Dendritic cell and Macrophage-Mediated Tolerance in Lupus-Prone Mice

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ABSTRACT

MILEKA RICHELLE GILBERT: Dendritic cell and Macrophage-Mediated Tolerance in
Lupus-Prone Mice
(Under the direction of Barbara Vilen)

During infection, immune cells respond to polyclonal activators, like bacterial and viral

antigens, through innate immune responses. Therefore, mechanisms to regulate the

activation of autoreactive B cells during polyclonal activation are necessary to prevent

autoimmunity. Previous studies into the mechanisms of B cell tolerance have focused on B

cell Receptor (BCR)-mediated regulation of autoreactive or chronically antigen-experienced

B cells. However, the regulation of chronically antigen-experienced B cells during

polyclonal activation is less understood. We recently identified a novel mechanism of

tolerance wherein DCs and MΦs repress Ig secretion by autoreactive B cells. Polyclonal

activators through Toll-like Receptors (TLRs) induce DCs and MΦs to secrete soluble

factors (IL-6, sCD40L, and TNFα) that differentially regulate naïve and chronically antigen-

experienced B cells. IL-6, sCD40L, and TNFα selectively repress chronically stimulated

autoreactive B cells while having no effect on naïve B cells. Thus, we have identified a

mechanism that prevents autoimmunity while allowing naïve B cells to respond during innate

immune responses. Significantly, TLR-activated DCs and MΦs from lupus-prone mice are

defective in repressing autoreactive B cells, coincident with defects in IL-6, sCD40L, and

TNFα secretion. This could allow autoreactive B cells to secrete autoantibodies during

innate immune responses, promoting autoimmune disease in lupus-prone mice. Determining

the TLR defect in DCs and MΦs from lupus-prone mice could identify a genetic signature

for individuals susceptible to lupus disease.

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Ars – *p*-azophenylarsonate

BCR – B cell Receptor

CM – conditioned media

DC – dendritic cell

HEL – hen egg lysozyme

Ig – immunoglobulin

IκBα – inhibitor of nuclear factor kappa-B kinase alpha

IL – interleukin

LPS – lipopolysaccharide

MyD88 – myeloid differentiation factor 88

MΦ – macrophage

NF-κB – nuclear factor-kappa B

RF – rheumatoid factor

RT-PCR – reverse transcriptase-polymerase chain reaction

SLE – Systemic Lupus Erythematosus

Sm – Smith antigen

SOCS – suppressor of cytokine signaling

Tg – transgenic

TLR – Toll-like Receptor

TNFα – tumor necrosis factor alpha

CHAPTER I

Introduction

1.1 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a multiorgan autoimmune disease characterized by the production of autoantibodies to nuclear components, including DNA and Smith antigen (Sm). Autoantibodies contribute to pathology either by directly binding tissue antigen or by forming immune complexes which activate complement or bind Fc receptors inducing inflammation (1). Symptoms and severity of disease vary but include malar or discoid rash, arthritis, glomerulonephritis, vasculitis, neurologic and hematologic disorders.

SLE afflicts up to 1.5 million people in the United States, with both genetic and environmental factors associated with disease. 90% of SLE patients are women, and disease disproportionately affects African-Americans. Ethnic background correlates with the production of certain autoantibodies and has been associated with different clinical and laboratory features of SLE. Several studies have linked autoantibodies against dsDNA, Sm, Ro, and phospholipids with increased disease damage in different ethnicities (2-9). The concordance of disease in identical twins is 25-50% compared to around 5% in dizygotic twins, and polymorphisms and homozygous deficiencies in some genes increases the risk of developing SLE (10). For example, HLA DR2 and DR3 and certain polymorphisms in TCR, FcγR, IL-6, and TNFα genes increase risk of disease (10). In addition, deficiencies in DNaseI (11) and complement C2, C4, and C1q (10) are highly associated with SLE. These observations suggest a strong genetic component of disease; however, most cases of SLE are sporadic. It is thought that environmental factors trigger disease or flare-ups in susceptible individuals. Certain drugs, infectious agents (especially Epstein Barr virus), and excess estrogens have been linked to disease (12). The interaction of these environmental factors

with predisposed genetic backgrounds could explain the heterogeneity in disease symptoms and severity.

Autoimmunity results from a breakdown in tolerance mechanisms that regulate autoreactive lymphocytes (10, 13). The breakdown of B cell tolerance mechanisms ultimately leads to the production of autoantibodies against nuclear components and immune complexes that are linked to the pathology of disease (10, 13). The etiology of SLE remains unknown; however, investigation into the breakdown of tolerance mechanisms has provided valuable insight into the causes of disease. SLE patients are unable to effectively clear dying cells, and thus exhibit an increased burden of apoptotic cells that serve as a source of nuclear antigens (14-17). The dysregulated presence of apoptotic cells promotes autoimmunity, and recently been shown to induce nephritis in mice (18). Currently, the standard of care for SLE is immunosuppressive or anti-inflammatory drugs, with recent therapies focusing on B cell depletion. Understanding the mechanisms that regulate autoreactive B cells would lead to more specific therapies that re-establish tolerance in SLE patients.

1.2 Mechanisms of B cell Tolerance

B cell development begins in the bone marrow where immature B cells generate B cell receptors (BCR) with diverse specificities. Tolerance mechanisms are critical in regulating the estimated 50-75% of newly produced B cells with autoreactive specificity in the bone marrow in order to prevent autoimmune disease (19). The study of these mechanisms began with the creation of immunoglobulin (Ig) transgenic mice, which increases the frequency of B cells having one specificity, thereby providing a tool to follow the development and regulation of autoreactive B cell clones *in vivo*. Using Ig transgenic

mice, several mechanisms of tolerance have been identified, including clonal deletion, anergy, positive selection into the B1 compartment, receptor editing, and ignorance.

A. Clonal deletion, anergy, and positive selection into the B1 compartment. The first reports describing B cell tolerance used anti-H-2^k and anti-hen egg lysozyme (HEL) Ig transgenic models to identify clonal deletion and anergy as mechanisms of tolerance. The H-2^k Ig transgenic model described deletion of autoreactive B cell clones in the bone marrow. In this model, an H-2^d mouse expressing an H-2^k specific B cell receptor (BCR) was crossed to an H-2^k mouse (20). The resulting mice lack anti-H-2^k B cells and serum anti-H-2^k antibody, in contrast to their H-2^d transgenic naïve counterpart that exhibits anti-H-2^k serum titers. The data suggest that these anti-H-2^k B cells are deleted in the bone marrow, and that autoreactive B cells are regulated by clonal deletion (20). However, Goodnow et al reported that anti-HEL B cells are regulated by another mechanism of tolerance. The authors created a double transgenic mouse expressing soluble HEL (sHEL) and the rearranged Ig heavy and light chain genes encoding a high affinity anti-lysozyme antibody (anti-HEL) (21). Anti-HEL B cells are present in the spleen but do not secrete Ig spontaneously, nor when stimulated with antigen in the presence of T cell help. In addition, anti-HEL B cells chronically exposed to sHEL downregulate membrane IgM, unlike naïve anti-HEL B cells (21). Further, unresponsiveness to HEL requires that a threshold number of receptors be occupied (22). Antigen-binding B cells normally migrate into lymphoid follicles where they receive survival signals. However, autoreactive B cells exposed to sHEL are excluded from the follicle, resulting in their premature death (23). The data indicate that autoreactive B cells in the periphery exist in a functionally silenced state termed anergy. Thus, these initial

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reports determined that autoreactive B cells generated in the bone marrow can be clonally deleted, but some escape into the periphery where they are regulated by anergy.

To understand why some autoreactive B cells are regulated by deletion versus anergy, the HEL model was used to alter the presentation of self-antigen. When sHEL is modified to encode an integral membrane protein (mHEL), mature B cells in the periphery are deleted (24). Subsequent experiments revealed that the elimination of anti-mHEL B cells proceeds in two stages. Upon initial encounter with self-antigen, developmental arrest occurs and B cells are deleted within 1-3 days (25). Thus, membrane-bound antigen induces deletion, whereas soluble antigen induces anergy. In addition to the form of antigen, cellular location can determine whether autoreactive B cells are deleted. HEL was modified to contain an endoplasmic reticulum retention signal (ER-HEL), sequestering antigen intracellularly. Unlike mHEL that causes deletion of B cells, ER-HEL positively selects B cells to differentiate into B1 cells (26). Collectively, the data show that the induction of deletion or anergy depends on the cellular location of antigen and the ability of antigen to crosslink receptors providing a stronger stimulus. The role of BCR affinity remains unclear.

B. Clonal deletion and anergy regulate B cells specific for disease-associated antigen.
The HEL and H-2^k models of tolerance proved to be invaluable in the early description of clonal deletion and anergy; however, one weakness is in their use of non-disease associated antigen. A transgenic model of autoimmune hemolytic anemia demonstrated that both deletion and anergy can regulate autoreactive B cells within the same mouse (27). Several models of tolerance exist to study the regulation of autoreactive B cells specific for lupus-associated antigens.

The majority of SLE patients have high anti-dsDNA serum titers and anti-dsDNA has been shown to cross react with the kidney protein α-actinin. To study the mechanisms that regulate anti-DNA B cells, the VH3H9 Ig heavy chain transgenic mouse was created (28). These mice do not exhibit anti-DNA serum titers higher than wildtype animals, indicating that regulatory mechanisms prevent the production of autoantibodies. The VH3H9 heavy chain can pair with multiple light chains to form antibodies of both ss- and dsDNA specificity. However, only anti-ssDNA, and not anti-dsDNA, B cells exist in the periphery. Collectively, the data suggest that anti-dsDNA B cells recognize self-antigen and are deleted, whereas anti-ssDNA B cells escape into the periphery but exist in an anergic state (28). Anti-ssDNA B cells from VH3H9/Vκ8 double transgenic mice are anergic, yet they do not down-regulate IgM unlike anergic anti-HEL B cells (28, 29). These cells show suboptimal proliferation in response to BCR and LPS stimulation; however, their turnover rate is similar to non-transgenic B cells (29). Thus, deletion and anergy exist in the anti-DNA model, and down-regulation of IgM is not always associated with anergy.

The regulation of anti-ssDNA B cells is studied in the Ars/A1 transgenic mouse where anti-*p*-azophenylarsonate (Ars) BCR cross reacts with ssDNA. There is no evidence of clonal deletion or receptor editing in the bone marrow, although these B cells have seen self-antigen. Mature B cells exist in an anergic state in the periphery, as determined by lack of calcium mobilization, tyrosine phosphorylation, and antibody production in response to BCR stimulation (30).

Anti-Sm titers are specific for lupus and correlate with poor prognosis. To study the regulation of anti-Sm B cells, 2-12 heavy chain Ig transgenic mice were generated. The 2-12 heavy chain pairs with multiple light chains to create Sm and ssDNA-specific B cells, as well as non-autoreactive specificities. Similar to other tolerance models, Sm-specific B cells are

present in the periphery, exhibit shortened lifespans, but fail to secrete autoantibodies (31). In addition, functional anti-Sm B cells are positively selected into the peritoneal cavity and differentiate into B1 cells (32). These cells secrete anti-Sm upon immunization with snRNPs (31). Thus, transgenic mice harbor functional and non-functional anti-Sm B cells. To study low-affinity anti-Sm B cells, 2-12 heavy chain Ig transgenic mice were crossed with Vκ8 light chain transgenic mice (33). 2-12H/Vκ8 antibodies have low affinity for Sm and do not bind ssDNA or dsDNA. Low-affinity Sm-specific B cells are found in the peripheral lymphoid organs and do not differentiate into B1 cells. They are unresponsive to LPS, do not down-regulate IgM, and fail to be excluded from the B cell follicle. This demonstrates a unique phenotype compared to other models (33). Anergic anti-Sm B cells in the spleen are regulated at an early pre-plasma cell stage, defined by intermediate expression of CD138 and normal levels of CD19 and B220. The tolerance checkpoint occurs upstream of Blimp-1 control of plasma cell differentiation (34).

C. Receptor editing. The anti-H-2^k (35) and anti-DNA (36, 37) models identified receptor editing as another mechanism of tolerance. Analysis of anti-DNA B cells showed that rearrangement of the endogenous light chain genes occurs, and they successfully compete with the transgenic light chain for heavy chain binding. As a result, a nonautoreactive receptor is created, thereby allowing the B cell to escape deletion (36).

D. Lack of T cell help. Antigen-stimulated B cells require T cell help to produce Ig. Activated T cells induce surface CD40 ligand (CD40L) expression, and lack of CD40L or blocking CD40 stimulation on B cells impairs Ig production (38). Anergic B cells can overcome tolerance by providing T cell help. In VH3H9/λ1 Ig transgenic mice, anti-dsDNA

B cells are held at the T-B interface. Injection of a source of cognate T cells leads to the

migration of anti-dsDNA B cells into the B cell follicle and production of autoantibody (39).

Thus, lack of T cell help is also a tolerance mechanism. However, the situation is somewhat

different for anergic HEL-specific B cells. These B cells do not respond to antigen and T cell

stimulation (40). T-dependent Ig responses require B cell signaling for the B cell to interact

with the T cell and receive T cell help. Significantly, anergic HEL-specific B cells exhibit a

block in sHEL-mediated BCR signaling (40) and decreased up-regulation of costimulatory

molecules (41), and thus can not activate T cells to provide help. However, they respond

normally to IL-4 and anti-CD40 stimulation *in vitro*, indicating that they are still capable of

responding to T cell help. Stimulation with the more potent mHEL antigen triggers the BCR

to signal, thereby restoring their ability to respond to T cell help and secrete Ig (40). The

absence of T cell help could result from the deletion of autoreactive cognate T cells by

central tolerance mechanisms in the thymus or CD4^{+}CD25^{+} regulatory T suppression of CD4^{+}

helper T cells. Some evidence shows that regulatory T cells can also directly suppress B cell

Ig production (42).

In summary, several mechanisms of tolerance regulate B cells specific for self-antigens.

Central tolerance mechanisms such as deletion and receptor editing regulate immature B cells

specific for high avidity, membrane-bound self-antigens (20, 24, 27, 35, 36). Peripheral

tolerance mechanisms regulate bone marrow emigrants through deletion (37), positive

selection into the B1 compartment (26, 32), functional ignorance of low affinity antigens (43,

44), lack of T cell help (38), and anergy (22, 28, 30, 33, 40). Some anergic B cells display

BCR signal transduction defects with diminished induction of phosphotyrosine yet elevated

intracellular Ca^{2+} and constitutive ERK activation (45). Anergic cells exhibit reduced surface

IgM (21), decreased lifespan (23), and exclusion of B cells from the follicle (23, 46). These findings lead to a model wherein BCR affinity coupled with the abundance, location, and avidity of self-antigen dictates the fate of autoreactive B cells (26, 47).

1.3 Loss of Tolerance in Autoimmune Mice

A. Mouse models of lupus. Regulating autoreactive B cell secretion of Ig is crucial in preventing lupus. To better understand human disease, murine models of SLE are used to study the defects in B cell tolerance mechanisms that lead to disease. Several strains of lupus-prone mice were generated as spontaneous serendipitous products of mouse breeding. The MRL strain resulted from the combination of four nonautoimmune strains: LG, C3H, C57BL/6, and AKR (48). During the breeding at Jackson Laboratories, a spontaneous mutation in the *lpr* gene occurred, resulting in a defective Fas receptor. MRL/*lpr* mice produce several autoantibodies specific for SLE, including anti-dsDNA, anti-Sm, anti-Su, and anti-ribosomal P. As a result, mice develop a systemic autoimmune syndrome consisting of vasculitis, dermatitis, and immune complex-mediated glomerulonephritis with proliferative changes in the glomeruli and Ig and complement deposition. Mice also exhibit lymphadenopathy due to the accumulation of $CD4^-CD8^-$ T cells and increased numbers of $CD4^+$ T cells, $CD8^+$ T cells, and B cells. Although there seems to be no general defect in deletion of autoreactive lymphocytes in lupus-prone mice (49, 50), massive lymphoproliferation may reflect the lack of a functional Fas receptor potentially preventing negative selection and clonal deletion through apoptosis. Median survival of MRL/*lpr* mice is 5 months. Without the *lpr* gene, the MRL strain still produces autoantibodies, but the development of lupus disease is delayed to about 1 year. Yet, the *lpr* gene is sufficient in causing autoimmunity as backcross onto nonautoimmune backgrounds leads to autoantibody

production. Thus, the MRL/*lpr* lupus mouse model exhibits several characteristics consistent with human disease.

B. Breakdown of B cell tolerance in lupus-prone mice. In order to study the breakdown of tolerance mechanisms, Ig transgenes were backcrossed onto autoimmune backgrounds to identify the steps leading to autoantibody production. Like human SLE (75%) (51), the majority of MRL/*lpr* mice (>90%) (48) develop anti-dsDNA antibodies. To study how anti-DNA B cells break tolerance in lupus, the Ig heavy chain VH3H9 transgene was bred onto the MRL and MRL/*lpr* backgrounds. VH3H9 transgenic B cells produce anti-DNA antibodies that stain nuclei in a homogenous nuclear (HN) fashion. VH3H9 trangenic MRL/*lpr* are seropositive by 3 months, while nonautoimmune transgenic mice actively delete anti-HN B cells (52). MRL/*lpr* mice fail to delete anti-HN B cells, and these cells undergo preferential clonal expansion. In addition, the VH3H9 transgene is less efficient at inhibiting heavy chain gene rearrangement on the autoimmune versus the nonautoimmune background (53). Somatic mutation and peripheral light chain rearrangement in anti-ssDNA B cells from transgenic MRL/*lpr* mice can generate pathogenic anti-dsDNA antibodies (54), demonstrating that receptor editing occurs in the periphery. The VH3H9 Ig heavy chain paired with the Vλ1 light chain is specific for dsDNA, allowing the ability to follow dsDNA antibodies specifically in the VH3H9 transgenic mouse. Before seroconversion, anti-dsDNA B cells in MRL and MRL/*lpr* mice exhibit a defect in maintaining developmental arrest, as they express levels of B220, HSA, CD22, and CD44 comparable to mature cells. In addition, anti-dsDNA B cells in MRL/*lpr* but not MRL mice are present in the splenic follicle (55).

Thus, the MRL background and *lpr* gene allow anti-dsDNA B cells to break tolerance, differentiate to antibody secreting cells, and produce autoantibody.

The prevalence of anti-Sm in human SLE and MRL/*lpr* mice is 25% (48, 51). To study how anti-Sm B cells break tolerance in lupus, the 2-12 Ig heavy chain was backcrossed onto MRL/*lpr* mice to increase the prevalence and frequency of anti-Sm B cells. The presence of the transgene accelerates the anti-Sm response and increases the prevalence of anti-Sm titers from 25% to 100% (56). Anti-Sm B cells are more activated and express higher levels of CD44, CD40, CD80, and CD86. The frequency of transitional anti-Sm B cells is similar to nonautoimmune mice. However, there are very few peritoneal anti-Sm B-1 cells in 2-12H transgenic MRL/*lpr* mice, suggesting that a defect in B-1 differentiation may be a factor in the loss of tolerance to Sm (56). The CD138^{int} pre-plasma cell stage was identified as a tolerance checkpoint in anti-Sm B cells (34). MRL/*lpr* mice exhibit an increased frequency of CD138^{int} B cells that secrete Ig, and anti-Sm CD138^{int} cells are more activated and differentiated than nonautoimmune mice. Moreover, anti-Sm B cells in 2-12 heavy chain transgenic MRL/*lpr* mice differentiate to CD138^{hi} pre-plasma and plasma cells, where they are absent on the nonautoimmune background (34). Thus, anti-Sm B cells break tolerance in MRL/*lpr* mice by progressing through the CD138^{int} checkpoint differentiating into antibody-secreting plasma cells.

C. Apoptotic cells and the break in tolerance. The role that apoptotic cells play in regulating the immune system is a critical issue in SLE considering that an increased burden of apoptotic cells is associated with both human and murine lupus (14-16, 57, 58). Further, mice deficient in the mer, axl, and tyro3 receptor tyrosine kinases involved in the signaling

and phagocytosis of apoptotic cells develop a severe lymphoproliferative disorder and a lupus-like disease (59-62). Injection of apoptotic cells in mer deficient mice induces a chronic anti-Sm response (63). Preliminary data also show that chronically antigen-experienced B cells from Ig transgenic 2-12H/Vκ8 mice spontaneously break tolerance when on a mer deficient background and exhibit a positive anti-Sm titer (Kilmon and Vilen, unpublished observations). Thus, the link between lupus and the inability to signal and/or phagocytose apoptotic cells through tyro 3 family tyrosine kinases is well established.

D. SLE congenic mice. Understanding the molecular defects that drive autoreactive B cells through tolerance checkpoints is critical in understanding the etiology of SLE. Several groups have approached this problem by studying the phenotypic effects of lupus susceptibility loci using congenic mice (64, 65). Interval mapping of F2 mice intercrossed between MRL/*lpr* and B6.*lpr* identified four loci (Lmb1-4) linked to lymphadenopathy and splenomegaly. *Lmb1*, *Lmb2*, and *Lmb3* are also associated with the production of anti-dsDNA, and *Lmb4* is associated with glomerulonephritis (66). *Nba2*, *Nba5*, and *Sgp3* loci, derived from the NZB lupus strain, are anti-gp70 autoantibody production, gp70 immune complex formation, and glomerulonephritis (67). *Nba2* alone increases the proportion of activated B cells and anti-ssDNA antibody production (68). The *Sle* congenic mice were derived from the NZM2410 lupus model. The *Sle1* locus is associated with increased activation of CD4$^+$ T cells that provide help to anti-chromatin B cells. Significantly, *Sle1a* and *Sle1c* are associated with decreased numbers of CD4$^+$CD25$^+$ regulatory T cells that precedes autoantibody production (69). *Sle1ab* splenic *ex vivo* B cells are aberrantly activated compared to wildtype, as demonstrated by upregulation of B cell activation marker

CD69, increased phosphorylation of STAT3 and ERK, and increased IL-6 production (70).

These data indicate that the *Sle1* locus causes a break in T cell tolerance that could contribute

to the activation of autoreative B cells. Other studies showed that Sle3 contributes to defects

in DCs and MΦs and in Ig gene recombination. B6.*Sle3*-derived DCs and MΦs are more

activated and better co-stimulators of T cells (71). Sle3/5 affects somatic Ig heavy chain

diversification and led to recombination of allelically excluded Ig heavy chain genes in the

periphery, possibly contributing to the development of autoreactive B cell repertoires (72).

Hence, murine models of lupus and congenic mice carrying lupus susceptibility loci have

provided valuable insight into the breakdown of tolerance causing disease.

1.4 Toll-like Receptors in SLE

The tolerance mechanisms described above address the regulation of autoreactive B

cells in the periphery during T-dependent immune responses and describe tolerance mediated

by antigen/BCR. However, flare-ups in SLE have been associated with infectious agents that

polyclonally activate immune cells. Significantly, many of the autoantibodies associated

with lupus react against nuclear antigens that also bind Toll-like Receptors (TLRs) important

in innate immune responses. Thus, polyclonal activation of immune cells during innate

immunity may contribute to SLE pathogenesis.

A. Characteristics of TLRs. TLRs play a major role in bridging the innate and adaptive

immune responses. The Toll signaling pathway was originally described in the anti-fungal

immune response in *Drosophila* (73) and parallels signaling through the IL-1 receptor in

mammalian cells (74). Identification of the human homolog to the *Drosophila* Toll protein confirmed that the Toll signaling pathway is an important evolutionarily conserved innate immune response (75). Toll is a transmembrane protein with an extracellular domain consisting of a leucine-rich repeat domain and a cytoplasmic domain homologous to that of the IL-1 receptor (75). There have been 10 TLRs identified in humans and approximately 13 in mice responding to different bacterial and viral components (also called pathogen-associated molecular patterns-PAMPs), including LPS (TLR4), CpG oligonucleotides (TLR9), dsRNA (TLR3), and viral ssRNA (TLR7). TLRs are found mostly on the cell surface in the plasma membrane, except for TLR3, TLR7, and TLR9 that are located on the membranes of intracellular endosomes (76-78).

B. TLRs and autoimmunity. Only recently has the role of innate immunity in tolerance and autoimmunity been investigated. Mice deficient in the key TLR signaling adaptor molecule MyD88 are not susceptible to autoimmune diseases like celiac disease and autoimmune myocarditis, uveitis, and encephalomyelitis (79-82), suggesting the involvement of TLRs in causing disease. *In vitro* studies suggest that TLR9 is required for activation of rheumatoid factor (RF) B cells through anti-chromatin immune complexes (83), and showed that dual ligation of TLR9 and BCR activates anti-chromatin B cells (84). Additionally, TLR7 is required for optimal proliferation of B cells specific for RNA-containing antigens (85). Studies *in vivo* tell a more complicated story. Production of anti-chromatin antibodies, but not anti-dsDNA or anti-Sm-RNP, in TLR9-deficient MRL/*lpr* mice is diminished; yet, these mice exhibit worse disease accompanied by more activated lymphocytes and plasmacytoid DCs and increased serum IgG and IFNα (86, 87). Similarly, TLR9 in the

absence of FcγRIIB is required for class switching to pathogenic anti-DNA antibodies (88).

Another study showed that TLR9-deficient MRL and MRL/*lpr* mice develop more severe

disease. However, unlike the previous studies, anti-dsDNA, anti-ssDNA, and RF antibody

production is increased (89). These studies suggest that TLR9 is protective against lupus,

and the effect on autoantibody production is controversial. On the other hand, one study

using alternative methods suggests that TLR9 promotes lupus. Injection of CpG DNA

triggers lupus nephritis in MRL/*lpr* mice accompanied by increased B cell proliferation, B

cell production of IL-12, anti-dsDNA production, and DC IFNα production (90). TLR7 has

also been implicated in lupus pathogenesis. TLR7-deficient mice are protected from disease

having decreased activation of lymphocytes and serum IgG production (86), suggesting that

TLR7 promotes lupus disease. Although the exact role of TLRs in disease is complicated

and controversial, the presence or absence of certain TLRs and stimulation through these

receptors are indeed having an effect. Thus, research into the role of TLRs in tolerance and

autoimmunity is critical in understanding SLE.

1.5 Toll-like Receptors in Innate Immunity

A. TLR signaling pathways. TLRs and their signaling pathways are generally characterized

as MyD88-dependent (TLR7 and TLR9) or TRIF-dependent (TLR3). The exception is

TLR4 which utilizes both MyD88 and TRIF-dependent pathways. In the MyD88-dependent

pathway, ligand binding induces MyD88 association with TLR, which recruits IRAK4 and

IRAK1. IRAK4 subsequently phosphorylates IRAK1 and recruits TRAF6 to the receptor

complex. Activated IRAK1 and TRAF6 dissociate from the receptor and IRAK1 is

degraded. TRAF6 is degraded in the cytosol, which leads to TAK1 phosphorylation of MAP

kinases and the IKK complex. MAP kinases (ERK, JNK, and p38) activate the transcription factor AP-1. The IKK complex phosphorylates IκB, causing IκB degradation and release of the transcription factor NF-κB. NF-κB then translocates to the nucleus where it induces transcription of inflammatory cytokines and costimulatory molecules (77, 78, 91-95). The TRIF-dependent pathway, ligand binding induces the association of TRIF with TLR. This activates IKKε and TBK1, which activate IRF3. Phosphorylation of IRF3 promotes it to dimerization and nuclear translocation. IRF3 dimers induce transcription of IFN-β and IFN-inducible genes. TRIF also induces TRAF6 activation, leading to IκB phosphorylation and late phase NF-κB activation (77, 78, 91, 92, 96). A defect in the activation of any of these signaling effectors or transcription factors could lead to defective cytokine secretion in response to TLR stimulation.

B. Negative regulation of TLR signaling. Regulation of TLR signaling is important in controlling inflammation. Endotoxin tolerance has been described in monocytes and macrophages as a mechanism to control responses to bacteria prevent overwhelming inflammation. Pretreatment with LPS induces SHIP phosphatase activity and decreases phosphorylation of ERK, JNK, p38, and IκB, preventing activation of AP-1 and NF-κB transcription factors. Subsequently, cells are rendered refractory to further TLR stimulation (97-103).

Other negative regulators of TLR responses have been described that bind early signaling intermediates and block further propagation of signal. The flightless I homolog prevents MyD88 from associating with TLR4 (104). MyD88 short (MyD88s) blocks the association of IRAK4 with MyD88. IRAK-M prevents the dissociation of the IRAK1-

IRAK4 complex from the receptor (91). Monarch-1 was the first CATERPILLER protein described to antagonize TLR signaling by preventing IRAK-1 phosphorylation and activation (105). MAPK phosphatase-1 and PI3K regulate TLR signaling by blocking activation of the MAPK p38 (106-109).

Suppressor of cytokine signaling (SOCS)-1/3 proteins inhibit cytokine and LPS signaling (110-114). These proteins are induced by the inflammatory signals they inhibit creating an autoregulatory feedback loop that functions to control inflammation. Specifically, IL-10, IFNγ, IL-6, and LPS induce SOCS1 and SOCS3 in a STAT dependent or independent manner, and SOCS proteins prevent LPS induced cytokine production (111-113, 115-117). Little is known about how SOCS proteins inhibit TLR signaling. Frobose et al showed that SOCS3 inhibits IL-1 signal transduction by inhibiting ubiquitination of TRAF6, thus preventing association and activation of TAK1 (118). SOCS1 inhibition of LPS signaling involves several mechanisms. SOCS1 associates with TLR molecules IRAK (111) and Mal (116). SOCS1 also inhibits phosphorylation of Jak2 and STAT5 involved in NF-κB activation (119).

In summary, several molecules and mechanisms exist that regulate innate immune responses mediated by signaling through TLRs.

1.6 Dendritic Cells and Macrophages Link Innate and Adaptive Immune Responses

Dendritic cells (DCs) and macrophages (MΦs) are professional antigen presenting cells characterized by location, cell surface phenotype, and function in directing immune responses or immune tolerance. They are important in linking the innate and adaptive immune responses through phagocytosis of infectious material or necrotic and apoptotic

cells, activation of TLRs, and presentation of intact or processed antigen to B cells and T cells respectively. MΦs also act as chronic inflammatory cells to clear cellular debris in the resolution of inflammation.

A. DC activation of T cells. There are several subpopulations of DCs; however those that help to direct immune responses in the spleen include myeloid DCs (myDCs), lymphoid DCs (lyDCs), and plasmacytoid DCs (pDCs) (120-122). MyDCs ($CD11c^{hi}/CD11b^+/CD8^-/CD4^{+/-}$) make up the majority of DCs in the spleen, while lyDCs ($CD11c^{hi}/CD11b^-/CD8^+/CD4^-$) and pDCs ($CD11c^{lo}/CD11b^-/CD4^+/B220^{hi}$) comprise only a small portion of splenic DCs. Antigen stimulation of myDCs leads to a Th2-type T cell response through the production of IL-4, IL-5, and IL-10, whereas antigen-stimulated lyDCs secrete IL-2, IL-12, and IFNγ inducing a Th1-type T cell response (123, 124). pDCs induce T cell activity by secreting IFNα in response to viral stimulation (122, 125).

Innate stimulation through TLR in DCs drives adaptive immunity in several ways. LPS induces functional maturation in DCs by upregulating surface expression of MHC Class II, CD40, CD80, and CD86 costimulatory molecules and inflammatory cytokine secretion (126-128). LPS-stimulated maturation enhances the ability of antigen (Ag)-loaded DCs to activate naïve Ag-specific T cells (126, 127) and blocks $CD4^+CD25^+$ regulatory T cell inhibition of naïve helper T cells (129). Although different DC subsets are intrinsically capable of inducing different T cell responses, innate stimulation can also influence a preferential Th1 versus Th2 response. *E. coli* LPS (TLR4), flagellin (TLR5), and CpG DNA (TLR9) induce IL-12 in DCs and elicit Th1 responses (130). However, Pam3cys (TLR2), schistosome egg Ags, Candida albicans, and P. gingivalis LPS fail to induce IL-12 in DCs

and stimulate Th2 responses (130). Pam3cys and schistosome egg Ags stimulate sustained

duration and magnitude of ERK phosphorylation that stabilizes the c-Fos transcription factor,

which suppresses IL-12 production (130). DCs indirectly promote B cell antibody secretion

through the activation of CD4$^+$ T cells that provide the necessary cytokines for B cell

activation.

B. DC activation of B cells. Recently, DCs were shown to directly interact with and

activate B cells. DCs interact with B cells through the presentation of intact antigen on their

surface. DCs pulsed with antigen *in vitro* or DCs isolated from mice that have been injected

with antigen can retain unprocessed antigen, form short-lived clusters with naïve B cells, and

induce Th2-type antibody responses (131-134). Antigen endocytosed by FcγRIIB can be

recycled back to the cell surface unprocessed (135). Follicular DCs present opsonized and

Ig-complexed antigen through complement and Fc receptors to B cells with high affinity

BCRs. This interaction provides survival signals and promotes memory cell differentiation

in the germinal center (136). In addition to direct cell contact, DCs secrete soluble factors

that promote plasma cell differentiation and immunoglobulin (Ig) secretion. An early study

showed that CD40-activated DCs secrete IL-12, that promotes plasma cell differentiation of

naïve B cells, and IL-6, which induces Ig secretion by pre-plasma cells (137). IFN or CD40-

activated DCs secrete BLyS and APRIL that induce Ig class switching and plasma cell

differentiation in naïve B cells (138). TLR-stimulated DCs also secrete BLyS and APRIL

that promote plasma cell differentiation of MZ and B1 B cells (139). Viral-stimulated pDCs

secrete IFNαβ and IL-6 that act on CD40-activated B cells to promote plasmablast formation

then Ig-secreting plasma cell differentiation (140). Nucleic acid-containing immune

complexes stimulate DCs to secrete proinflammatory cytokines in a TLR-dependent manner (141-143). Thus, DCs can activate B cells by T-independent mechanisms.

C. Effect of Environment and apoptotic cells on DC and MΦ responses. In addition to TLR stimulation, the lymphoid microenvironment is critical in determining the DC response. For example, antigen stimulation of DCs alone without a CD40 signal induces T cell tolerance (144). Splenic stromal cells, through contact and secretion of TGFβ, induce the differentiation of a regulatory DC subset. These DCs secrete IL-10 and nitric oxide that inhibit antigen-specific T cell responses promoting tolerance (145, 146). Exposure to necrotic versus apoptotic cells also regulates the DC response. Necrotic cells activate DCs by upregulating cell surface expression of CD86, MHC Class II, and CD40 and induce T cell responses, whereas DCs exposed to apoptotic cells do not mature (147, 148).

DCs and MΦs exposed to apoptotic cells are unable to respond to TLR stimulation (149) (Sykes and Matsushima, unpublished observations), whereas mer deficient DCs (149) and MΦs (150) exposed to apoptotic cells respond to TLR signals. This demonstrates the importance of apoptotic cells in regulating innate immunity. Subsequent experiments revealed that signaling through the mer receptor activates PI3K and AKT activity that inhibits activation of NF-κB and MAP kinases (ERK, JNK, and p38) in response to LPS (149). Therefore, dysregulated innate immune responses due to the inability to appropriately respond to and clear apoptotic cells may induce pathological autoimmune responses.

D. Defects in DCs and MΦs associated with lupus. Defects in DCs and MΦs have been described in lupus. Monocyte-derived DCs from SLE patients over express CD86 in the

absence of any activation signal (151). Splenic DCs from lupus-prone mice are more

apoptotic *in vivo* (152), a significant observation considering that a defect in DC apoptosis

can independently lead to autoimmunity (153). In addition, DCs from lupus-prone mice may

have defects in antigen presentation and stimulatory function in mixed lymphocyte reactions

(154). MΦs from several autoimmune strains exhibit defects in TLR-stimulated pro-

inflammatory cytokines, including IL-1, IL-6, IL-12, TNFα (155-157). Defective cytokine

production and increased adhesiveness to extracellular matrix proteins are triggered by

apoptotic cells (158, 159). Thus, DC and/or MΦ dysfunction may contribute to lupus

pathogenesis.

1.7 DC and MΦ-Mediated Tolerance

Previous studies into the mechanisms of B cell tolerance have focused on

antigen/BCR mediated regulation of autoreactive or chronically antigen-experienced B cells.

However, the regulation of chronically antigen-experienced B cells during polyclonal

activation is less understood. Recently, we described that chronically antigen-experienced B

cells are regulated by LPS-activated myDCs and MΦs, but not T cells, NK cells, NK T cells,

lyDCs or pDCs, and that repression of Ig secretion is readily reversed upon removal of these

populations (160). As few as 1 DC or MΦ could partially repress 100 Sm-specific B cells.

This potent repressive effect suggested that soluble mediators, in addition to a contact-

mediated mechanism, were involved in the regulation of Ig secretion. LPS-activated DC and

MΦ conditioned medium (CM) repressed Sm-specific Ig secretion, confirming that these

cells secrete soluble factors that regulate Ig secretion. Neutralization of IL-6 in DC CM

partially relieved the repressive effect and recombinant IL-6 inhibited LPS induced Ig

secretion. This result was surprising considering the role of IL-6 in promoting plasma cell differentiation in naïve B cells. However, the data showed that only chronically antigen-experienced, and not naïve B cells, were susceptible to repression by IL-6. IL-6 repressed LPS-stimulated Sm-specific B cells from 2-12H/Vκ8 mice, anti-ssDNA B cells from Ars/A1 mice, and anti-HEL B cells from HEL-Ig x sHEL mice, whereas B6 and naïve anti-HEL B cells were not affected. The data indicate that autoreactive B cells are regulated beyond chronic antigen-induced BCR desensitization, and suggest that chronic antigen stimulation reprograms the response to IL-6. Thus, these findings identified a novel B cell tolerance mechanism and a novel role for IL-6 in regulating Ig secretion, and suggested that overcoming tolerance in SLE may be associated with defects in the repression of autoreactive B cells by myDCs and/or MΦs.

It is important to note that neutralization of IL-6 in DC CM did not completely relieve repression and that neutralization of IL-6 in MΦ CM had no effect on the ability of MΦ CM to repress Ig secretion (160). The data suggest that DCs and MΦs secrete other repressive factors in addition to IL-6. Subsequent experiments have shown that MΦs also secrete a soluble form of CD40L (sCD40L) that represses Ig secretion (Kilmon et al, manuscript submitted). Neutralization of sCD40L in IL-6$^{-/-}$ MΦ CM, or in addition to IL-6 neutralization of B6 MΦ CM, relieves repression of LPS-stimulated Sm-specific B cells. Like IL-6, sCD40L only represses chronically antigen-experienced B cells and not naïve B cells. The mechanism of repression by sCD40L does not affect proliferation but inhibits the differentiation of B cells to antibody-secreting plasma cells, as sCD40L decreases the percent of intracellular IgM cells, the number antibody secreting cells, and Blimp-1 and XBP-1 transcription important in activating plasma cell differentiation. Significantly, MΦ-mediated

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tolerance is defective in lupus-prone MRL/*lpr* mice. MΦs and MΦ CM from MRL/*lpr* mice

are less efficient than B6 at repressing Sm-specific Ig secretion, coincident with a defect

secreting repressive soluble factors, IL-6 and sCD40L. The data suggest that defects in MΦ-

mediated tolerance play a role in autoimmunity.

Several questions remain in understanding DC and MΦ-mediated tolerance that are

currently under investigation. First, IL-6$^{-/-}$ DCs and IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦs are capable of

repressing Ig secretion, suggesting that a third repressive factor exists (Chapter 3). Secondly,

we have observed that DCs deficient in TLR4 partially repress LPS-induced Ig secretion

through a contact-dependent mechanism (Kilmon and Vilen, unpublished observations).

DCs display nuclear self-antigen on their surface (Carnathan et al, manuscript in

preparation), possibly interacting with the BCR. Whether DC surface antigen plays a role in

B cell tolerance is yet undetermined. Finally, we have shown that IL-6 and sCD40L repress

chronically antigen-experienced B cells that are isolated from a nonautoimmune background.

Thus, a potential defect in lupus is that autoreactive B cells from an autoimmune background

do not reprogram their responses to IL-6 and sCD40L. Therefore, it is important to

determine whether autoreactive B cells from lupus-prone mice are capable of being repressed

by these soluble factors.

1.8 Model of Tolerance During Innate Immune Responses

During infection, immune cells respond to polyclonal activators, like bacterial and

viral antigens, through innate immune responses. Therefore, mechanisms to regulate the

activation of autoreactive B cells during polyclonal stimulation are necessary to prevent

autoimmunity. Blood-born pathogens enter the spleen and peritoneal cavity where they first

encounter MΦs and B cells. Marginal zone (MZ) B cells in the spleen and B1 B cells in the

peritoneal cavity are primed for a quick response to bacterial and viral antigens to fight

infection as a first line of defense (161). We propose a model where autoreactive B cells that

chronically see antigen are repressed by polyclonal activators and maintained in an anergic

state, while naïve B cells are allowed to mount a response to infectious agents. We recently

identified a novel mechanism of tolerance wherein DCs and MΦs repress Ig secretion by

autoreactive B cells (160). Polyclonal activators stimulate DCs and MΦs through TLRs to

secrete soluble factors that differentially regulate naïve and chronically antigen-experienced

B cells. Significantly, IL-6 selectively represses TLR-stimulated autoreactive but not naïve

B cells. Thus, we have identified a mechanism that prevents autoimmunity while allowing

naïve B cells to respond during innate immune responses. As blood-born pathogens enter the

spleen and peritoneal cavity and polyclonally activate B cells, autoimmunity could result

from the lack of secretion of repressive soluble factors by DCs and/or MΦs or the inability of

autoreactive B cells to differentially respond to repressive factors. *Therefore, we hypothesize

that DC and/or MΦ-mediated tolerance mechanisms are dysfunctional in murine models of

lupus disease.*

The body of work presented here confirms this hypothesis in part. DCs and MΦs

from lupus-prone mice are defective in repressing autoreactive B cells when stimulated

through TLR, coincident with a defect in IL-6 and sCD40L secretion (Chapter 2; Kilmon et

al, manuscript submitted). We have identified TNFα as the final repressive factor secreted

by DCs and MΦs and found that these cells from lupus-prone mice are also defective in

secreting TNFα (Chapter 3). This could allow TLR-stimulated autoreactive B cells to

secrete autoantibodies during innate immune responses, promoting autoimmune disease in

lupus-prone mice. The molecular mechanism for the cause of the defects in the repressive

factors could be key to the etiology of lupus disease (Chapter 4).

CHAPTER II

Dendritic cells from Lupus-Prone Mice are Defective in Repressing Immunoglobulin Secretion

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 (10). The etiology of SLE remains

unknown; however, multiple immunoregulatory defects have been identified in lupus-prone

mice (61, 156, 158, 162-170), 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 (13).

Studies in immunoglobulin (Ig) transgenic (Tg) mouse models have defined anergy as

a state of unresponsiveness that regulate autoreactive B cells in the periphery (22, 28, 30, 33,

171). Anergic B cells fail to secrete antibody in response to LPS or antigen immunization

due to receptor unresponsiveness (30, 33, 172). Some anergic B cells exhibit reduced surface

IgM levels (21, 173), decreased lifespan (23, 172), and exclusion from the lymphoid follicle

(23, 46). 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 (33). 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 (160). 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 (160). 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 (139, 140, 174). Specifically, IL-6 was found to promote plasma

cell survival (175, 176). Although this seems paradoxical, the data indicate that IL-6

differentially regulates naïve and chronically antigen-experienced B cells (160). 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 (160). 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 (33, 160). 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) (160). 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 (160). BMDCs were >95% CD11c$^+$ (CRL 1969 hybridoma). Conditioned medium (CM) was made from 1×10^4 BMDCs (0.2 ml) cultured for an additional 4 days with or without Sigma LPS (30 µg/ml). 5×10^5 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 (177) and confirmed to be unable to induce IL-6 secretion by TLR4$^{-/-}$ DCs.

B cell Cultures

Splenocytes containing 1×10^5 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 (5×10^4; IgM^b) and 2-12H/Vκ8 (5×10^4; 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/Cκ$^{-/-}$) was captured with anti-κ (187.1), detected with

biotinylated anti-IgM^a (HB100) and Streptavidin-AP as previously described (33). 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/Cκ$^{-/-}$ 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 = $2^{\wedge}$ [-(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 (178).

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 (160). 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 2.1, 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 (160). 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 (160). 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 (160). 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 (179). 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 (180).

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

(160), 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 (160); 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 (160). 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 (160). 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 (181, 182). 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 (183). 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 (180). 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 (147). 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 (158), and mice functionally deficient in the phagocytosis of apoptotic cells get a lupus-like disease (61).

Increased production of pro-inflammatory cytokines such as IL-6, contribute to the inflammatory response and pathogenesis of lupus nephritis (184, 185). SLE patients (186-189) and diseased, lupus-prone mice (190-192) 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* (160). 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 (193), while endothelial cells (194-196), mesangial cells in the kidney (197, 198), and infiltrating monocytes/macrophages (199) 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 (186, 200-202). 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 (40, 172, 203, 204). In contrast, TLR-induced Ig secretion is regulated by soluble factors secreted from DCs and MΦs (160). 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 (45, 83). Similarly, chronic BCR exposure to self-antigen reprograms IL-6R signal transduction to repress Ig secretion (160). However, B cells that have been acutely stimulated and exposed to IFN-α/β induce Ig secretion in response to IL-6 (140). 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.

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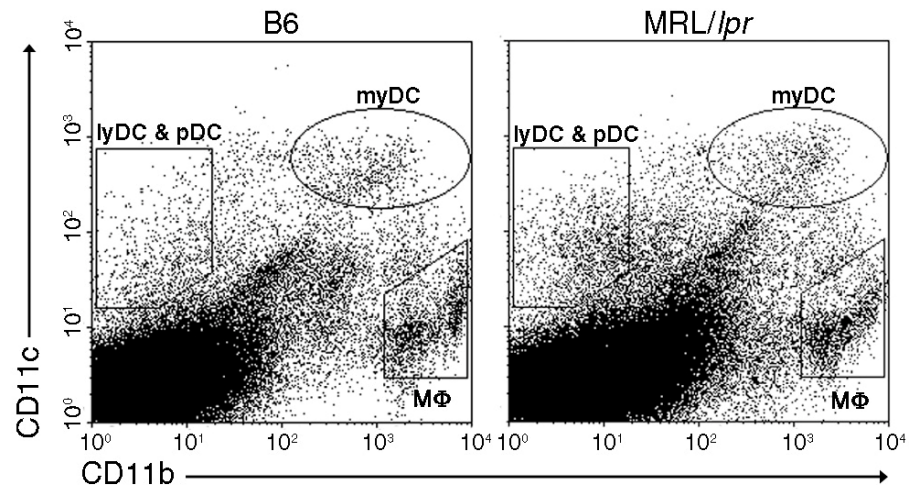
Figure 2.1. The distribution of splenic myDCs and MΦs are comparable between B6 and MRL/*lpr* mice. DC and MΦ subsets were separated based on CD11c and CD11b expression. Dot plots are representative of nine mice each.

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

	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.
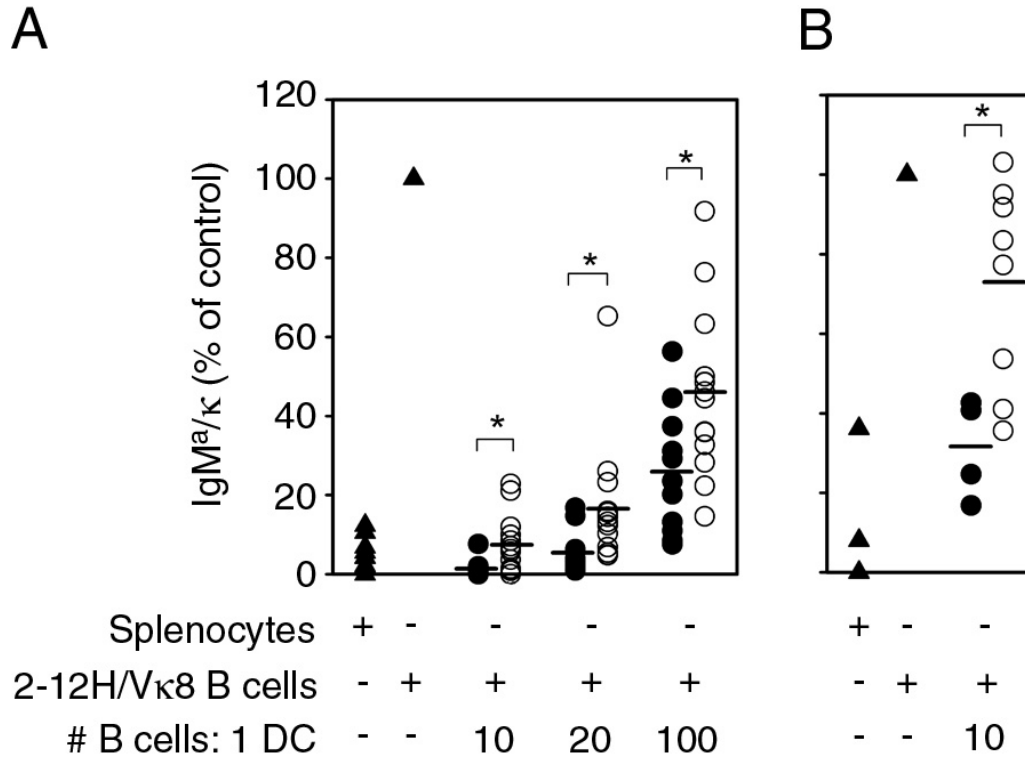
Figure 2.2. DCs from MRL/*lpr* mice fail to efficiently repress Sm-specific Ig secretion.

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*).
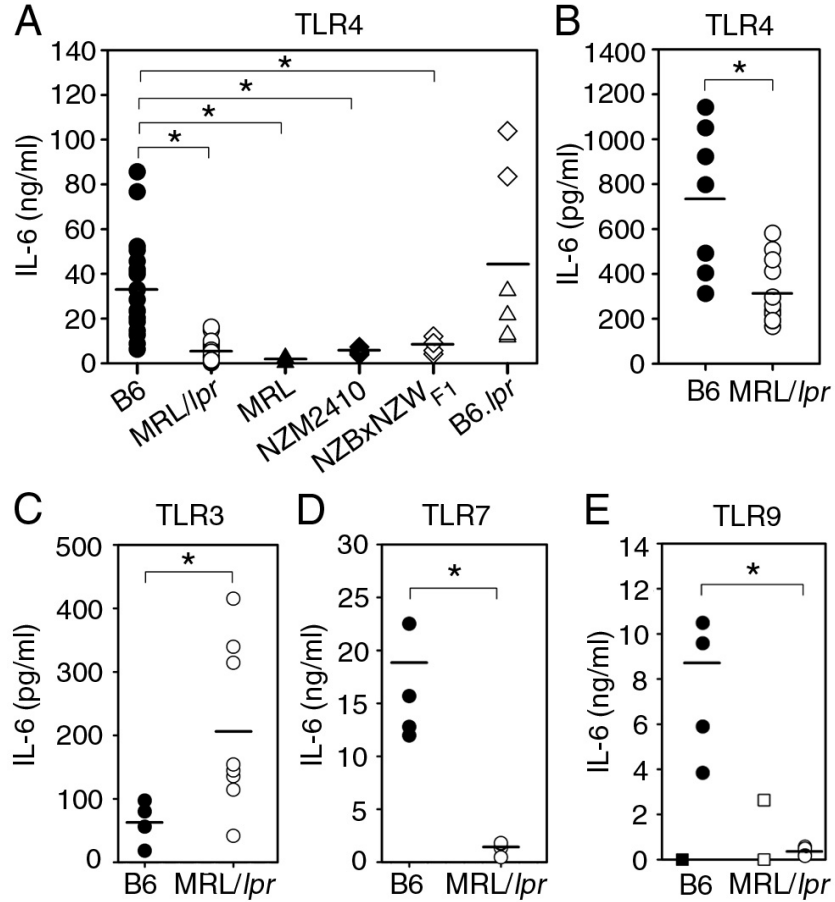
Figure 2.3. DCs from MRL/*lpr* mice are defective in IL-6 secretion upon TLR4, 7 and 9 stimulation, but not upon TLR3 stimulation. 1×10^4 BMDCs (A), or 1×10^5 e*x vivo* splenic DCs (B), were stimulated with LPS (30 µg/ml). 5×10^5 BMDCs were stimulated with poly (I:C) (50 µg/ml) (C), R848 (10 µg/ml) (D), 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$_{F1}$)
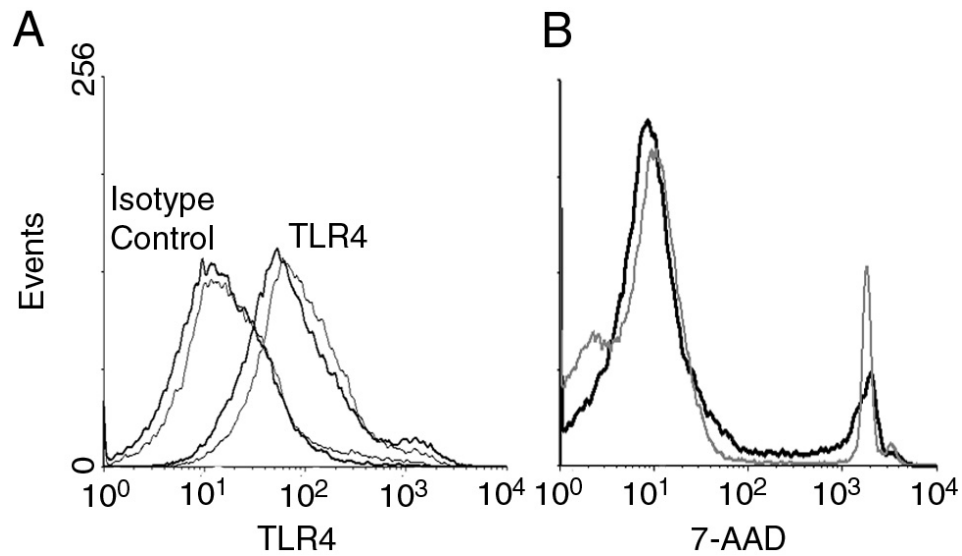
Figure 2.4. MyDCs from B6 and MRL/*lpr* mice have similar levels of TLR4 surface expression and no difference in survival. MyDCs within the CD11c$^+$ splenocyte population were gated as CD11c^{hi}/CD11b$^{int/hi}$, and then analyzed for TLR4 expression (A). LPS-stimulated BMDCs were stained with 7-AAD 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.
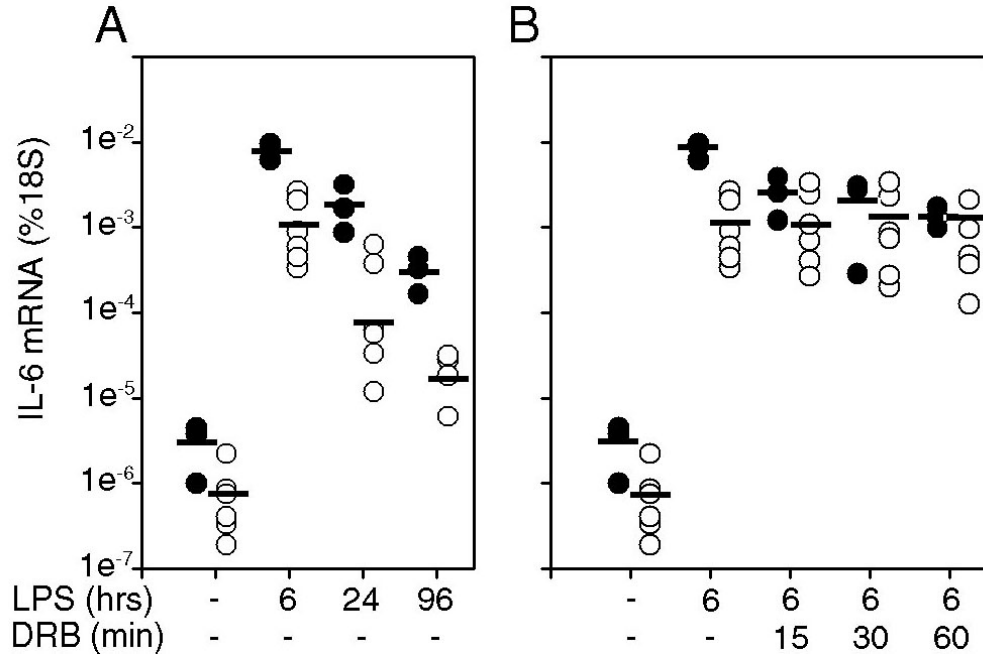
Figure 2.5. DCs from MRL/*lpr* mice show a decrease in synthesis and ability to sustain IL-6 mRNA levels. Real time-PCR was performed on RNA isolated from LPS-stimulated BMDCs (A) untreated or (B) treated with DRB at the indicated timepoints. The data from three individual B6 (●) and six MRL/*lpr* (○) mice are plotted as %18S.
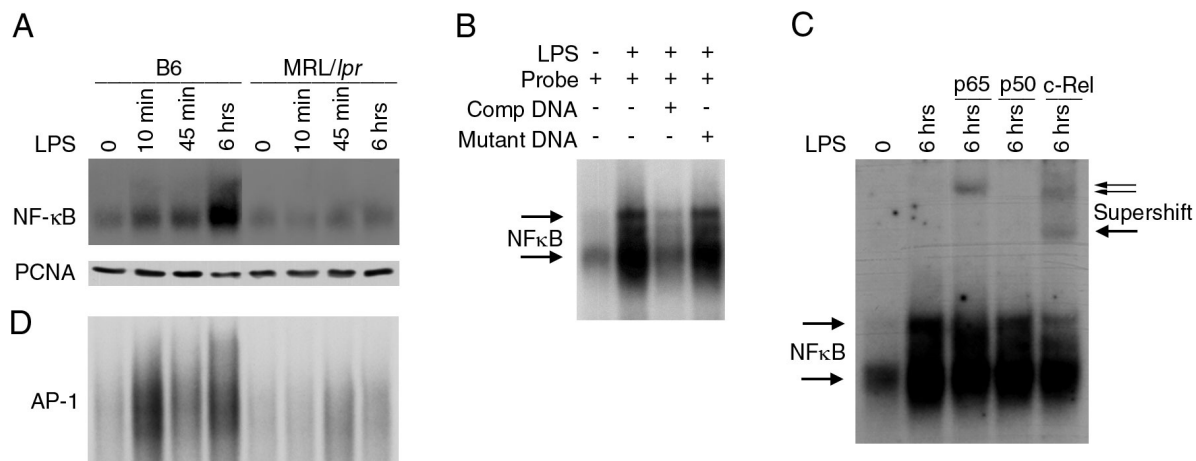
Figure 2.6. DCs from MRL/*lpr* mice fail to activate NF-κB and AP-1. BMDCs were stimulated with LPS (15 μg/ml) for the indicated times. Nuclear extracts were prepared, and NF-κB/DNA binding (A) or AP-1/DNA binding (D) was assessed by EMSA. Nuclear extracts prepared from unstimulated B6 BMDCs (lane 1) 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).
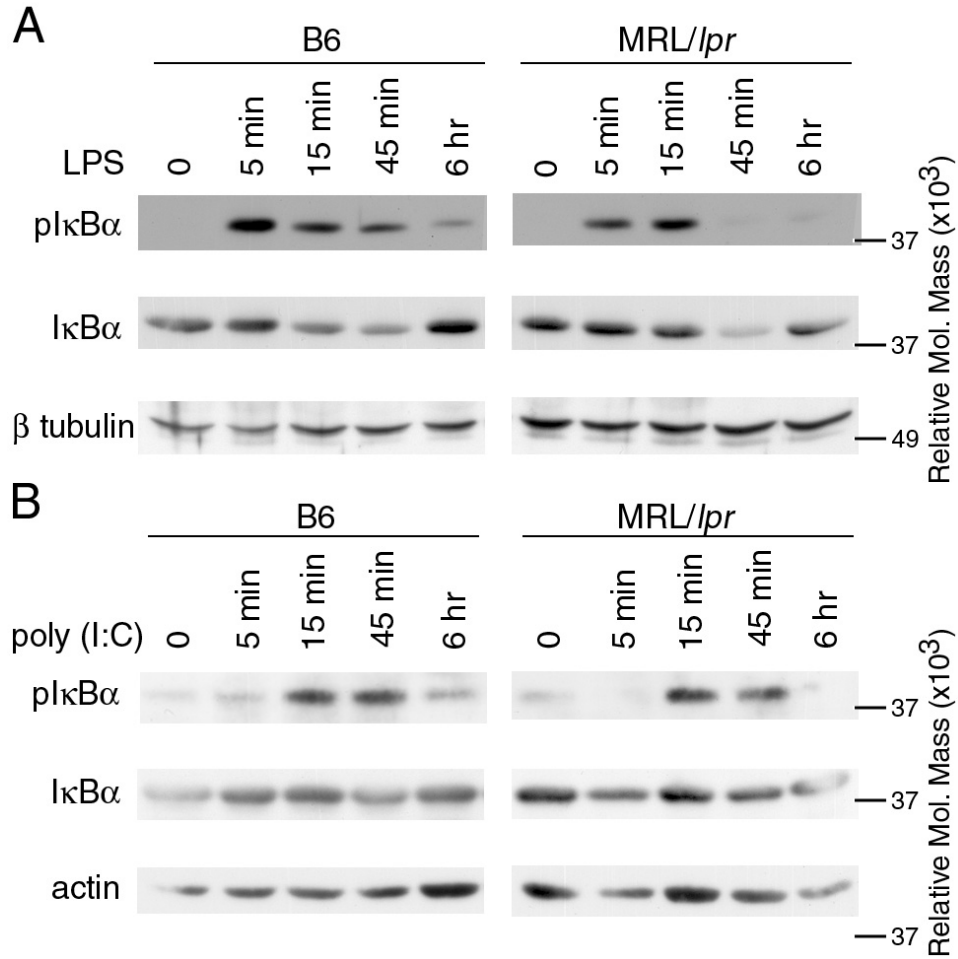
Figure 2.7. TLR4-stimulated DCs from MRL/*lpr* mice are unable to sustain IκBα phosphorylation. BMDCs ($2x10^6$) from B6 and MRL/*lpr* mice were stimulated with LPS (15 μg/ml) (A) or poly (I:C) (50 μg/ml) (B) for the indicated timepoints. Phospho-IκBα, IκBα, and β tubulin (A) or actin (B) expression in whole cell lysates was determined by immunoblotting. Data represent 7 (A) and 3 (B) experiments.
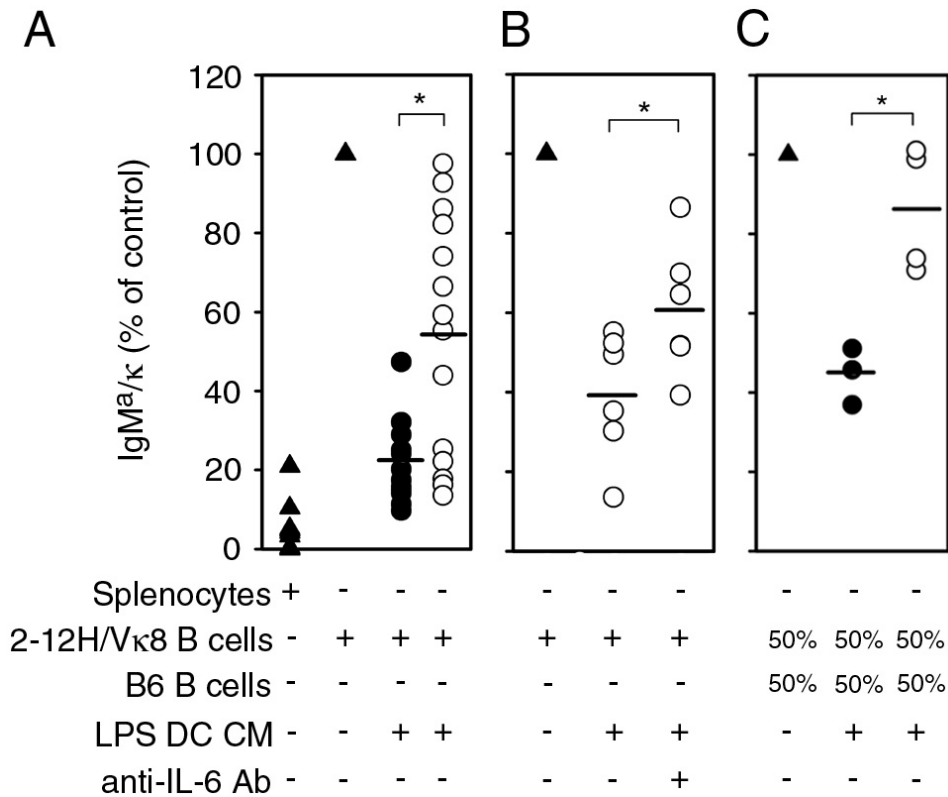
Figure 2.8. In addition to IL-6, other soluble factors regulate autoantibody secretion.

Purified B cells (1×10^5) 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). 5×10^4 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 Secrete TNFα that Regulates Smith Antigen-Specific B cells During Innate Immune Responses

Abstract

In the absence of B cell tolerance, polyclonal activation of B cells induces autoimmunity. Recently, we described that autoreactive B cells are regulated during innate immune responses by myeloid dendritic cells (myDCs) and macrophages (MΦs) through their secretion of IL-6 and soluble CD40L (sCD40L). DC/MΦ-mediated tolerance is unique in that immunoglobulin (Ig) secretion is repressed in B cells that are chronically exposed to antigen but not in naïve B cells. Analysis of DC/MΦ-mediated tolerance in murine models of SLE showed that DCs and MΦs from lupus-prone mice are defective in repressing autoreactive B cells, coincident with defects in IL-6 and sCD40L secretion. In this report, we identify TNFα as the final repressive factor secreted by TLR-activated DCs and MΦs. TNFα selectively repressed Smith (Sm)-specific B cells. Like IL-6 and sCD40L, DCs and MΦs from lupus-prone MRL/*lpr* mice were defective in secreting TNFα. Our data define a tolerogenic role for TNFα and suggest that defects in DC and MΦ-mediated tolerance contribute to autoimmunity during innate immune responses.

Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies that bind nuclear self-antigens. Autoimmunity results from a breakdown in tolerance mechanisms that regulate autoreactive lymphocytes (10, 13). Central tolerance mechanisms, such as deletion and receptor editing, regulate immature B cells specific for high avidity, membrane-bound self-antigens (20, 24, 27, 35, 36). Peripheral tolerance mechanisms regulate bone marrow emigrants through deletion (37), positive selection into the B1 compartment (26, 32), functional ignorance of low affinity antigens (43, 44), and anergy (22, 28, 30, 33, 40). Early studies indicated that dysregulated T-dependent immune responses are responsible for the break in tolerance because the pathogenic autoantibodies are somatically mutated, class switched IgGs. However, recent studies have revealed that T-independent immune responses may also lead to somatically mutated, class switched IgGs (205). Several studies implicate innate immune responses in the initiation of tolerance. First, bacterial or viral infection is correlated with the onset of disease (206). Second, inappropriate removal of apoptotic debris through complement and FcR and genetic defects in these receptors are associated with SLE. Last, activation of autoreactive B cells via Toll-like Receptors (TLRs) facilitates disease as TLR7-deficient mice are protected from disease (86). Hence, innate immune responses can play inflammatory or regulatory roles in lupus.

Recent studies have investigated the regulation of B cells during innate immune responses. TLR9 is required for activation of rheumatoid factor B cells through anti-chromatin immune complexes (83), and dual ligation of TLR9 and B cell receptor (BCR) activates anti-chromatin B cells (84). The mechanisms that regulate B cells specific for

nuclear self-antigens during TLR-mediated activation remain unclear. In the neo-self-antigen system where all B cells recognize hen egg lysoszyme (HEL), TLR9 and TLR4-induced Ig secretion was regulated by ERK. In this study, continuous binding of the BCR to self-antigen elevated ERK activity which inhibited TLR4- and TLR9-induced plasma cell differentiation (45, 207). Thus, chronic BCR stimulation regulates some anergic B cells during innate immune responses.

Recently, we described a novel mechanism of tolerance regulating autoreactive B cells during innate immune responses. Chronically antigen-experienced B cells are regulated by LPS-activated myeloid dendritic cells (myDCs) and macrophages (MΦs), and repression of Ig secretion is readily reversed upon removal of these populations (160). The repressive effect is mediated by the secretion of soluble factors, as LPS-activated DC and MΦ conditioned medium (CM) repressed Smith (Sm)-specific Ig secretion. Further investigation into the identity of the soluble repressive factors revealed that DCs secrete IL-6 (160) and MΦs secrete IL-6 and soluble CD40L (sCD40L) (Kilmon et al, manuscript submitted) that repress Ig secretion. These results were surprising considering the role of IL-6 in promoting plasma cell differentiation and CD40L in activating acutely-stimulated B cells. However, only chronically antigen-experienced, but not acutely-stimulated B cells, are repressed by IL-6 and sCD40L (160) (Kilmon et al, manuscript submitted). The data indicate that autoreactive B cells are regulated beyond chronic antigen-induced BCR desensitization, and suggest that the response of B cells to IL-6 and sCD40L is reprogrammed depending on the state of chronic BCR stimulation. Significantly, lupus-prone mice are defective in DC and MΦ-mediated repression, coincident with defects in IL-6 and CD40L secretion (208) (Kilmon et al, manuscript submitted). Thus, these findings suggested that overcoming

tolerance in SLE may be associated with defects in the repression of autoreactive B cells by myDCs and/or MΦs.

In this report, we show that IL-6-deficient DCs and IL-6 x CD40L-deficient MΦs partially repress Ig secretion, suggesting the presence of a third repressive factor. Neutralization of TNFα in CM from LPS-activated IL-6$^{-/-}$ DC and LPS activated IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ relieved repression of Sm-specific Ig secretion. Like previous findings with IL-6 and sCD40L, chronically antigen-experienced, but not naïve B cells are susceptible to repression by TNFα. Significantly, MRL/*lpr* DCs and MΦs are defective in secreting TNFα. Collectively, the data show that DCs and MΦs secrete soluble factors, IL-6, sCD40L, and TNFα, that prevent autoantibody production during innate immune responses, and that the lack of these factors contribute to autoimmunity.

Materials and Methods

Mice

C57BL/6J (B6) non-transgenic, C57BL/6-Tg(IghelMD4)4Ccg/J Ig (HEL-Ig) transgenic, C57BL/6-Tg(ML5sHEL)5Ccg/J (soluble HEL) transgenic (21), B6.129S2-$Il6^{tm1Kopf}$/J (IL-6$^{-/-}$), B6.129S2-$Tnfsf5^{tm1Imx}$/J (CD40L$^{-/-}$), B6;129S6-Tnf^{tm1Gkl}/J (TNFα$^{-/-}$), and MRL/MpJ-Fas^{lpr}/J (MRL/*lpr*) mice were purchased from The Jackson Laboratory. 2-12H/Vκ8/Cκ$^{-/-}$ Ig transgenic mice were previously described (33, 160). MRL/*lpr* mice were used at 6-10 weeks old. All other mice were used at 9-16 weeks old. All studies were approved by the Institutional Animal Care and Use Committee.

Reagents and Antibodies

Recombinant IL-6 (rIL-6) and antibodies to IL-6, CD40L, and hamster IgG$_3$ (isotype control for anti-CD40L) were purchased from BD Biosciences, TEPC 183 and *Escherichia coli* 055:B5 LPS from Sigma-Aldrich, mouse GM-CSF, IL-4, and M-CSF from PeproTech, and CFSE from Invitrogen. 54.1 (3-83 idiotype, isotype control for anti-IL-6), 187.1 (anti-κ), HB100 (anti-IgM^a), and B7.6 (anti-IgM) were purified from hybridoma culture supernatant. Recombinant TNFα (rTNFα) and recombinant sCD40L (rsCD40L) were purchased from R&D Systems. Rabbit polyclonal anti-TNFα was obtained from Vic Johnson (CDC/NIOSH/HELD, West Virginia) and purified by Protein A affinity. The rabbit polyclonal anti-Syk was prepared against residues 257-352 of the murine Syk linker region and provided by John Cambier (University of Colorado Health Science Center, CO).

Bone marrow-derived DC (BMDC) and MΦ (BMMΦ) Cultures

BMDCs (95% pure) and BMMΦs (98% pure) were generated as previously described (160). Conditioned medium (CM) was made from $1x10^4$ BMDCs and BMMΦs (0.2 ml) cultured 4 days with or without LPS (30 μg/ml).

TNFα expressing CHO cell line

RNA was prepared from B6 DCs treated with LPS (30 μg/ml) for 6 hours by solubilization in Trizol (Invitrogen). cDNA was generated by reverse transcription using random hexamer primers and M-MLV reverse transcriptase (Invitrogen). The TNFα cDNA was amplified by PCR using TNFα-specific primers (GenBank Accession #NM_013693; 5' – CCTCAGCGAGGACAGCAAGG – 3' and 5' – CCTTCACAGAGCAATGACTCC – 3'), subcloned into pCR2.1 TA TOPO vector (Invitrogen), and then cloned into the EcoR1 site of pHSPG-EGFP mammalian expression vector (Lishan Su, University of North Carolina). pHSPG containing the TNFα DNA sequence (5 μg) was transiently transfected in Chinese Hamster Ovary (CHO) cells ($5x10^6$) (American Type Culture Collection) by nucleofection according to manufacturer's instructions (Amaxa, Cell Line Nucleofector Kit T, Program U-023). CHO-TNFα supernatant was harvested on day 2, and the levels of TNFα quantitated to be 15 ng/ml (ELISA).

B cell Purification and Culture

B cells were isolated from spleens by negative selection (Easy Sep, StemCell Technologies) (160). B cells were 85-93% pure as determined by flow cytometry. $1x10^5$ purified B cells were cultured with LPS (30 μg/ml) for 4 days. rIL-6, rsCD40L, rTNFα, CHO-TNFα,

BMDC or BMMΦ conditioned media (CM) (25% of final volume) were added to B cell cultures on day 0. The IL-6 in CM was neutralized with either anti-IL-6 antibody or a control rat IgG_1 antibody (54.1). Soluble CD40L in CM was neutralized with either anti-CD40L or control hamster IgG_3 antibody. TNFα in CM was neutralized with either anti-TNFα or control anti-Syk.

ELISAs

$IgM^a/κ$ (encoded by 2-12H/Vκ8/$Cκ^{-/-}$) was captured with anti-κ (187.1) and detected with biotinylated anti-IgM^a (HB100) and Streptavidin-AP as previously described (33). IgM (encoded by B6) was captured with anti-IgM (clone 33-60) and detected with biotinylated anti-IgM (B7.6) and Streptavidin-AP. Purified mouse $IgM^a/κ$ (TEPC 183) served as the standard control. Anti-HEL IgM^a was captured with soluble HEL and detected with biotinulated anti-IgM^a (HB100) and Streptavidin-AP as previously described (22). Anti-HEL IgM^a (clone E1) was used as the standard control. $IgM^a/κ$, IgM, and anti-HEL IgM^a levels were plotted as "percent of control" defined by the level of Ig secretion in LPS-stimulated B cell cultures (100%). TNFα was quantitated by capturing with anti-TNFα (clone TN3-19, eBioscience) and detecting with biotinylated polyclonal anti-TNFα (eBioscience) and Streptavidin-AP. rTNFα served as the standard control.

CFSE-based Proliferation Assay

Purified B cells were resuspended at $1x10^6$ cells/ml in pre-warmed 0.1% BSA/PBS and labeled with CFSE at a final concentration of 0.4 μM for 10 minutes at 37^oC. The cells were then washed with IMBM containing 5% FCS. CFSE-labeled cells were stimulated with LPS

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(30 μg/ml) in the presence or absence of rTNFα (50 ng/ml) as described above. After 3

days, the cells were harvested and CFSE fluorescence intensity was analyzed by flow

cytometry.

Statistical Analysis

Statistical analysis was done using the Student's *t* test. p values < 0.05 were considered

significant and denoted by *.

Results

TNFα Secreted by DCs and MΦs represses Ig secretion.

Maintaining B cell tolerance during activation of the innate immune system is crucial in preventing autoimmunity. We previously showed that IL-6 and soluble CD40 ligand (sCD40L) secreted by TLR4-stimulated myeloid DCs (myDCs) and MΦs represses Ig secretion by chronically antigen-experienced B cells (160) (Kilmon et al, manuscript submitted). Although neutralizing antibodies to IL-6 restored IgM[a]/κ levels to 75% of control in B6 DC conditioned medium (CM) (160), we were surprised to find that IL-6^{-/-} DC CM repressed 45% of secretion (Figure 3.1A). This suggests that DCs secrete another repressive factor. To identify the third repressive factor, we neutralized IL-6^{-/-} DC CM with a panel of antibodies. As shown in Figure 3.1A, anti-TNFα restored 83% of Ig secretion, a level that was not statistically different from control cells (B cells + LPS; *p* value = 0.898); while an unrelated antibody failed to relieve repression. Consistent with the idea that TNFα contributes to the regulation of Ig secretion, CM derived from LPS activated TNFα^{-/-} DC repressed 33% of Ig secretion compared to 44% of Ig secretion by B6 DC CM (Figure 3.1B; *p* value = 0.010). Further, neutralization of CM from TNFα^{-/-} DCs with anti-IL-6 restored 127% of Ig secretion. These data suggest that DCs secrete TNFα as a second repressive factor that regulates autoreactive B cells

To corroborate the idea that TNFα regulates Ig secretion by autoreactive B cells we assessed if recombinant TNFα was repressive. Similar to the neutralization data, 50 ng/ml of rTNFα repressed 29% of Ig secretion (Figure 3.1C; *p* value = 0.00003). Although rTNFα repressed Ig secretion, it was significantly less potent compared to rIL-6, which repressed 75%, and rsCD40L, which represses 74% of Ig secretion (160) (Kilmon et al, manuscript

submitted). Further, the amount of recombinant protein required was beyond physiologic levels. This might be explained by low bioactivity in the commercial product because homotrimers of TNFα are required for biological activity. To address this possibility, we transiently expressed murine TNFα in Chinese Hamster Ovary (CHO) cells where TNFα is constitutively expressed in the culture supernatants at levels of 15 ng/ml. Coculture of Sm-specific B cells with 3.4 ng/ml of CHO-expressed TNFα repressed 39% of Ig secretion (data not shown), compared to 29% when the commercial recombinant product was used at 50 ng/ml (Figure 3.1C). Further titration of the bioactive TNFα did not repress additional IgM^a/κ levels indicating that approximately 30% repression is maximal.

To assess if the ability of TNFα to regulate Ig secretion was enhanced by IL-6, we cultured Sm-specific B cells with optimal levels of TNFα and suboptimal levels of IL-6 then assessed IgM^a/κ secretion. IL-6 at 5 ng/ml was chosen because this level represses 32% of secretion, approximately the same amount as optimal TNFα levels (50 ng/ml; 29% of secretion) (Figure 3.1C). Interestingly, the ability of rTNFα to repress was enhanced in the presence of suboptimal levels of rIL-6, although statistical significance was not reached in three experiments (Figure 3.1C; p value = 0.057). We are continuing to test this using the TNFα from CHO sup to see if this effect is significant. Collectively, the data indicate that DCs secrete IL-6 and TNFα to the regulate Ig secretion by Sm-specific B cells, but TNFα is a less potent repressor compared to IL-6. The ability of TNFα to repress may be enhanced by IL-6, although additional experiments are required.

To determine if IL-6 and TNFα were the only repressive factors secreted by DCs, we undertook two approaches. In the first, we used neutralizing antibodies to IL-6, TNFα, or

IL-6 + TNFα to abrogate the repressive ability of B6 CM. CM where IL-6 was neutralized

repressed 18% of Ig secretion, while CM with TNFα neutralized repressed 26% of secretion.

In contrast, neutralization of both IL-6 and TNFα restored Ig secretion to control levels (*p*

value = 0.695, Figure 3.2A). This supports the idea that IL-6 and TNFα are the only

repressive factors made by DCs. In a second approach, we generated IL-6$^{-/-}$ x TNFα$^{-/-}$ mice.

We predicted that if IL-6 and TNFα were the only repressive factors, LPS-stimulated DCs

from IL-6$^{-/-}$ x TNFα$^{-/-}$ mice would fail to repress Ig secretion. As shown in Figure 3.2B, CM

prepared from LPS-stimulated IL-6-deficient DCs repressed 45% of Ig secretion. Similarly

CM from TNFα-deficient DCs repressed 42%. In contrast, DC CM from IL-6$^{-/-}$ x TNFα$^{-/-}$

repressed 17% of Ig secretion, a value that was not statistically different than control cells (*p*

value = 0.106). The data confirm that IL-6 and TNFα are the only soluble mediators

secreted by TLR4-stimulated DCs to regulate autoreactive, Sm-specific B cells during innate

immune responses.

MΦs regulate autoreactive B cells by secreting IL-6 and sCD40L. Similar to the

findings with DCs, neutralizing B6 MΦ CM with IL-6 and sCD40L restored IgM^{a}/κ levels to

95% of control (Kilmon et al, manuscript submitted); however, IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM

repressed 48% of Ig secretion (Figure 3.3A). To assess the possibility that MΦ-derived

TNFα regulates autoreactive B cells, we co-cultured LPS-activated IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ

CM with Sm-specific B cells. As shown in Figure 3.3A, neutralization of TNFα in the IL-6$^{-/-}$

x CD40L$^{-/-}$ MΦ CM restored Ig secretion to 81% of control (*p* value = 0.060); a level that

again was not statistically different from control cells (LPS-stimulated B cells). To further

assess if other factors secreted by MΦs contributed to the regulation of autoreactive B cells

we neutralized sCD40L in IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ MΦ CM (Figure 3.3B). Ig secretion was

partially restored (from 69% to 81% secretion), indicating that TLR4-activated MΦs and

DCs regulate autoantibody secretion in part through the production of TNFα. It is of interest

to note that the dichotomy between the ability of IL-6$^{-/-}$ DCs and IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦs to

repress Ig secretion compared to the ability of TNFα-neutralized CM to repress Ig secretion

might suggest that MΦs compensate for the lack of IL-6 and CD40L by enhancing secretion

of TNFα. However, the amounts of TNFα in IL-6$^{-/-}$ DC CM and IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM

compared to B6 CM were not different (data not shown).

To definitively establish if IL-6, sCD40L and TNFα are the only repressive factors

secreted by MΦs to repress Ig secretion by autoreactive B cells, we generated IL-6$^{-/-}$ x

CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice. Although we have the mice, it remains to be determined whether

MΦ CM from these mice will repress LPS-induced Ig secretion. We predict that because

neutralizing TNFα relieved the repressive effect of IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM, that MΦ CM

from IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice will not repress Ig secretion. Collectively, the data

suggest that during innate immune responses DCs and MΦs secrete IL-6, sCD40L, and

TNFα that repress the secretion of Sm-specific autoantibody.

TNFα represses Sm-specific B cells but not high affinity lysozyme-specific cells.
We previously demonstrated that IL-6 (160) and sCD40L (Kilmon et al, manuscript

submitted) repress TLR4-stimulated chronically antigen-experienced B cells, but do not

affect acutely-stimulated naïve B cells. The data suggest that chronically-stimulated

autoreactive B cells reprogram their responses to IL-6 and sCD40L to prevent the secretion

of autoantibody during innate immune responses. TNFα is known to enhance naïve B cell activation and proliferation (209-213). Therefore, we hypothesized that TNFα, like IL-6 and sCD40L, would selectively repress chronically antigen-experienced B cells. rTNFα had no effect on Ig secretion by LPS-stimulated naïve B cells from nonautoimmune B6 mice. Likewise, LPS-induced Ig secretion by naïve HEL-specific B cells (HEL-Ig) was similar in the presence or absence of rTNFα (Figure 3.4A). Thus, TNFα does not affect LPS-stimulated Ig secretion by naïve B cells as predicted. Significantly, rTNFα did not repress Ig secretion by HEL-specific B cells chronically exposed to soluble HEL (HEL-Ig x sHEL) (Figure 3.4B). The data show that TNFα does not repress all chronically-stimulated B cells, and suggest that some autoreactive B cells are susceptible to repression by TNFα, whereas others are not. The BCR on Ig transgenic B cells from 2-12H/Vκ8 mice has a low to moderate affinity for Sm, and these B cells are susceptible to repression by TNFα. The BCR on Ig transgenic B cells from HEL-Ig x sHEL mice has a high affinity for sHEL. These B cells are repressed by IL-6 and CD40L, but not by TNFα. We determined that TNFα is less potent as a repressive factor than IL-6 and sCD40L (Figure 3.1C). Consequently, TNFα may not be strong enough to repress B cells with high affinity BCR specific for self-antigen. To confirm this hypothesis, we will test the ability of TNFα to repress autoreactive B cells from other low affinity Ig transgenic models.

TNFα does not affect proliferation of B cells as a means of repressing Ig secretion.
Plasma cell differentiation requires B cell proliferation and upregulation of transcriptional regulators, Blimp-1 and XBP-1. LPS-stimulated autoreactive B cells are allowed to proliferate in the presence of IL-6 and sCD40L; however, the production of Blimp-1 and

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XBP-1 mRNA is blocked preventing the formation of antibody secreting cells (Rutan et al, manuscript in preparation; Kilmon et al, manuscript submitted). To determine whether TNFα affects critical regulatory steps in plasma cell differentiation, we examined its effect on proliferation. To assess proliferation, we labeled B cells with CFSE, stimulated cells with LPS in the presence or absence of rTNFα, and analyzed proliferation by flow cytometry (Figure 3.5). TNFα did not affect proliferation of B cells from 2-12H/Vκ8 or HEL-Ig x sHEL mice, nor did it affect naïve B cells from B6 or HEL-Ig mice. The data show that LPS-stimulated B cells in the presence or absence of rTNFα had overlapping CFSE histogram plots, indicating that similar number of cells existed in each generation in the presence or absence of rTNFα. Thus, TNFα repression of Sm-specific B cells was not due to decreased proliferation. TNFα might block Blimp-1 or XBP-1 transcription, thereby inhibiting plasma cell differentiation and Sm-specific Ig secretion. Future experiments will determine the effect of rTNFα on Blimp-1 or XBP-1 transcription by real time RT-PCR.

DCs and MΦs from MRL/lpr mice are defective in secreting TNFα in response to TLR stimulation.

DCs and MΦs secrete IL-6, sCD40L, and TNFα that repress Ig secretion by chronically antigen-experienced B cells and suggest that overcoming tolerance in SLE may be associated with defects in the repression of autoreactive B cells by myDCs and/or MΦs. We previously showed that DCs and MΦs from lupus-prone mice are defective in repressing Ig secretion, coincident with defects in TLR4-stimulated secretion of IL-6 and sCD40L (208) (Kilmon et al, manuscript submitted). To determine if lupus DCs and MΦs were also defective in secreting TNFα, we measured TNFα secretion in culture by ELISA. LPS-stimulated

BMDCs from lupus-prone MRL/*lpr* secreted significantly less TNFα than B6 (p value = 0.00002, Figure 3.6A). BMMΦs from 7 of 9 MRL/*lpr* mice secreted less TNFα compared to B6 (Figure 3.6B). Thus, MRL/*lpr* mice also exhibit defects in secretion of TNFα. Collectively, the data show that defects in DC and MΦ-mediated regulation of autoantibody secretion exhibited by MRL/*lpr* mice are coincident with defects in all three repressive soluble factors.

Discussion

Previous studies into the mechanisms of B cell tolerance have focused on antigen/BCR mediated regulation of autoreactive or chronically antigen-experienced B cells. However, the regulation of chronically antigen-experienced B cells during polyclonal activation is less understood. Recently, we described that chronically antigen-experienced B cells are regulated by LPS-activated DCs and MΦs (160). In this report, we show that DCs and MΦs secrete TNFα, in addition to IL-6 and sCD40L, that selectively repress Ig secretion by chronically antigen-experienced B cells during innate immune responses. Thus, we have identified a novel mechanism of tolerance mediated by DCs and MΦs, and demonstrated novel roles for IL-6, sCD40L, and TNFα in Ig repression.

Neutralization studies suggest that all of the repressive soluble factors have been identified. IL-6$^{-/-}$ DC CM and IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM were just as capable of repressing chronically antigen-experienced B cells as DC CM and MΦ CM from B6 mice. Neutralizing TNFα in IL-6$^{-/-}$ DC CM (Figure 3.1) and IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM (Figure 3.3) relieved the repressive effect (DCs do not express CD40L). Hence, the data suggest that TNFα is responsible for Ig repression in the absence of IL-6 and sCD40L. However, when LPS-stimulated Sm-specific B cells were cultured with recombinant TNFα, Ig secretion was only repressed by 29% (Figure 3.2) in comparison IL-6$^{-/-}$ DC CM and IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM which repressed 45% and 48% of Ig secretion respectively (Figure 3.1 and 3.3). Thus, a cofactor secreted by DCs and MΦs may exist that allows TNFα to exert its full repressive potential on chronically antigen-experienced B cells. This cofactor is not capable of repression alone, as IL-6$^{-/-}$ x TNFα$^{-/-}$ DC CM did not repress Ig secretion (Figure 3.2). We expect that future experiments will also show that IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNFα$^{-/-}$ MΦ CM do

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not repress Ig secretion. It would be interesting to determine whether the possible cofactor is secreted by IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ DCs and present in CM. If so, we would predict that the cofactor in IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ DC CM could restore the full repressive potential of rTNFα, and that autoreactive B cells cultured in the presence of IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ DC CM and rTNFα would repress Ig secretion similar to B6 DC CM.

The repressive functions of IL-6, sCD40L, and TNFα during innate immune responses challenge what we currently understand about their roles in promoting B cell responses. B cells that have been acutely stimulated and exposed to IFN-α/β induce Ig secretion in response to IL-6 (140). In addition, IL-6 indirectly promotes B cell responses by blocking the repressive effect of regulatory T cells, thus activating CD4$^+$ T cells (129). Antigen-stimulated B cells require T cell help to produce Ig in the form of CD40L, and lack of CD40L or blocking CD40 stimulation on B cells impairs Ig production (38). TNFα enhances cytokine or mitogen-stimulated B cell proliferation and Ig secretion (209-213), and membrane TNFα expressed on viral-infected CD4$^+$ T cells mediates polyclonal B cell activation (214, 215). Thus, previous studies demonstrate IL-6, CD40L, and TNFα act as positive regulators of acutely-stimulated B cell responses. Importantly, these studies used B cells from non-transgenic models where the proportion of autoreactive B cells is either very low or nonexistent. Our data show that IL-6 (160), CD40L (Kilmon et al, manuscript submitted), and TNFα act as negative regulators of chronically antigen-stimulated B cells. Thus, the history of BCR ligation determines whether B cells are susceptible to IL-6, CD40L, and TNFα-mediated repression, and suggest that chronic stimulation of the BCR reprograms responses to these soluble factors.

Not only is the history of BCR ligation critical in determining whether B cells are susceptible to IL-6, CD40L, and TNFα-mediated repression, but the data suggest that BCR affinity may be important in this fate decision. TNFα repressed Ig secretion by low to moderate affinity, Sm-specific B cells, but did not affect Ig secretion by high affinity ($>10^9$ M), HEL-specific B cells chronically exposed to soluble HEL (HEL-Ig x sHEL) (Figure 3.3B). TNFα is less potent as a repressive factor than IL-6 and sCD40L. Consequently, TNFα may not be strong enough to repress B cells with high affinity BCR specific for self-antigen. To confirm this hypothesis, we plan to test the ability of TNFα to repress autoreactive B cells from other low affinity Ig transgenic models. One potential model is the anti-insulin 125 Ig transgenic model. Similar to anti-Sm 2-12H/Vκ8 Ig transgenic B cells, 125 Ig transgenic B cells express a BCR with low affinity for self-antigen (10^7 M) (216). These B cells do not undergo developmental arrest, but are anergic in the periphery and have characteristics of a B1-like subset (217, 218). Significantly, SLE patients have higher frequencies of serum anti-insulin IgM antibodies than healthy controls (219). Thus, the anti-insulin Ig transgenic model having a low affinity BCR should prove useful in studying tolerance mediated by TNFα.

The mechanisms of IL-6, sCD40L, and TNFα repression are currently under investigation. We have shown that IL-6 and sCD40L block transcription of Blimp-1 and XBP-1 important in plasma cell differentiation, thereby inhibiting or reducing LPS-stimulated Ig secretion (160) (Kilmon et al, manuscript submitted). Other studies have demonstrated that IL-6-mediated repression is ERK-dependent (Rutan et al, manuscript in preparation). Interestingly, some chronically-stimulated autoreactive B cells exhibit chronic ERK activation making them refractory to TLR9-induced Ig secretion (45). It is unknown,

however, whether chronic or acute ERK activation reprograms IL-6, CD40L, and TNFα responses in chronically-stimulated B cells.

To validate that DC/MΦ-mediated tolerance is important in disease, it is necessary to determine whether defective DC/MΦ-mediated tolerance can cause disease in nonautoimmune mice or whether functional DCs and MΦs can cure disease in autoimmune mice. Bone marrow transplantion involving fractionated total body irradiation has been used to prevent and treat lupus in MRL/*lpr* mice (220). This experiment can be modified to study the role of the repressive soluble factors in preventing disease. If hematopoietic stem cells from IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ are transplanted into irradiated MRL/*lpr* mice, we would predict from *in vitro* data that the mice will not be protected from disease because they lack the repressive soluble factors that regulate autoreactive B cells. Because wildtype stem cell transplantion protects from lupus, this data would suggest that the lack of IL-6, CD40L, and TNFα causes disease.

Our data show that lupus-prone mice are defective in DC and MΦ-mediated tolerance, coincident with defects in IL-6 (208), sCD40L (Kilmon et al, manuscript submitted), and TNFα, suggesting that the lack of repressive soluble factors would cause disease in nonautoimmune mice. However, it is difficult to predict the effect of IL-6, CD40L, and TNFα deficiencies on autoimmunity in triple knock-out mice for several complicating reasons. First, TNFα is important for proper formation of the splenic architecture (221, 222). TNFα is required for the formation of primary B cell follicles, follicular DC networks, and germinal centers. Secondly, CD40L is important in generating class-switched IgG pathogenic autoantibodies that cause disease. As a result, the presence or

absence of autoimmune disease in IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice would be difficult to interpret.

The proper formation of splenic follicles and germinal centers is a significant variable in studying autoimmunity in IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice. To eliminate disrupted splenic architecture as a variable, we propose to transplant stem cells from IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice into irradiated wildtype nonautoimmune mice. This would generate IL-6, CD40L, and TNFα deficient immune cells in the context of a properly structured spleen. We could then adoptively transfer Ig transgenic autoreactive B cells into these mice and follow their break in tolerance by the presence of Ig transgenic autoantibody in the serum. This experiment would prove that autoreactive B cells are regulated by IL-6, CD40L, and TNFα *in vivo* and that the lack of repressive soluble factors causes a break in tolerance.

The experiments described above do not address the source of the repressive factors *in vivo*. Thus, they would not directly address the role of DCs and MΦs in regulating autoimmunity. The only way to determine the role of DC and MΦ-derived IL-6, CD40L, and TNFα is to create DC and MΦ conditional knock-out mice for IL-6, CD40L, and TNFα. Using the cell specific conditional knock-out would allow for the proper formation of the splenic architecture and to restrict the deficiency of the repressive factors to DCs and MΦs. Although this mouse would be a useful tool, it would not be a trivial task to create.

The ultimate goal of understanding B cell tolerance mechanisms is to create more specific clinical therapies for the prevention and treatment of disease. Interestingly, the use of antibodies against IL-6, TNFα, and CD40L has been studied for the treatment of lupus for good reason. IL-6, TNFα, and CD40L all promote B cell activation, differentiation, and Ig production as previously described. In addition, IL-6 and TNFα are proinflammatory

cytokines that cause tissue damage. Significantly, lupus patients with active disease have increased serum concentrations of IL-6, TNFα, and CD40L (189, 223). Thus, it was logical to think that blocking their actions would decrease autoreactive B cell activation and symptoms of disease. However, our data suggest that these therapies would promote the break in tolerance of newly generated autoreactive B cells because IL-6, TNFα, and CD40L repress chronically antigen-experienced B cells. In fact, anti-TNFα therapy has been found to cause autoimmunity or lupus-like symptoms in patients with several types of autoimmune syndromes including rheumatoid arthritis, Chrohn's disease, and progressive multiple sclerosis (224-230). Yet, the findings that lupus patients with active disease have increased serum concentrations of IL-6, TNFα, and CD40L seem contradictary to our data. Preliminary studies suggest that autoreactive B cells from lupus-prone mice are not repressed by IL-6, sCD40L, and TNFα, similar to naïve B cells but unlike autoreactive B cells from nonautoimmune mice (N. J. Wagner and B. J. Vilen, unpublished observations). Thus, once B cells break tolerance in lupus patients, they may no longer be able to respond appropriately to the increased serum concentrations of repressive factors. It would be interesting to determine whether autoreactive B cells from lupus mice pre-disease are capable of being repressed by the soluble factors and if this corresponds with normal serum levels of IL-6, sCD40L, and TNFα.

In conclusion, therapies aimed at neutralizing the inflammatory effects of IL-6, sCD40L, and/or TNFα may have short-term benefits in treating lupus nephritis, however in some patients, they may promote loss of tolerance in newly emerging B cells during innate immune activation.

Figure 3.1. TNFα secreted by DCs represses Ig secretion. Purified B cells (1×10^5) from 2-12H/Vκ8 mice were stimulated with LPS (30 μg/ml) in the presence or absence of (A) LPS-activated B6 DC CM, IL-6$^{-/-}$ DC CM, or IL-6$^{-/-}$ DC CM neutralized with control Ab (324 μg) or anti-TNFα (324 μg), (B) LPS-activated B6 DC CM, TNFα$^{-/-}$ DC CM, or TNFα$^{-/-}$ DC CM neutralized with isotype-matched control Ab (50 μg/ml) or anti-IL-6 (50 μg/ml), (C) the indicated amounts of rIL-6, rsCD40L, or rTNFα. Secreted IgM^a/κ levels were quantitated by ELISA from day 4 culture supernatant. LPS-stimulated purified B cells (100%) secreted 1-10 μg/ml IgM^a/κ. Data represent triplicate samples in at least 3 experiments. *$p\leq0.05$.
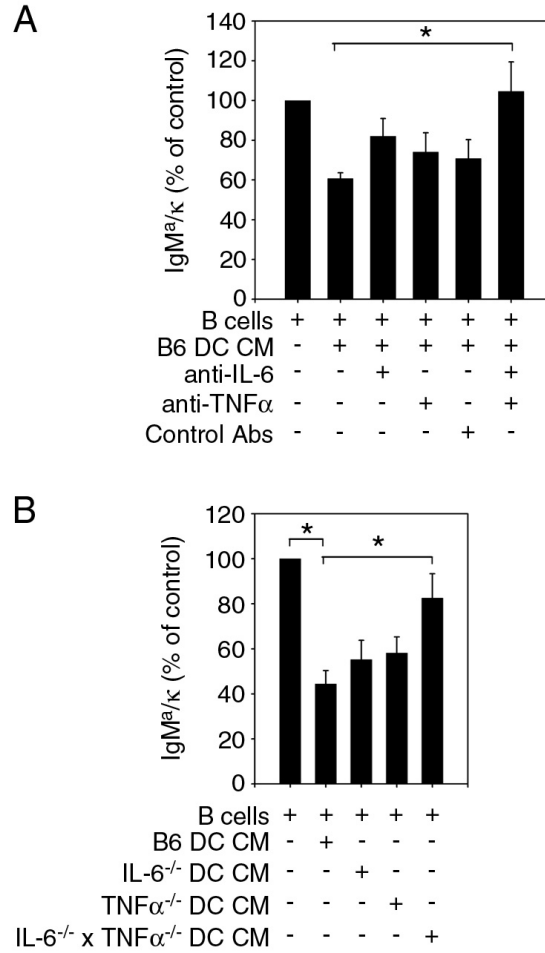
Figure 3.2. IL-6 and TNFα are the only repressive factors secreted by DCs. Purified B cells (1×10^5) from 2-12H/Vκ8 mice were stimulated with LPS (30 μg/ml) in the presence or absence of (A) LPS-activated B6 DC CM or B6 DC CM neutralized with control Abs, anti-IL-6 (50 μg/ml), anti-TNFα (324 μg), or both, (B) LPS-activated B6 DC CM, IL-6^{-/-} DC CM, TNFα^{-/-} DC CM, or IL-6^{-/-} x TNFα^{-/-} DC CM. Secreted IgM[a]/κ levels were quantitated by ELISA from day 4 culture supernatant. LPS-stimulated purified B cells (100%) secreted 1-10 μg/ml IgM[a]/κ. Data represent triplicate samples in each of at least 3 experiments. *$p\leq0.05$.
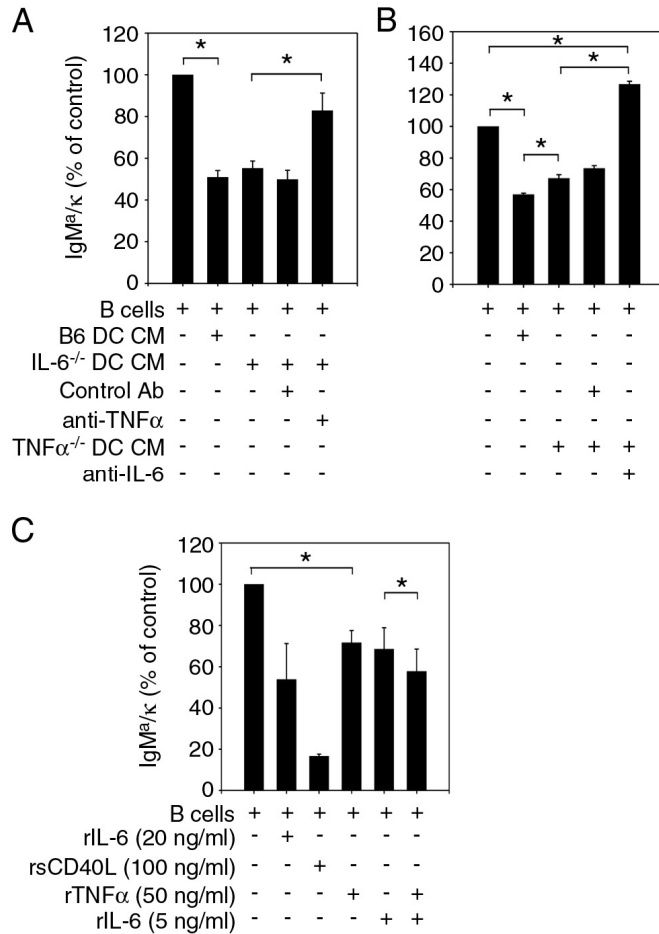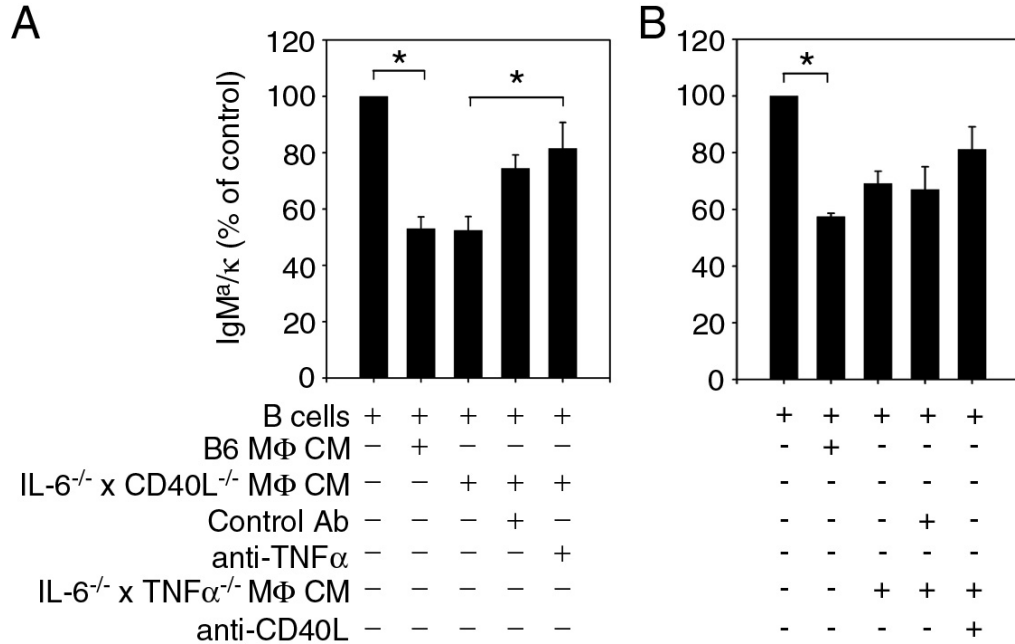
Figure 3.3. TNFα secreted by MΦs represses Ig secretion. Purified B cells ($1x10^5$) from 2-12H/Vκ8 mice were stimulated with LPS (30 μg/ml) in the presence or absence of (A) LPS-activated B6 MΦ CM, IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM, or IL-6$^{-/-}$ x CD40L$^{-/-}$ MΦ CM neutralized with control Ab (324 μg) or anti-TNFα (324 μg), (B) LPS-activated B6 MΦ CM, IL-6$^{-/-}$ x TNFα$^{-/-}$ MΦ CM, or IL-6$^{-/-}$ x TNFα$^{-/-}$ MΦ CM neutralized with control Ab (10 μg/ml) or anti-CD40L (10 μg/ml). Secreted IgM^a/κ levels were quantitated by ELISA from day 4 culture supernatant. LPS-stimulated purified B cells (100%) secreted 1-10 μg/ml IgM^a/κ. Data represent triplicate samples in each of at least 3 experiments. *$p \leq 0.05$.

A

Ig (% of control)

B6 B cells	+	+	-	-		
HEL-Ig B cells	-	-	+	+		
HEL-Ig x sHEL B cells	-	-	-	-	+	+
rTNFα (50 ng/ml)	-	+	-	+	-	+

B
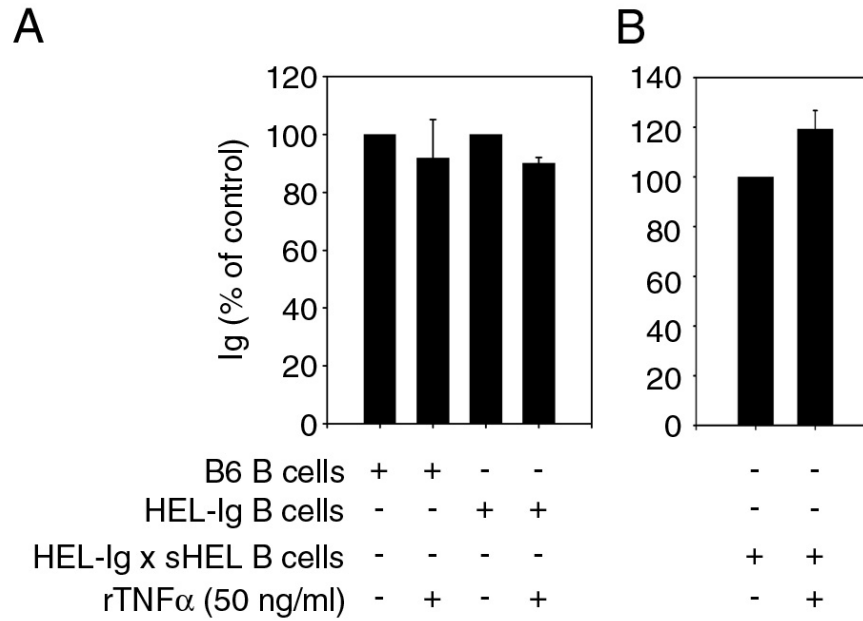
Figure 3.4. TNFα represses only Sm-specific B cells. Purified B cells (1×10^5) from (A) B6, HEL-Ig, or (B) HEL-Ig x sHEL mice were stimulated with LPS (30 μg/ml) in the presence or absence rTNFα for 4 days. Total IgM (B6) and anti-HEL IgM[a] (HEL-Ig and HEL-Ig x sHEL) were quantitated by ELISA. LPS-stimulated purified B cells (100%) secreted 44-65 μg/ml (B6), 11-17 μg/ml (HEL-Ig), and 3-5 μg/ml (HEL-Ig x sHEL). Data represent triplicate samples in each of at least 3 experiments. *$p \leq 0.05$.
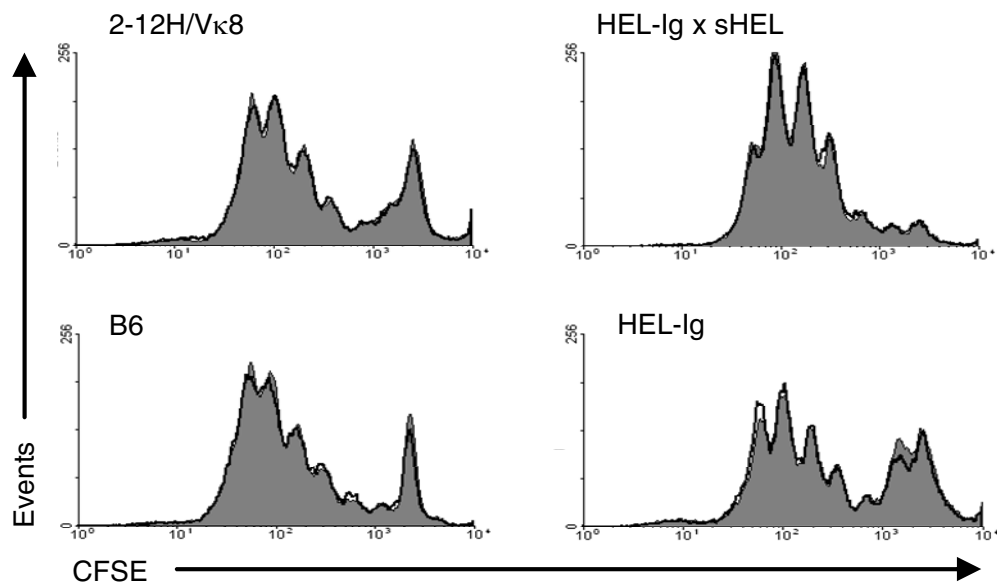
Figure 3.5. TNFα does not affect proliferation of B cells. Proliferation of LPS-stimulated (30 µg/ml), CFSE-labeled B cells from 2-12H/Vκ8, HEL-Ig x sHEL, B6, and HEL-Ig mice, in the presence (black line) or absence (gray fill) of rTNFα (50 ng/ml), was determined on day 3 by FACS analysis. Data is representative of 3 experiments.
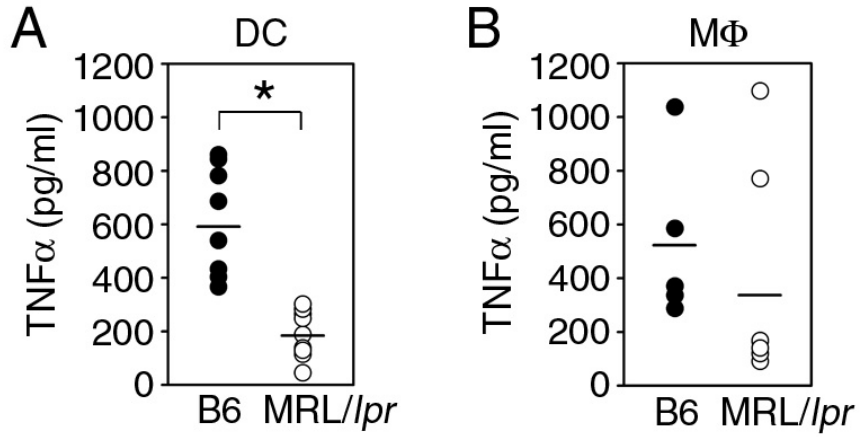
Figure 3.6. DCs and MΦs from MRL/*lpr* mice are defective in TNFα secretion. 1×10^4

(A) BMDCs or (B) BMMΦs were stimulated with LPS (30 μg/ml). TNFα was quantitated

by ELISA from the day 4 culture supernatants. Data represent at least 9 mice per group. (●

B6, ○MRL/*lpr*) *$p \leq 0.05$.

CHAPTER IV

Potential Mechanisms for the TLR Defect in Lupus-Prone Mice

Abstract

Toll-like Receptors (TLRs) polyclonally activate dendritic cells (DCs) and macrophages (MΦs) during innate immune responses. TLR stimulation induces secretion of pro-inflammatory cytokines, including IL-6, soluble CD40L (sCD40L), and TNFα. We recently described that these factors secreted by DCs and MΦs selectively repress chronically antigen-experienced B cells during innate immune responses. Significantly, DC and MΦ-mediated tolerance is defective in lupus-prone MRL/*lpr* mice, coincident with defects in TLR-induced IL-6, sCD40L, and TNFα secretion. Thus, the data suggest that defects in DC and MΦ-mediated tolerance contribute to autoimmunity. Investigation into the mechanism of the TLR defect showed that MRL/*lpr* DCs are unable to sustain signaling through the TLR, and suggest that lupus DCs quickly become tolerized to LPS. Several negative regulators of TLR signaling could play a role in this phenomenon; however, only a few of them have been associated with autoimmune disease. Potential mechanisms for the molecular defect involve STAT and SOCS proteins, apoptotic cells, and mechanisms of endotoxin tolerance. The purpose of this chapter is to discuss early observations as we investigate the TLR defect in DCs. Determining the cause of the TLR defect in lupus-prone mice could identify a genetic signature for individuals susceptible to lupus disease.

Introduction

B cell tolerance mechanisms are critical in regulating autoreactive B cells generated during development in the bone marrow. These tolerance mechanisms include deletion (37), positive selection into the B1 compartment (26, 32), receptor editing (35-37), functional ignorance of low affinity antigens (43, 44), and anergy (22, 28, 30, 33, 40). The breakdown of these mechanisms leads to uncontrolled autoimmunity and diseases like Systemic Lupus Erythematosus (SLE).

We recently described a novel of mechanism of tolerance involving dendritic cells (DCs) and macrophages (MΦs) that regulate chronically stimulated autoreactive B cells in the periphery (160). Polyclonal activators stimulate myeloid DCs (myDCs) and MΦs through TLRs to secrete soluble factors, IL-6, soluble CD40L (sCD40L), and TNFα, that differentially regulate naïve and chronically antigen-experienced B cells. IL-6, sCD40L, and TNFα selectively repress LPS-stimulated autoreactive B cells while having no effect on naïve B cells (160) (Kilmon et al, manuscript submitted; Chapter 3). We propose that chronic BCR ligation by self-antigen reprograms IL-6, sCD40L, and TNFα receptor-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 suggested that overcoming tolerance in SLE may be associated with defects in the repression of autoreactive B cells by myDCs and/or MΦs. Significantly, DC and MΦ-mediated tolerance is defective in lupus-prone MRL/*lpr* mice (208) (Kilmon et al, manuscript submitted). DCs and MΦs from MRL/*lpr* mice are less efficient than nonautoimmune mice at repressing autoantibody secretion. The defects in DC and MΦ-

mediated tolerance are coincident with defects in secreting the repressive soluble factors, IL-6, sCD40L, and TNFα. The data suggest that defects in DC and MΦ-mediated tolerance play a role in autoimmunity. Determining the cause of the TLR defect in lupus-prone mice could identify a genetic signature for individuals susceptible to lupus disease.

Investigation into the mechanism of the TLR defect showed that MRL/*lpr* DCs are unable to sustain signaling through the TLR. LPS stimulation of MRL/*lpr* DCs initially induces IκBα phosphorylation important for activation of the transcription factor NF-κB. However, IκBα phosphorylation is not sustained, resulting in reduced NF-κB DNA binding and IL-6 mRNA synthesis (208). The ability to sustain signaling through the TLR is critical for normal secretion of IL-6 by DCs (180). Hence, this defect in MRL/*lpr* DCs carries significant consequences for the production of IL-6 and other cytokines. Thus, the data suggest that lupus DCs quickly become tolerized to LPS causing defective production of TLR-stimulated cytokines.

There are several negative regulators of TLR signaling that could play a role in this phenomenon; however, only a few of them have been associated with autoimmune disease. Potential mechanisms for the molecular defect involve STAT and SOCS proteins, apoptotic cells, and mechanisms of endotoxin tolerance. The purpose of this chapter is to discuss early observations as we investigate the TLR defect in DCs.

Materials & Methods

Mice

MRL/MpJ-*Fas^{lpr}*/J (MRL/*lpr*) and C57BL/6J (B6) mice were purchased from The Jackson

Laboratory. All mice were used at 6-10 weeks old. All studies were approved by the

Institutional Animal Care and Use Committee.

Reagents and Antibodies

Recombinant IL-6 was purchased from BD Biosciences, *E. coli* 0111:B4 LPS from List

Biological Laboratories, mouse GM-CSF and IL-4 from PeproTech. Antibodies to phospho-

IκBα, phospho-STAT3 Y705, and STAT3 protein were purchased from Cell Signaling,

IκBα and β-tubulin from Santa Cruz, and IgG HRP from Promega.

Bone marrow-derived DC (BMDC) Cultures

Bone marrow single cell suspensions were prepared from the femurs and tibias of B6 and

MRL/*lpr* mice. Following red blood cell lysis, cells were cultured in GM-CSF (10 ng/ml)

and IL-4 (10 ng/ml) for 5 days. BMDCs were >95% $CD11c^+$.

Immunoblotting

BMDCs ($2x10^6$) were stimulated with recombinant IL-6 (10 ng/ml) and/or re-purified List

LPS (15 μg/ml) and solublized in lysis buffer containing 1% NP-40, 150 mM NaCl, 10 mM

Tris (pH 7.5), 2 mM sodium *o*-vanadate, 1 mM PMSF, 0.4 mM EDTA, 10 mM NaF, and 1

μg/ml each of aprotinin, leupeptin, and α1-anti-trypsin. Detergent insoluble material was

removed by centrifugation at 12,000 x g for 10 minutes. Proteins were resolved by SDS-

PAGE and transferred onto PVDF membrane. Phospho-IκBα, IκBα, phospho-STAT3 Y705, STAT3, and β tubulin were immunoblotted with HRP tagged antibodies and detected by enhanced chemiluminescence (ECL, Amersham Biosciences).

Real time RT-PCR

RNA was prepared from BMDCs treated with re-purified LPS (15 μg/ml) by solubilization in Trizol (Invitrogen). Reverse transcription was performed with random hexamer primers and M-MLV reverse transcriptase (Invitrogen). RT-PCR was performed on cDNA using ABgene QPCR SYBR Green Master Mix and the ABI 7000 sequence detection system with primers for SOCS1, SOCS3, and the 18s housekeeping gene (Table 4.1) as a control. SOCS1 and SOCS3 mRNA transcript levels were normalized to the amount of 18s ribosomal RNA transcription using the $2^{\Delta\Delta CT}$ method according to the following equation: relative RNA expression $= 2 \char`^ [-(\text{gene of interest} - 18s \text{ units})]$, and plotted as fold over background in unstimulated DCs.

Table 4.1. Real time RT-PCR primers

SOCS1 forward	5' – CACCTTCTTGGTGCGCG – 3'
SOCS1 reverse	5' – AAGCCATCTTCACGCTGAGC – 3'
SOCS3 forward	5' – TGAGCGTCAAGACCCAGTCG – 3'
SOCS3 reverse	5' – CACAGTCGAAGCGGGGAACT – 3'
18s rRNA forward	5' – TCAAGAACGAAAGTCGGAGGTT – 3'
18s rRNA reverse	5' – GGACATCTAAGGGCATCACAG – 3'

Results and Discussion

LPS-activated DCs and MΦs secrete IL-6, sCD40L, and TNFα that repress autoantibody production. Secretion of these repressive factors is an important component of DC and MΦ-mediated B cell tolerance during innate immune responses. Hence, defects in secretion of IL-6, sCD40L, and TNFα could contribute to autoimmunity. We have previously shown that DCs and MΦs from lupus-prone MRL/*lpr* mice are less efficient at repressing autoantibody secretion than nonautoimmune mice (208) (Kilmon et al, manuscript submitted). In addition, DCs and MΦs from several strains of lupus-prone mice are defective in secreting IL-6, sCD40L, and TNFα in response to TLR stimulation. In fact, a previous study showed that MΦs from several autoimmune strains are defective in expressing pro-inflammatory cytokines (158). However, the authors were unaware of the importance of MΦs in B cell tolerance and how defects in secretion of cytokines like IL-6 and TNFα are associated with autoimmunity.

Further investigation into IL-6 production by MRL/*lpr* DCs revealed differences in the ability to respond to different TLR ligands. Interestingly, IL-6 secretion is defective when stimulated through TLR4 (LPS), TLR7 (R848), and TLR9 (CpG ODN), however, secretion is increased when stimulated through TLR3 (poly I:C) (208). This indicates that not all TLRs are affected by this defect. To corroborate these findings, we examined the induction of IκBα phosphorylation in response to TLR4 and TLR3 stimulation. LPS induces early IκBα phosphorylation in MRL/*lpr* DCs but are unable to sustain phosphorylation like B6 DCs. In constrast, poly (I:C)-induced IκBα phosphorylation was comparable between DCs derived from B6 and MRL/*lpr* mice (208). Thus, signaling through TLR3 is not defective. TLR4, TLR7, and TLR9 are similar in that they use signaling pathways dependent

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on the MyD88 adaptor molecule, whereas TLR3 signals through a MyD88-independent pathway. Hence, the data suggest that the defect in TLR signaling is in the MyD88-dependent pathway.

The finding that IκBα phosphorylation is not sustained, resulting in reduced NF-κB and AP-1 DNA binding and IL-6 mRNA synthesis (208), is significant for the production of IL-6. The ability to sustain signaling through the TLR is critical for normal secretion of IL-6 by DCs. Yang et al showed that persistant TLR4 signals are required for normal DC secretion of IL-6 (180). Therefore, the inability to sustain TLR signaling results in diminished IL-6 secretion. Thus, it seems that lupus DCs quickly become tolerized to LPS causing defective production of TLR-stimulated cytokines.

Endotoxin tolerance is a normal regulatory mechanism controlling cellular responses to bacterial products. Upon ligand binding, MyD88 associates with the TLR and recruits IRAK4 and IRAK1. Activation of IRAK1 leads to downstream phosphorylation of IκBα, NF-κB activation, and transcription of cytokines. Endotoxin tolerance is associated with a reduction in IRAK1 expression, IRAK association with MyD88, and IκBα phosphorylation (231, 232). We previously demonstrated that MRL/*lpr* DCs exhibit decreased sustained amounts of IκBα phosphorylation (208). Here, we show that MRL/*lpr* DCs also exhibit reduced IRAK1 expression. IRAK1 is normally degraded after transducing a signal. BMDCs from B6 mice express IRAK1 protein, and once stimulated by LPS, the majority of IRAK1 protein is degraded by 30 minutes (Figure 4.1). In constrast, MRL/*lpr* BMDCs express reduced basal levels of IRAK1 with very little degradation (Figure 4.1), indicating minimal signal transduction through the TLR. Thus, reduced expression of IRAK1 mimicks what has been observed in endotoxin tolerance. The data suggest that MRL/*lpr* DCs have

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become tolerant to LPS signaling. We predict that IRAK association with MyD88 would also be diminished, correlating with reduced IκBα phosphorylation, NF-κB activation, and IL-6 mRNA levels.

MRL/*lpr* DCs are capable of transducing an early signal through the TLR, but are defective in sustaining signal transduction as previously described (208). One possible mechanism for the defect is that the activity of a negative regulator of TLR signaling is enhanced in MRL/*lpr* DCs. TLR-stimulated secreted products, including IL-6, could also feedback onto the cell in an autocrine manner to regulate TLR signaling. To determine if IL-6 affects TLR signaling, we first exposed DCs to IL-6 and then stimulated with LPS (Figure 4.2). BMDCs from B6 and MRL/*lpr* mice were pre-treated with IL-6 for 30 minutes, then stimulated with LPS for the indicated times. The effect of IL-6 on TLR signaling was determined by immunoblotting whole cell lysates for phospho-IκBα. The data showed that prior exposure to IL-6 in B6 DCs may enhance IκBα phosphorylation in response to LPS compared to B6 DCs stimulated with LPS alone. In contrast, prior exposure to IL-6 in MRL/*lpr* DCs decreased LPS-induced IκBα phosphorylation in compared to LPS stimulation alone. Thus, LPS signaling in lupus DCs exhibit a negative response to IL-6, whereas IL-6 may have a positive feedback on TLR signaling in nonautoimmune DCs. The data suggest that the IL-6 produced by lupus DCs in culture could act in an autocrine manner to prevent further signal transduction through the TLR.

MRL/*lpr* DCs are somehow responding differently to IL-6. IL-6 binding to its receptor activates the JAK-STAT signaling pathway. Receptor dimerization induces phosphorylation of JAK, which recruits and phosphorylates STAT3. Phosphorylated STAT3 dimerizes and translocates to the nucleus to function as a transcription factor. To determine

whether differences exist in IL-6 signaling in MRL/*lpr* DCs compared to B6 DCs, we immunoblotted whole cell lysates for phosphorylated STAT3 (Figure 4.3). Unstimulated B6 DCs exhibited very little basal phospho-STAT3. IL-6 stimulation induced phosphorylation of STAT3 peaking at 15 minutes and then diminishing thereafter. MRL/*lpr* DCs exhibited a similar pattern of STAT3 phosphorylation, however, significant basal levels of phospho-STAT3 indicated the presence of aberrantly activated STAT3. Phosphorylation of STAT3 was further enhanced by IL-6 stimulation with STAT3 protein levels similar to B6 DCs. Thus, the data suggest that MRL/*lpr* DCs have a hyperactive JAK-STAT signaling pathway. Figure 4.2 showed that prior exposure to IL-6 in MRL/*lpr* DCs decreased LPS-induced IκBα phosphorylation. Consequently, the aberrantly activated STAT3 in MRL/*lpr* DCs could be negatively affecting TLR signaling. Future experiments to confirm that IL-6 and STAT3 signaling pathways inhibit TLR signaling would involve blocking these pathways in MRL/*lpr* DCs to determine their effect on TLR signaling. IL-6 signaling can be inhibited by using an IL-6 receptor antibody blocking, and STAT3 signaling can be blocked by using the STAT3 pharmacological inhibitor JSI-124. We predict that TLR signaling in MRL/*lpr* DCs would be enhanced or sustained to a greater extent if IL-6 and STAT3 signaling are inhibited.

The molecular mechanism as to how IL-6 stimulation or hyperactive STAT3 could inhibit TLR signaling is unclear. IL-6 and LPS induce SOCS1 and SOCS3 in a STAT dependent or independent manner, and SOCS proteins prevent LPS induced cytokine production (111-113, 115-117). SOCS3 is the major SOCS protein induced by IL-6, although SOCS1 can also be stimulated. To determine if hyperactivated STAT3 translated into increased expression of SOCS proteins, SOCS1 and SOCS3 mRNA levels were measured in IL-6-stimulated BMDCs using real time RT-PCR (Figure 4.4). The data show

that IL-6 induced increased peak levels of SOCS1 mRNA at 15 minutes in MRL/*lpr* DCs compared to B6 DCs, but similar amounts of RNA transcripts were produced thereafter (Figure 4.4A). In contrast, IL-6 induced increased peak levels at 15 minutes and increased sustained amounts of SOCS3 mRNA out to 2 hours in MRL/*lpr* DCs (Figure 4.4B). Thus, hyperactive STAT3 translates into increased expression of SOCS transcription. It remains to be determined whether SOCS protein levels correlate with this trend.

In summary, MRL/*lpr* DCs quickly become tolerized to LPS causing defective production of TLR-stimulated cytokines. SOCS proteins are involved in the regulation of LPS-induced cytokine production (111-113, 115-117). We show here that prior exposure to IL-6 diminished TLR signaling in MRL/*lpr* DCs, coincident with hyperactive STAT3 and SOCS1 and SOCS3 gene expression. These findings present the possibility that STAT3/SOCS are involved in the excessive negative regulation of TLR responses in MRL/*lpr* DCs.

A previous study linked hyperactive STAT3 signaling to lupus. Liu et al have found that B cells from mice expressing the *Sle1ab* lupus susceptible locus produce antinuclear antibodies mediated by hyperactive STAT3 and ras-ERK signaling pathways (70). These B cells also exhibit increased expression of SOCS3. Significantly, we show here that DCs from MRL/*lpr* mice exhibit hyperactive STAT3 and increased SOCS3 expression, coincident with the inability to sustain TLR signaling and cytokine production and regulate autoantibody secretion. Thus, the STAT3/SOCS3 phenotype present in lupus DCs is similar to lupus B cells. The next step is to determine how hyperactive STAT3 and increased SOCS3 affect TLR signaling and whether this defect affects TLR tolerance in MRL/*lpr* DCs.

Little is known about the mechanisms of how SOCS proteins inhibit TLR signaling. Frobose et al showed that SOCS3 inhibits IL-1 signal transduction by inhibiting ubiquitination of TRAF6, thus preventing association and activation of TAK1 (118). SOCS1 inhibition of LPS signaling involves several mechanisms. SOCS1 associates with TLR molecules IRAK (111) and Mal (116). SOCS1 also inhibits phosphorylation of Jak2 and STAT5 involved in NF-κB activation (119). Further experiments are necessary to determine the role of SOCS proteins in regulating TLR responses in MRL/*lpr* DCs. Co-immunoprecipitation can be used to determine whether SOCS proteins interact with TLR signaling molecules. In addition, siRNA can be used to inhibit newly transcribed SOCS mRNA. Due to the rapid turnover of SOCS proteins (233), we expect there will be very little basal SOCS present. Thus, siRNA will inhibit SOCS activity in MRL/*lpr* DCs. We predict these experiments will show that in the absence of SOCS activity TLR signaling will be at least partially restored. Alternatively, the absence of restored TLR responses will be difficult to interpret. This result could be due to the lack of involvement of SOCS or compensation by any one of the several negative regulators of TLR signaling.

We have presented some evidence suggesting the involvement of STAT3/SOCS in the excessive regulation of TLR signaling in MRL/*lpr* DCs. However, it is important to investigate another important regulatory mechanism for TLR signaling associated with lupus. DCs and MΦs exposed to apoptotic cells are unable to respond to TLR stimulation (149) (Sykes and Matsushima, unpublished observations). Lupus in humans and mice is associated with an increased burden of apoptotic cells (14-16, 57, 58), and mice lacking receptor tyrosine kinases important in phagocytosis of apoptotic cells develop lupus (59-62). Thus, prolonged exposure to apoptotic cells *in vivo* could affect the ability of DCs and MΦs to

sustain TLR responses needed to produce repressive soluble factors. In fact, Koh et al described apoptotic cell inhibition of TLR signaling in MΦs from several strains of lupus-prone mice without understanding how the lack of inflammatory cytokines related to the break in B cell tolerance and lupus disease (158). Thus, there are several directions to take this investigation. Determining the cause of the TLR defect in lupus-prone mice could identify a genetic signature for individuals susceptible to lupus disease and make a significant contribution to the field.
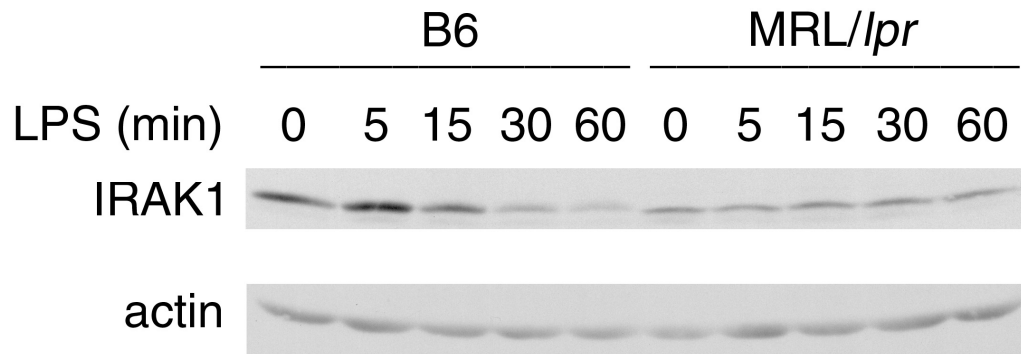
Figure 4.1. Lupus DCs exhibit defects in IRAK1 expression. BMDCs (2x10^6) from B6 or MRL/*lpr* mice were stimulated with LPS (15 µg/ml) for the indicated timepoints. IRAK1 or actin expression in whole cell lysates was determined by immunoblotting. Data represent 3 experiments.
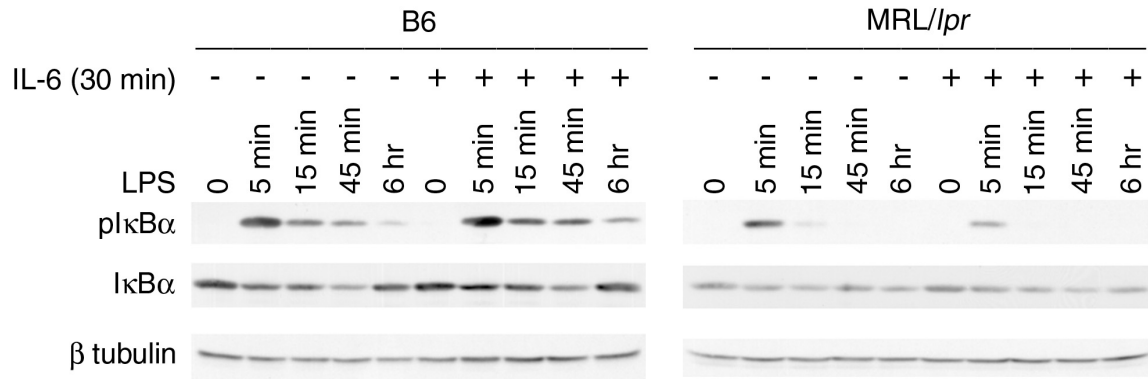
Figure 4.2. IL-6 affects TLR4 signaling in DCs. BMDCs ($2x10^6$) from B6 or MRL/*lpr*

mice were pre-treated with recombinant IL-6 (10 ng/ml) for 30 minutes then stimulated with

LPS (15 µg/ml) for the indicated timepoints. Phospho-IκBα, IκBα, and β tubulin expression

in whole cell lysates was determined by immunoblotting. Data represent 3 experiments.
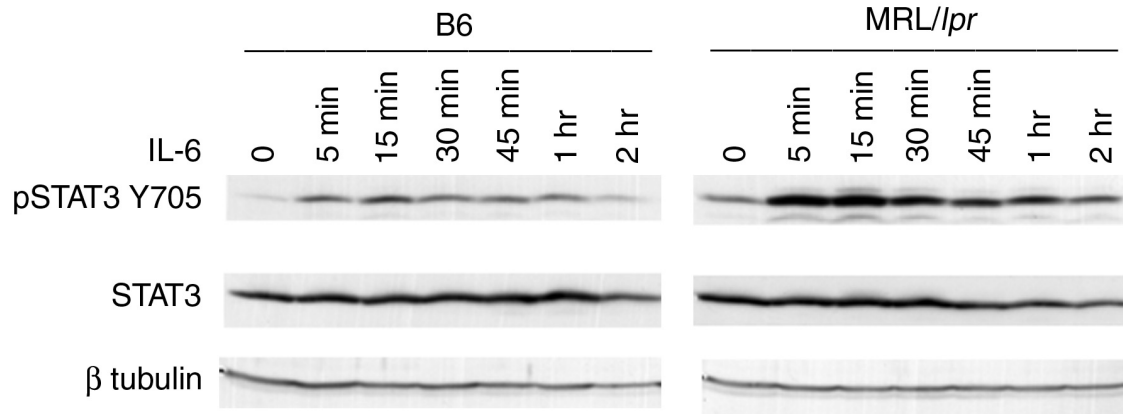
Figure 4.3. Lupus DCs exhibit aberrantly activated STAT3. BMDCs $(2x10^6)$ from B6 or

MRL/*lpr* mice were stimulated with IL-6 (10 ng/ml) for the indicated timepoints. Phospho-

STAT3 Y705, STAT3, and β tubulin expression in whole cell lysates was determined by

immunoblotting. Data represent 3 experiments.
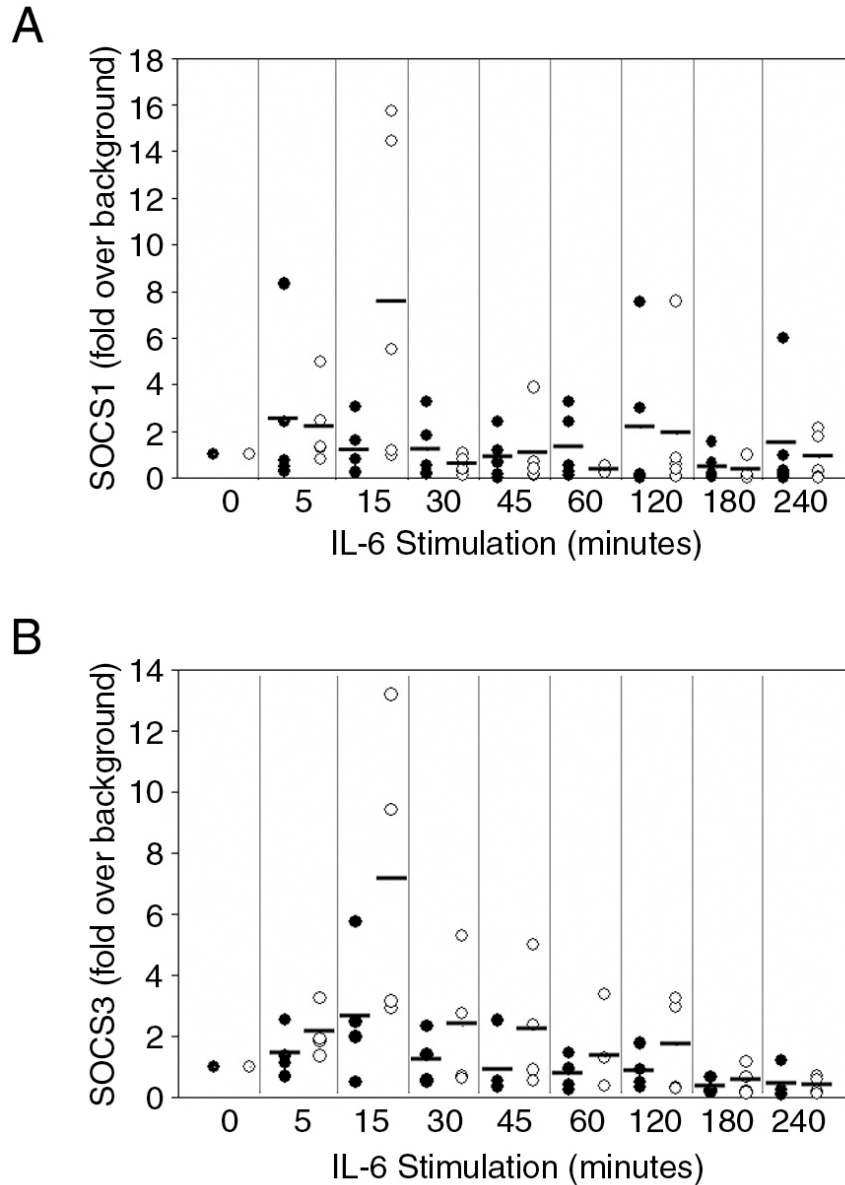
Figure 4.4. IL-6 induces increased expression of SOCS proteins in lupus DCs. BMDCs
($2x10^6$) from B6 (●) or MRL/*lpr* (○) mice were stimulated with IL-6 (10 ng/ml) for the
indicated timepoints. Real time RT-PCR was performed on RNA isolated from DCs.
SOCS1 and SOCS3 mRNA levels were quantitated and plotted as fold over background in
unstimulated DCs. Data represent at least 4 experiments.

CHAPTER V

Discussion

The ability to mount immune responses to infectious agents is critical in surviving and adapting to surrounding environments. Innate immunity has evolved to quickly and nonspecifically control the invasion and pathogenesis of microbes before the adaptive immune response can generate a targeted defense and memory against the infectious agent. The nonspecific response during innate immunity is effective in the early control of infection; however unregulated, polyclonal activation of immune cells can disrupt the balance between immunity and tolerance to self-antigens. Thus, polyclonal activation could promote autoimmunity in individuals susceptible to diseases like lupus.

The regulation of autoreactive lymphocytes during innate immune responses is important in understanding the breakdown of tolerance that occurs in lupus. We propose a model where autoreactive B cells that chronically see antigen are repressed during polyclonal activation and maintained in an anergic state, while naïve B cells are allowed to mount a response. We recently identified a novel mechanism of tolerance wherein DCs and MΦs repress Ig secretion by autoreactive B cells (160). Polyclonal activators through TLRs induce DCs and MΦs to secrete soluble factors (IL-6, sCD40L, and TNFα) that differentially regulate naïve and chronically antigen-experienced B cells. Significantly, IL-6, sCD40L, and TNFα selectively repress LPS-stimulated autoreactive B cells while having no effect on naïve B cells (160) (Kilmon et al, manuscript submitted; Chapter 3). Thus, we have identified a mechanism that prevents autoimmunity while allowing naïve B cells to respond during innate immune responses.

The data indicating that DCs and MΦs mediate tolerance through the secretion of IL-6, sCD40L, and TNFα may seem paradoxical at first glance. Myeloid DCs (myDCs) and plasmacytoid DCs (pDCs) 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 (139, 140, 174). Specifically, IL-6 was found to promote plasma cell survival (175, 176). CD40L is required for activation of B cells in response to T-dependent antigens (38). Soluble TNFα enhances cytokine or mitogen-stimulated B cell proliferation and antibody secretion (209-213, 234). Membrane-bound TNFα expressed on viral-infected T cells mediates polyclonal B cell activation (214, 215). Studies identifying IL-6 and TNFα as positive regulators focused on B cells from non-transgenic mice where the proportion of autoreactive cells is low. In contrast, the studies showing that IL-6, sCD40L, and TNFα repress autoantibody production used self-reactive Ig transgenic models where the B cells were constantly exposed to self-antigen (160) (Kilmon et al, manuscript submitted; Chapter 3). This effect is not limited to Ig transgenic B cells because autoreactive B cells from B6 mice are repressed by sCD40L. Thus, IL-6, sCD40L, and TNFα act as positive or negative regulators of B cells depending on the history of BCR ligation.

We propose that chronic BCR ligation by self-antigen reprograms IL-6, sCD40L, and TNFα receptor-mediated outcomes allowing naïve B cells to produce Ig in response to polyclonal stimulation while simultaneously repressing autoreactive B cells from producing autoantibody. Ig repression by IL-6 and sCD40L in chronically antigen-experienced B cells involves mechanisms upstream of Blimp-1 and XBP-1 expression. IL-6, sCD40L, and TNFα do not affect LPS-induced proliferation, but IL-6 and sCD40L inhibit the differentiation of B cells to antibody secreting plasma cells (Rutan et al, manuscript in preparation; Kilmon et al, manuscript submitted). Other studies have shown that IL-6 represses Ig secretion by an ERK-dependent mechanism (Rutan et al, manuscript in preparation). Thus, chronic BCR stimulation induces ERK activation that reprograms the IL-

6 response. As a result, instead of IL-6 promoting plasma cell differentiation, IL-6 prevents plasma cell differentiation in LPS-stimulated chronically antigen-experienced B cells.

Not only is the history of BCR ligation critical in determining whether B cells are susceptible to IL-6, CD40L, and TNFα-mediated repression, but the data suggest that BCR affinity is also important in this fate decision. TNFα repressed Ig secretion by low affinity, Sm-specific B cells, but did not affect Ig secretion by high affinity ($>10^9$ M), HEL-specific B cells chronically exposed to soluble HEL (HEL-Ig x sHEL) (Chapter 3). TNFα is less potent as a repressive factor than IL-6 and sCD40L. Consequently, TNFα may not be strong enough to repress B cells with high affinity BCR specific for self-antigen. To confirm this hypothesis, we plan to test the ability of TNFα to repress autoreactive B cells from other low affinity Ig transgenic models. One potential model is the anti-insulin 125 Ig transgenic model. Similar to anti-Sm 2-12H/Vκ8 Ig transgenic B cells, 125 Ig transgenic B cells express a BCR with low affinity for self-antigen (10^7 M) (216). These B cells do not undergo developmental arrest, but are anergic in the periphery and have characteristics of a B1-like subset (217, 218). Significantly, SLE patients have higher frequencies of serum anti-insulin IgM antibodies than healthy controls (219). Thus, the anti-insulin Ig transgenic model having a low affinity BCR should prove useful in studying tolerance mediated by TNFα.

Neutralization studies suggest that all of the repressive soluble factors have been identified. IL-6^{-/-} DC conditioned media (CM) and IL-6^{-/-} x CD40L^{-/-} MΦ CM were just as capable of repressing chronically antigen-experienced B cells as DC CM and MΦ CM from B6 mice. Neutralizing TNFα in IL-6^{-/-} DC CM and IL-6^{-/-} x CD40L^{-/-} MΦ CM relieved the repressive effect (DCs do not express CD40L) (Chapter 3). Hence, the data suggest that

TNFα is responsible for Ig repression in the absence of IL-6 and sCD40L. However, recombinant TNFα could not repress the majority of Ig secretion like IL-6 and sCD40L. Thus, a cofactor secreted by DCs and MΦs may exist that allows TNFα to exert its full repressive potential on chronically antigen-experienced B cells. This cofactor is not capable of repression alone, as IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ DC CM did not repress Ig secretion (Chapter 3). We expect that future experiments will also show that IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ MΦ CM do not repress Ig secretion. It would be interesting to determine whether the possible cofactor is secreted by IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ DCs and present in CM. If so, we would predict that the cofactor in IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ DC CM could restore the full repressive potential of rTNFα, and that autoreactive B cells cultured in the presence of IL-6$^{-/-}$ x TNF$\alpha^{-/-}$ DC CM and rTNFα would repress Ig secretion similar to B6 DC CM.

The findings that three different factors repress Ig secretion suggest redundancy in function. Certainly, the regulation of autoreactive B cells is important enough to have back-up mechanisms. This is the reason that several mechanisms of tolerance exist at different checkpoints of B cell development, activation, and differentiation. However, we have seen some differences in the ability of IL-6 and sCD40L to regulate different subsets of B cells. CD40L represses follicular and marginal zone B cells from 2-12 heavy chain Ig transgenic mice, whereas IL-6 represses only follicular B cells (Kilmon et al, manuscript submitted). Thus, the functions of IL-6 and CD40L are not completely redundant. Future experiments will determine the ability of these factors to repress pre-plasma cell and B1 subsets. In addition, we will determine the ability of IL-6, sCD40L, and TNFα to repress An1 positive naturally occurring anergic B cells in B6 mice.

In addition to tolerance mediated by repressive soluble factors, data indicate that a contact-mediated mechanism regulates Ig secretion. DC CM is less efficient at repressing Ig secretion compared to intact DCs indicating that a contact-dependent mechanism may partially regulate Ig secretion. In support of this, DCs deficient in TLR4 are unable to secrete repressive factors in response to LPS, yet they partially repress Ig secretion. However, repression is lost when DCs are separated from B cells by transwell apparatus (Kilmon and Vilen, unpublished observations). DCs display intact nuclear antigen on their surface and chronic antigen/BCR stimulation maintains B cells in an anergic state. Thus, the contact-mediated mechanism may involve antigen displayed on DCs. Alternatively, DCs interacting with B cells may engage inhibitory receptors that regulate BCR and/or TLR responses. Recent adaptation of 2-photon microscopy to visualize live interaction of cells *in vivo* now makes it feasible to determine whether DCs interact with autoreactive B cells *in vivo*.

During innate immune responses, autoimmunity could result if DC and MΦ defects diminish the secretion of repressive soluble factors or if autoreactive B cells are unable to differentially respond to repressive factors. Therefore, we hypothesize that DC and/or MΦ-mediated tolerance mechanisms are dysfunctional in murine models of lupus disease. We showed that MRL/*lpr*-derived DCs, MΦs, and their secreted factors were defective in repressing Ig secretion, coincident with defective secretion of IL-6, sCD40L, and TNFα. It remains to be determined whether autoreactive B cells from lupus-prone mice can be repressed by IL-6, sCD40L, and TNFα. Preliminary studies suggest that autoreactive B cells from 2-12 heavy chain Ig transgenic MRL and MRL/*lpr* mice are not repressed by IL-6, sCD40L, and TNFα. This would suggest that autoreactive B cells from lupus-prone mice fail to reprogram IL-6, sCD40L, and TNFα receptors to signal the repression of TLR

responses. Thus, one or more defects in DC/MΦ-mediated tolerance could lead to autoimmunity and lupus disease *in vivo*.

Investigation into the mechanism of the TLR defect in lupus DCs demonstrates their inability to sustain signaling through the TLR. The data suggest that lupus DCs quickly become tolerized to LPS. Endotoxin tolerance is a normal regulatory mechanism controlling cellular responses to bacterial products. Upon ligand binding, MyD88 associates with the TLR and recruits IRAK4 and IRAK1. Activation of IRAK1 leads to downstream phosphorylation of IκBα, NF-κB activation, and transcription of cytokines. Endotoxin tolerance is associated with a reduction in IRAK1 expression, IRAK association with MyD88, and IκBα phosphorylation (231, 232). Thus far, we have determined that MRL/*lpr* DCs exhibit decreased IRAK1 expression (Chapter 4) and decreased ability to sustain IκBα phosphorylation (208) in response to LPS. Thus, endotoxin tolerance may chronically disable MRL/*lpr* DCs making them inefficient at IL-6 and TNFα secretion. Determining the TLR defect in DCs and MΦs from lupus-prone mice could identify a genetic signature for individuals susceptible to lupus disease. There are several negative regulators of TLR signaling that could play a role in this phenomenon. However, only a few of them have been associated with autoimmune disease, including apoptotic cells and SOCS proteins.

DCs and MΦs exposed to apoptotic cells are unable to respond to TLR stimulation (149) (Sykes and Matsushima, unpublished observations). Lupus in humans and mice is associated with an increased burden of apoptotic cells (14-16, 57, 58), and mice lacking receptor tyrosine kinases important in phagocytosis of apoptotic cells develop lupus (59-62). Thus, prolonged exposure to apoptotic cells *in vivo* could affect the ability of DCs and MΦs to sustain TLR responses needed to produce repressive soluble factors. In fact, Koh et al

described apoptotic cell inhibition of TLR signaling in MΦs from several strains of lupus-prone mice without understanding how the lack of inflammatory cytokines related to the break in B cell tolerance and lupus disease (158).

SOCS proteins negatively regulate LPS signaling. Liu et al have found that B cells from mice expressing the *Sle1ab* lupus susceptible locus produce antinuclear antibodies mediated by hyperactive STAT3 and ras-ERK signaling pathways (70). These B cells also exhibit increased expression of SOCS-3. Significantly, we have found that DCs from MRL/*lpr* mice exhibit hyperactive STAT3 and increased SOCS3 expression (Chapter 4), coincident with the inability to sustain TLR signaling and cytokine production and regulate autoantibody secretion. Thus, the STAT3/SOCS3 phenotype present in lupus DCs is similar to lupus B cells. The next step is to determine if hyperactive STAT3 and increased SOCS3 dysregulate TLR signaling in MRL/*lpr* DCs. Whether STAT3/SOCS3 or apoptotic cells are inducing this response has yet to be determined.

To establish if DC/MΦ-mediated tolerance is important in disease, it is necessary to determine whether the lack of IL-6, sCD40L, and TNFα causes disease in nonautoimmune mice or whether functional DCs and MΦs can prevent onset or reverse disease in autoimmune mice. *In vitro* data suggest that the lack of repressive soluble factors would cause disease in nonautoimmune mice. However, it is difficult to predict the effect of IL-6, CD40L, and TNFα deficiencies on autoimmunity in triple knock-out mice for several complicating reasons. First, TNFα is important for proper formation of the splenic architecture (221, 222). TNFα is required for the formation of primary B cell follicles, follicular DC networks, and germinal centers. Secondly, CD40L is important in generating class-switched IgG pathogenic autoantibodies that cause disease. As a result, the presence or

absence of autoimmune disease in IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice is difficult to interpret. The presence of autoimmunity in triple knock-out mice could be contributed to the lack of repressive factors that regulate autoreactive B cells in the periphery. Alternatively, the presence of autoimmunity could be due to disruption of the splenic architecture such that autoreactive B cells are not in the proper location in the spleen to be regulated by extrinsic tolerance mechanisms other than IL-6, CD40L, and TNFα. Likewise, the absence of autoimmunity in triple knock-out mice could be contributed to disruption of the splenic architecture such that autoreactive B cells are not able to receive survival signals provided by follicular dendritic cells in the B cell follicle. Another possible scenario is that autoimmunity would result without disease. Although the splenic architecture would be disrupted due to the lack of TNFα, IgM responses are unaffected in TNF$\alpha^{-/-}$ mice. Despite the absence of B cell follicles, Ig class-switching can still occur, yet deregulated IgG responses are evident (221). However, the lack of CD40L could prevent the development of IgG class-switched autoantibodies that cause disease. Therefore, IgM autoantibodies could result without the presence of disease in triple knock-out animals. Thus, the absence of autoimmunity or disease in triple knock-out mice would not prove that IL-6, CD40L, and TNFα are not important in regulating autoreactive B cells *in vivo*.

The proper formation of splenic follicles and germinal centers is a significant variable in studying autoimmunity in IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice. To eliminate disrupted splenic architecture as a variable, we propose to transplant stem cells from IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ mice into irradiated wildtype nonautoimmune mice. This would generate IL-6, CD40L, and TNFα deficient immune cells in the context of a properly structured spleen. We could then adoptively transfer Ig transgenic autoreactive B cells into these mice and follow

their break in tolerance by the presence of Ig transgenic autoantibody in the serum. This experiment would prove that autoreactive B cells are regulated by IL-6, CD40L, and TNFα *in vivo* and that the lack of repressive soluble factors causes a break in tolerance.

The experiments described above do not address the source of the repressive factors *in vivo*. Thus, they would not directly address the role of DCs and MΦs in regulating autoimmunity. The only way to determine the role of DC and MΦ-derived IL-6, CD40L, and TNFα is to create a DC and MΦ conditional knock-out mouse for IL-6, CD40L, and TNFα. Using the cell specific conditional knock-out would allow for the proper formation of the splenic architecture and restrict the deficiency of the repressive factors to DCs and MΦs. Although this mouse would be a useful tool, it would not be a trivial task to create.

Future experiments will determine whether functional DCs and MΦs can prevent onset or reverse disease in lupus-prone mice. Bone marrow transplantion has been used to prevent and treat lupus in MRL/*lpr* mice. Total body irradiation, transplantation of allogeneic B6 bone marrow, plus bone grafts supplying stromal cells successfully prevent the development of autoimmune disease in young MRL/*lpr* mice (235). However, MRL/*lpr* mice after the onset of disease do not survive this regimen because they become extremely radiosensitive and susceptible to uremic enterocolitis. Therefore, bone marrow transplantation involving fractionated total body irradiation is used instead and results in survival of the mice and treatment of lupus disease (220). This experiment can be modified to study the role of the repressive soluble factors in preventing disease. B6 bone marrow transplantation protects MRL/*lpr* mice from disease. Thus, if hematopoietic stem cells from IL-6$^{-/-}$ x CD40L$^{-/-}$ x TNF$\alpha^{-/-}$ are transplanted into irradiated MRL/*lpr* mice, we would predict from *in vitro* data that the mice will not be protected from disease because they lack the

repressive soluble factors that regulate autoreactive B cells. Because wildtype stem cell transplantion protects from lupus, this data would suggest that the lack of IL-6, CD40L, and TNFα causes disease.

The ultimate goal of understanding B cell tolerance mechanisms is to create more specific clinical therapies for the prevention and treatment of disease. Interestingly, the use of antibodies against IL-6, TNFα, and CD40L has been studied for the treatment of lupus for good reason. IL-6, TNFα, and CD40L all promote B cell activation, differentiation, and Ig production as previously described. In addition, IL-6 and TNFα are proinflammatory cytokines that cause tissue damage. Significantly, lupus patients with active disease have increased serum concentrations of IL-6, TNFα, and CD40L. Thus, it was logical to think that blocking their actions would decrease autoreactive B cell activation and symptoms of disease. However, our data suggest that these therapies would promote the break in tolerance of newly generated autoreactive B cells because IL-6, TNFα, and CD40L repress chronically antigen-experienced B cells. As a matter of fact, anti-TNFα therapy has been found to cause lupus-like symptoms in several cases. Yet, the findings that lupus patients with active disease have increased serum concentrations of IL-6, TNFα, and CD40L seem contradictary to our data. Preliminary studies suggest that lupus autoreactive B cells are not repressed by IL-6, sCD40L, and TNFα. Thus, once B cells break tolerance in lupus patients, they may no longer be able to respond appropriately to the increased serum concentrations of repressive factors. It would be interesting to determine whether autoreactive B cells from lupus mice pre-disease are capable of being repressed by the soluble factors and if this corresponds with normal serum levels of IL-6, sCD40L, and TNFα.

The goals of therapy are to eliminate autoreactive B cells and/or restablish tolerance in autoreactive B cells. Currently, the standard of care for SLE is immunosuppressive or anti-inflammatory drugs, with recent therapies focusing on B cell depletion. Rituximab, the anti-CD20 monoclonal antibody that depletes B cells, has been used to treat lupus with some success for the last decade. The antibody depletes all B cells except antibody secreting plasma cells, yet some patients respond with better clinical outcomes. The rationale for B cell depletion is obviously to rid the immune repertoire of B cells that produce pathogenic antibodies. However, B cell depletion could also be used as a reset button, allowing bone marrow stem cells to regenerate the B cell repertoire. In conjunction, drugs that regulate autoreactive B cells could supplement B cell depletion to maintain tolerance of newly generated autoreactive B cells that escape central tolerance and enter the periphery. As more therapies are created to maintain tolerance of autoreactive B cells, the supplement to B cell depletion can be catered to the patient. This combination therapy would provide a more individualized treatment in comparison to the general immunosuppressive drugs.

The balance between immunity and tolerance is a very intricate system. Our work challenges the roles of DCs, MΦs, IL-6, CD40L, and TNFα in this balance. We submit that these cells and soluble factors are both positive and negative regulators of B cell activation and antibody production. Pleiotropic function of cells and cytokines in the immune system is not uncommon and depends on timing, location, and environment. For example, MΦs act as chronic inflammatory cells and are important in the resolution of inflammation. DCs are very efficient at priming T cell responses and can induce T cell tolerance (145, 146, 236). TNFα and CD40L have the potential to block or induce apoptosis depending on the cellular context (237, 238). Although new treatments have been helpful in many autoimmune

patients, we caution the use of anti-IL-6, CD40L, and TNFα therapies as their side effects

may involve lupus-like symptoms in the long-term.

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