Role of the major histocompatibility complex class II Ea gene in lupus susceptibility in mice

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ABSTRACT The gene(s) encoded within major histocompatibility complex (MHC) act as one of the major genetic elements contributing to the susceptibility of murine systemic lupus erythematosus (SLE). We have recently demonstrated that lupus susceptibility is more closely linked to the I-E^- H-2b haplotype than to the I-E+ H-2^d haplotype in lupus-prone BXSB and (NZB × BXSB)F1 hybrid mice. To investigate whether the reduced susceptibility to SLE in H-2^d mice is related to the expression of the MHC class II Ea gene (absent in H-2^d mice), we determined the possible role of the Ea gene as a lupus protective gene in mice. Our results showed that (i) the development of SLE was almost completely prevented in BXSB (H-2^d) mice expressing two copies of the Ea gene at the homozygous level as well as in BXSB H-2^k (I-E^k) congenic mice as for H-2^d BXSB mice, and (ii) the expression of two functional Ea gene (transgenic and endogenous) genes in either H-2^d (NZB × BXSB)F1 or H-2^k (MRL × BXSB)F1 mice provided protection from SLE at levels comparable to those conferred by the H-2^d or H-2^k haplotype. In addition, the level of the Ea gene-mediated protection appeared to be dependent on the genetic susceptibility to SLE in individual lupus-prone mice. Our results indicate that the reduced susceptibility associated with the I-E^- H-2^d and H-2^k haplotypes (versus the I-E^- H-2^d haplotype) is largely, if not all, contributed by the apparent autoimmune suppressive effect of the Ea gene, independently of the expression of the I-A or other MHC-linked genes.

Systemic lupus erythematosus (SLE) is a disorder of generalized autoimmunity characterized by the formation of a variety of autoantibodies and the development of lethal glomerulonephritis. It is now well established that SLE is under some form of polygenic control, in which multiple genetic factors independently contribute to the overall susceptibility of individuals to the disease (1–3). Studies in backcross and intercross mice as well as H-2 congenic mice have clearly demonstrated the major contribution of certain major histocompatibility complex (MHC) alleles to lupus susceptibility (for review, see refs. 4 and 5). However, the genes encoded within the MHC complex (MHC) alleles to lupus susceptibility (for review, see refs. 4 and 5). However, the expression of two copies of the functional Ea gene, either two Ea transgenes or a combination of the transgenic and endogenous Ea genes, is capable of providing protection from SLE at levels comparable to those conferred by the H-2^d and possibly H-2^k haplotypes. Our results indicate that the H-2^d haplotype is as protective as the H-2^k haplotype in the BXSB mice and that the reduced lupus susceptibility associated with the I-E^- H-2^d and H-2^k haplotypes is largely contributed by the apparent autoimmune suppressive effect of the Ea gene, independently of the expression of the I-A or other MHC-linked genes.

MATERIALS AND METHODS

Mice. BXSB-E-7 transgenic mice, which express a single copy of the Ea+ gene, and BXSB.H-2^d congenic mice were established, as described (6, 9). BXSB.H-2^d congenic mice were generated by transfer of the H-2^d gene complex of the C3H strain by backcross procedures. Inheritance of the H-2^d gene was monitored by immunofluorescence analysis of I-A^d and I-E expression. The congenic strain was made homozygous for the H-2^d after 12 generations. NZB and MRL mice were purchased from Bomholtgard (Ry, Denmark). BXSB.H-2^b/k, (NZB × BXSB)F1 and (MRL × BXSB)F1 mice were generated by local breeding. The presence of the transgene in F1 offsprings was screened by Southern blot analysis, as described

Abbreviations: SLE, systemic lupus erythematosus; MHC, major histocompatibility complex; gp70 1C, gp70-anti-gp70 immune complexes; Ea, I-Ea; Ea-I-Aa, Ea chain-derived peptide presented by I-Aa molecules.

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below. Mice were bled from the retroorbital plexus and resulting sera were kept at -20°C until use.

Southern and Northern Blot Analysis. Five micrograms of high molecular weight genomic DNA prepared from tails of mice were digested with KpnI or SacI, electrophoresed on a 1.0% agarose gel, and transferred to a nylon membrane. Hybridization was carried out under high stringency with a 32P-labeled 3.2-kb SacI–HindIII fragment containing exons 2–5 of the Eaα gene (11); this recognizes an additional 7.0-kb or 9.4-kb fragment of the injected Eaα gene, as a result of its integration in the host genome, in KpnI-digested DNA of (NZB × BXSBE-7)F1 or SacI-digested DNA of (MRL × BXSBE-7)F1 transgenic mice, respectively. Northern blot analysis on total RNA extracted from spleens was carried out, as described (9, 10).

Cytofluorometric Analysis. The expression of I-E or an Ea chain derived peptide presented by I-Aβ molecules (Eα-I-Aβ) in peripheral blood B cells was analyzed by staining with fluorescein isothiocyanate-conjugated anti-mouse μ chain (LO-MM-9) mAb (12) and then incubating with biotinylated anti-I-Eα (Ea) chain (H81.98.21.1) (13) or anti-Eα-I-Aβ (Y-Ae) (14), followed by phycoerythrin-conjugated streptavidin (Caltag, San Francisco, CA) and analyzed with FACScan (Becton Dickinson).

Serological Assays. Serum levels of IgG antibodies against single-stranded DNA were determined by ELISA as described (15). Serum levels of gp70–anti-gp70 immune complexes (gp70 IC) were quantified by an ELISA combined with the precipitation of serum with polyethylene glycol (average molecular weight, 6,000), as described (6).

Histopathology. Samples of all major organs were obtained at autopsy, and histological sections were stained with either periodic acid–Schiff reagent or with hematoxylin/eosin. Glomerulonephritis was scored blind on a 0–4 scale based on the intensity and extent of histological changes, as described (16). Grades 3 and 4 glomerulonephritis were considered significant contributors to clinical disease or death.

Statistical Analysis. Survival curves were estimated with BMDP statistical software (17). Statistical analysis for survival rates and serological parameters was performed with the Wilcoxon two-sample test. Probability values >5% were considered insignificant.

RESULTS

Protection of SLE in BXSB Male Mice Bearing the H-2k Haplotype. To determine the possible protective effect of another I-E-expressing H-2 haplotype on the development of a lupus-like autoimmune syndrome in BXSB male mice, in which a mutant gene, Yaa (Y chromosome-linked autoimmune acceleration), plays a critical role (18), BXSB.H-2k (I-E+) congenic mice were created by backcross procedures, and their clinical development of SLE (autoantibody production and glomerulonephritis) was assessed. As shown in Fig. 1, 50% of conventional BXSB male mice (H-2k, I-E−) died of glomerulonephritis by 8 months of age, but none of the BXSB.H-2k males died within the first year. Spontaneous production of IgG anti-DNA autoantibodies and gp70 IC correlated well with the survival rates of these two BXSB males. At 6 months of age, serum levels of both autoantibodies in BXSB.H-2k male mice were markedly limited, as compared with H-2k BXSB male mice (Fig. 1). Notably, the development of SLE in BXSB H-2k/k heterozygous male mice was essentially identical to that of H-2k BXSB male mice, as in the case of BXSB H-2k/k homoyzogous males (6).

Protection of SLE in BXSB-E-7 Eaα Homozygous Transgenic Male Mice. Previous studies on several lines of BXSB Eaα transgenic mice have shown that the development of SLE was almost completely prevented in the transgenic male mice expressing a high copy number of the transgene, but no significant protection from SLE was observed in the BXSB-E-7 transgenic mice, which express a single copy of the transgene at a heterozygous level (9, 10). To test whether a reduced susceptibility to SLE in H-2k and H-2b BXSB mice is related to the expression of two copies of the functional Ea gene, the BXSB-E-7 strain was made homozygous for the transgene. Circulating B cells from the BXSB-E-7 homozygous transgenic mice expressed I-E molecules at levels higher than those of the BXSB-E-7 hemizygous transgenics but comparable to those found in the BXSB.H-2d and BXSB.H-2k mice (Fig. 2). The surface density of Ea peptide presented by I-Aβ molecules, recognized by Y-Ae mAb, was significantly elevated in the homozygous transgenic mice, as compared with that of the hemizygous transgenics. Notably, expression levels of Ea mRNA in spleens from the homozygous transgenic mice were comparable to those found in the BXSB.H-2d and BXSB.H-2k mice but higher than that of the hemizygous transgenics (data not shown).

The lupus-like autoimmune syndrome developing in the BXSB-E-7 hemizygous transgenic males as well as conventional I-E− BXSB male mice was dramatically prevented in the BXSB-E-7 homozygous transgenic males (Fig. 3). None of the homozygous transgenic male mice died of glomerulonephritis

Fig. 1. Cumulative mortality with glomerulonephritis and serum levels of IgG anti-DNA and gp70 in H-2k ( ), H-2b/k (○), and H-2b (●) BXSB male mice. Eighteen H-2k, 24 H-2b/k, and 15 H-2b mice were followed for establishing the mortality rate. Results of serum levels of autoantibodies, determined at 6 months of age, are expressed as follows: units/ml, IgG anti-DNA antibodies; μg/ml, gp70 complexed with anti-gp70 antibodies.
within the first year; this contrasted with a 50% cumulative mortality rate at 10 months in the hemizygous transgenic males. The greatly prolonged survival in the homozygous transgenic male mice was reflected in serological parameters: their serum levels of IgG anti-DNA autoantibodies and gp70 IC at 6 months of age were far lower than those of conventional BXSB and BXSB-E-7 hemizygous transgenic males (Fig. 3). Notably, serum levels of autoantibodies in the BXSB-E-7 homozygous transgenic mice were comparable to those of the BXSB.H-2d and BXSB.H-2k mice.

**Contribution of the \( \text{Ea} \) Gene to a Reduced Lupus Susceptibility in H-2^d/d (NZB \times BXSB)F\(_1\) Female Mice.** We have previously shown that a lupus-like autoimmune syndrome occurring in the absence of the \( \text{Yaa} \) gene in H-2^d/b heterozygous (NZB \times BXSB)F\(_1\) hybrid females was markedly, though not completely, suppressed in the H-2^d/d homozygous F\(_1\) hybrid females (7). To investigate whether differences in the progression of SLE between H-2^d/b and H-2^d/d (NZB \times BXSB)F\(_1\) female mice is related to the number of the functional \( \text{Ea} \) gene, BXSB-E-7 hemizygous transgenic mice were crossed with NZB (H-2^d, I-E^d) mice. The development of SLE in H-2^d/b (NZB \times BXSB-E-7)F\(_1\) transgenic females expressing two \( \text{Ea} \) (transgenic and endogenous) genes was then compared with that of nontransgenic F\(_1\) female littermates expressing only a single copy of the endogenous \( \text{Ea} \) gene and with that of H-2^d/d (NZB \times BXSB.H-2^d)F\(_1\) female mice expressing two endoge-

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**Fig. 2.** Surface expression of I-E molecules and \( \text{Ea} \) peptide presented by I-A^b molecules (Eo-I-A^b) on IgM^+ B cells in three representative groups of BXSB-E-7 male mice: \( \text{Ea} \) hemizygous transgenics [H-2^b (Tg/+)], homozygous transgenics [H-2^b (Tg/Tg)], and nontransgenics (H-2^b). Peripheral blood mononuclear cells from 2-month-old mice were first stained with fluorescein isothiocyanate-conjugated anti-mouse \( \mu \)-chain mAb (LO-MM-9) and then incubated with biotinylated anti-Ea chain (H81.98.21.1) or anti-Eo-I-A^b (Y-Ae), followed by phycoerythrin-conjugated streptavidin. Results from BXSB.H-2^d mice (H-2^d) are also shown.

**Fig. 3.** Cumulative mortality with glomerulonephritis and serum levels of IgG anti-DNA and gp70 in BXSB-E-7 \( \text{Ea} \) hemizygous transgenic (\( \bigcirc \), Tg/+), homozygous transgenic (\( \bigotimes \), Tg/Tg), and nontransgenic (\( \bullet \)) male mice. Fifteen hemizygous transgenics, 20 homozygous transgenics, and 13 nontransgenics were followed for establishing the mortality rate. Results of serum levels of autoantibodies, determined at 6 months of age, are expressed as follows: units/ml, IgG anti-DNA antibodies; \( \mu \)g/ml, gp70 complexed with anti-gp70 antibodies. Serological results from H-2^d (\( \bigcirc \)) and H-2^d (\( \bigtriangleup \)) BXSB male mice are also shown.
Inbred BXSB.H-2k)F1 male mice with a 50% cumulative mortality rate; 5.5 months; \( P < 0.0005 \) (Fig. 5). Significantly, the survival curve of the H-2-\(^{ck} \) F1 males was essentially identical to that of the H-2-\(^{ck} \) F1 transgenic males (50% mortality rate; 8.5 months; \( P > 0.3 \)). The mortality rates of these three F1 hybrid males correlated well with serum levels of gp70 IC, although levels of IgG anti-DNA autoantibodies did not differ among them (Fig. 5). At 4 months of age, gp70 IC levels in H-2-\(^{ck} \) F1 males were comparable to those of H-2-\(^{kb} \) F1 transgenic males (\( P > 0.1 \)) but significantly lower than those of H-2-\(^{kb} \) F1 nontransgenic males (\( P < 0.01 \)).

**DISCUSSION**

The gene(s) encoded within MHC act as one of the major genetic elements predisposing to murine SLE. It has been postulated that the MHC class II genes encoding I-A and/or I-E molecules are likely candidates to determine the lupus susceptibility; however, the mechanism(s) by which certain MHC class II molecules regulate SLE is not known. The present studies were designed to determine the possible role of the MHC class II \( E_a \) gene as a lupus protective gene in mice. We demonstrate that (i) the development of SLE is almost completely prevented in BXSB (H-2\(^{b} \), I-E\(^{b} \)) mice expressing two copies of the \( E_a \) transgene at the homozygous level, as is the case of BXSB mice bearing the \( I-E \) expressing H-2\(^{a} \) and H-2\(^{k} \) haplotypes, and (ii) the expression of two functional \( E_a \) genes (one from the transgene and the other from the endogenous gene) in either H-2-\(^{kb} \) (NZB \( \times \) BXSB)F1 or H-2-\(^{kb} \) (MRL \( \times \) BXSB)F1 mice is capable of providing protection from SLE at levels comparable to those conferred by the H-2-\(^{id} \) or H-2-\(^{kk} \) haplotype. Our results, thus, indicate that the reduced susceptibility associated with the I-E-\(^{+} \) H-2\(^{a} \) and H-2\(^{k} \) haplotypes (versus the I-E-\(^{−} \) H-2\(^{a} \) haplotype) is largely, if not all, contributed by the \( E_a \) gene but not by the genes encoding the I-A molecules and the MHC-linked genes other than classical immune responses genes.

Our present conclusion is consistent with previous observations that the expression of the \( E_a \) transgenic or endogenous gene is capable of down-regulating autoimmune responses in several lupus-prone mice (9, 10, 19, 20). Although the precise mechanism responsible for the apparent autoimmune suppressive effect of the \( E_a \) gene has not been elucidated, it has been shown that B cells, but not T cells, are the major cellular site of the \( E_a \) transgene effect on the suppression of autoimmune responses (9, 20). Thus, it is unlikely that the MHC effect

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**FIG. 4.** Cumulative mortality with glomerulonephritis and serum levels of IgG anti-DNA and gp70 IC in H-2-\(^{kb} \) (NZB \( \times \) BXSB-E-7)F1 \( E_a \) transgenic (\( \bullet \); Tg) and nontransgenic (\( \bigcirc \)) female mice and H-2-\(^{id} \) (NZB \( \times \) BXSB.H-2\(^{a} \))F1, nontransgenic female mice (\( \bigtriangledown \)). Twenty-six H-2-\(^{kb} \) transgenics, 26 H-2-\(^{kb} \) nontransgenics, and 15 H-2-\(^{id} \) nontransgenics were followed for establishing the mortality rate. Results of serum levels of autoantibodies, determined at 4 months of age, are expressed as follows: units/ml, IgG anti-DNA antibodies; \( \mu g/ml \), gp70 complexed with anti-gp70 antibodies.

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**nous \( E_a \) genes. Notably, the I-E density on B cells from the H-2-\(^{kb} \) F1 transgenic mice increased to a level very close to that from the H-2-\(^{id} \) F1 mice (data not shown).

The expression of a single copy of the \( E_a \) transgene derived from the BXSB-E-7 strain, in addition to that of the endogenous \( E_a \) gene, resulted in a significant suppression of lupus-like autoimmune syndrome occurring in H-2\(^{kb} \) (NZB \( \times \) BXSB)F1 nontransgenic female mice. Whereas 50% of the F1 female nontransgenic littermates expressing I-E molecules at the heterozygous level died of glomerulonephritis within the first 9 months, the survival rate of the transgenic females was substantially prolonged, with a 50% cumulative mortality rate at 14 months of age (\( P < 0.0001 \)), almost comparable to that observed in H-2-\(^{kb} \) F1 female mice (50% mortality rate; 15 months; \( P > 0.1 \)) (Fig. 4). Serological analysis revealed that serum levels of IgG anti-DNA autoantibodies and gp70 IC in the H-2-\(^{kb} \) F1 transgenic female mice at 4 months of age were lower than those of the nontransgenic female littermates (\( P < 0.001 \)) but similar to those of H-2-\(^{id} \) F1 females (\( P > 0.1 \)) (Fig. 4).

In contrast, the \( E_a \) transgene was only slightly protective in (NZB \( \times \) BXSB)F1 males, in which the MHC effect was completely abrogated as a result of a remarkable \( Yaa \) gene-mediated acceleration of SLE in H-2-\(^{id} \) F1 males (7). All three different F1 male mice developed uniformly a very rapid course of SLE (50% mortality rates: 5.5 months in H-2-\(^{kb} \) nontransgenic and transgenic male hybrids; 6.5 months in H-2-\(^{id} \) male hybrids; \( P > 0.3 \)) with comparable levels of autoantibody production (data not shown).

**Contribution of the \( E_a \) Gene to a Reduced Lupus Susceptibility in H-2-\(^{ck} \) (MRL \( \times \) BXSB)F1 Male Mice.** A weak, but significant protection of SLE, as a result of the expression of a single copy of the \( E_a \) transgene derived from the BXSB-E-7 strain, has been shown in H-2-\(^{kb} \) (MRL \( \times \) BXSB)F1 hybrid male mice (10). We, therefore, determined whether the expression of two endogenous \( E_a \) genes in H-2-\(^{ck} \) homozygous (MRL \( \times \) BXSB.H-2\(^{ck} \))F1 males could confer a protection at a level comparable to that of (MRL \( \times \) BXSB.E-7)F1 transgenic males expressing two \( E_a \) genes (one from the \( E_a \) transgene and the other from the endogenous \( E_a \) gene). The development of a lupus-like syndrome was partially but significantly delayed in the H-2-\(^{ck} \) homozygous (MRL \( \times \) BXSB.H-2\(^{ck} \))F1 male mice with a 50% cumulative mortality rate at 8.5 months, as compared with H-2-\(^{kb} \) heterozygous (MRL \( \times \) BXSB.H-2\(^{ck} \))F1 nontransgenic males (50% mortality rate; 5.5 months; \( P < 0.0005 \)) (Fig. 5). Significantly, the survival curve of the H-2-\(^{ck} \) F1 males was essentially identical to that of the H-2-\(^{ck} \) F1 transgenic males (50% mortality rate; 8.5 months; \( P > 0.3 \)). The mortality rates of these three F1 hybrid males correlated well with serum levels of gp70 IC, although levels of IgG anti-DNA autoantibodies did not differ among them (Fig. 5). At 4 months of age, gp70 IC levels in H-2-\(^{ck} \) F1 males were comparable to those of H-2-\(^{kb} \) F1 transgenic males (\( P > 0.1 \)) but significantly lower than those of H-2-\(^{kb} \) F1 nontransgenic males (\( P < 0.01 \)).
observed among three H-2 haplotypes (H-2\textsuperscript{b}, H-2\textsuperscript{d}, and H-2\textsuperscript{a}) is a consequence of thymic selection of a harmful autoreactive T cell repertoire. The expression of the \textit{Ea} gene in B cells may lead to a reduced presentation of pathogenic self peptides, thereby inhibiting excessive activation of autoreactive B cells. Competition by \textit{Ea} chain derived peptides resulting in decreased self-peptide presentation by I-A molecules has been suggested as one of the possible mechanisms (9, 10). Notably, a peptide derived from the \textit{Ea} chains displays a high affinity to several different I-A molecules (21–23), and this peptide sequence is conserved in essentially all the \textit{Ea} chains, independently of the H-2 haplotypes (24).

It should be mentioned that the I-E molecules generated in mice carrying the \textit{Ea\textsuperscript{d}} transgene are not identical to those expressed in mice bearing the H-2\textsuperscript{a} or H-2\textsuperscript{b} gene complex. In H-2\textsuperscript{b} BXSB mice, the expression of the transgene results in the formation of \textit{Ea\textsuperscript{d}E\textsuperscript{b}} mixed-haplotype heterodimers (9), and H-2\textsuperscript{a,b} (MRL \times BXSB)F\textsubscript{1} transgenic mice potentially express two new mixed-haplotype heterodimers, \textit{Ea\textsuperscript{a}E\textsuperscript{b}} and \textit{Ea\textsuperscript{a}E\textsuperscript{b}}. Thus, one cannot exclude the possibility that the \textit{Ea\textsuperscript{d}} transgene-mediated protection can be due to the expression of new mixed-haplotype heterodimers, which are not expressed in nontransgenic littermates. However, it should be stressed that the transgene expression does not lead to the formation of novel I-E heterodimers in (NZB \times BXSB)F\textsubscript{1} mice. In addition, the complete identity in the amino acid sequences of the N-terminal \textit{e} domain between the \textit{Ea\textsuperscript{d}} and \textit{Ea\textsuperscript{b}} chains (24) argues against a potential protective role of \textit{Ea\textsuperscript{a}E\textsuperscript{b}} or \textit{Ea\textsuperscript{a}E\textsuperscript{b}} mixed-haplotype heterodimers, as compared with preexisting I-E (\textit{Ea\textsuperscript{a}E\textsuperscript{b}} and \textit{Ea\textsuperscript{a}E\textsuperscript{b}}) molecules, in (MRL \times BXSB)F\textsubscript{1} mice.

It is significant that the level of the protection associated with the expression of the \textit{Ea} gene markedly differs among the three H-2 haplotypes (H-2\textsuperscript{a}, H-2\textsuperscript{b}, and H-2\textsuperscript{d}) and inversely correlates with their genetic susceptibilities to SLE. This idea is also consistent with the findings that the \textit{Ea\textsuperscript{d}} transgene and the H-2\textsuperscript{a,b} are barely protective from SLE in (NZB \times BXSB)F\textsubscript{1} male mice, which are genetically more predisposed to SLE than their female counterparts because of the presence of the \textit{Yaa} gene (7), and that the H-2\textsuperscript{b} heterozygous expression is sufficient to suppress the autoantibody production induced by the \textit{lpr} (\textit{Fas}) mutation in C57BL/6 mice, which are poorly predisposed to SLE (26). Our present results show that the MHC class II \textit{Ea} gene apparently contributes to the reduced susceptibility of SLE by suppressing autoimmune responses in mice. However, this does not imply that the \textit{Ea} gene is the only gene encoded within MHC that determines the genetic susceptibility to murine SLE. Studies in New Zealand mice have demonstrated the association of the H-2\textsuperscript{d} heterozygosity or the \textit{bm12} mutation in the I-A\textit{\textalpha} chain, independently of the expression of I-E molecules, with the development of SLE (27–30). In view of particular sequence homologies of the \textit{A\beta}\textsubscript{nm12} and \textit{E\beta} chains in the region of the peptide-binding groove, it has been speculated that the generation of unique mixed-haplotype MHC class II molecules such as \textit{Ea\textsuperscript{a}E\textsuperscript{b}} may implicate in the development of SLE (31). However, the expression of several other H-2 heterozygosities (H-2\textsuperscript{a,b}, H-2\textsuperscript{b}, and H-2\textsuperscript{c}) also enhanced autoantibody production and incidence of lupus nephritis (1, 3, 32), and H-2\textsuperscript{d}\textit{b} (versus H-2\textsuperscript{d}\textit{a}) failed to promote the development of SLE in (BALB.H-2\textsuperscript{a} \times NZB) \times NZB backcross mice (33). These observations make it less likely that novel hybrid class II molecules create new lupus-predisposing MHC haplotypes. One possible explanation may be that the MHC-linked genes other than the classical immune response genes determine the contribution of the MHC genes to SLE in the New Zealand mice, as the \textit{Tnfa} allele of the NZW strain has been proposed as one of the lupus susceptibility genes present within the MHC region (34). Accordingly, the MHC likely encodes more than one lupus-associated gene, which can contribute to the development of SLE by acting at various levels of the disease process.

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