The Role of TLR3 in the Development of Lupus-Like Autoimmunity in B6.NZBc13 Mice

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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Abstract

The New Zealand Black (NZB) mouse chromosome 13 (c13) is linked to development of autoimmunity. B6 mice containing a portion of NZBc13 (B6.NZBc13 (c13)) develop a lupus phenotype that includes: autoantibody production, increased B and T cell activation, and marginal zone B cell and myeloid dendritic cell expansions. c13 mice have a B cell intrinsic dsRNA-sensing defect, leading to increased TLR3 expression and survival. The role of the aberrant dsRNA sensing in the generation of the c13 autoimmune phenotype was assessed by generating c13 mice with TLR3 knocked out (c13.TLR3KO). Marginal zone B cell expansion and B cell activation were attenuated in c13.TLR3KO mice, but other cellular phenotypes were not affected. Autoantibody production was partially reduced. These results indicate that altered dsRNA-sensing contribute to a portion of the altered cellular phenotypes in c13 mice, but that other susceptibility loci in the c13 interval are required for full development of autoimmunity.
Acknowledgments

Many people have contributed to my development as a researcher and to this thesis:

Thank you to:

My supervisor Dr. Joan Wither, for her support and her dedication to science.

Dr. Nan Chang for helping me with my large experiments and “always being right”.

Kieran Manion, for being an awesome friend.

Evelyn Pau, for being a great lab mentor.

Yuriy Baglaenko, for answering all of my questions.

Kimberley Lifeso, Nafiseh Talaei, Babak Noamani, and Dr. Carolina Landolt-Marticorena for keeping me entertained.

My supervisory committee, for their suggestions and thoughts.

Thomas de Haas, for putting up with my ridiculousness.
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1 Introduction

1.1 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a multifactorial autoimmune disorder where susceptibility is thought to be conferred through complex interactions between genetic predisposition, environmental factors, and hormonal influences (1–4). The hallmark of SLE is the generation of anti-nuclear autoantibodies (ANAs) (4). The diversity of clinical manifestations in lupus arises from autoantibody-autoantigen complexes, or immune complexes (ICs), that deposit in many different tissues or organs (1, 2, 5). Of the many organs affected in SLE, the kidneys and skin are the most studied (1). Kidney involvement is one of the most common disease manifestations in SLE affecting 30-60% of patients, where anti-double stranded (ds) DNA antibody and complement deposition have been shown to lead to recruitment of pro-inflammatory cells resulting in glomerulonephritis (GN). Currently, there is no curative therapy for SLE, resulting in significant morbidity and mortality. The 15-year survival rate is only 80% (6). The disease primarily affects women between 15 and 50 years of age, with a prevalence of approximately 50 cases per 100 000 people (1), although certain ancestries have higher incidence of disease than others (7).

The exact role that hormones play in SLE is unclear, but data suggests that they strongly influence disease development. SLE shows a strong sex bias, with approximately 90% of patients being female (1, 7). Patients using hormone replacement therapies have associated increases in developing lupus flares (7). Support for the role of hormones in disease development has also been demonstrated in mouse models where castration and administration of estrogen promotes lupus, and in patients with Kleinfelter’s syndrome, who have an increased prevalence of SLE (8, 9).

The role of environmental factors in the onset of SLE is equally ambiguous, but sun exposure has been shown to be a common trigger in disease exacerbation (1, 5). Epstein-Barr virus (EBV) may also play a role in the development of SLE in lupus-prone individuals; SLE
patients have higher frequencies of B cells infected with EBV and higher anti-EBV antibody titres in comparison to non-lupus prone individuals (1, 7). Mouse models have supported the role for EBV in the development of lupus; BALB/c mice expressing EBV proteins develop ANAs (10). Conversely, it has been proposed that higher rates of EBV activity in SLE patients may be due to immune defects rendering patients unable to combat the virus effectively (7).

There is strong evidence that genetic factors promote susceptibility to SLE. The risk of a sibling developing lupus is 15-20 times higher than that of the general population (2). Monozygotic twin studies have identified a 24% concordance rate, further affirming that genetic factors play a significant role in lupus susceptibility (1, 2). Both MHC and non-MHC genes have been shown to affect the risk for development of lupus. HLA-A1, B8 and DR3 have all been associated with SLE (1, 5, 7). In addition, a large number of candidate gene studies and extensive single nucleotide polymorphism (SNP) genome-wide association studies (GWAS) have linked at least 30 different non-MHC genes to the development of lupus (4). Current models suggest that individual genes have a modest effect on immune function and that an accumulation of predisposing loci up to a threshold number is required for disease susceptibility (5).

### 1.2 Mouse models of SLE

There are several spontaneous mouse models of lupus (11). The identification of susceptible genes in these models and their correlation to GWAS studies have indicated that similar pathways are defective in both mouse and human lupus (12). Understanding the exact mechanisms leading to spontaneously arising autoimmunity in these mouse models will provide insight into the defective molecular pathways involved in SLE.
1.2.1 MRL-\textit{lpr} autoimmune model

The MRL-\textit{lpr} strain is a spontaneously occurring lupus-prone mouse model that has a recessive mutation in the Fas receptor (lymphoproliferation-\textit{lpr}), which leads to reduced apoptosis in immune cells (11). The presence of the \textit{lpr} defect on the MRL background contributes to high mortality at an early age from glomerulonephritis, which strikes both sexes equally (11, 13). MRL-\textit{lpr} mice have high ANA titres and high circulating concentrations of immune complexes (11). In addition, the MRL-\textit{lpr} mouse has lymphadenopathy due to CD4\textsuperscript{+}, CD8\textsuperscript{+}, B220\textsuperscript{+} T cell expansion, and depletion of CD4\textsuperscript{+} T cells and B cells decreased autoimmunity (11).

Lupus susceptibility loci have been linked to chromosomes 4, 5, 7, and 10 in MRL-\textit{lpr} mice, independent of the \textit{lpr} mutation (14). B6 congenic mice, with a portion of MRL chromosome 1 have altered FcγIIIB and FcγIII expression, which is associated with development of GN (15). The \textit{Lmb3} lupus susceptibility locus on MRL-\textit{lpr} chromosome 7 is also associated with GN and anti-dsDNA autoantibody production (2, 14). Several studies of intracellular toll-like receptors (TLRs) have demonstrated essential roles for these receptors in the development of autoimmunity in MRL-\textit{lpr} mice (discussed in section 1.4).

1.2.2 BXSB autoimmune model

BXSB mice are another spontaneous lupus-prone strain that develop similar features to human SLE, including: IC-mediated GN, hypergammaglobulinemia, and ANAs (11). Autoimmunity is highly penetrant in BXSB mice. Unlike human SLE and other lupus-prone mouse models, however, disease severity and mortality are increased in male mice (11). The removal of either the testes or ovaries in BXSB mice neither ameliorated or exacerbated disease in male or female mice (11). The source of lupus-like autoimmunity in male BXSB mice was found to originate from the translocation of a section of the X chromosome onto the Y chromosome (11). The translocated locus, termed the Y-linked autoimmune accelerator (\textit{Yaa}),
contains 16 genes including the innate immune receptor Toll-like receptor 7 (TLR7), leading to its overexpression and facilitating autoimmunity (11). Other autoimmune susceptibility loci have been linked to chromosomes 1, 3, and 13 in BXSB mice (11). On chromosome 1, four susceptibility loci, termed Bxsl-4, are linked to nephritis and anti-dsDNA autoantibody production (12, 16). Bxs6, linked to chromosome 13, corresponds to the sgp3 locus in NZB mice and to the development of antibodies against the glycoprotein subunit gp70 and to subsequent gp70 immune complex formation (2).

1.2.3 New Zealand Black and New Zealand Black-derived autoimmune models

New Zealand Black (NZB) mice spontaneously develop a lupus-like disease (2, 17). In these mice, disease is characterized by development of anti-erythrocyte, -lymphocyte and -ssDNA autoantibodies which lead to hemolytic anemia, thymic lesions, and mild GN (2, 17–19). Although GN first appears at approximately 5-months of age, NZB mice do not succumb to kidney failure until 10- to 12-months of age (11). Studies of cellular function in NZB mice indicate that there is a fundamental B cell defect which causes high levels of polyclonal IgG and IgM antibody production (20–23).

Spontaneous autoimmunity in NZB mice resembles human lupus in that it is multifactorial, has similar functional cellular defects, and has a female bias (23). The NZB x NZW F1 hybrid (NZB/W) mouse is another classically studied spontaneous lupus mouse model (11). Linkage studies performed on these mice have revealed that the susceptibility loci responsible for disease are derived from each parent strain (12, 24). NZB/W mice produce ANAs against DNA, but not RNA-containing nucleoprotein complexes (11). Initial linkage studies performed on NZB/W mice indicated that only one susceptibility locus was derived from the NZB background; located on chromosome 4, this locus was correlated with glomerulonephritis and mortality (25). Subsequently, this was found to be incorrect when Drake et al. identified susceptibility loci on NZB chromosomes 1, 4, 7, 10, 13, and 19 (26). Since these original observations, multiple mapping studies, including those performed by the Wither laboratory on
the NZB model, have consistently linked susceptibility loci to chromosomes 1, 4, 7, and 13 (2, 17).

In addition to NZB and NZB/W mice, the New Zealand Mixed 2410 (NZM) mouse model were derived from NZB/W mice, and shares similar susceptibility loci to parental strains (11). NZM mice display a highly penetrant, early onset lupus-like disease (11), with susceptibility loci on chromosomes 1, 4, and 7 linked to this phenotype (27). Congenic mice in which these loci, named Sle1-3, have been introgressed onto the B6 background have provided many insights into the immunologic defects and genetic polymorphisms that encoded on this strain (11, 27, 28). Sle1, found on chromosome 1, is associated with a breach of B cell tolerance leading to autoantibody production, and reductions in the regulatory T cell (Treg) populations (11, 28, 29). Sle2, found on chromosome 4, produces polyclonal B cell activation as well as TH17 cell polarization (11, 28, 30), whereas Sle3, located on chromosome 7, leads to CD4+ T cell expansion and proliferation, as well as reduced T cell apoptosis (11, 27). Furthermore, these three loci, have been shown to interact epistatically, leading to progressively more severe autoimmune phenotypes with increasing number of loci, culminating in fatal GN in tricongenic mice (11, 28, 31).

Further refinement of the Sle1 locus by the generation of subcongenic mouse strains has revealed that there are at least three loci (a-c) within the Sle1 interval that facilitate autoimmunity, and genetic polymorphisms within candidate genes have been found for each locus (29). The Sle1a locus has been found to encode a novel splice variant of pre-B cell leukemia homeobox 1 (Pbx1) whose expression leads to the activation of autoreactive T cells, as well as decreases in Treg cells (32). Sle1b was shown to have a distinct gender bias and is associated with high titres of ANAs; combinations of this congenic strain with others lead to nephritis and high mortality (11, 33). Allelic variations of the signalling lymphocyte activation molecule (SLAM/Ly108) in the Sle1b interval are thought increase immune cell activation and lead to a breach of B cell tolerance in B6.Sle1b mice (33). The autoimmune phenotype of mice containing the Sle1c locus is thought to be controlled by three genes, where polymorphisms in CD21 lead to the generation of autoreactive B cells (29) as similar phenotypes were observed in CD21-deficient mice (11, 29). In addition to identifying the genetic variants associated with the NZM mouse strain, these experiments have also provided candidate genes for human
autoimmunity and there is some evidence that polymorphisms in CD21 and the SLAM locus may be associated with human SLE (11).

1.2.3.1 NZB congenic mouse strains

Congenic mice in which homozygous intervals from lupus-prone mouse strains have been introduced onto a non-autoimmune background, have not only been useful in confirming the presence of previously mapped lupus susceptibility loci, immune mechanisms, and genetic polymorphisms in NZM mouse strain, but also the NZB mouse strain (34). The Wither laboratory has used several congenic mouse models to determine the role of various NZB susceptibility loci in the generation of the spontaneous autoimmune phenotype (2).

Congenic mice, containing a portion of NZB chromosome 1 introgressed onto a B6 background (B6.NZBc1 (c1)), were first produced by Wither et al. in 2003 (35). Initially, two strains, c1 (35-106) and c1 (85-106), where numbers indicate the NZB chromosomal interval in centimorgans (cM), were generated (35). Studies of these mice demonstrated that a susceptibility loci in the c1 (82-106) interval promoted the activation of nucleosome specific self-reactive T cells, as well as memory T cell expansion (36). B and T cell defects from c1 (82-106) were found to be amplified by ancillary susceptibility loci in the c1 (35-106) interval, creating a positive feedback mechanism promoting autoimmunity (36). The c1 (35-106) interval was further subdivided by the generation subcongenic mice with smaller c1 intervals, and four susceptibility loci, as well as one suppressor locus have been identified (37). Loci located in c1 (96-100) are associated with breach of tolerance, while loci in c1 (88-96) correspond to anti-dsDNA autoantibody production and renal disease (37). Loci in c1 (70-88) are associated with increased mortality and auxiliary loci in the c1 (102-106) interval alter T cell function. The suppressor locus is thought to be located in either the c1 (35-70) or c1 (100-102) intervals (37).

A portion of NZB chromosome 4 has also been introgressed onto the B6 background (B6.NZBc4 (7.5-73cM) (c4)) (38). Interestingly, these mice have expanded B1a and natural killer T (NKT) cell populations and do not develop an autoimmune phenotype, despite several studies linking susceptibility loci to chromosome 4 (2). When c1 and c4 mice were intercrossed,
the resulting B6.NZBc1c4 (c1c4) bicongenic mice did not develop glomerulonephritis, pathogenic autoantibody production, and had decreased mortality relative to c1 mice (38). The presence of a suppressor locus on c4 is thought to quell the autoimmune loci on c1 (38).

The 24-73 cM interval of NZB chromosome 13 has also been introgressed onto the B6 background (B6.NZBc13 (c13)) by our lab. This mouse strain, which also develops lupus-like autoimmunity, is the focus of my studies and will be discussed in section 1.7 (39).

1.3 Mechanisms of lupus pathogenesis

SLE is a complex genetic disease where the inheritance of non-mendelian genetic factors play a significant role in pathogenesis (2, 12). In both humans and lupus-prone mice, multiple susceptibility alleles are present, where individual polymorphisms only modestly contribute to the disease state, but the accumulation of multiple defects results in pathogenic autoantibody production (2). In lupus, the fundamental immune abnormality appears to be a breach in tolerance to nuclear antigens leading to the production of ANAs (2). Defects in the clearance or response to apoptotic debris, lymphocyte signalling, and autoreactive lymphocyte survival can all contribute to the production of these ANAs, depending on the model studied or risk variants present (2). Studies of spontaneously arising lupus-prone mouse models, as well as single gene knockout mouse models that develop autoimmune phenotypes have aided in the delineation of the pathways responsible for ANA production and the genetic basis of SLE (2, 12).

1.3.1 Autoantibodies mediate tissue damage

There is strong evidence in lupus that the persistent presence of autoantigens results in IC formation, and that the deposition of these ICs into tissues causes damage (1, 40). Although the contributions of different Ig isotypes to the development of lupus are currently under investigation, high affinity IgG antibodies are strongly associated with tissue damage (1). There
are two proposed mechanisms for the development of kidney disease in SLE: in the first, IgGs form immune complexes with nuclear antigens, derived from nucleosomes found in circulating apoptotic debris, and settle on the glomerular basement membrane, leading to the activation of immune cells within the kidney, the fixation of complement, and the infiltration of activated macrophages (1, 40, 41). The resulting inflammation leads to tissue injury and kidney disease (40, 41). Evidence for this model of pathogenesis has been found in both mouse models and human lupus where extracellular chromatin has been shown to be bound by IgG autoantibodies in kidneys (1, 5, 41, 42). Parenchymal cells, mesangial cells, and kidney endothelial cells are also known to bind ICs (43).

In the second mechanism, autoantibodies bind to cross-reacting basement membrane antigen directly or through nuclear antigen bridges in the kidney itself (1). Several antibodies are known to directly bind the glomerular membrane, including: anti-α-actinin, anti-laminin 1, and anti-collagen antibodies (44). Furthermore, injections of α-actinin led to an autoimmune disease in lupus-resistant mice (44).

1.3.2 Apoptotic debris and clearance defects associated with SLE

One of the major classes of immune defects that leads to development of lupus is thought to be deficiencies in the ability to clear apoptotic debris (45). During apoptosis, the dying cell undergoes a stepwise series of death-related events to ensure that potential immunogenic antigens are not released, thereby preventing inflammation (46). The altered morphology of the cell membrane in early apoptotic bodies leads to the recruitment of professional phagocytes which upon engulfment of dying cells facilitates the secretion of IL-10 and TGFβ, stopping inflammation (2, 45, 47). If apoptotic cells are not efficiently cleared, they progress to late stage apoptosis, or secondary necrosis, where membrane integrity is lost, danger signals are released, and the debris becomes immunogenic (48). The majority of commonly observed autoantigens that are recognized in SLE are found in apoptotic debris and improperly cleared apoptotic debris is the most likely source of these autoantigens (49, 50).
Consistent with a role for impaired clearance of apoptotic debris in lupus pathogenesis, single-gene knockout mouse models of molecules involved in the apoptotic clearance pathway develop a lupus-like phenotype (45, 51). The loss of Mer, a TAM family tyrosine kinase, leads to the accumulation of apoptotic debris, and a pro-inflammatory response in cells binding debris. Similar results were observed in C1q knockout mice (2, 45). Mice deficient in milk fat globule EGF-8 (MFG-E8), a phosphatidylserine-binding molecule that facilitates phagocyte-debris interactions, also develop lupus-like autoimmunity (52). In addition, the loss of molecules that degrade or mask DNA released from late-apoptotic cells, such as serum amyloid P component (SAP), or DNaseI, lead to autoantibody production (2, 51). Impaired clearance of apoptotic debris has also been shown in several spontaneously arising SLE mouse models. Macrophages from both pre-autoimmune NZB/W and MRL-lpr mice demonstrated reductions in phagocytic capability after treatment with serum derived from autoimmune mice (53). A genetic locus associated with impaired clearance of apoptotic debris was found on NZB chromosome 13, as will be discussed in greater detail in section 1.7 (54).

Some SLE patients are defective in their ability to clear apoptotic debris. Muñoz et al. showed that although monocytes of SLE patients were defective in their ability to engulf opsonized materials, circulating phagocytes from many patients actively took up secondarily necrotic cell-derived material in the presence of anti-dsDNA antibodies (55). Accumulated apoptotic debris has been found in skin lesions and bone marrow from SLE patients (45). Similar to MFG-E8 knockout and B6.NZBc13 mice, some SLE patients exhibit increased levels of debris on the surface of follicular dendritic cells in germinal centres as well as defective engulfment of debris by tingible body macrophages (56).

1.3.3 T cell defects associated with SLE

T cells are known to play several pathogenic roles in lupus. T cells of SLE patients infiltrate and damage tissues, secrete large amounts of inflammatory cytokines, and activate both DCs and B cells to augment autoimmunity (57). CD4+ T cells have been shown in both mouse models and human SLE to regulate autoreactive B cell responses, and treatments with anti-CD4
antibodies in lupus-prone mice alleviated IgG autoantibody production (5, 41). CD8\(^+\) T cells have divergent roles in SLE patients, in some patients CD8\(^+\) T cells have decreased cytotoxic activity, while in patients with lupus nephritis, CD8\(^+\) T cells infiltrate the kidney where they may mediate damage (57).

CD4\(^+\) T cell subsets (T\(_{H1}\), T\(_{H2}\), T\(_{H17}\), and follicular helper T (T\(_{FH}\)) cells) have all been found to play a role in SLE pathogenesis (58). T\(_{H1}\) infiltrates and IFN\(_\gamma\) production are frequently found in patients with diffuse proliferative lupus nephritis, and MRL-\(lpr\) mice with IFN\(_\gamma\) deficiencies have reduced kidney disease (58). SLE has long been associated with altered T\(_{H2}\) responses due to class switching and the production of pathogenic autoantibodies; one of the major mechanisms of lupus pathogenesis is thought to be a T\(_{H2}/T_{H1}\) imbalance (58). Increased proportions of T\(_{H2}\) CD4\(^+\) T cells have been found in the peripheral blood of some patients, as well as T\(_{H2}\) infiltrates in patients with lupus nephritis (59). T\(_{H17}\) cells are also found in kidney infiltrates (57), and increased proportions of circulating T\(_{H17}\) cells have been observed in patient subsets (60). Lupus-prone mouse models have also demonstrated T\(_{H17}\) cell defects, where the expansion IL-17 secreting cells were shown to be critical for development of GN in MRL-\(lpr\) mice (61), B6.NZBc1 mice (Talaei, in press), and B6 mice with the \(Sle2cI\) locus (30). Studies of c1 mice by our laboratory have shown that the increased proportions of T\(_{H17}\) and T\(_{H1}\) cells results from a combination of T cell and DC defects that lead to increased production of and responses to the cytokines that drive differentiation of these T cells subsets in a positive-feedback manner (Talaei, in press).

More recently, T\(_{FH}\) cells have been shown to play an important role in lupus pathogenesis. In the sanroque mouse model of lupus, Roquin, a ring-type E3 ubiquitin ligase that represses inducible T cell costimulator (ICOS), is mutated leading to an intrinsic T cell defect that results in T\(_{FH}\) cell expansion (62). Subsequently, these mice were shown to have increased numbers and sizes of germinal centres (GC) and autoantibody production (62, 63). Expansion of T\(_{FH}\) cells is also seen in MRL-\(lpr\), BXSB.Yaa and NZB/W lupus-prone mouse models (63–66). In B6.NZBc1 mice, the increased proportion of T\(_{FH}\) cells results from a dendritic cell defect that leads to increased production of cytokines promoting T\(_{FH}\) differentiation (Talaei, in press). In humans, increases in the proportion of circulating T\(_{FH}\) cells are also seen in approximately ⅓ of SLE patients and have been correlated with more severe disease (63).
Extrafollicular CD4+ T helper cells have also been found to provide support for the activation of B cells outside of germinal centres in autoimmune mouse models, as well as in human SLE, leading to the production of autoantibodies in an IL-21 dependent manner (65, 67, 68). These findings suggest that a blockade of the second signals provided by T cells may be of therapeutic benefit in lupus. Although treatment of lupus-prone mice with anti-CD40 antibodies successfully mitigated disease symptoms, unexpected thromboembolic events occurred in humans (41, 57, 63).

1.3.4 B cell defects in signalling and survival associated with lupus

Although the production of ANAs by autoreactive B cells is a hallmark of SLE (28), autoreactive B cells can promote autoimmunity by both antibody-dependent and antibody-independent mechanisms (69).

Alterations in B cell signalling leading to autoimmunity may occur either by overexpression of signalling molecules that positively regulate BCR signalling, or by underexpression of signalling molecules that negatively regulate BCR signalling (28). Several mouse models with gene mutations, deletions, polymorphisms, or transgenes of the BCR signalling pathway have been found to develop autoimmunity (2, 28). Mutations to the BCR negative regulatory molecules Lyn and SHP-1, are associated with autoimmunity (70). Similarly, the loss of CD22 was found to lead to the development of anti-dsDNA autoantibodies in older mice (71). B6 mice lacking the BCR inhibitory molecule, FCRγIIB, also develop autoantibodies to chromatin and dsDNA, as well as kidney disease, leading to early mortality due to a loss in peripheral tolerance (72). Further studies revealed that TLR9 was responsible for pathogenic autoantibody class switching in these mice, and disruptions of either TLR9 or myeloid differentiation primary response gene (88) (MyD88) arrested class switching in autoreactive B cells (73). Increases in the co-receptor molecule CD19 of only 15% can induce the production of anti-nuclear autoantibodies (74), while CD19 knockout mice have lower levels of anti-ssDNA autoantibodies (28).
Defects in the apoptotic pathway promote SLE by augmenting self-reactive B cell survival. Conditional mutations of *Fas* in non-autoimmune mice cause increased autoantibody titres, and double deficient Fas/Bim mice developed severe lupus-like autoimmunity (11, 75). Relief of the *Fas* mutation in MRL-<i>lpr</i> mice, by introducing functional *Fas*, delayed disease onset but did not ameliorate disease, indicating that several other genes on the MRL background are important for lupus-like susceptibility (11). Similar to MRL-<i>lpr</i> mice, mice with mutations to Fas ligand (FasL), also known as *gld* mutants develop lupus-like autoimmunity (11). NZB mice were also found to have alterations to the apoptotic pathway. NZB T1 B cells did not undergo apoptosis as efficiently as B cells from non-autoimmune mice after IgM crosslinking. Subsequently, augmented expression of the anti-apoptotic protein Bcl-2 was found to cause this increase in T1 B cell survival (76). Furthermore, Bcl-2 expression was increased post-antigen encounter, leading to the persistence of autoreactive B cells in these mice (76). In human lupus, increased expression of several anti-apoptotic factors, and two different polymorphisms in the *Fas* promoter have been found in a small subset of patients (75, 77). In addition, lupus patients have elevated levels of B cell activating factor (BAFF) (78), which plays a critical role in setting the threshold for deletion of self-reactive B cells prior to entry into the mature B cell compartment (79). Overexpression of BAFF in transgenic mouse models with self-reactive Ig transgenes has demonstrated that BAFF promotes the survival of self-reactive T2 B cells and when increased leads to elevated numbers of self-reactive B cells in the marginal zone and follicular B cell compartments (80).

### 1.3.5 The role of marginal zone B cells in SLE

Marginal zone (MZ) B cells are innate-like lymphocytes that respond quickly to blood-borne antigens, producing low affinity antibodies early after antigen detection (81, 82). MZ B cells are non-circulating; they are retained in the marginal sinus where they can be activated either by interacting with APCs presenting large blood-borne antigens, or by directly binding smaller blood-borne antigens (81). Although follicular B cells are the major B cell type responsible for T-dependent (TD) antigen responses, MZ B cells are able to respond to both T-independent (TI) and TD antigens (82, 83). MZ B cells naturally express high levels of CD80,
CD86, and MHCII, pre-priming these cells for activation. Upon BCR/TLR co-engagement MZ B cells are rapidly and efficiently stimulated, differentiating into plasmablasts and producing large amounts of low affinity IgM antibodies in TI antigen responses (81, 83, 84). MZ B cells express TLRs 1, 3, 7, and 9 in higher proportions than other B cell subsets (85). When MZ B cells were stimulated with either TLR 3 or 9 agonists, cells were induced to migrate into the red pulp and adjacent follicles in a S1P$_1$ dependent manner, facilitating TD antigen responses (86). It has recently been found that patients deficient in the signalling molecules of various TLR pathways have reduced MZ B cell compartments (87).

Several lupus-prone mouse strains display alterations to the marginal zone compartment. NZM2410 mice have MZ B cells that are re-distributed into follicles, which are then able to activate CD4$^+$ T cells (11, 88). This re-distribution was found to be the result of the Sle2 susceptibility locus, located on chromosome 4 (88). Conversely, Sle1.Tg7 mice, containing the Sle1 susceptibility locus and extra copies of TLR7, have decreased proportions of marginal zone cells due to increased TLR7 expression (89). Over-expression of BAFF causes the development of a lupus-like disease where signalling through the nuclear factor-κB (NFκB) pathways promotes long-term autoreactive cell survival, activation, and the retention of B cells in the marginal zone (90). In NZB and NZB/W mice, the proportion of B cells retained in the marginal zone is much higher than in non-autoimmune mice (22, 91). Furthermore, the MZ B cells of these autoimmune mice express higher levels of CD80, CD86, and ICAM-1, relative to B6 controls (22). Although initial genetic studies showed that MZ B cell expansion was linked to the Nba2 locus on chromosome 1 of NZB mice, further elucidation of this pathway with B6.Nba2 mice indicated that Nba2 was responsible for early B cell activation, and not innate-like lymphocyte expansion (22, 92). Importantly, B6.NZBc13 mice had expansions in the MZ B cell compartment (39).

1.4 The role of TLR signalling in lupus

TLRs are innate immune pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) (93–95). PAMPs are highly conserved molecular
structures that can be found on the cell surface of a pathogen, such as LPS, which is recognized by TLR4, or, are common nucleic acid motifs found in viruses and bacteria (94). There are 12 TLR family members in mice and 10 in humans (93). TLRs act as immune adjuvants by inducing pro-inflammatory cytokine production and recruiting surrounding cells to produce cytokines and adhesion molecules (94). Surface TLRs induce the inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNFα), while intracellular TLRs lead to the induction of type I interferons (IFNs) (94). Signalling through TLRs leads to rapid changes in inflammatory gene expression, eliciting an efficient immune response (95).

TLRs 3, 7, 8 and 9 are considered intracellular TLRs, as they are sequestered to the endosomes of antigen presenting cells (APCs) in mice (96–98). TLRs 3, 7, 8, and 9 all recognize nucleic acid ligands: TLR3 recognizes double stranded (ds) RNA; TLR7 recognizes single stranded (ss) RNA and short dsRNA fragments; TLR8 recognizes ssRNA, short dsRNA fragments, and oligoribonucleotides (98); and TLR9 recognizes hypomethylated CpG motifs (99). Intracellular TLRs are located within the endosomal pathway and may only access extracellular ligands when they are sequestered to the same compartments (97). Restraining the expression of nucleic acid sensing TLRs to the endosomal pathway is thought to stop aberrant signalling (96, 97). Intracellular TLRs have the potential to bind self-ligands by recognizing apoptotic and necrotic cell debris and sequestering these receptors internally physically limits autoantigen exposure (97).

1.4.1 Previous evidence of aberrant TLR signalling B cells

TLR signalling in B cells plays an important role in the pathogenesis of lupus (83, 100, 101). The potential role of nucleic-acid sensing TLRs in the activation of B cells that bind antigenic complexes containing RNA or DNA, was first described by Leadbetter et al. in 2002 (81, 83, 100). These investigators noted that transgenic rheumatoid factor (RF)+ B cells, which recognize and bind IgG2a, proliferated when given chromatin containing immune complexes but not complexes with conventional antigens (102). When this transgene was crossed onto a MyD88 gene-deleted background, proliferation in response to chromatin-containing ICs was
abrogated, suggesting it occurred through a TLR-dependent pathway (102). Further studies revealed that hypomethylated CpG motifs were required to activate transgenic RF+ B cells in a TLR9-dependent manner (99). Since these initial studies, dual TLR/BCR engagement has been found to: increase B cell size, produce a variety of cytokines, induce proliferation, initiate differentiation into plasmablasts, and induce class switching to pathogenic autoantibodies in autoreactive B cells (73, 100, 103). Furthermore, TLR signalling has been shown to be required for optimal antibody responses to T-dependent antigens (104).

To further elucidate the role of nucleic acid sensing TLRs in the generation of the lupus-like phenotype, MRL-lpr autoimmune mice deficient in TLR9 were generated and assessed for disease (105). TLR9 knockout mice were no longer able to produce either anti-chromatin or anti-dsDNA autoantibodies. Importantly, while dsDNA autoantibodies are one of the hallmarks of SLE, and it was initially thought that abrogation of TLR9-mediated dsDNA antibody production would eliminate lupus-like symptoms in MRL-lpr mice, disease severity was actually magnified in TLR9-deficient mice (101, 105). The sustained disease activity in MRL-lpr TLR9 knockout mice appeared to arise from increased production of RNA-associated autoantibodies, such as anti-Sm and anti-small nuclear ribonucleoprotein (snRNP), as well as anti-cardiolipin autoantibodies, which deposited in the kidneys and produced elevated levels of circulating IFNα (105). TLR9 was subsequently found to cross-regulate TLR7-associated autoantibody production, supporting a role for TLR9 in the suppression of autoimmunity (106). These results indicated that although TLR9 is an important mediator of SLE, RNA-sensing and RNA-associated ICs may be more important in disease pathogenesis than anti-DNA antibodies in the context of at least one lupus-prone mouse model (83, 101).

TLR7 is critical for the induction of autoimmunity in many autoreactive B cell models. The mere overexpression of TLR7 in B6 mice can lead to an autoimmune phenotype (107). BXSB.Yaa lupus-prone mice have a two-fold increase in TLR7 expression and autoimmunity can be controlled by reducing gene dosage (107). When the expression of transgenic TLR7 was controlled in lupus-susceptible B cells by a Cre recombinase system, RNA-associated autoantibody production was lost and the severity of kidney disease was reduced (89). Similarly, the pristane mouse model of lupus, where 2, 6, 10, 14 – tetramethylpentadecane (TMPD/pristane) injections lead to lupus-like autoimmunity, with RNA-associated autoantibodies and severe GN is TLR7-mediated (11). Knocking out TLR7 in MRL-lpr mice had
a protective effect on the generation of lupus (108). TLR7-deficient MRL-\textit{lpr} mice did not make anti-RNA antibodies, had decreased lymphocyte activation and amelioration of renal disease (108).

Recently, several mouse models have begun to shed light on the role of TLR3 in lupus pathogenesis. Transgenic RF\textsuperscript{+} B cells can be induced to secrete antibodies in a TLR3-dependent manner when incubated with complexes containing endogenous, highly self-complementary RNA sequences (109). Furthermore, antigen specific anti-snRNP B cells are stimulated to produce autoantibodies upon stimulation with TLR3 ligands (110). However, although long term exposure to polyinosinic-polycytidic acid (poly (I:C)) exacerbates autoimmunity in MRL-\textit{lpr} mice, poly (I:C)-mediated disease activity was found to be B cell independent (111). These results were confirmed when MRL-\textit{lpr} TLR3 knockout mice were generated and found to have no defects in autoantibody production, or IFN\textalpha secretion (105). In contrast to the results found in the MRL-\textit{lpr} mouse model, studies by Loh et al. have demonstrated the presence of a dsRNA-sensing B cell intrinsic defect in B6.NZBc13 congenic mice (discussed in section 1.7) (3).

### 1.4.2 TLR signalling in pDCs

Plasmacytoid dendritic cells (pDCs) are a well characterized subset of DCs that rapidly and efficiently produce IFN\textalpha and IFN\textbeta (112–114). pDCs are thought to be one of the major contributing cell types to the IFN signature associated with SLE patients (113, 115, 116). TLRs 7 and 9 are perpetually expressed by pDCs, and signalling through these two receptors is thought to lead to type I IFN production (116–118). Retinoic acid-inducible gene I product (RIG-I) and melanoma differentiation-associated-antigen 5 (MDA5), two viral RNA cytosolic sensors, are also expressed in pDCs, but their activation is not associated with type I IFN induction in this cell type (119). pDCs are inefficient antigen presenting cells; they are unable to uptake antigen effectively and only become activated by autoantigen after Fc\gammaRIIa (CD32)-mediated endocytosis delivers autoantigens to TLR7- and 9-containing endolysosomes (113). Once sequestered to the endolysosomal pathway, however, autoantigens are retained for long periods of time, leading to sustained signalling and type I IFN production (113). The continuous expression
of IRF7 in pDCs gives these cells the distinctive ability to quickly produce IFNα and β (113, 120).

Immune complexes consisting of SLE patient derived IgG and cellular debris were initially shown to induce the production of IFNα in natural interferon producing cells, now known to be pDCs (121, 122). Similar results were found when pDCs were exposed to ICs containing snRNPs (117). Type I IFNs were not produced by pDCs, however, when ICs were treated with RNase, indicating that stimulation through RNA sensing pathways was essential for IFNα production (122). Platelets of SLE patients are also known to induce IFNα production by pDCs by becoming activated and forming monocyte-IC-pDC-platelet complexes (113).

Elucidating whether the function of pDCs in SLE is altered has proven to be somewhat difficult, with different groups reporting wildly differing results (113). While myeloid DCs are reduced in some SLE patients, no increases or reduction in pDC numbers are clearly associated with SLE (113). The genetic heterogeneity, inherent in the human population, makes the attribution of functional defects to specific genotypes difficult (123). Furthermore, prednisone, a steroid commonly given to SLE patients is known to affect the functional capacity and number of pDCs (113). Nevertheless, genetic studies have pointed to a role for alterations in TLR signalling pathways in the development of human SLE. Polymorphisms in interleukin-1 receptor-associated kinase 4 (IRAK4), TLR8, and tumor necrosis factor, alpha-induced protein 3 (TNFAIP3/A20) are all associated with SLE susceptibility and predicted to impact on disease genesis through augmented TLR signalling (101). Notably, patients deficient in either IRAK4 or MyD88 have increased numbers of naïve autoreactive B cells but do not produce elevated levels of ANA, likely because TLR7 and 9 signalling is required for these cells to differentiated into autoantibody producing cells (83).

The TLR signalling pathways have long been a target for treatment in SLE. Antimalarial drugs, one of the first therapies used in the treatment of SLE, inhibits the acidification of lysosomes, where intracellular TLRs also happen to reside (69). Small molecule inhibitors of TLR7 and 9 signalling have also been tested for their ability to inhibit IFNα production (124). Herpes simplex virus and influenza virus, shown to induce IFNα by pDCs through TLR9- and 7-dependent signalling, respectively, were unable to induce IFNα production when cells were also treated with non-stimulatory DNA sequences for TLR9 and 7 (124). These small molecules were
subsequently tested in NZB/W mice, where IFNα is thought to play a prominent role in the development of lupus-like pathogenesis (124, 125). Mice were treated continuously from the time of disease onset and found to have decreased autoantibody production, decreased kidney damage, and a reduction in the appearance of proteinuria (124, 125). Finally, peripheral blood mononuclear cells (PBMCs), cultured with SLE patient derived IC, did not produce anti-DNA and -RNA autoantibodies when treated with the small molecule TLR7 and 9 inhibitors (124, 126).

1.4.3 The role of type I IFNs in lupus

Type I interferons are a class of cytokines that is induced upon viral infection (95, 113, 116). The type I interferon family consists of 13 IFNα subclasses, and one of each of IFNβ, IFNω, IFNε, and IFNκ in humans, of which IFNα and IFNβ are the most biologically relevant to systemic autoimmune disease (116, 117, 127). pDCs are the largest producers of type I interferons, although other cell types have also been shown to produce type I IFNs (113, 116, 128). Type I IFNs have a wide range of effects on many different immune cells, all of which can contribute to SLE pathogenesis (113). IFNα and β cause increased T cell activation, increased T cell survival, the induction of T cell memory, and Th1 cross priming (113, 116). In B cells, type I IFNs cause the expansion of B1 cells, and promote B cell activation and isotype switching, as well as the differentiation of plasmablasts (113, 116). Type I IFNs also enhance TLR7 expression and B cell responses to TLR7 ligands (129). Myeloid DCs have a variety of responses to type I IFNs including: induction of maturation, upregulation of co-stimulatory molecules, increased antigen presentation to T cells, increased survival, induction, and secretion of the B cell survival factors BAFF and a proliferation inducing ligand (APRIL) (113, 116, 129). Type I IFNs also provide positive feedback to pDCs resulting in increased activation and upregulation of TLR7 and IRF7 expression (113, 116, 117). pDCs are also responsible for cross-regulating DC maturation (113). Unlike other cell types, pDCs constitutively express IRF7 and are not subject to the IFNβ feedback mechanism for IFNα induction found in mDCs, lending further support to the idea that these are the primary IFNα producing cell types (112, 130). Finally, type
IFNs can increase the amount of autoantigen available by having cytotoxic effects on several cell types, thus increasing the amount of apoptotic debris (129, 131).

The first evidence of type I IFN involvement in lupus came from hepatitis C patients who upon treatment with IFNα developed a lupus-like systemic disease complete with ANAs (113, 117, 131). IFNα had also been found in the sera of some SLE patients, and positively correlated with anti-dsDNA antibody titres (113, 129). Subsequently, it was found that many patients with SLE have an IFN signature, a set of activated genes that are typically induced by type I IFNs, suggesting that elaboration of type I IFN is a frequent feature of SLE (114, 130). Similar to the serum levels of type I IFN, the IFN signature correlated with disease activity (113, 129, 131). WISH reporter cell lines bathed in patient sera have also shown IFN-regulated gene induction (129). As outlined previously, SLE patient immune complexes, containing IgG and apoptotic debris, have been shown to be taken up by pDCs, via CD32, and induce the production and secretion of IFNα (116). This observation has led to the concept that the IC in lupus patients lead to activation of their pDCs, resulting in the high levels of type I IFNs. However, it has been difficult to directly demonstrate this. Indeed, many SLE patients have decreased circulating numbers of pDCs, and studies suggest that these cells are refractory to TLR activation. This phenomenon has been ascribed to TLR tolerance, or the process by which cells that have been signalled through TLR receptors do not respond to further TLR activation (132). It is possible that pDCs become activated in the skin of lupus patients, as increased numbers of pDCs are found in many of the skin lesions of many SLE patients, and these cells bear features consistent with type I IFN stimulation (112, 116).

A number of polymorphisms in genes regulating type I IFN production appear to lead to enhanced production and/or responses to type I IFN and promote development of lupus. Trisomy of type I IFN genes or SNPs in either IRF5 or TYK2 have been associated with lupus-like disease symptoms (116). Several alternate IRF5 isoforms, a signalling molecule downstream of TLR7 (117), have been discovered and are associated with elevated IRF5 expression, IFNα production, and increased risk of SLE (133). 15 variants of IRF3 were also found in Japanese patient populations. Interestingly, two polymorphisms were associated with decreased IRF3 expression and type I IFN induction, and are thought to confer potential resistance to SLE (134).
Very recent studies have begun to target IFNα for treatment of SLE (69). The first anti-IFNα monoclonal antibody studied was sifalimumab (135). Sifalimumab therapy was found to cause the neutralization of IFNα in a dose-dependent manner and to decrease the induction of IFNα/β-induced inflammatory proteins and genes. Furthermore, reductions in the levels of BAFF, GM-CSF, IFNγ, IL-10, and TNFα transcripts were also observed, indicating that these proteins are affected and may lie downstream of IFNα (135). A second anti-IFNα monoclonal antibody, rontalizumab, has shown similar results from phase I trials (136).

1.5 TLR3 signalling pathway

Recently, studies by the Wither laboratory have suggested a role for aberrant dsRNA sensing in B cells in the development of lupus (3). Thus, understanding the molecules and pathways involved in dsRNA sensing may aid in delineating the contribution of dsRNA hyper-responsiveness to lupus-like pathogenesis.

1.5.1 Origin and uptake of dsRNA

TLR3 recognizes dsRNA (94, 96, 97, 137, 138). Although TLR3’s major role is to recognize exogenous sources of dsRNA from dsRNA viruses, such as mouse cytomegalovirus (97) or viruses that create dsRNA during their replicative cycle (97, 137), recently bulges in the stem structures of mammalian ssRNA have been found to be recognized by TLR3 (139). In humans, dying cells are the main endogenous source of dsRNA (97, 137), and ribonucleoproteins released from these cells have been shown to activate auto-reactive B cells through both TLR3 and TLR7 (109). TLR7 recognized highly uridine rich ssRNA molecules, whereas TLR3 recognized ssRNA molecules with high self-complementarity in B cells (109).

Like other intracellular TLRs, TLR3 requires an acidic pH to induce signalling, becoming activated in the endolysosome (137, 138). When cells transfected with TLR3 are treated with
inhibitors of lysosomal acidification, such as chloroquine or bafilomycin, these cells are unable to mount an immune response to poly (I:C) (137). Different mechanisms of dsRNA uptake are utilized depending on the cell type. As outlined above, uptake by B cells is mediated through their BCR (102). For bone marrow derived macrophages (BMDMs), the cell surface molecule CD14 has been shown to interact with both TLR3 in the endosome and bind poly (I:C) at the cell surface (140). Mice lacking CD14 were found to be severely impaired in their ability to induce inflammatory cytokine production after poly (I:C) stimulation (140). CD14 likely internalizes poly (I:C) and co-localizes with TLR3 in the endosome to induce the TLR3 signalling cascade in BMDMs (140, 141). CD11b has also been found to facilitate the internalization of poly (I:C) in peritoneal macrophages and RAW 264.7 cells (142). After stimulation with poly (I:C), CD11b knockout mice were shown to be deficient in their levels of phosphorylated IRF3 as well as their production of IFNβ, two down-stream effects of TLR3 activation (142). When pre-treated with bafilomycin A, macrophages from CD11b knockout mice were also found to be deficient in their production of IFNβ, indicating that CD11b helps shepherd dsRNA into the endolysosomal pathway in macrophages (142). Experiments performed on mDCs, which do not express CD14, have shown, however, that the clathrin-mediated endocytic pathway is essential for dsRNA internalization in this cell type (141). Furthermore, when monocyte derived DCs were bathed in the TLR9 ligands B and C type oligonucleotides (ODN) prior to the addition of poly (I:C), these cells were unable to produce IFNβ, indicating that these ligands use the same receptor to enter the clathrin-mediated endocytic pathway (141). In addition to the clathrin-mediated endocytic pathway, the lipid raft protein, Raftlin, mediates dsRNA uptake in human mDCs (141). Raftlin is critical for the formation of lipid rafts, and in B cells, is an essential component of BCR signal transduction (143).

1.5.2 TLR3 expression and proximal signalling

TLR3 is similar to other TLRs in that it is a transmembrane protein where the extracellular portion (in TLR3’s case facing the interior of the endosome) contains a series of leucine rich repeats and the cytosolic portion contains the toll/interleukin-1 receptor (TIR) domain (94, 96, 137). The binding of dsRNA to TLR3 induces the receptor to homodimerize,
causing a structural rearrangement where the TIR domains come into close contact and provide a “platform” for the adaptor protein TIR domain-containing adapter protein inducing IFNβ (TRIF) (Figure 1) (94, 138). Humans and mice both express TLR3 on immune and non-immune cells (137). In both species, TLR3 expression has been found in fibroblasts and epithelial cells at the cell surface, as well as intracellularly (97, 137). Both species have also demonstrated intracellular expression of TLR3 in T cells as well as weak cell surface expression in γδ T cells (137). After stimulation, both γδ T cells and human macrophages upregulate TLR3 at the cell surface (137). Human dendritic cells, specifically mDC and monocyte-derived dendritic cells (MDDCs), are the immune cells with the highest levels of TLR3, and TLR3 expression is restricted to the endosome in these cell types (137). In contrast to humans, mouse macrophages have the highest levels of TLR3 expression, and expression is only found intracellularly (97, 137). In humans and mice, mDCs, but not pDCs express TLR3 (116, 144). Both species upregulate TLR3 expression upon exposure to type I IFNs and mouse cells also increase TLR3 expression after exposure to LPS (97, 137, 138). B cell subsets also differentially express TLR3. Marginal zone B cells show the highest levels of TLR3 expression in mice (85). In addition, human plasma cells, but not other B cell types also express TLR3 (145).
1.5.3 The IRF3 molecular pathway induced by TLR3 signalling

TLR3 directly binds and signals through the adaptor protein TRIF (Figure 1) (94, 97, 137), after which, TLR3 signalling diverges into several distinct arms. The first pathway leads to IRF3 phosphorylation and type I IFN production, while two other pathways lead to NFκB expression, as well as MAPK activation. Finally TRIF signalling can induce apoptosis by initiating the formation of the ripoptosome (138, 148). The only other TLR to utilize TRIF is TLR4 and LPS signalling is able to induce type I IFNs in a TRIF-dependent manner (94, 97, 137, 138, 149, 150). TRIF is an essential component of the TLR3 signalling pathway; TRIF knockout mice are unable to produce IFNβ in response to either TLR3 or TLR4 ligands (94, 95, 97, 138). B cells from TRIF knockout mice were also found to have reduced expression of the activation molecules CD69, CD86, and MHCII (150). TRIF activates tank binding kinase 1 (TBK1) by forming a complex with the proteins NAK-associated protein 1 (NAP1) and TNF receptor associated factor 3 (TRAF3) (Figure 1) (95, 137, 138). Only the association of these proteins is required to activate TBK1, which subsequently leads to the phosphorylation IRF3 (137). TRIF is negatively regulated by another TIR-adaptor molecule, sterile alpha- and armadillo-motif-containing protein (SARM) (138). TRIF is also negatively regulated by disintegrin and metalloproteinase domain-containing protein 15 (ADAM15) (151). Viruses have also evolved mechanisms to inhibit IFNβ production by controlling signalling through TRIF (138).

TBK1 is one point of convergence for the endosomal and cytosolic dsRNA sensing pathways (96, 152). TBK1 directly binds to and phosphorylates serine residues of IRF3 (Figure 1) (95, 97). IκB kinase ε (IKKε) is another molecule capable of phosphorylating IRF3. Studies have shown, however, that IKKε is not essential for signalling through this pathway, as only knockouts of TBK1 left cells completely unable to respond to TLR3 or TLR4 stimuli (97). Several endogenous inhibitors regulate TBK1: suppressor of IKKε (SIKE) acts as a TBK1 substrate and inhibits TBK1-IRF3 phosphorylation (153); SH2-containing protein tyrosine phosphatase 2 (SHP-2) directly binds to and inhibits TBK1 phosphorylation (153, 154); and TNFAIP3 is recruited to TBK1 by Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1) to obstruct its interaction with TRAF3 (155). In addition, NLR family, pyrin domain
containing 4 (NLRP4) causes the destruction of TBK1 via polyubiquitination with the E3 ubiquitin-ligase deltex 4 homolog (DTX4) (156).

Phosphorylated IRF3 (pIRF3) dimerizes and translocates into the nucleus (95, 138). The co-activator protein cAMP responsive element binding protein (CBP) and p300 interact with pIRF3 to bind interferon response elements (ISREs) in the promoter region of type I IFN genes (95, 137, 153). This leads to the production of IFNβ and IFNα4 (94, 95, 97, 100, 137, 138, 150). IRF3 is part of a nine-member family of IRF molecules, all of which induce type I IFNs (95). IRF3 and IRF7 are critical for type I IFN production, where IRF3 is constitutively expressed in all cell types, while IRF7 is restricted to immune cells (95, 137). Phosphoinositide 3-kinase (PI3K) is also important for the phosphorylation of IRF3. IFNβ produced through IRF3-TLR3 signalling leads to IRF7 upregulation (96). PI3K is recruited to TLR3, but is not sufficient for TLR3 signalling. Instead, PI3K seems to play a role in phosphorylating some of the residues on IRF3, although TBK1 is sufficient for generating pIRF3 that is able to dimerize and translocate into the nucleus (97). dsRNA sensing leads to the activation of type I IFNs, type I IFN stimulated genes (ISGs), and other genes involved in anti-viral responses (97, 157). In all, there are 175 genes upregulated, and 95 downregulated by pathways responsive to dsRNA (97). Type I IFN genes also positively feedback onto each other, multiplying the genes induced and the effects of type I IFN signalling (95, 97). In support of a role for aberrant TLR3 signalling leading to increased type I IFN production, PBMCs of some juvenile SLE populations have increased levels of TLR3, and TRIF inhibition reduced IFNα secretion (158).

1.5.4 Other molecular pathways induced by TLR3 stimulation

The transcription factor NFκB is induced through TLR3 stimulation by two different activation pathways that diverge at the point of TRIF binding (94, 97, 137, 138, 144). Receptor interacting protein 1 (RIP1) interacts with the RIP homotypic interaction motifs on TRIF and facilitates the release of NFκB from inhibitors of nuclear factor κB (IκB) (Figure 1) (97, 137, 138, 159, 160). Activation of NFκB leads to IFNβ, IL-6, TNF, IL-12 and chemokine (C-C motif) ligand 3 (CCL3) production (137). RIP3 acts as a negative regulator of NFκB induction by
disrupting RIP1-TRIF binding (94, 97, 137). TRAF6 is the other NFκB activating pathway recruited by TRIF. The exact mechanism by which TRAF6-TRIF binding leads to NFκB activation is currently unknown, but is thought to follow an activation pathway similar to that of MyD88-TRAF6. TRAF6 polyubiquitinates transforming growth factor β activated kinase 1 (TAK1) and IκB kinase-related kinase γ (IKKγ) (96). IKKγ directly associates with IKKα and IKKβ, while TAK1 forms a complex with TAK1 binding proteins 1, 2, and 3 (TAB1, 2, and 3) and then phosphorylates IKKβ (96, 161). The activated IKK complex then phosphorylates IκBα facilitating its ubiquitination and degradation, and the release and activation of NFκB (96, 137, 159).

TAK1, activated by TRAF6, also activates p38, extracellular receptor activated kinase (ERK), and c-Jun N-terminal kinase (JNK) MAPK family members, as well as IKKs to activate their respective transcription factors, AP-1, and NFκB, and induce IFNβ and cytokine production (97, 161, 162). AP-1 is a ubiquitously expressed complex composed of Jun and FOS family members which can bind regulatory sites leading to pro-inflammatory gene transcription and activation upon TLR3 signalling (163, 164). NFκB and AP-1 activation collectively upregulate pro-inflammatory gene expression (96).

PI3K has been shown to be differentially recruited to either TLR3 or TRIF depending on the cell type, and leads to the activation or inhibition of protein kinase B (PKB/AKT) (97, 137, 165). PI3K interacts with TRIF in MDDC, acting as a negative regulator of TLR3-induced gene expression by impairing NFκB activity (165). PKB directly interacts with both TBK1 and IRF3, leading to its phosphorylation and the regulation of IRF3 in RAW 264.7 cells (Figure 1) (166). In contrast to MDDCs and RAW 264.7 cells, however, PI3K has been shown to both directly interact with TLR3 and positively regulate IRF3 activation in HEK 293 cells transfected with TLR3 (157). In HEK 293 cells, PI3K is recruited to phosphorylated TLR3 and is required for the full activation of IRF3 (97). While TBK1 was sufficient to produce an activated form of IRF3 in HEK 293 cells, it was unable to tightly bind ISREs and induce type I IFNs without the presence of PI3K to activate PKB which fully phosphorylated IRF3 (97, 157). PKB is also phosphorylated by c-Src tyrosine kinase in a TLR3-dependent manner (137). Finally, phosphorylation of PKB through TLR3 dependent signalling has been found to induce caspase-9-dependent cell death, arrest cell growth and cell cycling, and initiate cytoprotective autophagy (167).
TLR3-TRIF signalling can induce apoptosis or necroptosis, which is regulated necrotic cell death, is generally stimulated upon inflammation (138, 146). The loss of either TLR3 or TRIF can block TLR3-induced apoptosis and subsequent intestinal damage in mouse models (168). Importantly, the induction of apoptotic or necroptotic pathways via TLR3 signalling is independent of TNF, TRAIL, or BAX related death signalling pathways (146, 168). In the apoptotic mechanism, TRIF, caspase 8, Fas associated protein with death domain (FADD), RIP1, and TLR3 form a complex, known as the ‘ripoptosome’, which induces the non-classical pathway of caspase 3 induction (169). The loss of cellular inhibitors of apoptosis proteins (cIAPs) can lead to the spontaneous formation of the ripoptosome and mediate necroptosis (148, 170). RIP1 and RIP3 can also form a complex initiating cell death in the absence of caspase 8 in a TNF-dependent manner (146, 170). Most recently, FADD has been shown to be dispensable in inducing death from this complex, as its inhibition did not prevent TLR3 signalling-induced cell death (169). Double stranded RNA-sensing through TLR3 has also been found to play a role in cell migration in a TRIF independent manner (147). After binding TLR3, c-Src becomes activated and has been shown to initially increase the levels of cell migration in several mouse-derived cell lines, as well as BMDMs; upon the sequestration of TLR3-activated c-Src into lipid rafts, however, migration is reduced (147). The initial increases and subsequent decrease in the migration of TLR3 activated cells is thought to modulate immune responses (147).

1.6 Other dsRNA sensors

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are a family of cytosolic proteins able to sense viral RNA products. There are three family members: RIG-I, MDA5, and laboratory of genetics and physiology 2 (LGP2) (96, 171). These proteins are ubiquitously expressed at low concentrations, allowing for all cell types to sense viral infection and are upregulated upon type I IFN treatment (96, 172). RIG-I and MDA5 both contain two caspase recruitment domains (CARDs) and a DExD/H box-containing RNA-helicase domain (95). When these molecules bind dsRNA by their helicase domain, a conformational change is induced to expose the CARD regions, which then allows these molecules to bind IFNβ-promoter-stimulator 1 (IPS1/CARDIF/VISA/MAVS), located on the cytosolic face of the outer mitochondrial
IPS1 is then recruits TRAF3 and FADD/RIP1 to induce signalling events leading to IRF3- and NFκB-mediated induction of various genes, including those for type I IFNs and pro-inflammatory cytokines (96, 172). Unlike its family members RIG-1 or MDA5, LGP2 does not contain any CARD regions, making it unable to interact with IPS1 (96). The current function of LGP2 is uncertain, but some recent studies have shown that it is able to negatively regulate RIG-I signalling in response to RIG-I specific-viruses, while enhancing MDA5 signalling in response to MDA5-specific viruses (96, 172).

RIG-I recognizes short dsRNA and 5’ triphosphate ssRNA molecules in the cytosol (172). MDA5, like TLR3, is able to recognize both long strands of dsRNA and poly (I:C) (173). While both TLR3 and MDA5 can recognize the same ligands, they are differentially expressed in cell subsets, sequestered to different compartments of the cell, and have different pathophysiological outputs upon their respective activation (172). For example, mouse natural killer (NK) cells, rely predominantly on MDA5 activation in their cognate APCs to induce anti-tumor responses, while TLR3 expression and response is found in the splenic B cell zone (173). Through the use of bone marrow chimeras, MDA5 has been found to act as the primary dsRNA sensor in stromal cells, while TLR3 is sequestered to the hematopoietic lineage (172).

Importantly, the addition of exogenous poly (I:C) to B cells of TLR3 knockout mice, did not lead to the induction of activation markers; similar results were found in knockout macrophages which when treated with naked poly (I:C) were unable to produce inflammatory cytokines (164).

1.7 B6.NZBc13 mouse model and phenotype

NZB and NZB/W mice have increased levels of co-stimulatory molecules on their splenic B cells (17), reflecting the polyclonal B cell activation in these mice. Linkage studies have demonstrated that chromosomes 1 and 13 contain susceptibility loci associated with development of nephritis and anti-dsDNA autoantibody production (17). Notably, epistatic interactions between the same regions on chromosomes 1 and 13 were found to correlate with the upregulation of CD44 and co-stimulatory molecules on B cells (17), suggesting that the immune mechanisms leading to polyclonal B cell activation are relevant to disease. On chromosome 13,
the upregulation of CD44 and co-stimulatory molecules was most tightly linked to D13Mit39 marker. Although, anti-gp70 antibodies and gp70 levels have also been linked to sgp3, an overlapping locus on chromosome 13, and TLR7 stimulation, this antigen system is not thought to be important for the generation of lupus-like autoimmunity in B6.NZBc13 mice (2, 174, 175).

Congenic mice with a portion of the NZB chromosome, centered on D13Mit39 develop a lupus-like phenotype (2, 39). B6.NZBc13 mice, termed c13 in this thesis for convenience, were also found to have increased levels of the co-stimulatory markers CD80, CD86, ICAM-1, and CD69, at both 4- and 8-months of age (39), recapitulating to some extent the results of previous mapping experiments. Surprisingly, however the proportion of CD44^{high} B cells, the linkage marker most associated with chromosome 13, was not consistently elevated in c13 mice (39). This B cell activation phenotype demonstrated a partial sex-bias, as male mice displayed intermediately activated B cells (39).

In addition to enhanced B cell activation, c13 mice displayed a number of additional cellular phenotypes. These included: 1) splenomegaly; 2) increased proportions of myeloid dendritic cells (mDCs), but not pDCs; 3) an altered distribution of B cell subsets with an increased population of marginal zone B cells, B1a B cells, plasmablasts, and centroblasts, together with a reduction in the proportion of follicular B cells; 4) increased numbers and size of germinal centres; 5) increased proportions of CD4^{+} and CD8^{+} T cells expressing of the activation marker CD69 and memory cell markers; and 6) increased proportions of macrophages (39).

The autoimmune phenotype in c13 mice differed somewhat from that observed for NZB mice (39). Similar to NZB mice, 4- and 8-month old mice had increased proportions of IgM producing plasma cells, as determined by ELISPOT, and a 3- to 4-fold increase in total serum IgM, as well as increases in total IgG in 8-month old mice (39). They also demonstrated increases in IgM autoantibodies to histones, chromatin, Sm/RNP, ssDNA, and dsDNA in 8-month old mice (39). However, in contrast to NZB mice they produced IgG autoantibodies to histones, Sm/RNP, and chromatin (39). Only mild renal disease was seen in c13 mice, which did not result in development of proteinuria or increase mortality (2, 39).

In 2011, Loh *et al.* performed a series of bone marrow transfer experiments where it was discovered that B6 mice reconstituted with c13 bone marrow were able to reproduce many of the autoimmune phenotypes of the c13 mouse (3). Hematopoietic radiation chimeras with mixtures
of B6 and either B6 or c13 bone marrow cells were then generated, and while B6:c13 mixed chimeric mice had increased B cell activation, B cell subset distribution and DC expansion, T cells were not activated and autoantibodies were not produced. Subsequent mixing experiments where a non-reactive B cell receptor (BCR) was introduced onto the c13 background indicated that autoreactive B cells were required to initiate these phenotypes (3). To elucidate the role these defects played in B cell tolerance of c13 mice, anti-HEL and sHEL transgenes were crossed onto the c13 background (2, 3). No breaches of tolerance were noted, but there were increased proportions of receptor edited B cells, indicating that intrinsic defects in c13 mice may lead to altered peripheral selection and survival (3). Stimulation through the BCR led to similar levels of calcium flux and phosphorylation of signalling molecules, indicating that BCR signalling was not responsible for altered B cell selection in c13 mice (3). Functional studies of c13 splenocytes and B cells then led to the discovery that the TLR3 ligand poly (I:C) could enhance B cell proliferation, frequency, and survival (3). The response of mixed B6 and c13 B cells then revealed that these defects were B cell intrinsic (3). Finally, TLR3 expression, which is upregulated after stimulation with poly (I:C) was found to be markedly increased in c13 CD21Intermediate (Int) B cells as compared to B6 (3). This hyper-responsiveness to poly (I:C) also occurred basally in CD21High (Hi) B cells, in accordance with the increased expression of TLR3 in marginal zone B cells (3, 85).

Since mice with impaired apoptotic clearance pathways develop a lupus-like autoimmune disease, similar to that observed in c13 mice (2, 45, 54), c13 mice were assessed for their ability to clear apoptotic debris. Peritoneal macrophages from c13 mice were shown to be unable to efficiently associate with or take up apoptotic debris (54). In addition, the tingible body macrophages in the germinal centres of c13 mouse spleens were also shown to be associated with an increased amount of cellular debris, similar to mouse strains with impaired function of this cell population (54). No defect was seen for c13 mouse bone marrow-derived macrophages however, indicating that the clearance defect in c13 mice is likely the result of altered cell surface expression or function of a receptor that is differentially expressed in these macrophage populations, or differences between the environment of peritoneum and germinal centres as compared to the bone marrow (54).

To further refine the polymorphisms associated with the c13 interval, a series of subcongenic mice, with small and partially overlapping regions of the NZB c13 interval, were
generated (54). Mice were aged to 6-months and then their splenic cellular phenotypes were assessed (54). All of the subcongenic mice tested were found to have increased expression of the activation markers CD86, CD80, CD69, and ICAM-1 on their B cells and an expansion of the CD11c+ or total dendritic cell population in their spleens (54). In addition, increased expression of the activation marker CD69 on CD4+ T cells, and an increased proportion of centroblasts (PNA+ CD21− B cells) were found in many of the subcongenic strains, and trended towards significance in all subcongenic lines tested (54). As the only overlapping region for all of the subcongenic mouse strains is the 81-94 megabase (Mb) interval (region c on Figure 2), it is likely that these phenotypes arise from the susceptibility locus in this region. Notably, anti-chromatin autoantibody production, as well as splenomegaly, were only found in mice containing the c interval together with the proximal a-b interval, indicating that another locus may be responsible for these autoimmune phenotypes (54). From these observations, the concept of at least two autoimmune susceptibility loci present on the c13 interval was formed. Functional experiments indicated that the impaired clearance of apoptotic debris localized to the c region, suggesting that this defect is responsible for much of the altered cellular activation and dendritic cell expansion in c13 mice.
Figure 2. Subcongenic c13 mouse strains. Subcongenic mice containing smaller portions of the c13 interval were generated and housed in the TWRI animal facility. All mice with * notation were generated after Pau et al. (2013) publication (54). B6.NZBc13 (47-94Mb) (c13a-c) and B6.NZBc13 (72-120Mb) (c13b-e) strains did not survive and were lost shortly after the Pau et al. (2013) publication (54).
1.8 Objectives of thesis

Systemic lupus erythematosus is a multifactorial autoimmune disorder where disease susceptibility is conferred through complex environmental, hormonal and polygenic interactions (2). The NZB mouse model closely recapitulates many of the autoimmune phenotypes found in human lupus and disease susceptibility is conferred through multiple loci (17).

The aim of this study was to refine the locus responsible for poly (I:C) hyper-responsiveness and to investigate the role of the dsRNA-sensing defect in the pathogenesis of the altered immunologic phenotype observed in c13 mice. To determine the location of the dsRNA-sensing defect on chromosome 13, I used various subcongenic c13 mice, containing shortened intervals of the NZB c13 locus, to look for the region encoding the dsRNA-sensing abnormality. To study the role of the B cell dsRNA-sensing defect on the overall autoimmune phenotype of c13 mice, a TLR3 knockout was backcrossed onto the c13 background disrupting all TLR3 signalling and TLR3-mediated dsRNA sensing. I then characterized the cellular phenotype of these mice. Initial reports by previous lab members suggested that at least two susceptibility loci were located on chromosome 13. The first locus, located in the proximal interval, was associated with B cell activation and IgM autoantibody production, while the second interval, located in the distal interval of c13, was associated with anti-chromatin IgG antibody production (2). Work by Pau et al. then demonstrated several autoimmune cellular defects and anti-chromatin autoantibody production co-localized with the apoptotic clearance defect found in the c interval. I therefore hypothesized that abrogating dsRNA sensing would affect the defects associated with the susceptibility locus in the proximal interval, while leaving those associated with distal defect relatively unaffected.
2 Materials and methods

2.1 Generation and housing of B6.NZBc13.TLR3\textsuperscript{KO} mice

B6.NZBc13 congenic mice were produced using the speed congenic technique, as described previously in Wither et al. 2005 (39). Subcongenic mice were generated by backcrossing the B6.NZBc13 mouse onto the B6 genetic background and selecting offspring with smaller intervals of the c13 locus. Mice were then intercrossed to produce a homozygous background (54). Mice were genotyped with polymorphic microsatellite markers, spaced approximately 4 cM apart, to determine B6 or c13 genetic background (54). C57BL/6 (B6) mice (product code: 000664) and B6.TLR3\textsuperscript{KO} mice (product code: 005217) were initially purchased from Jackson Laboratory (Bar Harbor, ME, USA). Knockouts were bred to B6 mice to generate heterozygotes and the resulting breeding lines were then maintained within the facility. To generate c13.TLR3\textsuperscript{KO} mice, B6.TLR3\textsuperscript{Het} mice were crossed with c13 mice containing the full length interval. The resulting heterozygotes were backcrossed onto the full length c13 background to produce homozygous c13.TLR3\textsuperscript{Het} mice. Mice were then intercrossed to produce knockouts. All mice used in experiments were female, and all litters were aged to 6-months in microisolators at the Animal Resource Centre of the Toronto Western Research Institute. Mice were maintained and experiments were performed in accordance with the Animal Use Protocol #123, from the University Health Network’s Animal Care Committee.

2.2 Flow cytometry

All flow cytometric analyses were performed using a BD\textsuperscript{TM} LSR II instrument (Becton Dickenson (BD) Biosciences, Franklin Lakes NJ, USA). For \textit{de novo} splenocyte stains, $5 \times 10^5$ cells were analyzed per stain set, and for two-day splenocyte cultures, $6 \times 10^5$ cells were analyzed per stain set. Fc receptors on the cells were blocked with 10 $\mu$g/mL of mouse IgG
(product code: I5381, Sigma-Aldrich, Saint Louis, MO, USA) prior to staining. A list of the various monoclonal antibodies used to determine cellular phenotypes can be found in Table 1. In addition, peanut agglutinin (PNA) conjugated to biotin was purchased from Sigma-Aldrich (product code: L-6135). Two different stains were used to determine cell viability: propidium iodide (PI; product code: 81845, Sigma-Aldrich) was used at a final concentration of 0.6 μg/mL to discriminate between live and dead cells on the Per CP Cy5.5 channel of the flow cytometer when cells were only assayed for surface markers; and Fixable Viability Stain 450 (FVS450) (product code: 562247, BD Horizon™) was used to discriminate between live and dead cells on the Pacific Blue channel for intracellular TLR staining at a final dilution of 0.5 μL per 1 million cells.
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Table 1. List of antibodies used in flow cytometry for characterizing splenocytes in de novo and two day cultures.
2.3 Splenocyte culture

Splenocytes were isolated from 6-month-old B6 and c13 TLR3 gene-deleted mice and their littermates, or 10-14-week-old B6, c13 full length, and c13 subcongenic mice. Red blood cells were depleted using Gey’s solution. The resultant cells were cultured for two days at 37°C in U-bottom 96 well plates containing complete media (RPMI 1640 with 10% decomplemented fetal bovine serum (product code: 12483020, Invitrogen), 100 IU/mL penicillin and 100 μg/mL streptomycin (product code: 15140-122, Invitrogen), 0.1 mM MEM nonessential amino acids (product code: CA12001-634, VWR International Radnor, PA, USA), 2 mM L-glutamine (product code: 25030-81, Invitrogen) and 2-mercaptoethanol (product code: 21985-023, Invitrogen) alone or with TLR ligands. Various TLR ligands were added to the culture medium to stimulate the cells: CpG oligonucleotide (ODN) 1826 (CpG B 1826) (product code: ttrl-1826, Invivogen, San Diego, CA, USA) at a final concentration of 250 nM; CpG ODN 1826 control (CpG B 1826 Ctrl) (product code: ttrl-1826c, Invivogen) at a final concentration of 250 nM; or polyinosinic-polycytidylic acid (Poly (I:C)) (product code: ttrl-pic, Invivogen) at a final concentration of either 25 μg/mL (Poly (I:C) 1x), or 50 μg/mL (Poly (I:C) 2x).

2.4 ELISA

Serum samples were collected from the mice at the time of their sacrifice. IgM and IgG antibodies were measured with alkaline phosphatase conjugated anti-IgM (clone 1B4B1) (product code: 1140-04, Southern Biotech, Birmingham, AL, USA) and anti-IgG (product code: M30108, Invitrogen, Fredrick, MD, USA), respectively. dsDNA was prepared from calf thymus DNA (product code: 1501, Sigma Aldrich). ssDNA was made by boiling dsDNA for 10 minutes and then quick cooling on ice for 2 minutes. H1-stripped chromatin was made from whole chicken blood as outlined by Yager et al. (176). Immulon™ 2 HB microtiter plates (product code: Thermo 3455, Immunochemistry Technologies, Bloomington, MN, USA) were coated with: 8 μg/mL chromatin, 40 μg/mL dsDNA, 20 μg/mL ssDNA, or 1 unit/well Sm/RNP (product code: Sm/RNP, Invivogen).
code: SRC-3010, ImmunoVision, Springdale, AR, USA) antigens diluted in PBS and left at 4°C Celsius overnight. 4-nitrophenyl phosphate disodium salt hexahydrate (product code: 9839, Sigma Aldrich) was used as the substrate and the absorbance read at 405nm.

2.5 Statistics

The Mann-Whitney U non-parametric two-tailed test was used for making a comparison between only two groups of mice (eg. B6.TLR3WT and c13.TLR3WT mice) and is denoted as a specific p value in the text, or as a specific p value on graphs comparing the induction of TLRs or activation markers after TLR ligand stimulation. For comparisons of multiple strains where normality tests indicated a non-Gaussian distribution of data sets, a Kruskal-Wallis one way analysis of variance test with a Dunn’s post-test was performed. For data that was normalized or where all groups passed normality testing, a one-way analysis of variance (ANOVA) with a Bonferroni’s post-test comparing multiple selected columns was performed. These results are denoted as: ns for not significant, * for a p value of between 0.05 and 0.01, ** for a p value of between 0.01 and 0.001, and *** for a p value < 0.001 on graphs where significance was achieved.

2.6 Calculations

In order to compare the mean fluorescence index (MFI) of various markers between experiments, samples were normalized to the average mean MFI of B6 mice used per experiment, as denoted in Equation 1 below.
Normalized MFI = \( \frac{\text{Mean MFI of sample}}{\text{Average mean MFI of B6 mice (minimum 2/experiment)}} \)

Equation 1. Normalization of MFI values to compare between experiments.

To calculate the relative specificity of the anti-TLR3 and anti-TLR9 antibodies, the mean MFI of each sample in media alone was divided by the mean MFI of the isotype control in media alone, as denoted in Equation 2 below.

\[ \text{Induction of Marker} = \frac{\text{Mean MFI of anti-TLR antibody in media alone/mouse}}{\text{Mean MFI of isotype control in media alone/mouse}} \]

Equation 2. Calculating antibody specificity. MFI of anti-TLR antibodies were divided by the isotype control for each sample, to determine binding specificity.

To calculate the fold induction, or increase in the expression of a marker after stimulation, the mean MFI of the marker after stimulation was divided by the mean MFI in media alone as denoted in Equation 3.

\[ \text{Fold induction} = \frac{\text{Mean MFI of marker in stimulatory condition}}{\text{Mean MFI of marker in media alone}} \]

Equation 3. Fold induction, or change in expression of marker, post activation.
3 Results

3.1 Mapping the poly (I:C) hyper-responsiveness with subcongenic mice

Previous work by Loh et al. found that pre-autoimmune c13 mice had an intrinsic B cell defect which led to a hyper-responsiveness to the TLR3 ligand poly (I:C) (3). One of the consequences of this defect was increased expression of TLR3 on CD21\text{Int} splenic B cells after two days of stimulation with poly (I:C) in c13 mice, relative to B6 controls (3). In order to localize the loci associated with the poly (I:C) hyper-responsiveness, subcongenic mice, containing smaller portions of the c13 interval, were assessed following poly (I:C) stimulation using the same method to assess the same cell populations as previously reported (3). As in the previous study, B cells were stained with anti-B220 and anti-CD21 to roughly stratify B cells into CD21\text{Lo} (T1, germinal centre, and plasmablast), CD21\text{Int} (T1 and follicular), and CD21\text{Hi} (marginal zone precursor and marginal zone B cell) subsets. Only mice containing the full-length c13 interval had increased relative expression of TLR3 on the CD21\text{Int} B cell subset after poly (I:C) stimulation, while none of the subcongenic strains tested were found to be hyper-responsive to poly (I:C) (Figure 3). These results indicate that although this assay produced similar results to those seen in Loh et al. for the full-length c13 interval, the hyper-responsive defect did not appear to localize to any of the subcongenic c13 mouse strains. This finding is compatible with the possibility that multiple susceptibility loci spread across several different subcongenic intervals are required to generate the hyper-responsive defect, or alternatively, that the defect is localized to the a” region, an interval not found in any of the subcongenic mouse strains (Figure 2).
Figure 3. Relative TLR3 expression on CD21<sup>int</sup> splenic B cells of young mice. 10-14-week-old mouse splenocytes were cultured in either media or media containing poly (I:C) for 2 days. To normalize the MFI results between experiments, results are expressed as expression relative to the mean of TLR3 expression of B6 mice, contained within each experiment, as outlined in the Methods using Equation 1. Each point symbolizes the results from an individual mouse. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by a one-way ANOVA with a Bonferroni’s multiple comparison post-test.
3.2 TLR3 knockout mice were unable to respond to the TLR3 ligand poly (I:C)

In order to determine the role dsRNA sensing plays in the generation of the cellular autoimmune phenotype of c13 mice, c13.TLR3\(^{ KO}\) mice were generated using the speed congenic technique as described previously (39). Mice were first genotyped to assess the status of TLR3, and then upon sacrifice, stimulated with poly (I:C) to confirm a lack of TLR3 function. As seen in Figure 4a, staining with the anti-TLR3 Ab was not much higher than staining seen for the isotype control when cells were left in media only, and did not appear to be markedly different between TLR3 sufficient and deficient mice (Figure 4b). This finding suggested that both c13 and B6 B cells expressed low levels of TLR3 basally as staining was not detectable above background staining for the Ab. Therefore, to further assess whether mice genotyped as TLR3 knockouts lacked functional TLR3, splenocytes were tested for their ability to upregulate TLR3 after stimulation with poly (I:C) in the same manner as described by Loh et al. (3). As shown Figure 4a and as previously reported, TLR3 expression was induced by stimulation with poly (I:C) for B6.TLR3\(^{ WT}\) and c13.TLR3\(^{ WT}\) mice (Figure 4c), but remained near basal levels for B6.TLR3\(^{ KO}\) and c13.TLR3\(^{ KO}\) total B cells (Figure 4c), with B6.TLR3\(^{ Het}\) and c13.TLR3\(^{ Het}\) demonstrating intermediate phenotypes. In contrast to TLR3, TLR9 expression was much higher basally in the B cells of both c13 and B6 mice (Figure 4b), indicating that TLR9 is highly expressed in resting B cells. Upon stimulation with the TLR9 ligand CpG B 1826, TLR9 expression was induced 2-4 fold over basal levels of expression in all groups tested (Figure 4d), indicating that the expression of other intracellular TLRs were not affected by knocking out TLR3.
a

Media TLR3 / Isotype Control

Media TLR9 / Isotype Control

MFI of Media TLRs in B220+, CD19+

Live Cells over Isotype

Poly (I:C) 1x

p = 0.0027

p = 0.0081

p = 0.0012

p = 0.0022

b

Media TLR3 / Isotype Control

Media TLR9 / Isotype Control

Poly (I:C) 1x

Poly (I:C) 2x

c

p = 0.0027

p = 0.0022

p = 0.0081

p = 0.0012

d

CpG B 1826

CpG B 1826 Ctrl
**Figure 4. TLR expression in splenic B cells.** a) Representative histograms of TLR3 expression on B cells (B220+, CD19+) from different mouse groups in various media conditions. b) MFI of either TLR3 or TLR9 in media over MFI of isotype control in media (values determined as described in the Methods using **Equation 2**. c) Induction of TLR3 expression; MFI of TLR3 after poly (I:C) stimulation over MFI of basal TLR3 expression (media control), as described in the Methods using **Equation 3**. d) Induction of TLR9 expression; MFI of TLR9 after CpG B 1826 or CpG B 1826 Ctrl stimulation over MFI of basal TLR9 expression (media control). 6-month-old mouse splenocytes were cultured in either media, or media containing poly (I:C) (1x), poly (I:C) (2x), CpG B 1826, or CpG B 1826 Ctrl. Each point symbolizes the results from an individual mouse. Horizontal lines represent the mean of each group tested. A Mann-Whitney U test was used as the statistical analysis to compare the induction of TLR3 expression between groups.
B6.TLR3KO splenic B cells are reported to be unable to upregulate the expression of the activation molecules CD69, CD80, or CD86 with poly (I:C) stimulation (164). Therefore to further investigate the impact of the TLR3 gene deletion on B cell function in c13 mice, splenocytes were stimulated with poly (I:C), and their activation was assessed. In accordance with previous results, B6.TLR3KO B cells did not upregulate CD69 after stimulation with poly (I:C), and similar findings were obtained for c13.TLR3KO splenic B cells (Figure 5a-b). In contrast, stimulation with the TLR9 ligand, CpG B 1826, resulted in equivalent upregulation of CD69 on B cells for all mouse groups examined (Figure 5b). CD80 and CD86 expression were also assessed after poly (I:C) stimulation, and were only induced in TLR3-sufficient B cells (data not shown). These results confirm that mice which were genotyped as TLR3 knockouts did not express functional TLR3 on either the B6 or c13 background. Importantly, although TLR3 knockout mice retained the cytosolic poly (I:C) sensor MDA5, the addition of exogenous poly (I:C) did not induce B cell activation in the absence of TLR3 for either knockout mouse strain (Figure 5a-b). One interesting consequence of knocking out TLR3 on the c13 background was the increased response to the TLR9 ligand CpG B 1826 (Figure 5b). The increase in CD69 expression was only apparent on c13.TLR3KO B cells, relative to c13.TLR3WT, and not in B6.TLR3KO B cells relative to B6.TLR3WT. It is possible that the dsRNA sensing defect in c13 mice may facilitate TLR3 mediated repression of TLR9 pathways, similar to TLR9 repression of TLR7 pathways in MRL-lpr mice. It should also be noted, that as previously reported, older c13 wildtype mice lacked the enhanced upregulation of TLR3 and B cell activation molecules relative to B6 mice, presumably due to prior activation in-vivo.
**Figure 5. Induction CD69 expression on splenic B cells.**

**a)** Representative histograms of CD69 expression on B cells from different genotypes in response to different media conditions.

**b)** Induction of CD69 in various culture conditions over media, calculated as described in the Methods using Equation 3. 6-month-old mouse splenocytes were cultured in either media alone, or media containing poly (I:C) (1x), poly (I:C) (2x), CpG B 1826, or CpG B 1826 Ctrl for two days. Each point symbolizes the results from an individual mouse. Horizontal lines represent the mean of each group tested. A Mann-Whitney U test was used as the statistical analysis to compare the induction of CD69 expression between groups.
3.3 Splenic cellular phenotype of c13.TLR3\textsuperscript{KO} mice

3.3.1 Knocking out TLR3 on the c13 background differentially affected B cell activation markers.

In the original study of c13 mice, the activation markers CD69, CD80, CD86, and ICAM-1 were all up regulated on all B cell and B cell subsets separated by their relative expression of CD21 (39). Subsequent studies by Loh \textit{et al.} demonstrated CD21 intermediate and high expressing B cells had altered responses to poly (I:C) (3). To determine the role of aberrant dsRNA-sensing in the increased activation of c13 splenic B cell subsets \textit{in-vivo}, spleens were isolated and analyzed by flow cytometry for expression of these activation markers, after partitioning populations with their relative expression of CD21. As seen previously, there was increased expression of CD69 on c13.TLR3\textsuperscript{WT} total B cells (CD19\textsuperscript{+}, B220\textsuperscript{+}) (Figure 6a-b), as well as increased expression on CD21\textsuperscript{Hi} B cells (Figure 6c), and CD21\textsuperscript{Int} B cells (Figure 6d) (39). For the total B cell population no significant differences in the proportion of CD69\textsuperscript{+} cells were found between c13.TLR3\textsuperscript{WT} and c13.TLR3\textsuperscript{KO} mice (Figure 6b-d). There was a trend towards a decreased proportion of CD69\textsuperscript{+} cells in CD21\textsuperscript{Hi} and CD21\textsuperscript{Int} B cells, however this did not achieve statistical significance when corrections were made for multiple comparisons. These findings suggest the possibility that the increased CD69 expression in c13 mice arises from interactions between both susceptibility loci in the c13 interval.
%B220+; CD19+; CD69+
of Live Cells

---

%B220+; CD19+; CD21Hi
CD69+
of Live Cells

---

%B220+; CD19+; CD69+
of Live Cells

---

%B220+; CD19+; CD21Hi
CD69+
of Live Cells

---
Figure 6. Expression of the activation marker CD69 on the total B cell population and B cell subsets. a) Representative contour plots showing the expression of CD69 on total B cells and of the proportions of cells within the CD21-stratified B cell subsets. Plots were gated on B220⁺, CD19⁺, and live cells. b) Proportion of CD69⁺ cells within the B220⁺, CD19⁺ total B cell populations. c) Proportion of CD69⁺ cells within the B220⁺, CD19⁺, CD21Int B cell subset. d) Proportion of CD69⁺ cells within the B220⁺, CD19⁺, CD21Hi B cell subset. Each point symbolizes the result from an individual mouse. Horizontal lines represent the mean of each group tested. The Kruskal-Wallis test with a Dunn’s multiple comparison test of groups is shown as the statistical analysis.
Similar trends were seen for expression of ICAM-1, however in contrast to CD69 expression, the differences were significant (Figure 7a-e). Expression of ICAM-1 was increased on the total B cell population (CD19⁺, B220⁺) (Figure 7c), CD21<sup>Hi</sup> (Figure 7d), and CD21<sup>Int</sup> B cell subsets (Figure 7e) of c13.TLR3<sup>WT</sup> mice as compared to B6.TLR3<sup>WT</sup> mice. Although, no significant changes were found between B6 mice with and without TLR3, comparison of c13.TLR3<sup>WT</sup> and c13.TLR3<sup>KO</sup> mice revealed a significant decrease in ICAM-1 expression, with c13.TLR3<sup>KO</sup> mice having near B6 levels of ICAM-1 expression (Figure 7a-b). This phenomenon was observed in the total splenic B cell population (CD19⁺, B220⁺ cells) (Figure 7c), and CD21<sup>Hi</sup> (Figure 7d), and CD21<sup>Int</sup> B cell subsets (Figure 7e). Thus the increased expression of ICAM-1 observed on the B cells of c13 mice is dependent upon TLR3 signalling.
**Figure 3:**

- **c:** MFI of ICAM-1 of B220+, CD19+ cells as compared to average of B6 per experiment.
- **d:** MFI of ICAM-1 of B220+, CD19+, CD21^hi^ cells as compared to average of B6 per experiment.
- **e:** MFI of ICAM-1 of B220+, CD19+, CD21^int^ cells as compared to average of B6 per experiment.

Significance levels:
- ***: p < 0.001
- **: p < 0.01
- *: p < 0.05
- ns: not significant
Figure 7. Expression the activation marker ICAM-1 on the total B cell population and B cell subsets normalized to the average MFI of ICAM-1 expression of B6.TLR3WT per experiment. a) Representative histograms showing ICAM-1 expression on B220⁺, CD19⁺, live B cells from each mouse strain with results for the different genotypes overlayed. b) Representative contour plots showing the expression of ICAM-1 on various B cell subsets stratified based upon their expression of CD21. Plots were gated on B220⁺, CD19⁺, and live cells. c) Relative expression of ICAM-1 on live cells within the B220⁺, CD19⁺ total B cell population. d) Relative expression of ICAM-1 on cells within the B220⁺, CD19⁺, CD21^{Hi} B cell subset. e) Relative expression of ICAM-1 on cells within the B220⁺, CD19⁺, CD21^{Int} B cell subset. Relative expression of ICAM-1 was normalized to the average MFI of B6 mice per experiment, as described in the Methods using Equation 1. Each point symbolizes the result from an individual mouse. Horizontal lines represent the mean of each group tested. A one-way ANOVA with a Boneferroni’s multiple comparison test of groups shown was used for statistical analysis.
Similar findings were not observed, however, for expression of CD80 and CD86. Consistent with previous studies, there was increased expression of CD80 on the total B cell population and CD21^int B cell subset of c13.TLR3^{WT} mice as compared to B6.TLR3^{WT} mice when making a single comparison between groups, using the Mann-Whitney U test (p = 0.0071), but was lost when making multiple comparisons (Figure 8a-b,d) (3). Increased expression of CD80 was not seen on the CD21^Hi B cell subset (Figure 8c), indicating that in the current study the main B cell subset with increased CD80 expression in c13 mice is likely the follicular B cell subset. In contrast to ICAM-1 and CD69 expression, no significant differences or even trends towards decreased CD80 expression were observed between c13.TLR3^{WT} and c13.TLR3^{KO} mice for either the total B cell population (Figure 8a-b), CD21^Hi, or CD21^int B cell subsets (Figure 8c-d). CD86 expression was also assessed, and while the increase in expression between c13 and B6 wild-type mice was not significant, there was a trend towards increased expression (data not shown). Similar to CD80, the trend of increased CD86 expression in c13 mice was not affected when TLR3 was knocked out (data not shown).
Figure 8. Expression of the activation marker CD80 on the total B cell population and B cell subsets. a) Representative contour plots showing the expression of CD80 on B cell subsets stratified based upon expression of CD21. Plots were gated on B220+, CD19+, and live cells. b) Proportion of CD80+ cells within the B220+, CD19+ total B cell population. c) Proportion of CD80+ cells within the B220+, CD19+, CD21HI B cell subset. d) Proportion of CD80+ cells within the B220+, CD19+, CD21INT B cell subset. Each point symbolizes the result from an individual mouse. Horizontal lines represent the mean of each group tested. The Kruskal-Wallis test with a Dunn’s multiple comparison test of groups is shown as the statistical analysis.
In summary, B cell activation markers appeared to be differentially regulated in c13 mice. TLR3 signalling plays a definite role in ICAM-1 overexpression and possibly co-regulates CD69 expression in conjunction with the susceptibility locus in the c interval. TLR3 signalling, however, appears to have little impact on expression of CD80 and CD86, suggesting that the susceptibility locus found in the c interval is predominantly responsible for driving the overexpression of this family of co-stimulatory molecules in c13 mice.

### 3.3.2 Marginal zone B cell expansion is lost in c13.TLR3\(^{\text{KO}}\) mice

Expansion of the marginal zone B cell compartment has been demonstrated in several different lupus-prone mouse models, as well as the full length c13 mouse (39, 91). Previous experiments indicate that TLR3 mRNA expression is almost exclusively localized to marginal zone B cells in a resting state (85). Therefore, we postulated that altered dsRNA sensing in the B cells of c13 mice might play a role in the expansion of this B cell subset. To address this possibility, c13.TLR3\(^{\text{KO}}\) mice were assessed for alterations to their B cell subsets by flow cytometry. The relative expression of CD21 and CD23 were used to differentiate between the different B cell subsets (Figure 9a). Comparison of B6 mice with and without TLR3 revealed no significant changes in the proportion of cells in any of the B cell subsets examined (Figure 9a-e). Comparison of B6 and c13 wild type mice, revealed that there was no significant difference in the proportion of marginal zone precursor B cells (Figure 9b) (B220\(^+\), CD19\(^+\), CD23\(^+\), CD21\(^{\text{Hi}}\)), in accordance with previous studies of the c13 mouse strain (3, 39, 54). Similar to previous studies, the proportion of CD21\(^-\), CD23\(^-\) B cells was expanded in c13.TLR3\(^{\text{WT}}\) mice in comparison to B6.TLR3\(^{\text{WT}}\) mice (\(p = 0.0240\)), but was lost when correcting for multiple statistical comparisons, while no differences were observed between c13.TLR3\(^{\text{WT}}\) and c13.TLR3\(^{\text{KO}}\) strains (Figure 9c) (39). The proportion of T2/follicular B cells was significantly contracted (Figure 9d) and the proportion of marginal zone B cells (B220\(^+\), CD19\(^+\), CD23\(^-\), CD21\(^{\text{Hi}}\)) was significantly expanded (Figure 9e) in c13.TLR3\(^{\text{WT}}\) mice as compared to B6.TLR3\(^{\text{WT}}\) mice (39). A comparison of c13.TLR3\(^{\text{WT}}\) and c13.TLR3\(^{\text{KO}}\) mice revealed that there was a restoration of the follicular contraction (Figure 9d). The marginal zone B cell expansion of c13.TLR3\(^{\text{WT}}\) mice was lost in c13.TLR3\(^{\text{KO}}\) mice, and this statistically significant contraction
neared marginal zone B cell proportions seen in B6 mice (Figure 9e). Thus the dsRNA-sensing defect appears to play an important role in driving the marginal zone expansion observed in c13 mice.
a

B6.TLR3 WT

B6.TLR3 Het

B6.TLR3 KO

c13.TLR3 WT

c13.TLR3 Het

c13.TLR3 KO

CD23

CD21
% B220⁺, CD19⁺, CD23⁺, CD21⁺ of live cells

**b**

% B220⁺, CD19⁺, CD23⁻, CD21⁻ of live cells

**c**

% B220⁺, CD19⁺, CD23⁻, CD21⁻ of live cells

**d**

% B220⁺, CD19⁺, CD23⁻, CD21⁻ of live cells

**e**

% B220⁺, CD19⁺, CD23⁻, CD21⁻ of live cells

WT

B6.TLR3⁺/−

B6.TLR3⁻/−

c13.TLR3⁺/−

c13.TLR3⁻/−

KO

c13.TLR3⁻/−

KO
Figure 9. Proportions of splenic B cell subsets from 6-month-old mice. a) Representative contour plots, gated on B220+, CD19+, live cells, showing expression of CD21 and CD23, with the regions used to differentiate between the different B cell subsets indicated. Scatter plots showing the proportion of b) marginal zone precursor B cells (B220+, CD19+, CD23+, CD21^{hi}), c) CD21^-CD23^- B cells (B220+, CD19+, CD23-, CD21^-), d) follicular and transitional 2 B cells (B220+, CD19+, CD23+, CD21^{int}), and e) marginal zone B cells (B220+, CD19+, CD23-, CD21^{hi}). Each point symbolizes the results from an individual mouse. Horizontal lines represent the mean of each group tested. Significance was determined by Kruskal-Wallis one-way ANOVA with a Dunn’s post-test comparing groups shown.
3.3.3 CD21⁻ B cell subsets were not altered in c13.TLR3KO mice

In the previous study (39), CD5⁺ B cells, germinal centre B cells, and plasmablasts were all found to be expanded within the CD21⁻, CD23⁻ B cell compartment. To determine if aberrant dsRNA sensing plays a role in the expansion of these populations, B cells were stained with combinations of anti-CD21 and either anti-PNA, anti-AA4.1, anti-CD138, or anti-CD5 and then assessed by flow cytometry. As seen in Figure 10a, germinal centre B cells (CD21⁻, PNA⁺, B220⁺, CD19⁺) were expanded in c13.TLR3WT as compared to B6.TLR3WT mice. Although there was a slight trend to decreased proportions of germinal centre cells in c13.TLRKO as compared to c13.TLR3WT, this did not achieve statistical significance. Transitional 1 B cell populations (CD21⁻, AA4.1⁺, B220⁺, CD19⁺) were originally found in similar proportions in B6 and c13 mice at 4- and 6-months of age (39). In accordance with these findings, no differences were observed in the proportion of transitional 1 B cells between mice of the B6 genetic background and mice of the c13 genetic background (Figure 10b). However, in contrast to previous studies, no expansion of CD5⁺ B cells (CD21⁻, CD5⁺, B220⁺, CD19⁺) (Figure 10c), or plasmablasts (CD21⁻, CD138⁺, B220⁺, CD19⁺) (Figure 10d) were observed in any mice with the c13 genetic background, as compared to all mice with the B6 genetic background. One important difference between this study and the previous studies examining c13 mice, was the age of the mice used to examine these populations. In the previous study, the proportions of plasmablasts, CD5⁺ B cells, and germinal centre B cells were assessed in mice ranging from 5- to 12-months of age, whereas only 6-month-old mice were analyzed in the current study (39). It is possible that the expansions of these populations accumulate with age, and that the mice examined in this study were simply not old enough to show these expanded phenotypes. Consequently, it is unclear whether the dsRNA sensing defect contributes to the generation of these phenotypes.
% CD21-, PNAHI, CD19+, B220+, of Live Cells

% CD21-, CD19+, B220+, of Live Cells

% CD21-, CD5+, CD19+, B220+, of Live Cells

% CD21-, AA4.1+, CD19+, B220+, of Live Cells

WT
B6.TLR3
Het
B6.TLR3
KO
B6.TLR3
WT
c13.TLR3
Het
c13.TLR3
KO
c13.TLR3
Figure 10. Proportions of various B cell subsets within the CD21⁻, CD23⁻ B cell population of 6-month-old mice. a) Proportion of germinal centre cells (CD21⁻, PNA<sup>Hi</sup>, CD19<sup>+</sup>, B220<sup>+</sup> cells). b) Proportion of plasmablasts (CD21⁻, CD138<sup>+</sup>, CD19<sup>+</sup>, B220<sup>+</sup> cells). c) Proportion of CD5<sup>+</sup> B cells (CD21⁻, CD5<sup>+</sup>, CD19<sup>+</sup>, B220<sup>+</sup> cells). d) Proportion of transitional 1 B cells (CD21⁻, AA4.1<sup>+</sup>, CD19<sup>+</sup>, B220<sup>+</sup> cells). Horizontal lines represent the mean of each group tested. Significance between groups was determined by Kruskal-Wallis one-way ANOVA with a Dunn’s post-test. For graphs b-d no significance was achieved by Kruskal-Wallis statistical testing when comparing the groups shown in a, and thus are not recorded.
3.3.4  Dendritic cell populations were not affected by knocking out TLR3

Dendritic cells are expanded in c13 mice (54). Careful analysis of splenic DC populations revealed that the mDC compartment, but not the pDC compartment was expanded in c13 mice (39). mDCs express all of the intracellular nucleic acid-sensing TLRs, while pDCs express only TLRs 7 and 9 (113). The exclusive expression of TLR3 in MZ B cells, and the subsequent contraction of the MZ B cell compartment in c13.TLR3KO mice led to the inference that aberrant TLR3 signalling might also drive mDC expansion (85). To investigate this possibility, the proportion of splenic DCs was analyzed by flow cytometry. For B6 mice, the proportion of total DC and mDC were similar in the presence or absence of TLR3. As seen in Figure 11a, the proportion of total DC (gated as CD11c+) was expanded in c13.TLR3WT mice as compared to B6.TLR3WT mice, and a similar trend towards total DC expansion was seen in c13.TLR3KO mice (Figure 11a). To further analyze the proportions of DC subsets, cells were stained with a combination of anti-CD11b and anti-B220 in conjunction with anti-CD11c. Similar to total DC populations, the proportion of mDCs were increased in c13.TLR3WT in comparison to B6.TLR3WT mice (Figure 11b). Again, in contrast to the original expectations, the mDC cell expansion was maintained c13.TLR3KO mice (Figure 11b). These results indicate that TLR3 signalling is not responsible for the DC expansion found in c13 mice and further support the evidence presented by Pau et al. that a susceptibility locus within the c interval causes this expansion. These findings also support previous evidence that the dsRNA-sensing defect is B cell-specific (3).
Figure 11. Proportions of splenic dendritic cell subsets in 6-month-old mice. Scatterplots showing the proportion of a) total DCs (CD11c+) and b) mDCs (CD11c+, CD11b+, B220−). Each point symbolizes the results from an individual mouse. Horizontal lines represent the mean of each group tested. Statistical differences between groups were determined by a one-way ANOVA with a Bonferroni’s multiple comparison test.
3.3.5 CD4+ T cell populations were not affected by knocking out TLR3

CD4+ T cells are activated in c13 mice (39, 54). Full-length c13 mice and all of the subcongenic mice containing the c interval had increased proportions of CD69 expressing CD4+ T cells, strongly suggesting that a susceptibility locus in this region was responsible for the increased T cell activation (54). Therefore it was hypothesized that the TLR3 gene deletion would have little effect on altered T cell activation in c13 mice. To assess this possibility, splenic T cells were stained and analyzed by flow cytometry (Figure 12a). A significant expansion of the proportion of CD69 expressing CD4+ T cells was found when B6.TLR3WT and c13.TLR3WT mice were compared using the Mann-Whitney U test (p = 0.0106) in the same fashion as the original c13 study (39). When correcting for multiple comparisons, however, significance was lost (Figure 12b). Similar to mDCs, no contraction of the activated CD4+ T cell population was observed between c13.TLR3WT and c13.TLR3KO T cells either by Kruskal-Wallis or Mann-Whitney U tests.
Figure 12. Proportions of CD69+ cells within the live CD3+, CD4+ splenic T cell population of 6-month-old mice. a) Representative contour plots showing the expression of CD69 gated on CD4+ T cells. Boxes indicate the proportion of CD4+ T cells that are CD69+. b) Scatter plots showing the proportion of CD69+, CD4+ T cells in the different mouse groups. Each point symbolizes the results from an individual mouse. Horizontal lines represent the mean of each group tested.
3.3.6 Spleen size and cell number were increased in mice with the c13 genetic background

In the original study of c13 mice, 4- and 8-month old mice had increased spleen size, and 8-month old mice had increased numbers of splenocytes (39). Pau et al. further refined this phenotype by mapping it to the proximal interval (a-c) with subcongenic mice. Therefore it was postulated that this phenotype might be partially dependent upon the dsRNA sensing defect. To examine this possibility, spleens were weighed immediately after sacrifice and the number of splenocytes counted following removal of red blood cells. There was a significant increase in spleen weight between B6.TLR3<sup>WT</sup> and c13.TLR3<sup>WT</sup> when compared by the Mann-Whitney U test (p = 0.0104), in accordance with previous studies (39), although this significance was lost when correcting for multiple statistical comparisons. Surprisingly, the spleens of c13.TLR3<sup>KO</sup> mice were still significantly increased in size, similar to c13.TLR3<sup>WT</sup> mice, as compared to B6.TLR3 sufficient, heterozygous, and deficient mice (Figure 13). A general trend to increased splenocyte numbers was also observed in all mice with the c13 genetic background (data not shown). Taken together these findings indicate that although the proximal interval contains the locus responsible for splenomegaly, altered dsRNA sensing is not the driving mechanism behind this abnormality.
Figure 13. Spleen weights of 6-month-old experimental mice. Each point symbolizes the results of an individual mouse. Horizontal lines indicate the mean of each group tested. The Kruskal-Wallis test with a Dunn’s multiple comparison post-test is shown as the statistical analysis.
3.4 IgM autoantibody production was partially ameliorated in c13.TLR3\textsuperscript{KO} mice

In the original observations of c13 mice, 8-month-old mice had increased anti-chromatin, and to a lesser extent, anti-ssDNA, and -histone autoantibody production of both IgM and IgG isotypes, while mice were only able to produce IgM anti-dsDNA autoantibodies (39). Pau et al. further refined this phenotype by mapping IgG and IgM anti-chromatin autoantibody production to the proximal interval (a-c) with 6-month-old subcongenic mice. The localization of this phenotype to the proximal interval indicated that autoantibody production might be partially dependent upon the dsRNA-sensing defect. To examine this possibility, serum was collected from the mice upon sacrifice, frozen, and then tested for the presence of specific autoantibodies at a later time point by ELISA. Although there was a trend to increased IgM autoantibody production in c13.TLR3\textsuperscript{WT} mice as compared to B6.TLR3\textsuperscript{WT} mice against all antigens tested, statistical significance was only achieved for anti-chromatin and -dsDNA antibody production when corrected for multiple comparisons. Decreased IgM anti-chromatin (p = 0.0181) (Figure 14a), and IgM anti-ssDNA (p = 0.0241) (Figure 14b) levels were seen in c13.TLR3\textsuperscript{KO} mice as compared to c13.TLR3\textsuperscript{WT} mice when making a direct comparison using the Mann-Whitney U test, however this significance was lost when corrected for multiple comparisons. Surprisingly, the levels of IgM autoantibodies were higher in c13.TLR3\textsuperscript{Het} mice than c13.TLR3\textsuperscript{WT} mice, and were markedly elevated as compared to c13.TLR3\textsuperscript{KO} mice (Figure 14a-d). While the mechanism leading to increased autoantibody production in c13.TLR3\textsuperscript{Het} mice is currently unclear, the differences between these mice and TLR3 knockout mice strongly suggests that the IgM autoantibody production in c13 mice is TLR3-dependent. It is likely, that the lack of statistically significant differences between c13.TLR3\textsuperscript{WT} and c13.TLR3\textsuperscript{KO} mice, reflects the low levels of autoantibody production in c13.TLR3\textsuperscript{WT} mice. This may be due to the age of the mice assayed. In the original study of c13 mice, only IgM anti-chromatin autoantibodies were produced at 4-months of age, and the other autoantibody specificities were not seen until 8-months of age. Thus, 6-month-old mice may still be too young to produce a full complement of autoantibodies, as originally observed (39).
Figure 14. IgM autoantibody levels in 6-month-old B6 and c13 TLR sufficient, heterozygous, and deficient mice. Serum samples were assayed for the presence of a) IgM anti-chromatin, b) IgM anti-ssDNA, c) IgM anti-dsDNA, and d) IgM anti-Sm/RNP autoantibodies by ELISA. Each point denotes the circulating autoantibodies present from an individual mouse. Horizontal lines denote the mean of each group. Significance was determined by Kruskal-Wallis one-way ANOVA with a Dunn’s post-test used to make comparisons between the groups shown.
While statistically significant increases in anti-chromatin IgG autoantibody production were observed between B6.TLR3\textsuperscript{KO} and c13.TLR3\textsuperscript{WT} mice (\textbf{Figure 15a}), and between B6.TLR3\textsuperscript{WT} and c13.TLR3\textsuperscript{WT} mice when analyzed by Mann-Whitney U test (p = 0.0045) in accordance with previous studies of subcongenic mice (54), increased c13.TLR3\textsuperscript{WT} IgG autoantibody production was not observed for any of the other antigens tested (\textbf{Figure 15b-d}). Although not significant, there was a trend towards decreased production of anti-chromatin antibodies in c13.TLR3\textsuperscript{KO} mice in comparison to c13.TLR3\textsuperscript{WT} mice. Again, the reduction in disease phenotype in this study, in comparison to previous studies of the c13 mouse may be the result of using 6-month-old mice whose disease has not progressed far enough to produce the full complement of autoantibodies previously observed (39). Despite trends towards decreased IgG anti-chromatin autoantibody production in c13.TLR3\textsuperscript{KO} mice pathogenic immunoglobulin production persisted, suggesting that the dsRNA sensing defect controls primarily IgM autoantibody production. This fits with previous evidence of the reduction in the proportion of natural IgM producing-marginal zone B cells in c13.TLR3\textsuperscript{KO} mice, and the lack of reduction of CD4\textsuperscript{+} T cells in c13.TLR3\textsuperscript{KO} mice. However, the specific role that aberrant dsRNA sensing plays in the generation of IgG autoantibodies cannot be conclusively determined in the absence of examining older mice due to the general lack of increased autoantibody production in the 6-month-old c13.TLR3\textsuperscript{WT} mice.
Figure 15. IgG autoantibody levels in 6-month-old B6 and c13 TLR sufficient, heterozygous, and deficient mice. Serum samples were assayed for the presence of a) IgG anti-chromatin, b) IgG anti-ssDNA, c) IgG anti-dsDNA, and d) IgG anti-Sm/RNP autoantibodies by ELISA. Each point denotes the circulating autoantibodies present from an individual mouse. Horizontal lines denote the mean of each group. Significance was determined by Kruskal-Wallis one-way ANOVA with a Dunn’s post-test comparing groups. For graphs b-d no significance was achieved by either Kruskal-Wallis or by Mann-Whitney U test when comparing the groups shown in a, and thus are not recorded.
4 Discussion and future directions

This study sought to localize the aberrant dsRNA sensing defect seen in c13 mice and to define the role of the poly (I:C) hyper-responsiveness in the development of the c13 cellular autoimmune phenotype. Knocking out TLR3 on the c13 background led to the loss of the expanded marginal zone B cell compartment, a partial loss of B cell activation, and partial reductions in IgM autoantibody production, while other phenotypes remained unaffected. These results support a role for at least two independent susceptibility loci within the c13 interval being required to develop an autoimmune phenotype.

The depletion of the high proportion of marginal zone B cells in c13 mice was clearly TLR3-dependent, as the loss of this receptor and corresponding dsRNA sensing by B cells reduced the proportions of MZ B cells back to non-autoimmune levels. However, the exact role dsRNA sensing plays in the development of this compartment is unknown. Previous reports have found that patients with abnormal IRAK4 and MyD88 have decreased proportions of MZ B cells and increased proportions of naïve autoreactive B cells, indicating that TLR signalling plays a role in MZ B cell development as well as in B cell selection (87, 177). Recently, Ori et al. demonstrated that the loss of TAB2, a polyubiquitin-binding protein located downstream of TLR signalling (Figure 1), decreased the proportion of MZ B cells and was essential for B cell activation in a MAPK-dependent manner (178). While TLR3 utilizes the TAK1-TAB1-TAB2-TAB3 complex for signalling, poly (I:C) was not used to stimulate TAB2KO mice and it currently remains unknown as to whether TLR3 signalling is involved in MZ B cell development through this particular mechanism. Alternatively, aberrant TLR3 signalling may regulate cell survival. Stimulation through the MyD88-dependent TLR pathways in MZ B cells increases the expression of BAFFR and TACI (83); TLR3 signals may work in a similar manner to promote autoreactive B cell survival in c13 mice. Future studies looking at altered B cell selection and survival, will aid in understanding how TLR3 signalling promotes alterations to B cell subsets.

Another striking difference between c13.TLR3 wildtype and knockout mice was in their relative expression of various activation markers. ICAM-1 was found to be significantly lower in c13.TLR3KO mice, while the proportion of CD69 expressing B cells was slightly reduced in c13.TLR3KO mice, in comparison to c13.TLR3WT mice. In contrast, proportions of CD80+ B cells
were not altered in any mice containing the c13 genetic background. These results indicate that
the B cell activation markers may be differentially regulated by the abnormal cellular defects
found within the c interval, and the dsRNA-sensing defect (likely located in the a” interval), and
may arise through differential regulation of transcription factors or the actions of different
interferons. The mechanisms leading to enhanced upregulation of the various co-stimulatory
markers in c13 mice remain to be elucidated, and further studies delineating the altered pathways
associated with TLR3 signalling will reveal its exact role in these autoimmune phenotypes.

While the primary function of ICAM-1 is mediating leukocyte trafficking through blood
vessels, it also acts as a co-stimulatory molecule on B cells, and blocking ICAM-1 can
moderately inhibit T cell proliferation (179–181). ICAM-1 is regulated through different dsRNA
sensing mechanisms in different cell types; in Sertoli and intestinal epithelial cells, poly (I:C)
stimulation leads to ICAM-1 induction in a TLR3-dependent manner (182, 183). The deletion of
TRIF, however, did not stop ICAM-1 induction after poly (I:C) stimulation in murine endothelial
cells, indicating a role for MDA5 in the upregulation of adhesion molecules in these cells (184).
The results of this study indicate that the increase in ICAM-1 expression on B cells of the
autoimmune c13 mouse is TLR3-dependent. TLR3 signalling induces IFNγ (185). The reduction
of aberrant ICAM-1 expression in c13.TLR3KO mice may be a result of lost IFNγ production.
Since CD69 expression is dependent on IFNα/β, the loss of both type I and type II IFN
production in c13.TLR3KO mice from the loss of aberrant TLR3 signalling may account for the
reduced levels of these two molecules. Evaluating the expression of type I and II IFNs in c13
mice will help elucidate how these activation markers are regulated.

The results of this study indicate that dsRNA sensing does not play a role in several key
cellular autoimmune phenotypes of the c13 mouse, including: CD4+ T cell activation, myeloid
dendritic cell expansion, plasma cell expansion, splenomegaly, and the production of IgM anti-
Sm/RNP and anti-dsDNA autoantibodies, as well as IgG autoantibody production. In light of the
recent study by Pau et al. that found that most of these phenotypes map to the c interval, these
results further support the hypothesis that at least two susceptibility loci are required to develop
the full c13 autoimmune phenotype (54). The initial hypothesis for this study proposed that
splenomegaly and all autoantibody production would be abrogated in c13.TLR3KO mice, as these
two phenotypes were only found in c13 full length and c13 a-c mice. While knocking out TLR3
relieved B cells of the aberrant poly (I:C) hyper-responsiveness, the fundamental defect leading
to altered dsRNA sensing in c13 B cells was not actually targeted. It is possible that the loci responsible for dsRNA hyper-responsiveness plays a role in both TLR3-dependent and TLR3-independent pathways. Abrogating TLR3 signalling would affect the TLR3-dependent immune phenotypes, but the TLR3-independent phenotypes would remain unaffected and still promote autoimmunity in c13 mice. Alternatively, it is possible that there is another susceptibility locus in the a-b interval, and that all loci are required to generate these two phenotypes.

TLR3 is required for the generation of anti-chromatin and -ssDNA IgM antibodies, as evidenced by the differences in autoantibody secretion between c13.TLR3WT and c13.TLR3KO mice. It was interesting to note the increased production of all specific IgM autoantibodies assayed in c13.TLR3Het mice, relative to both c13.TLR3WT and c13.TLR3KO mice. Increased autoantibody production could be a result of increased signalling through other intracellular TLRs. In studying the stimulation of c13.TLR3KO mice with various TLR ligands, there was a conspicuous upregulation of activation markers in c13.TLR3KO B cells in the presence of TLR9 ligands (Figure 5b). These results indicate that TLR3 expression may cross-regulate TLR9 stimulatory pathways, similar to the cross-regulation of TLR7 by TLR9 in MRL-lpr B cells (101). The partial loss of TLR3 in the c13.TLR3Het group may exacerbate TLR9 signalling, while still allowing for autoantibody production by TLR3-dependent mechanisms in autoreactive B cells, leading to the results observed for IgM autoantibody production. Knocking out TLR3 is known to protect gut epithelium from dsRNA-induced apoptosis, and the partial loss of TLR3 may modify the presence of autoantigens in c13.TLR3Het mice (168). Examining potential TLR3-TLR9 cross-regulation in c13 B cells is an area of future interest and may help in deciphering the augmented IgM autoantibody production observed in c13.TLR3Het mice.

The lack of increased TLR3 expression following poly (I:C) stimulation in any of the subcongenic mice analyzed strongly suggests that the genes involved in the dsRNA sensing defect may be located within the a” region, located between 61 and 72 Mb, (Figure 2). The most promising candidate gene in the c13 a” interval is nucleotide-binding oligomerization domain (NOD), leucine rich repeat and pyrin domain containing protein 4f (Nlrp4f). Nlrp4f is one of seven highly homologous genes found in the NLRP4 family (186). NLRP4 is a NOD-like receptor (NLR), and has been shown to interact with Beclin-1 to prevent autophagy, and upon bacterial infection, NLRP4 transiently dissociates from Beclin-1 (187). NLRP4 was also shown to prevent autophagosome maturation by binding the class C vacuolar protein-sorting complex
In addition to regulating autophagy, NLRP4 has been recently found to control type I interferon production in dsRNA sensing cells (156). Homologs to Nlrp4f, Nlrp4b and Nlrp4g, were recently shown to regulate IFNβ signalling in macrophages (156). NLRP4 proteins bind activated TBK1 and recruit the E3 ubiquitin ligase DTX4, facilitating the polyubiquitination and degradation of TBK1 (156). It is possible that the expression of Nlrp4f is altered in c13 mice; studies of resting and activated c13 B cells may reveal dysregulated expression of Nlrp4f.

Alternately, mutations in the Nlrp4f gene may not alter levels of expression, but instead perturb protein function. While checking the binding capacity of NLRP4f is not currently possible as no antibodies exist that can specifically target NLRP4f, Nlrp4f knock down assays in B6 B cells could be studied to mimic the predicted altered protein function of c13 Nlrp4f, and determine whether this leads to hyper-responsive dsRNA sensing. Additionally, Nlrp4f could be knocked down in c13 c-d B cells. This would indicate whether a single locus is responsible for aberrant dsRNA sensing, or multiple loci across the c13 a-b interval are required. Sequencing the c13 interval would reveal polymorphisms in the c13 interval and greatly refine the susceptibility loci responsible for the c13 phenotype, and would be the most direct way of determining if there are potential alterations in Nlrp4f that could affect function.

Mediator of RNA polymerase II transcription, subunit 10 (Med10/NUT2) is another potential candidate gene in c13 mice. MED10 is part of the mediator complex, a large, multiprotein unit that facilitates transcription by bridging transcription factors and RNA polymerase II (188). The mediator complex has been found to bind the promoters of stress-induced genes, such as heat shock factors, and acts as an initiating signal for RNA polymerase II recruitment, allowing for rapid and specific responses to environmental pressures (189). How alterations to Med10 expression in c13 mice would lead to aberrant dsRNA sensing is currently unknown, but excess transcription could be a factor in dsRNA autoantigen persistence.

An alternative approach to identifying the mechanism responsible for aberrant dsRNA sensing would be to specifically inhibit the different pathways induced by TLR3 signalling. The inhibitors SP600125, U0126, and SB203580 specifically inhibit JNK, MEK1/2-ERK, and p38 MAPKs (137, 190), while Amlexanox inhibits TBK1 and IKKε (191), and caffeic acid phenethyl ester specifically inhibits NFκB (192). The amelioration of the dsRNA hyper-responsiveness defect after the addition of one or a combination of these chemical inhibitors to poly (I:C)-treated
and non-treated B cells would refine the role played the different branches of TLR3 pathways play in the generation in this B cell intrinsic defect. Furthermore, identifying inhibitors of autoreactive B cell TLR signalling could lead to a greater understanding of possible future therapeutics for these types of defects in SLE.

This study shows that dsRNA hyper-responsiveness contributes to alterations in B cell subsets, some IgM autoantibody production, and partial B cell activation. This work contributes to the working knowledge of the c13 autoimmune phenotype, and supports the hypothesis of two susceptibility loci segregating to two different locations on chromosome 13 which contribute to the overall autoimmune phenotype. As seen in Figure 16, in the proposed mechanism of pathogenesis in c13 mice, improper clearance of apoptotic debris would lead to immune cell expansion and activation. In addition, the presence of excess apoptotic debris would facilitate dsRNA hyper-responsive B cells activation, subset expansion, and IgM autoantibody production. Immune complexes would then be generated with superfluous apoptotic debris which would then deposit in various tissues causing organ damage.
Figure 16. Proposed mechanism of pathogenesis in c13 mice. Susceptibility loci in the c interval of c13 mice leads to: (1) inefficient apoptotic clearance, and the persistence of autoantigens; additionally, immune defects found in the c interval lead to expansions of the proportions of DCs, macrophages, CD80^+ and CD86^+ activated B cells, and activated T cells (2). These cells are able to produce IgG autoantibodies, and IgM autoantibodies to dsDNA and Sm/RNP (3/not shown). Susceptibility loci in the a” interval lead to aberrant TLR3 signalling in autoreactive B cells due to the persistence of autoantigens derived from c interval defects (4), leading to B cell subset expansion and activation (5). These cells produce IgM autoantibodies particularly against chromatin and ssDNA (6). The presence of autoantibodies along with the persistence of apoptotic debris in c13 mice leads to immune complex formation (7), which deposit into organs mediating inflammation and tissue damage (8).
The current work adds to the literature by demonstrating that not only altered TLR7 and TLR9, but also TLR3 signalling can contribute to the development of lupus. This finding suggests that perturbations in all of the pathways by which nucleic acids can signal immune cells to become activated and/or differentiate are likely to play a role in the pathogenesis of lupus. Thus targeting of molecules that are shared between all of the nucleic acid sensing pathways may be preferable for treatment of lupus to targeting just TLR7 or TLR9, as has been done previously.
5     References


120. Dai, J., N. J. Megjugorac, S. B. Amrute, and P. Fitzgerald-Bocarsly. 2004. Regulation of IFN regulatory factor-7 and IFN-alpha production by enveloped virus and lipopolysaccharide in


