The modulation of autoimmune diseases progression in mouse models

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ABSTRACT

B cells play crucial roles in the development of the two human autoimmune diseases, type 1 diabetes (T1D) and systemic lupus erythematosus (SLE). In the past decade, numerous studies showed positive responses of B cell depletion therapies in these two diseases. However, the beneficial effects are temporary and accompanied with adverse events. In this dissertation, we aimed to identify novel targets for a better modulation of disease development using mouse models. These diseases have circulating autoantibodies that are mostly mutated with an IgG isotype, indicating B cells that are producing them have been through the process of affinity maturation. Activation-induced cytidine deaminase (AID) is a core enzyme that regulates somatic hypermutation (SHM) and class switch recombination (CSR), the two key mechanisms in affinity maturation. We showed that genetic ablation of AID significantly inhibited the development of TID in NOD mice. Homologous recombination (HR) pathway is important for the repair of AID-induced DNA double strand breaks during CSR. 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid, also known as DIDS, is a small molecule that inhibits HR pathway and subsequently leads to apoptosis of class switching cells. DIDS treatment remarkably retarded the progression of TID, even when started at a relatively late stage, indicating the potential of this treatment for disease reversal. In both approaches, we observed a notable expansion of $CD73^+$ B cells, which exerted an immunosuppressive role and could be responsible for T1D resistance. Next we examined the effect of targeting affinity maturation through these two approaches in lupus-prone mice. The

genetic abrogation of AID in BXSB mice significantly ameliorated lupus nephritis and prolonged their lifespan. AID-deficient mice also exhibited improvement on disease hallmarks with increased marginal zone B cells and more normal splenic architecture. DIDS treatment notably reduced class switching when B cells were stimulated *in vitro*. However, the administration of DIDS did not strikingly alter the course of SLE in either BXSB mice or MRL/*lpr* mice. These findings demonstrated that affinity maturation could be a potential target for T1D and SLE, while further explorations into targeting other components in the repair pathway are warranted for SLE. Lastly, we assessed the effect of maternal AID modulation on the SLE development in the offspring using BXSB mouse model. Interestingly, the absence of maternal AID resulted in offspring that developed significantly more severe lupus nephritis compared to control. The offspring born to AID-deficient dams also exhibited elevated levels of pathogenic autoantibodies and exacerbated disease features. Therefore, the modulation of maternal AID could influence the SLE development in the offspring, and future investigations are needed to determine the underlying mechanisms responsible for the disease acceleration.

GENERAL AUDIENCE ABSTRACT

The failure of the immune system to differentiate self from non-self leads to the development of autoimmune diseases. Type 1 diabetes (T1D) and systemic lupus erythematosus (SLE) are complex autoimmune diseases affecting millions of people in the world. Despite intensive research regarding these two diseases, no known cure is available indicating an imperative need for the development of novel therapies. With the importance of B cells in the development of these two diseases, intensive research focused on immune therapies that deplete B cells. However, these therapies exhibited high risks of infections as a result of depleting all the B cells. In this dissertation, we sought to selectively target specific B lymphocyte subsets that are crucial contributing factors in the development of T1D and SLE. While the effect of therapeutic treatment varied among different mouse models, the genetic manipulation of specific B cells successfully retarded the progression of both T1D and SLE and extended the lifespan of the mice. Further studies shed light on the possible mechanisms that are responsible for the disease inhibition. These data proved that targeting specific B cell compartment could be a potential disease management in T1D and SLE patients. In addition, using the established mouse model, we demonstrated the deficiency of maternal antibodies significantly impact the SLE development in the offspring. Future experiments to identify the underlying mechanisms could provide more targets for the therapeutic development.

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Table of Contents

List of Figures

Chapter I

Introduction

The complex immune system develops to protect hosts from infectious antigens, such as bacteria and viruses (1). However, the immune system can mistakenly attack the host as a result of the failure to differentiate self from non-self, which is also known as a breach of self-tolerance (2). This dysregulation of the immune system leads to the development of autoimmune diseases, which is a devastating problem with the chronic nature and the associated medical cost (3). It has been reported that around 3-5% of the population are affected by autoimmune diseases, the majority of which are young and middle-aged individuals during the peak of reproductive years (2, 3). There are more than 100 types of autoimmune diseases with distinct clinical manifestations and complications, among which type 1 diabetes (T1D) and systemic lupus erythematosus (SLE) are the most common ones and are the main topics in this dissertation (2).

T1D is an organ specific autoimmune disease characterized by the infiltration of autoreactive T cells into pancreas and the subsequent destruction of insulin-producing beta cells (4). Insulin deficiency results in hyperglycemia and associated complications which can cause severe damage to other organs such as the retina, brain and kidneys (4). While T1D used to be known as juvenile diabetes, increasing newly cases diagnosed with T1D are people over 30 years of age (5). Current therapies primarily focus on the management of the glucose level with a lifelong need for insulin treatment (4), which necessitates the development of novel therapies. The importance of B lymphocytes as antigen presenting cells in the pathogenesis of T1D has been appreciated for decades, as demonstrated by the T1D resistance in mouse models deficient of B lymphocytes (4). Although the presence of autoantibodies is dispensable for T1D development, accumulating evidence supports the power of autoantibodies for predicting disease progression (6).

It has been revealed that highly mutated IgG autoantibodies are associated with high risks of progression to T1D (7), indicating autoreactive B lymphocytes that produce these antibodies have undergone affinity maturation. However, little is known regarding the role of affinity maturation in the development of T1D. A better understanding of the relationship between affinity maturation and disease progression can help identify potential targets for T1D prevention and reversal.

In contrast to T1D, SLE is a systemic autoimmune disease in which multiple organs are affected by the aberrant immune responses (8). It is a very remarkably heterogeneous disease with various clinical manifestations. Lupus nephritis is the most severe manifestation and is one of the leading causes of death in SLE patients (8). Current disease management mainly relies on high doses of corticosteroids with serious adverse events and an undesirable response rate (9). Significant progress in the development of immune intervention has been made in the past two decades with the approval of Belimumab, a monoclonal antibody to deplete B cells, in SLE patients (8). However, total B cell depletion is accompanied with an increased risk of infectious complications, which warrants further explorations to identify innovative strategies for immune intervention. While autoantibodies are not involved in the development of T1D, the pathogenicity of mutated IgG autoantibodies in the SLE development have been illustrated in several murine studies (10-12). For example, IgG anti-DNA antibodies derived from SLE patients were demonstrated to deposit in the kidney and induce proteinuria in SCID mice (10). Therefore, it would be of great value to investigate the effect of targeting affinity maturation, which is responsible for high affinity and the class switch to IgG, on the development of SLE.

In this dissertation, a comprehensive literature review on the pathogenesis, treatment and mouse models of T1D and SLE is provided in chapter II. Further discussion focuses on the modulation of T1D and SLE in mouse models through genetic and therapeutic approaches. In

Chapter III, we employed NOD mice as a model for T1D to examine the efficacy of genetically and therapeutically targeting affinity maturation in the development of T1D. Similarly, these approaches were assessed in lupus-prone mouse models, BXSB and MRL/*lpr* mice, in chapter IV. It is known that maternal factors can influence the development of neonatal immune system (13). However, little is known about their ability to impact the SLE development in the genetic susceptible offspring. The genetic knockout mice we established in chapter I provided an excellent model to evaluate the effect of maternal natural antibodies on the SLE development in the offspring. We sought to determine whether the absence of maternal antibodies would make a difference in the SLE development of the offspring in chapter V. Lastly, the chapter VI includes the implications of the findings and the possible directions for future investigations.

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Chapter II

Review of Literature**Introduction**

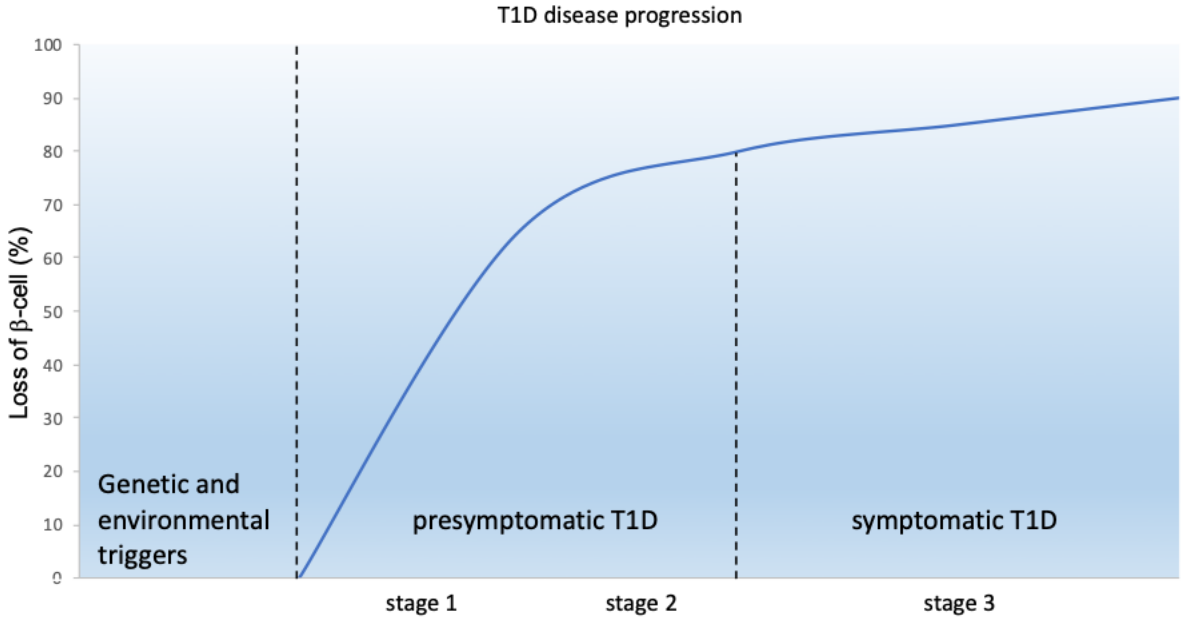
With the continual exposure to various microorganisms in daily life, the immune system is dedicated to the defense against invaders and the elimination of pathogenic antigens. The innate immune system responds rapidly to a wide range of antigens, while the adaptive immune system develops at a much slower rate for highly specific responses and immune memory to the encountered pathogens. An imbalance, however, of the immune system will result in two distinct areas of diseases: immunodeficiency and autoimmunity. Deficiency of the immune system, either a significant decrease of immune cell numbers or the impaired function of immune components, makes patients extremely vulnerable to infections. On the other hand, the breach of immune tolerance, also known as the inability to distinguish self-antigens from foreign antigens, leads to the generation of autoreactive lymphocytes attacking the body's own organs. It has been reported that around 3-5% of the population is affected by autoimmune diseases (1). There are more than 100 types of autoimmune diseases which can be divided into organ-specific ones such as type I diabetes (T1D) and systemic ones in which multiple organs are affected by aberrant immune responses, such as systemic lupus erythematosus (SLE) (1). Genome-wide association studies (GWAS) reveal numerous genetic susceptibility loci linked to each disease, while the discordance in twin studies indicate the involvement of various other factors such as environmental triggers and comorbidities (2-6). These other factors complicate research of autoimmune diseases. Nevertheless, advances in establishing animal models and developing novel technologies in gene editing as well as sophisticated assays enhance the comprehension of disease pathogenesis and improvements in therapeutic treatments. In this review, the main focus will be systemic lupus

erythematosus and type 1 diabetes. A detailed description of disease pathogenesis, murine models and treatments will be provided.

Type 1 Diabetes (T1D)

T1D is an organ specific autoimmune disease in which insulin-producing pancreatic beta cells are attacked by the infiltrating autoreactive $CD4^+$ and $CD8^+$ T cells. While intensive studies elucidated the pathogenic role of T cells in T1D development, the role of autoantibodies is less clear. Different autoantibodies are detected months or years before the onset of symptomatic T1D (7). Characteristic T1D autoantibodies include the ones against insulin, glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA2) and zinc transporter 8 (ZNT8), among which anti-insulin and/or anti-GAD are the first detected antibodies in most T1D patients (5). T1D is recognized as a continuum that progresses sequentially through three different stages (Figure 2.1), and the presence of two or more autoantibodies marks stage 1 of T1D. Stage 2 is defined as the appearance of both autoantibodies and hyperglycemia. While stage 1 and 2 are presymptomatic, stage 3 is characterized by the presence of symptoms including polyuria, thirst, hunger and weight loss.

The pancreas is the only organ targeted by the immune system in T1D, but this attack causes beta cell loss and insulin deficiency, resulting in hyperglycemia and subsequently complications affecting the retina, brain, kidneys and heart. For example, hyperglycemia results in oxidative stress and impaired blood flow, which stimulate the proliferation of retinal vessels as a compensation (5). However, the newly formed vessels are fragile and susceptible to hemorrhages and leakages of proteins into the retina, which may ultimately lead to blindness (5). These long-termed complications are the major contributors to mortality for T1D patients.



	stage 1	stage 2	stage 3
the presence of autoantibodies	present	present	present
β-cell loss	present	present	present
dysglycemia	absent	hyperglycemia	hyperglycemia
symptoms	absent	absent	present

FIGURE 2.1 T1D stages. The presence of autoantibodies precedes the presence of dysglycemia and marks the start of β cell loss in stage 1. Stage 2 is characterized by the detection of hyperglycemia. The appearance of symptoms in T1D patients is a hallmark of stage 3. (Adapted from Katsarou, A. et al. 2017. Type 1 diabetes mellitus. Nat Rev Dis Primers 3: 17016.)

Epidemiology

It has been estimated that more than one million people under the age of 20 years old suffer from T1D worldwide, with an increase of approximately 2-3% every year (6). Although the peak of disease detection is at or around puberty, more and more newly diagnosed cases are people older than 30 years of age, indicating that the incidence of T1D is underestimated (6). The incidence rate varies from country to country. It is highest among European countries such as Finland (more than 60 cases per 100,000 people every year) and Sardinia (around 40 cases per 100,000 people every

year), followed by North America and Australia (7). In contrast, T1D is a rare disease in Asian countries including China, South Korea and Japan with around 0.1 case per 100,000 people every year (7). This variation suggested the potential involvement of genetic susceptibility in T1D development. GWAS identified over 50 loci linking to T1D, among which the HLA region on chromosome 6 was proven to be the major genetic contributor to T1D (5). The HLA class II haplotypes *DRB1*0401-DQB1*0302* and *DRB1*0301-DQB1*0201* show the greatest susceptibility, whereas *DRB1*1501* and *DQA1*0102-DQB1*0602* are protective (7). These high-risk genotypes are more common in European countries than in Asian countries (5). In addition, it was reported that the development of T1D in Mexico is strongly associated with European HLA-DR-DQ genotypes (5). Collectively this evidence confirmed the contribution of genetic factors to T1D development.

Recently, accumulating evidence indicates that environmental factors also play a role in triggering T1D. The incidence rates of T1D are significantly different between neighboring countries even though they have similar frequency of high-risk genotypes (6). Moreover, the risk of developing T1D was increased for people, who migrated from a low-incidence area to a high-incidence area, and also for their offspring (5). Further studies in murine models suggest that potential environmental factors include diet, vitamin D intake, infections and gut microbiota. Gluten free diet, for example, significantly reduced the infiltration of T cells and decreased the T1D incidence in non-obese diabetic (NOD) mice, a classic mouse model of T1D, supporting a pathogenic role of gluten in inducing T1D (8). Vitamin D has been demonstrated to regulate the immune system by generating a tolerogenic environment. The administration of active vitamin D protected NOD mice from disease development, possibly by reducing effector T cells and increasing regulatory T cells (9). Infections with certain viruses were evidenced to induce T1D

development (10). In one virus-induced T1D mouse model, vaccination not only enhanced neutralizing antibody responses to the virus but also protected the mice from T1D (10). In consistence with the importance of gut microbiota in multiple other autoimmune diseases, the intervention of microbiota showed promising results in regulating T1D. For instance, the administration of probiotics such as *Bifidobacteria* and *Lactobacilli* significantly ameliorated insulitis in NOD mice (11). These environmental factors have been suggested to modulate immune responses and autoimmunity through epigenetic mechanisms (6). Precise details about these mechanisms, however, remain to be defined. Together, these findings support the significant contribution of numerous environmental factors in the progression of T1D.

For the past few decades, the management of T1D has mainly focused on the insulin therapy accompanied with close monitoring of the blood glucose levels. Various clinical trials showed that improvement in glycemic control by this therapy reduced hemoglobin A1c (HbA1c) level, which serves as an indicator of average blood glucose concentration for the past three months. Additionally, this therapy alleviated certain complications during the trial and even during the follow-up, indicating that the benefits of this therapy could last for years (5). However, there exist significant obstacles related to insulin therapy. For one, the continuous demands of daily treatments and monitoring the glucose level are burdens for patients physically, psychologically and financially. Further, disparities in insulin access is another obstacle for this therapy. While people in most western countries have adequate access to insulin, T1D patients in some parts of the world like sub-Saharan African countries would die due to lack of access to insulin (7).

One alternative to insulin therapy is immuno-intervention. In the past decade, the interest has grown in preventing the immune system's attack on beta cells and preserving the functions of residual beta cells in T1D patients. Monoclonal anti-CD3, anti-CD20, anti-IL-2, mammalian target

of rapamycin (mTOR) inhibitor and other immunosuppressive agents have shown promising results in phase I and II clinical trials (5). Unfortunately, none of them passed phase III due to either failure to meet primary endpoints or severe adverse events (5). It is crucial to gain a better understanding of T1D pathogenesis to optimally approach interventions for beta cell autoimmunity.

Roles of B lymphocytes in T1D

As it has been mentioned above, the destruction of beta cells is mediated by the autoreactive $CD4^+$ and $CD8^+$ T cells. However, the activation of these autoreactive T cells requires help from other cells, specifically APCs (Figure 2.2). With the presence of autoantibodies against islet cell-related antigens and the infiltration of B lymphocytes in the pancreas of diabetic mice, mounting studies have investigated the role of B lymphocytes in the pathogenesis of T1D. First, the Shultz and the Naji groups demonstrated the requirement of B lymphocytes in the initiation of T1D. They showed that the depletion of B lymphocytes by the genetic ablation of functional Igμ allele or the anti-μ treatment from birth both exerted a protective effect in NOD mice (12, 13). Further study revealed that autoantibodies secreted by B lymphocytes might be an epiphenomenon rather than a primary drive for the disease initiation, as the treatment with autoantibodies from overtly diabetic donors failed to abrogate T1D resistance in the B cell-deficient NOD mice (14). In addition, emerging evidence strongly supports the contribution of B lymphocytes as APCs to process and present islet antigens to autoreactive T cells (Figure 2.2). For example, blocking the antigen-presenting function of B lymphocytes *in vitro* significantly inhibited T cell responses to islet antigens (15). Also, the disruption of MHC I or MHC II on B lymphocytes in NOD mice successfully prevented T1D development (16, 17). Taken together, these findings support the importance of B lymphocytes as APCs in the pathogenesis of T1D.

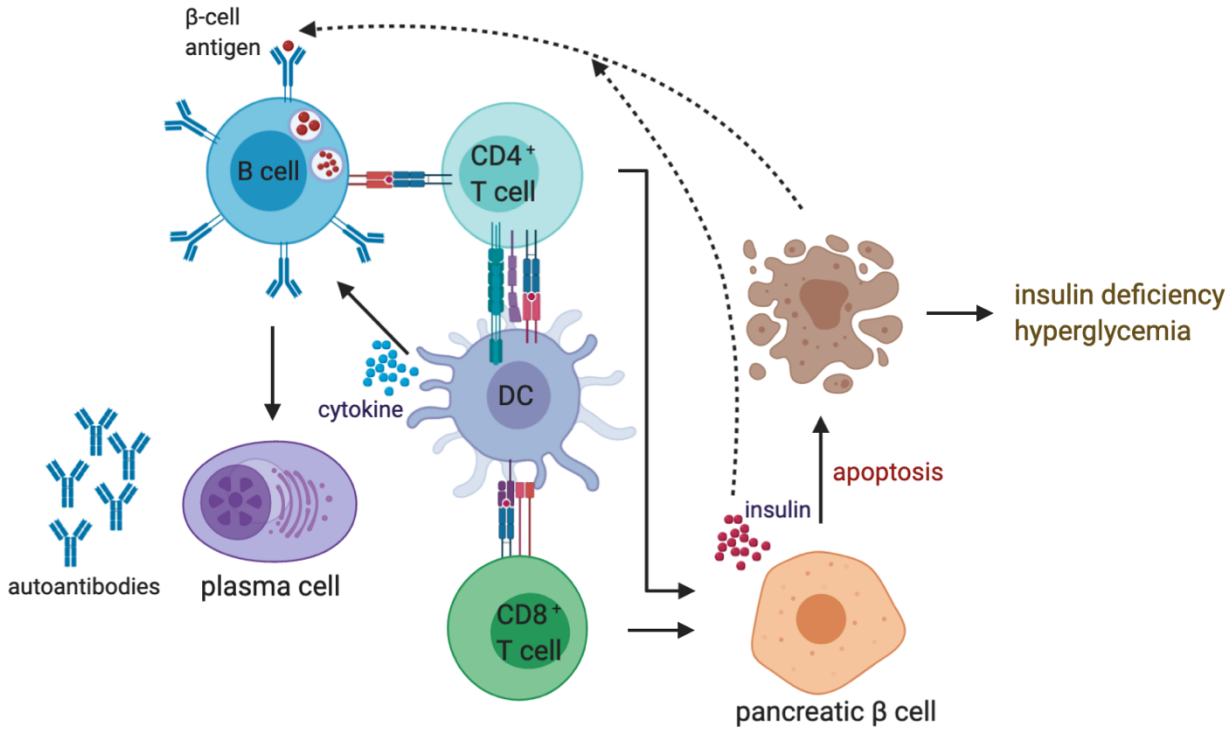


FIGURE 2.2 T1D pathogenesis. Autoreactive B lymphocytes, with the activating signals from T cells and DCs, differentiate into plasma cells and secrete autoantibodies. Antigen presentation by either DCs or B lymphocytes activates autoreactive T cells, leading to an attack on pancreatic beta cells and a deficiency in insulin secretion. The reduction of circulating insulin results in an elevated level of blood glucose, facilitating multiple complications. (Adapted from Katsarou, A. et al. 2017. Type 1 diabetes mellitus. Nat Rev Dis Primers 3: 17016.

As antigen presenting cells, B lymphocytes capture β cell antigens through the membrane-bound Ig molecules and internalize them for processing and subsequent presentation to autoreactive T cells (15). It is plausible that the specificity for β cell antigens is indispensable for the contribution of B lymphocytes to T1D. Indeed, the introduction of a transgenic Ig molecule restricts B lymphocyte repertoire to express antigen receptors specific for an irrelevant protein, hen egg lysozyme (HEL), and significantly retards the development of T1D in NOD mice (18). VH281.NOD mice which have a B lymphocyte repertoire with limited insulin binding potential are also resistant to the disease (19). In contrast, the introduction of VH125, shown to contain mutations necessary for insulin binding, skewed the B lymphocyte repertoire towards insulin and accelerated T1D development in NOD mice (19). The same group then investigated the antigen

presenting function of 125Tg B lymphocytes *in vitro* and showed that insulin-specific 125Tg B lymphocytes, though phenotypically anergic, were more efficient to activate T cells under conditions of transient exposure to antigens than naïve counterparts (20). Therefore, B lymphocyte specificity is important for their ability to activate autoreactive T cells and thus facilitating the pathogenesis of T1D.

Although circulating autoantibodies are not a major contributor to the disease development, numerous studies have recognized them as valuable predictors of disease progression. Currently, IgG autoantibodies with high affinity to β cell antigens are the most accurate predictors of the T1D development (21, 22). The presence of these autoantibodies indicates that B lymphocytes responsible for the production of these autoantibodies have undergone affinity maturation, including somatic hypermutation and class switch recombination. Activation-induced cytidine deaminase (AID) is an enzyme that is required for these two mechanisms (23). AID can deaminate cytidine to uridine on the single-strand DNA thus introducing mutations by replacing the uridine along with the adjacent nucleosides or generating single-strand breaks by removing the uridine (24). The single-strand breaks will be converted to double-strand breaks for subsequent class switching (24). Whether AID-mediated somatic hypermutation and class switch recombination contributes to the pathogenesis of T1D is poorly understood and warrants further explorations.

T1D mouse models

NOD strain

There are several different mouse models established for T1D research. The NOD strain, a spontaneous model, resembles human T1D in many aspects, leading to the wide use of this model in autoimmune research. A phenotyping study in the Jackson Laboratory showed that 90% of

female mice develop the disease by 30 weeks old, whereas male mice develop at a much slower rate, with only 52% of males being diabetic by 30 weeks old. As early as 3 weeks of age, the infiltration of DCs, macrophages and neutrophils was detected in the pancreas of NOD mice, followed by the infiltration of lymphocytes in the next one or two weeks (25). While $CD4^+$ and $CD8^+$ T cells are the dominant cell populations that penetrate the pancreas, it was noted that their migration to and retention in the islets were regulated by DCs and macrophages (25). MHC II, mostly expressed on APCs such as DCs and B cells, is the most important genetic factor contributing to T1D development in NOD mice. NOD mice express I-A^{g7} (MHC II haplotype) but not I-E. The substitution of one amino acid to change into a more common polymorphism or the expression of I-E prevented NOD mice from developing the disease (26). There are many other genetic loci that were demonstrated to be crucial in the T1D development, but they will not be elucidated in detail here. The incidence rate of T1D in NOD mice varies from facility to facility (27), suggesting the involvement of other factors such as gut microbiota in disease pathogenesis. The integration of various factors in T1D pathogenesis hampers the identification of underlying mechanisms and the development of innovative therapies for autoimmune diabetes.

Other T1D mouse models

Another model used for T1D is the double transgenic strain, characterized by the expression of an antigen X specifically on beta cells along with the expression of a TCR specific for this antigen X. Currently, the established models include ovalbumin (OVA), influenza haemagglutinin (HA) and lymphocytic choriomeningitis virus glycoprotein (LCMV-GP). In the OVA model, beta cells in the pancreas express OVA, and T cells with transgenic TCR specific for OVA react with beta cells and attack them. These double transgenic mice develop diabetes by 10

weeks of age, much faster than NOD mice (27). In addition, the transgenic TCR simplify the research with easy access to the antibody which recognizes the specific T cells.

The induced model is also available for T1D, which is partially similar to double transgenic model. In the induced model, only LCMV-GP is introduced under the control of an insulin promoter for the specific expression in pancreatic beta cells (27). The initiation of T1D requires the infection with the virus to induce the generation of LCMV-GP-specific T cells, recognizing both virus-infected cells and pancreatic beta cells. The destruction of islets normally occurs within 1-2 weeks post infections. The flexible time frame and rapid immune responses make this system a great tool for prevention research.

Aim of the T1D study

With the critical role of B lymphocytes in the pathogenesis of T1D, B lymphocyte depletion therapies have been assessed by multiple groups. Indeed, the depletion of B lymphocytes by either anti-CD20 or anti-BAFF treatment from an early age led to T1D resistance in NOD mice (28-30). However, the protective effect did not last after the first year of anti-CD20 transient treatment in newly onset T1D patients (31, 32). Another limitation of anti-CD20 treatment is that these antibodies target not only autoreactive B lymphocytes but also normal B lymphocytes, resulting in the loss of humoral responses and high risks of infections in patients. It will be of great significance to investigate the contribution of affinity maturation in activated B lymphocyte to progression of T1D; this investigation would promote the development of more specific therapies for T1D.

In Chapter III, we targeted AID or the related pathway to address the importance of affinity maturation in the T1D development of NOD mice. Genetic ablation of AID significantly inhibited

the progression to overt T1D in NOD mice, despite few changes in lymphocyte infiltration and comparable insulitis. 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid, also termed DIDS, is an inhibitor of the RAD51 complex, which is the core factor in homologous recombination pathway for double-strand break (DSB) repair. Our collaborator showed that DIDS treatment can result in the failure of DSB repair and subsequent cell apoptosis. In our study, the administration of DIDS remarkably retarded the development of T1D, even when the treatment was started at a stage with the presence of certain β cell autoimmunity. Interestingly, both approaches induced the expansion of $CD73^+$ B lymphocytes which exerted a regulatory role in inhibiting T cell functions. This work adds to our understanding of the disease pathogenesis, allowing for the further development of novel therapies that are more specific but still potent for T1D interventions.

Systemic lupus erythematosus

SLE is a complex autoimmune disease with diverse clinical symptoms. In addition to the well-known butterfly rash on the cheek, SLE patients can also experience severe fatigue, joint pain, joint swelling, headaches and anemia depending on which part of the body is affected. It is also a remarkably heterogeneous disease and different patients can show relatively distinct manifestations (33). For example, some patients predominantly develop skin rashes and do not have kidney involvement, while other patients demonstrate severe kidney inflammation with little sign of skin rashes. Detailed criteria have been established to facilitate the diagnosis and classification of SLE (34). However, this heterogeneity remains to be the top barrier for disease management. Among all the SLE complications, renal dysfunction, also known as lupus nephritis (LN), is the most severe one and is present in over 50% of SLE patients (35). Kidneys are a key regulator to control blood pressure, electrolyte balance and red blood cell production. Abnormal

kidney function leads to proteinuria, edema, high blood pressure and, most severely, end-stage renal disease (36). Kidney failure has been indicated as one of the leading causes of death in SLE patients (37).

SLE incidence

SLE affects 1.5 million people in the United States and at least 5 million people in the world (38). Although SLE can affect both men and women of all races and ages, it does have a strong preference for women of child-bearing age and African Americans (2). The female to male ratio ranges from 2:1 to 15:1 depending on the age group (39-41). The female predominance is greatest during peak reproductive years and declines towards both extremes of age (39). Several explanations were proposed to illustrate the female predominance of the disease. Evidence shows a pathogenic role for estrogens and a protective role of androgens in regulating SLE development (41, 42). In addition, the prevalence of SLE in men with Klinefelter's syndrome, which is characterized by the extra copy of X chromosome in males, is increased 14 fold compared with the prevalence in men with normal karyotypes, which leads to a similar prevalence with women (43). This implicates the X chromosome gene-dose effect as a potential contributor to the SLE susceptibility. The incidence of SLE also varies in different ethnic groups. In one recent study conducted in the United States, the incidence of SLE was found to be highest among African Americans (31.19 per 100,000 person-years) and Native Americans (30.01 per 100,000 person-years) (40). Hispanic people have a relatively lower incidence rate (22.21 per 100,000 person-years). The incidence was reported to be lowest for Caucasian people (17.96 per 100,000 person-years) and Asian people (16.68 per 100,000 person-years). While recent GWAS identify numerous genes involved in the pathogenesis of SLE (3, 44), increasing evidence suggests that SLE is likely

a result from an interaction between genetic predisposition and environmental factors. Monozygotic twins only showed a concordance rate of 22-40% for SLE (45-47). Furthermore, cigarette smoking, alcohol consumption, diet and infection with certain viruses have been linked to the development of SLE (47). Epigenetic regulation was proposed to be a possible mechanism by which these environmental risk factors contribute to the disease (4). The wide range of diverse factors involved in the pathogenesis of SLE has challenged research on a more comprehensive understanding of the disease and the advancement of therapies.

SLE pathogenesis

The innate immune system in the pathogenesis of SLE

SLE is characterized by the breakdown of tolerance to self-antigens, especially nuclear components such as double stranded DNA (dsDNA), histones and nucleosomes (2). Both innate and adaptive immune system dysregulation have been implicated as important contributors to the pathogenesis of SLE (Figure 2.3). The innate immune system is the first line of defense when encountering pathogens and thus, it is necessary for rapid response to a wide range of invaders. Toll-like receptors (TLRs) are one type of pattern recognition receptors that the innate immune system utilizes to help achieve the immediate reaction. They recognize structurally conserved molecules shared by microbes with 13 TLRs identified up to date, among which TLR1-10 have been demonstrated to be expressed in humans (48). GWAS suggest that TLR7 and TLR9 are strongly associated with disease susceptibility (49). The binding of single-stranded RNA and CpG-enriched DNA, self-antigens which are reported to be more numerous in SLE patients, to TLR7 and TLR9 respectively induces the activation of downstream signaling pathways and ultimately increases the production of type I interferon (IFN) (48, 50).

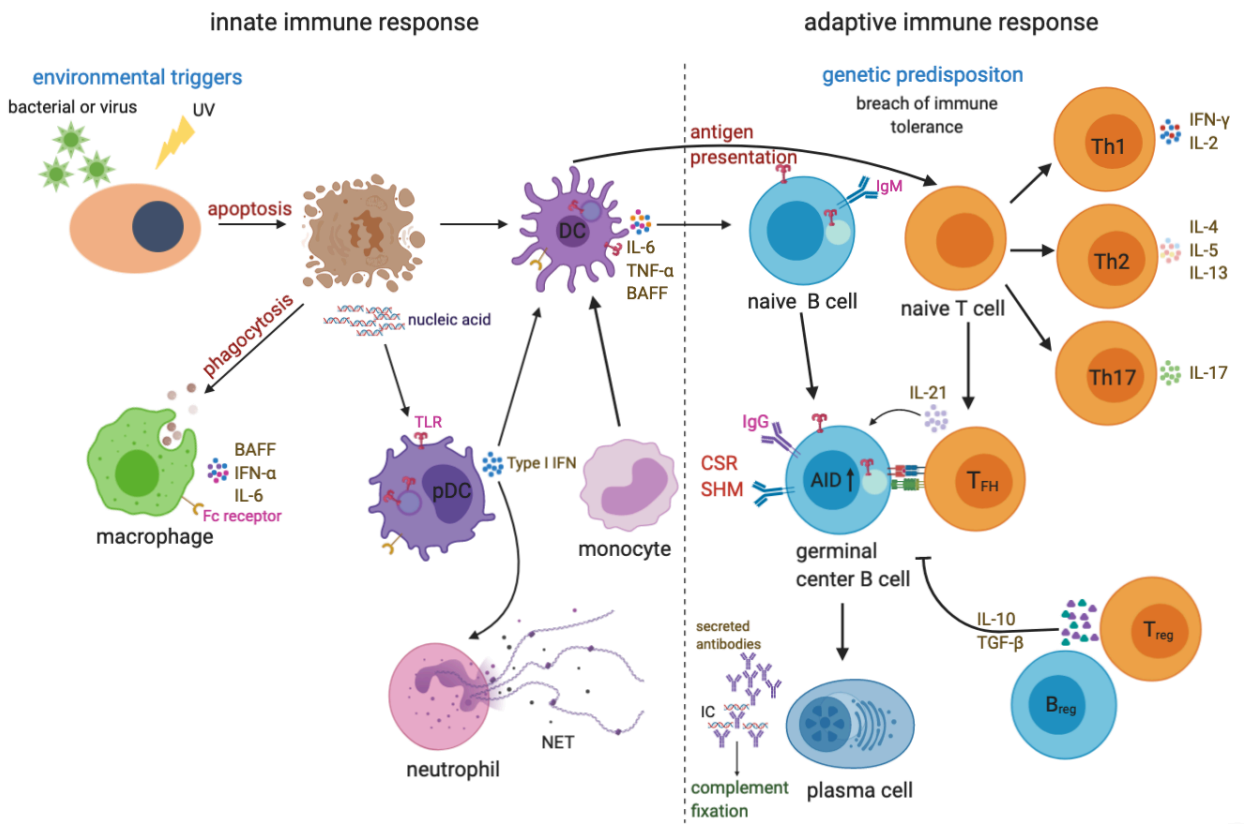


FIGURE 2.3. Both innate and adaptive immune system are involved in the pathogenesis of SLE. Increased cell apoptosis by environmental triggers or deficiency in removal of cell debris by phagocytes results in activation of antigen presenting cells such as dendritic cells (DCs) leading to the production of proinflammatory cytokines. Autoreactive B lymphocytes activated by self-antigens and cytokines, with the help of activated autoreactive T cells, differentiate into antibody-producing plasma cells. The immune complexes formed by autoantibodies and self-antigens initiate complement fixation. The production of proinflammatory cytokines and the activation of several effector mechanisms ultimately cause organ damage.

The increased serum level of type I IFN and the identification of numerous type I IFN-induced gene transcripts in peripheral blood mononuclear cells and damaged tissues, also known as the “IFN signature”, from SLE patients (51-55) suggested type I IFN as a crucial mediator in the disease. Accumulation of plasmacytoid dendritic cells (pDC), the main producers of type I IFN, were found to infiltrate the kidney in patients with active LN (56, 57). Research groups showed that type I IFN can stimulate human monocytes to differentiate into dendritic cells (DCs) *in vitro* and enhance the antigen presenting function by increasing the expression of major histocompatibility complex (MHC) I and MHC II on DCs (55, 58). Furthermore, type I IFN

treatment significantly induces the production of B-cell activating factor (BAFF) by monocytes and neutrophils, which can promote B cell differentiation and antibody secretion (59). It was also reported that the addition of type I IFN *in vitro* decreased the number and suppressed the function of natural regulatory T (T_{reg}) cells isolated from lupus patients (60). All these findings demonstrate the pathogenic roles of type I IFN in SLE development.

The complement system is also involved in the pathogenesis of SLE. While mounting evidence support the pathogenic role of aberrant complement cascade activation in tissue inflammation and damages (61-63), the classical pathway plays a protective role since defects in early components of classical pathway were strongly associated with the development of SLE (64-66). These paradoxical results are explained by the ability of classical pathway to remove apoptotic debris (67), which are a primary source of self-antigens. Thus, caution is warranted for therapies targeting the complement pathways.

The roles of B cells in SLE

The importance of the adaptive immune system in SLE pathogenesis has been recognized for decades. While a well-known role of B cells is to produce specific antibodies against pathogens in the immune responses, they also contribute to SLE by secreting autoantibodies against self-antigens, especially nuclear components (68). High titers of autoreactive antibodies are a defining characteristic of SLE and have been linked to the progression of the disease (69). These autoantibodies are crucial contributors to the occurrence of tissue damage (70). For example, autoantibodies can form immune complexes (ICs) with autoantigens and the deposition of ICs in the kidney are positively related to LN (71). Activation of the complement system and effector cells by ICs leads to the formation of membrane attack complexes, upregulation of

proinflammatory cytokines and recruitment of leucocytes, all of which facilitate nephritis (35, 72). An *in vivo* murine study also showed that human anti-DNA antibodies passively transferred into the mice can deposit in glomeruli, inducing kidney inflammation and proteinuria (70). In addition to aberrant inflammation following glomerular deposition, recent studies have shown that autoantibodies can cross-react with other self-antigens and mimic their functions, resulting in aberrant signaling. It has been demonstrated that anti-DNA antibodies can bind to receptors on neurons and lead to excessive excitation-induced neuronal death independent of complement-mediated cytotoxicity, facilitating damage in the central nervous system (73).

Some recent advances in murine SLE models have shed light on another role of B cells: presentation of antigens to T cells, and its contribution to the pathogenesis of SLE. Lupus-prone mice deficient in antibody production but intact in other functions of B cells show comparable T cell activation and severe cellular infiltration in the kidney, indicating B cells can contribute to the disease in an antibody-independent way (74). The capacity of B cells to bind low concentration antigens suggests the potential for them to act as potent antigen presenting cells (APCs) and activate T cells with a limited presence of autoantigens (75). Indeed, B cell-specific deletion of MHC II, a molecule important for antigen presentation, in SLE mouse model inhibited the production of autoantibodies, reduced the percentage of activated T cells and ameliorated nephritis (76). This study illustrates a nonredundant role of antigen presentation by B cells in SLE pathogenesis.

Besides the above pathogenic roles in driving the development of the disease, a small population of B cells does negatively regulate SLE. These B lymphocytes are called regulatory B (B_{reg}) cells and are characterized by the production of anti-inflammatory cytokines such as interleukin 10 (IL-10) (77). The proliferation of T cells upon stimulation *in vitro* was significantly

inhibited in the presence of human B_{reg} cells (78). In addition, when activated $CD4^+$ T cells were co-cultured with human B_{reg} cells, their ability to secrete inflammatory cytokine was compromised in a manner dependent on a combination of IL-10, CD80 and CD86. (79). Another study demonstrates that B_{reg} cells suppress Type I IFN production by pDCs in an IL-10 dependent mechanism (80). Interestingly, various groups show an elevated level of circulating B_{reg} cells in SLE patients (77). Further studies explained that these increased B_{reg} cells from SLE patients produced less IL-10 and were less efficient in exerting immunosuppressive functions (78-80).

The involvement of T cells in SLE

SLE has been well-known as a mainly B cell-mediated autoimmune disease. However, the importance of T cells in the development of SLE has been increasingly recognized. $CD4^+$ T cells are the most potent drivers for B cell differentiation and antibody production. In the germinal center where B cells are educated for affinity maturation, there is a special subset of T cells, called follicular helper (T_{FH}) T cells. They consistently select and provide survival signals to B lymphocytes with high affinity by interaction between co-stimulatory molecules (CD40/CD40 ligand) and the production of IL-21 (81, 82). A remarkable increase of T cells with T_{FH}-like phenotype was shown in peripheral blood from SLE patients in various studies (83-85). Moreover, the upregulated expression of CD40 ligand (CD40L) upon stimulation of T cells isolated from patients with SLE was sustained for a longer period compared with that of T cells isolated from healthy controls, facilitating excessive help for B cell proliferation and differentiation (86).

Besides T_{FH} cells, intensive research also show evidence for the involvement of T_{reg} cells and T helper 17 (Th17) cells in the SLE pathogenesis. T_{reg} cells can regulate self-tolerance and suppress immune responses through the surface checkpoint molecule, cytotoxic T-lymphocyte-

associated protein 4 (CTLA4), and the secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β) (87). Fewer $CD4^+$ T_{reg} cells are detected in patients with active SLE, possibly due to a favored microenvironment for effector T cells over T_{reg} cells (88). The importance of IL-2 in the maintenance of T_{reg} cells has been appreciated by the finding that administration of low doses of IL-2 in SLE patients showed an expansion of T_{reg} cells and an alleviation of the disease (89). Th17 cells are characterized by the production of IL-17, a pro-inflammatory cytokine. Several studies showed an increased frequency of circulating Th17 cells, an elevated level of IL-17 in peripheral blood and an infiltration of Th17 cells in damaged organs from SLE patients (81). However, conflicting results for the pathogenicity of Th17 cells are documented partially due to their heterogeneity. Human Th17 cells show the capacity to secrete IL-10 *in vitro* under certain conditions (90), indicating the potential suppressive role of these cells. Further investigations are warranted for a more comprehensive understanding of Th17 cells.

SLE treatment

While intensive studies focusing on SLE have led to numerous breakthroughs and a more profound knowledge of the disease pathogenesis, advances in developing new SLE treatments have been rather slow. In the 1950s, treatment with high doses of corticosteroids was identified to improve SLE, instating this treatment as the standard-of-care for SLE patients. This therapy increased the survival rate strikingly from 17% at 5 years to 55% at 5 years (91). Then, in the 1970s, the combination of cytotoxic agents such as cyclophosphamide and azathioprine with corticosteroids further improved the survival rate to 80% at 5 years, however long-term high-dose immunosuppressive treatment has serious adverse events and patients still develop renal failure (91). Progress in disease management has been hampered since then with few alternative therapies

providing superior benefits. In the past two decades, mounting evidence supports the efficacy of B cell depletion therapies in SLE patients (92). Remarkably, Belimumab, a monoclonal antibody inhibiting the binding of BAFF to B cells, was the first drug approved by FDA for SLE patients in more than 50 years. Certain patients, however, were not included in the clinical trials that proved the efficacy of Belimumab, specifically those with active LN. Thus, many groups have been focusing on examining the efficacy of B cell depletion therapies specifically in SLE patients with active LN. Several small uncontrolled trials of B cell depletion therapies in LN patients exhibited high response rates and promising benefits (93-95). Unfortunately, two randomized controlled trials assessing the efficacy of Rituximab and Ocrelizumab, anti-CD20 monoclonal antibodies depleting B cells, in treating active LN failed to meet the primary end point, showing no superiority to placebo in response rates (96, 97). The ongoing phase 3 study of Belimumab, however, conducted by GlaxoSmithKline (GSK) in active LN patients announced its achievement of the primary endpoint and all major second endpoints in December 2019. This breakthrough provides great promise for LN patients with inadequate responses to the conventional treatment.

Targeting of other immune cells or cytokines also exhibit beneficial effects for SLE patients. Immunization with inactivated autoreactive T cells in SLE patients induced the reaction against specific autoreactive T cells and significantly decreased SLE disease activity index (SLEDAI) scores (98). Ustekinuma, targeting IL-12/23 which is the upstream of IL-17, was evaluated in SLE patients and showed strikingly improvement of anti-dsDNA, complement component 3 (C3) levels and SLEDAI scores (99). In a small clinical trial, the treatment with Infliximab, a tumor necrosis factor alpha (TNF-α) blockade, showed remarkably long-term improvement on the LN in over 50% of treated patients (100). Regardless of these very promising results, most agents failed to meet the primary endpoint in further clinical trials with improved

designs and larger sample sizes. The heterogeneity of SLE patients remains a substantial roadblock to advancing novel therapies.

SLE murine models

GWAS and analysis of peripheral blood and biopsy samples from SLE patients provides significant breakthroughs and advances for a deeper insight of SLE pathogenesis. However, there are many limitations in treatments and use of novel technologies when human subjects are involved in the research. Heterogeneity in SLE patients is another significant hurdle for improvement in clinical research. SLE mouse models serve as an excellent tool for further investigations to understand the cellular and molecular changes in SLE. On average, the protein-coding regions of mouse genomes are 85% identical to that of human genomes (101). Mice are small, have a short lifespan and breed relatively well, all of which make them an economical choice. The brother-sister mating system also significantly limits the genetic diversity in inbred mouse strains. More importantly, with the current knowledge and technologies, researchers are capable of easily manipulating mouse genomes to target specific mechanisms underlying disease pathogenesis.

There are multiple murine models that have been utilized in SLE studies, including NZB/W, MRL/*lpr* and BXSB/MpJ strains. While they all develop SLE spontaneously, each has their unique disease characteristics and genetic background for disease development. There is another pristane-induced mouse model that has been commonly employed in some groups. This thesis mainly focuses on BXSB/MpJ strain, illustrating the genetic background, disease signatures and important research advancements in SLE pathogenesis of this mouse strain.

BXSB/MpJ mouse model

The BXSB/MpJ (henceforth referred to as "BXSB") mouse strain was a recombinant inbred strain, first discovered and characterized by Murphy and Roths in 1978 (102). The strain originated from a backcross of C57BL6/J (B6) to SB/Le mice. The mice began developing a spontaneous SLE-like autoimmune disease and have since been utilized in many studies for deciphering the disease. While females and males both spontaneously develop lupus-like syndrome, the onset of the male, in contrast to other lupus-prone strains, is strikingly earlier than the onset of the female. The average survival is about 5 months for males and 14 months for females (103). The disease hallmarks for BXSB strain include splenomegaly, lymphadenopathy, monocytosis, marginal zone B cell depletion, high levels of IgG antibodies, especially anti-nuclear antibodies (ANA), and IC glomerulonephritis.

The Y-linked autoimmune accelerator (Yaa) mutation

The fact that the disease is accelerated in males suggests that an element on Y chromosome or sex hormone might be highly involved in the SLE development. However, further studies excluded the indispensable role of sex hormone in regulating SLE. Castration of both BXSB males and females did not show any effect on the progression of the disease compared with sham-operated controls (104). Furthermore, transfer of male or female bone marrow cells into lethally irradiated BXSB mice of either sex illustrated that only the sex of donor cells was important to determine the rate of disease development (105). Recipients, both males and females, receiving male cells developed the disease rapidly, whereas recipients of female cells had the disease in a much slower rate (105). The transfer of spleen cells showed consistent results with bone marrow chimera study, supporting that SLE development is not hormonally mediated in BXSB strain (105).

In addition, consomic mouse strains carrying BXSB-derived Y chromosome with MRL or NZW backgrounds, both of which parallel sex preference in human and exhibit rapid progressive disease in females, showed exacerbated SLE development in male mice (103). An element, known as Y-linked autoimmune accelerator (*Yaa*), was determined to contribute to the acute disease development in BXSB males.

The *Yaa* on the Y chromosome of BXSB male mice is due to a translocation of an approximately 4Mbp segment of the X chromosome onto Y chromosome near the pseudoautosomal region, leading to the duplication of at least 19 genes and around 2-fold expression of several genes in BXSB males (Figure 2.4) (103). Among these genes, *Tlr7* was found to be the major candidate for the autoimmune phenotype development in BXSB mice. TLR7 is a pattern recognition receptor expressed on B cells and APCs. The binding of its ligand, single stranded RNA, to TLR7 activates the cells via a MyD88-dependent pathway leading to the activation of nuclear factor (NF)-κB signaling and the expression of proinflammatory cytokines such as type I IFN (103).

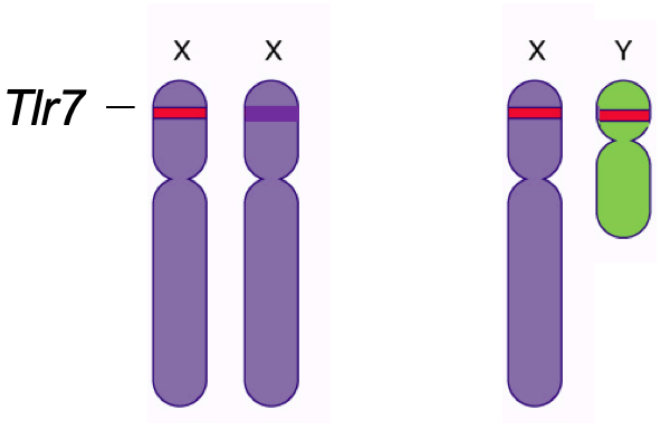


FIGURE 2.4 Sex chromosomes of BXSB mice. Female mice only have one copy of the segment, including *Tlr7* gene, due to one X chromosome inactivation. Male mice have two active copies of the segment as a result of the translocation.

Numerous studies demonstrated that disease activity is highly correlated to TLR7 expression. The disruption of TLR3, 7 and 9 signaling via depleting the protein required for the trafficking of these receptors to bind to their ligands significantly reduced the production of anti-nuclear IgG antibodies, suppressed lymphoproliferation with remarkable decreases in splenomegaly and lymphadenopathy, and increased survival rate in BXSB mice (106). Moreover, reduction of *Tlr7* gene dosage to one copy either by *Tlr7* null mutation on X chromosome or breeding *Yaa* males to *Tlr7* knockout females abolished the *Yaa* phenotype (107, 108). One transgenic mouse study elaborated that an increase of 4-8 fold of Tlr7 expression on the B6 background was sufficient to induce phenotypes shown in BXSB mice, including marginal zone B cell depletion, B and T lymphocyte hyperactivation, DC expansion, high autoantibody secretion and glomerulonephritis (107). Collectively this evidence implicates the *Tlr7* gene as a crucial contributor for increased lupus susceptibility in BXSB male mice.

While the *Yaa* mutation is the most characterized hallmark in BXSB mouse model, it requires combination with other susceptibility alleles for the development of overt disease. This is supported by studies of consomic mice carrying BXSB Y chromosome with none autoimmune backgrounds, such as B6 and CBA mice, and with autoimmune backgrounds, such as MRL and NZW mice (103, 109, 110). Y chromosome alone was not able to initiate overt SLE development in nonautoimmune strains, and mice only develop autoimmune syndrome in the presence of other autoimmune susceptibility genes (109). Furthermore, the MHC H-2 haplotype was proven to be important in SLE pathogenesis of BXSB mice. When BXSB (H-2^b) males were backcrossed to create H-2^d BXSB males, these congenic mice exhibited a much less severe autoimmune phenotype, which was similar to that of BXSB females with either H-2^b or H-2^d haplotype (111). In contrast, MHC class I family proteins exerted a suppressive role in SLE pathogenesis. BXSB

mice deficient in beta-2-microglobulin (B2M), a molecule required for the expression of most MHC class I family proteins, exhibited an acceleration of disease development (112). Other non-MHC-linked autosomal genes in BXSB genome have been investigated and suggested to contribute to the disease activity. Several susceptibility loci have been identified on 4 different chromosomes in quantitative trait linkages analysis of BXSB males. *Bxs1-3* on chromosome 1 were associated with autoantibody production, glomerulonephritis and splenomegaly, while *Bxs4* on the same chromosome was linked to lymphadenopathy (113). Another locus that is linked to lymphadenopathy is located on chromosome 4 and overlaps with the one found in NZW mice, termed *Sles2* (113). *Bxs*5 on chromosome 3 and *Bxs6* on chromosome 13 were also associated with different SLE traits (113). The identification of these susceptibility alleles confirmed that the autoimmune syndrome developed in BXSB males is a result of collaboration between *Yaa* mutation and many other autosomal regions.

Marginal zone B cell reduction

The loss of marginal zone B cells is another hallmark of BXSB strain. The marginal zone (MZ) is a border interposed between red pulp and white pulp. There are various types of cells locating in MZ, including specific B lymphocytes, macrophages and DCs (114). With the blood passing through MZ at a slow rate, MZ cells interact with circulating antigens in the blood and readily initiate immune responses if needed (114). MZ B cells are known to immediately respond to blood-borne antigens, both T cell-dependent and T cell-independent, and differentiate into antibody-producing cells (114). Thus, they are also called “innate-like” lymphocytes that integrate features of innate and adaptive immunity.

While mounting studies have investigated the mechanisms underlying the significant reduction of MZ B cells in BXSB strain, no definite conclusions have been drawn in the literature. There are three possible but not exclusive explanations: 1) the change in B cell receptor (BCR) signaling favoring differentiation into follicular (FO) B cells, which share the same precursors with MZ B cells; 2) the disturbed maintenance of MZ B cells; 3) migrating out of MZ due to activation. It is well-established that the FO versus MZ B cell fate choice is finely regulated by the integration of the strength of BCR signaling and other factors such as neurogenic locus notch homolog protein 2 (Notch2) signaling and the canonical NF-κB pathway (115). The defect in proteins involved in the downstream of BCR signaling led to a remarkable decrease of FO B cells, and the mutations in the protein that functions as a negative regulator of BCR resulted in a significant reduction in MZ B cells. Both demonstrated strong BCR signaling induces differentiation into FO B cells, while weak BCR signaling favors development of MZ B cells (115). Interestingly, BXSB B cells showed a hyperreactive phenotype, possibly through a lower expression of CD21 and a decreased threshold for BCR signaling (116). This finding suggests that the change in BCR signaling in BXSB B cells favors maturation towards FO B cells and contributes to the reduction of MZ B cells. The signaling for B cell retention in the MZ is also crucial for MZ B cell development. One study illustrated that the interaction between macrophage receptor with collagenous structure (MACRO) expressed on MZ macrophages and an undefined receptor on MZ B cells is required to maintain MZ B cells in the MZ (117). The decreased expression of MACRO on macrophages at both the mRNA and protein level has been reported in BXSB strain (118). Thus, it could potentially explain the loss of MZ B cells by the decreased retention signaling due to limited interaction between the MZ macrophages and MZ B cells. DCs are another cell type located in MZ and researchers highlighted their importance in regulating MZ B cells. Loss of DCs by impairing their development restored

the MZ B cell population in B6.*Yaa* mice, and expansion of DCs resulted in the depletion of MZ B cells in B6 mice (119). It was reported that DCs can transfer antigens to naïve B cells and induce B cell activation for antibody production both *in vitro* and *in vivo* (120). These findings indicate that enhanced activation and differentiation of MZ B cells by DCs might be responsible for the loss of MZ B cells in *Yaa* mice. In conclusion, the mechanisms proposed above and other undetermined mechanisms might work exclusively or collaboratively to contribute to the reduction of B cells in the MZ compartment of BXSB males. Further investigations are warranted for a more profound insight of MZ B cell development in BXSB strain.

The germinal center and extrafollicular responses

Spontaneous germinal center (GC) development is one of the characteristics of autoimmune mouse strains. However, the GC structure is disrupted in BXSB males. In contrast to the compact and organized center of highly proliferating B cells in other autoimmune strains, germinal center B (GCB) cells in BXSB males are dispersed and scattered (121). It is well known that GCs are the main sites where B cells differentiate into antibody-producing plasma cells. Thus, the presence of high levels of IgG antibodies in BXSB males despite disorganized GCs indicates that organized GC structure might not be required for plasma cell differentiation and/or an extrafollicular response occurs to contribute to the production of pathogenic autoantibodies (122). Sections of the spleen from BXSB males showed the localization of plasma cells outside follicles (112), suggesting the existence of extrafollicular responses in BXSB mice. However, whether confined GC structure is indispensable for the differentiation into plasma cells remains unclear and warrants future explorations.

Monocytosis

Monocytosis, i.e. the expansion of monocytes in the spleen, is also a characteristic of BXSB males. There are two functionally and phenotypically distinct subtypes of circulating monocytes in mice and it was shown that only Gr-1^{-}CD62L^{-}CD11b^{+}CD11c^{+} monocytes were expanded in BXSB males (123). Monocytosis appears as early as 8 weeks of age in BXSB males (124). While *Yaa* mutation can induce autoantibody production and glomerulonephritis alone, monocytosis requires combining *Yaa* with the *Bxs*3 locus (125). In addition, irradiated mice reconstituted with both *Yaa* bone marrow cells and non-*Yaa* bone marrow cells revealed a similar contribution of *Yaa* and non-*Yaa* monocytes to monocytosis (123). This indicates that *Yaa*-associated monocytosis is not a consequence of intrinsic abnormality in monocytes.

Th1 vs. Th2 responses

Both Th1 and Th2 responses have been involved in the pathogenesis of SLE. While it is well-accepted that the Th1 response, characterized by IFN-γ production, induces the inflammatory response and accelerates disease in multiple different murine SLE models, the role of the Th2 response in SLE pathogenesis remains to be elucidated due to conflicting results in different studies (126). Research has suggested the BXSB strain primarily develops a Th1-mediated autoimmune disease. For example, higher secretion of IgG2a and IgG3 over IgG1 and the elevated production of IFN-γ are detected in BXSB males as early as 10 weeks of age (127, 128). Furthermore, the deficiency of IL-4, a signature cytokine of the Th2 response, in BXSB mice did not show significant changes in mortality rate, nephritis severity or autoantibody production (126), indicating that Th2 cells might not play a critical role in BXSB SLE development. Nevertheless, some cytokines not designated into either Th1 or Th2 profiles have also been demonstrated as

critical to the disease pathogenesis. For instance, IL-21 is a potent cytokine that regulates the adaptive immune system by activating B cells, inducing plasma cell differentiation, shifting B cells to a regulatory status, driving T_{FH} cell differentiation and suppressing the functions of T_{reg} cells (122). It was reported that BXSB mice lacking the IL-21 receptor specifically on B cells were resistant to SLE development, while the removal of the IL-21 receptor on $CD8^+$ T cells accelerated the disease (122), suggesting a complex role for IL-21 in SLE. Thus, SLE is a complicated autoimmune disease with different cell types and cytokines acting in a cooperative and interactive manner and it would be overly simplistic to define it as a solely Th1 or Th2 type response.

Other SLE mouse models

MRL/lpr mouse model

MRL/*lpr* strain is another classical SLE model and has been widely employed in research. In contrast to the BXSB strain, both MRL/*lpr* females and males are strikingly affected by the SLE-like syndromes, with an average lifespan of 17 weeks for females and 22 weeks for males (129). This strain develops systemic autoimmunity, including a high concentration of anti-dsDNA antibodies, enormous lymphadenopathy associated with double negative ($CD4^-CD8^-$) T cells, arthritis and IC glomerulonephritis (103). These exacerbated phenotypes are attributed to a homozygous mutation, termed lymphoproliferation (*lpr*), on chromosome 19 which leads to a defect in the transcription of the Fas receptor (103).

The Fas receptor (CD95) is a surface-bound receptor ubiquitously expressed in numerous tissues and interaction with its ligand, Fas ligand (FasL), induce programmed cell death (130). FasL is primarily expressed on activated T cells and natural killer cells (130). Apoptosis induced by Fas-FasL plays an important role in peripheral tolerance by depleting circulating autoreactive

lymphocytes (130). The lack of Fas receptor expression leads to a defect in apoptosis and thus an accumulation of pathogenic autoreactive lymphocytes followed by progressive autoimmune syndromes in MRL/*lpr* mice. Interestingly, the introduction of *lpr* mutation fails to induce overt SLE symptoms not only in B6 mice, an autoimmune resistant strain, but also in C3H/HeJ mice, an autoimmune sensitive strain (131), indicating the involvement of other genes on the MRL background in the SLE development. Four susceptibility loci, *Lmb1- Lmb4*, have been identified and linked to the increase of anti-dsDNA antibody production and glomerulonephritis (103). Several genes located within *Lmb4* locus were proved to regulate lymphocyte proliferation and apoptosis (103). Further investigations will be needed to reveal more candidate genes in the MRL background. These studies will provide a clearer picture of the SLE development in this strain and make advances in developing novel therapies.

NZB/W F1 mouse model

NZB/W F1 strain is the oldest classical mouse SLE model, first described in the early 1960s (132). They are a hybrid of NZB and NZW mice. While both NZB and NZW strains show limited autoimmunity, NZB/W F1 mice develop severe autoimmune syndromes resembling human SLE patients, including lymphadenopathy, splenomegaly, a high level of circulating ANA, and IC-mediated glomerulonephritis (133). Consistent with human SLE, the incidence of SLE in NZB/W F1 hybrids has a female predominance, with an average lifespan of 9 months for female mice and 15 months for male mice (133). Estrogens, unlike that in the BXSB strain, are suggested to be pathogenic and play an essential role in the SLE development in NZB/W F1 mice (103). Many other contributors such as genetic factors and environmental triggers are also identified for disease susceptibility in NZB/W F1 mice (103, 113). This model serves as a great tool for the genetic and

preclinical studies although it presents two drawbacks: the slow progression of SLE and the need to breed two strains to obtain the mice for lupus study.

New Zealand Mixed (NZM) strains resulted from a backcross between NZB/W F1 and NZW with subsequent brother-sister mating. These inbred recombinant strains vary in their susceptibility to SLE development (134). While some strains develop disease at an accelerated rate, others show a significant delay on disease development or become resistant to SLE. Among all the NZM strains, NZM2410 strain has been selected and intensively studied as a SLE model. NZM2410 strain exhibits an earlier onset without a strong gender bias (103). 80% of NZW2410 mice, both males and females, develop severe LN by 6 months of age (134). The homozygosity of NZM strains and the possible enrichment for the most potent lupus susceptibility loci from parental strains facilitate the wide use of NZM2410 mice in genetic analysis. Lupus linkage analysis reveal that *Sle1-3* loci are strongly associated with LN (134). *Sle1* on chromosome 1 contributes to the breach of tolerance to nuclear antigens inducing the generation of autoreactive lymphocytes and the production of autoantibodies (103). *Sle2* on chromosome 4 is associated with B cell hyperactivity and IgM polyreactivity (103). *Sle3* on chromosome 7 is linked to a deficiency in activation-induced cell death of $CD4^+$ T cells (103). The threshold liability model is validated in NZM2410 mice, in that each susceptibility locus alone is not able to induce overt SLE syndromes while the copresence of these three major loci is indispensable and sufficient for SLE development (134). Numerous other susceptibility loci and their contribution to lupus pathogenesis have been identified and reviewed in detail in (103).

Other SLE mouse models

The BXD2 strain is a mouse model that spontaneously develops generalized autoimmune syndromes such as LN, arthritis, splenomegaly, and autoantibody production (135). It is derived from an intercross of B6 and DBA/2J strains. The development of both rheumatoid arthritis and SLE features in adult BXD2 mice makes this strain an excellent mouse model for understanding autoantibody-mediated diseases and distinguishing factors that could be responsible for the disease. Several previous identified autoimmune susceptibility loci such as *Sle*, *Sles*, *Lmb* and *Asm2* have been reported to be present in BXD2 mice and contribute to autoimmune phenotypes (136). With the spontaneous development of SLE syndromes, BXD2 mice have been used in various lupus studies.

Another commonly used SLE mouse model is the pristane-induced lupus model. Intraperitoneal injection of pristane is a standard technique to induce ascites for the collection of antibodies. Intriguingly, the treatment with pristane in BALB/C mice induced a significant level of autoantibodies, even comparable to MRL/*lpr* mice. The treatment also caused IC deposition in the kidney, facilitating proteinuria and severe nephritis (103). Other strains were also demonstrated to be susceptible, though to a various level, to pristane-induced SLE manifestations (132). Induced lupus models provide flexible "time windows" for researchers to examine the efficacy of preventive and therapeutic treatments.

The role of affinity maturation in SLE

While most lymphocytes with self-reactive antigen receptors are deleted, re-edited or anergized during the central and peripheral self-tolerance, a few self-reactive lymphocytes escape and mature, promoting the production of autoantibodies. It was reported that the majority of

pathogenic autoantibodies in SLE patients were highly mutated IgG antibodies, and numerous studies have implicated these IgGs as a main driver of SLE (70, 137, 138). The education for affinity maturation, including somatic hypermutation (SHM) to increase the specificity for antigens and class switch recombination (CSR) to gain different effector functions, occurs when B lymphocytes encounter antigens and interact with T helper cells and DCs. Thus, targeting of affinity maturation by disrupting the interaction between B cells and T cells or depleting SHM and CSR could be a potential therapy for SLE patients.

B cell development

Hematopoietic stem cells in the bone marrow give rise to all blood cells, including both lymphoid and myeloid cells. The commitment to B-cell or T-cell lineage depends on signals produced by stromal cells that have close interaction with developing lymphocytes in the microenvironment (24). While the precursors of T cells migrate to the thymus for maturation, B cells remain within the bone marrow, where they rearrange their immunoglobulin genes and develop into immature B cells (24). Immature B cells are tested for autoreactivity before leaving the bone marrow, followed by a second examination in the circulation, to ensure the elimination of self-reactive B cells (139). The surviving immature B cells develop into mature B cells in peripheral lymphoid organs, such as the spleen. Mature B cells encountering foreign antigens are activated and differentiate into plasma cells for antibody production against the antigen and memory cells for further protection against the same antigen (Figure 2.5).

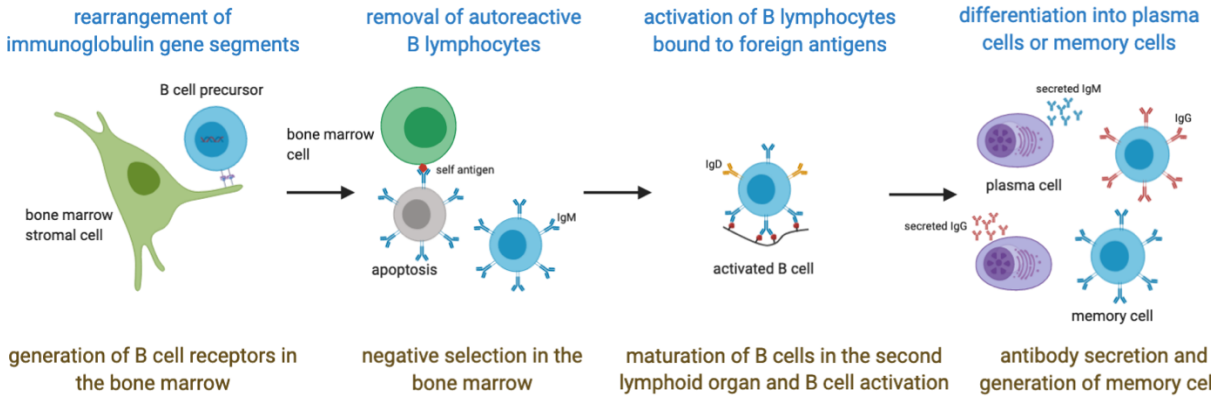


FIGURE 2.5 Main steps in B cell development. Stem cells differentiate into immature B cells in the bone marrow and migrate to second lymphoid organs for further maturation, where they encounter antigens and become activated. Activated B cells can differentiate into either antibody-producing plasma cells or long-lived memory cells. (Adapted from Janeway's Immunobiology, 9th Edition. *Janeway's Immunobiology, 9th Edition*: 296.)

During the early B-cell development in the bone marrow, rearrangement of the heavy chain and light chain loci is initiated to enrich the diversity of BCR repertoire. There are two different gene segments, variable (V_L) and joining (J_L) gene segments, in the light chain that are involved in the process of rearrangement. The heavy chain contains an additional segment called diversity (D_H) gene segment, which lies between V_H and J_H segments. In pro-B cells, a D_H gene segment is rearranged to join a J_H gene segment, followed by a recombination of an upstream V_H gene segment to the combined DJ_H segment (139). The last step in the assembling of the heavy chain is completed by joining the arranged VDJ_H segment to the constant (C) region gene (139). The next phase is the pre-B cell stage: V_L and J_L gene segments in the light chain are rearranged to form a κ chain or a λ chain. The expression of κ chains versus λ chains varies from species to species. While 95% of B cells express κ chains in mice and rats, only 65% of B cells express κ chains in humans and only 5% in cats (24). The diverse ratios are attributed to the different number of functional V_κ and V_λ gene segments in different species (24). The expression of an IgM molecule on the cell surface (also termed as BCR), a molecule that is formed by the rearranged heavy chain and light chain, characterizes the entry into the next stage, immature B cells.

The rearrangement of immunoglobulin genes generates a diverse repertoire with B cells expressing BCR capable of recognizing a wide range of pathogens. However, the rearrangement results in unpredicted specificity that could include BCRs with self-reactivity. During the B-cell development, there are two checkpoints, central tolerance and peripheral tolerance, that remove autoreactive B cells from the repertoire and avoid damages to the self. The newly formed BCR on the immature B cells will be screened for autoreactivity before they leave the bone marrow. If they react with self-antigens, there are three different fates for these autoreactive immature B cells. The first fate is known as receptor editing followed by clonal deletion. The strong cross-linking of IgM with a multivalent self-antigen can lead to the arrest of B cell development, inducing further rearrangements in the light chain until a novel non-autoreactive BCR is produced, a process known as receptor editing (24). With the limited number of gene segments, it is possible that further recombination fails to generate a non-autoreactive receptor, and these autoreactive cells are subjected to undergo apoptosis, termed clonal deletion. This mechanism has proved to be crucial since defects in this process appear to cause elevated production of autoantibodies and the development of autoimmune diseases such as SLE (24). The second mechanism to ensure a repertoire purged of autoreactive B cells is called anergy. When the interaction between an immature B cell and the self-antigen is weak, this autoreactive B cell enters a state of anergy. They are inactivated and do not respond to their specific antigens, even with the help of T cells. In addition, studies demonstrated that anergic B cells fail to migrate to follicles and locate in the T-cell areas, restricting their ability to compete with non-autoreactive B cells for further maturation (24). The final possible fate for autoreactive immature B cells is immunological ignorance. While these B cells have affinity for self-antigens, they survive and migrate to the peripheral due to various reasons, such as limited access to their specific antigens and a rather low concentration of

these antigens. Differing from anergic cells, these immunologically ignorant cells can be activated in certain situations, such as inflammation or an accumulation of the self-antigen (24).

While a large number of autoreactive immature B cells are removed before they leave the bone marrow, a small number of lymphocytes specific for antigens, confined to express in certain organs such as thyroglobulin in the thyroid, escape central tolerance and migrate into the peripheral. Peripheral tolerance is the second checkpoint to eliminate autoreactivity from the B cell repertoire. The immature B cells that encounter self-antigens in the peripheral have quite similar fates to the ones in the bone marrow. The only difference is that there is no receptor editing in the peripheral; immature B cells with strong affinity for self-antigens undergo apoptosis directly (24). After surviving these two checkpoints, immature B cells enter second lymphoid organs for final maturation, where they differentiate into FO B cells or MZ B cells and can be activated by binding to their specific foreign antigens. The activation of B cells induces affinity maturation for the production of antibodies with high affinity against foreign antigens.

Class switch recombination and somatic hypermutation

While rearrangements of gene segments enrich the diversity of the BCR repertoire for adaptive immune responses against a wide range of foreign antigens, the generation of antibodies with high affinity and distinct effector functions requires SHM and CSR. CSR is the replacement of the heavy-chain constant region in order to switch to other isotypes. There are five isotypes of antibodies: IgM, IgD, IgG, IgE and IgA. Each of these isotypes exhibits specific effector functions. All naïve B cells express IgM and IgD on the cell surface, and IgM is the first produced antibodies. IgM is primarily presented as pentamers and is a potent activator of the complement system. Some B cells can rapidly produce IgM upon encountering pathogens to provide protection before the

adaptive system fully develops (24). IgG is the most predominant antibody in blood and is highly mutated to gain increased affinity to antigens (24). In contrast to the pentamers form of IgM, IgG is mainly monomeric and diffuses easily into tissues, facilitating opsonization of antigens for phagocytosis and the activation of the complement system (138). IgA, the most abundant isotype in the body, is mostly present on mucosal surfaces and operates as a neutralizing antibody. IgE is normally present at a rather low level in blood and is significantly associated with allergies. SHM is a process to introduce mutations in the variable region of both heavy chains and light chains to change the affinity of the antibody against the antigen. While random mutations give rise to unpredictable alternations in affinity, only B cells with higher affinity to the antigen are selected to survive and cycle back for further mutations (140). CSR and SHM are two indispensable mechanisms for B cells to produce highly specific antibodies against antigens.

Activation-induced cytidine deaminase (AID) was first reported to be required for CSR and SHM by the Honjo group in 2000 (23). They showed that the overexpression of AID could lead to class switching even in the absence of cytokine stimulation *in vitro*. Additionally, mice deficient in AID failed to undergo CSR and SHM upon immunization (23). Further investigations elucidated the mechanisms by which AID regulates these two processes. AID can attack the cytidine exposed on the transcribing single-stranded DNA (not double-stranded DNA) and deaminate it into uridine (Figure 2.6) (24). The newly formed uridine is foreign to the DNA and also a mismatch for guanosine, triggering different DNA repair pathways. In the mismatch repair pathway, uridine nucleotide is removed along with some adjacent nucleotides, followed by the repair from error-prone DNA polymerases to introduce mutations (24). The base-excision repair pathway removes the uracil base, followed by either a random insertion at the next round of DNA replication to cause mutations or an excision of the abasic residue to create a single-strand nick

(Figure 2.6) (24). The mutations generated in AID-induced repair pathways are termed SHM. Single-strand nicks on both strands of DNA in the switch region are converted to double-strand breaks (DSBs), recruiting repair proteins involved in the DSB repair. The two switch regions with DSBs are aligned by the repetitive sequences shared by different switch regions, and the intervening sequences are deleted by DSB repair pathways to bring the selected constant region to the VDJ region, finalizing the process of CSR (24).

AID is highly expressed in the GC, a special structure where activated B cells rapidly proliferate and mutate. It has long been proposed that both CSR and SHM occur in GCs until an interesting finding by Carola Vinuesa and colleagues challenged this assumption. With the

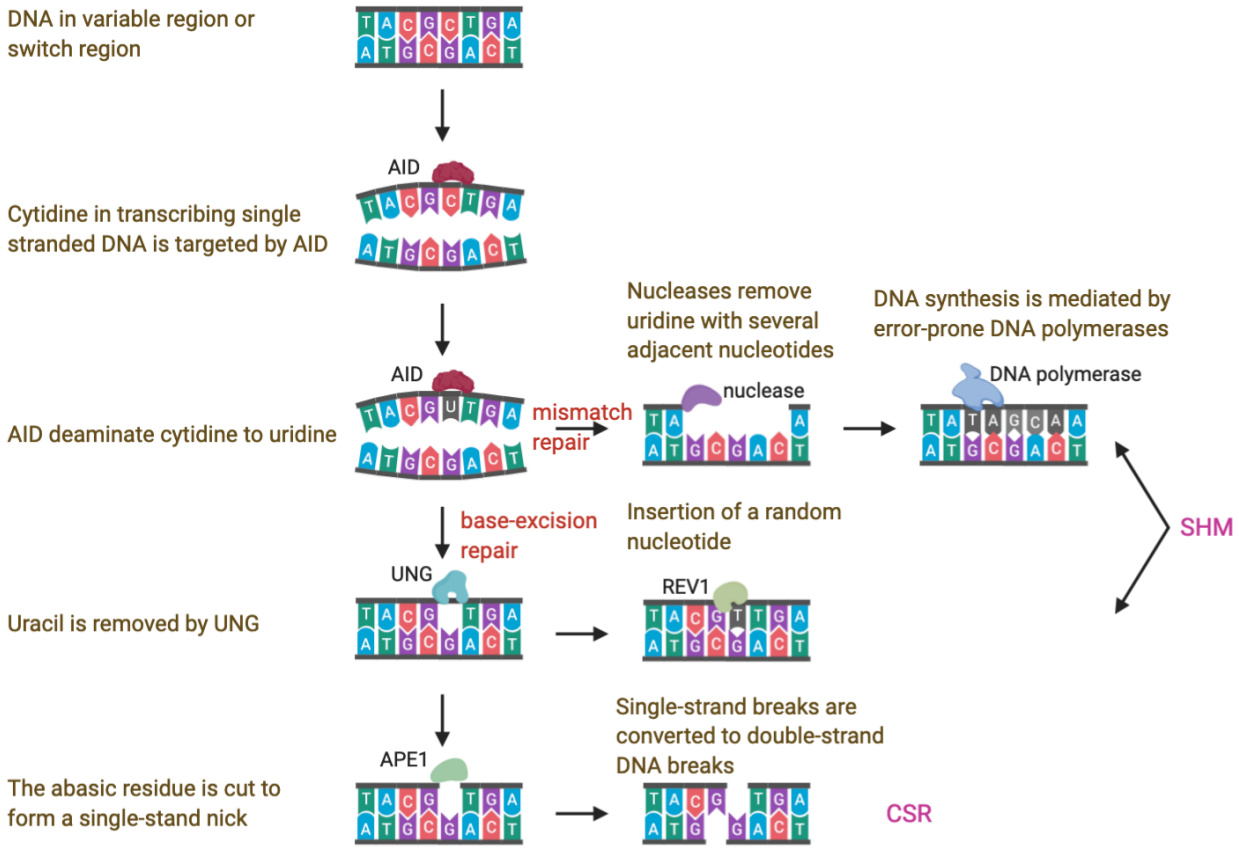


FIGURE 2.6 SHM and CSR are mediated by AID. Different repair pathways result in different mutation outcomes. UNG, uracil-DNA glycosylase; APE1, apurinic/apyrimidinic endonuclease 1; REV1, a DNA repair enzyme that can insert cytidine or recruit other DNA polymerases. (Adapted from Janeway's Immunobiology, 9th Edition. *Janeway's Immunobiology, 9th Edition*: 414-415.

adoptive transfer of transgenic B cells specific for hen egg lysozyme (HEL) and mutated HEL protein conjugated to sheep red blood cells, they demonstrated CSR predominantly takes place prior to GC formation and rapidly declines with the appearance of GC and the onset of SHM (140). Their data also suggested that a relatively low level of AID expression in combination with apurinic/apyrimidinic endonuclease (APE) 1 can be sufficient for the initiation of CSR compared with the requirement of high expression of AID and APE2 in GC B cells for SHM (140). These results indicate that different mechanisms are involved in CSR and SHM, though they share the crucial enzyme AID. Thus, the elimination of highly mutated IgG autoantibodies can be achieved by either targeting AID directly or disrupting the related mechanisms.

Targeting AID in different SLE mouse models

With the importance of autoantibodies in the development of SLE, the abrogation of AID has been achieved in different murine SLE models and showed beneficial results (Table 1). For example, AID-deficient MRL/*lpr* mice showed an increase in survival and an improvement in nephritis (141). Further investigation illustrated that the protective role was mediated by anti-dsDNA IgM since adoptive transfer of anti-dsDNA IgM significantly delayed the onset of SLE and ameliorated nephritis in MRL/*lpr* mice (142). Likewise, the inhibition of AID expression either by therapeutically targeting upstream positively regulating proteins or by increasing microRNAs that silence AID expression in MRL/*lpr* mice impaired CSR and SHM, reduced autoantibody production, alleviated nephritis and extended lifespan (143-145). In BXD2 mice, inhibition of SHM and CSR functions by single base pair mutations in AID also led to less autoantibody production, decreased GC response and ameliorated glomerulonephritis (146). However, one conflicting result was reported in B6/*lpr* mice deficient in AID. They produced

dramatically more pathogenic autoreactive IgM and showed more severe nephritis (147), raising concerns for the impact of genetic backgrounds on the benefits of AID abrogation.

Table 1. Targeting AID in different SLE murine models.

Strain	Manipulation	Results					
		survival	nephritis	ANA	anti-dsDNA	IgG deposition	proteinuria
MRL/*lpr*	B6.*Aicda*$^{-/-}$ were backcrossed to MRL/*lpr* for at least 5 generations	↑	↓	↓	IgG↓ IgM↑	↓	↓
MRL/*lpr*	block Rab7, which promotes AID expression	↑	↓	↓	IgG↓ IgM NC	NA	↓
MRL/*lpr*	B6.*HoxC4*$^{-/-}$ were backcrossed to MRL/*lpr* for at least 5 generations. HoxC4 directly activates the promoter of the *Aicda* gene	NA	↓	NA	IgG↓ IgM NA	↓	NA
MRL/*lpr*	upregulate microRNAs that silence AID expression	↑	↓	↓	IgG↓ IgM NC	↓	NA
BDX2	site mutations in AID catalytic domain	NA	↓	NA	IgG↓ IgM NC	↓	↓
B6/*lpr*	B6.*Aicda*$^{-/-}$ were backcrossed to B6/*lpr* for at least 6 generations	NA	↑	NA	IgG↓, IgM↑	NC	↑

(*Aicda*: gene encoding AID; Rab7: Ras-related in brain 7; HoxC4: Homeobox C4; ANA: anti-nuclear antibody; NC: no change; NA: not applicable)

Aim of the SLE study

Consistent with the heterogeneity in different SLE patients, different murine SLE models have distinct genetic backgrounds and SLE pathogenesis. The resemblance of BXSB mice to SLE patients warrants further investigations to determine the role of AID in SLE development in the BXSB strain. In this study, first we utilized the cutting-edge technology, CRISPR/Cas9, in the BXSB strain to genetically target exons in *Aicda*. This standard lab tool allows us to directly edit the gene in the BXSB zygote, thus excluding the possibility of donor genome effect and avoiding the time-consuming backcrossing for several generations in the classic genetic manipulation method. The smaller size of the target region in genotyping and failure to produce class-switched antibodies upon *in vitro* B cell stimulation confirmed the success of *Aicda* gene targeting and the abrogation of AID functions. We examined the disease development by analyzing the changes in

survival, kidney pathology, ANA and other hallmarks of the BXSB model. More details will be elaborated in chapter IV.

With the beneficial effects of targeting AID shown in different SLE murine models, we also aimed to explore the potential of a therapeutic treatment to retard SLE development by intervening AID pathway and reducing autoantibody production. In our previous work, the treatment with 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), a small molecule that inhibits the repair pathway recruited by AID-induced DSBs, was proven to prevent class switching and decrease diversity of antibody profile *in vivo* (148). In chapter IV, we examined the effects of DIDS treatment on SLE development in both BXSB males and MRL/*lpr* females, illustrating the possibility of clinical transition for this treatment.

The genetic knockout mice we established in the chapter IV provided a good model for investigating the role of maternal antibodies in the SLE development of the offspring. In chapter V, we employed this model and showed the absence of maternal antibodies altered the disease progression in the offspring. Various disease hallmarks were assessed and detailed in chapter V.

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Chapter III

Targeting AID/RAD51 pathway significantly inhibits T1D development in NOD mice

Abstract

B lymphocytes play a crucial role in the pathogenesis of type 1 diabetes (T1D), as demonstrated by their ability to present islet antigens to diabetogenic T cells. While the production of autoantibodies has been proved to be dispensable for the initiation of the disease, the strong correlation between the presence of high-affinity autoantibodies and the disease onset raises the question whether affinity maturation contributes to the T1D development. Activation-induced cytidine deaminase (AID) is an enzyme required for somatic hypermutation (SHM) and class switch recombination (CSR), two processes that B lymphocytes undergo to achieve affinity maturation. In this study, we demonstrated the genetic ablation of AID significantly retarded the disease progression in NOD mice. 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid, also termed DIDS, is a small molecule that inhibits one of the major repair pathways recruited to fix AID-induced DNA double stranded breaks in CSR, thus leading to cell apoptosis and defects in CSR. The administration of DIDS starting from different time points, even when initiated with the presence of high levels of anti-insulin antibodies, successfully slowed the progression to overt T1D. Both approaches exhibited similar cellular changes including a remarkable increase of memory cells and $CD73^+$ B lymphocytes, the latter of which exerted an immunosuppressive role in numerous studies and thus could be responsible for the T1D prevention. Together, these results suggest that AID and the related pathways can be potential targets for alternative T1D treatments.

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease associated with the destruction of insulin-producing pancreatic β cells by autoreactive T cells, which leads to insulin deficiency and hyperglycemia. More than one million people (less than 20 years of age) suffer from T1D worldwide (1). T1D patients with long-term hyperglycemia can develop numerous complications affecting the brain, heart, kidneys and retina (2). Despite many advancements in the development of novel therapies, treatments to cure the disease are not available. The current management of T1D primarily depends on monitoring the blood sugar level and lifetime insulin injections, which can be burdens both physically and financially (2). Given that T cells are the main contributors to the damages of the pancreatic β cells, many studies have focused on immune therapies that target T cells to find a real cure. In clinical trials, treatments that reduced T cell number or function significantly slowed the progression of T1D (2, 3). However, these immunomodulatory strategies only showed a transient disease remission and failed to preserve β cell function after a short period (2, 3). As the pathogenesis of T1D involves a complex integration of different immune components, other components could be better targets for alternative treatments in T1D patients.

Accumulating studies has emerged to recognize the critical role of B lymphocytes in the development of T1D. For example, researchers found that the deficiency of B lymphocytes protected nonobese diabetic (NOD) mice, a well-characterized model that resembles human T1D from many aspects, from developing T1D (4, 5). B lymphocyte reconstitution from a bone marrow chimera study restored T1D susceptibility in B lymphocyte-deficient NOD mice, whereas the transfer of wildtype sera failed to abrogate the T1D resistance in B lymphocyte-deficient NOD mice (6). This indicates that autoantibodies are not required for the initiation of the disease. In addition to the production of antibodies, B lymphocytes can also function as antigen presenting

cells (7). Further studies demonstrated that the disruption of the antigen presenting function in B lymphocytes by targeting MHC I or MHC II both prevented the development of T1D in NOD mice (8, 9). Collectively, this evidence suggest that B lymphocytes promote β cell autoimmunity through the presentation of autoantigens to diabetogenic T cells.

Although autoantibodies alone are not able to drive disease progression, they have been proven to be an accurate predictor for the disease in both NOD mice and genetically susceptible people (10). The appearance of autoantibodies against β cell antigens occurs months to decades before clinical diagnosis (11). The detection of multiple types of autoantibodies is strongly associated with a high risk for T1D, while low titers of insulin autoantibodies (IAAs) and slow progression to multiple islet autoantibodies could predict a delay onset of symptoms (12). Furthermore, in a group of IAA-positive children followed up to 6 years, Bonifacio and colleagues showed that 50% of the children with high-affinity IAAs developed diabetes, whereas children with low-affinity IAAs were free from diabetes (13). This suggests that high-affinity IAAs may characterize children with the highest risk for T1D. In the same study, they also showed the majority of the β cell autoantibodies were of an IgG isotype (13).

The production of IgG autoantibodies with high affinity by B lymphocytes indicates that these cells have been through affinity maturation. There are two mechanisms that are involved in affinity maturation. Somatic hypermutation is a process to introduce mutations in the variable region to generate Ig with different affinity, while class switch recombination is a process to switch from IgM to other isotypes for different effector functions (14). Activation-induced cytidine deaminase (AID, coded by *Aicda* gene), primarily expressed in germinal center B (GCB) lymphocytes, is an enzyme that plays an indispensable role in regulating these two processes (15). AID can target cytidine on the transcribing single-strand DNA and deaminate it to uridine, thus

recruiting repair pathways (14). The mismatch will either be replaced by other nucleotides to introduce mutations or will be removed to form single-strand breaks followed by conversion to double-strand breaks (DSBs) (14). Homologous recombination (HR) is one of the major pathways that mammalian cells use to repair DSBs (16). 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid, also known as DIDS, can bind to RAD51, a core complex in HR, and inhibit its DNA binding ability (17). A previous study showed that *in vitro* and *in vivo* treatment of DIDS impaired DSB repair and promoted the apoptosis of stimulated B lymphocytes (17). In addition, the cytotoxicity induced by DIDS was demonstrated to be AID-dependent since no changes in apoptosis were shown with AID-deficient B lymphocytes (17). With the importance of IgG autoantibodies in T1D, targeting AID or related pathways can be a potential therapy for T1D patients.

In this study, we first genetically abrogated AID on the NOD background using CRISPR/Cas9 technology. While no significant difference in lymphocyte infiltration was shown, AID-deficient NOD mice exhibited a notable delay in the development of T1D. Then we investigated the ability of DIDS to attenuate the progression of T1D in NOD mice. DIDS treatment significantly retarded the T1D development and showed similar cellular changes to AID-deficient NOD mice. Together, these findings provide evidence supporting the potential for a clinical transition of this novel therapeutic approach.

Material and methods

Mice

Mice were housed under specific pathogen-free conditions and the animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University. NOD mice were obtained from Dr. David V. Serreze (The Jackson Laboratory). NOD.*Aicda*$^{-/-}$ mice were generated using CRISPR/Cas9 technology by microinjection of Cas9 mRNA and single-guide RNAs into NOD zygotes in the Dr. David V. Serreze laboratory (The Jackson Laboratory). The founder mice with mutations in the targeted region were then backcrossed to NOD mice and the resulting N1 progeny were analyzed for germline mutations. The selected mutant mice were intercrossed to generate homozygous mice followed by brother-sister mating. Mice in this study were matched by age and gender.

T1D development monitoring

Mice were examined weekly for glycosuria using Diastix (Bayer HealthCare). The color reading was performed 30 seconds after wetting the strip with fresh catch urine. Diabetes was confirmed with two consecutive reading > 0.25% (250mg/dL).

Immunofluorescence staining

Pancreas were collected and fully embedded in OCT. The embedding molds were placed on dry ice for 10 minutes and then stored at -80°C until processing. Samples were cut at 8μm using a Microm HM550 Cryostat (Thermo Fisher Scientific). Slides were fixed in cold acetone (catalog A18-4; Fisher Chemical) at -20°C for 10 minutes followed by 3 times of 5-minute PBS (Life Technologies) wash. Then the slides were blocked with 3% FBS (HyClone; Thermo Fisher

Scientific) at room temperature for 1 hour followed by primary anti-insulin antibody (ab181547, Abcam) incubation at 4°C overnight. Slides were washed for 3 times with PBS and then incubated with fluorochrome-conjugated antibodies against rabbit IgG (ab150077, Abcam), B220 (RA3-6B2, Biolegend), CD3 (17A2, Biolegend) for 1 hour at room temperature. Slides were examined using an Eclipse T*i* microscope with NIS-Elements software (Nikon).

Flow cytometry

Splenocytes were prepared by smashing the spleen in a 70μm bag. Then single cell suspension was treated with ACK lysis buffer to remove red blood cells followed by counting using a Nexcelom cell counter. Cells were resuspended in FACS buffer (2% FBS with sodium azide) at 2×10^7cells/ml and stained with fluorochrome-conjugated antibodies for 30 min at 4°C. Propidium iodide (BioLegend) was used to differentiate live and dead cells. The following antibodies (clone) were used in this study: B220 (RA3-6B2), CD4 (GK1.5), CD8 (53-6.7), CD21 (7E9), CD23 (B3B4), GL7 (GL7), CD73 (TY/11.8), CD138 (281-2), CD25 (3C7), CD80 (16-10A1), CD273 (TY25) (BioLegend) and FAS (Jo2) (BD Biosciences). Apoptosis was characterized using Apoptosis Kit (BioLegend) following the manufacturer's instructions. For intracellular staining, cells were fixed using Fixation/Permeabilization kit (BD Biosciences) per manufacturer's instructions. Briefly, cells were resuspended in 250μL of Fixation/Permeabilization solution for 20 minutes at 4°C followed by 2 times of wash with Perm/Wash buffer. Cells were then stained with intracellular antibodies against Ki67 (B56, BD Biosciences) and Foxp3 (FJK-16s, Invitrogen) for 30 minutes at 4°C. All samples were evaluated on an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Data were analyzed using Flowjo software (TreeStar). All analyses were performed after gating on the single cells.

DIDS treatment

DIDS (Tocris Bioscience) was weekly injected i.p. at 10 mg/kg beginning at 10 weeks of age until disease onset or 30 weeks of age, whichever comes first. In another experiment, NOD mice were injected with 50 mg/kg DIDS starting at 8 weeks of age weekly for a total of eight injections. DIDS was reconstituted in 0.1 M potassium bicarbonate to a concentration of 0.1 M and further diluted for injection in sterile PBS. Control mice received 0.1M potassium bicarbonate diluted in sterile PBS at the same injection interval.

Statistics

All statistical analyses were performed in Prism (GraphPad). Mann–Whitney U test were used for two groups comparison (*$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$). Log rank was used for diabetes assessment.

Results

AID deficiency significantly delays the onset of T1D in NOD mice

To investigate whether affinity maturation is an important contributor to the pathogenesis of T1D in NOD mice, we employed CRISPR/Cas9 technology to target *Aicda* gene directly in NOD zygotes. This gene editing technique remarkably reduces the time required for genetic modification and excludes the potential effect of the donor genome compared with the conventional technologies using embryonic stem cells. Mice with homozygous mutations were sequenced and the one with a 2-bp deletion and 309-bp insertion in the exon 1 of *Aicda* gene was selected to be used in this study (Figure 3.1A). When AID-deficient B lymphocytes were isolated

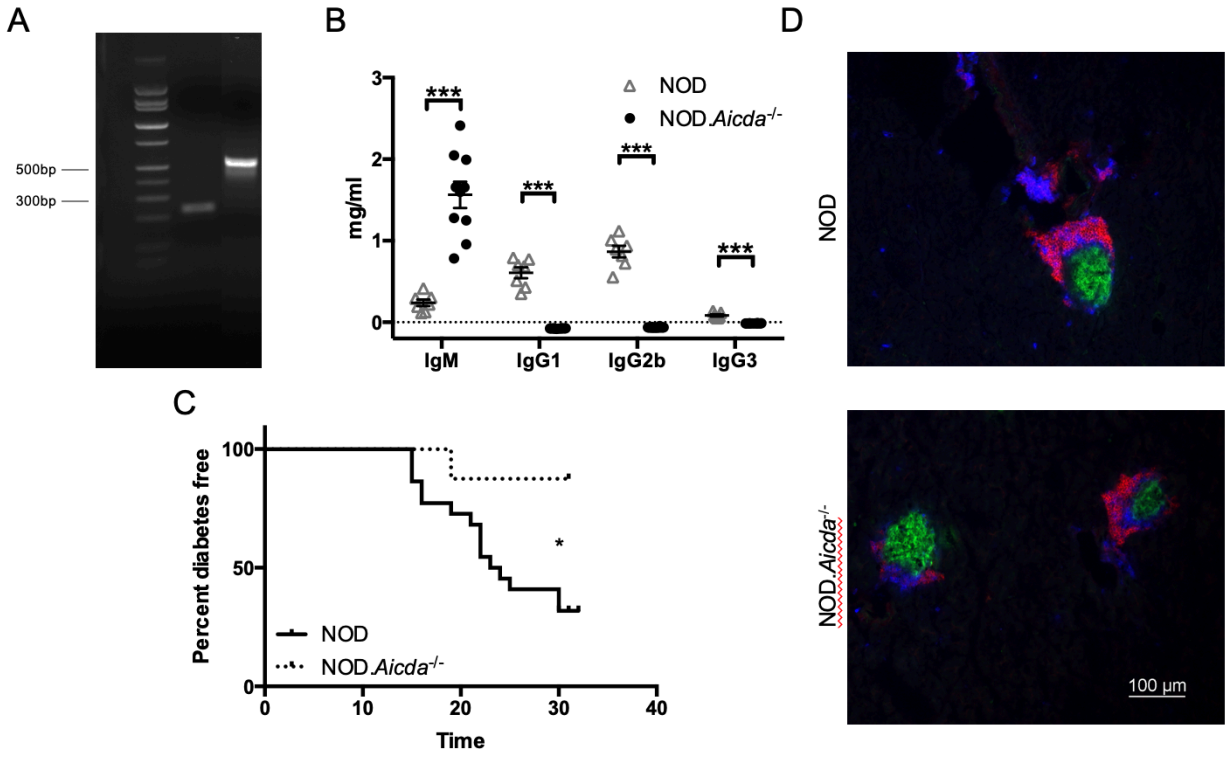

FIGURE 3.1 AID deficiency significantly delays the onset of T1D. (**A**) CRISPR-Cas editing extends *Aicda* in NOD mice. Lane 1, wt; lane 2, homozygous mutation. (**B**) The levels of IgM and IgG subisotypes in the sera from 6-7 weeks old NOD (n=7) and NOD.*Aicda*$^{-/-}$ (n=10) females. Data were shown as mean±SEM. Statistical significance was determined by Mann–Whitney U test. ***, $p<0.001$. (**C**) The percent of diabetes free for NOD (n=22) and NOD.*Aicda*$^{-/-}$ (n=8) females. Log-rank test was used for statistical analysis. *, $p<0.05$. (**D**) Representative immunofluorescence staining images of pancreas sections from 18-week-old NOD and NOD.*Aicda*$^{-/-}$ mice. Green: insulin, red: B220 and blue: CD3.

and stimulated with CD40 plus IL-4 *in vitro*, they failed to class switch to IgG1 (18). Consistently, no detectable IgG antibodies were shown in the serum of NOD.*Aicda*$^{-/-}$ mice (Figure 3.1B). These results confirmed the successful targeting of *Aicda* gene and the loss of AID function in the mutant mice. In addition, the lack of IgG in NOD.*Aicda*$^{-/-}$ mice was accompanied with an elevated level of IgM similar to previously established AID-deficient mice (Figure 3.1B) (15). When the T1D development was monitored by assessing the glucose level in the urine, AID-deficient NOD mice showed a significant delay in the onset of the disease (Figure 3.1C). Interestingly, the insulitis scores were comparable between the wildtype and knockout aged-matched females (18). Immunofluorescence staining also indicated that AID abrogation did not significantly change lymphocyte infiltration into the islet (Figure 3.1D). Together, these results demonstrate that the abrogation of AID in NOD mice retards the development of T1D, despite the failure to regulate lymphocyte infiltration.

AID ablation in NOD mice expands memory-like B lymphocytes

To investigate the potential mechanisms that contribute to the protection from T1D, we assessed the effects of AID depletion on GCB cell development. Consistent with other AID-deficient strains, we appreciated a notable increase of GCB cells in NOD.*Aicda*$^{-/-}$ mice (Figure 3.2A). Since previous reports indicate that AID-deficient B lymphocytes are hyperproliferative upon stimulation *in vitro* compared with wildtype B lymphocytes (15, 19), we examined the proliferation of GCB cells using Ki67, a marker highly associated with cell proliferation. While we did not observe an increased proliferation of AID-deficient GCB cells, a trend toward decreased proliferation was appreciated (Figure 3.2B). In the germinal center, B lymphocytes undergo clonal selection (14). High-affinity B lymphocytes are selected to differentiate into plasma cells or

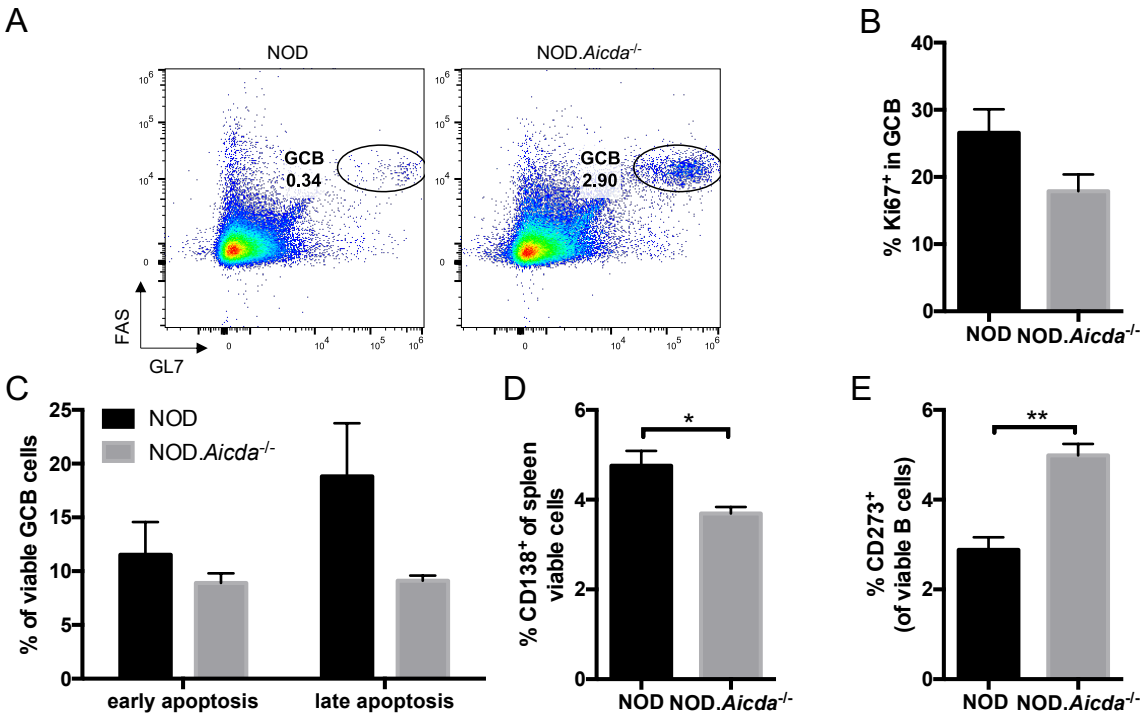

FIGURE 3.2. AID deficient GCB cells are more likely to differentiate into memory cells than plasma cells. Splenocytes from 13-week-old NOD (n=4) and NOD.*Aicda*$^{-/-}$ (n=6) females were analyzed by flow cytometry. (**A**) Representative FACS images for germinal center B (GCB) cells (Fas^{+}GL7^{+}) was shown. (**B**) The percentage of Ki67^{+} cells in the viable GCB cell population was shown. (**C**) The percentages of early apoptosis (Annexin V^{+}7-AAD^{-}) and late apoptosis (Annexin V^{+}7-AAD^{+}) were compared between NOD and NOD.*Aicda*$^{-/-}$ mice. (**D**) The percentage of plasma cells (CD138^{+}) in the viable splenocytes was shown. (**E**) The percentage of memory cells (CD273^{+}) in the viable B cell population was shown. Data were shown as mean±SEM. Statistical significance was determined by Mann–Whitney U test. *, $p<0.05$; **, $p<0.01$.

memory cells, while low-affinity B lymphocytes are selected to experience apoptosis (14). With the importance of AID in the affinity maturation, we investigated the effects of AID deficiency on the apoptosis of GCB cells. Apoptosis in the early stage or in the late stage was assessed for GCB cells using Annexin V and 7-AAD. No differences were shown in the percentage of GCB cells that were in the early stage of apoptosis (Figure 3.2C). Interestingly, we observed a decrease trend for GCB cells that were in the late stage of apoptosis, which could partially explain the increase of GCB cells (Figure 3.2C). A significant decrease of plasma cells was shown in the AID-deficient NOD mice, whereas a remarkable increase of memory-phenotype cells was appreciated (Figure

3.2D and 3.2E). Thus, although AID depletion results in the loss of affinity maturation, GCB cells exhibits a reduction in apoptosis and a favored differentiation into memory-phenotype cells.

An expansion of CD73$^+$ B lymphocytes in the absence of AID

T regulatory (T_{reg}) cells, which control the peripheral immune tolerance, are critical regulators in the pathogenesis of T1D (20). Loss of T_{reg} phenotype or function has been linked to accelerated disease progression, while T_{reg} expansion or promoting T_{reg} function significantly protects the mice from developing T1D in different models (21-24). We next investigate the effects of AID depletion on the T_{reg} cells. No difference in the T_{reg} proportion was detected in AID-deficient NOD mice (Figure 3.3A). Recently, a subpopulation of T cells, characterized with the expression of CD73, exhibited a suppressive role in dampening effector T cell proliferation (25, 26). CD73 is an ectoenzyme that converts AMP to adenosine. Adenosine has been recognized as an anti-inflammatory molecule with its ability to suppress immune responses by activating its receptors on the effector cells (27, 28). Of note, CD73$^+$ B lymphocytes were also reported to inhibit activated T cell functions *in vitro* and ameliorate colitis when transferred into dextran sulfate sodium salt-induced colitis murine model (29, 30). While the percentages of CD73$^+$ in CD4 T cells were comparable between wildtype and knockout mice, a notable increase of CD73$^+$ B lymphocytes was observed in multiple locations, such as the spleen, islets, peripheral and peritoneal cavity of NOD.*Aicda*$^{-/-}$ mice (Figure 3.3B-F, and (18)). It is plausible the expanded CD73$^+$ B lymphocytes in the AID-deficient mice exert an immunosuppressive role and retard the T1D development by regulating T cell functions.

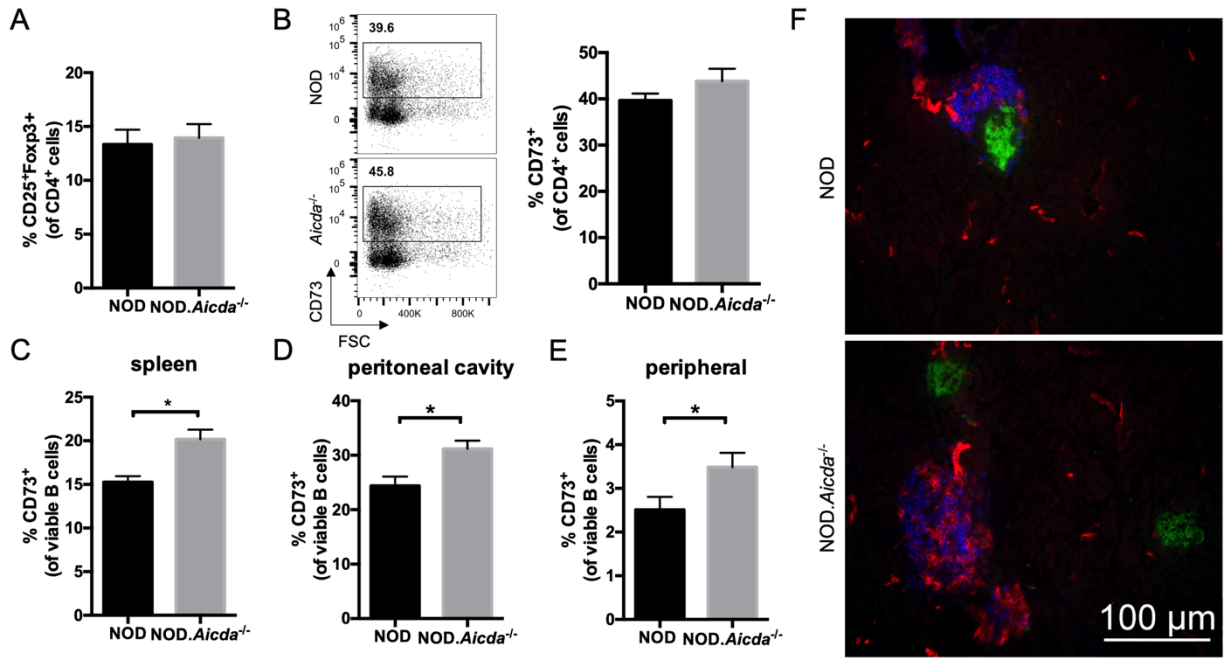

FIGURE 3.3. Regulatory CD73+ B cells expanded in the NOD.*Aicda-/-* mice. Splenocytes from 13-week-old NOD (n=4) and NOD.*Aicda*$^{-/-}$ (n=6) females were analyzed by flow cytometry. (**A**) The percentage of T regulatory cells (CD25^{+}Foxp3^{+}) in the CD4^{+} T cell were compared between wildtype and AID-deficient mice. (**B**) Representative dot plots of CD73^{+} in CD4^{+} T cell were shown on the left and the percentage of CD73^{+} cells in the CD4^{+} T cell population was shown. (**C**, **D**) The percentages of CD73^{+} cells in the viable B cell population in the spleen (C), and the peritoneal cavity (D) were shown. (**E**) The percentages of CD73^{+} cells in the viable B cell population in the peripheral were compared between wildtype (n=4) and knockout (n=5) mice. Data were shown as mean±SEM. Statistical significance was determined by Mann–Whitney U test. *, $p<0.05$; **, $p<0.01$. (**F**) Representative immunofluorescence images of pancreas sections. Green: insulin, blue: B220, red: CD73.

DIDS treatment significantly inhibits the T1D development in NOD mice

With the success of genetic abrogation of AID in protecting NOD mice from T1D, we next sought to determine whether therapeutically targeting affinity maturation can lead to similar beneficial effects. NOD mice were treated weekly with either vehicle or DIDS of 10mg/Kg or 50mg/Kg starting from 6, 8 or 10 weeks old. Despite the dosage or the start point, DIDS treatment was capable to exert protective effects from developing T1D in NOD mice (Figure 3.4A and (18)). We analyzed the splenocytes after 8 times of injections with vehicle or 50mg/Kg DIDS to evaluate the cellular changes underlying the T1D protection by this small molecule. While DIDS treatment

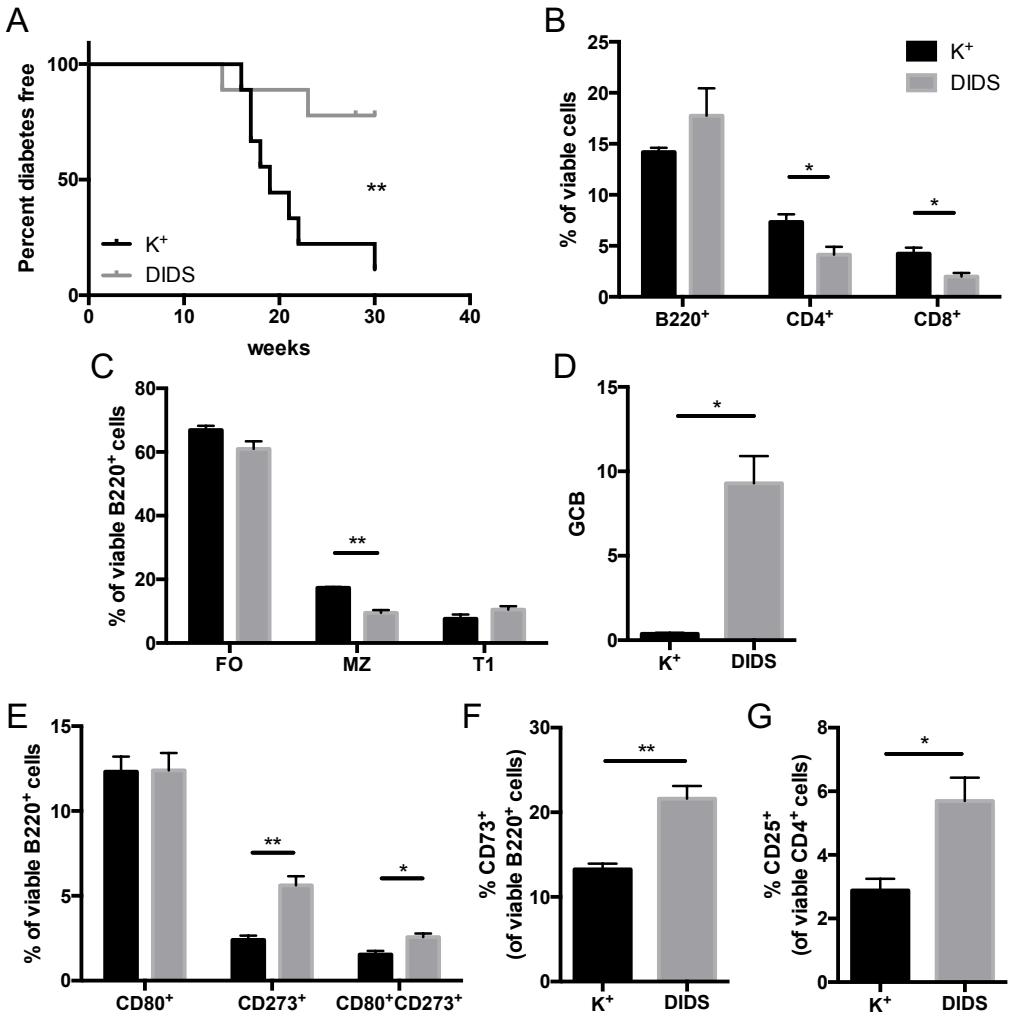

FIGURE 3.4 DIDS treatment significantly increases regulatory cells and retards the development of T1D in NOD mice. (**A**) The percent of diabetes free for NOD mice treated with 50mg/Kg DIDS (n=9) or potassium (K^+, n=9) from 10 weeks old until 30 weeks old or tested diabetic, whichever comes first. Log-rank test was used for statistical analysis. **, $p<0.01$. (**B-G**) NOD mice were treated with 50mg/Kg DIDS (n=4) or potassium (K^+, n=3) from 8 weeks old for 8 times. Splenocytes were analyzed at the end of the study. The percentages of B cells, CD4 T cells and CD8 T cells were shown in B. The percentages of follicular (FO; $CD23^+CD21^+$), MZ ($CD23^{low/-}CD21^+$), and transitional 1 (T1; $CD23^-CD21^-$) B cells in the B lymphocyte population were compared between control and treated mice in C. The percentage of GCB cells were shown in D. Memory cells were analyzed in E. Special regulatory B cells ($CD73^+B220^+$) were evaluated in F. T regulatory cells ($CD25^+CD4^+$) were compared between two groups in G. Data were shown as mean±SEM. Statistical significance was determined by Mann–Whitney U test. *, $p<0.05$; **, $p<0.01$.

did not significantly change the percentage of B lymphocytes, we observed a notable reduction of both CD4 and CD8 T cells (Figure 3.4B). Marginal zone B (MZB) lymphocytes, a special B lymphocyte subset that located between the red pulp and the white pulp of the spleen, has been demonstrated to be enriched for autoreactive specificities (31). Previous studies exhibited an

expansion of MZB cells in NOD mice as early as 9 weeks old (32). We appreciated a significant decrease of MZB cells in the DIDS-treated mice (Figure 3.4C). In addition, DIDS treatment remarkably increased the frequencies of GCB cells and memory cells in NOD mice, which are consistent with the genetic manipulation of AID (Figure 3.4D and 3.4E). Previously characterized $CD73^{+}$ B lymphocytes shown to have potential immunosuppressive capacity were also found to be elevated in the treated mice (Figure 3.4F). In addition, an expansion of regulatory T cells was shown with the DIDS treatment (Figure 3.4G). Together, these results indicate that DIDS treatment significantly alters the course of T1D in NOD mice, and this T1D protective effect is possibly exerted by inducing B regulatory cells.

Discussion

In this study, we first employed CRISPR/Cas9 technology to directly target *Aicda* gene in NOD mice. Although the ablation of AID did not significantly diminish lymphocyte infiltration into islets, the lack of affinity maturation slowed the progression to overt T1D with the majority of mice diabetes free up to 30 weeks of age. Likewise, therapeutically targeting AID-related pathway, which results in the apoptosis of class-switching cells, remarkably delayed the onset of the disease in NOD mice. The point of this intervention is very distinct from the genetic modulation. Both of them, however, induced similar cellular changes in NOD mice, including a notable expansion of GCB cells, memory-phenotype cells and $CD73^+$ B lymphocytes. $CD73^+$ B lymphocytes have recently been recognized as a new subset of regulatory B lymphocytes with the ability to inhibit T cell functions (29, 30). Thus, while the link between affinity maturation and regulatory B lymphocytes warrants further investigations, these findings suggest that affinity maturation contributes to the pathogenesis of T1D and could be a potential target for alternative treatments.

The expansion of GCB cells in NOD.*Aicda*$^{-/-}$ mice is consistent with other AID-deficient strains (15, 33). Affinity is one of the key factors that determine the fate of GCB cells in the clonal selection and expansion (34). High-affinity GCB cells survived from iterative rounds of mutation and selection will ultimately differentiate into plasma cells and give rise to the secretion of highly specific antibodies (34). The majority of low-affinity GCB cells are selected to undergo apoptosis due to the lack of survival signals from follicular helper T cells, while some GCB cells with low-affinity differentiate into memory cells and enter the circulation in the early stage of the immune response (14, 35). The formation of memory cells before affinity maturation is to maintain the flexibility in antigen binding thus ensuring a broader range of protection (35). In this study, we

showed the loss of affinity maturation resulted in a significant decrease of plasma cells accompanied with a notable increase of memory cells in NOD mice. Recently, several studies have implied the main producers of IL-10 were characterized with a memory B lymphocyte phenotype (36-38). Further investigations are needed to explore the possibility that the expansion of memory cells contributes to the T1D protection by the production of IL-10.

Although it is well-accepted that regulatory B lymphocytes exert an anti-inflammatory role predominantly through the secretion of IL-10, it is becoming apparent that there are other mechanisms, independent of IL-10, which are responsible for the suppression of the immune responses. The production of adenosine is one of the mechanisms responsible for the suppression of immune responses (28). Adenosine inhibits effector T cells through the interaction with its receptors on the target cells and the subsequent modulation of downstream pathways (27, 28). A new subset of B cells characterized with the expression of CD73, an enzyme that hydrolyze AMP to adenosine, has been reported to suppress T cell functions and ameliorate autoimmune diseases in mice (29, 30). We examined this cell population and found that a remarkable increase of this regulatory-phenotype B lymphocytes was observed in multiple sites of the T1D resistant mice, including the spleen, the islet and the pancreatic lymph nodes. It is very likely that expanded $CD73^+$ B lymphocytes are the major contributors to the amelioration of T1D. Indeed, our collaborators demonstrated the adoptive transfer of B lymphocytes depleted of $CD73^+$ B lymphocytes was less efficient in preventing the disease compared with the transfer of total B lymphocytes (18). Additionally, the presence of CD73 inhibitor significantly diminished the ability of $CD73^+$ B lymphocytes to suppress T cells (18). Collectively, these results suggest the expansion of regulatory B lymphocytes in NOD mice as a consequence of the disruption of affinity maturation could, at least in part, explain the T1D resistance.

This study mainly focuses on the effects of targeting AID or related pathways on the B lymphocyte maturation in the spleen. However, the manipulation of affinity maturation results in the loss of IgA which plays a crucial role in mucosal immunity. Secretory IgA constantly surveil the microenvironment to block the colonization of microbial pathogens and ensure mucosal immune homeostasis (39). The deficiency of IgA has been linked to dysbiosis and increased frequency of gut-associated infections (39). Changes in the gut microbiota composition can dramatically alter the course of T1D (1). For instance, the administration of probiotics, *Bifidobacteria* and *Lactobacilli*, significantly alleviated insulitis and β cell autoimmunity (40). Therefore, understanding how microbiota changes due to AID deficiency impact the development of T1D is an important area for future investigations.

In conclusion, this study show that genetic modulation or therapeutic intervention of affinity maturation successfully retard the development of T1D in NOD mice, at least in part by inducing regulatory B lymphocytes. These findings indicate that targeting activated B lymphocytes rather than total B cell depletion could prevent the disease and provide support for a clinical transition of this work. Because many other components, besides RAD51 complex, are involved in the AID-related pathways, further exploration may uncover more competent targets for immune interventions. Moreover, the identification of mechanisms that induce an immunoregulatory state is warranted and may ultimately represent a novel approach for T1D prevention. With the critical role of B lymphocytes in autoimmunity and inflammation, it will also be of great scientific and clinical interest to examine the potential effects of targeting affinity maturation in regulating the pathogenesis of those diseases.

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Chapter IV

Abrogated AID Function Prolongs Survival and Diminishes Renal Pathology in the BXSB Mouse Model of Systemic Lupus Erythematosus

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Abstract

Almost a decade has passed since the approval of belimumab, a monoclonal antibody (mAb) directed against B lymphocyte stimulation and the first targeted therapy approved for systemic lupus erythematous (SLE) in over 50 years. Although well tolerated, the efficacy of belimumab remains limited and is not labeled for patients suffering from nephritis, the leading cause of patient mortality. We sought to explore alternative targets of autoreactive B lymphocytes through manipulation of affinity maturation. The BXSB/MpJ mouse, a well-established model of human SLE, develops elevated antinuclear Abs and immune complex–mediated nephritis along with other manifestations of SLE-like disease. To limit interfering with critical background genetics, we used CRISPR-Cas9 to disrupt activation-induced cytidine deaminase (AID; *Aicda*) directly in BXSB zygotes. Homozygous null mice demonstrated significantly prolonged survival compared with wild-type. Although mice continued to develop plasma cells, splenic follicular structure was restored, and renal pathology was reduced. Mice developed expanded germinal center B lymphocyte populations as in other models of AID deficiency as well as increased populations of $CD73^{+}$ B lymphocytes. Treatment with the small molecule inhibitor of RAD51, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid, resulted in minimal changes in disease markers in BXSB mice. The prolonged survival in AID-deficient BXSB mice appears attributed primarily to the reduced renal pathology, warranting further exploration, as current therapeutics targeting lupus nephritis are limited and, thus, in great demand.

Introduction

Systemic lupus erythematosus (SLE) is a complex, B lymphocyte–driven autoimmune disease in which high levels of circulating autoreactive Abs exert toxic effects that lead to organ damage, most significantly severe nephritis. SLE has no effective cure, and current treatments are based on global immunosuppression, often insufficiently controlling symptoms coupled with significant side effects. Advances in therapeutics for SLE have been slow, with the notable exception of belimumab, a mAb targeting B lymphocytes, which became the first Food and Drug Administration–approved drug for SLE in decades (1-3). The success of belimumab establishes the clinical effectiveness of targeting pathogenic B lymphocytes in SLE; however, only ∼30% of lupus patients benefited from this treatment in clinical trials (4) with no current labeling for patients with nephritis. This warrants further investigations into novel therapeutics targeting B lymphocytes for lupus patients.

A defining hallmark of SLE is the presence of copious amounts of antinuclear autoantibodies (ANA) generated by autoreactive B lymphocytes (5, 6). The accumulation of ANA and other autoantibodies results in the formation of immune complexes that activate downstream effector mechanisms, including the fixation of complement, activation of monocytes, and the abundant secretion of proinflammatory cytokines, most notably type 1 IFNs (7, 8). These events ultimately lead to multisystem organ erosion, in particular, renal failure, which is the leading cause of death among SLE patients (9). The majority of pathogenic autoantibodies in human patients with SLE are highly mutated and isotype switched from IgM and IgD to IgG (5, 6). This indicates the autoantibodies are produced by affinity-matured B lymphocytes that have undergone class-switch recombination (CSR) and somatic hypermutation. Both CSR and somatic hypermutation are driven by activation-induced cytidine deaminase (AID), an enzyme that initiates double-strand

DNA breaks (DSB) predominantly in Ig genes (10-14). These DSB are then repaired via homologous recombination by the RAD51 protein family (15-20). Failure to repair DSB leads to cell apoptosis and loss of CSR, thus providing a potential approach for disrupting CSR and abrogating pathogenic autoantibody production (21).

AID is expressed principally in B lymphocytes residing within germinal centers (GCs) (http://www.immgen.org), and its expression is increased in the MRL/MpJ-Fas^{lpr}/J (MRL/*lpr*) mouse model of SLE (22). Previous studies show disrupting CSR through introduction of an *Aicda* null gene in MRL/*lpr* mice results in marked reduction in disease symptoms, including decreased nephritis and decreased levels of IgG autoantibodies, leading to increased survival (23). These mice also had high levels of autoreactive IgM that exerted a protective effect (24), suggesting that preservation of circulating IgM may be beneficial. However, when *Aicda* was knocked out in C57BL/6 mice carrying the *lpr* mutation, SLE-like disease was rapidly accelerated, driven by autoreactive IgM Abs (25). These conflicting studies raise the important question of whether IgM autoantibodies can substitute for their IgG counterparts in the development of SLE. The BXSB/MpJ mouse offers a model in which SLE-like disease develops through the effects of multiple SLE genetic loci and duplicated expression of *Tlr7* (26, 27). This model exhibits a spontaneous, robust extrafollicular response with reduced marginal zone B lymphocyte numbers and copious plasma cell generation (28, 29). BXSB mice, with a disease pathogenesis distinct from MRL/*lpr* mice, provide a good model to resolve controversy regarding the role of AID in SLE development.

4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), a disulfonated *trans*-stilbene derivative, has previously been shown to impair the DNA binding ability of the RAD51 complex, a core component in HR pathway, thus preventing the repair of AID-induced DSBs and inducing

the apoptosis of class-switching cells (21, 30, 31). *In vitro* and *in vivo* use of DIDS inhibits DNA break repair and leads to B cell death without evidence of off-target toxicity (21). The deficiency of AID abrogates the changes in apoptosis with DIDS treatment, confirming that DIDS-induced cytotoxicity is AID-dependent (21). In addition, our previous study demonstrated that treatment with DIDS dramatically diminished the diversity of the antibody repertoire in NOD mice in a manner similar to the genetic depletion of AID (32). Given the presence of hypergammaglobulinemia and the pathogenicity of IgG autoantibodies in lupus-prone mice (33), it is worthwhile to examine the effect of the DIDS treatment on SLE development.

In this study, we used CRISPR-Cas gene–editing techniques to ablate AID expression directly in the BXSB background. This technique allowed for gene targeting with minimal disruption to background genetics critical for the study of complex traits. We found SLE-like disease diminished in BXSB mice lacking AID with significant improvements in lupus nephritis, a rebound in marginal zone B lymphocyte populations and a restoration of splenic and GC architecture. We then investigated the ability of an inhibitor RAD51, previously found successful in limiting autoimmune disease (32), to attenuate the progression of lupus-like disease in our mice.

Material and Methods

Mice

Mice were maintained under specific pathogen-free conditions with the approval of the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University. BXSB/MpJ mice (henceforth referred to as "BXSB") were provided by Dr. D. Roopenian (JR000740, available from The Jackson Laboratory). BXSB.*Aicda*$^{-/-}$ mice were generated using CRISPR-Cas 9 technology as described (32). Briefly, the following single-guide RNAs were used to target *Aicda* exon 1 or 2 respectively: 5′-gaaattaatacgactcactataggAGTCAC-GCTGGAGACCGATAgttttagagctagaaatagc-3′ or 5′-gaaattaatacgactcactataggACTTCTTTTGC-TTCATCAGAgttttagagctagaaatagc-3′ (the uppercase letters being the complementary sequences to the targeted region). The resulting progeny were backcrossed to wild-type (wt) BXSB mice. N1 progeny were sequenced, and a founder with a 101-bp deletion on exon 1 was identified. This founder was backcrossed one additional time to BXSB to further reduce the chance of off-target effects with the resulting progeny then intercrossed to homozygosity. The line was maintained by brother–sister mating (referred to as BXSB.*Aicda*$^{-/-}$ in the text). Genotyping primers (Integrated DNA Technologies) used for *Aicda* were forward, 5′-TCACACAACAGCACTGAAGC-3′ and reverse, 5′-ACCCAAAAGACCTGAGCAGA-3′. PCR products were run on a 1.5% agarose (Lonza) gel and imaged on ChemiDoc XRS+ (Bio-Rad Laboratories) using Image Lab Software. The band size for wt is 230 bp and for *Aicda* knockout is 129 bp. Ear pinna collected at the time of notching for identification was used for genotyping.

In vitro CSR assay

Splenocytes from 8- to 9-wk-old BXSB and BXSB.*Aicda*$^{-/-}$ male mice were treated with ammonium–chloride–potassium (ACK) lysis buffer and then incubated with biotin–CD43 (S7; BD Biosciences) for 30 min at 4°C, followed by Streptavidin MicroBeads (Miltenyi Biotec) for 15 min at 4°C. Cells were washed and passed through MACS LD Columns (Miltenyi Biotec) according to the manufacturer's instructions. Enriched B lymphocytes were resuspended at 1×10^6/ml in X-VIVO (Lonza) supplemented with 2-ME (Sigma-Aldrich). Cells were then stimulated with IL-4 (50 ng/ml; PeproTech) and anti-CD40 (2 μg/ml, HM40-3; BD Biosciences) at 37°C (5% CO2) for 48 h. After 48 h, cells were restimulated with IL-4 (25 ng/ml) and anti-CD40 (1 μg/ml). At 96 h, cells were incubated with anti-IgG1 (A85-1; BD Biosciences) for 30 min at 4°C and collected on an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Results were analyzed using FlowJo software (FlowJo).

RT-PCR

Splenocytes were isolated from 12- to 15-wk-old BXSB and BXSB.*Aicda*$^{-/-}$ males and enriched for B lymphocytes as described in CSR assay. These B lymphocytes were resuspended at 1×10^6/ml in X-VIVO (Lonza) supplemented with 2-ME (Sigma-Aldrich). Cells were then stimulated with IL-4 (10 ng/ml; PeproTech) and anti-CD40 (0.1 μg/ml, HM40-3; BD Biosciences) at 37°C (5% CO2) for 72 h. Cells were harvested for RNA extraction using Quick-RNA MicroPrep Kit (catalog ZR1050; Zymo Research). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Primers for *Aicda* RT-PCR are 5′-CAGGGACGG-CATGAGACCT-3′ and 5′-TCAGCCTTGCGGTCTTCACA-3′ and primers for *Gapdh* are 5′-GAGAAACCTGCCAAGTATGATGAC-3′ and 5′-TGATGGTATTCAAGAGAGTAGGGAG-

3′ (32). PCR products were run on a 1.5% agarose (Lonza) gel and imaged on ChemiDoc XRS+ (Bio-Rad Laboratories) using Image Lab Software. The band size for *Aicda* is 302 bp and for *Gapdh* is 421 bp.

In vitro plasma cell generation

Splenocytes from 8-wk-old BXSB males were isolated to gain enriched B lymphocytes as described in CSR assay. Enriched B lymphocytes were stimulated with IL-4 (4 ng/ml; PeproTech), TGF-β (2 ng/ml; R&D Systems), anti-Igδ/dex (100 ng/ml; Fina Biosolutions) and retinoic acid (10 mM; Sigma-Aldrich) plus either anti-CD40 (2 μg/ml, HM40-3; BD Biosciences) for T cell–dependent response or LPS (5 μg/ml; Sigma-Aldrich) for T cell–independent response. After 66 h, cells were stained with Abs and analyzed using flow cytometry as described.

Flow cytometry

Splenocytes were treated with ACK lysis buffer. (For the 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid [DIDS] experiments, splenocytes were not lysed, because DIDS-exposed RBCs are refractory to lysis. RBC were excluded by gating for size in those experiments.) Splenocytes and femoral bone marrow were counted using a Nexcelom cell counter and resuspended at 2×10^7cells/ml. Cells were then stained with fluorochrome-conjugated Abs for 30 min at 4°C, and propidium iodide (BioLegend) or 7-aminoactinomycin D (7-AAD) (BioLegend) was added before analyzing to differentiate live and dead cells. The following fluorochrome-conjugated Abs (clones) were used: B220 (RA3-6B2), CD4 (GK1.5), CD8 (53-6.7), CD21 (7E9), CD23 (B3B4), GL7 (GL7), CD73 (TY/11.8), CD138 (281-2), CD11b (M1/70), CXCR5 (L138D7), ICOS (C398.4A), and PD-1 (RMP1-30) (BioLegend) and FAS (Jo2) and IgG1 (A85-1) (BD

Biosciences). Apoptosis was characterized using Apoptosis Kit (BioLegend) following the manufacturer's instructions. All experiments were performed on an Attune NxT Flow Cytometer (Thermo Fisher Scientific), and data were analyzed using FlowJo software (FlowJo). All analyses were done after gating on single cells.

ANA analysis

Antinuclear Abs in the sera were detected using an ANA Test Kit (Antibodies). Either FITC anti-mouse κ L chain (187.1; BD Biosciences) or FITC anti-mouse IgM (RMM-1; BioLegend) was used as secondary Ab. Slides were imaged with an Eclipse T*i* microscope using NIS-Elements software (Nikon). Intensity was measured using ImageJ software (National Institutes of Health).

Urine albumin and creatinine assay

Free catch urine samples were collected from BXSB (n = 8) and BXSB.*Aicda*$^{-/-}$ (n = 6) males at 6 and 16 wk of age. Samples were stored at -20°C until processed using the Mouse Albumin ELISA Kit (Bethyl Laboratories) and Creatinine Colorimetric Assay Kit (catalog 500701; Cayman Chemical) per manufacturer's instructions. The albumin to creatinine ratio was calculated by dividing albumin concentration in milligrams per deciliter by creatinine concentration in milligrams per deciliter.

Sera Ab ELISA

Different Ab isotypes were quantified by ELISA as described (28). Briefly, plates were incubated with appropriate coating Ab at 4°C overnight; diluted sera were added to the plate and incubated for 1h at room temperature (RT), followed by detection Abs for 1h at RT. Anti-dsDNA Abs were

quantified as described (34). The plate was coated with calf thymus DNA (Sigma-Aldrich) at 4°C overnight. After blocking for 1h at RT, diluted sera were added to the plate and incubated for 1h at RT, followed by detection Abs for 1h at RT. Detection Abs used in this study were either goat anti-mouse κ-chain (polyclonal; SouthernBiotech) or anti-mouse IgM (polyclonal; Bethyl Laboratories) conjugated to alkaline phosphatase. Plates were developed with 1-Step *p*-nitrophenyl phosphate disodium salt (Thermo Fisher Scientific) and read on an Infinite M200 PRO plate reader using Magellan 7.0 software (Tecan).

Immunofluorescence staining

Tissues were embedded in OCT (Thermo Fisher Scientific), frozen on dry ice for 10 min, and stored at -80°C until processing. Frozen tissues were sectioned at 8 μm using a Microm HM550 Cryostat (Thermo Fisher Scientific). Slides were fixed with acetone (catalog A18-4; Fisher Chemical) at -20°C for 10 min and washed with PBS (Life Technologies) for three times. A PAP pen (MilliporeSigma) was used to circle the sections and blocking buffer (3% FBS, HyClone; Thermo Fisher Scientific) was added onto the slides and incubated in a moist chamber for over 1 h. Slides were washed with PBS for three times and incubated with fluorochrome-conjugated Abs against B220 (RA3-6B2), CD4 (RM4-5), CD73 (TY/11.8), and GL7 (GL7) (BioLegend) and κ-chain (187.1) (BD Biosciences) and C3c (polyclonal, Nordic-MUbio) at RT for 1h. CD16/32 (93; BioLegend) was used for Fc Block prior to Ig labeling for 30 min at RT. DAPI was used for nuclear counterstaining. Slides were imaged using an LSM 880 Confocal or Zeiss Axio Observer (ZEISS) microscope.

Renal pathology

Kidneys were fixed in 10% formaldehyde (Fisher Chemical) overnight and transferred to 70% ethanol (Decon Laboratories). Samples were sent to Histo-Scientific Research Laboratories for processing and H&E staining. The sections were analyzed in a blinded fashion and scored as described by a trained pathologist (35). Briefly, kidneys were scored for the severity of glomerular mesangial proliferation and immune complex deposition, tubular degeneration with protein casts, interstitial inflammation, and vasculitis.

ELISPOT

ELISPOT for dsDNA-producing plasma cells was performed as described (36). Briefly, ELISPOT MultiScreen plates (catalog MSIPS4W10; MilliporeSigma) were precoated with 10 μg/ml methylated BSA (Sigma-Aldrich) at 37°C for 2 h, followed by 10 μg/ml calf thymus dsDNA (Sigma-Aldrich) or PBS (control group) at 4°C overnight. Femurs and spleen were collected from 12-wk-old BXSB (n = 5) and BXSB.*Aicda*$^{-/-}$ (n = 6) males. Bone marrow cells were flushed using syringes and resuspended at 5×10^6/ml in RPMI 1640 (Life Technologies) supplemented with 2 g/L sodium bicarbonate (Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 1% penicillin–streptomycin (Sigma-Aldrich), and 10% FBS and 100 mM 2-ME (Sigma-Aldrich). Splenocytes were treated with ACK lysis buffer and resuspended at 2×10^6/ml in complete RPMI 1640. After blocking with complete RPMI 1640 RT for 2 h, cells were seeded and cultured at 37°C (5% CO2) overnight. After washing with PBS three times and PBS with Tween-20 three times, plates were incubated with either goat anti-mouse κ (polyclonal; SouthernBiotech) or anti-mouse IgM (polyclonal; Bethyl Laboratories) conjugated to alkaline phosphatase for 1h. Spots were developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Thermo

Fisher Scientific), and plates were read on an automated ELISPOT reader and analyzed by the software (AID Autoimmun Diagnostika). The number of anti-dsDNA–secreting cells was calculated by subtracting the number in the control group (no dsDNA coating) from the number in the experimental group.

In vitro DIDS treatment

Splenocytes from 7-wk-old BXSB males (n = 4) were stimulated with IL-4 (50 ng/ml; PeproTech) and anti-CD40 (0.2 μg/ml, HM40-3; BD Biosciences) in the presence of DIDS (Tocris Bioscience) (0, 50, 100, 150, and 300 μM) or potassium bicarbonate (Fisher Chemical) at 37°C (5% CO2) for 48 h. After 48 h, cells were restimulated with IL-4 (25 ng/ml) and anti-CD40 (0.1 μg/ml). At 96 h, cells were harvested and incubated with fluorochrome-conjugated antibodies (clones) against B220 (RA3-6B2), CD80 (16-10A1), CD86 (GL-1), MHC class II (M5/114.15.2), CD11c (N418), F4/80 (BM8), CD4 (GK1.5), and CD8 (53-6.7, all from BioLegend) and IgG1 (A85-1; BD Biosciences) for 30 min at 4°C and collected on an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Results were analyzed using FlowJo software (FlowJo). DIDS was reconstituted in 0.1 M potassium bicarbonate and added to the cells on day 0 of culture.

DIDS treatment

DIDS (Tocris Bioscience) was injected i.p. at 50 mg/kg beginning at 6 weeks of age in BXSB male mice or MRL/*lpr* female mice. Mice were injected weekly for a total of eight injections. DIDS was reconstituted in 0.1 M potassium bicarbonate to a concentration of 0.1 M and further diluted for injection in sterile PBS. Control mice received 0.1M potassium bicarbonate diluted in sterile PBS at the same injection interval.

Statistics

Statistical significance was determined by Mann–Whitney U test using Prism (GraphPad). In the figures, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Log rank was used for survival assessment.

Results

Disruption of AID prohibits CSR and retards lupus-like disease progression in BXSB mice

To determine the effect of affinity maturation on the pathogenesis of the lupus-like disease in the BXSB mouse, we employed CRISPR-Cas gene editing to target the *Aicda* gene directly in BXSB zygotes. Because of the complex background genetics of lupus-like disease, we chose this technology to preserve the background genetics of our model. Resulting PCR product sizes are shown in Fig. 4.1A. B lymphocytes were cultured under conditions to induce CSR (37). RT-PCR confirmed that lack of transcript produced in BXSB.*Aicda*$^{-/-}$ B lymphocytes under stimulatory conditions (Fig. 4.1B). Although wt BXSB B lymphocytes successfully produced a subset of cells expressing IgG1, the BXSB.*Aicda*$^{-/-}$ B lymphocytes remained unswitched (Fig. 4.1C). Sera Ig levels in BXSB.*Aicda*$^{-/-}$ mice reflect this deficiency as well, because these mice lack IgG isotypes and exhibit elevated IgM levels as seen previously in *Aicda*$^{-/-}$ mice (Fig. 4.1D) (23, 32, 38). Male BXSB.*Aicda*$^{-/-}$ exhibited extended lifespans with 100% of mice surviving past 36 wk, whereas wt BXSB males averaged 15-wk survival (Fig. 4.1E).

Given that male BXSB.*Aicda*$^{-/-}$ mice survived significantly longer than their wt BXSB counterparts, we sought to phenotype disease progression in these two strains. BXSB lupus-like disease is characterized by elevated sera IgG (Fig. 4.1D), glomerular disease with IgG and C3

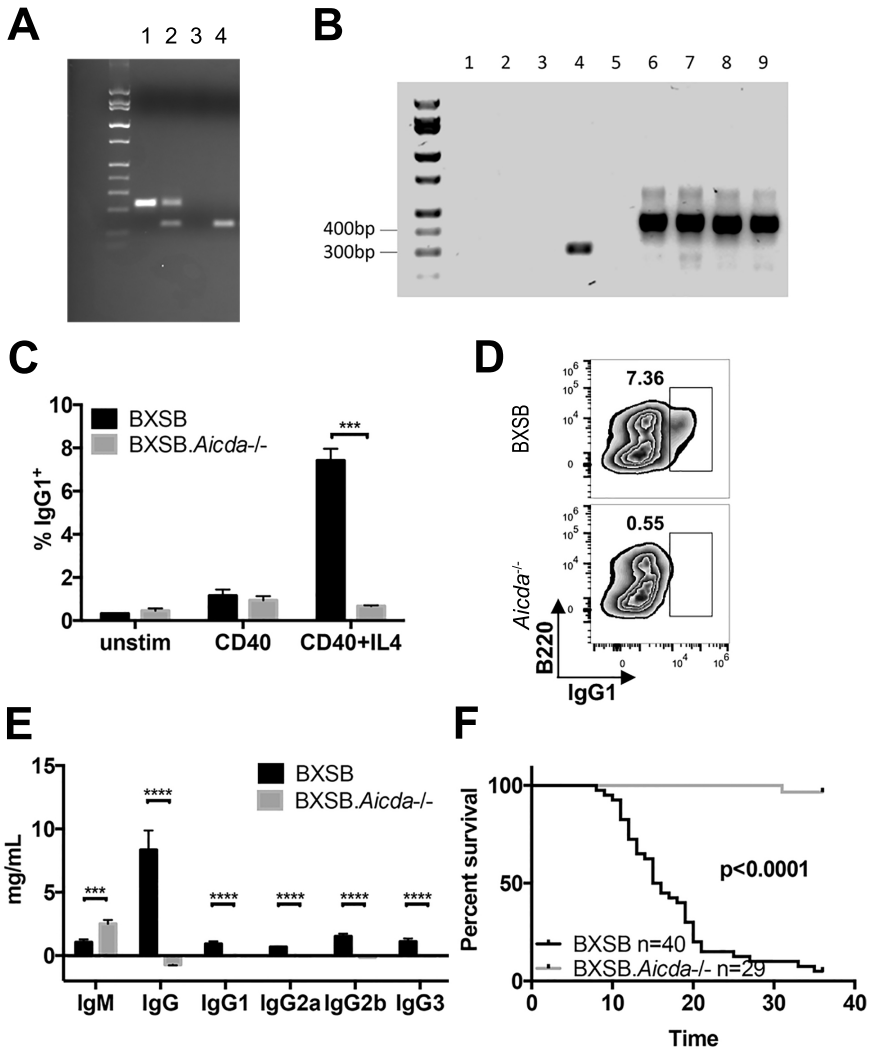

FIGURE 4.1 Disruption of *Aicda* extends survival in BXSB mice. (**A**) CRISPR-Cas deletion shortens *Aicda* in BXSB mice. Lane 1, wt; lane 2, heterozygous; lane 3, water; lane 4, homozygous mutation. (**B**) Representative data of AID expression using RT PCR upon stimulation of B cells *in vitro*. Lane 1, water; lanes 2–5, *Aicda*; lanes 6–9, *Gapdh*; lanes 2 and 6, BXSB unstimulated; lanes 3 and 7, BXSB.*Aicda*$^{-/-}$ unstimulated; lanes 4 and 8, BXSB CD40 + IL-4 stimulated; lanes 5 and 9, BXSB.*Aicda*$^{-/-}$ CD40 + IL-4 stimulated. (**C**) *In vitro* stimulation of B cells to induce class switch to IgG1 is abrogated in BXSB.*Aicda*$^{-/-}$ mice. The percentage of IgG1^{+} cells in the viable cell population were shown (n = 3 for both groups). Data are presented as mean±SEM. Unpaired t test was used for statistical analysis. ***p<0.001. (**D**) Representative FACS zebra plots of (C) data. (**E**) IgM and IgG isotypes present in sera from 12-wk-old BXSB (n = 15) and BXSB.*Aicda*$^{-/-}$ males (n = 11). Data are presented as mean±SEM. Statistical significance was determined by Mann–Whitney U test. ***p<0.001, ****p<0.0001. (**F**) Survival curve for BXSB and BXSB.*Aicda*$^{-/-}$ males. Log-rank test was used for statistical analysis.

deposition and the presence of antinuclear autoantibodies (28, 39-41). Histologic renal pathology seen in BXSB.*Aicda*$^{-/-}$ mice was significantly less than in wt BXSB mice (Fig. 4.2A, Fig. 4.3). The primary renal lesions in wt BXSB mice were membranoproliferative glomerulonephritis and

tubular protein casts. Interstitial inflammation was predominantly minimal, and there was no evidence of vasculitis. As BXSB.*Aicda*$^{-/-}$ mice lack IgG, renal Ig deposition was evaluated using

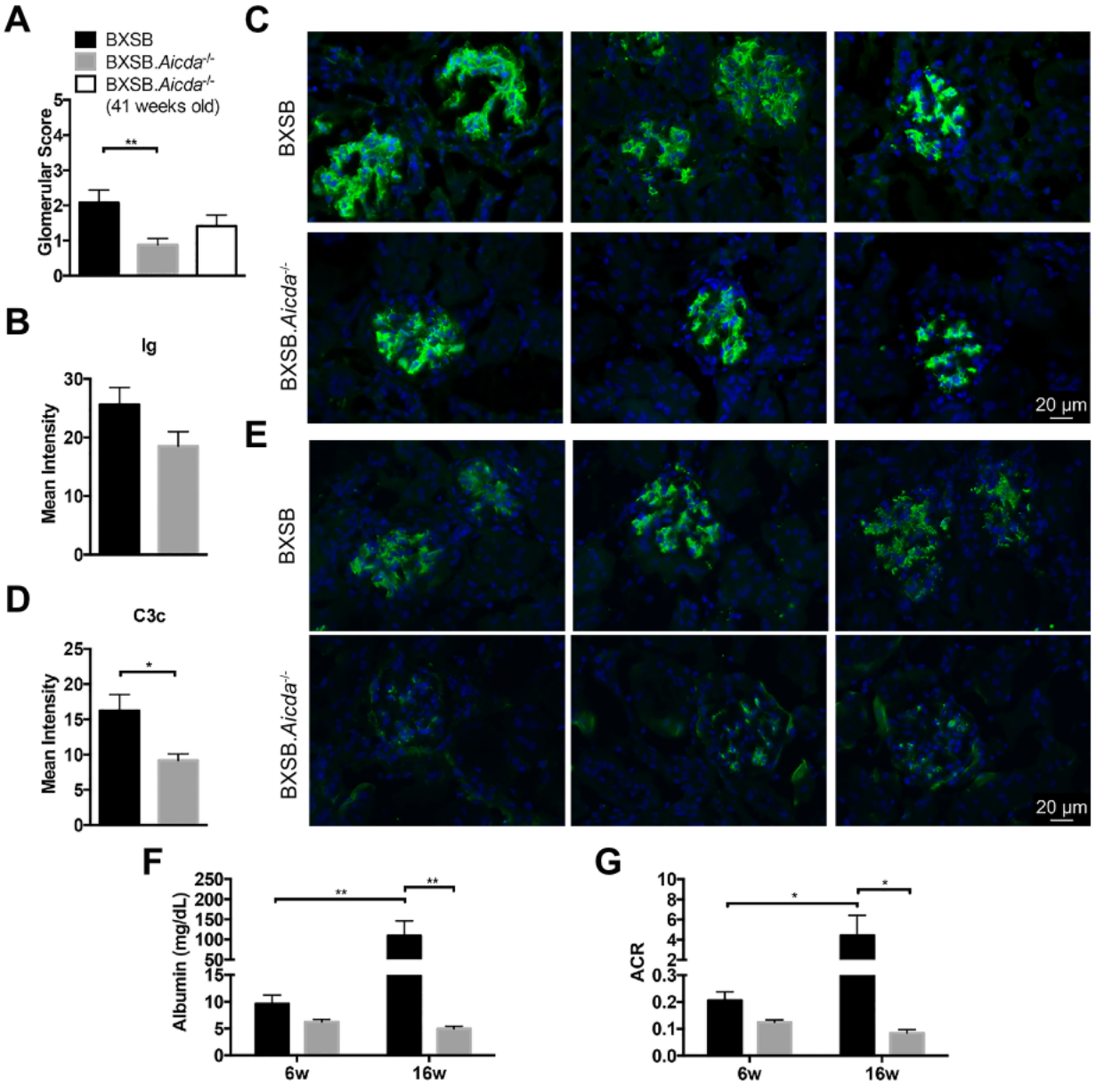

FIGURE 4.2 Complement activation was attenuated, and kidney function was improved in the absence of AID. (**A**) Glomeruli scores were compared between 12-wk-old BXSB (n = 12), 12-wk-old BXSB.*Aicda*$^{-/-}$ (n = 16), and 41-wk-old BXSB.*Aicda*$^{-/-}$ (n = 12) mice. (**B**–**E**) Kidney images from 12-wk-old BXSB (n = 5) and BXSB.*Aicda*$^{-/-}$ (n = 5) mice. Ig (green), C3c (green), and DAPI (blue). Images were evaluated for mean intensity in (B) and (D). Representative Ig staining images were shown in (C), and C3c staining was shown in (E) (original magnification ×400). Statistical significance was determined by Mann–Whitney U test. *p<0.05, **p<0.01. (**F** and **G**) Free catch urine samples were collected from 6-wk-old and 16-wk-old BXSB (n = 8) and BXSB.*Aicda*$^{-/-}$ (n = 6) mice. Albuminuria is shown in (F), and the albumin-to-creatinine ratio (ACR) is shown in (G). Statistical significance was determined by two-way ANOVA. *p<0.05, **p<0.01.

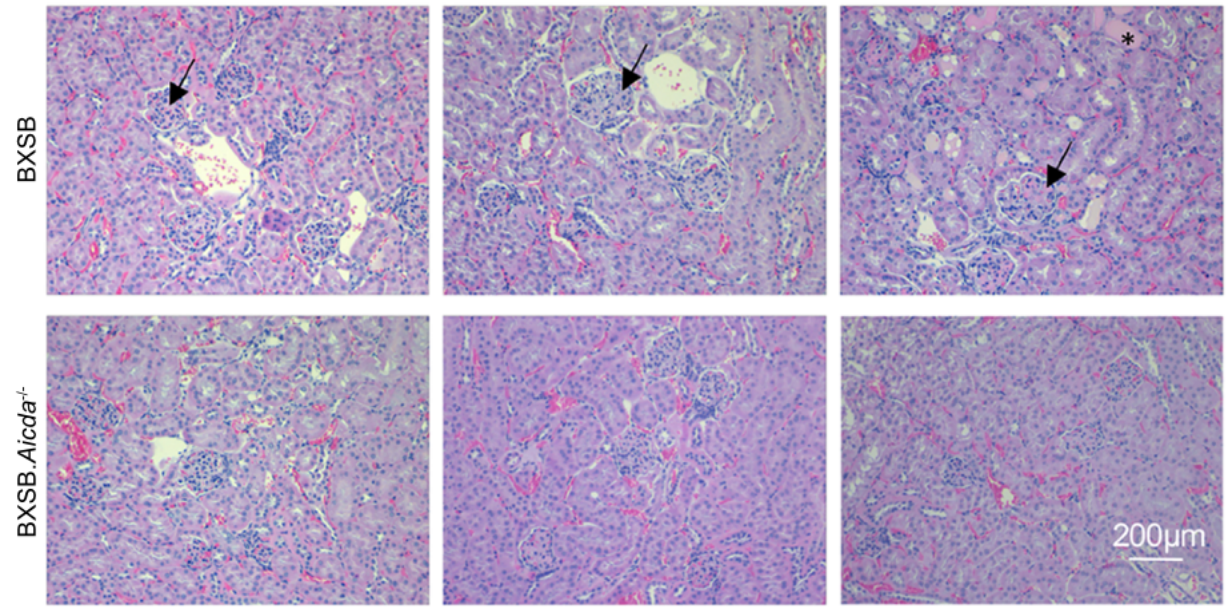

FIGURE 4.3 Significant improved renal pathology in BXSB.*Aicda*$^{-/-}$ mice. Representative images of H&E stained kidney slides from 12-week-old BXSB (n=12) and BXSB.*Aicda*$^{-/-}$ (n=16) mice were shown (magnification 200×). The arrows show the mesangioproliferative glomerulonephritis and the asterisk is on a protein cast in the tubules.

an all isotype (pan)-Ig detection Ab. The mean intensity of glomerular Ab deposition between the two strains did not significantly differ; however, staining appears to be confined to the mesangial regions in the BXSB.*Aicda*$^{-/-}$ mice and extends to the glomerular loops in wt BXSB (Fig. 4.2C, 2D, Fig. 4.4). Despite no measured differences in renal Ig staining, C3c deposition was significantly reduced in BXSB.*Aicda*$^{-/-}$ mice compared with wt BXSB mice (Fig. 4.2E, 4.2F). To evaluate levels of ANA, a pan-Ig Ab was used with BXSB.*Aicda*$^{-/-}$ mice exhibiting reduced ANA staining intensity compared with wt BXSB mice (Fig. 4.5A). To assess levels of autoreactive IgM Abs, ANA intensity was measured using an IgM detection Ab, and no differences in labeling intensity were seen in the BXSB.*Aicda*$^{-/-}$ compared with wt BXSB mice (Fig. 4.5A). The predominant pattern seen with the IgM ANA was perinuclear localization, whereas the pan-Ig Ab showed principally nuclear staining. (Fig. 4.6). Anti-dsDNA Abs were also quantitated using a pan-Ig Ab as well as an IgM Ab; however, no significant differences were observed with low

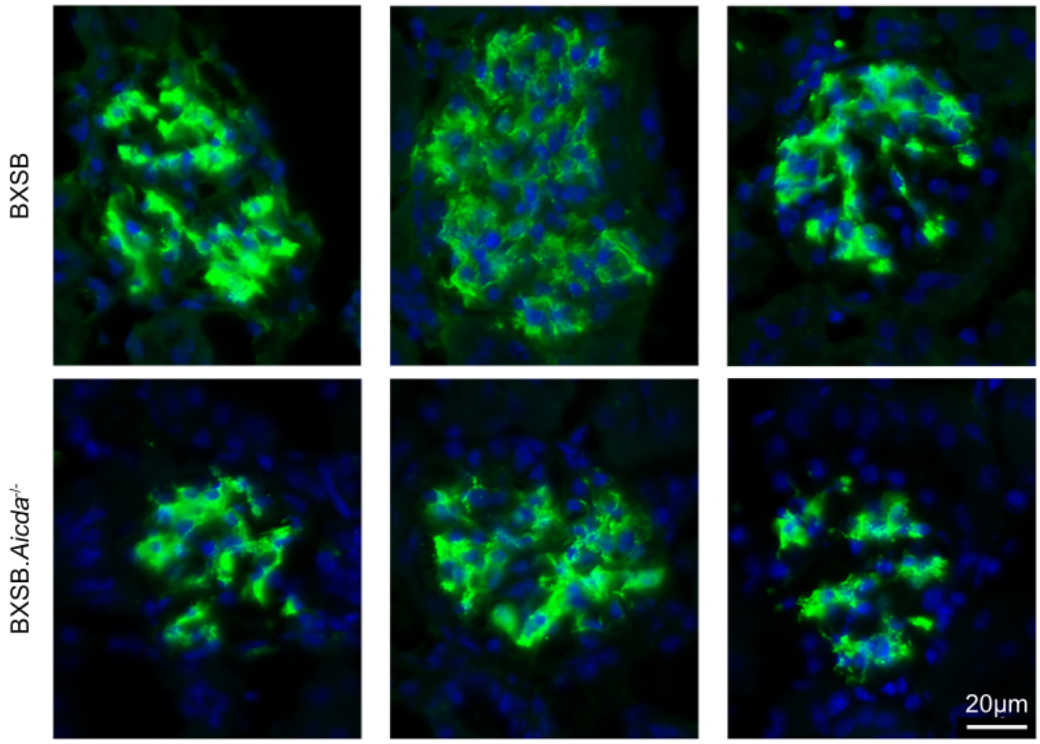

FIGURE 4.4 Magnified glomerular Ig staining pattern in BXSB and BXSB.*Aicda*$^{-/-}$ mice. Representative immunofluorescence images of Ig deposition in glomeruli of 12-week-old BXSB (n=5) and BXSB.*Aicda*$^{-/-}$ (n=5) mice are shown. Green: Ig, blue: DAPI.

levels detected in both strains (Fig. 4.5B). Anti-dsDNA ELISPOT showed no differences in bone marrow or splenic plasma cell numbers between the two strains (Fig. 4.5C, 4.5D).

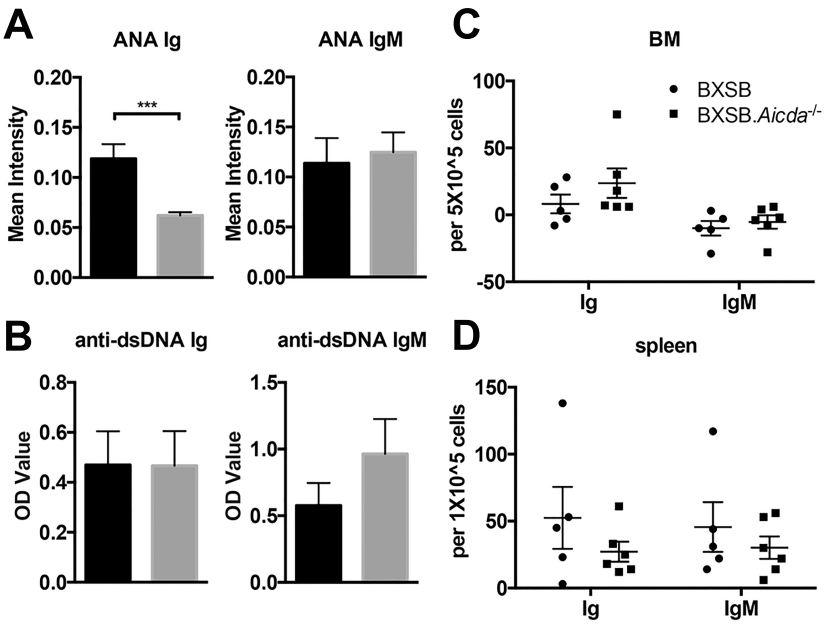

FIGURE 4.5 Levels of circulating antinuclear Abs were reduced in AID-deficient BXSB mice. (**A**) Sera ANA intensity detection of pan-Ig and IgM autoantibodies in 12-wk-old BXSB (n = 15) and BXSB.*Aicda*$^{-/-}$ (n = 11) mice. (**B**) Sera anti-dsDNA autoantibody detection for pan-Ig and IgM isotypes in these same mice. Anti-dsDNA plasma cell ELISPOT analysis of bone marrow (**C**) and spleen (**D**) from 12-wk-old BXSB (n = 5) and BXSB.*Aicda*$^{-/-}$ (n = 6) mice. Spot numbers in (C) and (D) are normalized to control wells. Data are presented as mean±SEM. Statistical significance was determined by Mann–Whitney U test. ***p<0.001.

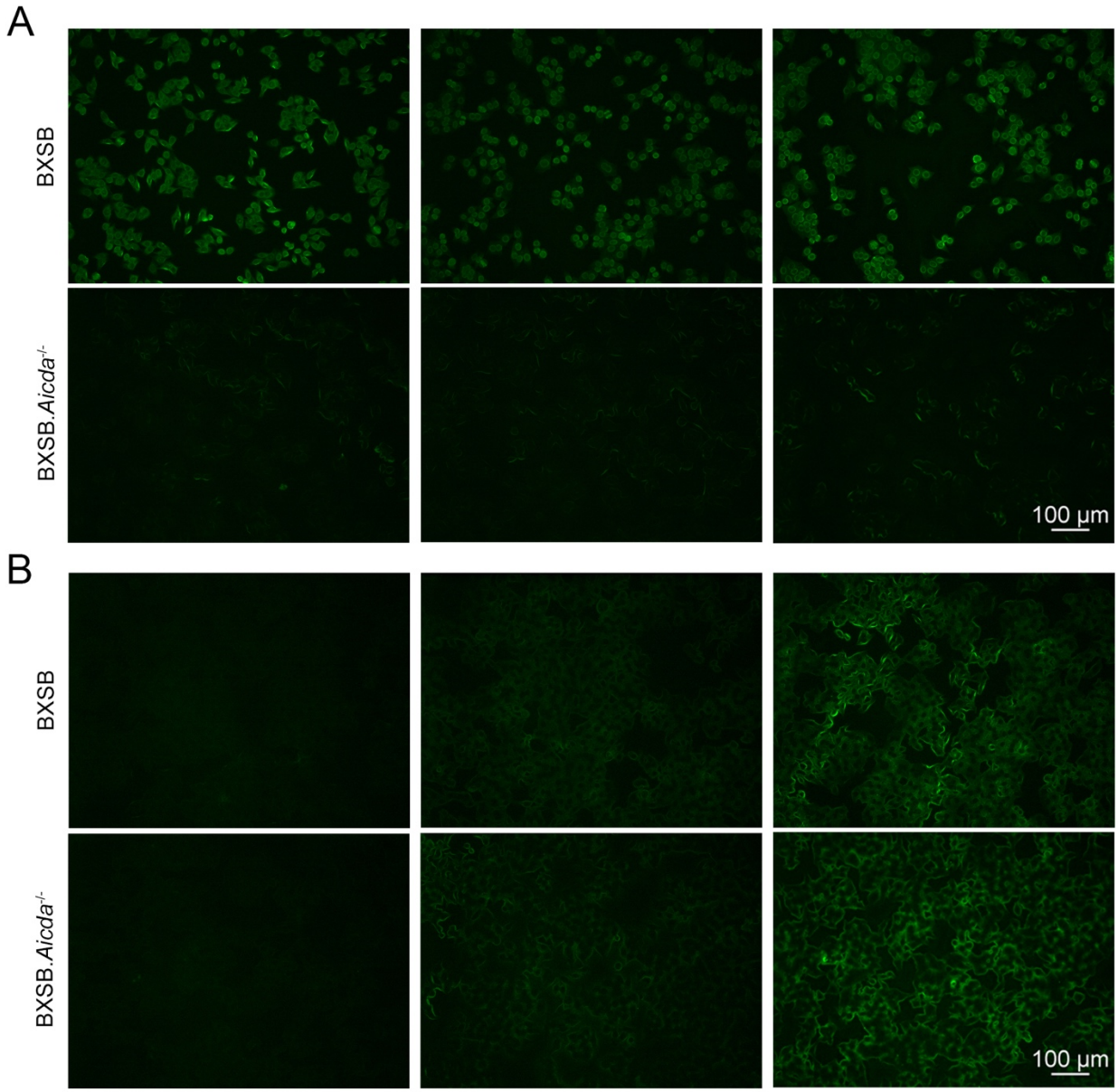

FIGURE 4.6 Reduced anti-nuclear antibodies (ANA) in sera of AID-deficient mice. Sera from 12-week-old BXSB (n=15) and BXSB.*Aicda*$^{-/-}$ (n=11) mice were assessed by ANA slides. Representative images of ANA slides stained with pan Ig (**A**) or IgM (**B**) are shown (magnification 100×).

Splenic cellularity as assessed using flow cytometry did not vary dramatically between the two strains. B and T lymphocyte percentages were unchanged (Fig. 4.7A). The marginal zone B lymphocyte compartment is known to be reduced in male BXSB mice (28, 41), and this reduction was at least partially restored in mice deficient in AID (Fig. 4.7B). Consistent with other *Aicda*$^{-/-}$

strains (32, 42), the GC B cell compartment was expanded in the BXSB.*Aicda*$^{-/-}$ mice (Fig. 4.7C); however, a corresponding increase in T follicular helper (Tfh) cells was not appreciated (Fig. 4.7D). The characteristic expansion of splenic monocytes (CD11b^{+}) did not differ between the two strains (Fig. 4.7E). Splenic architecture appears distorted in BXSB mice, with follicular and GC structure difficult to determine; however, AID deficiency resulted in restoration of this architecture with more discernable follicles and GCs (Fig. 4.7F)

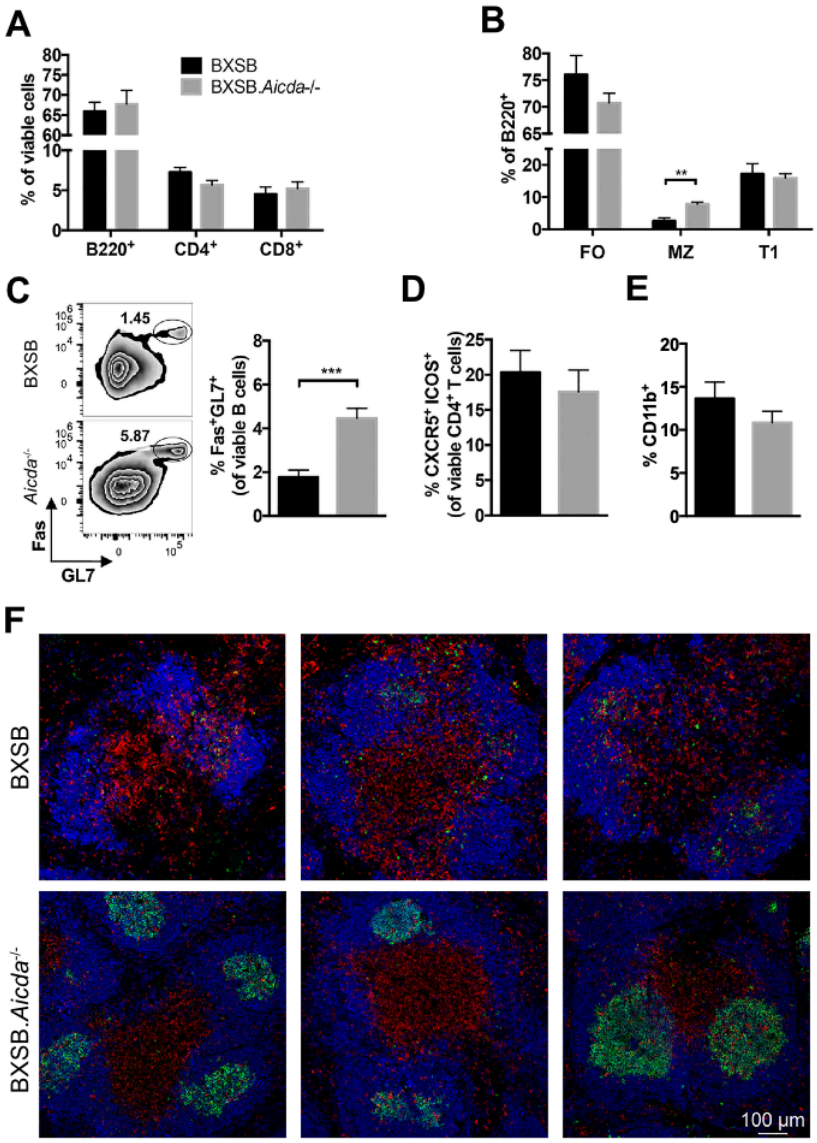

FIGURE 4.7 Marginal zone (MZ) B cell depletion and GC integrity restored in BXSB.*Aicda*$^{-/-}$ mice. Splenocytes of 12-wk-old BXSB (n = 7) and BXSB.*Aicda*$^{-/-}$ (n = 9) males were analyzed. (**A**) The percentages of B220^{+}, CD4^{+}, and CD8^{+} cells in the viable cell population were compared between wt and *Aicda*$^{-/-}$ mice. (**B**) The percentages of follicular (FO; CD23^{+}CD21^{+}), MZ (CD23$^{low/-}$CD21^{+}), and transitional 1 (T1; CD23^{-}CD21^{-}) B cells in the B lymphocyte population are shown. (**C**) Representative flow cytometric zebra plots showing the percentage of GC B cells (Fas^{+}GL7^{+}) in the viable B lymphocyte population, with summary data shown on the right. (**D**) The percentage of Tfh cells (CXCR5^{+}ICOS^{+}). (**E**) The percentage of monocytes (CD11b^{+}). (**F**) Representative confocal images showing the follicles in the spleen. Blue, B220; green, GL7; and red, CD4. Data are presented as mean±SEM. Statistical significance was determined by Mann–Whitney U test. Results are representative for at least three independent experiments. **p<0.01, ***p<0.001.

The ability of BXSB B lymphocytes to mature into plasma cells remained unhampered in the absence of AID

Extrafollicular plasma cell generation is a hallmark of lupus-like disease in BXSB mice (28, 29). We hypothesized that inability to undergo CSR would inhibit plasma cell generation in this model. We found no significant differences in splenic or bone marrow $CD138^+$ populations between wt BXSB and BXSB.*Aicda*$^{-/-}$ (Fig. 4.8A). To determine if this response was intrinsic to B lymphocytes, we performed *in vitro* stimulation for plasma cell generation. We found that both wt BXSB and BXSB.*Aicda*$^{-/-}$ B lymphocytes were able to differentiate into plasma cells under both T cell–independent and T cell–dependent culture conditions (Fig. 4.8B, 4.8C). As negative

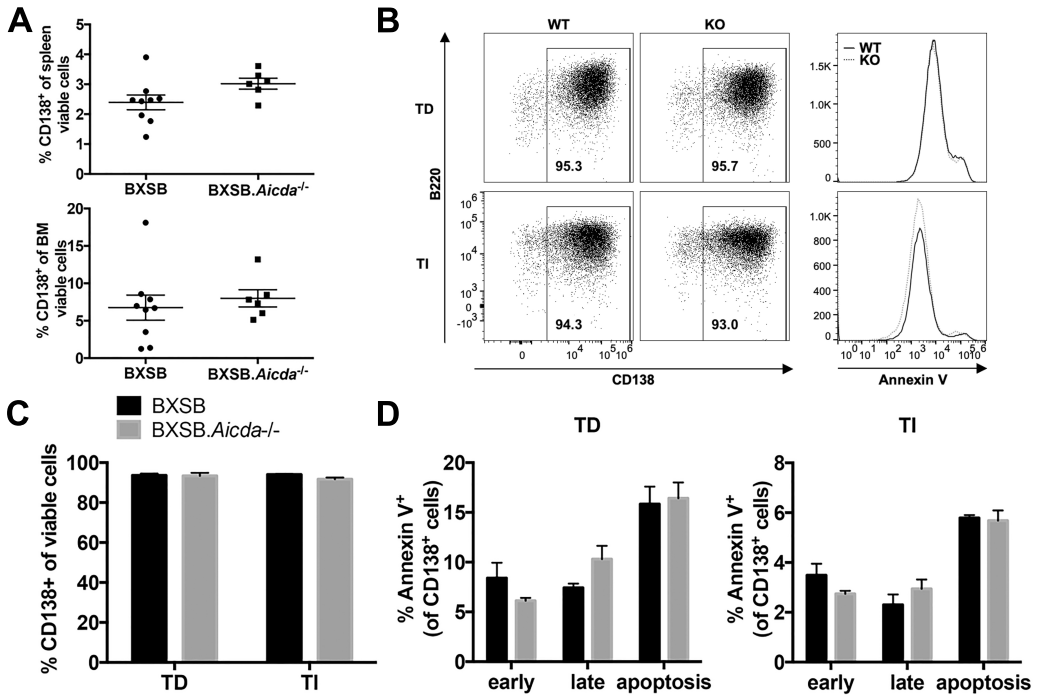

FIGURE 4.8 No effect on plasma cell generation both *in vivo* and *in vitro* in the absence of AID. (**A**) The percentage of plasma cells ($CD138^+$) in either viable splenocytes or viable bone marrow cells were compared between 12-wk-old BXSB (n = 9) and BXSB.*Aicda*$^{-/-}$ (n = 6) males. (**B**–**D**) Enriched B lymphocytes from 8-wk-old BXSB (n = 3) and BXSB.*Aicda*$^{-/-}$ (n = 3) males were stimulated to differentiate into plasma cells either with anti-CD40 for T cell–dependent (TD) response or LPS for T cell–independent (TI) response. Representative plasma cell staining in viable cells and annexin V staining in $CD138^+$ cells is shown in (B), and data are summarized in (C) and (D). Early apoptosis (early) and late apoptosis (late) were differentiated by annexin V and 7-AAD, respectively. Early (annexin V^+7-AAD^-), late (annexin V^+7-AAD^+), and all apoptosis (apoptosis; annexin V^+) were compared in (D). Data are presented as mean±SEM. Statistical significance was determined by Mann–Whitney U test. Results are representative for at least three independent experiments.

selection during B lymphocyte maturation is thought to lead to apoptosis in low-affinity B cells (43), we asked if BXSB.*Aicda*$^{-/-}$ B lymphocytes unable to undergo somatic hypermutation would experience an increase in cell death. We found no increase in apoptosis indicators in the AID-deficient BXSB B lymphocytes (Fig. 4.8D).

Memory B lymphocytes are expanded in AID-deficient BXSB mice

Previously, we have reported expansion of B memory cells in AID-deficient mice on the NOD background (32). These memory B cells were also expanded in BXSB.*Aicda*$^{-/-}$ mice, with the majority of GC B cells being CD73^{+} (Fig. 4.9A, 4.9B). A change in CD73 mean fluorescence

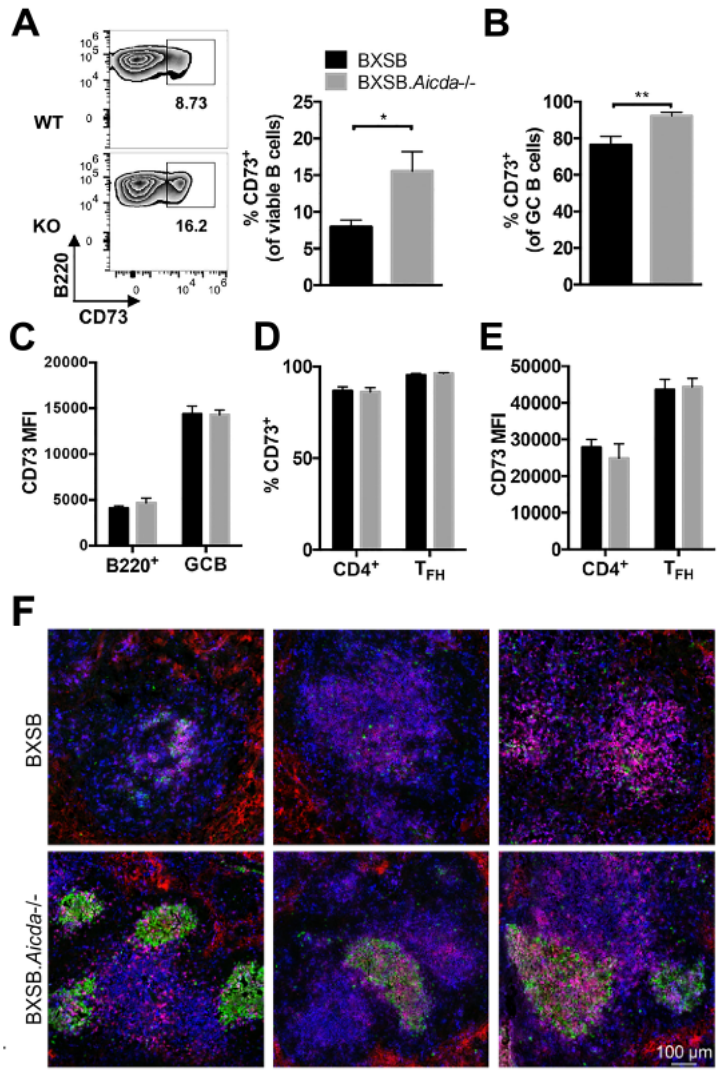

FIGURE 4.9 Memory B cell population expanded in BXSB.*Aicda*$^{-/-}$ mice. Splenocytes of 12-wk-old BXSB (n = 6) and BXSB.*Aicda*$^{-/-}$ (n = 6) males were analyzed. (**A**) Representative flow cytometric zebra plots showing the percentage of CD73^{+} in the viable B lymphocyte population, with summary data shown on the right. (**B**) The percentages of CD73^{+} GC B cells. (**C**) MFI of CD73 on B cells and GC B cells. (**D**) The percentages of CD73^{+} cells in the viable CD4^{+} and Tfh (PD-1^{+}ICOS^{+}) cell populations. (**E**) MFI of CD73 in CD4^{+} cells and Tfh cells. (**F**) Representative confocal images of spleen showing the distribution of CD73. Blue, CD4; green, GL7; and red, CD73. Data are presented as mean±SEM. Statistical significance was determined by Mann–Whitney U test. Results are representative for at least three independent experiments. *p<0.05, **p<0.01.

intensity (MFI) on B lymphocytes was not appreciated (Fig. 4.9C). CD73 was also present on a large proportion of $CD4^+$ lymphocytes and Tfh cells (Fig. 4.9D). No change in CD73 MFI was seen on these $CD4^+$ T lymphocytes (Fig. 4.9E). These $CD73^+$ cells were generally associated with the distorted follicular structures seen in wt BXSB, whereas BXSB.*Aicda*$^{-/-}$ mice showed localization of CD73 mainly in GC structures (Fig. 4.9F).

Disruption in repair of AID-induced DNA breaks resulted in minimal attenuation of lupus-like disease in BXSB and MRL/lpr mice

Previous work demonstrated that DIDS inhibits RAD51 function and induces apoptosis of class-switching cells. We recently showed that use of DIDS significantly delayed the development of type 1 diabetes in NOD mice (32). We sought to investigate the effect of DIDS treatment in BXSB model of SLE. *In vitro* assessment of DIDS on B lymphocytes stimulated to undergo CSR showed a dosage dependent loss of total B cells and $IgG1^+$ cells (Fig. 4.10A, 4.10B) without a corresponding decrease in T lymphocytes (Fig. 4.10C). Macrophages and dendritic cells (DCs) also showed a dosage-dependent decrease in cell numbers (Fig. 4.10D), and whereas B cell activation markers increased with dosage of the DIDS (Fig. 4.10E–G), DC expression of CD80 and CD86 declined (Fig. 4.10H–J). To assess if these results translated *in vivo*, BXSB mice were treated weekly, beginning at 6 wk of age with 50 mg/kg DIDS for eight injections. mice were euthanized 1 wk following the final injection. $CD4^+$ T and B lymphocyte percentages in the spleen were decreased with treatment (Fig. 4.11A); however, no differences were seen in splenic B cell subsets (Fig. 4.11B). GC B cell percentage was unaffected, whereas the Tfh cell population trended toward reduction in the presence of DIDS (Fig. 4.11C, 4.11D). The percentage of splenic monocytes was significantly reduced with DIDS administration (Fig. 4.11E). ANA intensity

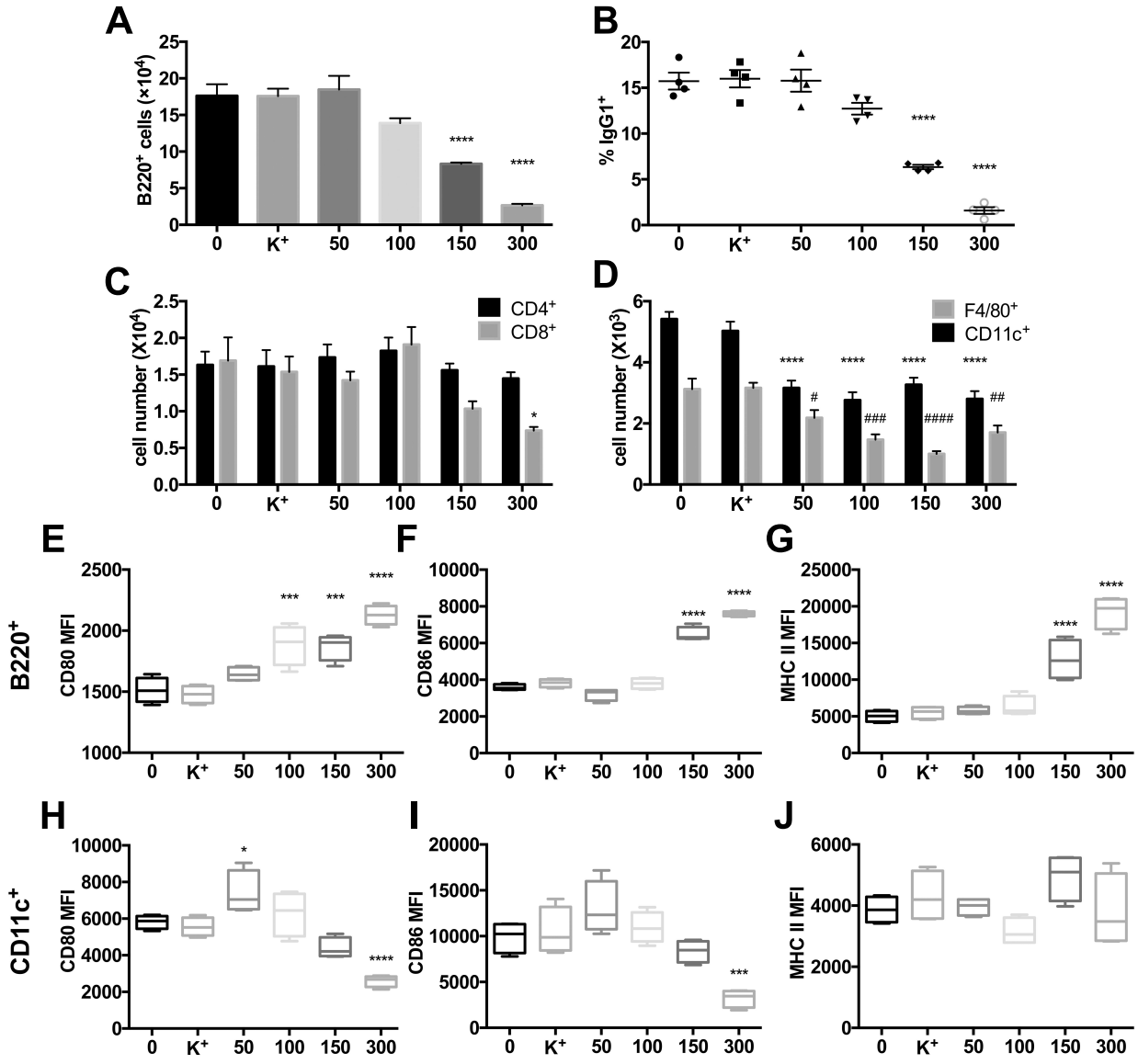

FIGURE 4.10 B lymphocytes undergo cell death and fail to class switch upon stimulation in the presence of DIDS *in vitro*. Wt BXSB splenocytes were stimulated with CD40 and IL-4 along with DIDS (0, 50, 100,150 or 300μM) or potassium bicarbonate (K^+) (n=4). (A, C, D) B and T lymphocyte, dendritic cell and macrophage cell numbers after 96 hours of culture. (B) The percentage of $IgG1^+$ cells after 96 hours of culture. Data are presented as mean±SEM. (E-J) MFI of CD80, CD86 and MHC II were analyzed for $B220^+$ gated cells (E-G) and $CD11c^+$ gated cells (H-J). (E-J) Data are presented as minimum to maximum. Statistical significance was determined by One-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are representative of three independent experiments.

remained consistent between the treated and control groups (Fig. 4.11F), whereas the circulating IgG levels over the course of the experiment trended toward reduction but did not reach

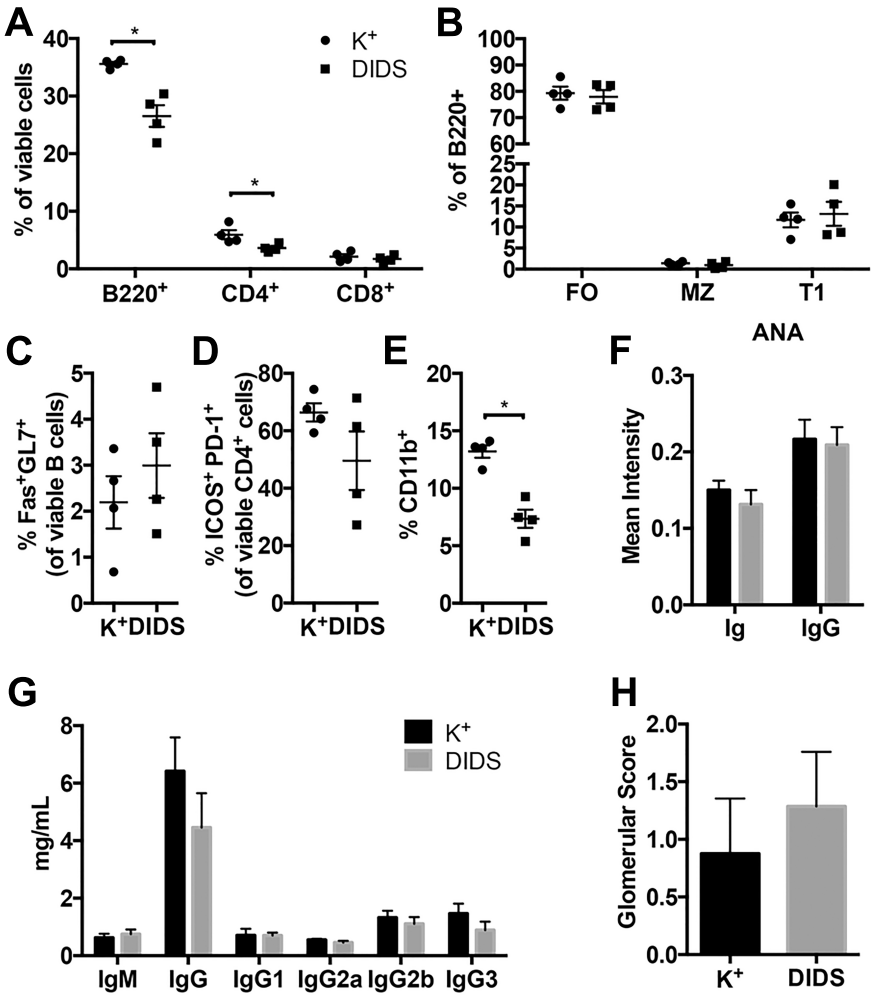

FIGURE 4.11 A high dose of DIDS treatment to block DNA repair of attempted CSR does not significantly alter the course of SLE-like disease in BXSB mice. (**A**–**E**) Six-week-old BXSB males received either 50 mg/kg DIDS (n = 4) or vehicle (K^+, potassium bicarbonate; n = 4) weekly for eight injections. Data are representative for three independent experiments. (A) Percentage of lymphocyte populations. (B) The percentages of follicular (FO; $CD23^+CD21^+$), MZ ($CD23^{low/-}CD21^+$), and transitional 1 (T1; $CD23^-CD21^-$) B cells in the B lymphocyte population. (C) Percentage of GC B cells. (D) Percentage of Tfh cells. (E) Percentage of monocytes. (**F**–**H**) Fifty milligrams per kilogram DIDS (n = 7) or vehicle (n = 8) treated mice were analyzed. (G) Ig isotypes in sera. (F) Mean intensity of ANA staining for pan-Ig and IgG isotypes. Glomerular scores were evaluated in (H). Data are presented as mean±SEM. Statistical significance was determined by Mann–Whitney U test. *$p<0.05$.

significance (Fig. 4.11G). No change was seen in renal pathology scoring between the two groups (Fig. 4.11H).

We also examined the effect of DIDS in MRL/*lpr* female mice, another classical lupus-prone mouse model. Likewise, there is a significant decrease of B cell percentage with DIDS treatment (Fig. 4.12A). No differences in B cell subsets were observed (Fig. 4.12B). The

percentages of GCB and Tfh cells were unaffected by the treatment of DIDS. While levels of circulating antibodies were comparable between DIDS treated and vehicle control groups (Fig. 4.12E and Fig. 4.12F), the intensity of ANA, especially with the IgG isotype, showed a trend towards decline in the treated group (Fig. 4.12E). No improvement was shown in the renal pathology when mice were treated with DIDS (Fig. 4.12G).

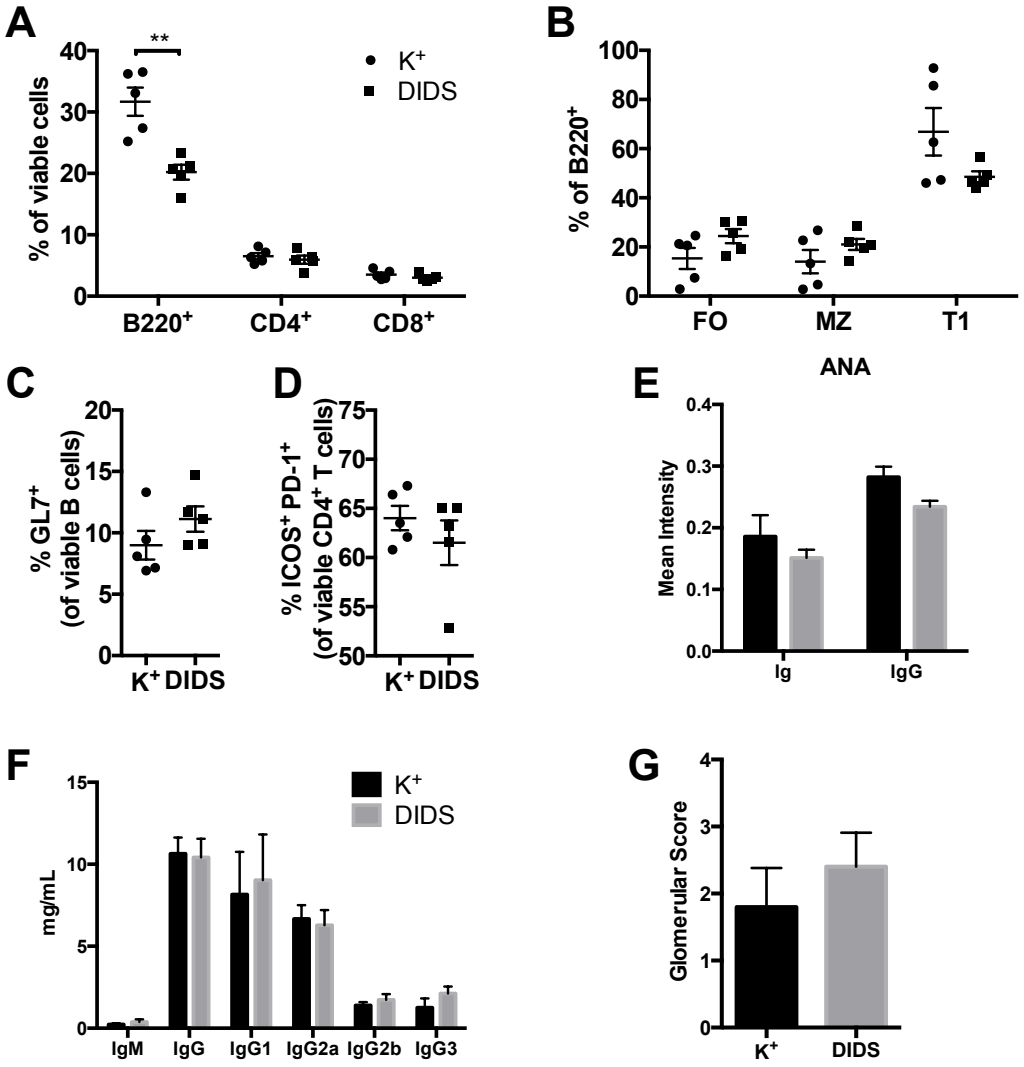

FIGURE 4.12 No improvement in SLE development was observed in MRL/*lpr* mice treated with DIDS. Six-week-old MRL/*lpr* females received either 50 mg/kg DIDS (n = 5) or vehicle (K^+, potassium bicarbonate; n = 5) weekly for eight injections. (**A**) Percentage of lymphocyte populations. (**B**) Percentages of B cell populations (gating strategy the same as in Figure 4). (**C**) Percentage of GC B cells. (**D**) Percentage of Tfh cells. (**F**) Ig isotypes in sera. (**E**) Mean intensity of ANA staining for pan-Ig and IgG isotypes. Glomerular scores were evaluated in (**G**). Data are presented as mean±SEM. Statistical significance was determined by Mann–Whitney U test. *p<0.05, **p<0.01.

Discussion

The disruption of affinity maturation of B lymphocytes through ablation of AID in BXSB mice significantly prolonged survival with decreased renal damage and restoration of splenic architecture. Pathogenic glomerular changes were diminished, as was urine protein concentrations. Immune complexes were still present in the *Aicda*$^{-/-}$ mice; however, complement activation appeared absent. These results suggest that these IgM Abs lacking somatic hypermutation are not as adept at activating the inflammatory profile characteristic of lupus nephritis. It is generally accepted that IgG isotypes initiate IFN production fueling renal damage (44), and a potential explanation needing further exploration in this model is the inability of IgM to induce IFN production. Although hyper-IgM syndrome appears to exist in BXSB.*Aicda*$^{-/-}$ mice as in other AID-deficient models (23, 32, 38), antinuclear IgM Ab levels do not differ between wt BXSB and BXSB.*Aicda*$^{-/-}$ mice, suggesting no increase in autoreactivity. However, the pattern of Ig and IgM ANA binding is altered between the strains. BXSB.*Aicda*$^{-/-}$ sera presents a cytoplasmic, dense, fine-speckled pattern, whereas wt sera shows a predominately homogeneous nuclear pattern (https://www.anapatterns.org). This suggests altered Ag affinity that would be of interest to explore further.

Although the cellular phenotype of spleens by flow cytometry remained mostly consistent between the two strains, the organization of these cells within the spleen was noticeably altered. GC B cell percentages were increased as previously reported in mice with targeted AID deficiency (32, 38). A corresponding increase in Tfh cells was not observed, suggesting that the creation of GC B cells may not be accelerated, but perhaps their movement out of this compartment is hampered. A reduction in marginal zone B cells is a consistent change seen in BXSB mice, and abrogation of AID restored this population. This lack of detectable changes using traditional flow

cytometry in a model with significantly prolonged survival cautions against relying on these markers as an indicator of disease improvement. Further exploration in situ revealed, despite little change in cellular percentages and numbers, splenic architecture was restored in the spleens of the knockout mice with discernable follicles and GCs readily visible. Previously, we reported that BXSB mice exhibit a robust extrafollicular response (39), which has also been appreciated in other lupus models (45, 46) as well as suspected in human patients (47). The return to more-normal architecture in the AID-deficient mice suggests a shift from an extrafollicular response to a GC response. However, no differences were seen in plasma cell generation, including anti-dsDNA Ab–producing plasma cells. BXSB historically has low production of anti-dsDNA Abs (48), and assay sensitivity may be clouding these results. The possibility exists that although follicular structure is distorted in the wt BXSB mice, the plasma cell–generating interactions between B and T cells are still occurring in a similar manner between the two models. Further studies are needed to investigate this possibility.

BXSB.*Aicda*$^{-/-}$ B lymphocytes expressed increased CD73, a memory B cell marker, which is similar to what we showed in the NOD.*Aicda*$^{-/-}$ model (32). In the NOD model, these CD73^{+} memory B cells exert a protective effect (32). The regulatory role of CD73^{+} memory B cells has yet to be tested in the BXSB model. The literature presents a model in which low-affinity GC B cells move into the memory compartment (49). These results support this model, as B lymphocytes unable to undergo affinity maturation would presumably remain as lower affinity, increasing the memory B cell compartment.

Given the reduced production of Igs and increase in the GC B cell compartment in BXSB.*Aicda*$^{-/-}$ mice, we anticipated the generation of plasma cells would be reduced. With diminished autoreactive Ab levels in these mice, we hypothesized that the *Aicda*$^{-/-}$ B lymphocytes

would have limited survival because of low affinity and would not undergo plasma cell differentiation. We did not, however, find a statistically significant drop in the percentage of plasma cells. In fact, we saw that BXSB.*Aicda*$^{-/-}$ B cells were able to differentiate into plasma cells as readily as their wt counterparts *in vitro* and that these cells were not subject to early or late apoptosis. Further study into the generation of these cells and the reduction in pathogenic B lymphocyte development in this model are warranted.

Because disruption of the AID pathway led to significantly improved life span in BXSB mice, targeting this pathway therapeutically may prove beneficial. The small molecule inhibitor of RAD51, DIDS, blocks the ability of B cells to repair the DSB induced by AID, causing these cells to undergo early cell death (21). The point of intervention in the pathway is, therefore, different from targeted knockout of the *Aicda* gene. The NOD model of type 1 diabetes showed success with this therapy, and diabetes onset was greatly delayed (32). Here we explored the ability of DIDS to inhibit CSR *in vitro* and its capacity to retard SLE development *in vivo*.

In vitro assays with DIDS successfully decreased B lymphocyte population and reduced isotype-switched B cells. Interestingly, *in vitro* stimulation revealed increased expression of activation markers on B cells in the presence of DIDS, leading us to examine the possibility of DIDS to act as an antigen to activate B cells. However, further investigation into the other two major antigen presenting cells, dendritic cells and macrophages, exhibited significant diminished expression of activation markers. The numbers of these two cell populations were also found to decline with the DIDS treatment. While the effect of decreasing B lymphocytes was consistent with earlier studies, a toxic effect on DCs and macrophages of BXSB origin has not been previously reported (21). DIDS has also been commonly used as an anion channel antagonist that inhibits Cl^- uptake and bicarbonate transport (50-52). One recent study proved the importance of

bicarbonate in regulating inflammatory responses of macrophages upon stimulation (53). While the presence of bicarbonate enhances the expression of inflammatory genes, the addition of DIDS in the cell culture abrogates the effects (53). Thus, it is plausible that the sensitivity to ion concentrations might be responsible for the suppression of dendritic cells and macrophages by DIDS treatment.

We next assessed whether the effect of DIDS on preventing CSR can be extended to *in vivo* studies. A high dosage of 50 mg/kg (21) was investigated in two distinct lupus-prone murine models, BXSB and MRL/*lpr* mice. We appreciated a significant decrease in splenic B lymphocytes accompanied with a trend toward reduced levels of pathogenic antibodies with the DIDS treatment of both strains. However, we saw no remarkable improvement *in vivo* in either BXSB males or MRL/*lpr* females, namely glomerular pathology was not significantly altered. With the average lifespan of 5 months and rapid disease progression for these two SLE mouse models (54, 55), it is possible that SLE has been largely developed when the treatment starts, thus limiting the inhibitory role of DIDS and leading to the lack of efficacy. Studies on an earlier start point or using NZB/W mice, a model develops SLE much slower, are needed to elucidate the effect of DIDS on SLE development.

Another explanation for the lack of response to the DIDS treatment is that other DSB repair pathways compensate for the loss of HR *in vivo*. DIDS inhibits the HR repair pathway by preventing the binding of its core factor, the RAD51 complex, to DNA (21). However, accumulating studies have provided evidences for the predominant roles of NHEJ and another new pathway, termed alternative end joining or microhomology, in repairing AID-induced DSBs (56-60). While HR requires a homologous sequence as the template to extend DNA with high fidelity, NHEJ can ligate DNA ends without the homologous template (58). The lack of long homologous

sequences in the switch region suggests that HR might not be the major repair pathway in CSR (61). Furthermore, the deficiency of XRCC4 and DNA ligase IV (Lig4), two factors exclusively involved in NHEJ, in B cells results in 50-75% loss of CSR compared with wildtype B cells (62). This finding not only confirms the importance of NHEJ in class switching but also indicates that the repair of AID-induced DSBs is not entirely dependent on NHEJ. The Martin group revealed that 5′ DSBs were favored in the switching, and that the polarity of DSBs influenced the choice of the repair pathway with the increased use of microhomology in 5′ DSBs repair (56). Taken together, these results prove the contribution of NHEJ and microhomology to CSR, which could be a possible explanation for the loss of DIDS effects *in vivo*. Although targeting with the DIDS molecule appeared unsuccessful in diminishing glomerular disease in our mice, we feel our success in genetically targeting of this pathway warrants further exploration for potential therapeutic development.

In conclusion, targeting B lymphocyte maturation in the BXSB model alleviated lupus-like nephritis and prolonged survival. One limitation of this work is that only a single founder was used to develop the BXSB.*Aicda*$^{-/-}$ line, and thus, off-target effects cannot be ruled out. The authors did perform an additional backcross to BXSB to limit this possibility when creating these mice. Our results are supported by previous work in the MRL model targeting this same molecule (23). Because these two mouse models of SLE vary greatly in disease pathogenesis, these similar findings indicate greater promise that this work could translate to human patients, particularly those with nephritis. Further explorations into ways to target this pathway for the treatment of SLE are warranted.

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Chapter V

Maternal antibodies modulate the SLE development in the offspring

Abstract

Maternal factors play an essential role in shaping the immune system of the newborn, yet it is unknown whether maternal factors could modulate the development of systemic lupus erythematosus (SLE) in the offspring. Activation-induced cytidine deaminase (AID) is an enzyme required for somatic hypermutation and class switch recombination. Given that IgG and IgA isotypes account for the vast majority of passive immunity in rodents, our previously established AID-deficient BXSB mice provide a model in which few maternal antibodies are transferred to the offspring. In this study, we compared genotypically identical mice born to either AID-sufficient dams or AID-deficient dams and evaluated the effects of maternal antibodies in disease progression. We revealed the offspring of knockout dams developed the disease at a faster rate, as shown by more severe nephritis. These maternal antibody-deficient mice also exhibited elevated pathogenic autoantibodies compared to their counterparts. In addition, the splenic structure was intensively disrupted with disorganized follicles and fewer discernible germinal centers. Despite a significant decrease of germinal center B cells, a comparable level of plasma cells was shown in the offspring of knockout dams. An expansion of GC-like B cells was identified and might be responsible for plasma cell generation. Lastly, a more activated T cell phenotype was characterized in maternal antibody-deficient mice. These findings demonstrate the absence of maternal antibodies accelerates the SLE development in the offspring of BXSB mice.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by high titers of circulating autoantibodies and aberrant inflammation in various tissues and organs (1). Various immunological abnormalities have been demonstrated to be involved in the pathogenesis of SLE, including breach of self-tolerance, uncontrolled activation of B and T cells, defective clearance of apoptotic debris and increased pro-inflammatory cytokine production (2). While the etiology of SLE is not fully understood, it is well-accepted that SLE is a complex disease that results from an integration of genetic predisposition and environmental factors (2-4). These factors lead to alternations in the immune system and subsequently modulate the development of SLE (2). Previous research primarily focused on individuals, and little is known about the generation effects, for instance, whether maternal or paternal factors play a role in the pathogenesis of SLE.

As placental mammals, the intensive interaction between the mom and the newborn during gestation and lactation provides the newborn with nutrients, passive immunity and other maternal components (5). Recently, accumulating evidence has demonstrated the importance of different maternal factors in shaping the immune system of the neonates. For instance, several groups found that neonates exhibited dampened responses to vaccination (6-9). A recent study revealed that the presence of maternal antibodies inhibited mucosal T cell responses and resulted in a correspond decline of germinal center formation in the neonates (10). Along with nutrients and antibodies, antigens can also cross the placenta (5). In utero exposure to antigens induced immune tolerance and impaired the immune response to subsequent postnatal infections (11). The newborns of the dam infected with *Dipetalonema viteae* during the pregnancy were more susceptible to the infection by this parasite than the offspring of uninfected dams (11). Similar to

maternal antibodies and exposure to antigens, the maternal microbiota has been reported to impact neonatal immunity. Neonates born to transiently colonized dams showed an expansion of intestinal group 3 innate lymphoid cells and $F4/80^{+}CD11c^{+}$ cells and an enhanced response to infections compared with the neonates born to germ-free dams (12). With the large amount of studies emphasizing the importance of maternal factors in reprogramming the immune system of the newborn, we hypothesized that maternal factors can influence the SLE development in the offspring.

In our previous study, we established BXSB.*Aicda*$^{-/-}$ mice using CRISPR/Cas9 technology. *Aicda* gene encodes an RNA editing enzyme, termed activation-induced cytidine deaminase (AID) (13). This enzyme is highly expressed in germinal center B cells and plays an essential role in class switch recombination and somatic hypermutation (13). The AID-deficient mice fail to class switch and secrete only IgM antibodies. IgG is the only isotype that can cross placenta in rodents (14). The vast majority of antibodies present in breastmilk is of IgA and IgG isotypes (14). Therefore, AID-deficient dams are not capable to provide maternal antibodies to the newborns. It should be stressed that unlike other lupus-prone mouse strains, the BXSB strain shows SLE acceleration in male mice. BXSB females exhibit a greatly dampened disease phenotype, with very few pathogenic autoantibodies detected in the circulation (15). Using AID-deficient BXSB females can better illustrate the function of maternal antibodies in that the presence of pathogenic autoantibodies may confound the effect of maternal antibodies. To address the role of maternal factors on the development of SLE in the offspring, we bred AID-deficient females to wildtype males and breed wildtype females to AID-deficient males to generate genetically identical offspring. By comparing the heterozygous (het) mice from different dams, we found het mice from AID-deficient dams developed more severe nephritis with significantly more renal inflammation

and damages, despite no differences in immune complex deposition or complement activation in the kidney. Consistently, a notable increase of anti-nuclear antibodies, especially anti-dsDNA antibodies, was detected in these mice. Other disease hallmarks were also found to be worse in maternal antibody-deficient mice. These results indicate maternal antibodies alter the SLE development in the offspring.

Material and methods

Mice

The generation of BXSB.*Aicda*$^{-/-}$ mice were described in chapter III. Mice are bred and maintained under specific antigen free environment. All the animal studies were performed in accordance with the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University.

Renal pathology

Formaldehyde-fixed kidneys were processed in the Histo-Scientific Research Laboratories (Mount Jackson, Virginia). Samples were embedded in paraffin and cut to 5μm for the H&E staining. Sections were graded according to the criteria with modification by an experienced pathologist blinded to the treatment (16). The assessment mainly focuses on the severity of glomerular mesangial proliferation and immune complex deposition, tubular degeneration with protein casts, interstitial inflammation, and vasculitis.

Urine albumin and creatinine assay

Fresh urine samples were collected at 12 weeks of age and stored at -20°C until further processing. The concentrations of albumin and creatinine in the urine were determined with the Mouse Albumin ELISA Kit (Bethyl Laboratories) and Creatinine Colorimetric Assay Kit (catalog 500701; Cayman Chemical) following manufacturer's instructions. The albumin to creatinine ratio was calculated by dividing albumin concentration in milligrams per deciliter by creatinine concentration in milligrams per deciliter.

Immunofluorescence staining

Tissue samples were embedded in OCT (Thermo Fisher Scientific) and frozen in liquid nitrogen for 1-2 minutes. Frozen samples were cut at 8 μm onto the microscope slides using a Microm HM550 Cryostat (Thermo Fisher Scientific). Slides were fixed with cold acetone (catalog A18-4; Fisher Chemical) at -20°C for 10 min followed by 3 times of PBS (Life Technologies) wash. Then slides were blocked with 3% FBS (HyClone; Thermo Fisher Scientific) in a moist chamber at room temperature for 1 hour. After blocking, slides were incubated with fluorochrome-conjugated antibodies at room temperature for 1 hour. The following antibodies were used in this study: B220 (RA3-6B2), CD4 (RM4-5), and GL7 (GL7) (BioLegend), κ-chain (187.1, BD Biosciences) and C3c (polyclonal, Nordic-MUbio). Prior to κ-chain staining, slides were treated with CD16/32 (93; BioLegend) for 30 minutes at room temperature to block Fc receptors. DAPI was added before the coverslip for a nuclear counterstain. All the slides were imaged using a Zeiss Axio Observer (ZEISS) microscope. The mean intensity was analyzed using ImageJ software (National Institutes of Health).

ELISA

The levels of antibodies with different isotypes were determined by ELISA as previously described (17). Briefly, plates with incubated with coating antibodies at 4°C overnight. After 2 times of wash, plates were blocked for 1 hour followed by the incubation with appropriately diluted serum samples at 37°C for 1 hour. After another hour incubation with alkaline phosphatase (AP)-conjugated detection antibody at 37°C, 1-Step *p*-nitrophenyl phosphate disodium salt (Thermo Fisher Scientific) was added to the plates for color development. For the detection of anti-dsDNA antibodies, the plates were coated with calf thymus DNA (Sigma-Aldrich) at 4°C overnight followed by the same procedures described above. All the plates were read on an Infinite M200 PRO plate reader using Magellan 7.0 software (Tecan).

ANA

The circulating antinuclear antibodies were quantified with an ANA Test Kit (Antibodies) per manufacturer's instructions. The secondary antibodies used in this study were either FITC anti-mouse κ L chain (187.1; BD Biosciences) or FITC anti-mouse IgM (RMM-1; BioLegend). Slides were assessed by an Eclipse T*i* microscope with the NIS-Elements software (Nikon). The immunofluorescence intensity was analyzed using CellProfiler (Broad Institute).

Flow cytometry

Spleens were minced and treated with ACK lysis buffer for single cell suspension. Splenocytes were counted using a Nexcelom cell counter. Cells were then stained with fluorochrome-conjugated antibodies for 30 minutes in the dark fridge. Propidium iodide (BioLegend) or 7-aminoactinomycin D (7-AAD) (BioLegend) were used to differentiate live and dead cells. The

following antibodies were used in this study: B220 (RA3-6B2), CD4 (GK1.5), CD5(53-7.3), CD8 (53-6.7), CD19 (6D5), CD21 (7E9), CD23 (B3B4), CD44 (IM7), CD62L (MEL-14), GL7 (GL7), CD138 (281-2), CD11b (M1/70), CXCR4 (L276F12), CXCR5 (L138D7), ICOS (C398.4A), and (BioLegend) and FAS (Jo2) and PSGL-1 (2PH1) (BD Biosciences). All the experiments were conducted on an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Data were analyzed using FlowJo software (FlowJo). All analyses were done after gating on single cells.

Statistics

For the comparison of 2 groups, Mann–Whitney U test was used to determine statistical differences. One-way ANOVA was conducted for comparison of more than 2 groups. In the survival assessment, Log-rank test was employed. Significant was determined when the value of p is less than 0.05.

Results

Heterozygous mice born to AID-deficient moms develop more severe nephritis

12-week-old het mice born to AID-deficient (KO) moms exhibited more severe nephritis with significantly higher glomerular scores compared to het mice born to wildtype (WT) moms (Figure 5.1A). To examine the kidney function, we collected fresh urine samples to measure the albumin and creatinine levels in the urine. Consistent with the glomerular score, het mice born to

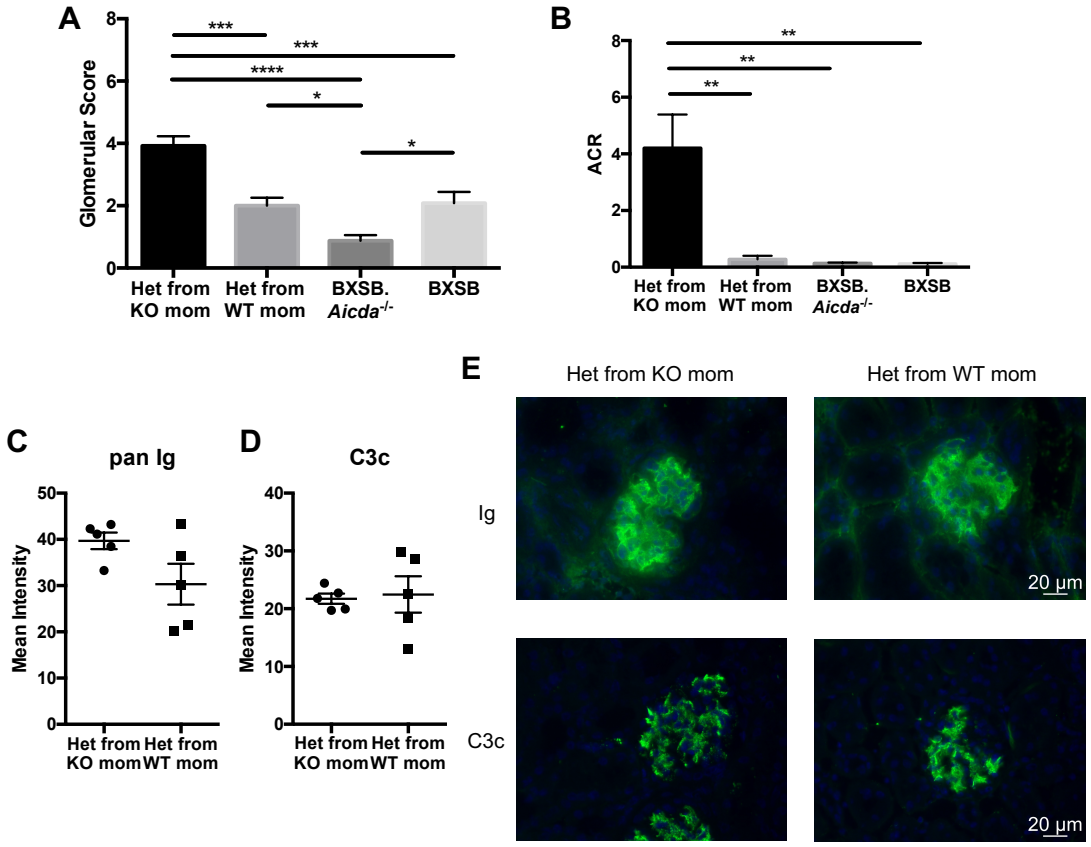

FIGURE 5.1 Heterozygous neonates born to AID KO moms exhibited an accelerated progression of nephritis. (**A**) Glomeruli scores were compared between 12-wk-old het mice born to AID KO moms (n=13) and WT moms (n=10), BXSB.*Aicda*$^{-/-}$ mice (n=16) and BXSB mice (n=12). (**B**) Urine samples from 12-wk-old het mice born to AID KO moms (n=13) and WT moms (n=10), BXSB.*Aicda*$^{-/-}$ (n=9) and BXSB (n=8) mice were analyzed for albumin-to-creatinine ration (ACR). (**C-E**) Kidney samples from 12-wk-old het mice born to AID KO moms (n=5) and WT moms (n=5) were examined for Ig and C3c deposition. Mean intensity was shown. Representative images were shown in E. Data are presented as mean±SEM. Statistical significance was determined by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

AID-deficient moms exhibited an elevated albumin to creatinine ratio, confirming more severe

damage in the kidney (Figure 5.1B). Given that immune complexes and the complement system are crucial mediators for nephritis, we next sought to determine Ig and C3c deposition in the kidney. While there was a trend towards increased Ig deposition in the kidney of het mice born to AID-deficient moms, offspring from different groups showed comparable complement activation in the kidney (Figure 5.1C-E). Together, these results indicate the lack of AID in the dam significantly accelerated the disease progression in the het offspring.

Pathogenic antibody levels were elevated in het mice from KO moms

With the importance of antibodies in the development of SLE, we examined the changes in the serology. Despite no significant changes in IgG antibodies, het mice from KO moms exhibited an increase of IgM antibodies compared to their counterparts (Figure 5.2A, 5.2B). High levels of circulating anti-nuclear antibodies (ANAs) are one of the hallmarks in the SLE (1). Het mice from KO moms showed a significant increase of IgG ANA as well as IgM ANA (Figure 5.2C, 5.2D). Additionally, we noticed a significant increase of total anti-dsDNA antibodies, one type of ANAs that has been highly associated with nephritis activity (18), in the serum of het mice from KO moms (Figure 5.2E). In the literature, anti-dsDNA IgM antibodies show conflicting roles in the development of nephritis. While anti-dsDNA IgM treatment significantly improved nephritis in MRL/*lpr* mice, the accumulation of anti-dsDNA IgM as a result of AID abrogation in B6/*lpr* mice was accompanied with exacerbated nephritis (19, 20). The level of anti-dsDNA IgM was increased in het mice from KO moms with more severe nephritis, suggesting possible

pathogenicity of these antibodies (Figure 5.2F). Collectively, these findings reveal a more pathogenic antibody profile in het mice from KO moms.

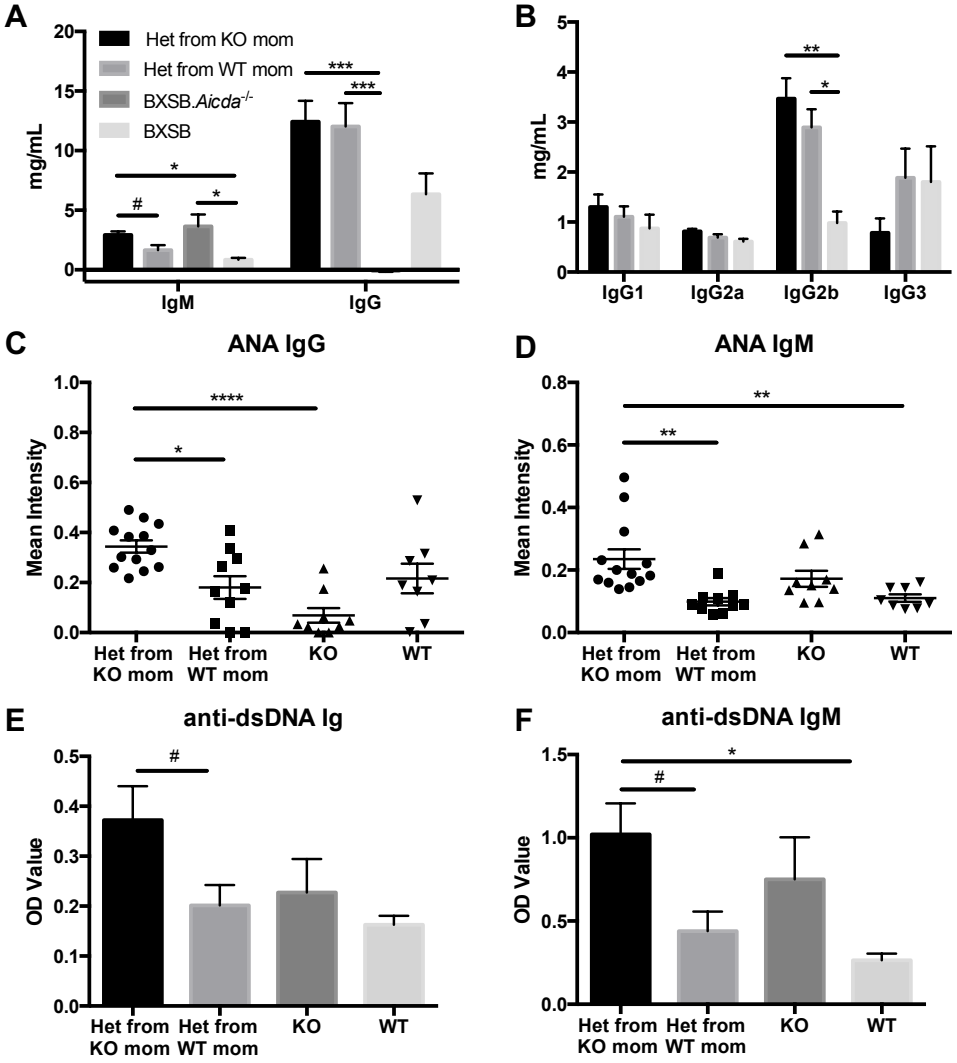

FIGURE 5.2 Het mice born to AID KO moms produced more pathogenic antibodies. Sera from 12-wk-old heterozygous mice born to AID KO moms (n=13) and WT moms (n=10), BXSB.*Aicda*$^{-/-}$ mice (n=7) and BXSB mice (n=6) were analyzed. (**A**) Antibodies of IgM and IgG isotypes were shown. (**B**) Antibodies of different IgG subisotypes were shown. (**C**, **D**) The levels of ANA with IgG and IgM isotypes were measured and compared in C and D, respectively. (**E**, **F**) Anti-dsDNA antibodies of pan-Ig and IgM isotypes were shown in E and F. Data are presented as mean±SEM. Statistical significance was determined by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Mann–Whitney U test was used between heterozygous mice born to AID KO moms and WT moms. #p<0.05.

Disrupted splenic architecture in het mice from KO moms

We next characterized the cellular changes in het mice born to different moms. Surprisingly, a notable decrease of the B cell percentage was detected in het mice born to KO moms (Figure 5.3A). Immunofluorescence staining of spleen sections confirmed reduced follicles and disrupted structures in het mice from KO moms (Figure 5.3B). The accumulation of monocytes in the circulation and the spleen is a disease characteristic of the BXSB model (21). Interestingly, the

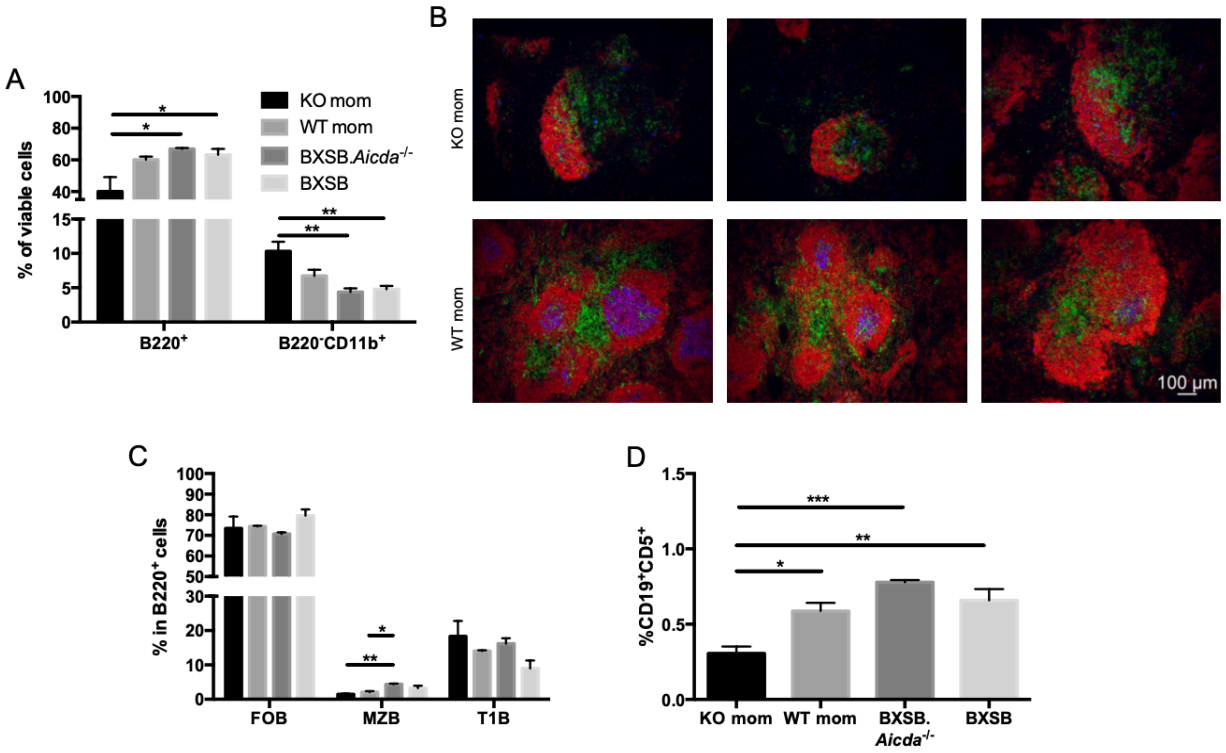

FIGURE 5.3 Het mice from KO moms showed disorganized splenic structure. Splenocytes from 12-wk-old heterozygous mice born to AID KO moms (n=4) and WT moms (n=3), BXSB.*Aicda*$^{-/-}$ mice (n=4) and BXSB mice (n=4) were analyzed. (**A**) The percentages of B220^{+}, and B220^{-}CD11b $^{+}$ cells in the viable cell population. (**B**) Representative immunofluorescence images showing the follicles in the spleen. Red, B220; blue, GL7; and green, CD4. (**C**) The percentages of follicular (FO; CD23^{+}CD21^{+}), MZ (CD23$^{low/-}$CD21^{+}), and transitional 1 (T1; CD23^{-} CD21^{-}) B cells in the B lymphocyte population. (**D**) The percentage of B1a cells (CD19^{+}CD5^{+}) in the viable cell population. Data are presented as mean±SEM. Statistical significance was determined by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.

increase of monocyte percentage in the spleen coincided with the diminished B cell percentage in het mice from KO mom (Figure 5.3A). The reduction of marginal zone (MZ) B cells, an innate-

like B cell population, is known to be another disease hallmark of the BXSB strain (21). Here, we observed decreased MZ B cells in het mice from KO moms (Figure 5.3C). B1 cells, which can be further divided into B1a and B1b cells, are also called innate-like B cells. B1a cells in the spleen were found to be reduced in the het mice from KO moms (Figure 5.3D). These results suggest the absence of maternal antibodies results in striking changes in spleen cell components of the offspring and subsequently causes the loss of the normal spleen structure.

An expansion of germinal center-like phenotype B cells was observed

Given that germinal centers (GCs) are the major sites where plasma cells arise, we then assessed whether germinal center responses were affected. We showed a significant decrease of GCB cells in het mice from KO moms (Figure 5.4A). The impaired GC confinement is one of the characteristics in the BXSB strain (17). Interestingly, GC structures were restored in het mice from WT moms, while few organized GCs were detected in the spleen section of het mice from KO moms (Figure 5.3B). Despite the notable reduction of GCB cells, het mice from KO moms showed a comparable level of plasma cells compared to het mice from WT moms, suggesting the contribution of other cells to the plasma cell compartment (Figure 5.4B). Of note, we observed an expansion of $GL7^{+}Fas^{-}$ cells in het mice from KO moms (Figure 5.4C). Given the Fas-Fas ligand pathway regulates cell apoptosis to maintain homeostasis of the immune system (22), we next assessed whether the downregulation of Fas in the $GL7^{+}$ B cells affected the apoptosis of these cells. No significant differences in apoptosis were found between different het groups (Figure 5.4D). Taken together, het mice from KO moms exhibited diminished GCB cells accompanied with a new GC-like phenotype cell population.

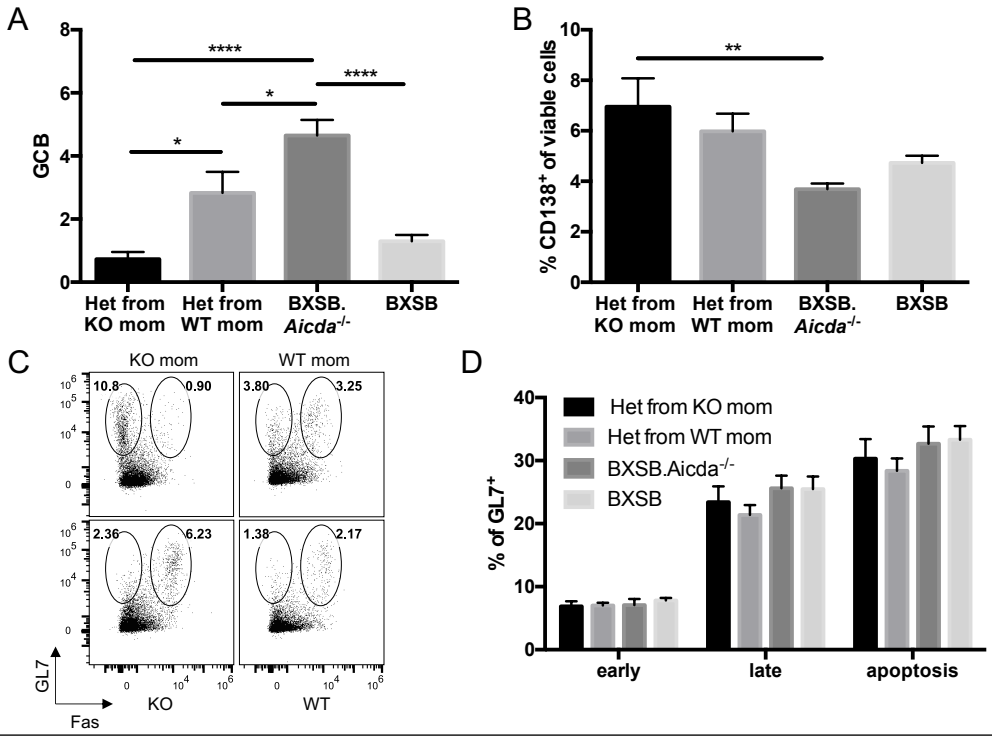

FIGURE 5.4 GL7$^+$Fas$^-$ B cells are increased in het mice from KO moms. Splenocytes from 12-wk-old heterozygous mice born to AID KO moms (n=7) and WT moms (n=4), BXSB.*Aicda*$^{-/-}$ mice (n=8) and BXSB mice (n=10) were analyzed. (**A**) The percentages of germinal center B cells (Fas$^+$GL7$^+$) in the viable B cell population. (**B**) Representative flow cytometry dot plot images showing the GCB and GL7$^+$Fas$^-$ B cells. (**C**) The percentages of plasma cells (CD138$^+$) in the viable cell population. Data are presented as mean±SEM. Statistical significance was determined by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

A more activated T cell phenotype was characterized in het mice from KO mom

We next explored whether the maternal absence of AID impacts T cell phenotypes in the offspring. In the germinal center, follicular helper T cells are essential for GCB cell selection and survival (23). While GCB cells were significantly decreased in het mice from KO moms, the percentages of follicular helper T cells were similar (Figure 5.5B). Extrafollicular responses have been reported in various lupus-prone mouse models, and extrafollicular helper T cells are characterized as CD4$^+$CXCR4$^+$PSGL-1^{low} (24, 25). Although het mice from KO moms exhibited a significant increase of extrafollicular helper T cells compared to BXSB mice, the difference was not shown between het mice from different moms (Figure 5.5B). Likewise, the decrease of CD4$^+$

and CD8$^+$ T cells was only observed when compared to BXSB mice. Further analysis of naïve and memory cells indicated a more activated T cell phenotype with more effector memory T cells and reduced naive T cells in het mice from KO moms (Figure 5.5C, 5.5D).

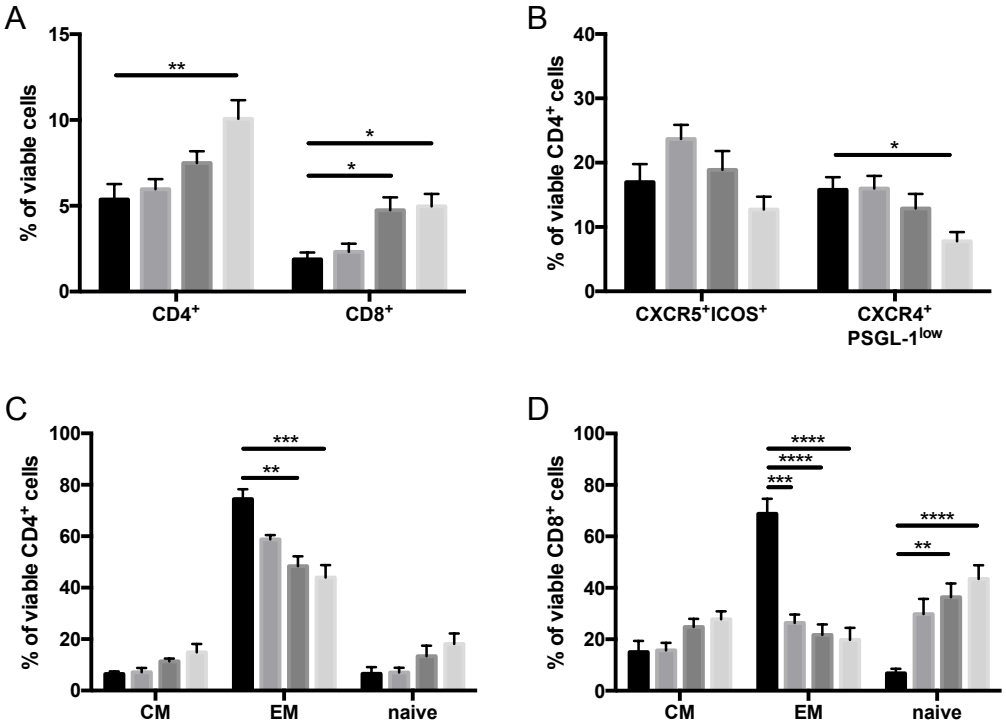

FIGURE 5.5 More activated T cells were shown in het mice from KO moms. Splenocytes from 12-wk-old heterozygous mice born to AID KO moms (n=7) and WT moms (n=4), BXSB.*Aicda*$^{-/-}$ mice (n=8) and BXSB mice (n=10) were analyzed. (**A**) The percentages of CD4 and CD8 T cells in the viable cell population. (**B**) The percentages of extrafollicular helper T cells (CXCR4$^+$PSGL-1^{low}) and follicular helper T cells (CXCR5$^+$ICOS$^+$) in the viable CD4 T cell population. (**C, D**) The percentage of central memory (CM, CD44$^+$CD62L$^+$), effector memory (EM, CD44$^+$CD62L$^-$) and naïve (CD44$^-$CD62L$^+$) in the viable CD4 (C) and CD8 (D) T cell population. Data are presented as mean±SEM. Statistical significance was determined by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

In this study, we employed the previously established BXSB.*Aicda*$^{-/-}$ mouse model to investigate the importance of maternal antibodies in the SLE development of the offspring. AID-deficient females are not capable to provide maternal antibodies to the offspring as they only produce IgM. We revealed that het mice from KO moms developed more severe nephritis with significantly higher glomerular scores compared to het mice from WT moms. In line with the increased inflammation, kidney function was intensively damaged in het mice from KO moms, as shown by the increase of the protein concentration in the urine. We also observed notably elevated levels of anti-dsDNA antibodies and anti-nuclear antibodies in the het mice from KO moms. Further, the disease characteristics of BXSB model, such as monocytosis, marginal zone B cell reduction and disrupted germinal center structures, were exacerbated in het mice from KO moms. These findings suggest the difference in maternal antibodies due to the modulation of AID results in differential SLE development rates in the offspring.

Marginal zone B cells and B1 cells are recognized as innate-like B cells as they share various properties with innate immune cells, including rapid response to antigens and the production of IgM antibodies with broad reactivity (26). It has been demonstrated that they are the main producers of naïve IgM antibodies (26). In contrast to the significant increases of IgM antibodies in the sera, we observed notable decreases of marginal zone B cells and B1a cells in het mice from KO moms, suggesting other cell population contribute to the secretion of IgM. Another known feature of innate-like B cells is their capacity to regulate immune responses by producing IL-10 (26). While conflicting results regarding the role of IL-10 have been reported in different lupus murine models, genetically defective IL-10 production lead to an accelerated disease progression in BXSB mice (27-29) (https://www.jax.org/strain/021872). It is reasonable to

propose that maternal antibodies modulate the SLE development in the offspring by altering the development of IL-10 producing B cells. Further studies evaluating IL-10 producing B cells and the IL-10 concentration in het mice from KO moms are needed.

Germinal center B cells undergo somatic mutation and clonal selection to differentiate into plasma cell with the ability to produce antibodies with high affinity (23). Pathogenic autoantibodies are highly mutated, indicating the contribution of GC responses to the SLE pathogenesis (30). We showed significantly diminished GC B cells with disrupted splenic architecture in het mice from KO moms. Surprisingly, a corresponding reduction of plasma cells was not appreciated, with comparable plasma cell percentages seen between different het groups. An expansion of GC-like phenotype cells was observed in het mice from KO moms. These cells maintained the upregulation of GL7 as conventional GC B cells; however, they exhibited a decreased expression of Fas. A previous study showed the deficiency of Fas specifically in B cells led to disrupted lymphoid organ structures and remarkable increase of plasma cells (31), indicating the expression of Fas is dispensable for GCB cells to differentiate into plasma cells. Thus, the expanded cell compartment in het mice from KO moms might share GC B cell properties and give rise to plasma cells. Given the Fas-Fas ligand pathway is crucial for maternal-fetus tolerance (32), it is possible that maternal antibodies are involved in the modulation of Fas-Fas ligand pathway. Future investigations are needed to determine the underlying mechanisms responsible for the downregulation of Fas in the absence of maternal antibodies.

Another explanation for similar plasma cells is that extrafollicular responses compensate for the reduced germinal center responses and contribute to plasma cell differentiation. In addition, extrafollicular plasma cells primarily secrete IgM (25), which could explain the increased IgM in het mice from KO moms. When evaluating extrafollicular T helper cells, no significant differences

were observed between het mice from different moms. However, it has been reported that extrafollicular plasma cells can arise independent of T cell help (33). Thus, further characterization of extrafollicular B cells would help to determine whether the extrafollicular responses are altered in the absence of maternal antibodies.

Upon antigen exposure, naïve T cells differentiate into effector T cells, central memory T (T_{CM}) cells and effector memory T (T_{EM}) cells (34). Abnormal T cell activation and differentiation are associated with the development of SLE (35, 36). In this study, we revealed significant increases of both $CD4^+$ T_{EM} and $CD8^+$ T_{EM} cells in the offspring of AID-deficient dams, indicating a more activated T cell phenotype. This finding is consistent with a recent study showing maternal IgG and IgA antibodies cooperate to inhibit T cell activation in the newborn (10). Hence, this suggests maternal antibodies could regulate the disease progression by altering T cell activation and differentiation in the offspring.

In conclusion, we demonstrate maternal antibodies modulate the development of SLE in the offspring, which imply the impact is not transient and could sustain in the long term. It is worth noting we propose the influence on the SLE development in the offspring could be a result of direct modulation of the immune system by maternal antibodies or a result of indirect regulation – maternal antibodies modulate the immune system through altering maternal microbiota. With the importance of IgA in microbiota homeostasis (37, 38), a detailed screen for differential bacteria between AID-deficient females and AID-sufficient females can provide candidates responsible for the accelerated disease progression and advance the identification of underlying mechanisms. Our data expands the current understanding of the role of maternal antibodies in reprogramming neonatal immune system and suggests intervention of maternal antibodies or underlying

mechanisms in the early life could potentially modulate the SLE development in genetically susceptible individuals.

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Chapter VI

Conclusion and Future Directions

The critical roles of B lymphocytes in the pathogenesis of type 1 diabetes (T1D) and systemic lupus erythematosus (SLE) have been illustrated throughout the dissertation. While B cell depletion therapies show significant positive responses in both T1D and SLE patients, there are limitations for these therapies, such as the loss of effects in the long term and accompanied high risks of infections (1, 2). Thus, it is of great value to obtain a deeper understanding of the disease pathogenesis and identify novel treatments that can be more specific. In this dissertation, we gained insight into the roles of activation-induced cytidine deaminase (AID), a key enzyme in B cell affinity maturation, and the related pathway in the development of these two diseases and provided potential targets for clinical transition of this work.

The first section of this dissertation research focused on the effects of targeting affinity maturation on the T1D development of NOD mice. The genetic ablation of AID significantly inhibited the progression to overt T1D in NOD mice, despite substantial infiltration of lymphocytes into the pancreas. This indicates that affinity maturation does not regulate the infiltration of B lymphocytes into the pancreas but might substantially modulate the diabetogenic capacity of B lymphocytes. Next we therapeutically targeted the repair of AID-mediated double strand DNA (dsDNA) breaks to induce apoptosis of class-switching cells. DIDS is an inhibitor of homologous recombination pathway, which is employed by mammalian cells to repair the dsDNA breaks (3). The administration of DIDS prevented NOD mice from developing the disease. It is noteworthy that even when started at a stage with the presence of destructive insulitis, DIDS treatment was able to keep the mice free from diabetes. These results indicate that DIDS has the potential for disease reversal in NOD mice. Interestingly, a remarkable expansion of $CD73^{+}$ B

lymphocytes were revealed in both approaches. CD73 is an enzyme that result in the production of adenosine, and CD73/adenosine pathway has been well characterized in the tumor microenvironment to suppress the immune responses (4). Evidence of the regulatory properties of $CD73^+$ B lymphocytes was revealed by their capacity to inhibit the proliferation of effector T cells (5). Further studies by our collaborators proved the contribution of $CD73^+$ B lymphocytes to T1D resistance through the production of adenosine and IL-10. The importance of CD73/adenosine pathway in dampening immune responses has also been highlighted in other autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (6). There are 4 different receptors for adenosine that are widely distributed and have distinct functions, among which A2AR has been tightly linked to the anti-inflammatory effects (7, 8). Therefore, it would be interesting to assess whether the stimulation of A2AR with its agonist can downregulate the immune responses and retard the development of T1D in NOD mice.

One limitation of the NOD study is the lack of examination on whether the microbiota change as a result of the absence of AID is involved in the modulation of T1D development. One study showed the deficiency of AID in B6 mice induced the enrichment of segmented filamentous bacterium (SFB) in their small intestine (9). It has been reported the colonization of SFB protected NOD females from developing the disease through the expansion of Th17 cells in the lamina propria (10). Thus, it is plausible that AID-deficient NOD mice also exhibit the outgrowth of SFB, which could be a contributing factor to the T1D resistance. Further evaluations on the alteration of microbiota compositions, especially SFB, in AID-deficient NOD mice are warranted.

Unlike the dispensable role of autoantibodies in the pathogenesis of T1D, highly mutated autoantibodies play pathogenic roles and contribute to tissue damage in the development of SLE. The genetic abrogation of AID in BXSB mice significantly alleviated lupus nephritis and

prolonged the lifespan. AID-deficient BXSB mice also exhibited restored marginal zone B cell compartment and normal splenic architecture. However, DIDS treatment did not remarkably alter the course of SLE in either BXSB mice or MRL/*lpr* mice. The examination of the effect of DIDS on BXSB splenocytes *in vitro* showed that while the presence of DIDS notably inhibited class switching and decreased the B cell numbers in a dose-dependent manner, macrophages and dendritic cells were strikingly impacted in all doses. The unexpected effect on macrophages and dendritic cells might be partly responsible for the differential responses to DIDS treatment in NOD mice and lupus-prone mice since these cells are classic antigen presenting cells which are crucial in the pathogenesis of T1D. Despite the lack of responses to DIDS treatment in lupus-prone mice, it is worth investigating other targets in this pathway since the depletion of AID inhibits SLE progression in different lupus-prone mouse models.

Lastly, we examined whether the absence of maternal AID would influence the SLE development in the genetically susceptible offspring. Heterozygous (het) mice born to AID-deficient dams developed much more severe nephritis compared to het mice born to AID-sufficient dams. Numerous disease hallmarks of the BXSB strain, including marginal zone B cell reduction and monocytosis, were exacerbated in the absence of maternal AID. Additionally, B1a cells were significantly diminished both in the spleen and in the peritoneal cavity. Marginal zone B cells and B1 cells are major contributors to IL-10 producing B cells. Thus, it would be interesting to assess the capacity of these cells to secrete IL-10, as well as whether the restoration of these cells would ameliorate the disease in the offspring born to AID-deficient dams. Another interesting finding in this study is the elevated levels of anti-dsDNA IgM and anti-nuclear IgM in the het mice born to AID-deficient dams. The role of anti-dsDNA IgM is paradoxical in different lupus-prone mouse

models. Further investigations on the pathogenicity of these autoreactive IgM and their contribution to the accelerated disease are needed.

While AID-deficient dams provide few maternal antibodies to the offspring, the lack of IgA also leads to significant changes in microbiota. To determine whether the modulation of the SLE development in the offspring by maternal antibodies depends on the changes in maternal microbiota, the following experiments are proposed: 1) injection of IgG into AID-deficient dams during pregnancy and lactation to investigate whether restoration of maternal IgG would attenuate SLE in the offspring; 2) antibiotics treatment for both AID-deficient and AID-sufficient dams to examine whether eliminating the differences in microbiota would abrogate the differential SLE development. These experiments will gain a better understanding of maternal modulation of the SLE development in the offspring and provide potential targets for the therapeutic development.

In summary, one of the major barriers in the development of therapies for autoimmune diseases is to find a balance between efficient immunosuppression and less adverse effects. The identification of more specific targets could help accomplish this goal. In this dissertation, we demonstrated the targeting of antibody diversification during B cell maturation successfully inhibits the development of T1D and SLE. This implies the potential of this strategy in modulating other autoimmune diseases and inflammatory diseases in which B cells play a critical role. In addition, we highlighted the importance of maternal factors in the regulation of SLE development in the offspring. Future studies are needed for the clinical transition of this dissertation research to benefit the patients with autoimmune diseases and potentially patients with inflammatory diseases.

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