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Control of TLR7 expression is essential to restrict autoimmunity and dendritic cell expansion

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Summary

Nucleic acid-binding innate immune receptors such as TLR7 and TLR9 have been implicated in the development of some autoimmune pathologies. The Y chromosome-linked genomic modifier *Yaa*, which correlates with a duplication of *TLR7* and 16 other genes, exacerbates lupus-like syndromes in several mouse strains. Here we demonstrate that duplication of the *TLR7* gene is the sole requirement for this accelerated autoimmunity, as reduction of TLR7 gene dosage abolishes the Yaa phenotype. Further, we describe new transgenic lines that overexpress TLR7 alone, and find that at levels beyond a 2-fold increase, spontaneous autoimmunity develops. While a modest increase in *TLR7* gene dosage promotes autoreactive lymphocytes with RNA specificities and myeloid cell expansion, a substantial increase in TLR7 expression causes fatal acute inflammatory pathology and profound dendritic cell dysregulation. These results underscore the importance of tightly regulating expression of TLR7 to prevent spontaneous triggering of harmful autoreactive and inflammatory responses.

Introduction

Innate immune responses initiated by Toll-like receptors (TLRs) are a critical first line of defense against pathogens and also serve to enhance adaptive responses (Kawai and Akira, 2007). In pathological conditions, however, innate responses can exacerbate autoimmune conditions and trigger harmful inflammatory pathologies. Engagement of TLRs in dendritic cells induces the production of inflammatory cytokines such as IL-6, TNF-α and type I interferons (Bekeredjian-Ding et al., 2005; Honda et al., 2005; Hornung et al., 2005; Lund et al., 2004; Pascual et al., 2006; Savarese et al., 2006; Vollmer et al., 2005). TLRs in B cells act in synergy with the antigen receptor to induce proliferation, isotype switching and plasma cell differentiation (Berland et al., 2006; Lartigue et al., 2006; Lau et al., 2005; Leadbetter et al., 2002; Pasare and Medzhitov, 2005; Savarese et al., 2006; Viglianti et al., 2003). Thus, tight regulation of TLR-induced responses is necessary to maintain a healthy and tolerant immune environment.

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The role of TLRs in the onset of autoimmune pathologies has been effectively addressed in the context of murine models of systemic lupus erythematosus (SLE). In a number of these models of lupus it has been shown that inhibitory and pro-apoptotic receptors such as FcγRIIB, Fas and SLAM family members regulate lymphocyte activation and prevent the expansion of self reactive B cells (Bolland and Ravetch, 2000; Wandstrat et al., 2004; Watanabe-Fukunaga et al., 1992). Both in lupus patients and in mouse models of lupus, systemic autoimmunity is directed against multiple self-antigens that commonly include DNA, histones, RNA and ribonucleoproteins (D'Cruz, 2006). A synergistic effect between nucleic acid-binding TLRs and the antigen receptor of self-reactive B cells has been proposed to explain the preference for nuclear specificities in autoantibodies (Christensen and Shlomchik, 2007; Deane and Bolland, 2006; Marshak-Rothstein and Rifkin, 2007). With regard to RNA-related specificities, B cell receptor transgenic models have been used to show that TLR7, which recognizes ssRNA of viral origin, mediates activation of RNA-specific B cells (Barrat et al., 2005; Berland et al., 2006; Savarese et al., 2006). Deletion of the *TLR7* gene was shown to be protective in the MRL/lpr lupus mouse model and to reduce the amount of antibodies against RNA-related antigens (Christensen et al., 2006).

Mice bearing the *Yaa* locus provide a prime example of how important it is to control the expression of innate receptors. In *Yaa* mice, a hyperactive phenotype in B and dendritic cells correlates with a genomic translocation that results in duplication of at least 17 genes, one of which being *TLR7* (Pisitkun et al., 2006; Subramanian et al., 2006). The *Yaa* locus, which stands for Y chromosome-linked autoimmune accelerator, produces a striking acceleration of autoimmunity when bred to other models of lupus such as the FcγRIIB-deficient mouse (Amano et al., 2003; Bolland et al., 2002). In a healthy genetic background, such as the C57BL/6 strain, addition of the *Yaa* locus diminishes the marginal zone B cell population in the spleen and induces a moderate myeloid cell expansion. While initial reports about this genomic duplication focused on hyperresponsiveness to TLR7 ligands in this strain, the nature of how this allele accelerates systemic autoimmunity remains unclear because of the fact that multiple genes are duplicated. For example, *TLR7* could function with one or more of the other duplicated genes to potentiate the acceleration in pathology, or the increase in TLR7 expression may be merely correlative with the development of autoimmunity. Thus, the precise importance of *TLR7* gene duplication in the *Yaa* mouse remains undetermined (Marshak-Rothstein and Rifkin, 2007). In this study, we used two approaches to answer this question, by either decreasing or increasing *TLR7* gene dosage. When we lowered *TLR7* gene dosage we were able to ablate the hyperresponsiveness caused by the *Yaa* allele, and when bred to FcγRIIB-deficient mice we were able to eliminate the nucleolar antibody response, enhancement in pathology and mortality that is seen in the *Yaa* mouse. On the other hand, increasing *TLR7* gene dosage via the generation of TLR7 transgenic mice resulted in the development of acute systemic autoimmune disease characterized by anti-RNA autoantibody production, glomerulonephritis, dendritic cell expansion, anemia, and a pro-inflammatory interferon signature in the dendritic cells. The combination of these two techniques allows us to conclude that *TLR7* gene dosage is both necessary and sufficient to promote autoantibody production and pathological disease associated with lupus.

RESULTS

Increased *TLR7* gene dosage is necessary for the Yaa phenotype

To determine if TLR7 is the critical gene for the Yaa phenotype, we bred B6.*Yaa* males to *TLR7*-ko female mice so that the two copies of the *TLR7* gene were reduced to one copy as in wild type male mice. Because of its location on the X-chromosome, the *TLR7*-ko allele eliminates X-chromosomal expression of TLR7 while maintaining TLR7 expression on the *Yaa* Y-chromosome. Thus, we will refer to wild type male mice as $TLR7^{+/Y}$, TLR7-deficient

males as *TLR7*$^{-/Y}$, while the combination of *Yaa* and the *TLR7*-ko on the X chromosome produces *TLR7*$^{-/Yaa}$ mice. While the marginal zone (MZ) B cell compartment was lacking in the spleen of *TLR7*$^{+/Yaa}$ mice as previously reported (Amano et al., 2003), we observed wild type levels of MZ B cells in *TLR7*$^{-/Yaa}$ mice, and even slightly increased levels in *TLR7*$^{-/Y}$ (*TLR7*-null) mice (Fig. 1A, B). The hyperresponsiveness to the TLR7 ligand imiquimod seen in the *Yaa* strain was also reduced to wild-type levels in *TLR7*$^{-/Yaa}$ splenocytes; no proliferation was observed in *TLR7*-null mice in these conditions (Fig. 1C). To examine the consequences of lowered *TLR7* gene dosage on the development of a lupus-like syndrome, we bred the *FcγRIIB*$^{-/-}$*Yaa* males (hereafter referred to as *R2 TLR7*$^{+/Yaa}$) to female mice bearing the *TLR7*-ko allele. Previous studies showed that addition of the *Yaa* allele to *FcγRIIB*$^{-/-}$ mice shifts their autoantibody pattern from a homogenous nuclear to a nucleolar pattern, and that mortality is significantly accelerated(Bolland et al., 2002). We observed that reduction of *TLR7* gene-copy number resulted in increased survival of these mice. As *R2 TLR7*$^{+/Yaa}$ mice became moribund at 5 months of age, kidney disease and splenomegaly were significantly decreased in the *R2 TLR7*$^{-/Yaa}$ littermates of the same age (Fig. 2A, B, C). In contrast to the nucleolar pattern seen in the antibodies from *R2 TLR7*$^{+/Yaa}$ sera, the production of autoantibodies in the *R2 TLR7*$^{-/Yaa}$ mice appeared of the homogenous nuclear type (Fig. 2C). This change in autoantibody pattern correlated with a reduction of anti-RNA specificities in the serum. Anti-RNA antibodies detected by ELISA were reduced in *R2 TLR7*$^{-/Yaa}$ compared to those found in *R2 TLR*$^{+/Yaa}$ sera. This reduction resulted in levels similar to those found in *R2*$^{-/-}$ mice but slightly above the levels of wild type mice (Fig. 2D). Overall, R2 TLR7$^{-/Yaa}$ mice died from a lupus-like syndrome at a later age and in lower numbers than *R2 TLR7*$^{+/Yaa}$ mice (Fig. 2D). Cellular analysis comparing these mice showed reductions in activation in *R2 TLR7*$^{-/Yaa}$ splenocytes and reduced number of antibody producing cells, as compared to *R2 TLR7*$^{+/Yaa}$ splenocytes (Supplementary Fig. 1). Thus, increases in TLR7 expression levels are necessary for the accelerated development of systemic autoimmunity seen in the *Yaa* mouse.

Increased *TLR7* dosage is sufficient to induce systemic autoimmunity

To examine whether TLR7 was sufficient to produce the Yaa phenotype, we generated transgenic mice that overexpressed TLR7 alone. Starting with the RP23-139P21 BAC construct that contains the *TLR7*, *TLR8* and *Tmsb4x* genes, we replaced the first 83 kb with a neomycin cassette so that *TLR7* would be the only gene expressed from this clone. The final construct contained approximately 40kb both upstream and downstream of *TLR7*, as it was designed to maintain tissue specific expression comparable to that of the endogenous gene (Fig. 3A). Microinjection of C57BL/6 zygotes with this DNA produced 6 founder lines that had varying copy number of TLR7 at the genomic level, ranging from 4 to 32 copies of the gene. Expression at the mRNA level ranged between 4 and 16 fold higher than the wild type level, as judged by quantitative PCR on follicular B cell cDNA (Fig. 3B). Increased expression of TLR7 was also detected to an even greater extent in purified dendritic cell mRNA, while CD4 T cells did not show similar patterns of overexpression (data not shown). While our initial goal was to cross these animals with *FcγRIIB*$^{-/-}$ animals, we observed lethality to varying degrees in the various TLR7 transgenic (hereafter TLR7.Tg) founder lines, which correlated with the extent of TLR7 overexpression at the mRNA level (Fig. 3C). Additionally, in the five studied strains autoantibody production and glomerulonephritis could be detected as the mice aged (Fig. 3D, E), indicating that TLR7 overexpression results in an autoimmune syndrome. Further analysis of autoantibody production in these mice showed that while ANA patterns varied somewhat, where most sera appeared either mixed cytoplasmic or nucleolar, an ELISA showed that all strains analyzed were positive for anti-RNA IgG production (Fig. 3D). The penetrance and nature of the autoimmune pathology noted in the examined founder lines is summarized in Table I. Overall, the creation of mice overexpressing TLR7 alone was able to produce an autoimmune syndrome on its own.

Moderately increased TLR7 gene dosage recapitulates the Yaa phenotype and promotes activated lymphocytes

We first tested the extent of TLR7-mediated activation in splenocytes from TLR7.Tg mice with medium levels of overexpression (i.e., 4–8 fold overexpression) and compared them to B6 littermates. FACS analysis of various splenic cell compartments in TLR7.Tg.6 mice showed that young (2–3 month old) transgenic animals had a marginal zone defect, similar to what was observed in the *Yaa* mice (Fig. 4A). Both B and T lymphocytes from TLR7.Tg.6 mice showed spontaneous activation, as CD69 expression was found more frequently in TLR7.Tg.6 splenocytes. Furthermore, the $CD11c^+$ dendritic cell population was expanded significantly in young TLR7.Tg6 mice, similar to what was found in previous studies on aging *Yaa* mice (Amano et al., 2005). These data on cellular subsets in TLR7.Tg.6 mice are quantitated in Supplementary Table I. Using splenocytes from the TLR7.Tg.6 line, we found increased responsiveness to the TLR7 ligand imiquimod but similar stimulation using CpG when compared to wild type splenocytes in the same conditions (Fig. 4C). Because we had detected autoantibody production in serum from all the TLR7.Tg.6 mice, we analyzed the B cells in these mice and noted that the $B220^{low}CD138^+$ population, most likely representing antibody producing cells, was expanded (Fig. 4D). A large number of immunoglobulin-loaded cells was detected by immunohistochemistry of splenic sections using anti-kappa chain antibody staining (Fig. 4E). Thus, a 4–8 fold increase in expression of TLR7 produces a phenotype that resembles the Yaa phenotype but is more severe, in that in TLR7.Tg.6 mice dendritic cells expand even at a young age, both B and T cells are spontaneously activated, and antibody producing B cells are substantially increased.

High level of TLR7 expression produces a massive expansion of inflammatory dendritic cells and anemia

We next characterized immune development in the two TLR7.Tg strains that overexpressed TLR7 at levels between 8 and 16 fold over wild-type littermates. In these studies, we focused on the TLR7.Tg.1 line, though similar results were found with TLR7.Tg.4 mice (data not shown). At a young age ($n<8$ weeks), the phenotype of TLR7.Tg.1 mice was quite similar to those in TLR7.Tg strains that expressed lower levels of TLR7, with increases in the degree of the severity of the phenotype but general similarities between all strains of mice (data not shown). However, as we allowed the strains to age beyond two months, we noted high incidences of early lethality with high levels of TLR7 overexpression (Fig. 3C). Analysis of morbid animals showed severe splenomegaly (Fig. 5A, Supplementary Table II) that could be identified as early as 2 months of age. Cellular and histological analysis of blood and spleen showed a severe expansion of $CD11c^+$ cells (Fig. 5B). Phenotypically, these $CD11c^+$ cells comparably separated in terms of expression of other molecules such as CD11b, CD8, and B220. TLR7.Tg.1 dendritic cells had increased expression of molecules that are indicative of enhanced activation, such as class II MHC and CD80 (Fig. 5B, Supplementary Table II). Highly elevated levels of the inflammatory cytokines TNF-α, IL-6, and MCP-1 were detected in sera from TLR7.Tg.1 mice (Fig. 5C). Expression analysis of dendritic cell cDNA from TLR7.Tg. 1 mice also showed a significant increased in the expression of IRF5 and SOCS-1, which are two signaling molecules whose upregulation is associated with inflammation mediated through type I interferons (Fig. 5D). A severe anemia also accompanied these traits that included marked reductions in RBCs and severe deficiencies in platelets (Fig. 6A, B). While sera from these mice were negative for anti-RBC antibodies as assessed by direct and indirect Coombs' assays (data not shown), some sections did show what appeared to be erythrophagocytosis (Supplementary Fig. 2). On the other hand, there were signs of compensatory erythropoiesis, as $Ter\text{-}119^+$ erythroblasts were also expanded in spleens of anemic TLR7.Tg.1 mice (Fig. 6D). This erythropoiesis was found to be restricted to the periphery, as bone marrow from TLR7.Tg. 1 mice did not show similar enhancements in erythroblasts (data not shown). Additionally, megakaryocyte development was not abnormal in either the spleen or the bone marrow,

suggesting that the decreases in RBCs and platelets were not due to a decreased production of these cells (data not shown). Histological analysis showed that as myeloid cells expanded there was a concomitant loss of lymphocytes, as white pulp regions were severely atrophied in spleens of dying TLR7.Tg.1 mice (Fig. 6C). Thus, while increased TLR7 gene dosage produced an environment that stimulated autoantibody-secreting B cells, higher levels of gene dosage result in a shift to a highly inflammatory and lymphopenic environment as dendritic cells expressing TLR7 expand in an uncontrolled fashion and promote anemia.

DISCUSSION

A synergistic effect between antigen receptors and TLRs has been proposed to drive autoimmune responses by activating self-reactive lymphocytes in the presence of endogenous nucleic acids (Deane and Bolland, 2006; Marshak-Rothstein and Rifkin, 2007). This theory stems from the observation that B cells with DNA or RNA specificities are activated by nucleic acids *in vitro* (Fields et al., 2006; Krieg et al., 1995; Lau et al., 2005; Leadbetter et al., 2002; Viglianti et al., 2003). Genetically, it has been shown that deletion of TLR9 reduces the amount of DNA-related antibodies in serum of lupus-prone mice but how this correlates with the inflammatory pathology is still unclear (Christensen et al., 2005; Christensen et al., 2006; Ehlers et al., 2006; Wu and Peng, 2006). Knockout mouse studies have also shown that deletion of TLR7 is protective from anti-RNA B cell responses (Berland et al., 2006; Christensen et al., 2006). While these studies reveal that TLR function is necessary to develop certain B cell autoreactivities, they do not address the precise role of TLR dysfunction in the spontaneous onset of disease or in the inflammatory pathology associated with autoimmune disorders. The *Yaa* modifier, suggested to be caused by a duplication of the *TLR7* gene (Pisitkun et al., 2006), provides the best example of TLR dysregulation leading to autoreactivity and inflammatory pathology. Data presented in this manuscript unmistakably show that an increase in *TLR7* gene dosage can potentiate systemic autoimmunity, as the phenotype of the *Yaa* mouse was completely restored through lowering *TLR7* gene dosage. The 16 other duplicated genes were thus not required to produce the accelerated autoimmunity. Further, it was even more intriguing to find that, at levels above a 2-fold overexpression, production of anti-RNA autoantibodies and inflammatory cytokines could be observed in a mouse that lacked any genetic lesions such as a null allele of *FcγRIIB*. Thus, while TLR7 has been described as a modifier of systemic autoimmunity, the present work underscores the fact that dysregulation of TLR7 on its own can break tolerance. Whether this will be true of other genetic backgrounds such as BALB/c, which has shown resistance to developing autoimmunity in other instances, or even in human subjects, remains to be seen.

Future studies exploring cellular interactions in TLR7.Tg mice may clarify the way that increased *TLR7* gene dosage serves to intensify the severity of autoimmunity on a cellular level. We observe that multiple insertions of the *TLR7*-containing BAC clone results in augmented expression of TLR7 in the same cell types (B cells and DCs) as what is detected for the endogenous gene (Bekeredjian-Ding et al., 2005; Lund et al., 2004; Pisitkun et al., 2006). The preference for anti-RNA specificities and the spontaneous activation of lymphocytes observed in these transgenic mice is most likely due to increased expression of TLR7 in B cells, since this phenotype was shown to be B cell intrinsic in the Yaa mouse (Pisitkun et al., 2006). Overexpression of TLR7 also leads to severe expansion of the $CD11c^+$ population, a cell type that is very restricted in numbers when it develops in healthy conditions (Liu et al., 2007; Steinman et al., 2005). Whether this dysregulated expansion of DCs occurs as a result of increased expression of TLR7 in DCs with consequent hyperactivity of these cells, or whether it happens in response to a highly inflammatory environment is still undetermined.

Understanding what endogenous ligands serve to activate these receptors and promote autoimmunity will clarify how altered gene dosage has such a strong effect on the maintenance

of tolerance. Recent studies on TLR9 indicate that binding a specific stimulatory ligand is essential for a conformational change in the TLR, which leads to activation of the NF-κB signalosome (Latz et al., 2007). If this is true of TLR7, we can rule out that overexpression leads to constitutive activation in the absence of ligand. Is affinity for host RNA low in a tolerant host with normal levels of TLR7 such that increased expression crosses a threshold of sensitivity where autoimmunity results? The production of better reagents will allow these sorts of questions to be addressed in the future.

Studies shown here on TLR7.Tg mice with the highest levels of expression revealed the importance of restricting TLR activity to avoid the development of an extreme inflammatory condition where dendritic cell expansion dominates resulting in lymphoid atrophy, anemia and other pathologies associated with autoimmunity. Finding elevated levels of IRF5 and SOCS1 was particularly notable, as the interferon signature has been argued to be an important indicator of the severity of lupus, particularly in the case of IRF5 (Graham et al., 2006; Pascual et al., 2006). Because interferon alpha itself serves to upregulate TLR7 levels, our model mirrors what may happen at the RNA level when an interferon signature is developed (Bekeredjian-Ding et al., 2005; Pascual et al., 2006). Thus, TLR7 overexpressing transgenic mice provide not only evidence for a role of TLRs in the onset of autoimmunity, but also a useful and highly simplified genetic system to study the development of severe autoimmune disease with concomitant production of RNA autoantibodies and myeloid dysregulation.

EXPERIMENTAL PROCEDURES

Mice

Mice lacking TLR7 and FcγRIIB, and the Yaa strain of mice have already been described (Lund et al., 2004; Murphy and Roths, 1979; Takai et al., 1996). They were maintained on a C57BL/6 background for at least 10 generations. Transgenic mice overexpressing TLR7 (TLR7.Tg) mice were generating by recombineering BAC clone RP23-139P21 using the Quick and Easy BAC modification kit (Open Biosystems). DNA was injected into C57BL/6-derived zygotes and positive founders were identified using Southern Blots for a neomycin resistance cassette.

Cellular Analysis

Collagenase D treated splenocyte suspensions were stained with antibodies to the following molecules: B220, CD4, CD8a, CD11c, CD11b, CD21, CD23, CD40, CD69, CD138, IA-b, Ter-119 (BD Biosciences). These cells were analyzed on a FACS Calibur flow cytometry machine, and were later analyzed further using FlowJo (Tree Star, Ashland, OR). Follicular B cells were purified using an AutoMACS machine (Miltenyi) by negative selection, using biotinylated antibodies to CD11c, CD43 and CD9 (BD Biosciences) and anti-Biotin magnetic beads (Miltenyi). Imiquimod, CpG ODN1826, and control ODN186 (Invivogen) were added to evaluate proliferation in splenocytes in 3H-Thymidine incorporation assays, which were performed as described(Pisitkun et al., 2006).

Pathology

Necropsies were performed on selected mice. Tissues were fixed neutral buffered formalin, embedded in paraffin and sections stained with hematoxylin and eosin. Frozen or paraffin-embedded sections of spleen, lymph nodes, kidneys and were analyzed using a variety of antibodies, including H&E, anti-MOMA-1 FITC, anti-B220-APC. Kappa Light Chains, Catalog # A0191 from Dako Corporation, Carpinteria Ca, were used on paraffin embedded sections, at a dilution of 1:1000. The Kappa Light Chain stain Utilized Pro-K, catalog # S3020, enzyme digestion from Dako Corporation (Carpinteria, CA). They were then stained with the Anti-Rabbit envision + polymer system from Dako Corporation (Carpinteria, CA). All slides

used the Cardassian DAB chromogen counterstained using the modified Harris hematoxylin from Biocare Medical (Concord, CA).

Serological Analysis

Serum was tested for autoantibody production using a standard autoantibody test using Hep-2 cells fixed on slides (Bion). Sera were diluted 1:200 and after two washes, a goat anti-mouse IgG secondary antibody (Sigma) was used to identify autoreactive sera. Sera were also used for an ELISA to detect anti-RNA autoantibodies using a kit from Immco Diagnostics (Buffalo, NY). Additionally, sera were analyzed for cytokines using the BD CBA Inflammation kit.

Hematological Analysis

Whole blood in EDTA was analyzed using a Cell Dyn 3500 System from Abbott Laboratories (Abbott Park, Illinois).

Real-Time Quantitative PCR

Quantitative PCR on genomic DNA and cDNA were used to measure levels of the following genes: TLR7, and β-Actin, as described previously(Pisitkun et al., 2006). For measuring levels of IRF-4, IRF-5 and SOCS1, a Sybr green based assay was performed, using primers for IRF-4, IRF-5, SOCS1 and β-Actin, as described previously(Honma et al., 2005; Takaoka et al., 2005).

Statistical Analysis

To determine statistical significance, p-values were calculated using a paired Student's t-test. Values less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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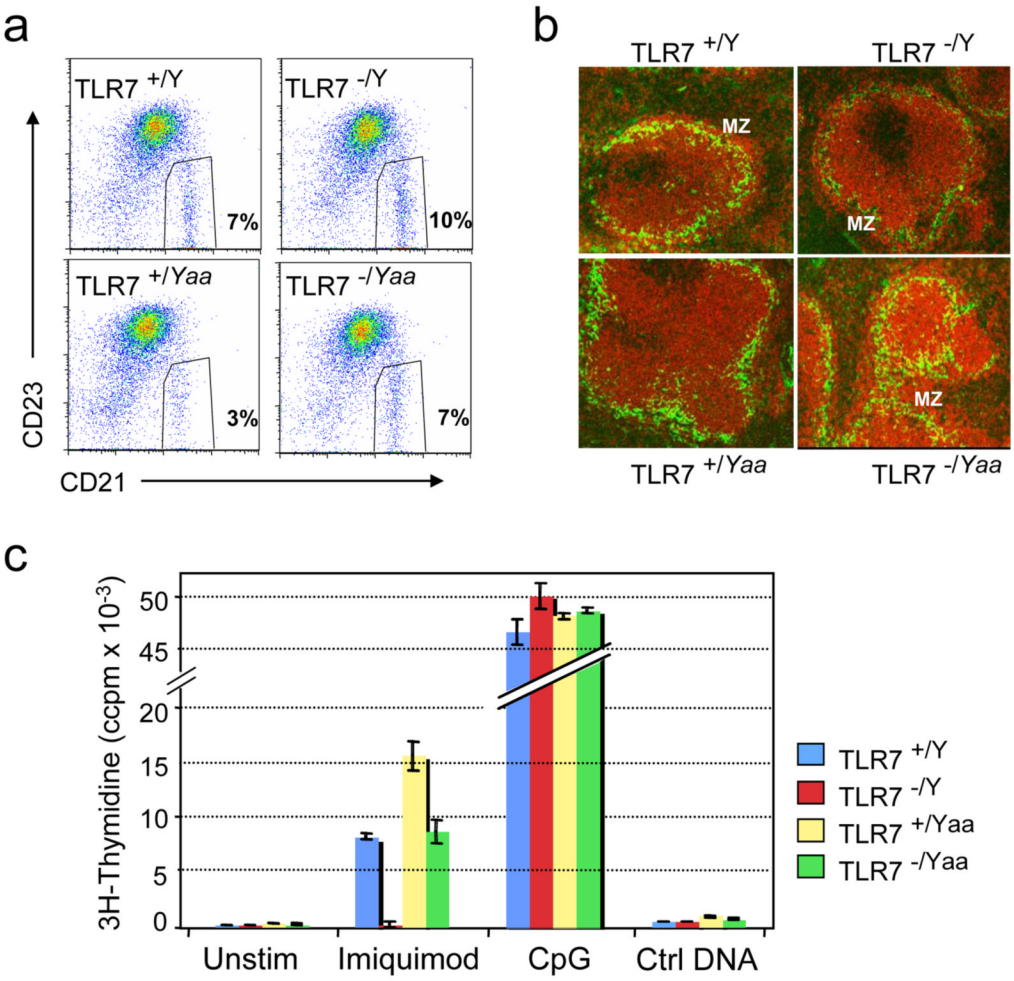

Figure 1.

Increased TLR7 gene dosage is necessary for hyperresponsiveness in the Yaa strain

A) FACS analysis of CD21 and CD23 expression in splenocytes shows marginal zone B cell gates.

B) Histological assessment of the marginal zone B cell population using antibodies to marginal zone macrophages (anti-MOMA1, shown in green) and B cells (anti-B220, shown in red).

C) Proliferation assay of splenocyte responsiveness to Imiquimod and CpG using 3H-Thymidine incorporation. Data are representative of four independent experiments using mice aged 2–3 months.

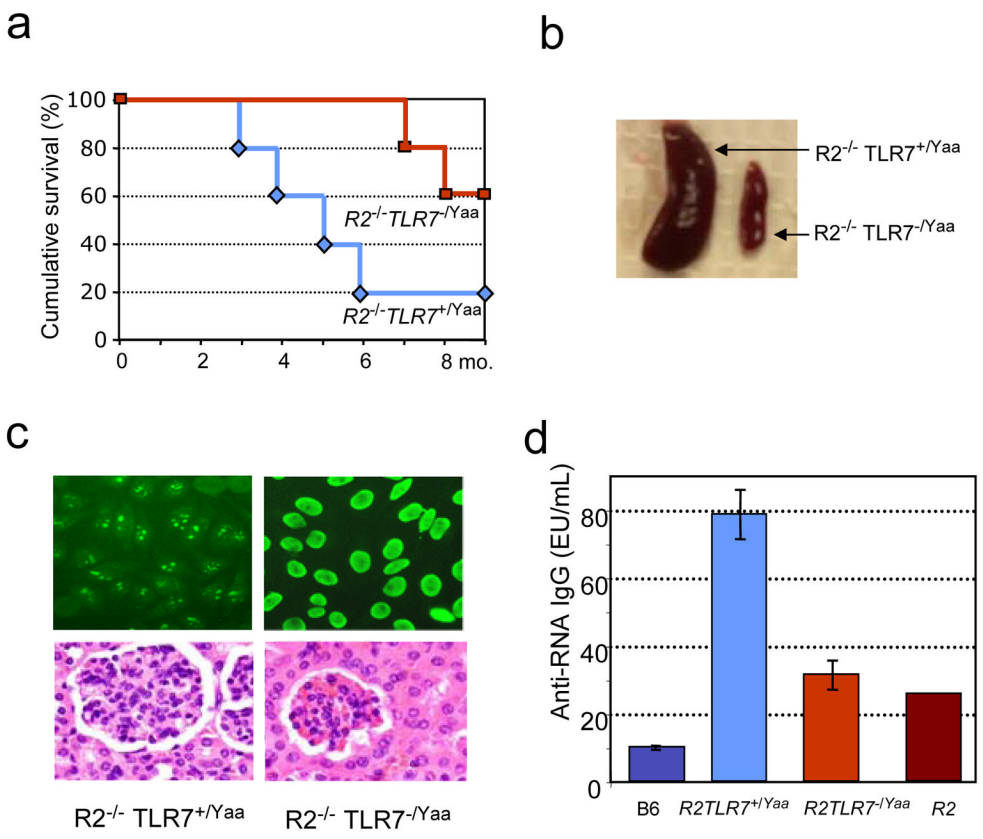

Figure 2.

Increased *TLR7* gene dosage is necessary for the accelerated pathology in the Yaa mouse.

A) Mortality curves of $R2^{-/-}TLR7^{+/Yaa}$ (blue) and $R2^{-/-}TLR7^{-/Yaa}$ (red) mice, where n=5 in each group.

B) Spleens from $R2^{-/-}TLR7^{+/Yaa}$ (left) and $R2^{-/-}TLR7^{-/Yaa}$ mice. Image is representative of 4 independent experiments using mice aged 4–6 months.

C) ANA tests detect nucleolar staining antibodies in serum from 5 month old $R2^{-/-}TLR7^{+/Yaa}$ mice (left) and homogeneous nuclear pattern in serum from $R2^{-/-}TLR7^{-/Yaa}$ mice (right). Histological analysis of glomeruli from 5 month-old $R2^{-/-}TLR7^{+/Yaa}$ (left) and $R2^{-/-}TLR7^{-/Yaa}$ (right) kidneys stained with Hematoxylin and Eosin. Data are representative of at least 4 independent experiments.

D) RNA-specific antibodies were quantitated in sera from B6, $R2^{-/-}TLR7^{+/Yaa}$ and $R2^{-/-}TLR7^{-/Yaa}$, and $R2^{-/-}$ mice aged 4–6 months. Data are shown in terms of units, based on control serum provided by the ELISA manufacturer. N=6, except for the $R2^{-/-}$ sera, which is from one independent experiment.

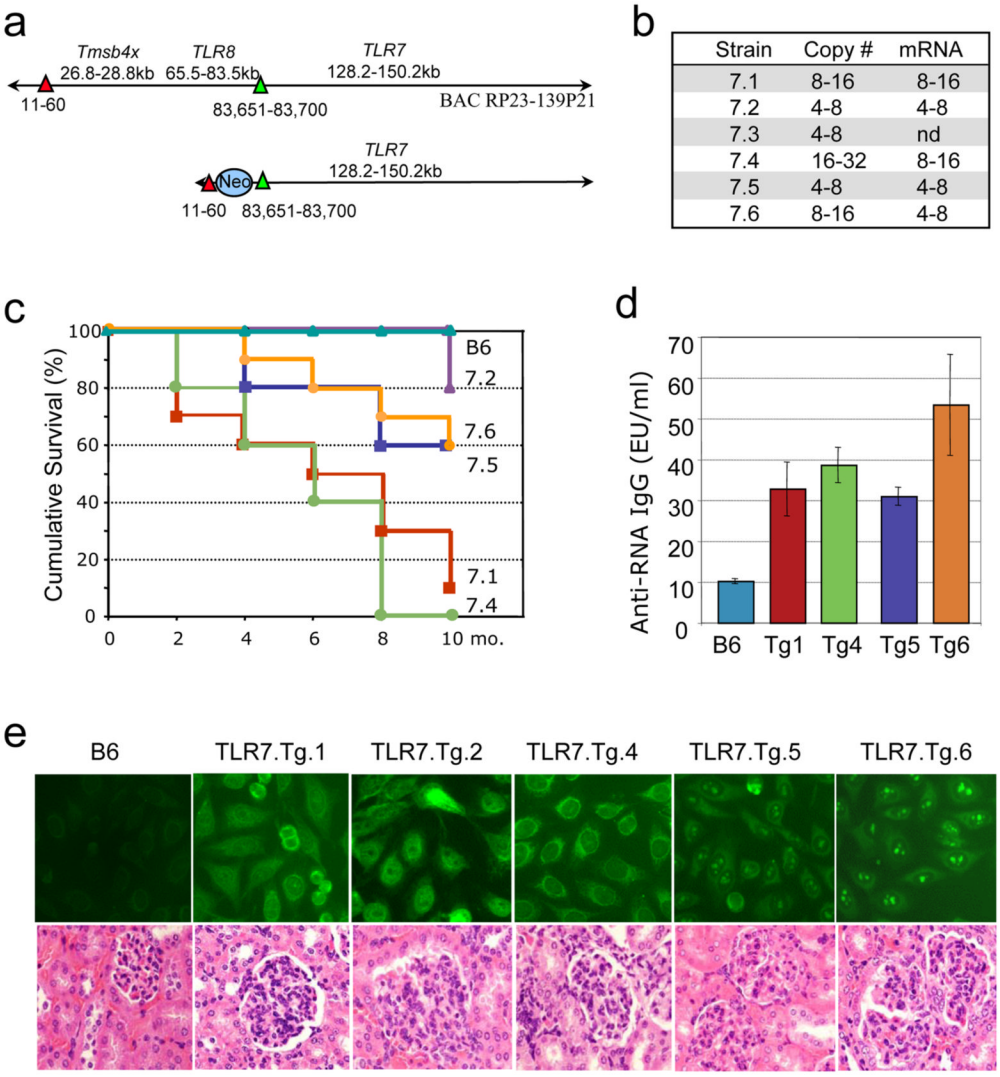

Figure 3.

Construction and characterization of BAC transgenic mice overexpressing TLR7.

A) Layout of BAC clone RP23-139P21 and recombineering strategy for producing TLR7 transgenic lines. Primers used to excise sequences containing *TLR8* and *Tmsb4x* are indicated as triangles.

B) Table of Genomic DNA/mRNA levels in founder strains. Numbers were calculated by comparing TLR7 threshold cycle values between B6 control littermates and Tg mice, using tail DNA or follicular B cell mRNA from young mice (<3 months).

C) Spontaneous mortality in TLR7.Tg lines. Survival curves of B6 or TLR7.Tg strains were measured over the course of 10 months. Data are based on 5 mice for Tg7.1 and Tg7.6 or 10 mice for all other strains.

D) RNA-specific antibody production in TLR7.Tg strains. Quantification of units was performed as in figure 2. Sera from at least 4 mice per strain were used to detect anti-RNA antibodies using an ELISA.

E) Autoantibody production shown by ANA tests and glomerulonephritis shown by hematoxylin and eosin stained kidney sections from TLR7.Tg strains. Data are based on at least 5 mice per transgenic line, and were obtained from mice that were 3–9 months.

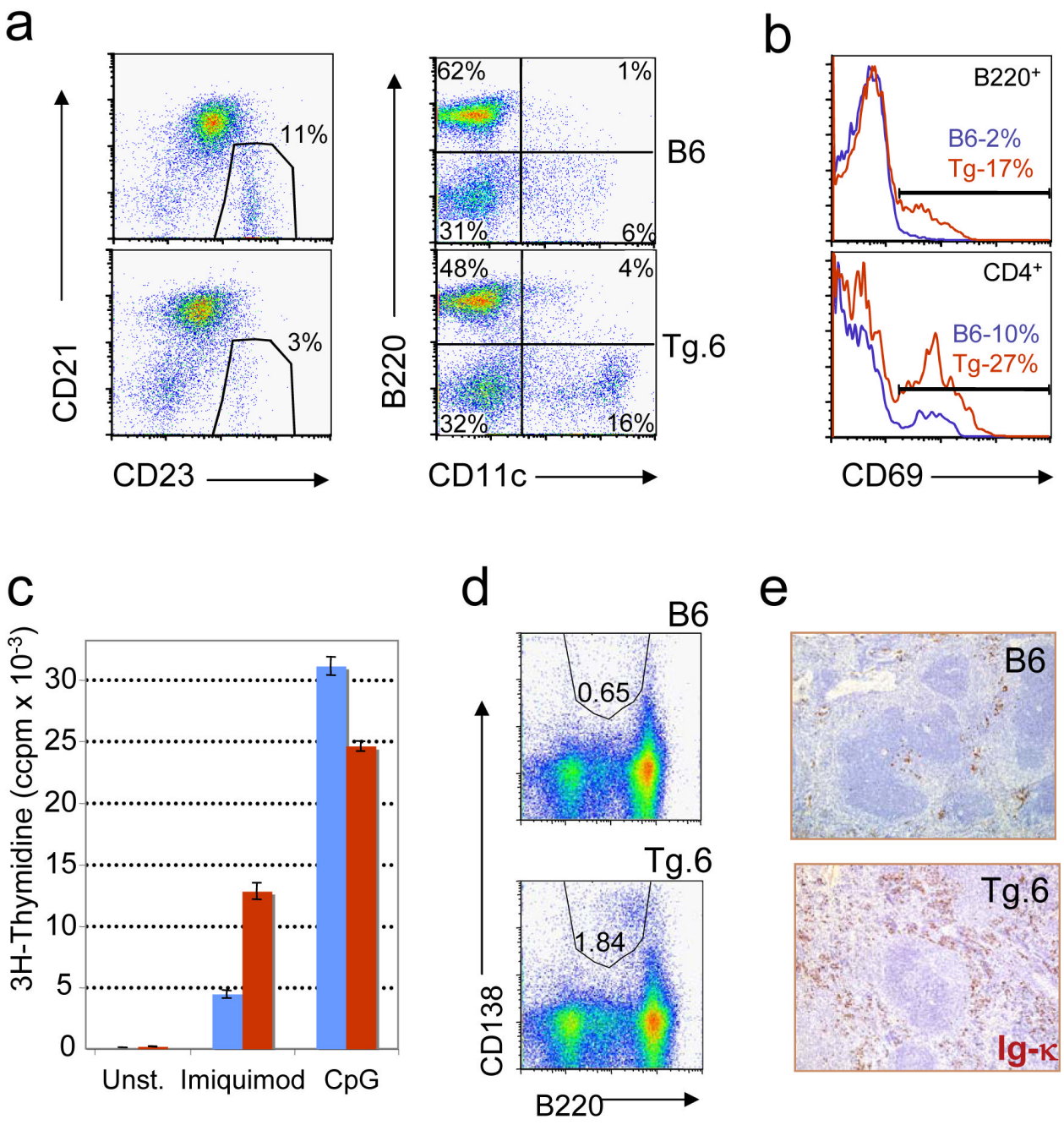

Figure 4.

TLR7 transgenic mice recapitulate the enhanced responses seen in the *Yaa* strain and promote lymphocyte activation.

A) Flow cytometric analysis of CD21 and CD23 expression shows impaired marginal zone B cell development, (left) and dendritic cell ($CD11c^+$) expansion (right) in TLR7.Tg.6 mice.

B) Spontaneous lymphocyte activation in TLR7.Tg.6 mice shown by FACS analysis using antibodies to CD69. $B220^+$ gated cells are overlayed in the top histogram and CD4 gated cells are shown on the bottom.

C) Proliferation assay using 3H-Thymidine incorporation in response to Imiquimod or CpG in both B6 and TLR7.Tg.6 splenocytes shows enhanced sensitivity to TLR7 ligands in TLR7.Tg cells.
D) Flow cytometric analysis of splenocytes from B6 and TLR7.Tg.6 splenocytes shows expanded $B220^{low}CD138^{+}$ population.
E) Histological analysis of spleen sections from B6 (top) and TLR7.Tg.6 (bottom) mice shows increased numbers of antibody-producing lymphocytes, as judged by Ig-Kappa chain stains. Data were obtained from mice that were 2–3 months of age.

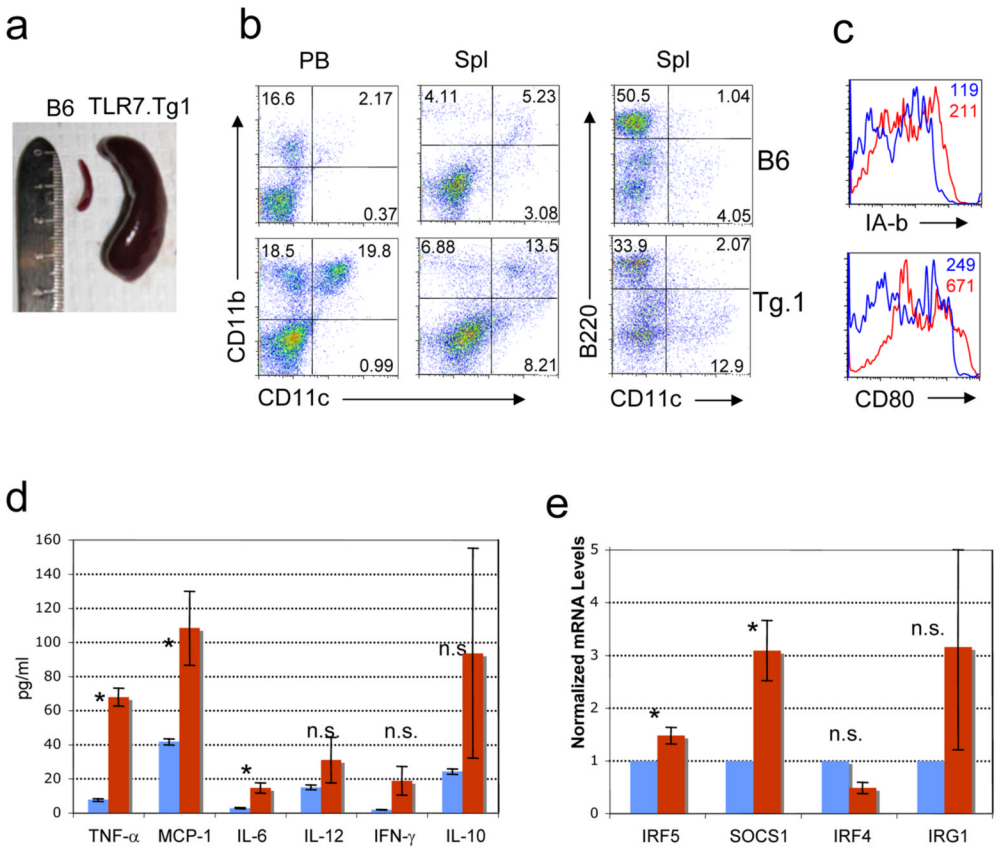

Figure 5.

TLR7 overexpression produces a highly inflammatory environment.

A) Severe splenomegaly in TLR7.Tg.1 mice. Picture is representative of aging (>3 month) old TLR7.Tg.1 mice, where spleen weight is at least 30 times greater than that of a B6 control.

B) Dendritic cell expansion observed in peripheral blood (PB) and spleens (Spl) of TLR7.Tg. 1 mice, as compared to B6 splenocytes by measuring CD11c vs. either CD11b or B220.

C) Enhanced expression of costimulatory molecules in TLR7.Tg.1 dendritic cells. CD11c+ gated cells were stained with antibodies for IA-b (top) or CD80 (bottom). Overlays of TLR7.Tg. 1 (red) and B6 (blue) littermates are shown, with numbers indicating MFI values.

D) Elevated inflammatory cytokines in serum from TLR7.Tg.1 mice. Sera from B6 (blue) or TLR.7.Tg.1 (red) mice shows statistically significant increases in TNF-a, MCP-1, and IL-6 in TLR7.Tg.1 sera, whereas other cytokines are not significantly different (n.s.).

E) Elevated levels of interferon-signature genes in TLR7.Tg.1 dendritic cells. Quantitative-PCR analysis of molecules related to interferon signaling shows elevated levels of IRF5 and SOCS1, while IRF4 and IRG1 were not statistically different. Asterisk represents $p<0.05$. Data were obtained from mice that were 3–5 months of age.

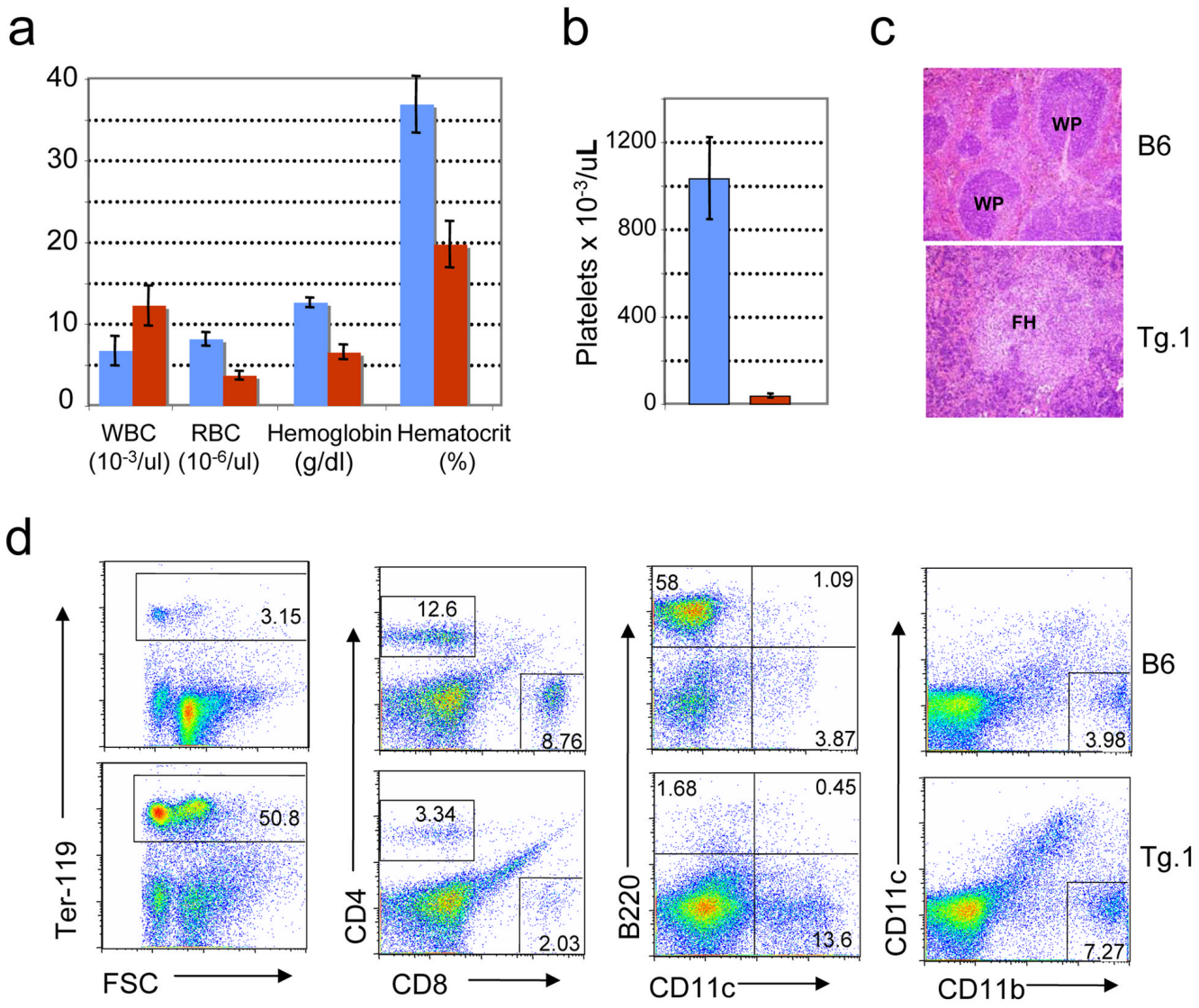

Figure 6.

Highest levels of TLR7 overexpression result in anemic disease.

A) Measurement of RBCs, hemoglobin and hematocrit in moribund (>3 month-old) TLR7.Tg. 1 mice (red) vs. B6 littermates (blue).

B) Platelet counts in moribund TLR7.Tg.1 mice (red) vs. B6 littermates (blue).

C) Histological analysis of spleens from B6 or moribund TLR7.Tg.1 mice shows normal white pulp (WP) architecture in B6 mouse, with depletion of WP in TLR7.Tg.1 mice, and white pulp focal histiocytosis (FH).

D) Flow cytometric analysis of spleens from B6 or moribund TLR7.Tg.1 mice shows an abundance of Ter-119^{+} erythroblasts, lymphoid depletion (as judged by the decrease in CD4^{+}, CD8^{+}, and B220^{+} cells) and expansion of dendritic cells and myeloid cells. Data are shown as the average of 4 independent pairs of mice (a,b) or as representative from four independent experiments (c,d).

Table 1

Autoantibody Profiles and Pathological Manifestations in TLR7.Tg Mice

Strain	ANA^{+} (nuclear, nucleolar, mixed)	Splenomegaly[a]	GN[b]	DC expansion[c]	Liver inflamm[d].	Lung inflamm.[e]
7.1	5 (1,0,4)/7	6/6	3/6	6/6	5/6	0/6
7.2	5 (1,0,4)/6	4/5	4/5	4/5	3/5	0/5
7.4	7 (0,1,6)/7	6/6	5/6	6/6	5/6	0/6
7.5	7 (4,3,0)/8	5/5	3/5	5/5	3/5	0/5
7.6	8 (1,3,4)/9	8/8	6/8	8/8	3/8	2/8

[a] spleen size >300 mg

[b] kidney histology shows a majority of glomeruli being increased in size and cellularity

[c] >10% CD11c+ cells in blood

[d] presence of inflammatory cells in liver or lung