Dendritic cell regulation of B cells

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ABSTRACT

DIANE GAIL CARNATHAN: Dendritic cell regulation of B cells
(Under the direction of Barbara Vilen)

The innate and adaptive immune responses protect from autoimmunity during infection through B cell tolerance mechanisms. We previously showed that during innate immune responses dendritic cells (DCs) and macrophages (MΦs) repress autoantibody secretion in part through their secretion of soluble factors (IL-6 and CD40L). Herein I describe that DCs from lupus-prone mice are deficient in repressing autoreactive B cell coincident with their inability to secrete IL-6. This defect results from defective Toll-Like Receptor signal transduction.

We further describe that DCs repress innate and adaptive immune responses independent of DC/MΦ-derived soluble factors. We show that DCs display endogenous nuclear self antigens and affect B cells responses as evidenced by upregulation of CD69 expression, induction of IκB phosphorylation and destabilization of the BCR. Despite evidence of stimulation, DCs inhibit BCR-derived signals and LPS-induced Ig secretion in autoreactive and naïve B cells suggesting that DCs regulate B cell responses independent of self-antigen.

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LIST OF ABBREVIATIONS

Abbreviations

ANA anti-nuclear antibodies

APC antigen presenting cell

Ars p-azophenylarsonate

ASC antibody secreting cell

B6 C57BL/6

B6.lpr B6.Fas^{lpr}

BCR B cell receptor

BLys B lymphocyte stimulating factor

BM bone marrow-derived

CM conditioned medium

DC dendritic cell

DRB 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

FcγR Fcγ Receptor

FO follicular B cells

HEL hen egg lysozyme

IC immune complexes

ICOS-L inducible costimulator ligand

IFN interferon

Ig immunoglobulin

ITAM immunoreceptor tyrosine-based activation motif

ITIM immunoreceptor tyrosine-based inhibitory motif

LPS lipopolysaccaride

MΦ macrophage

MRL/*lpr* MRL/MpJ-Fas^{lpr}

MRL MRL/MpJ

MyD88 myeloid differentiation factor 88

MyDC myeloid dendritic cell

MZ marginal zone B cell

NZM New Zealand Mixed

pDC plasmacytoid dendritic cell

RNP nuclear ribonucleoprotein

SLE systemic lupus erythematosus

Sm Smith protein

sn small nuclear

snRNP small nuclear ribotnucleoprotein

SOCS suppressor of cytokine signaling

Tg transgenic

TLR toll-like receptor

Yaa Y chromosome-linked autoimmune accelerator

μm μ-heavy chain

CHAPTER I. INTRODUCTION

Systemic Lupus Erythematosus

Autoimmunity is characterized by the breakdown in tolerance mechanisms that control the autoreactive lymphocytes. Systemic lupus erythematosus (SLE) is a chronic form of autoimmune disease that results from the production of antibodies to a wide array of cellular autoantigens including phospholipids and the nuclear components DNA, chromatin, RNA, Smith (Sm) and nuclear ribonucleoproteins (RNP) such as Ro and La (1, 2, 3). The concept of epitope spread, or the ordered emergence of autoreactivity to multiple autoantigen epitopes, has been proposed as a mechanism for the development of disease. This hypothesis was confirmed by clinical data indicating that loss of tolerance to phospholipids and anti-nuclear antibodies (ANA) occured first, followed by activation of Ro, La, DNA specific B cells and finally by anti-Sm and RNP (4). These antibodies form immune complexes (IC) that deposit in various tissues, leading to inflammatory diseases like glomerulonephritis, vasculitis, dermatitis, arthritis, and nervous system disorders (5). The cause of SLE is unknown, however, a combination of genetic and environmental factors are believed to play a role. For instance, hormones are thought to influence the disease and its onset because SLE disproportionately affects women at a ratio of nine females to one male. Many susceptibility genes have been identified through the study of lupus-prone animals and patients with SLE. It is thought that around 30 different genes contribute to the pathogenesis of this disease (6). There are a variety of manifestations of this disease which complicates its diagnosis.

The anti-Sm antibody titer is a key diagnostic tool for SLE (2). Sm and RNPs are composed of small nuclear (sn) RNAs that are rich in uridylate and when associated with

proteins make up the small nuclear ribonucleoprotein particles (snRNPs) (7). snRNPs form the spliceosomal complex that removes the non-coding RNA from pre-mRNA (8). The response to an unknown nuclear component was initially described in 1966 from the serum from a young SLE patient, Stephanie Smith (1). The response to Sm is of particular interest because it is unique to SLE, however, not all patients diagnosed with the disease have this response (2, 9, 10). Interestingly, the presence of these antibodies are associated with renal complications and increased disease activity and severity (2, 9, 10). The anti-Sm response is an essential aspect of the diagnosis and understanding of SLE.

The pathogenesis of SLE is complex and largely unknown; however, it is hypothesized that apoptotic cells play an important role in the disease. These cells display nuclear antigens such as dsDNA, nucleosomes, histones, and Sm on their surface and provide a ligand for autoreactive B cells and possibly toll-like receptors (TLRs) (11, 12, 13, 14). In murine models, studies show that an acculumation of apoptotic cells leads to autoreactive B cells activation (13, 15). In SLE elevated levels of apoptotic cells have been described in blood and bone marrow (16, 17). Additionally, dysfunction in the uptake and clearance of apoptotic cells has also been implicated in the disease (18, 19). Interestingly, a study of non-autoimmune mice immunized with antigen from apoptotic cells resulted in development of SLE by the epitope spread model (20). This increase in available antigen could contribute to the loss of autoreactive B cell tolerance in patients resulting in serum ANA titers and disease.

Murine models of SLE

Murine models are utilized to study the pathogenesis of SLE. These models can be divided into three categories: engineered models, congenic models, and mice that spontaneously develop lupus-like disease. Genetically engineered mice eliminate genes that encode for components of the immune system thought to be involved in SLE. However, because the entire gene is eliminated, other possible phenotypes of the deletion must be considered. Thus, gene knockouts may not reflect actual mutations that occur in SLE patients. There is a wide variety of molecules involved in clearance of autoantigens that are of particular interest. For example, mice deficient in DNase1, or the complement components, C1qa and C4, display SLE symptoms such as ANA and nephritis (21, 22, 23). Mice that are deficient in cytokines are important to assess their ability to regulate the disease Lupus prone mice deficient in IFN-α and TGF−β, have exacerbated disease indicating that it has protective effects, however, mice lacking IFN-γ have a reduction in symptoms (24, 25, 26). Mice that lack molecules, such as Src family kinases, FcγRIIb, and CD45, important in regulating B and T cell signaling also develop SLE (27, 28, 29, 30). Congenic models provide a tool to isolate individual susceptibility loci and understand their contribution to the disease. Of interest, the *Sle1*, *Sle2*, and *Sle3* susceptibility loci were discovered in an inbred model of SLE, NZM2410 (30, 31, 32, 33, 34). Each of these loci confer unique contributions to the onset or manifestations of disease and are influenced by other loci. *Sle1* on the C57BL/6 (B6) background has a strong anti-chromatin response to the H2A/H2B/DNA subnucleosomes, however, the mice do not develop disease (32). Adoptive transfer experiments demonstrate that the loss of tolerance by B cells to chromatin is mediated by *Sle1* expression (35). *Sle2* mice

has hyper-reactive B cells and an increase in peritoneal and splenic B1 B cells, in contrast, on the B6 background they do not have an ANA titer (31). The *Sle3* loci in B6 mice, confers activated T cells with reduced apoptosis and the development of autoantibodies (33). When mice are generated that express both *Sle1* and either Y chromosome-linked autoimmune accelerator (*Yaa)* or the FAS^{lpr}, glomerulonephritis develops more rapidly (36, 37). From the BXSB lupus-prone mouse other loci have been identified. *Bxs1*, *Bxs2/3*, and *Bxs1/4* are all important in the development of nephritis (36, 37, 38, 39). BXSB/*Yaa* mice exhibit accelerated onset of SLE in males on an autoimmune background (40). The *Yaa* gene is associated with monocytosis characterized by an expansion of CD11c-expressing cells (41, 42). The increase in monocytes correlates with an increase in production of autoantibodies (43). B cells in these mice have increased TLR7 expression due to a duplication in the gene encoding that receptor (44). New Zealand Black crossed to New Zealand White (NZB x NZW) mice present with lupus-like symptoms, but also have other autoimmune diseases (45). Closely related to this background are NZM2410 mice. In-breeding between the progeny of the NZB x NZW formed the New Zealand Mixed (NZM) mice (46). These mice are particularly useful because they are homozygous at all loci and both males and females develop lupus symptoms at a young age (47). All of these mouse models can be utilized to study the immune dysregulation that occurs in SLE.

Another spontaneous murine model, MRL/*lpr*, has an autosomal recessive *lpr* mutation, which results in a loss of function defect in Fas(48). Signaling through Fas initiates apoptosis, therefore, its disruption leads to an increase in survival by lymphocytes (48).

Defective Fas results in aberrant T cell selection in the thymus and increased lymphocyte survival in the periphery, which contributes to an expansion or persistence of autoreactive cells (49, 50). Because of their predisposition to autoimmunity, the MRL/*lpr* animals spontaneously develop an increased ANA, glomerulonephritis, lymphosplenomegaly, and joint and skin disorders. Furthermore, approximately 25% of the mice develop an anti-Sm titer, which is similar to the response in SLE patients (3, 51, 52). This murine model of lupus is particularly useful because many aspects of their disease development and symptoms mirror that of humans.

Fc Receptors and SLE

Fcγ receptors (FcγR) play an essential role controlling the immune response and loss results in autoimmune disease. Increased autoantibody production leads to the formation of IC leading to inflammation. FcγR bind to Fc domains of immunoglobulin (Ig) G (IgG) with varying affinities and promote the clearance of IC. The activating receptors, FcγRI and FcγRIII on antigen presenting cells (APCs) bind to monomeric IgG and IC containing IgG and enhance the autoantibody response by influencing the epitopes that are presented (53). Binding of chromatin-IC by FcγRIII on dendritic cells (DCs) induces secretion of B lymphocytes stimulator (BLyS), a TNF family member associated with lupus (54, 55). FcγRIIa and FcγRIII are essential in the transfer of IC from erthrocytes to macrophages (MΦs) and for engulfment of these complexes (56). DNA-containing IC activates plasmacytoid DCs (pDCs) through FcγRIIa and the toll-like receptor 9 to secrete the proinflammatory cytokines IFN-α and IL-8 (57). The downregulation of activating FcγR results in decreased inflammatory response (58, 59). FcγRIIb is an

inhibitor of B cell signaling and can also suppress autoantibody production by the B cell (60). Deletion of the FcγRIIb gene in C57BL/6 mice causes an increase in IC, autoantibody production and lupus symptoms (27). Restoring this receptor expression by retroviral transduction reverses disease (61). Studies show that the expression of FcγRIIb on germinal center B cells is reduced by ten-fold in autoimmune-prone mice (NZB x NZW) $F_1$and expression is decreased on memory B cells in SLE patients (62, 63). This defect was directly linked to the failure to downregulate calcium signaling in B cells (62). In support of a role for dysregulated FcγRIIb in SLE, a polymorphism (FcγRIIbT_{232}) was found to exclude the receptor from the lipid raft resulting in aberrantly activated B cells (64). The expression of FcγR on DCs in SLE has not been studied, however, FcγRIIb on non-autoimmune DCs captures IC and recycles them as intact complexes back to the cell surface to activate B cells (65). Thus, FcγRIIb can provide a source of antigen to autoreactive B cells. The role of FcγRs is important in the clearance of IC and the inhibition of BCR signaling.

Complement and SLE

Another molecule that has been implicated in SLE is complement. Apoptotic cells can activate the complement cascade leading to complement molecules then depositing in sites of inflammation. C1, C4, and C3 bind to IC to maintain solubility for clearance; and CR4 and CR1 transfer IC from erthrocytes to macrophages for engulfment (56, 65, 66, 67, 68). Defective clearance of apoptotic cells or IC, results in acculumation and they become immunogenic. In humans, a homozygous complement deficiency in C1q, C1r, C1s, C4 or C2 predisposes those affected individuals to develop SLE (69). MΦs from

patients are defective in the clearance of apoptotic cells because of decreased amounts of C1q, C4, and C3 in their serum (18). C3aR is essential in the development of nephritis and treatment with an agonist in lupus-prone mice ablates the inflammatory properties of this receptor (70). Mice that lacking C1qa or C4 have increased ANA, apoptotic cells, IC, and develop glomerulonephiritis (22, 23). The increase in production of antibodies to C1q and decrease in the levels of C1q is found in patients with the SLE and correlates with an acquired C1-inhibitor deficiency (71, 72). The binding of C1q to apoptotic cells that are not eliminated results in the production of autoantibodies to this complement component (69). Elimination of C3 in MRL/*lpr* causes no change in ANA but an increase in IC deposition in the glomeruli (73). MRL/*lpr* mice deficient in C5aR have reduced disease, anti-DNA titers and Th-1 responses (74). Complement is implicated in B cell development through the expression of CD19 and CD81. These receptors could bind to apoptotic bodies facilitating the ligation of autoreactive B cell receptor (BCR) and resulting in deletion. A deficiency in this mechanism would cause autoreactive B cells to escape tolerance (75). Because patients who lack components of complement pathway develop SLE, it can be concluded that complement is essential in the maintenance of tolerance.

Toll-like receptors and SLE

One way the body can distinguish an invading microbe during innate immunity is the recognition of pathogen-associated molecular patterns. These components are conserved elements of infectious agents identified by TLRs. Eleven TLRs have been identified and they recognize a variety of pathogens such as viral RNA, CpG DNA, and other

components of bacteria, viruses, or fungi. In particular, TLR7/8, 9 and 3 recognize ssRNA, unmethylated CpG DNA, and dsRNA respectively. In response to microbial stimulation the TLR initiates signal transduction that activates both the innate and adaptive immune responses in order to quickly respond to infection. This stimulation results in the production of inflammatory cytokines, chemokines, anti-microbial factors, proliferation and upregulation of costimulatory and adhesion molecules. Activation of the innate immune system through TLRs can induce an autoimmune response (76, 77, 78). Lipopolysaccaride (LPS), a TLR4 ligand, augments an immune response of normal mice to apoptotic antigens (20). It has also been shown that immunization of lupus-prone mouse models with either unmethylated CpG DNA or polyinosic polycytidylic acid RNA (ssRNA), cause exacerbation of the disease symptoms (79, 80, 81). This data indicates that stimulation of the innate immune system through TLRs might initiate or exacerbate SLE.

TLRs are important in both the regulation and the development of SLE. A common stop codon polymorphism of TLR5, that recognizes bacterial flagellin, is associated with protection from lupus symptoms supporting a role for infection in the development of innate immunity (82). Females with SLE produce higher amounts of IFN-α compared to males in response to TLR7 stimulation (83). Genetic abnormalities in TLR7 have been implicated in the skewing of autoreactive B cells to respond to RNA-associated antigens and the aggravation disease (44, 84). Lupus-prone mouse models that lack TLR7 do not display symptoms of disease, in particular, these mice failed to produce antibodies to RNA-containing antigens (85). In contrast, lupus-prone mice that are deficient in TLR9

demonstrate exacerbated disease (85, 86). These results indicate that TLR7 promotes disease and TLR9 regulates it. However, the role of TLR9 in SLE still remains controversial as some studies indicate a pathogenic role. SLE patients with active disease had an anti-dsDNA titer and increased proportion of plasma cells, memory B cells, and monocytes that expressed TLR9 (87). Further, in lupus-prone mice TLR9 and myeloid differentiation factor 88 (MyD88) is required for class switching in anti-DNA B cells to pathogenic IgG2a and 2b autoantibodies (88). Therefore, the role TLR regulation and dysfunction in SLE remains to be elucidated.

The primary role for TLRs is to recognize foreign antigens, however, they interact with nuclear self-antigens as well. Because of abnormalities in the clearance of apoptotic cells and the increase in immune complexes in SLE patients more antigen is available to the innate immune system. pDCs are activated to secrete IFN-α by IC containing either mammalian DNA-IC through TLR9 or RNP-IC through TLR7 (89). DCs are activated by the co-ligation of FcγRIII and TLR9 by chromatin-IC to produce the proinflammatory cytokine TNF-α (55). pDCs can bind to DNA-IC through TLR9 and FcγRIIb (90). The regulation of autoreactive B cells by stimulation of both the BCR and TLR has also been characterized utilizing B cells from rheumatoid factor mice (91, 92). Studies show that autoreactive B cells are induced to proliferate by the dual ligation of DNA or RNA-IC to both the BCR and TLR9 or 7 respectively (91, 92). It is hypothesized that the binding of antigen to the BCR or the FcR, leads to endocytosis of the antigen and provide a mechanism to present the antigen to the TLRs located in endosomal compartments. The ability of TLRs to be cross-reactive with mammalian nuclear components may result in

functionally ignorant, autoreactive B cells secreting autoantibodies because of costimulation through the TLR (93). This cooperation between the two receptors may be one mechanism to break tolerance to nuclear self-antigens.

Cytokines and SLE

The aberrant production of cytokines, important in regulating immune functions contributes to SLE. Interferons (IFN) are increased in lupus patients and have been shown to affect the pathogenesis of SLE (94). Lupus-prone mice that are deficient in IFN-α (type I) have exacerbated disease, however, mice lacking either IFN-γ (type II) or IL-4 do not develop disease or have a reduction of symptoms (24, 25). Studies show that IL-21 promotes autoantibody production in MRL/*lpr* mice (95). MΦs from lupus-prone animals are defective in secretion of TNF-α, resulting in reduced IL-1 and IL-6 production(96). MZ B cells from lupus-prone mice produced IL-10 in response to CpG-ODN, inhibiting the production of IL-12p40 and IFN-γ in response to TLR9 stimulation in other splenocytes (97). Patients with active SLE and some murine lupus models have increased levels of IL-6, and anti-IL-6 treatment in mice reduced the production of anti-dsDNA antibodies (98, 99). It is not apparent how each cytokine abnormality contributes to SLE, however, these molecules clearly play a role in the broader dysregulation that results in the disease.

DC/MΦs secrete a variety of cytokines to affect the regulation of the immune system. Many of these molecules are typically thought of as proinflammatory, however, they also

have the ability to be repressive depending on the target cell. DCs can promote B cell growth and differentiation by secreting IL-6 and IL-12 (100). Further, virally-stimulated pDCs secrete IL-6 and IFN-αβ activating naïve B cells acutely stimulated with antigen to secrete virus-specific Ig (101). Evidence indicates that polyclonal stimulation of DCs results in IL-6 secretion, which relieves the repressive effect of T regulatory cells to allow T cells to be activated in the antigen-specific adaptive immune response (102). Other studies show that IL-6 and CD40L repress Ig secretion by LPS-stimulated, chronically antigen-experienced autoreactive B cells (103, 104). These studies indicate that cytokines have multiple roles in the control of the immune response. Further, the regulation of cytokine production by other cytokines promotes a balance in the immune system, and their dysregulation results in the development of autoimmunity.

B cells and SLE

B cells are key participants in the humoral immune response. When activated they secrete Ig, or antibodies, which are essential in neutralizing and destroying pathogens. These antibody secreting cells can develop into long-lived memory cells that are important in the response against repeat infection. They can also process and present antigen to activate T cells and to receive T cell help. The role for B cells in immunity is diverse, and their dysregulation is a key component in the development of SLE.

Because SLE is characterized by the production of autoantibodies to nuclear components, it is clear that hyper-responsive, autoreactive B cells play a central role in the

development and pathogenesis of the disease. Studies show the transfer of pre-B cells from lupus-prone (NZB x NZW) F_1 mice into mice lacking B cells results in lupus symptoms (105). Further, the development of disease is prevented in lupus-prone animals lacking B cells (106, 107). Patients with SLE have elevated serum levels of BLyS which promotes B cell survival and activation (108, 109). Thus, the abnormal activation and survival of B cells results in their targeting for therapy in SLE treatment (110).

The process of B cell development and regulation is important in controlling and eliminating autoreactive cells. In lupus-prone mouse models, autoreactive B cells escape deletion usually occurs after an encounter with high-affinity antigen (111). Further, in SLE patients, it has been found that early B cell tolerance checkpoints as well as memory B cell development are defective (112, 113, 114). Studies also show that in lupus-prone mice, anti-Sm, autoreactive B cells escape the pre-plasma cell checkpoint and become antibody secreting cells (ASC) (115). In mice with Sm-specific B cells that also have impaired apoptotic cell clearance, there is a loss of the MZ and B-1 cell populations coincident with the ability to circumvent the pre-plamsa cell checkpoint (13, 116). Mice expressing the Yaa mutant gene associated with lupus have a significantly reduced transitional 2 and marginal zone (MZ) B cell population (117). While the authors conclude that this is a result of defective development of MZ B cells, it could also be due to premature development into plasma cells. Studies of the NZM Tan mice, who have the same susceptibility loci as NZM2410, indicate that these mice do not develop disease symptoms, however, the MZ appears to be defective because they do not migrate to the

follicle when stimulated and fail to interact with T-independent antigens (118). In the (NZB x NZW) F_1 mice the MZ is shown to be expanded and associated with disease (119, 120, 121). These defects in the development and control of autoreactive B cells are critical in the development of disease.

Because the SLE B cells are aberrantly activated to produce autoantibodies, it is not surprising that B cell signal transduction is abnormal. Upon activation these cells show enhanced tyrosine phosphorylation and intracellular calcium flux compared to normal B cells (122). In lupus-prone animals, resting B cells are shown to be hyper-responsive, and express increased costimulatory molecules when stimulated with T-cell associated stimuli (123). As discussed above, FcγRIIb is a negative regulator of BCR signaling. Some SLE patients have a defective version of this receptor, while others fail to upregulate its expression, contributing to an increase in calcium flux upon activation compared to healthy controls and correlates with increased IgG autoantibodies in (NZB x NZW) F_1 (63, 124). The expression of inducible costimulator ligand (ICOS-L) is downregulated in SLE B cells and is thought to be a result of interaction with ICOS high-expressing T cells to induce plasma cell differentiation (125). CD72 can both be a positive and negative regulator of BCR signaling. A study of SLE patients revealed that expression of CD72 is reduced and over 95% of patients had mutations in mRNA. Roughly half of the mutations were in the tyrosine-based inhibitory motif (ITIM) indicating a loss in repressive ability (126). CD19 is an enhancer of BCR signaling and in most SLE patients CD19 expression is lower than normal, however, a population of individuals that have high levels of expression on antigen-selected, activated memory B

cells (127). All of these defective components of signaling act to enhance the BCR response to stimulation contributing to the hyper-reactivity of these cells.

Mechanisms of B cell Tolerance

Regulation of autoreactive B cells is essential to prevent an immune response to self-antigens. Developing autoreactive B cells in the bone marrow and the periphery are controlled by tolerance mechanisms including: deletion or receptor editing; and are subjected to a checkpoint at the pre-plasma cell stage and another during memory B cell development (113, 115). Low-affinity antigens may not be recognized by the autoreactive B cell or may not transduce a strong enough signal through the BCR to induce a response, and thus tolerance is maintained by functional ignorance (128). During the adaptive immune response B cells can be regulated by the lack of T cell help or being positively selected into the B-1 compartment (129, 130, 131). Exposure of the BCR to antigen leads to failure to renew signal transduction, which results in desensitization of the BCR signaling complex (132).

Antigen ligation can lead to the destabilization of the BCR signaling complex, which is thought to be another regulatory mechanism of B cell tolerance. The BCR complex consists of μ-heavy chain (μm) and Ig-α/β heterodimer. This signaling complex mediates the BCR signal transduction through their immunoreceptor tyrosine-based activation motif (ITAM) in their cytosolic tail. During BCR-mediated signal transduction, the Igα/β physically dissociates from the μm indicating a destabilized BCR complex. BCR destabilization was identified as the inability to coprecipitate

stoichiometric amounts of Ig-α/β with μm following antigen stimulation (133). The distance of this separation is approximately 200 nm, as quantitated by electron and confocal microscopy (134). Upon antigen ligation to the BCR, autoreactive B cells become unresponsive to further stimulation (132). Destabilization was shown to be coincident with receptor desensitization, indicating the destabilization plays a role in maintaining autoreactive B cell unresponsiveness. Further studies showed that if signal-competent and signal-incompetent receptors were co-aggregated, then signal transduction is negatively affected (135). These studies also showed that if just 15% of the surface receptors were signal-incompetent, then the competent receptors were also unable to initiate signal transduction (135). Taken in conjunction with the required constant receptor occupancy for B cell anergy (136), this data suggests that a constant receptor ligation by antigen would lead to destabilization, aggregation, and attenuation of signal transduction that would maintain autoreactive B cells unresponsiveness.

The activation of the innate immune system can cause activation of autoreactive B cells, however, there are mechanisms to prevent breaking tolerance each time it encounters infection. B cells that have constant receptor occupancy with antigen will remain angeric (136). Hen egg lysozyme (HEL)-specific B cells that have chronically ligated BCR have constitutive ERK activation, which represses Ig secretion induced by CpG, TLR9 stimulation. DCs or MΦs are able to repress Sm-specific, low-affinity B cell Ig secretion in response to TLR4 stimulation by LPS by secreting soluble mediators, IL-6 and CD40L respectively that act on the B cell to attenuate the autoantibody response (103, 104). This mechanism of tolerance is not limited to Sm-specific B cells, because HEL-specific B

cells from soluble HEL expressing mice and *p*-azophenylarsonate-specific B cells, that are cross reactive with ssDNA, are also regulated by DCs and MΦs during activation of the innate immune response (103). If DC/MΦs are removed from the culture then, the B cells are able to secrete in response to LPS indicating that this tolerance is reversible. These mechanisms are essential to prevent activating autoreactive cells during innate immune stimulation

B cell Tolerance to Sm Murine Models 2-12H and 2-12H/Vκ8

The response to Sm has been characterized in both humans and the MRL/*lpr* mouse model of SLE (3, 52). In order to study the regulation of B cells specific for the antigen, transgenic mice were developed. The transgene in these mice is a BCR heavy chain that was derived from a hybridoma from MRL/*lpr* (137). The 2-12H mouse has a fixed heavy-chain that can pair with variable light chains to create a varied, autoreactive B cell repertoire (137). Approximately 30% of the B cells are specific for Sm at varying affinity, while the remaining B cells may bind to ssDNA, dsDNA, or have an unknown specificity (129, 138, 139). Despite the large number of B cells specific for nuclear self-antigen, these mice do not develop autoimmune disease (137). It is thought these B cells are regulated by a variety of tolerance mechanisms including ignorance, anergy, developmental arrest and the formation of early pre-plasma cells (115). In order to pinpoint the role of affinity in this Sm-specific response, an Ig-transgenic mouse of 2-12H is crossed with a mouse expressing Vk8 light chain to generate B cells with low-affinity for Sm (140). These mice have low levels of serum transgenic-antibodies, indicating that the B cells are not spontaneously activated *in vivo* (140). Because these

mice do not develop autoimmunity it makes them ideal for studying tolerance mechanisms that regulate low-affinity, autoreactive B cells.

Dendritic cells and Macrophages and SLE

DCs and MΦs have similar roles in the immune response. They are responsible for obtaining antigen and presenting it to activate T cells. The immunoregulatory effects of DC/MΦs are important in the balance between activation of the innate immune system and maintaining tolerance to autoantigens. DCs regulate self-reactive T cells in the periphery by inducing their proliferation and apoptosis (141). MΦs have also been shown to repress T cells during infection (142). MΦ ingestion of necrotic cells causes enhanced antigen-presentation (143). Further, engulfment of necrotic cells results in DC maturation and will induce $CD4^+$ and $CD8^+$ T cell responses (144). DCs that phagocytose apoptotic cells, will not be able to efficiently present antigen to T cells; however, if they are matured by another stimulus then they are able to cross-present to $CD8^+$ T cells (144). This inability to present stimulatory antigen can lead to induction of peripheral tolerance in T cells during steady state conditions (145). Tolerogenic DCs appear to be mature by the expression of cell surface markers, however, they lack of production of IL-12 and the ability to produce IL-10 (146). These cells act to drive the development of T regulatory cells (147). Therefore, DC/MΦs not only stimulate the T cell response but also have the ability to regulate it as well.

Several studies have demonstrated dysregulation of DCs in both lupus patients and lupus-prone murine models. Interestingly, DCs accumulate in older, diseased lupus-prone mice

(148, 149). However, it has been shown that these cells can be defective. In NZM2410 mice, CD40 expression on the surface of splenic DCs is increased abnormally prior to disease development and it is thought to be a result from stimulation *in vivo* because upregulation does not occur on bone marrow-derived DCs (149). This may indicate that the DCs are being stimulated to mature and survive which could explain the accumulation of DCs in diseased animals (150). CD40 can rescue B cells stimulated with high-affinity antigen from cell death which may override the tolerance mechanism of inducing the death of autoreactive B cells (151). Another study demonstrated that myeloid DCs from SLE patients have increased costimulatory markers, MHC class II, and proinflammatory cytokine IL-8; and these DCs caused proliferation and activation of T cells (152). Further, mature DCs would not be able to respond to new stimulation by pathogens and therefore contribute to prolonged infection. The upregulation of the costimulatory molecule CD80 is deficient in some patients and NZM2410 and (NZB x NZW) F_1 (149, 153, 154). CD80 is able to activate regulatory T cells, and if these cells cannot be stimulated by DCs then autoreactive B cells may become activated. The dysregulation of DCs' ability to maintain a balance between activation of the immune system and induce tolerance to self-antigen contributes to the development of SLE.

MΦs are also a key participant in SLE. In particular, MΦs and T cells accumulate in the glomeruli, contributing to glomerulonephritis (155). Studies of MΦs in lupus-prone mice have shown that they have certain defects as well. Interestingly, their morphology and cytoskeleton appears to be quite different from normal mice. The amount of lupus-prone MΦs that adhere *in vitro* is greatly increased compared to non-autoimmune mice and the

reduced activity of the cytoskeleton regulator, Rho, results in a larger cell size (156, 157). Extensive studies in murine lupus models show that their MΦs are defective in their production of IL-1α, IL-1β, IL-12, and IL-6 in response to stimulation which is intrinsic to the MΦs as demonstrated by bone marrow chimeras (158). Both the defect in IL-1 production and the differences in morphology are coincident with the presence of lipids from fetal bovine serum or apoptotic cells (156, 159). Given their role in regulation of the immune system, defects in MΦ regulation are important in the pathogenesis of SLE.

The clearance of apoptotic cells is essential to maintain tolerance to self-antigen. Apoptotic cells display nuclear self-antigen on their surface (11, 13, 14). Therefore, these cells can provide a source of self-antigen to autoreactive BCRs and to TLRs that may aberrantly activate the immune system. Apoptotic cells form IC with soluble antibody, resulting in inflammation. (NZB X NZW) F_1 mice have DC/MΦs that are defective in their uptake of and destruction of DNA leading to an increase in the formation and accumulation of anti-DNA IC (160). The increased apoptotic load in SLE has been shown to be a result of defective uptake by MΦs on the Fas^{lpr} background (116, 161, 162). DC/MΦs that engulf apoptotic cells present antigen through MHC molecules to T cells without upregulating costimulatory molecules to maintain tolerance (145). Data indicate MΦs that phagocytose apoptotic cells produce anti-inflammatory response upon LPS stimulation (163, 164). In contrast, DCs stimulated with LPS after engulfing apoptotic cells secrete the proinflammatory cytokine TNF-α (144). During infection in patients with SLE where polyclonal activation and exposure to apoptotic cells may occur congruently, presentation of apoptotic antigens may become immunostimulatory.

Understanding DC/MΦ-mediated tolerance is essential to elucidating how their dysregulation contributes to the development of autoimmunity.

DC: B cell interaction

DCs can display antigen on their surface by mechanisms other than MHC peptide presentation. When pulsed with exogenous antigens, such as HEL or human serum albumin, they internalize, maintain intracellular pools of antigen, and recycle them, intact, back to their surface (165). In another mechanism, FcγRIIb has been shown to mediate the uptake and recycling of IC with OVA to the DC surface (65). In fact, it was demonstrated that this presentation of intact exogenously loaded antigen to results in DCs interacting with antigen-specific B cells (65, 165, 166, 167). Both *in vitro* and *in vivo* experiments showed that B cells can recognize and acquire surface-antigen on DCs. This interaction results in T cell independent signal transduction and T cell dependent class switching to produce Ig (65, 165, 166, 167, 168). Therefore, DCs display exogenous native antigen in a form that B cells may encounter through their BCR. The contact between DCs and B cells through intact antigen interacting with the BCR is of interest in the study of B cell tolerance and activation mechanisms.

Model for DC/MΦ-Mediated B cell Tolerance

Our previous studies show that during polyclonal activation, antigen-experienced, autoreactive B cells are regulated by myeloid (my)DCs and MΦs. LPS-stimulated DC/MΦs secrete soluble mediators that prevent the B cells from secreting Ig, specifically, IL-6 and TNF-α while MΦs produce CD40 ligand (CD40L) ((103, 104)

Gilbert MR and Vilen BJ manuscript in preparation). This mechanism regulates several different BCR transgenic mouse models that are specific for self-antigen, including: low-affinity Sm (2-12H/Vκ8), p-azophenylarsonate (Ars), and HEL (103). Only chronically antigen experienced mice are sensitve to the DC/MΦ-mediated regulation, therefore, we believe that the constitutive ligation of the BCR with antigen modulates the response to the soluble mediators, and this mechanism is dependent on ERK (Rutan JA manuscript in preparation). Autoreactive, follicular (FO) B cells are repressed by both DCs and MΦs while MZ B cells are only susceptible to inhibition by MΦs (104). The MΦ secreted CD40L inhibits autoreactive MZ B cell Ig by preventing their development into plasma cells by downregulating the transcription factors Blimp-1 and XBP-1 required for differentiation into antibody secreting cells (104). Understanding this DC/MΦ-mediated mechanism to regulate LPS-stimulated autoreactive cells is important in further elucidating how self-tolerance is maintained to prevent autoimmune disease.

Chapter II. Dendritic cells from lupus-prone mice are defective in repressing immunoglobulin secretion

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Contributions

Diane Carnathan contributed firgues 2.1, 2.2B, 2.4, 2.5, 2.7, and Table 2.1. She wrote the Materials and Methods, portions of results section, and edited the manuscript. Mileka Gilbert contributed figures 2.2A, 2.3, 2.7, and 2.8 wrote the remaining portions and edited manuscript. Patricia Cogswell contributed figure 2.6. Li Lin did the statisitical analysis. Albert Baldwin supported Patricia Cogswell. Barbara Vilen is the Primary Investegator and edited the manuscript.

Abstract

Autoimmunity results from a breakdown in tolerance mechanisms that regulate autoreactive lymphocytes. We recently showed that during innate immune responses, secretion of IL-6 by dendritic cells (DCs) maintained autoreactive B cells in an unresponsive state. Here we describe that TLR4-activated DCs from lupus-prone mice are defective in repressing autoantibody secretion, coincident with diminished IL-6 secretion. Reduced secretion of IL-6 by MRL/*lpr* DCs reflected diminished synthesis and failure to sustain IL-6 mRNA production. This occurred coincident with lack of NF-κB and AP-1 DNA binding and failure to sustain IκBα phosphorylation. Analysis of individual mice showed that some animals partially repressed Ig secretion despite reduced levels of IL-6. This suggests that in addition to IL-6, DCs secrete other soluble factor(s) that regulate autoreactive B cells. Collectively, the data show that MRL/*lpr* mice are defective in DC/IL-6-mediated tolerance, but that some individuals maintain the ability to repress autoantibody secretion by an alternative mechanism.

Introduction

Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease characterized by the production of autoantibodies to nuclear components. Alternating periods of flares and remissions are associated with an increased burden of apoptotic cells, the formation of immune complexes, and inflammation (169). The etiology of SLE remains unknown; however, multiple immunoregulatory defects have been identified in lupus-prone mice (69, 96, 159, 170, 171, 172, 173, 174, 175, 176, 177, 178), including complement deficiencies, TCR signal transduction anomalies, and dysfunctional cytokine secretion by macrophages (MΦs). These defects contribute to the onset and/or pathogenesis of SLE, while a breakdown in tolerance leads to the formation of autoantibodies and immune complexes that may play a role in vasculitis, glomerulonephritis, and cerebritis (179).

Studies in immunoglobulin (Ig) transgenic (Tg) mouse models have defined anergy as a state of unresponsiveness that regulate autoreactive B cells in the periphery (128, 140, 180, 181, 182). Anergic B cells fail to secrete antibody in response to LPS or antigen immunization due to receptor unresponsiveness (140, 181, 183). Some anergic B cells exhibit reduced surface IgM levels (184, 185), decreased lifespan (183, 186), and exclusion from the lymphoid follicle (186, 187). In the case of B cells specific for the lupus-associated antigen, Smith (Sm), a partially anergic phenotype is evident. Sm-specific B cells from 2-12H/Vκ8 Ig Tg mice are unable to secrete Ig in response to LPS, yet maintain surface IgM levels, exhibit a normal lifespan, and remain competent to enter the B cell follicle (140). Recently, we described that Sm-specific B cells purified from myeloid dendritic cells (myDCs) and MΦs regain the ability to secrete Ig in response to

LPS (103). The data show that secretion of IL-6 by DC/MΦs represses LPS-induced Ig secretion by autoreactive B cells without repressing acutely stimulated naïve B cells. This mechanism of tolerance is not limited to Sm-specific B cells as chronically antigen-experienced HEL- and Ars/A1-specific B cells are similarly affected (103). These findings identify a unique mechanism of B cell tolerance wherein DCs and MΦs play a central role in regulating autoimmunity during innate immune responses.

MyDCs and plasmacytoid DCs have been described as positive regulators of immunity promoting growth and differentiation of some B cells through the secretion of IL-12, IL-6, BLyS, and APRIL (101, 188, 189). Specifically, IL-6 was found to promote plasma cell survival (190, 191). Although this seems paradoxical, the data indicate that IL-6 differentially regulates naïve and chronically antigen-experienced B cells (103). Studies identifying IL-6 as a positive regulator focused on B cells from non-Tg mice where the proportion of autoreactive cells is low. In contrast, the studies showing that IL-6 represses autoantibody production used self-reactive Ig Tg models where the B cells were constantly exposed to self-antigen (103). Thus, IL-6 acts as a positive or negative regulator of B cells depending on the history of BCR ligation. We propose that chronic BCR ligation by self-antigen reprograms IL-6R-mediated outcomes allowing naïve B cells to produce Ig in response to polyclonal stimulation while simultaneously repressing autoreactive B cells from producing autoantibody. These findings identify a novel B cell tolerance mechanism, and suggest that overcoming tolerance in SLE might be associated with defects in the repression of autoreactive B cells by myDCs and/or MΦs.

In this report, we show that LPS-activated DCs from MRL/*lpr* mice inefficiently repress Sm-specific Ig secretion, coincident with diminished IL-6 secretion. Mechanistically, diminished secretion of IL-6 resulted from decreased synthesis of IL-6 mRNA coincident with decreased IκBα phosphorylation and reduced DNA binding by NF-κB and AP-1. These data identify signal transduction defects in DCs that occur coincident with diminished IL-6 secretion and failure to repress Ig secretion by autoreactive B cells. Further analysis of DC-mediated tolerance mechanisms revealed that DC conditioned medium (CM) from some MRL/*lpr* mice repressed Ig secretion despite low levels of IL-6. This suggested that additional soluble factors are involved in repressing autoantibody secretion. These findings implicate DC defects in the breakdown of tolerance in lupus-prone mice and suggest that defects in multiple factors may be required for the complete breakdown of tolerance associated with autoimmunity.

Materials and Methods

Mice

2-12H/Vκ8/Cκ$^{-/-}$ immunoglobulin transgenic mice were previously described (103, 140). MRL/MpJ-*Fas*^{lpr}/J (MRL/*lpr*) and C57BL/6J (B6) mice were purchased from The Jackson Laboratory, and NZM2410 mice from Taconic. NZBxNZW$_{F1}$ mice were obtained from Trine Jorgensen (University of Colorado), MRL/MpJ (MRL) and B6.*Fas*^{lpr} (B6.*lpr*) from Stephen Clarke (University of North Carolina). 2-12H/Vκ8/Cκ$^{-/-}$ mice were used at 9-17 weeks of age. All other mice were used at 6-10 weeks old. All studies were approved by the Institutional Animal Care and Use Committee.

Reagents and Antibodies

7-AAD, rIL-6, and antibodies to CD11c, CD11b, B220, and IL-6 were purchased from BD Biosciences, GR1 and TLR4 from eBiosciences, phospho-IκBα from Cell Signaling, IκBα and β-tubulin from Santa Cruz, and IgG HRP from Promega. Streptavidin-AP was purchased from Southern Biotech, anti-actin, TEPC 183, and *Escherichia coli* 055:B5 LPS from Sigma Aldrich, 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) from Calbiochem, *E. coli* 0111:B4 LPS from List Biological Laboratories, mouse GM-CSF and IL-4 from PeproTech, poly (I:C) and R848 from InvivoGen, and CpG oligodeoxynucleotides (ODN) and non-CpG ODN from Coley Pharmaceutical Group. JA12.5, 54.1, 187.1, HB100, and CRL 1969 were purified from hybridoma culture supernatant.

Cell Purification

B cells were purified from 2-12H/Vκ8/Cκ$^{-/-}$ spleens by negative selection (StemCell Technologies) (103). Biotinylated CD3 antibody was added to the antibody cocktail to increase the efficiency of T cell depletion. B cells were 86-93% pure with <3% T cells and <7% DCs/MΦs. Splenic CD11c^{+} cells (~70% pure) were purified by positive selection (Miltenyi Biotec) and found to contain 20% lymphocytes and 10% MΦs.

Bone marrow-derived DC (BMDC) Cultures

Bone marrow-derived DCs were generated as previously described (103). BMDCs were >95% CD11c^{+} (CRL 1969 hybridoma). Conditioned medium (CM) was made from 1x10^{4} BMDCs (0.2 ml) cultured for an additional 4 days with or without Sigma LPS (30 μg/ml). 5x10^{5} BMDCs (0.2 ml) were cultured for an additional 4 days with or without poly (I:C) (50 μg/ml), R848 (10 μg/ml), CpG ODN (1 μg/ml), or non-CpG ODN (1 μg/ml). In experiments where RNA was isolated or nuclear extracts were prepared, BMDCs were stimulated with *E. coli* 0111:B4 LPS (List Biological Laboratories) that was re-purified (192) and confirmed to be unable to induce IL-6 secretion by TLR4$^{-/-}$ DCs.

B cell Cultures

Splenocytes containing 1x10^{5} B cells, or the equivalent number of purified B cells, were cultured with Sigma LPS (30 μg/ml) for 4 days. In the mixed B cell experiments, purified B6 (5x10^{4}; IgM^{b}) and 2-12H/Vκ8 (5x10^{4}; IgM^{a}) B cells were cocultured with LPS for 4 days as above. BMDCs, CD11c^{+} splenocytes, or BMDC CM (25% of final

volume) were added to B cell cultures on day 0. The IL-6 in DC CM was neutralized with either anti-IL-6 antibody or a control rat IgG_1 antibody (54.1).

ELISA

IgM^a/κ (encoded by 2-12H/Vκ8/$C\kappa^{-/-}$) was captured with anti-κ (187.1), detected with biotinylated anti-IgM^a (HB100) and Streptavidin-AP as previously described (140). Purified mouse IgM^a/κ (TEPC 183) served as the standard control. IgM^a/κ levels were plotted as "percent of control" defined by the level of Ig secretion in LPS-stimulated cultures of purified 2-12H/Vκ8/$C\kappa^{-/-}$ B cells (100%). IL-6 was quantitated by capturing with anti-IL-6 (clone MP5-20F3) and detecting with biotinylated anti-IL-6 (clone MP5-32C11) and Streptavidin-AP. Recombinant IL-6 served as the standard control.

Real time (RT)-PCR

RNA was prepared from BMDCs treated with re-purified LPS (15 μg/ml) by solubilization in Trizol (Invitrogen) and treatment with Turbo DNase (Ambion). Reverse transcription with oligo(dT) primers was performed with Superscript II (Invitrogen). The amount of IL-6 message was determined using the TaqMan Assay-On-Demand primer-probe sets (Applied Biosystems) and the ABI 7000 sequence detection system. IL-6 mRNA transcript levels were normalized to the amount of 18S ribosomal RNA transcription according to the following equation: %18S = 2^ [-(IL-6 – 18S units)]. To measure IL-6 mRNA stability, BMDCs were stimulated with re-purified LPS (15μg/ml) for 6 hrs and then treated with 50 μM DRB for 15, 30, and 60 min to block transcription. mRNA was quantitated by RT-PCR as described above.

Electrophoretic Mobility Shift Assay (EMSA)

BMDCs were stimulated with re-purified LPS (15 μg/ml) and gel shift assays were performed as previously described (193).

Statistical Analysis

Exact Wilcoxon rank sum test was used for most unpaired two-sample comparisons. When total sample size was small (<8), t test was used instead. For test differences between paired observations, exact Wilcoxon signed rank test was used. p values < 0.05 were considered significant and denoted by *.

Results

The frequencies of splenic myDCs and MΦs are not diminished in MRL/lpr mice.

Maintaining B cell tolerance during activation of the innate immune system is crucial in preventing autoimmunity. We have previously shown that stimulation through TLR4 activates myDCs and MΦs to secrete soluble factors thereby repressing Ig secretion by chronically antigen-experienced (autoreactive) B cells (103). To determine if the breakdown of tolerance in lupus-prone mice was associated with the lack of a repressive cell type, we compared the frequency of splenic MΦ and DC subsets in MRL/*lpr* and B6 mice. As shown in Figure 2.1 and Table I, the frequencies of myDCs ($CD11c^{hi}/CD11b^{int/hi}$) and plasmacytoid DCs (pDCs, $CD11c^{lo}/CD11b^{-}/B220^{+}/GR1^{+}$) were not significantly different. The lymphoid DCs (lyDCs, $CD11c^{lo}/CD11b^{-}/B220^{-}/GR1^{-}$) were significantly decreased in MRL/*lpr*, however this population is not involved in DC/MΦ-mediated tolerance (103). The $CD11c^{-}/CD11b^{hi}$ and $CD11c^{-}/CD11b^{lo}$ populations were increased in MRL/*lpr* mice, raising the possibility that these populations might secrete an activator that enhances Ig secretion. However, when isolated by cell sorting, these populations did not augment LPS-induced Ig secretion or affect the ability of B6 DCs to regulate Ig secretion by Sm-specific B cells (data not shown), suggesting that neither population promotes the loss of B cell tolerance. Thus, neither diminished frequency of myDCs and MΦs nor secretion of an activator accounts for the loss of tolerance in MRL/*lpr* mice.

DCs from MRL/lpr mice fail to efficiently repress Sm-specific B cells.

LPS-activated DCs from B6 mice regulate chronically antigen-experienced B cells (103). To assess if DCs from MRL/*lpr* mice were capable of repressing Ig secretion, we cocultured Sm-specific B cells with bone marrow-derived DCs (BMDCs) from B6 or MRL/*lpr* mice (Figure 2.2A). Compared to B6 DCs, MRL/*lpr* DCs were less efficient at repressing Sm-specific B cells when cultured at B cell: DC ratios of 10:1, 20:1, and 100:1 ($p = 0.016$, 0.004, and 0.015 respectively). These differences were not due to contaminating cells, because BMDCs from B6 and MRL/*lpr* mice contained >95% myDCs, and sorted B cells compared to negatively selected B cells from 2-12H/Vκ8 mice cultured with DCs from MRL/*lpr* mice exhibited similar results (data not shown). To determine if splenic DCs were also defective in repressing autoreactive B cells, splenic $CD11c^+$ cells were isolated from B6 and MRL/*lpr* mice, and cocultured with B cells from 2-12H/Vκ8 mice (B cell: DC ratio 10:1). As shown in Figure 2.2B, *ex vivo* B6 DCs repressed significantly better than DCs purified from MRL/*lpr* mice ($p = 0.015$), indicating that the defect was not specific to BMDCs. Collectively, the data indicate that myDCs from MRL/*lpr* mice are present at a normal frequency, but they are defective in repressing Ig secretion by autoreactive B cells.

DCs from MRL/lpr mice are defective in IL-6 secretion

We previously showed that IL-6 secreted by DCs repressed autoreactive B cells (103). To determine if diminished IL-6 was associated with the inability of MRL/*lpr* DCs to repress Sm-specific Ig secretion, we measured IL-6 secretion. LPS-activated BMDCs (Figure 2.3A) and splenic $CD11c^+$ cells (Figure 2.3B) from MRL/*lpr* mice secreted significantly less IL-6 compared to B6 controls ($p < 0.001$ and $p = 0.003$ respectively).

To assess if this defect was unique to MRL/*lpr* mice, we quantitated LPS-induced IL-6 secretion from BMDCs from several other lupus-prone models. As shown in Figure 2.3A, BMDCs from MRL, NZM2410, and NZBxNZW$_{F1}$ were defective in secreting IL-6 when compared to B6 ($p < 0.0001$, $p < 0.0001$, and $p = 0.002$ respectively). Interestingly, B6.*lpr* mice were not defective in secreting IL-6 ($p = 0.932$), suggesting that the inability to secrete IL-6 is associated with the MRL background. Defective IL-6 production was not secondary to IL-10 inhibiting TLR signaling, as MRL/*lpr* DCs secreted decreased levels of IL-10 and neutralizing IL-10 did not restore IL-6 levels (data not shown). To determine whether defective IL-6 secretion was limited to stimulation through TLR4, we measured IL-6 secretion from MRL/*lpr*-derived DCs in response to other TLR ligands. As shown in Figures 2.3C-E, IL-6 secretion was increased when MRL/*lpr* BMDCs were stimulated through TLR3 (poly (I:C), $p = 0.006$); however, secretion was defective when stimulated through TLR7 (R848, $p = 0.028$) and TLR9 (CpG ODN, $p = 0.016$). This indicates that not all TLRs are affected by this defect, and that mutation within the IL-6 structural gene is unlikely to explain the reduced levels of IL-6. Collectively, the data indicate that DCs from multiple strains of autoimmune mice exhibit defects in cytokine secretion induced through some TLRs.

Diminished IL-6 secretion is not due to decreased TLR4 expression or survival.

Expression of TLRs ensures that DCs are activated during innate immune responses. It was possible that the decreased secretion of IL-6 from MRL/*lpr* DCs reflected a reduced expression of surface TLR4. As shown in Figure 2.4A, the expression of TLR4 on myDCs from B6 (MFI 58.9 $\pm$ 12.6) and MRL/*lpr* (MFI 68.1 $\pm$ 10.9) mice was not

significantly different. Likewise, BMDCs from B6 and MRL/*lpr* mice did not differ in TLR4 expression (data not shown), nor did they differ in viability as determined by 7-AAD staining at day 4 (Figure 2.4B). Thus, diminished surface expression of TLR4 or decreased survival do not account for the decreased IL-6 secretion by LPS-activated DCs from MRL/*lpr* mice.

Defective IL-6 secretion is associated with failure to sustain IL-6 transcription. Transcriptional regulation of IL-6 depends on several signal transduction pathways that activate multiple transcriptional regulators including NF-κB and AP-1. To determine if the diminished secretion of IL-6 by MRL/*lpr* DCs was due to defective transcriptional regulation, we LPS-stimulated BMDCs from B6 and MRL/*lpr* mice and quantitated IL-6 mRNA levels by real time (RT)-PCR. The basal level of IL-6 mRNA in the MRL/*lpr* mice was slightly lower than in B6 mice (Figure 2.5A). Upon stimulation with LPS, IL-6 mRNA levels in B6 and MRL/*lpr* DCs were dramatically increased; however, the magnitude of the response by MRL/*lpr* BMDCs was 7-fold lower. Further, the sustained levels of IL-6 mRNA production were higher in B6 compared to MRL/*lpr* mice (24 hr and 96 hr timepoints). To determine if decreased mRNA stability contributed to the decreased production of IL-6 message, BMDCs were LPS-stimulated for 6 hours followed by pharmacological attenuation of transcription. The levels of IL-6 mRNA in BMDCs from B6 and MRL/*lpr* mice were quantitated by RT-PCR. As shown in Figure 2.5B, the rates of mRNA degradation in the MRL/*lpr* DCs did not change over time; however, the IL-6 mRNA levels in B6 DCs were reduced by 3-fold within 15 minutes of attenuating new transcription. This indicates that IL-6 message is inherently unstable and

that sustained production of IL-6 mRNA requires continual synthesis. Further, given that degradation was not observed in DCs from MRL/*lpr* mice, the data indicate that increased degradation does not contribute to the diminished IL-6 mRNA levels. This suggests that MRL/*lpr* DCs harbor a defect at or upstream of transcriptional initiation that reduces the level of IL-6 mRNA and protein.

To assess if decreased IL-6 mRNA levels were associated with defects in NF-κB or AP-1 activation, we compared the DNA binding activity in nuclear extracts prepared from B6 and MRL/*lpr* DCs. The DNA binding activity of NF-κB from LPS-stimulated B6 DCs occurred within 10 minutes, with robust binding at 6 hours. In contrast, the DNA binding activity of NF-κB from MRL/*lpr* DCs was diminished at these same timepoints (Figure 2.6A). This was not a reflection of unequal protein loading, as the levels of an unrelated nuclear protein (PCNA) were comparable. The specificity of NF-κB for the DNA probe was confirmed by diminished complex formation in the presence of unlabelled probe (competitor DNA), and failure of a mutant competitor DNA (mutant DNA) to reduce complex formation (Figure 2.6B). To identify the NF-κB subunits involved in DNA binding, we supershifted the DNA/protein complex with subunit-specific antibodies. As shown in Figure 2.6C, p65 and c-Rel, but not p50, were identified as components of the NF-κB complex formed in B6 DCs following 6 hour LPS stimulation. p65 and c-Rel anti-sera were specific for these components as pre-immune serum failed to supershift a protein/DNA complex (data not shown). Similar to NF-κB, DNA binding by AP-1 was also markedly diminished in DCs from MRL/*lpr* compared to B6 mice (Figure 2.6D).

Thus, LPS-stimulated MRL/*lpr* DCs fail to activate key transcriptional regulators required for IL-6 gene transcription.

Nuclear translocation of NF-κB is dependent on phosphorylation and degradation of IκB (194). To assess if the lack of NF-κB DNA binding was associated with defects in IκB phosphorylation/degradation, we immunoblotted whole cell lysates from LPS-stimulated B6 and MRL/*lpr* BMDCs. B6 DCs showed induced phosphorylation of IκBα at 5 minutes that was sustained through 6 hours (Figure 2.7A, left panel). In contrast, MRL/*lpr* DCs induced IκBα phosphorylation at 5 minutes with maximal phosphorylation at 15 minutes. Phosphorylation was not evident at 45 minutes or 6 hours (Figure 2.7A, right panel). Similarly, IκBα degradation was delayed following LPS stimulation of MRL/*lpr* DCs, indicating that defects in TLR4-induced signal transduction correlate with lack of IL-6 mRNA production and protein secretion. To assess if other TLR pathways in MRL/*lpr* DCs were similarly affected, we assessed IκBα phosphorylation in response to TLR3 ligation. We showed in Figure 2.3 that despite defects in TLR4-, TLR7- and TLR9-induced IL-6 production, TLR3-induced IL-6 production was enhanced. This revealed that the defect in IL-6 production by MRL/*lpr* DCs did not affect all TLRs. To correlate TLR-induced protein secretion with TLR-mediated signal transduction, we assessed IκBα phosphorylation in response to poly (I:C). As shown in Figure 2.7B, poly (I:C)-induced IκBα phosphorylation was comparable between DCs derived from B6 and MRL/*lpr* mice. Collectively, the data suggest failure to sustain IκBα phosphorylation reduces NF-κB activation, diminishes IL-6 transcription, and ultimately decreases IL-6 protein synthesis by MRL/*lpr* DCs. This supports the idea that continuous TLR4 signal

transduction is required to maintain IL-6 secretion and suggest this is defective in DCs from lupus-prone mice (195).

Autoantibody secretion is repressed by IL-6 and other soluble factors.

We have previously shown that IL-6 repressed 75% of Ig secretion by Sm-specific B cells. Here, we show that DCs from lupus-prone MRL/*lpr* mice exhibit markedly decreased IL-6 levels coincident with their inability to regulate Ig secretion. To determine the importance of decreased IL-6 in the breakdown of tolerance, we assessed the ability of conditioned medium (CM) from B6 and MRL/*lpr* DCs to repress Ig secretion. CM allowed us to distinguish the effects of soluble mediators from the effects of cell contact. As shown in Figure 2.8A, DC CM from most B6 mice repressed 70-90% of Ig secretion. In contrast, the ability of DC CM from individual MRL/*lpr* mice to repress Ig secretion was extremely variable (10-90% repression, p= 0.004). Given the central role for IL-6 in repressing autoantibody secretion (103), we reasoned that if IL-6 were the sole repressive factor, there would be a direct correlation between IL-6 in DC CM and Ig secretion. However, this broad range of repression only partially correlated with IL-6 levels (data not shown). Despite the fact that all MRL/*lpr* mice exhibited low levels of IL-6, four individuals still repressed 80-90% of Ig secretion (Figure 2.8A). To assess if the low levels of IL-6 secreted by MRL/*lpr* mice contributed to Ig repression, we neutralized any remaining IL-6 in the DC CM of mice retaining repressive function, then assessed the ability of the CM to regulate Ig secretion. As shown in Figure 2.8B, neutralization partially restored Ig secretion (p = 0.031), confirming that that the low levels of IL-6 regulated Ig secretion. Interestingly, secretion comparable to controls

(100%) was never attained, suggesting that in addition to IL-6, other DC-derived soluble mediators regulate Ig secretion. It was possible that the variability in repression by MRL/*lpr* DC CM was due to the secretion of an activating factor by the MRL/*lpr* DCs. We addressed this in two ways. First, we added recombinant IL-6 (rIL-6) to the MRL/*lpr* DC CM, and then assessed Ig secretion by Sm-specific B cells. When added to the CM from three individual mice, rIL-6 repressed Ig secretion indicating that if activating factors were present, they did not override the repressive effect of IL-6 (data not shown). In a second experiment, we assessed if MRL/*lpr* DCs secreted an activator by determining if MRL/*lpr* DC CM activated naïve B6 B cells. We previously showed that DC CM did not repress naïve B cells (103); thus, the presence of an activator may be more evident when Ig secretion is not simultaneously being repressed by the low levels of IL-6 in the MRL/*lpr* DC CM. The data indicate that MRL/*lpr* DC CM did not increase Ig secretion of naïve B6 B cells, indicating that the dysregulated production of an activator is unlikely (data not shown).

Collectively, the data indicate that during innate immune responses, IL-6 and another repressive factor(s) regulates B cells chronically exposed to antigen. Further, this mechanism appears defective in lupus-prone mice coincident with diminished secretion of IL-6. However, it remained unclear if soluble factors secreted by LPS-activated DCs repressed autoreactive B cells when present in mixed populations with naïve cells. To assess this, we cocultured naïve (B6) and autoreactive (2-12H/Vκ8) B cells with DC CM prepared from B6 and MRL/*lpr* DCs. As shown in Figure 2.8C, DC CM prepared from B6 cells, but not MRL/*lpr* cells, repressed Ig secretion in the mixed B cell cultures (p =

0.009) (Figure 2.8C). The data suggest that DC-mediated repression regulates mixed populations of autoreactive and naïve B cells.

Discussion

The defects leading to the breakdown in B cell tolerance remain a central focus in understanding SLE. Previous studies showed that during innate immunity Sm-specific B cells were regulated by myDCs and MΦs through the secretion of soluble mediators (103). We propose a model where polyclonal activators stimulate myDCs and MΦs to secrete IL-6, which selectively represses autoreactive B cells, while naïve B cells mount a polyclonal antibody response to bacterial and viral antigens. In this report, we show that DCs from lupus-prone mice are less efficient at repressing autoreactive B cells coincident with a defect in secreting IL-6. This DC defect was not due to decreased survival or TLR4 expression, lack of a regulatory DC subpopulation, or the secretion of factors that enhance Ig secretion. Instead, the reduced IL-6 secretion resulted from the inability of MRL/*lpr* DCs to induce or maintain IL-6 transcription in response to LPS. Analysis of upstream signaling effectors showed that, although LPS induced IκBα phosphorylation, it was not sustained. Further, DNA binding by NF-κB and AP-1 were markedly decreased. These findings indicate that MRL/*lpr* DCs exhibit a TLR4 signal transduction defect at, or upstream of, IκB kinase (IKK)/IκB/NF-κB activation that results in diminished IL-6 mRNA production and protein secretion.

Previous data showed that rIL-6 effectively regulated chronically antigen-experienced B cells (103). At several B cell: DC ratios, MRL/*lpr* DCs were less efficient at repressing Ig secretion compared to B6 DCs. However, despite significant defects in IL-6 secretion, they still repressed 53% of anti-Sm secretion at a ratio of 100:1 (Figure 2.2A). Further, DC CM was less efficient at repressing Ig secretion compared to intact DCs indicating

that a contact-dependent mechanism might partially regulate Ig secretion. In support of this, we have observed that DCs deficient in TLR4 partially repressed LPS-induced Ig secretion; however, repression was lost when the cells were separated in a transwell apparatus (Kilmon and Vilen, unpublished observations).

The finding that repression of Ig secretion by DCs is multifaceted fits well with the heterogeneity of human disease. We propose that defects in any regulatory component may predispose to autoimmunity, but complete loss of tolerance requires multiple defects. Our data show that the repressive ability of LPS-activated MRL/*lpr* DCs was variable. Some DCs efficiently repressed Ig secretion, despite diminished IL-6 production (Figure 2.2, 2.3A/B, 2.8A), while others failed to repress secretion coincident with reduced IL-6 levels. Compared to the contact-dependent mechanism described above, this repressive activity was apparent in the CM from some MRL/*lpr* mice, indicating that DCs secrete additional repressive factors that contribute to the regulation of Ig secretion. Thus, despite markedly decreased IL-6 secretion by DCs from all mice analyzed, some likely harbor defects in another repressive factor(s) making them more susceptible to autoimmunity during innate stimulation. Although a direct correlation between Ig secretion and IL-6 levels in MRL/*lpr* mice was not evident, we favor the interpretation that IL-6 and another repressive factor regulates Ig secretion because IL-6 deficient DCs repress LPS-induced Ig secretion (unpublished observations) and neutralizing IL-6 only partially restored Ig secretion (Figure 2.8B). This indicates that the low levels of IL-6 secreted by MRL/*lpr* DCs partially represses Ig secretion, but that IL-6 is not the sole means of regulating autoimmunity during innate immune responses.

The inability of LPS-stimulated MRL/*lpr* DCs to produce IL-6 and efficiently repress Ig secretion suggests that defects in innate immune responses contribute to autoimmunity. Our data show that DCs derived from MRL/*lpr* mice are unable to sustain IκB phosphorylation, thereby reducing NF-κB DNA binding and IL-6 mRNA synthesis. This suggests an intrinsic defect where lack of sustained TLR-mediated signal transduction leads to decreased IL-6 protein secretion. This could reflect a defect in the TLR signaling pathway or possibly the selective formation of NF-κB complexes that are less transcriptionally active. Aberrant cytokine production and abnormal NF-κB activity in T cells and MΦs from lupus-prone mice and lupus patients have been associated with decreased p65, increased p50 homodimers which are more inhibitory to gene transcription, reduced binding of p50/c-Rel and p65 NF-κB complexes, and increased activity of histone deacetylases (196, 197). Unfortunately we could not identify the NF-κB subunits formed by MRL/*lpr* DCs because DNA binding was not observed at levels sufficient for supershifting.

MyD88-dependent, TLR-induced activation of NF-κB and AP-1 is mediated through TRAF6 (198). Thus, the findings that both NF-κB and AP-1 DNA binding activity are reduced (Figure 2.6), and that IL-6 secretion and IκBα phosphorylation are defective only upon stimulation through MyD88-dependent TLRs (TLR4, 7, and 9, but not TLR3), suggest a defect in the MyD88-dependent signaling pathway possibly at or upstream of TRAF6. Alternatively, a defect at the level of the TLR4 receptor may occur. Yang et al showed that persistant TLR4 signals are required for normal DC secretion of IL-6 (195).

In the case of dysfunctional MRL/*lpr* DCs, the TLR4 receptor may become desensitized to LPS following an initial stimulus, mimicking LPS removal and causing the decreased phospho-IκBα and IL-6 mRNA levels seen at later timepoints (Figures 2.5 and 2.7). In addition, exposure to apoptotic cells may affect the TLR4 response. Apoptotic cells fail to induce inflammatory responses, in part by repressing DC activation (199). Thus, the increased burden of apoptotic cells associated with SLE may dysregulate some of the TLRs, rendering them incapable of secreting cytokines that are needed to repress autoantibody secretion. In support of this, others have shown that apoptotic cells cause defective IL-6 secretion by macrophages (159), and mice functionally deficient in the phagocytosis of apoptotic cells get a lupus-like disease (177).

Increased production of pro-inflammatory cytokines such as IL-6, contribute to the inflammatory response and pathogenesis of lupus nephritis (200, 201). SLE patients (99, 202, 203, 204) and diseased, lupus-prone mice (205, 206, 207) exhibit elevated serum IL-6 levels (2-19 pg/ml), yet fail to repress Ig secretion. Although elevated, this level of systemic IL-6 is insufficient to repress autoreactive B cells *in vitro* (103). Therefore, we propose that colocalization of DCs and B cells is necessary to provide sufficient IL-6 to repress Ig secretion. Our findings showed that DCs derived from MRL/*lpr* mice secrete reduced levels of IL-6, coincident with lack of Ig repression. We propose that once tolerance is overcome, autoantibody secretion and immune complex formation induce systemic production of pro-inflammatory mediators, promoting inflammation and pathogenesis. Consistent with this model, CpG-stimulated dendritic cells from SLE patients produced lower levels of IL-6 (208), while endothelial cells (209, 210, 211),

mesangial cells in the kidney (212, 213), and infiltrating monocytes/macrophages (214) secrete elevated levels of IL-6. This suggests that IL-6 plays a beneficial role when released in a local microenvironment between myDCs and autoreactive B cells, yet when elevated systemically, it induces inflammation, tissue destruction, and spontaneous Ig production by activated B cells (99, 215, 216, 217). Therapies aimed at neutralizing the inflammatory effects of IL-6 may have short-term benefits in treating lupus nephritis, however, they are likely to promote loss of tolerance in newly emerging B cells during innate immune activation.

Immunoglobulin secretion by B cells is induced by ligation of the TLR and/or BCR. BCR-induced Ig secretion is regulated by lack of T cell help and sustained BCR-induced calcium signaling and prolonged Erk activation (133, 183, 218, 219). In contrast, TLR-induced Ig secretion is regulated by soluble factors secreted from DCs and MΦs (103). Although the mechanisms regulating the BCR and TLR are unique, signals derived from chronic BCR stimulation impact TLR-induced activation. For example, the chronic Erk activation associated with continuous exposure to self-antigens represses TLR9-induced Ig secretion, whereas, acute Erk activation following BCR stimulation of naïve B cells promotes TLR9-induced Ig secretion (91, 220). Similarly, chronic BCR exposure to self-antigen reprograms IL-6R signal transduction to repress Ig secretion (103). However, B cells that have been acutely stimulated and exposed to IFN-α/β induce Ig secretion in response to IL-6 (101). Our data expand our understanding of IL-6 to include a role in repressing Ig secretion by autoreactive B cells. During autoimmunity, the tolerance mechanisms that regulate autoreactive B cells become dysregulated. For many B cells

with autoreactive specificities, it remains unclear if BCR and/or TLR responses facilitate autoantibody production. Our studies of TLR-mediated responses in Sm-, HEL- and Ars/A1-specific autoreactive B cells identify DCs and MΦs as key regulatory cells during innate immune responses, and show that DC-mediated tolerance is defective in lupus-prone MRL/*lpr* mice. These findings implicate dysregulated innate immune responses in the autoantibody production associated with SLE.

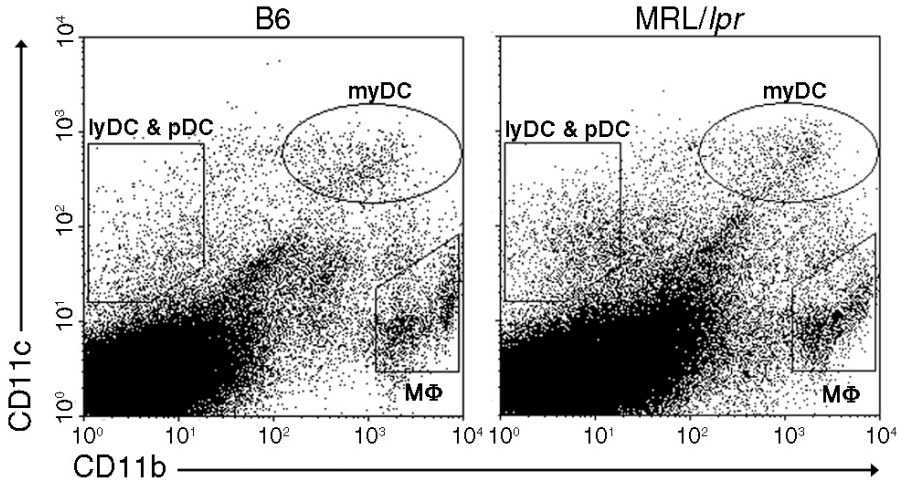

Figure 2.1. The distribution of splenic myDCs and MΦs are comparable between B6 and MRL/*lpr* mice. DC and MΦ subsets were separated based on CD11c and CD11b expression. Dot plots are representative of nine mice each.

	B6	MRL/*lpr*	*p* value
myDCs	1.02 ± 0.12	0.86 ± 0.07	0.658
lyDCs	0.13 ± 0.01	0.25 ± 0.03	0.001 [b]
pDCs	0.03 ± 0.01	0.10 ± 0.02	0.168
MΦs	1.68 ± 0.25	4.38 ± 0.96	0.002 [b]

[a] Splenic DC and MΦ subsets were analyzed based on CD11c, CD11b, B220, and GR1 expression. The data depict the average percent of total splenocytes ± SEM from 9 mice. The average number of splenocytes from B6 was $1.4 \times 10^8 \pm 0.1 \times 10^8$ and from MRL/*lpr* was $1.3 \times 10^8 \pm 0.1 \times 10^8$.

[b] Significantly different.

Table 2.1. The frequencies of splenic myDCs and MΦs are not diminished in MRL/*lpr* mice.[a]

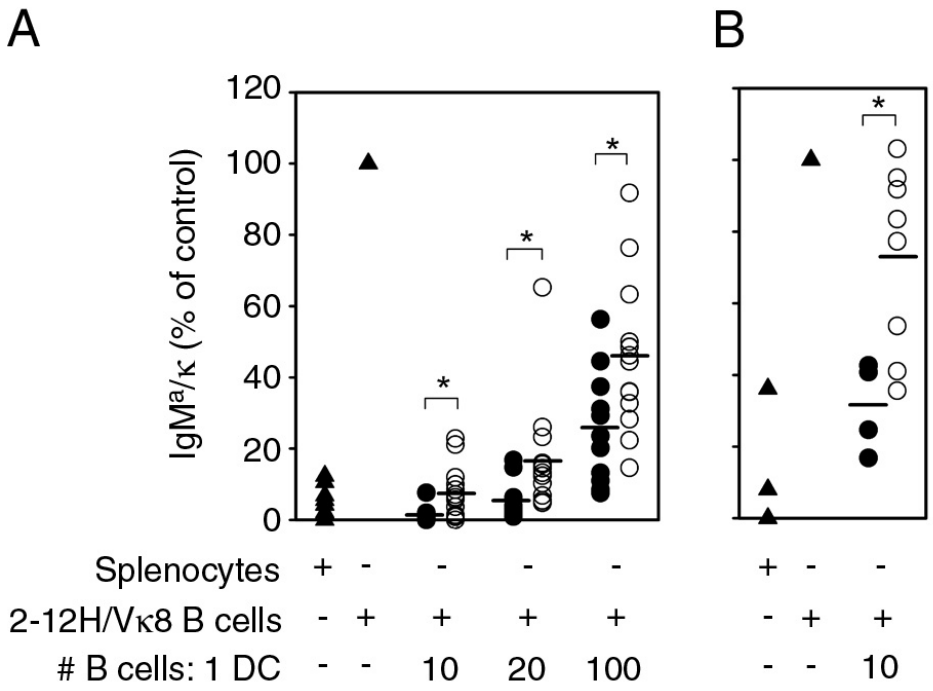

Figure 2.2. DCs from MRL/*lpr* mice fail to efficiently repress Sm-specific Ig secretion. LPS-stimulated (30 μg/ml) splenocytes ($1x10^5$ B cells) or purified B cells ($1x10^5$) were cocultured with the indicated ratios of BMDCs (A), or *ex vivo* splenic DCs (B). Secreted IgM^a/κ levels were quantitated by ELISA from the day 4 culture supernatant. LPS-stimulated purified B cells (100%) secreted 1-10 μg/ml IgM^a/κ. Data represent 14 (A) and 8 (B) MRL/*lpr* mice. (▲Controls, ● B6, ❍MRL/*lpr*).

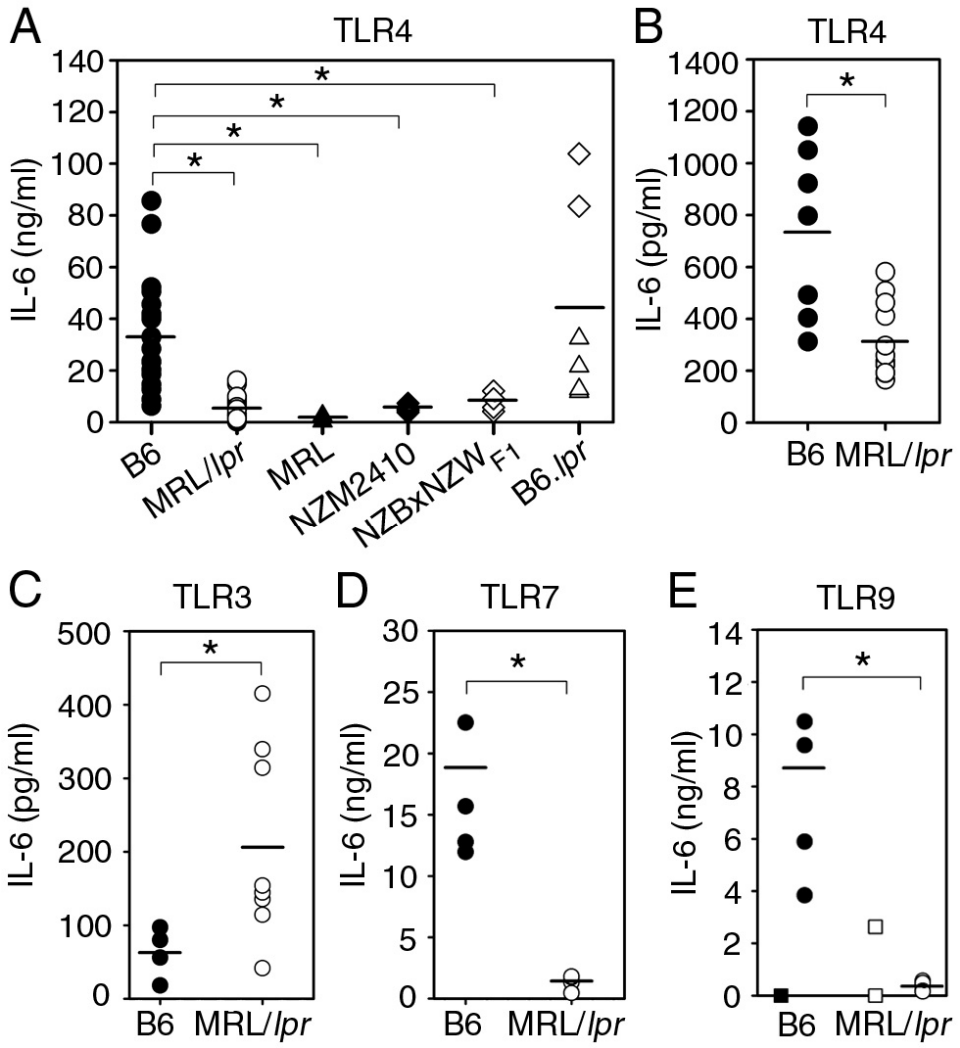

Figure 2.3. DCs from MRL/*lpr* mice are defective in IL-6 secretion upon TLR4, 7 and 9 stimulation, but not upon TLR3 stimulation. $1x10^4$ BMDCs (A), or $1x10^5$ e*x vivo* splenic DCs (B), were stimulated with LPS (30 μg/ml). $5x10^5$ BMDCs were stimulated with poly (I:C) (50 μg/ml) (C), R848 (10 μg/ml) (D), and non-CpG ODN (■/❑) or CpG ODN (● / ❍)(1μg/ml) (E). IL-6 was quantitated by ELISA from the day 4 culture supernatants. Data represent at least 5 mice per group. (● B6, ❍MRL/*lpr*, ▲MRL, △B6.*lpr*, ◆NZM2410, ◇$NZBxNZW_{F1}$)

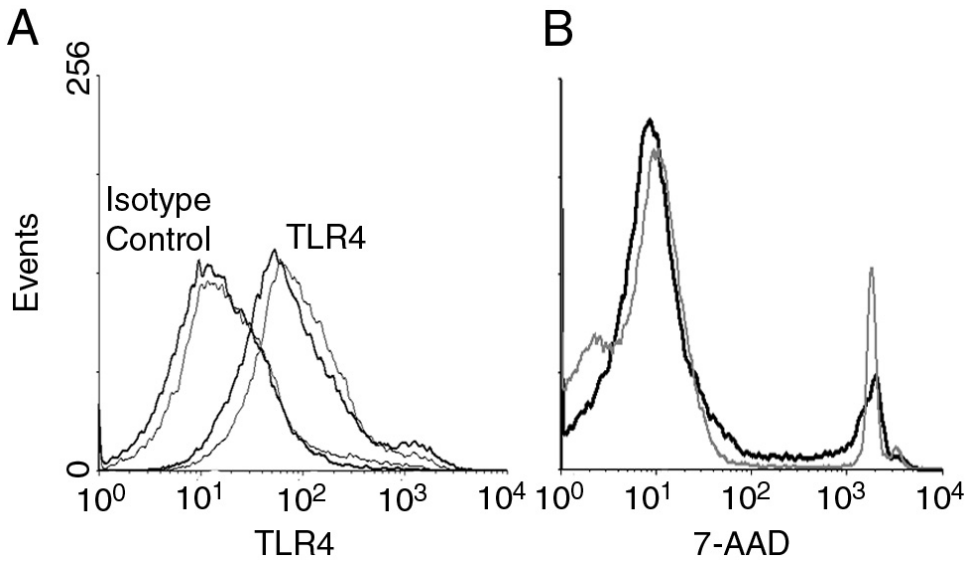

Figure 2.4. MyDCs from B6 and MRL/*lpr* mice have similar levels of TLR4 surface expression and no difference in survival. MyDCs within the $CD11c^+$ splenocyte population were gated as $CD11c^{hi}/CD11b^{int/hi}$, and then analyzed for TLR4 expression (A). LPS-stimulated BMDCs were stained with 7-AAD on Day 4 (B). The thick black line represents B6 mice. The thin gray line represents MRL/*lpr* mice. Histogram shows a representative plot from three experiments.

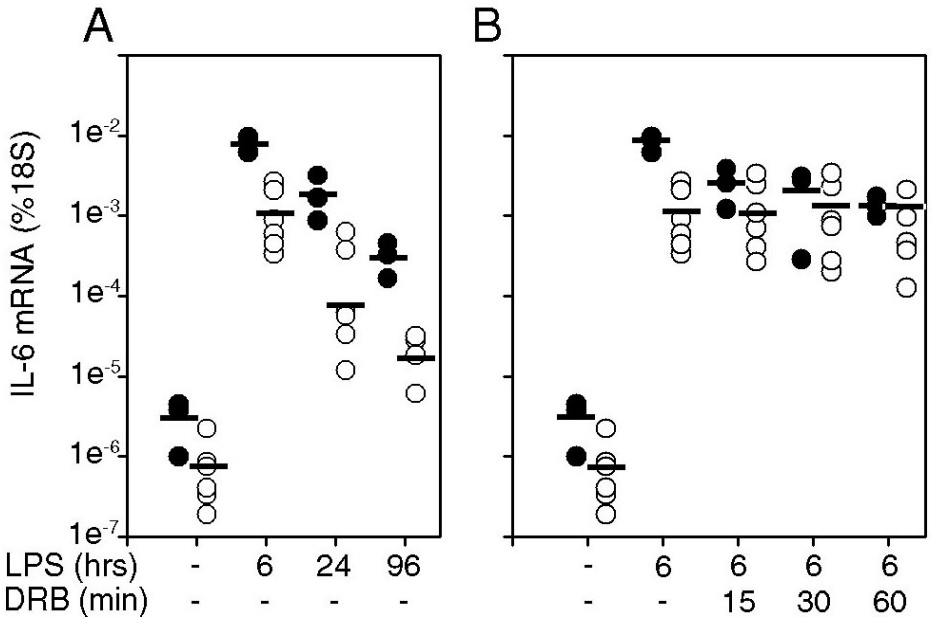

Figure 2.5. DCs from MRL/*lpr* mice show a decrease in synthesis and ability to sustain IL-6 mRNA levels. Real time-PCR was performed on RNA isolated from LPS stimulated BMDCs (A) untreated or (B) treated with DRB at the indicated timepoints. The data from three individual B6 (●) and six MRL/*lpr* (❍) mice are plotted as %18S.

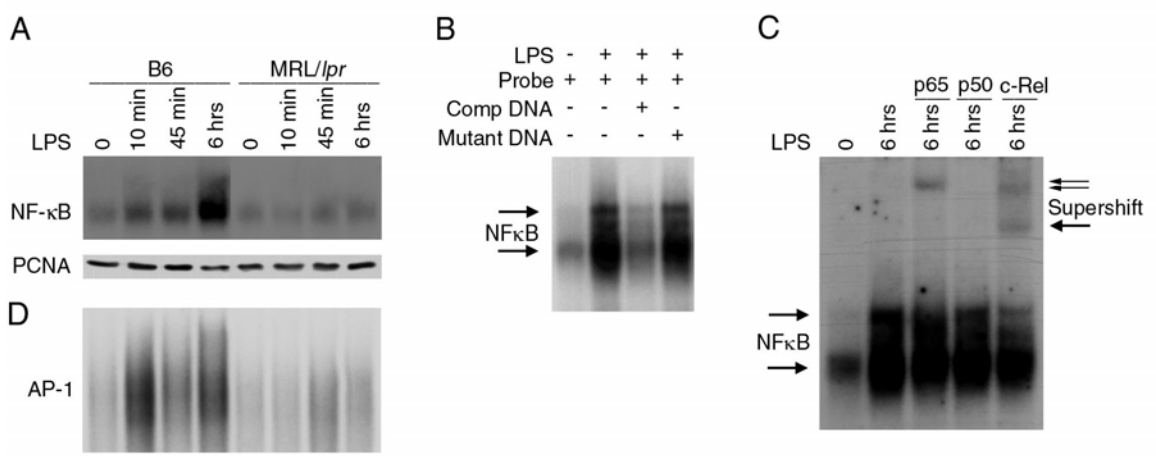

Figure 2.6. DCs from MRL/*lpr* mice fail to activate NF-κB and AP-1. BMDCs were stimulated with LPS (15 μg/ml) for the indicated times. Nuclear extracts were prepared, and NF-κB/DNA binding (A) or AP-1/DNA binding (D) was assessed by EMSA. Nuclear extracts prepared from unstimulated B6 BMDCs (lane 1) or from DCs stimulated 6 hours with LPS (lanes 2-4) were incubated with radiolabeled DNA probe (lanes 1-4), unlabeled competitive DNA (lane 3), or mutant DNA (lane 4), and NF-κB DNA binding was assessed by EMSA (B). NF-κB/DNA complexes in the nuclear extracts from unstimulated B6 DCs (lane 1) or from DCs stimulated 6 hours with LPS (lane 2-5) were supershifted using p65 (lane 3), p50 (lane 4), or c-Rel antiserum (lane 5) (C).

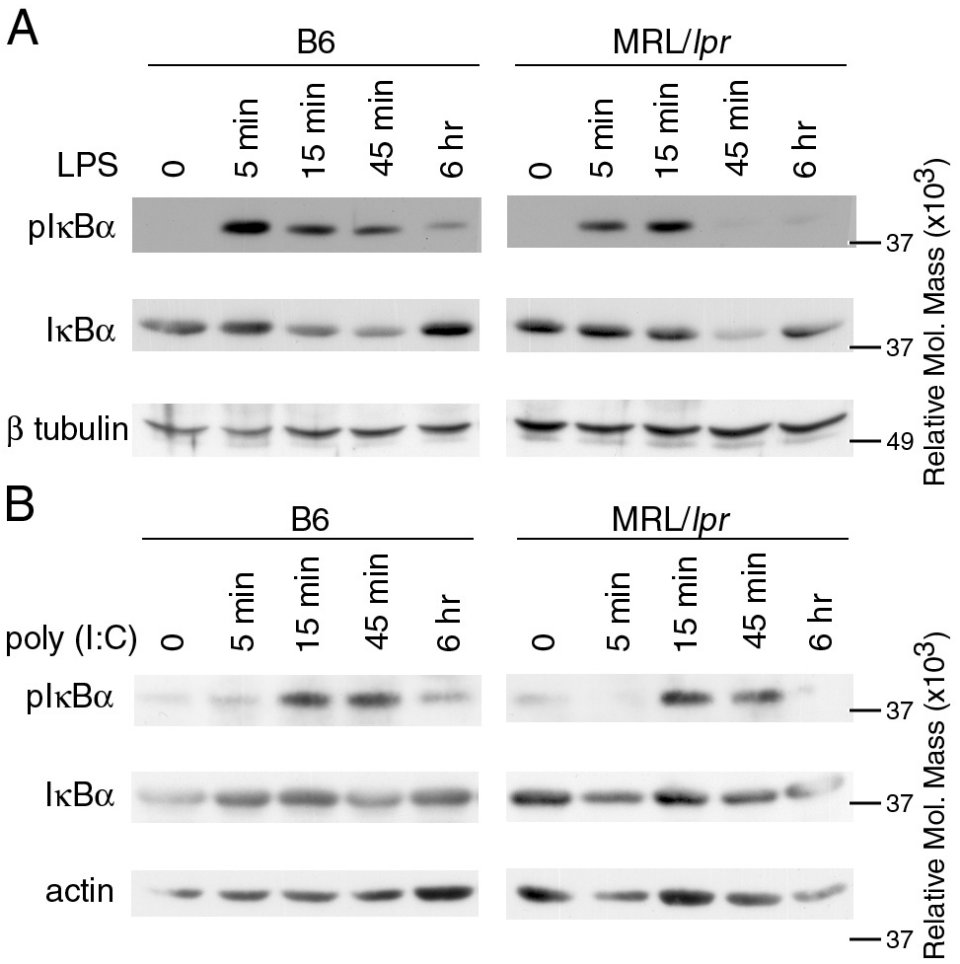

Figure 2.7. TLR4-stimulated DCs from MRL/*lpr* mice are unable to sustain IκBα phosphorylation. BMDCs ($2x10^6$) from B6 and MRL/*lpr* mice were stimulated with LPS (15 μg/ml) (A) or poly (I:C) (50 μg/ml) (B) for the indicated timepoints. Phospho-IκBα, IκBα, and β tubulin (A) or actin (B) expression in whole cell lysates was determined by immunoblotting. Data represent 7 (A) and 3 (B) experiments.

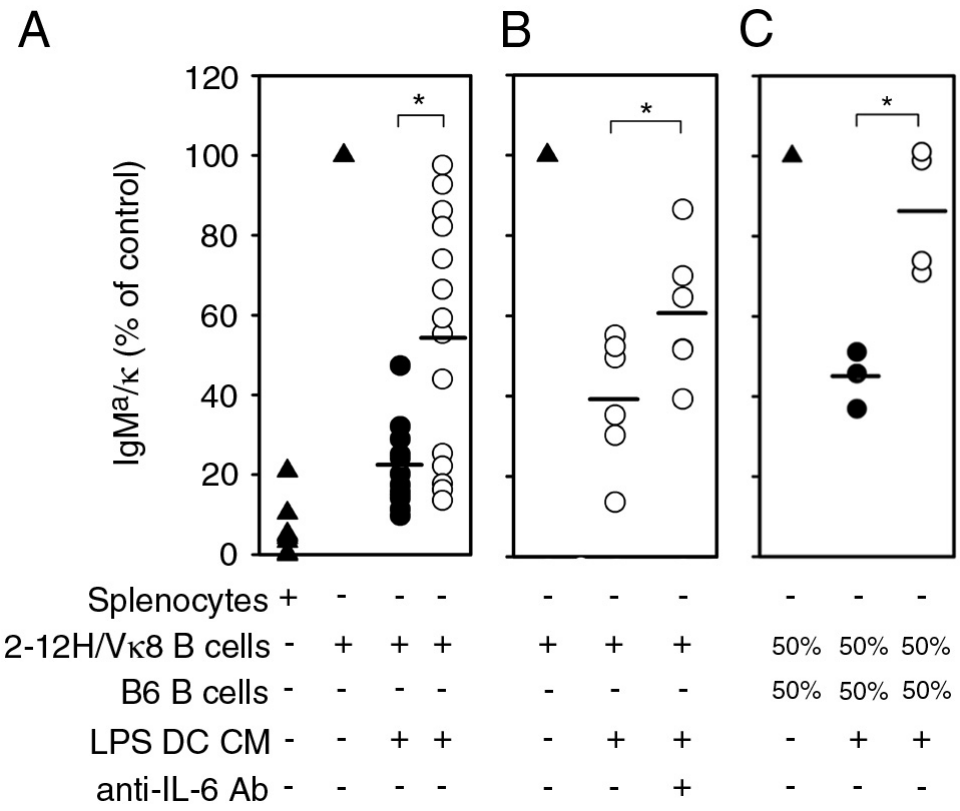

Figure 2.8. In addition to IL-6, other soluble factors regulate autoantibody secretion. Purified B cells ($1x10^5$) from 2-12H/Vκ8 mice were stimulated with LPS (30 μg/ml) in the absence (▲) or presence of DC CM (25% of final volume) from B6 (●) or MRL/*lpr* (❍) mice (A). DC CM from individual MRL/*lpr* mice (❍) was untreated or neutralized with anti-IL-6 antibody (50 μg/ml) prior to coculture with B cells from 2-12H/Vκ8 mice (B). $5x10^4$ purified B cells from 2-12H/Vκ8 and B6 mice were stimulated with LPS (30 μg/ml) in the absence (▲) or presence of DC CM from B6 (●) or MRL/*lpr* (❍) mice (C). Secreted IgM^a/κ levels were quantitated by ELISA from the day 4 culture supernatant. LPS-stimulated purified B cells (100%) secreted 1-10 μg/ml IgM^a/κ. Data represent 15 (A), 5 (B), and 4 (C) MRL/*lpr* mice.

Chapter III. Dendritic cells and macrophages repress innate and adaptive B cell responses coincident with the display nuclear self-antigen.

Diane G. Carnathan, Michelle A Kilmon, Michelle Borrero, Barbara J. Vilen

Contributions

Diane Carnathan contributed firgues 3.2, 3.3, 3.4, 3.5, 3.6, 3.8, and 3.9. She wrote and edited the manuscript. Michelle Kilmon contributed figures 3.1 and 3.7. Michelle Borerro contributed figure 3.8. Barbara Vilen is the Primary Investegator and edited the manuscript.

Abstract

B cell tolerance during innate immune responses requires chronic exposure to self-antigen to allow IL-6 and CD40L to repress Ig secretion. In this report, we show that murine B cells (2-12H/Vκ8) specific for the nuclear self-antigen Smith (Sm) fail to repress LPS-induced Ig secretion when chronically exposed to soluble Sm, small nuclear ribonucleoprotein particles (snRNPs), or apoptotic cells. Instead, these B cells recognize self-antigen displayed on the surface of dendritic cells (DCs) and macrophages (MΦs) as evidenced by constitutive destabilization of the BCR complex. The display of nuclear self-antigen by DCs and MΦs is not limited to Sm as DNA and histones are also present. In assessing if these self-antigens were functional in regulating Ig secretion we discovered a contact-dependent mechanism of regulating Ig seretion that occurs independent of BCR engagement of self-antigen. In addition, autorective and naïve BCR-mediated signaling is repressed by DCs. Collectively, our data demonstrate that innate and adaptive immune responses of B cells are regulated by DC independent of the nuclear self-antigen displayed on their surface.

Introduction

The dysregulation of B cells specific for nuclear antigens is a hallmark of many autoimmune diseases. Nuclear antigens become accessible to the immune system when they are displayed on the surface of apoptotic cells or released as soluble proteins upon cell lysis (11, 13, 14, 221). How different forms of nuclear self-antigens induce B cell tolerance remains unclear; however, the strength of the BCR signal and the availability, and/or location of antigen directly affects the differentiation of autoreactive B cells in antibody secreting cells (131, 180, 222, 223). This is exemplified in the hen egg lysozyme (HEL) model of B cell tolerance where, BCR interaction with soluble HEL produce anergic B cells while membrane bound HEL causes deletion of autoreactive B cells (224). Further, in nuclear antigen-specific models, an increased burden of apoptotic cells is implicated in autoimmune disease, and injection or accumulation of apoptotic cells activates some autoreactive B cells, including Smith antigen (Sm)-specific B cells (13, 15, 177). Depending on the affinity of the BCR for antigen Sm-specific B cells are regulated by B1 cell formation or peripheral anergy, (137, 140). However, since B cell anergy requires tolerizing antigen to constantly occupy the BCR (136), it remains unclear how the low concentration of soluble small nuclear ribonucleoprotein particles (snRNPs) or the transient appearance of apoptotic cells regulate Sm-specific B cells.

During innate and adaptive immune responses, autoreactive B cells are regulated by multiple mechanisms. Sm-specific B cells are regulated during innate immune responses through IL-6 and CD40L secreted by DCs and/or MΦs (103, 104). HEL-specific B cells repress TLR9-induced Ig secretion by constitutive ERK activation resulting from chronic

exposure to high-affinity self-Ag (220). In another model, immune complexes induce B cell proliferation when the BCR and Toll Like Receptor-9 or 7 (TLR9, TLR7) are coligated, yet antigen alone fails to initiate a response (55, 92). During adaptive immune responses, autoreactive B cells remain unresponsive to self-antigen by modulating surface BCR levels (180), inducing B1 cell formation (131), failing to obtain T cell help (130), and desensitizing the BCR to renewed signal transduction (132). In addition, antigen destabilizes the BCR complex (μm/Ig-α/β), dissociating Ig-α/β from μm by distances of approximately 200 nm (133, 134). The observation that BCR destabilization occurs following receptor ligation suggests it contributes to the unresponsive state of autoreactive B cells (135). Thus, multiple mechanisms have evolved to regulate autoreactive B cells stimulated by self-antigen or TLR ligation.

In this report we show that some soluble self-antigens effectively repressed LPS-induced Ig secretion, while nuclear self-antigens such as Sm, SnRNPs, and apoptotic cells failed to tolerize autoreactive cells. Analysis of other possible sources of tolerizing antigen revealed that DCs and MΦs displayed endogenous nuclear self-antigens including Sm, DNA and histones. Since soluble mediators secreted by DC/MΦs repress autoantibody secretion during the innate immune responses, we hypothesized that this surface-antigen might also be tolerogenic. However, in the absence of soluble factors, we found that Ig secretion by autoreactive B cells is repressed through a contact-dependent mechanism, independent of BCR ligation. Sm-specific B cells recognized the self-antigen displayed on DC/MΦs demonstrated by their destabilized BCR. However, DCs were not able to induce BCR signaling in Sm-specfic B cells. Indeed, these cells repressed BCR-mediated

signal transduction in both autoreactive and naïve B cells. The data show that although Sm-specific B cells recognize self-antigen on the surface of DC/MΦs, contact mediated repression of the innate and adaptive immune response occur independent of BCR engagement of antigen.

Materials and Methods

Mice

2-12H/Vκ8/Cκ$^{-/-}$ immunoglobulin transgenic mice were previously described (225). sHEL (ML5) mice, HEL-Ig x sHEL (MD4 x ML5) and C57BL/6 (B6) mice were purchased from Jackson Laboratory. Animals were used at 8-16 weeks of age and maintained in an accredited animal facility.

Reagents and Abs

Antibodies to CD3, CD19, CD69, CD11c, CD11b, and phosphorylated -tyrosine were obtained from BD Biosciences, canine distemper virus (CDV) from Biodesign, IgM Cy3 and IgG Cy3 from Jackson ImmunoResearch, phosphorylated-Syk from Cell Signaling and Streptavidin Alexa 465 and 647 from Molecular Probes. Sm was obtained from Immunovision. 2.12.3 and Vκ31T were gifts from Steve Clarke (University of North Carolina, Chapel Hill, NC), and PA4 and LG2-2 gifts from Marc Monestier (Temple University, Philadelphia, PA). 2.4G2, HO13, B7.6 (anti-μ), and anti-HEL (HyHEL10) were purified from hybridoma supernatant.

B cell purification and culture

Resting B cells (ρ>1.066) were isolated by percoll density centrifugation as previously described (133). Splenic B cells were purified by negative selection (Stem Cell Technologies) as previously described (225) and cultured with LPS and Sm, snRNPs or apoptotic cells for 4 days.

Preparation of DCs and MΦs

Preparation of BMDC/MΦs was performed as previously described (225). $CD11c^+$ splenocytes were purified by positive selection (Miltenyi Biotech).

Apoptotic cells

Apoptotic cells were prepared by irradiating thymocytes with 600 rads and culturing overnight. After co-culture with DCs they were removed by lympholyte-m (Cedarlane) density centrifugation.

ELISA

Quantitation of IgM^a/κ was described previously (225). Ig levels were plotted as "percent control" calculated as the percent secretion relative to LPS-stimulated B cells.

Immunoprecipitation and immunoblotting

BCR destabilization was detected as previously described (133). B cells ($3x10^6$) were stimulated with anti-m or BMDC solublized in lysis buffer containing 1% NP-40 lysis buffer. Proteins were resolved by SDS-PAGE. Phosphorylated-IkBa and phosphorylated-Syk were immunoblotted with HRP tagged antibodies and detected by chemiluminescence (GE Biosciences). Densitometry was performed utilizing Image J (National Institute of Health). Briefly, we multipled the area by the density and subtracted the background.

Immunofluorescence staining

$1x10^6$ B6 BMDC/MΦs, $CD11c^+$ splenocytes, T cells, or B cells were stained with Vκ31T (anti-Sm), 2.12.3 (anti-Sm), LG2-2 (anti-histone), PA4 (anti-DNA) or isotype controls (HO13 or CDV) followed by fluorochrome-conjugated secondary antibodies. BMDC/MΦs were treated with trypsin (2.5 mg/ml) or DNase (25 μg/ml) prior to PA4 staining. Cells were plated onto coverslips and images obtained using the Zeiss Axioplan 2 fluorescence microscope or the Olympus Fluoview 500 microscope and deconvolved using SlideBook (Intelligent Imaging Innovation) and/or Image J (NIH).

Statistical Analysis

Student's *t* test was used. Values of $p < 0.05$ were considered significant and denoted by an asterisk (*).

Results

Soluble self-antigens and apoptotic cells do not tolerize Sm-specific B cells.

Previous data demonstrate that autoreactive B cells must be chronically exposed to self-antigen for tolerance mechanisms to regulate innate immune responses (103, 104). However, the location and form of the self-antigens that tolerize these cells remains unclear. To determine if some self-antigens are sufficient to regulate B cell responses we LPS-stimulated HEL-specific B cells cocultured with cognate antigen (Figure 3.1A). Purified B cells from HEL-Ig x sHEL mice secrete Ig upon LPS stimulation because they were disengage self-antigen during the purification (226). However, LPS-induced Ig secretion is completely repressed when HEL antigen is present (Figure 3.1B) (220, 225). This corroborates that self-antigen regulates TLR responses (220, 226). To determine if any of the known forms of Sm regulate TLR-induced Ig secretion, we LPS-stimulated Sm-specific B cells (2-12H/Vκ8) in the presence of soluble Sm, snRNPs, and apoptotic cells, then assessed autoantibody secretion. Figure 3.1B demonstrates LPS-induced Ig secretion was unaffected by the presence of soluble Sm or by the snRNP complex. In contrast, Sm on the surface of apoptotic cells enhanced Ig secretion (13, 15). These data indicate that some self-antigens fully regulate TLR responses, but that in the case of Sm, the known forms of Sm are either ignored, or induce B cell activation.

Nuclear Self-Antigen is Displayed on DC/MΦs

DC and MΦs are responsible for clearing apoptotic cells. Since apoptotic cells display nuclear self-antigen on their surface, we hypothesized that DC/MΦs might acquire self-antigens from apoptotic cells during phagocyotsis providing a source of tolerizing antigen

while simultaneously colocalizing DC/MΦs with autoreactive B cells to allow soluble mediators to repress autoantibody secretion (103). To investigate this possibility, we stained BMDCs, BMMΦs, and $CD11c^+$ splenocytes for Sm. As demonstrated in Figure 3.2, BMDCs, BMMΦs, and *ex vivo* $CD11c^+$ cells showed the presence of surface Sm. Trypsin treatment removed the antigen, indicating that Sm was displayed as a surface protein. To confirm the specificity of the anti-Sm antibody for Sm, we absorbed the antibody with recombinant Sm. As shown in Figure 3.2, antibody absorbed with Sm failed to stain BMDCs while antibody absorbed with BSA stained these cells. These data indicate that the anti-Sm antibody recognizes Sm on DCs and MΦs.

Previously, others reported that histones and DNA were present on the surface of apoptotic cells (14). If Sm displayed by DCs and MΦs originated from apoptotic cells, we reasoned that other nuclear antigens might also be present. To assess this, BMDCs and BMMΦs were stained with antibodies to DNA and histones. As shown in Figure 3.3, BMDCs and BMMΦs displayed histones and DNA on their surfaces. Further, treatment of cells with DNase abrogated staining, confirming the specificity of the antibody and that the antigen was located on the cell surface. The data show that several nuclear self-antigens are displayed on the surface of DCs and MΦs.

To address if apoptotic cells are a source of the nuclear self-antigen found on the surface of DC/MΦs, BMDCs were cultured with apoptotic cells, the dying cells were removed by density centrifugation, and the DCs were stained for DNA. We observed that after coculture with apoptotic cells, DCs displayed a two-fold ($p=0.002$) increase in DNA on

their surface compared to untreated cells (Figure 3.4A). This shows that apoptotic cells are a source of nuclear self-antigen for display on the surface of DC/MΦs. The observations that nuclear self-antigens are displayed by DCs and MΦs raised the possibilities that other cells may display self-antigen and that all self-antigens, regardless of source, might be present on these cells. To address the first possibility, splenic T and B cells were sorted for the expression of CD3 or B220 and then stained for surface Sm. As shown in Figure 3.4B, neither T nor B cells displayed surface Sm. To assess if all self-antigens were displayed on DCs and MΦs, we stained the $CD11c^+$ splenocytes from mice expressing a HEL transgene (ML5). These mice constitutively secrete HEL with serum levels approximating 15 ng/ml, levels sufficient to induce a state of unresponsiveness in HEL-specific B cells. As shown in Figure 3.4C, $CD11c^+$ splenocytes failed to display the soluble antigen, HEL, on their surface. This was not due to the failure of the anti-HEL antibody to recognize HEL because loading exogenous HEL onto a HEL-specific B cell line (K46/D1.3) resulted in HEL-specific staining. The data indicate that nuclear, but not soluble, self-antigens are displayed by DCs and MΦs, but not by lymphocytes.

B cell coculture with DCs leads to activation.

It has been previously demonstrated that intact antigen exogenously loaded onto DCs causes BCR-mediated signal transduction (65, 165, 166, 167). DC/MΦs regulate chronically antigen-experienced B cells during innate immune responses and display nuclear self-antigen on their surface, therefore, we hypothesized that this form of self-antigen might tolerize Sm-specific B cells. Alternatively, in the absence of functional

tolerance mechanisms, the antigen on the DC/MΦs might activate Sm-specific B cells and promote autoimmunity. To first assess if DCs had any effect on B cell responses, we cocultured B cells with DCs and monitored downstream responses. As shown in Figure 3.5A, B6 cells cocultured with DCs increased IκBα phosphorylation after five minutes. Similarly, autoreactive B cells upregulated CD69 when cocultured with DCs for six hours (Figure 3.5B). This effect was also apparent when B6 (non-autoreactive) B cells were cocultured with DCs, indicating that DCs interact with B cells inducing B cell responses that are independent of surface antigen binding to the BCR. The finding that B6 B cells were activated by DCs suggested that activation might be induced through TLRs since the percent of autoreactive B cells in the polyclonal repertoire of B6 mice would be very small. Thus, it was unclear if BCR and/or TLR-mediated responses activated 2-12H/Vk8 B cells.

BCR-mediated signal transduction is not induced by coculture with DCs.

BCR or TLR ligation induces antibody secretion by B cells. To test if DCs activate B cells through the BCR we monitored the ability of DCs to induce Syk phosphoryation since this effect is only activated upon BCR ligation (Figure 3.6 A). Given that marginal zone (MZ) B cells to undergo rapid BCR-mediated responses we chose to use the 2-12H model because it contains this population. Purified 2-12H B cells do not exhibit detectable Syk phosphorylation in the absence of stimulation. In contrast, anti-μ induces robust Syk phosphorylation demonstrating that the cells are capable of responding to BCR ligation. Addition of DC/MΦs failed to induce detectable Syk phosphorylation,

suggesting that the display of Sm on the DC/MΦs does not activate BCR-mediated signal transduction.

To define if the absence of Syk phosphorylation was due to our inability to detect low level signals we examined if a downstream consequence of BCR signaling was evident. During adaptive immune responses, one consequence of BCR-mediated signal transduction is the accumulation of intracellular μ. FO and MZ B cells from 2-12H mice cocultured with DCs for 12 or 24 hours failed to exhibit increased intracellular μ (Figure 3.6B). This corroborates the lack of Syk phosphorylation and suggests that the Sm antigen displayed on the DC fails to elicit BCR-mediated signal transduction. However, it raised the possibility that DCs might actively inhibit BCR- and/or TLR-derived responses.

DCs repress LPS-induced Ig secretion in a contact-dependent mechanism.

To determine if DCs repress innate responses by mechanisms other than the secretion of soluble mediators, we assessed if their contact with autoreactive B cells regulated secretion. TLR4-deficient DCs in contact with autoreactive B cells repressed 50% of LPS-induced Ig (Figure 3.7A). However, the ability of DCs to repress Ig secretion was abolished when the B cells were separated from the DCs in a transwell apparatus indicating a contact-dependent mechanism of repression. To evaluate if the antigen displayed by the DCs was essential for contact-dependent repression, we blocked the Sm on the surface of the TLR4-deficient DCs with an anti-Sm $F(ab')_2$. Blocking surface Sm on TLR4-deficient DC did not change the levels of LPS-induced Ig secretion (Figure

3.7B). This indicates that contact-dependent regulation of TLR-induced Ig secretion is independent of BCR engagement by self-antigen. To further clarify if surface antigen on the DC regulates LPS-induced Ig secretion we tested if LPS-induced Ig secretion by HEL-specific B cells was repressed by DCs. Because DCs do not display HEL antigen, we reasoned that if contact-dependent regulation of innate responses was mediated in an antigen-independent manner, HEL-specific B cells would be repressed by TLR4-deficient DCs. As shown in Figure 3.7C, TLR4-deficient DCs repressed 50% of Ig secretion by LPS-stimulated HEL-Ig x sHEL B cells. Collectively, the data show that nuclear self-antigens are displayed on the surface of DCs, however, they repress Ig secretion during innate immune responses in a contact-dependent manner that is independent of antigen binding to the BCR.

DC/MΦs induce Sm-specific BCR destabilization.

Although the data identify a novel contact-dependent mechanism of repression of Ig secretion, the regulation of the innate immune response was found to be independent of the BCR ligation of antigen. However, it was unclear if surface-antigen on DC/MΦs affects B cell responses. The unresponsive state that protects autoreactive B cells from dysregulated adaptive immune responses requires the BCR to be constantly occupied by self-antigen (136). A consequence of constant receptor occupancy in low-affinity B cells is destabilization of the BCR. We reasoned that if 2-12H/Vk8 B cells constitutively recognized Sm on the DC, receptor destabilization might be evident. We assessed the amount of Ig-α/β associated with μm in B cells from 2-12H/Vκ8 mice compared to B cells from control mice (Vκ8 transgenic). We utilized B cells copurified with DC/MΦs

by T-depletion and density centrifugation or highly purified B cells with less than 5% contaminating DC/MΦs obtained by negative selection. Splenic B cells isolated by negative selection showed stoichiometric amounts of Ig-α/β coprecipitated with μm indicating that they display an intact BCR (Figure 3.8; left panel). In contrast, B cells purified by T cell depletion and density centrifugation consistently showed a two-fold ($p=0.01$) decrease in the amount of coprecipitated Ig-α/β in μm immunoprecipitates, signifying destabilization of the receptor complex when DCs and MΦs are present (Figure 3.8; right panel). Previous studies identified that BCR-derived signals are required for destabilization, indicating that Sm-specific B cells encounter self-antigen on the surface of DC/MΦs, thus inducing BCR destabilization (133). Combined with the data in Figure 3.6, a model emerges wherein an autoreactive B cell recognizes antigen and destabilizes Ig-α/β from μm but do not elicit detectable positive signals via the BCR. This suggested the possibility that DCs may actively inhibit BCR-derived signals.

DCs negatively regulate BCR-dervied signaling.

Signal transduction through the BCR is regulated at multiple levels but coligation of inhibitory receptors sets the threshold of BCR signaling and protects from prolonged signaling. If DCs actively inhibit BCR signal transduction anti-μ induced Syk phosphorylation would decrease when DCs were present in the B cell cultures. Preincubation of 2-12H B cells with B6 BMDCs lead to a two-fold ($p=0.01$) reduction in anti-μ-induced Syk phosphorylation, establishing that DCs inhibit BCR signaling in autoreactive B cells (Figure 9A). To assess if DC-mediated repression was exclusive to autoreactive B cells we induced Syk phosphorylation in B6 B cells after incubation with

DCs. As shown in Figure 9B, DCs also repressed BCR-mediated signaling in B6 B cells by a two-fold reduction ($p < 0.0001$). This is the first evidence that DCs negatively regulate BCR signal transduction and raised the possibility that ligands for inhibitory receptors were selectively expressed on DC/MΦs, thereby regulating basal and antigen-induced signaling thresholds. Collectively, the data show that although DCs and MΦs provide a unique source of self-antigen to B cells as demonstrated by destabilization of the BCR. We cannot define if this antigen tolerizes or activates autoreactive B cells because a cell contact-dependent inhibitory mechanisms precludes such analysis.

Discussion

The regulation of low-affinity autoreactive B cells during innate immune responses requires DCs and MΦs to secrete IL-6, TNF- α, and CD40L ((103, 104) Gilbert MR and Vilen BJ manuscript in preparation). These soluble mediators repress LPS-induced Ig secretion by B cells chronically exposed to self-antigen, but not acutely-stimulated, naïve B cells. In this report, we show that DCs and MΦs display nuclear self-antigens that were recognized by autoreactive B cells. DNA, histones, and Sm were found on the surface of $CD11c^+$ splenocytes and BMDC/MΦs, suggesting that their display *in vivo* was constitutive. Apoptotic cells were a source of antigen to the DCs because their coculture resulted in increased surface antigen. Although several nuclear self-antigens were present on DCs and MΦs, a soluble antigen, HEL, was not evident. This demonstrates that not all self-antigens are displayed on DCs and MΦs. BCR destabilization of Sm-specific B cells indicates recognition of the endogenous antigen on the surface of DC/MΦs; however, there is no evidence of BCR-mediated signal transduction. Indeed, the DCs are repressive of BCR mediated signaling and Ig secretion resulting from LPS stimulation in a manner independent of BCR engagement of antigen.

The data indicate that the DCs repress both the adaptive and innate immune responses. However, there is still much to understand about these mechanisms. It is of interest to assess if the repression of BCR signaling is contact-dependent and how this affects BCR induced Ig secretion. We know that contact is essential in regulating the innate immune response, but it is unclear if this mechanism exclusively affects chronically antigen-experienced autoreactive B cells or if it also affects naïve B cells. The repression of BCR

signaling suggests that inhibitory receptor(s) might be involved. It is unclear if the same receptor(s) would regulate the TLR stimulated cells because few studies have addressed the role of inhibitory receptors in innate responses. Defining the molecular mechanisms of the DC-mediated repression will also further our understanding of the role for surface self-antigen.

Despite the interaction of the Sm-specific B cell and the antigen on the surface of DC/MΦs, there was no detectable signal transduction. BCR destabilization has been shown to cause receptor desensitization *in vitro*, therefore, the lack of signaling could be from prolonged interaction with nuclear self-antigen *in vivo* (65, 165, 166, 167). The lack of BCR mediated signaling in Sm-specific B cells raised several other possibilities. First, that the BCR may be of such low affinity that the antigen is recognized but it does not transduce a signal. Second, the signal is below the limit of detection. Third, the DC is actively repressing the B cell from signaling. While investigating these possibilities we discovered that the pre-treatment of non-transgenic, B6 and Sm-specific 2-12H B cells with DCs resulted in decreased phosphorylation of Syk after stimulation of the BCR. Thus, DCs repress BCR-mediated signal transduction in both autoreactive B cells and naïve cells. It is unclear what is the importance of DC-repression of non-autoreactive B cells is in the adaptive immune response. *In vitro* can repress naïve B cell signaling. However, *in vivo* this regulation may only occur when DCs and B cells encountered each other for prolonged periods of time as a result of the autoreactive BCR engaging its cognate endogenous antigen on the surface of DC/MΦs. Thereby, facilitating the inhibition conferred by the DC. This regulation of B cell signaling is yet to be

understood, but could have broader implications in the maintenance of tolerance. Collectively these data show that DC/MΦs can regulate both the adaptive and innate immune responses.

Nuclear self-antigens on the surface of DC/MΦs are recognized by autoreactive B cells and induce BCR destabilization. Using high-affinity antigen-pulsed systems, several groups reported that DCs displaying cognate antigen interact with B cells and transduce signals that promote class switching, and antigen processing (65, 165, 166, 167) . However, the physiological relevance of these antigen-pulsed DCs is unclear since we were unable to detect endogenous HEL on the surface of *ex vivo* $CD11c^+$ splenocytes from mice expressing a HEL transgene (Figure 3.4B). It is possible that DCs pulsed with HEL exhibit a much higher concentration of self-antigen compared to antigen displayed *in vivo* or that HEL expressed on $CDllc^+$ splenocytes may be below our limit of detection. Jenkins et al, demonstrated that HEL-specific B cells could obatin with HEL antigen in the follicle without display on the surface of DCs (227). Therefore, antigen loaded exogenously onto DCs may not represent a physiologically relevant mechanism of display.

We demonstrated that apoptotic cells provide a source of antigen to DCs. However, other antigens, such as immune complexes or soluble antigen, would be present *in vivo* and may also contribute to the antigen on the surface of these cells. The mechanisms by which DC/MΦs acquire nuclear self-antigens remain unclear; however, a variety of surface receptors are used to clear apoptotic cells. For example, immune complexes are

recognized by FcγRIIb and recycled to the DC surface (65). Similarly, nuclear self-antigens may be displayed on DC/MΦs via complement receptors since apoptotic antigens are readily coated with complement components (228, 229). However, BMDCs from various mouse strains lacking complement component 4, FcγRIIb, or complement receptors 1, 2, or 3 display surface Sm (Carnathan DG unpublished observations). One or a combination of receptors may be responsible for obtaining antigen and when one is deficient other receptors may compensate. Another possibility is that donor cells transfer antigen to DCs through membrane lipids. Others have reported that human DCs acquire HLA molecules by the transfer of membrane lipid from donor cell lines and transferred proteins activate tumor-specific $CD4^+$ T cells (230, 231). Apoptotic cells express surface self-antigen and DC/MΦs efficiently clear apoptotic debris, providing a possible mode of membrane transfer.

The regulation of the innate and adaptive immune responses is important in maintaining B cell tolerance. DCs repress LPS-induced Ig secretion through the secretion of repressive factors, IL-6 andCD40L, and by a contact-dependent mechanism. Further, they are able to repress the BCR-mediated signal transduction in order to maintain unresponsiveness. Therefore, we propose a model wherein antigen displayed by DCs and MΦs is recognized by the BC R, inducing destabilization, thus, prolonging the DC/MΦ interaction with the B cell. This colocalization results in repression of both BCR-derived signaling as well as Ig secretion resulting from TLR-stimulation. This ensures that low-affinity autoreactive B cells maintain quiescence to self-antigen, such as apoptotic cells, and repress Ig secretion during innate immune response. Lastly, chronic exposure to self-

antigen reprograms IL-6 receptor and CD40L responses, allowing autoreactive and acutely-stimulated B cells to be differentially regulated by IL-6 and CD40L. This promotes immunity in the absence of autoimmunity.

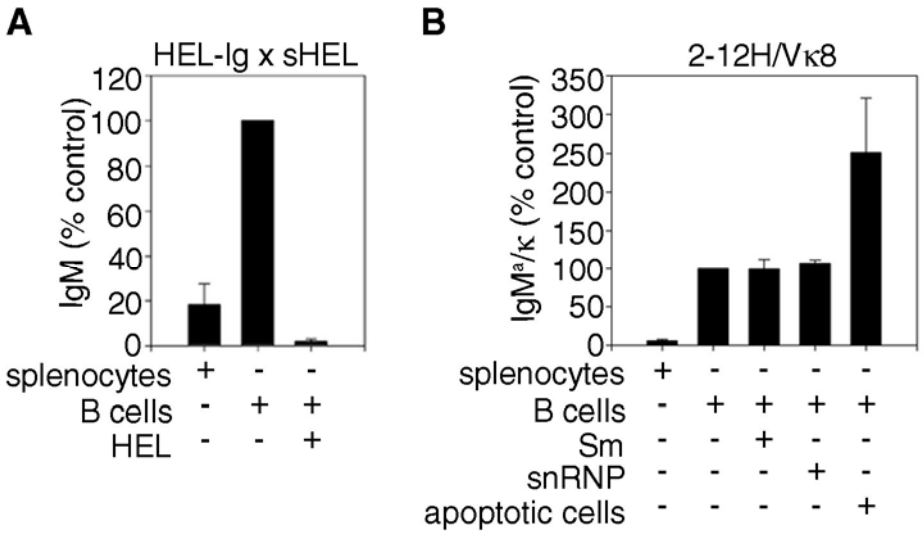

Figure 3.1. Soluble Sm and apoptotic cells fail to negatively regulate Sm-speficic B cells. (A) Splenic B cells ($1x10^5$) purified by negative selection from HEL-Ig x sHEL mice were LPS stimulated (30 μg/ml) in the presence or absence of soluble HEL (100 μg/ml) **(B)** 2-12H/Vκ8 B cells ($1x10^5$), purified by negative selection, were LPS stimulated (30 μg/ml) in the presence or absence snRNPs (10 μg/ml), soluble Sm (10 U/ml) or apoptotic cells ($5x10^5$). Ig secretion was quantitated by ELISA at day 4.

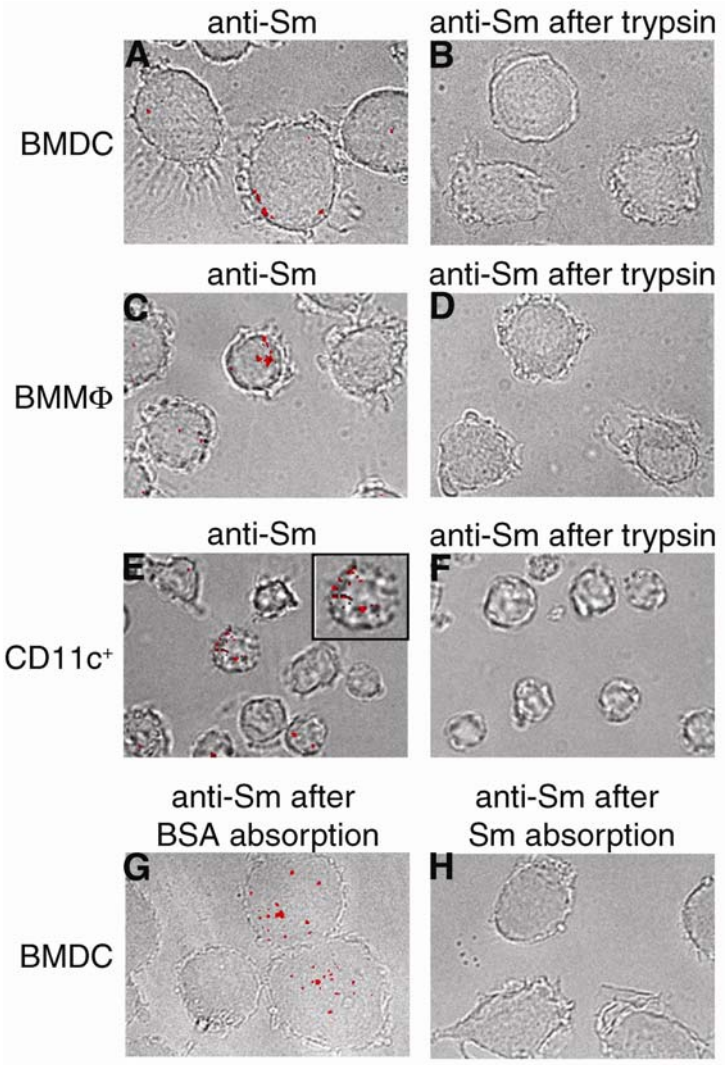

Figure 3.2. BMDCs, BMMΦs, and CD11c$^+$ splenocytes display surface Sm. B6 BMDCs **(A, B)**, BMMΦs **(C, D)**, and CD11c$^+$ splenocytes **(E, F)** were untreated or trypsin treated, then stained with anti-Sm (2.12.3). Forty-six percent of untreated BMDCs, 44% of untreated BMMΦs, and 40% of untreated CD11c$^+$ splenocytes displayed Sm (100 cells analyzed). In contrast, 2% BMDC/MΦs and 4% CD11c$^+$ cells showed Sm following trypsinization. BMDCs **(G, H)** were stained with anti-Sm absorbed with BSA or recombinant SmD. All images shown at a magnification of 63x. The insert contains a 1.5x magnification of a Sm-expressing CD11c$^+$ cell.

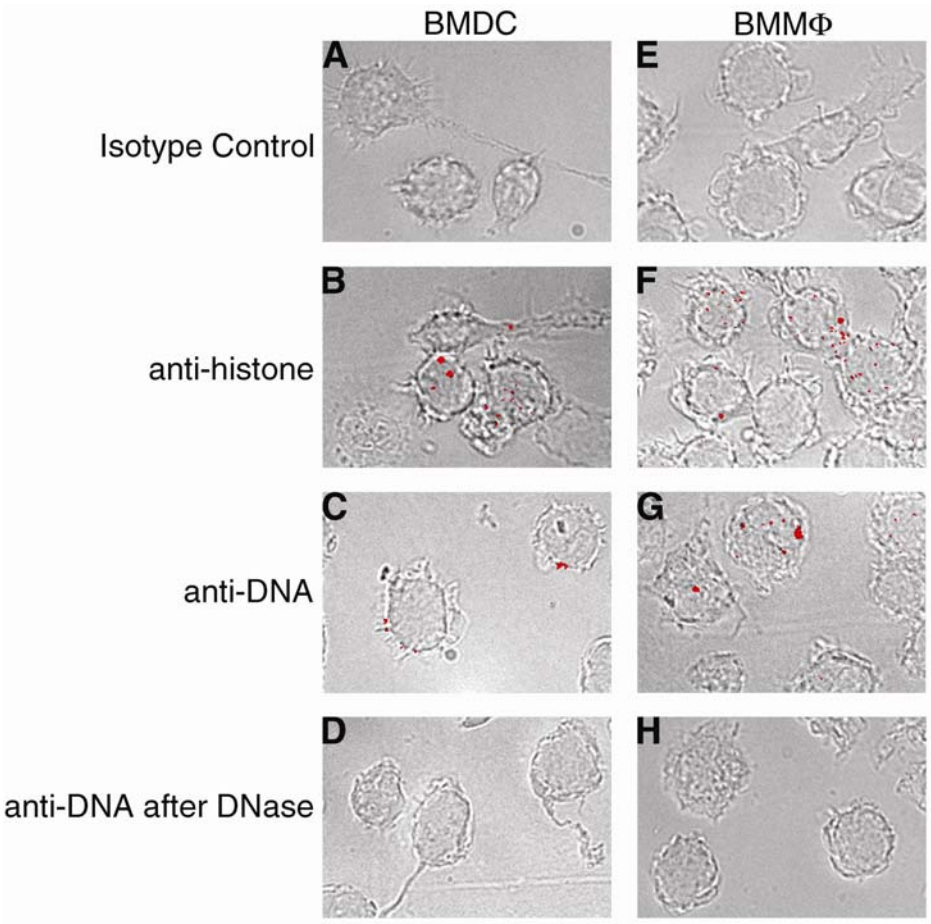

Figure 3.3. BMMΦs and BMDCs display surface histones and DNA. B6 BMDCs **(A-D)** and BMMΦs **(E-H)** were untreated or treated with DNase **(D, H)** and stained with isotype control **(A, E)**, anti-histone **(B, F)**, or anti-DNA **(C, G)**. Thirty-three percent of BMDCs and BMMΦs exhibited histone staining, 48% of BMDCs, and 44% of BMMΦs showed DNA (100 cells analyzed). In contrast, 5% BMDCs and 7% BMMΦs displayed Sm following DNase treatment. All images shown at a magnification of 63x.

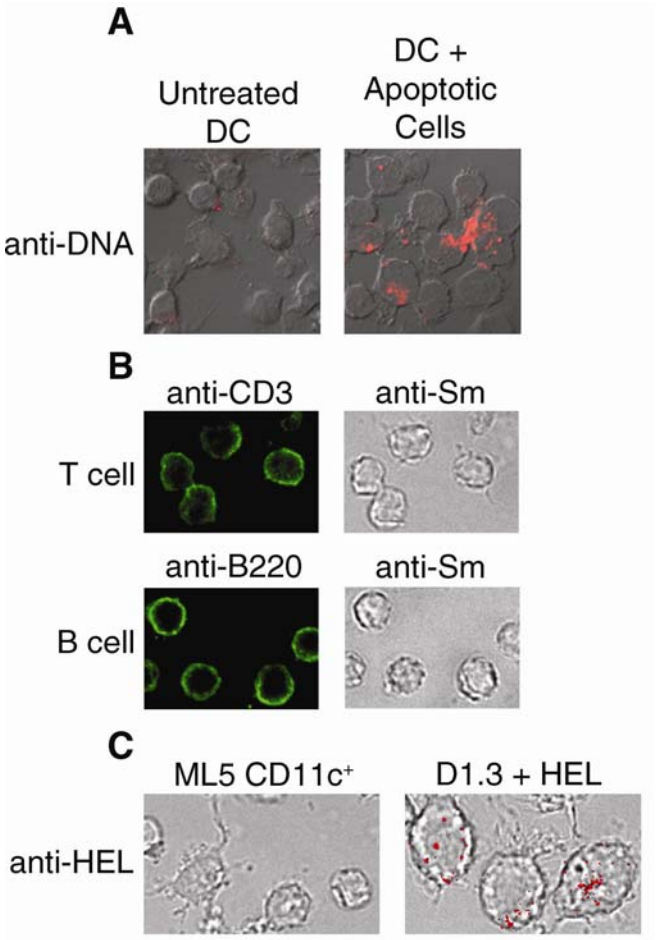

Figure 3.4. Nuclear, but not soluble, self-antigens are displayed by DCs and MΦs, but not lymphocytes. (A) B6 BMDCs were cultured with apoptotic cells for 4 hours. The apoptotic cells were removed by density centrifugation and the cells were stained for the presence of DNA. The DCs cultured with apoptotic cells (right panel) displayed a 2-fold (p=0.002) increase in antigen on their surface compared to DCs alone (left panel). **(B)** Sorted $CD3^+$ splenic T cells and $B220^+$ B cells were stained with anti-Sm. **(C)** *Ex vivo* $CD11c^+$ splenocytes (left panel) from mice expressing soluble HEL (ML5) were stained with anti-HEL. The HEL-specific D1.3 cell line (right panel) was loaded with HEL (500 ng/ml) and stained with anti-HEL. Seventy-three percent of D1.3 cells displayed HEL (100 cells analyzed). All images shown at a magnification of 63x.

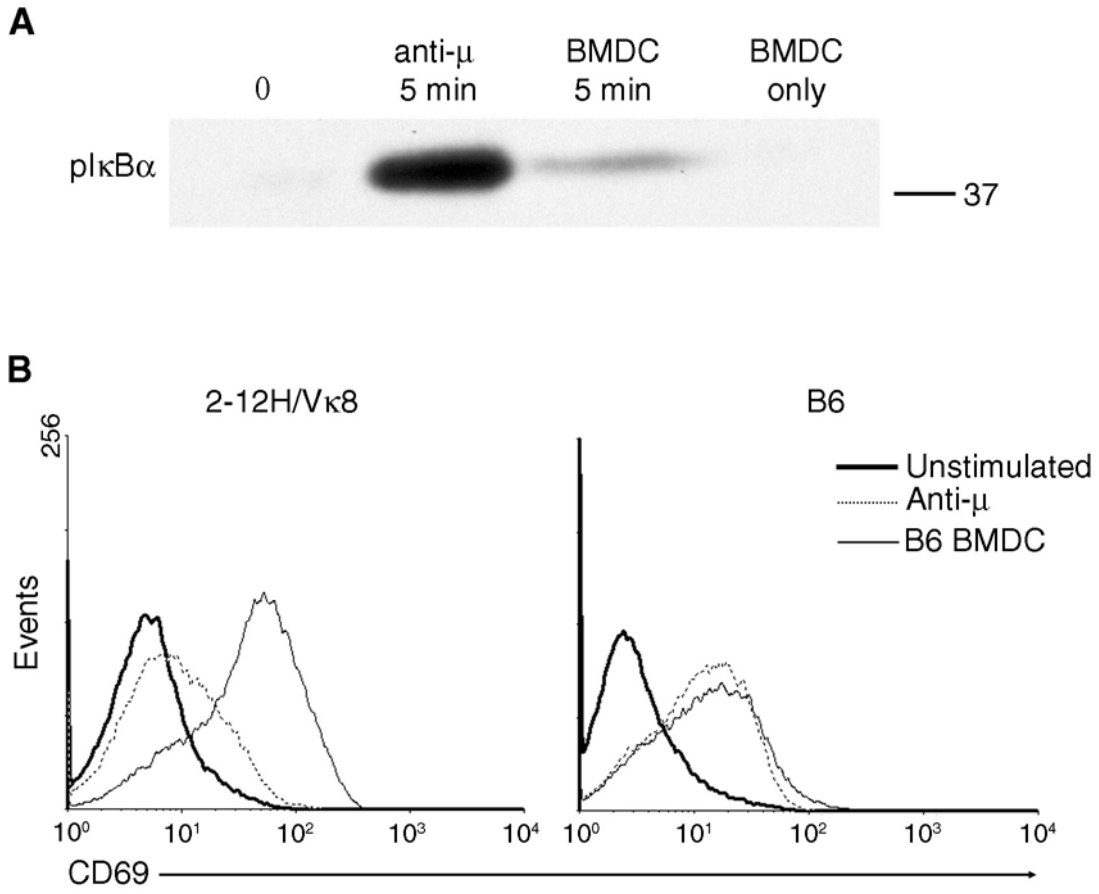

Figure 3.5. Coculture of DCs and B cells leads to B cell activation. (A) B6 B cells were stimulated with anti-μ or B6 BMDCs for 5 minutes. The amount of phosphorylated- IκB-α in whole cell lysate was determined by immunoblotting. **(B)** 2-12H/Vk8 (left panel) and B6 (right panel) purified B cells were stimulated with anti-μ (dashed lines) or B6 BMDCs (thin line), or left unstimulated (thick line) for 6 hours. Cells were then stained with CD19 and CD69 and analyzed by flow cytometry. Immunoblots and histograms are representative of 3 experiments.

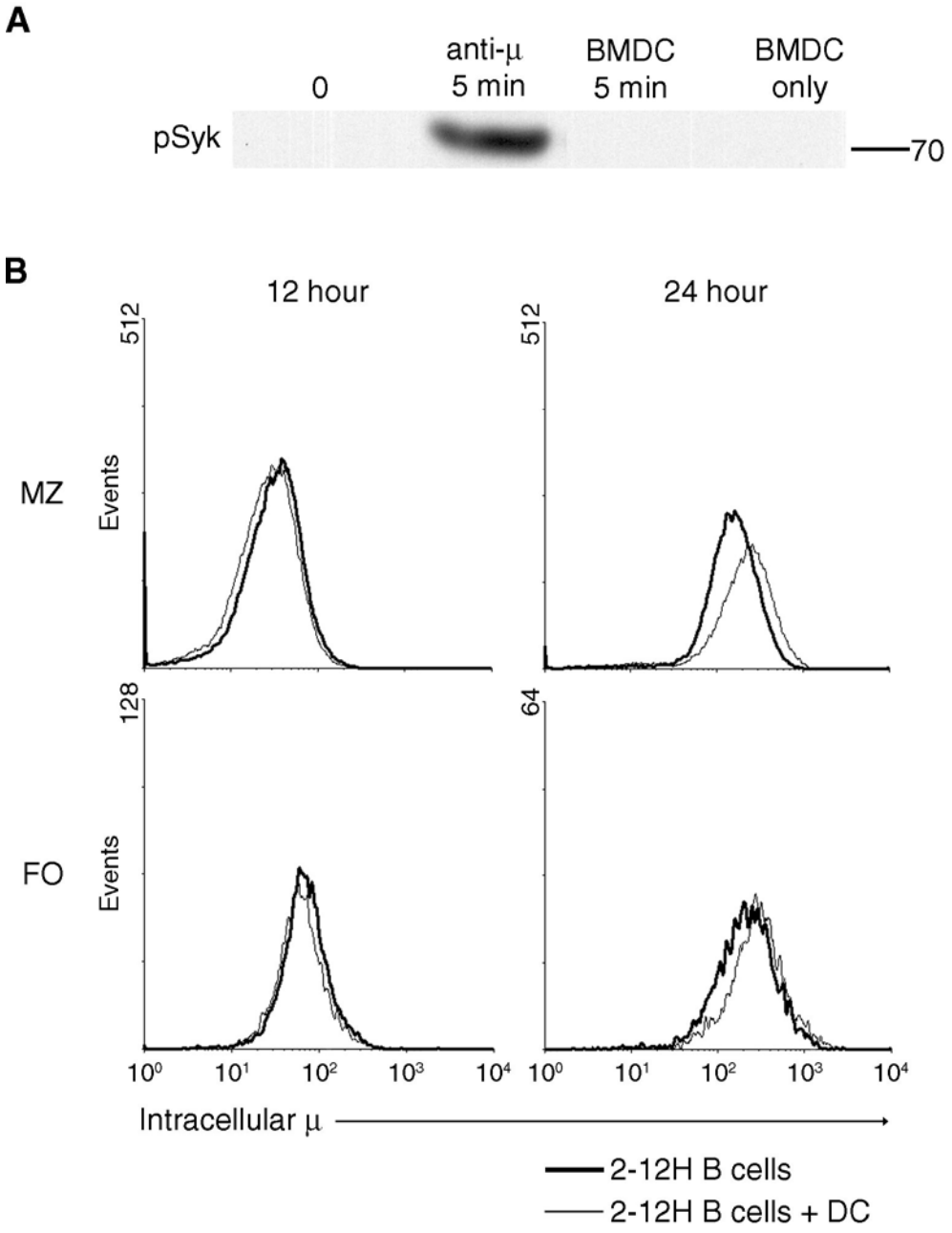

Figure 3.6. DCs do not induce BCR-mediated signal transduction in Sm-specific B cells. (A) Purified 2-12H B cells were stimulated with anti-μ or BMDCs for 5 minutes. The amount of phosphorylated-Syk in whole cell lysate was determined by immunoblotting. **(B)** Purified 2-12H B cells were co-cultured with DCs and harvested at time points indicated. Cells were fixed and stained for expression of intracellular

μ in MZ (top panels) and FO (bottom panels) B cells. In both populations, B cells cultured with or with out DCs had the same amount of intracellular μ after 12 (left panels) and 24 hours (right panels). Data is representative of 3 experiments.

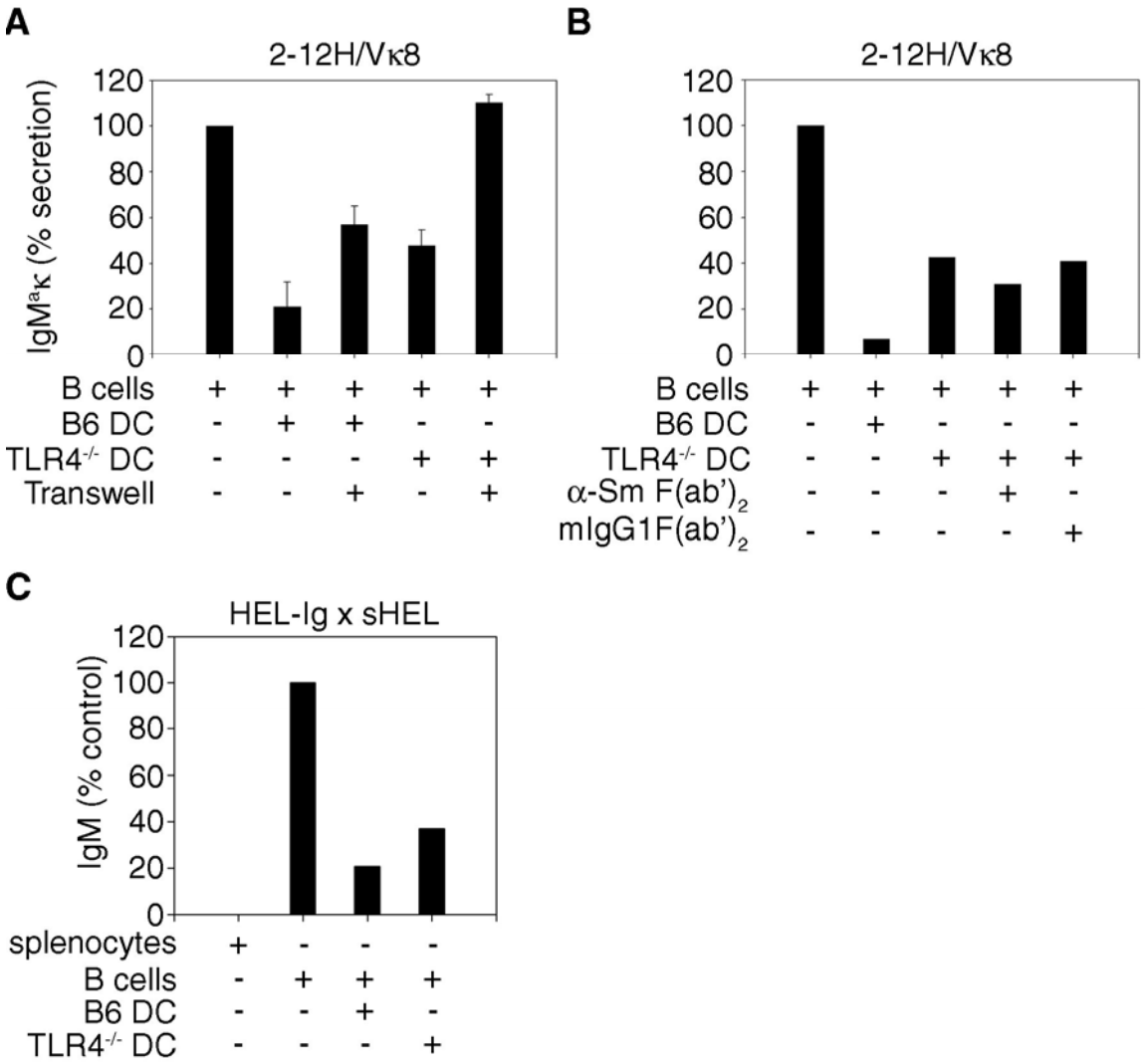

Figure 3.7. DCs repress LPS-induced Ig secretion by autoreactive B cells by an antigen- independent contact dependent mechanism. (A) 2-12H/Vk8 B cells ($1x10^5$) were LPS stimulated (30 μg/ml) in the presence or absence of B6 or TLR4-deficient BMDCs ($1x10^4$) with or without a transwell apparatus. **(B)** 2-12H/Vk8 B cells ($1x10^5$) were LPS stimulated (30 μg/ml) in the presence or absence of B6 or TLR4-deficient BMDCs ($1x10^4$). B cells were treated with TLR4-deficient DCs with or without anti-Sm $F(ab')_2$ (2.12.3) or isotype control murine IgG1 $F(ab')_2$. (C) HEL-Ig x sHEL B cells were LPS stimulated (30 μg/ml) in the presence or absence of B6 or TLR4-deficient BMDCs ($1x10^4$). Ig secretion was quantitated by ELISA at day 4.

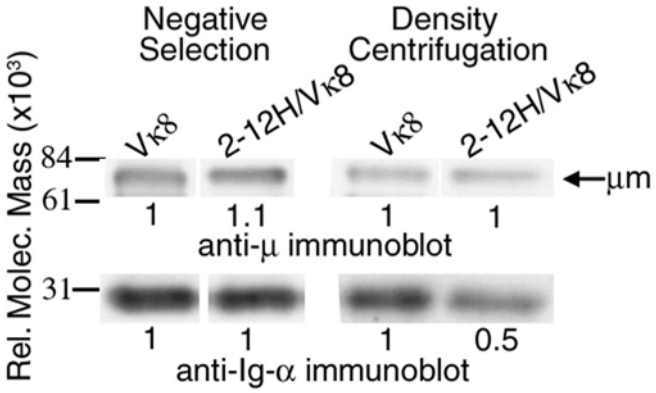

Figure 3.8. Sm-specific B cells recognize self-antigen and destabilize their BCR. The BCR complex was immunoprecipitated from $30x10^6$ unstimulated, B cells were isolated by either negative selection or by percoll density centrifugation (ρ>1.066). Proteins resolved by SDS-PAGE were immunoblotted for μ-heavy chain and Ig-α. For each purification method the intensity of Ig-α in 2-12H/Vκ8 relative to Vκ8 was determined by densitometry (displayed below each band). Data is representative of 5 experiments.

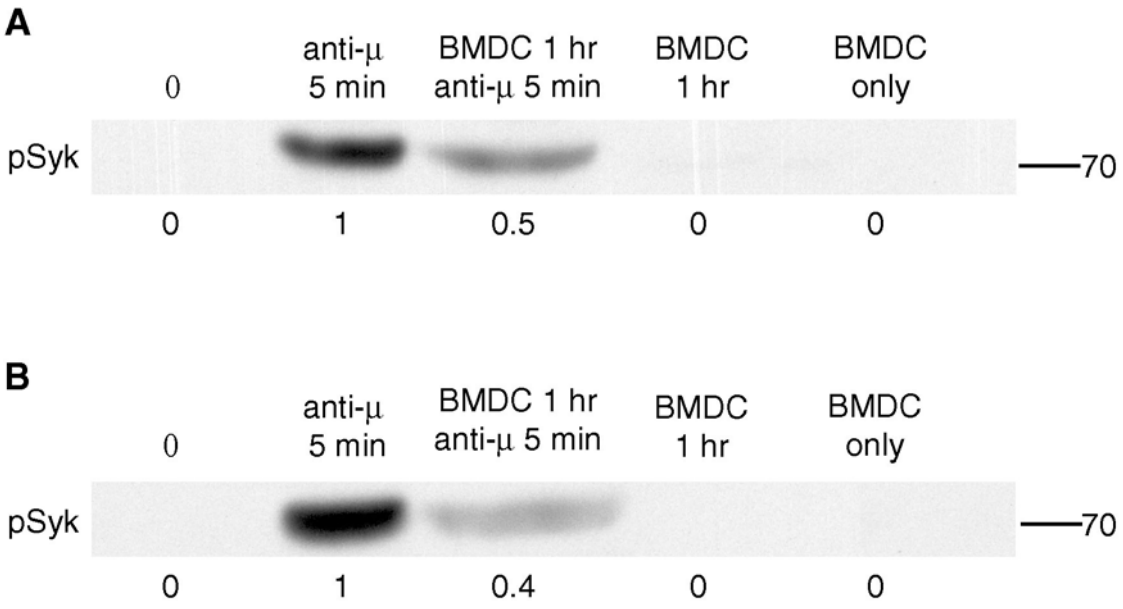

Figure 3.9. DCs repress BCR signaling upon anti-μ stimulation. 2-12H **(A)** or B6 **(B)** B cells were cultured with or without B6 BMDCs for 1 hour and stimulated with anti-μ for 5 min. The amount of phophorylated-Syk in whole cell lysate was determined by immunoblotting followed by densitometry. The 2-12H and B6 B cells cultured with DCs have two-fold ($p=0.03$) less p-Syk than B cells without DCs. The intensity of pSyk in B cells treated with DCs and anti-μ relative to B cells treated with anti-μ alone was determined by densitometry (displayed below each band). Data is representative of 3 and 5 experiments respectively.

CHAPTER IV. DISCUSSION

Regulation of both the innate and adaptive immune responses is important in maintaining autoreactive B cell tolerance. Microbial stimulation of TLRs provides a rapid inflammatory response involving the secretion of cytokines and production of antibodies. Further, B cell response to foreign antigen during the adaptive immune response is also essential in the clearance of infection. However, the activation of autoreactive B cells by either self-antigen or microorganisms causes the production of autoantibodies and contributes to the development of autoimmune disease. Therefore, SLE is characterized by hyperactive B cell activation resulting from abnormal tolerance mechanisms.

We previously demonstrated that DCs repress LPS-induced Ig secretion by autoreactive B cells through soluble mediators. Now we show that they can repress these B cells in the absence of soluble factors in a contact-dependent mechanism. DCs from lupus-prone mice are defective in responding to LPS stimulation resulting in a deficient ability to repress the innate B cell response. We also showed that DCs repress BCR-mediated signal transduction in naïve and autoreactive cells. The mechanism of this repression is unknown, but one interesting possibility is an inhibitory receptor on the surface of B cells. Our data show that DC/MΦs display nuclear self-antigen on their surface. It is known that self-antigen is important in regulating B cell tolerance to antigen and microbial stimulation. While the role of this antigen is unclear, we hypothesize that it is important in modulating autoreactive B cells susceptibility to regulation by soluble repressors and inducing colocalization.

The onset and later flares of autoimmune disease are often associated with bacterial or viral infection (76, 77, 78). In particular, Epstein-Barr viral infection has been implicated in the onset and exacerbation of SLE (78). Lupus-prone mouse models that are immunized with bacterial or viral components have aggravated symptoms (79, 80, 81). Further, several studies focusing on TLR-deficient mice and TLR expression in patients have demonstrated the importance of these receptors in the initiation and progression of the disease (85, 86, 87, 88). Therefore, regulation of the innate immune response in autoreactive cells is critical in maintaining tolerance. Previously, we described that DC/MΦs secrete IL-6 and CD40L and repress chronically antigen-experienced, autoreactive B cells; these B cells are then unable to secrete Ig in response to polyclonal activation (103, 104). The cytokines produced by these cells are sufficient to repress Ig secretion by autoreactive B cells because the supernatant from stimulated cells or individual recombinant cytokines alone are repressive (103). We studied this repression of innate responses in lupus-prone MRL/*lpr* mice to determine if this mechanism is dysregulated in disease. Compared to B6 mice, the DCs from MRL/*lpr* were defective in their IL-6 mRNA production and secretion of IL-6 (Figure 2.3 and 2.5). While overall the DCs were less efficient in their repression of Sm-specific B cells, their lack of IL-6 production did not always correlate with deficient repression of Ig secretion (Figure 2.2). In order to make up for the lack of IL-6, other mechanisms of tolerance may be utilized. For instance, TNF-α, which is also secreted by DCs, represses Ig secretion by autoreactive B cells and, if produced at normal levels, could compensate for the defective production of other soluble mediators (Gilbert MR manuscript in preparation). We also have demonstrated that in the absence of the tolerizing cytokines, DCs are able to

partially repress LPS-stimulated, autoreactive B cell secretion in a contact-dependent manner (Figure 3.7). Cell contact-mediated inhibition of autoreactive B cells during an innate immune response is important when the DCs fail to be activated by the same pathogen as the B cell or are defective in secreting soluble factors as in the MRL/*lpr* mice. However, because the MRL/*lpr* DCs have an overall defect in their ability to repress autoreactive cells, they may also lack the component(s) essential for the contact mediated repression, thereby exacerbating the lack of soluble repressors. Therefore, investigating the ability of MRL/*lpr* DCs to mediate contact-dependent repression will help us to fully describe their contribution to dysregulation of B cells in lupus-prone mice. Our data confirm that the regulation of the TLR response in autoreactive cells is important in preventing their aberrant activation leading to the breakdown in tolerance and disease.

Numerous studies characterize DCs as dysfunctional or abnormal in patients and lupus-prone murine models. We demonstrated MRL/*lpr* DCs compared to B6 were defective in the production of IL-6 mRNA and secretion of IL-6 upon LPS stimulation. Because several components of the TLR4 signaling pathway were shown to be defective, we concluded that the diminished IL-6 production was due to dysfunctional TLR signaling (Figure 2.6 and 2.7). Further, DCs were also defective in IL-6 production upon stimulation of TLR7 and 9, but not TLR3, indicating that the defect is in the MyD88-dependent pathway (Figure 2.3). We are unsure exactly where the dysregulation of signaling occurs. It may be a defective component of the signaling pathway upstream of IκBα, or perhaps the receptor itself is unable to sustain the signal. Signaling components

that act to repress TLR signaling may contribute to defective signal transduciton. Suppressors of cytokine signaling (SOCS) family proteins regulate TLR signaling (232). We have evidence that expression of SOCS3 is increased in MRL/*lpr* DCs (Gilbert MR unpublished observations). Therefore, the response to TLR stimulation could be diminished due to overexpression or activation of repressive mediators. DCs poor response to polyclonal activation might be due to an increase in apoptotic cells found in SLE (16, 17, 116, 161, 162). DCs are responsible for the clearance of these cells, however, after phagocytosis they are not stimulated by polyclonal activation (199). The antigen displayed on the surface of apoptotic cells may also contribute to defective TLR activation. Exposure of cross-reactive TLRs 7 and 9 to self-antigen may desensitize the TLR signaling pathway (55, 89). These receptors are not on the surface of the cell, but could interact with the antigen if it was endocytosed through another receptor delivered to the TLR endosome. This could be mediated by receptors that are involved in phagocytosis, such as FcRs or complement (55). Multiple factors may contribute to the dysregulation of TLR signaling. The abnormal response of DCs to polyclonal activation leads to reduced cytokines secretion resulting in defective repression of autoreactive B cell Ig secretion.

We demonstrated that the interaction between normal DCs and B cells results in repression of BCR-derived signaling in naïve and autoreactive B cells (Figure 3.9). This is the first evidence that DCs repress BCR signal transduction. Non-autoreactive B cells are sensitive to this repression; therefore, BCR desensitization by exposure to antigen is eliminated as a possible mechanism. However, there are several other potential

mechanisms by which this repression could be mediated. One interesting possibility is that DCs might activate inhibitory receptors on the surface of B cells that negatively regulate BCR signaling. The inhibition by these receptors is mediated through the activation of protein phosphatases such as SHP-1, SHP-2, and SHIP. CD72, PIR-B, CD5 and CD22 associate with SHP-1 to attenuate BCR-derived signals (233, 234, 235, 236). FcγRIIb activates SHIP and PD-1 and PIR-B recruit SHP-2 to prevent downstream signaling from the BCR (235, 237, 238). Of particular interest are CD22 and FcγRIIb, because of their involvement with Lyn (106, 239). Lyn has been shown to be able to repress both BCR- and TLR-mediated responses (240, 241). In this scenario, DC-mediated repression of the innate and adaptive immune responses could be mediated through the same receptor.

In order for this regulation to occur through one of these inhibitory molecules, the DC must engage the receptor on the B cell. If the antigen on the surface of DCs is in IC, then FcγRIIb would be a candidate for repression. The receptors CD22, CD72, Plexin B-1, PIR-B, and PD-1 have known ligands on the surface of DCs (237, 242, 243, 244, 245, 246, 247). However, not all of the ligands for these inhibitory receptors have been identified. There may be an unknown receptor-ligand combination that promotes negative regulation of the BCR when in contact with the surface of DCs. It is also thought that some receptors and ligands for negative regulation are both present on the surface of the B cell and can interact in cis (248, 249). Instead of providing a ligand, the DC may act as a bridge to bring these two molecules together. Because several of these receptors utilize the same phosphatases, multiple receptors may be involved.

Determining if inhibitory receptors are involved in DC-mediated repression of BCR signaling will further our understanding of this mechanism.

Another reason that inhibitory receptors are potential candidates for this repression is that several of the molecules discussed above have been implicated in the development of autoimmune disease. The expression of FcγRIIb, CD72, and Lyn is reduced in murine lupus models and in patients, which results in activated B cells (62, 63). Mice lacking FcγRIIb, CD22, and Lyn develop disease (27, 240). We would like to investigate if lupus-prone DCs can mediate this general repression of BCR signaling and if lupus-prone B cells are susceptible to it. The ability of DCs to repress BCR signal transduction implies that it could be important in regulating autoreactive B cells' response to cognate antigen to prevent an autoimmune response

We demonstrated that naïve B cell signaling is also negatively regulated by DCs (Figure 3.9). Repression of both naïve and autoreactive B cells was not complete. It was diminished by half compared to B cells stimulated with anti-μ alone. It is possible that the DCs are only regulating a specific population of B cells. Another potential role for this repression is that DCs may provide a developmental checkpoint by increasing the threshold of stimulation for the cognate antigen. This mechanism could prevent B cells from becoming antibody secreting cells. Therefore, it is important to examine if DCs can repress antigen-induced Ig secretion and proliferation in both autoreactive and naïve B cells. These studies will help us to determine the role for DC-mediated repression of BCR-signal transduction.

The ability of DCs to display intact antigen resulting in prolonged interaction with B cells has been demonstrated in a variety of studies. DCs exogenously loaded with HEL cause antigen-specific B cells to signal (165, 166, 167). Follicular DCs hold IC on their surface in germinal centers to prevent apoptosis and promote proliferation of B cells (250, 251). Blood DCs capture and transport antigen to interact with splenic MZ B cells and help induce the T-independent immune response (188). These data indicate that DCs activate B cells by providing a cognate antigen. However, HEL-specific B cells can bind to soluble antigen in the follicle without DCs (227). Further, most of these studies examined exogenously-loaded, high-affinity antigens, infectious antigens, or synthesized immune complexes. Our confocal studies demonstrate that endogenous, nuclear self-antigen is constitutively displayed on the surface of DC/MΦs (Figure 3.2 and 3.3). Low-affinity Sm-specific B cells can recognize Sm on the surface of DC/MΦs, demonstrated by their destabilized BCR (Figure 3.8). However, this interaction does not result in activation or BCR signaling as it does in the studies described above (Figure 3.6). Because DC/MΦs display nuclear antigens other than Sm, such as DNA and histones, it would be of interest to study the stability of BCRs in other autoreactive B cell models, specifically Ars/A1 that recognizes ssDNA and VH3H9H that have anti-ss and dsDNA B cells (181, 252). This would confirm that other nuclear antigens on the surface of DC/MΦs can induce a similar response. If multiple autoreactive cells recognize low affinity self-antigens, it would corroborate the idea that the DCs and autoreactive B cells maintain contact with each other. Further, we hypothesize that the antigen is important in colocalizing DC/MΦs and autoreactive B cells specific for nuclear antigen in order to

mediate repression to both antigen and infectious stimuli and maintain anergy. While *in vitro* DCs can repress BCR signaling in B cells of a varied repertoire, *in vivo* the DCs may only regulate B cells that can maintain a prolonged interaction through BCR engagement of self-antigen. In order to study the duration and nature of the interaction between autoreactive B cells and DCs *in vivo*, we would utilize two-photon microscopy imaging studies as in Qi et al. (167). The surface antigen may not be directly responsible for repression; however, it could be influencing what B cells are being affected by the regulation by promoting colocalization with autoreactive cells.

Antigen plays an important role in the maintenance of B cell tolerance to both adaptive and innate stimulation. Constant BCR occupancy is required to maintain anergy in response to further stimulation (136). During the innate response, antigen stimulation of HEL-specific B cells results in unresponsiveness to TLR9 and 4 stimulation. Additionally, chronic antigen experience is necessary for LPS-stimulated, autoreactive B cells to be susceptible to repression by soluble mediators (103, 104, 220, 226). These data demonstrate that engagement of the BCR modulates the B cell response to other stimuli in order to maintain tolerance. Therefore, we investigated the source of tolerizing antigen for low-affinity Sm-specific B cells. We demonstrated that high-affinity soluble antigen, HEL, was sufficient in repressing LPS-induced Ig secretion by HEL-specific B cells. However, Sm-specific B cells were unaffected by treatment with their cognate soluble antigens (Figure 3.1). Further, the antigen on the surface of DCs was not important in contact-depedent repression of LPS-induced Ig secretion (Figure 3.7). However, during the innate response, chronic antigen experience is necessary to be

sensitive to repression by soluble factors (103). Therefore, we propose that surface self-antigen provides a source of antigen that can engage the BCR without activating the cell. This engagement of the BCR causes reprogramming of cell signaling that confers susceptibility for IL-6 and CD40L-mediated tolerance, while the DC/MΦs that secrete these factors negatively regulate BCR-mediated signal transduction.

We demonstrated that apoptotic cells, which display nuclear self-antigen on their surface, enhanced LPS-stimulation of Sm-specific B cells (Figure 3.1). In lupus-prone mice, an accumulation of apoptotic cells leads to activation of autoreactive B cells to produce autoantibody (13, 15). Further, some SLE patients have elevated levels of apoptotic cells (16, 17). Previously, it was shown in the HEL system that antigen-experienced autoreactive B cells can be eliminated in a Fas-dependent manner when they encounter T cell help; however, they may be protected from apoptosis by interacting with highly cross-linking antigen (253, 254). Thus, an increase in stimulatory form of self-antigen on apoptotic cells could contribute to a loss of tolerance. Further, we have evidence that $CD11c^+$ splenocytes and BMDCs from MRL/*lpr* display four-fold ($p<0.0001$) and nine-fold ($p=0.001$) more Sm compared to B6 DCs, respectively (Carnathan DG unpublished observations). This increase in surface antigen on DCs could contribute to the defect in the DC-mediated repression of autoreactive B cells, or it may provide more stimulatory antigen to lupus-prone, autoreactive B cells protecting them from Fas-dependent apoptosis. Further experiments to investigate the role for increased surface-antigen in defective tolerance mechanisms are necessary. Our data corroborate previous studies

demonstrating that the form of antigen is important in the regulation of autoreactive B cells.

Our data focus on DC repression of the innate and adaptive immune response and their dysregulation in lupus-prone mice. Previously, we examined the ability of MΦs to repress Ig secretion by antigen-experienced autoreactive B cells in response to LPS sitmulation (104). This study demonstrates that much like DCs, MΦs from lupus-prone mice are also defective in their secretion of soluble factors and deficient in the ability to repress autorective B cells. These cells are defective in their production of the soluble repressors IL-6 and CD40L. Several reports have indicated that DCs and B cells are able to interact; however, our study introduces the concept of nuclear self-antigen on the surface of MΦs playing a role in regulating B cells through direct contact, demonstrated by BCR destabilization that was evident when Sm-specific B cells were purified with by DC/MΦs (Figure 3.2, 3.3, and 3.8). It is of interest to confirm that, like DCs, MΦs are also capable of repressing BCR-mediated signal transduction and Ig secretion. In our previous studies of DC/MΦ regulation of autoreactive B cells, it was determined that FO B cell LPS-induced Ig secretion can be repressed by both MΦs and DCs while MZ B cells are only susceptible to suppression by MΦs (104). It would be of interest to study how the MΦs differentially regulate B cell subset signaling and autoantibody secretion compared to DCs. Determining the role of MΦs in the repression of B cell responses will help us to understand how they contribute to tolerance.

We have demonstrated that DC/MΦs are capable of regulating both the innate and adaptive immune responses by B cells. We propose a model in which the antigen displayed on the surface of these cells might act to colocalize the DC/MΦs with autoreactive B cells and induces receptor destabilization. The combination of destabilization and the ability of DCs to repress BCR signaling results in anergy**.** *In vivo,* autoreactive cells are subject to continuous ligation of the BCR with self-antigen, which may cause the B cell to reprogram their response to IL-6, CD40L, and TNF-α secreted by DC/MΦs upon LPS stimulation resulting in their repression. These cytokines are sufficient to regulate Ig secretion of LPS-stimulated autoreactive B cells; however, a DC mediated contact-dependent mechanism is utilized if the DC is not activated or is defective in secretion of repressive factors. In MRL/*lpr* mice, the defective DCs fail to secrete the soluble mediators and display abnormal amounts of surface self-antigen; their dysregulation results in their inability to repress autoreactive B cell Ig secretion. Thus, DC/MΦ-regulation of adaptive and innate responses is essential in maintaining tolerance and preventing autoimmunity.

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