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B Cell TLR7 Expression Drives Anti-RNA Autoantibody Production and Exacerbates Disease in Systemic Lupus Erythematosus-Prone Mice

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B Cell TLR7 Expression Drives Anti-RNA Autoantibody Production and Exacerbates Disease in Systemic Lupus Erythematosus-Prone Mice

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Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease characterized by the production of antinuclear autoantibodies. Antinuclear autoantibody development is recognized as one of the initial stages of disease that often results in systemic inflammation, kidney disease, and death. The etiology is complex, but it is clear that innate pathways may play an important role in disease progression. Recent data have highlighted an important role for the TLR family, particularly TLR7, in both human disease and murine models. In this study, we have presented a low copy conditional TLR7 transgenic (Tg7) mouse strain that does not develop spontaneous autoimmunity. When we combine Tg7 with the *Sle1* lupus susceptibility locus, the mice develop severe disease. Using the CD19^{Cre} recombinase system, we normalized expression of TLR7 solely within the B cells. Using this method we demonstrated that overexpression of TLR7 within the B cell compartment reduces the marginal zone B cell compartment and increases B and T cell activation but not T follicular helper cell development. Moreover, this enhanced B cell TLR7 expression permits the specific development of Abs to RNA/protein complexes and exacerbates SLE disease. *The Journal of Immunology*, 2012, 189: 5786–5796.

Systemic lupus erythematosus (SLE) is a complex chronic autoimmune disease that is classically associated with the production of pathogenic autoantibodies to a wide spectrum of nuclear Ags (1, 2). These antinuclear Abs can also be detected in a high frequency of individuals in the general population (3). A subset of individuals with high-titered antinuclear autoantibodies (ANAs) transition to severe disease, and this progression is accompanied by increased numbers of Abs (4). We have focused our recent efforts in understanding how this transition from benign autoimmunity to severe disease occurs.

We have used the B6.*Sle1* model as the first step in disease, which develops a mild splenomegaly, enhanced B and T cell activation, and high titers of ANAs while rarely developing glomerulonephritis (GN) (5). *Sle1*, present on chromosome 1, was one of three lupus susceptibility regions originally identified from the NZM2410 lupus-prone mouse strain through a backcrossing strategy. In these original analyses, *Sle2* and *Sle3* were also identified, and data since then have shown that the presence of these loci leads to enhanced B cell activation and TLR hyperactivity, respectively (6). Over time, it has become increasingly apparent that the region encompassing *Sle1* is fundamental for driving autoimmunity in a number of other autoimmune-prone strains (termed *Nba1*, *Sbw1*, *Lbw1*, *Cgnz1*, *Bxs1*, and *Bxs2*; reviewed in Ref. 7). However, the progression of *Sle1*-associated benign autoimmunity to severe disease depends on an additional immune alteration. Combination of *Sle1* with other disease susceptibility loci, particularly with those affecting innate pathways, such as Y-linked autoimmune accelerating (*Yaa*) and *Sle3*, results in severe disease (6, 8).

The *Yaa* locus is located on the Y chromosome, driving an aggressive SLE-like disease in male mice. This mutation was proven to be a translocation of ~16 genes from the X chromosome onto the Y chromosome (8, 9), which resulted in a two-fold increase in expression and function of the translocated genes. In particular, there were several interesting immune genes present, including *Tlr7*, *Tlr8*, and *Rab9*, in the translocated DNA. Therefore, we and others went on to show that the upregulation of TLR7 was necessary for the progression to severe disease in different *Yaa*-containing SLE murine models by using knockout strategies (10–12). Our studies and those of the Izui group also suggested that other genes within this region play a role, as not all severe autoimmune phenotypes were prevented with TLR7 normalization.

Data demonstrating the importance of TLR7 in disease progression is not restricted to *Yaa* models. Inhibition of TLR7 with

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Abbreviations used in this article: ANA, antinuclear autoantibody; BAC, bacterial artificial chromosome; GN, glomerulonephritis; SLAMF, signaling lymphocyte activation molecule family; SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein; T_{FH}, T follicular helper cell; *Yaa*, Y-linked autoimmune accelerating; YFP, yellow fluorescent protein.

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immunoregulatory sequences inhibits disease in both NZB/W and MRL^{lpr} mice (13, 14). Furthermore, when TLR7 is overexpressed to 8–16 gene copies, it results in acute severe inflammation, kidney disease, and mortality (11).

Although genomic analysis of adult patients has not supported a role for a translocation of TLR7 (15), recent studies have shown an association of SLE with an X chromosome-linked TLR7 single nucleotide polymorphism in Asian males (16, 17). Additionally, an examination in pediatric patients from Mexico revealed a higher copy number of TLR7 in both male and female patients, which could point to an explanation of why pediatric patients demonstrate such an aggressive disease compared with adults (18). Other data suggest that TLR7 mRNA levels are increased in adult patients with SLE, correlating with increased RNA expression of IFN- α (19). Furthermore, additional evidence suggests that enhanced activation of this pathway is important in human disease, with polymorphisms in its downstream transcription factor, IRF5, being found in multiple studies (20–23). Immunological studies have also demonstrated a role for TLR7 in NETosis, the process by which neutrophils empty their nuclear contents trapping pathogenic material (24). NETosis is enhanced in SLE, and this may be amplified by stimulation with anti-small nuclear ribonucleoprotein (snRNP) Abs (25, 26). This may provide the antigenic material that contributes to the propagation of the inflammatory response.

Because earlier work has focused on eliminating the additional copy of TLR7 to demonstrate its necessity, and given the presence of other immune candidate genes within the *Yaa* locus (e.g., *Rab9*, *Tlr8*), we sought to determine 1) whether moderate upregulation of TLR7 alone is sufficient to drive disease progression, and 2) how B cells contribute to the later stages of disease progression. We developed a modified bacterial artificial chromosome (BAC) transgenic mouse strain carrying a low copy number of TLR7 (Tg7). The TLR7 gene is flanked by loxP sites so that the transgene can be deleted by Cre-recombinase. When the Tg7 is present on the B6 background and in the absence of Cre, TLR7 mRNA levels are similar to the *Yaa* strain, and mice do not exhibit any evidence of disease. However, when combined with the *Sle1* locus (B6.*Sle1*Tg7) the mice display disease characteristics similar to the B6.*Sle1**Yaa* strain. To investigate the role of increased TLR7 expression in B cells, we crossed the B6.*Sle1*Tg7 strain with CD19^{Cre} mice (CD19^{Cre}.B6.*Sle1*Tg7). When the additional TLR7 copies encoded in the transgene were deleted within B cells, expression of TLR7 was normalized solely within this population. Our data demonstrate that many autoimmune traits associated with disease are dependent on an intrinsic upregulation of TLR7 within B cells. Moreover, we show a requirement of increased B cell TLR7 expression for Abs to RNA/protein complexes, without affecting anti-DNA/chromatin ANAs. This increase in B cell TLR7 expression exacerbates inflammatory infiltrate into the kidney and subsequent disease progression.

Materials and Methods

Reagents and mice

Mice were bred in the University of Texas Southwestern Medical Center or in the Biomedical Resource Centre, Singapore. Breeding pairs for C57BL/6J (B6), B6.129.CD19^{Cre}, and *Gt(ROSA)26Sor^{tm1(SmcrEYFP)Amc}* (B6.ROSA.EYFP) mice were originally obtained from The Jackson Laboratory (27, 28). Cre mice were reported to be backcrossed 10 generations (n10) to B6 and were also checked for any contaminating 129 autoimmune alleles using single nucleotide polymorphism analyses. None was found near known autoimmune susceptibility regions. The derivation of the B6.*Sle1* and B6.*Yaa* strains has already been described (8, 29). For aging studies to detect the development of disease, mice were 6–9 mo old. For all other studies mice used were 6–8 wk old. The modified TLR7 BAC transgene

was introduced by pronuclear injection into fertilized eggs and was performed by the Transgenic Core at the University of Texas Southwestern Medical Center. The care and use of laboratory animals conformed to the National Institutes of Health guidelines, and all experimental procedures conformed to an Institutional Animal Care and Use Committee-approved animal protocol. All Abs were purchased from BD Biosciences or eBioscience with the exception of PE-Texas Red and Qdot conjugates, which were from Invitrogen, and Neu-7/4, which was purchased from Serotec.

Modification of BAC RP23-92P

The B6-derived BAC clone RP23-92P6 was purchased from the BACPAC Resources Center at Children's Hospital Oakland Research Institute. It contained the full sequences for both TLR7 and phosphoribosylpyrophosphate synthetase subunit II. It was modified using a Counter Selection BAC Modification Kit by Red/ET Recombination produced by Gene Bridges (Dresden, Germany). The 50-bp homology primer sequences for TLR7 were as follows: 5' forward, AGC TCA AAG GCT CTG CGA GTC TCG GTT TTC TGT TGC CTT CTC TCT GTC TC; 5' reverse, TAA AAT ACC TTT CTT GAA GGC ATA GAG TGG TTC TAT AGA TGG AGT CCT CT; 3' forward, CTC TCT GAA GAA TGT CAC CAC CTA GGA CAT GCC TTG GTA CCT GAA GTT TT; 3' reverse, ATG ACA ACT GTT AGG AAA AAT TCA GAC CTT TCT CAT TTA TGG AAA CCT TTA TG. The loxP sequence inserted into the BAC consists of a [core] flanked by palindrome sequences: 5'-ATAACTTCGTATA [GCATACAT] TATAC-GAAGTTAT-3' and 3'-TATTGAAGCATAT [CGTATGTA] ATATGCTT-CAATA-5'. Efficient modification of the BAC was examined using genomic sequencing and multiple enzyme digestion with pulsed-field gel analyses. Integration of the modified BAC by pronuclear injection into C57BL/6J fertilized eggs was completed at the University of Texas Southwestern Transgenic Core.

Assessment of renal disease

Blood urea nitrogen was assessed using the QuantiChrom urea assay kit (BioAssay Systems (Hayward, CA)). Following euthanasia, kidneys were fixed in formalin and embedded in paraffin for blinded analysis by an independent pathologist, as previously described (30). The severity of GN was graded using the following guidelines set by the World Health Organization, based on light microscopy: 0, normal; 1, mild increase in mesangial cellularity and matrix; 2, moderate increase in mesangial cellularity and matrix, with thickening of the glomerular basement membrane; 3, focal endocapillary hypercellularity with obliteration of capillary lumina and a substantial increase in the thickness and irregularity of the glomerular basement membrane; and 4, diffuse endocapillary hypercellularity, with segmental necrosis, crescents, and hyalinized end-stage glomeruli. Tubular interstitial nephritis was graded on a 0–4 scale, as described (30).

Serology

Serum autoantibodies were analyzed using ELISAs for 1) ANAs, detecting histones and dsDNA, 2) anti-dsDNA, and 3) anti-U1 snRNP with B6.*Sle1**Yaa* pooled serum as standard, as described previously (10, 30). Further autoantibody specificity of serum was measured in the Immunomonitoring Core at the Singapore Immunology Network using the AtheNA Multi-Lyte ANA-III test system (Zeus Scientific), as per the manufacturer's instructions, using a donkey anti-mouse IgG detection Ab (Jackson ImmunoResearch). Hep2 staining was performed on selected samples at a 1:200 dilution as per the manufacturer's instructions, with a goat anti-mouse IgG-DyLight 488 secondary Ab. Cytokine multiplex analysis was performed with the Bio-Plex cytokine assay kit (Bio-Rad) as per the manufacturer's protocol at Baylor Institute for Immunology Research Luminex Core (Dallas, TX).

Cell purification and RT-PCR

TLR7 copy number from tail DNA lysates was assessed using premixed RT-PCR TaqMan primer sets for GAPDH (Mm99999915_g1) and custom-designed primers and probe TLR7 from Applied Biosystems: TLR7 forward, 5'-CAGAGGCCCATGTGATCGT-3', reverse, 5'-GCCCTCAGG-GATTTCGTGCA-3', probe (TAMRA), 5'-ACTGCACAGACAAGC-3'. B cells were labeled using the CD19 positive selection kit from Miltenyi Biotec and separated using an autoMACs. Purity was determined to be >97%. Cells were processed for RNA using the TRIzol/chloroform extraction and a Qiagen RNeasy kit. RNA message was analyzed using Applied Biosystems TaqMan gene expression assays using RNA-specific primers for β_2 -microglobulin (Mm00437762_m1), TLR7 (Mm00446590_m1), and TLR9 (Mm00446193_m1). Absolute MAX quantitative RT-PCR mix from Thermo Scientific was used for amplification, as per the manufacturer's

instructions. An Applied Biosystems 7300 real-time System, using Applied Biosystems sequence detection software (version 1.2.3) was used for amplification and analysis. The message levels for TLR7 and TLR9 were expressed after normalization to β_2 -microglobulin expression levels.

Cell preparation, flow cytometry, and microscopy

Peripheral blood was taken retro-orbitally or by cardiac puncture. Kidneys were prepared as described previously (10, 31). Briefly, they were minced and resuspended into 0.75 ml PBS. Cells were spun down and the supernatant was kept at -20°C for cytokine analysis. This is referred to as kidney plasma in Fig. 2F. Cells were resuspended in digestion buffer consisting of 1 mg/ml collagenase IV (Sigma-Aldrich) and DNase I (1 $\mu\text{g/ml}$) in RPMI complete media and incubated at 37°C for 30 min. Cells were centrifuged, filtered through a $70\text{-}\mu\text{m}$ mesh, and then mixed 1:1 with 40% Percoll solution. This was centrifuged at 3000 rpm for 20 min at room temperature with the brake off. The loose pellet was washed and then counted using a Cellometer Auto T4 from Nexcelom Bioscience.

Splenic or kidney cells were resuspended in staining buffer and stained with a combination of up to nine directly conjugated Abs (FITC, PE, PE-Texas Red, PE-Cy5 or PerCP-Cy5.5, PE-Cy7, allophycocyanin, Alexa 700, allophycocyanin-Cy7, Pacific Blue) and one biotinylated Ab. CFSE and yellow fluorescent protein (YFP) were evaluated in the FITC channel (in the absence of a monoclonal FITC marker). Secondary detection of the biotinylated Ab was with streptavidin Qdot 655. RBC lysis was completed using BD FACS lysing solution (BD Biosciences) as per the manufacturer's instructions. Acquisition and analysis was completed using a BD LSR II with FlowJo 7.6 for Windows (Tree Star). Confocal images were captured with an Olympus FV1000 confocal system and processed with Olympus FV10-ASW Fluoview version 2.0b.

Statistical analyses

Results are expressed as the arithmetic means \pm SEM. Normality was tested using the Kolmogorov-Smirnov test. For comparing two groups, an unpaired two-way Student *t* test was used, and for multiple comparisons,

a one-way parametric ANOVA and Tukey post hoc comparison were used. When the normality test failed, a Mann-Whitney *U* test compared two groups, and one-way nonparametric ANOVA and a Dunn's multiple comparison test were used to test multiple comparisons. For time/treatment or time/strain comparisons, a two-way ANOVA with Bonferroni post hoc analyses was used. Analyses of survival were completed using the log-rank Mantel-Cox test. Comparisons were made between Tg7 or Yaa containing strains and their wild-types (either B6 or B6.Sle1). Analyses were completed using Prism 5.0 for Windows (GraphPad Software, San Diego, CA) or Microsoft Excel.

Results

TLR7 transgenic (Tg7) mice are similar in expression and function to Yaa mice

To determine whether an upregulation of TLR7 was sufficient to cause disease progression, we generated a conditional BAC transgenic mouse (Tg7) using the BAC vector shown in Fig. 1A. Analysis of genomic DNA from four initial founder lines (Fn) showed two additional copies of TLR7 within these mice (Fig. 1B), with three exhibiting a similar doubling in B cell mRNA expression to the Yaa strain (Fig. 1C). Two lines were studied in parallel (Fn1 and Fn2) to ensure that there were no differences between them with regard to disease manifestations, and then we concentrated only on one Tg7 strain (Fn1) for the remaining investigations. The increase in B cell TLR7 transcription was associated with an increase in response to TLR7 ligands R837 and R848 in B cells, as determined by the significant upregulation of CD69 overnight, and an associated trend in proliferation at 72 h (Fig. 1D, 1E). Additional data from a mixed sex population (Fn1 and Fn2) showed similar trends ($n = 4$, $p = 0.076$; Supplemental

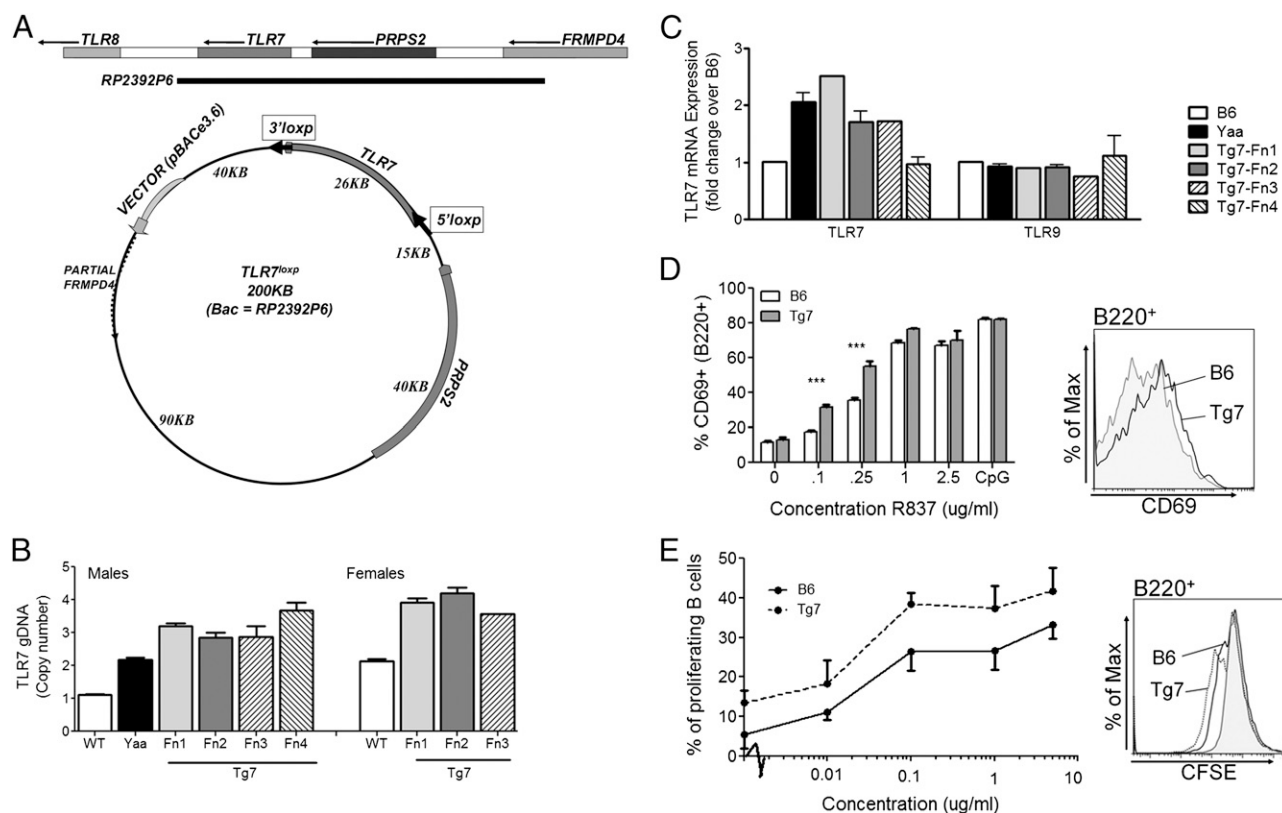


FIGURE 1. TLR7 transgenic (Tg7) mice are similar in expression and function to Yaa mice. (A) BAC RP23-92P6 was modified as described in *Materials and Methods* to include two loxP sites flanking the TLR7 gene. (B) Mice carrying the transgene carried two additional genomic DNA copies. (C) Multiple founders (Fn) show two-fold TLR7 expression compared with B6 mice. Male mice (Fn1 shown) were examined for an increase in the TLR7 functional response. Tg7 (Fn1) mice demonstrate an increase in CD69⁺ B cells (B220⁺ cells) after TLR7 stimulation by R837 (D) and enhanced proliferation of B cells by R848 as detected by CFSE dilution (E). Two-way ANOVA demonstrated significance for both (D) and (E). *** $p < 0.001$ for post hoc comparisons. Bars represent SEM; $n = 3$ mice/strain in each separate experiment.

Fig. 1A). These Tg7 mice did not develop significant levels of ANAs, or glomerulonephritis (Fig. 2A, 2B).

Thus we developed a low-copy TLR7 transgenic, similar to the *Yaa* strain, that did not exhibit any overt autoimmune traits.

An epistatic interaction between Sle1 and Tg7 results in severe autoimmune pathology

We then crossed our TLR7 transgenic strain (Tg7) to the B6.*Sle1* to examine whether the upregulation of TLR7 recapitulated the effects observed in the B6.*Sle1Yaa* strain. We have previously shown that the addition of *Yaa* onto B6.*Sle1* increases the penetrance and titer of autoantibodies to nucleosomes (histones/dsDNA) (32). Our B6.*Sle1*/Tg7 mice developed a similar ANA profile in both female and male mice, which was significant in male mice analyzed (Fig. 2A). Pathological analysis indicated that female and male B6.*Sle1*/Tg7 mice also developed severe GN, similar to their B6.*Sle1Yaa* counterparts (Fig. 2B). Analysis of mortality demonstrated that male B6.*Sle1*/Tg7 mice survived significantly longer than did their B6.*Sle1Yaa* counterparts (Fig. 2C), suggesting that additional genes within the *Yaa* locus may contribute to disease. When examining female B6.*Sle1*/Tg7 mice, we observed an increase in mortality when compared with B6.*Sle1* females and B6.*Sle1*/Tg7 males, which is probably due to the previously

reported female sex bias of the *Sle1* region, as evidenced by the sex bias in IgG ANA levels (Fig. 2A).

We have previously shown that the CD45⁺ leukocyte infiltration associated with kidney disease in experimental and spontaneous SLE models is measurable using flow cytometry (10, 31, 33). Using this technique we were able to confirm that the B6.*Sle1*/Tg7 mice had increased CD45⁺ cells within the kidney, and that this infiltrate was composed of CD4⁺ and CD8⁺ T cells, as well as CD11b⁺ myeloid cells, in a similar manner to B6.*Sle1Yaa* mice (Fig. 2D, 2E). Analysis of the supernatant from freshly isolated kidney cells using multiplex Luminex determined several inflammatory cytokines and chemokines that were significantly increased in the B6.*Sle1Yaa* compared with B6.*Sle1* controls, with B6.*Sle1*/Tg7 mice showing similar trends (Fig. 2F). These included IL-12p40, keratinocyte chemoattractant, and MCP-1, as we detected in an earlier cohort of B6.*Sle1Yaa* mice (10).

Therefore, we demonstrated that the epistatic interaction of the *Sle1* lupus susceptibility region and our TLR7 transgene resulted in severe kidney pathology, in a similar manner to the B6.*Sle1Yaa* strain.

B6.Sle1Tg7 mice develop splenic disease similarly to the B6.Sle1Yaa strain

Splenomegaly is a common feature of lupus-prone murine models, including the B6.*Sle1Yaa* model, and this was also detected in the

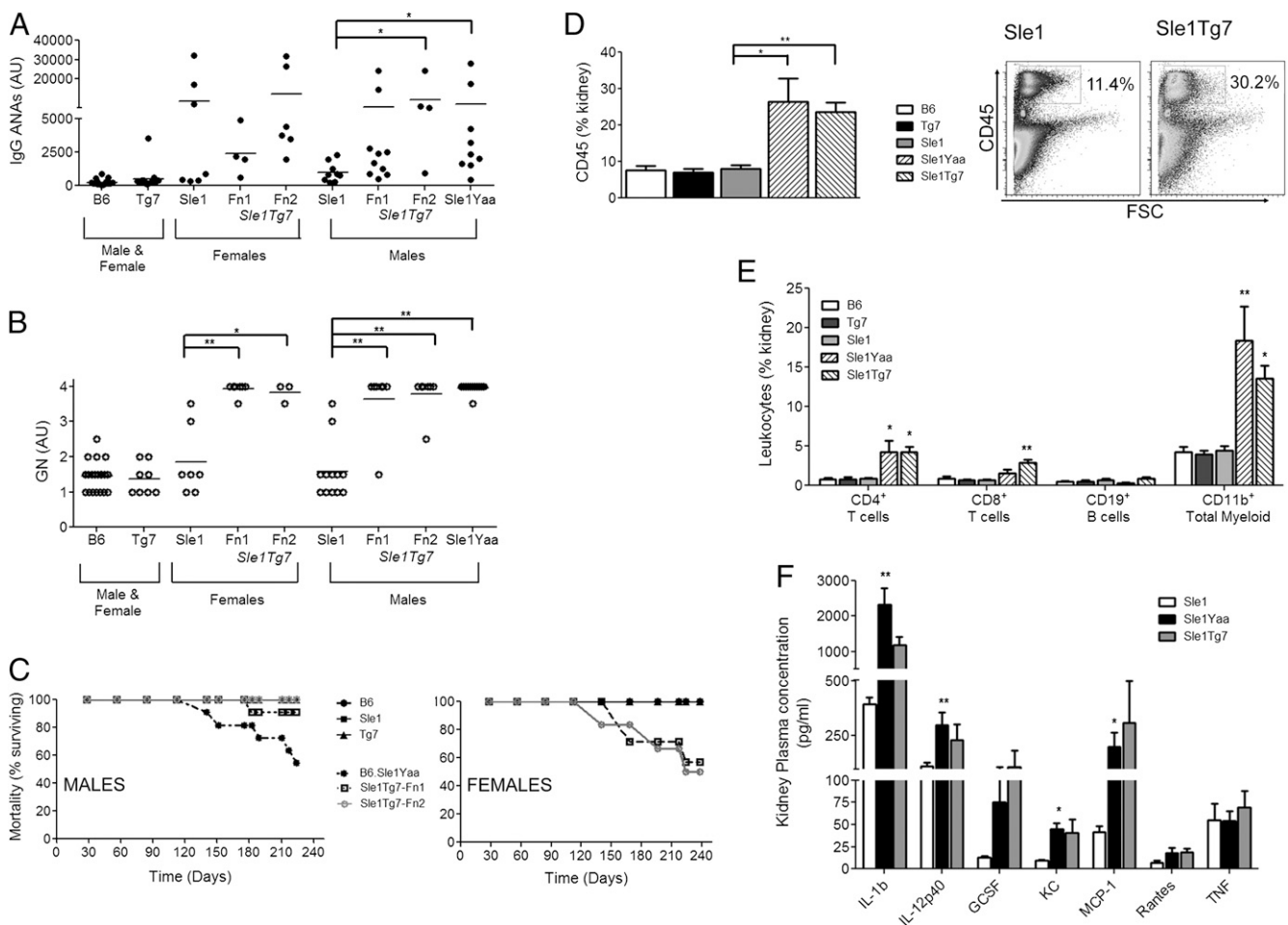


FIGURE 2. An epistatic interaction between *Sle1* and Tg7 results in severe autoimmune pathology. (A) The Tg7 was crossed to the autoimmune-prone B6.*Sle1* strain and IgG antichromatin/dsDNA Abs (ANAs) were measured by ELISA (Tg7 alone, Fn1/Fn2). (B) GN was analyzed by an independent pathologist. (C) Mortality curves of male and female mice were significantly different. Total leukocyte infiltration into the kidney was measured by CD45 expression. The cumulative data ($n \geq 6$ /strain) and gating strategy is shown in (D). (E) Specific infiltrating leukocytes into the kidney were identified with the lineage markers CD4, CD8, CD19, and CD11b ($n \geq 6$ /strain). (F) Inflammatory cytokines released by the kidney were measured by luminex ($n = 3$ /strain). Horizontal bars in (A) and (B) represent the mean of the data. Results in (D)–(F) are from male mice (Tg7 = Fn1/2, mean \pm SEM). Significant differences are shown compared with B6.*Sle1* control. * $p < 0.05$, ** $p < 0.01$.

B6.*Sle1*/Tg7 strain (Fig. 3A). Cellular expansion involved CD4⁺ T cells, CD19⁺ B cells, and, in particular, CD11b⁺ myeloid cells (Fig. 3B). Spleens from B6.Tg7 mice were also slightly larger than B6 wild-type controls, but there was no detectable leukocyte population expanding (Fig. 3A, 3B). An expansion of splenic CXCR5⁺ T follicular helper cells is characteristic of *Yaa* models, and this was also found to be a trait of the B6.Tg7 itself and was also evident on the B6.*Sle1* background, with no observable difference when comparing the B6.*Sle1*Yaa and B6.*Sle1*Tg7 (Fig. 3C). Other mouse models containing the *Yaa* translocation also show decreases in the percentage of marginal zone B cells (34), and it has been hypothesized that this may play a significant role in disease progression through the associated reduction in IL-10 (35). This reduction in B cell marginal zone cells was also apparent in aged B6.*Sle1*Tg7 mice (Fig. 3D).

Therefore, our data so far demonstrated that the Tg7 mice that carry additional copies of TLR7 behave similarly to the corresponding *Yaa* SLE model in terms of all investigated autoimmune traits. Using Tg7 SLE mice we showed that an upregulation of TLR7 is sufficient to promote autoimmunity in B6.*Sle1* mice. Comparison of female and male B6.*Sle1*Tg7 mice did not identify any other differences in autoimmunity other than an increased rate of mortality.

Effective elimination of the additional TLR7 copy in B cells

Because our transgenic system promotes disease progression in a similar manner comparable to the B6.*Sle1*Yaa model, we then sought to use the loxP modifications of this transgene to determine the necessity of the upregulation of TLR7 within the B cells to drive full pathogenicity. To eliminate the additional copies of TLR7 introduced by the BAC, we used the Cre recombinase system (36). When the B6.*Sle1*Tg7 strain was crossed with the CD19^{Cre} (28) strain to generate CD19.B6.*Sle1*Tg7, the normal mRNA expression of TLR7 was restored only within the B cells, without a corresponding reduction in CD11b⁺ myeloid populations (Fig. 4A). Analysis of the deletion using a YFP reporter, using the ROSA/EYFP strain (27), showed that <1% of non-B cells expressed YFP, confirming high specificity, and that >90% of B cells did express YFP, showing high efficiency (0.96 ± 0.11 and 90.03 ± 2.46 , respectively; representative plots in Fig. 4B).

Increases in B cell TLR7 result in enhanced splenic B and T cell activation as well as marginal zone B cell depletion

Using this effective deletion of excess copies of TLR7 by CD19^{Cre}, we analyzed disease progression in female CD19.B6.*Sle1*Tg7 mice and compared them to B6.*Sle1* and B6.*Sle1*Tg7 counterparts to determine the necessity of B cells in the augmentation of autoimmune phenotypes. Analysis of splenomegaly, overall splenic

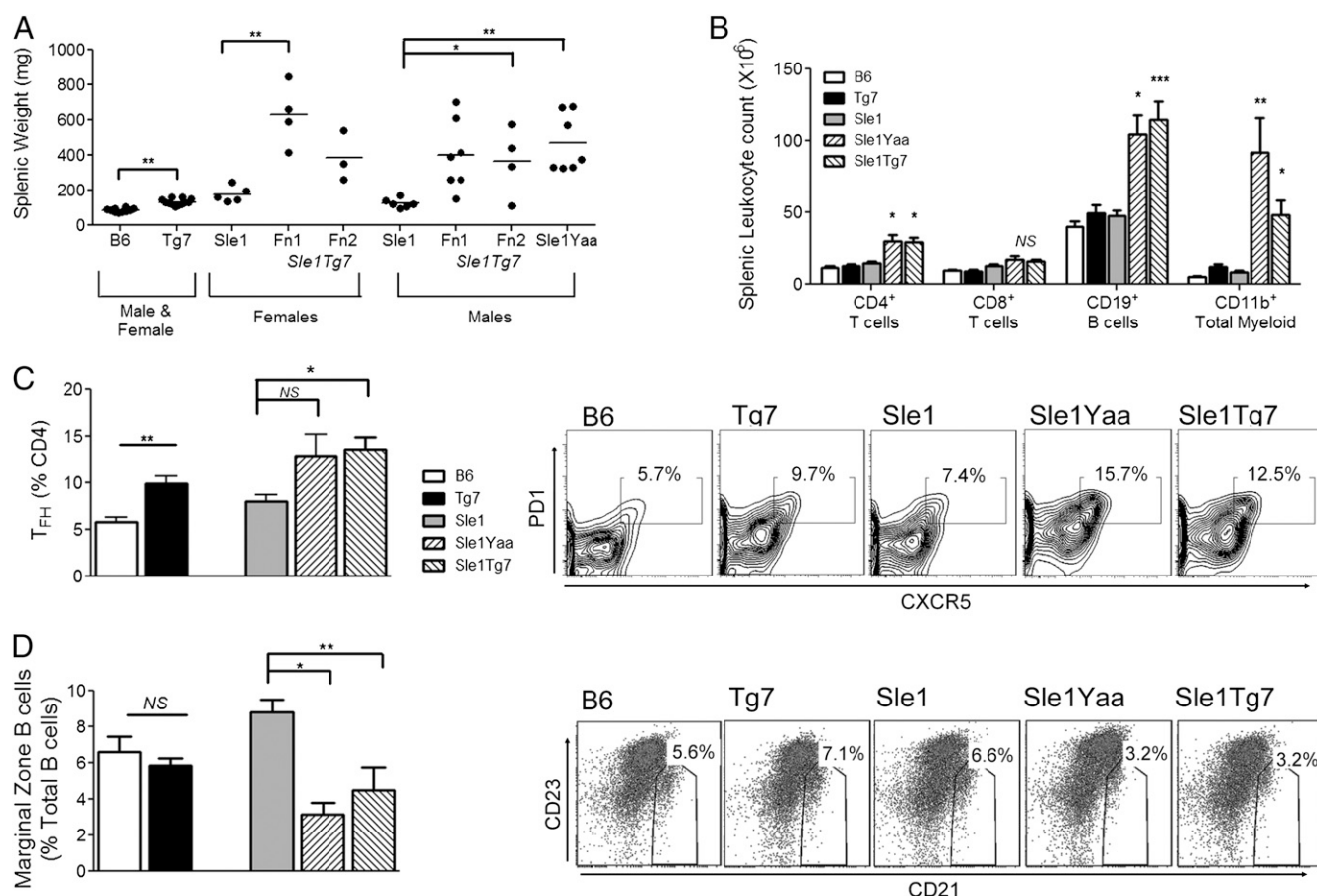


FIGURE 3. B6.*Sle1*/Tg7 mice develop splenic disease characteristics associated with the B6.*Sle1*Yaa strain. (A and B) Male mice were aged to 7–9 mo and splenic autoimmune traits were analyzed. (A) B6.*Sle1*/Tg7 mice exhibit splenomegaly in a similar manner to B6.*Sle1*Yaa over their B6.*Sle1* counterparts. (B) Expanding leukocytes in the spleen were analyzed using flow cytometry with Abs to CD3, CD4, CD8, CD19, and CD11b to identify CD4 and CD8 T cells, B cells, and the total myeloid population (CD11b⁺). (C) T_{FH} were identified by PD-1 and CXCR5 expression on CD4⁺ T cells. (D) Marginal zone B cells (Mz) were identified using B220, CD23, and CD21 mAbs. Data are represented as means \pm SEM. Data are cumulative from two independent aging cohorts, with a minimum of three mice per group for each cohort ($n = 6$ for B6, B6.*Sle1*; $n = 7$ for B6.Tg7 (Fn1/2); $n = 11$ for B6.*Sle1*Tg7; $n = 7$ for B6.*Sle1*Yaa). Data from (B) and (C) are representative from one cohort. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

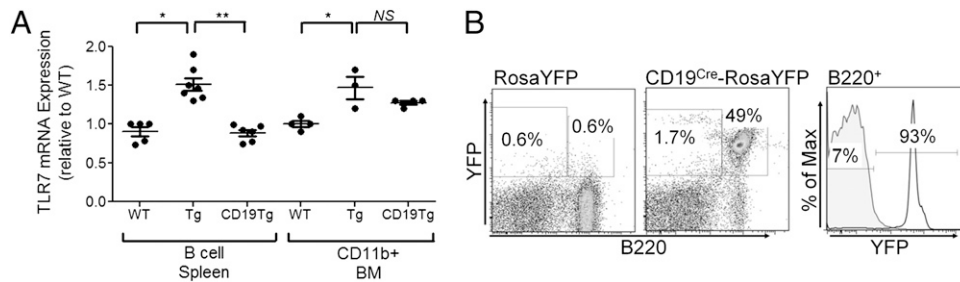


FIGURE 4. Effective elimination of the additional TLR7 copy in B cells. **(A)** Transgenic mice (Tg7) were crossed with Cre recombinase mice (CD19^{Cre}). B cells or CD11b⁺ cells from bone marrow (BM) were purified in Tg7, wild type (WT), or CD19Tg7 and the mRNA was purified and assessed for TLR7 expression. **(B)** CD19^{Cre} mice were crossed with a ROSA-enhanced YFP reporter strain, and the fluorescence was evaluated by flow cytometry using B220 mAbs and YFP emission. **p* < 0.05, ***p* < 0.01.

numbers, and leukocytes using common lineage markers CD19, CD3, CD4, CD8, and CD11b by flow cytometry showed that B cell normalization of TLR7 did not alter the disease profile of the spleen (Table I). Analysis of splenic weights from 7- to 9-mo-old male mice also replicated these findings and determined that there was no observable effect from the CD19^{Cre} itself (Supplemental Fig. 1B).

Further flow cytometry analysis showed that the restoration of normal TLR7 expression within B cells prevented the loss of the marginal zone compartment (Fig. 5A, Supplemental Fig. 1B). Additionally, immunohistochemistry of spleen confirmed a completely disrupted follicular architecture in B6.*Sle1*Tg7 with no real marginal B cell area. CD19.B6.*Sle1*Tg7 spleens had marginal B cells, but the zone was discontinuous around the follicle. Both flow and microscopy demonstrated no differences in either germinal center cells (GL7⁺) or plasma cells within these mice (Fig. 5A, 5B).

Analysis of activation markers in splenic B and T cells revealed that B6.*Sle1*Tg7 lymphocytes were activated, with increased CD69 and ICOS, compared with B6.*Sle1* controls (Fig. 5C). When TLR7 was normalized by CD19^{Cre} the upregulation of CD69 in both lymphocyte populations, as well as ICOS in CD4⁺ T cells, was prevented (Mann–Whitney comparison, *p* < 0.05; Fig. 5C, 5D).

Normalization of TLR7 in B cells specifically decreases Abs to RNA/protein complexes

The ANA production by the B6.*Sle1* model has been measured repeatedly over the years by ELISA detecting IgG autoantibodies to histones, chromatin, and dsDNA, as these appear to be predominant. We determined that the increase in titers in the B6.*Sle1*Tg7 strain was dependent on overexpression of TLR7 in B cells (IgG ANA, Fig. 6A), as levels in CD19.B6.*Sle1*Tg7 6-mo-old female mice were similar to B6.*Sle1*. However, when we analyzed the penetrance of IgG anti-dsDNA in CD19.B6.*Sle1*Tg7

mice, we observed no difference when compared with the B6.*Sle1*Tg7 controls (anti-dsDNA). Because TLR7 has been associated with U1 snRNP autoreactivity, we determined serum levels of these autoantibodies using an ELISA (anti-snRNP) (37–39). The increase in anti-snRNP (U1) shown in the B6.*Sle1*Tg7 strain was prevented by B cell TLR7 normalization (Fig. 6A). Furthermore, analysis of 7- to 9-mo-old male mice showed identical findings (Supplemental Fig. 1B).

Assessment of Abs to 10 different autoantigens by Luminex showed a significant reduction in the titers of anti-SSA52 autoantibodies (Fig. 6B). In this assay, the snRNP analyte includes both anti-U1 and the anti-B/B core proteins, unlike the ELISA, which detects anti-U1 alone. Further analysis of antinuclear Abs using staining of the HEp-2 cell line confirmed that the CD19.B6.*Sle1*Tg7 had similar titers to the B6.*Sle1* mice and there was no change in staining pattern (Fig. 6C).

Overall, these data indicate that the B cell TLR7 expression is responsible for the high titers of Abs to RNA/protein complexes.

Normalization of B cell TLR7 expression moderately reduces kidney inflammation

Blinded analysis of renal histology by an independent pathologist demonstrated a marginal reduction in GN from CD19.B6.*Sle1*Tg7 mice when compared with B6.*Sle1*Tg7 control mice (Fig. 7A; *p* < 0.05). However, 85% of the mice still developed severe GN (score of ≥3; six of seven mice), suggesting that other cell types are critical for initiation of kidney disease. Assessment of the blood urea nitrogen suggested that the increase in levels shown by the B6.*Sle1*Tg7 mice was prevented in the CD19.B6.*Sle1*Tg7 mice, although a comparison between the two strains was not significant (Fig. 7B).

Examination of the leukocyte infiltrate in the kidney using flow cytometry and microscopy showed a reduction in CD45⁺ cells in the CD19.B6.*Sle1*Tg7 model when compared with the B6.*Sle1*Tg7 model, although there was still considerable infiltrate in both

Table I. Normalization of TLR7 in CD19⁺ cells does not impact splenic leukocyte expansion

	B6	Tg7	<i>Sle1</i>	<i>Sle1</i> Tg7	CD19 <i>Sle1</i> Tg7
Spleen weight	84 ± 4	114 ± 7	134 ± 6	467 ± 28	335 ± 31
Count (×10 ⁷)	12.9 ± 1.0	15.0 ± 1.1	17.8 ± 1.3	55.4 ± 8.2	37.1 ± 4.5
Sample size (<i>n</i>)	18	19	25	26	14
Splenic leukocytes (% CD45)					
CD19	50.3 ± 7.2	47.9 ± 6.4	48.1 ± 7.3	38.3 ± 8.4***	33.7 ± 6.9***
CD4 T	18.1 ± 3.7	20.6 ± 4.1	19.2 ± 4.6	17.5 ± 2.8	19.7 ± 4.8
CD8 T	13.3 ± 2.2	13.4 ± 3.2	13.0 ± 2.3	7.7 ± 1.7***	10.6 ± 3.9
CD11b	7.4 ± 1.8	9.2 ± 1.9	9.8 ± 2.3	22.1 ± 7.4***	26.0 ± 6.2***
Sample size (<i>n</i>)	13	19	20	23	11

****p* < 0.001 compared with B6.*Sle1* mice. Comparisons between B6.*Sle1*Tg7 and CD19.B6.*Sle1*Tg7 mice did not reveal any significant differences.

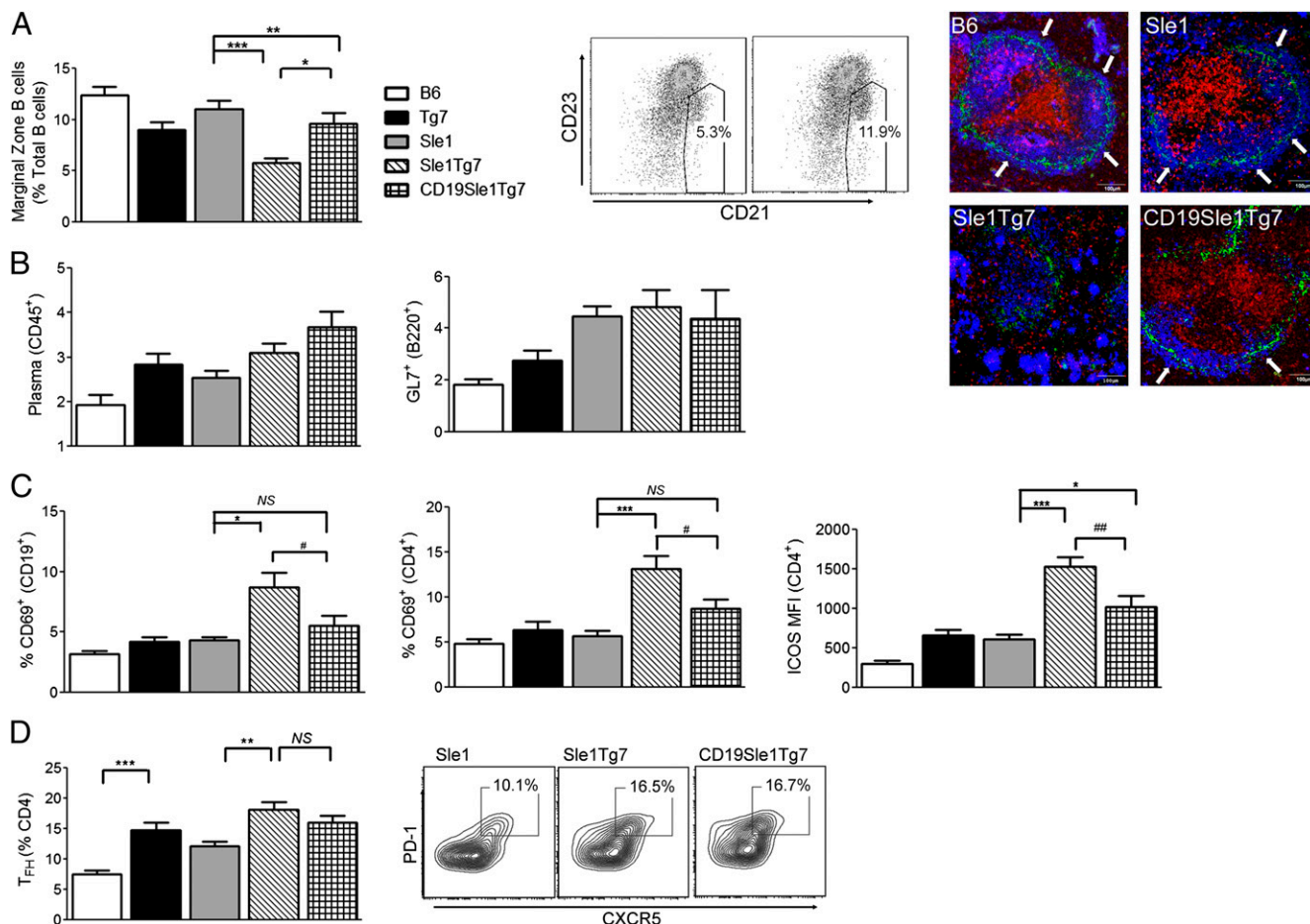


FIGURE 5. Increases in B cell TLR7 result in enhanced splenic B and T cell activation and marginal zone B cell depletion. **(A)** Female mice were aged to 6 mo and the percentage of splenic B cell marginal zone cells were determined as described earlier (with representative plot). Microscopy using MOMA-1 (green), CD5 (red), and IgM (blue) shows continuous IgM⁺ marginal B cell zone (white arrows) in B6 and B6.*Sle1* controls. There is a completely disrupted follicular architecture in B6.*Sle1*Tg7 with no definitive marginal zone. The IgM bright cells are plasma cells, which are numerous in B6.*Sle1*Tg7. In B6.CD19.*Sle1*Tg7 there are marginal B cells although the zone is discontinuous around the follicle. **(B)** FACS analysis of plasma cells (B220⁺CD138⁺) and GL7⁺ germinal center B cells showed no difference between B6.*Sle1*Tg7 and B6.CD19.*Sle1*Tg7. **(C)** The expression of CD69 B cells and CD4⁺ T cells, as well as ICOS on CD4⁺ T cells, shows a decrease in activation with CD19^{Cre} B cell normalization. **(D)** Analyses of splenic CD4⁺ T_{FH} population gated using PD-1 and CXCR5 show no difference between B6.*Sle1*Tg7 and B6.CD19.*Sle1*Tg7. Data are representative of means \pm SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 by one-way ANOVA (Mann–Whitney, # p < 0.05, ## p < 0.01 by Mann–Whitney test when B6.CD19.*Sle1*Tg7 is compared with B6.*Sle1*Tg7) ($n \geq 8$ /strain).

strains (Fig. 7C, 7D). Further characterization revealed that CD11b⁺ and CD19⁺ cellular recruitment to the kidney was lower in the CD19^{Cre}-expressing mice than in the parental B6.*Sle1*Tg7 strain, with CD4⁺ recruitment showing a similar trend (Fig. 7E, $p = 0.067$ [CD4]). When we calculated mortality of mice aged >200 d, we determined that the CD19.*Sle1*Tg7 strain had a penchant to survive longer than did their B6.*Sle1*Tg7 counterparts, although this was not significant (Fig. 7F).

Thus, we had demonstrated that an increase in TLR7 expression within B cells enhances leukocyte infiltrate, resulting in an exacerbation of kidney disease and increased mortality.

Discussion

In this study, we have described a new conditional TLR7 BAC transgenic mouse strain to investigate the role of increased TLR7 expression in SLE. These mice carry two additional copies of the TLR7 gene that results in a two-fold increase in TLR7 mRNA levels. Without the addition of other susceptibility loci, these mice do not develop disease. When we combined the TLR7 transgene with the B6.*Sle1* susceptibility region, B6.*Sle1*Tg7 mice developed a severe autoimmune profile almost indistinguishable from the B6.

*Sle1*Yaa strain. Because the TLR7 transgene is floxed it can be used to assess the contribution of upregulated TLR7 in specific leukocyte lineages. In the present studies we have assessed the contribution of TLR7 upregulation in CD19⁺ cells. We have demonstrated that an increase of TLR7 specifically in B cells is responsible for high titers of Abs to RNA/protein complexes. Furthermore, we have shown that increased B cell TLR7 expression results in increased inflammation of the kidney, leukocyte recruitment, and exacerbation of disease.

Since the discovery of the translocation of TLR7 in the *Yaa* model there has been a burgeoning interest in the role of this receptor in SLE. Recent genetic studies in humans have demonstrated TLR7/TLR8 associations with SLE at genome-wide significant levels in multiple populations (16, 17). Furthermore, immunological analyses have shown that anti-snRNP Abs can increase NETosis, a process that is enhanced in SLE (25, 26). Data from recent investigations using knockout mice demonstrate an underlying requirement for TLR7 for the development of autoimmunity (40, 41). Our findings from the B6.*Sle1* model also support these findings (T. Celhar, N. Panday, H. Lee, E.K. Wakeland, and A.-M. Fairhurst, manuscript in preparation). In our

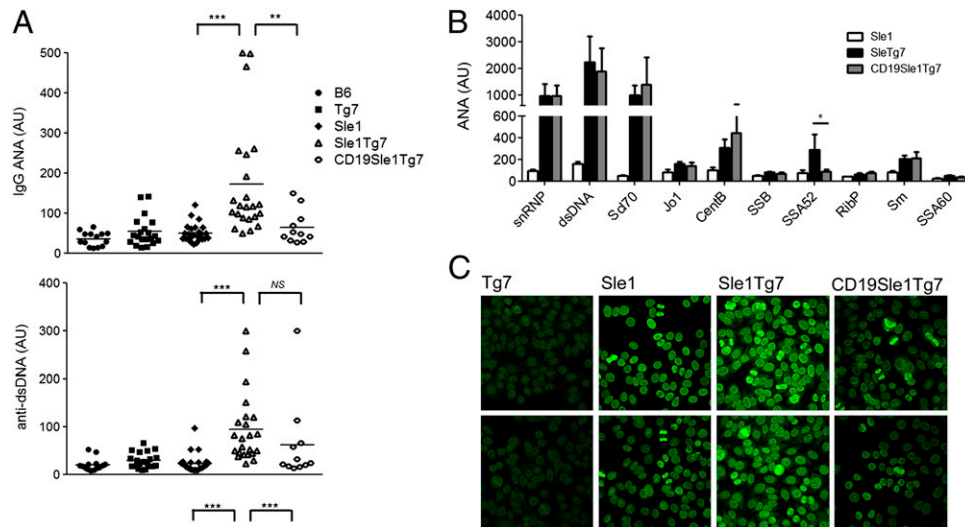


FIGURE 6. Normalization of TLR7 in B cells specifically decreases Abs to RNA/protein complexes. **(A)** Sera were analyzed for IgG dsDNA/histone/chromatin autoantibodies (IgG ANAs), IgG anti-dsDNA Abs (anti-dsDNA), or IgG anti-snRNP Abs (anti-snRNP) by ELISA. The standard curve for each ELISA was done with pooled B6.*Sle1Yaa* sera to give arbitrary units (AU) for each ELISA. **(B)** Multiple ANAs were also assayed using Luminex. Hep-2 staining with secondary Dylight-488 was completed on five to six mice per strain and analyzed by confocal microscopy. Original magnification $\times 60$. **(C)** Representative images are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bars represent SEM.

original multistep hypothesis of how SLE progresses there is an initial loss of tolerance to self that results in the production of autoantibodies (7). Thus, previous data support a dependency for TLR7 at this stage. The second step to a progression of severe disease depends on an additional immune alteration. Studies using *Yaa*-containing SLE models have shown that an increase in TLR7 expression is required for the initiation of severe disease (10–12). Furthermore, other non-*Yaa* SLE models show a role for TLR7 in pathogenesis. Inhibition of both TLR7 and TLR9 or single inhibition of TLR7 reduces autoimmune pathology in both the NZB/W strain and the MRL^{lpr} strain (13, 14, 42). Furthermore, exogenous administration of a TLR7 ligand, imiquimod, results in an augmentation of kidney disease (43).

In this study, we demonstrated that a moderate chronic upregulation of TLR7 is sufficient to drive kidney pathogenesis in the lupus susceptible strain, B6.*Sle1*. Because we know that IFN- α induces TLR7 expression, this suggests that a viral initiation or exacerbation of severe disease is a plausible mechanism in SLE (44, 45).

A moderate increase in TLR7 alone is not sufficient for the development of severe disease, and the additional susceptibility loci *Sle1* is required. Polymorphisms of the signaling lymphocyte activation molecule family (SLAMF^Z) are responsible for the mild autoimmune traits conferred by this region (46, 47). Recent studies have supported a role for both Ly108 and CD84 in this model (46–48). Ly108 is important for B and T cell autoreactivity, with an autoimmune-resistant haplotype (Ly108-H1) existing in CD4 T cells in B6 mice that prevents ANA production (47). Additionally, CD84 is critical for optimum germinal center formation, efficient T and B cell interaction, and the generation of T follicular helper cells (49). Moreover, differential expression or signaling of SLAMF members have been associated with human disease. Different haplotypes of two family members, CS1 and

2B4, exist in the human population and differential expression is associated with disease (50–52). Furthermore, associations in Ly9 have been shown to correlate with T cell differences in United Kingdom and Canadian SLE populations, and ligation of CD352 (SLAMF6) results in impaired cytokine responses in SLE T cells (53, 54).

Therefore, there is increasing evidence that SLAMF and its downstream signaling adaptors are significant immunomodulatory receptors, regulating normal B, T, and NK cell function, and this may play a significant role in SLE (55). Our murine data have shown us that an epistatic interaction exists between the SLAMF and TLR7, as an immune alteration in either does not result in more severe disease, but the combination of SLAMF polymorphisms and TLR dysregulation results in early onset aggressive nephritis. It is less clear how these receptors interact with the TLR pathway and impact immune cell function.

A comparison of mortality between B6.*Sle1Tg7* and B6.*Sle1Yaa* mice clearly demonstrates that additional genes within the *Yaa* locus contribute to disease. These findings are consistent with our earlier investigations and with the results from Izui and colleagues (12), which show that additional *Yaa*-genes may contribute to ANA titer and autoimmune pathology (10). These data are in contrast to those reported by another group, who described a deletion of all autoimmune phenotypes with TLR7 normalization (11). This inconsistency may be due to the additive autoimmune effect from the Fc γ RIIb deletion, which is not present in the other two murine strains.

In these studies we also examined the contribution of B cells to the autoimmunity traits conferred by increased TLR7 expression. We demonstrated that the level of TLR7 expression, in the context of SLAMF^Z, dictates the capacity for Ag specificity to RNA. Additionally, in this environment, TLR7 confers an increase in the expression of ICOS, CD69, and CXCR4 on B and T cells, which

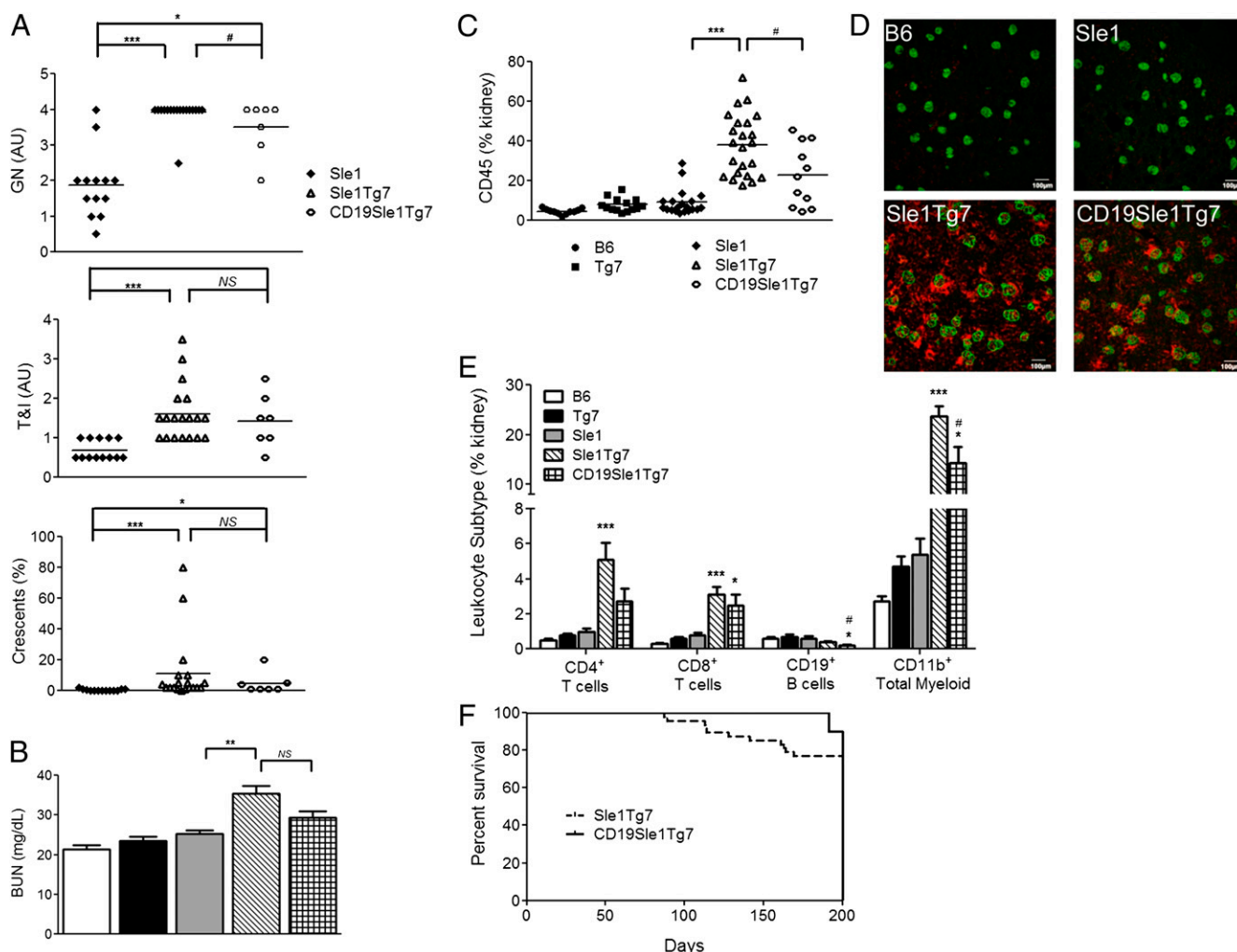


FIGURE 7. Normalization of B cell TLR7 expression moderately reduces kidney inflammation. **(A)** Female mice were aged to 6 mo and kidneys were assessed for histological nephritis. **(B)** Blood urea nitrogen (BUN) was also assessed as a measure of kidney function ($n \geq 6$ /strain). Total CD45⁺ leukocyte infiltration into the kidney was assessed using flow cytometry as described earlier ($n \geq 6$ /strain) **(C)**, with microscopy demonstrating equivalent infiltration into the glomeruli between B6.Sle1Tg7 and B6.CD19Sle1Tg7 mice, CD45-allophycocyanin (red), and nephrin-Alexa 488 (green). Original magnification $\times 100$ **(D)**. **(E)** Analyses of leukocyte lineages infiltrating the kidney show a reduction in CD3⁺CD4⁺, CD19⁺, and CD11b⁺ cells when B6.CD19Sle1Tg7 mice are compared with B6.Sle1Tg7. $^{\#}p < 0.05$. **(F)** There was a slight reduction in mortality in the B6.CD19Sle1Tg7 ($n = 10$) mice compared with B6.Sle1Tg7 mice ($n = 48$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for comparisons to B6.Sle1 control. Graph bars represent SEM.

are molecules important in leukocyte emigration and activation. However, B cell normalization of TLR7 did not significantly reduce splenomegaly or the cellular composition of the spleen. Furthermore, T follicular helper cell (T_{FH}) development was unaffected by TLR7 normalization. A recent study by Tangye and colleagues (56) demonstrated that although the SLAM adaptor protein SAP is required for the B/T cell-dependent T_{FH} development, in the case of excess Ag, the T/dendritic cell interaction becomes dominant and overrides the necessity of SAP for T_{FH} production. However, SAP is required for the T-dependent Ab response. Therefore, in SLE, where there is persistent Ag activation from immune complexes containing nuclear components, the dendritic cells may be driving T_{FH} development and may be the central player of disease.

Aside from the role the dendritic cells may play, it is also possible that there are TLR7-dependent inflammatory effects intrinsic to the kidney that may contribute to pathogenic disease, as we have previously shown that the kidney itself can produce IFN- α in response to autoantibody-mediated GN (33). However, recent studies suggest that TLR7 is expressed by renal macrophages and not kidney mesangial renal cells (14, 43). The combination of our

model with the Vav1^{Cre} system will normalize TLR7 expression in all leukocytes and help to elucidate the contribution of the target organ (57).

Overall, we have demonstrated that a chronic moderate up-regulation of TLR7 is sufficient to drive severe disease in a murine model of lupus. Furthermore, we demonstrated that upregulation of TLR7 within B cells results in a high titer of Abs to RNA/protein complexes, a B cell marginal zone defect, and an escalation of disease. Our findings have important consequences for clinical therapeutics. SLE is a heterogeneous polygenic disease with patients displaying a wide range of disease characteristics. Therefore, it is likely that aberrant activation of different inflammatory pathways may lead to disease. Based on our findings we hypothesize that patients who exhibit Abs to RNA/protein complexes possess an upregulated TLR7 signaling pathway within their B cells and possibly within other leukocytes. If we could preselect patients for specific autoimmune mechanisms, such as enhanced TLR7 expression or function, using clinical parameters (e.g., anti-snRNP ANAs), we could move to a personalized medicine approach based on the individual needs of the patients, thereby reducing side effects and improving efficacy.

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Disclosures

The authors have no financial conflicts of interest.

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