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Role of MHC-Linked Genes in Autoantigen Selection and Renal Disease in a Murine Model of Systemic Lupus Erythematosus

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We previously described a renal protective effect of factor B deficiency in MRL/lpr mice. Factor B is in the MHC cluster; thus, the deficient mice were H2b, the haplotype on which the knockout was derived, whereas the wild-type littermates were H2k, the H2 of MRL/lpr mice. To determine which protective effects were due to H2 vs factor B deficiency, we derived H2b congenic MRL/lpr mice from the 129/Sv (H2b) strain. Autoantibody profiling using autoantigen microarrays revealed that serum anti-Smith and anti-small nuclear ribonucleoprotein complex autoantibodies, while present in the majority of H2 k/k MRL mice, were absent in the H2 b/b MRL/lpr mice. Surprisingly, 70% of MRL/lpr H2 b/b mice were found to be serum IgG3 deficient (with few to no IgG3-producing B cells). In addition, H2 b/b IgG3-deficient MRL/lpr mice had significantly less proteinuria, decreased glomerular immune complex deposition, and absence of glomerular subepithelial deposits compared with MRL/lpr mice of any H2 type with detectable serum IgG3. Despite these differences, total histopathologic renal scores and survival were similar among the groups. These results indicate that genes encoded within or closely linked to the MHC region regulate autoantigen selection and isotype switching to IgG3 but have minimal effect on end-organ damage or survival in MRL/lpr mice. The Journal of Immunology, 2006, 177: 7423–7434.

Major histocompatibility complex haplotypes encode scores of immunologically important genes, including the highly polymorphic MHC class I and class II gene clusters, complement cascade components (C4, C2, and factor B), cytokines (Tnfα and Lta), proteins involved in Ag processing (Lmp2 and Tap1), and chaperones (Hsp70) (1, 2). The murine H2 region, located on chromosome 17, is orthologous with the human MHC on chromosome 6. An exception is the class I region in which the composition and arrangement of class Ib genes differ markedly. MHC haplotypes are genetic determinants of susceptibility to multiple diseases with an autoimmune etiology, including type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE) (3, 4).

MHC is a prototypic autoimmune disease characterized by production of autoantibodies, as well as inflammation in target organs, including the kidneys (5). A host of autoantigens are targeted in humans and in murine models of SLE, including components of the nucleosome (e.g., histones and DNA), the U1 small nuclear ribonucleoprotein complex (U1-snRNP), the Ro/La complex, and phospholipids. Genome-wide scans of SLE multiplex families revealed that MHC is the strongest of candidate lupus susceptibility loci. Using a variety of MHC gene markers, population-based studies showed that the MHC class II DR-B1 alleles DR2 and DR3 are associated with SLE in European-Caucasian populations (6, 7). However, MHC gene associations with SLE are complicated by the existence of extended HLA haplotypes in which class II genes are in significant linkage disequilibrium with other potential lupus susceptibility genes, including C4, C2, Tnfα, and other MHC class III genes (6).

New Zealand Black (NZB)/New Zealand White (NZW), BXSB, and MRL/lpr are inbred mouse strains that spontaneously develop a disease similar to human SLE (8). The genetic basis of disease in lupus-prone mice is complex. However, data from backcross and intercross experiments demonstrated linkage of disease with genes encoded within or closely linked to the MHC locus in NZB/NZW and BXSB mice (9). However, similar studies in MRL/lpr mice have not established a link between MHC and disease expression (10). Class II expression appears required for disease in MRL/lpr mice because MHC class II-deficient MRL/lpr mice (H2b) did not produce serum anti-DNA Abs or develop proliferative renal disease in contrast to their wild-type (H2b) counterparts (11). However, a direct link between these findings and MHC class II-deficiency is still

1 Abbreviations used in this paper: SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein complex; NZB, New Zealand Black; NZW, New Zealand White; RF, rheumatoid factor; Sm, Smith; DFU, digital fluorescence unit; SAM, significance analysis of microarray; GA, glomerular Ag; TMB, 3,3',5,5'-tetramethylbenzidine; BBS, borate-buffered saline; EM, electron microscopy; GBM, glomerular basement membrane.

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unclear because the extended H2 of the MHC class II-deficient H2\(b\) mice was different from the control H2\(\alpha\) MRL/lpr mice.

Lupus nephritis is a major cause of morbidity and mortality in human SLE, as well as in murine models of disease (12, 13). Deposition of glomerular immune complexes is the initiator of the inflammatory process in lupus nephritis (14, 15). Anti-DNA Abs are the predominant autoantibody eluted from glomeruli both in human SLE and MRL/lpr mice (16, 17). IgG3 is the dominant IgG isotype eluted from the kidneys of MRL/lpr mice (18), and IgG3 cryoglobulins with rheumatoid factor (RF) activity are associated with renal disease in this murine model (19, 20). We previously reported that MRL/lpr mice deficient in the alternative complement pathway component factor B develop less renal disease, survive longer, and a majority were serum IgG3 deficient. As the gene for factor B is in the MHC locus, the H2 of the factor B-deficient MRL/lpr mice was H2\(b\), the genotype of the strain (129/Sv) on which the factor B knockout was derived originally. The wild-type littermates were the H2 of MRL/lpr mice, H2\(k\). To determine which of the observed differences in factor B-deficient MRL/lpr mice were due to H2 vs factor B deficiency, we derived H2\(b\) MRL/lpr mice.

By comparing H2\(b/k\) wild-type MRL/lpr mice with the H2\(b/k\) congenic MRL/lpr mice, we provide evidence that genes within or closely linked to MHC are critical for specific features of disease. Protein microarray analysis demonstrated that H2\(b/k\) MRL/lpr mice lacked autoantibodies directed against Smith (Sm) and other snRNP autoantigens. The majority of MRL/lpr H2\(b\) mice was IgG3-deficient with decreased glomerular immune complex deposition, basement membrane subepithelial immune complex deposits, and proteinuria. These profound differences in phenotype, however, had no impact on proliferative renal disease, vasculitis, or survival.

Materials and Methods

Mice

129X1/SvJ (129/Sv; H2\(b\)), C57BL/6J (B6; H2\(b\)), BALB/cJ (BALB/c; H2\(b\)), and MRL/MpJ-lpr mice were purchased from The Jackson Laboratory. Congenic MRL/lpr mice were developed from The Jackson Laboratory. Congenic MRL/lpr mice were derived in seven generations to derive F9 H2\(b/k\), H2\(b/k\), and H2\(k/k\) MRL/lpr mice. At each generation, the haplotype of H2 was determined with four microsatellite markers (86 markers on chromosome 17; 50 markers on the other chromosomes) were screened for genotyping and verified that all of the markers outside of MHC region were MRL/lpr. Phenotypic confirmation of genetic markers was determined by flow cytometric analysis of H2 class I (K) and class II (I-A and I-E) expression on peripheral mononuclear cells. All F9 congenic mice were confirmed homozygous for the lpr mutation in the Fas gene by PCR (21). Mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Ralph H. Johnson Veterans Affairs Medical Center. The Ralph H. Johnson Veterans Affairs Institutional Animal Care and Use Committee approved the protocols used for experiments with mice.

Array production and probing

Screening of mouse serum for autoantibodies directed against major autoantigens in autoimmune rheumatic disease was performed using autoantigen microarray technology (22). A robotic arrayer was used to attach peptides and proteins to poly-l-lysine-coated slides (CEL Associates) in an ordered array (22, 23). Four to 12 replicate features of each peptide or protein were printed on each array. Spotted Ags included 36 recombinant or purified proteins, including Ro52, La, Jo-1, serine/arginine proteins, histones H1, H2A, H2B, and H3 plus H4, U1snRNAP-BB\(c\), U1–68 kd, U1snRNAP-A, InRNP-B1, Sm/RNP complex, Sm Ags, topoisomerase I, centromere protein B, and pyruvate dehydrogenase; 6 nucleic acid-based putative Ags, including dsDNA and ssDNA; and 154 overlapping synthetic peptides derived from snRNP proteins, Sm proteins, polypeptide-ribosome polymerase, and histone H1, H2A, H3, and H4. Arrays were circumscribed with a hydrophobic marker, blocked overnight at 4°C in PBS containing 3% FCS and 0.05% Tween 20, incubated with 1/150 dilutions of mouse serum in blocking buffer for 1 h at 4°C, and washed twice in blocking buffer. Arrays were incubated with 1/4000 dilutions of cytochrome c dye-conjugated goat anti-mouse IgG/M (Jackson ImmunoResearch Laboratories) for 1 h at 4°C and then washed twice in blocking buffer, PBS, and water. Arrays were then dry and scanned with a Gene Pix 4000B scanner (Axon Instruments). Detailed protocols are published (22) and are available online (www.stanford.edu/group/antigenarrays/). False-color images derived from the scanned digital images are presented.

Analysis of array data

The median feature and background pixel intensities for each Ag feature were determined using GenePix Pro 3.0 software (Axon Instruments), from which the net fluorescence intensity (expressed as digital fluorescence units (DFU)) was calculated for individual features. For each Ag, a raw value was generated from the median of net fluorescence values for all features representing that Ag (4–12 features per Ag). For statistical analysis, an adjustment of raw values was performed to eliminate negative values. If the raw value for an Ag was < 1.0 on any array in the data set, the values for that Ag were adjusted for all arrays in the data set as follows: 1.0 plus the absolute value of the lowest raw value was added to the raw value for that Ag for all arrays in the data set to generate adjusted raw values. Normalization between arrays was then performed. For each array, the median of raw values for eight identical anti-mouse IgG features was used to normalize data sets between arrays. Adjusted raw values for each array were multiplied by a normalization factor so that the normalized median for the anti-mouse IgG reactivity was equal to 17,500. Normalized values were analyzed using the significance analysis of microarrays (SAM) algorithm (24).
Threshold parameters were selected so that negative control Ags were excluded and false discovery rates (q values) were <0.05%. Two-class SAM identified Ags with significant differences in reactivities between 20-wk-old H2b/b and H2k/k MRL/lpr mice. Thresholds for positive values included an absolute difference in normalized DFU between groups >500 and a false discovery rate (q) of <0.05 (25). Ags meeting these criteria were designated as positive and selected for cluster analysis. Positive SAM hits were subjected to hierarchical clustering using Cluster software (26) and results displayed using TreeView software (26).

Autoantibody ELISA

Serum autoantibody levels against dsDNA and glomerular Ags (GA) were measured as described previously (27). For the anti-DNA ELISA, plates were coated overnight at 37°C with double-stranded calf thymus DNA (5 μg/ml; Sigma-Aldrich) diluted in SSC buffer. For the anti-GA ELISA, plates were coated for 90 min with rat GA (40 μg/ml) diluted in PBS. After washing, serum was added in serial dilutions starting at 1/100 dilution. The assay was then performed as described above with HRP-conjugated goat anti-mouse IgG (Southern Biotechnology Associates). Results for histones were performed similarly. The anti-Sm ELISA was performed as described previously (28). Briefly, ELISA plates were coated with 10 U/ml Sm Ag (Immunovision) in borate-buffered saline (BBS) at 4°C overnight, washed, and blocked with BBS containing 1% BSA. Serum diluted in BBS-Tween 20/1% BSA was incubated in the plates for 2 h. The assay was then performed as described above with HRP-conjugated goat anti-mouse IgG or anti-mouse IgG subclasses, except for using BBS instead of PBS. Assays for snRNP were performed similarly (Immunovision).

Measurement of serum Ig isotype levels

Serum Ig levels were measured by ELISA. Briefly, microtiter plates were coated overnight at 4°C with goat anti-mouse Ig (M plus G plus A) (2 μg/ml; Southern Biotechnology Associates) or goat anti-mouse IgG (1 μg/ml; Southern Biotechnology Associates) diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Sera and Abs were diluted in

FIGURE 2. Connective tissue disease arrays. Ordered Ag arrays were produced using a robotic microarrayer to attach lupus-associated Ags and Abs directed against mouse IgG (α-IgG) to poly-L-lysine-coated microscope slides. Individual arrays were probed with diluted mouse sera. The positions of select autoantigens and control features are indicated. A, An array probed with normal control C57BL/6 mouse serum showed no autoantibody reactivity. B, Representative images of arrays probed with sera from two 20-wk-old F9 H2b/b and H2h/k MRL/lpr mice. C, Representative images of arrays probed with sera from two F9 H2b/b MRL/lpr mice. D, Quantitative analysis of A–C. Results are expressed as normalized DFU (see Materials and Methods).
PBS-Tween 20 (PBS-T) (0.05%) containing 1% BSA (PBS-T/1% BSA). Serum dilutions were then incubated in the coated wells for 1 h, and bound Abs were detected with HRP-conjugated anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates) and developed with TMB (Sigma-Aldrich), 0.1 M citrate buffer (pH 4.0), and 0.015% H₂O₂. Purified mouse Ig isotypes (Southern Biotechnology Associates) were used as standards. Absorption at OD₅₀₀ was determined on a flow microtiter plate reader (Dynatech Laboratories). Standard curves and serum Ab concentrations were calculated using DeltaSoft 3 (version 2.2; BioMetallics), and statistical analysis was performed with GraphPad Prism software (version 3.0; GraphPad).

**ELISPOT assay**

The number of IgM, IgG1, IgG2a, IgG2b, or IgG3 secreting cells in the spleen was assessed by ELISPOT. Ninety-six-well microtiter plates (Millipore) were coated overnight with 1 µg/ml goat anti-mouse IgG (M plus G plus A; Southern Biotechnology Associates) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and then washed with endotoxin-free Dulbecco’s PBS and blocked with 200 µl/well of PBS containing 3% BSA. The plates were washed with DMEM containing 3% BSA and incubated in triplicate with spleen cells in serial dilutions starting at 1 × 10⁸ cells/well in 100 µl of DMEM with 3% BSA, 2.5% FCS, streptomycin, and penicillin. Following a 2-h incubation, the plates were washed with 1 M NaCl and PBS-T (0.25%). Then, the plates were incubated with 100 µl/well biotinylated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (each 0.5 µg/ml; Southern Biotechnology Associates) in 1% BSA/PBS-T, followed by 50 µl/well streptavidin-HRP (0.2 µg/ml; Jackson ImmunoResearch Laboratories). After washing, the plates were developed with 50 µl/well of a substrate solution containing 0.3 mg/ml 3-amino-9-ethylcarbazole (Sigma-Aldrich) in 0.05 M sodium acetate (pH 5.0) and 0.015% H₂O₂. The reaction was stopped by washing with tap water, and the number of spots were counted using a dissecting microscope.

**Measurement of urine albumin excretion**

Mice were placed in metabolic cages for 24-h urine collection every 2 wk beginning at 12 wk of age. Urinary albumin excretion was determined by ELISA using a standard curve of known concentrations of mouse albumin (Cappel). Briefly, 96-well ELISA plates were coated with 2.5 µg/ml goat anti-mouse albumin (Research Diagnostics) overnight at 4°C. After washing with PBS-T, PBS-0.25% gelatin (Sigma-Aldrich) was added to each well to block nonspecific binding. Urine samples were added in serial dilutions, starting at a 1/10 dilution, and incubated for 1 h at room temperature. After washing with PBS-T, HRP-conjugated goat anti-mouse albumin (Research Diagnostics) was added. After additional washing, the plates were developed with TMB solution, and urine albumin concentration was calculated using DeltaSoft 3 (version 2.2; BioMetallics), and statistical analysis was performed with GraphPad Prism software (version 3.0; GraphPad). Data are reported as milligrams of albumin/mouse/day.

**Renal pathology**

At the time of sacrifice (32 wk of age), the kidneys were removed. One kidney was fixed with 10% buffered formalin, embedded in paraffin, then sectioned before staining with H&E. The other half kidney was snap-frozen in liquid nitrogen and placed in OCT medium. Four-micrometer-thick frozen sections were stained with fluorescein-conjugated anti-mouse total IgG, IgG3 (Southern Biotechnology Associates), or C3 (Cappel). The remaining kidney was diced into small pieces (~2 mm), dipped in glycolaldehyde buffer, and prepared for electron microscopy (EM) analysis by standard procedure. The H&E kidney slides were graded for glomerular inflammation, proliferation, crescent formation, and necrosis. Scores from
0 to 3+ (0, none; 1+, mild; 2+, moderate; 3+, severe; scores of crescent formation and necrosis were doubled) were assigned for each of these features and then added together to yield a final renal score. Immunofluorescence slides were graded 0 to 3+ (0, none; 1+, mild staining; 2+, moderate staining; 3+, high staining) for fluorescence intensity. EM samples were assessed for morphological changes of glomerular cells, glomerular immune electron-dense deposit localization (i.e., mesangial, subendothelial, and subepithelial deposition) and podocyte foot process fusion, and were graded 0 to 3+ (0, none; 1+, mild; 2+, moderate; 3+, severe). All pathological assessments were performed in a blinded fashion.

Statistics

The unpaired Student’s t test was used to test for significant differences between groups. The Mann-Whitney two-tailed U test or Kruskal-Wallis test was used to determine the significance of changes in glomerular IgG, IgG3, and C3 deposition (see Fig. 6B), renal score (see Fig. 6C), and glomerular electron-dense deposits (see Fig. 6D). A value of p < 0.05 was considered significant.

Results

Generation of study mice

129/Sv (H2b) mice were backcrossed for nine generations to MRL/lpr mice. After nine generations of backcrossing, H2b heterozygous MRL/lpr mice were intercrossed to obtain F0 H2b/K, H2b/K, and H2b/B MRL/lpr mice. The termini of the H2b interval of F0 congenic MRL/lpr mice were mapped with microsatellite markers located near the selected four H2 markers. As shown in Fig. 1, the H2b interval extended 1 cM centromeric of D17Mit16 and 0.5 cM telomeric of D17Mit177. Thus, the centromeric crossover point on H2 was between D17Mit198 (16.0 cM) and D17Mit61 (16.4 cM), and the telomeric crossover point was between D17Mit105 (24.5 cM) and D17Mit108 (24.7 cM). Extensive genotyping to confirm lack of recombinational events in H2 and to exclude admixture outside of the H2 is detailed in Materials and Methods and summarized in Fig. 1. Phenotypic confirmation of genetic markers was determined by flow cytometric analysis of H2 class I (K) and class II (I-A, and I-E) expression on PBMC from mice of each F9 H2k/K, H2b/K, and H2b/B MRL/lpr strain (data not shown).

To assess the effect of H2 haplotype on disease expression in MRL/lpr mice, we evaluated the mice biweekly for development of skin rash, ear necrosis, and lymphadenopathy beginning at 8 wk of age until the time of sacrifice (32 wk old; n = 12–16 in each group). No differences were observed between the three groups in these external manifestations of disease (data not shown). At the time of sacrifice, there were no differences in body weight or spleen weight between the three groups (data not shown).

Serum autoantibody assays

To assess the effect of H2 haplotype on the spectrum of autoantibody production in MRL/lpr mice, we used recently developed
autoantigen microarray technology. Using 1152-feature arrays composed of 140 Ags or Ag fragments, we analyzed serum derived from six different H2b/b and H2k/k mice at 20 wk of age. Arrays were scanned and images analyzed as described in Materials and Methods. The data sets were analyzed using the SAM algorithm, applying highly stringent false discovery rates (5%) as cutoffs. Using these criteria, SAM identified seven Ags (U1snRNP-A, U1snRNP-BB, Sm/RNP complex, Sm Ag, H2glycoprotein I, histone H2B, and histone H3+H4) that were differentially recognized by sera from H2k/k compared with H2b/b MRL/lpr mice (Figs. 2 and 3). To confirm these findings, ELISA for these Ags were performed. Although the Ags used in the microarray experiments were the same as those used for the confirmatory ELISA, the differences between the two groups in serum levels of Abs to H2glycoprotein I, histone H2B, and histone H3+H4, although present, were not significant when analyzed by ELISA (data not shown). However, microarray results for four of the Ags—U1snRNP-A, U1snRNP-BB, Sm/RNP complex, and Sm Ag—were validated by ELISA (Fig. 3B). These results indicate a profound effect of MHC on autoantigen selection in MRL/lpr mice. These results also demonstrate that autoantigen arrays are a useful tool for discovery of novel autoantibody reactivities in murine SLE; testing that is impractical for individual Ags by ELISA techniques.

Serum anti-dsDNA Ab, anti-GA Ab, and IgG3 RF with cryoglobulin activity are major nephritogenic autoantibodies in MRL/lpr mice (20, 29, 30). To determine whether H2 impacts the serum levels of key pathogenic autoantibodies in MRL/lpr mice, we analyzed serum levels of these autoantibodies by ELISA. Although serum from all animals contained anti-dsDNA and
anti-GA Abs, levels were significantly decreased in the H2b/b MRL/lpr mice compared with the other two groups (Fig. 4, A and C). We next analyzed anti-dsDNA levels of each IgG isotype and found that IgG3 was the only IgG anti-dsDNA isotype that was reduced in the H2b/b MRL/lpr mice (Fig. 4B). Serum IgG3 anti-IgG2a RF levels were also significantly lower in the H2b/b MRL/lpr mice compared with the other two groups (Fig. 4D), similar to our findings in the factor B-deficient MRL/lpr mice (31). These results suggested that lower levels of anti-dsDNA Ab, anti-GA Ab, and IgG3 anti-IgG2a RF in the H2b/b MRL/lpr mice were due to IgG3 deficiency alone.

To determine whether the lack of serum anti-Sm levels in the H2b/b MRL/lpr mice was also due to decreased IgG3 alone, we analyzed serum anti-Sm levels for each IgG isotype. We found none of the H2b/b MRL/lpr mice had anti-Sm Abs of any IgG isotype above background, whereas the H2k/k MRL/lpr mice had.

FIGURE 6. Renal disease assessment in MRL/lpr mice. A, Urinary albumin excretion levels in the H2k/k, IgG3+ H2b/b, and IgG3- H2b/b MRL/lpr mice. Data presented are the mean 24-h albumin excretion (mg/mouse/day) ± SEM in each group. n = 11, 8, and 11 in the H2k/k, IgG3+ H2b/b, and IgG3- H2b/b MRL/lpr mice, respectively. B, Albuminuria development (Kaplan-Meier curve) in the H2k/k, IgG3+ H2b/b, and IgG3- H2b/b MRL/lpr mice. Mice excreting urinary albumin (>0.1 mg/mouse/day) were counted until the time of sacrifice (32 wk). n = 11, 8, and 11 in the H2k/k, IgG3+ H2b/b, and IgG3- H2b/b MRL/lpr mice, respectively. Albuminuria in H2k/k mice was similar to the IgG3+ producing groups (data not shown). C, Immunofluorescence assessment of glomerular IgG, IgG3, or C3 deposition of 32-wk-old MRL/lpr mice. D, Pathologic renal scores of 32-wk-old MRL/lpr mice. E, Glomerular immune electron-dense deposit localization and podocyte foot process fusion. EM analysis revealed no glomerular subepithelial dense deposits and reduced levels of foot process fusion in the IgG3- H2b/b MRL/lpr mice. n = 6–8 in each group. *, p < 0.05; **, p < 0.01.
detectable levels of serum anti-Sm Abs of each IgG isotype (Fig. 4, E and F), indicating that lower serum anti-Sm levels in the H2b/b MRL/lpr mice was not due to IgG3 deficiency alone.

**Serum Ig levels**

Given the effects of H2 on specific IgG isotype levels of anti-DNA Abs, we determined whether global serum Ig levels were affected by H2 genotype in the MRL/lpr strain. Serum IgM levels were statistically higher in the H2b/b MRL/lpr mice compared with the H2k/k and H2b/b groups (Fig. 5A, upper panel). Levels of serum IgG2a, the dominant IgG isotype in the serum of MRL/lpr mice (18), were also significantly higher in the H2b/b compared with H2k/k MRL/lpr mice (Fig. 5A, middle panel). Strikingly, 11 of the 16 H2b/b MRL/lpr mice had undetectable levels of serum IgG3 (Fig. 5A, lower panel), similar to our prior findings in MRL/lpr factor B-deficient mice (31). To confirm whether serum Ig isotype levels reflect the population of each Ig isotype-producing cell, we analyzed the population of each Ig isotype-producing cell in the spleen of F3 H2b/b MRL/lpr mice by ELISPOT analysis. As shown in Fig. 5B, 7 of 10 H2b/b MRL/lpr mice had no or very few (0–50/10^6 spleen cells) IgG3-producing cells in the spleen, whereas it was a readily evident in the spleen of H2k/k and H2b/b MRL/lpr mice as well as other Ig isotype-producing cells. Although it did not reach statistical differences, there was a trend toward increased numbers of IgM- or IgG2a-producing cells in the spleen of H2b/b MRL/lpr mice compared with H2k/k MRL/lpr mice. These results of ELISPOT analysis were consistent with the results of serum Ig measurement.

To verify that IgG3 deficiency was due to the H2b haplotype, we analyzed serum IgG3 levels in F3, B6 strain-derived H2b (H2b-B6) and BALB/c strain-derived H2b (H2b-BALB/c) MRL/lpr mice. The F3 H2b/B6 MRL/lpr mice had significantly decreased serum IgG3, with 7 of 15 being totally deficient (Fig. 5C). F3, H2b-k129/Sv MRL/lpr mice also had significantly decreased serum IgG3 levels (Fig. 5C). Serum derived from F3, H2b-BALB/c MRL/lpr mice had lower levels of serum IgG3 than H2b/k MRL/lpr mice; however, unlike H2b/B6 MRL/lpr mice, no H2d/B6 MRL/lpr mice were completely deficient in serum IgG3 (Fig. 5C). From these results, we concluded that the combination of H2b haplotype and unidentified genes within the MRL/lpr genetic background result in the observed deficiency in IgG3 production in 70% of H2b MRL/lpr mice.

**Assessment of glomerular nephritis**

IgG3 autoantibodies are reported to be pathogenic in glomerulonephritis in MRL/lpr mice. Total IgG, IgG3, and C3 glomerular deposition as assessed by immunofluorescence are depicted in the left three panels. Proliferative glomerular disease and inflammation are demonstrated in H&E-stained paraffin sections. B–D, EM analysis of kidneys from F9 H2b/b (B), IgG3−/−H2b/b (C), and IgG3+/−H2b/b (D) MRL/lpr mice. Both the H2b/b and IgG3+/−H2b/b mice had extensive subepithelial (red arrows), subendothelial (blue arrows), and mesangial (yellow arrows) electron-dense deposits, a thickened GBM (bm), and extensive fusion of podocyte foot processes (⁎). In contrast, in the glomeruli of the IgG3−/−H2b/b MRL/lpr mice, there were subendothelial and mesangial dense deposits, but no subepithelial deposits and little or no basement membrane thickening or foot process fusion. Each image is representative of the results obtained from one kidney of six to eight mice per group. us, urinary space; cl, capillary lumen; md, endothelial cell; m, mesangial cell; and ec, erythrocyte. Scale bars, 2 μm.

**FIGURE 7.** Histological, immunofluorescence, and EM studies of kidneys from MRL/lpr mice. A, Representative sections of kidneys from MRL/lpr mice differing in H2 and IgG3 production. Total IgG, IgG3, and C3 glomerular deposition as assessed by immunofluorescence are depicted in the left three panels. Proliferative glomerular disease and inflammation are demonstrated in H&E-stained paraffin sections. B–D, EM analysis of kidneys from F9 H2b/b (B), IgG3−/−H2b/b (C), and IgG3+/−H2b/b (D) MRL/lpr mice. Both the H2b/b and IgG3+/−H2b/b mice had extensive subepithelial (red arrows), subendothelial (blue arrows), and mesangial (yellow arrows) electron-dense deposits, a thickened GBM (bm), and extensive fusion of podocyte foot processes (⁎). In contrast, in the glomeruli of the IgG3−/−H2b/b MRL/lpr mice, there were subendothelial and mesangial dense deposits, but no subepithelial deposits and little or no basement membrane thickening or foot process fusion. Each image is representative of the results obtained from one kidney of six to eight mice per group. us, urinary space; cl, capillary lumen; md, endothelial cell; m, mesangial cell; and ec, erythrocyte. Scale bars, 2 μm.

To investigate the discrepancy between albuminuria, glomerular immune complex deposition, and renal score, we performed EM analysis on renal sections from all groups. The IgG3−/−H2b/b MRL/lpr mice had electron-dense deposits in the glomerular subendothelial space and mesangial matrix similar to the IgG3−/− groups (Figs. 6E and 7, B–D). Notably, none of the sections from IgG3+/−H2b/b mice had glomerular subepithelial deposits. Such deposits were readily evident in glomeruli from mice in the IgG3−/− groups regardless of H2 haplotype (Figs. 6E and 7, B–D). Furthermore, podocyte foot process fusion was significantly more evident in the IgG3−/− H2k/k mice compared with the IgG3−/− H2b/b mice (Figs. 6E and 7, B–D).
We also assessed a limited number of IgG3+ H2b/b MRL/lpr mice (n = 3) and observed intermediate changes compared with pathological features observed in H2k/k and H2b/b MRL/lpr mice (data not shown). These EM changes likely explain most of the differences in proteinuria between the groups.

Discussion
The data presented herein indicate that gene(s) encoded within or closely linked to the MHC region regulates autoantibody targeting of specific autoantigens in MRL/lpr mice and IgG3 isotype switching. The critical role of IgG3 in localization of glomerular immune complex deposition, and albuminuria, there was no difference in survival among the groups likely reflecting the similarities in proliferative renal disease in the three groups (Fig. 8; data of H2b/b mice is not shown).

An intriguing result in comparing H2b and H2k MRL/lpr mice was the stark difference in autoantibody specificity. Protein microarrays were only recently applied to the study of autoimmune disease, particularly to large-scale autoantibody profiling. Robinson et al. (22) demonstrated the use of autoantigen microarrays for measuring human autoantibodies that characterize eight distinct systemic rheumatic diseases. These studies were extended to two other animal models of human disease: the experimental autoimmune encephalomyelitis model of multiple sclerosis (32) and a monkey model of acquired immunodeficiency syndrome (33), as well as to the study of human patients with rheumatoid arthritis (34). Zhen et al. (35) recently reported the discovery of predictive autoantibody profiles in a congenic mouse model of SLE, further suggesting that multiplex autoantibody profiling might aid in uncovering pathogenic mechanisms that contribute to SLE pathogenesis.

In the current study, we used the previously validated connective tissue disease arrays (22) as a discovery tool to identify differences in autoantibody production between groups of animals expressing unique H2 haplotypes. This led to the novel discovery that a subset of prominent SLE autoantigens are frequently targeted in H2k/k but are completely absent in H2b/b MRL/lpr mice. It is unlikely that all these differences would have been identified using low-throughput assays such as ELISA to screen the hundreds of known candidate SLE Ags. Thus, these results demonstrate the use of autoantigen microarray technology as a discovery tool.

The differences between the H2k/k and H2b/b groups in serum levels of autoantibodies to three SAM-identified Ags—β-2-glycoprotein I, histone H2B, and histone H3—although present, were not statistically significant when analyzed by ELISA. However, it is worth noting that autoantigen arrays offer the ability to detect reactivity to autoantigens with sensitivity that is potentially superior to ELISA (22). The SAM algorithm was originally developed for analysis of DNA microarray data, and it has proven useful recently in the analysis of autoantigen array data (33, 34). SAM is capable of detecting differences between groups, which, although subtle, may nevertheless be clinically relevant. Analysis of larger numbers of samples for those Ags identified as significant by SAM will be required to directly test the validity of the algorithm. The current generation arrays are being expanded to include overlapping peptides with a goal to identify epitopes that we predict might bind to disease-specific MHC gene products.

In this study, both protein microarray analysis and conventional ELISA clearly demonstrated strong recognition of Sm Ag and snRNP proteins by sera from H2k/k MRL/lpr mice, whereas none of the H2b/b MRL/lpr mice had reactivity to these Ags above background activity in serum of BALB/c mice. Abs to Sm Ag are currently regarded as the most specific, although insensitive, marker of SLE. Abs to snRNP are also observed in sera from lupus patients, but they are present in much higher titers in sera from patients with mixed connective-tissue disease (36). Abs to Sm Ag and snRNP Ags are often linked in both patients and murine models of lupus (37). Our results indicate that gene(s) encoded within or closely linked to the MHC region (i.e., H2b interval in H2 congenic mice) regulates anti-Sm/RNP production in MRL/lpr mice. Indeed, in previous studies of MHC and human lupus, it has been suggested that reactivity to major autoantigens (Ro (SS-A), La (SS-B), ribosomal P, β-2-glycoprotein I, Sm Ag, and U1-RNP) is linked with HLA class II alleles (DQ and DR), except for antinuclear Ab and anti-sDNA (38, 39). HLA class II haplotypes carrying certain HLA-DR2 and -DR3 (and their linked HLA-DQ alleles) also have been associated with susceptibility to SLE. However, HLA class II alleles show stronger associations with specific autoantibody responses than with lupus itself or clinical features thereof (40). An association between HLA class II alleles and specific autoantibody generation is consistent with the concept of an Ag-driven process involving Th cell recognition. In previous studies of MHC and murine lupus, autoantibody production and lupus susceptibility were linked to the class II I-E-deficient haplotype carried in H2b/b mice in BXSB (41), (NZB × BXSB)F1 (42, 43), (MRL × BXSB)F1 (44), and B6/lpr mice (37). I-Eα, which is a homolog of human DRα-chain, is deficient in H2b mice due to a deletion of the promoter region of the Ea gene (45). In contrast to these studies, we found no accelerated autoantibody production in the I-E− H2b/b MRL/lpr mice. Indeed, there were reduced autoantibody levels and a more limited autoantibody spectrum in the I-E− H2b/b compared with the I-E+ H2b/b mice. Whether the observed differences in autoantibody specificity in this study are directly linked to I-E deficiency or other uncharacterized differences between H2b and H2k remains to be determined.

IgG3 deficiency accounts for some of the autoantibody differences between the H2b/b and H2k/k mice (i.e., anti-dsDNA and anti-H2b). An intriguing result in comparing H2b and H2k MRL/lpr mice is that despite these profound effects on disease parameters, an additional novel finding in these studies. The critical role of IgG3 in localization of glomerular immune complex deposition, and albuminuria, there was no difference in survival among the groups likely reflecting the similarities in proliferative renal disease in the three groups (Fig. 8; data of H2b/b mice is not shown).

FIGURE 8. Survival of MRL/lpr mice differing in H2 and IgG3 production. Mortality was observed until the time of sacrifice (32 wk). There were no differences in survival between the groups. n = 13–16 in each group.
IgG3 RF) but not all (i.e., the anti-Sm Ag response). Our results also differ from a recent study of the effect of the H2a genotype on the Fas-intact MRL +/+ H2a strain (46). Similar to our findings, Kong et al. (46) observed decreased levels of anti-dsDNA Abs, although they did not perform Ig isotype analysis. In contrast to our findings, these investigators did not observe differences in anti-U1-snRNP Abs between the H2 disparate strains. These contrasting findings may reflect differential effects of H2 on Fas-intact vs Fas-deficient strains and/or the enhanced sensitivity of the protein microarrays.

A prior study using F2 intercrosses of MRL/lpr and B6/lpr mice demonstrated four non-MHC-linked lupus susceptibility loci—“Lmb1, -2, -3, and -4,”—that localized to chromosomes 4, 5, 7, and 10, respectively (10). Lmb1, -2, and -3 were linked to the production of anti-dsDNA Abs, but not glomerulonephritis, whereas Lmb4 was associated with glomerulonephritis. No significant links between autoantibody production or pathologic disease and H2 were reported in this study. The authors did not, however, assess proteinuria, serum IgG isotypes, or anti-Sm/RNP Abs.

Despite significant differences in serum autoantibody levels, there was no difference in pathologic renal scores between H2kb and H2b/b MRL/lpr mice, suggesting circulating autoantibodies do not directly correlate with proliferative renal disease in MRL/lpr mice. Our results are consistent with the observations of Chan et al. (47) that serum autoantibodies are not required for full development of renal disease in MRL/lpr mice. Our results, taken together with these previous reports, suggest that the H2b genotype has minimal effect on production of nephrogenic autoantibodies, proliferative glomerulonephritis, or mortality in MRL/lpr mice. However, H2 does influence the selection of autoantigens for autoantibody targeting.

The association of MHC haplotype with IgG isotype spectrum is not well understood. Recently, an association between H2 haplotype and serum IgG2a levels was demonstrated in H2 congenic B10 and BALB/c mice (48, 49). The phenotypic marker of high IgG2a levels, denoted Ig isotype-1 (Igis1), was mapped to the central MHC class III region. Linkage of specific Ab isotype deficiency with the MHC is also present in humans (50, 51). It is unclear whether the previous reports and our findings are caused by common or independent gene(s). It is possible that we are describing common gene(s) that affect isotype switching to either IgG3 or IgG2a depending on interactions with other background genes.

In our study, it is unclear why most, but not all, F0 H2b/b MRL/lpr mice (>99.8% MRL/lpr background) had undetectable levels of serum IgG3. Analysis of IgG3 production within litters suggested that IgG3 deficiency in H2b/b MRL/lpr mice was stochastic in nature. Mice within a litter were IgG3− or IgG3+ regardless of the IgG3-producing status of their parents (data not shown). The “random” deficiency of IgG3 production in the majority of MRL/lpr H2b mice is similar to the stochastic control of the anti-Sm response in MRL/lpr H2b mice (52).

Previous studies implicated IgG3 autoantibodies in renal disease in MRL/lpr mice. Indeed, IgG3 Abs are the predominant isotype eluted from diseased MRL/lpr kidneys (18). Additionally, IgG3 anti-IgG3a RF derived from MRL/lpr mice induced glomerulonephritis when injected into normal mice (53, 54). In our study, IgG3 anti-IgG3a RF levels were significantly lower in H2b/b MRL/lpr mice due to the lack of IgG3 production. We predicted that the IgG3-deficient mice would have less severe proliferative renal disease, lack vasculitis, and have improved survival based on previous studies linking longevity of survival MRL/lpr mice with decreased production of IgG3 (19, 20). It is possible that if we sacrificed mice at an earlier time point, we may have detected differences between the groups; however, the similar survival in the three groups suggests that any differences would not have been clinically relevant. Based on our results, we believe that, although IgG3 autoantibodies may be sufficient to induce proliferative renal disease and vasculitis in MRL/lpr mice, they are not required for proliferative renal disease development or vasculitis and have no effect on survival.

Of interest in our study, the IgG3− H2b/b MRL/lpr mice had significantly reduced albuminuria but had renal scores and survival similar to IgG3+ littermates, regardless of H2 genotype. This observation indicates that the presence and/or amount of albuminuria does not necessarily parallel or predict the severity of proliferative renal disease as the IgG3− mice developed proliferative glomerulonephritis despite minimal proteinuria. To gain insight into the mechanisms underlying reduced albuminuria in the IgG3− mice, we performed EM analysis. The IgG3− H2b/b MRL/lpr mice, which had low levels of albuminuria, had electron-dense immune deposits in the subendothelial space of the glomerular capillary wall, but not in the subepithelial area. The IgG3+ MRL/lpr mice, which developed severe albuminuria, had electron-dense deposits in the subepithelial space, as well as in the subendothelial areas. In murine models of nephrotic syndrome and in human nephrotic syndrome, where proteinuria is a key measure of disease, subepithelial immune deposits, and podocyte foot process effacement are a near universal finding (55). In our results, the extent of podocyte foot process effacement was significantly less in the IgG3− MRL/lpr mice compared with the IgG3+ MRL/lpr mice. In these models and human kidney disease, the occurrence and severity of proteinuria and podocyte effacement is associated with the presence of glomerular subepithelial electron-dense deposits (56–58). The severity of proliferative renal disease in lupus nephritis, however, correlates with the presence of subendothelial deposits that were similar in the different MRL/lpr H2 groups (56, 59). The mechanisms of immune deposition on, and transport through, the glomerular basement membrane (GBM) are largely unknown. Our results suggest that IgG3 plays an important role in the specific localization of immune deposits in the kidneys of MRL/lpr mice and secondarily in the development of proteinuria. How IgG3 influences the location of immune complex deposition and subsequent albuminuria remains unclear. In MRL/lpr mice, IgG3 is the only IgG subclass that exhibits a cationic shift between isoelectric point 7.5 and 8.0 in isoelectric focusing (18). The initial deposition of cationic IgG3 immune complexes in the subendothelial space may interfere with the negatively charged selective barrier of the GBM by electrical neutralization. This may allow immune complexes to pass through the GBM to the subepithelial space where they can induce podocyte effacement and subsequent protein leakage into the urinary space. The influence of IgG3 on complement activation and/or FcR activation may also play a role in immune complex localization (60–62).

In conclusion, our results demonstrate that in MRL/lpr mice: 1) there are striking differences in autoantibody specificity for nuclear protein Ags between MRL/lpr H2b and H2b mice, identified by microarray screening techniques; 2) H2b expressed on the MRL/lpr background leads to IgG3 deficiency in the majority of mice, a finding that is likely due to complex genetic interactions; 3) IgG3 plays an important role in determining localization of glomerular immune complex deposits and secondary excretion of albumin into the urine; and 4) IgG3 autoantibodies are not necessary for development of proliferative renal disease or vasculitis in MRL/lpr mice and do not impact survival. These studies provide a novel
insight into the role of MHC in autoantigen selection and in the disease association of IgG3 with lupus nephritis. Additional studies are planned to define the genetic factors in H2 that control autoantigen selection, the genetic factors in H2 that control isotype switching to IgG3, and the mechanistic role of IgG3 in immune complex localization in the glomerulus.

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Disclosures

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