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Immunity 25, 417–428, September 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.07.013

Toll-like Receptor 7 and TLR9 Dictate Autoantibody Specificity and Have Opposing Inflammatory and Regulatory Roles in a Murine Model of Lupus

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Summary

Antibodies (Abs) to RNA- and DNA-containing autoantigens are characteristic of systemic lupus erythematosus (SLE). We showed previously that Toll-like receptor (TLR) 9, recognizing DNA, is required for the spontaneous generation of DNA autoantibodies, but not for the development of lupus nephritis in susceptible mice. We report that lupus-prone mice deficient in TLR7, a receptor for ssRNA, failed to generate Abs to RNA-containing antigens (Ags) such as Smith (Sm) Ag. TLR9 and TLR7 also had dramatic effects on clinical disease in lupus-prone mice. In the absence of TLR9, autoimmune disease was exacerbated, lymphocytes and plasmacytoid DCs were more activated, and serum IgG and IFN-α were increased. In contrast, TLR7-deficient mice had ameliorated disease, decreased lymphocyte activation, and decreased serum IgG. These findings reveal opposing inflammatory and regulatory roles for TLR7 and TLR9, despite similar tissue expression and signaling pathways. These results have important implications for TLR-directed therapy of autoimmune disease.

Introduction

Production of autoantibodies is a cardinal feature of systemic lupus erythematosus (SLE) (Tan et al., 1982). Disease-related autoantibodies in SLE focus on particular targets, such as double-stranded DNA (dsDNA) and chromatin and RNA-containing Ags such as Smith (Sm) Ag and ribonucleoprotein (RNP) or ribosomal components (Egner, 2000; Muro, 2005). The correlation of DNA, Sm, and RNP Abs with disease activity and distinct clinical features of SLE highlights the potential pathogenic roles of these autoantibodies (Alba et al., 2003; Kirou et al., 2005). Despite long-standing knowledge of the specificity of the autoimmune response in SLE, little is known about why autoantibodies are focused on these particular targets (Plotz, 2003).

An important new insight in this area was the implication of Toll-like receptors (TLRs) in autoreactive B cell stimulation. Studies showing that rheumatoid factor B cells were stimulated in vitro in a DNase-sensitive and MyD88-dependent way led to the proposal that TLR9, a receptor for CpG DNA, is required to activate these B cells through anti-chromatin immune complexes (Leadbetter et al., 2002). This was extended to chromatin-specific B cells, which can be activated by the dual ligation of the Ag-specific BCR on the cell surface and TLR9 in intracellular compartments (Viglianti et al., 2003). Moreover, analogous data indicate that TLR7, a receptor for ssRNA, is required for optimal in vitro proliferation of B cells specific for RNA-containing Ags, thus potentially explaining a second major class of autoantibodies (Lau et al., 2005).

Until recently, the in vivo significance of these findings was unknown. The fact that TLR9 and TLR7 are expressed on B cells, macrophages, plasmacytoid DCs (pDCs), and (in mice) myeloid DCs (mDCs) (Edwards et al., 2003; Iwasaki and Medzhitov, 2004) could lead to a potentially complicated situation in vivo, particularly because TLR signaling differs in pDCs and other cell types (Honda et al., 2005). Indeed, a role for TLRs in stimulating dendritic cells (DCs) in autoimmune disease was suggested by the finding that DCs secrete inflammatory cytokines via a TLR9- or TLR7-dependent mechanism upon stimulation with nucleic acid-containing immune complexes (Boule et al., 2004; Means et al., 2005; Savarese et al., 2006). By using an F2 breeding strategy to cross MRL/Mp$^{lpr/lpr}$ with B6/129 TLR9-deficient mice, we initially found that TLR9 was required for the production of chromatin Abs in vivo (Christensen et al., 2005). This validated the notion that TLRs, presumably recognizing self-Ags, played a key role in systemic autoimmunity. We also noted that despite the lack of anti-chromatin, clinical disease was not ameliorated, and certain parameters of immune activation were actually greater in *Tlr9*$^{-/-}$ than in wild-type mice (Christensen et al., 2005). Subsequently, an effect of TLR9 in regulating autoimmune disease was reported in a study of a small number of backcrossed MRL/Mp mice (Wu and Peng, 2006). However, with ELISA-based assays, these investigators did not find an effect of TLR9 on DNA Abs (Wu and Peng, 2006). A third study, using a model in which all B cells express a high-affinity anti-DNA H chain in the context of FcγRIIB deficiency, reported that TLR9 was required for the production of IgG2a/2b DNA Abs, although these investigators did not assess clinical disease or total anti-DNA and anti-chromatin (Ehlers et al., 2006). Thus, the effect of TLR9 on DNA and chromatin Abs, as well as various parameters of immune activation and clinical disease, remains controversial.

Antibodies to DNA and chromatin represent one class of dominant autoantibodies in human and murine lupus; Abs to RNA-containing molecules are another. Although Abs to RNA-related Ags are not as common as anti-chromatin, they tend to be associated with more severe disease in lupus patients (Kirou et al., 2005). We previously reported that neither TLR3 nor TLR9 were required for production of Sm Abs in vivo (Christensen et al., 2005). Since all classes of nuclear Abs were absent in MyD88-deficient autoimmune-prone mice, however, it seems likely that anti-Sm and other RNA Abs are

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controlled by another TLR (Lau et al., 2005). Recent work has implicated TLR7 in the pathogenesis of SLE (Pisitkun et al., 2006; Savarese et al., 2006; Subramanian et al., 2006; Vollmer et al., 2005), but whether this receptor is required for autoantibody formation and whether it affects clinical disease progression in vivo have not been determined.

Here we first clarify the situation with regard to TLR9 and autoimmune disease, in light of the three conflicting reports, by performing extensive analysis of mice in which the disrupted TLR9 allele was backcrossed to the autoimmune MRL/Mp$^{lpr/lpr}$ genetic background. These data support and extend our original findings in autoimmune F2 hybrid mice, particularly the clear effect on chromatin Abs (Christensen et al., 2005). We confirm that MRL/Mp$^{lpr/lpr}$ mice lacking TLR9 developed more severe clinical disease and show that it leads to early mortality. In addition, we report that TLR7 was required for the generation of autoantibodies to RNA-containing Ags in vivo. Surprisingly, despite the similarities between TLR7 and TLR9 in terms of sequence, specificity, tissue expression, and signaling, TLR7 had the opposite effect on disease from TLR9. These findings together account for the targeting of the major classes of autoantibodies in SLE and have important implications for therapeutic strategies based on manipulating TLR recognition and signaling.

Results

Impaired Generation of Antibodies to DNA Ags in TLR9-Deficient Mice

In order to analyze the effects of TLR9 on murine SLE in a controlled genetic experiment, *Tlr9*$^{+/-}$ hybrid mice were backcrossed eight generations to lupus-prone MRL/Mp$^{lpr/lpr}$ mice (Cohen and Eisenberg, 1991). Backcrossed heterozygotes were then intercrossed, and wild-type littermates were used as controls for *Tlr9*$^{-/-}$ MRL/Mp$^{lpr/lpr}$ mice. We used the fluorescent ANA assay as a sensitive detection method for Abs to both RNA-containing and DNA-containing autoantigens (Emlen and O'Neill, 1997; Muro, 2005; Tan et al., 1982). Typical of MRL/Mp$^{lpr/lpr}$ sera, the majority of *Tlr9*$^{+/+}$ sera produced a homogenous nuclear ANA pattern, corresponding to DNA and chromatin autoantibodies (Figures 1A and 1B). Moreover, 25 of 26 wild-type mice produced specific chromatin Abs, as determined by equatorial staining of chromosomes in metaphase cells (Figures 1A and 1C). In contrast, none of the sera from 21 *Tlr9*$^{-/-}$ mice produced homogenous staining, and only two mice generated Abs capable of binding to mitotic chromatin (Figures 1A–1C, $p < 0.0001$). These results confirm our earlier findings in F2 mice, demonstrating severe impairment in the generation of Abs to native DNA and chromatin in the absence of TLR9.

Staining patterns generated by TLR9-deficient sera revealed a potential shift in repertoire specificity. Although an equivalent fraction of sera from both groups produced speckled nuclear staining patterns (indicative of Abs to RNA splicing complexes such as Sm and RNP), sera from 15 of 21 *Tlr9*$^{-/-}$ mice, and none of the wild-type mice, produced a primarily cytoplasmic pattern (Figures 1A and 1B, $p < 0.0001$ for comparison of pattern distribution between *Tlr9*$^{+/+}$ and *Tlr9*$^{-/-}$). Autoantibodies to cytoplasmic RNA complexes are common in SLE sera and lead to fine granular or homogenous cytoplasmic staining of ANA substrates (Lau et al., 2005; Muro, 2005), such as was observed in *Tlr9*$^{-/-}$ sera (Figure 1A, bottom right). Although cytoplasmic staining can also be produced by Abs to organelles such as endosomes, the Golgi apparatus, or mitochondria, these Abs stain discrete regions of the cytoplasm in a characteristic pattern (Muro, 2005) that was not observed in sera from *Tlr9*$^{-/-}$ mice (Figure 1A and data not shown). Thus, although TLR9-deficient and wild-type sera both produced nuclear speckled patterns characteristic of Sm and RNP Abs, only TLR9$^{-/-}$ mice generated Abs reacting with cytoplasmic Ags that may include RNA.

The lack of homogenous nuclear and mitotic chromatin staining patterns in *Tlr9*$^{-/-}$ sera indicated a block in the generation of Abs to DNA-containing Ags. Because of the high specificity of *Crithidia luciliae* immunofluorescence in the detection of Abs to dsDNA (Isenberg et al., 1987), we used this assay to confirm our ANA findings. *Tlr9*$^{-/-}$ sera showed a substantial decrease in Abs binding the dsDNA of the *C. luciliae* kinetoplast (Figure 1D, $p < 0.0001$). This decrease was highly significant but did not represent a complete block in dsDNA Ab production, because some *Tlr9*$^{-/-}$ samples specifically stained the kinetoplast, albeit with lower intensity. This low level of dsDNA Ab production in *Tlr9*$^{-/-}$ mice was in contrast to the almost complete absence of Abs to native DNA and chromatin as determined by the ANA assay. It is probable that this difference reflects the nature of endogenous DNA, which exists in a macromolecular chromatin complex. Indeed, many autoantibodies in SLE preferentially bind to continuous DNA-histone epitopes rather than isolated dsDNA, suggesting that chromatin is the physiologically relevant autoantigen (Losman et al., 1992).

We used enzyme-linked immunosorbent assays (ELISA) to quantitate the decrease in Abs to DNA-containing Ags in *Tlr9*$^{-/-}$ sera. An anti-nucleosome ELISA, in which the detection Ag was the combination of histones and dsDNA, demonstrated an order-of-magnitude decrease in specific DNA Abs in *Tlr9*$^{-/-}$ sera (Figure 1E, $p < 0.0001$). When we employed a standard ELISA for dsDNA in which the detection Ag was the combination of poly-L-lysine and dsDNA, however, there was not a significant decrease in *Tlr9*$^{-/-}$ sera (Figure 1F). These apparently conflicting findings illustrate two points. First, they highlight the low specificity of ELISA-based assays, which are known to be susceptible to high false positive rates in the detection of DNA Abs (Emlen and O'Neill, 1997; Isenberg et al., 1987). Second, they again implicate the chromatin complex of DNA and histones as the endogenous Ag recognized by TLR9 in SLE, while Abs binding to purified dsDNA may represent cross-reactivity with other nucleic acid autoantigens, or charge-based nonspecific association. The anti-dsDNA ELISA appeared to be particularly susceptible to nonspecific binding, because values from this assay were significantly correlated with total IgG ($p = 0.0163$), while antinucleosome values were not (see Figure S1 in the Supplemental Data available online).

We also determined amounts of Sm Abs in *Tlr9*$^{-/-}$ sera. Unlike Abs to DNA-containing Ags, we did not

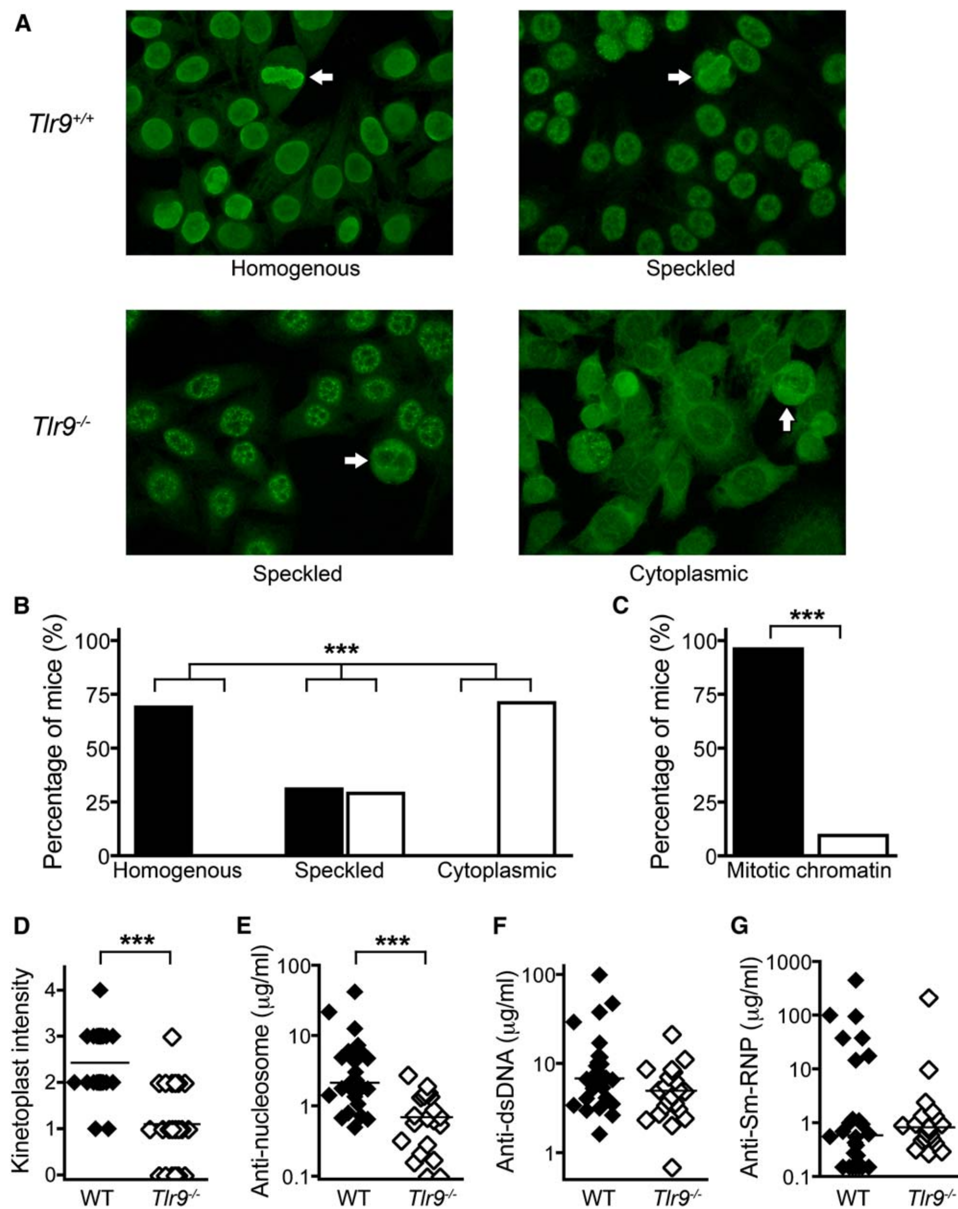

Figure 1. Impaired Generation of Abs to DNA Ags in TLR9-Deficient Mice

(A) ANAs from $Tlr9^{+/+}$ sera are shown at top (left, homogenous nuclear pattern; right, speckled nuclear pattern), and $Tlr9^{-/-}$ sera at bottom (left, speckled nuclear pattern; right, cytoplasmic pattern). White arrows indicate cells in metaphase that demonstrate positive (top, $Tlr9^{+/+}$) or negative (bottom, $Tlr9^{-/-}$) staining of mitotic chromatin.

(B) Serum ANAs were classified as either nuclear homogenous, nuclear speckled, or cytoplasmic staining patterns. Black bars indicate $Tlr9^{+/+}$ (n = 26), and white bars indicate $Tlr9^{-/-}$ (n = 21) sera.

(C) As in (B), but ANAs were classified as either positive or negative for mitotic chromatin staining.

(D) dsDNA Abs in $Tlr9^{+/+}$ (black symbols, n = 26) and $Tlr9^{-/-}$ (white symbols, n = 21) sera detected by *C. luciliae* immunofluorescence. Intensity of staining *C. luciliae* kinetoplast DNA was scored from 0 to 4.

(E–G) Nucleosome (E), dsDNA (F), and Sm-RNP (G) Abs determined by ELISA in $Tlr9^{+/+}$ (black symbols, n = 26) and $Tlr9^{-/-}$ (white symbols, n = 21) sera. Bars represent median values (E–G). ***$p < 0.0001$ by either Chi square analysis (B), Fisher's exact test (C), or Mann-Whitney U test (D and E).

detect a significant difference in Abs to Sm or the Sm-RNP complex between wild-type and $Tlr9^{-/-}$ mice (Figure 1G and data not shown). Of note, however, is that $Tlr9^{-/-}$ mice appeared to have an elevated baseline level of anti-Sm-RNP (Figure 1G), suggesting that, had these mice survived longer, they may have developed higher anti-Sm titers, analogous to our earlier findings in a cohort of older $Tlr9^{-/-}$ F2 mice (Christensen et al., 2005).

TLR9 Regulates Plasmacytoid Dendritic Cell Activation in SLE

Because TLR9 is expressed by murine plasmacytoid and myeloid DCs (Edwards et al., 2003), we hypothesized that the lack of this receptor could influence the function of DC subsets in autoimmune disease. By identifying pDCs as $CD11c^{int}$ pDCA-1^{+} cells (Krug et al., 2004), we found that splenic pDCs from $Tlr9^{-/-}$ mice appeared to be more activated than wild-type pDCs based

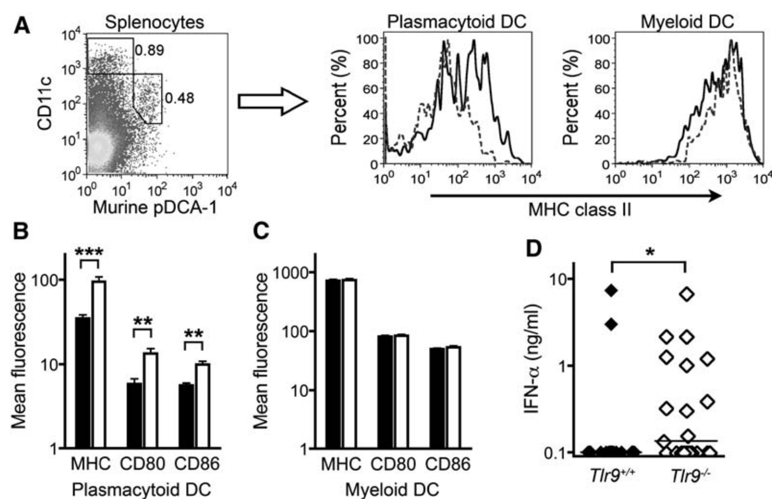

Figure 2. TLR9 Regulates pDC Activation in SLE

(A) Left: splenic pDCs (CD11c^{int} pDCA-1^{+}) and myeloid DCs (CD11c^{hi} pDCA-1^{-}) were identified by FACS. Right: MHC class II expression in pDCs (left) and myeloid DCs (right) from *Tlr9*$^{+/+}$ (dashed line) and *Tlr9*$^{-/-}$ (solid line) mice. Numbers on gates indicate percentage of cells.

(B and C) Expression of MHC class II, CD80, and CD86 in pDCs (B) and myeloid DCs (C) from *Tlr9*$^{+/+}$ (black bars, n = 26) and *Tlr9*$^{-/-}$ (white bars, n = 21) mice. Data are presented as mean ± SEM.

(D) IFN-α levels in serum from *Tlr9*$^{+/+}$ (black symbols, n = 26) and *Tlr9*$^{-/-}$ (white symbols, n = 21) mice. Limit of detection was 0.1 ng/ml. Two of 26 *Tlr9*$^{+/+}$ and 11 of 21 *Tlr9*$^{-/-}$ sera had IFN-α > 0.1 ng/ml. Bars represent median values. *p < 0.05; **p < 0.01; ***p < 0.0001 by Mann-Whitney U test.

on the increased expression of MHC class II (Figures 2A and 2B, p < 0.0001). Plasmacytoid DCs from *Tlr9*$^{-/-}$ mice also had increased expression of the activation markers CD80 and CD86 (Figure 2B, p = 0.0007 and 0.0002, respectively). This effect was specific to pDCs; conventional, CD11c^{hi} myeloid DCs from both strains had similar expression of these molecules (Figures 2A and 2C).

The increased expression of activation markers by pDCs in *Tlr9*$^{-/-}$ mice suggested a specific effect of TLR9 on pDC function in SLE. Because a major functional consequence of pDC activation by TLRs is the secretion of type I interferons (IFN-I), we determined the serum concentration of IFN-α in *Tlr9*$^{-/-}$ mice. In agreement with the increased pDC activation state, we found that more than half of *Tlr9*$^{-/-}$ sera had detectable amounts of IFN-α, compared with only 2 of 26 wild-type sera (Figure 2D, p = 0.0009 by Fisher's exact test).

Increased Immune Activation in TLR9-Deficient Mice

Having observed the clear effects of TLR9 deficiency on autoantibody production and pDC activation, we then determined whether the absence of this receptor had a significant impact on the manifestation of clinical autoimmune disease, as had been suggested by previous studies (Christensen et al., 2005; Wu and Peng, 2006). We found that *Tlr9*$^{-/-}$ mice had a significant increase in the incidence and severity of autoimmune skin disease compared to wild-type littermates (Figure 3A, p = 0.0136). Concordant with this were increased lymphadenopathy and splenomegaly in *Tlr9*$^{-/-}$ mice (Figure 3B, p = 0.0043 and 0.0023, respectively).

Increased spleen and lymph node weight in MRL/Mp$^{lpr/lpr}$ mice is accompanied by accumulation of lymphocytes expressing an activated phenotype in these organs (Cohen and Eisenberg, 1991). As expected from their increased spleen and lymph node weight, *Tlr9*$^{-/-}$ mice had an increased number of CD4^{+} helper T cells as well as a 3-fold increase in the number of CD4^{-} CD8^{-} double-negative T cells in the spleen (Figure 3C, p = 0.0096 and 0.002, respectively). This aberrant population of double-negative T cells is thought to arise from the inability to delete activated cells in Fas-deficient animals (Cohen and Eisenberg, 1991). Analysis of T cell activation status in the lymph node revealed a similar effect of TLR9 deficiency. *Tlr9*$^{-/-}$ mice had fewer naive phenotype CD4^{+} cells, with a significant increase in activated CD44^{+} cells (Figure 3D, p = 0.0001). Similar results were observed in the spleen (data not shown). Finally, deficiency of TLR9 also affected global B cell activation. Splenic B cells from *Tlr9*$^{-/-}$ mice had increased expression of the activation markers CD44 and CD69 (Figure 3E, p = 0.0002 and 0.0333, respectively).

As another measure of global B cell activation, we determined total amounts of serum IgG. *Tlr9*$^{-/-}$ sera had increased concentrations of every IgG isotype, but the most prominent increases were in IgG2a and IgG3, which were 2-fold greater than in wild-type sera (Figure 3F, p = 0.0038 for IgG1, 0.0043 for IgG2a, 0.0006 for IgG2b, and p < 0.0001 for IgG3). Interestingly, the isotypes profoundly affected by the absence of TLR9 are the immunoglobulin isotypes frequently associated with inflammation and autoimmunity (Nimmerjahn and Ravetch, 2005). Indeed, IgG3 has been shown to play a central role in the development of nephritis in MRL/Mp$^{lpr/lpr}$ mice (Takahashi et al., 1991). Taken together, these data indicate that the genetic absence of TLR9 led to increased disease activity and global immune activation in MRL/Mp$^{lpr/lpr}$ mice.

Accelerated Lupus Nephritis and Mortality in the Absence of TLR9

The definitive measure of end-organ pathology in SLE is the presence of lupus nephritis. In agreement with other evidence of increased disease activity, TLR9-deficient mice developed exacerbated kidney disease. We observed increased glomerular size, cellularity, and protein deposition in *Tlr9*$^{-/-}$ kidney sections, leading to a histologic glomerulonephritis score that was significantly greater than wild-type littermates (Figures 4A and 4B, p = 0.0024). Kidneys from MRL/Mp$^{lpr/lpr}$ mice also show a characteristic interstitial infiltration of lymphocytes and monocytes, primarily centered around major vessels, and occasionally invading the cortical parenchyma. There was a trend toward increased severity of interstitial infiltrates in *Tlr9*$^{-/-}$ mice, but it did not reach statistical significance (Figure 4B). However, the composite score of both interstitial and glomerular disease revealed a significant

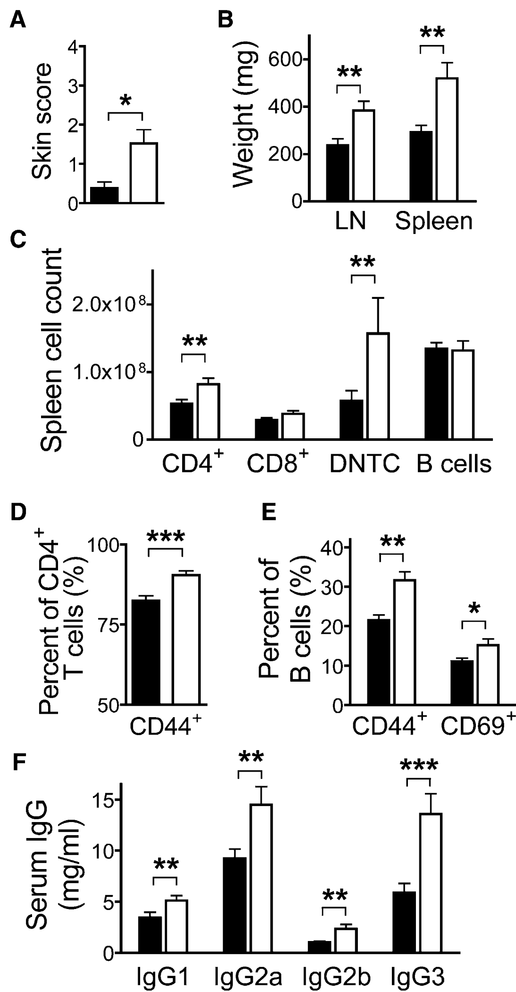

Figure 3. Increased Immune Activation in TLR9-Deficient Mice

Tlr9$^{+/+}$ (black bars, n = 26) and *Tlr9*$^{-/-}$ (white bars, n = 21) mice were assayed for various parameters of disease activity and immune activation.

(A) Severity of skin disease.

(B) Weight of spleens and the two largest axillary lymph nodes.

(C) Numbers of splenic CD4^{+} T cells, CD8^{+} T cells, CD4^{-}/CD8^{-} double-negative T cells (DNTC), and B cells.

(D) CD44 expression by lymph node CD4^{+} T cells.

(E) Expression of CD44 and CD69 on splenic B cells.

(F) Serum IgG isotype concentrations. Data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.0001 by Mann-Whitney U test.

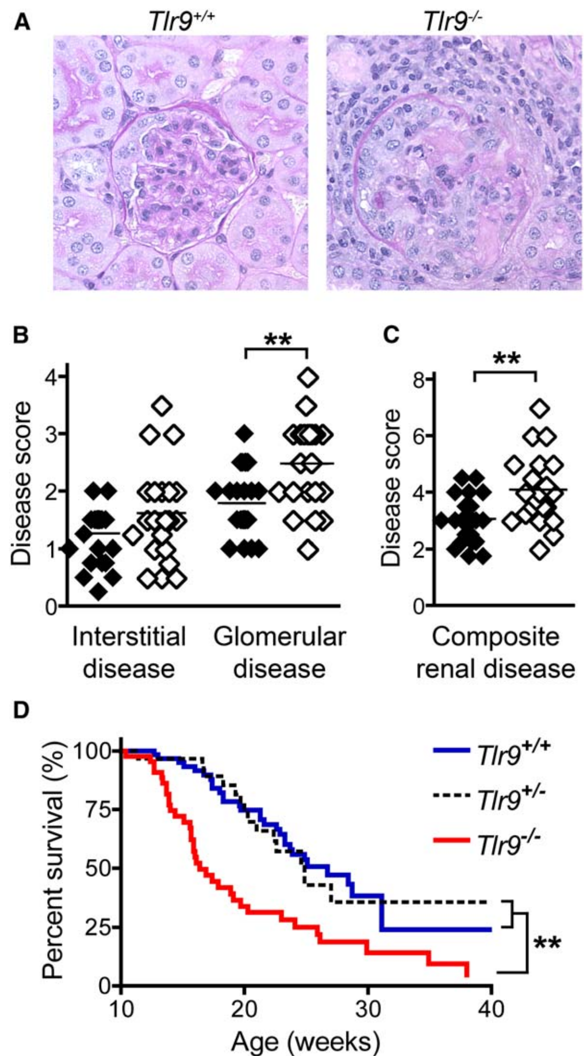

Figure 4. Accelerated Lupus Nephritis and Mortality in TLR9-Deficient Mice

(A) Representative PAS-stained *Tlr9*$^{+/+}$ (left) and *Tlr9*$^{-/-}$ (right) glomeruli.

(B) Interstitial and glomerular renal disease were scored from 0 to 4 for *Tlr9*$^{+/+}$ (black symbols, n = 26) and *Tlr9*$^{-/-}$ (white symbols, n = 21) mice.

(C) As in (B), but the sum of interstitial and glomerular scores for each mouse was plotted to determine composite renal disease.

(D) *Tlr9*$^{+/+}$ (blue line, n = 61), *Tlr9*$^{+/-}$ (black dashed line, n = 29), and *Tlr9*$^{-/-}$ (red line, n = 44) mice were observed until the time of death. **p < 0.01 by Mann-Whitney U test (B and C) or logrank test (D).

exacerbation of nephritis in the absence of TLR9 (Figure 4C, p = 0.0029).

Although we observed an increase in nearly every marker of disease severity in autoimmune *Tlr9*$^{-/-}$ mice, it remained unclear whether these disease markers truly affected mortality. We therefore allowed a second cohort of *Tlr9*$^{+/+}$, *Tlr9*$^{+/-}$, or *Tlr9*$^{-/-}$ MRL/Mp$^{lpr/lpr}$ littermates to develop spontaneous disease, and we monitored them without intervention until the time of death or irreversible morbidity. We found that TLR9-deficient mice had accelerated mortality relative to wild-type controls; median survival was reduced from 25.1 weeks in *Tlr9*$^{+/+}$ mice and 24.9 weeks in *Tlr9*$^{+/-}$ mice to 16.4 weeks in *Tlr9*$^{-/-}$ mice (Figure 4D, p = 0.0002 and 0.0047 for comparison of *Tlr9*$^{-/-}$ with *Tlr9*$^{+/+}$ and *Tlr9*$^{+/-}$, respectively). There was no difference in survival between wild-type and *Tlr9*$^{+/-}$ heterozygote mice, suggesting that a single copy of TLR9 is sufficient to regulate fatal SLE in this model.

Association of Disease Severity with Antibodies to RNA Ags

Abs to RNA-containing Ags have been associated with disease severity in human SLE patients (Alba et al., 2003; Kirou et al., 2005; Migliorini et al., 2005). Because we observed a shift in autoantibody specificity and exacerbated disease in *Tlr9*$^{-/-}$ mice, we determined

whether the presence of RNA complex Abs was associated with disease severity. *Tlr9*$^{+/+}$ and *Tlr9*$^{-/-}$ mice were grouped based on the presence of Sm or Sm-RNP Abs. The presence of Abs to RNA Ags was indeed correlated with increased immune activation in our cohort. In both the wild-type and *Tlr9*$^{-/-}$ groups, mice with Sm-RNP Abs exhibited an increase in MHC class II expression by pDCs compared to mice without anti-Sm-RNP (Figure S2A). In *Tlr9*$^{-/-}$ mice, higher titers of RNA-related Abs were also associated with exacerbated skin disease (Figure S2B). We also observed increased splenic CD4^{+} T cell activation and interstitial lymphocytic infiltrates in the kidneys of TLR9$^{-/-}$ mice with Sm-RNP Abs (data not shown).

Impaired Generation of Antibodies to RNA Ags in TLR7-Deficient Mice

Analogous to TLR9, we generated TLR7-deficient autoimmune-prone mice by backcrossing three to six generations to MRL/Mp$^{lpr/lpr}$ mice. Because TLR7 is located on the X chromosome, we compared male *Tlr7*$^{-/y}$ mice with male *Tlr7*$^{+/y}$ littermates. Analysis of ANA staining by *Tlr7*$^{-/y}$ or wild-type sera revealed patterns typical of SLE. The majority of mice in both groups produced DNA Abs, as determined by homogenous nuclear staining, and all serum samples contained chromatin Abs (Figures 5A–5C). There were, however, subtle differences in ANA staining patterns derived from wild-type and *Tlr7*$^{-/y}$ mice. Sera from some wild-type mice produced speckled staining in the absence of any other patterns, indicative of specific Abs to Sm and RNP. *Tlr7*$^{-/y}$ sera, in contrast, produced speckled patterns that were obscured by superimposed homogenous staining (Figure 5A). 7 of 36 wild-type sera produced this "pure" speckled pattern, which was not observed in any of 35 *Tlr7*$^{-/y}$ sera ($p = 0.0113$ by Fisher's exact test). This suggested impairment in the generation of Abs to RNA complexes in *Tlr7*$^{-/y}$ mice.

Consistent with the ANA staining patterns, the generation of high-titer Sm Abs was blocked in *Tlr7*$^{-/y}$ mice (Figure 5D, $p = 0.0309$). However, because Sm Abs occur with a relatively low prevalence (Cohen and Eisenberg, 1991; Migliorini et al., 2005), only 6 of 36 *Tlr7*$^{+/y}$ sera had high titers of anti-Sm, in addition to 3 sera with intermediate levels, compared with 0 of 35 *Tlr7*$^{-/y}$ samples. We therefore also used a more sensitive Ag, the U1 small nuclear ribonucleoprotein complex, composed of both Sm and RNP protein Ags, to detect Abs to RNA-containing Ags (Migliorini et al., 2005). The relative decrease in these Abs to RNA Ags in *Tlr7*$^{-/y}$ mice was highly significant (Figure 5E, $p = 0.0035$). This blockade of autoantibody production in *Tlr7*$^{-/y}$ mice was specific for Abs to RNA-containing Ags, as shown by the fact that there was no decrease in DNA or chromatin Abs as detected by either ANA or ELISA-based assays (Figures 5A–5C, 5F, and 5G).

Reduced Immune Activation in TLR7-Deficient Mice

There was a significant decrease in both lymph node and spleen weight in *Tlr7*$^{-/y}$ mice compared to wild-type littermates (Figure 6A, $p = 0.0005$ and 0.0295, respectively). The decrease in spleen weight among *Tlr7*$^{-/y}$ mice was paralleled by a decrease in the accumulation of double-negative T cells (Figure 6B, $p = 0.0155$). Similarly, *Tlr7*$^{-/y}$ mice had a decreased proportion of CD4^{+} T cells expressing CD44, both in the lymph node (Figure 6C, $p = 0.0009$) and the spleen (data not shown). Lack of TLR7 also inhibited general B cell activation, as indicated by the fact that CD44 expression was decreased in splenic B cells from *Tlr7*$^{-/y}$ mice, although CD69 expression was unaffected (Figure 6D, $p = 0.0026$ for CD44). Serum IgG levels, another marker of B cell activation, were decreased in *Tlr7*$^{-/y}$ mice, but only for IgG2a and IgG3 (Figure 6E, $p < 0.0001$ for both), the two isotypes most elevated in *Tlr9*$^{-/-}$ serum. Interestingly, these two immunoglobulin isotypes are most potently induced by IFN-I (Le Bon et al., 2001).

Unlike TLR9, which regulated pDC activation, TLR7 appeared to promote pDC activation in the context of SLE. Plasmacytoid DCs from *Tlr7*$^{-/y}$ mice exhibited a more immature phenotype, with decreased expression of MHC class II (Figures 6F and 6G, $p = 0.0144$), although expression of CD80 and CD86 were unaffected (Figure 6G). The activation state of myeloid DCs appeared unaffected by the absence of TLR7 (Figures 6F and 6H). Although TLR7 deficiency inhibited the activation of pDCs, it did not completely prevent systemic IFN-I production, as shown by the fact that a small subset of sera from both *Tlr7*$^{+/y}$ and *Tlr7*$^{-/y}$ mice contained detectable levels of IFN-α (data not shown). These findings correlate with the reciprocal effects of TLR7 and TLR9 on serum IgG levels and suggest the possibility of B cell-pDC interactions in the pathogenesis of autoantibody formation.

Ameliorated Clinical Disease in the Absence of TLR7

The effects of TLR7 deficiency were also evident in the kidney. *Tlr7*$^{-/y}$ mice appeared to have ameliorated renal disease, with decreased glomerular protein deposition and preserved glomerular structure (Figure 7A). Although the trend toward decreased severity of individual parameters of nephritis did not reach statistical significance (Figure 7B), the composite score of renal disease was significantly reduced in *Tlr7*$^{-/y}$ mice (Figure 7C, $p = 0.0109$). In addition, there was very little skin disease in *Tlr7*$^{-/y}$ mice at 16 weeks of age. Although skin disease was evident in *Tlr7*$^{+/y}$ mice, it occurred with a relatively low frequency such that the difference between the two groups did not reach statistical significance ($p = 0.0878$, data not shown). All 20 mice in each group survived to analysis at 16 weeks of age; a formal analysis of survival in a new cohort is ongoing. Overall, the effects of TLR7 deficiency on clinical disease were less pronounced than the effects of TLR9 deficiency. This could be due to subtle differences between the TLR7 and TLR9 cohorts, or could be a result of the overwhelming predisposition toward autoimmune disease in MRL/Mp$^{lpr/lpr}$ mice, which is more prone to exacerbation than inhibition.

Discussion

The findings presented here help to clarify the controversy in the literature regarding the role of Toll-like receptors and innate immunity in the generation of specific autoantibodies in SLE and the contribution of autoantibodies and TLRs to clinical disease manifestations. We found that the requirement for TLR9 in the

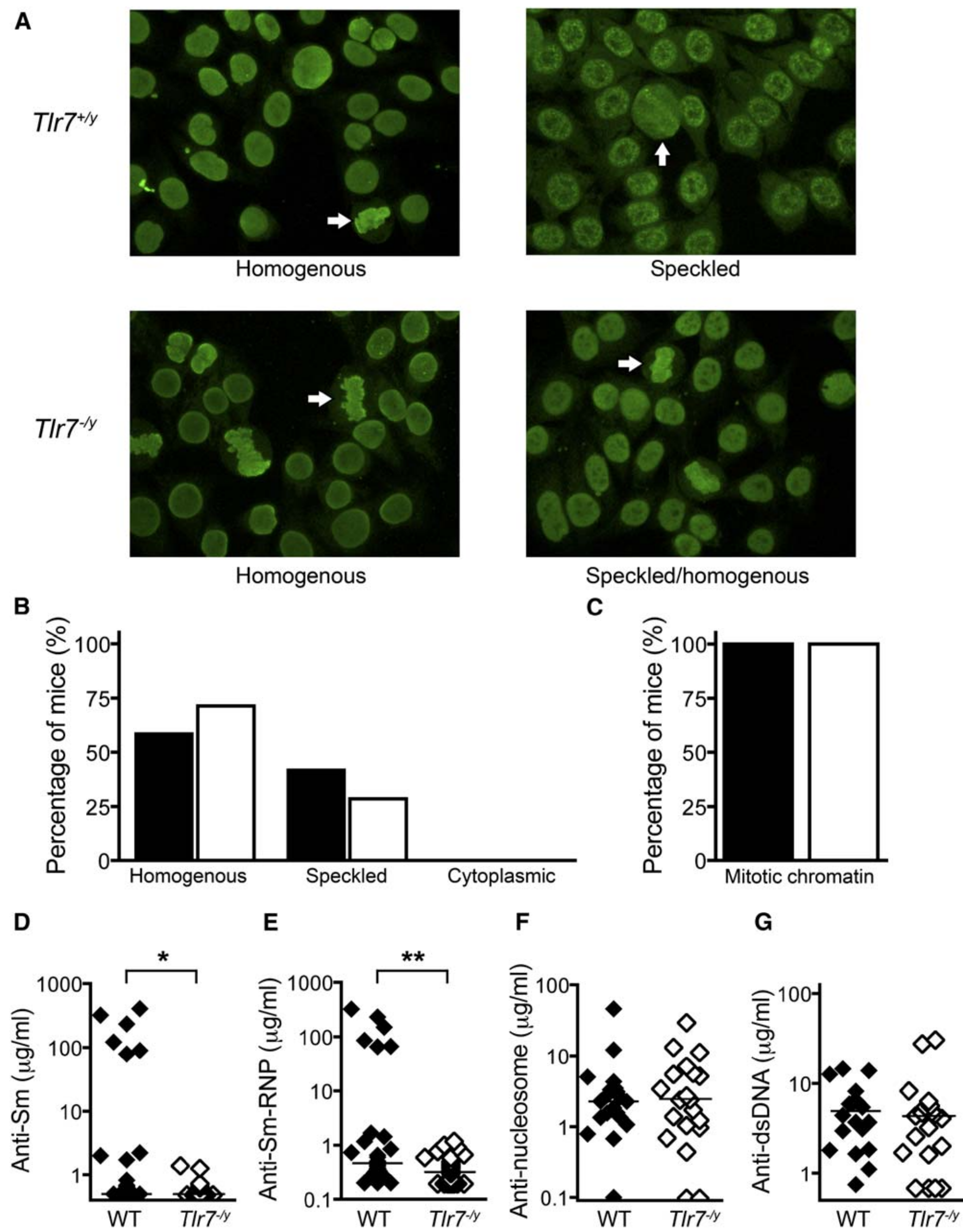

Figure 5. Impaired Generation of Abs to RNA Ags in TLR7-Deficient Mice

(A) ANAs from *Tlr7*$^{+/y}$ sera are shown at top (left, homogenous nuclear pattern; right, speckled nuclear pattern), and *Tlr7*$^{-/y}$ sera at bottom (left, homogenous nuclear pattern; right, speckled nuclear pattern). Speckled patterns in *Tlr7*$^{-/y}$ sera were a mix of speckled staining with superimposed homogenous staining. White arrows indicate cells in metaphase that demonstrate positive staining of mitotic chromatin.

(B) Serum ANAs were classified as either nuclear homogenous, nuclear speckled, or cytoplasmic staining patterns. Black bars indicate *Tlr7*$^{+/y}$ (n = 36), and white bars indicate *Tlr7*$^{-/y}$ (n = 35) sera.

(C) As in (B), but ANAs were classified as either positive or negative for mitotic chromatin staining.

(D and E) Sm (D) and Sm-RNP (E) Abs determined by ELISA in *Tlr7*$^{+/y}$ (black symbols, n = 36) and *Tlr7*$^{-/y}$ (white symbols, n = 35) sera.

(F and G) Nucleosome (F) and dsDNA (G) Abs determined by ELISA in *Tlr7*$^{+/y}$ (black symbols, n = 20) and *Tlr7*$^{-/y}$ (white symbols, n = 20) sera. Bars represent median values (D–G). $^{*}p < 0.05$; $^{**}p < 0.01$ by Mann-Whitney U test.

production of autoantibodies to DNA-containing Ags, first observed in lupus-prone F2 mice (Christensen et al., 2005), also applied to *Tlr9*$^{-/-}$ mice fully backcrossed to the MRL/Mp$^{lpr/lpr}$ strain. This is in contrast to a reported increase in anti-DNA titers in TLR9-deficient MRL/Mp mice (Wu and Peng, 2006). These conflicting findings can most likely be attributed to the assays used to detect DNA Abs. Wu and Peng used an ELISA-based assay to determine anti-DNA titers, despite the documented lack of specificity of such assays for autoantibodies in the context of SLE (Emlen and O'Neill, 1997; Isenberg et al., 1987) (Figure S1). Moreover, this study, with a limited number of six mice per group, did not adequately document immunofluorescent ANA patterns or *C. luciliae* immunofluorescence with *Tlr9*$^{-/-}$ sera (Wu and Peng, 2006). By using these more specific assays in *Tlr9*$^{-/-}$ mice of a homogenous and defined genetic background, we clearly show that

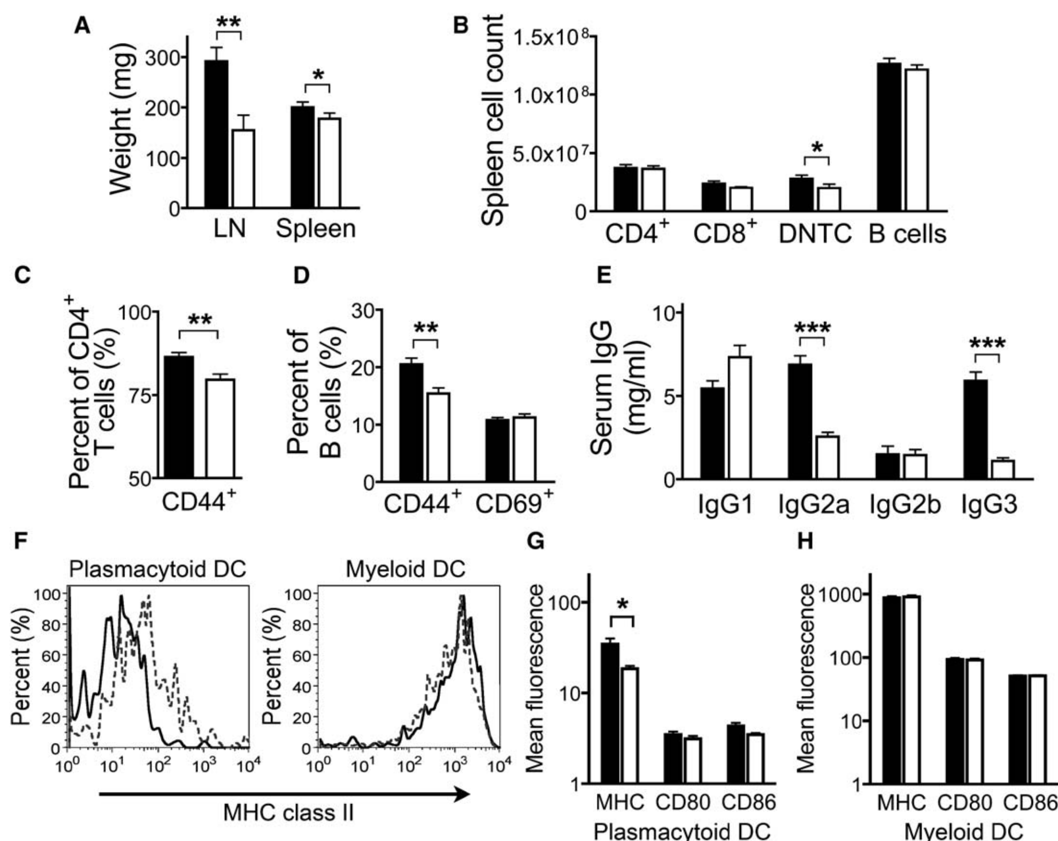

Figure 6. Decreased Immune Activation in TLR7-Deficient Mice

Tlr7$^{+/y}$ (black bars, n = 20) and *Tlr7*$^{-/y}$ (white bars, n = 20) mice were assayed for various parameters of disease activity and immune activation.

(A) Weight of spleens and the two largest axillary lymph nodes.

(B) Numbers of splenic $CD4^+$ T cells, $CD8^+$ T cells, $CD4^-$/$CD8^-$ double-negative T cells (DNTC), and B cells.

(C) CD44 expression by lymph node $CD4^+$ T cells.

(D) Expression of CD44 and CD69 on splenic B cells.

(E) Serum IgG isotype concentrations.

(F) MHC class II expression in pDCs (left) and myeloid DCs (right) from *Tlr7*$^{+/y}$ (dashed line) and *Tlr7*$^{-/y}$ (solid line) mice.

(G and H) Expression of MHC class II, CD80, and CD86 in pDCs (G) and myeloid DCs (H). Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$ by Mann-Whitney U test.

TLR9 is required for effective production of Abs to the relevant DNA-containing autoantigens in SLE. This is consistent with the impaired generation of class-switched DNA Abs in *Tlr9*$^{-/-}$ *Fcgr2b*$^{-/-}$ mice (Ehlers et al., 2006).

In the absence of anti-DNA and anti-chromatin, *Tlr9*$^{-/-}$ mice developed an autoantibody repertoire shifted toward other nuclear and cytoplasmic Ags, producing novel ANA staining patterns. This is reminiscent of our findings in *Tlr9*$^{-/-}$ F2 mice, which developed increased anti-Sm titers in the absence of anti-DNA (Christensen et al., 2005). Although the *Tlr9*$^{-/-}$ mice presented here did not have elevated serum titers of anti-Sm (perhaps due to the younger age of this cohort), ANA patterns from these mice suggested an increase in Abs to cytosolic autoantigens, most likely containing RNA. It is possible that in the absence of TLR9, anti-DNA B cells persist in an ignorant or anergic state. This could then allow other autoreactive B cells to better compete for Ag or other survival and differentiation factors, leading in turn to a compensatory increase in RNA or Sm Abs.

The importance of TLR7 in autoimmunity was recently suggested by the finding that the Y-linked autoimmune accelerator (*Yaa*) locus is composed of a duplication of a 4 Mb region of the X chromosome containing TLR7, as well as several other immune response genes (Pisitkun et al., 2006; Subramanian et al., 2006). Our data, a report of genetic deficiency of TLR7 in a model of spontaneous SLE, complements these results. Whereas a duplication of this locus on the X chromosome was shown to be sufficient to induce autoantibody formation, the data presented here indicate that TLR7 is required for the generation of autoantibodies to canonical RNA Ags in vivo. Thus, our results indicate that the two major classes of autoantigens in SLE, nuclear complexes containing either DNA or RNA, are specifically controlled by TLR9 and TLR7, respectively. TLR8 may also play a role in the recognition of RNA autoantigens in humans (Vollmer et al., 2005). The lack of any nuclear Abs in autoimmune-prone mice deficient in MyD88 further emphasizes the central role of TLRs and innate immunity in directing autoantibody specificity (Lau et al., 2005).

We had previously reported an increase in certain parameters of immune activation in *Tlr9*$^{-/-}$ mice of a mixed genetic background (Christensen et al., 2005). Similarly, Wu and Peng found increased lymphadenopathy and

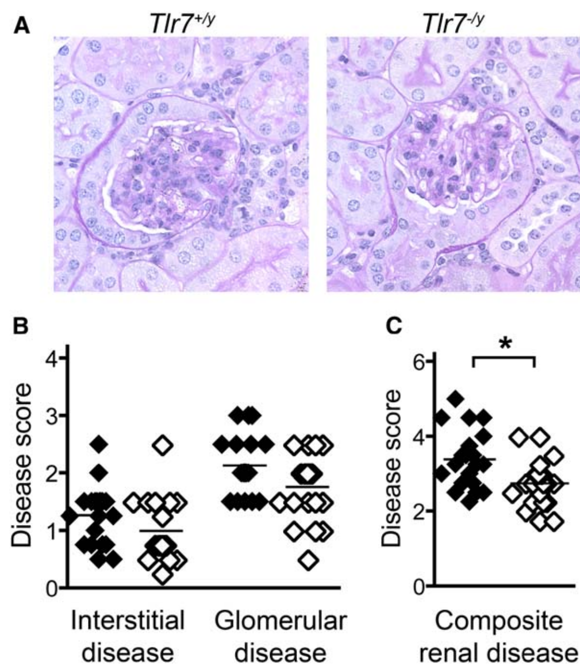

Figure 7. Ameliorated Lupus Nephritis in TLR7-Deficient Mice

(A) Representative PAS-stained *Tlr7*$^{+/y}$ (left) and *Tlr7*$^{-/y}$ (right) glomeruli.

(B) Interstitial and glomerular renal disease were scored from 0 to 4 for *Tlr7*$^{+/y}$ (black symbols, n = 20) and *Tlr7*$^{-/y}$ (white symbols, n = 20) mice.

(C) As in (B), but the sum of interstitial and glomerular scores for each mouse was plotted to determine composite renal disease. *$p < 0.05$ by Mann-Whitney U test.

end-organ disease in both Fas-intact and Fas-deficient *Tlr9*$^{-/-}$ mice (Wu and Peng, 2006). We confirm those findings of increased spleen/lymph node weight and renal disease and extend those findings here in several ways. First, we demonstrate a role for TLR9 in lupus-induced mortality, accompanied by exacerbated skin disease. Second, we provide new insights into the mechanism with the description of increased pDC activation and IFN-α production in *Tlr9*$^{-/-}$ MRL/Mp$^{lpr/lpr}$ mice. The lack of chromatin Abs coupled with increased IFN production and exacerbated disease pathology in *Tlr9*$^{-/-}$ mice provides a potent example of how autoantibodies and disease manifestations are not necessarily directly linked.

Despite the analogous roles of TLR7 and TLR9 in directing autoantibody specificity, we found that TLR7-deficient lupus-prone mice were relatively protected from autoimmune disease. In addition to decreased lymphocyte and pDC activation, renal disease was also ameliorated in *Tlr7*$^{-/y}$ mice. These findings suggest that therapeutic inhibition of TLR pathways could have beneficial effects on end-organ disease in SLE patients. Indeed, the mitigating effect of inhibitory oligodeoxynucleotides (ODN) on lupus nephritis has already been demonstrated in mice (Dong et al., 2005; Patole et al., 2005). Although these synthetic inhibitors were initially shown to inhibit signaling through TLR9, the data presented here, in concert with the observation that ODNs can also inhibit immune responses to TLR7 ligands (Barrat et al., 2005; Lau et al., 2005), indicate that the primary effect of these molecules in vivo may be mediated through inhibition of TLR7. Similarly, the therapeutic effects of chloroquine in the treatment of autoimmune and rheumatic diseases may be due to inhibition of endosomal TLR activation (Vinuesa and Goodnow, 2002).

The opposing effects of TLR7 and TLR9 on immune activation and disease severity were unexpected, since these two receptors are expressed in similar cell types and share downstream signaling pathways (Akira, 2003; Iwasaki and Medzhitov, 2004; Uematsu et al., 2005). Multiple mechanisms for downregulating TLR-induced signals have now been described (Liew et al., 2005), and differential effects of these mechanisms on TLR7 or TLR9 could explain the disparate knockout phenotypes. The adaptor protein IL-1 receptor-associated kinase-M (IRAK-M) is induced after prolonged TLR activation and appears to preferentially inhibit TLR9, although its effect on TLR7 has not been determined (Kobayashi et al., 2002). Interferon regulatory factor 4 (IRF-4) has also been identified as an inducible inhibitor of signaling from both TLR7 and TLR9 (Negishi et al., 2005). Induction of these regulatory mechanisms may also allow for crossinhibition between TLR7 and TLR9 when both receptors are ligated in the same cell (Sato et al., 2002). Additionally, a direct role for TLR9 in immune regulation was recently suggested by the observation that stimulation of human pDC with CpG ODN promotes regulatory T cell development (Moseman et al., 2004).

The autoantibodies induced by TLR7 and TLR9 may also contribute to the phenotypes of their respective genetic knockouts. In this scenario, Abs to RNA-containing autoantigens act as potent mediators of inflammation, independent of DNA Abs (Alba et al., 2003; Savarese et al., 2006). Decreased Sm-RNP Abs in *Tlr7*$^{-/y}$ mice and the appearance of additional RNA complex Abs in *Tlr9*$^{-/-}$ mice could then account for ameliorated or exacerbated disease, respectively. Indeed, we found that several parameters of disease severity and immune activation were correlated with Sm-RNP Abs in *Tlr9*$^{-/-}$ mice. In addition, the presence of DNA or chromatin Abs may serve a regulatory function in SLE by promoting clearance of cell debris and thereby reducing the availability of endogenous inflammatory mediators. Impairment in the phagocytosis of apoptotic and necrotic cells is known to induce autoimmune disease (Cohen et al., 2002), and the protective effects of IgM DNA Abs have been clearly demonstrated in autoimmune NZB/W mice (Werwitzke et al., 2005).

The importance of IFN-I in autoimmune disease has been extensively documented (Baechler et al., 2003; Bennett et al., 2003; Santiago-Raber et al., 2003; Theofilopoulos et al., 2005). The ability of autoantibodies to induce IFN-I may also contribute to divergent phenotypic effects in *Tlr7*$^{-/y}$ and *Tlr9*$^{-/-}$ mice. Although autoantibodies to both DNA- and RNA-containing Ags can induce IFN-I production by pDCs, RNA Abs may be more potent in this regard (Barrat et al., 2005; Lovgren et al., 2004). Moreover, Abs to RNA-containing Ags have been correlated with increased IFN-I production and exacerbated disease in human SLE (Kirou et al., 2005). Enhanced IFN-I production stimulated by RNA-containing immune complexes thus provides a potential

mechanistic link between the autoantibody profile, pDC activation, and clinical disease phenotypes observed in *Tlr7*$^{-/y}$ and *Tlr9*$^{-/-}$ mice. Finally, the ability of IFN-I to induce expression of TLR7 in B cells may allow for a positive feedback cycle that is not present for TLR9 (Bekeredjian-Ding et al., 2005).

Our findings support the notion of two critical and potentially pathologic functions of TLRs in SLE. First, activation of the innate immune system by TLRs directs the autoantibody response to the characteristic lupus autoantigens; without appropriate TLR signaling, these Abs are not generated. Second, TLRs can have a dramatic impact on disease pathogenesis, either by promoting disease in the case of TLR7 or by regulating disease in the case of TLR9. These results suggest that inhibition of TLR-signaling pathways may have therapeutic benefit in autoimmune disease, but highlight the need for specific targeting of such therapy because of the surprisingly divergent effects of different TLRs.

Experimental Procedures

Generation of Autoimmune TLR-Deficient Mice

Tlr9$^{+/-}$ hybrid mice (Christensen et al., 2005) were backcrossed eight generations to Fas-deficient, lupus-prone MRL/Mp$^{lpr/lpr}$ mice (Jackson Laboratory), at which point more than 99.8% of the genome was statistically derived from the MRL/Mp strain, and expression of the MHC$^{k/k}$ and Ig$^{a/a}$ haplotypes derived from MRL/Mp was verified by FACS analysis of peripheral blood cells. These *Tlr9*$^{+/-}$ mice were then intercrossed to produce 26 *Tlr9*$^{+/+}$ and 21 *Tlr9*$^{-/-}$ littermates, which were analyzed between 13 and 14 weeks of age. For mortality assessment, additional *Tlr9*$^{+/-}$ mice were intercrossed to produce a second cohort of *Tlr9*$^{+/+}$ (n = 61, with 32 deaths during the observation period), *Tlr9*$^{+/-}$ (n = 29, with 16 deaths), and *Tlr9*$^{-/-}$ (n = 44, with 35 deaths) mice, which were observed without intervention until the time of death. Severely moribund animals were sacrificed for humane concerns and were included as deaths in the analysis.

Hybrid *Tlr7*$^{+/-}$ females were generated by an initial cross of TLR7-deficient mice (mixed C57BL/6 and 129Sv genetic background) with male MRL/Mp$^{lpr/lpr}$ mice. These hybrids were then backcrossed three to six generations to male MRL/Mp$^{lpr/lpr}$ mice, selecting only those mice that were homozygous for the Fas$^{lpr/lpr}$ mutation as well as the MHC$^{k/k}$ and Ig$^{a/a}$ haplotypes. After the third backcross generation, 20 *Tlr7*$^{+/y}$ and 20 *Tlr7*$^{-/y}$ male littermates were analyzed at 16 weeks of age. For analysis of the autoantibody repertoire, serum was taken from an additional 16 *Tlr7*$^{+/y}$ and 15 *Tlr7*$^{-/y}$ littermates of backcross generation 6 at 16 weeks of age. Importantly, no locus from an undefined genetic background could be homozygous in these mice, because every locus harbored at least one copy of the MRL/Mp allele derived from the male parent. All animal work was approved by the Yale Institutional Animal Care and Use Committee.

Determination of Autoantibody Profiles

ANA and *Crithidia luciliae* immunofluorescence were performed as previously described (Christensen et al., 2005), with serum at 1:200 dilution for ANA and 1:50 dilution for *C. luciliae*, and scored by an observer blinded to the genotype of the mice. ANA images were captured at 400× magnification with a constant 2.5 s exposure time. For anti-nucleosome and anti-dsDNA ELISAs, polystyrene plates were coated with either bovine histones (Sigma) or poly-L-lysine (Sigma), respectively. Plates were then incubated with phenol-extracted and S1 nuclease-treated dsDNA from calf thymus (Sigma). After blocking with 1% BSA in PBS, serial dilutions of serum from 1:200 to 1:5400 were added. Specific Abs were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotech), and absorbance at 405/630nm was compared with either PL2-3 nucleosome (Losman et al., 1992) or PL9-6 dsDNA (Losman et al., 1993) monoclonal Abs to quantitate. Anti-Sm ELISA was performed with serial dilutions of serum from 1:200 to 1:5400 as previously described (Christensen et al., 2005). Anti-Sm-RNP ELISA was performed similarly, but plates were coated with Sm/RNP complex Ag (Immunovision).

Analysis of Dendritic Cells and Global Immune Activation

Plasmacytoid and myeloid DCs were identified with anti-CD11c (HL3, BD Biosciences) and anti-mpDCA-1 (Miltenyi Biotec). Anti-MHC class II (M5/114.152, Biolegend), anti-CD80 (16-10A1, BD Biosciences), and anti-CD86 (GL1, BD Biosciences) were used to assess DC activation. Serum IFN-α ELISA was performed as previously described (Christensen et al., 2005), with serum added at 1:5 dilution. Limit of detection was 0.1 ng/ml.

Spleen and lymph node cells were isolated, and T and B cell subsets were enumerated and activation markers determined as previously described (Christensen et al., 2005). CD69 Ab (H1.2F3, BD Biosciences) was used as an additional marker for B cell activation. Levels of serum IgG isotypes were determined by Beadlyte Mouse Immunoglobulin Isotyping Kit (Upstate) according to the manufacturer's instructions. Serum was tested at 1:250,000 or 1:200,000 dilution for TLR9 and TLR7 samples, respectively.

Determination of Clinical Disease

For skin disease, mice were scored for the extent of typical lesions on the dorsum of the neck and back. Macroscopic surface area was scored from 0 to 4 for affected area up to 4 cm^2, with up to 1 additional point for the presence of ear dermatitis. For kidney disease, formalin-fixed and paraffin-embedded sections, stained with either H&E or PAS, were scored for interstitial and glomerular disease by two independent observers who were blinded to the genotypes of the mice.

Supplemental Data

Two Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/3/417/DC1/.

Acknowledgments

These studies were supported by NIH grants P01-AI36529 and P01-AR050256. S.R.C. was supported by NIH medical scientist training grant GM07205. We thank Yale Animal Resources Center for their outstanding animal husbandry and Ann Marshak-Rothstein for stimulating discussions. We thank Dr. Shizuo Akira for generously providing the TLR9-deficient mice.

Received: June 21, 2006
Revised: July 6, 2006
Accepted: July 27, 2006
Published online: September 14, 2006

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