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Opposing Impact of B Cell–Intrinsic TLR7 and TLR9 Signals on Autoantibody Repertoire and Systemic Inflammation

Shaun W. Jackson,*† Nicole E. Scharping,‡ Nikita S. Kolhatkar,§ Socheath Khim,‡ Marc A. Schwartz,†‡ Quan-Zhen Li,§ Kelly L. Hudkins,‖ Charles E. Alpers,‖ Denny Liggitt,‖ and David J. Rawlings*†§

Systemic lupus erythematosus is a multisystem autoimmune disease characterized by autoantibodies targeting nucleic acid–associated Ags. The endosomal TLRs TLR7 and TLR9 are critical for generation of Abs targeting RNA- or DNA-associated Ags, respectively. In murine lupus models, deletion of TLR7 limits autoimmune inflammation, whereas deletion of TLR9 exacerbates disease. Whether B cell or myeloid TLR7/TLR9 signaling is responsible for these effects has not been fully addressed. In this study, we used a chimeric strategy to evaluate the effect of B cell–intrinsic deletion of TLR7 versus TLR9 in parallel lupus models. We demonstrate that B cell–intrinsic TLR7 deletion prevents RNA-associated Ab formation, decreases production of class-switched Abs targeting nonnuclear Ags, and limits systemic autoimmunity. In contrast, B cell–intrinsic TLR9 deletion results in increased systemic inflammation and immune complex glomerulonephritis, despite intact TLR signaling within the myeloid compartment. These data stress the critical importance of dysregulated B cell–intrinsic TLR signaling in the pathogenesis of systemic lupus erythematosus.


Despite numerous potential autoantigen targets, patients with systemic lupus erythematosus (SLE) frequently develop a restricted autoantibody repertoire targeting nucleic acid–associated Ags. In addition to exogenous pathogens, the TLR family of germline-encoded, pattern-recognition receptors are able to recognize endogenous ligands. Nucleic acid–containing apoptotic particles promote activation of autoreactive B cells via dual BCR/TLR-mediated signals, thereby explaining the prominence of antinuclear Abs (ANAs) in autoimmunity (1–3). The Myd88-dependent, endosomal receptors TLR7 and TLR9 are critical in this context, with TLR7 required for the generation of Abs targeting RNA and RNA-associated proteins, whereas TLR9 activation promotes production of Abs targeting dsDNA and chromatin (4–7).

Importantly, two alternate, but not mutually exclusive, mechanisms may explain the role of TLR7 and TLR9 in autoimmune pathogenesis in vivo. Either dual BCR/TLR activation may promote a B cell–intrinsic break in tolerance, or immune complex (IC)–mediated TLR activation of plasmacytoid dendritic cells may promote autoimmunity via increased type 1 IFN production (4, 8). In addition, trl9−/− MRL.Mp/Jpr/Jpr mice develop greater plasmacytoid dendritic cell activation and increased serum IFN-α levels, suggesting that loss of trl9 in the myeloid compartment exacerbates autoimmunity on the MRL.Mp/Jpr/Jpr background (6).

Although several models (9–13) have implicated B cell Myd88 signaling in autoimmune pathogenesis, the B cell–intrinsic impact of TLR7 and TLR9 has been addressed in only a limited number of studies. The role of B cell–intrinsic TLR7 signaling was evaluated in two studies using the TLR7 transgenic (trl7Tg) model of spontaneous autoimmunity. First, using a chimeric transplant strategy comparing wild-type (WT) with TLR7 transgenic (trl7Tg) hematopoietic cells, Walsh et al. (14) demonstrated that trl7Tg B cells are preferentially recruited into germinal centers (GCs) and generate CD138+ plasmablasts. Second, Hwang et al. (15) used a CD19Cre recombine system to normalize B cell TLR7 expression in a low-copy trl7Tg strain crossed with the Sle1 lupus susceptibility locus. B cell–intrinsic TLR7 normalization decreased RNA-associated anti–small nuclear ribonucleoprotein (RNP) titers, but did not have an impact on GC and plasma cell formation and only moderately reduced autoimmune glomerulonephritis. To address the B cell–intrinsic impact of TLR9, a recent study used MRL.Fas−/− mixed bone marrow (BM) chimeras in which trl9 deficiency was primarily limited to the B cell compartment and demonstrated a specific reduction in antinucleosome

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reactivity. Whether B cell–intrinsic tlr9 deletion accelerated systemic autoimmunity, however, was not addressed in that study (16).

We recently developed a murine model of autoimmunity that provides important information regarding how self-reactive B cells are initially activated and can drive generation of pathogenic Abs (12). In this model, B cells, but not other hematopoietic lineages, harbor a mutation that abolishes the expression of Wiskott–Aldrich syndrome (WAS) protein (WASP). In the absence of WASp, peripheral B cells are rendered mildly hyperresponsive to both BCR and TLR ligands. In this setting, WAS-null (was−/−) B cells drive the development of humoral autoimmunity that is characterized by spontaneous GCs, class-switched IgG2c autoantibodies, and IC glomerulonephritis. Disease development is dependent on WT CD4+ T cells and MyD88-dependent B cell–intrinsic TLR signaling. An important advantage of the WASp chimera model is that dysregulated immune responses are limited to the B cell compartment, allowing genetic manipulation in a B cell–intrinsic fashion. In the current study, we detail the relative contributions of B cell TLR signaling in humoral autoimmunity and demonstrate that B cell–intrinsic TLR7 versus TLR9 activation is sufficient to alter the autoantibody repertoire. In addition, we demonstrate that B cell TLR7 and TLR9 signals exert opposing pathogenic and protective effects on systemic inflammation and autoimmune disease.

Materials and Methods

**Mice**

Ly5.1+ and Ly5.2+ C57BL/6, μMT, was−/−, tlr7−/−, and tlr9−/− mice were bred and maintained in the specific pathogen–free animal facility of Seattle Children’s Research Institute (Seattle, WA). All animal studies were conducted in accordance with Seattle Children’s Research Institute Institutional Animal Care and Use Committee–approved protocols.

**BM transplantation**

WT, was−/−, tlr7−/−, tlr9−/−, or was−/−×tlr9−/− donor BM and B cell–deficient (μMT) BM (20:80 ratio, 6 × 10^6 total BM) were injected into lethally irradiated (450 cGy × 2 doses) μMT recipients. Chimeras were sacrificed at 24–36 wk post transplant. For CD4 depletion assays, mice were treated weekly with i.p. injection of 250 μg anti-CD4 (GK1.5) or isotype control (rat IgG2b) Ab (University of California, San Francisco, Monoclonal Antibody Core) from 5 to 24 wk post transplant, as described (12). Data are representative of four (B WT, B WAS−/−, and B WT×TLR9−/−), three (B WT×TLR7−/−), or two (B WAS−/− CD4 depletion) independent experimental cohorts.

**Flow cytometry and Abs**

Flow cytometry was performed as described (12). Abs used were as follows: B220 (RA3-6B2), CD4 (RM4-5), Thy1.2 (53-2.1), CD138 (281-2), CD7 (16F12), CD44 (IM7) from BioLegend; PNA (Fl-1071) from Vector Labs; control, unlabeled, or isotype from Southern Biotechnology; CD19 and Leica Application Suite Advanced Fluorescence software at 40× with a constant 5 s (ANA) or 2 s (kinetoplast) exposure. ANAs were scored as nuclear homogeneous, nucleolar, cytoplasmic or a combination of these patterns. Kinetoplast reactivity was defined by colocalization of DAPI–nuclear homogeneous, nucleolar, cytoplasmic or a combination of these patterns. ANA patterns and kinetoplast intensity were scored by two independent observers blinded to genotype.

**Measurement of autoantibodies**

For ANA assays and determination of dsDNA reactivity by kinetoplast staining, diluted serum (1:200 for ANA or 1:50 for kinetoplast) was added to fixed Hep-2 ANA slides (Bio-Rad 30472) or Crithidia luciliae slides (Bio-Rad 31069). FITC-conjugated goat anti-mouse IgM–IgG, IgG2c–HRP conjugated, unlabeled, or isotype from Southern Biotechnology; CD19 (ID3), CD44 (IM7) from BioLegend; PNA (Fl-1071) from Vector Labs; and Fas (Jo2) from BD Pharmingen.

**For specific Ab ELISAs,** 96-well Immuno plates (Nunc) were coated with the following: calf thymus dsDNA (100 μg/ml; Sigma-Aldrich D3664-5×2MG); sm/RNP (5 μg/ml; Arotec Diagnostics ATR01-10); phosphoryl cholines (PC)–BSA (10 μg/ml; Biosearch Technologies PC-10110); or malondialdehyde conjugated with low-density lipoprotein (MDA-LDL) (10 μg/ml; Academy Bio-medical 20P-MD-L110). Plates were blocked for 1 h with 1% BSA/PBS prior to addition of diluted serum for 2 h. Specific Abs were detected using goat anti-mouse IgM–IgG– or IgG2c–HRP (1:2000 dilution; Southern Biotechnology Associates), and peroxidase reactions were developed using OptiEIA TMB substrate (BD Biosciences). Absorbance at 450 nm was read using a Victor 3 plate reader (PerkinElmer), and data were analyzed using GraphPad Prism (GraphPad Software). Autoantigen microarrays were performed at the Seattle Children’s Research Institute.

**Figure 1.** B cell–intrinsic TLR7 and TLR9 signals promote ANA production. (A) *Upper panel.* ANA staining patterns were scored as homogenous, nuclear, or cytoplasmic by two independent, blinded observers. Unfilled portion of circle represents ANA-negative animals. The number in the circle represents the total number of mice analyzed. *Lower panel.* Representative Hep-2 ANA immunofluorescence staining showing combined homo-meaningous/nuclear staining in B WAS−/−, homogenous pattern with nuclear sparing in B WT×TLR9−/−, and nuclear and cytoplasmic staining in B WT×TLR7−/−. Scale bars, 50 μm. (B) Anti-dsDNA IgG Ab by ELISA and kinetoplast staining. (C) Anti-sm/RNP IgG and IgG2c Ab by ELISA. (B and C) Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. (A–C) Total mice analyzed: B WT (n = 6), B WAS−/− (n = 18), B WT×TLR7−/− (n = 11), and B WT×TLR9−/− (n = 16), pooled from four independent experimental cohorts.
Spleen and kidney immunofluorescence staining

Mouse spleens and kidneys were embedded in OCT compound and snap frozen over liquid nitrogen. Then 10-μm sections were cut on a cryostat, mounted on Superfrost Plus slides, and fixed in −20°C acetone for 20 min. After rehydration in staining buffer (PBS, 1% goat serum, 1% BSA, 0.1% Tween 20), slides were stained with the following: B220-PE, CD4-FITC, and GL7-allophycocyanin (spleen); or, IgG-FITC, IgG2c-FITC, or C3- FITC (kidney). Images were acquired using a Leica DM6000B microscope, Leica DFL300 FX camera, and Leica Application Suite Advanced Fluorescence software. For glomerular IC quantification, images were obtained using a constant exposure and scored from 0 to 3 by two independent observers blinded to genotype.

Histopathology

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin; tissue sections were stained with H&E (lung, liver, pancreas) or Jones’ methenamine silver–periodic acid–Schiff (kidney) according to standard practices. Immunohistochemistry staining was performed using a Leica Bond–automated immunostainer and HRP-conjugated secondary Abs. Histology images were acquired with a Nikon Optiphot-2 microscope and a Canon Eos 5D Mark II camera. Tissue sections were examined by a board-certified veterinary pathologist (D.L.), who was blinded to study design. Kidney sections were analyzed by two observers blinded to genotype (K.L.H. and C.E.A.) and scored from 0 to 2 based on degree of mesangial expansion, glomerular basement membrane (GBM) thickening/reduplication, and glomerular hypercellularity. For quantification of MAC-2 area, glomeruli were manually delineated in more than four independent kidney sections and MAC-2 area as a percentage of glomerular area determined using Image Pro Plus (Media Cybernetics, Rockville, MD).

Statistical evaluation

The p values were calculated using one-way ANOVA, followed by the Tukey multiple comparison test (GraphPad Software).

Results

B cell–intrinsic TLR7 and TLR9 signals alter the autoantibody repertoire

To test the impact of B cell–intrinsic TLR7 and TLR9 deletion in humoral autoimmunity, we generated mixed BM chimeras by transplanting a mix of 20% WT, WAS-null (was−/−), double-deficient was−/−tlr7−/−, or was−/−tlr9−/− BM with 80% B cell–deficient μMT BM into lethally irradiated (450 Gy × 2 doses) μMT recipients. After reconstitution, all B cells were donor derived (WT, was−/−, was−/−tlr7−/−, or was−/−tlr9−/−), whereas ~80% of myeloid cells and >97% CD4+ T cells were WT (not shown). With this strategy, in the TLR-deficient cohorts, all B cells, but only ~20% of myeloid cells, lacked TLR7 or TLR9. Respective B cell chimeras will henceforth be referred to as BWT, B WAS−/−, BWT/LR7−/−, and BWT/LR9−/−.

We initially screened for autoimmunity using fluorescent ANA assays. The majority of B WAS−/− were ANA positive with mixed homogeneous and nucleolar staining patterns, consistent with both DNA and RNA Ab reactivity. In contrast, BWT/LR7−/− ANA staining was homogeneous with no nucleolar reactivity, whereas BWT/LR9−/− showed nucleolar and cytoplasmic, but not homogeneous, ANA reactivity (Fig. 1A). These altered ANA staining patterns suggested a B cell–intrinsic impact of TLR7 versus TLR9 on RNA- and DNA-associated Abs, which we evaluated using autoantibody reactivity to apoptotic and disease-associated Ags.

FIGURE 2. B cell–intrinsic TLR7 and TLR9 signals affect autoantibody reactivity to apoptotic and disease-associated Ags. (A) Left panel, Anti–MDA-LDL IgG. Right panel, Anti–MDA-LDL IgG2c in B WT, B WT/LR7−/−, B WT/LR9−/−, and CD4-depleted B WAS−/− chimeras (B WAS−/− CD4 DEP). Data normalized to B WT anti–MDA-LDL IgG2c titer. (B) Left panel, Anti-PC IgG. Right panel, Anti-PC IgG2c in B WT, B WT/LR7−/−, B WT/LR9−/−, and CD4-depleted B WAS−/− chimeras (B WAS−/− CD4 DEP). Data normalized to B WT anti-PC IgG2c titer. (A and B) Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Total mice analyzed: B WT (n = 7), B WAS−/− (n = 13), B WT/LR7−/− (n = 13), B WT/LR9−/− (n = 14), and B WAS−/− CD4 DEP (n = 10). (C) Serum IgG Abs from B WT (n = 4), B WAS−/− (n = 6), B WT/LR7−/− (n = 4), and B WT/LR9−/− (n = 8) chimeras determined using an autoantibody array chip containing 88 specific autoantigens. Data are represented as a heat map of Z-scores ranging from −1 (blue) to 3 (red). Representative of two independent microarray analyses.
specific autoantigen ELISAs. Prior reports have demonstrated persistent anti-dsDNA Ab in tlr9-deficient murine lupus models, an effect attributed to a lack of DNA specificity of standard anti-dsDNA ELISA assays (6, 18). Although we noted anti-dsDNA Ab by ELISA in B\(^{\text{W/TLR9}^{+/+}}\), DNA reactivity by highly specific \textit{Cricthidia luciliae} kinetoplast staining was abrogated in B\(^{\text{W/TLR9}^{+/+}}\), but unaffected in B\(^{\text{W/TLR7}^{+/+}}\) (Fig. 1B, Supplemental Fig. 1A).

In contrast, RNA-associated anti-sm/RNP Abs were abolished in B\(^{\text{W/TLR7}^{+/+}}\). Further, anti-sm/RNP Ab titers of the pathogenic IgG2c subclass were significantly increased in B\(^{\text{W/TLR9}^{+/+}}\) versus B\(^{\text{WAS}^{+/+}}\) chimeras, an observation consistent with elevated anti-RNA titers in tlr9\(^{-/-}\) MRL.Mp\(^{lpr/lpr}\) mice (Fig. 1C). Therefore, the requirement for TLR7 in anti-RNA and TLR9 in anti-DNA Ab production is B cell intrinsic; and lack of TLR9 enhances anti-RNA Ab formation in a B cell–intrinsic manner.

Generation of Abs targeting nonnuclear Ags was recently shown to be Myd88 dependent (11, 19), but the specific TLRs responsible for these Ab specificities have not been determined. For this reason, we measured IgG reactivity to apoptotic cell epitopes in CD4\(^+\) T cells and monocyte/macrophages relative to B cell–intrinsic TLR9 deletion resulted in statistically greater expansion of activated B and T cell populations; whereas B cell TLR9 signaling serves to limit these processes.

FIGURE 3. B cell–intrinsic TLR7 and TLR9 exert opposing signals on immune activation. Splenic monocyte subsets were analyzed in B\(^{\text{WT}}\), B\(^{\text{WAS}^{+/+}}\), B\(^{\text{W/TLR7}^{+/+}}\), and B\(^{\text{W/TLR9}^{+/+}}\) chimeras at sacrifice. (A) Spleen weight and total splenocyte numbers. (B-D) Number of splenic (B) CD19\(^+\) B cells, (C) CD4\(^+\) T cells, and (D) CD11b\(^+\)GR1\(^{lo}\) monocyte/macrophages and CD11b\(^+\)GR1\(^{hi}\) neutrophils. (A–D) Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Total mice analyzed: B\(^{\text{WT}}\) (n = 5), B\(^{\text{WAS}^{+/+}}\) (n = 12), B\(^{\text{W/TLR7}^{+/+}}\) (n = 12), and B\(^{\text{W/TLR9}^{+/+}}\) (n = 13), pooled from four independent experimental cohorts.

CD138\(^+\) plasma cells/plasmablasts, were markedly expanded in spleens of B\(^{\text{WAS}^{+/+}}\) and B\(^{\text{W/TLR9}^{+/+}}\) chimeras (Fig. 4A, 4C). In addition, splenic immunofluorescence staining revealed spontaneous GL7\(^+\) GCs in B\(^{\text{WAS}^{+/+}}\) and B\(^{\text{W/TLR9}^{+/+}}\) chimeras, which were absent in B\(^{\text{W/TLR7}^{+/+}}\) (Fig. 4D). Relative to B\(^{\text{WAS}^{+/+}}\), B\(^{\text{W/TLR9}^{+/+}}\) animals exhibited a higher percentage of GC B cells with greater numbers of splenic CD138\(^+\) plasma cells/plasmablasts, suggesting that B cell–intrinsic TLR9 signals serve to limit B cell activation. Consistent with this, total serum IgM, IgG, and IgG2c Ab titers were markedly elevated in B\(^{\text{W/TLR9}^{+/+}}\) mice relative to B\(^{\text{W/TLR7}^{+/+}}\) mice, with a trend toward greater Ab titers than B\(^{\text{WAS}^{+/+}}\) chimeras (Fig. 4E).

B\(^{\text{WAS}^{+/+}}\) and B\(^{\text{W/TLR9}^{+/+}}\) also developed significant expansion of autoimmune disease–associated CD11b\(^+\)CD11c\(^+\) “age-associated B cells” (ABCs), with \(\leq 75\%\) of total B cells expressing CD11b and CD11c in B\(^{\text{W/TLR9}^{+/+}}\), exceeding the percentage of ABCs previously reported in aged NZB/WF1 and Mer\(^{-/-}\) autoimmune strains (20, 21). In keeping with the importance of chronic TLR7 activation in ABC generation, this population was absent in B\(^{\text{W/TLR7}^{+/+}}\).

In addition to B cell activation, CD4\(^+\) effector/memory (EM) subsets (CD44\(^hi\)CD62L\(^lo\) and CD44\(^hi\)CD62L\(^hi\)) were increased in B\(^{\text{WAS}^{+/+}}\) and B\(^{\text{W/TLR9}^{+/+}}\) (with relatively greater T cell activation in B\(^{\text{W/TLR9}^{+/+}}\)), whereas the number of CD44\(^lo\)CD62L\(^hi\) naive CD4 T cells did not differ between experimental groups (Fig. 5A). Consistent with greater GC B cell numbers, PD1\(^+\) CCR5\(^+\) Th follicular cells (T\(^F\)) were also expanded in B\(^{\text{WAS}^{+/+}}\) and B\(^{\text{W/TLR9}^{+/+}}\) (Fig. 5B). B cell–intrinsic TLR7 activation appears critical for T cell activation in this model, as CD4\(^+\) EM and T\(^F\) cell subset expansion is abrogated in B\(^{\text{W/TLR7}^{+/+}}\).

In conclusion, B cell–intrinsic activation of autoreactive was\(^{+/+}\) B cells via TLR7 promotes global immune activation and expansion of activated T and B cell populations; whereas B cell TLR9 signaling serves to limit these processes.
B cell–intrinsic TLR7 signaling promotes systemic autoimmunity

Aged B<sup>WAS<sub>2/2</sub></sup> and B<sup>W/TLR7<sub>2/2</sub></sup> chimeras demonstrated diffuse inflammation and lymphoid infiltrates involving multiple organs—in particular, the lungs and liver—findings that were absent in all B<sup>WT</sup> and B<sup>W/TLR9<sub>2/2</sub></sup> animals evaluated (Fig. 6A). Immunohis-tochemistry staining demonstrated that these lymphoid infiltrates were consistent with ectopic lymphoid follicles comprising separate B220<sup>+</sup> B cell and CD3<sup>+</sup> T cell zones (Fig. 6B). The presence of ectopic lymphoid tissues within target organs has been reported in several human autoimmune diseases, including rheumatoid arthritis and type 1 diabetes, and is correlated with disease severity in autoimmunity (22). We demonstrate that hyperresponsive was<sup>2/2</sup> B cells are sufficient to recruit WT T cells to ectopic lymphoid follicles in a TLR7-dependent manner, reinforcing the critical role of B cell–intrinsic TLR7 signals in the pathogenesis of systemic autoimmunity.

B cell TLR7 deletion eliminates, whereas TLR9 loss exacerbates, IC glomerulonephritis

Given divergent impacts on Ab production and systemic inflammation, we evaluated the role of B cell–intrinsic TLR signaling in autoimmune glomerulonephritis. B<sup>WAS<sub>2/2</sub></sup> chimeras develop inflammatory glomerulonephritis characterized by deposition of class-switched Ab, activated complement, and recruitment of MAC-2<sup>+</sup> macrophages (12). In keeping with an overall decrease in Ab titer and systemic inflammation, B<sup>W/TLR7<sub>2/2</sub></sup> chimeras did not develop glomerulonephritis (Fig. 7A), an observation consistent with decreased glomerulonephritis in <sup>tlr7<sub>2/2</sub></sup> MRL/Mp<sup>lpr/lpr</sup> mice (6). Despite absent histologic glomerulonephritis, evaluation of glomerular Ig deposition by immunofluorescence staining showed persistent glomerular IgG in B<sup>W/TLR7<sub>2/2</sub></sup> chimeras (albeit at lower levels than in B<sup>WAS<sub>2/2</sub></sup> and B<sup>W/TLR9<sub>2/2</sub></sup>) (Fig. 7B). However, deposition of the pathogenic IgG2c subclass was absent in B<sup>W/TLR7<sub>2/2</sub></sup> (Fig. 7C). In keeping with the role for IgG2c in complement and Fc receptor activation (23), C3 complement deposition was absent in B<sup>W/TLR7<sub>2/2</sub></sup> and glomerular MAC-2<sup>+</sup> macrophages were not increased over B<sup>WT</sup> controls (Fig. 7D, 7E).

In contrast to the protective effect of B cell TLR7 deletion, B<sup>WAS<sub>2/2</sub></sup> and B<sup>W/TLR9<sub>2/2</sub></sup> chimeras developed significant glomerulonephritis characterized by mesangial expansion, GBM thickening, and glomerular hypercellularity (Fig. 7A). In keeping with increased serum IgG2c Ab, B<sup>W/TLR9<sub>2/2</sub></sup> mice demonstrated statistically greater glomerular IgG2c deposition than did B<sup>WAS<sub>2/2</sub></sup>, with a trend toward greater C3 complement deposition and abundant infiltration by MAC-2<sup>+</sup> macrophages, likely recruited by

### FIGURE 4

Divergent impact of B cell–intrinsic TLR7 and TLR9 on B cell activation. (A and B) Representative FACS plots (left), total number (middle), and percentage (right) of (A) PNA<sup>+</sup>FAS<sup>+</sup>GL7<sup>+</sup>GC B cells (gated on CD19<sup>+</sup> B cells) and (B) CD11b<sup>+</sup>CD11c<sup>+</sup> ABC cells (gated on CD19<sup>+</sup> B cells). (C) Representative FACS plots (left) and total number (right) of B220<sup>+</sup>CD138<sup>+</sup> plasmablasts/plasma cells (gated on total splenocytes). (A–C) Number in FACS plot represents percentage within gated population. (D) Representative examples of splenic sections stained with B220 (red), CD4 (green), and GL7 (blue). Stars denote spontaneous GCs in B<sup>WAS<sub>2/2</sub></sup> and B<sup>W/TLR9<sub>2/2</sub></sup> chimeras. Images were taken with ×10 objective. Scale bars, 100 μm. (E) Total IgM, IgG, and IgG2c serum titers in B<sup>WT</sup>, B<sup>WAS<sub>2/2</sub></sup>, B<sup>W/TLR7<sub>2/2</sub></sup>, and B<sup>W/TLR9<sub>2/2</sub></sup> chimeras. (A–C, E) Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Total mice analyzed: B<sup>WT</sup> (n = 5), B<sup>WAS<sub>2/2</sub></sup> (n = 12), B<sup>W/TLR7<sub>2/2</sub></sup> (n = 12), and B<sup>W/TLR9<sub>2/2</sub></sup> (n = 13), pooled from four independent experimental cohorts.
glomerular IgG2c ICs binding activating Fc receptors (Fig. 7B–E). Increased IC glomerulonephritis in B W/TLR9-/- parallels accelerated autoimmune disease in tlr9-/- MRL/Mp[+lpr] mice, but demonstrates that the disease-exacerbating effects of TLR9 deletion can occur independently of the lack of TLR9 expression in the majority of the myeloid compartment.

Discussion
The importance of the Myd88-dependent TLRs TLR7 and TLR9 in lupus pathogenesis has been clearly established (1–3). In this context, global deletion of TLR7 in murine lupus models protects against autoimmunity, whereas TLR9 deficiency paradoxically exacerbates disease (4–7). The mechanisms underlying these divergent effects of TLR7 and TLR9 remain unclear, in part because studies involving globally gene-deficient autoimmune strains are unable to address the differential impacts of TLR signaling on T cells versus type 1 IFN–producing plasmacytoid dendritic cells. Our study provides the novel observation that B cell–intrinsic TLR7 and TLR9 exert opposing impacts on T cell activation. (A) Representative FACS plots (left) and total number (right) of splenic (A) naive (CD44LoCD62LHi) and EM (CD44HiCD62LLo) and CD44HiCD62LHi CD4+ T cells and (B) PD1+CXCR5+ TFH cells. Number represents percentage within gated population (gated on splenic CD4+ T cells). Mean ± SEM. ***p < 0.001. Total mice analyzed: B WT (n = 5), B WAS-/- (n = 12), B W/TLR7-/- (n = 10), and B W/TLR9-/- (n = 13), pooled from four independent experimental cohorts.

B cell–intrinsic TLR7 and TLR9 signals in lupus models, B cell–intrinsic TLR7 and TLR9 signals are required for in vivo generation of RNA- and DNA-reactive Ab, respectively. In addition, we also establish TLR7 as the major Myd88-dependent receptor responsible for B cell–intrinsic breaks in tolerance to a broad range of nonnuclear, tissue-specific autoantigens.

Second, we identify a critical role for B cell–intrinsic TLR7 signals in sustaining spontaneous autoimmune GC responses. Although the absence of anti-sm/RNP Abs in B WT/TLR7-/- may have been predicted from in vitro studies (24), the observation that Abs targeting apoptotic and phospholipid epitopes develop in B WT/TLR7-/-, but fail to undergo efficient class switch recombination, suggests an additional role for B cell TLR7 signaling in enhancing GC responses during autoimmune disease—a finding that parallels the requirement for TLR7 in Ab responses to chronic viral infection (25).

Third, we document a striking expansion of effector and T FH CD4+ T cells in B WAS-/- and B W/TLR9-/- animals. Similar expansion of the EM T cell compartment has been documented on the lupus-prone MRL/Mp[+lpr] background (6), although it should be noted that CD4+ T cells in our model are genetically WT and not Fas deficient. Our data, therefore, suggest that BCR/TLR-mediated activation of autoreactive B cells directly promotes T cell proliferation and activation during autoimmunity. These data are consistent with recent reports describing decreased EM T cell expansion in the setting of B cell–intrinsic Myd88 deletion in the MRL,Fas[+lpr] and Lyn-deficient models of autoimmunity (11, 13).
Our data suggest that B cell TLR7 is likely the major Myd88-dependent receptor driving this CD4⁺ T cell activation. Surprisingly, dual BCR/TLR7 B cell activation was also sufficient for the establishment of ectopic lymphoid follicles within target organs, as have previously been described in several human autoimmune diseases (22). We predict that, although not formally demonstrated, these expanded EM T cell compartments are enriched for autoreactive T cell clones, supporting the emerging hypothesis that activated autoreactive B cells can initiate breaks in T cell tolerance.

Fourth, we demonstrate the importance of autoantibody isotype (in particular, IgG2a/c) in promoting autoimmune nephritis. Despite producing anti-dsDNA Ab, B<sup>W/TLR7⁻/⁻</sup> fail to develop IC glomerulonephritis. These renal protective effects of TLR7 deletion, despite persistent DNA-reactive Ab, are surprising given clinical data implicating anti-dsDNA Abs in lupus nephritis (26). In keeping with this paradox, we observed glomerular IgG deposition by immunofluorescence in B<sup>W/TLR7⁻/⁻</sup>, in the absence of histologic glomerulonephritis. However, deposition of the pathogenic Ig subclass IgG2c was abrogated in the absence of B cell TLR7. In keeping with the role for IgG2c in promoting complement activation and activation of myeloid Fc receptors (23), glomerular C3 deposition and recruitment of inflammatory macrophages were absent in B<sup>W/TLR7⁻/⁻</sup>. These data emphasize the need to consider IgG subclass in addition to Ab specificity in mediating end-organ damage in SLE.

Finally, we show that B cell–intrinsic TLR9 deletion enhances class-switched Abs targeting RNA-associated Ags and broadens the autoantibody repertoire; exacerbates systemic inflammation and expansion of activated CD4⁺ T cell and myeloid populations; and promotes glomerular deposition of pathogenic IgG2c IC. Although protective roles for TLR9 in autoimmunity have been described in several murine autoimmune models, how TLR9 deletion promotes autoimmunity has not yet been determined. Potential underlying mechanisms include the following: enhanced interaction of TLR7 with the shared endoplasmic reticulum trafficking protein Um93bl in the setting of TLR9 deletion (27); facilitation of entry of autoreactive cells into the mature B cell repertoire (16); or decreased generation of TLR9-dependent regulatory B cells (2). Our study makes the important observation that B cell–intrinsic TLR9 deletion can accelerate autoimmunity, despite largely intact myeloid TLR signaling.

Importantly, however, our data do not exclude the possibility that myeloid TLR7 and TLR9 signals may exert additional effects on lupus pathogenesis. Two recent studies, using a cre-recombinase strategy to delete the TLR adapter Myd88 in CD11c⁺ dendritic cells, demonstrated that myeloid TLR activation accelerates autoimmunity, likely via enhanced type I IFN production by IC-mediated activation of plasmacytoid dendritic cells (11, 13). Given this observation, it will be important to evaluate whether deletion of myeloid TLR7 versus TLR9 exerts similar divergent effects on autoimmune pathogenesis. In this regard, a potential caveat of our study is that reconstitution with an 80:20 ratio of µMT/donor (was⁻/⁻, was⁻/⁻,tlr7⁻/⁻, or was⁻/⁻,tlr9⁻/⁻) BM, will result in ~20% myeloid cells also lacking TLR7 or TLR9. However, because the majority of myeloid cells (~80%) in our model are TLR sufficient, it is highly likely that our data reflect the B cell–intrinsic effects of TLR7 versus TLR9 in autoimmune pathogenesis.

In conclusion, our study provides critical new insights into how dual BCR and TLR signals promote B cell activation resulting in class-switched Ab production, CD4⁺ T cell activation, and the development of IC glomerulonephritis. B cell TLR7 and TLR9 exert opposing effects in this process, reinforcing the importance of dysregulated B cell TLR signaling in autoimmunity and informing efforts to therapeutically target TLR signals in SLE and other disorders characterized by autoantibody production.

**Disclosures**

The authors have no financial conflicts of interest.

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**FIGURE 7.** B cell–intrinsic TLR7 and TLR9 exert opposing effects on IC glomerulonephritis. (A) **Left panels,** Representative examples of glomeruli from B<sup>WAS⁻/⁻</sup>, B<sup>W/TLR7⁻/⁻</sup>, and B<sup>W/TLR9⁻/⁻</sup> chimeras stained with Jones’ methenamine silver–periodic acid–Schiff stain. **Right panel,** Glomerular inflammation scored as follows: (0+) minimal mesangial expansion consistent with radiation injury; (1+) focal glomerular changes with moderate mesangial expansion, GBM thickening/reduplication, and glomerular hypercellularity; or (2+) diffuse glomerular changes with severe mesangial expansion, GBM thickening/reduplication, and glomerular hypercellularity. Pathologic change was scored by two observers blinded to genotype. (B–D) Glomerular IC deposits were determined by immunofluorescence staining for (B) IgG, (C) IgG2c, and (D) complement C3. Representative images are shown (left), together with intensity of glomerular fluorescent staining (right) scored from 0 to 3+ by two independent, blinded observers. (E) **Left panels,** Representative images of immunohistochemistry staining for glomerular MAC-2⁺ macrophages. **Right panel,** MAC-2⁺ area as a percentage of total glomerular area determined using Image Pro Plus (Media Cybernetics). More than four independent kidney sections were examined per mouse. Scale bars, 50 µm. (A–E) Results are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Total mice analyzed (A–D, E): B<sup>WT</sup> (n = 6, 4), B<sup>WAS⁻/⁻</sup> (n = 14, 6), B<sup>W/TLR7⁻/⁻</sup> (n = 11, 9), and B<sup>W/TLR9⁻/⁻</sup> (n = 15, 8), from three (A–D) and two (E) independent experimental cohorts.
References


