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B cell-TLR7 expression drives anti-RNA autoantibody production and exacerbates disease in SLE-prone mice

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Abstract

Systemic Lupus Erythematosus (SLE) is a chronic systemic autoimmune disease characterized by the production of anti-nuclear autoantibodies (ANA). ANA development is recognized as one of the initial stages of disease which 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 has highlighted an important role for the TLR family, particularly TLR7 in both human disease and murine models. In the studies presented here, we have presented a low copy conditional TLR7 transgenic (Tg7) mouse strain which 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 antibodies to RNA/protein complexes and exacerbates SLE disease.

Introduction

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 antigens (1, 2). These antinuclear antibodies can also be detected in a high frequency of individuals in the general population (3). A subset of individuals with high titered ANA transition to severe disease and this progression is accompanied by increased numbers of antibodies (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 3 lupus susceptibility regions originally identified from the NZM2410 lupus prone mouse strain

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through a backcrossing strategy. In these original analyses *sle2* and *sle3* were also identified and data since then has shown that the presence of these loci leads to enhanced B cell activation and Toll like receptor (TLR) hyperactivity respectively (6). Over the years 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 earlier (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 *Yaa* (y-linked autoimmune accelerating) 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 approximately 16 genes from the X-chromosome, onto the Y-chromosome (8, 9), which resulted in a 2-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, using knock-out strategies (10–12). Studies by ourselves and the Izui group also suggested that other genes within this region play a role, since 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 immunoregulatory sequences (IRS) inhibits disease in both NZB/W and MRL^{lpr} mice (13, 14). Further, 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 (SNP) in Asian males (16, 17). In addition, 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 to adults (18). Other data suggests TLR7 mRNA levels are increased in adult patients with SLE, correlating with increased RNA expression of IFNα (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-snRNP antibodies (25, 26). This may provide the antigenic material which contributes to the propagation of the inflammatory response.

Since 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 (eg *Rab9, Tlr8*), we sought to determine (a) whether moderate upregulation of TLR7 alone is sufficient to drive disease progression, and (b) 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.*Sle1Tg7*) the mice display disease characteristics similar to the B6.*Sle1Yaa* 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 demonstrates 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 antibodies 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 B6.ROSA.EYFP [*Gt(ROSA)26Sor*$^{tm1(Smo/EYFP)Amc}$] mice were originally obtained from the Jackson Laboratory (27, 28). Cre mice were purchased at *n10* B6, but were also checked for any contaminating 129 autoimmune alleles using SNP analyses. None were 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 months old. For all other studies mice used were 6–8 weeks. The modified TLR7 BAC transgene was introduced by pronuclear injection into fertilized eggs and was performed by the Transgenic Core at UT 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 IACUC approved animal protocol. All antibodies were purchased from BD Biosciences or Ebiosciences 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, RP2392P6 was purchased from the BACPAC Resources Center at Children's Hospital Oakland Research Institute, CA. It contained the full sequence for both TLR7 and PRPS2 (phosphoribosylpyrophosphate synthetase subunit 2). It was modified using a "Counter-Selection BAC Modification Kit" by Red®/ET® Recombination produced by Gene Bridges, Dresden, Germany. The 50bp homology primer sequences for TLR7 were as follows: ***5′Fwd***: AGC TCA AAG GCT CTG CGA GTC TCG GTT TTC TGT TGC CTT CTC TCT GTC TC; ***5′Rev***: TAA AAT ACC TTT CTT GAA GGC ATA GAG TGG TTC TAT AGA TGG AGT CCT CT; ***3′Fwd***; CTC TCT GAA GAA TGT CAC CAC CTA GGA CAT GCC TTG GTA CCT GAA GTT TT; ***3′Rev***: ATG ACA ACT GTT AGG AAA AAT TCA GAC CTT 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] TATACGAAGTTAT-3′ and 3′-TATTGAAGCATAT [CGTATGTA] ATATGCTTCAATA-5′. Efficient modification of the BAC was examined using genomic sequencing and multiple enzyme digestion with pulse field gel analyses. Integration of the modified BAC by pronuclear injection into C57BL/6J fertilized eggs was completed at the UT Southwestern Transgenic Core.

Assessment of Renal Disease

Blood Urea Nitrogen (BUN) was assessed using the QuantiChrom Urea Assay Kit (BioAssay Systems, 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

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mesangial cellularity and matrix; 2, moderate increase in mesangial cellularity and matrix, with thickening of the glomerular basement membrane (GBM); 3, focal endocapillary hypercellularity with obliteration of capillary lumina and a substantial increase in the thickness and irregularity of the GBM; and 4, diffuse endocapillary hypercellularity, with segmental necrosis, crescents, and hyalinized end-stage glomeruli. Tubular interstitial nephritis (T&N) 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.*Sle1Yaa* 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 (SIgN), using the AtheNA Multi-Lyte ANA-III Test System (Zeus Scientific), as per manufacturer's instructions, using a donkey anti-mouse IgG detection antibody (Jackson Immunoresearch). Hep2 staining was performed on selected samples at a 1:200 dilution as per manufacturer's instructions, with a goat-anti-mouse IgG-DyLight®488 secondary antibody. Cytokine Multiplex Analysis was performed the Bio-Plex cytokine assay kit (Bio-Rad) as per manufacturer's protocol at BIIR Luminex Core, Dallas.

Cell Purification and RT-PCR

TLR7 copy number from tail DNA lysates was assessed using premixed RTPCR Taqman® primer sets for GAPDH, Mm99999915_g1 and custom designed primers and probe TLR7 from ABI TLR7F: CAGAGGCCCATGTGATCGT; TLR7R: GCCCTCAGGGATTTCTGTCA; TLR7Probe (TAMRA™): ACTGCACAGACAAGC. 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 RNA-easy kit. RNA message was analyzed using Applied Biosystems Taqman® Gene Expression Assays using RNA-specific primers for B2M (Mm00437762_m1), TLR7 (Mm00446590_m1) and TLR9 (Mm00446193_m1). Absolute MAX QRT-PCR mix from Thermo Scientific was used for amplification, as per the manufacturer's instructions. An ABI 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 B2M 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 0.75ml PBS. Cells were spun down and the supernatant was kept at −20°C for cytokine analysis. This is referred to as kidney plasma in Figure 2f. Cells were resuspended in digestion buffer, consisting of 1mg/ml collagenase IV (Sigma-Aldrich) and DNase I (1ug/ml) in RPMI Complete Media (RPMI CM) and incubated at 37°C for 30mins. Cells were centrifuged, filtered through a 70uM mesh and then mixed 1:1 with 40% Percoll solution. This was centrifuged at 3000 rpm for 20 minutes at RT with the brake off. The loose pellet was washed, 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 9 directly conjugated antibodies (FITC, PE, PE-Texas-Red, PE-Cy5 or PerCP-Cy5.5, PE-Cy7, allophycocyanin (APC), Alexa700, APC-Cy7, Pacific Blue) and one biotinylated antibody. CFSE and YFP were evaluated in the FITC channel (in the absence of a monoclonal-FITC marker). Secondary detection of the biotinylated antibody was with streptavidin QDOT655. Red blood cell lysis was completed using BD FACS Lysing solution

as per manufacturer's instructions. Acquisition and analysis was completed using a BD LSR II with Flowjo 7.6 for Windows (Treestar). Confocal images were captured with an Olympus FV-1000 Confocal system and processed with Olympus FV10-ASW Fluoview v2.0b.

Statistical Analyses

Results are expressed as the arithmetic mean ± standard error of the mean (SEM). Normality was tested using the Kolmogorov and Smirnov test. For comparing 2 groups, an unpaired 2-way Student's t-test was used and for multiple comparisons, a 1-way parametric ANOVA & Tukey post hoc comparison was used. If normality test failed, a Mann-Whitney U test compared two groups, and 1-way non-parametric ANOVA and Dunn's multiple comparison test was used to test multiple comparisons. For time-treatment or time-strain comparisons, a 2-way ANOVA with Bonferroni posthoc analyses was used. Analyses of survival was 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, USA) or Microsoft Excel.

Results

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

In order 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 Figure 1A. Analysis of genomic DNA from 4 initial founder lines (Fn) showed 2 additional copies of TLR7 within these mice (Figure 1B), with 3 exhibiting a similar doubling in B cell mRNA expression to the *Yaa* strain (Figure 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 associated trend in proliferation at 72hrs (Figure 1D and E). Additional data from a mixed sex population (Fn1 and Fn2) showed similar trends (n=4, p=0.076, supplemental figure 1A). These Tg7 mice did not develop significant levels of ANAs, or glomerulonephritis (Figure 2A and 2B).

Thus we developed a low copy TLR7 transgenic, similar to the *Yaa* strain, which 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, this was significant in male mice analyzed (Figure 2A). Pathological analysis indicated that female and male B6.*Sle1*Tg7 mice also developed severe glomerulonephritis (GN), similar to their B6.*Sle1Yaa* counterparts (Figure 2B). Analysis of mortality demonstrated that male B6.*Sle1*Tg7 mice survived significantly longer than their B6.*Sle1Yaa* counterparts (Figure 2C) suggesting 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 to 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 (Figure 2A).

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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, and $CD11b^+$ myeloid cells, in a similar manner to B6.*Sle1Yaa* mice (Figure 2D and 2E). Analysis of the supernatant from freshly isolated kidney cells using multi-plex luminex determined several inflammatory cytokines and chemokines that were significantly increased in the B6.*Sle1Yaa* compared to B6.*Sle1* controls, with B6.*Sle1*Tg7 mice showing similar trends.(Figure 2F). These included IL-12p40, KC (keratinocyte chemoattractant) and MCP-1 (monocyte chemoattractant protein-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* and this was also detected in the B6.*Sle1*Tg7 strain (Figure 3A). Cellular expansion involved $CD4^+$ T cells, $CD19^+$ B cells and in particular, $CD11b^+$ myeloid cells (Figure 3B). Spleens from B6.Tg7 mice were also slightly larger than B6 wild-type controls but there was no detectable leukocyte population expanding (Figure 3A and 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.*Sle1Yaa and* B6.*Sle1*Tg7 (Figure 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 (Figure 3D).

Therefore, our data so far demonstrated that the Tg7 mice which 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

Since our transgenic system promotes disease progression in a similar manner comparable to the B6.*Sle1Yaa* model, we then sought to utilize 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 (Figure 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 over 90% of B cells did express YFP, showing high efficiency (0.96±0.11, 90.03±2.46 respectively; representative plots Figure 4b).

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Increases in B cell TLR7 result in enhanced splenic B and T cell activation, and 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 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 1). Analysis of splenic weights from 7–9 month old male mice also replicated these findings and determined that there was no observable effect from the $CD19^{Cre}$ itself (Supplemental Figure 1B).

Further flow cytometry analysis showed that the restoration of normal TLR7 expression within B cells prevented the loss of the marginal zone compartment (Figure 5A and supplemental Figure 1B). In addition, 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 however, 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 (Figure 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 to B6.*Sle1* controls (Figure 5C). When TLR7 was normalized by $CD19^{Cre}$ the upregulation of CD69 in both lymphocyte populations, and ICOS in $CD4^+$ T cells was prevented (Mann-Whitney comparison, $p<0.05$; Figure 5C and 5D).

Normalization of TLR7 in B cells specifically decreases antibodies 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 double stranded DNA, since 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, Figure 6A), since levels in CD19.B6.*Sle1*Tg7 6 month 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). Since 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 (Figure 6A). Furthermore, analysis of 7–9 month old male mice showed identical findings (Supplemental Figure 1B). Assessment of antibodies to 10 different autoantigens by Luminex, showed a significant reduction in the titers of anti-SSA52 autoantibodies (Figure 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 anti-nuclear antibodies using staining of the HEp2 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 (Figure 6C).

Overall this data indicates that the B-cell TLR7 expression is responsible for the high titers of antibodies 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 to B6.*Sle1*Tg7 control mice

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(Figure 7A; $p<0.05$). However, 85% of the mice still developed severe GN (≥score 3; 6/7 mice) suggesting that other cell types are critical for initiation of kidney disease. Assessment of the blood urea nitrogen (BUN) suggested that the increase in levels shown by the B6.*Sle1*Tg7 mice, was prevented in the CD19.B6.*Sle1*Tg7, although a comparison between the 2 strains was not significant (Figure 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 to the B6.*Sle1*Tg7, although there was still considerable infiltrate in both strains (Figure 7C and D). 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 (Figure 7E, $p=0.067$ (CD4). When we calculated mortality of mice aged beyond 200 days, we determined that the CD19.*Sle1*Tg7 strain had a penchant to survive longer than their B6.*Sle1*Tg7 counterparts, although this was not significant (Figure 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 report, 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 which 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 develop a severe autoimmune profile almost indistinguishable from the B6.*Sle1Yaa* strain. Since the TLR7 transgene is floxed it can be used to assess the contribution of upregulated TLR7 in specific leukocyte lineages. In the studies presented here 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 antibodies to RNA/protein complexes. Furthermore, 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). Further, immunological analyses have shown that anti-snRNP antibodies can increase NETosis, a process which is enhanced in SLE (25, 26). Data from recent investigations using knockout mice, demonstrates an underlying requirement for TLR7 for the development of autoimmunity (40, 41). Our findings from the B6.*Sle1* model also support these findings (manuscript in preparation). In our original multi-step hypothesis of how SLE progresses there is an initial loss of tolerance to self which results in the production of autoantibodies (7). Thus, previous data supports 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 reduce autoimmune pathology in both the NZB/W strain and the MRL^{lpr} (13, 14, 42). Furthermore, exogenous administration of a TLR7 ligand, imiquimod, results in an augmentation of kidney disease (43).

In our studies presented here, we demonstrated that a moderate chronic upregulation of TLR7 is *sufficient* to drive kidney pathogenesis in the lupus susceptible strain, B6.*Sle1.*

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Since we know that IFNα 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, such *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 which 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 2 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 UK and Canadian SLE populations, and ligation of CD352 (SLAMF6) results in impaired cytokine responses in SLE T cells (53, 54). Therefore, there is an increasing evidence that the SLAM family 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 has shown us that an epistatic interaction exists between the SLAMF and TLR7, since 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 on how these receptors interact with the TLR pathway and impact immune cell function.

A comparison of mortality between B6.*Sle1*Tg7 and B6.*Sle1Yaa* mice clearly demonstrate 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, which show that additional *Yaa*-genes may contribute to ANA titer and autoimmune pathology (10, 12). These data are in contrast to those reported by another group, who describe a deletion of all autoimmune phenotypes with TLR7 normalization (11). This inconsistency may be due to the additive autoimmune effect from the FcγRIIb 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 antigen specificity to RNA. In addition, in this environment, TLR7 confers an increase in the expression of ICOS, CD69, CXCR4 on B and T cells, which 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 demonstrated that although the SLAM adaptor protein SAP is required for the B-T cell dependent T_{FH} development, in the case of excess antigen, the T-DC interaction becomes dominant and overrides the necessity of SAP for T_{FH} production. However, SAP is required for the T dependent antibody response (56). Therefore, in SLE, where there is persistent antigen activation from immune complexes containing nuclear components, the DCs may be driving T_{FH} development and may be the central player of disease.

Aside from the role the DC may play, it is also possible that there are TLR7-dependent inflammatory effects intrinsic to the kidney which may contribute to pathogenic disease, since we have previously shown that the kidney itself, can produce IFNα in response to autoantibody-mediated glomerulonephritis (33). However recent studies suggest that TLR7

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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 upregulation of TLR7 is sufficient to driving severe disease in a murine model of lupus. Further, we demonstrated that upregulation of TLR7 within B cells results in a high titer of antibodies 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 heterogenous 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 might hypothesize that patients who exhibit antibodies 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, reducing side effects and improving efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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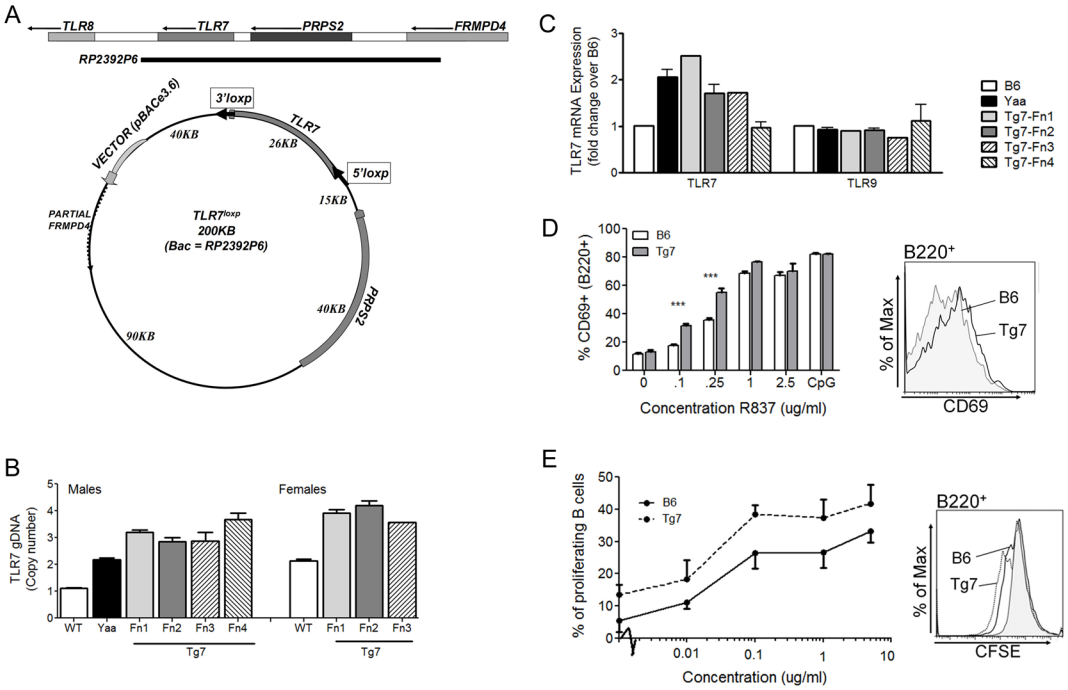

Figure 1. TLR7 Transgenic (Tg7) mice are similar in expression and function to Yaa mice

BAC RP23-92P6 was modified as described in the methods to include 2 loxP sites, flanking the TLR7 gene (A). Mice carrying the transgene carried two additional genomic DNA copies (B). Multiple founders (Fn) show 2x TLR7 expression compared to B6 mice (C). 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). 2 way ANOVA demonstrated significance for both D and E, post hoc significance is shown by *, $p<0.05$. Bars represent SEM, *n=3* mice per strain in each separate experiment.

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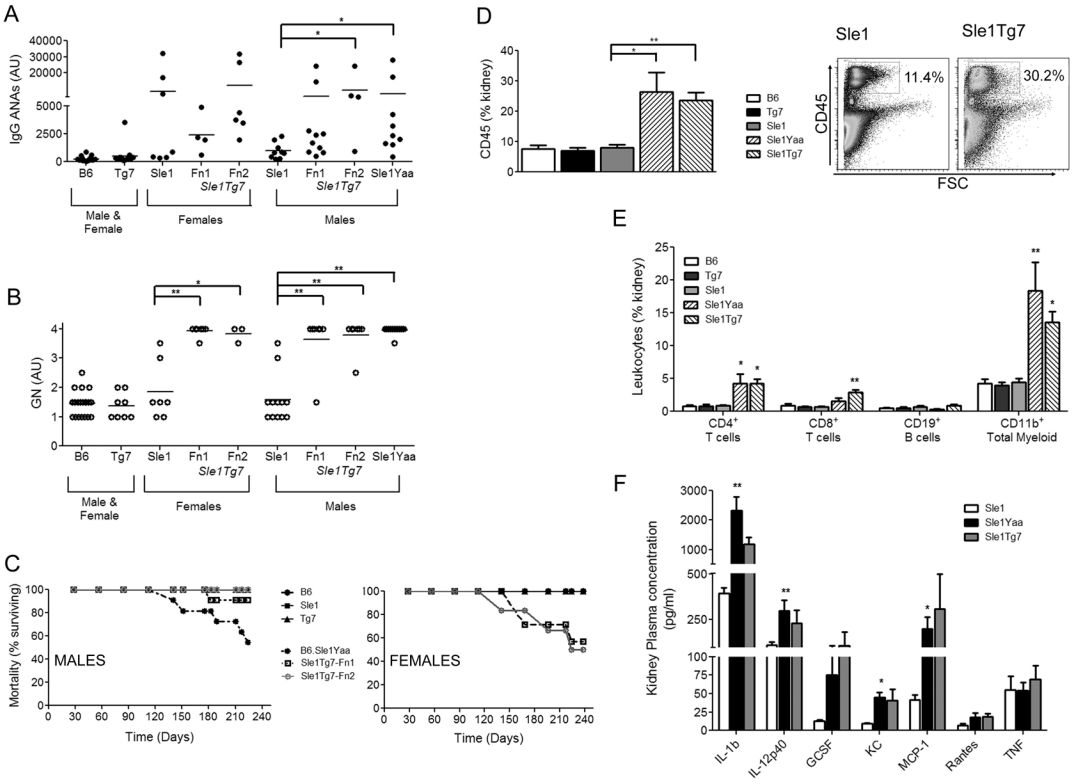

Figure 2. An epistatic interaction between Sle1 and Tg7 results in severe autoimmune pathology

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

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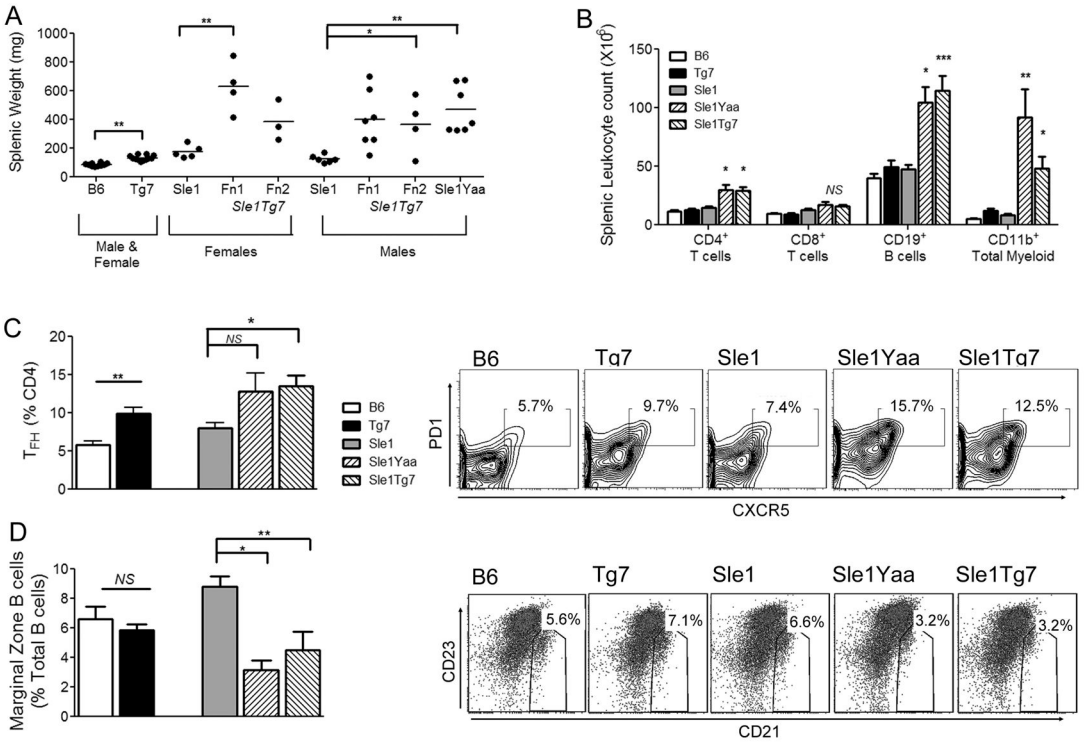

Figure 3. B6.Sle1Tg7 mice develop splenic disease characteristics associated with the B6.Sle1Yaa strain

Male mice were aged to 7–9 months and splenic autoimmune traits were analyzed. (Figure 3A and 3B). B6.*Sle1*Tg7 mice exhibit splenomegaly in a similar manner to B6.*Sle1Yaa* over their B6.*Sle1* counterparts (A). Analysis of expanding leukocytes in the spleen was analyzed using flow cytometry with antibodies to CD3, CD4, CD8, CD19 and CD11b to identify CD4 and CD8 T cells, B cells and the total myeloid population ($CD11b^+$) (B). T follicular helper (T_{FH}) cells were identified by PD-1 and CXCR5 expression on $CD4^+$ T cells (C). Marginal zone B cells (Mz) were identified using B220, CD23 and CD21 monoclonal antibodies (D). Data are represented as mean +/− SEM. Data are cumulative from 2 independent aging cohorts, with a minimum of 3 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.*Sle1Yaa*) Data from (B) and (C) are representative from one cohort. *p<0.05, **p<0.01.

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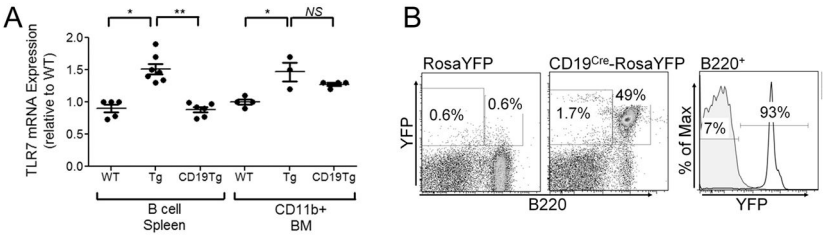

Figure 4. Effective elimination of the additional TLR7 copy in B cells

Transgenic mice (Tg7) were crossed 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 purified and assessed for TLR7 expression. (A). $CD19^{Cre}$ mice were crossed with a ROSA-eYFP reporter strain, and the fluorescence evaluated by flow cytometry using B220 monoclonal antibodies and YFP emission (B).

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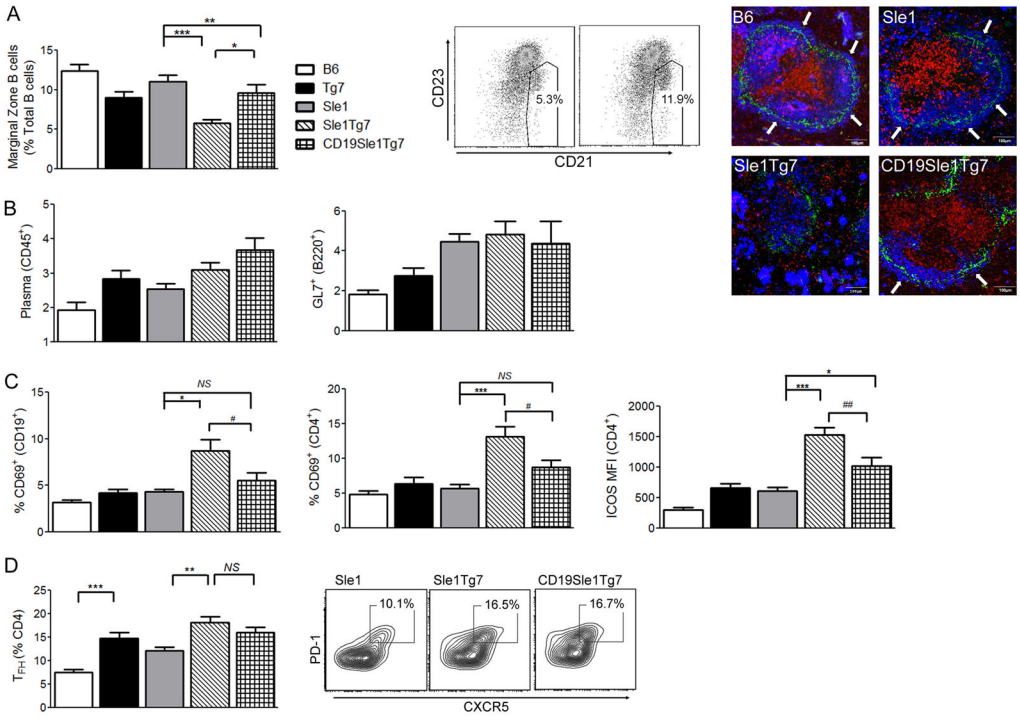

Figure 5. Increases in B cell TLR7 result in enhanced splenic B and T cell activation, and marginal zone B cell depletion

Female mice were aged to 6 months and the percentage of splenic B cell marginal zone cells were determined as described earlier (a, with representative plot). Microscopy using MOMA-1 (green), CD5 (red) 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. 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 (b). The expression of CD69 B cell and $CD4^+$ T cells, and ICOS on $CD4^+$ T cells shows a decrease in activation with $CD19^{Cre}$ B cell normalisation (c). Analyses of splenic $CD4^+$ T_{FH} population, gated using PD-1 and CXCR5 shows no difference between the B6.*Sle1*Tg7 and B6.CD19*Sle1*Tg7 (d). Data is representative of mean +/− SEM; 1-Way ANOVA, *p<0.05, **p<0.01, ***p<0.001. (Mann-Whitney, # p<0.05 when B6.CD19*Sle1*Tg7 is compared to B6.*Sle1*Tg7). NS, not significant. (*n*≥*8* per strain)

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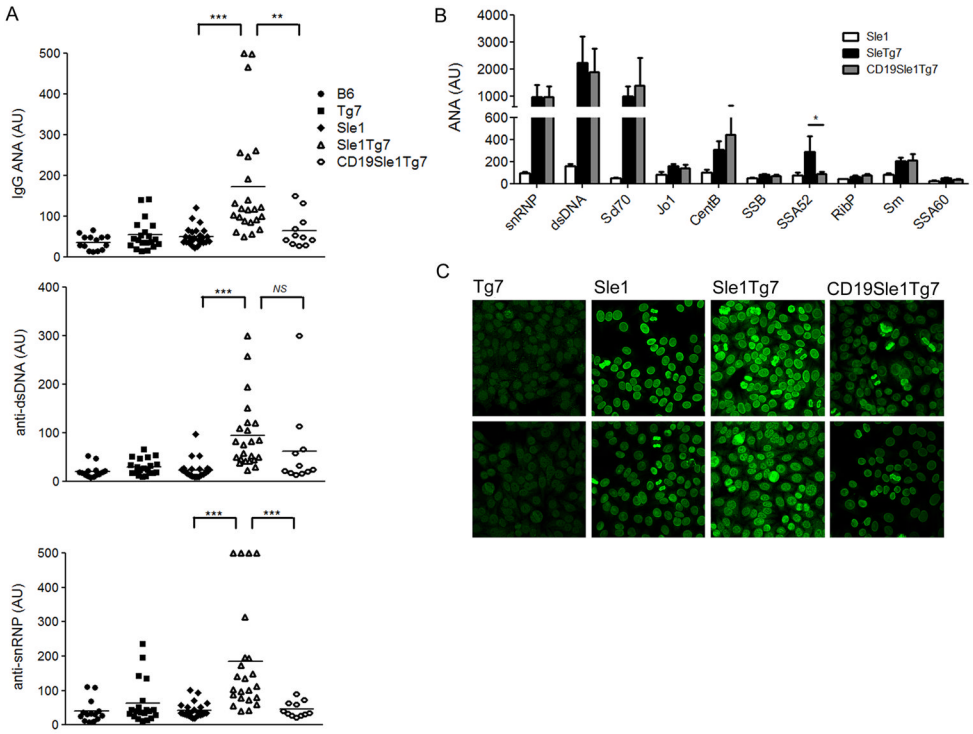

Figure 6. Normalization of TLR7 in B cells specifically decreases antibodies to RNA/protein complexes

Sera were analyzed for IgG dsDNA/histone/chromatin autoantibodies (IgG ANAs) or IgG anti-dsDNA antibodies (anti-dsDNA), or IgG anti-snRNP antibodies (anti-snRNP) by ELISA (a). The standard curve for each ELISA was done with pooled B6.*Sle1Yaa* sera to give arbitrary units (AU) for each ELISA. Multiple ANAs were also assayed using luminex (b). Hep-2 staining was completed on 5–6 mice per strain and analyzed by confocal microscopy. Representative images are shown (c). Comparisons to B6.*Sle1* control, *p<0.05, ***p<0.001. # p<0.05. Bars represent SEM, NS, not significant.

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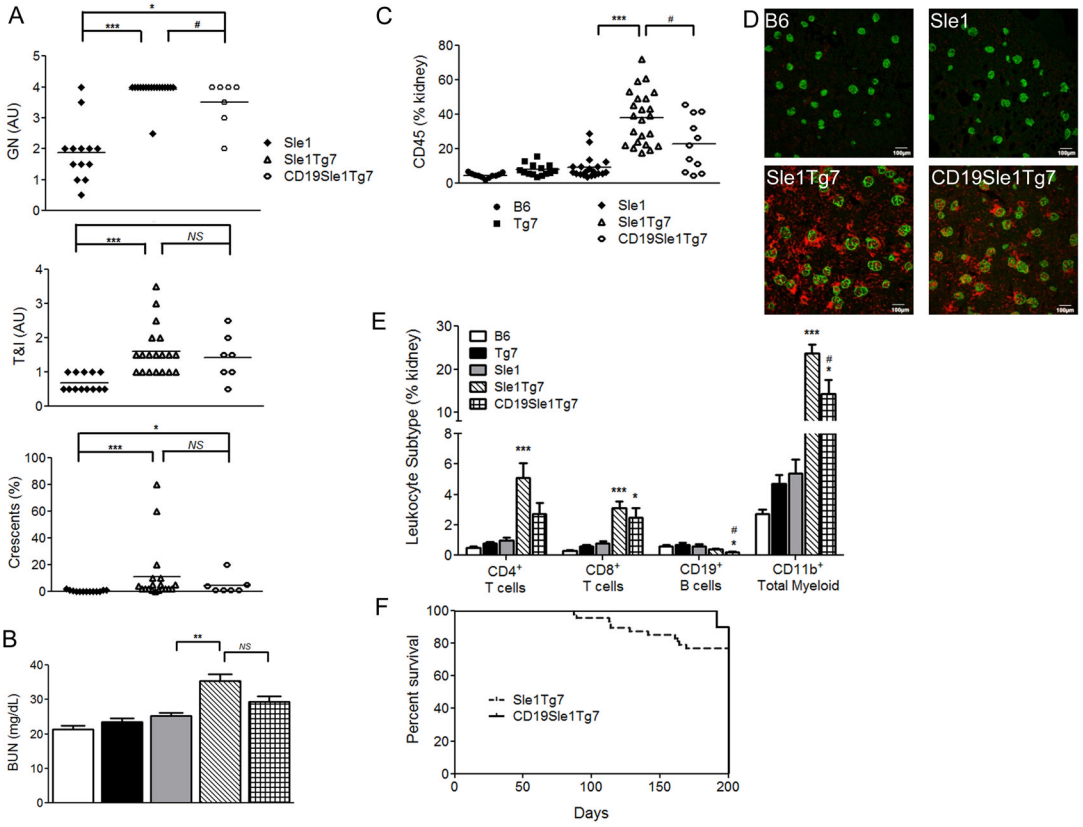

Figure 7. Normalization of B cell TLR7 expression moderately reduces kidney inflammation

Female mice were aged to 6 months and kidneys were assessed for histological nephritis (a). BUN was also assessed as a measure of kidney function ($n \geq 6$ per strain) (b). Total $CD45^+$ leukocyte infiltration into the kidney was assessed using flow cytometry as described earlier ($n \geq 6$ per strain) (c), with microscopy demonstrating equivalent infiltration into the glomeruli between B6.*Sle1*Tg7 and B6.CD19*Sle1*Tg7 mice, CD45-apc (red), nephrin-Alexa488 (green) (d). Analyses of leukocyte lineages infiltrating the kidney shows a reduction in $CD3^+CD4^+$, $CD19^+$ and $CD11b^+$ cells when B6.CD19*Sle1*Tg7 mice are compared to B6.*Sle1*Tg7 (#, $p<0.05$) (e). There was a slight reduction in mortality in the B6.CD19*Sle1*Tg7 (*n=10*) mice compared to B6.*Sle1*Tg7 (*n=48*) (f). Comparisons to B6.*Sle1* control, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Graph bars represent SEM.

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Table 1

Normalization of TLR7 in CD19^{+} cells does not impact splenic leukocyte expansion

	B6	Tg7	*Sle1*	*Sle1*Tg7	CD19*Sle1*Tg7
Spleen Weight	84 ± 4	114 ± 7	134 ± 6	467 ± 28	335 ± 31
Count (x10^{7})	12.9 ± 1.0	15.0 ± 1.1	17.8 ± 1.3	55.4 ±8.2	37.1 ±4.5
Sample size (*n*)	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 (*n*)	13	19	20	23	11

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