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Allelic variants alter distinct stages of SLE pathogenesis in the CD45E613R murine model of SLE

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Allelic variants alter distinct stages of SLE pathogenesis in the CD45E613R murine model of SLE

by

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ALLELIC VARIANTS ALTER B CELL TOLERANCE CHECKPOINTS IN THE CD45E613R MURING MODEL OF SLE

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ABSTRACT

Systemic lupus erythematosus (SLE) is a highly heterogeneous and prevalent autoimmune disease. The development of anti-nuclear autoantibodies (ANAs) is a hallmark of SLE and many patients will go on to develop glomerulonephritis (GN), a potentially fatal destruction of the kidneys. In the CD45E613R murine model of SLE, a single point mutation in the juxtamembrane wedge region of the protein tyrosine phosphatase CD45, results in hyperactivated immunoreceptor tyrosine-based activation motif (ITAM) signaling. Interestingly, despite the mutation functioning biochemically identically, different strains expressing the mutation result in different phenotypic consequences. CD45E613R B6 and 129 mice develop normally, while CD45E613R B6-129 F1 mice develop double stranded (ds)DNA autoantibodies, lymphoproliferative disease (LPD) and GN. CD45E613R BALB/c mice develop dsDNA autoantibodies, slight LPD, and no GN. This indicated genetic modifiers on these backgrounds modulated disease phenotype, and a single nucleotide polymorphism (SNP) screen performed between B6 and BALB/c mice for the development of ANAs identified two susceptibility loci. One locus, termed *Wedge Associated Modifier (Wam) 1* on the BALB/c background identified *tlr9* as the putative modifier gene. The second locus identified on the B6 background corresponded to the MHC H2 region.

Here we examine the role of these two susceptibility regions in the CD45E613R BALB/c and B6 models of SLE. Previous studies from our lab identified two alleles of TLR9, the B6 ($TLR9^{B6}$) and BALB/c ($TLR9^{Ba}$) alleles, hypothesized to result in the signaling

difference observed between the two backgrounds. In CD45E613R BALB/c TLR9-/- mice, B cell expression of CD45E613R and TLR9 is required for ANA development. Interestingly, CD45E613R B6 TLR9-/- mice acquired ANA development, suggesting the two alleles are regulating ANA and B cell tolerance in opposing ways. In Chapter II, we developed TLR9 congenic mice on the B6 and BALB/c backgrounds with the CD45E613R polymorphisms to determine if signaling difference and autoantibody development observed in intact mice were intrinsic to the TLR9 alleles. We show here that the $TLR9^{Ba}$ allele positively regulates autoreactive B cell development and is required for the development of ANAs, GN, and results in hyperresponsive B cell activation upon stimulation resistant CD45E613R B6 mice. Furthermore, the $TLR9^{B6}$ allele negatively regulates ANA production, resulting in tolerance in susceptible CD45E613R BALB/c mice. The TLR9 alleles also dictate the signaling differences, such that the $TLR9^{B6}$ allele results in hyperresponsive signaling and the $TLR9^{Ba}$ allele results in hyporesponsive signaling, regardless of genetic background, potentiating the differences in disease phenotypes. Together, these data demonstrate the dual role two allelic variants of TLR9 plays in regulating B cell tolerance.

In Chapter III, we determine the role of the B6 susceptibility loci, MHC $H2^{b}$ (MHC^{B6}), in the development of pathogenesis in the CD45E613R BALB/c model. We hypothesized the protective endogenous BALB/c MHC allele was preventing development of the severe GN observed in the CD45E613R B6-129 F1 model. In order to test this, we developed congenic CD45E613R BALB/c mice congenic for the MHC H2 region. CD45E613R BALB/c MHC^{B6} mice developed accelerated autoantibody production compared to CD45E613R BALB/c mice and eventually GN upon aging. B cells from CD45E613R BALB/c MHC^{B6} mice became dysregulated during development and were hyperactivated upon stimulation compared to CD45E613R BALB/c B cells. Further investigation revealed

the B6 MHC $H2^b$ allele resulted in increased MHC class II expression on the surface of B cells compared to the BALB/c MHC $H2^d$ allele, but not other cell populations prior to disease onset. These data demonstrate one mechanism by which MHC susceptibility alleles identified in autoimmune patient populations are required for disease progression from autoreactivity to autoimmunity. Taken together with the TLR9 studies, we hypothesize the MHC^{B6} allele, in cooperation with the $TLR9^{Ba}$ allele and the sensitizing CD45E613R mutation results in the severe progression of autoimmunity in the CD45E613R B6 $TLR9^{Ba}$ model.

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CHAPTER I: INTRODUCTION

Systemic lupus erythematosus: Autoreactivity to Autoimmunity

Systemic lupus erythematosus (SLE) is a chronic, and often devastating autoimmune disease that affects over 250,000 people in the United States[1]. Twin studies have demonstrated that SLE has both a genetic and environmental component, as only approximately 25% of monozygotic twins, and fewer than 2% of dizygotic twins are concordant for the disease[2]. While there are clearly some heritable genetic mutations that confer SLE, such as patients with identified defects in the complement pathway or the DNase TREX1[3], these genetic mutations only account for a small minority of SLE patients. Newer techniques such as genome sequencing and GWAS studies have failed to determine a single gene or group of genes, other than the HLA loci [4], whose risk alleles predispose to SLE in a large proportion of patients. The stochastic and heterogeneous nature of SLE manifestation and penetrance supports a model of multigenic contributions to disease pathogenesis[5-9]. It has been postulated that, similar to cancer models, autoimmune diseases develop through multiple cooperating "hits" to the immune system that are required to breach self-tolerance, resulting in the development of autoantibodies, deposition of C3 and immunoglobulins in target organs, and immune-mediated end organ damage[10]. Although there is consensus that multiple genes interact to influence SLE, how and by what specific mechanisms this occurs is less certain.

The current working model in the field is that the pathogenesis of SLE is a multistep process[5]. The first step in SLE pathogenesis is believed to be a break in central tolerance that permits development and retention of autoreactive lymphocytes. Next, these autoreactive lymphocytes migrate to the periphery, where they become activated, differentiate, and facilitate production of autoantibodies. These first two stages are

considered autoreactivity. Autoimmunity ensues when these autoantibodies bind or deposit into tissues, precipitating tissue destruction (Chapter I, Figure 1). Genetic polymorphisms or dysregulated gene expression due to epigenetic changes in response to environmental insults could theoretically influence any step in this process. Consistent with the polygenic nature of SLE, the data in the field to date suggests that distinct mechanisms can impact each stage of SLE pathogenesis. It is still unclear which hematopoietic cell compartment drives which disease stages and phenotypes in SLE, but understanding and clarifying disease pathogenesis has critical therapeutic implications for development of directed therapies and precision medicine approaches.

Most attention has focused on the role of the adaptive immune system in SLE pathogenesis. The role of T cells in SLE is controversial. Some studies have found that T cells of SLE patients are characterized by general polyclonal hyperactivation[11, 12] and abnormal T regulatory cells[13]. Other studies have demonstrated that T cells of SLE patients are hyporesponsive to TCR stimulation and exhibit defective ERK pathway signaling[14, 15]. Upon ex vivo stimulation, SLE patients T cells have also been shown to have altered cytokine production[11]. As dsDNA autoantibody production is a hallmark of SLE, B cells are thought to play a central role in SLE pathogenesis at both the initial autoreactive and autoimmune stages of disease. Supporting this, B cell receptor (BCR) signaling is reportedly enhanced in SLE patients, demonstrated by increased Ca^{2+} flux and downstream signaling[16]. Additional alterations in this pathway have been identified, such as decreased protein tyrosine kinase Lyn [17].

SLE patients fail to delete autoreactive B cells, instead allowing for their proliferation and expansion [5, 18, 19]. Studies in SLE patients have also demonstrated defects in central and peripheral B cell tolerance mechanisms, revealing the presence of two

checkpoints intended to delete autoreactive B cells during development [5, 18, 20, 21]. The first checkpoint, termed the central checkpoint, occurs in the bone marrow as B cells transition between the preB and immature B cell stages (Chapter I, Figure 2). SLE patients have been shown to have a defect in clearance of autoreactive B cells at this tolerance checkpoint, allowing a much higher proportion of B cells to pass through to the periphery than healthy human controls [18]. The second tolerance checkpoint occurs in the periphery, termed the peripheral checkpoint, between peripheral transitional/new emigrant B cell and follicular/marginal zone B cell development. SLE murine models have also been found to have defects in this checkpoint as well[5, 22]. Together, these data suggest that multiple concurrent mechanisms during B cell development may allow for the aberrant development of autoreactive B cells, and for their expansion in the periphery.

Based on these data, it was surprising that the efficacy of T- and B- cell directed therapies such as anti-CD40L and CTLA-4-Ig [23, 24] or anti-CD20 [19, 25] has been limited in a majority of SLE patients. This body of literature taken together supports the notion that SLE pathogenesis is more complicated than just aberrations in the adaptive immune system. Rather, alteration of both the innate and the adaptive immune system may be required for SLE pathogenesis. Therefore, we hypothesize that the convergence of innate and adaptive immune signaling plays a fundamental role in SLE development and progression.

CD45E613R murine model of SLE

Demonstrating its critical role in immune function, deficiency of CD45 in both mice and humans results in a severe combined immunodeficiency phenotype[26]. Additionally, altered CD45 expression and function has been implicated in autoimmune disease[27]. A

point mutation, CD45E613R, in the negative regulatory juxtamembrane wedge of CD45 results in altered substrate specificity in all hematopoietic cells[28, 29]. Mice homozygous for the CD45E613R 'wedge' mutation have hyperresponsive ITAM-mediated signaling in T, B, NK, and myeloid cell lineages [28, 30, 31]. Advantages of this model are that the mutation is expressed in both the innate and adaptive cells of the immune system and that the downstream signaling networks regulated by CD45 are relatively well understood. To begin to address the hypothesis that genetic polymorphisms in the context of another mutation are sufficient to break tolerance and cause autoimmunity, we utilized a tractable genetic system, the CD45E613R mutant mouse model of SLE.

Despite indistinguishable signaling profiles in multiple inbred genetic backgrounds, the phenotypic consequences of this aberrant signaling vary dramatically with genetic background [22, 30, 31]. On the initial mixed C56BL6 (B6) – 129/Sv background the mice were generated in, approximately 50% of mice developed autoantibodies and immune complex mediated glomerulonephritis (GN). However, CD45E613R mice backcrossed 9 or more generations to the B6 or 129/Sv parental strains failed to develop high-tiers of autoantibodies or proteinuria. In contrast, CD45E613R BALB/c mice develop high titers of anti-dsDNA antibodies with 100% penetrance. While CD45E613R BALB/c mice have immune complex deposition in their kidneys, they fail to develop GN or proteinuria. Interestingly, T cells were dispensable for autoantibody production in this model while B cells were required for autoantibody production[22]. Surprisingly, F1 CD45E613R mice generated from an intercross of B6 x 129/Sv mice develop full-blown SLE-like autoimmune disease with 100% penetrant high-titer autoantibodies and severe GN. These data highlight the importance of genetic modifiers in the multi-step pathogenesis of SLE. They also suggest that distinct genes may mediate each step SLE pathogenesis. To identify potential

loci contributing to disease, a genetic modifier screen for autoantibody production was performed. Single nucleotide polymorphisms (SNPs) highlighting two loci, termed wedge associated modifier (WAM) 1 and 2 were identified (Chapter 1, Figure 3)[22]. The WAM1 susceptibility loci located to the distal end of chromosome 9 from the BALB/c background, and the gene *tlr9* was identified as the putative modifier gene. WAM2 was located on chromosome 17 from the B6 background and was identified as the MHC H2 region.

Genetics of SLE: Susceptibility and Resistant alleles of MHC (HLA) H2

Upon the advent of genome and whole exome sequencing, determining genetic aberrations in SLE patient populations has been of great interest[32]. Of note, nearly every genetic population study has identified the HLA region as important in SLE pathogenesis, with many considering it an important internal positive control [33, 34]. Specific HLA haplotypes have been associated with both decreasing and increasing risk in various ethnic populations [35]. HLA haplotypes have also been shown to positively correlate with levels of anti-Ro and anti-dsDNA autoantibodies in SLE [36, 37]. Despite the great number of overall genes that have been identified by these techniques, few subsequent studies have determined how these polymorphisms, including differences in the MHC region, contribute to disease, [38].

MHC Gene Cluster

The MHC H2 region is comprised of several immune related genes, including the MHC Class I (MHCI) and Class II (MHCII) complexes. However, several potentially immunologic relevant genes are also included within this region including the complement cascade genes complement C2, C4 and factor B, TNF family members, lymphotoxin cytokines, and other non-classical MHC genes. Means by which these genes could contribute to SLE

pathogenesis include alterations in complement production, increased cytokine production due to TNF or LTA polymorphisms, altered leukocyte maturation by the Ly6 gene cluster, altered stress responses by the heat shock protein genes, or alterations in MHCI expression. For example, decreased levels of complement C3 and C4 are associated with increased risk in subsets of SLE patients[39]. C4 deficient mice also develop a SLE-like phenotype[40, 41]. By what mechanism of action this may result in SLE is still unclear, but increased antigenic availability may be one explanation. LTA polymorphisms in an additive model showed a weak association with increased SLE risk[42], as did TNF promotor polymorphisms[43], but neither showed functional alterations in gene expression or cellular effects. Mice deficient in MHCI develop worse disease[44], implicating a protective role for MHCI, though alterations or polymorphisms within MHCI have not been widely associated with SLE.

While any of these genes could potentially be contributing to disease pathogenesis, MHCII is the candidate gene most frequently identified in studies of autoimmune disease. Antigenic availability and presentation are critical for several aspects of immune activation, and implicated in several autoimmune diseases besides SLE [33, 45]. The role of MHCII alleles in SLE pathogenesis and progression though, remains unclear and our studies aim to address some of these missing links. As B cells are critical for SLE autoantibody production, the focus of this section will be on B cells and MHCII expression, though potential MHCII contributions of the myeloid compartment in SLE are not to be discounted [46-48].

One potential mechanism of action in the development of glomerulonephritis in SLE was the discovery of increased expression in MHCII expression in renal parenchymal cells of MRL^{lpr} mice[49]. Genetic deletion of MHCII in these mice resulted in complete prevention

of nephritis development[50]. These studies underscore the importance of MHCII in SLE pathogenesis. However, the broad immunosuppression and ablation of a critical signaling molecule in immune cell development could have many potential effects that contribute to the phenotypes observed, including a failure in T cell development[51]. Therefore, studies examining modulation of MHCII expression, rather than deletion, may be of greater value in SLE models.

A role for increased MHCII-peptide presentation is also highlighted by work in other models of autoimmune disease. A Type 1 Diabetes model has shown that modulation of MHCII on the surface of B cells alters the T:B cell interaction time[21]. By decreasing MHCII levels, the authors showed that T:B cell interaction time was shortened, resulting in less efficient germinal center formation. One could hypothesize conversely that increased MHCII expression on B cell surfaces would increase germinal center formation and B cell selection efficiency. Consistent with this notion, studies in murine B cells expressing a mutated MHCII revealed that increased abundance of MHCII resulted in increased T cell-mediated selection of B cells and promoted effective germinal center responses[52]. One might hypothesize then that SLE patients with their increased T cell and B cell activation would also have increased MHCII expression levels. Work evaluating genomic transcripts of an SLE cohort revealed patients experiencing a flare also had increased B cell expression of MHCII[53]. While these studies confirm the expected phenotype, no studies have been done to test whether increased levels of MHCII on B cells predisposes to SLE or modulate disease penetrance.

Based on this literature, we hypothesize that an intrinsic increase in MHCII expression on B cells converges with another driving mutation prior to disease onset to result in progression from autoreactivity to glomerulonephritis and an overall worsening of

disease. This hypothesis is directly addressed by our work presented here, and aims to fill the gap in the field as to whether the MHC H2 allele is required for progression of disease and if increases in MHCII seen in SLE patients is a byproduct of immune activation or a predisposition to disease development.

Nucleic Acid sensing TLRs contribution to SLE

Links between autoimmunity and microbial products are currently being investigated in the context of precision medicine and microbiome studies. However, long before interest in gut flora developed, it was clear that viral and bacterial infections frequently precede diagnosis and disease flares in SLE[54]. This suggests an association between autoimmunity and the innate immune system. Studies in germ-free mice have further solidified this link. For example, germ-free mice , such as the T cell-dependent IL-1Ra-/- [55] and K/BxN models[56], are resistance to autoimmune diseases. These data support a role for microbial sensing receptors in contributing to inappropriate immune cell activation and initiation of autoimmunity.

Toll like receptors (TLRs) are innate germline encoded pattern recognition receptors present on most immune cells. Most TLRs are constitutively expressed on both mouse and human B cells[57]. Cell surface TLRs are programmed to recognize common pathogen products such as flagellin, lipopolysaccharides, and peptidoglycans. Endosomal TLRs 3, 7, 8, and 9 recognize nucleic acids and are capable of sensing viral, bacterial, endogenous retroelements, and self-nucleic acids[58-60]. They are transmembrane proteins comprised of an ectodomain containing leucine-rich repeats (LRRs) which bind ligands, a transmembrane domain, and a cytoplasmic tail containing a Toll/Interleukin-1 receptor homology (TIR) domain[61] and a chaperone binding site. Upon engagement with their

ligand, TLRs signal through an adaptor protein that binds to its TIR domain. The most common adaptor is MyD88, although TLR3 signals through TRIF, and TLR1,2, and 6 use TIRAF[62]. Downstream signaling occurs through the Interferon regulatory factor (IRF)-3, NFkB and MAPK pathways, inducing a pro-inflammatory response that includes chemokine, cytokine, and interferon (IFN) release, cell survival, and B cell proliferation[61, 63]. Dysregulation of these signaling pathways can result in both progression and amelioration of disease phenotypes, emphasizing the multifaceted roles TLRs play in autoimmunity [20, 62, 64, 65].

Studies of individuals deficient in TLR signaling molecules offer interesting clues into how TLR signaling may be influencing disease progression. Numerous risk alleles and polymorphisms within the MyD88 signaling pathway have been linked to SLE and RA [65-67], although their functional contributions are still unexplored. Patients lacking MyD88 or IRAK4, a signaling molecule downstream of MyD88, demonstrated these proteins are required for the removal of autoreactive B cells[20]. Deletion of the endosomal TLR chaperone protein Unc93B1 also showed a similar phenotype[20]. Surprisingly, these MyD88 and IRAK4-deficient patients have defective central checkpoints, but failed to develop any autoantibodies. These data also suggest that distinct genes may regulate the different steps along SLE pathogenesis. Interestingly, IRF7-/- patients also failed to develop autoimmunity, but predictably had significant defects in the Type I IFN amplification loop downstream of the TLR-MyD88 pathway upon influenza infection, resulting in severe infections [68]. This suggests that the IFN signature found in SLE patients [69, 70] may require an IRF7-mediated amplification for disease progression. This is further supported by the fact that IRF7-/- mice in a SLE serum transfer model fail to develop an IFN response

or autoimmunity compared to the IRF7+/+ controls[47]. Together, these data support a critical role for TLRs, particularly endosomal TLRs, in B cell tolerance and development.

Endosomal TLRs

Endosomal TLRs have been the focus of SLE and RA treatments for decades with the long-term first-line use of the anti-malarial drug Chloroquine and its derivative Hydroxychloroquine. The canonical mechanism of action is thought to be inhibition of endosomal acidification and thereby inhibition either directly or indirectly of TLR activation [71-73]. New studies have identified further mechanisms of action including decreased mitochondrial activity[74]. While focus is mainly on the role of TLR7 and TLR9 in B cell-mediated pathogenesis, recent studies have also highlighted the role of TLRs in the myeloid compartment [46, 75]. Of these endosomal TLRs, most attention has been focused on TLRs 7 and 9 in B cells. The ligands for TLR7 and 9 are double-stranded (ds)RNA and dsDNA, and are also the target of the most common autoantibodies in RA and SLE, respectively. However, their roles in autoimmune disease are complex as murine studies have demonstrated paradoxical roles for these TLRs in both preventing and promoting SLE.

TLR7

Several lines of evidence indicate that activation of TLR7 and increased strength of signal through this pathway play a role in mediating SLE. TLR7 has been implicated in mediating end organ damage in several murine models. TLR7 gene duplication was found to mediate the SLE phenotype of Yaa mice. This increase in TLR7 activity results in B cell-mediated dysregulation of T and dendritic cells [48, 76-78]. The B cell intrinsic effect of this duplication promoted germinal center formation and plasmablast differentiation that is

dependent on activating autoreactive T cells[79], specifically though CD40 and SAP signals[80]. This phenotype could be reversed by the overexpression of ribonucleases[81], demonstrating that augmented TLR7 activation through increased antigen availability or receptor expression can lead to inappropriate B cell activation. This activation mediates a systemic inflammatory immune activation and fatal GN in this model. Genetic deletion of TLR7 in the pristine induced SLE model prevents IFN production, autoantibodies, and nephritis [82, 83]. Moreover, inhibitory oligodeoxynucleotide (ODN) ligands for TLR7 or those cross-reactive to TLR7/9 reduce nephritis in the MRL^{lpr} model of SLE[84]. Similarly, in the K/BxN serum transfer model of RA, low dose TLR7 agonist results in hyporesponsive TLR7 signaling and prevention of autoimmunity[85]. Studies of TLR7 in human SLE patients are also suggestive. TLR7 mRNA is increased in SLE patients, and SNPs within TLR7 are associated with increased disease risk in Asian SLE populations[86].

The role of TLR7 in mediating these B cell-driven phenotypes is thought to be through TLR7-BCR signal convergence. Dual activation TLR7 and the BCR in B cells in this model promotes IL-6 production and autoreactive B cell retention [87]. This B cell derived IL-6 drives autoantibody production in BXSB.Yaa mice, presumably through the enhanced B cell TLR7-BCR signaling and is increased by type I IFN production[88]. Complete B cell activation by TLR7 ligands has been shown to require an IFN-alpha and -beta positive feedback loop, demonstrated by decreased TLR7 activation in IFNAR1-/- B cells[89] and MyD88-/- mice[90]. TLR7-BCR co-stimulation also results in IRF4 activation and pushes B cells towards plasma cell differentiation[91].

One signaling molecule potentially mediating this TLR7-BCR effect is Btk, an essential component of the BCR signaling network. BXSB.Yaa mice can be cured by genetic deletion of Btk. Btk inhibition of B cells blocks TLR7-dependent activation by immune

complexes (ICs). Treatment of BXSB.Yaa or pristane-induced DBA/1 arthritic mice with a Btk inhibitor also results in decreased autoantibody production, arthritis, nephritis, and overall mortality [92]. This TLR7-BCR costimulatory pathway was shown to be active in patients as well, as dual stimulation can lead directly to increased B cell activation and induce B cell necroptosis in SLE PBMCs, contributing to the lymphopenia phenotype that is also characteristic of SLE in some patients[93]. Together, these data present a mechanism by which TLR7 promotes autoimmunity in a B cell and Btk-dependent manner.

TLR7-TLR9 combined effects

One of the main regulatory mechanisms used by immune cells to prevent TLR7-dependent autoimmunity is through competitive binding between TLR7 and TLR9 to their shared chaperone protein Unc93B1. This can be explained by studies investigating the role of the TLR7 chaperone protein Unc93B1. Unc93B1 was shown to traffic not only TLR7, but also TLR9, from the endoplasmic reticulum to the endosome[94]. Yet, Unc93B1 preferentially binds to TLR9, restricting TLR7 protein expression[95], and thereby decreasing the potential for TLR7-induced autoimmunity. The suboptimal GC content of the TLR7 codons contributes to this regulation, as Unc93B1 binding affinity is biased towards the optimized TLR9[96]. Dysregulation of the Unc93B1-mediated balance by the D34A mutation results in loss of TLR9 affinity and triggers a B cell-intrinsic, TLR7-dependent autoimmune phenotype in mice [94, 95, 97]. Consistent with this data, knocking out Unc93B1 in MRL or BXSB mice ameliorates disease [98], indicating a driving role for TLR7 and TLR9 in disease development.

TLR9

The other endosomal TLR implicated in multiple disease processes is TLR9. In the case of SLE and RA mouse models, the development of anti-dsDNA autoantibodies requires TLR9 expression [99-101], implicating it in promoting disease development. Contrary to TLR7 though, TLR9 seems to play a dual role in SLE pathogenesis: A pathogenic role through induction of dsDNA autoantibodies, and a protective role by preventing TLR7-mediated disease[99]. Reducing TLR9 activity by the Unc93B1$^{3d/3d}$ mutation in MRL-Fas^{lpr} mice also results in decreased levels of anti-dsDNA autoantibodies and more severe GN [102]. TLR9-/- B cells alone can drive autoimmunity[79], similar to the TLR9-/- BXSB.Yaa mice[98]. Paradoxically, FcγRIIB-/- B6 mice develop autoantibodies and autoimmunity upon TLR9 deficiency[103].

TLR9 has been shown in several murine models of SLE to regulate B cell tolerance thresholds. In MRL mice, TLR9 stimulation can also restrict the survival of autoreactive B cells, by preventing TLR7 activation[101]. TLR9 is expressed much earlier than TLR7, during the proB cell stage [104], implicating a role for TLR9 in B cell selection. Any alterations to this TLR7-TLR9 balance results in a discernable effect on TLR9-mediated autoimmune phenotypes as well. In the mouse bone marrow, CpG stimulation can induce autoreactive B cell retention by preventing the negative selection-induced apoptosis of immature B cells[104]. In hyper-IgM patients and B cells from murine models of SLE, TLR9 stimulation also induces transitional B cells to undergo somatic hypermutation, increasing affinity maturation, and inducing differentiation into IgM+ memory B cells[105, 106]. In mice, this induces a B cell-intrinsic increase in germinal center output though a TLR9-mediated positive selection[107], further altering class switching to the pro-inflammatory IgG2a subclass, and increasing the memory B cell antibody-mediated response. This results in an overall increase in B cell responsiveness to antigen by murine

B cells[106]. In AM14 mice, BCR-TLR9 co-engagement depletes autoreactive B cells in the periphery. These BCR-TLR9-induced, but not BCR-TLR7-induced, autoreactive B cells can then escape deletion through a BAFF-mediated pathway[91]. Conversely, IC stimulation through a BCR-TLR9-dependent pathway induces rheumatoid factor reactive MRL, B6. Sle123, or AM14 B cells to differentiate [108-110], likely due to the continuous exposure of antigen and the cyclic generation of ICs[107]. This notion is further supported by evidence correlating strong ligand binding to the BCR with increased ability to endocytose and traffic to TLR9-containing endosomes [111-113]. These sets of data would indicate that unlike TLR7, TLR9 plays two roles: limiting autoreactive B cells during development, while simultaneously activating autoreactive B cells that have escaped deletion in the periphery. Murine studies seem to support this conclusion as TLR9 expression in MRL B cell regulates tolerance through the restriction of autoreactive B cell development, but can effectively stimulates these autoreactive B cells in the periphery[101].

Differences in TLR9 signaling potentially influencing disease outcomes also reflects work done in SLE patient populations. The strength of TLR9-BCR signaling can also be augmented by cytokines, whereby Type I IFNs can increase BCR signal strength and lower the TLR7[114] or TLR9 threshold [111], allowing retention of autoreactive B cells instead of inducing anergy or death. Numerous risk-associated polymorphisms in TLR9s promoter region have been identified in RA and SLE populations [115-117], though few have identified a functional impact on TLR9. The TLR9 G1174A polymorphism is associated with increased risk and is frequently co-inherited with the T1486C polymorphism[118]. This AT haplotype decreased TLR9 transcription, suggesting the risk associated with this haplotype may be due to decreased signal strength resulting from decreased TLR9 transcription. Additionally, B cells from SLE patients with active or severe disease upon TLR9

stimulation produced less cytokines [119], diminished signaling, and reduced TLR9 upregulation[120], indicating hyporesponsive TLR9 signaling present in SLE B cells can modulate B cell activity. Interestingly, overall increases in B cell TLR9 expression in this patient population positively correlated with kidney damage [120, 121], indicating a potential role for TLR9 in mediating end organ disease as well. This is also supported by patients with common variable immune deficiency who demonstrate decreased BCR signaling and defective TLR9 signaling, impairing B cell function[122, 123].

Additional regulatory mechanisms preventing TLR9-mediated B cell autoreactivity post development have also been demonstrated in mice. Several of these mechanisms center on limiting inappropriate ligand binding. TLR9 requires several cleavage events to occur before full activation and signaling is possible. Firstly, both TLR7 and TLR9 require cleavage within the endosome to become functional [124, 125]. Both Cathepsins and AEP are required for this cleavage event, but different cell types rely on one more than the other[126]. For instance, Cathepsin L and S are required in macrophage, but only Cathepsin L is required in B cells[127]. Upon cleavage, the N-terminus cleavage product remains associated with the C-terminus, and is required for complete TLR9 activation[128], likely by preventing further enzyme degradation of the C-terminus, while also negatively regulating homodimerization until ligand binding[129]. Upon DNA ligation, utilizing a TLR9-Fc fusion receptor, Latz et al. showed that dimerization with a stimulatory CpG induced an allosteric change in the cellular signaling portion of TLR9, decreasing the distance between what would be the two TIR domains. Importantly, this distance to closure does not occur with inhibitory CpG stimulation[130].

Further TLR9 regulatory mechanisms may limit overall antigen availability. One example is the restriction of TLR9 to the mature endosome. Forcing TLR9 to the cell

surface through a chimeric CD4-TLR9 transmembrane fusion [131], resulted in TLR9 recognition of self-nucleic acids and induction of cell activation. Consequently, these cells had significantly reduced recognition of viral DNA and a dampened response to infection [132], indicating an evolutionary advantage to endosomal localization as well. Mice expressing a TLR3-TLR9 fusion, also forcing cell surface expression, developed a severe SLE-like autoimmunity and mortality by 4 weeks of age [133]. In the MRL model, it was shown that the endolysosomes of these mice have a defect in maturation, allowing for leakage of IgG-ICs, activating TLR9[134]. Furthermore, anergic B cells which normally block antigenic accessibility to endosomes in non-susceptible strains, still traffic BCR complexes in the SLE susceptible MRL strain [113]. Defects in cellular debris clearance also result in inappropriate TLR9 activation, as accumulations of extracellular DNA can also stimulate the BCR pathway and induce autoreactivity[135]. Finally, the type of CpG content within the DNA itself results in altered TLR9 signal strength. In AM14 B cells, which express a transgenic autoreactive BCR, cells stimulated with CpG-rich endogenous DNA results in a robust TLR9-dependent signal[136]. CpG-poor ligands results in a weak signal[137] that is downregulated through FcγRIIB-dependent inhibitory signaling[136]. Together, these studies highlight the importance of antigenic accessibility, a major mechanism by which inappropriate TLR9 activation is avoided by immune cells.

Model

How genetic elimination of TLR9 can result in amelioration of autoantibodies in one model and development of disease in another may be explained by our previous work describing two allelic variants of TLR9. Knockouts of TLR9 in the CD45E613R B6 model of SLE resulted in the manifestation of autoantibodies in otherwise normal mice. However, TLR9 deficiency on the CD45E613R BALB/c background resulted in the amelioration of dsDNA

autoantibody development. This phenotype was intrinsic to B cells as phenotypes were the same, even when TLR9-/- was restricted to the B cell compartment[22], highlighting the importance of B cell TLR9 expression. These allelic differences were attributed to a difference in downstream signaling. Hyporesponsive signaling phenotype by the BALB/c TLR9 allele was postulated to positively regulate autoantibody formation, while the B6 TLR9 allele resulted in hyperresponsive TLR9 signaling, and negatively regulation of autoantibody formation. It could be hypothesized that in the 3d mutants, decreased but not abolished TLR9 signaling may mimic the signaling of the BALB/c TLR9 allele, while the TLR9-/- FcγRIIB-/- B6 mice containing the B6 TLR9 allele appears to phenocopy the CD45E613R B6 TLR9-/- mice. The BXSB.Yaa mice would presumably contain the BALB/c TLR9 allele as TLR9-/- on the CD45E613R BALB/c background ameliorated disease. Further work to confirm this hypothesis is needed. These data suggest that TLR9 signal strength may modulate disease phenotype through synergy with the BCR signaling, regulating B cell development and tolerance.

How TLR7 contributes to disease progression while TLR9 both negatively and positively regulate SLE disease pathogenesis has been an interesting paradox raised by several murine models. Multiple studies investigating modulation of BCR-TLR convergence and signal strength have revealed a model whereby strong synergy through the BCR-TLR9 signaling pathways induces deletion of autoreactive B cells and anergy [22, 99, 136], but slight alterations in signal strength can lower the threshold parameters, allowing autoreactive cells to escape death and promote autoimmunity in a TLR9- and TLR7-dependent mechanism[22, 79, 119, 120, 138-140].

SLE is a polygenic autoimmune disease with a multi-step pathogenesis[5]. Multiple common genetic polymorphisms cooperate to contribute to the heterogeneous nature of

disease symptoms[6] and may explain the difference in disease prevalence between ethnicities[141]. Human and murine model of SLE have identified the TLR9-BCR signaling pathway as significant to the development and dysregulation of B cells, resulting in autoreactive lymphocytes and autoimmunity. However, model systems in which these stages of pathogenesis can be delineated and modulated are lacking. Here we present the tractable CD45E613R model system by which contributions of common allelic variants of TLR9 and MHC H2 individually modulate each stage of disease pathogenesis.

Figures

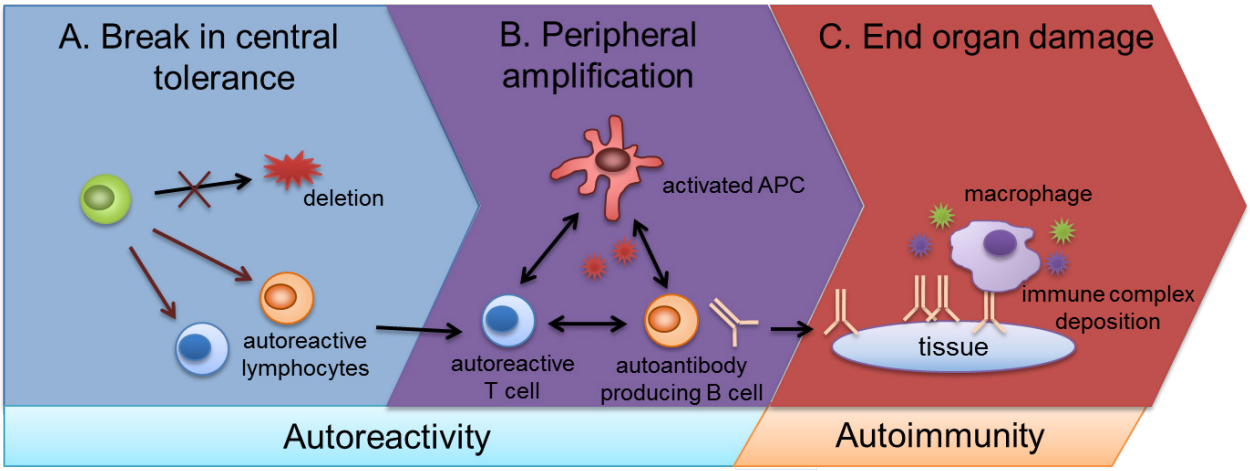


Figure 1. Multistage pathogenesis of SLE.

(**A**) Initially, autoreactive lymphocytes are allowed to develop and are retained due to a break in central tolerance. (**B**) These autoreactive lymphocytes move to the periphery where they become activated and produce autoantibodies. These first two steps are considered autoreactivity. (**C**) When these autoantibodies deposit in the target tissues, such as immune complexes in the kidneys, they are then able to mediate targeted tissue destruction.

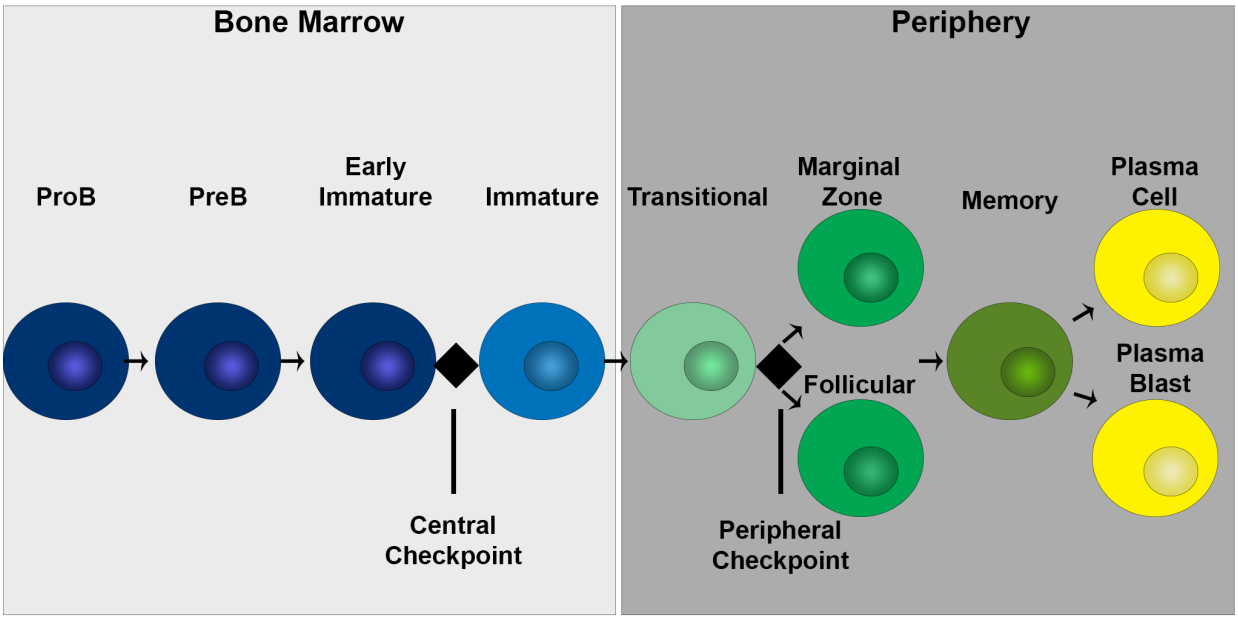


Figure 2. Tolerance checkpoints during B cell development.

At least two major checkpoints exist during B cell development to rid the population of autoreactive B cells. The first occurs during the transition from preB cells to immature B cells in the bone marrow, termed the central checkpoint. The second occurs when transitional B cells differentiate into the follicular or marginal zone B cells of the germinal center.

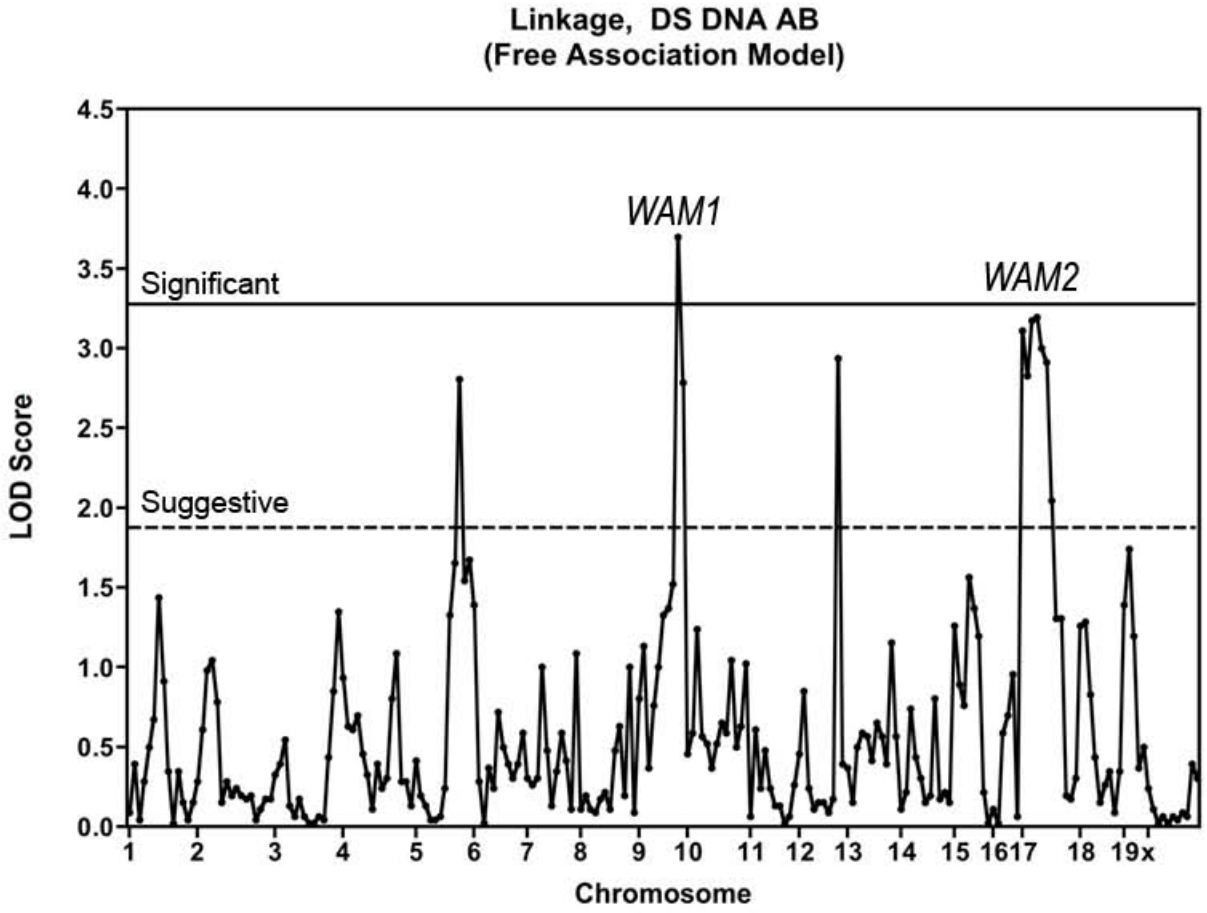


Figure 3. *Wam1* and *Wam2* susceptibility loci LOD scores.

SNP analysis for autoantibody production identified two susceptibility loci. *WAM1* corresponded to the distal end of chromosome 9, and the putative modifier candidate from the BALB/c background was identified as *tlr9*. *WAM2* was identified as the B6 MHC H2 region on chromosome 17.

CHAPTER II: ALLELIC VARIANTS OF TLR9 CONFER AUTOANTIBODY PRODUCTION AND AUTOIMMUNITY DUE TO ALTERED DOWNSTREAM SIGNALING IN A MURINE MODEL OF SLE

Abstract

Systemic lupus erythematosus (SLE) is a highly heterogeneous and polygenic autoimmune disease in which several common polymorphisms cooperate to result in disease pathology. In the CD45E613R murine model of SLE, the phenotypic outcome is modulated by background dependent genetic modifiers, similar to SLE patients. Previous work identified TLR9 as a putative modifier, and knockout experiments suggested that the B6 and BALB/c TLR9 alleles regulate tolerance in opposing ways. Here, we show through a CD45E613R congenic model that the B6 TLR9 allele negatively regulates autoantibody production, preventing autoreactive B cell development, while the BALB/c allele positively regulates autoantibody production, and is permissive for development of autoreactive B cell and glomerulonephritis. These phenotypic outcomes are due to intrinsic MAPK and NFkB hyperresponsive signaling by the B6 TLR9 allele and hyporesponsive signaling by the BALB/c TLR9 allele. This work indicates one mechanism by which common polymorphisms may be individually innocuous, but together mediate development of SLE through potentially therapeutically targetable signaling pathways.

Introduction

SLE is a common and highly heterogeneous autoimmune disease characterized by development of anti-nuclear autoantibodies (ANAs), predominantly against double-stranded (ds)DNA [142]. Despite its prevalence, little progress has been made in the

development of treatment options or in stratifying patients for more individualized therapy based on the broad array of symptoms patients may present with. Current front line treatment continues to involve broad immunosuppression, which presents several risks for patients, and inhibition of endosomal TLRs with the anti-malarial chloroquine [143]. One main challenge in treating patients has been determining who will respond to therapy and who will progress to glomerulonephritis (GN), potentially resulting in a need for kidney transplantation or even death [144].

Utilizing genetics to stratify patients has proven to be useful in the field of cancer, and since SLE has a significant genetic contribution to disease development [145], understanding how genetics influence SLE susceptibility and progression is vital to the identification of potential diagnostic and therapeutic targets. Unfortunately, the genetics underlying SLE pathogenesis are not clear for every patient. Ethnic backgrounds clearly influence risk and outcome [141], but there is no common genetic basis for this difference. Genomic studies have repeatedly failed to identify rare mutations or allelic variants that broadly contribute to SLE susceptibility [3, 146, 147], instead finding only small effect sizes often only within some patient populations. Taken together, these finding suggest a polygenic hypothesis of disease [148], whereby several common polymorphisms cooperate to produce the diversity of phenotypes and severity observed in SLE patients.

The CD45E613R murine model of SLE has a single point mutation in the juxtamembrane "wedge" region of the protein tyrosine phosphatase CD45, expressed on all nucleated hematopoietic cells. This mutation results in dysregulated Src family kinase activity [29] and a lupus like disease in mice [28]. Despite similar biochemical effects on B cell receptor (BCR) signaling, the phenotypic consequences vary depending on the genetic strain of mice. CD45E613R B6 and 129 mice are developmentally normally, while BALB/c mice develop

dsDNA autoantibodies. CD45E613R B6-129 F1 mice develop autoantibodies, lymphoproliferative disorder (LPD), and GN with early mortality, indicating that genetic modifiers modulate disease outcome. A genetic modifier screen identified two susceptibility regions associated with the development of autoantibodies, *Wedge associated modifier (Wam) 1* and *Wam2* [22]. *Wam2* corresponds to the MHC H2 region on chromosome 17, while *Wam1* was identified as the *tlr9* gene on the distal end of chromosome 9.

While toll-like receptor 9 (TLR9) has long been associated with SLE, as its ligand is dsDNA and the quintessential marker of SLE is development of dsDNA autoantibodies, its specific role in disease pathogenesis is controversial. This endosomal TLR is tightly regulated through both chaperone [94, 95] and structural regulatory mechanisms [124, 126, 149]. Disrupting these regulatory mechanisms through forced surface expression, altered chaperone binding, or knockout models have demonstrated a role for TLR9 in modulating the disease phenotype in murine models of SLE [95, 99, 132]. Further highlighting the importance of TLR9, SLE patients have been shown to have altered TLR9 signaling [119], and studies in SLE populations have identified risk associated polymorphisms within the promoter region of TLR9 [116, 117]. Additionally, SLE patients are often treated with the anti-malarial drug chloroquine, which is thought to inhibit endosomal TLR signaling [150], though other TLR-independent mechanisms have been demonstrated [74, 151]. Together these data suggest that strength of TLR9 signaling may contribute to disease susceptibility, but the mechanistic basis for this remains poorly understood.

We previously identified two alleles of *tlr9*, termed the BALB/c ($TLR9^{Ba}$) and B6 ($TLR9^{B6}$) alleles[22]. Five non-synonymous coding differences were noted; four polymorphisms flank the extracellular cleavage region, while one is adjacent to the intracellular Toll/Interleukin-1 Receptor homology (TIR) domain. TLR9-/- in the context of the CD45E613R mutation

resulted in opposing phenotypic outcomes in these two murine strains: the previously non-autoreactive CD45E613R B6 TLR9-/- mice developed autoantibodies, while the autoreactive CD45E613R BALB/c TLR9-/- mice lost dsDNA autoantibody production. The TLR9 allelic variants were hypothesized to result in the hyper- and hyporesponsive signaling, respectively, seen in intact mice, indicating the B6 TLR9 allele may be negatively regulating autoantibody production, while the BALB/c allele may be positively regulating autoantibody production [22].

To formally test the hypothesis that the TLR9 alleles affect tolerance in opposing ways, we created congenic mice harboring the alternate allele of TLR9 on the CD45E613R background. Here we demonstrate that the $TLR9^{B6}$ allele induces B cell tolerance in the bone marrow during development, inducing loss of autoantibody production in CD45E613R BALB/c mice. Conversely, the $TLR9^{Ba}$ allele results in a break in B cell tolerance in the otherwise autoantibody resistant CD45E613R B6 background, resulting in hyperresponsive B cells upon stimulation, ANAs production, lymphadenopathy, and GN upon aging. We demonstrate here that this is due to the hyporesponsive MAPK and NFkB signaling by the $TLR9^{Ba}$ allele, while the $TLR9^{B6}$ allele induces tolerance by hyperresponsive signaling, regardless of genetic background. These data provide one mechanism by which functional allelic variants of TLR9 in the context of secondary mutations can synergize to alter B cell tolerance mechanisms to induce variable phenotypic outcomes, mimicking the diverse phenotypes observed across SLE patients.

Materials and Methods

Mice

Mice were obtained from the following sources: C57BL/6.CD45.2 (000664), BALB/c.CD45.2 (001026) (The Jackson Laboratory). CD45E613R TLR9 (wildtype) mice were generated as previously described[22] and backcrossed at least 9 generations onto B6 or BALB/c backgrounds. TLR9 congenic mice were generated through a speed congenics whereby the most backcrossed mouse was used to breed the next generation 7 generations to the wt background and 9 generations for the CD45E613R mutation. Mice were bred and housed in a specific pathogen-free facility and experiments were performed according to University of California San Francisco Institutional Animal Care and Use Committee and National Institutes of Health guidelines. For aging cohorts, mice were bled twice monthly beginning at 8 weeks of age to monitor serum autoantibodies.

SNP PCR and Sanger Sequencing

Mice were verified for containing the identified congenic regions by the University of California San Francisco Genomics Core using the mouse medium density linkage analysis SNP array (Illumina). Neither wt nor CD45E613R congenic strains contained SNPs of the alternate strain outside of the identified congenic region. TLR9 SNPs of the five polymorphisms were used to confirm allele type. All loci were of the backcrossed background except between the flanking SNPs as indicated. The target variation is screened by Sanger sequencing. PCR primers are designed (Primer3) to amplify a region containing the variation. The PCR product is processed post-reaction with SAP and exoI enzymes. The product is used as a template for the sequencing reaction with BigDye Terminator (Applied Biosystems). The sequencing reaction is cleaned up with X-Terminator (Applied Biosystems) and analyzed on the 3730xl DNA Analyzer (Applied Biosystems). The resulting sequencing data are viewed through Sequencher (GeneCodes) to genotype for variations. The final concentrations of the PCR components were 2.5 mM $MgCl_2$, 0.1 mM

dNTPs, 0.025 U of Platinum Taq polymerase (Invitrogen), 2% DMSO, 1X PCR Buffer, 200uM PCR primers and 10 ng of DNA template. The 2uL reaction was run with the following conditions: 95C for 5min, [94C for 20s, 65C for 20s (0.5C decrease per cycle), 72C for 45s; 14 cycles],[94C for 20s, 58C for 20s, 72C for 45s; 35 cycles],72C for 10min. The PCR products were treated with 0.5U of SAP and 0.5U of exoI at 37C for 60min, then 90C for 15min. The sequencing reaction consists of final concentrations of sequencing buffer, BigDye Terminator mix, 500uM sequencing primer and PCR product template. The running conditions for the sequencing reaction were: 96C for 1min, [96C for 10s, 55C for 5s, 60C for 4min; 25 cycles].

Flow Cytometry

Single cell suspensions were plated at 10^6 per well following RBC lysis and Fc receptor blockade. Cells were stained, and fixed in 1% paraformaldehyde before being processed on a BD FACSVerse and analyzed using FlowJo v10.1r5 (Tree Star). The following antibodies were used: B220-APC Cy7, CD11b Pacific Orange, CD19 APC-Cy7, CD138 PE, IgM PerCPCy5.5 (BD Pharmingen), AA4.1 APC, B220 APC, CD3 PE, CD3 Pacific Blue, CD3e PerCP, CD4 APC 750, CD8 PerCPCy5.5, CD11b APC, CD11c Pacific Blue, CD21 Pacific Blue, CD23 FITC, CD45R PerCPCy5.5, CD69 PECy7, F4/80 e450, IgD PE, IgD e450, panNK FITC, Ter119 APC (eBiosciences), CD3 APC, CD11c Pacific Blue (Tonbo Biosciences), CD45R Pacific Orange (Caltag Laboratories), IkBa 44D4, pERK P-p44/42 MAPK (Cell Signaling).

Pathology Staining and Imaging

Aged kidneys were fixed in 10% formalin (Fisher Scientific) overnight, dehydrated in 70% ethanol, and paraffin embedded. 5um sections were stained for hemotoxilin and eosin

(H&E) by the University of California San Francisco Mouse Pathology Core, and pathology evaluated and scored by a blinded renal pathologist. Images were taken on an AxioImager Z2 (Zeiss) at 10x. For electron microscopy, kidneys were fixed in 10% formalin and sent to the UCSF Electron Microscopy Core, and imaged for 900ms with an XR611 (AMT) transmission electron microscope at 1300x magnification.

Immunofluorescence

IF was performed on 5um thick sections of OCT frozen (Sakura Finetek) kidneys, sectioned on a Leica CM1950 cryostat. Sections were fixed by -20°C acetone over dry-ice for 2min, air-dried for 1hr at room temperature, rehydrated for 15min in PBS, and blocked for 30min. Slides were stained with DAPI (Roche) at 1:10000 dilution, FITC anti-mouse C3 (Cappel) at 1:75 dilution or FITC anti-mouse IgG (Jackson Immunoresearch) at 1:100 dilution, washed three times, cover slipped and sealed. Slides were analyzed on DM2500 light microscope (Leica) at 20x using Open Lab software.

Autoantibody Assays

ANAs were performed as previously described[22]. Briefly, HEp-2 and *Crithidia luciliae* slides (Inova Diagnostics) were stained with 1:40 serum dilution, washed and detected with FITC donkey anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories). Images were acquired on a DM2500 light microscope (Leica) at 20x or 40x oil immersion with OpenLab software. Anti-dsDNA IgG ELISA was performed in 96-well flat bottom plates were coated with poly-L-lysine (Sigma-Aldrich, P2636), then poly(deoxyadenylic-thymidylic) acid (Sigma-Aldrich, P0883) and blocked. Serum was diluted 2-fold starting at a dilution of 1:64, and Abs were detected with HRP-goat anti-mouse pan IgG, IgG1, IgG2a, IgG2b, IgG3, or pan IgM (Southern Biotech) and tetramethylbenzidine (Sigma-Aldrich). Anti-Sm ELISA

was performed similarly except coated with Sm/RNP Ag (ImmunoVision SRC-3000) and serum diluted 4-fold starting at a dilution of 1:40. Pooled CD45E613R 129-B6 F1 6mos sera were used as positive control.

Cell Stimulation and Signaling

Single cell suspensions from lymph nodes of 8 week mice were resuspended in 10% serum complete RPMI at $5x10^6$ and 200ul plated in U-bottom 96 well plate. Cells were stimulated with LPS 500ng/ml, PolyI:C at 500ng/ml, Imiquimod 1ug/ml, CpG1558 500nM, CpG1668 500nM (InvivoGen) or complete media alone for 18 hours, then analyzed as indicated. Signaling was performed as described previously[22]. Briefly, $1x10^6$ lymph node cells rested in serum free media for 1 hour prior to stimulation, then stimulated with CpG1668 3uM, anti-mouse IgM $F(ab')_2$ 10ug/ml (Jackson ImmunoResearch Laboratories) for 20 minutes, fixed, washed, permeabilized in MeOH, stained and analyzed by FACS as indicated.

Analysis

Statistical analyses were performed using Prism v6 (GraphPad Software). All p values ≤ 0.05 were considered significant and are shown in figures. A Student t test was used for pairwise comparisons. A one-way ANOVA or two-way ANOVA was used for comparing more than 2 groups as indicated. Post-Hoc analyses are as stated in figure legends.

Results

Autoantibody production relies on BALB/c TLR9 alleles in the CD45E613R murine model

A single nucleotide polymorphism (SNP) screen identified TLR9 as a putative modifier of autoantibody production in the CD45E613R model [22], and five non-synonymous coding differences were noted between B6 and BALB/c strains (Fig. 1A). We also screened for these

polymorphisms in several other SLE susceptible mouse strains to determine TLR9 allele status (Table 1). Most of these autoimmune strains mapped to the $TLR9^{Ba}$ allele, with the exception the MRL/MpJ^{lpr} and the B6.NZMc1 *Sle1* strains which contained the $TLR9^{B6}$ allele. Breeding lineages to develop these lines (Fig. 1B) tracks the segregation of the TLR9 alleles[152]. To test the hypothesis that the TLR9 alleles were responsible for the autoantibody status previously seen in CD45E613R mice, we used speed congenics to develop B6 and BALB/c mice congenic for the distal end of chromosome 9, crossed these to CD45E613R mutants, and confirmed TLR9 allele status using SNP sequencing (Fig. 1C). The LOD peak identified in the initial screen was reproduced for this analysis, and we confirmed the presence of the congenic regions derived in CD45 wild type (wt) and CD45E163R B6 $TLR9^{Ba}$ and BALB/c $TLR9^{B6}$ congenic mice.

These congenic mice enabled us to ask if the different TLR9 alleles modify the autoantibody production seen in the endogenous backgrounds in the presence of the CD45E613R mutation. In concordance with our hypothesis, the CD45E613R mutation in the presence of $TLR9^{Ba}$ was associated with HEp-2 positivity at 24 weeks of age, signifying the presence of autoantibodies in the serum, while the $TLR9^{B6}$ was associated with low levels of HEp-2 staining (Fig. 2A). The autoantibodies produced in the context of the $TLR9^{Ba}$ allele include antibodies that are highly specific to dsDNA, as determined by *C. luciliae* staining (Fig. 2B). By 24 weeks, the CD45E613R B6 $TLR9^{Ba}$ mice developed a variety of HEp-2 staining patterns, indicating the breadth of autoantigens targeted in both the cytoplasm and nucleus (Fig. 2C), including development of anti-Smith (Sm) IgG autoantibodies (Fig. 2E). In contrast, CD45E613R BALB/c $TLR9^{B6}$ mice showed significantly reduced levels of anti-dsDNA IgG autoantibodies relative to CD45E613R BALB/c mice with the endogenous TLR9 allele (Fig2D). One copy of the BALB/c allele was sufficient to break tolerance, as

CD45E613R BALB/c TLR9$^{B6/Ba}$ and CD45E613R B6 TLR9$^{B6/Ba}$ mice developed autoantibody levels equivalent to TLR9^{Ba} homozygous mice (Fig. 2D,E).

We then asked if specific dsDNA Ig subtypes were mediating the differences in autoantibody production in the congenic mouse strains. CD45E613R B6 TLR9^{Ba} mice developed IgG1 dsDNA autoantibodies that were absent in the CD45E613R B6 mice, while CD45E613R BALB/c TLR9^{B6} mice showed significantly reduced IgG1 production relative to CD45E613R BALB/c mice (Fig. 2F). Trends towards higher IgG2a, IgG2b, IgG3 and IgM dsDNA and Sm antibody production in CD45E613R B6 TLR9^{Ba} and decreased production of these Ig subtypes in CD45E613R BALB/c TLR9^{B6} mice (sup. Fig. 1A,B), including significant decreases in IgG1 anti-Sm production were also observed (sup. Fig. 1C). These data indicate that the TLR9^{Ba} allele is sufficient to confer loss of tolerance and autoantibody production on the otherwise resistant CD45E613R B6 background, while the TLR9^{B6} allele induces tolerance in the susceptible CD45E613R BALB/c background with loss of autoantibody production.

CD45E613R B6 TLR9^{Ba} mice develop kidney disease

To identify the phenotypic consequences of autoantibody production, we followed mice to 24 weeks of age. Upon aging, CD45E613R B6 TLR9^{Ba} mice developed lymphadenopathy and splenomegaly (Fig. 3A, B). Surprisingly, 30% of mice also developed crescentic, necrotizing, prolific GN (Fig. 3C, right) with C3 (Fig. 3D) and IgG (Fig. 3E) deposition in the glomeruli. Concurrent with the autoantibody production observed by ELISA, one copy of TLR9^{Ba} was sufficient to induce kidney deposition (Fig. 3D, E), though these mice failed to develop GN (data not shown). These results imply an immune complex (IC)-mediated GN.

To directly determine if ICs are present in the glomeruli, electron microscopy (EM) of aged mice with pathology-confirmed GN was evaluated. EM confirmed a proliferative, sub-endothelial IC-mediated glomerulonephritis (Fig. 3F), with features typical of an aggressive human connective tissue disease such as in lupus nephritis. The lumen of the glomeruli is nearly completely lost due to these IC depositions, with large deposits located in the mesangial region in addition to subendothelial deposits. These data support the conclusion that the TLR9^{Ba} allele on the CD45E613R B6 background is sufficient to induce both autoantibody production and interestingly, the development of IC-mediated GN.

TLR9 alleles alters immune compartment composition prior to disease onset

The cellular composition of immune cell compartments is often altered in the context of autoimmune disease. To investigate how the TLR9 alleles might impact B and myeloid cell development at a cellular level, we characterized the immune compartment of 8 week mice, prior to disease onset. The prevalence of the preB cell bone marrow (BM) population in CD45E613R BALB/c TLR9^{B6} mice resembles that of wt BALB/c mice, despite the significant reduction in overall percentage conferred by the CD45E613R mutation (Fig. 4A). The proportions of the preB cell and immature B cell populations were also altered in CD45E613R BALB/c TLR9^{B6} returning to near BALB/c wt levels (Fig. 4B), This alteration of B cell population composition also extended to the periphery, as an increased frequency of splenic (Sp) T1 transitional B cells in CD45E613R BALB/c TLR9^{B6} mice (Fig. 4C). Additionally, we observed decreased transitional stage 2-3 (T2/3) B cell numbers in the spleen (Fig. 4D), lymph node CD19+ B cells (Fig. 4E), and lymph node T2-follicular B cells (Fig. 4F).

In addition, the CD45E613R B6 TLR9^{Ba} mice also developed alterations in the composition of the B cell compartment. There was a reduction in the preB population relative to CD45E613R B6 mice (Fig. 4G) and further decreases in the splenic T1 B cell population (Fig. 4H). In the spleen, the ratio of follicular B cells to T2/3 B cells was consistently increased in mice carrying the BALB/c TLR9 allele relative to CD45E613R B6 mice (Fig. 4I). Increases in the overall CD19+ B cell population in the lymph node (Fig. 4J) and increased splenic CD11b+CD11c+ myeloid cells (sup. Fig. 2E) were also observed. In conjunction, these results highlight a potential alteration in B cell composition in the context of the TLR9^{B6} allele on the susceptible BALB/c background, and conversely, by the TLR9^{Ba} allele on the resistant B6 background.

The TLR9^{Ba} allele results in hyperresponsive B cells upon stimulation

To determine the cellular mechanism by which the TLR9^{Ba} allele may be modulating tolerance at a cellular level, we hypothesized that B cells expressing the TLR9^{Ba} allele may display a hyperresponsive cellular profile by CD69 expression upon TLR9 stimulation. To test this, we stimulated lymph node B cells from 8 week mice through TLR9 with two different backbones of the TLR9 ligand CpG: type A CpG1585, which mainly targets dendritic cells, and type B CpG1668 [7], which mainly targets B cells. CD45E613R B6 TLR9^{Ba} B cells showed increased expression of the B cell activation marker CD69 when stimulated with CpG1668 (Fig. 5A, B, D), with levels similar to those of CD45E613R BALB/c B cells (sup. Fig. 3A, B). No difference was seen with stimulation by CpG1585 or with ligands to TLR3, 4 or 7. The percentage of B cells that upregulated CD69 in response to CpG1668 also increased in CD45E613R B6 TLR9^{Ba} B cells (Fig. 5C). We did not observe a difference in CD69 expression when these cells were stimulated with LPS or PolyI:C (data not shown). These results highlight the different phenotypic effects that the TLR9^{B6} and

TLR9^{Ba} alleles have on a compartmental and cellular level, perhaps reflecting an innate difference in TLR9-induced downstream signaling.

The TLR9^{B6} allele confers a hyperresponsive signaling phenotype, while the TLR9^{Ba} allele confers a hyporesponsive signaling phenotype

To directly determine whether the TLR9 alleles conferred the signaling phenotype previously established in intact mice, lymph node B cells from intact or congenic CD45wt mice were stimulated with CpG1668 with and without concurrent IgM stimulation. Hyperresponsive pERK was observed in B cells containing the TLR9^{B6} allele and hyporesponsive signaling in those containing the TLR9^{Ba} allele (Fig. 6A, B). Hyperresponsive signaling by the TLR9^{B6} allele and hyporesponsive signaling by the TLR9^{Ba} allele was also observed by decreases in IkBa levels, a surrogate for the NFkB pathway which degrades upon activation (Fig. 6C, D). The signaling phenotype was not dependent on the CD45 status, as lymph node B cells from B6 background mice stimulated with CpG and stained for pERK showed hyporesponsive signaling only when the TLR9^{Ba} allele was present (Fig. 6E, F). IgM signaling is known to be altered by the CD45E613R mutation [22], and when stimulated with IgM alone, wt or CD45E613R B6 B cells pERK signaling segregated high and low responders by CD45 status, not by the TLR9 allele (Fig. 6G, H).

To determine if one TLR9 allele is dominant over the other, we stimulated 8 week CD45wt B6 and BALB/c TLR9$^{B6/Ba}$ lymph node B cells with CpG1668 and interrogated the MAPK and NFkB pathways. One copy of the B6 allele was sufficient to significantly increase BALB/c signaling above TLR9$^{Ba/Ba}$ levels, though not sufficient to recapitulate TLR9$^{B6/B6}$ levels for pERK and IkBa (sup. Fig. 4A, B). Conversely, there is no significant difference

between the B6 TLR9$^{B6/B6}$ and the B6 TLR9$^{B6/Ba}$ mice. In combination with the fact that one copy of TLR9^{Ba} is sufficient for autoantibody production, these studies indicate that the TLR9^{B6} allele is dominant, but not sufficient, as there may be a threshold for signaling required to prevent autoantibody development not obtained by one TLR9^{B6} allele alone. Overall, these data indicate that the TLR9 allele directly determines the signaling phenotype initially observed in intact mice.

Discussion

Genetic factors have long been known to have a significant impact on SLE development and disease phenotype. Several genetic screens for rare variants have failed to identify genetic alterations in significant proportions of SLE patients, indicating that common variants present in both healthy populations and patients with SLE may instead have a greater role in lupus development. It is thought that similar to the development of cancer, SLE is a polygenic disease where multiple "hits" consisting of these common polymorphisms are required to induce loss of tolerance [148]. The development of SLE pathogenesis is currently thought to consist of three components. Firstly, the initial break in tolerance results in autoreactive lymphocytes. These lymphocytes then proliferate in the periphery to induce autoantibodies and autoinflammation. Finally, autoimmunity occurs when these autoreactive lymphocytes and autoantibody-immune complexes mediate destruction of tissue, such as in GN [5]. In the CD45E613R model of SLE, we have a tractable model in which each of these distinct stages is modulated by genetic modifiers [22], allowing us to genetically dissect how each modifier contributes to individual stages of disease pathogenesis. BCR signal strength is similar between the B6 and BALB/c CD45E613R

strains, and is hyperresponsive compared to CD45 wt. This hyperresponsive BCR signaling results in altered B cell development [28, 30, 153, 154]. While there are no documented clinical cases of SLE harboring this specific CD45 mutation, defects in BCR signaling and B cell development have been implicated in SLE patients [12] and is commonly associated with SLE phenotypes in murine models [155-157].

In previous studies, a quantitative trait locus conditioning autoantibody production identified *tlr9* as a putative genetic modifier between the resistant CD45E613R B6 and the susceptible CD45E613R BALB/c strains. Five polymorphic differences between these backgrounds identified two distinct alleles of TLR9. This putative involvement of TLR9 in the CD45E613R model of SLE is consistent with the field, as several murine models have identified a role for TLR9, as well as TLR7, the other endosomal TLR which shares TLR9's chaperone protein Unc93B1 [94, 95]. Further linking TLR9 to SLE, dsDNA is not only a hallmark autoantibody in SLE, but is also the ligand for TLR9. Upon sequencing of *tlr9* in other lupus susceptible strains, we identified a predominance of the $TLR9^{Ba}$ allele. The modulation of disease phenotype upon TLR9 knockout in these models is consistent with our model, in which we previously demonstrated a role for the $TLR9^{Ba}$ allele in positively regulating autoantibody production, and has implications for future use of these model systems for the identification of mechanisms governing TLR9 biology.

Previous studies using CD45E613R TLR9$^{-/-}$ mice suggested that the $TLR9^{B6}$ allele results in induction of a tolerance checkpoint due to hyperresponsive signaling in response to CpG compared to cells expressing the $TLR9^{Ba}$ allele, which allows for autoreactive B cells to escape deletion due to the hyporesponsive TLR9 signaling [22]. In this study we show that by generating congenic strains containing the TLR9 allele of the opposing genetic background in the context of the CD45E613R mutation, we can recapitulate the

autoantibody levels seen in the endogenous strain (Fig. 1). While we cannot rule out the possibility that other genes within this congenic region may play a role, analysis of the region identified few immune related genes with non-synonymous coding differences, and none were highly expressed in B cells nor were they associated with autoimmunity or autoantibody production.

Consistent with other B6 SLE models, the CD45E613R B6 TLR9^{Ba} mice develop anti-dsDNA as well as anti-Sm autoantibodies, indicating a breadth of autoantigens targeted, confirmed by multiple HEp-2 staining patterns (Fig. 2D-F). The consistent alteration in the IgG1 subtype of dsDNA autoantibodies is interesting. The closest human homologs of mouse IgG1 are IgG2 and IgG4, and studies in patients with rheumatic autoimmune diseases have shown that human IgG2 is mainly targeted against anti-Sm/RNPs, while human IgG4 is mainly anti-DNA [158, 159]. In line with this human data, a significant proportion of CD45E613R B6 TLR9^{Ba} mice develop high titers of anti-Sm/RNP and anti-dsDNA IgG1 upon aging (Fig. 3), whileCD45E613R BALB/c TLR9^{Ba} mice decrease both of these IgG1 autoantibodies. Interestingly, while two copies of the TLR9 alleles confer the respective autoantibody phenotype, one copy of TLR9^{B6} is insufficient to induce complete tolerance in either the CD45E613R BALB/c or B6 strains.

Deposition of IgG and complement C3 are associated with the TLR9^{Ba} allele and not the TLR9^{B6} allele. Consistent with the autoantibody production, one copy of TLR9^{B6} is insufficient to prevent kidney deposition. Yet, despite the presence of autoantibodies and kidney deposition in the heterozygous mice, this was inadequate to induce GN (data not shown). This is in line with the BALB/c model however, as these mice develop non-pathogenic autoantibodies and glomerular deposition as well. Since the CD45E613R B6 TLR9^{Ba} mice do go on to develop GN unlike the CD45E613R BALB/c mice, and these mice

did not show increased TLR7 signaling as other SLE model do upon loss of TLR9 signaling, this would indicate a role for a second genetic modifier on this background that allows for the progression of disease. In the following chapter, we demonstrate that the second region identified by the SNP study was the MHC H2 region, in which the B6 $H2^b$ allele (MHC^{B6}) is shown to be an additive modifier to autoantibody production and ultimately resulted in the development of GN in CD45E613R BALB/c MHC^{B6} mice. Therefore, we propose that the endogenous B6 MHC $H2^b$ allele to the CD45E613R B6 $TLR9^{Ba}$ strain is the additional hit to the immune system required for the progression of disease from an autoinflammatory phenotype to an autoimmune GN.

To determine how these changes in autoantibodies may occur, we investigated immune cell development and tolerance checkpoints. It is well established that alterations in BCR signal strength can favor development of certain B cell subsets over others, particularly follicular vs marginal zone and B1 and B2 B cells [160-162]. More recently, studies have begun to demonstrate how interactions between TLR9 and BCR signaling may also influence B cell development [107, 163-165]. We provide evidence suggesting that the TLR9 alleles may be altering the development of B cells, potentially regulating the development and clearance of autoreactive B cells. In the CD45E613R BALB/c $TLR9^{B6}$, the ratio of immature to preB cells returns to a nearly 1:1 balance, with corresponding increase in the preB cell population compared to the CD45E613R BALB/c mice. One interpretation of this data may be that the majority of B cells that are generated in the BM are able to successfully transition to immature B cells as they are appropriately tolerized to self-antigen or become anergized, as demonstrated by the decreased autoantibody production upon aging. Moving to the periphery, we see a decrease in the proportions of cells in the later stages of B cell development. These data could potentially be explained as the tolerized CD45E613R

BALB/c TLR9^{B6} B cells no longer inappropriately recognizing self-antigen and proliferating in the periphery (Fig. 4C-F). Alternatively, it could indicate an overall dampening down of the immune system, as decreases in other peripheral immune cell populations are also noted. Conversely, CD45E613R B6 TLR9^{Ba} mice show a reduction in the proB-preB and T1 B cell populations in the BM (Fig. 4G-H). If the TLR9 alleles are functioning in B cells by ridding autoreactive cells at this cell transition, this result could be an indication of a central checkpoint tolerance mechanism ineffectively ridding the population of autoreactive B cells. This hypothesis is reflected in the increased ratio of follicular to T2-3 B cells, increased LN B cells (Fig. 4I-J), and the resultant development of autoantibodies. These data could also be explained by ineffectual silencing of anergized B cells in the periphery by a similar mechanism as proposed in Lyn-/- B cells, in which inhibitory CD22 and FcgRIIb signaling is impaired, and mice develop a lupus-like disease [166, 167]. Strikingly, these immune compartment differences are discernable as early as 8 weeks of age. These data suggest a model by which convergence of TLR9-BCR signal strength influences tolerance checkpoints and the development of autoreactive B cells. Further work identifying and following autoreactive B cell clones and repertoire through development in this model are required to directly address this hypothesis.

These studies led us to investigate the cellular effect the TLR9^{Ba} allele has on B cells upon activation. We demonstrate that stimulation of TLR9 by B cell specific Type B ODN-CpG1668 results in a hyperstimulatory phenotype, as measured by CD69 upregulation. To rule out the possibility that TLR9 alleles affect TLR7 responsiveness, we also stimulated TLR7 with the small molecule Imiquimod, as well as stimulating other TLRs, and we observe no difference between cells expressing the TLR9^{B6} or TLR9^{Ba} alleles. Further

studies are required to identify the mechanisms by which these hyperactivated B cells may go on to induce GN.

Finally, we show that these TLR9 alleles induce different signaling phenotypes regardless of the genetic background they are expressed on or CD45 status (Fig. 6). The $TLR9^{Ba}$ allele is hyporesponsive to TLR9 stimulation when compared to $TLR9^{B6}$, and this deficit is not overcome by combined BCR-TLR9 stimulation through either the MAPK or NFkB pathways. Signaling in $TLR9^{B6/Ba}$ B cells reveals a B6 dominant phenotype, implying that unlike TLR4 [168], allelic exclusion does not seem to occur for TLR9 in this system, concurrent with our previous $TLR9^{+/-}$ heterozygous knockout data [22]. Taken together, we propose a model in which the $TLR9^{B6}$ allele is protective by inducing tolerance through increased TLR9 signal strength, which cooperates with the increased CD435E613R-induced BCR signaling, resulting in deletion of autoreactive B cells at the central checkpoint. In contrast, the $TLR9^{Ba}$ allele is permissive due to its hyporesponsive signaling, and with the increased BCR signaling due to the CD435E613R mutation, allows autoreactive B cells to escape tolerance mechanisms.

Collectively, these data indicate the $TLR9^{Ba}$ allele alters B cell developmental checkpoints, resulting in a hyporesponsive phenotype upon stimulation of TLR9, and ultimately results in autoantibody production and GN in the CD45E613R B6 background. In contrast, the $TLR9^{B6}$ allele tolerizes B cells during the central checkpoint, decreasing autoantibody production on the BALB/c background. Finally, these phenotypic differences are due to alterations in MAPK and NFkB signaling downstream of the respective TLR9 alleles. Consistent with the field, this study demonstrates the importance of TLR9 signaling in the development and progression of SLE. Further investigations into TLR9 signaling profiles in individual patients may reveal pathway components amenable to agonist or antagonist

therapies. As several TLR9 inhibitors are currently in development[169, 170], and our data would suggest that assaying individual patients TLR9 signaling responses may be of therapeutic value prior to treatment with these inhibitors, as inhibiting TLR9 in patients with normal signaling may be deleterious and promote disease progression.

Acknowledgements & Footnotes

We would like to acknowledge M. Katz, and D. Tobler for their technical assistance.

Figures

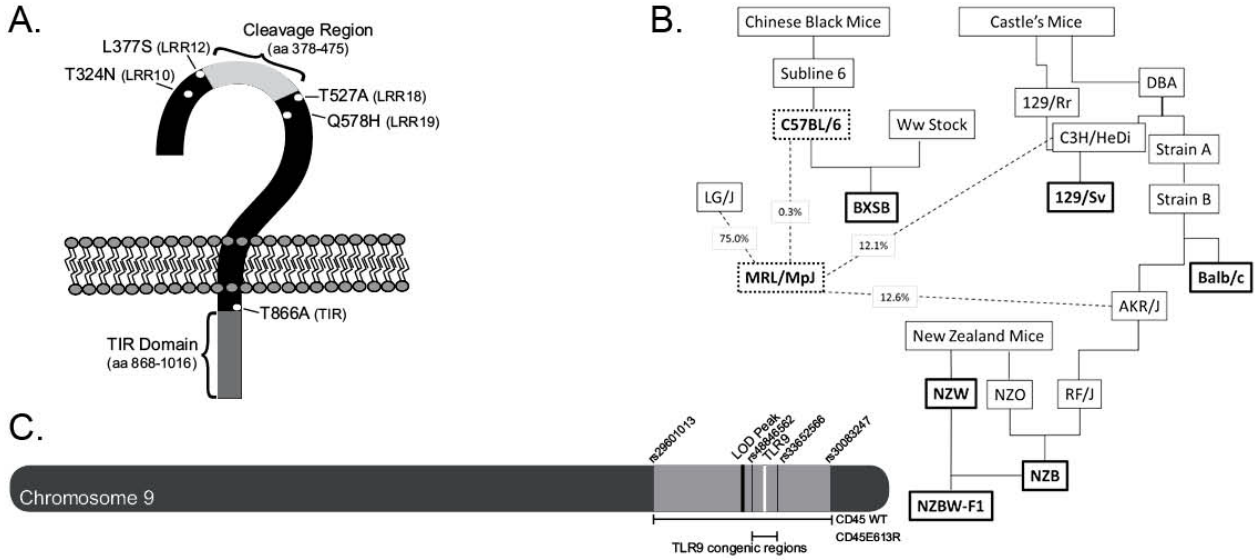


Figure 1. Congenic mice and TLR9 allele mapping.

(**A**) Schematic of TLR9 identifying the 5 polymorphisms (B6:BALB/c) and location in leucine rich repeat (LRR), the cleavage region, and TIR domain. (**B**) Lineage tracing common mouse strains and their TLR9 alleles by SNP genotyping. Bolded boxed strains contain the BALB/c allele, dashed boxed strains contain the B6 allele. Breeding data derived from (Beck et. al, 2000) (**C**) Representation of chromosome 9 congenic regions flanked by four SNPs, rs29601013-rs30083247 for the CD45 wild type strains and rs48846562-rs33652566 for the CD45E613R strains. LOD score peak from SNP analysis and TLR9 genomic position marked.

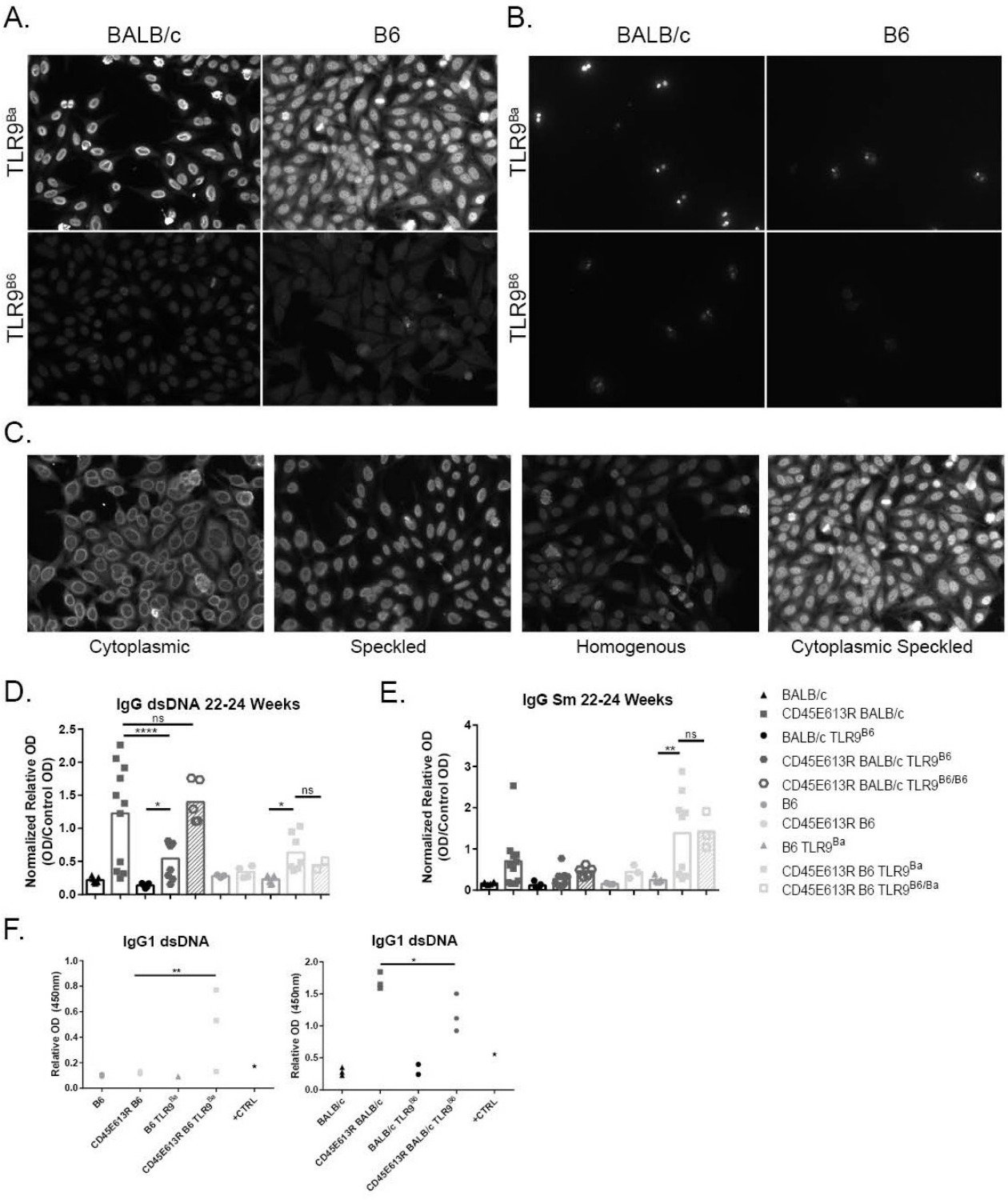


Figure 2. Autoantibody development linked to TLR9^{Ba} allele in CD45E613R model.

(**A-F**) CD45E613R mice with indicated genetic background and TLR9 allele were aged to 24 weeks and (**A**) serum stained HEp-2 and (**B**) *C. luciliae* assays with anti-IgG. (**C**) CD45E613R B6 TLR9^{Ba} 24 week serum staining patterns as labeled. One way ANOVA, then Turkey's multiple comparisons test shown. (**D**) IgG anti-dsDNA or (**E**) IgG anti-Sm ELISAs on 22-24 week serums and (**F**) IgG1 anti-dsDNA autoantibodies assayed by ELISA with 24 week serums shown with relative OD at 450nm. Students T test. +CTRL is 129-B6 CD45E613R 24 week serum. Representative of 2-3 independent experiments, n=2-11 mice per condition, Standard Deviation (SD), *$p<0.05$, **$p<0.005$, ***$p<0.0001$.

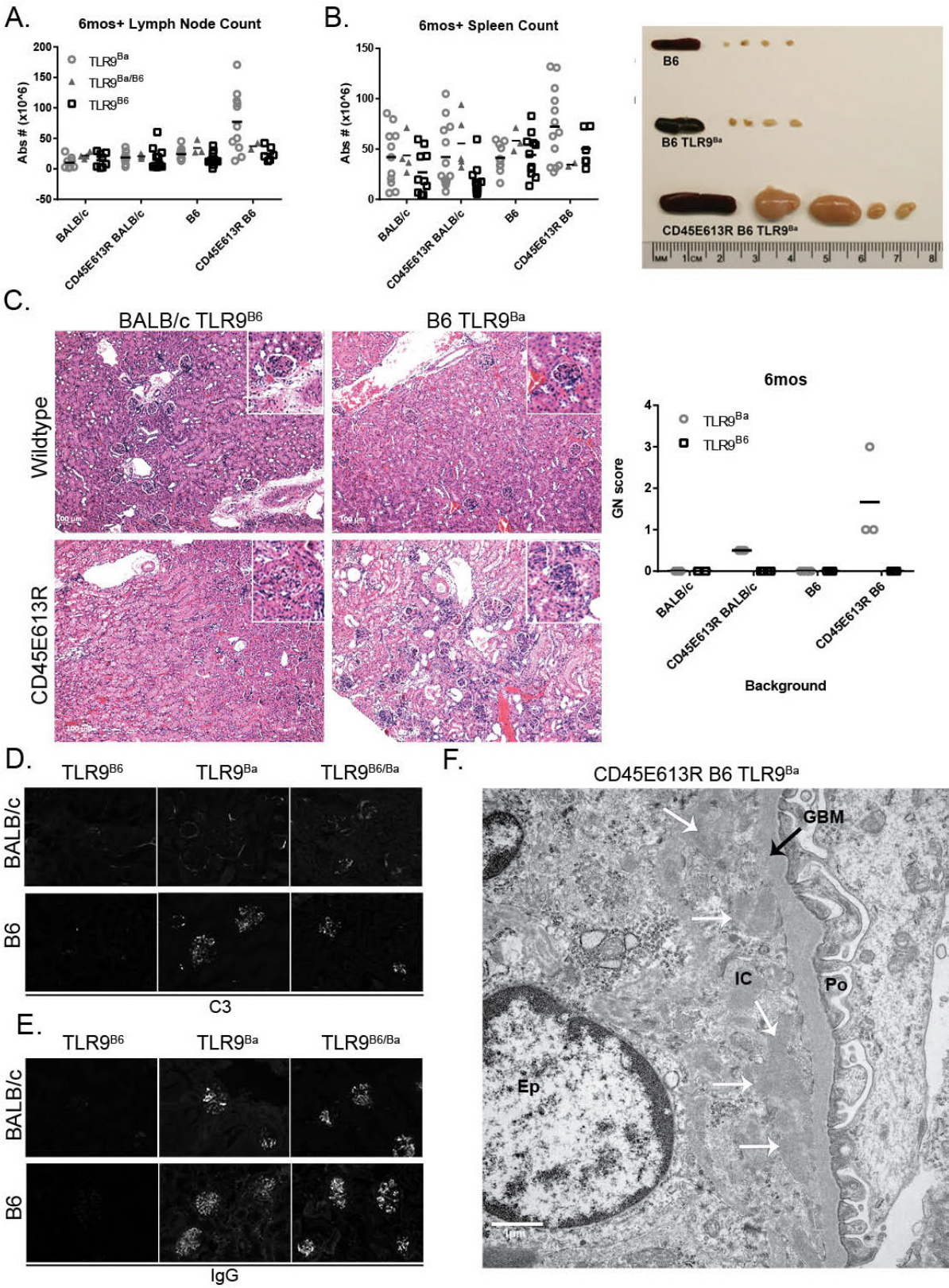

A.
6mos+ Lymph Node Count
Abs # (x10^6)
TLR9Ba
TLR9Ba/B6
TLR9B6
BALB/c
CD45E613R BALB/c
B6
CD45E613R B6
B.
6mos+ Spleen Count
Abs # (x10^6)
BALB/c
CD45E613R BALB/c
B6
CD45E613R B6
B6
B6 TLR9Ba
CD45E613R B6 TLR9Ba
C.
BALB/c TLR9B6
B6 TLR9Ba
Wildtype
CD45E613R
6mos
GN score
TLR9Ba
TLR9B6
BALB/c
CD45E613R BALB/c
B6
CD45E613R B6
Background
D.
TLR9B6
TLR9Ba
TLR9B6/Ba
BALB/c
B6
C3
E.
TLR9B6
TLR9Ba
TLR9B6/Ba
BALB/c
B6
IgG
F.
CD45E613R B6 TLR9Ba
GBM
IC
Po
Ep

Figure 3. CD45E613R B6 TLR9^{Ba} mice develop LPD and GN.

(**A**) Live cell counts of lymph nodes and (**B**) Spleens of each genetic background at 6 months or older. Photo of 12 month B6 wt (*top*), B6 TLR9^{Ba} (*middle*), and CD45E613R B6 TLR9^{Ba} (*bottom*) spleen and lymph nodes. (**C**) H&E stained kidney. Inset is enlarged glomeruli. Graph of 6 month cohort, GN score by pathologist. (**D**) C3 and (**E**) IgG deposition in kidneys from 24 week kidneys. Representative of at least 2 independent experiments, n=3-5 mice per condition. (**F**) Electron microscopy of 12mos CD45E613R B6 TLR9^{Ba}. Glomerular basement membrane, GMB; podocytes, Po; epithelial cell, Ep; immune complex deposition, IC. White arrows indicate areas of dense IC deposits.

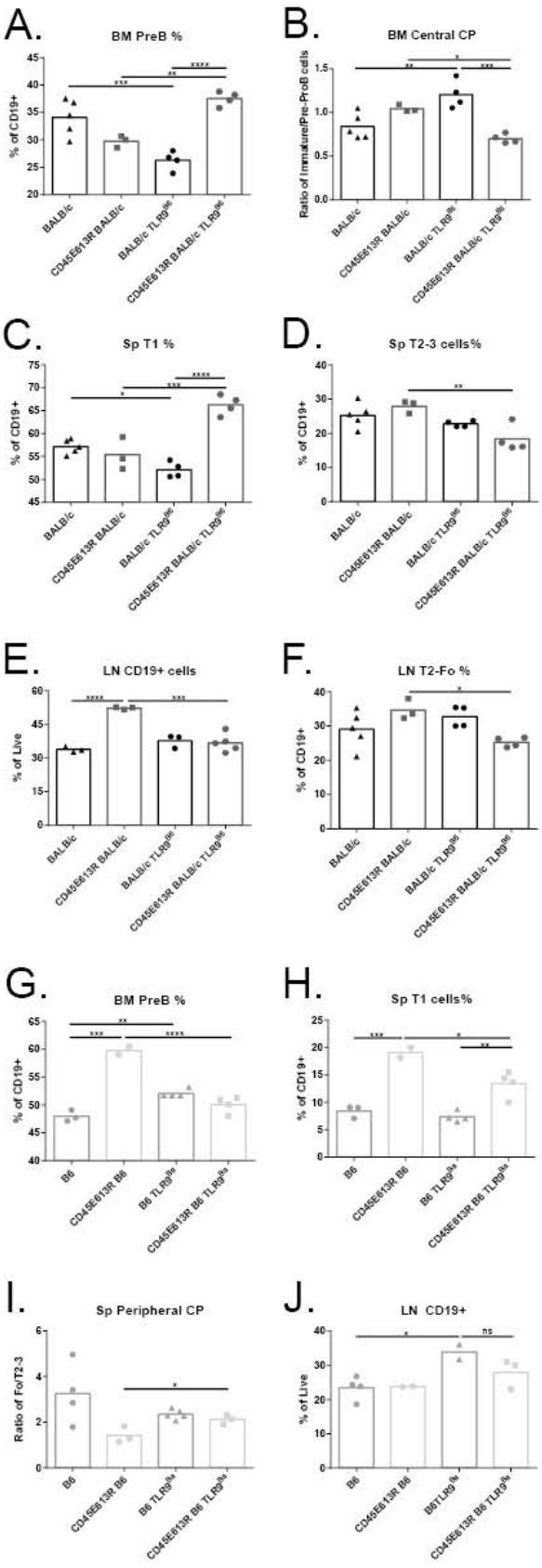


Figure 4. TLR9 alleles alter immune compartment composition prior to disease.

(**A-F**) B cell populations from 8 week mice of the BALB/c and (**G-J**) B6 background. (**A**) Bone marrow (BM) cells gated on Pre-Pro B cells (CD19+ IgD^{lo} IgM^{lo}). (**B**) Bone marrow central checkpoint (CP); ratio of Immature (CD19+ IgM+ IgD^{lo}) over Pre-Pro B cells from bone marrow 8 week mice. (**C**) Splenic (Sp) transitional 1 (T1) B cells (CD19+ CD23- CD21-), (**D**) transitional 2-3 (T2/3) B cells (CD19+ AA4.1+ CD23+), (**E**) CD19+ Lymph node (LN) B cells, and (**F**) LN transitional 2-follicular B cells (T2-Fo; CD19+ CD23+ CD21+). (**G**) BM Pre-Pro B cells, (**H**) Sp T1 B cells, (**I**) Splenic peripheral checkpoint; ratio of Fo over T2/3 B cells from spleen, and (**J**) LN CD19+ B cells. Representative of 3 independent experiments, n=2-5 mice per condition, SD. One-way ANOVA, then Turkey's multiple comparisons, *p<0.05, **p<0.005, ***p<0.0001.

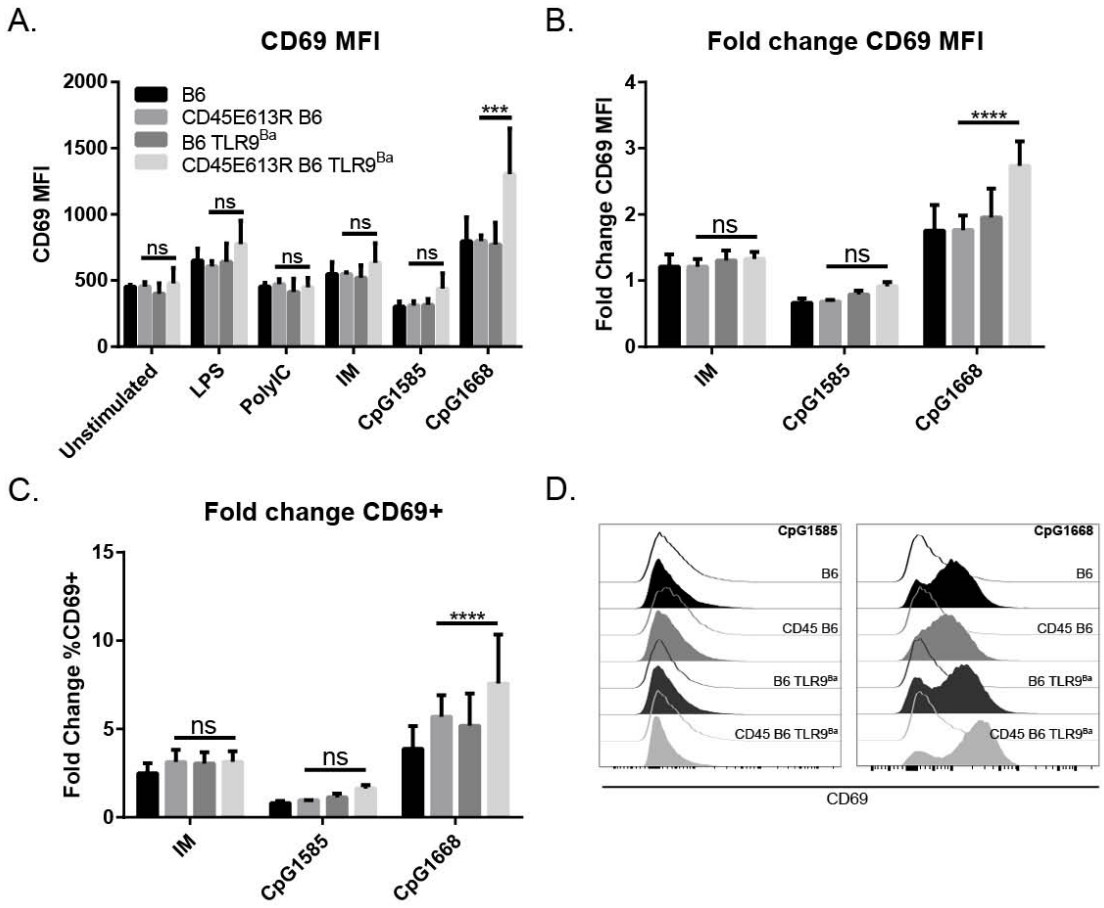


Figure 5. TLR9^{Ba} allele confers hyperactive B cell phenotype.

(**A**) Lymph node cells stimulated for 18hrs in the presence of CpG 500nM, Imiquimod (IM) 1ug/ml, LPS 500mg/ml, PolyI:C 500ng/ml, or complete media alone, then stained for CD69. (**B**) Fold change in CD69 MFI over unstimulated or (**C**) fold change in percentage of B220+ CD69+ cells. (**D**) Representative histograms of CD69 MFI upon stimulation with CpG1585 (*left*) or CpG1668 (*right*); (▫) unstimulated and (▪)stimulated as labeled. Representative of two independent experiments, n=2-5 mice per condition, SD. Two-way ANOVA, then Turkey's multiple comparisons test, *p<0.05, **p<0.005, ***p<0.0001.

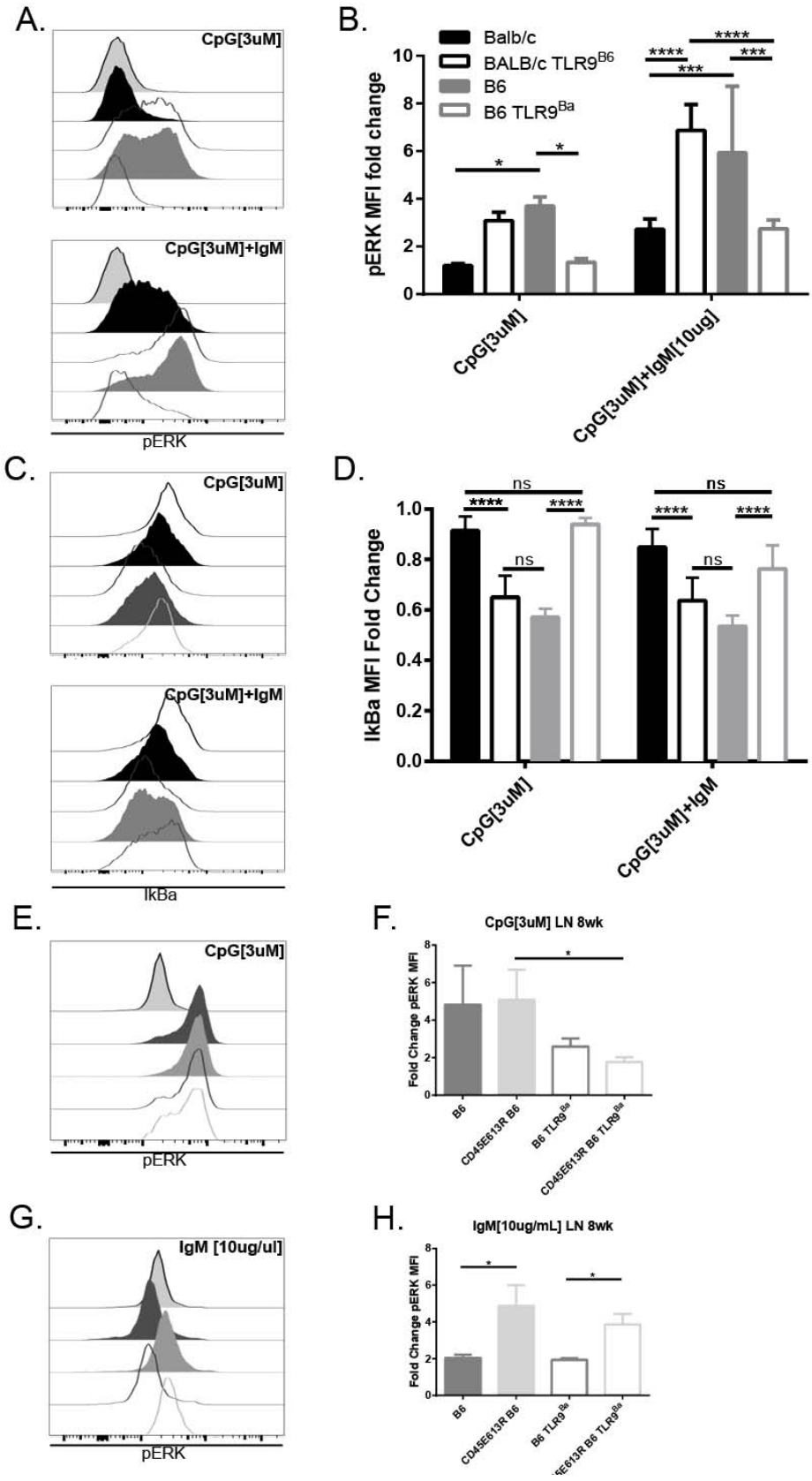
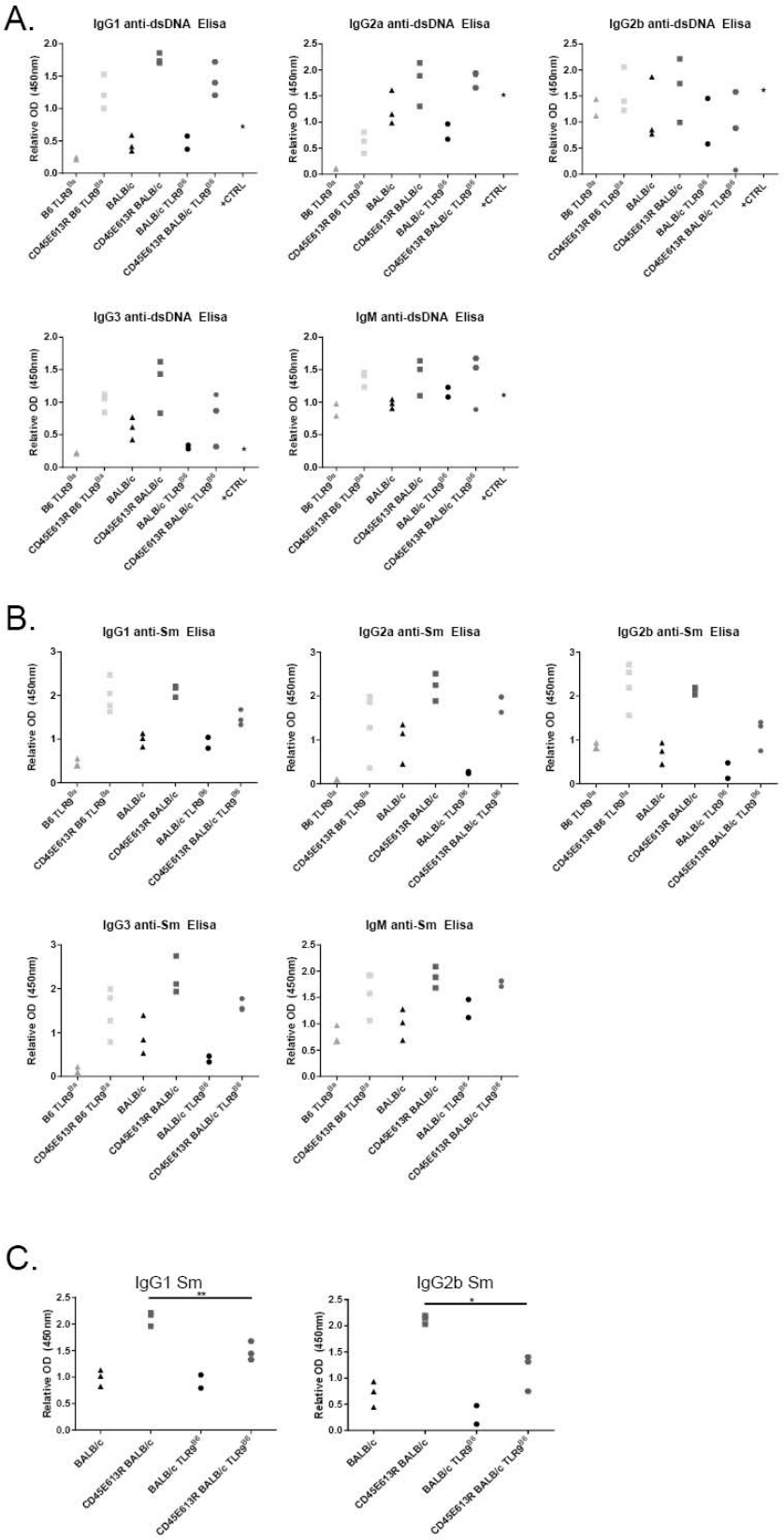

A.
CpG[3uM]
CpG[3uM]+IgM
pERK
B.
Balb/c
BALB/c TLR9B6
B6
B6 TLR9Ba
pERK MFI fold change
CpG[3uM]
CpG[3uM]+IgM[10ug]
C.
CpG[3uM]
CpG[3uM]+IgM
IkBa
D.
IkBa MFI Fold Change
CpG[3uM]
CpG[3uM]+IgM
E.
CpG[3uM]
pERK
F.
CpG[3uM] LN 8wk
Fold Change pERK MFI
B6
CD45E613R B6
B6 TLR9Ba
CD45E613R B6 TLR9Ba
G.
IgM [10ug/ul]
pERK
H.
IgM[10ug/mL] LN 8wk
Fold Change pERK MFI
B6
CD45E613R B6
B6 TLR9Ba

Figure 6. Signaling profile in B6 and BALB/c mice correlates with TLR9 allele.

(**A**) Representative histograms of pERK induction in lymph node B220+ cells from 8 week old mice stimulated with CpG1668 at 3uM with (bottom) or without IgM at 10ug/ml (top). Black shaded histogram is basal BALB/c, black filled is stimulated BALB/c, black open is BALB/c TLR9B6, grey filled is B6, and grey open is B6 TLR9Ba. (**B**) Fold change of pERK induction over unstimulated. One-way ANOVA, then Sidak's multiple comparisons test. (**C**) Representative histograms of IkBa degradation. (**D**) Fold change of IkBa degradation. One-way ANOVA, then Turkey's multiple comparisons test. (**E**) Representative histograms of B220+ lymph node B cells stimulated with CpG1668 3uM or (**G**) IgM 10ug/ml and stained for pERK. Black shaded is B6 unstimulated, dark grey filled is B6, light grey filled is CD45E613R B6, dark grey open is B6 $TLR9^{Ba}$, light grey open is CD45E613R B6 $TLR9^{Ba}$. (**F**,**H**) Fold change in pERK. Unpaired t-test. Representative of 3 independent experiments, n=2-5 mice per condition, SD, *$p<0.05$, **$p<0.005$, ***$p<0.0001$.

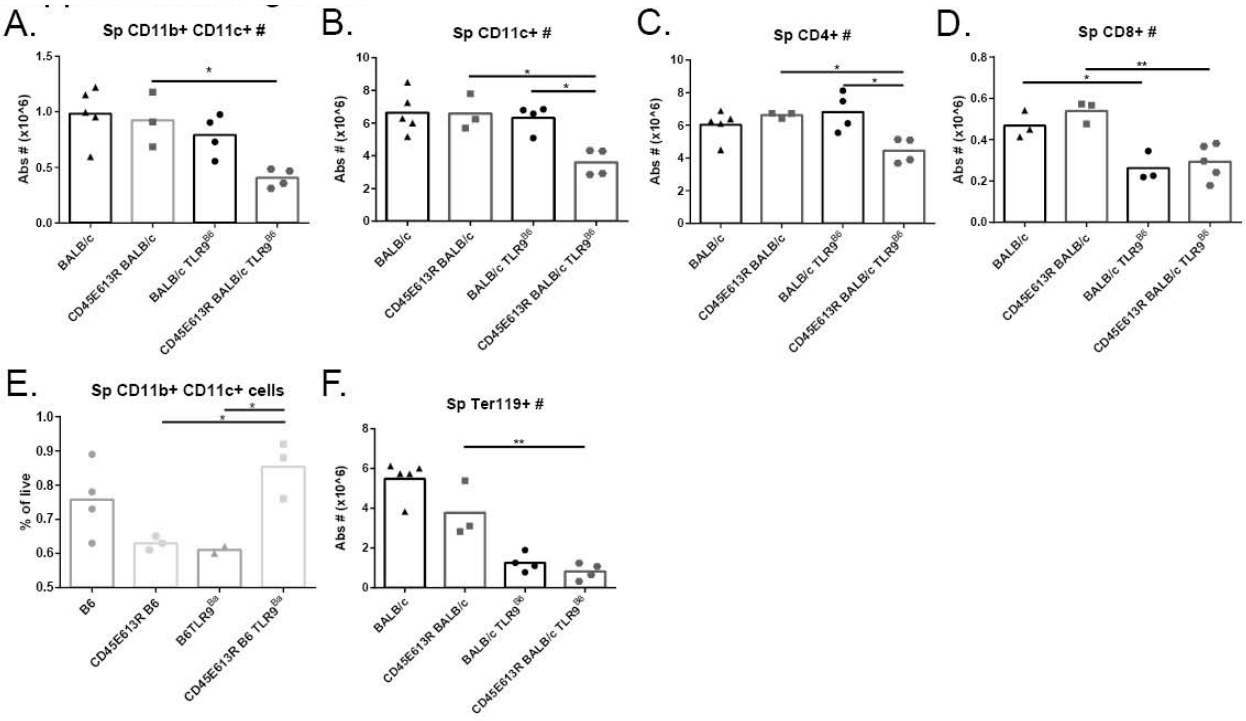
AMINO ACID	325	378	573	579	867
B6	**T**	**L**	**T**	**Q**	**T**
MRL/MPJ (LPR)	T	L	T	Q	T
B6.NZMC1 (SLE1)	T	L	T	Q	T
BALB/c	**N**	**S**	**A**	**H**	**A**
129/SV	N	S	A	H	A
NZB	N	S	A	H	A
NZW	N	S	A	H	A
BXSB	N	S	A	H	A
HUMAN	**T**	S	A	H	A

Table 1. TLR9 polymorphisms in autoimmune susceptible strains.



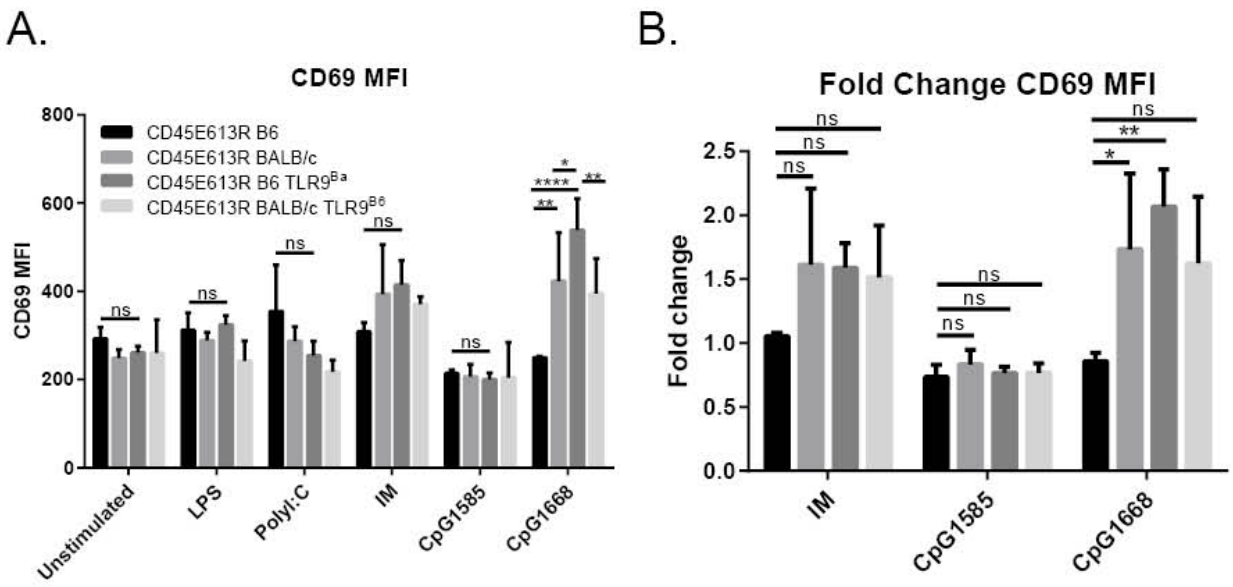
Supplemental Figure 1. Autoantibody subtypes.

(A) 24 week serums from indicated genetic backgrounds tested by ELISA at 450nm for anti-dsDNA autoantibodies or (B) anti-Sm autoantibodies. (C) IgG subtypes in BALB/c background congenic mice at 24 weeks of age. Representative of at least 2 independent experiments, n=2-5 mice per condition. Student's t-test, *p<0.05, **p<0.005, ***p<0.0001.



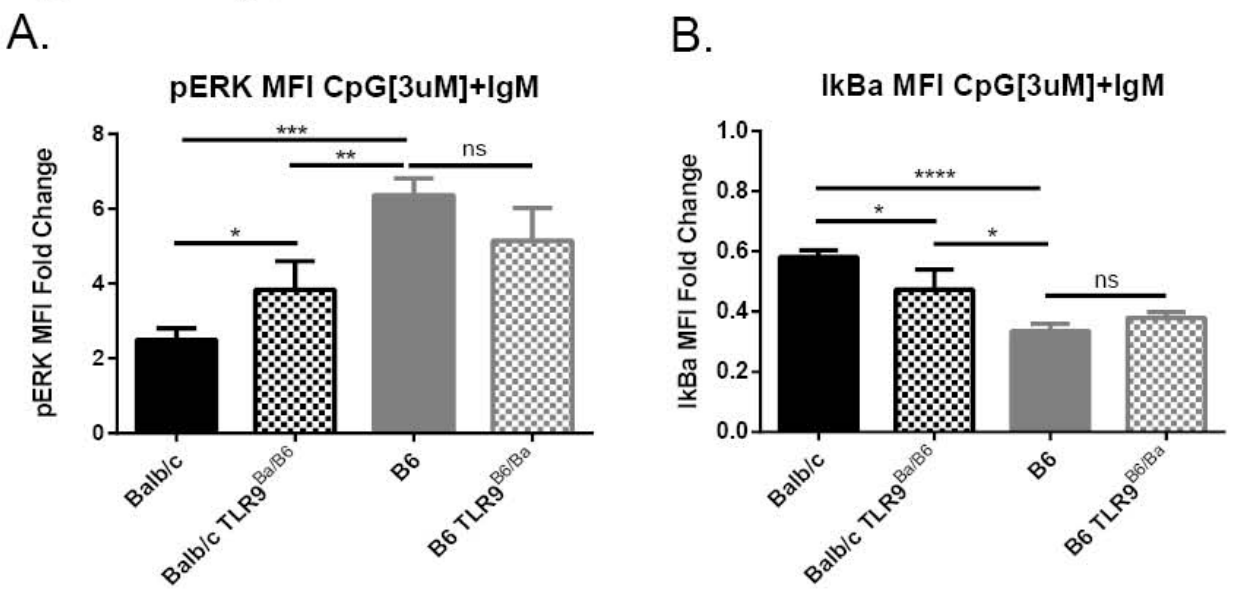
Supplementary Figure 2. Splenic immune cell populations altered by TLR9 allele.

(**A-D, F**) Immune cell populations from 8 week old mice from the BALB/c and (**E**) B6 background. (**A**) Absolute numbers of splenic CD11b+CD11c+ (**B**) CD11c+, (**C**) CD4+ T cells (CD3+ CD4+), (**D**) CD8+ T cells (CD3+ CD8+), (E) percentage of CD11b+ CD11c+ cells, and (**F**) absolute numbers of Ter119+ cells. Representative of 3 independent experiments, n=2-5 mice per condition, SD. One-way ANOVA, then Turkey's multiple comparisons test, *p<0.05, **p<0.005, ***p<0.0001.



Supplementary Figure 3. TLR9^{Ba} allele induces hyperesponsiveness regardless of background.

Lymph node cells from 8 week mice were stimulated with ligands as previously described for 18hrs, gated on B220+ and analyzed for CD69 surface staining. (**A**) CD69 MFI and (**B**) fold change of MFI over unstimulated are shown. n=2-5 mice per condition, SD. One-way ANOVA, then Turkey's multiple comparisons test, *p<0.05, **p<0.005, ***p<0.0001.



Supplementary Figure 4. B6 TLR9 allele is dominant for signaling phenotype.

Lymph node cells stimulated with CpG1668 3uM and IgM 10ug/ml, gated on B220+ and stained for (**A**) pERK and (**B**) IkBa fold change over unstimulated. n=2-5 mice per condition, SD. One-way ANOVA, then Sidak's multiple comparisons test, *p<0.05, **p<0.005, ***p<0.0001.

CHAPTER III: SUSCEPTIBILITY ALLELE MHC $H2^B$ ACCELERATES AUTOANTIBODY PRODUCTION AND INDUCES GLOMERULONEPHRITIS IN THE CD45E613R MODEL OF SLE

Abstract

Despite the multiplicity of MHC alleles among phenotypically diverse autoimmune patients, repeated genetic analyses consistently identify the MHC locus as contributing to disease. Unfortunately, autoimmune models demonstrating a role for MHC H2 in disease pathogenesis are unclear. Here we present a model of systemic lupus erythematosus (SLE) in which addition of the MHC $H2^b$ susceptibility allele accelerates the development of autoreactivity and autoantibodies, and is required for the progression of disease to glomerulonephritis in the context of the permissive CD45E613R mutation. Additionally, the $H2^b$ allele induces alterations in immune cell compartments, most notably the B cell compartments, with B cells uniquely expressing increased cell surface MHC class II prior to disease onset and displaying a hyperresponsive signaling phenotype upon stimulation. These data demonstrate how allelic variants can cooperate with genetic mutations to modulate autoimmune phenotypes, mimicking the phenotypic heterogeneity seen in patients with SLE.

Introduction

Autoimmunity arises from an inappropriate reactivity to self-antigens. Case studies and animal models have identified a wide variety of genes and pathways that can contribute to the breakdown of immune tolerance, leading to the development of autoimmunity. The genetic diversity seen across populations of patients with autoimmune diseases would

suggest little overlap in the genes that contribute to disease pathogenesis, yet genetic analyses have repeatedly identified the MHC locus as a putative genetic contributor to disease development [4, 171]. Allelic variants of the HLA D region for example, corresponding to MHC class II (MHCII), have been shown to modify disease risk for a variety of autoimmune diseases, including SLE[36]. In spite of this, few models have conclusively demonstrated a contribution of MHC H2 susceptibility alleles to disease pathogenesis or progression.

The phenotype of the CD45E613R murine model of SLE was previously shown to be heavily influenced by genetic modifiers associated with different strain backgrounds [22, 30]. A single point mutation in the juxtamembrane "wedge" region of CD45 results in increased immunoreceptor tyrosine activating motif (ITAM) signaling though altered Src family kinase regulation [29], but the phenotypic outcome is dependent on genetic background, despite biochemically identical CD45 activity across these different strains. CD45E613R B6 and CD45E613R 129 mice do not develop a lupus-like autoantibody production, while CD45E613R BALB/c mice develop anti-double stranded (ds)DNA autoantibodies, and a mild lymphoproliferative disorder (LPD), but lack kidney disease. In contrast, CD45E613R B6-129 F1 mice develop dsDNA autoantibodies, LPD and glomerulonephritis (GN) at 100% penetrance. These data demonstrate that genetic modifiers on these strains influence disease phenotype. A SNP screen for autoantibody production identified two susceptibility loci between the B6 and the BALB/c strains contributing to disease. One locus was identified in the BALB/c strain at the distal end of chromosome 9 and the second was identified in the B6 strain corresponding to the MHC H2 region on chromosome 17 [22]. While identification of the MHC H2 region as a susceptibility locus is often emblematic of a successful genetic screen, as it encompasses MHCII along with several non-classical MHC

and immunologically important genes, we sought to determine the contribution of this genetic locus to disease progression in the CD45E613R murine model of SLE using a congenic model system.

SLE is thought to arise through a multi-step pathogenesis[5] whereby genetic or epigenetic alterations result in the development of autoreactive lymphocytes. These autoreactive lymphocytes migrate to the periphery, where they encounter self-antigen, activate and facilitate autoantibody production. Autoimmunity ensues when these autoantibodies deposit in target tissues and precipitate tissue destruction. MHC has been associated with both of the initial steps in autoantibody production[37], and linked with some autoimmunity phenotypes[38, 49] in human and mouse models. While multiple mechanisms have been proposed for MHC in SLE pathogenesis [21, 53], few studies have tested if MHC plays a role in each stage of pathogenesis. Here we demonstrate the B6 MHC $H2^b$ allele in the CD45E613R BALB/c murine model of SLE significantly accelerates autoantibody development, and is required for the progression of disease to GN, resulting in altered B cell development, and increased MHCII expression prior to disease onset. We illustrate here how a risk associated MHC H2 allele can target B cells, leading to a significantly worse clinical outcome in a murine model of SLE.

Materials and Methods

Genetic modifier linkage analysis

Genomic DNA was extracting using the GenePure kit according to manufacturer's protocol. Single nucleotide polymorphism analysis was performed on B6-BALB/c F2 mice as previously described[22]. Linkage analysis mapping was performed using Mapmaker/QTL and National Center for Biotechnology Information m36 mouse genome assembly. In silico

mapping of candidate loci and analyses were performed using Ensemble (http://www.ensembl.org/Mus_musculus/index.html), University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/), and the Jackson Laboratories Mouse Genome Informatics database (http://www.informatics.jax.org/).

Flow Cytometry

Single cell suspensions were plated at 10^6 per well following RBC lysis and Fc receptor blockade. Cells were stained, and fixed in 1% paraformaldehyde before being processed on a BD FACSVerse and analyzed using FlowJo v10.1r5 (Tree Star). The following antibodies were used: B220-APC Cy7, CD11b Pacific Orange, CD19 APC-Cy7, IgM PerCPCy5.5, PD-1 PE (BD Pharmingen), AA4.1 APC, B220 APC, CD3 PE, CD3e PerCPCy5.5, CD3 Pacific Blue, CD4 APC 750, CD5 PE, CD8 PerCPCy5.5, CD11b APC, CD21 Pacific Blue, CD23 FITC, CD45R PerCPCy5.5, CD69 PECy7, CD80 FITC, CD86 PE, F4/80 e450, FoxP3 e450, IgD PE, IgD e450, Ki67 PECy7, MHC II PE, MHC II APC, panNK FITC, PD-1 FITC, Ter119 APC, (eBiosciences), CD3 APC, CD11c Pacific Blue, CD25 PE (Tonbo Biosciences), CD45R Pacific Orange (Caltag Laboratories), IkBa 44D4, pERK P-p44/42 MAPK (Cell Signaling).

B cell Stimulation

Cells were stimulated as previously described[22]. Briefly, single-cell suspensions from lymph nodes of 8 week old mice were rested for 1hr in serum-free media at 37°C and stimulated in 96-well conical plates at 37°C with anti-mouse CD40 (1C10; eBiosciences), CpG1668 (Invivogen), $F(ab')_2$ anti-mouse IgM (Jackson ImmunoResearch Laboratories) for times indicated in figures. Cells were fixed, washed, stained and analyzed as described

above. When indicated, isolated B cells were derived from lymph nodes of 8 week old mice using Mouse CD43 Untouched B cells (DynaBeads) following manufacture's protocol.

Immunofluorescence

IF was performed on 5um thick sections of OCT frozen (Sakura Finetek) kidneys, sectioned on a Leica CM1950 cryostat. Sections were fixed by -20°C acetone over dry-ice for 2min, air-dried for 1hr at room temperature, rehydrated for 15min in PBS, blocked with 2% BSA(Fisher Scientific) and 0.1% Tween20 (BioRad) in PBS for 30min. Slides were stained with DAPI (Roche) at 1:10000 dilution, FITC anti-mouse C3 (Cappel) at 1:75 dilution or FITC anti-mouse IgG (Jackson Immunoresearch) at 1:100 dilution, washed in 0.5%BSA-0.1% Tween20 in PBS for 5min, and sealed. Slides were analyzed on DM2500 light microscope (Leica) at 20x using Open Lab software.

H&E and IHC

Aged kidneys were fixed in 10% formalin (Fisher Scientific) overnight, dehydrated in 70% ethanol, and paraffin embedded. 5um sections were stained for hemotoxilin and eosin (H&E) by the University of California San Francisco Mouse Pathology Core, and pathology evaluated by a blinded renal pathologist. Images were taken on an AxioObserver Z1 (Zeiss) using Slidebook 6 software at 10x. F4/80 IHC was performed on 6 month or older kidneys fixed and blocked as above by HistoWiz Inc. (histowiz.com) using a Standard Operating Procedure and fully automated workflow. Samples were processed, embedded in paraffin, and sectioned at 4μm. Immunohistochemistry was performed on a Bond Rx autostainer (Leica Biosystems) with enzyme treatment (1:1000) using standard protocols. Antibodies used were rat monoclonal F4/80 primary antibody (eBioscience, 14-4801, 1:200) and rabbit anti-rat secondary (Vector, 1:100). Bond Polymer Refine Detection (Leica Biosystems) was

used according to manufacturer's protocol. After staining, sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems) and analyzed by Aperio ImageScope v12.2.2.5015 (Leica).

Mice

Mice were obtained from the following sources: C57BL/6.CD45.2 (000664), BALB/c.CD45.2 (001026), B6.C-H2d/bByJ (000663), B10-H2b/LilMcdJ (001952) (The Jackson Laboratory). CD45E613R mice were generated as previously described[22] and backcrossed at least 9 generations onto B6 or BALB/c backgrounds. Mice were bred and housed in a specific pathogen-free facility and experiments were performed according to University of California San Francisco Institutional Animal Care and Use Committee and National Institutes of Health guidelines. For aging cohorts, mice were bled twice monthly beginning at 8 weeks of age to monitor serum autoantibodies. Proteinuria was determined as previously described (*). Briefly, urine was evaluated by Uristix (Siemens) from 0 to ++++, where a score of +++ or ++++ (corresponding to >300mg/dl) was considered positive and ++ (corresponding to 100mg/dl) was considered low.

Autoantibody Assays

ANAs were performed as previously described[22]. Briefly, HEp-2 and Crithidia luciliae slides (Inova Diagnostics) were stained with 1:40 serum dilution, washed and detected with FITC donkey anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories). Images were acquired on a DM2500 light microscope (Leica) at 20x or 40x oil immersion with OpenLab software. Anti-dsDNA IgG ELISA was performed as previously described[22]. Briefly, 96-well flat bottom plates were coated with poly-L-lysine (Sigma-Aldrich, P2636),

then poly(deoxyadenylic-thymidylic) acid (Sigma-Aldrich, P0883) and blocked. Serum was diluted 2-fold starting at dilution of 1:64, and Abs were detected with HRP-goat anti-mouse pan IgG, IgG1, IgG2a, IgG2b, IgG3, or pan IgM (Southern Biotech) and tetramethylbenzidine (Sigma-Aldrich). Pooled CD45E613R 129-B6 F1 6mos sera were used as the positive control.

Analysis

Statistical analyses were performed using Prism v6 (GraphPad Software). All p values $\leq$0.05 were considered significant and are shown in figures. A Student t test was used for pairwise comparisons. A one-way ANOVA or two-way ANOVA and post-hoc analyses used as stated in figure legends.

Results

The $H2^b$ allelic variant is associated with accelerated autoantibody production

In previous work in the CD45E613R BALB/c model, we established that CD45E613R BALB/c mice have altered B cell development that leads to the production of dsDNA autoantibodies, as opposed to the CD45E613R B6 mice which remain tolerant and lack autoantibody development. Although the CD45E613R BALB/c mice develop autoantibodies, they fail to progress to GN. In order to unravel the complexities of the genetic modifiers impacting the development of autoantibodies and end organ disease, a genome wide SNP analysis of BALB/c-B6 F2 mice was performed as previously described [22]. A suggestive peak on chromosome 17 corresponds to the MHC H2 region (Fig. 1A). Mice were sorted based on MHC H2 genotype and interestingly, the B6 $H2^b$ allele associated with autoantibody production in an additive fashion (Fig. 1B).

Given that the CD45E613R B6 mice failed to develop autoantibodies, we next asked if the BALB/c $H2^d$ allele prevents progression to rampant autoimmunity as was seen in the original B6-129 background[28]. To test whether there is a role for the MHC H2 region in autoantibody production, we crossed the CD45E613R mutation onto the BALB/c $H2^b$ (MHC^{B6}) congenic background and monitored mice for 6 months. By 10 weeks of age, CD45E613R BALB/c MHC^{B6} mice developed significantly higher titers of autoantibodies than did CD45E613R BALB/c mice (Fig. 1C). Congenic mice developed higher titers of IgG2a and IgG2b, and acquired the IgG1 subtype, which was not seen in CD45E613R BALB/c mice (Fig. 1D). CD45E613R BALB/c MHC^{B6} antibody titers were also significantly higher by dilution series (Fig. 1E). Positive HEp-2 staining, a test for autoantibody production, confirmed the presence of serum autoantibodies as early as 8 weeks of age (Fig. 1F), and these antibodies were found to have a high specificity for dsDNA demonstrated by *C. luciliae* staining at 6 months. Aged mice also showed a wide variety of HEp-2 cellular staining patterns, suggesting the presence of a broad spectrum of targeted autoantigens (sup. Fig. 1A).

CD45E613R BALB/c MHC^{B6} mice develop glomerulonephritis and increased lymphoproliferative disease

As early as 8 weeks of age, when autoantibodies are first detected, CD45E613R BALB/c MHC^{B6} mice show increased splenic weight (Fig. 2A), and at 6 months develop splenomegaly and lymphadenopathy. Interestingly, even the CD45 wild type MHC^{B6} mice have significantly increased splenic weight by 6 months, though they remain negative for autoantibodies (Fig. 2B). Upon aging, CD45E613R BALB/c MHC^{B6} develop endo- and extra capillary proliferative and necrotizing GN, defined and scored by a pathologist blinded to genotype (Fig. 2C, D), with low levels of proteinuria (data not shown). Staining nephritic

kidneys revealed an influx of F4/80+ cells surrounding glomeruli, absent in BALB/c MHC^{B6} (sup. Fig. 1B) and CD45E613R BALB/c aged mice (Hai and Hermiston, unpublished data). Aged CD45E613R BALB/c mice developed IgG (Fig. 2E) and C3 (Fig. 2F) kidney depositions upon aging, which were retained in the MHC^{B6} congenics. The positive IgG staining in kidneys, along with the subtype of GN ultimately suggests an immune complex-mediated GN [147]. Interestingly, some mice aged to 12 months or older developed infiltrating B cell lymphomas (data not shown), consistent with the observation that several human autoimmune diseases are associated with increased risk for hematologic malignancies [172, 173], presumably due to the dysregulated BCR signaling that often accompanies autoimmunity. These data indicate the MHC^{B6} allele is required for the progression of disease from autoreactivity to autoimmunity.

Alterations in immune cell compartments precedes the development of GN

To determine if allelic variation of the MHCII allele alters immune cell development, we phenotypically characterized the immune compartment by examining mice at 6 or 8 weeks of age, prior to disease onset. We observed a decrease in the splenic CD8+ T cell subset in CD45E613R BALB/c MHC^{B6}, with an increase in CD4+ T helper cells (Fig. 3A,B), and NK cells (Fig. 3C) by 8 weeks of age. No significant changes in T cell activation, as measured by CD69 staining, were observed (data not shown). Further analysis of T cell subsets showed a significant decrease in percentage of FoxP3+ T regulatory cells in the MHC^{B6} lymph nodes (Fig. 3D), but not in the spleen (data not shown). These minor alterations in T regulatory cells are suggestive of a decreased ability to suppress an inflammatory state, but are unlikely to be sufficient to drive the development of autoimmunity. These observations are in line with prior work indicating T cells were not required for autoantibody production (Mills and Hermiston, unpublished data).

In the CD45E613R BALB/c model, B cells were previously shown to be the dominant drivers of autoantibody production[22]. To investigate further whether alterations in B cell subsets lead to accelerated autoantibody production, we examined splenic CD19+ B cell subsets. At 6 and 8 weeks of age, there were no obvious changes in B cell numbers, but by 6 months B cells were increased, as expected (Fig. 4E). When examining the distribution of CD19+ B cells between tissues at 8 weeks of age, there is a significant reduction in bone marrow (BM) production of B cells, and a trend towards increased lymph node B cells in CD45E613R BALB/c MHC^{B6} mice (Fig. 3F). Further dissecting the BM B cell subsets, we observed an alteration in the proportions of preB and immature B cells to (Fig. 3G-I). Taken together with the reduced percentage of CD19+ cells in the BM, these data may suggest that there is an alteration in B cell development. As autoantibodies are first produced between 8 and 10 weeks of age, it is presumed some autoreactive cells escape to the periphery where they presumably begin to proliferate.

Examination of more mature B cell populations in the CD45E613r BALB/c MHC^{B6} mice at 8 weeks of age revealed a decrease in follicular B cells (Fig. 3J) and an increase in marginal zone B cells in the spleen (Fig. 3K). As the development of the B2 cell population is likely influenced by the enhanced signal strength provided by the CD45E613R mutation, we also looked at peritoneal B1 and B2 B cell populations. Consistent with this, we noted an increased prevalence of B2 cells and a decreased prevalence of B1a B cells in CD45E613R BALB/c MHC^{B6} as compared to CD45E613R BALB/c mice (Fig. 3L). These alterations in the B cell compartment suggest that the CD45E613R mutation and MHC allelic variant cooperate to influence the development and selection of autoreactive B cell prior to disease onset.

MHCII H2^{b} expression is increased in B cells from CD45E613R BALB/c MHC^{B6} mice

To determine if distinct MHCII alleles are associated with differences in cell surface protein expression, we quantified MHCII expression in lymph node B cells from 6 week old mice. We observed an allelic dose-dependent increase in cell surface expression on BALB/c MHC^{B6} B cells, which was enhanced by the presence of the CD45E613R mutation (Fig. 4A), but only in the CD19+ subset (sup. Fig. 2A). Upon stimulation with the TLR9 ligand CpG1886, we also saw a significant increase in MHCII expression on CD45E613R BALB/c MHC^{B6} B cells (Fig. 4B). We investigated the possibility that antibody affinity could artificially increase the cell surface expression by flow cytometry, but early work on the M5/114 clone showed no affinity or specificity differences between the $H2^b$ and the $H2^d$ (BALB/c endogenous) alleles[174]. Additionally, naïve B6 splenic B220+ B cells stained with the same antibody clone showed increased expression of MHCII when compared to B6 MHC^{Ba} mice (supp. Fig. 2B), indicating that this increased expression is specific to the B6 MHCII allele.

CD45E613R BALB/c MHC^{B6} B cells have an increased stimulatory capacity

We hypothesized that altered B cell development and an increased capacity for antigen presentation by the $H2^b$ allele might increase B cell responsiveness to ligand stimulation. Isolated B cells from lymph nodes of 8 week CD45E613R BALB/c MHC^{B6} mice were stimulated for 18 hours with anti-CD40 antibody, CpG1668, or IgM $F(ab')_2$. This stimulation resulted in an increase in basal levels of CD80 and CD86, and relative increases were calculated by determining the fold change in expression. The fold change in the $CD80^{hi}$ $CD86^{hi}$ population increased in CD45E613R BALB/c MHC^{B6} B cells upon stimulation (Fig. 5A). These increases were primarily driven by the increased expression of CD80 on B cells (Fig. 5B-D), potentially suggesting a preference for upregulation of a costimulatory pathway. No changes in proliferation were seen by 18 hours (data not

shown). Collectively, these data demonstrate a hyperresponsive B cell phenotype upon stimulation in the CD45E613R BALB/c MHC^{B6} background.

Discussion

Autoimmunity is the result of the breakdown of intrinsic immune tolerance mechanisms leading to reactivity against self-antigens. While environmental triggers may influence the pathogenesis of autoimmune disease by inducing epigenetic alterations[147], both classical genetic and twin studies have suggested that genetics likely play dominant role in influencing disease susceptibility and progression. More recent genetic manipulation involving model systems and advanced in-depth sequencing technologies of patient samples have corroborated these findings [141, 147, 148]. A commonality of these studies is the identification of the MHC region as a disease modifying locus [4, 171], but no clear link has been established between expression of MHC variants and the development of an SLE phenotype. This work establishes that expression of an MHC susceptibility allele is required for the progression of disease from autoreactivity to a fulminant autoimmune phenotype.

Utilizing the CD45E613R SLE model, the influence of individual susceptibility loci to disease pathogenesis can be studied at each stage in the progression from autoreactivity to complete autoimmunity [5]. In this study, we exploit the tractability of this model to identify the distinct role of genetic modifiers associated with differences in strain background in modulating the progression of SLE. A SNP screen identified the B6 MHC H2^{b} allele as a susceptibility locus in an additive model (Fig. 1B) that predisposes to autoantibody development. To confirm this, we crossed the MHC^{B6} allele onto the CD45E613R BALB/c strain, resulting in significantly accelerated autoantibody production

(Fig. 1C, F). CD45E613R BALB/c MHC^{B6} demonstrated increased production of dsDNA autoantibody Ig subtypes seen in CD45E613R BALB/c mice, but also develops the IgG1 subtype. Consistent with our hypothesis, the CD45E613R BALB/c MHC^{B6} mice go on to develop GN, indicating a potential role of IgG1 in disease pathogenesis. Further studies investigating the relationship between IgG1 production and disease progression in this model are required before a causative role can be ascribed.

Early signs of immune dysfunction in this model are noted in 8 week old mice from either wild type or CD45E613R mutants and manifest first as increased splenic weight (Fig. 2A) and development of dsDNA autoantibodies (Fig 1F). By 6 months of age, LPD is evident and GN develops with IgG and C3 complement deposition (Fig. 2B-E). F4/80+ cells appear to surround the diseased glomeruli, consistent with a role for these cells in mediating the development of GN in other models of SLE [175, 176]. Additional studies investigating hyperresponsive signaling of the myeloid compartment and the relationship to the development of GN in the CD45E613R model system are forthcoming (Hai and Hermiston, unpublished).

Previous work in this model indicated a predominant role of aberrant B cell activity in the development of autoantibodies. Upon phenotypic analysis of the B cell compartment, we observed differences in B cell population's composition. Previous work in SLE patients [18] and murine models [177] has identified a central tolerance checkpoint between preB cells and immature B cells in the BM, prior to entry into the periphery. Looking at these BM B cell compartments, we see a dramatic increase in pre B cell levels and significant decreases in immature B cell levels in the MHC^{B6} mice (Fig. 3F-H). These data may suggest increased numbers of autoreactive B cells in 8 week mice, correlating with the development of autoantibodies confirmed by HEp-2 staining of sera. Additional phenotypic analysis of B

cells present in a variety of different tissues demonstrated a shift towards increased lymph node B cells in CD45E613R BALB/c MHC^{B6}, indicative of the increased splenocyte numbers and presumably of the proliferation of B cells upon antigen stimulation in the periphery.

Peritoneal B cells are a population of peripheral B cells in that contain both B2 and B1 B cells which are known to be derived from the fetal yolk sac, rather than from the BM [178]. Development towards the B1 or B2 lineages is dependent on BCR signal strength[162]. B2 and B1b are CD5- peritoneal B cells, a negative regulator of B cell activation [179]. As the CD45E613R BALB/c MHC^{B6} mice show an increased prevalence of these populations, this suggests a breakdown of the negative regulation of activated B cells as CD45E613R-mediated autoreactivity is acquired. Future studies are needed to investigate the specific mechanisms by which the MHC H2 region and BCR signaling converge to modulate B cell development.

T cells in the CD45E613R BALB/c model were previously shown have little role in autoantibody production, as TCR-/- CD45E613R BALB/c mice still developed autoantibodies upon aging (Mills and Hermiston, unpublished data). However, while T cells may not be necessarily required for autoantibody production, they may be required for the enhancement of autoantibodies and for the development of end organ damage observed in these mice. To determine if the MHC alleles were altering T cell populations, we examined splenic populations and observed an increase in the percentage of CD4+ and a decrease in the percentage of CD8+ T cell percentages, suggesting a role for T cells in this genetic context. Further investigation revealed a decrease in the prevalence of T regulatory cells in the lymph nodes, indicating a potential defect in the ability to suppress inflammation in the MHC^{B6} mice. Therefore, larger numbers of CD4+ T helper cells in combination with the altered B cell development led us to investigate possible alterations in B cell antigen

presentation function, potentially leading to increased T cell help. As differences in MHCII cell surface expression have been shown to alter B cell response to antigen stimulation [21], and SLE patients are known to have increased expression of MHCII [53], we examined MHCII expression levels. Interestingly, lymph node B cells from 6 week mice, obtained prior to autoantibody development, but not CD19- cells, were shown to have increased MHCII levels (Fig. 4A, sup. Fig. 2A). These results could indicate several mechanisms of action, one of which may be that the MHC^{B6} allele is revealing a T cell epitope not present in the MHC^{Ba} repertoire. This new epitope could potentially trigger the immune activation, increased MHCII on B cells and result in an amplification loop, and the disease progression observed. Future work will investigate the exact role of T cells in mediating disease progression.

To determine if these altered MHCII levels affected B cell phenotypes upon stimulation, B cells were stimulated with the TLR9 agonist CpG, which resulted in a significant increase in MHCII upregulation in the CD45E613R BALB/c MHC^{B6} cells compared to CD45E613R BALB/c (Fig. 4B). As the MHC H2 region contains several immunologically important genes, including MHCII, MHC Class I, TNF superfamily genes, as well as complement components, we cannot rule out a possible role for these genes in modulating the observed phenotype. The increased prevalence of NK cells in the periphery could be indicative of a process in which autoimmunity begins in the periphery, but may also indicate a role for MHC class I variants in disease development. Studies of B6 MHCII-/- chimeras demonstrated a requirement for MHCII on kidney epithelial cells for development of GN, though the role of non-BM derived myeloid populations cannot be ruled out[180]. However, as the increased expression of MHCII was seen prior to the development of autoantibodies at 6 weeks, and not seen in other cell types, we hypothesize that the MHCII levels on B

cells may play an important role in overcoming the barrier between the development of autoantibodies and the progression to fulminant GN in the CD45E613R BALB/c mice.

Based on this work and the aforementioned studies of the role of MHCII in autoimmunity, we would predict that altered MHCII levels would induce an increased activation capacity by B cells upon stimulation. Consistent with this hypothesis, stimulation of B cells from CD45E613R BALB/c MHC^{B6} mice with anti-CD40 antibody, CpG8, or IgM antibody caused increased CD80 expression, reflected by an increased CD80 MFI, fold change in CD80 MFI, and an increased proportion of $CD80^{hi}$ $CD86^{hi}$ B cells (Fig 5). The co-stimulatory molecule CD80 has been shown to be used preferentially over CD86 to inhibit B cell activation. In conjunction with the decreased CD5+ B cell populations in the peritoneum, these data suggest a mechanism by which a tolerance checkpoint fails to quench autoreactivity, resulting in peripheral amplification and mediating end organ damage.

We propose that the additive effect of the susceptible B6 MHC $H2^b$ susceptibility locus and the CD45E613R BALB/c background allows for accelerated progression of autoreactivity and is required for progression to an autoimmune phenotype. This second hit to the immune system in conjunction with the sensitizing CD45E613R mutation, tips the immune system to breach the last barrier in the development of end organ damage. Further studies are needed to determine the precise mechanism by which MHCII and B cells may be exerting these effects. Together, these studies support a model whereby increased CD45E613R-mediated BCR signal strength in conjunction with the causative B6 $H2^b$ allele, alters immune cell development, and B cell co-stimulatory potential, resulting in peripheral amplification and a fulminant autoimmune phenotype. By demonstrating a role for MHC class II levels on modulating B cell activity, leading to the development of autoimmunity, we highlight one pathway by which common variants of MHC H2 can combine with

secondary mutations present in the genome to augment disease initiation, progression, and severity.

Acknowledgements & Footnotes

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Figures

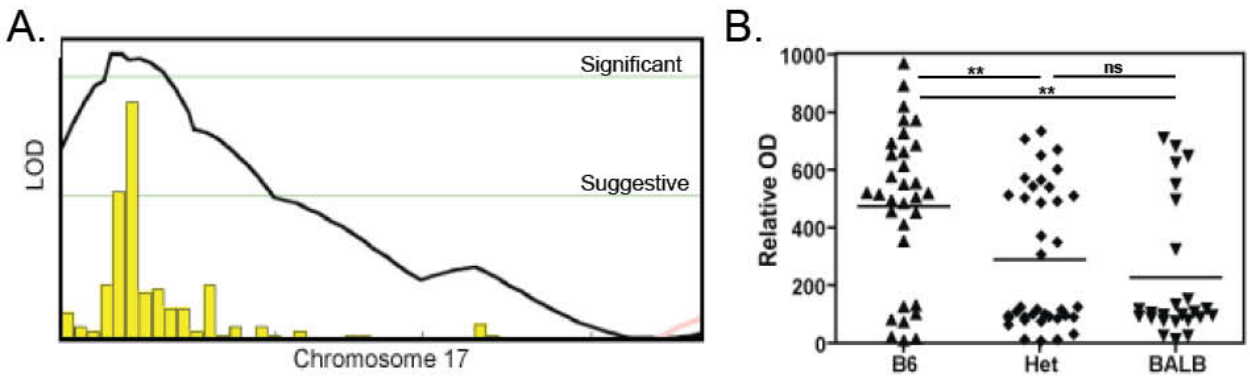


Figure 1. B6 MHC $H2^b$ allele associated with autoantibody production.

(**A**) Interval mapping of locus *Wam2* on chromosome 17 from the SNP analysis. (**B**) Anti-dsDNA IgG relative OD of cohort segregated by *Wam2* genotype, as published in [22].

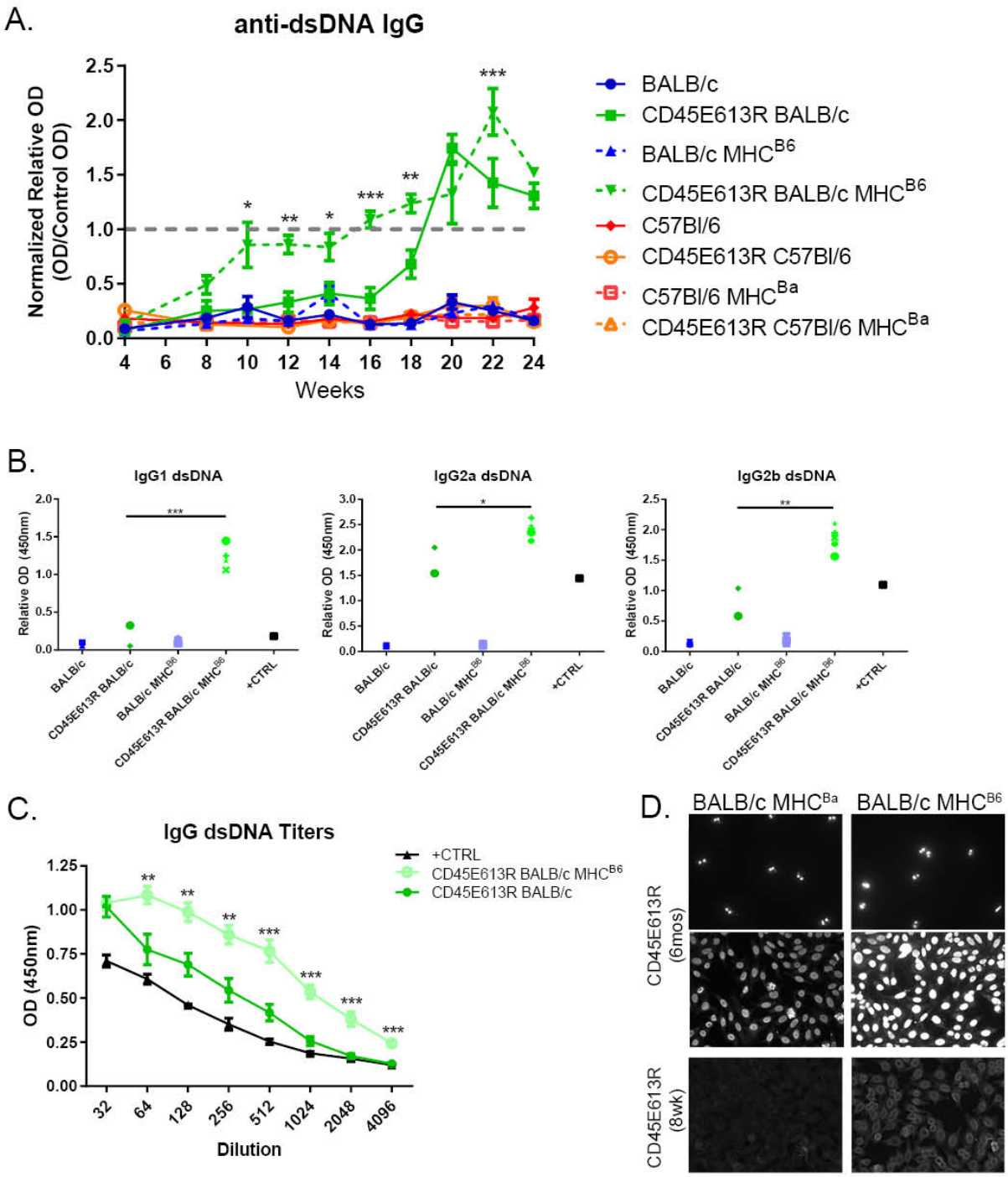


Figure 2. MHC^{B6} allele accelerates autoantibody production in CD45E613R BALB/c mice.

(A) Normalized relative OD of IgG dsDNA autoantibody production over time (n>10 per genotype). Statistical comparison by multiple t-tests is between CD45E613R BALB/c and

CD45E613R BALB/c MHC^{B6}, *p*-value indicated at each time point. (**B**) Relative OD of IgG subtypes ELISA for dsDNA autoantibody production of 6 month sera. One-way ANOVA, then Turkeys multiple comparisons test. (**C**) Titration of 6 month sera (n=5). Statistical comparison by multiple t-tests is between CD45E613R BALB/c and CD45E613R BALB/c MHC^{B6}, *p*-value indicated at each time point. (**D**) *C. luciliae* staining, top, and HEp-2 staining, middle, of 6 month sera and of 8 week sera, bottom from CD45E613R BALB/c with indicated MHC allele. SD, *p<0.05, **p<0.005, ***p<0.0001; representative data from three independent experiments.

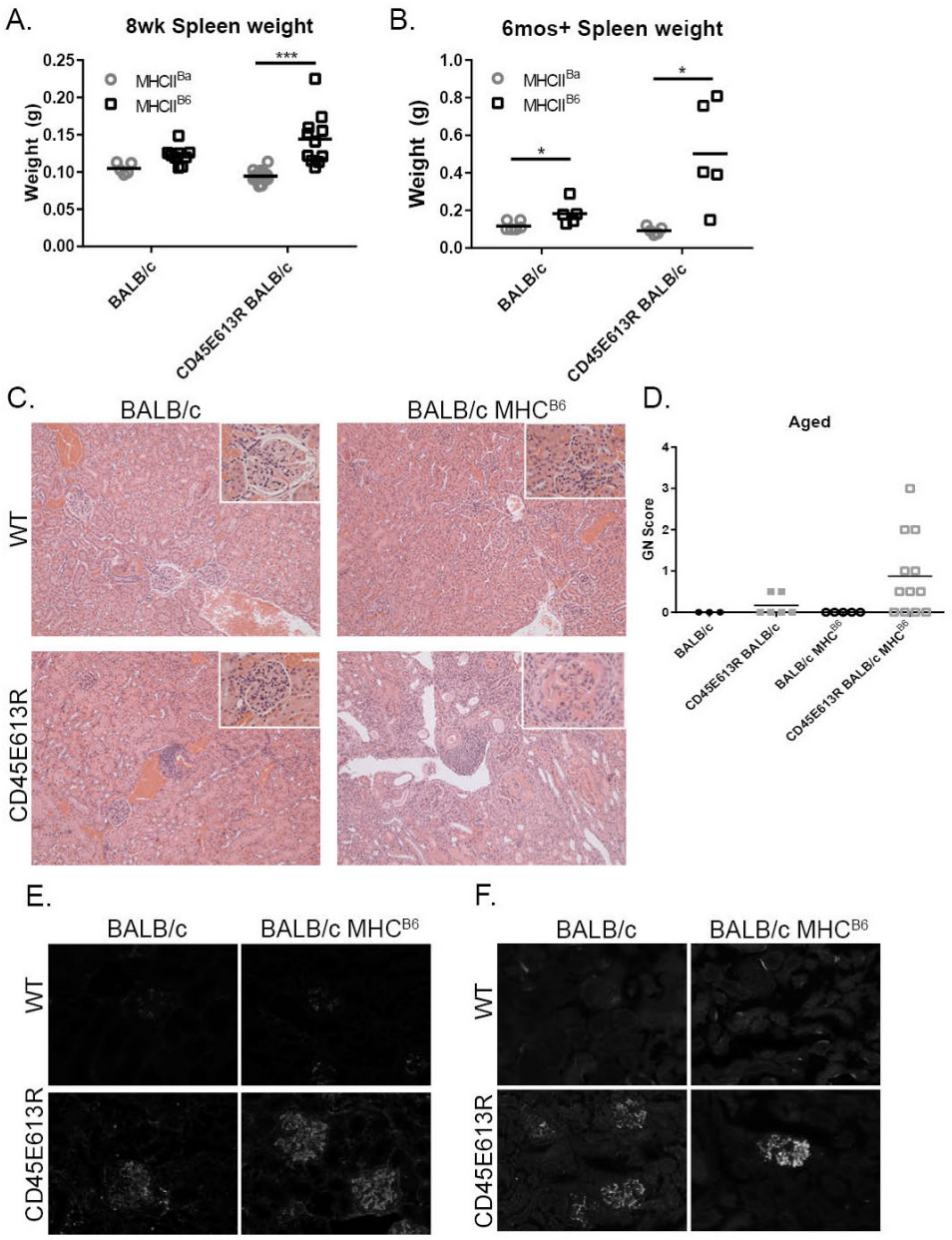


Figure 3. CD45E613R BALB/c MHC^{B6} mice develop glomerulonephritis.

(**A**) Spleen weight of each genotype at 8 weeks and (**B**) 6 months and older. (**C**) H&E stains of 6 month old kidneys, inset is of enlarged glomeruli. (**D**) Glomerular nephritis (GN) scores of individual mice, where each dot is one mouse. (**E**) IgG and (**F**) C3 deposition in kidneys of 6 month old mice. Statistical comparison by multiple t-tests, *p<0.05, **p<0.005, ***p<0.0001; representative data from three independent experiments.

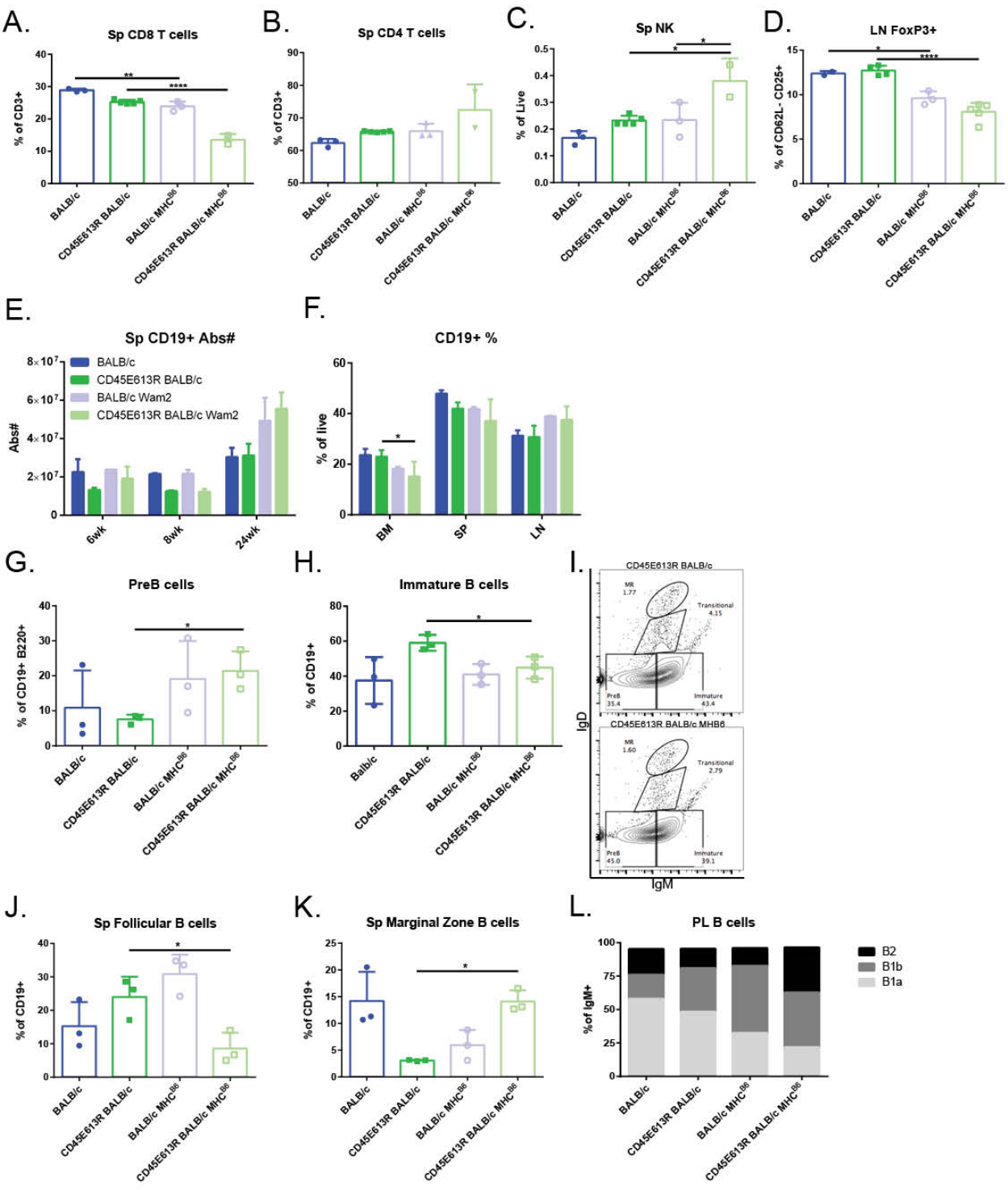


Figure 4. MHC H2^{b} allele results in altered immune cell compartments.

8 week mice were analyzed for (**A**) splenic CD3+ CD8+ T cells and (**B**) CD3+ CD4+ T cells, (**C**) splenic NK cells (panNK+). (**D**) Lymph node FoxP3+ cells gated on CD45+ CD4+ and

shown as a fraction of CD25+ CD62L- T cells. (**E**) Absolute number of genotypes splenic B cell populations over time (Sp; CD19+). 8 week old mice (**F**) percentage of cells CD19+ in bone marrow (BM), spleen, and lymph node (LN). (**G**) BM pre-proB cells, and (**H**) immature B cells gated as shown in (**I**), representative plots of CD45E613R BALB/c (*top*) and CD45E613R BALB/c MHC^{B6} (*bottom*) (**J**) splenic follicular (CD19+ CD23+ AA4.1-) and (**K**) splenic marginal zone (CD19+ CD23+ CD21+) B cells percentage of CD19+. (**L**) Peritoneal (PL) B cell populations B1a (IgM+ CD5+ B220+), B1b (IgM+ CD5- B220-), and B2 (IgM+ CD5- B220+). SD. One-way ANOVA, Turkey's multiple comparisons test, *p<0.05, **p<0.005, ***p<0.0001; representative data from three independent experiments, n=2-5 mice per genotype.

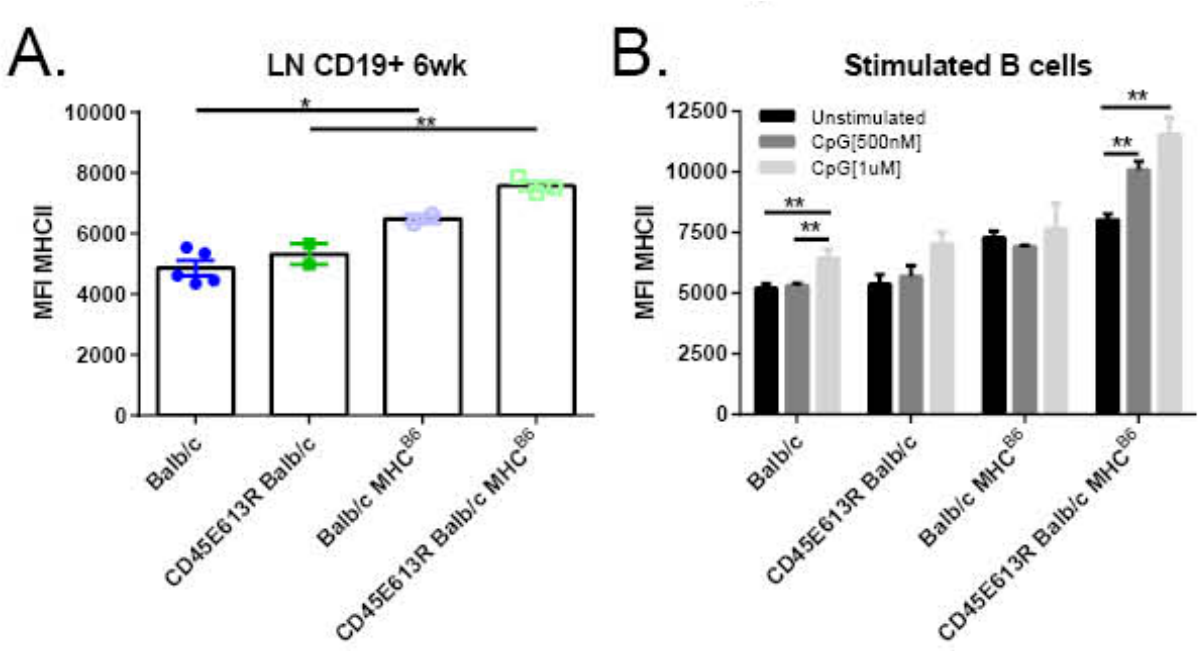


Figure 5. B6 MHC Class II allele associated with increased expression level prior to disease onset.

(**A**) Isolated 6 week lymph node B cells (CD19+) stained for MHCII at basal. One-way ANOVA, Turkeys multiple comparisons test. (**B**) MHCII MFI upon stimulation for 18hrs. SD. Two-way ANOVA, Sidak's multiple comparisons test, *p<0.05, **p<0.005, ***p<0.0001; n=3-5.

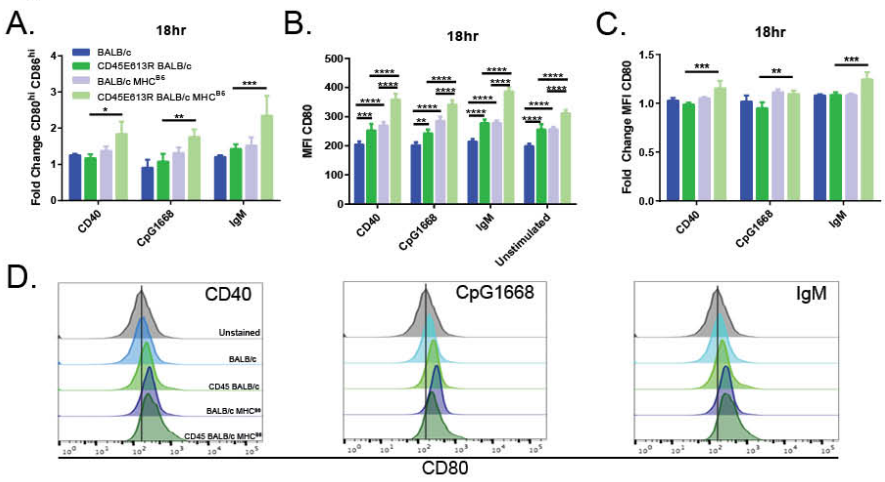
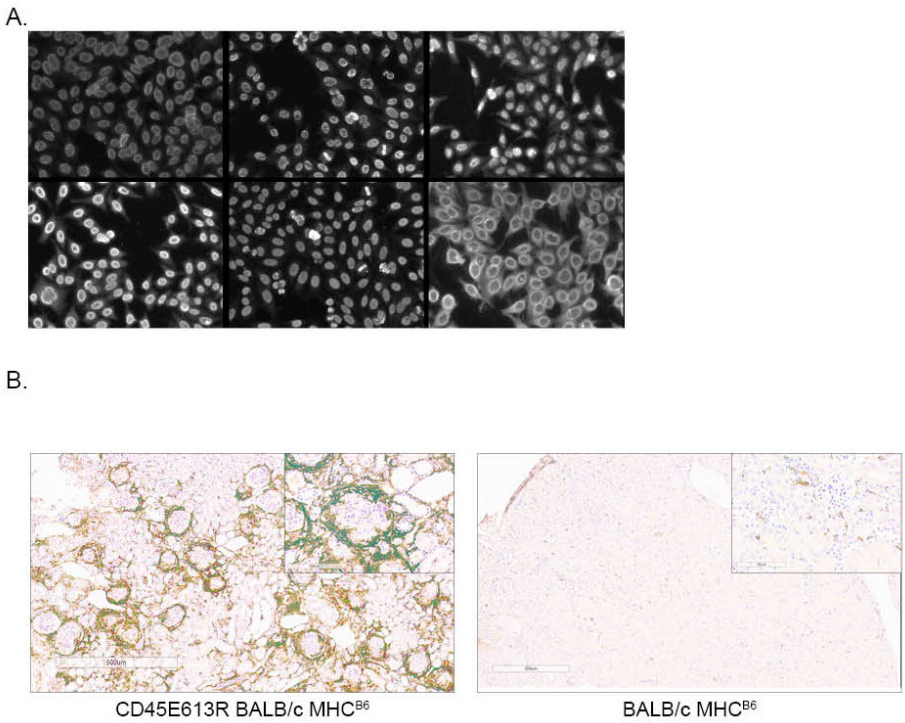


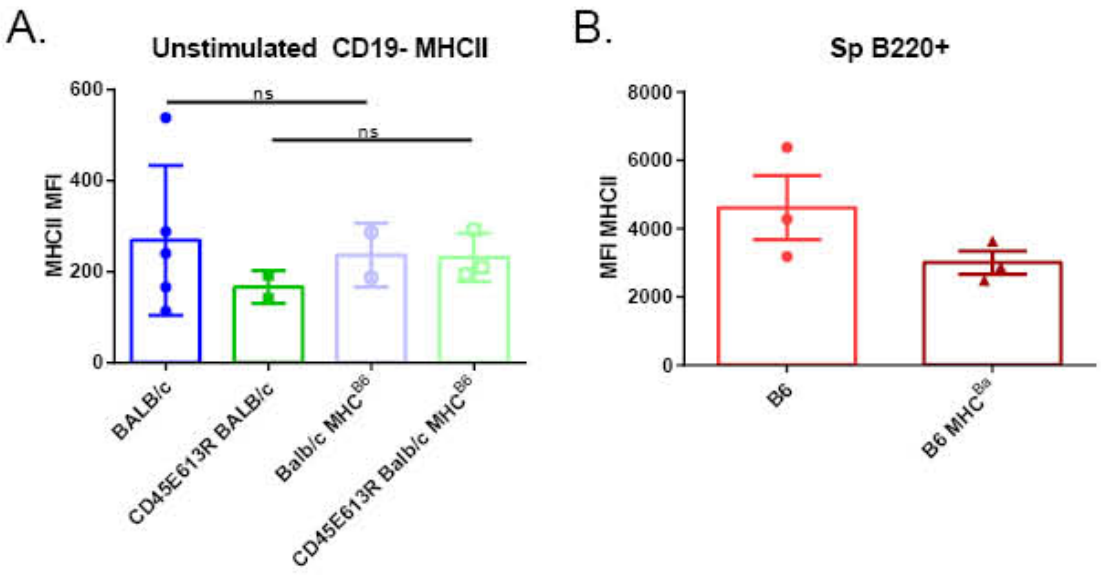
Figure 6. CD45E613R BALB/c MHCB6 B cells have augmented stimulatory capacity potential.

(**A**) Fold change in percentage of isolated lymph node B cells (B220+) $CD80^{hi}$ $CD86^{hi}$ population, (**B**) CD80 MFI (**C**) CD80 MFI fold change after 18 hours of stimulation as indicated. (**D**) Representative plots of CD80 upregulation upon stimulation indicated. SD, Two-Way ANOVA, Turkeys multiple comparisons test, *p<0.05, **p<0.005, ***p<0.0001; representative data from two independent experiments, n=3-5.



Supplementary Figure 1. Autoantibody and GN phenotype in CD45E613R BALB/c MHC^{B6} mice.

(**A**) Sera from 6 month mice were used to stain HEp-2 slides. (**B**) Immunohistochemistry of F4/80+ cells from 6 month CD45E613R BALB/c MHC^{B6} (*left*) and BALB/c MHC^{B6} mice kidneys (*right*). Inset of a glomeruli. Representative of 3 independent experiments, n=3-5 mice per genotype.



Supplementary Figure 2. Altered MHCII expression is allele and cell type specific.

(**A**) 6 week lymph node cells gated on CD19- and stained for MHCII. One-way ANOVA, Turkey's multiple comparisons test. (**B**) Splenic B220+ cells from 8 week mice stained for MHCII and plotted by MFI intensity. Student's t test. SD; representative of two independent experiments, n=2-5 mice per genotype.

CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

The breadth of symptoms constituting diagnostic criteria under the American College of Rheumatology guidelines for SLE presents difficulties for both diagnosis and individualized treatments. Current therapeutic approaches employ broad immunosuppression for most patients and use of B cell targeted therapies in a select subset of patients. [143]. Unfortunately, the lack of therapeutic effectiveness for a significant number of patients remains disappointing. To address these gaps in the field, enhanced understanding of the genetic and environmental alterations that contribute to the development and progression of SLE are needed. This will enable current therapies to be more effectively tailored to patients that would benefit from them and enhance development of novel targeted therapies.

The genetic contributions to SLE are complex. Only a small subset of patients has been found to harbor monogenic mutations linked to disease. To explain this finding, the current view in the field is that SLE is a polygenic disease with a multi-step pathogenesis[5]. Initially, genetic polymorphisms that impact tolerance checkpoints permit development and retention of autoreactive lymphocytes. These autoreactive lymphocytes migrate to the periphery where they encounter self-antigen, become activated, and proliferate or differentiate, resulting in development of autoantibodies. Finally, these autoantibodies form immune complexes that deposit in target tissues, such as the kidneys, skin, or lung, and initiate immune cell-mediated tissue destruction (Chapter I, Figure 1). It is hypothesized that the contribution of multiple polymorphisms within each patient, potentially differing between individual patients or ethnic backgrounds, could explain the heterogeneous nature of SLE[6, 141] and the skewing of disease severity in Hispanic and African-American populations[141]. Unfortunately, identification of polymorphisms

contributing to SLE pathogenesis has been difficult, due to the frequency of these common polymorphisms within the healthy population.

Due to the potential for several common polymorphisms to alter multiple immune pathways, murine models have been instrumental in identifying individual contributions to disease. One such model is the CD45E613R model of SLE. The advantage of this model compared to other SLE models is that the phenotypic consequences of disease depend on the genetic background in which the mutation is placed. This indicates a role for genetic modifiers present in each mouse strain in determining the disease phenotype. This model allows us to dissect each stage of the multi-step pathogenesis of disease in these mice, such that CD45E613R B6 or 129 are normal, with no signs of disease. CD45E613R BALB/c mice develop autoreactive B cells and autoantibodies, corresponding to the first and second stages of pathogenesis. Finally, F1 CD45E613R B6-129 mice develop high titer autoantibodies and GN with complete penetrance, reaching that final stage of disease pathogenesis. Utilizing this model, we show here the individual contributions of seemingly innocuous alleles of TLR9 and MHC H2 in a wildtype background, that, when combined with the sensitizing CD45E613R allele, contribute to each step of SLE pathogenesis.

TLR9 ALLELES CONFER EITHER AUTOANTIBODY PRODUCTION OR TOLERANCE

A genetic modifier screen identified TLR9 as a potential disease-modifying locus in CD45E613R mice. The role of TLR9 in the development of autoantibodies in SLE models has been shown to be both protective and causative, depending on the experimental model system. Consistent with those findings, in the CD45E613R model of SLE, previous work has shown two alleles of TLR9 from the B6 and the BALB/c genetic strains had opposing roles. Genetic deletion of the B6 allele revealed a protective role whereby the CD45E613R

B6 TLR9-/- mice developed autoantibodies. In contrast, genetic deletion of the BALB/c allele revealed a causative role whereby CD45E613R BALB/c TLR9-/- mice lost dsDNA autoantibody production. Interestingly, the magnitude of response to TLR9 stimulation differed between these alleles such that BALB/c B cells had a much weaker response relative to B6 B cells. We hypothesized that the B6 allele functioned as a negative regulator of autoantibodies, while the BALB/c allele functioned as a positive regulator of disease, each converging in B cells with the hyperresponsive BCR signaling induced by the CD45E613R mutation to delete or retain autoreactive B cells, respectively. However, it was possible that genetic polymorphisms other than TLR9 could mediate these responses. To definitively prove that the allelic variations in TLR9 between these strains were mediating the differential responses at the B cell tolerance checkpoints, I swapped these alleles in congenic mice. In Chapter II, we presented data that conclusively demonstrates that the BALB/c allele of TLR9 is a positive regulator of autoantibodies, conferring the production of autoantibodies in the CD45E613R model, whereby each allele of TLR9 conferred the TLR9 signaling phenotype observed in intact mice.

Based upon our prior work, we hypothesized that the observed differences in TLR9 signal strength would alter tolerance and autoantibody production. To test this, we generated mice either heterozygous for the TLR9 alleles or homozygous for one allele and determined signaling and autoantibody production in the context of the CD45E613R mutation. We found that the $TLR9^{Ba}$ allele was dominant in the autoantibody phenotype, as only one copy was required for development of dsDNA autoantibodies at equivalent levels to the homozygous mice. However, one copy was insufficient to phenocopy BALB/c's hyporesponsive B cell signaling, as B cells heterozygous for the B6 and BALB/c alleles on the BALB/c background had an intermediate signaling phenotype. We speculate that this

intermediate signaling, though increased over TLR9$^{Ba/Ba}$, is still insufficient to result in the deletion of autoreactive B cells, hence the development of autoantibodies. One possible explanation for these results is that the TLR9 alleles may utilize the downstream NFkB and MAPK pathways differently, relying on one more than the other. The intermediate signal strength in response to CpG stimulation in TLR9$^{Ba/B6}$ B cells is consistent with this notion. However, initial experiments inhibiting each of these pathways independently upon TLR9 stimulation in CD45E613R TLR9 wt, congenic, or heterozygous B cells failed to produce consistent results. Further work exploring this hypothesis by repeating this experiment in the context of CD45wt is warranted as this may minimize the effect increased ITAM signaling may have with CD45E613R present. Alternatively, generating B cells lines with the various alleles using CRISPR-Cas9 technology could also more cleanly address this hypothesis.

Future work determining the threshold signaling required for tolerance induction would be interesting to follow up. One approach would be to utilize the Nur77-GFP reporter mice developed by Dr. Julie Zikherman crossed onto the TLR9 congenic strains. Nur77 is an immediate early gene[181] that is used as a surrogate marker for BCR signal strength, as increased GFP expression positively correlates with increased BCR signaling [182]. Co-stimulation of BCR and TLR9 in B cells from congenic mice with the Nur77-GFP reporter may reveal a directly observable alteration in downstream signal strength between the two alleles. We would predict that the B6 allele is the minimal signaling threshold required for tolerance induction. It is possible that the importance of strong TLR9 signaling in regulating B cell tolerance checkpoints could fluctuate during each B cell developmental stage. This may explain why we see differences in some, but not all developmental stages of

both the BALB/c and B6 TLR9 congenic mice compared to their wt counterparts. This approach could be used to evaluate these possibilities.

Do the TLR9 polymorphisms impact B cell development?

Our working model is that signal strength determines whether autoreactive B cells are deleted or maintained. Monitoring the frequency of B cell populations through development is one way to observe this. Meffre and colleagues have shown through BCR sequencing and single cell hybridomas from autoimmune patients and healthy controls [18, 20], that at least two tolerance checkpoints exist during B cell development, one between the preB and immature B cell stages in the bone marrow, referred to here as the central checkpoint, and one in the periphery between T2 and follicular B cells, referred to here as the peripheral checkpoint. They revealed a purging of autoreactive cells as they transition between these stages of development, indicated by a decrease in the proportion of B cells post checkpoint in healthy controls relative to individuals with autoimmune disease. Monitoring the ratio of these cell populations before and after the checkpoint purging can reveal a defect or alteration in this tolerance checkpoint. Increased ratios relative to control would indicate a failure to delete autoreactive B cells, while a decreased ratio would suggest increased deletion of autoreactive B cells. Correlating these ratios with autoantibody production could provide further evidence to whether deletion was functionally successful or not. We used this approach to investigate how these two TLR9 alleles may be altering B cell development tolerance checkpoints.

Our prior work identified a driving role for B cells in autoantibody development in CD45E613R mice [22]. Using mixed bone marrow chimeras, an increase in the ratio of B cells at the central tolerance checkpoint was observed in CD45E613R BALB/c but not

CD45E613R B6 mice. Consistent with this finding, we observed that CD45E613R B6 mice homozygous for the TLR9^{Ba} allele also demonstrated an increase in the ratio of B cells at the central checkpoint as early as 8 weeks of age, prior to any disease onset, supporting an intrinsic B cell defect. We speculate that failure to delete autoreactive B cells between the preB and immature B cell stages ultimately results in the development of the autoantibodies observed in these mice. Conversely, the TLR9^{B6} allele in the CD45E613R BALB/c mice resulted in a decrease in the ratio of B cells at the central tolerance checkpoint. To more definitively evaluate whether these alleles alter the B cell repertoire, one could perform single cell sequencing of the preB and immature B cell compartments to determine whether the proportion of B cells were being deleted or rearranged from all CD45E613R strains. Furthermore, developing B cell hybridomas from these two compartments would reveal what proportion of deleted B cells were autoreactive.

Determining the intrinsic and extrinsic effects the TLR9 alleles have on B cell development and autoantibody production utilizing mixed bone marrow chimeras is in progress. We aim to determine if the tolerogenic or permissive nature of each allele functions in cis or trans. Non-congenic strains carrying endogenous TLR9 alleles containing marked Ig antibodies (IgH^{a} or IgH^{b}) are mixed with bone marrow from the congenic strains, injected into host mice, and recipients monitored for autoantibody development. Depending on the IgH^{a} or IgH^{b} antibodies that develop, this will help to address whether the development of autoantibodies is B cell intrinsic. This approach would not definitively rule out influences by the myeloid compartment. Additional experiments will be performed in which CD45E613R congenic or CD45E613R TLR9 wt mice are adoptively transferred with equal amounts of TLR9^{Ba} or TLR9^{B6} bone marrow of which the endogenous allele is IgH marked and monitored for autoantibody production. This experimental design will confirm

the cis or trans data from the previously mentioned chimera experiments, but will also serve to determine the role of TLR9 on stromal cells. TLR9 is also expressed on kidneys and some epithelial subsets and is implicated in various autoimmune phenotypes including SLE [183, 184]. While we do not expect the stromal compartment to alter autoantibody production, it could influence development GN, so these mice will be monitored for GN development as well.

Myeloid alterations by the TLR9 alleles

The TLR9 alleles also resulted in alterations in additional cellular compartments, including myeloid cells. We observed an increase in myeloid cell populations in 8 week old CD45E613R B6 $TLR9^{Ba}$ mice. The opposite phenotype was observed in CD45E613R BALB/c $TLR9^{B6}$ mice, with decreased myeloid and Ter119+ cells in the spleen, consistent with a decrease in extramedullary hematopoiesis. One possible explanation is that the $TLR9^{Ba}$ allele results in a decrease in clearance of autoreactive B cells, which could potentiate the myeloid expansion in the CD45E613R B6 $TLR9^{Ba}$ mice.

Future work examining the requirement of the T and myeloid cellular compartments is required to determine if the TLR9 alleles work in cis or trans to alter cellular composition. Utilizing TCR-/- TLR9 congenics may aid in answering the importance of T cells in this model. Mice deficient for TCR, and therefore mature T cells, would not be expected to alter autoantibody production or GN development in the CD45E613R B6 $TLR9^{Ba}$ mice, as previous work has shown that CD45E613R BALB/c TCR-/- mice still developed autoantibodies. We do not believe GN development would be altered in this context because of the lack of T cell involvement in autoantibody production, but also because immunohistochemistry performed on nephritic kidneys from CD45E613R B6

TLR9^{Ba} mice showed no T cell infiltration. Rag-/- mice, which lack T and B cells, but retain the myeloid compartment, crossed to the congenic lines could indicate the importance of TLR9 within the myeloid compartment alone. Bone marrow chimeras reconstituting BALB/c TLR9^{B6} congenic Rag-/- with CD45E613R BALB/c TLR9^{Ba} or BALB/c Rag-/- mice with CD45E613R BALB/c TLR9^{B6} bone marrow would aim to address if TLR9 functioned in cis or trans for the myeloid effect. The same experiment would be conducted with the B6 strains. While these chimera mice would still retain a small proportion of myeloid cells containing the adoptively transferred TLR9 allele, a vast majority would contain the endogenous allele. We would predict that the myeloid expansion in the TLR9^{Ba} mice is a trans effect due to the B cell expression of TLR9 because previous work with chimeras did not show an influential role for the myeloid compartment when sufficient or deficient in TLR9 in the CD45E613R model. Alternatively, it could be that intrinsic TLR9^{Ba} expression in the myeloid compartment is mediating the LPD and myeloid expansion, which could be explained by the high expression of TLR9 within the myeloid compartment.

How do the TLR9 polymorphisms dictate differences in signal strength?

The two TLR9 alleles differ in 5 non-synonymous coding differences between the B6 and BALB/c strains: T325N, L378S, T573A, Q579H, and T867A. How these 5 TLR9 polymorphisms may be mediating this effect is still unclear, but altered MyD88 binding ability, cleavage of inactive TLR9 to the active form, or interference with ligand binding are all possibilities. These polymorphisms lie within two regions of TLR9, the leucine-rich repeats and the TIR domain. To address the potential contributions of these polymorphisms to the altered signaling observed between these alleles, studies using the CRISPR-Cas9 system to target the point mutations are currently in progress. We would predict that the polymorphism adjacent to the TIR domain at amino acid 867 may mediate the signaling

difference between the two alleles. The adaptor protein MyD88 binds to the intracellular TIR domain to mediate downstream signaling, and if mutated, may result in the altered signaling we observe. Early indications using this system in a BALB/c derived B cell line suggest that this polymorphism may be responsible for the decreased signaling in the $TLR9^{Ba}$ allele, in line with our hypothesis. However, additional sequencing and signaling studies are needed to confirm this observation.

The other four polymorphisms cannot be discounted in also contributing to the signaling phenotype. The leucine-rich repeats are important in both ligand binding and the cleavage required for the full activation of TLR9. If mutating the TIR domain polymorphism does not alter signaling, it may be that the other polymorphisms are altering ligand binding or preventing complete cleavage events to occur and are therefore responsible for the signaling phenotype. Additional mutations targeting each of the extracellular polymorphisms would be necessary to address this. It may be that an intermediate phenotype occurs upon targeting the TIR polymorphism, which would then indicate combinations of polymorphisms are responsible for the signaling phenotype. Cell lines that have had multiple polymorphisms targeted at once would be required to fully address this issue.

TLR9-BCR signals synergize to alter central tolerance checkpoints

Our working model is that TLR9 regulates the development and repertoire of autoreactive B cells by acting as a rheostat for the threshold of tolerance checkpoints in BCR-mediated cell death and autoimmunity. We propose that the CD45E613R mutation increases BCR signaling in B cells during development and the balance between elimination and retention of autoreactive B cells is further modulated by TLR9 signaling. This dual nature of TLR9,

as protective and negatively regulating tolerance or as permissive and positively regulating tolerance, has been a point of confusion in the field. Here we show two allelic versions of TLR9 delineate between these two roles in individual models. In the context of the B6 TLR9 allele, which is hyperresponsive to ligand stimulation, autoreactive B cells are eliminated, with the combined BCR-TLR9 signaling breaching the upper limit of threshold tolerances in the bone marrow. Conversely, the increased BCR signaling from the CD45E613R mutation, combined with the hyporesponsive BALB/c TLR9 allele results in signaling under the deletion threshold, allowing for retention of autoreactive B cells. Inhibiting TLR9, either through knockouts or knockdowns, often paradoxically results in a TLR7-mediated worsening of disease, and, an amelioration of dsDNA autoantibodies. Interestingly, we did not see an increased in TLR7-mediated B cell activation with the hyporesponsive $TLR9^{Ba}$, potentially indicating a TLR7-independent role for TLR9 in mediating disease progression.

Furthermore, we postulate that modulation of these tolerance checkpoints by additional increased signaling in the periphery can initiate the switch between autoreactivity to autoimmunity. If defects present at the bone marrow checkpoint allow for the retention of autoreactive B cells, once in the periphery, they may be able to inappropriately respond to self-antigens, inducing end organ damage.

CONTRIBUTION OF THE SUSCEPTIBLE MHC H2 LOCI

The MHC region has repeatedly been associated with the development of several autoimmune diseases, including SLE. How and by what mechanism these MHC alleles may influence SLE has not been elucidated. A potential mechanism has been suggested by studies examining SLE patient peripheral blood mononuclear cells[53], and by examining other autoimmune murine models[21]. These data propose that increased MHCII

expression on B cells may result in increased T cell help and thus inappropriate B cell activation, resulting in disease pathogenesis. However, a limitation of these studies is that MHCII expression was evaluated after disease onset so whether it was a cause or consequence of disease was unclear. Unfortunately, no studies have shown whether altering MHCII expression prior to disease onset alters disease pathogenesis or progression. In our CD45E613R BALB/c MHC^{B6} model of SLE, we are able to demonstrate that increases in B cell-specific cell surface MHCII expression precedes autoantibody production and disease onset. Upon aging, these mice developed severe GN, while CD45E613R BALB/c mice did not, indicating that the MHC H2^{b} allele is required for progression from autoreactivity to autoimmunity in the multistep pathogenesis of SLE model, resulting in immune-mediated kidney destruction.

B cells are hyper-responsive to activation

We were curious whether the MHCII alleles impacted the B cell compartment. Our working hypothesis predicted that the MHC^{B6} allele would result in altered B cell function, potentially by increased activation and function by the increased MHCII expression. The increased MHCII protein expression on BALB/c MHC^{B6} B cells, with or without the CD45E613R mutation, is consistent with this model. Upon TLR9 stimulation, MHC class II expression was further upregulated in CD45E613R BALB/c MHC^{B6} relative to CD45E613R BALB/c B cells. These B cells also had significantly increased CD80 expression after 18hrs of stimulation with CD40, IgM, or CpG1668. Together, these data suggest that the MHC^{B6} allele modulates B cell activation and suggests potentially altered interaction with T cells. This altered B:T cell interaction is also supported by the increase in CD4+ helper T cells and decrease in T regulatory cells in the spleen and lymph nodes. To directly test that whether increased MHC class II production would result in altered MHC-peptide

presentation to T cells, and thus the altered T cell proportions seen in mice, studies examining antigen presentation by B cells, T cell activation upon peptide presentation, and characterization of peptides presented are needed. One way to test this would be to use B cells from CD45E613R BALB/c MHC^{B6} or CD45E613R BALB/c mice loaded with antigen and presented to CD45 wt T cells, measuring T cell proliferation and B cell activation levels. If we observed differences in B or T cell activation or proliferation, this would suggest altered peptide presentation could be responsible for the phenotypes we observed. Mass spectrometry of the MHC-peptide complexes could reveal differences in peptides presented between the two strains as well.

Alterations in B cell compartments and tolerance

In Chapter III, we also demonstrated how the susceptible MHC^{B6} allele results in further alterations of the B cell compartment in the CD45E613R BALB/c model. We hypothesized that the MHC^{B6} allele would result in a defect in the peripheral checkpoint as cells transition from T2 to follicular B cells, based on MHC's role in activating naïve B cells upon antigen encounter, resulting in a further inability to clear autoreactive B cells. Splenic B cell compartments in 8-week-old CD45E613R BALB/c MHC^{B6} mice displayed a skewing towards retention of the majority of the T2 cell population, potentially suggesting few autoreactive B cells are being deleted. Finally, we also noted a marked increase in MHCII protein expression on B cells with the MHC^{B6} allele, but not with the MHC^{Ba} allele or in other cellular compartments. The modulation of the peripheral immune compartments is consistent with the role MHC class II plays in B cells during activation and presentation of antigens to T cells, and we hypothesize MHC^{B6} B cells may have increased ability to present self-antigen to T cells, which may provide increased T cell help, allowing for pathogenic autoantibodies and end organ damage to occur.

Determining the alteration in the BCR repertoire and autoantigen specificities in CD45E613R BALB/c MHC^{B6} mice, as suggested by the altered HEp-2 staining patterns, may also be of value. This could be explored through the use of autoantibody arrays to determine the autoantigens targeted by the CD45E613R MHC^{B6} congenic mice. We hypothesize that the broad autoantigens targeted by CD45E613R BALB/c mice (Hai et al, unpublished), would be further expanded in the congenic mice, and would show increased affinity by ELISA. Additionally, sequencing BCRs and developing single-cell hybridomas to determine percentages of overall B cell autoreactivity at early and late time points could indicate an expansion of autoreactive B cells over time. The presence of more diverse IgG subclasses in the congenic strains further supports this hypothesis, suggesting that germinal center B cells may be further activated in the CD45E613R BLAB/c MHC^{B6} mice.

Further work in the MHC congenic mice utilizing various chimeric experiments would be useful to determine the role of other cellular compartment to disease progression. As decreases in FoxP3+ T regulatory cells were observed in these mice, we predict that the role for T cells in our working model would be as providing B cells with help and promoting expansion and survival of autoreactive B cells. As previous work in the TLR9 context showed little role for T cells in autoantibody production, we hypothesize that T cells may be instead mediating the peripheral alterations we observe, and potentiating the GN development in these mice. We would predict TCR-/- CD45E613R BALB/c MHC^{B6} mice would fail to develop GN as a result of the lack of peripheral activation and T cell help provided to B cells and any myeloid mediated effects from their antigen presenting ability.

An increase in myeloid cells was also observed. We hypothesize that the exacerbation in GN seen in the MHC congenic mice that develops with age is likely myeloid-dependent. The F4/80+ IHC staining of glomeruli from GN-positive mice, but not

GN-negative mice are consistent with this hypothesis. Myeloid intrinsic and extrinsic effects may also play a role in GN development. Bone marrow chimeras reconstituting irradiated mice containing endogenous MHC alleles with the congenic MHC alleles could be useful in parsing these myeloid differences apart. We would anticipate that bone marrow chimeras in which equal proportions of bone marrow cells from IgM-/-CD45E613R BALB/c and CD45E613R BALB/c MHC^{B6} mice injected into irradiated wt BALB/c recipients would result in the same acceleration of autoantibody production and GN development due to the increased MHCII expression on B cells. We would also predict that if these proportions were heavily skewed towards IgM-/- CD45E613R BALB/c cells, such that a majority of myeloid cells contained the MHC^{Ba} allele, we would not see the myeloid expansion observed in the congenic mice, nor the development of GN. Unfortunately, this setup does not rule out the possibility that radiation-resistant tissue myeloid populations may be influencing GN development as well. Alternatively, it may be that CD45E613R BALB/c myeloid cells containing the endogenous MHC^{Ba} allele were still able to infiltrate kidneys in the presence of myeloid cells containing MHC^{B6} allele, we would infer that the GN and kidney infiltration may be a myeloid extrinsic effect, potentially mediated by radiation-resistant myeloid populations or the small percentage of MHC^{B6} myeloid cells present in these chimeric mice.

INTERPLAY BETWEEN TWO SUSCEPTIBILITY LOCI

In the CD45E613R B6 $TLR9^{Ba}$ mice, we expected that the presence of the $TLR9^{Ba}$ allele would result in autoantibody production. As these mice unexpectedly developed GN upon aging, we hypothesize that this may be explained by the presence of a secondary modifier gene on the B6 background. In the CD45E613R BALB/c MHC^{B6} congenic mice we showed that these mice were able to progress from autoreactivity and the development of

autoantibodies in the CD45E613R BALB/c model to development of GN in the presence of the B6 MHC $H2^b$ allele. In light of the results from the MHC^{B6} congenic mice, we hypothesized that the combination of the endogenous MHC^{B6} allele with the $TLR9^{Ba}$ allele could result in the hyperactivation of peripheral B cells and ultimately resulting in the GN observed in both CD45E613R BALB/c MHC^{B6} and CD45E613R B6 $TLR9^{Ba}$ mice (Chapter IV, Figure 1). The phenocopying between these two strains is significant and warrants further investigation to determine whether the susceptible B6 MHC allele is truly mediating this progression of disease. Future work crossing the resistant MHC^{Ba} allele onto the CD45E613R B6 $TLR9^{Ba}$ strain would directly answer this question. We would predict that these double congenic CD45E613R B6 $TLR9^{Ba}$ MHC^{Ba} mice would retain autoantibody production, but no longer develop GN.

CLINICAL IMPLICATIONS

Frontline therapy for patients includes the use of glucocorticoids and the endosomal TLR inhibitor hydroxychloroquine or chloroquine. Even with current treatment options, TLR9 activation by self-antigens induces glucocorticoid resistance in SLE patient dendritic cells[75], eventually rendering this category of immunosuppressant's ineffective for many patients. Consistent with the field, our data would suggest that patients with decreased TLR9 signaling would benefit from the TLR9 inhibition by these drugs, decreasing dsDNA autoantibodies and potentially some TLR7-mediated effects, as in the $TLR9^{B6}$ containing strains. Conversely, our model also suggests that inhibiting TLR9 signaling in patients with normal or high signaling phenotypes may instead be detrimental, and potentially lead to disease progression, akin to the $TLR9^{Ba}$ allele. Considering several TLR9 inhibitors are in various stages of development, and the potential side effects these therapies may present, including worsening of disease or malignancies [169], we would propose that

assaying TLR9 signaling prior to treatment may provide personalized therapeutic options for patients.

SUMMARY

The CD45E613R model of SLE is a tractable model to delineate normal development from autoreactivity and autoimmunity. A major benefit of this model is the ability to genetically modulate each stage of disease development and pathogenesis. We now have a genetic system where addition of susceptibility or resistant alleles allows for the amelioration or progression of disease. Current views on SLE are that it is a polygenic disease of common polymorphisms, cooperating at multiple junctions in immune development to perturb tolerance, culminating in disease pathogenesis. This fluctuation in genetic permissiveness to SLE in our model mimics the broad array of genetic contributions observed in SLE patients. Here we show through two different susceptibility genes, how multiple otherwise innocuous polymorphisms can combine to result in alterations at distinct stages of SLE pathogenesis in the context of a sensitizing mutation, in this case CD45E613R. We hypothesize that during tolerance induction of B cells during development, TLR9 signaling synergizes with BCR signaling to result in retention or deletion of autoreactive B cells. Further, in the periphery, in the context of the permissive $TLR9^{Ba}$ allele, and with the addition of the MHC^{B6} susceptibility allele, autoreactive B cells accelerate autoantibody production, become inappropriately activated in the periphery, and develop a myeloid-mediated GN. Current therapies for SLE aim to decrease endosomal TLR signaling, particularly that of TLR7 and TLR9. Given the results presented here, we would caution the use of TLR9 inhibitors in broad patient populations, but rather propose an individualized treatment plan whereby patient TLR9 signaling is assessed prior to therapy induction, especially those targeting TLR9 signaling.

Figures

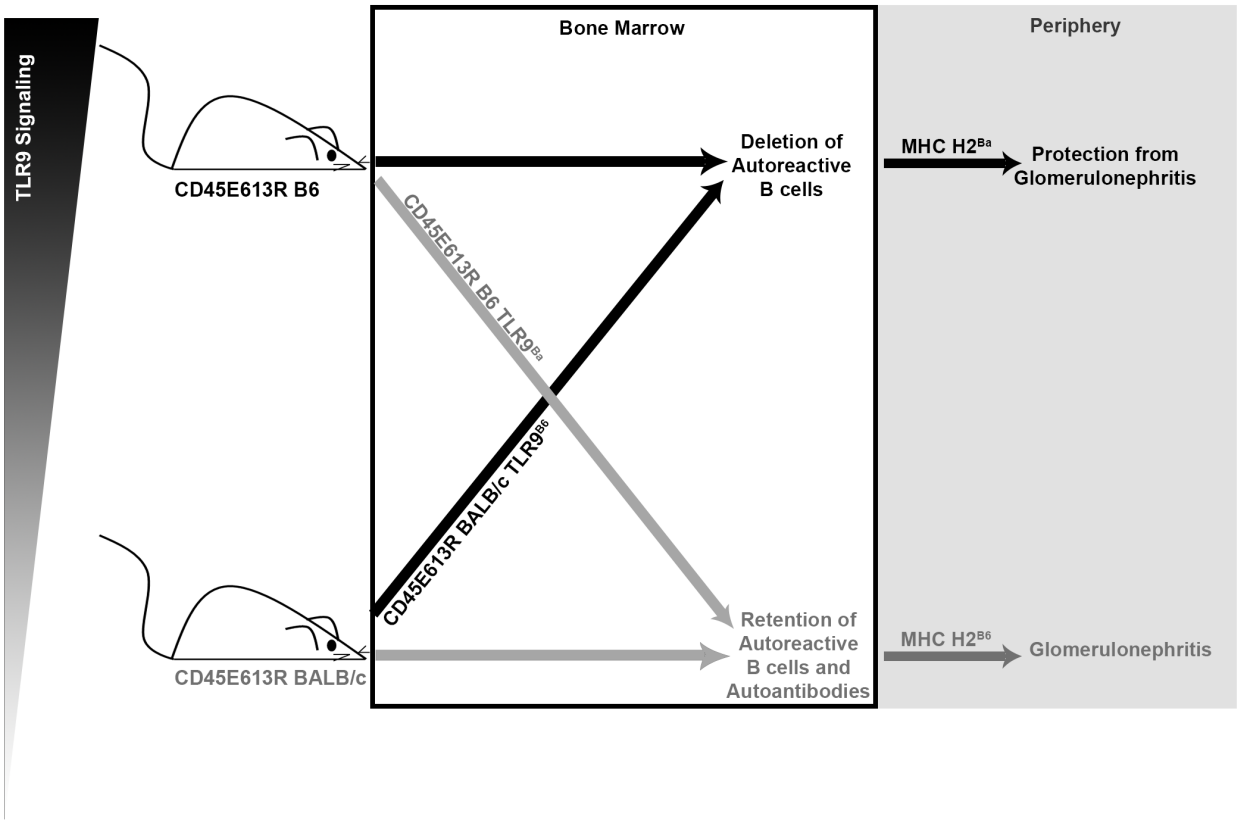


Figure 1. TLR9-BCR synergizes to alter signal strength and tolerance threshold.

High TLR9 signaling by the B6 allele in the context of the CD45E613R-mediated increase in BCR signaling results in deletion of autoreactive B cells, while low TLR9 signaling by the BALB/c allele results in retention of autoreactive B cells in the bone marrow, and autoantibody production. In the periphery, addition of the B6 MHC H2 allele results in the development of glomerulonephritis in either B6 or BALB/c backgrounds, while the BALB/c MHC H2 allele protects from development of glomerulonephritis.

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