and endothelial cells, were significantly increased in glomeruli of lpr mice and were decreased with prednisolone treatment (Fig. 1, Table 1). Infiltrating leukocytes into the glomeruli were identified by immunohistochemistry (Fig. 2). The number of infiltrating CD3ε-positive T cells, CD68-positive macrophages, and neutrophils increased markedly in lpr mice, and these increases were significantly attenuated with prednisolone treatment (Fig. 2).

Up-regulated gene expressions in glomeruli of lpr mice

Five hundred and sixty-seven genes including 54 expressed sequence tags fulfilled the criteria described in the Methods section as up-regulated genes in the glomeruli of lpr mice. The full list of these up-regulated genes is given in the supplemental table (online version only). Among these up-regulated genes, more than a hundred of genes were judged by the detection algorithm as absent or marginal in all of five control mice. In these cases, since basal expression level is close to or below the detection limit, the robustness of fold change cannot be assured. Therefore, these genes were shown in italic. Despite such limitation, this does not affect the statistical significance among the groups. As inflammatory cells such as T cells, macrophages, and neutrophils accumulated within the glomeruli, interpretation of microarray data should be done with caution. In fact, among these up-regulated genes, 137 genes are known to be preferentially or exclusively expressed in the hematopoietic cell lineage, presumably reflecting leukocytes infiltration. These genes are listed and marked in the supplemental table (online version only). This is a minimal estimate since 567 up-regulated genes contain a considerable number of genes that have not been well characterized. More than half of the genes from hematopoietic cell lineage are cell surface molecules, that is, various types of receptors (Fc receptors, chemokine receptors, pattern recognition receptors), MHC molecules, adhesion molecules, and a number of CD antigens. Enzymes and transcriptional regulators are also included in this group. For instance, Cybb/bp91phox, Ncf1/p47phox, and Ncf4/p40phox, components of phagocyte NADPH

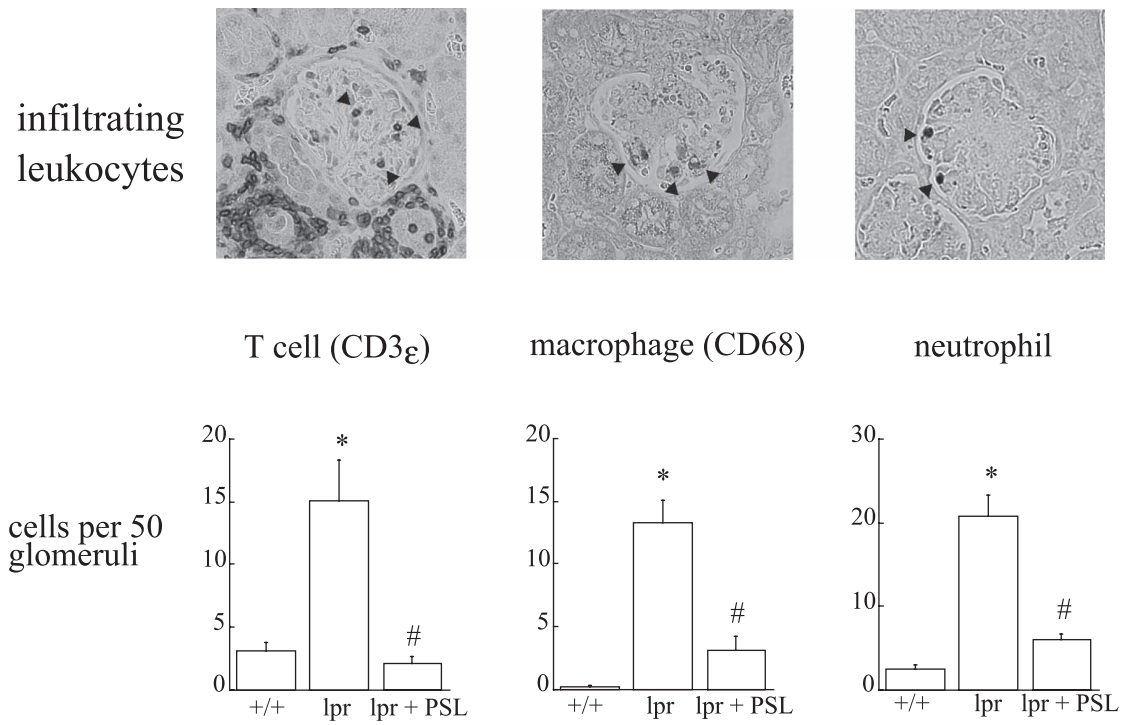

Fig. 2. Glomerular infiltration of T cells, macrophages, and neutrophils. Upper panel: immunohistochemistry of infiltrating leukocytes. Arrow heads in each photograph indicate the glomerular infiltrating CD3ε-positive T cells, neutrophils, and CD68-positive macrophages in MRL/lpr mice at the age of 16 weeks. Magnification: ×400. Lower panel: Quantification of infiltrating leukocytes. +/+: MRL+/+ mice, lpr: MRL/lpr mice, lpr + PSL: MRL/lpr mice treated with prednisolone. Results were expressed as the mean ± S.E.M. *P<0.05, compared to MRL+/+ mice; $^{\#}P$<0.05, compared to MRL/lpr mice.

Table 2. Representative genes up-regulated in glomeruli of lpr mouse found by microarray analysis

Common name	Full name	Fold change		PSL effect
		lpr/control	lpr + PSL/control	
Complement and coagulation				
C1qa	Complement component 1,q subcomponent, alpha polypeptide	7.6	7.4	
C1qb	Complement component 1,q subcomponent, beta polypeptide	8.0	4.8	*
C1qc	Complement component 1,q subcomponent, C chain	22.8	8.1	*
C1qr1	complement component 1, q subcomponent, receptor 1, CD93	5.2	2.1	*
C2	Complement component 2	5.8	0.9	*
C3	Complement component 3	4.5	1.2	*
C3ar1	Complement component 3a receptor 1	8.4	6.4	
F10	Coagulation factor 1	10.5	2.5	*
Serping1	Serine (cysteine) peptidase inhibitor, clade G, member 1; C1 inhibitor	4.2	2.0	*
Chemokines and chemokine receptors				
Ccl2	Chemokine (C-C motif) ligand 2, MCP-1	4.9	2.1	
Ccl3	Chemokine (C-C motif) ligand 3, MIP-1alpha	150.3	19.9	*
Ccl4	Chemokine (C-C motif) ligand 4, MIP-1beta	11.9	1.5	*
Ccl5	Chemokine (C-C motif) ligand 5, RANTES	199.6	16.1	*
Ccl6	Chemokine (C-C motif) ligand 6, C10	17.5	5.5	*
Ccl8	Chemokine (C-C motif) ligand 8, MCP-2	164.9	9.2	*
Ccl9	Chemokine (C-C motif) ligand 9	15.2	6.7	*
Ccl12	Chemokine (C-C motif) ligand 12	48.1	1.3	*
Cxcl2	Chemokine (C-X-C motif) ligand 2, GRO beta	42.7	3.7	*
Cxcl4	Chemokine (C-X-C motif) ligand 4, platelet factor 4	2.9	3.3	
Cxcl9	Chemokine (C-X-C motif) ligand 9, Mig	942.6	55.7	*
Cxcl10	Chemokine (C-X-C motif) ligand 10, IP-10	7.3	0.8	*
Cxcl11	Chemokine (C-X-C motif) ligand 11, I-TAC	4.1	1.9	*
Cxcl16	Chemokine (C-X-C motif) ligand 16, small inducible chemokine B6	5.2	1.2	*
Ccr2	Chemokine (C-C motif) receptor 2	3.6	1.1	*
Ccr5	Chemokine (C-C motif) receptor 5	18.5	3.6	*
Cx3cr1	Chemokine (C-X3-C motif) receptor 1	9.0	1.4	*
Cxcr3	Chemokine (C-X-C motif) receptor 3	2.7	1.4	*
Cxcr4	Chemokine (C-X-C motif) receptor 4	15.1	3.7	*
Cxcr6	Chemokine (C-X-C motif) receptor 6, BONZO	4.7	0.4	*
Cklf	Chemokine-like receptor	14.7	1.3	*
Adhesion molecules				
Alcam	Activated leukocyte cell adhesion molecule, CD166	3.1	2.0	
Itgal	Integrin alpha L (CD11a)	13.0	4.8	*
Itgam	Integrin alpha M (CD11b)	15.6	7.3	
Itgax	Integrin alpha X (CD11c)	8.3	2.1	*
Itgb2	Integrin beta 2 (CD18)	26.1	2.2	*
Icam1	Intercellular adhesion molecule 1, ICAM-1	6.5	0.6	*
Vcam1	Vascular cell adhesion molecule 1, VCAM-1	3.5	0.9	*
Antigen presentation and processing				
MHC class I molecules and antigen processing molecule				
H2-D1	Histocompatibility 2, D region locus 1	7.1	3.9	
H2-K1	Histocompatibility 2, K region locus 1	2.2	1.4	*
H2-T10	Histocompatibility 2, T region locus 10	6.2	2.2	*
H2-T23	Histocompatibility 2, T region locus 23	6.9	3.6	

Common name	Full name	Fold change		PSL effect
		lpr/control	lpr + PSL/control	
H2-T24	Histocompatibility 2, T region locus 24	8.9	1.1	*
b2m	Beta-2 microglobulin	2.8	2.2	
Psmb8	Proteasome subunit, beta type 8, LMP7	9.4	3.5	
Psmb9	Proteasome subunit, beta type 9,LMP2	15.8	5.8	*
Psmb10	Proteasome subunit, beta type 10, MECL1	3.3	1.5	*
Tap1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	6.4	2.4	*
Tapbp	TAP binding protein	4.9	2.5	*
MHC class II molecules and antigen processing molecules				
H2-Aa	Histocompatibility 2, class II antigen A alpha	3.4	1.6	*
H2-DMb1	Histocompatibility 2, class II, locus Mb1	3.7	1.3	*
H2-Ea	Histocompatibility 2, class II antigen E alpha	5.9	1.6	*
H2-Eb1	Histocompatibility 2, class II antigen E, beta 1	3.3	1.4	*
H2-Ab1	Histocompatibility 2, class II antigen A, beta 1	7.3	2.4	*
Ctss	Cathepsin S	10.2	4.5	*
Extracellular matrix and related molecules				
Bgn	biglycan	2.4	1.8	
Col3a1	Procollagen, type III, alpha 1	8.7	2.9	*
Col4a1	Procollagen, type IV, alpha 1	2.4	1.8	*
Col6a1	Procollagen, type VI, alpha 1	4.2	0.8	*
Col8a1	Procollagen, type VIII, alpha 1	5.6	1.6	*
Col12a1	Procollagen, type XII, alpha 1	5.0	2.3	
Mmp13	Matrix metallopeptidase 13	11.7	2.9	*
S100a4	S100 calcium binding protein A4, Fibroblast specific protein-1	3.5	0.8	*
Serpine1	Serine (cysteine) peptidase inhibitor, clade E, member 1, PAI-1	111.3	18.8	*
Timp1	Tissue inhibitor of metalloproteinase 1	20.1	4.3	
Dcn	Decorin	7.7	1.2	*
Fn1	Fibronectin 1	4.6	1.9	*

Fold change indicates the ratio of average gene expression signals of the SLE (MRL/lpr) or PSL (MRL/lpr + prednisolone treatment) group to those of the control (MRL+/+) group. PSL effect indicates significantly attenuated gene expression of lpr mouse with prednisolone treatment ($P<0.05$). Genes shown in italics were judged by the detection algorithm as absent or marginal in all of five control mice. In these cases, fold change should be regarded as a reference.

oxidase were up-regulated more than 10 times compared to control mice. Prednisolone markedly attenuated this increased mRNA expression concomitantly with the reduction of neutrophil and macrophage infiltration. This may merely mean that NADPH oxidase-expressing neutrophils and/or macrophages accumulated in lupus glomeruli and disappeared with prednisolone. However, it is also possible that infiltrating neutrophils and/or macrophages over-expressed these molecules or alternatively these gene expressions were induced in the glomerular resident cells. In fact, nephritogenic anti-DNA antibodies induce a number of transcripts including inflammatory molecules in cultured mesangial cells derived from MRL/lpr mice (10). To confirm the relative role of each cell population in individual gene expression, further studies are needed. It should be pointed out that reactive parietal cells had minimal influence on our gene expression data since glomerular microdissection was performed with caution not to include Bowman's capsule or crescent.

Among these up-regulated genes, we chose 5 functional gene groups that are listed in Table 2: 1) complement and coagulation, 2) chemokines and chemokine receptors, 3) adhesion molecules, 4) antigen processing and presentations, and 5) extracellular matrix.

Genes of complement components and their receptors, such as C1q, C1qr, C2, C3, and C3ar1 were highly expressed in the glomeruli of lpr mice. These gene expressions were suppressed by prednisolone but to different degrees, and C1qa and C3ar1 expression was not affected significantly. A number of C-C and C-X-C chemokines and their receptors were highly expressed in

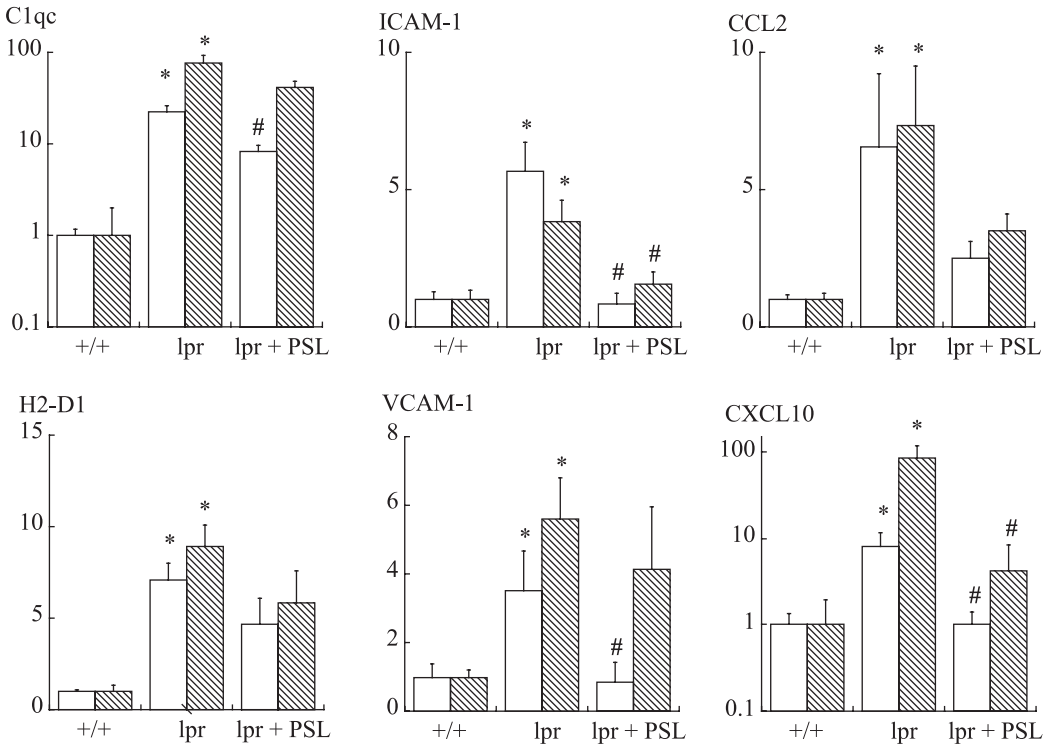

Fig. 3. Glomerular mRNA expression of representative genes determined by microarray and real-time RT-PCR. Vertical axis indicates the expression levels of individual genes. Open column: microarray data. Hatched column: RT-PCR data. Average values of expression levels in MRL+/+ mice were assigned to a unity. Results were expressed as the mean ± S.E.M. **P*<0.05, compared to MRL+/+ mice; #*P*<0.05, compared to MRL/lpr mice.

glomeruli of lpr mice. Cx3cr1 was also up-regulated in lpr mice. The expression of these chemokines and their receptors were in most cases attenuated by prednisolone. Among adhesion molecules, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were up-regulated in lpr mice. Expressions of integrins alpha L (CD11a), alpha M (CD11b), alpha X (CD11c), and beta 2 (CD18) were also increased in the glomeruli of lpr mice, suggesting that LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18, CR3), counterparts of ICAM-1, and CR4 (CD11c /CD18) were up-regulated. These changes were attenuated by prednisolone. A number of MHC class I and class II molecules showed high expression in lupus glomeruli. There were increased gene expressions in lpr glomeruli of immunoproteasomes (Psmb 8/LMP7, Psmb 9/LMP2, and Psmb 10/MECL1) (11), Tap1, and Tapbp that are implicated in processing for MHC class I–restricted antigen presentation. These changes were attenuated by prednisolone. There were increased gene expressions of extracellular matrices (ECM) such as several species of procollagen, biglycan, and fibronectin; inhibitors of ECM degradation; tissue inhibitor of metalloproteinase-1 (TIMP-1), plasminogen activator inhibitor-1 (PAI-1/Serpine 1) and S100A4 (fibroblast specific protein-1), possibly reflecting mesangial proliferation and ECM deposition in lpr mice. These changes were attenuated by prednisolone.

Real-time RT-PCR in representative genes

To verify the validity of microarray analysis, real-time RT-PCR assays were performed for selected genes (Fig. 3). Although magnitudes of changes in gene expression are not necessarily and completely the same, the patterns of the changes are almost the same. These results confirmed the validity of the microarray data obtained in the present experiments.

Discussion

In the present experiments, we found that mRNA expressions of over 500 genes were up-regulated in the glomeruli of lupus mice. These genes include a number of 1) complement components, 2) adhesion molecules, 3) chemokines and chemokine receptors, 4) genes involved in antigen processing and presentation, and 5) extracellular matrix and related molecules. Although some of the genes categorized in these functional groups

have been separately shown to participate in the pathogenesis of lupus (6, 12 – 16), we also identified a number of genes not previously associated with lupus glomeruli. These increased gene expressions in the glomeruli were accompanied by mesangial proliferation and leukocytes infiltration. Prednisolone treatment attenuated most of these mRNA increases with concomitant reduction in enhanced cellular proliferation and leukocytes infiltration within the glomeruli. This study is the first to examine the global gene expression profile of the glomeruli of lupus-prone mice, MRL/lpr, and also to study the therapeutic effects of glucocorticoid on this profile. Liu et al. (4) recently performed genome-wide mRNA expression analysis in the kidneys of MRL/lpr mice throughout the disease course. Although their study provided valuable information on the renal disease process, they used whole kidney tissue. The architecture of the kidney is complex and different structural regions of the kidney exhibit distinctive gene expression profiles (5). Furthermore, pathologic features of lupus nephritis are diverse, variably affecting the different renal compartments. Thus, our study has the merit of elucidating molecular mechanisms of lupus-induced glomerular lesions separately from different renal compartments such as tubules, blood vessels, and renal interstitial space.

One of the new findings of the present study is an additional evidence supporting glomerular accumulation of T helper 1 (Th1) cells and their role in the pathogenesis of glomerular injury observed in MRL/lpr mice (17, 18). Particularly, important chemokines for Th1 cells were the CXCR3 ligands, CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (I-TAC), and the CCR5 ligands, CCL3 (macrophage inflammatory protein-1α), CCL4 (macrophage inflammatory protein-1β), and CCL5 (RANTES) (19 – 21), all of which gene expressions were markedly up-regulated in the glomeruli of lpr mice. Furthermore, corresponding chemokine receptors also showed elevated expression. There was an up-regulation of Bonzo/CXCR6 that is also closely associated with type-1-polarlized T cells (22) with concomitant elevation of its interacting ligand CXCL16. This is the first time to detect increased glomerular transcripts of the above-mentioned chemokines and chemokine receptors except CCL5 and CCR5 that were previously reported (15). Thus, our results suggest that glomeruli of lpr mice produced various chemokines that stimulated Th1 cell migration and resulted in glomerular accumulation of Th1 cells carrying corresponding chemokine receptors. Therefore, therapy aiming to prevent Th1 cell recruitment by interrupting these chemokines–chemokine-receptor interactions would be promising in treating lupus nephritis. In fact, a CXCL10 antagonist ameliorated the progression of autoimmune sialadenitis of MRL/lpr mice in which lesion up-regulation of Th1-associated CXCL9, CXCL10, and CXCL11 mRNA was evident, whereas Th2-associated chemokines were almost undetectable (23). Unfortunately, these authors did not mention whether renal injury was also affected. It has been suggested that CXCL9 and CXCL10 not only act as chemoattractants for infiltrating mononuclear cells in the inflamed tissue but also directly induce the proliferation of mesangial cells, suggesting the role of these chemokines in the development of proliferative glomerulonephritis (24). Such mechanisms may partly explain the glomerular injury observed in lupus mice.

Th1 responses induce the activation of macrophages and cytotoxic T-lymphocytes and also elicit immunoglobulin IgG subclass switching to favor complement fixation and opsonization. This Th1 subset is characterized by the production of interferon-γ (IFN-γ) (25) that is a potent macrophage activator. It was reported that MRL/lpr mice lacking IFN-γ receptor develop no obvious glomerulonephritis but elicit IgG subclass switching (26). Our microarray data revealed that glomerular IFN-γ expression was about 10-fold higher in lupus mice than in controls and decreased with glucocorticoid treatment, although these differences did not reach statistical significance due to a large variation of data in lupus glomeruli (Fig. 4). It was reported that renal IFN-γ mRNA expression of MRL/lpr mice is higher than that of control mice (4, 27). Furthermore, a number of IFN-γ gene "signatures" were identified from our microarray data. Possible IFN-γ gene signatures include chemokines (CXCL9, CXCL10, and CXCL11); MHC class I molecules and related antigen-processing molecules [immunoproteasome (Psmb8, Psmb9 and Psmb10), Tap1, and Tapbp]; MHC class II molecules and adhesion molecules (ICAM-1 and VCAM-1) (28 –

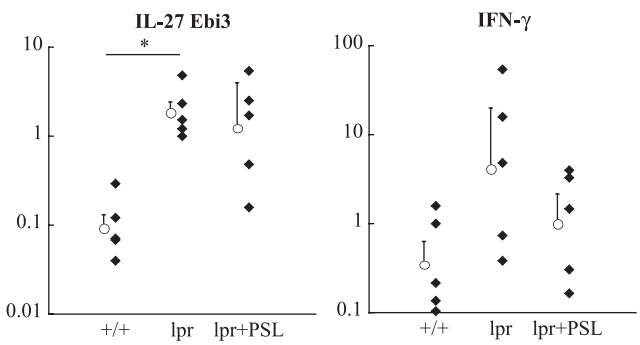

Fig. 4. Glomerular expression of IL-27 Ebi3 and interferon-γ determined by microarray. Vertical axis indicates normalized expression signals. Individual expression signals were plotted. Open circle indicates average expression signal of each group and error bar indicates S.E.M. *P<0.05

30). Genes that were shown to be induced by IFN-γ are listed in the supplemental table. These 58 genes are minimal estimates of the genes that are inducible by IFN-γ. Liu has previously documented such IFN-γ gene signatures in the whole kidney extract of MRL/lpr mice. We extended their findings and demonostrated IFN-γ gene signatures within the glomeruli of lupus mice. thereby further supporting the role of Th1 cells and IFN-γ in the development of proliferative glomerulonephritis in MRL/lpr mice.

Of note is the novel finding that an IL-12p40-related protein, Ebi3, is expressed at high level in MRL/lpr glomeruli. Ebi3 dimerizes with p28 to form IL-27 (31). IL-27 is a newly described member of the IL-12 family. Although it is well established that IL-12 is a prerequisite for committing CD4 T cell into a Th1 cell subset, IL-12 deficiency in MRL/lpr mice had minimal effects on glomerular pathology (32). Shimizu recently reported that deletion of the IL-27 receptor (WSX-1) dramatically changed the pathophysiology of glomerulonephritis developing in MRL/lpr mice and prolonged survival (17). Glomerular inflammatory cell infiltration and proliferative lesions became absent and as a result, renal lesion resembling diffuse proliferative glomerulonephritis (World Health Organization class IV) disappeared in the IL-27–receptor knockout mice. In turn, all of the glomeruli showed diffuse thickening of the basement membrane, indicating membranous changes occurring in IL-27–receptor deficient MRL/lpr mice. Such glomerular lesions observed in IL-27–deficient mice resemble human membranous glomerulonephritis (World Health Organization class V). These authors concluded that IL-27–receptor deficiency favors the Th2-type autoimmune response and suggested that Th1/Th2 balance may be a pivotal determinant of the phenotype of lupus nephritis. Although glomerular overexpression of Ebi3 may be involved in the glomerular injury in MRL/lpr mice, further detailed study is required that is beyond the scope of the present study.

It has been demonstrated that type I IFN-inducible transcripts are highly expressed in peripheral blood of SLE patients especially during active renal disease (33). Type I IFNs, including INF-α, as well as INF-β play a critical role in innate immunity and defense against viruses. The pathologic importance of type I IFN-inducible genes were well appreciated in human SLE (34). Peterson et al. (35) performed microarray experiment and analyzed the glomerular gene expressions of focal/diffuse proliferative lupus glomerulonephritis from clinical biopsy samples. They found high expression of a gene cluster containing type I IFN response elements in a subset of samples that were associated with reduced expression of fibrosis-related genes and milder pathological features. Thus, the role of type I IFN-inducible gene transcripts within lupus glomeruli remains unclear. Interestingly, glomerular injury in MRL/lpr mice was reduced in IFN-γ–receptor knockouts and enhanced in IFN-α/β–receptor knockouts (36), suggesting the protective role of type I IFNs in the glomerular injury observed in MRL/lpr mice.

In the present study, we found up-regulation of several complement components (C1q, C2, and C3) and their receptor (C3ar1) gene expressions within the glomeruli of MRL/lpr. Deposits of immunoglobulin and complement components, particularly C3, are identified in the kidney with progression of the disease. Immune complex–mediated activation of complement through the classical pathway is traditionally believed to be a major mechanism by which glomerular injury occurs (37). C3a anaphylatoxin receptor (C3ar) antagonism significantly reduced renal disease with concomitant reduction of IL-1β and CCL5 mRNA in MRL/lpr mice (38), supporting the pathologic role of the complement system. In contrast, genetic deletion of C3 in MRL/lpr mice did not affect glomerular pathology (39). Thus, it requires further study to clarify the role of locally produced complements within the lupus glomeruli.

Adhesion molecules, various chemokines, and chemokine receptors were markedly up-regulated with a concomitant increase in glomerular leukocyte influx, suggesting a cause-and-effect relationship. LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) are members of the β2 integrins and mainly expressed in neutrophils, macrophages, and/or T cells. It is reasonable to speculate that over-expressed β2 integrins participated in firm adhesion and extravasation of leukocytes in concert with up-regulated ICAM-1, presumably expressed on endothelial cells. This idea was supported by the previous finding that MRL/lpr mice deficient in LFA-1 but not Mac-1developed attenuated glomerular injury with less neutrophil infiltration (40). In contrast, although ICAM-1–deficient lpr mice showed milder glomerular lesion, these mice were not protected from leukocyte infiltration, indicating other adhesion molecules are able to facilitate transendothelial migration of leukocytes (41). Chemokines also helped to control the selective migration and activation of inflammatory cells. Each chemokine binds to several different receptors and differential expression of the receptors by distinct leukocyte subset is an important component of the specificity of chemokine action (20, 42). There have been direct evidences that CCL2 and CCR2 are involved in glomerular influx of macrophages and T cells in MRL/lpr mice (43 – 46). In the present experiment, we found increased expression of many chemokines and chemokine receptors that have not been previously

reported. For instance, increased gene expression of CXCL2 (47) observed in the present study may explain neutrophil accumulation in the glomeruli of lupus mice. Pathophysiological roles of individual chemokines in glomerular injury should be tested in the future. The increased glomerular expression of transcripts in the pathway of MHC class I and II antigen presentation suggests a glomerular potential to present peptide antigen to T cells.

In the present study, prednisolone treatment markedly attenuated glomerular injury observed in MRL/lpr mice. The beneficial effects were accompanied by inhibition of most of enhanced gene expressions including the complement system, adhesion molecules, chemokines and chemokine receptors, and molecules related to the pathways of antigen presentations. These results would possibly provide the fundamental information to understand the mechanism of the beneficial effects of glucocorticoid on glomerular injury of MRL/lpr mice.

In summary, we found that glomerular proliferative lesions and inflammatory cellular influx were associated with increased gene expressions including a number of complement components, adhesion molecules, chemokines and their receptors, and molecules related to antigen processing and presentation, suggesting the role of these molecules in the development of glomerular injury in MRL/lpr mice. We observed an increased gene expression of various chemokines and their receptors that is suggestive of glomerular Th1 cell accumulation where locally expressed IL-27 may play a role. Enhanced expression of IFN-γ signature also supports the role of Th1 cells in the pathogenesis of glomerular injury in MRL/lpr mice. Since our current knowledges about the individual genes and their network are not sufficient, our ability to interpret the entire data is so far limited at this moment. Nevertheless, we believe that our study provides an important resource for seeking new therapeutic targets for the treatment of lupus nephritis.

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