B cell-intrinsic TLR9 expression is protective in murine lupus

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Graphical abstract

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Abstract:
Toll-like receptor 9 (TLR9) is a regulator of disease pathogenesis in systemic lupus erythematosus (SLE). Why TLR9 represses disease while TLR7 and MyD88 have the opposite effect remains undefined. To begin to address this question, we created two alleles to manipulate TLR9 expression, allowing for either selective deletion or overexpression. We used these to test cell type-specific effects of Tlr9 expression on the regulation of SLE pathogenesis. Notably, Tlr9 deficiency in B cells was sufficient to exacerbate nephritis while extinguishing anti-nucleosome antibodies, whereas Tlr9 deficiency in dendritic cells (DCs), plasmacytoid DCs, and neutrophils had no discernable effect on disease. Thus, B cell-specific Tlr9 deficiency unlinked disease from autoantibody production. Critically, B cell-specific Tlr9 overexpression resulted in ameliorated nephritis, opposite of the effect of deleting Tlr9. Our findings highlight the non-redundant role of B cell-expressed TLR9 in regulating lupus and suggests therapeutic potential in modulating and perhaps even enhancing TLR9 signals in B cells.
Introduction:

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by loss of tolerance to nuclear antigens, immune cell activation, autoantibody production, and multi-organ damage. SLE is a complex disease involving the dysregulation of multiple immune cell lineages, and numerous cellular and genetic mediators of SLE pathogenesis have been elucidated. However, none have been so robustly recapitulated in multiple mouse models as components of the toll-like receptor (TLR) signaling pathways (1, 2). Among these, several components of the Toll-like receptor (TLR) signaling pathway have been identified as risk alleles in SLE patients, including TLR7, TLR8, TLR9, IRAK1, IRAK4, OPN, and ACP1 (3-5).

TLRs are a family of conserved surface and endosomal pattern recognition receptors, critical for innate immunity by recognition of microbial products. Ligation of TLRs culminates in the activation of transcription factors including NF-κB and interferon regulatory factors. Genetic studies in murine models of SLE highlight the endosomal TLRs TLR7, 8 and 9 as critical regulators of SLE pathogenesis (1, 2). TLR7/8 are cellular sensors for ssRNA while TLR9 senses dsDNA (1, 6). We have previously shown that TLR7, TLR9, and their shared signaling adaptor MyD88 are indispensable for autoantibody production, with TLR9 driving anti-DNA antibodies (i.e. anti-double stranded DNA and anti-nucleosome) and TLR7 driving anti-RNA antibody (i.e. anti-Sm) production (7, 8). Both anti-DNA and anti-RNA classes of autoantibody were absent in MyD88-deficient lupus-prone mice.

Genetic overexpression of Tlr7 is a driver of SLE pathogenesis and is a major contributor to disease in Y-chromosome–linked autoimmune accelerator (Yaa) associated models of disease (9). Moreover, genetic deletion of Tlr7 in the MRL/lpr
mouse model of lupus resulted in ameliorated kidney disease and reduced immune activation (8). Strikingly, and in contrast to the prevailing hypothesis at the time, global Tlr9 deficiency instead resulted in decreased survival with exacerbated nephritis and dermatitis, despite the loss of anti-DNA and specifically anti-nucleosome autoantibodies (8). Furthermore, Tlr9-regulated disease—manifested by worse disease in its absence—is dependent on Tlr7 (7). The protective role of Tlr9 in SLE was counterintuitive given that: TLRs are thought of as proinflammatory receptors; TLR9 signaling is responsible for anti-DNA antibody production (8) which in turn was long thought to be a pathogenic mediator of disease; and TLR9 signals similarly to TLR7, an accelerator of disease (10). Since the protective role of TLR9 in SLE was first reported, this finding has been confirmed in at least seven models of lupus, including MRL/lpr, MRL+, B6/lpr, Ali5 B6, Nba2.Yaa, WASp and pristane (11-17). In each of these models, when Tlr9 was deleted severity of renal disease was increased. In nearly all the lupus models examined, Tlr9 deficiency led to a loss of anti-DNA autoantibodies.

Despite substantial research, it remains unclear is why TLR9 and TLR7 have paradoxical effects on SLE pathogenesis, especially as these two receptors are thought to engage nearly identical downstream signaling pathways (10). Insights into this issue will be important for understanding lupus pathogenesis, designing lupus therapy, and possibly for understanding lupus patient heterogeneity. Importantly, deciphering why TLR7 and TLR9 play such different roles in SLE will provide basic insights into the biology of these critical TLRs and TLR signaling in general. One hypothesis to explain the dichotomous effects of TLR7 and TLR9 is that there are cell type-specific roles for each TLR, and while TLR9 may be protective due to its effects in one cell type, TLR7 may accelerate disease due to its effects in another cell type. Alternatively, and non-
exclusively, TLR9 may regulate TLR7 in a cis fashion within the same cell type by competing for shared rate limiting downstream signaling components.

To unravel the mechanisms behind this unsolved paradox, a key step is to determine the cell specific role of the TLRs in SLE—the goal of this study. Here, we evaluated the effects of TLR9 expression in multiple target populations to identify which, if any, regulate SLE in a TLR9-dependent fashion. This was important because multiple hematopoietic cell lineages express TLR9 and could modulate disease. These cell lineages include B cells (18-20), neutrophils (21, 22), macrophages (23, 24), DCs (25) (26), and pDCs (26, 27).

To address this, we generated two alleles to manipulate TLR9 expression, allowing either selective deletion or overexpression using different cell type-specific Cre-expressing lines. These alleles were crossed onto appropriate autoimmune-prone genetic backgrounds for this study. We evaluated the effect of TLR9 loss using a conditional Tlr9 knock-out (Tlr9<sup>fl</sup>) in B cells (CD19-Cre), DCs (CD11c-Cre), neutrophils (MRP8-Cre) and myeloid cells, both macrophages and neutrophils (LysM-Cre). Based on the results of these studies, we evaluated the reciprocal effect of Tlr9 overexpression in B cells. This strategy allowed us to delineate the contributions of TLR9 signaling in various hematopoietic cell types in lupus pathogenesis. Indeed, we found striking cell-type specific dependence on Tlr9 expression for the regulation of lupus. Either over- or under-expression in B cells led to suppression or exacerbation of lupus phenotypes, respectively. Whereas, deletion of Tlr9 via any of the other tested Cre lines failed to show a phenotype, together suggesting that B cell TLR9 expression was both necessary and sufficient to modulate SLE pathogenesis.
Results

**TLR9 is expressed in B cells, myeloid cells, and DC lineages**

Previously others and we demonstrated that global *Tlr9* deficiency results in exacerbated disease in murine lupus. Employing a Cre-lox approach, we set out to determine which hematopoietic cell type(s) mediated this acceleration/exacerbation of disease.

To date a comprehensive analysis has not yet shown which cell lineages express TLR9 during autoimmunity, the process of which could alter or induce expression. Therefore, we evaluated TLR9 expression in varying immune cell subsets in non-autoimmune and lupus prone both pre-disease and diseased MRL/lpr mice at the protein level using a relatively new antibody reagent (28). RNAseq data compiled on ImmGen, from non-autoimmune mice, suggested transcription of *Tlr9* in DC, B cell and monocyte subsets but no detectable expression on T cell populations (29). We extended these data at the protein level by intracellular flow cytometry and further delineated TLR9 expression on specific lymphocyte and myeloid subsets (Figure 1). As expected, BALB/c mice had TLR9 expression that correlated with the previously published transcriptional data. Given that TLR9 expression may have been altered in the setting of autoimmunity, we aimed to determine if the expression patterns autoimmune MRL/lpr mice differed from either pre-autoimmune (5 wk old MRL/lpr) and non-autoimmune mice (BALB/c). In all groups, the highest expression was observed in both DCs lineages (cDC and pDCs), in which expression was 2.2-3.3 fold higher than in B cells. Within the B cell compartment, expression of TLR9 was similar in both marginal zone and follicular B cells (Figure 1); however, plasmablasts did not express TLR9 when compared to *Tlr9*−/− controls (not shown). Lack of *Tlr9* mRNA expression in T cells was confirmed at the protein level, and despite reports to the contrary (30, 31), neutrophils did not exhibit TLR9 expression
BALB/c mice had statistically higher TLR9 expression in the cDC and macrophage compartments compared to diseased MRL/lpr mice; however, the overall pattern of TLR9 expression when comparing between cell types was the same among different mouse strains (Figure 1). Furthermore, pre-autoimmune MRL/lpr mice exhibited no significant differences in TLR9 expression compared to older diseased MRL/lpr counterparts (Figure 1).

**Generation of Tlr9 conditional allele**

Based on prior studies highlighting B cell specific MyD88 as a major driver of SLE kidney disease (32), we postulated that Tlr9 exerts its protective effects through its expression in B cells. Thus, to evaluate the role of Tlr9 in B cells, we generated a Tlr9-floxed allele and backcrossed it to the MRL/lpr lupus prone background (Figure 2A). We then generated homozygous MRL/lpr Tlr9-floxed mice that were also heterozygous for CD19-Cre. Cre-negative Tlr9-floxed littermates served as a negative control in this cohort. Tlr9 deletion in CD19-Cre Tlr9^{fl/fl} mice was assessed by qPCR of genomic DNA from FACS-sorted immune cell populations in 16-week-old mice. Tlr9 was efficiently deleted in B cells (96.3% allele deletion) (Supplemental Table 1). As expected, TLR9 deletion was <10% in all other immune cell populations (Supplemental Table 1). Consistent with effective Tlr9 DNA deletion, CD19-Cre Tlr9^{fl/fl} B cells stimulated with CpG DNA demonstrated significant reduction in IgM secretion compared to controls (Figure 2B).

**B cell-specific deletion TLR9 results in acceleration of murine SLE**

To evaluate the impact of cell lineage-specific Tlr9 deletion on disease state, several pathologic indicators of disease were assessed in 16-week-old mice. Similar to MRL/lpr mice with global Tlr9 deficiency (7, 8), the CD19-Cre Tlr9^{fl/fl} mice exhibited significant increases in proteinuria and exacerbated glomerular and interstitial nephritis compared
to Cre-negative littermate controls (Figures 2C-F). No differences in dermatitis were identified across genotypes (Figure 2G). Global Tlr9-deficient MRL/lpr exhibit pronounced splenomegaly and lymphadenopathy, while CD19-Cre Tlr9\(^{fl/fl}\) exhibited modest but statistically significantly decreased lymph node weight compared to littermate controls and demonstrated no differences in spleen weight (Figure 2H,I). This suggests that the observed exacerbated renal disease is regulated at least in part independently of lymphoproliferation. To control for a possible effect of haploinsufficiency of CD19 (as the CD19-Cre is a knock-in allele resulting in a deletion of one copy of CD19), CD19-Cre MRL/lpr mice were compared to MRL/lpr wild-type mice for disease outcomes. CD19-Cre MRL/lpr mice did not exhibit any significant differences in renal disease, dermatitis, lymph node weight or spleen weight (Supplemental Figure 1A).

**B cell-specific TLR9 deletion alters the autoantibody response**

Serum anti-nuclear antibody (ANA) profiles were significantly altered by B cell-specific Tlr9 deletion with complete loss of homogenous nuclear staining typical of anti-chromatin antibodies and increased cytoplasmic, nucleolar and fine speckled staining indicative of RNA-related specificities (Fig 3A,B), mirroring the ANA alterations previously documented in globally Tlr9-deficient lupus prone strains (7). Consistent with the loss of homogenous staining pattern, the anti-nucleosome antibody titers were significantly reduced in the CD19-Cre Tlr9\(^{fl/fl}\) group compared to controls. Titers of RNA-associated autoantibodies, such as anti-Smith and anti-RNA, remained unchanged (Figure 3C). These autoantibody patterns were attributable to Tlr9 deletion, as they were not observed in mice carrying the CD19-Cre allele alone (Supplemental Figure 1B). Thus, TLR9 expression in B cells is required for the development of autoantibodies to DNA-associated autoantigens.
Effects of B cell-specific TLR9 deletion on Splenic T cell activation markers

CD19-Cre \( Tlr9^{fl/fl} \) mice exhibited fewer CD19\(^+\) cells as a percentage of the total splenocytes (Supplemental Table 2) attributable to a concomitant increase in the frequency of double negative T cells (not shown). Notably, \( Tlr9 \)-intact control mice had increased percentages of CD44\(^{low}\)CD62L\(^{high}\) naïve CD4 and CD8 cells compared to CD19-Cre \( Tlr9^{fl/fl} \) mice, suggesting increased activation of the T cell population when B cells lack TLR9 (Supplemental Table 2). Since CD19-Cre \( Tlr9^{fl/fl} \) mice had larger spleens, there was no net change in absolute cell number of naïve CD4 and CD8 cells (Supplemental Table 3). Mice carrying the CD19-Cre allele without the \( Tlr9^{fl/fl} \) genotype were not significantly different from wild-type MRL/lpr mice in the T cell compartment or in any of the disease assessments (Supplemental Figure 1C).

Myeloid-specific TLR9 deficiency did not alter clinical parameters of SLE pathogenesis

Although the CD19-Cre \( Tlr9^{fl/fl} \) mice recapitulated the exacerbated nephritis observed in global \( Tlr9 \)-deficient MRL/lpr mice, it remained possible that \( Tlr9 \) expression in other cell lineages contributes to SLE pathogenesis. TLR9 expression has been documented in myeloid lineages including cDC, pDC, neutrophils, and macrophages (10). Therefore, we examined three additional promoter-driven Cre lines with myeloid tropism to assess the effect of TLR9 in myeloid lineages on clinical parameters and immune activation, including autoantibody production, in SLE. We used CD11c-Cre to delete TLR9 in cDCs and pDCs, and MRP8-Cre to target neutrophils. To date, there is no Cre line that specifically and efficiently targets all macrophage subpopulations. Therefore, we employed LysM-Cre, which is predominantly expressed in macrophages and neutrophils, and MRP8-Cre, which is predominantly expressed in neutrophils, to deduce
the differential contribution of macrophage and neutrophil TLR9 in lupus. For each
cohort, mice were homozygous for the Tlr9<sup>fl</sup> allele and either heterozygous or negative
for the indicated Cre.

LysM-Cre was previously reported to target a reporter gene in 80% of
polymorphonuclear cells, 40-50% of inflammatory monocytes and 30-40% of splenic
macrophages (33). To assess the deletion of Tlr9 in our MRL/lpr system, we used qPCR
of genomic DNA prepared from FACS-sorted splenocytes. In LysM-Cre Tlr9<sup>fl/fl</sup> mice,
deletion of TLR9 evaluated by qPCR on genomic DNA was 58.2% in macrophages
(CD11b<sup>+</sup>F4/80<sup>+</sup>) 69.3% in neutrophils (CD11b<sup>+</sup>Ly6G/C<sup>+</sup>) 26.5% in cDCs and <15% in
other assessed lymphoid lineages (Supplemental Table 1). Notably, these efficiencies
may underestimate deletion in tissue-resident macrophages, since targeting rates of
90% or more were reported for LysM-Cre in peritoneal and alveolar macrophages (33).

We assessed clinical parameters of SLE in LysM-Cre Tlr9<sup>fl/fl</sup> mice, including renal
disease, dermatitis, and lymphoproliferation. No differences in dermatitis were identified
across genotypes (Figure 4A). A trend towards reduced proteinuria (p=0.057) was
observed in LysM-Cre Tlr9<sup>fl/fl</sup> when compared to Cre-negative littermate controls. This
trend toward improved proteinuria in the setting of Tlr9 deficiency is opposite of what is
seen in global Tlr9 deficiency. Moreover, we did not identify differences in either
glomerulonephritis or interstitial disease (Figure 4A). Lymph node and spleen weights
were similar amongst all genotypes (Figure 4A). No changes in autoantibody formation
were observed in these mice (Figure 5A), nor did Tlr9 deletion by LysM-Cre alter the
composition of the myeloid or lymphoid compartments in MRL/lpr mice (Supplemental
Table 2).
Although we observed no basal expression of TLR9 in neutrophils in MRL/lpr mice (Fig 1F/G), we felt it prudent to evaluate the role of neutrophil Tlr9 in lupus given numerous reports suggesting Tlr9 is expressed and functional in neutrophils (22, 34, 35). Using a neutrophil-specific Cre (MRP8-Cre), Tlr9 deletion efficiency was 90.6% in neutrophils with only minor effects on other cell lineages (Supplemental Table 1). No alteration of pathophysiology in the MRP8-Cre Tlr9fl/fl mice was noted when compared to littermate controls for any of the assessed parameters, including renal disease, dermatitis and lymphadenopathy or splenomegaly (Figure 4B). MRP8-Cre Tlr9fl/fl mice did exhibit mildly reduced anti-nucleosome titers. However, no differences were observed for anti-Sm and anti-RNA autoantibodies (Figure 5B). Tlr9 deletion in neutrophils did not substantially alter the composition of the myeloid or lymphoid compartments. Minor but significant alterations were observed in the CD4 T cell compartment with an increase in the percentage of CD4 T cells, as well as naïve CD4 T cells within the CD4 T cell compartment (Supplemental Table 2). None of these minor changes would be explanatory of the global Tlr9 deletion phenotype of exacerbated disease.

Plasmacytoid DCs (pDCs) and cDCs express high amounts of TLR9 protein and mRNA compared to other cell types (Figure 1). pDCs have been implicated in SLE pathogenesis as major producers of type 1 IFNs following stimulation by TLR7 or TLR9. To assess the role of these lineages in TLR9-mediated SLE pathogenesis, we crossed the Tlr9fl allele to the CD11c-Cre allele on the MRL/lpr background. The CD11c-Cre depleted greater than 90% of Tlr9 alleles as assessed by qPCR in sorted cDCs and pDCs. As previously reported, this Cre line can be promiscuous and also targets B cells (33, 36). In accordance with these data, we observed 93.2% deletion efficiency in plasmablasts, and 62.8% allele deletion in the B cell compartment. Despite the extensive deletion of Tlr9 in pDCs, cDCs and plasmablasts, the CD11c-Cre Tlr9fl mice did not
exhibit significant alterations in any of the measured clinical parameters of SLE, including proteinuria, glomerulonephritis, interstitial inflammation, dermatitis or lymphoproliferation (Figure 4C). CD11c-Cre Tlr9<sup>fl/fl</sup> mice had a substantial reduction in anti-nucleosome antibodies (a subtype of anti-DNA antibody) and an altered ANA pattern, comparable to the antibody pattern observed in CD19-Cre Tlr9 depleted mice (Figure 5C,D and Figure 3). This is consistent with extensive deletion of Tlr9 mediated by CD11c-Cre in plasmablasts. There was also a significant increase in plasmablasts in CD11c-Cre Tlr9<sup>fl/fl</sup> mice, despite high deletion levels in this compartment, consistent with prior data (37) (Supplemental Table 1 and 2).

**B cell-specific overexpression of TLR9 ameliorates disease in murine SLE**

Given that suppression of TLR9 signaling via genetic deletion resulted in exacerbated disease, we hypothesized that TLR9 overexpression would result in ameliorated disease. To test this hypothesis, we created what we believe to be a novel murine model in which a Tlr9 conditional allele was targeted to the rosa26 locus (Figure 6A). The targeted allele has a floxed region comprising eGFP, a neomycin resistance cassette, and a transcriptional stop sequence, which is followed by HA-tagged TLR9. Upon Cre mediated recombination the floxed region is excised resulting in loss of eGFP and Tlr9 over-expression driven by the rosa26 promoter. The absence of eGFP denotes successful recombination and serves as a surrogate for TLR9 expression.

As B cell specific loss of Tlr9 resulted in exacerbated disease, we chose to overexpress Tlr9 in the B cell lineage using the CD19-Cre allele. The TLR9 overexpressor strain will be referred to as CD19-Cre Rosa<sup>Tlr9</sup>. Tlr9 gene expression was 2.0 ± 0.15-fold higher in FACS sorted B cells from CD19-Cre Rosa<sup>Tlr9</sup> mice compared to control mice as measured by quantitative real-time PCR (Figure 6B), and the HA tagged TLR9 was
found in both the cleaved and uncleaved form (Figure 6C). To test the function of the Rosa<sup>Tirg</sup> conditional allele, CD19-Cre Rosa<sup>Tirg</sup> B cells were stimulated with varying doses of TLR9 agonist CpG ODN 1826, which resulted in 1.7 to 2.5-fold increase in IgM secretion (Figure 6D). Using eGFP as a surrogate for TLR9 expression, the percent of B cells escaping the complete TLR9 overexpression phenotype was found to be 16 ± 9% (Figure 6E). This is of particular importance as B cell escape has been responsible for altered or loss of phenotype in several other genetic deficiency models of SLE. Previously, selective pressure in the CD19-Cre Myd88<sup>fl/fl</sup> mice and CD19-Cre MHCII<sup>floxflox</sup> mice resulted in only 50% and 29% of AFCs respectively having deletion of target genes compared to 90% of naive B cells (32, 38), suggesting that autoreactive B cells without protective mutations undergo increased expansion and differentiation. Despite this technical caveat, we aimed to determine whether even incomplete overexpression of TLR9 in B cells would result in altered disease.

We generated homozygous MRL/lpr Rosa<sup>Tirg</sup> mice that were also heterozygous for CD19-Cre. In these experiments CD19-Cre negative littermates served as controls. Indeed, as hypothesized, B cell-specific Tlr9 overexpression ameliorated disease in MRL/lpr mice. CD19-Cre Rosa<sup>Tirg</sup> mice exhibited a significant reduction in proteinuria and glomerulonephritis but no difference in interstitial disease (Figure 7A,C,D,E). There was no difference between the groups in dermatitis, splenomegaly, or lymphadenopathy (Figure 7B,F,G). The composition of both the lymphoid and myeloid compartments were similar amongst all the groups (Supplemental Table 2). CD19-Cre Rosa<sup>Tirg</sup> mice demonstrated no change in serum anti-nucleosome autoantibodies (Figure 7H) nonetheless, they exhibited a modest but significant decrease in anti-RNA antibodies (Figure 7I). Notably, the ratio of anti-nucleosome:RNA antibodies within each animal
was increased in B-cell specific \textit{Tr}9 overexpressing mice (Figure 7J), suggesting that the balance between TLR7 and 9 signaling had been altered.

To further confirm our findings in the MRL\textit{lpr} background and to determine whether overexpression of TLR9 could protect against a TLR7-driven disease, we crossed the \textit{Rosa}^{\textit{Tr}9} allele to the B6.Fcgr2b\textsuperscript{−/−}.\textit{Yaa} SLE model. In this model, the \textit{Yaa} modifier that harbors a duplication of TLR7 is responsible for amplifying the lupus pathology in the \textit{Fcgr2b}^{−/−} mouse strain (39). Similar endpoints were assessed in 24 wk old male mice, with the exception of dermatitis, which is not a feature of this genetic background. As with the MRL\textit{lpr} model, \textit{Fcgr2b}^{−/−}.\textit{Yaa- Rosa}^{\textit{Tr}9} mice had a significant decrease in renal disease as assessed by proteinuria and glomerulonephritis; in addition they also demonstrated reduced interstitial nephritis (Figure 8A-D). Spleen and lymph node weight remained unchanged, recapitulating the data from the MRL\textit{lpr} model (Figure 8E-F and Figure 7F-G). There was no observed difference in autoantibody production, although all antibodies were observed at much lower titers than in the MRL\textit{lpr} model (Figure 7, and data not shown). Altogether our results demonstrate that the severe glomerulonephritis manifested in \textit{Fcgr2b}^{−/−}.\textit{Yaa} mice due to a 2-fold increase in TLR7 was ameliorated by a 2-fold overexpression of TLR9 in B cells.

One potential mechanism for the regulation of TLR7 by TLR9 is that TLR9 may suppress TLR7 at the transcriptional level. To assess this, TLR7 mRNA was measured in B cells of TLR9 deficient and overexpressing mice. While TLR9 mRNA was expressed at 2-fold increased levels in the overexpressing mice, there was no effect on the expression of TLR7 (Supplemental Figure 2).
Discussion:

The roles of TLRs and MyD88 have been well established in numerous models of SLE as well as in SLE patients (1, 2, 40, 41). A major unsolved puzzle with respect to TLR signals and disease pathogenesis is why and how TLR9 actually protects from disease, even as it promotes the hallmark lupus-associated anti-chromatin antibody response. To gain further insight into this paradox, we examined the cell-type specific effects of Tlr9 deficiency. Despite the broad expression pattern of Tlr9—and the use of multiple Cre lines that targeted lineages including B cells, DCs, neutrophils, and macrophages—only B cell-specific Tlr9 deficiency resulted in acceleration of lupus nephritis. Further, we found that in two independent models of SLE, Tlr9 overexpression in the B cell compartment resulted in ameliorated renal disease. Taken together these results identify B cells as a primary and possibly unique site of TLR9-based regulation of multiple aspects of lupus-like autoimmunity.

Specific promoter-driven Cre lines commonly used to target immune cell lineages in fact affect multiple lineages and also often affect only a fraction of the intended target cells, making interpretation more complicated. Moreover, not all target floxed loci are equally affected by a given Cre line (42). Here we have been careful to directly measure extent of deletion in relevant lineages using qPCR of genomic DNA to help clarify conclusions. In addition, our strategy of using multiple Cre lines that inevitably overlap is helpful when taking the data together for interpretation.

From these data we can conclude that TLR9 expression in both pDC and cDC is not likely to be important in regulating lupus in MRL.Fas<sup>lof</sup> mice. Deletion was extensive in both lineages using the CD11c-Cre, yet no significant clinical effects were observed. A
potential complication of CD11c-Cre is substantial off-target effects in B lineage cells, in particular plasmablasts. But as our CD19-Cre mice did show a phenotype while the CD11c-Cre did not, we can conclude that TLR9 expression in pDCs and cDC TLR9 does not contribute to disease regulation. Neutrophils were also extensively targeted by LysM-Cre; yet we saw no effect on disease. This was corroborated by the MRP8-Cre mice that targeted neutrophils with reasonable efficiency but much more specifically. From these two crosses we can conclude that neutrophil-expressed TLR9 is also unlikely to regulate lupus.

The role of Tlr9 in macrophages is harder to interpret, since only one Cre (LysM) affected them, and then only to a partial degree. This Cre line is reported to variably affect macrophages depending on their location, so it is conceivable that there was more extensive deletion in some tissue macrophages that we did not assay (33). Nonetheless, it remains possible that macrophage-expressed Tlr9 could still regulate disease in addition to B cell-expressed Tlr9. This will not be resolvable until the advent of Cre lines with both greater efficiency and specificity within the macrophage lineages. Additionally, our data does not rule out a role for Tlr9 in non-hematopoietic lineages; however, recent work showed that Tlr9 expression on renal tubular epithelial cells promotes acute kidney injury, albeit in a non-autoimmune model, suggesting parenchymal Tlr9 is likely not renal-protective (43).

The importance of TLR regulation in B cells, though supported by other data, was not completely anticipated. For example, TLR7 overexpression in cDC was identified as an important driver of nephritis in two different B6-based models (44, 45). However, overexpression can show what is sufficient, but does not necessarily pinpoint what is happening at more physiological levels of expression. In our case, to the extent that
TLR7 is constrained by TLR9, deletion of TLR9 in cDCs was not sufficient to drive more disease in a model where global TLR9 deletion was sufficient to drive disease in a TLR7-dependent manner (7). Nonetheless, others and we do identify a unique role for DCs per se in promoting nephritis (25, 45). Previously, we deleted MyD88 in B cells and found markedly reduced disease; this indicated that pro-inflammatory TLR effects in B cells were important (32). Presumably these were driven by TLR7 in the B cell, though that has yet to be directly tested. Our current results nicely complement these prior studies by showing that TLR9 is required in the B cell to restrain disease.

Previously, using a Wiskott Aldrich Syndrome protein (WASp) model of autoimmunity, Jackson et al. showed that B cell intrinsic loss of TLR9 resulted in exacerbated disease, consistent with our findings (13). However, we note that this model depends on the loss of a single gene that is not implicated in lupus (in contrast to our multigenic MRL model) and most importantly that this loss of WASp was itself restricted to B cells in this model. The use of a model driven solely by the loss of a single gene in B cells may render it as a foregone conclusion that TLR expression in B cells would also be important in this context. For these reasons, it would be hard to generalize from this model to more polygenic models in which multiple cell types play a role in pathogenesis. Nonetheless, agreement of disparate murine models tends to support the relevance of the basic mechanisms elucidated in the model systems.

Our studies, along with others, suggest that B cells that respond to TLR ligands control not just autoantibodies, but also nephritis and T cell activation. This conclusion comes from results of manipulating MyD88, TLR7 or TLR9 in B cells (13, 32), which should affect only nucleic acid-specific B cells, yet has broad global effects on disease. These
considerations in turn imply there is a mechanism of tissue pathogenesis that stems from initial activation of nucleic acid-specific B cells.

At the molecular level, TLR9 likely regulates TLR7 in the same B cell, consistent with in vitro data suggesting disparate roles for TLR7 and TLR9 in B cell-specific activation, proliferation, death and differentiation upon stimulation of both BCR and TLR (37, 46). Specifically, studies of TLR signaling in the B cell compartment suggest that TLR9 stimulation limits expansion of autoreactive B cell populations (46) and is required for spontaneous peripheral activation of anti-DNA B cells, as well as their differentiation into Ab-producing cells (37, 47). Therefore, the observed Tlr9 protective effect we observe herein may be due, at least in part, to regulation of these autoreactive B cells. It has also been suggested that TLR9 competes with TLR7 for the chaperone Unc93b that is required for proper processing, transport and expression of both TLRs, and that in the absence of TLR9, there is enhanced TLR7 activity (48-52). However, these studies have been conducted in cell lines or using global mutations in vivo. Most studies of TLR9 signaling per se have focused on macrophages, although studies in pDC and one study in B cells suggests marked cell-type specificity in the nature of the signaling pathways (53-55). These differences could underlie in part why we only observed a regulatory role in vivo in B cells. Our implication of TLR9 as a key regulator operating directly in B cells emphasized the importance of studying TLR9-TLR7 interactions in B cells, which have been scarcely investigated to date (53).

TLR9 could also be regulating TLR7-driven autoimmunity in trans, by promoting the production of protective anti-chromatin antibodies. This concept may seem to disagree with the consensus in the lupus field that anti-DNA and related autoantibodies are disease mediators (56, 57). However, the current study and our prior work suggest that
this may not always be the case. First, B cell-specific \textit{Tlr9} deficient mice exhibit exacerbated disease, despite the fact that they do not produce any anti-nucleosome antibodies. Similar observations were made in the context of \textit{global Tlr9} deficiency in multiple murine mouse models of SLE (11-17). It is thought that the specific loss of anti-nucleosome antibodies likely is due to the fact that the BCR and TLR9 receptors signal in concert and loss of TLR9 prevents the second signal needed for the BCR ligation to result in autoantibody production (58). Additionally, \textit{Tlr9} overexpression ameliorated disease without a concomitant change in anti-nucleosome antibodies and only a slight decrease in anti-RNA antibodies. Of interest in this regard from the current work is the observation that both CD19-cre and CD11c-cre \textit{Tlr9}\textsuperscript{fl/fl} mice exhibit a loss of dsDNA antibodies but only CD19-cre mice exhibit ameliorated pathology. This unlinking of the autoantibody response from end-organ damage is consistent with some prior results, for example that lupus prone mice that cannot make circulating autoantibodies but do have B cells still develop lupus nephritis (59). Moreover, some human and murine studies suggest that IgM anti-dsDNA antibodies are protective (60-62). Hence it remains an intriguing possibility that some TLR9-driven antibodies actually have a dominant protective effect, perhaps by promoting autoantigen clearance, partly explaining the regulatory role of TLR9.

While TLR9 overexpression had a significant protective effect in two models of disease, the extent of this protection may have been limited by some technical aspects of the model system used. Both TLR9 overexpression alleles were not expressed in an average of 15.6\% of the B cells, so there were some B cells that lacked the suppressive effect; these cells could have dominantly promoted disease, for example by serving as APC for autoreactive T cells. There is precedent for this effect from “escapee” B cells when CD19-Cre was used to conditionally delete MHCII in MRL/\textit{lp}r mice(38). Second,
expression of two copies of the Rosa allele resulted in only a 2-fold increase in expression of TLR9; even higher expression of TLR9 may have had greater protective effects. It is theoretically possible that the CD19-Cre allele and consequent CD19 haploinsufficiency may confer some protective effects, potentially accounting for some of the ameliorated disease in the CD19-Cre Rosa^Tlr9 mice. However, there was no significant disease amelioration or effects on any measured immunologic parameter when the CD19-Cre allele was studied alone in the MRL/lpr background. These data suggest that the effects of CD19-Cre per se in ameliorating disease in the context of promoting TLR9-overexpression would be limited, and thus, based on the data we have available, we conclude that TLR9 overexpression has a protective role in models of SLE.

In summary, we have delineated the role of B cell-specific TLR9 in SLE pathogenesis. B cell-specific Tlr9 deficiency is a disease accelerator in SLE pathogenesis. Further, our study is unique in demonstrating that B cell-specific Tlr9 overexpression resulted in ameliorated renal disease. These conclusions relate to emerging human studies demonstrating that B cells from lupus patients are hyporesponsive to TLR9 stimulation (63, 64). Our studies in turn suggest that TLR9 hyporesponsiveness in B cells of these patients may represent loss of a protective mechanism, which results in initiation and/or potentiation of autoimmunity. Our findings further suggest the counterintuitive notion that TLR9 agonism may suppress lupus. Systemically administered TLR9 agonists have been used in the clinic in the context of cancer treatment, and were generally well-tolerated (65, 66). It may be feasible to devise a strategy to deliver such agonists just to B cells or even to DNA-specific B cells. Hence, understanding how discrete cell populations regulate lupus may allow for more targeted therapeutic design.
**Methods:**

*Flow Cytometry*

Flow cytometry was performed as previously described (67). Spleens were processed via mechanical dissociation and lysed using Ammonium-Chloride-Potassium buffer (prepared in house). Cells were resuspended in Phosphate Buffered Saline (PBS) with 3% calf serum and the FcR-blocking antibody 2.4G2. Ethidium monoazide (EMA) or Ghost 510 (Tonbo) was used for live-dead discrimination. Cells were fixed in 1% paraformaldehyde or Cytofix/Cytoperm (BD) where appropriate. Flow cytometry was performed on a BD LSRII and cell sorting on BD FACS Aria II. Analysis was performed in FlowJo 10. Surface and intracellular staining antibodies are listed below.

*Measurement of serum antibodies*

Hep2 immunofluorescence assays (Antibodies Inc or Bio-Rad) were performed as previously described (8) with serum diluted at 1:200. Images were captured on a Zeiss LSM 510 microscope and processed in Adobe Photoshop. Anti-nucleosome, anti-Sm and anti-RNA concentrations were measured by ELISA as previously described (68). Specific antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotech [1030-04]). The monoclonal antibodies Y2, BWR4, 400tµ23, or PL2-3 (in-house) were used as standards for the anti-Sm, anti-RNA, rheumatoid factor, and anti-nucleosome measurements respectively.

*qRT-PCR*

For assessing deletion efficiency of TLR9, qRT-PCR was performed on genomic DNA extracted from purified cells. The amount of TLR9 in each sample was normalized to the unaffected gene IL10 or GAPDH. Primer sequences are TLR9 Forward 5’ ACTCCGACTTCGTCCACCT, Reverse 5’ GGCTCAATGGTCATGTGGCA; IL-10
Forward 5’ ATAACTGCACCCACTTCCCA, Reverse 5’ GT CCTGCATTAAGGAGTCGG;
GAPDH Forward 5’ TCCCACTCTTCCACCTTCGA, Reverse 5’
AGTTGGGATAGGGCCTCTTT. qRT-PCR was performed with Agilent Brilliant II SYBR Green qPCR kit on a Stratagene Mx3000P or Roche LightCycler 96.

**Ex Vivo Stimulation:**
To assess TLR9 functionality, splenocytes from indicated mice as noted in individual figures were isolated as per flow cytometry methods, with the exception that all media was azide free. Splenic single cell suspensions were sorted using a BD FACS Aria II. Isolated B cells were plated at a concentration of 250,000 cells per well in a 96 well plate. Cells were stimulated with the indicated concentration of CPG DNA, CpG ODN 1826 (Hokkaido System Science) for 72 hours. Supernatants were collected at 72 hours and used for anti-IgM ELISA as described (59).

**Evaluation of Clinical Disease**
Proteinuria was measured by Albustix strips. Kidneys were formalin-fixed, paraffin embedded, and H&E stained. Glomerular and interstitial nephritis were scored by a pathologist (M.K or S.B.) in a blinded manner. Kidneys were removed, bisected, formalin-fixed, paraffin embedded, and H&E stained. Glomerulonephritis was scored on a scale of 1-6 where (1= normal kidney, 2= mesangial expansion and increased mesangial cellularity and patent capillary loops, 3= enlarged glomeruli with moderate endocapillary hypercellularity, 4= 3+ with marked endocapillary hypercellularity and loss of patency of most capillary loops, 5 = few glomeruli with necrosis (karyorrhexis) or few active (cellular or fibrocellular) or organized (fibrous) crescents, 6= many active (cellular or fibrocellular) or organized (fibrous) crescents, necrosis (karyorrhexis), obliteration of glomerular architecture, segmental / global sclerosis. Interstitial nephritis was scored on
a scale of 1-4 in a blinded manner where 1= minimal inflammation (lymphocytes and plasma cells) confined to the perivascular area, 2= expansion of inflammation throughout the interstitial space but maintained in discrete area, 3= diffuse infiltrates in >40% of high powered fields, 4 = diffuse infiltrate throughout the entire interstitial space. Dermatitis was scored based on the extent of dermatitis on the dorsum of the neck and back. Macroscopic surface area was scored from 0 to 5 for an affected area up to 9.1 cm², with up to 1 additional point for the presence of ear (1/4 point each) and muzzle (1/2 point) dermatitis.

Reagents:

Antibodies used for FACS staining:

Antibodies used for FACS surface and intracellular staining were as follows: IA/E-PE (Biolegend, M5/114.15.2), Bst-2-biotin (in-house conjugated, 927), CD11c-PE/Cy7 (BD Pharmigen, HL3), CD45R-APC/Cy7 (BD Pharmigen, RA3-6B2), SiglecH-Al647 (eBioscience, eBio440c), CD19-Pacblue (in-house conjugated, 1D3.2), Ly6G-Al488 (in-house conjugated, 1A8), Gr1-PE/Cy7 (Biolegend, RB6-8C5), Gr1-PE (Biolegend, RB6-8C5), CD11b-APC/Cy7 (Biolegend, M1/70), CD11b-PE (Biolegend, M1/70), F4/80-Al647 (in-house conjugated, BM8), F4/80-APC (Biolegend, BM8), CD44-Al488 (in-house conjugated, 1M7), CD44-APC-Cy7 (Biolegend, 1M7), TcRβ-APC/Cy7 (Biolegend, H57-597), TCRβ-PE/Cy7 (Biolegend, H57-597), CD62L-PE/Cy7 (Biolegend, Mel-14), CD8-Al647 (in-house conjugated, TIB 105), CD4-PE (in-house conjugated, GK1.5), CD138-PE (BD Pharmigen, 281-2), CD19-Al647 (in-house conjugated, 1D3.2), kappa-Al488 (in-house conjugated, 187.1), and Ly6B.2-Fitc (AbD Serotec, 7/4). TLR9-PE (BD Pharmingen J15A7).

Mice
A conditional allele of TLR9 was generated using homologous recombination in ES cells by Ingenious Targeting Labs (Figure 2A). Mice carrying the TLR9 floxed allele (TLR9 $^{\text{fl/fl}}$) were crossed to MRL-MpJ-Fas$^{\text{pr}}$/J (The Jackson Laboratory) 10 times. CD11c-Cre (69) and CD19-Cre (70) mice were backcrossed to MRL-MpJ-Fas$^{\text{pr}}$/J as previously described (32), and LysM-Cre (The Jackson Laboratory) and MRP8-Cre (The Jackson Laboratory) were backcrossed to MRL-MpJ-Fas$^{\text{pr}}$/J at least 9 generations. Experimental cohorts were generated by intercrossing individual promoter-driven Cre$^+$ TLR9$^{\text{fl/fl}}$ with TLR9 $^{\text{fl/fl}}$ MRL-MpJ-Fas$^{\text{pr}}$/J mice and were aged to the indicated time points. To generate mice conditionally overexpressing TLR9, HA-tagged TLR9 was knocked into the Rosa26 locus (R26 FL ST TLR9-HA$^{+/+}$ referred to as Rosa$^{729}$) as described in Figure 6 and (71) were backcrossed to MRL-MpJ-Fas$^{\text{pr}}$/J for 7 generations. Resulting offspring were crossed to CD19-Cre MRL/pr mice to generate the experimental cohort. To delineate the effect of B cell overexpression of TLR9 in lupus disease, R26 FL ST TLR9 HA$^{+/+}$ mice were also crossed to B6.FcγRIIB$^{-/-}$ Yaa mice (a kind gift from Dr. Silvia Bolland) and the resulting progeny crossed to CD19-Cre mice to generate the experimental cohort. Disease pathology was evaluated in male mice at 24 weeks of age. Balb/c mice controls were purchased (The Jackson Laboratory) and TLR9$^{-/-}$ of mixed genetic background (72) were bred to Balb/c in our colony.

**Statistical Analysis**

Statistics were calculated in GraphPad Prism by one-tailed or two-tailed Mann-Whitney U test or Student’s t-test, and chi-squared tests were used as indicated throughout, with p values represented as * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

**Study Approval:** All work was approved by either University of Pittsburgh’s or Yale University’s Institutional Animal Care and Use Committee.
**Author contributions:** SJ, RAG, JST and MJS conceived the project and designed experiments. SJ designed and validated the $Tlr9^\beta$ and $Rosa^{Tlr9}$ constructs. JST analyzed the flow data. SJ and RAG analyzed the qPCR data. KMN and RAG assisted with data interpretation and experimental design. JST, SJ, and MJS wrote the manuscript. MK and SB conducted pathologic analysis of the kidney tissue. There are three co-first authors for this manuscript, JST was responsible for the majority of the writing of the manuscript and completed the final analysis as well as the initial experiments on one cohort, and thus was listed first; SJ was listed second as she designed and made the $Tlr9^\beta$ and $Rosa^{Tlr9}$ constructs used for the majority of the experiments and completed initial analysis of three of the cohorts, while RG completed the majority of the experiments and completed initial analysis for two of the cohorts.

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Figure 1: **TLR9 is expressed on B cell, DC, and myeloid populations.**

Quantification of TLR9 MFI is shown in for each of the defined populations representing *Tlr9*−/− BALB/c (n=5), BALB/c (n=7), 5-week-old pre diseased MRL/lpr (n=5), diseased 21 week old MRL/lpr mice (n= 5). Scatter plots represent individual mice with bars identifying means and error bars representing standard deviations. * p<0.05; ** p<0.01; *** p<0.001, as determined by ANOVA with Tukey’s Multiple Comparison Test evaluating for differences within each cell type.
Figure 2
Figure 2: **B cell specific TLR9 deficiency results in exacerbated renal disease:**

(A) Schematic representation of *Tlr9* floxed allele generation. Exons (open rectangles), LoxP sites (black triangles), FRT sites (block open arrow heads) are also shown. (B) Sorted B cells from control or CD19-Cre *Tlr9*fl/fl mice were stimulated with CpG ODN 1826 (5μg/ml) for 3 days and IgM secretion was quantified by ELISA (n=2 per group). Scatter plots display data from individual mice with black lines showing means, ** indicates p<0.01 using the two-tailed Student’s t-test. Phenotypic markers were assessed in 16-week-old MRL/lpr mice from each indicated genotype including (C) proteinuria, (D) glomerular renal disease, (E) interstitial and perivascular renal infiltrates, with (F) representative images of H&E kidney sections from mice of indicated genotype, where black arrow heads indicate interstitial inflammation and white arrows show glomeruli. Additional phenotypic endpoints were assessed for each noted genotype including (G) dermatitis, (H) spleen weight, and (I) lymph node weight. Controls *n=24-31* and CD19-Cre *Tlr9*fl/fl *n=19-24*. Scatter plots display data from individual mice with black lines showing median values. * p<0.05, two-tailed Mann-Whitney U test.
Figure 3

A

Homogenous

Fine speckled

CD19-Cre Tlr9<sup>fl/fl</sup>

Nucleolar

Cytoplasmic

B

N=27

7.4%

11.11%

81.49%

N=19

65%

10%

20%

CD19-Cre Tlr9<sup>fl/fl</sup>

Anti-nucleosome IgG (ug/ml)

Anti-Sm IgG (ug/ml)

Anti-RNA IgG (ug/ml)

C

****
Figure 3: **B cell-intrinsic deletion of TLR9 alters autoantibody patterns**

(A) Representative Hep-2 ANA staining patterns from serum of control or CD19-Cre $Tlr9^{fl/fl}$ mice. Arrows indicate mitotic chromatin staining. (B) Frequency of ANA staining patterns produced by sera from control and CD19-Cre $Tlr9^{fl/fl}$ mice with numbers in the circles indicating the number of mice analyzed and patterns were compared using chi-squared analysis. (C) Serum concentrations of anti-nucleosome, anti-Sm and anti-RNA IgG of control (n= 27) and CD19-cre $Tlr9^{fl/fl}$ (n=19) measured by ELISA. Scatter plots display data from individual mice with black lines showing median values. **** p<0.0001, two-tailed Mann-Whitney U test.
Figure 4
Figure 4: **Myeloid specific TLR9 deficiency does not alter clinical parameters of SLE pathogenesis.**

Evaluation of phenotypic markers of disease including proteinuria, glomerulonephritis, interstitial and perivascular renal infiltrates, dermatitis, spleen weight, and lymph node weight for the indicated Cre lineages. (A) $\text{Tlr9}^{-/-}$ vs LysM-Cre $\text{Tlr9}^{-/-}$, n=37-38 and 36 per group respectively (B) $\text{Tlr9}^{-/-}$ vs MRP8-Cre $\text{Tlr9}^{-/-}$, n=40 per group and (C) $\text{Tlr9}^{-/-}$ vs CD11c-Cre $\text{Tlr9}^{-/-}$, n=26 and 32 per group respectively for phenotypic markers and n=22 and 29 for histologic scoring. Scatter plots display data from individual mice with black lines representing median values. No comparisons were statistically significant by two-tailed Mann-Whitney test.
Figure 5
Figure 5: Alterations in autoantibody production after Tlr9 deletion in myeloid cell lineages. Serum concentrations of anti-nucleosome, anti-Sm and anti-RNA IgG from (A) Tlr9<sup>fl/fl</sup> vs LysM-Cre Tlr9<sup>fl/fl</sup>, n=37 and 36 per group (B) Tlr9<sup>fl/fl</sup> vs MRP8-Cre Tlr9<sup>fl/fl</sup>, n=40 and 39 respectively per group and (C) Tlr9<sup>fl/fl</sup> vs CD11c-Cre Tlr9<sup>fl/fl</sup>, n=22 and 29 per group respectively. Scatter plots display data from individual mice with black lines representing median values. * p<0.05; **** p<0.0001, two-tailed Mann-Whitney test. (D) Frequency of ANA staining patterns produced by sera from control (Tlr9<sup>fl/fl</sup>) and CD11c-Cre Tlr9<sup>fl/fl</sup> mice with numbers in the circles indicating the number of mice analyzed. * p<0.05; **** p<0.0001, using Chi-Squared analysis.
**Figure 6**

- **A**
  - Diagram showing the genetic modification of the TLR9 locus in ROSA26 mice, including the ROSA26 locus and ROSA26 eGFP TLR9 allele, with and without Cre-mediated excision.

- **B**
  - Graph showing TLR9 mRNA fold expression in WT and CD19-Cre Rosa^Tyr^ mice.

- **C**
  - Immunoblot analysis showing FL-TLR9 and C-TLR9 expression levels.

- **D**
  - Graph showing IgM secretion (μg/ml) in response to CpG ODN 1826 (μg/ml) treatment, comparing WT and CD19-Cre Rosa^Tyr^ mice.

- **E**
  - Flow cytometry analysis showing eGFP expression in T cells and B cells, with TCRβ expression levels.

Legend:
- *: Significant difference
- **: Highly significant difference
- ****: Extremely significant difference
- Ctrl: Control
- CD19-Cre: CD19-Cre Rosa^Tyr^ mice
- IgM secretion: IgM secretion levels in response to CpG ODN 1826
- eGFP expression: eGFP expression levels in T cells and B cells
- T cells: Red dots
- B cells: Blue dots
- TCRβ: TCRβ expression levels

**TLR9 mRNA (fold expression)**

**IgM secretion (μg/ml)**

**% eGFP expression (CD19+ B cells)**
Figure 6: **Generation and validation of a conditional TLR9 overexpression allele**

Diagram of the TLR9 overexpression plasmid and insertion into the ROSA locus. The plasmid contains two ROSA26 homology arms flanking the expression vector. The vector is composed of a floxed region (demarcated by black triangles) containing eGFP, a Neo cassette, and a transcriptional stop sequence. This floxed sequence is followed by an HA-tagged Tlr9. PGK-DTA was used as a negative selection marker for ES cells; bPA represents the bovine growth hormone polyadenylation site. The top panels show the targeting plasmid and ROSA26 locus, and the bottom two panels show the Rosa<sup>Tyr</sup> locus prior to and after Cre mediated excision. (B) qPCR analysis of TLR9 expression in sorted B cells from control (n=7) and CD19-Cre Rosa<sup>Tyr</sup> mice (n=4) mice. (C) Representative western blot showing TLR9-HA expression in CD19-Cre Rosa<sup>Tyr</sup> mice but not control mice. Sorted B cells were immunoprecipitated (IP) with isotype control antibody (rat IgG1) or anti-HA antibody and immunoblotted with anti-HA antibody. Arrows depict full-length (FL-TLR9) and cleaved (C-TLR9) form of TLR9. (D) Sorted B cells from control and CD19-Cre Rosa<sup>Tyr</sup> mice were stimulated with CpG ODN 1826 (at indicated concentrations) for 3 days and IgM secretion measured by ELISA. Scatter plots display data from individual mice with black lines showing means. (E) Left panel shows representative FACS plots showing GFP expression in CD19+ (red) and TCRβ+ cells derived from CD19-Cre Rosa<sup>Tyr</sup> mice with the right panel showing summary data from CD19-Cre+ and Cre- Rosa<sup>Tyr</sup> mice (n=42, and n=46 respectively). For tabulated data each dot denotes an individual mouse and horizontal lines represent the mean and standard deviation. * p<0.05; ** p<0.01, and **** p<0.0001 using Student's t-test.
Figure 7
Figure 7: B cell specific overexpression of Tlr9 results in ameliorated renal disease and altered antibody profile in MRL/lpr mice

CD19-Cre Rosa$^{Tlr9}$ MRL/lpr and Cre-negative Rosa$^{Tlr9}$ MRL/lpr controls were aged until 19 weeks of age (female) and 21 weeks of age (male). Phenotypic markers were assessed including (A) proteinuria, (B) dermatitis, (C) glomerular renal disease, (D) interstitial and perivascular renal infiltrates, with (E) representative images of H&E kidney sections from mice of indicated genotypes, where black arrow heads denote interstitial inflammation and white arrows show glomeruli. Additionally (F) spleen weight, and (G) lymph node weight were assessed as markers of lymphoproliferation. Serum concentrations of (H) anti-nucleosome, (I) anti-RNA antibody formation and (J) ratio of anti-nucleosome/anti-RNA antibodies of Rosa$^{Tlr9}$ controls ($n=45$) and CD19-Cre Rosa$^{Tlr9}$ ($n=46$). Scatter plots display data from individual mice with black lines showing median values. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, one-tailed Mann-Whitney U test.
Figure 8
Figure 8: **B cell specific overexpression of Tlr9 results in ameliorated renal disease and altered antibody profile in Fcgr2b−/−.Yaa mice.**

CD19-Cre Rosa<sup>Tirg</sup> Fcgr2b<sup>−/−</sup>.Yaa and Cre-negative Rosa<sup>Tirg</sup> Fcgr2b<sup>−/−</sup>.Yaa male controls were aged until 24 weeks. Phenotypic markers assessed included: **(A)** proteinuria, **(B)** glomerular renal disease, **(C)** interstitial and perivascular renal infiltrates, with **(D)** representative images of H&E kidney sections from mice of indicated genotype, where black arrow heads indicate interstitial inflammation and white arrows show glomeruli. Additionally **(E)** spleen weight, and **(F)** lymph node weight were measured as markers of lymphoproliferation. Scatter plots display data from individual mice with lines showing median values. * p<0.05; **** p<0.0001, two-tailed Mann-Whitney U test.