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CELLULAR AND MOLECULAR CONTRIBUTIONS OF TLR9 TO AUTOREACTIVE B CELLS IN A MURINE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

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CELLULAR AND MOLECULAR CONTRIBUTIONS OF TLR9 TO AUTOREACTIVE B CELLS IN A MURINE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

by

Robyn Mills

DISSERTATION

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CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

Chapter II of this dissertation is based on a submitted manuscript "Signal strength determines whether Toll-Like Receptor 9 positively or negatively regulates autoantibody production." The co-authors on this publication will be Viola C. Lam[1], Julie Zikherman[2], Arthur Weiss[2,3], and Michelle L. Hermiston[1]. Viola C. Lam and Julie Zikherman performed immunoglobulin-tagged bone marrow chimera experiments and Viola C. Lam also provided technical assistance. Arthur Weiss supervised preliminary experiments and contributed to experimental design. Michelle L. Hermiston supervised the work.

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ABSTRACT

One of the hallmarks of the autoimmune disease systemic lupus erythematosus (SLE) is the production of pathogenic anti-nuclear antibodies (ANA). These autoantibodies are the result of a break in tolerance that allows for the activation and differentiation of autoreactive B cells into antibody-secreting plasma cells. ANA can form immune complexes with self nucleic acids, which can bind to and deposit in small blood vessels and instigate immunopathology. The CD45E613R knock-in model of SLE causes different phenotypic consequences on several murine genetic backgrounds, indicating that this signaling mutation is sensitive to genetic modifiers. Despite similar dysregulation of phosphatase activity, CD45E613R mice on a C57BL/6 (B6) background are resistant to SLE phenotypes, while CD45E613R.BALB/c are sensitive to ANA but not end organ damage. In an unbiased screen for genetic modifiers of ANA, we identify TLR9 as a putative modifier for the production of a specific ANA subtype, anti-dsDNA, in the context of CD45E613R.

Here, we examine whether TLR9 modifies autoantibody production in the CD45E613R model between the resistant CD45E613R.B6 and sensitive CD45E613R.BALB/c model. Since CD45E613R alters Src family kinase and immunoreceptor signaling in several immune lineages, we generated a series of mixed bone marrow chimeras in the sensitive BALB/c genetic background to test which lineages require CD45E613R. TLR9 is also broadly expressed, so we

examined which cell lineages require TLR9 and whether CD45E613R and TLR9 are necessary in cis or trans for anti-dsDNA IgG production in the BALB/c background. Upon finding that both CD45E613R and TLR9 are necessary in B cells for autoreactivity, we examined B cells from both genetic backgrounds. TLR9 stimulation induces elevated signaling in B cells from B6 mice compared to BALB/c, and these signals are not mediated by CD45E613R or co-stimulation via the BCR. We find that TLR9 negatively regulates ANA production in resistant CD45E613R.B6 mice by altering central B cell tolerance. In contrast, TLR9 positively regulates ANA production in BALB/c mice, likely because the strength of signal downstream of TLR9 is not sufficient for tolerance. These results indicate that TLR9 is a genetic modifier of autoreactivity in the context of dysregulated CD45 signaling.

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CHAPTER I: INTRODUCTION

Recent advances in SLE: B cell depletion and modulation therapies

The autoimmune disease systemic lupus erythematosus (SLE) has a complex etiology that includes both genetic and environmental influences(1). Prevalence of SLE ranges from 20 to 70 per 100,000 depending on the population studied, and SLE has a strong gender bias, since approximately 90% of patients are female(2). Though the survival rate has increased dramatically over the past 50 years, the 15-20 year survival rate is still only 80%(2). A serologic hallmark of SLE is the presence of antibodies against nuclear antigens, which can affect several organ systems, depending on the patient(3). These antibodies can bind to self-antigens and form immune complexes that deposit in small vessels. Once deposited, these immune complexes recruit and activate other inflammatory cells, causing tissue destruction and pathology(4). The clinical and genetic heterogeneity of this disease make it difficult to treat, but most treatment regimens rely on some form of immunosuppression. Frequently treatment choice is made based on the affected organ system, the level of damage, and which drug will have the fewest negative repercussions in the affected organ(1). Improved understanding of the consequences of genetic heterogeneity on the cellular and molecular mechanisms of disease pathogenesis should lead to better targeted therapies and improved patient care.

Though the clinical diversity of presentation makes SLE more difficult to treat, anti-nuclear antibodies (ANA) are a common serological feature(1). Since B cells are the source of pathogenic ANA in SLE, targeted therapeutics that deplete or modulate B cells have recently been an attractive avenue for treatment(5). However, despite initially promising results in open label trials for patients with refractory SLE, the B cell depleting agent rituximab failed to achieve

primary and secondary endpoints in the EXPLORER and LUNAR clinical trials(6). In contrast, modulation of B cell development has recently shown more promise. Modulation of transitional B cell development by depletion of B cell activating factor (BAFF, also known as BLyS) via the monoclonal antibody belimumab (Benlysta) was approved for SLE treatment in 2011(7). Furthermore, epratuzumab, an antibody against CD22 that is thought to negatively regulate BCR signaling, is currently in clinical trials for SLE(8). Use of belimumab during the repopulation of B cells following rituximab-mediated depletion has also been proposed to impair the development of new autoreactive B cells(7). More monoclonal therapeutics that modulate B cell development or activation are in development, so it is of great importance to understand how abnormalities in B cell development, activation, and antibody production result in SLE and how these abnormalities are modulated by genetic differences between patients.

Systemic lupus erythematosus: Multi-step pathogenesis

The current paradigm in the field is that development of SLE is a multi-step process that involves dysregulation of both innate and adaptive immune cells(4). First, central tolerance against nuclear antigens must be breached, leading to the survival of autoreactive B and T cells. In the periphery, these cells are inappropriately activated, allowing for the production and secretion of ANAs that form immune complexes and deposit in tissues. Finally, innate cells are recruited to the immune complex depositions and become activated, causing tissue-damaging inflammation.

Mouse models have been instrumental in the dissection of the multi-step pathogenesis of SLE(9-11). Spontaneous mouse models of SLE have been useful in identifying pathways frequently dysregulated in this disease, such as the (NZB/NZW)-derived NZM2410, BXSB, or

MRL/*lpr* mouse strains. Unbiased approaches have also utilized these spontaneous models to identify novel loci that contribute to disease pathogenesis. For example, the Wakeland group identified several loci from the NZM2410 that contribute to disease pathogenesis via linkage analysis(12). Backcrossing these disease-associated loci to autoimmune resistant genetic backgrounds like B6 has permitted genetic dissection of the multi-step pathogenesis of disease(13). Combining SLE-associated loci in an autoimmune resistant background can recapitulate a complete SLE-like phenotype, underlying the polygenic and multi-step nature of SLE. Furthermore, genetic deletion of specific immune cell lineages and antibody-mediated cell depletion strategies in the context of these autoimmune prone loci has helped define the contributions of various cell lineages and identify new mechanisms of disease pathogenesis(4).

More recently, genetically engineered mice containing mutant signaling molecules have been useful in studying how lymphocytes with defective tolerance mechanisms interact with each other and other inflammatory cells to mediate tissue damage(14). In many cases, introduction of such mutations caused a lupus-like phenotype in mixed background B6 x 129/Sv mice. However, genetic contributions from these two strains can lead to spontaneous autoimmunity, leading to confounding results(15). Backcrossing these engineered mutations to a non-autoimmune prone genetic background has been essential for better understanding how these mutations alter tolerance in the absence of confounding genetic perturbations. As with lupus-associated loci identified in spontaneous models, genetically engineered mutant mice on an autoimmune resistant background have slower disease onset or become resistant to disease entirely. However, these engineered mutations can also cooperate with other lupus-prone alleles and loci from spontaneous models to recapitulate lupus-like phenotypes on resistant backgrounds,

allowing for better understanding of the requirements for autoimmunity(10). This is the case with the model used here, the CD45E613R mutant mouse model.

CD45E613R murine SLE model: Genetic separation of autoreactivity and autoimmunity

CD45 is a receptor-like protein tyrosine phosphatase (RPTP) expressed on all nucleated hematopoietic cells. This phosphatase primarily dephosphorylates the negative regulatory tyrosine of Src family kinases(16). Despite the high levels of CD45 expressed on the surface of most hematopoietic cells, no ligand has been identified for this molecule. Introduction of a point mutation in a highly conserved domain across many RPTPs, the juxtamembrane wedge domain, altered phosphatase activity. The CD45E624R mutation maps to the tip of human CD45's juxtamembrane wedge domain, and was initially shown to prevent negative regulation of phosphatase activity upon forced dimerization of chimeric EGFR-CD45 *in vitro*(17). When the analogous murine mutation, CD45E613R, was introduced into the germline of mice, homozygotes developed a lymphoproliferative disorder and a subset developed as a lupus-like phenotype including anti-dsDNA IgG and lupus nephritis(18).

Backcrossing CD45E613R to defined genetic backgrounds revealed that this phenotype is highly sensitive to genetic context. While the initial cohort of CD45E613R mice on a mixed B6 and 129/Sv background developed lymphoproliferative disease and lupus nephritis, most phenotypic consequences were blunted upon backcrossing 10 generations to the autoimmune-resistant B6 or 129/Sv genetic backgrounds(19). However, F1 (B6 x 129/Sv) CD45E613R mice develop a lupus-like phenotype, including autoantibodies, glomerulonephritis, and accelerated mortality at 100% penetrance, indicating that contributions from genetic modifiers promote CD45E613R-mediated pathology(20). Interestingly, CD45E613R mice on the B6 background

can cooperate with established lupus risk alleles to exacerbate disease(21, 22), indicating that the signaling consequences of introducing CD45E613R can alter phenotypic consequences in combination with other genetic perturbations. In contrast, we found that CD45E613R mice on the BALB/c genetic background develop anti-dsDNA IgG antibodies at 100% penetrance but no end organ disease, thereby providing a tractable model of autoreactivity without the interference of immune complex-mediated tissue damage.

An unbiased screen was performed to identify modifiers of serum anti-dsDNA IgG production between ANA-resistant CD45E613R.B6 and ANA-susceptible CD45E613R.BALB/c mice. This screen identified a novel modifier locus of BALB/c origin on the distal end of chromosome 9, *Wam1* (Figure 1). An *in silico* analysis of *Wam1* identified Toll-like receptor 9 (TLR9) as a putative modifier based on two non-synonymous coding SNPs between the two strains. No other genes of immunological relevance reported in the Mouse Genome Informatics database contained non-synonymous coding SNPs. Moreover, TLR9 was a particularly intriguing potential modifier, since it contributes to autoreactive B cell activation as well as type I interferon (IFN) production in plasmacytoid dendritic cells (pDCs) (23). Beyond the reported SNPs, five promoter polymorphisms and three other amino acid changes between *Tlr9* alleles from these two strains were reported (Table 1). These alterations in TLR9 were associated with differential responses to *Helicobacter felis* infection and caused elevated CpG-induced NF-κB activation in cell lines expressing the B6 allele compared to the BALB/c allele *in vitro*(24), but had not been examined in the context of autoimmune disease.

Nucleic-acid sensing TLRs in SLE

TLR9 is a member of a family of innate pattern recognition receptors that recognize a variety of conserved bacterial and viral motifs(25). A subset of these receptors primarily recognizes foreign nucleic acids from bacteria and viruses. This subset includes TLR7, a receptor for ssRNA, and TLR9, a receptor for unmethylated CpG islands in DNA. Activation of either of these molecules in plasmacytoid dendritic cells (pDCs) leads to large amounts of type I interferon production, leading to an anti-viral host response. However, when improperly regulated, these receptors can also recognize self nucleic acids(26).

Several mechanisms play important roles in protecting against self-nucleic acid recognition by this subset of nucleic acid-sensing TLRs. Nucleic acid-sensing TLRs are endosomally-restricted to protect against inappropriate activation by self nucleic acids(27, 28). Upon ligand binding in the endosome, the ectodomain of TLR9 undergoes a cathepsin-mediated cleavage event that allows for dimerization of the intracellular TIR signaling domain and recruitment of the downstream signaling adapter MyD88(29-32). This requirement for cleavage is mediated by the transmembrane domain and prevents signaling from the cell surface(33). However, interactions with immune complexes formed by nucleic acid-associated proteins or ANA can subvert endosomal protection and allow self nucleic acids to access the endosomal compartment(26, 34). In addition to preventing intact self-nucleic acids from accessing the endosome, cells also control access of these TLRs to the endosome. The trafficking adapter UCN93B1 interacts with the cytoplasmic domain of endosomal TLRs, allowing them to traffic to the endosome(35) and controlling the relative amounts of each receptor that access that compartment(31, 36).

It has become clear that nucleic-acid sensing TLRs play an important role in the pathogenesis of SLE. Autoreactive B cells that generate ANA require signaling from both the

BCR and a nucleic acid-sensing Toll-like receptor (TLR) to become activated and to secrete pathogenic autoantibodies(37, 38). Furthermore, self nucleic acids can be protected from degradation in autoreactive B cells by binding to the autoreactive BCR and bypassing the cellular barriers to self nucleic acid recognition by this subset of TLRs(26, 37). Individual TLRs have been associated with particular ANA specificities: TLR7, a receptor for ssRNA, is associated with autoantibodies against RNA and RNA-associated proteins, whereas TLR9, a receptor for unmethylated CpG islands in dsDNA, is associated with autoantibodies against chromatin and DNA-containing antigens(23).

The importance of the endosomal TLRs in the pathogenesis of SLE has been verified in murine models. Genetic deletion of the signaling and trafficking adaptors MyD88 and UNC93B1 eliminates most disease in murine SLE models(38-40). However, retaining the balance of TLR7 and TLR9 signaling is also important, since introduction of a point mutation in UNC93B1 that promotes TLR7 at the expense of TLR9 causes a lethal inflammatory response in mice(41, 42). TLR7 is also pathogenic in SLE, since deletion of TLR7 in the MRL/*lpr* murine SLE model was protective while elimination of TLR9 exacerbated disease(43). Furthermore, TLR7 is one of several genes duplicated in the lupus-associated *Yaa* locus derived from the spontaneous BXSB mouse model of SLE(44, 45). Over-expression of TLR7 promotes disease in lupus models, with the level of overexpression correlating to the level of disease acceleration(46). TLR7 expression is also increased in response to Type I IFN signaling, which is also associated with more severe disease(47, 48). In contrast, several groups have demonstrated that genetic ablation of TLR9 promotes end organ damage in murine models(49-52). However, these studies found different alterations in autoantibody repertoire in the absence of TLR9. These findings indicate that the

TLR9 plays a protective role in end organ damage but the role of TLR9 in autoantibody specificity is sensitive to other perturbations depending on genetic background.

In several recent studies, deletion of TLR7 in addition to deletion of TLR9 resolved the increased disease observed in TLR9 deficient animals, indicating that the pathology in TLR9 deficient mice is TLR7 mediated(53, 54). TLR9 negatively regulates TLR7 activation in B cells(55) and is necessary in B cells for homogenous nuclear ANA staining, an indication of DNA or chromatin specificity, in the MRL/lpr model(56). However, the mechanisms by which TLR9 restricts TLR7-mediated ANA production remain unclear. Here, we explore the role of TLR9 as a genetic modifier of anti-dsDNA IgG production between CD45E613R mice on the B6 and BALB/c backgrounds.

FIGURES

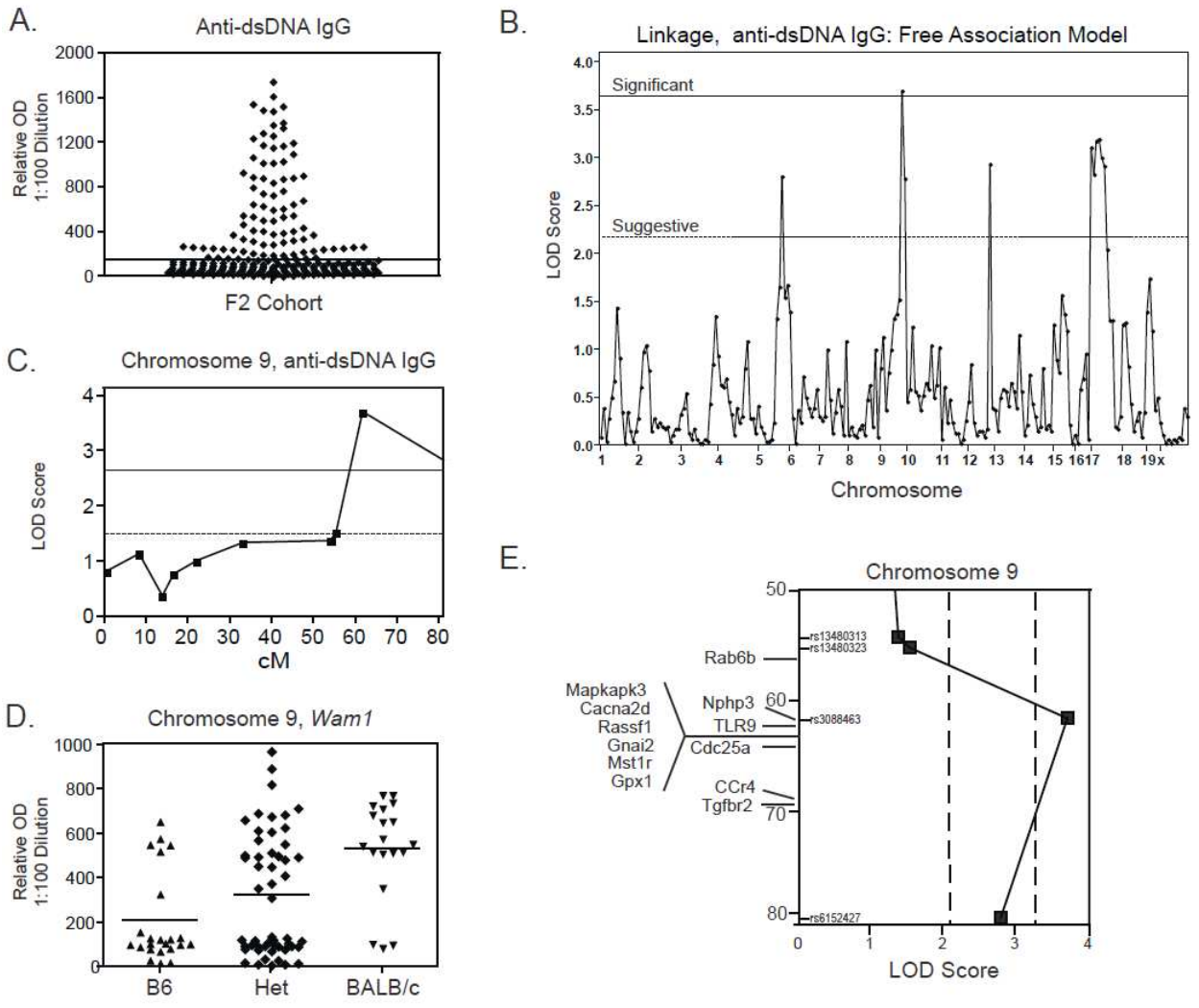

Figure 1. Identification of *Wam1*, a novel ANA susceptibility locus.

(A) Relative OD of 1:100 dilutions of sera for anti-dsDNA IgG for all animals in F2 cohort (n = 255). Each symbol represents one mouse. (B) Linkage analysis for anti-dsDNA IgG was performed on 96 F2 (B6 x BALB/c) CD45E613R mice. The solid line indicates the threshold for a significant LOD score and the dashed line indicates the threshold for a suggestive LOD score as based on permutation testing (1000 permutations) for each model. (C) Interval mapping of locus *Wam1* on chromosome 9. (D) Anti-dsDNA IgG relative OD of cohort segregated by *Wam1*

genotype. (E) Genes of immunological interest and relative position within *Wam1* are displayed on chromosome 9.

TABLES

Table 1. Polymorphisms in TLR9

Residue (murine/human)	**B6**	**BALB/c**	**Human**
325/324	T	N	T
378/377	L	S	S
573/572	T	A	A
579/578	Q	H	R (H)
867	T	A	A

CHAPTER II: SIGNAL STRENGTH DETERMINES WHETHER TOLL-LIKE RECEPTOR 9 POSITIVELY OR NEGATIVELY REGULATES AUTOANTIBODY PRODUCTION

Abstract

Anti-nuclear antibodies (ANA) are a hallmark of Systemic lupus erythematosus. Here, we utilize a mouse model in which an activating mutation (CD45E613R) causes ANA in CD45E613R.BALB/c but not CD45E613R.B6 mice. In ANA-sensitive CD45E613R.BALB/c mice, B cell intrinsic expression of both CD45E613R and TLR9 are required for ANA. However, despite similar hyper-responsiveness to B cell receptor (BCR) stimulation in both strains, responses to TLR9 stimulation are elevated in CD45E613R.B6 B cells relative to CD45E613R.BALB/c B cells. Surprisingly, genetic ablation of TLR9 permits ANA in resistant CD45E613R.B6 mice but eliminates ANA in CD45E613R.BALB/c mice. Furthermore, $TLR9^{-/-}$ CD45E613R B cell precursors persist better than $TLR9^{+/+}$CD45E613R B cell precursors when competing with wildtype B cells in the B6 but not BALB/c background. These data demonstrate that TLR9 signal strength determines whether B cells sharing a common BCR signaling mutation become autoreactive. While a strong TLR9 signal negatively regulates autoreactivity, a weak signal permits autoantibody production.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of diverse clinical presentation, with both genetic and environmental influences (1). Circulating autoantibodies against nuclear antigens are a hallmark of SLE and can be specific for nucleic acids or associated proteins such as double stranded DNA (dsDNA), chromatin, ribonuclear protein (RNP), or single stranded (ss) DNA (1, 3). These anti-nuclear antibodies (ANA) form immune complexes with debris from dying cells and other proteins, deposit in organs, and promote inflammation and tissue damage (1). Inappropriately activated autoreactive B cells secrete these pathogenic autoantibodies, and murine models of dysregulated BCR signaling frequently result in a lupus-like phenotype (14). Understanding the break in tolerance that permits generation and activation of autoreactive B cells that produce pathogenic autoantibodies has been an area of intense research for decades, but remains incompletely understood.

Autoreactive B cells require signaling from both the BCR and nucleic acid-sensing Toll-like receptors (TLRs) to become activated and to secrete pathogenic autoantibodies (23). The nucleic-acid sensing subset of TLRs is endosomally-restricted to protect against inappropriate activation by self nucleic acids (26, 33). Genetic deletion of signaling and trafficking adaptors common to several nucleic-acid sensing TLRs, such as MyD88 and UNC93B1, eliminates most disease in murine SLE models (38-40). Humans deficient in either of these adapters also do not develop serum ANA despite defects in elimination of autoreactive B cells during development, demonstrating the essential role for TLRs in activation of autoreactive human B cells (57). Individual TLRs have been associated with particular ANA specificities: TLR7, a receptor for ssRNA, is associated with autoantibodies against RNA and RNA-associated proteins, whereas

TLR9, a receptor for dsDNA, is associated with autoantibodies against chromatin and DNA-containing antigens (23). Recent evidence suggests that TLR9 may alter autoantibody specificity by negatively regulating TLR7-mediated activation of autoreactive B cells (53, 55), although the mechanisms by which activating receptors like TLR9 negatively regulate autoantibody specificity remain unclear.

To address the mechanisms mediating development of ANA, we used a murine model where a germline point mutation in the phosphatase CD45 (CD45E613R) causes dysregulation of Src family kinase and immunoreceptor signaling, resulting in a lupus-like phenotype on a mixed C57BL/6 (B6) and 129/Sv genetic background (18-20, 58). Interestingly, CD45E613R mice on the B6 background are resistant to SLE phenotypes but can cooperate with established lupus risk alleles to exacerbate disease (21, 22). In contrast, CD45E613R mice on the BALB/c genetic background develop anti-dsDNA IgG antibodies at 100% penetrance but no end organ disease, thereby providing a tractable model of autoreactivity without the interference of immune complex-mediated tissue damage. We identified TLR9 as a putative modifier of autoreactivity in the absence of autoimmunity in a screen for modifiers of serum anti-dsDNA IgG production between ANA-resistant CD45E613R.B6 and ANA-susceptible CD45E613R.BALB/c mice (Hermiston et al, manuscript in preparation). Between TLR9 alleles from these two strains, there are five promoter polymorphisms and five amino acid changes, resulting in elevated CpG-induced NF-κB activation in cell lines expressing the B6 allele compared to the BALB/c allele *in vitro* (24). Based on these studies, we hypothesized that TLR9 modifies CD45E613R-induced phenotypes in these two genetic backgrounds.

Here, we assess how TLR9 modulates autoreactivity in two non-autoimmune prone strains of mice bearing the same risk allele, CD45E613R. In ANA-susceptible

CD45E613R.BALB/c mice, TLR9 ablation eliminates ANA. We establish that B cell intrinsic CD45E613R and TLR9 are required for autoantibodies in this background. Surprisingly, signaling downstream of TLR9 is decreased in BALB/c follicular B cells compared to B6, inconsistent with the established role of TLR9 as an activating receptor in autoreactive B cells. In fact, genetic elimination of TLR9 permits ANA development in the CD45E613R.B6 mice and TLR9 deficiency permits increased persistence of CD45E613R.B6 B cell precursors compared to TLR9 sufficient CD45E613R.B6 B cell precursors in a competitive bone marrow microenvironment. These data support a model in which TLR9 regulates the central B cell tolerance checkpoint. The stronger B6 TLR9 signal prevents autoreactivity in CD45E613R mice while the weaker BALB/c TLR9 signal is not sufficient for tolerance, permitting CD45E613R to cause TLR9-dependent ANA. Our findings reveal a previously unappreciated role for TLR9 signal strength in setting the threshold for tolerance against anti-nuclear antigens in the context of hyper-responsive BCR signaling.

Materials and Methods

Mice

C57BL/6.CD45.1 (002014), C57BL/6.CD45.2 (000664), BALB/c.CD45.1 (006584), BALB/c.CD45.2 (001026), BALB/c.IgH^{b} (001107), and BALB/c.Actin.GFP+ (007075) strains were obtained from The Jackson Laboratory. BALB/c.TCR$\alpha^{-/-}$ and BALB/c.$J_H^{-/-}$ were obtained from Dr. A. Abbas (UCSF). C57BL/6.TLR9$^{-/-}$ mice (59) were obtained from Dr. J. DeRisi (UCSF) and BALB/c.TLR9$^{-/-}$ were obtained from Dr. I. Rifkin (Boston University) and used with permission from Dr. S. Akira. CD45E613R mice were generated as previously described (18), and backcrossed at least nine generations to C57BL/6 and BALB/c backgrounds. Fidelity of backcrossing was verified in the UCSF Genomics Core using the Mouse MD Linkage Analysis SNP Array (Illumina). Mice were bred and housed in a specific-pathogen free facility and experiments were performed according to UCSF IACUC and NIH guidelines. For aging cohorts, mice were bled monthly beginning at 8 weeks of age to monitor serum autoantibodies. In some cases, mice were monitored for proteinuria with Uristix (Siemens).

Bone Marrow Chimeras

Donor bone marrow was isolated from femurs of 5-10 week old mice of the indicated genotypes, resuspended at 1.5-2x10^{7} cells/ml in sterile PBS, combined at the indicated ratios, and 100μl injected intravenously into lethally irradiated 6-8 week old hosts. Recipients were treated with antibiotic pellets (Bio-Serv S0443) for two weeks following bone marrow transfer. Beginning 8 weeks following transfer, mice were bled monthly to monitor for engraftment and serum autoantibodies. ANA titers from the terminal time point for each experiment are shown.

Autoantibody Assays

ANA were performed as previously described (21). Briefly, HEp-2 slides (Inova Diagnostics) were stained with a 1:40 serum dilution, washed and detected with FITC-conjugated donkey anti-mouse IgG secondary (Jackson Immunoresearch). Images were acquired on an Olympus BX51 epifluorescence microscope with OpenLab software and scored according to staining intensity. Pooled CD45E613R.BALB/c serum was used as a positive control.

Anti-dsDNA IgG ELISA was performed as previously described (18). Briefly, 96 well flat bottom plates were coated with Poly-L-lysine (Sigma P2636), then Poly dA:dT (Sigma P0883), then blocked. Serum was diluted 2-fold starting at a dilution of 1:100, and antibodies were detected with HRP-Goat-anti Mouse pan IgG (Southern Biotech) and TMB (Sigma). Pooled CD45E613R.BALB/c serum was used as a positive control and run in serial dilution on each plate as a quantitation control. Anti-RNP IgG was similarly detected using plates coated with Sm/RNP antigen (Immunovision SRC-3000). Sm/RNP-positive TLR9$^{-/-}$CD45E613R.B6 serum was used as a positive control on all plates as shown in Figure 1D.

Antibodies and flow cytometry

Following red blood cell lysis and Fc receptor blockade, cells were stained with antibodies recognizing CD4-V500, CD11c-PE-Cy7, CD19-V450, CD19-APC-Cy7, IgM-PerCP-Cy5.5, CD45.2 V500, CD45.2-APC (BD Biosciences), IgD-PE, CD45.1 FITC, CD11b-PerCP-Cy5.5, CD21-PB, CD23-PE-Cy7, AA4.1-APC (eBiosciences), CD45.1-PB (BioLegend). Cells were acquired on BD FACSVerse or LSR II flow cytometer and analyzed using FlowJo v9.6.4 (Treestar).

B cell signaling flow cytometry

Single cell suspensions were generated from lymph nodes of 8-10 week old mice, resuspended at $2x10^7$ cells/ml, rested in serum free media at 37°C for 1 hour, and stimulated in 96 well V-bottom plates at 37°C with vehicle, CpG 1668 (Invivogen) or $F(ab')_2$ anti-mouse IgM (Jackson Immunoresearch) for the times indicated in the figure legend. Cells were fixed with pre-warmed 1% paraformaldehyde, washed, permeabilized with ice cold 100% methanol, washed, rehydrated in PBS, stained with antibodies against pERK or IκBα (Cell Signaling Technologies), washed, stained with PE-donkey anti-rabbit IgG (Jackson Immunoresearch) and anti-B220-APC (eBiosciences), and analyzed as described above.

Statistics

Statistical analyses were performed using Prism v5.0a (GraphPad). All p values ≤ 0.05 were considered significant and are shown in the figures. Student's t test was used for pair-wise comparisons. In experiments comparing more than two groups, a one-way ANOVA test was first performed to evaluate significance, followed by pair-wise t test indicated in the figures.

Results

TLR9 is necessary for ANA in CD45E613R.BALB/c mice

The phenotypic consequences of the CD45E613R mutation depend on genetic context. CD45E613R mice on a B6 background do not develop autoantibodies or glomerulonephritis (21, 22) while CD45E613R mice on a mixed B6/129 background or an F1 B6 x 129/Sv background do (18, 20). On a BALB/c background, CD45E613R mice developed serum ANA at 100% penetrance as assessed by HEp-2 cell nuclear staining but no glomerulonephritis (Fig 1A,B; data not shown). Consistent with the homogenous nuclear staining pattern of the ANAs, further quantitation of serum antibodies by ELISA indicated that CD45E613R.BALB/c mice produce high titer anti-ds DNA IgG and much lower titer anti-RNP IgG (Fig 1C,D).

Anti-dsDNA specificity has been associated with the pattern recognition receptor TLR9, which recognizes unmethylated CpG islands in dsDNA (23). To test whether TLR9 is required for ANA production in our model, we compared $TLR9^{+/+}$, $TLR9^{+/-}$ and $TLR9^{-/-}$ CD45E613R.BALB/c mice. While aged $TLR9^{+/+}$ and $TLR9^{+/-}$CD45E613R.BALB/c mice developed ANA and anti-dsDNA IgG with a trend towards a gene dosage effect, $TLR9^{-/-}$ CD45E613R.BALB/c mice did not (Fig 1B,C). Interestingly, consistent with observations in the $TLR9^{-/-}$MRL/lpr model (43, 53), cytoplasmic staining was seen in 3 of the 9 CD45E613R.BALB/c mice (Fig 1A). However, unlike $TLR9^{-/-}$MRL/lpr mice, $TLR9^{-/-}$ CD45E613R.BALB/c mice did not develop anti-RNP antibodies or any stigmata of end organ disease (Fig 1D, data not shown). We conclude that TLR9 can positively regulate ANA in autoantibody-sensitive CD45E613R.BALB/c mice.

B cell intrinsic CD45E613R is necessary for ANA in CD45E613R.BALB/c mice

While B cells are the source of autoantibodies, the inappropriate activation, differentiation, and survival of autoreactive B cells can be driven by dysregulation of other immune cell lineages (60-62). Since both CD45 and TLR9 are expressed in many immune cell subtypes (16, 23), we used a mixed bone marrow chimera approach to methodically evaluate which cell types require CD45E613R and/or TLR9 for ANA production in CD45E613R.BALB/c mice.

We initially focused on defining which cell lineage(s) required the CD45E613R mutation for ANA production. We generated chimeras with congenically marked immunoglobulin by transferring a 1:1 ratio of IgH^{b}CD45WT.BALB/c and IgH^{a}CD45E613R.BALB/c marrow into lethally irradiated CD45 wildtype (WT) BALB/c hosts. Six months following transfer, all ANA were IgG^{a} but not IgG^{b} (Fig. 2A). While this indicated that ANA are of CD45E613R B cell origin, it did not exclude the possibility that other CD45E613R cell types were required for development and/or activation of autoreactive CD45E613R B cells.

To more rigorously examine whether B cell intrinsic CD45E613R is necessary for anti-dsDNA IgG, a 1:4 ratio of B cell sufficient ($J_H^{+/+}$) to B cell deficient ($J_H^{-/-}$) CD45WT or CD45E613R marrow was transferred into lethally irradiated BALB/c hosts to generate chimeras where all B cells expressed only the CD45WT or CD45E613R allele in the context of an immune compartment primarily derived from the opposite CD45 genotype. Confirming a B cell intrinsic requirement for CD45E613R, recipients with CD45WT B cells in a predominantly CD45E613R immune compartment (B^{WT}) did not develop anti-dsDNA IgG, similar to negative control chimeras generated from 20% CD45WT and 80% CD45WT $J_H^{-/-}$ marrow (All^{WT}) (Fig. 2B). In contrast, recipients with CD45E613R B cells in a predominantly CD45WT immune

compartment (B^{E613R}) produced anti-dsDNA IgG at similar titers and frequencies to positive control chimeras generated from 20% CD45E613R B cell sufficient marrow combined with 80% CD45E613R B cell deficient marrow (All^{E613R}) (Fig. 2B).

The capacity of CD45E613R B cells to generate anti-dsDNA IgG in the context of a primarily CD45WT immune compartment suggests that B cell intrinsic CD45E613R is sufficient for ANA. However, potentially 20% of the other immune compartments in these chimeras could be derived from CD45E613R marrow. Since dysregulation of the myeloid compartment has also been implicated in autoantibody production (60, 62), we assessed the proportion of CD45E613R $CD11b^+$ cells in the spleen to ensure that CD45E613R myeloid cells were not skewing results by preferentially expanding in aged chimeras. CD45E613R $CD11b^+$ cells were out-competed by CD45WT cells in both B^{WT} and B^{E613R} chimeras, resulting in less than 20% of $CD11b^+$ cells of CD45E613R origin in B^{E613R} recipients (Fig. 2C). Moreover, the observation that all ANA were IgG^a in the $IgH^bCD45WT$:$IgH^aCD45E613R$ mixed chimeras (Fig 2A) further indicated that a common microenvironment is sufficient to permit autoreactivity in CD45E613R but not CD45WT B cells.

Since αβ T cells are also implicated in SLE pathogenesis (1) and dysregulated in CD45E613R mice (58), we tested whether CD45E613R αβ T cells were required for autoantibody production. Chimeras were generated using congenically marked CD45WT (CD45.1) marrow and CD45E613R (CD45.2) $TCR\alpha^{+/+}$ or $TCR\alpha^{-/-}$ marrow. Because CD45E613R B cells were slightly out competed by CD45WT cells, a 2:3 ratio of CD45WT to CD45E613R marrow was used to generate chimeras containing approximately 50% CD45E613R $CD19^+$ B cells in the peripheral blood 8 weeks post transfer (Fig. 2E). Aged chimeras lacking CD45E613R αβ T cells developed anti-dsDNA IgG antibodies at similar frequencies and titers

to chimeras containing both CD45E613R and CD45WT αβ T cells (Fig 2D), demonstrating that CD45E613R αβ T cells are dispensable for autoantibody production. Together these data indicate that B cell intrinsic CD45E613R is necessary and likely sufficient for autoreactivity.

CD45E613R.BALB/c B cells require TLR9 for anti-dsDNA IgG production

While CD45 expression is restricted to hematopoietic cells, TLR9 expression is not. Interestingly, non-hematopoietic TLR9 expression has been implicated in lupus pathogenesis (63, 64). To test whether hematopoietic TLR9 is required for ANA in CD45E613R.BALB/c mice, we transferred $TLR9^{+/+}$ or $TLR9^{-/-}$Actin.GFP^{+}CD45E613R.BALB/c marrow into lethally irradiated $TLR9^{+/+}$ or $TLR9^{-/-}$CD45WT recipients. Neither $TLR9^{+/+}$ nor $TLR9^{-/-}$CD45WT recipients of $TLR9^{-/-}$ CD45E613R marrow developed serum ANA or anti-dsDNA antibodies (Fig 3A-C). Consistent with observations in $TLR9^{-/-}$CD45E613R.BALB/c mice, $TLR9^{-/-}$CD45E613R marrow also did not give rise to anti-RNP IgG (Fig 3D). In contrast, both lethally irradiated CD45WT $TLR9^{+/+}$ and $TLR9^{-/-}$ recipients of $TLR9^{+/+}$CD45E613R marrow developed ANA and anti-dsDNA IgG (Fig 3A-C), indicating that hematopoietic TLR9 is required for ANA.

TLR9 is expressed in several immune cell types, including B cells. Having established a B cell intrinsic role for the CD45E613R mutation (Fig 2), we next tested whether anti-dsDNA IgG production also requires B cell intrinsic TLR9. CD45E613R chimeras in which all B cells were $TLR9^{-/-}$ but 80% of the other immune cells were $TLR9^{+/+}$ were monitored monthly for serum ANA (Fig 3E). Chimeras with $TLR9^{-/-}$CD45E613R B cells (B^{9KO}) never developed anti-dsDNA IgG (Fig 3F). However, half the positive control chimeras containing $TLR9^{+/+}$CD45E613R B cells (B^{9WT}) developed anti-dsDNA IgG (Fig 3F). Neither B^{9KO} nor B^{9WT} chimeras had detectable serum anti-RNP IgG (Fig 3G). The absence of anti-dsDNA IgG in

chimeras containing TLR9$^{-/-}$CD45E613R B cells in the context of a primarily TLR9$^{+/+}$CD45E613R hematopoietic compartment indicates a B cell intrinsic requirement for TLR9.

TLR9 signaling differs in B cells depending on genetic background

The observation that ANA production in BALB/c mice requires both the CD45E613R mutation and TLR9 was quite intriguing to us. A genetic modifier screen between ANA-resistant CD45E613R.B6 and ANA-susceptible CD45E613R.BALB/c mice identified TLR9 as a putative genetic modifier of anti-dsDNA antibodies (Hermiston et al., manuscript in preparation). Since autoreactive B cells require signaling via the BCR and a TLR to become activated and secrete autoantibodies (23), we were curious whether primary B cells from B6 and BALB/c mice signaled differently via TLR9.

Since CD45E613R.BALB/c mice develop ANA and CD45E613R.B6 mice do not, we predicted that BALB/c B cells would have an elevated response to CpG relative to B6 B cells. However, we observed the opposite: IκBα degradation, a measure of NF-κB activity, was *increased* in B6 relative to BALB/c follicular B cells when stimulated *ex vivo* with low doses of the synthetic TLR9 ligand CpG 1668 (Fig 4A). This difference in signaling was masked at saturating doses of CpG (Fig 4B). Likewise, ERK was *hyper*phosphorylated in CpG-stimulated B6 B cells compared to BALB/c (Fig 4C). However, unlike NF-κB signaling, pERK was elevated in B6 B cells at both low and high doses of CpG (Fig 4D). Interestingly, the response to CpG was not modulated by the presence or absence of the CD45E613R mutation (Fig 4B,D).

BCR and TLR9 co-stimulation does not alter the magnitude or kinetics of ERK phosphorylation

While our results were consistent with a prior *in vitro* study comparing B6 and BALB/c B cell responses to CpG stimulation (24), they were perplexing given the field's current paradigm requiring both BCR and TLR signals for autoreactive B cell activation (23). We hypothesized that co-stimulation via TLR9 and the BCR would differentially modulate a common signaling node, pERK, in B6 and BALB/c CD45E613R follicular B cells. To test this, cells were stimulated with anti-IgM or CpG alone or in combination. Following BCR stimulation with a sub-optimal dose of anti-IgM, pERK peaked at 2.5 minutes and was similarly elevated in B6 and BALB/c CD45E613R B cells relative to CD45WT B cells (Fig 5A,B). In contrast, pERK peaked at 20 minutes following TLR9 stimulation and was significantly elevated in B6 but not BALB/c B cells regardless of the presence or absence of the CD45E613R mutation (Figure 5C). Surprisingly, co-stimulation with both BCR and TLR9 ligands did not modulate the magnitude or timing of these kinetically distinct waves of pERK in either genetic background (Fig 5D). We further tested co-stimulation with a range of CpG and anti-IgM doses (data not shown) but never observed a synergistic increase in pERK. Furthermore, the two waves of pERK induced by BCR or TLR9 ligation remained temporally distinct. We conclude the CD45E613R mutation dictates ERK hyperphorphorylation in response to BCR stimulation regardless of genetic background. In contrast, the B6 background dictates ERK hyperphorphorylation in response to TLR9 stimulation regardless of the presence or absence of the CD45E613R mutation.

TLR9 prevents CD45E613R.B6 mice from ANA development

Given the essential role for TLRs in autoreactive B cell activation (23), we expected to observe increased TLR9 signaling in B cells from mice that develop anti-dsDNA IgG. However, we observe the opposite: mice with stronger TLR9 signals, CD45E613R.B6, are resistant to serum ANA production while mice with weaker TLR9 signals, CD45E613R.BALB/c, develop ANA. How do we reconcile this paradox? Recent studies have demonstrated an important role for TLR9 in negative regulation of TLR7-mediated autoantibody development (53, 55, 65). We hypothesized that perhaps elevated TLR9-mediated signaling in CD45E613R.B6 mice is protective and negatively regulates ANA. To test this, we generated $TLR9^{+/+}$, $TLR9^{+/-}$ and $TLR9^{-/-}$CD45E613R.B6 mice.

As previously observed (21, 22), $TLR9^{+/+}$CD45E613R.B6 mice did not develop ANA (Fig 6A,B). However, loss of one allele of TLR9 permitted ANA in aged $TLR9^{+/-}$ CD45E613R.B6 mice, and complete deficiency for TLR9 permitted ANA production in 2 of 8 mice tested (Fig 6A,B). An additional 4 $TLR9^{-/-}$CD45E613R.B6 mice developed anti-cytoplasmic antibodies, similar to observations of $TLR9^{-/-}$MRL/lpr mice (43) and $TLR9^{-/-}$ CD45E613R.BALB/c mice (Fig 6A). Further analyses of ANA specificity revealed that both $TLR9^{+/-}$ and $TLR9^{-/-}$ CD45E613R.B6 mice failed to develop high titer anti-dsDNA antibodies (Fig 6C). However, significantly more $TLR9^{+/-}$CD45E613R.B6 mice than $TLR9^{+/+}$CD45E613R.B6 mice developed anti-RNP IgG. A similar trend was observed in $TLR9^{-/-}$ CD45E613R.B6 mice, but did not reach statistical significance (Fig 6D). While ANA penetrance was incomplete, these data clearly demonstrate that decreasing the gene dosage of TLR9 confers a permissive capacity for autoantibody production and supports the argument that strong TLR9 signals are protective while weak TLR9 signals are permissive. The observed increase in anti-RNP IgG is consistent with TLR7-driven activation of autoreactive B cells in the absence of

sufficient tolerogenic TLR9 signaling in CD45E613R.B6 mice. Contrasting its role as a positive regulator of ANA in autoantibody-sensitive CD45E613R.BALB/c mice, we conclude that TLR9 acts as a negative regulator of ANA production in autoreactivity-resistant CD45E613R.B6 mice.

TLR9 negatively regulates CD45E613R.B6 but not CD45E613R.BALB/c B cell development in a competitive microenvironment.

Hyper-responsive BCR signaling alters B cell selection in several murine models, including CD45E613R (14). We previously observed that CD45E613R.B6 mice with an unrestricted BCR repertoire have decreased mature B cell numbers relative to controls. However, restricting the repertoire with a BCR transgene results in increased antigen specific B cell numbers in the absence of cognate antigen but decreased antigen specific B cell numbers when exposed to cognate antigen *in vivo* (19). These data support a model where increased BCR signal strength conferred by the CD45E613R mutation results in enhanced deletion of mature B cells by endogenous ligands. Based on the observations that TLR9 signaling is elevated in B6 compared to BALB/c B cells and TLR9 deficiency permits autoreactivity in CD45E613R.B6 mice (Fig 4,6), we hypothesized that the combined strength of signal from the BCR and TLR9 alters B cell tolerance checkpoints in CD45E613R.B6 but not CD45E613R.BALB/c mice.

We analyzed B cell development in the bone marrow, spleen, or lymph node of 8-week-old TLR9$^{+/+}$, TLR9$^{+/-}$, or TLR9$^{-/-}$CD45E613R.B6 mice directly *ex vivo* but failed to observe any TLR9-mediated differences (data not shown). To eliminate potential interference of compensatory B cell expansion, we tested our hypothesis in a more stringent setting: the competitive microenvironment of mixed bone marrow chimeras. We generated chimeras in both

the B6 and BALB/c genetic backgrounds using a 2:3 ratio of congenically marked CD45WT (CD45.1) to $TLR9^{+/+}$ or $TLR9^{-/-}$CD45E613R (CD45.2) marrow.

An important B cell development checkpoint occurs in the spleen, where immature autoreactive B cells are eliminated or anergized and prevented from becoming follicular mature B cells (66). While there was a CD45E613R-mediated loss of cells at the splenic transitional-mature B cell checkpoint relative to CD45WT in both genetic backgrounds, it was not affected by TLR9 (Fig S1). We next examined the central tolerance checkpoint between pro- and pre-B versus immature B cells in the bone marrow. We noted a significant decrease in the reconstitution efficiency of $IgM^{+}IgD^{-}CD19^{+}$ immature B cells compared to $IgM^{-}IgD^{-}CD19^{+}$ pro- and pre-B cells in chimeras generated using $TLR9^{+/+}$CD45E613R.B6 marrow that was not observed in chimeras containing $TLR9^{-/-}$CD45E613R.B6 marrow (Fig 7A,B). In contrast, a TLR9-mediated decrease at this checkpoint was not observed in BALB/c chimeras (Fig 7C). These differences were even more striking when we calculated the engraftment ratio of CD45E613R immature to pro- plus pre-B cells. This ratio was significantly increased when the CD45E613R cells lacked TLR9 in the B6 but not BALB/c genetic background (Fig 7D). In the bone marrow of both strains, the CD45E613R-derived recirculating mature $IgD^{+}IgM^{-}$ B cell fraction was decreased compared to the $IgM^{+}IgD^{lo}$ T2-like B cell compartment, consistent with the CD45E613R-mediated loss of follicular cells in the spleen (Fig S1).

Our observation that TLR9 modulates the central B cell development checkpoint for B cells reacting with nuclear antigens is consistent with the peak in *Tlr9* expression reported in Hardy Fraction E immature B cells of B6 mice by the Immunological Genome Project Consortium (67). We propose that the increased signal strength via TLR9 is sufficient to eliminate autoreactive immature CD45E613R B cells in the bone marrow of B6 but not BALB/c.

Our results indicate a previously unappreciated tolerogenic role for TLR9 during B cell development that depends on genetic context-determined TLR9 signal strength.

Discussion

Autoantibodies against nuclear antigens are both hallmarks of SLE and mediators of disease pathogenesis (1). To become activated and secrete pathogenic ANA, autoreactive B cells require a second signal from endosomal nucleic acid-sensing TLRs in addition to BCR signals (23). Here, we demonstrate that the endosomal nucleic acid-sensing TLR9 is a positive regulator of ANA in CD45E613R.BALB/c mice. However, we also provide genetic evidence that TLR9 can negatively regulate autoreactivity, since deletion of one or both copies of *Tlr9* in CD45E613R.B6 mice permits autoreactivity. In B6 B cells, the strength of signal downstream of TLR9 activation is elevated compared to BALB/c B cells. Furthermore, $TLR9^{+/+}$CD45E613R.B6 B cell precursors are eliminated to a greater extent than $TLR9^{-/-}$CD45E613R.B6 B cell precursors when competing with CD45WT B cells. Based on these data, we propose that TLR9 can negatively regulate autoreactivity at the central B cell tolerance checkpoint. This capacity to promote B cell tolerance depends on strength of TLR9 signal, where $TLR9^{B6}$ is sufficient but $TLR9^{BALB/c}$ is not for tolerance against anti-nuclear antigens in the context of hyper-responsive BCR signaling.

Controversy over the role of TLR9 in autoreactivity stems from several groups reporting different alterations to autoantibody specificity upon genetic ablation of TLR9 in several mouse models of SLE (43, 50-52). However, in these models ablation of TLR9 did not alter the susceptibility of lupus-prone mice to autoreactivity, but instead shifted the specificities of their circulating autoantibodies. Here, we demonstrate that loss of TLR9 can shift the permissiveness of mice to autoreactivity in addition to altering specificity. Therefore, we conclude that TLR9 has a previously unappreciated role in B cell tolerance that depends on genetic background. We

find that genetic ablation of the same molecule, TLR9, in the context of the same risk allele, CD45E613R, results in completely opposite phenotypes in two distinct genetic backgrounds. Our results highlight the importance of understanding the genetic context in which risk alleles exist for their function. In human populations, TLR9 polymorphisms have been shown to be risk alleles for SLE in some populations, but meta-analyses of these studies have generated conflicting results (68-70). Perhaps the human polymorphisms in *Tlr9* have opposing effects in the context of other risk alleles, similar to the mouse models described here.

In the B6 genetic background, loss of one or both copies of TLR9 permits the development of anti-RNP and cytoplasmic autoantibodies, consistent with TLR7-driven autoantibodies described in other TLR9$^{-/-}$ murine lupus models (53, 55, 65). SLE phenotypes are also promoted in mice by increasing the gene dosage of TLR7 (44-46) or by introducing a point mutation into the trafficking adapter UNC93B1 that promotes TLR7 at the expense of TLR9, causing lethal systemic inflammation (41). We observe that TLR9 heterozygosity in CD45E613R.B6 B cells permits development of autoantibodies of TLR7-associated specificities. Our results are consistent with a model in which autoreactivity is determined by the balance of TLR9 and TLR7 signaling, where loss of one allele of TLR9 or increasing the gene dosage of TLR7 can cause disease. It is unclear why the absence of TLR9 does not permit TLR7-mediated anti-RNP IgG in TLR9$^{-/-}$CD45E613R.BALB/c mice. Other TLRs differ between B6 and BALB/c (71), so perhaps the BALB/c *Tlr7* allele is not sufficient for autoreactivity. According to expression data from the Immunological Genome Project Consortium, *Tlr9* is highly upregulated in immature bone marrow B cells, while *Tlr7* expression remains much lower (67). Since this expression data was collected in B6 mice, it will be of interest to assess the expression levels of *Tlr7* and *Tlr9* in B cell subsets and precursors in BALB/c and other murine strains.

We observed loss of TLR9$^{+/+}$CD45E613R.B6 B cell precursors in the bone marrow at the IgM^{+}IgD^{-}CD19^{+} immature B cell stage of development when in competition with WT cells. However, it remains unclear how TLR9 negatively regulates autoreactive CD45E613R B cells in a non-competitive microenvironment. A possibility is that self-reactive pre-B cells stimulated via TLR9 undergo receptor editing, leading them to be out-competed in mixed bone marrow chimeras but not in unmanipulated animals. However, we did not observe TLR9-mediated changes in the frequency of mature B cells that expressed surface IgL lambda chain in CD45E613R.B6 mice, though we did observe increased use of lambda in CD45E613R B cells compared to CD45WT in both genetic backgrounds (data not shown). While this observation does not formally rule out the possibility of TLR9-mediated increased receptor editing in CD45E613R.B6 B cell precursors, we believe it is unlikely.

To our surprise, genetic ablation of one copy of *Tlr9* in CD45E613R.B6 resulted in a higher frequency of mice developing ANA compared to TLR9$^{-/-}$ CD45E613R.B6 mice. This is likely due to the importance of TLR9 signaling in the activation and expansion of autoreactive B cells in the periphery. The B cell intrinsic requirement for TLR9 for autoreactivity in CD45E613R.BALB/c is also likely due to the role of this receptor in peripheral activation of autoreactive B cells (23). The observation that TLR9 can negatively regulate CD45E613R.B6 B cell development in a competitive microenvironment is consistent with published data indicating that TLR signaling can regulate the development and purging of autoreactive B cells (57, 72). Patients deficient for MYD88, IRAK4, or UNC93B1, important downstream signaling and trafficking adapters for endosomally restricted nucleic-acid sensing TLRs, have defects in purging autoreactive B cells at the central B cell tolerance checkpoint (57). Consistent with these human studies, we also observe TLR9-mediated loss of CD45E613R.B6 B cell precursors at the

central B cell tolerance checkpoint in the context of a competitive microenvironment. Patients deficient for these TLR-associated proteins also do not develop serum autoantibodies, since these signaling and trafficking adapters are also necessary for activation of autoreactive B cells. This may parallel the role of TLR9 in CD45E613R.BALB/c mice, where we observe loss of autoreactivity in the absence of TLR9.

Currently many SLE patients are treated with hydroxychloroquine, an inhibitor of endosomal acidification that prevents activation of several endosomal nucleic acid-sensing TLRs, including TLR9 and TLR7. It has also been demonstrated that activation of endosomal nucleic acid-sensing TLRs contributes to glucocorticoid resistance in SLE patients via increased type I IFN production (73). For these reasons, there has been increased interest in more specific TLR7 and TLR9 antagonists as possible therapeutics (74). Here, we demonstrate an important tolerogenic role for TLR9 in B cell development in a murine lupus model. Our results indicate that distinct alleles of TLR9 can have opposing effects on the development of ANA, an important mediator of tissue damage in SLE. Therefore, further research into possible negative repercussions of TLR9 inhibition on B cell development is warranted.

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Footnotes

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FIGURES

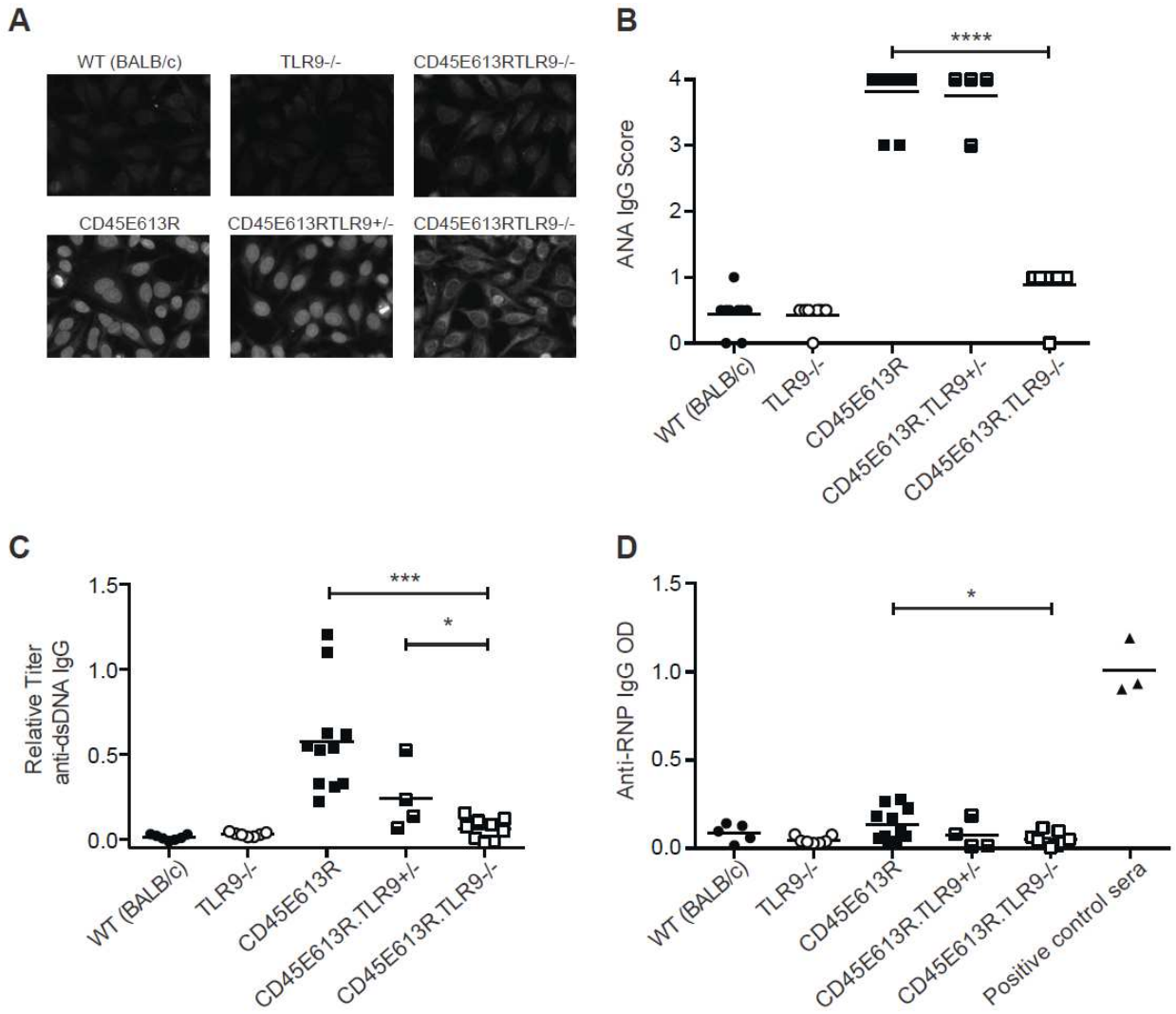

Figure 1. TLR9 is necessary for ANA in CD45E613R.BALB/c mice.

(A) Representative ANA staining pattern (original magnification 40X) of 1:40 diluted sera and **(B)** staining intensity of serum collected from 6-8 old mice of the indicated genotypes on the BALB/c background relative to pooled 6-8 month CD45E613R.BALB/c positive control sera. **(C)** Anti-dsDNA IgG and **(D)** Anti-RNP IgG titers from the same mice relative to pooled 6-8 month CD45E613R.BALB/c as assessed by ELISA. Data are pooled from three independent aging cohorts. * *p<0.05*; *** *p<0.005*; **** *p<0.001*

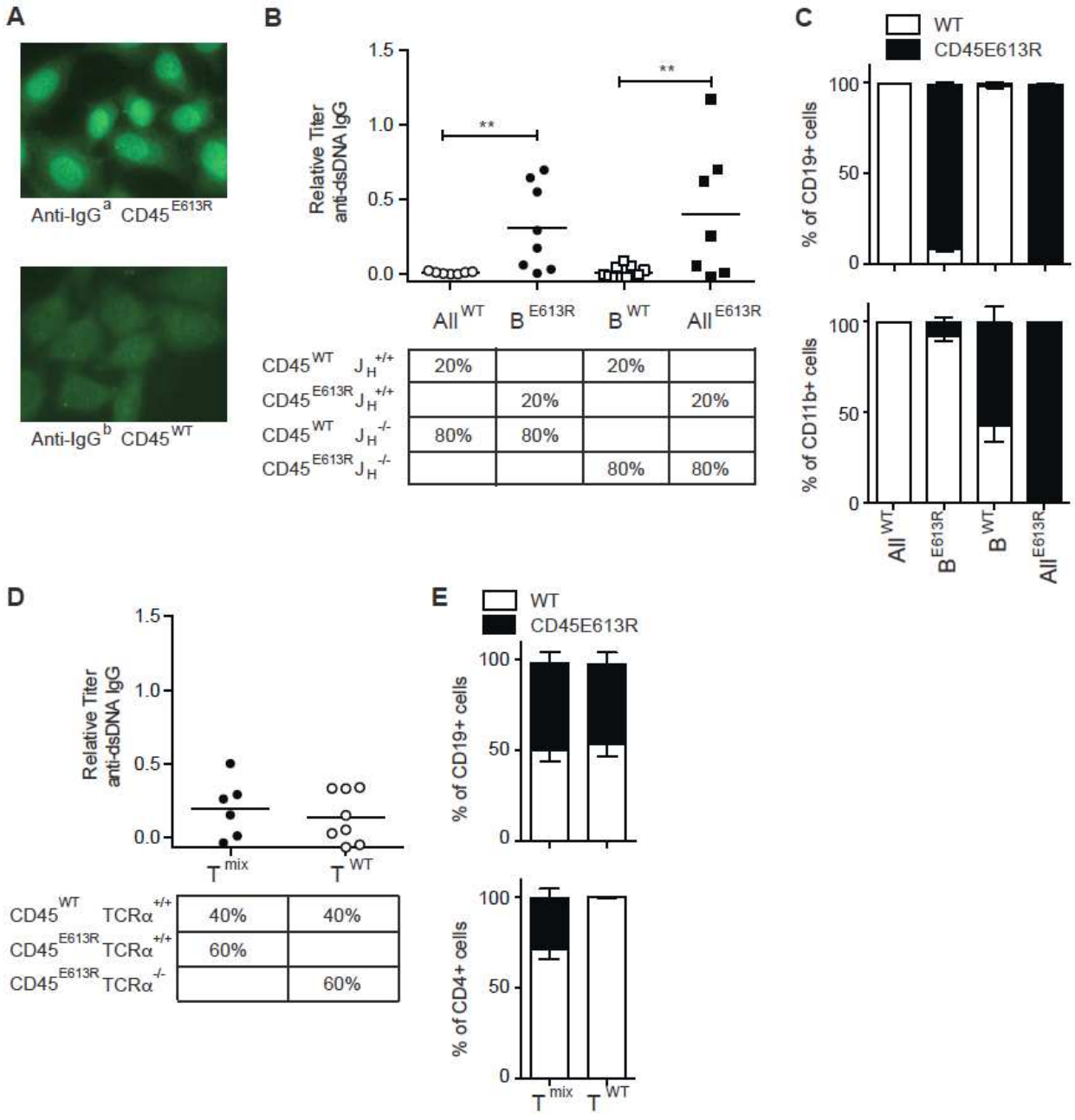

Figure 2. B cell intrinsic CD45E613R is necessary for ANA in CD45E613R.BALB/c mice.

(A) To determine whether ANA are CD45E613R B cell intrinsic, mixed bone marrow chimeras were generated from IgH^{b} CD45WT and IgH^{a} CD45E613R.BALB/c donors. HEp-2 cells were incubated with recipient sera and detected by fluorescent anti-IgG^{a} or anti-IgG^{b} secondary antibodies. Representative ANA staining pattern (original magnification 40X) of 1:40 diluted

sera is shown for two independent experiments of 5 mice. **(B-C)** Mixed bone marrow chimeras were generated using the schema in **(B, lower panel)** to examine the role of CD45E613R B cells in autoreactivity. **(B)** Anti-dsDNA IgG serum titers detected by ELISA from chimeras 24-32 weeks after marrow transfer relative to pooled 6-8 month CD45E613R.BALB/c positive control sera. **(C)** Composition of splenic B cell and monocyte populations from the same chimeras 24-32 weeks after transfer. Data are pooled from two independent experiments of 3-4 mice per condition. **(D-E)** Bone marrow chimeras were generated using the schema in **(D, lower panel)** to examine the role of CD45E613R αβ T cells in autoreactivity. **(D)** Anti-dsDNA IgG serum titers from indicated chimeras at 20-24 weeks after marrow transfer relative to pooled 6-8 month CD45E613R.BALB/c positive control sera. **(E)** Blood composition of congenically marked CD45WT (CD45.1) and CD45E613R (CD45.2) B and T cells from the chimeras in **(D)** at 8 weeks after marrow transfer. Data are pooled from three independent experiments of 2-3 mice per condition. ** $p<0.02$

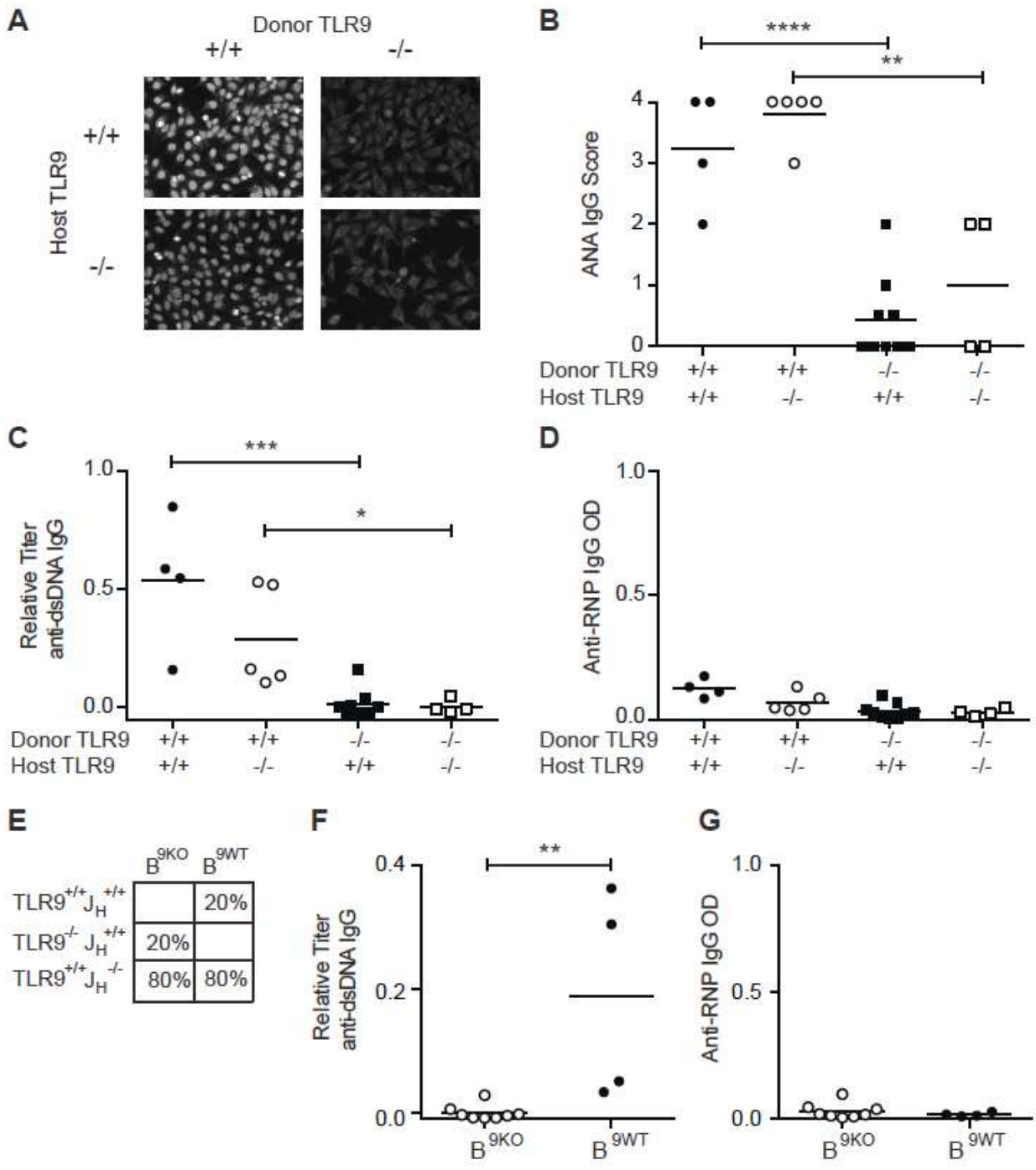

Figure 3. CD45E613R.BALB/c B cells require TLR9 for anti-dsDNA IgG production. (A-D) To test whether non-hematopoietic TLR9 contributes to ANA, $TLR9^{+/+}$ or $TLR9^{-/-}$ Actin.GFP^{+}CD45E613R marrow was transferred into lethally irradiated $TLR9^{+/+}$ or $TLR9^{-/-}$

CD45WT.BALB/c hosts as indicated. **(A)** Representative ANA staining pattern (original magnification 40X) of 1:40 diluted sera and **(B)** quantification of relative fluorescence intensity of sera from individual chimeras of the indicated genotypes**. (C)** Relative anti-dsDNA IgG or **(D)** anti-RNP IgG titers determined by ELISA as in figure 1. Data are pooled from two independent experiments of 2-4 mice per condition. **(E-G)** To test whether CD45E613R B cells require TLR9 for autoreactivity, bone marrow chimeras were generated with CD45E613R marrow of the indicated TLR9 genotypes transferred into lethally irradiated CD45WT hosts . **(E)** Scheme of mixed bone marrow chimeras and **(F)** anti-dsDNA IgG or **(G)** anti-RNP IgG serum titers from 20-30 weeks following marrow transfer. Data are pooled from three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.005$; **** $p<0.001$

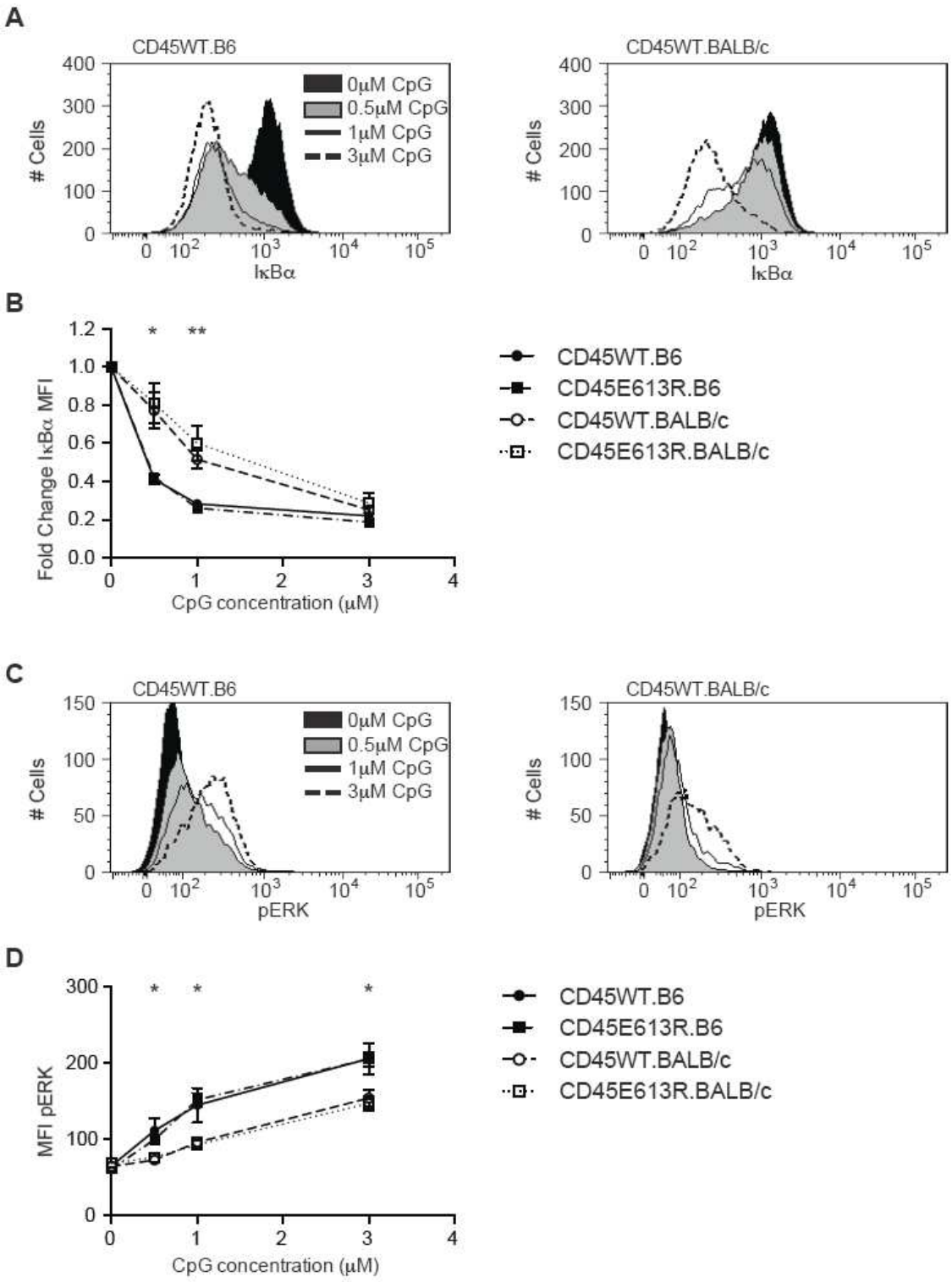

Figure 4. TLR9 signaling differs in B cells depending on genetic background. Lymph node B cells from 8-10 week old mice of the indicated genotypes were stimulated for 20 minutes with

the indicated concentration of CpG 1668, fixed, permeabilized, and stained for IκBαor pERK. **(A)** Representative histograms of IκBα expression in B220+ LN B cells following stimulation with indicated dosage of CpG. **(B)** Geometric mean fluorescence intensity (GMFI) of IκBα for each CpG dosage was divided by GMFI of unstimulated cells from the same mouse and plotted as the mean ± SD of three mice/genotype. **(C)** Representative histograms of pERK in B220+ LN B cells following stimulation with indicated dosage of CpG. **(D)** GMFI for each CpG dosage was plotted as mean ± SD of three mice/genotype. Data are representative of at least two independent experiments. * $p<0.05$; ** $p<0.01$; comparing CD45WT.B6 to CD45WT.BALB/c using student's *t* test at the indicated time point.

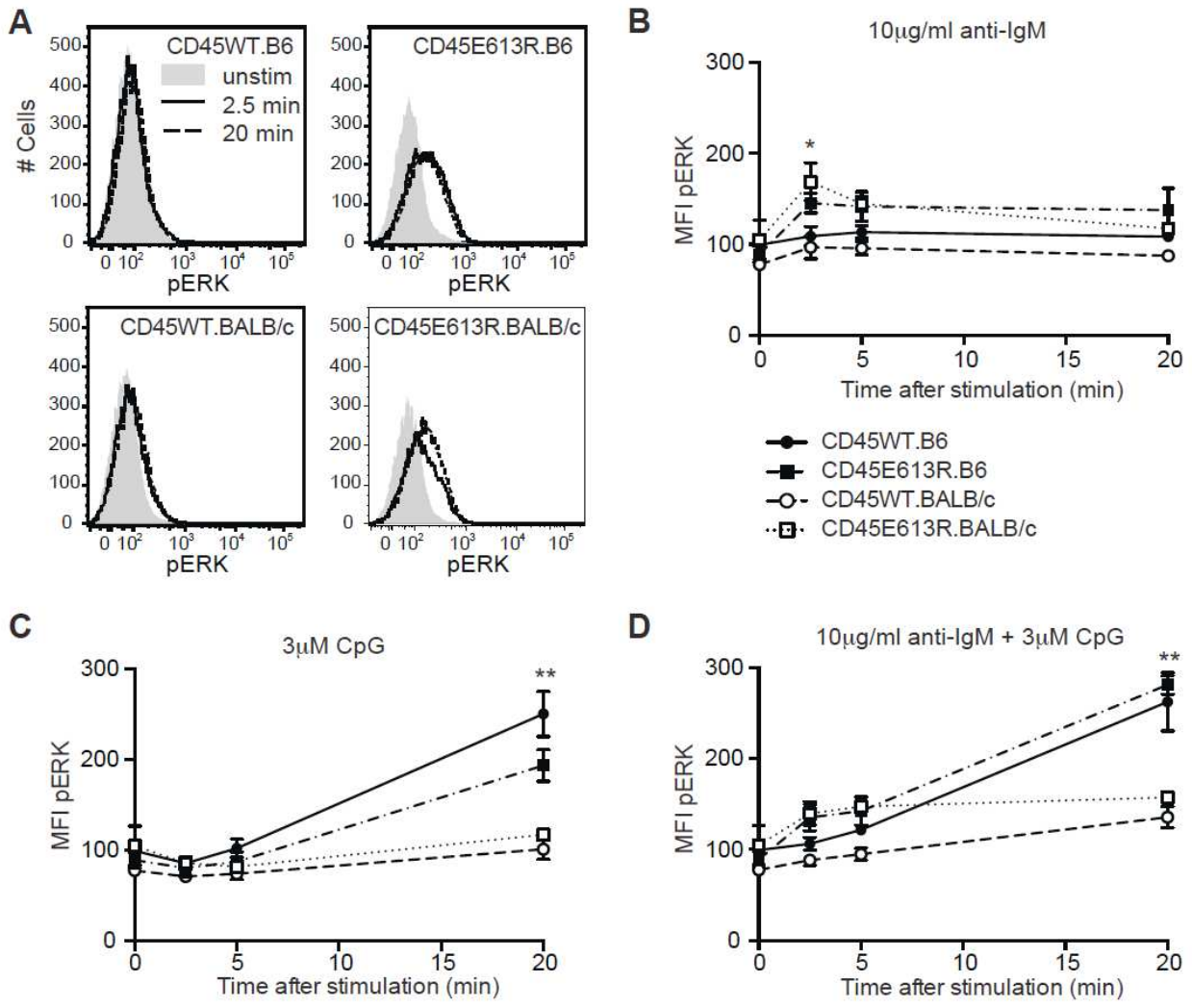

Figure 5. BCR and TLR9 co-stimulation does not alter the magnitude or kinetics of ERK phosphorylation. Lymph node B cells from 8-10 week old mice of the indicated genotypes were stimulated with 3μM CpG 1668 or 10μg/ml anti-IgM $F(ab)_2$ and stained for pERK. **(A)** Representative histograms of pERK expression in $B220^+$ LN B cells of the indicated genotypes following stimulation with anti-IgM. **(B-D)** Time course of GMFI of pERK in $B220^+$ B cells following 10μg/ml anti-IgM **(B)**, 3μM CpG 1668 **(C)**, or both **(D)**. Data points represent mean ± SD of three mice/genotype and are representative of at least three independent experiments. In **(B)**, * *p<0.05* comparing CD45WT and CD45E613R regardless of genetic background. In **(C, D)** ** *p<0.01* comparing B6 to BALB/c regardless of CD45WT or CD45E613R status.

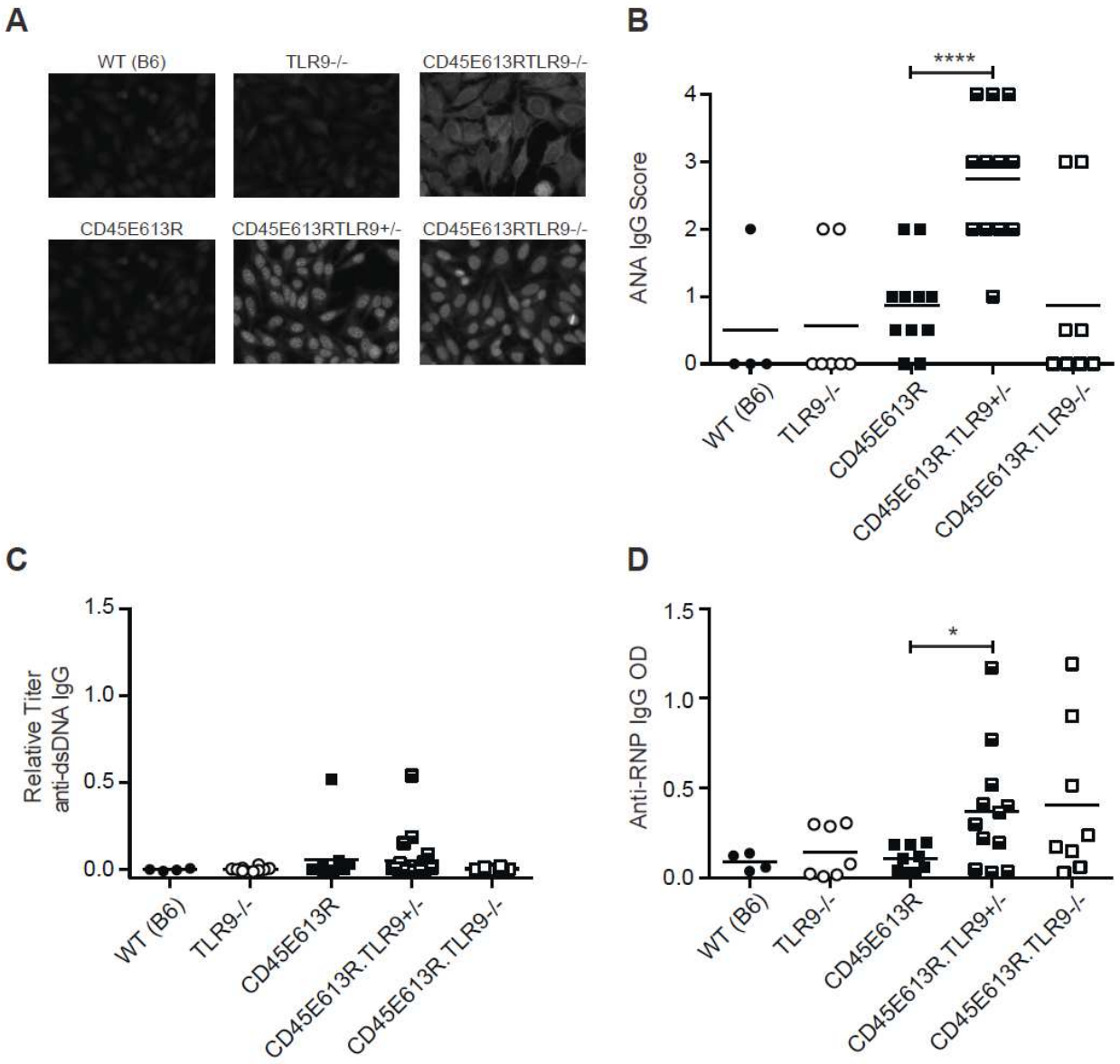

Figure 6. TLR9 protects CD45E613R.B6 mice from ANA. **(A)** Representative ANA staining pattern (original magnification 40X) of 1:40 diluted sera and **(B)** staining intensity of serum collected from 6-8 old mice of the indicated genotypes on the B6 background relative to pooled 6-8 month CD45E613R.BALB/c positive control sera. **(C)** Anti-dsDNA IgG and **(D)** anti-RNP IgG titers as assessed by ELISA from sera from the same mice relative to pooled 6-8 month CD45E613R.BALB/c positive control sera. Data are pooled from at least three independent aging cohorts. * *p<0.05*; **** *p<0.001*

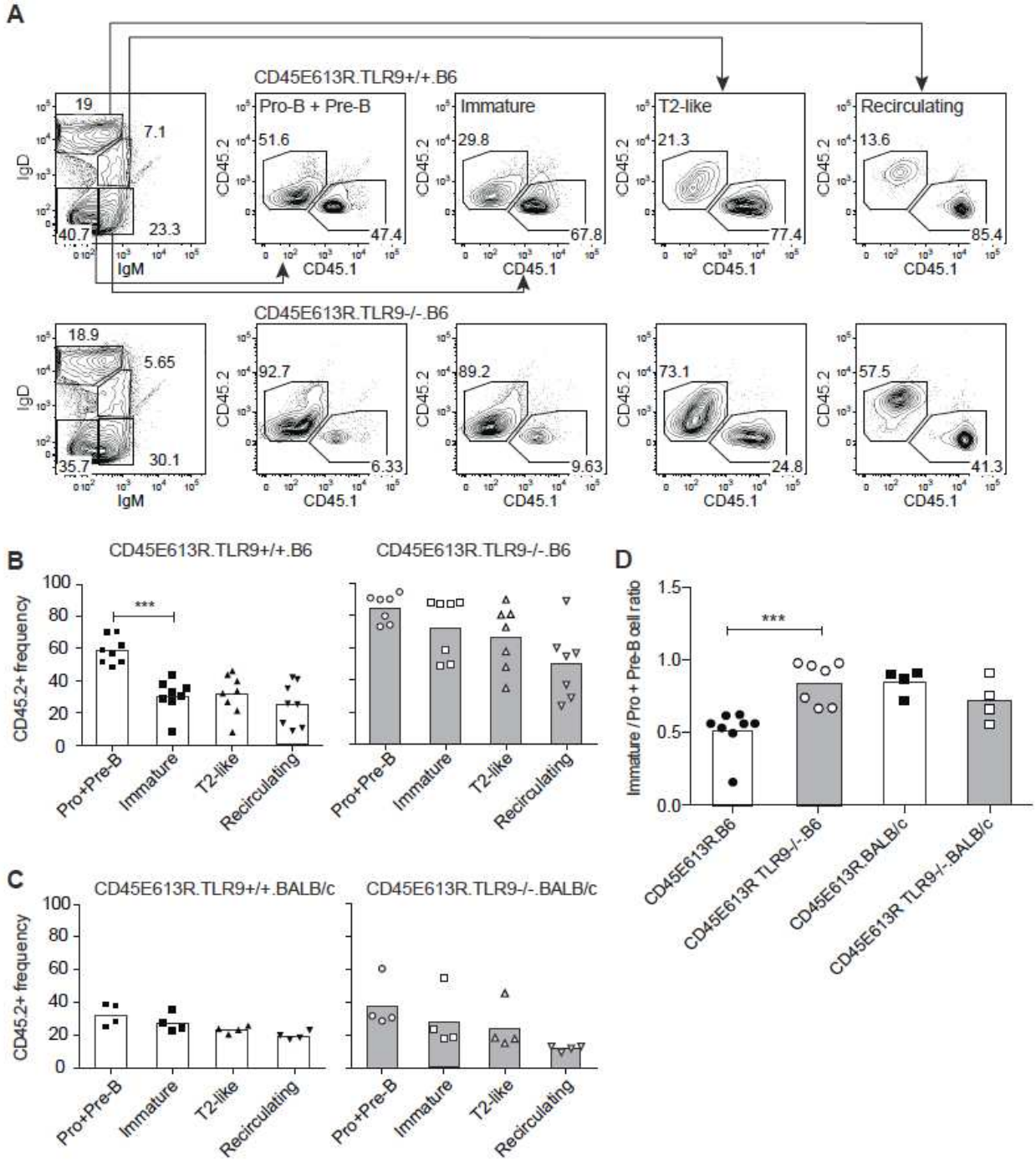

Figure 7. TLR9 negatively regulates CD45E613R.B6 but not CD45E613R.BALB/c B cell development in a competitive microenvironment. Bone marrow chimeras were generated using 40% CD45WT (CD45.1) and 60% TLR9$^{+/+}$ or TLR9$^{-/-}$CD45E613R (CD45.2) marrow transferred to lethally irradiated B6 or BALB/c CD45WT hosts (heterozygous for the CD45.1/2

congenic markers). **(A)** B cell developmental subsets in the bone marrow were defined using IgM and IgD staining of cells in the $CD19^+$ gate. The frequency of $CD45.1^+$ (CD45WT) and $CD45.2^+$ (CD45E613R) cells in the indicated B cell subset was then assessed. **(B)** Frequency of $CD45.2^+$ cells in each B cell compartment of chimeras generated using $TLR9^{+/+}$ (left) or $TLR9^{-/-}$ CD45E613R (right) marrow on the B6 background. **(C)** Frequency of $CD45.2^+$ cells in each B cell compartment of chimeras generated using $TLR9^{+/+}$ (left) or $TLR9^{-/-}$CD45E613R (right) marrow on the BALB/c background. **(D)** Ratio of the $CD45.2^+$ frequency of immature ($IgM^+IgD^-CD19^+$) to pro- plus pre-B ($IgM^-IgD^-CD19^+$) cells for chimeras of the indicated genotype. Data in **(B)** are compiled from three independent experiments, and in **(C)** from two independent experiments. *** *p<0.005*

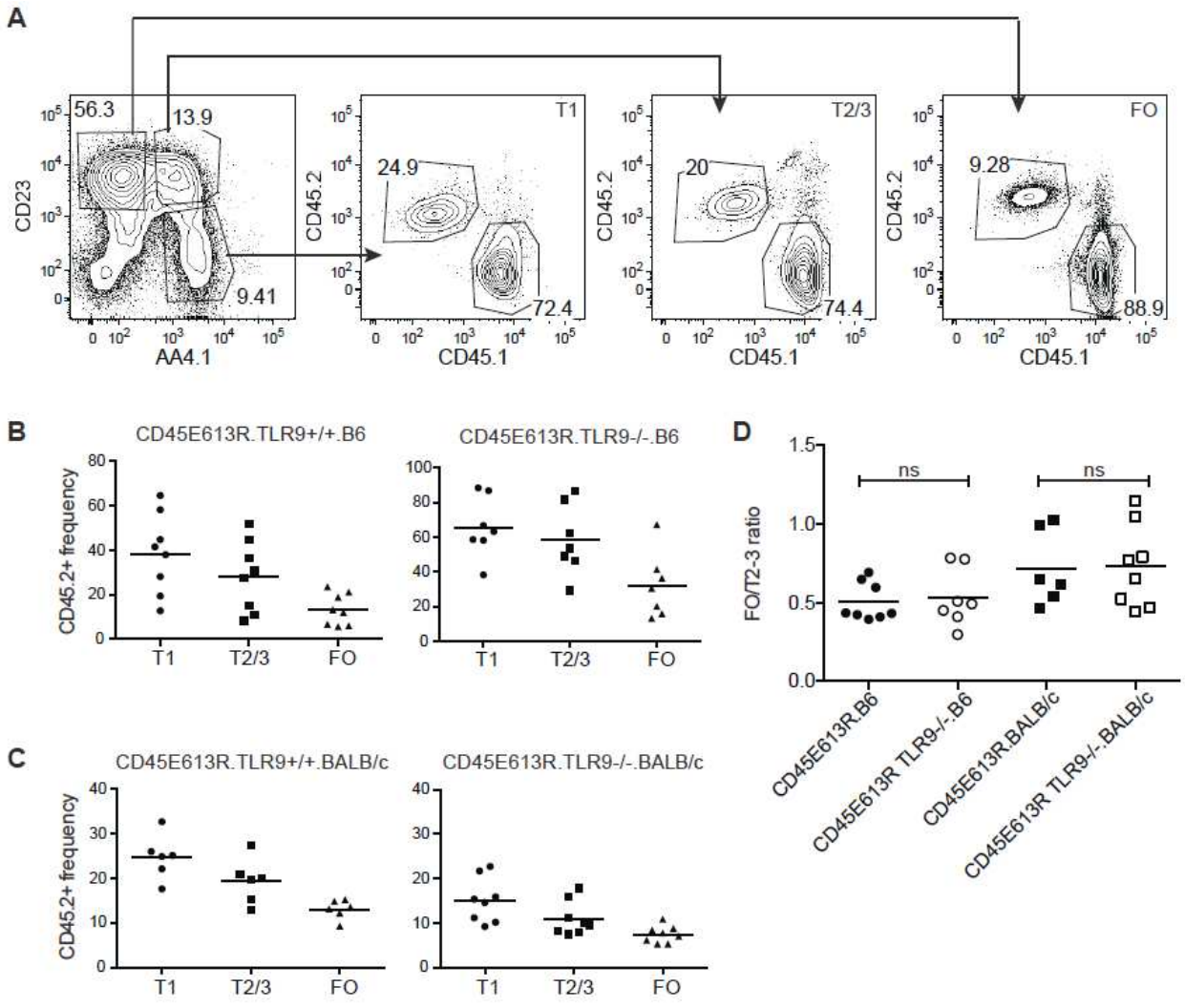

Figure S1. TLR9-independent negative regulation of CD45E613R B cell development in a competitive splenic microenvironment. Bone marrow chimeras were generated as described in Fig 7. **(A)** The CD45E613R proportion of developing B cell subsets in spleen was determined by $CD45.2^+$ frequency of cells in the indicated compartments as identified by IgM/IgD staining of cells in the $CD19^+$ gate in the spleen according to gating strategy shown. **(B)** $CD45.2^+$ frequency of the indicated B cell compartment in spleens of chimeras of the indicated genotypes on the B6 background. **(C)** $CD45.2^+$ frequency of indicated B cell compartment in spleen of chimeras of the indicated genotypes on the BALB/c background. **(D)** $CD45.2^+$ frequency of follicular (FO) compartment was divided by $CD45.2^+$ frequency of the T2/3 compartment for each chimera and

plotted as ratio. Data in **(B)** are compiled from three independent experiments, and in **(C)** from three independent experiments. * $p<0.05$.

CHAPTER III. CONCLUSIONS AND FUTURE DIRECTIONS

Dissecting the cell lineages responsible for loss of tolerance in CD45E613R.BALB/c mice

Several cell lineages have been implicated in the multi-step pathogenesis of SLE(4). Since the initial break in central tolerance and peripheral amplification steps are almost exclusively caused by perturbations to immune cells, the CD45E613R model is particularly useful because CD45 is expressed in all nucleated hematopoietic cells(16). The differences in phenotypic consequences between CD45E613R mice highlight the importance of genetic modifiers on SLE pathogenesis in the context of the same activating mutation and provide a model system in which to better understand their contribution. Furthermore, the CD45E613R.BALB/c model provides a useful system for studying the contributions of CD45E613R (and thereby dysregulated ITAM-containing receptor signaling) to SLE in several immune lineages.

Since B cells are the source of autoantibodies, B cell tolerance has been a heavily studied aspect of this disease(5). B cells from CD45E613R mice exhibit altered signaling, with elevated Ca^{2+} and Erk phosphorylation downstream of BCR engagement(19). B cell development is also altered in CD45E613R.B6 mice, resulting in decreased numbers of follicular mature naïve B cells, increased numbers of B1-a cells, and a loss of the marginal zone (MZ) B cell population(19). We have observed similar alterations in B cell development in the BALB/c background (data not shown) and signaling (Chapter 2, Figure 5). Also, regardless of genetic background, B cells from aged CD45E613R mice have elevated CD69 and CD86 levels, indicative of increased activation (data not shown). However, the phenotypic consequences of introducing CD45E613R differ between B6 and BALB/c genetic backgrounds. We sought to

determine which cell lineages require CD45E613R in BALB/c mice to better understand the break in tolerance that allows for the development of autoreactivity.

In order to class switch and differentiate into an antibody secreting plasma cell, B cells usually require help from T cells in a germinal center reaction(75). Under some circumstances B cells can class switch and produce antibodies without T cell help(76), but the prevailing view in the field is that the autoantibodies observed in patients are the result of T cell help. T cells also become inappropriately activated in SLE, contributing to disease pathogenesis(1, 14). Here we sought to better understand the contribution of αβ T cells in the CD45E613R.BALB/c model of autoreactivity through mixed bone marrow chimeras. It has been previously established that altering T cell activity can contribute to a lupus-like phenotype in CD45E613R.B6 mice. In the absence of Pep, the murine homolog of PTPN22, CD45E613R.B6 mice develop autoimmunity, presumably due to the combination of hyporesponsive T cells due to Pep deficiency and hyperresponsive B cells due to CD45E613R(22). CD45E613R also alters T cell signaling, rendering peripheral αβ T cells hyporesponsive compared to CD45WT T cells(58), albeit to a lesser degree than $Pep^{-/-}$. Interestingly, we observed ANA in bone marrow chimeras in the BALB/c background that contained only CD45WT αβ T cells, while the rest of the hematopoietic compartment was partially of CD45E613R origin (Chapter 2, Figure 2D). Furthermore, the presence of CD45WT αβ T cells did not affect autoreactivity incidence in these mixed bone marrow chimeras, indicating that CD45E613R.BALB/c B cells do not require hyporesponsive αβ T cells to break tolerance.

Inappropriate activation of myeloid cells, especially neutrophils, has been implicated in SLE disease pathogenesis as well(77). Dysregulated myeloid cells can secrete high levels of cytokines that promote the survival or activation of autoreactive B cells, promoting their break in

tolerance(62). We were surprised that the CD45E613R myeloid cell population did not expand in aged mixed bone marrow chimeras generated to examine whether CD45E613R in B cells was sufficient for autoreactivity (Chapter 2, Figure 2C). Aged CD45E613R.BALB/c mice have an expanded CD11b+ population that contributes to splenomegaly (data not shown), and we expected a similar phenotype in chimeras. However, CD45E613R CD11b+ cells were somewhat out-competed in these chimeras, perhaps by proper regulation by a population of CD45WT cells. It may be of interest to further investigate how CD45WT cells negatively regulate CD45E613R myeloid cells, and whether this contributes to autoreactivity or autoimmunity in this model.

The CD45E613R mutation can also contribute to lupus pathogenesis and cooperate with other lupus risk alleles to break tolerance and cause a severe lupus-like disease in B6 mice. When combined with the Fas^{lpr} mutation, CD45E613R caused a B cell-driven lupus like phenotype in the B6 genetic background(21). Here we show that the anti-dsDNA antibody phenotype in CD45E613R.BALB/c mice requires CD45E613R B cells, which may be sufficient for the phenotypic consequence (Chapter 2, Figure 2). Based on these observations in mixed bone marrow chimeras, the CD45E613R risk allele promotes B cell activation and hyper-responsiveness, which is sufficient for ANA production in the BALB/c genetic background but not the B6 genetic background (Chapter 3, Figure 1).

CD45E613R modulates peripheral B cell tolerance independent of genetic background

Since B cells play such an essential role in the development of autoreactivity in CD45E613R.BALB/c mice, we sought to determine whether CD45E613R differentially mediated B cell development in the resistant B6 and sensitive BALB/c genetic backgrounds. In mixed bone marrow chimeras, we find that CD45E613R impairs splenic B cell development in

the context of a competitive microenvironment. CD45E613R B cells are outcompeted by their CD45WT counterparts during splenic development in both BALB/c and B6 genetic backgrounds (Chapter II, Figure S1). This observation is consistent with recent evidence that has demonstrated that the CD45E613R mutation alters the specificity of this phosphatase for individual Src family kinases (20).

In B cells, the E613R mutation impairs CD45 interaction with and activation of the SFK Lyn. Lyn is unique among SFKs in B cells, as it can positively regulate BCR signaling, but also has a negative regulatory role(78). While Lyn's activating functions are redundant with other B cell SFKs Fyn and Blk, its inhibitory functions are unique. Following activation, Lyn phosphorylates both the ITAMs in CD79a and b, but also phosphorylates ITIM-containing receptors like CD22 and FcγRIIB. ITIM phosphorylation recruits the phosphatases SHIP-1 and SHP-1, which then dephosphorylate ITAMs and end BCR signaling(78). Without this negative regulation, BCR signaling is elevated and sustained, as observed in Lyn-deficient and, to a lesser extent, CD45E613R B cells.

Unlike CD45E613R, Lyn deficiency in B cells is sufficient for autoantibody production and end organ damage in the B6 genetic background(79). Lyn alters B cell tolerance, and deficiency for Lyn reduces follicular mature B cell numbers(80). Recent studies have demonstrated that Lyn deficiency impairs B cell maturation and survival in the spleen due in part to developmentally regulated acquisition of the CD22-SHP-1 inhibitory pathway(81, 82). SHP-1 and SHIP-1 mediated dephosphorylation of BCR ITAMs is further upregulated in germinal center B cells and thought to contribute to increasing affinity during the germinal center reaction (83). Clearly, developmental differences in the expression of negative regulators contribute to the capacity of Lyn to negatively regulate BCR signaling. It is likely that these differences contribute

to the differential consequences of BCR engagement in developing B cell subsets. Since the E613R mutation impairs the capacity of CD45 to activate Lyn and its inhibitory consequences, it is likely that CD45E613R exerts differential effects on BCR signaling depending on the maturation state of the B cell and the level of negative regulation of BCR signaling necessary to prevent autoreactivity. While we did observe a loss of CD45E613R B cells between the T2/3 and FO developmental stages in our mixed bone marrow chimeras, this loss was not modulated by TLR9. Instead, TLR9 modulated the central B cell tolerance checkpoint in the bone marrow.

TLR9 and CD45E613R cooperate to modulate central B cell tolerance

We found that TLR9 is a genetic modifier of autoreactivity between resistant CD45E613R.B6 and sensitive CD45E613R.BALB/c mice (Chapter 2, Figure 1, 6). Furthermore, TLR9 strength of signal contributes to central B cell tolerance in the context of CD45E613R (Chapter 2, Figure 4,5). In competitive mixed bone marrow chimeras, loss of CD45E613R TLR9$^{+/+}$.B6 precursors primarily occurred between the Pro + Pre-B cell subset and the immature IgM+IgD- subset. We did not observe differences in the frequencies of B cell precursors in unmanipulated CD45E613R.B6 mice upon genetic ablation of TLR9. However, autoreactive B cells in the immature bone marrow subset usually are not eliminated, but instead primarily undergo receptor editing(5). To broadly examine whether CD45E613R B cells undergo increased receptor editing compared to CD45WT B cells, we stained bone marrow and splenic B cell subsets with IgL κ and IgL λ specific antibodies and measured their frequencies by flow cytometry. We did observe increased levels of usage of the IgL λ chains in CD45E613R B cells, indicating that CD45E613R B cells undergo increased receptor editing compared to CD45WT B cells. We did not observe TLR9-mediated differences in IgL λ usage that would indicate TLR9

mediates receptor editing. However, these experiments were performed in mice with an unrestricted BCR repertoire. It is possible that the frequency of ANA-specific B cell precursors that would be selected against at this point is not detectable. It the future, it may be of interest to determine the role of TLR9 in mediating anti-DNA B cell tolerance in CD45E613R mice using the anti-DNA 3H9 and 56R heavy chain transgenic models. Previous studies utilizing BCR Tg mice specific for a neo-self antigen have demonstrated that CD45E613R B cells undergo increased negative selection in the spleen in the context of cognate antigen, at least in the B6 background(19). We hypothesized that the decreased mature B cell numbers observed in the spleen of non-transgenic mice were due to negative selection against self-antigens. With anti-DNA BCR Tg models, perturbations that are masked in the context of an unrestricted repertoire might be detectable.

In the 3H9 transgenic model, the 3H9 heavy chain generates anti-DNA antibodies when paired with most light chains. An introduced point mutation altering residue 56 to arginine (56R) generates anti-DNA B cells with higher affinity for DNA(84, 85). Previous studies have demonstrated that TLR9 mediates transgenic anti-DNA B cell development in a lupus-prone mouse model. Introducing 3H9 on MRL/lpr lupus-prone genetic background accelerates disease in a TLR9-dependent fashion(56). In fact, though TLR9 was dispensable for receptor editing, TLR9 promoted an anergic phenotype in DNA-specific B cells. However, in aged animals, TLR9 was necessary in B cells for the production of anti-DNA antibodies. In this model, an anti-idiotype antibody can detect these anti-DNA specific B cells bearing the introduced heavy chain transgene paired with the Igλ1 light chain. Interestingly, anti-DNA B cells using this receptor have different phenotypes in the MRL/lpr, MRL, and BALB/c backgrounds(86). In the BALB/c background, transgenic B cells maintained an immature phenotype, were excluded from the

follicle, and did not secrete serum anti-DNA antibodies. In contrast, MRL/lpr B cells had a mature phenotype, were not excluded from the follicle, and secreted anti-DNA IgG into the serum. Interestingly, in the absence of the Fas^{lpr} mutation, MRL-background anti-DNA B cells were excluded from the follicle and did not produce serum anti-DNA IgG, but had a mature phenotype(86), indicating that other genetic modifiers prevent the maturation of Ag-experienced anti-DNA B cells between the MRL and BALB/c backgrounds. Therefore it would be of interest to sequence $TLR9^{MRL}$ to assess whether it is more similar to $TLR9^{B6}$ or $TLR9^{BALB/c}$ and might contribute to the genetic differences that dictate these altered phenotypic consequences.

The higher affinity 56R anti-DNA transgene model is also sensitive to genetic modifiers in the non-lupus prone backgrounds examined here, where the 56R transgene undergoes effective tolerance in BALB/c mice but not in B6 (83). This is due to acquisition of an editor light chain in the BALB/c but not B6 background. As a consequence 56R.B6 B cells are not efficiently excluded from the follicle, leading to inappropriate activation and autoantibody production. In the B6 background, SLE-associated loci can accelerate 56R-mediated disease, as demonstrated by *Sle2*.56R.B6 mice(87). Like CD45E613R, the *Sle2* locus is associated with abnormal B cell differentiation, promoting B1a development or expansion in the B6 background(19), so one might predict that CD45E613R.56R.B6 mice might have accelerated disease similar to *Sle2*.56R.B6 mice. Furthermore, it may be of interest to examine how the phenotypic consequences of CD45E613R are modulated by introduction of anti-DNA heavy chain transgenes of differing affinity. If CD45E613R alters tolerance by increasing BCR signal strength, one would predict that introducing the 3H9 heavy chain into CD45E613R.BALB/c mice would phenocopy the increased editing observed in 56R.BALB/c mice.

This model system might also be useful in dissecting how TLR9 and BCR-induced signals are able to alter B cell development. Here, we establish differences in the kinetics of Erk phosphorylation downstream of activation via the BCR and TLR9 in mature naïve B cells with an unrestricted BCR repertoire (Chapter 3, Figure 1). It is possible that these kinetically distinct waves of Erk phosphorylation represent different pools of Erk within the B cells. Activation of different pools of Erk via different mechanisms is thought to result in different cellular outcomes. For example, in bone marrow immature and transitional B cells, Ca^{2+} mediated Erk activation downstream of BCR activation leads to apoptosis, whereas DAG-mediated Erk activation downstream of BCR activation in mature B cells leads to proliferation and survival(88). Clearly BCR-mediated activation of Erk via different mechanisms leads to different cellular outcomes depending on the maturation state of the B cell being activated. If the kinetic differences between BCR and TLR9-mediated Erk activation correspond to different mechanisms of Erk activation, they may have different functional consequences. Furthermore, it will be of interest to examine whether immature and transitional B cell subsets have kinetic differences in Erk activation that might represent different mechanisms of Erk activation, leading to different functional outcomes in less mature cells.

We observed differences in signaling outcomes between mature follicular naïve B cells from B6 and BALB/c genetic backgrounds despite similar expression levels of intracellular of TLR9 protein (Chapter 3, Figure 1). However, according to the ImmGen database, TLR9 is highly upregulated at the immature stage of B cell development in the B6 genetic background(67). This corresponds to the stage in which we observe a TLR9-mediated enrichment of CD45WT.B6 cells at the expense of CD45E613R.B6 cells in competitive bone marrow chimeras. Since a similar loss of CD45E613RTLR9$^{+/+}$.BALB/c precursors was not

observed, it will be of interest to examine whether TLR9 expression is similarly upregulated in immature bone marrow B cells in the BALB/c genetic background.

Allelic variance of TLR9 from B6 and BALB/c

Between $TLR9^{B6}$ and $TLR9^{BALB/c}$, there are five amino acid substitutions (B6/BALB/c): 325 T/N, 378 L/S, 573 T/A, 579 Q/H, and 867 T/A. Four of these polymorphisms map to the leucine rich repeats in the ectodomain of TLR9, and the 867 T/A polymorphism maps to the cytoplasmic domain, just upstream of the TIR domain. The TIR domain is the primary signaling domain of TLR9, mediating recruitment of the adapter MyD88, which in turn recruits IRAK4 and leads to NF-κB and MAPK signaling(25).

Comparison to the published human sequence in NCBI demonstrated that three of the five BALB/c polymorphisms are synonymous with the human sequence: human 377S and BALB/c 378S, human 572A and BALB/c 573A, and human 867A and BALB/c 867A. The human 324T corresponds to B6 325T, and the human 578R doesn't correspond to either B6 579Q or BALB/c 579H. However, a human polymorphism occurs at residue 578, where a missense mutation changes the R to an H, rendering this polymorphism similar to the BALB/c allele. Overall, the published human TLR9 sequence more closely resembles the BALB/c variant of murine TLR9 and no other variants in human TLR9 reported by the 1000 genomes map to the polymorphisms found in $TLR9^{B6}$ and $TLR9^{BALB/c}$. Furthermore, the murine polymorphism in the cytoplasmic domain, 867 A/T has a synonymous SNP in humans and is adjacent to three synonymous variants in humans: 868L, 870Y, and 873F. All three of these residues are conserved between human and murine TLR9 as well, which may indicate functional importance

of this segment of TLR9. It will be of great value to compare the individual contributions of the B6 polymorphisms to the increased NF-κB and Erk activation we observed in murine B cells.

Experiments are underway to determine which these polymorphisms affect signaling. An obvious candidate for mediating differential signaling is the cytoplasmic 867 T/A polymorphism. Since this residue is adjacent to the TIR domain, which is responsible for recruiting the downstream signaling adapter MyD88 and initiating signaling, substituting a polar residue for a non-polar residue might alter the TIR-TIR interaction between TLR9 and MyD88(25). MyD88 recruitment to TLR9 following stimulation could be measured *in vitro* by immunoprecipitation of a C-terminal epitope tagged TLR9 allele and Western blot for recruited MyD88.

TLR9-mediated interaction with UNC93B1 is also mediated in part via the intracellular domain. Residue 34D in UNC93B1 localizes to N-terminal cytoplasmic tail, and altering this residue to an alanine alters the balance of TLR7 and TLR9 trafficking from the endoplasmic reticulum to the endosome, favoring TLR7 association at the expense of TLR9(42). If the TLR9 867 T/A polymorphism affects the capacity of TLR9 to associate with UNC93B1, proper trafficking of TLR9 to the endosome upon activation may be impaired. This could be examined by fluorescent microscopy using a C-terminal epitope tagged TLR9 allele and organelle specific markers.

If the TLR9 polymorphisms in the ectodomain of TLR9 alter signaling, they are most likely to alter ligand recognition, but could also alter TLR9 interactions with the cathepsins and asparagine endopeptidase that are important for ectodomain cleavage in the endosome(30, 32). The polymorphisms 325 T/N and 378 L/S are located in the N-terminal fragment that is cleaved from full-length TLR9, and 573 T/A and 579 Q/H are retained in the C-terminal fragment (29, 31). Recent evidence demonstrated that the N-terminal cleavage product associates with the C-

terminal receptor upon cleavage and both fragments are necessary for proper TLR9 signaling (89), so any of the ectodomain residues might alter ligand interaction and TLR9 activation. It will be of interest to determine how each of these polymorphisms affects signaling.

Balancing of TLR9 and TLR7 signaling restrains SLE phenotypes

Recent studies have demonstrated an important role for TLR9 in the restriction of TLR7-mediated pathology(53-55). However, two of these studies were performed in the B6 genetic background, and the other in the MRL/lpr background. In one of these recent studies, TLR8$^{-/-}$ TLR9$^{-/-}$B6 mice were found to develop TLR7-mediated lupus like phenotypes in the absence of other lupus-associated loci (55). These authors also observed partial lupus-like phenotypes in TLR9$^{-/-}$B6 mice. We also observed some low titer anti-RNP IgG in the serum of aged TLR9$^{-/-}$B6 control mice, but did not observe similar phenotypes in the BALB/c genetic background. Perhaps the two murine alleles have differential effects on UNC93B1-mediated restriction of TLR7.

Another possibility is that TLR7 differs between B6 and BALB/c mice. However, no sequence for a BALB/c allele of TLR7 has been reported, and the SNPs in the region of the X chromosome that contains TLR7 do not distinguish B6 and BALB/c. It may be of interest to sequence *Tlr7* from these two strains to determine whether similar allelic variations are present between the two strains that might account for the observed differences in TLR7-mediated autoantibody production in the absence of TLR9. Also, since the BALB/c allele more closely resembles human TLR9, it may be of interest to examine whether TLR9 restricts TLR7-mediated pathology in the BALB/c genetic background, perhaps by introducing the *Yaa* autoimmune locus that contains duplication of TLR7(45).

Human implications

Despite strong evidence of the essential role of B cells in SLE, B cell depletion therapy using rituximab did not achieve primary or secondary endpoints in the LUNAR or EXPLORER trials (6). This result was particularly puzzling due to the reports of effective use of rituximab in patients with SLE refractory to conventional immunosuppression. However, various open-label trials have provided evidence that rituximab can be useful in refractory SLE with either renal, CNS, or hematological involvement(90), indicating that B cell depletion therapy should not be abandoned as a possible therapeutic avenue. Interestingly, patients of African American/Hispanic ancestry receiving rituximab in EXPLORER had a slight improvement over placebo at the primary endpoint, with an observed major clinical response in 13.8% and a partial clinical response in 20.0% of treated patients compared to 9.4% and 6.3% of placebo treated patients, respectively(91). Perhaps genetic differences in these groups predispose them to better responses to B cell modulation therapeutics. Improved understanding of possible subgroups of SLE patients and their response to therapy may be of clinical benefit.

Studies of human B cells have demonstrated two checkpoints that prevent the escape of autoreactive naïve B cells: a central checkpoint in the bone marrow, where polyreactive B cells are eliminated, and a peripheral checkpoint in the spleen, where nuclear-reactive B cells are eliminated(72). Humans deficient in MYD88 and UNC93B1 have defects in purging polyreactive B cells at the central B cell tolerance checkpoint(57, 72). However, these patients also do not develop serum ANA despite defects in elimination of autoreactive B cells during development, demonstrating the essential role for TLRs in activation of autoreactive human B cells(57). In contrast, the recently approved anti-BAFF agent belumimab is thought to modulate the transitional B cell checkpoint in the spleen(7). Since BAFF is primarily produced by stromal

cells independent of the number of maturing B cells present, high levels can promote the inappropriate survival of transitional B cells that would otherwise perish. Administration of belimumab following B cell depletion therapy has also been proposed to alter the microenvironment in which newly developing B cells are developing to repopulate the B cell compartment(7). The expression pattern of receptors for BAFF and a family member, APRIL (A Proliferation Inducing Ligand), has made modulation of these B cell survival and growth factors an attractive target for new therapeutics(7).

Signaling from receptors for BAFF can also alter TLR expression, indicating that a potentially complex interplay between these signaling cascades and BCR stimulation might alter B cell tolerance in humans and be an attractive therapeutic target. Modulation of endosomal TLR activity is an attractive therapeutic avenue, since many patients are treated with the anti-malarial drug hydroxychloroquine, which inhibits endosomal acidification and the activation of endosomal TLRs like TLR7 and TLR9. Activation of these TLRs leads to increased type I IFN production and inhibits the activity of glucocorticoids in disease(73). More specific TLR7 and TLR9 antagonists are being studied as possible therapeutics to potentiate the activity of glucocorticoids(74). However, our results suggest that the impacts of potential new therapeutics that alter TLR activity on B cell development should also be examined.

Another approach currently being pursued is modulation of BCR signaling via activation of CD22 with the monoclonal antibody epratuzumab(8). This negative regulator of BCR signaling is thought to be activated by the monoclonal antibody, promoting B cell tolerance. It is clear that improved understanding of B cell development will be essential to therapeutic design strategies in the future, so a greater understanding of the dual nature of TLR9 signaling on

autoreactive B cell development and activation should contribute to improved therapeutic strategies.

Summary

ANAs are important mediators of disease in the autoimmune disease SLE. Here we have demonstrated that TLR9 is a genetic modifier of ANA production in the context of dysregulated CD45 signaling in a murine model of SLE. We further demonstrate that B cell intrinsic CD45E613R and TLR9 are necessary for autoreactivity in a sensitive genetic background. However, elevated TLR9 signal strength can negatively regulate autoreactivity by modulating central tolerance in a resistant genetic background. Our results should provide improved understanding of how nucleic-acid sensing TLRs can alter B cell tolerance and highlight a B cell-intrinsic role for strength of TLR9 signal in modulating tolerance to nuclear antigens.

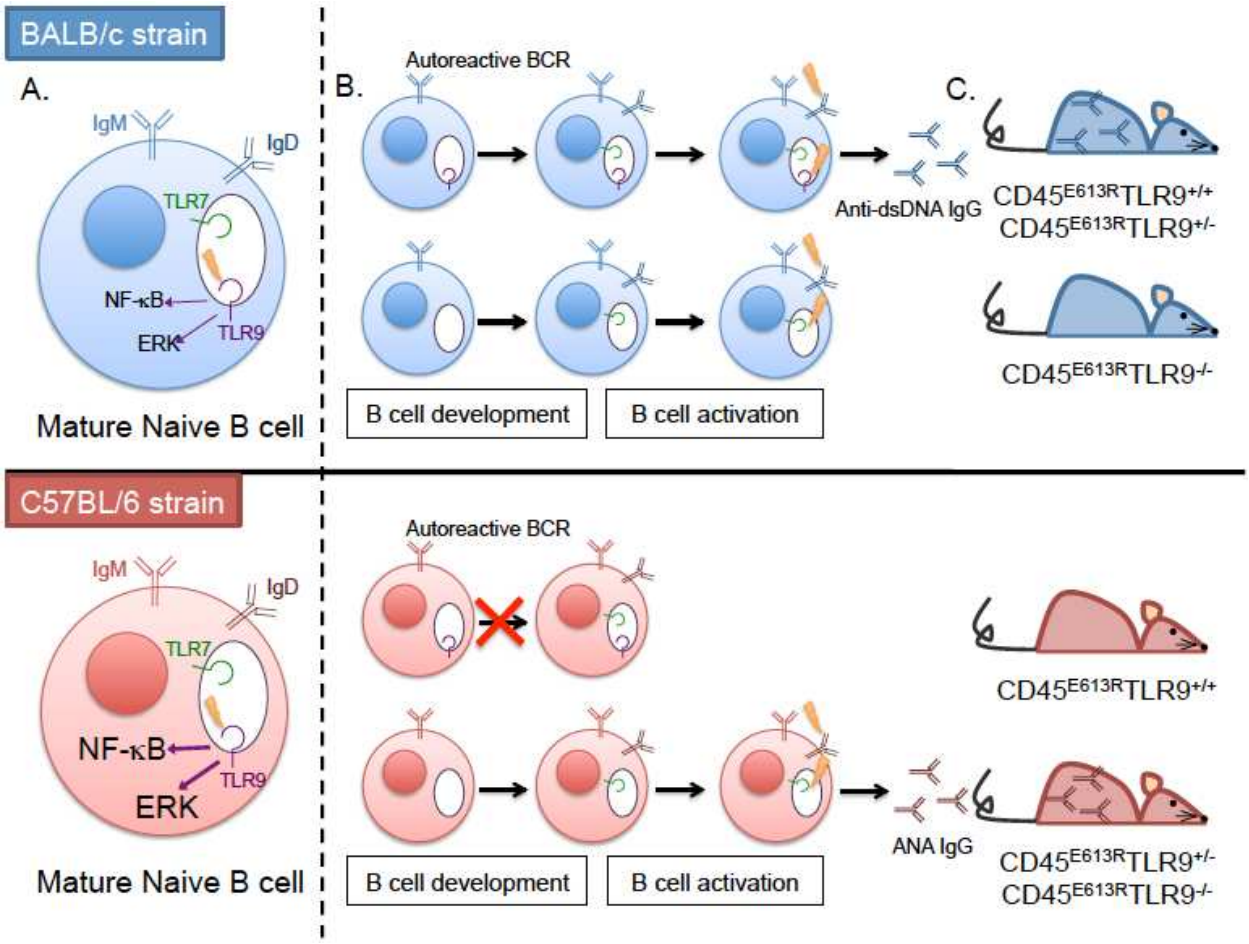

Figure 1. Signal strength determines role of TLR9 in B cell development and ANA production. **A.** Stimulation via TLR9 in B cells from B6 mice results in increased NF-κB signals and increased Erk phosphorylation. **B.** TLR9 negatively regulates bone marrow B cell development in the B6 and not BALB/c background in the context of hyper-responsive IgM signals. **C.** Altered B cell selection permits ANA in TLR9 sufficient CD45E613R.BALB/c, but not in TLR9 sufficient CD45E613R.B6. In contrast, elimination of one or both copies of TLR9 in CD45E613R.B6 permits ANA.

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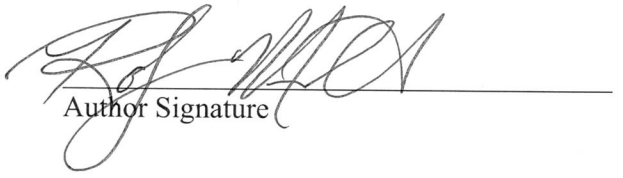

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