THE ROLE OF APOPTOTIC CELLS IN THE BREAKDOWN OF B CELL TOLERANCE

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ABSTRACT

KRISTEN N. KRUM: The role of apoptotic cells in the breakdown of B cell tolerance
(Under the direction of Barbara Vilen)

We have recently shown that dendritic cells (DCs) and macrophages (MΦs) are able to produce IL-6, sCD40L, and TNFα that regulate autoreactive B cells. In systemic lupus erythematosus (SLE) autoreactive B cells are dysregulated allowing for their activation, autoantibody production, and loss of susceptibility to DC/MΦ-mediated regulation. Apoptotic cells have been found to contribute to dysregulation and disease pathogenesis in SLE patients and lupus-prone mice, such as *MerTK*^{kd} or *lpr* mice. We hypothesized that if an apoptotic cell burden is responsible for dysregulation of autoreactive B cells, then removal of the apoptotic cell burden by injected C57BL/6 DCs and MΦs should restore susceptibility of autoreactive B cells to DC/MΦ-mediated regulation. We found that autoreactive B cells from mice with clearance defects, *MerTK*^{kd} and *lpr*, were not susceptible to DC/MΦ-mediated regulation. Injecting C57BL/6 DCs and MΦs into 2-12H/*lpr* mice restored autoreactive B cells to be susceptible to CD40L and increased basal pERK levels, while not reducing the apoptotic cell burden in the spleen. Also, DC/MΦ-treated 2-12H/*lpr* mice showed reduced autoantibody levels. In DC/MΦ treatedB6/*lpr* mice, susceptibility to IL-6 was restored in autoreactive B cells, but there was no change in apoptotic cell burden in the spleen. In addition, autoantibody production by B6/*lpr* mice was not affected by DC/MΦ

treatment. Further optimization of apoptotic cell removal and autoantibody production could make DC/MΦ injection a potential therapeutic for SLE.

DEDICATION

To my Mom, Lori, Gary, Leah, Jasmine, Belle, Sequoia, Cheddar, Cara, Rosie, Lexi, and Callie

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ABBREVIATIONS

Ab – Antibody

ASC – Antibody-Secreting Cell

B6 – C57BL/6

BAFF – B cell-Activating Factor (also known as BLyS)

BCR – B cell receptor

Breg – Regulatory B cell

DC – Dendritic cell

dsDNA – double-stranded deoxyribonucleic acid

ELISA – Enzyme-linked immunosorbent assay

ELISpot – Enzyme-linked immunosorbent spot

FACS – Fluorescent-activated cell-sorting

FBS – Fetal Bovine Serum

HRP – Horseradish peroxidase

Ig – Immunoglobulin

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IL-6 – Interleukin 6

IL-10 – Interleukin 10

IC – Immune Complex

IFN – Interferon

i.v. – intravenous

lpr – Fas^{lpr}

LPS – Lipopolysaccharide

MΦ – Macrophage

MΦCM – Macrophage-conditioned media

MRL/*lpr* – MRL/MpJ-Fas^{lpr}

PAMP – Pathogen-associated molecular pattern

pDC – Plasmacytoid Dendritic cell

PRR – Pattern recognition receptor

rIL-6 – recombinant interleukin 6

rsCD40L – recombinant soluble CD40 ligand

sCD40L – soluble CD40 ligand

SLE – Systemic lupus erythematosus

Sm – Smith Antigen

Tg – transgenic

TLR – Toll-like receptor

TNFα – Tumor Necrosis Factor α

Treg – Regulatory T cell

CHAPTER 1

INTRODUCTION

B cell tolerance

During development of the B cell repertoire, there is expression of a vast array of B cell receptors (BCRs). These BCRs can recognize foreign antigens of pathogens and as a by product there will also be the expression of receptors that recognize self antigens. About 50% of BCRs are specific for self-antigens [1]. Therefore, mechanisms are required to silence these autoreactive B cells. Many mechanisms have been identified that regulate autoreactive B cells, including receptor editing, deletion, and anergy. Without the ability to regulate autoreactive B cells, autoimmune diseases could occur, such as systemic lupus erythematosus, rheumatoid arthritis, or diabetes.

Receptor editing and clonal deletion are the main mechanisms to regulate the B cell repertoire in the bone marrow [2]. Affinity of the BCR contributes to B cell fate [3-5]. Stronger affinity to antigens drive B cells toward receptor editing or clonal deletion while lower affinity to antigens induces anergy [3, 4, 6].The initial response of self-reactive BCRs is receptor editing, which occurs through rearrangement of the variable, diversity and joining segments of the B cell receptor[2, 7]. The goal of receptor editing is to produce a new and less autoreactive BCR [2, 8]. Receptor editing continues until the receptor is either no longer autoreactive or when rearrangements are not possible [1]. Clonal deletion results when no more BCR rearrangements are possible or the BCR affinity to auto-antigen is too high [7, 8]. However, despite these mechanisms, some autoreactive B cells escape into the periphery where they are silenced through anergy, a process that impedes signaling through the B cell receptor [1, 6, 8]. Additionally, autoreactive B cells in the periphery can go through BCR editing and deletion [9-12].

Innate and Cellular B cell Regulation

The innate immune system allows for the rapid response to pathogens through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) [13]. There are many PRRs, such as NOD-like receptors and RIG-I-like receptors, but of particular interest are toll-like receptors (TLRs) of which there are 13 identified [14]. These receptors are displayed on macrophages, dendritic cells, B cells, T cells, natural killer cells, neutrophils, and many other cells including those within tissues [15, 16]. TLRs are able to recognize viral and bacterial components, including both single- and double-stranded RNA and DNA, through TLR7 and TLR9 respectively [13-17]. Activation of NFκB and IRF transcription factors occur when TLRs bind their respective ligand [13, 15, 16]. These transcription factors promote the production of inflammatory cytokines and type I interferon, which induces an immune response and brings about clearance of the pathogen [13, 15, 16, 18]. There are also intracellular negative regulators of the TLR-signaling, such as TOLLIP and IRAK-M, which interact directly with molecules in the signaling pathway to inhibit pro-inflammatory cytokine production and the inflammatory response [13, 15, 16, 18].

In addition to recognition of self-antigens by the BCR, B cells can detect self-antigens through TLRs. Of particular interest are TLRs 7 and 9, which are expressed by B cells and recognize viral RNA and microbial DNA along with their ability to bind self-RNA and -DNA, respectively. This ability to recognize self-antigens can promote autoimmune responses if they are not properly regulated. Regulation of endosomal TLR-signaling in anergic autoreactive B cells is important in preventing the production of autoantibodies [19, 20].

When an autoreactive BCR binds self-antigen internalization occurs, however, through a JNK-mediated mechanism, the BCR-antigen endosome is unable to merge with a TLR7 or TLR9 containing endosome, ultimately blocking TLR stimulation [21]. On a lupus-prone MRL/*lpr* background, this TLR-signaling regulation is lost indicating that B cell tolerance during innate immune responses in part, relies on regulation within the endocytic pathway [21].

Autoreactive B cells can also be controlled through additional receptors. One inhibitory receptor that regulates B cell-mediated immune responses is FcγRIIb. These responses are regulated through co-ligation of FcγRIIb and BCR by IgG containing immune complexes. This co-ligation inhibits BCR-signaling, activation, antigen presentation, proliferation, and antibody production [22-24]. It has been found that FcγRIIb is a major regulator of autoreactive B cells, since mice lacking this receptor display a lupus-like phenotype including autoantibody production and glomerulonephritis [25, 26].

In addition to anergy, peripheral B cells specific for self antigens are regulated by regulatory B and T cells (Bregs and Tregs, respectively), plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (DCs), and macrophages (MΦs) [27-30]. B regulatory cells promote the formation of Tregs and tolerize autoreactive T cells [31]. The autoreactive T cells are therefore inactivated, which prevents their interaction with autoreactive B cells, thus blocking T-dependent autoantibody production [31]. In addition, both Bregs and Tregs produce IL-10, an anti-inflammatory cytokine, which inhibits T helper cell type 1 and T helper cell type 2 responses and the production of pro-inflammatory cytokines by dendritic cells and macrophages [27-30]. pDCs promote enhanced survival and autoantibody

production of autoreactive B cells in a TLR, soluble factor, and contact-dependent manner [32].

We have recently found that DCs and MΦs repress autoreactive B cells in a TLR-dependent mechanism [33, 34]. When DCs and MΦs are LPS-stimulated, they produce soluble factors, IL-6, sCD40L, and TNFα, that repress TLR4-induced Ig secretion (Wagner, et al. in preparation) [33, 34]. Efficacy of DC/MΦ-mediated regulation was obtained using mice deficient in IL-6, sCD40L, and TNFα. LPS-stimulated autoreactive B cells injected into mice lacking the three repressive factors were unable to be regulated, while those injected into wild type mice were regulated (Wagner et al. in preparation). These results indicate a function of TLR4-induced repression *in vivo* by DCs and MΦs. Mechanistically, the soluble repressive factors prevented autoreactive B cells from differentiating into antibody secreting plasma cells (Wagner et al. in preparation)[33, 34]. Through this mechanism of repression, onset of autoimmunity is prevented by blocking the production of autoantibodies by autoreactive B cells.

Repression of Ig secretion by these soluble factors is selective in that only B cells chronically stimulated by self-Ag repress Ig secretion while naive B cells secrete antibody [33, 34]. The selective DC/MΦ-mediated regulation of autoreactive B cells suggests that chronic stimulation of the BCR is involved in the cells response to stimulation [35]. Chronic BCR stimulation promotes phosphorylation of ERK increasing basal levels and promoting an anergic phenotype [35]. When ERK phosphorylation in autoreactive B cells is blocked by MEK inhibitors, susceptibility to the repressive factors is lost (Lee et al. in preparation). This

indicates that DC/MΦ-mediated regulation of autoreactive B cells is dependent on MEK/ERK pathway.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic multisystemic autoimmune disease. It is characterized by the production of autoantibodies, which occurs when autoreactive B cells break tolerance and secrete autoantibodies specific for nuclear antigens, such as DNA, histones, snRNPs, and RNA. These autoantibodies bind to apoptotic cells and create immune complexes, which are deposited in tissues throughout the body causing inflammation, tissue damage, and organ failure [36-39]. The organs affected by SLE are the kidneys, skin, blood vessels, joints, and the nervous system [40]. Additionally, autoantibodies to DNA and Smith (Sm) are used diagnostically since they associate with SLE clinical symptoms and disease activity [38, 41-48]. Anti-dsDNA antibodies are found in individuals with nephritis and tend to increase when a patient is about to have a flare in disease activity [37, 38, 45, 49]. Anti-Sm levels are prevalent in approximately 25-30% of SLE patients and lupus-prone mice and correlate with poor prognosis [50-52]. Unfortunately, the etiology for SLE is unknown. However, genetic and environmental components have been found to contribute to disease susceptibility. Many susceptibility loci have been identified in SLE patients that correlate with TLR and interferon (IFN) signaling pathways and apoptotic cell clearance that contribute to dysregulation and disease pathogenesis [53-55]. SLE patients present with a variety of symptoms and

exposure to different environmental factors and the presence of varied genetic components could contribute to this diversity.

Murine models of SLE

There are many murine models of SLE used to determine components of disease activity. Two of the main models are the MRL/Mp$^{lpr/lpr}$ (MRL/*lpr*) and the F1 generation, NZB/W, of the New Zealand Black (NZB) x New Zealand White (NZW). Both of these models spontaneously develop SLE and produce autoantibodies and develop glomerulonephritis, lesions, and joint inflammation [56] These mice are good models for SLE because their disease manifestations are similar to human SLE clinical symptoms.

The *lpr* gene encodes for the Fas protein, which is involved in apoptosis. In *lpr* (for lymphoproliferation) mice, there is a defect in the Fas protein, which makes the cells from these mice deficient in apoptosis. This apoptosis defect allows autoreactive B cells that would have been deleted to escape into the periphery [56]. In addition, mice with the *lpr* gene have lympadenopathy, splenomegaly, and produce autoantibodies [56-58]. Recently, *lpr* mice have been shown to have an increase in apoptotic cells due to a phagocytosis defect in macrophages [59]. MRL/Mp mice have a genetic predisposition to develop autoimmune disease [57]. When MRL mice were being created, the *lpr* mutation arose spontaneously and was found to accelerate the time to disease onset in these MRL/*lpr* mice[57].

One antigen of specific interest in SLE is Smith (Sm). SLE is the only autoimmune disease to have antibodies towards this antigen [50, 51]. About 25-30% of SLE patients

present with anti-Sm antibodies [44, 46, 52]. In addition, SLE patients who have anti-Sm antibodies tend to have a more severe disease and as a result have a poorer prognosis [44, 46, 50, 51]. Ig-transgenic mice have been made that have the 2-12 heavy chain specific to Sm antigen [60]. Since they are only transgenic for the heavy chain, they are able to pair with endogenous light chains allowing for a variety of affinities to Sm. These Ig Tg mice maintain anergy on non-autoreactive backgrounds [33, 34, 60]. When the 2-12H mice were crossed with *lpr* mice, making 2-12H/*lpr* mice, the autoreactive B cells break tolerance and secrete autoantibodies [59]. This same result was also seen when the 2-12H was placed on an MRL/*lpr* background [60].

Apoptotic cells

Apoptosis, a form of programmed cell death, occurs continuously in organisms to allow for replacement of old and damaged cells. During apoptosis, cells display nucleic antigens on their surface and release signals which promote their rapid clearance by multiple cell types including macrophages and dendritic cells [61, 62]. The ingestion of apoptotic cells promotes the production of anti-inflammatory cytokines, preventing a response to auto-antigens and inflammation [61, 63]. However, when rapid clearance does not occur, apoptotic cells can become necrotic or opsonized by auto-antibodies, leading to engulfment through receptors that promote pro-inflammatory cytokine production by phagocytes and inflammation [61, 63, 64].

An inability to clear apoptotic cells allows for increased amounts of auto-antigen, since nuclear antigens are displayed on the surface of cells undergoing apoptosis [62].

Delayed clearance allows apoptotic cells to undergo secondary necrosis, which leads to blebbing of the membrane and release of potent auto-antigens into the extracellular space [62, 65-68]. This provides a source of nuclear antigen that can bind to TLRs and the BCR, leading to B cell activation and autoantibody production [69-71]. Production of autoantibody and the presence of auto-antigen via apoptotic cells allows for the formation of immune complexes (ICs). Immune complexes are deposited in tissues throughout the body promoting an inflammatory response through binding to FcγRs [36, 37, 39, 72-77].

Many murine models of lupus harbor defects in apoptotic cell clearance by macrophages. In mice with Fas^{lpr} or $MerTK^{kd}$ deficiencies, macrophages are unable to phagocytose apoptotic cells [59, 78, 79]. In $MerTK^{kd}$ mice the defect was through the MerTK receptor and not FcγR dependent, since there was no difference in clearance of opsonized apoptotic thymocytes between macrophages from $MerTK^{kd}$ mice and wild type mice [78, 79]. Both Fas^{lpr} and $MerTK^{kd}$ mice have an increased apoptotic cell burden through TUNEL staining of the spleen and lymph nodes as compared to a wild type mouse [59, 78, 79]. Increased apoptosis and clearance defects have been observed in SLE patients [36, 66, 67, 75, 80-83]. One example is that cells in the bone marrow from SLE patients die more quickly than observed in cells from healthy controls by increased CD40 expression [84]. Therefore, clearance defects have are common in lupus-prone mice and SLE patients and may contribute to disease pathogenesis.

Inflammation in autoimmunity is caused by self-antigens and their continual presence through a lack of clearance that promotes chronic inflammation. Plasma cells have been found to migrate to inflamed kidneys in NZB/W F1 mice and increase the

antibody concentrations and immune complex deposition present in the kidney [85-87]. Upregulation of chemokines, such as CXCL9, CXCL10, and CXCL11, by cells in the inflamed tissue promote migration of immune cells to the site and survival niches for infiltrating cells [86, 88]. IL-5, IL-6, TNFα, BAFF, and CXCL12 production is required for plasma cell survival [85, 86, 88-90]. Plasma cell survival niches will be maintained through chronic inflammation driven by immune complex deposition and sustained presence of infiltrating immune cells [86, 87]. Furthermore, autoantibody-secreting plasma cells in the tissue enhance and perpetuate the pathogenesis occurring through formation and deposition of immune complexes within the tissue [86-88, 91]. The persistence of inflammation in the kidneys and other tissues in lupus patients will provide a permanent home for plasma cells producing both auto- and non-autoantibodies and leading to destructive damage of the organ [86-88].

Regulatory Defects in SLE

A breakdown in any of the mechanisms regulating autoreactive cells can contribute to the development of autoimmune diseases/autoimmunity. Defective regulation of TLR7, TLR9, and FcγRIIb, contribute to autoimmunity through the production of autoantibodies [18, 25, 92-94]. Upregulation of TLR expression increases signaling and activation while promoting plasma cell differentiation to produce anti-RNA and anti-DNA autoantibodies [18, 20, 93]. Mice lacking TLR7 showed amelioration of disease symptoms [20, 93]. Similarly, anti-RNA antibodies in human SLE is driven by TLR7 [20, 93]. Mice lacking TLR9 have exacerbated disease symptoms [93]. Similarly in human SLE expression of TLR9 is increased on B cells and monocytes from patients with active disease and lupus nephritis

[95]. In many SLE patients, a loss of function defect has been found in FcγRIIb and contributes to a loss in inhibitory signals and activation of many cell types promoting inappropriate or sustained signaling [24, 25, 96]. In addition, a defect in FcγRIIb is present in a majority of murine autoimmune models, such as NZB and MRL, and when displayed in B6 mice, there is an increase in autoantibody production, indicating its role in immune regulation [26].

Regulatory cells and cytokines are also dysregulated in SLE and contribute to disease pathogenesis. Lupus-prone mice display an increase in IL-10 producing Breg cells. Bregs reside in the marginal zone and are more than doubled in frequency in lupus-prone mice compared to wild type control mice [97]. The presence of Bregs in lupus patients show a similar trend where both the frequency and the absolute number of Bregs is increased [98]. However, Bregs from SLE patients have been shown to produce to little or too much IL-10 when compared to healthy controls, indicating that they can contribute to SLE disease [99, 100]. Defects in apoptotic cell phagocytosis lead to an increased amount of apoptotic cells and impact cytokine production by dendritic cells and macrophages. Macrophages from lupus-prone mice exhibit decreased pro-inflammatory cytokine production and an increase in IL-10 production compared to non-autoimmune prone mice [82, 97, 101]. Interestingly, macrophages cultured from lupus-prone mice without fetal bovine serum (FBS) showed cytokines produced at levels comparable to those from non-autoimmune mice [102]. The role of FBS in cytokine dysregulation of lupus-prone mice macrophages implicates the apoptotic cell burden in the mice, since FBS contains anionic lipids found on the apoptotic cell surface and delipidation of the FBS can fully remove the defect [102].

The repressive factors produced by DCs and MΦs through TLR4 stimulation are reduced in lupus-prone mice (Wagner et al. in preparation)[34, 103]. Stimulation through TLR4, TLR7, and TLR9 showed defects in IL-6 production by DCs from MRL, MRL/*lpr*, NZM2410, and NZB/W F1 lupus-prone mice along with reduced production of TNFα by TLR4-stimulated MRL/*lpr* DCs (Wagner et al. in preparation) [103]. Similar defects in IL-6, sCD40L, and TNFα production were seen in TLR4-stimulated MΦs from MRL/*lpr* mice (Wagner et al. in preparation) [34]. However, DCs from B6/*lpr* mice secrete IL-6 at levels comparable to C57BL/6 DCs. This indicates that the cytokine secretion defect is related to the MRL background and not to defects in Fas production [34, 103]. The inability of DCs and MΦs from MRL/*lpr* mice to secrete the repressive factors suggests a point of dysregulation in autoreactive B cells. Indeed, autoreactive B cells fail to repress Ig secretion in culture with DCs or MΦs from MRL/*lpr* mice [34, 103]. This indicates that diminished secretion by lupus-prone DCs and MΦs contribute to the break in tolerance.

Natural and Pathogenic Autoantibodies

Antibodies play an important role in immunity and autoimmunity by binding to foreign and self antigens and promoting removal of immune complexes. Antibodies are produced in IgM, IgD, IgG, IgA, and IgE isotypes each having unique functions in pathogen neutralization and hypersensitivity. Production of autoantibodies has been associated with a number of autoimmune diseases. Of particular interest due to their protective and pathogenic roles are the IgM and IgG isotype autoantibodies.

Natural antibodies are produced by B cells that recognize both self and non-self antigens with a low affinity [104]. The majority of natural antibodies are IgM, but IgG and IgA have been found [105, 106]. Natural antibodies are beneficial in the clearance of pathogens and apoptotic cells [107, 108]. Natural antibodies promote clearance of apoptotic cells in a non-inflammatory manner through use of a C1q or mannose-binding lectin dependent pathway along with many other pathways that prevent the production of pro-inflammatory cytokines [107, 109, 110]. Prevention of activation and maturation of phagocytes is accomplished by natural antibody-mediated clearance [107].

In lupus, autoantibodies contribute to inflammation, disease progression, and tissue damage. IgG isotype antibodies are pathogenic in lupus patients and are produced by antigen-stimulated B cells [111, 112]. Through DNA sequencing, IgG autoantibodies had higher amounts of somatic mutations as compared to IgM antibodies, indicating affinity maturation of the BCR [113]. A portion of the dsDNA IgG antibodies bind to multiple antigens, especially glomerular proteins like α-actinin and laminin involved in kidney structure [113-116]. Impact of IgG and IgM autoantibodies on lupus disease symptoms were determined through studies on their presence in affected tissues and disease activity in lupus patients, and mouse studies with NZB/W F1 and activation-induced deaminase (AID) deficient MRL/*lpr*. SLE patients with higher IgM autoantibodies had a less severe disease as compared to patients with an increased amount of IgG autoantibodies [117-119]. NZB/W F1 mice showed a change from IgM to IgG autoantibodies as their age increased [120]. Finally, MRL/*lpr* deficient in AID, required for class-switching and somatic hypermutation, did not develop glomerulonephritis or cellular infiltration of the kidneys

and had an increase in anti-dsDNA IgM and survival as compared to MRL/*lpr* mice [121]. IgM autoantibodies show a protective role in lupus by preventing nephritis or other lupus symptoms along with blocking anti-dsDNA IgG [116, 118-121].

Therapeutics

There are many treatment options available for SLE patients, most of which focus on suppressing the immune system. Some of the main treatments are NSAIDs, corticosteroids, antimalarials, azathioprine, cyclophosphamide, and methotrexate. Although these treatments are effective, they possess side effects with prolonged use. Corticosteroids, such as prednisone, inhibit cytokine secretion and are very common in treating SLE patients, since it is used for skin lesions, arthritis, and systemic disease including nephritis and vasculitis with the negative aspects being weight gain, diabetes, glaucoma, and increased risk of infection [40]. The benefits of antimalarials, hydroxychloroquine and quinacrine, is the sparing of steroids while providing synergistic effects through their combined usage and work by inhibiting antigen-processing and cytokine production[40, 122]. Side effects associated with antimalarials are skin discoloration and ophthalmologic damage [40]. Cyclophosphamide is a chemotherapeutic agent and the main treatment for SLE patients with severe organ damage [40]. Nevertheless it can cause fertility problems and increased risk of infections [40]. Finally, azathioprine, which prevents nucleic-acid synthesis and spares the use of steroids, is used to treat nephritis and other SLE symptoms with toxic side effects in the bone marrow and gastrointestinal system [40].

The toxicity of the conventional treatments has lead to recent advances in treating SLE. Antibody to CD20 (Rituximab) removes B cells from the patient to prevent flares and improve clinical symptoms. The chimeric antibody contains mouse components which can induce an immune response to the antibody [123, 124]. In addition, patients with incomplete B cell depletion show no improvement of symptoms [123-127]. Generation of a fully human anti-CD20 antibody will remove the concern for responses toward the mouse contributions to the current antibody. Mixed results were seen in serum autoantibody titers, where those to RNA products were unaffected while those against dsDNA were reduced [123-126]. Since plasma cells do not express CD20, the inability of the Rituximab antibody to bind plasma cells and drive their depletion can explain these effects on serum autoantibody titers [128].

There are many ideas for transplantation of stem cells or bone marrow to alleviate disease or even cure lupus patients. However, these studies in mice have resulted in mixed abilities to treat and prevent SLE pathogenesis [127]. In two studies looking at the ability of mesenchymal stem cells to treat disease symptoms in lupus-prone mouse models, one study found that NZB/W mice had worsened disease, while the study using MRL/*lpr* mice showed a decrease in nephritis symptoms [129, 130]. There are also studies determining whether hematopoietic stem cell transfer could be a method of treating lupus [127]. The use of mesenchymal stem cells along with bone marrow transplant showed an improvement in survival, renal condition, and a reduction in macrophages and double-negative T cells in the spleen [131]. Glomerulonephritis improved in mixed chimera BXSB mice that were also treated with anti-CD40L and depleted of T cells [132]. Finally, a lupus

patient received a bone marrow transplant and was symptom free for 15 years post-transplant [133]. All of these studies bring hope to this method of treatment as being a potential cure for lupus once efficacy is proved.

A final method of treatment is the neutralization of cytokines. Cytokines being targeted are IL-10, B cell activating factor (BAFF), TNFα, CD40L, IL-6 and IFNα [124, 125, 134]. Implications of IL-10 produced at a higher rate in SLE patients are unknown, but contributes to plasma cell differentiation and increased apoptosis [82, 124]. Anti-IL-10 given to NZB/W mice and SLE patients provided a protective effect in mice by preventing onset of autoimmunity while patients showed a quick response with clinical improvement [124]. BAFF is an important cytokine in B cell survival and plasma cell differentiation allowing autoreactive B cells to survive and become antibody-secreting cells that perpetuates disease [89, 124, 125]. In BAFF depleted mice, survival was enhanced and the disease was halted; however in patients, while reduction in B cells in the blood, anti-dsDNA antibodies and disease severity was not reduced [124, 134]. Lastly, TNFα can have both a protective and detrimental effect on disease, since giving TNFα to TNFα low-producing NZB/W mice slows disease pathogenesis, while its increased production in kidneys with glomerulonephritis correlates with disease severity [124]. When patients were treated with anti-TNFα, there was an improvement in kidney proteinuria and only a temporary increase in autoantibodies [124, 125]. However, when undergoing this treatment, patients needed to be treated with an immunosuppressive agent to prevent a severe reaction. While all of these cytokines reduced disease pathogenesis in open-label trials, more closed-label trials

are needed to confirm efficacy [135]. In addition, the previously mentioned treatments could potentially be used as a combination therapy to block multiple contributors.

CHAPTER 2

RESTORATION OF SUSCEPTIBILITY TO DC/MΦ-MEDIATED REGULATION BY DC/MΦ INJECTION

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that affects multiple organ systems and is characterized by the production of anti-nuclear antibodies. These autoantibodies bind to apoptotic cells and debris to form immune complexes deposited throughout tissues that promote inflammation and tissue damage [36, 37, 39, 73, 74, 81, 136-138]. Flares in disease activity correlate with increased titers of anti-dsDNA and inflammation [38, 41-46, 139, 140]. Many regulatory defects have been found in SLE, such as production and response to cytokines, complement, and apoptotic cells [61, 63, 71, 83, 96, 99, 101, 102, 125, 141]. Ultimately, the etiology of SLE is unknown, but both genetic and environmental factors contribute to disease pathogenesis. One factor that may contribute to SLE are apoptotic cells since they are found at increased levels in lupus patients and lupus-prone mice and contribute to immune complexes [36, 66, 67, 73, 75, 80-82, 142, 143].

In healthy individuals, innate immune cells clear apoptotic cells quickly and promote an anti-inflammatory response through the production of IL-10 and TGF-β ([61, 64, 72, 144]. This prevents the activation of autoreactive cells, maintains homeostasis, and promotes the formation of Bregs [144, 145]. SLE patients exhibit defects in clearing apoptotic cells [36, 37, 66, 67, 73-75, 80-82, 84, 137, 142, 143, 146, 147]. The inability to clear apoptotic cells allows for secondary necrosis which is the loss of membrane integrity and subsequent leaking of nuclear components into the periphery [39, 62, 66, 137, 139, 147]. Presentation of auto-antigens stimulates autoreactive B cells through both a TLR- and BCR-dependent manner promoting autoantibody production [24, 92, 95, 148, 149]. The *MerTK*^{kd} and *lpr*

mouse models have shown similarities to SLE patients through phagocytosis defects and increased apoptotic cell burden compared to wild type mice [59, 78, 79]. In addition, these mice also spontaneously produce autoantibodies indicating a dysregulation of autoreactive B cells [59, 78, 79].

Regulation of autoreactive B cells during innate immune responses is extremely important to prevent their activation. One mechanism that we have identified is the production of IL-6, sCD40L, and TNFα by TLR4 stimulated dendritic cells (DCs) and macrophages (MΦs) (Wagner et al. in preparation) [33, 34]. We found that TLR4-stimulated B cells from 2-12H and 2-12H/Vκ8 Ig transgenic (Tg) mice showed markedly diminished immunoglobulin (Ig) secretion when cultured with DCs, MΦs, or recombinant IL-6, sCD40L, or TNFα [33, 34]. We recently confirmed a role for the soluble repressive factors in B cell regulation *in vivo* and have found that IL-6 and sCD40L regulate TLR-induced Ig secretion by excluding phospho-ERK (pERK) from the nucleus (Wagner et al. and Lee et al. in preparation). Defects in DC/MΦ-mediated tolerance have been identified at both the level of IL-6, CD40L, and TNFα production by lupus-prone DCs and MΦs and in the ability of autoimmune prone B cells to respond to those factors and repress Ig secretion [33, 34, 103]. Whether this is a consequence of ERK-mediated defects remains unclear.

In this study, we show that coincident with an increased apoptotic cell burden autoreactive B cells from *MerTK*^{kd} and *lpr* mice fail to repress Ig. We also show that the diminished susceptibility to these repressive factors occurs coincident with decreased basal pERK. This suggested that reducing the apoptotic cell burden may aid in restoring tolerance. To test this, we injected DCs and MΦs from C57BL/6 bone marrow. We found

that autoreactive B cells from 2-12H/*lpr* mice injected with DCs and MΦs regained susceptibility to sCD40L, but not to IL-6. Surprisingly, we saw no change in the amount of apoptotic cells present by TUNEL analysis, or VAD-FMK staining. In sero-positive 2-12H/*lpr* mice, DC/MΦ treatment reduced serum autoantibodies. However, in sero-negative mice they failed to prevent the onset of autoantibody production. We found that autoreactive B cells from B6/*lpr* mice injected with DCs and MΦs regained susceptibility to IL-6. In B6/*lpr* mice, DC/MΦ treatment showed no reduction in apoptotic cells by TUNEL staining. In both sero-positive and sero-negative B6/*lpr* mice, DC/MΦ treatment did not reduce or prevent serum autoantibody. These findings suggest that the injection of C57BL/6 DCs and MΦs are able to induce tolerance to soluble repressive factors while not having an impact on the apoptotic cell burden, indicating that the apoptotic cell burden may not be the cause of the dysregulation of autoreactive B cells.

Materials and Methods

Mice. C57BL/6 (B6) and MRL/*lpr* non-transgenic mice were purchased from the Jackson Laboratory and bred in house (Bar Harbor, ME). B6/*lpr*, 2-12H/B6 Tg, 2-12H/*lpr* Tg, and 2-12H/Vκ8 Tg mice have been described [56-59]. 2-12H/Vκ8 *MerTK*^{kd} mice were created in house by breeding mice containing the *MerTK*^{kd} gene from Glenn Matsushima with 2-12H and Vκ8 mice and then crossing the two mice. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Reagents and Antibodies. LPS, TEPC 183, dsDNA, Phosphatase substrate, and 3-Amino-9-ethyl-carazole from Sigma-Aldrich (St. Louis, MO), LPS was also purchased from Invivogen (San Diego, CA), mouse GM-CSF from PeproTech (Rocky Hill, NJ), Recombinant sCD40L (rsCD40L) was purchased from R&D Systems (Minneapolis, MN), Recombinant IL-6 was purchased from BD Pharmingen (San Jose, CA), SMA3000 (Sm) and histones were purchased from Immunovision (Springdale, AR). 33-60 and B7.6 (anti-IgM) were purified from hybridoma culture supernatants, M-CSF was produced by L Cells provided by Jeffrey Frelinger (University of Arizona), and IL-4 was produced by mIL-4 cells. Anti-CD19-PE, Anti-CD3-FITC, Anti-CD11b-FITC, and Anti-CD11cPE antibodies purchased from BD PhosFlow (San Jose, CA), FITC-VAD-FMK purchased from Promega (Madison, WI), polyclonal Donkey anti-mouse IgG from Abcam (Cambridge, MA), Strep-HRP from BD Pharmingen (San Jose, CA), and Strep-AP and goat anti-mouse Ig-AP was purchased from Southern Biotech (Birmingham, AL).

Bone marrow-derived DC (BMDC) and MΦ (BMMΦ) Culture. Bone marrow-derived DCs (BMDCs) (75% pure as $CD11c^{+}$) were generated by culturing bone marrow cells with GM-CSF and IL-4 and bone marrow-derived MΦs (BMMΦs) (95% pure) were generated by culturing with M-CSF in L cell media. BMDCs were grown for 5 days and BMMΦs were grown for 8 days.

Macrophage Condition Media (MΦ CM). Macrophages were sorted from C57BL/6 spleens using MoFlo (98% pure) and plated at $1x10^5$/well in a 96-well plate with 30μg/ml LPS for 4 days. Supernatants were harvested on day 4 and frozen for later use in B cell culture.

B cell purification. Splenic B cells were isolated through negative selection by the StemSep B cell enrichment kit (StemCell Technologies, Vancouver, BC). B cells were isolated from B6 (95-99% pure), B6/*lpr* (88-99% pure), 2-12H/B6 (94-98% pure), 2-12H/*lpr* (76-98% pure), 2-12H/*lpr* DC/MΦ injected (65-90% pure), and B6/*lpr* DC/MΦ injected (95-99% pure). FICOLL gradient enrichment along with T cell depletion using biotinylated anti-CD3 was used to increase purity of B cells from B6/*lpr* and 2-12H/*lpr* mice.

B cell culture. Purified B cells ($1x10^5$ per well in a 96-well plate) were cultured with 30 μg/ml (Sigma) or 15μg/ml (Invivogen) LPS alone or with rIL-6, rsCD40L, or MΦ CM for 3 (ELISpot) or 4 (ELISA) days.

ELISAs. Total IgM was captured with anti-IgM (33-60) and detected with biotinylated anti-IgM (B7.6) antibody and Streptavidin-AP as previously described [24]. Anti-nucleosome antibodies were captured with histones and dsDNA, and the detected with either polyclonal anti-IgG-AP (Abcam), polyclonal anti-Ig-AP (Southern Biotech) or biotinylated anti-IgM (B7.6) antibodies and Streptavidin-AP. Anti-dsDNA antibodies were captured with dsDNA and detected with either polyclonal anti-IgG-AP (Abcam) or biotinylated anti-IgM (B7.6) antibodies and Streptavidin-AP. Purified mouse IgM^a/κ (TEPC 183) served as the standard

control for Total IgM, anti-nucleosome IgM, anti-dsDNA IgM, and anti-nucleosome (PL2-6) antibodies served as the standard control for anti-nucleosome IgG, Ig, and anti-DNA (PA4) antibody served as the standard control for anti-dsDNA IgG antibody.

ELISpot. ELISpots were performed using plates (Millipore, Billerica, MA) coated with 10U/ml Sm (Immunovision, Springdale, AR). B cells were harvested 3 days after culture with LPS, IL-6 and sCD40L. Cells were plated at $8x10^4$ cells per well and diluted 1:2 into 3-5 additional wells. After 8 hour incubation on Sm-coated plates, cells were washed off the plates and secreted antibody remaining on the plates was detected using biotin-labeled anti-IgM antibody (B7.6), followed by Streptavidin-HRP (BD Biosciences). Spots were developed using the substrate 3-amino-9-ethylcarbazole (Sigma-Aldrich). Plates were scanned and analyzed using the Immunospot ELISpot analyzer (Cellular Technology Ltd., Cleveland, OH).

BMDC/BMMΦ injection. 2-12H/*lpr* and B6/*lpr* mice were injected i.v. every 5 days with $6x10^6$ BMDCs and $2x10^6$ BMMΦs in 200μl of PBS or 200μl only (controls) for 6-11 weeks. Mice were bled every 1-2 weeks to determine serum autoantibody production.

TUNEL. Spleen tissues were fixed in 10% formalin, embedded in paraffin, and sectioned. Deoxynucleotidyl-transferase-mediated UTP nick-end labeling (TUNEL) assays were performed using *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Germany) according to manufacturer's instructions. Hoechst staining was used for counterstaining to visualize nuclei. Images were obtained with Zeiss710 with a 10x objective. Image processing and

analysis was done using ImageJ. Quantification of apoptotic cells in each mouse spleen was determined through counting apoptotic cells from 5-10 images and averaging the number of apoptotic cells from all images counted.

Statistical Analysis. Data was analyzed using the Mann-Whitney Rank Sum test. Significant *p* values < 0.05 are denoted by *, p values < 0.01 are denoted by **, p values < 0.001 are denoted by ***.

Results

Dysregulation of DC/MΦ-mediated tolerance of autoreactive B cells from MerTK^{kd} *and* lpr *mice*

During innate immune responses, autoreactive B cells are repressed by IL-6, sCD40L and TNFα secreted by TLR4-activated DCs and MΦs [33, 34, 103]. In lupus-prone mice, the susceptibility of autoreactive B cells to the repressive factors is lost allowing autoantibody secretion. We reasoned that perhaps the increased burden of apoptotic cells and the exposure of nuclear self-antigens on dying cells might disrupt the mechanisms that regulate autoreactive B cells during innate immune responses. To address this, we co-cultured LPS-stimulated B cells from 2-12H/Vκ8 *MerTK^{kd}* and 2-12H/Vκ8 mice with *ex vivo* DCs and MΦs sorted from C57BL/6 mice. At B cell:DC ratios of 10:1 and 20:1, we found that Ig secretion from 2-12H/Vκ8 was repressed in a dose dependent fashion as previously reported [33, 34]. However, Ig secretion from those B cells exposed to an increased burden of apoptotic

debris (2-12H/Vκ8 *MerTK*^{kd}) was significantly less repressed than 2-12H/Vκ8 B cells (Figure 2.1A).

To confirm that the secreted products of DCs and MΦs conferred the loss of susceptibility to the repressive factors, we cultured LPS-stimulated B cells from 2-12H/Vκ8 and 2-12H/Vκ8 *MerTK*^{kd} mice with MΦ CM. This CM was prepared from sorted *ex vivo* C57BL/6 MΦs cultured for 4 days with LPS. It was previously shown to contain IL-6, sCD40L, and TNFα and to repress TLR4-induced Ig secretion [33, 34]. While Ig secretion from LPS-stimulated B cells from 2-12H/Vκ8 mice was repressed 26-82%, Ig secretion from 2-12H/Vκ8 *MerTK*^{kd} mice was only repressed 0-53% (Figure 2.1B). Similarly, recombinant IL-6 and sCD40L were unable to repress B cells from the 2-12H/Vκ8 *MerTK*^{kd} mice. This suggests that during innate immune responses, B cells from mice harboring an increased burden of apoptotic cells are less susceptible to regulation by the secreted products of DCs and MΦs.

Although the *MerTK*^{kd} mice are known to have a defect in clearing apoptotic debris, it is possible that other secondary affects were impacting the B cell response to innate stimuli. To address this, we assessed whether B cells from Fas-deficient mice could be repressed. Fas-deficient mice are known to harbor an increased burden of apoptotic debris [59]. To determine whether B cells from these mice exhibited similar defects, we LPS-stimulated B cells from 2-12H/B6 and 2-12H/*lpr* mice with MΦ CM and recombinant cytokines. As shown in Figure 1C, anti-Sm antibody secreting cell (ASC) formation from 2-12H/B6 B cells was repressed 56-65%, however, B cells from 2-12H/*lpr* mice were only repressed 3-43% by MΦ CM or recombinant cytokines.

Apoptotic cells increased and pERK levels decreased in MerTKkd *and* lpr *mice*

We predicted that if apoptotic cells were responsible for the diminished susceptibility to DC/MΦ-mediated tolerance, these cells would be evident by TUNEL. Comparison of spleens from 2-12H/Vκ8 and 2-12H/Vκ8 *MerTK*^{kd} mice confirmed our prediction by the increased TUNEL-positive cells present in mice with the *MerTK*^{kd} gene (Figure 2.2A and B). We saw trends of increased apoptotic cell levels in Fas-deficient mice that showed similar defects in B cell susceptibility (Figure 2.2C and D). Lupus-prone MRL/*lpr* mice were also examined and found to display a trend in increased burden of apoptotic cells (Figure 2.2C and D). These results indicate a role for apoptotic cells to dysregulate DC/MΦ-mediated tolerance of autoreactive B cells.

The ability of IL-6 and sCD40L to repress Ig secretion is in part dependent on activation of ERK 1 and 2. Blockade of ERK phosphorylation by MEK inhibitors in autoreactive B cells prevents their susceptibility to DC/MΦ-mediated tolerance, indicating a role for pERK 1 and 2 in reprogramming of the response by IL-6R and CD40 (Lee et al. in preparation). To determine whether ERK phosphorylation was dysregulated in the B cells that lacked susceptibility to IL-6, sCD40L and MΦ CM, we measured the basal levels of pERK in B cells from 2-12H/Vκ8 *MerTK*^{kd} and 2-12H/*lpr* mice. B cells from both mice that harbored decreased susceptibility and increased apoptotic cells had reduced basal pERK levels as compared to their anergic B cell controls (Figure 2.3 A and B). Their levels of pERK were comparable to naïve B cells from C57BL/6 mice, which are not susceptible to regulation by the repressive factors. Our findings indicate that an over abundance of

apoptotic cells contributes to dysregulation of autoreactive B cell susceptibility to DC/MΦ-mediated tolerance likely through a pERK dependent mechanism.

Injection of DC/MΦ restores susceptibility of autoreactive B cells to CD40L

Loss of susceptibility to IL-6, sCD40L, and TNFα in autoreactive B cells is correlated with an increased presence of apoptotic cells. We hypothesized that removal of apoptotic cell burden from *lpr* mice would restore susceptibility of autoreactive B cells to the soluble repressive factors. To test this, we injected 2-12H/*lpr* mice with C57BL/6 DCs and MΦs every five days for six weeks and then assessed whether B cells from these mice regained susceptibility to rIL-6, rsCD40L, and MΦ CM. As shown in Figure 2.4A, autoreactive B cells from 2-12H/*lpr* mice treated with DCs and MΦs were repressed in response to rsCD40L and MΦCM, but not in response to IL-6 as compared to the PBS injected and 2-12H/B6 controls (Figure 2.4A). After 8 weeks of DC/MΦ treatment, basal pERK levels of B cells were found to be increased to levels comparable to those seen in B cells from 2-12H/B6 mice (Figure 2.4E). This indicates that injection of C57BL/6 DCs and MΦs can induce susceptibility of previously unsusceptible autoreactive B cells to DC/MΦ-mediated regulation and restore pERK to levels associated with anergy.

We hypothesized that the increased amount of apoptotic cells was the trigger for dysregulation of autoreactive B cells from *MerTK*^{kd} and *lpr* mice. To assess whether injecting DCs and MΦs into 2-12H/*lpr* mice reduced apoptotic cell burden, we performed TUNEL analysis on spleen sections. Apoptotic cells are identified through broken DNA by TUNEL staining or by any activated caspases with VAD-FMK staining. TUNEL analysis

revealed no reduction in apoptotic cells in the spleens of DC/MΦ treated 2-12H/*lpr* compared to PBS treated 2-12H/*lpr* mice (Figure 2.4 B and D). Apoptotic cell analysis by VAD-FMK staining of splenocytes indicated no reduction of apoptotic cells in the DC/MΦ compared to PBS treated 2-12H/*lpr* mice (Figure 2.4C). Therefore, the induced susceptibility of autoreactive B cells from DC/MΦ-treated 2-12H/*lpr* mice appear to not be due to the apoptotic cell burden.

*Anti-Sm antibodies reduced in DC/MΦ-treated sero-positive 2-12H/*lpr *mice*

We and others have shown that B cells from mice with *MerTK*^{kd} or *lpr* genes are dysregulated allowing for the production of autoantibodies [59, 78, 79]. Autoantibodies in SLE patients and lupus-prone mice are able to exacerbate symptoms and correlate with flares in disease activity [38, 41-46, 48, 139, 140]. Certain treatments for SLE have been shown to reduce autoantibody production along with disease symptoms [133, 150-162]. Therefore, one of our goals was to test whether the injection of DCs and MΦs would be able to reduce autoantibody levels or prevent them from being produced, particularly of the pathogenic IgG class [121]. Anti-Sm antibodies are produced by 2-12H mice and in SLE patients correlates with a more severe disease and worse prognosis [41-46, 140]. When testing DC/MΦ injection in 2-12H/*lpr* mice, we found that sero-positive mice treated with DCs and MΦs had reduced anti-Sm antibodies and when a couple of mice stopped receiving injections, they continued to remain sero-negative or making very low levels (Figure 2.5A). On the other hand, sero-negative mice were induced to make anti-Sm antibodies after the first injection, which were maintained at a constant concentration throughout the entire

experimental period (Figure 2.5B). Unfortunately, in both of these experiments the controls were not sufficient to make a clear conclusion, since only one PBS-treated 2-12H/*lpr* mouse made antibodies in each cohort. In addition, the PBS-treated mouse in the sero-negative cohort never produced antibody at the levels seen in mice in the sero-positive cohort. Therefore, our results indicate a potential role for DCs and MΦs to regulate IgM autoantibody production in Tg *lpr* mice.

*Defect in DC/MΦ-mediated susceptibility of autoreactive B cells from B6/*lpr *mice*

We previously used 2-12H/*lpr* mice that produced B cells specific for Sm. In order to determine the impact on a B cell repertoire that was more comparable to SLE patients, we utilized B6/*lpr* mice. These non-Tg mice were similar to the 2-12H/*lpr* in their spontaneous production of autoantibody and increased apoptotic cell burden. However, they had a B cell repertoire that contained autoreactive and non-autoreactive B cells. To determine whether the autoreactive B cells from B6/*lpr* mice were not susceptible to DC/MΦ-mediated tolerance, we isolated B cells from C57BL/6 and B6/*lpr* mice. We found that autoreactive B cells from B6/*lpr* mice were not susceptible to DC/MΦ-mediated regulation while those from C57BL/6 mice were regulated (Figure 2.6B). In addition, Total IgM production was unaffected by rIL6 and rsCD40L in B cells from both C57BL/6 and B6/*lpr* mice (Figure 2.6A). This indicates that the loss of susceptibility to the soluble repressive factors is maintained in non-Tg mice.

*DC/MΦ-mediated susceptibility restored in autoreactive B cells from DC/MΦ injected B6/*lpr *mice*

B6/*lpr* mice were utilized to determine the impacts this DC/MΦ treatment would have on a non-Tg mouse with increased apoptotic cells. Since these mice had both autoreactive and non-autoreactive B cells, there could be a difference in the outcomes identified from the previously treated 2-12H/*lpr* mice. B6/*lpr* mice were injected with DCs and MΦs as described above for 10 weeks. When tested for susceptibility to IL-6 and CD40L, autoreactive B cells from DC/MΦ treated B6/*lpr* mice displayed susceptibility when cultured with rIL-6, however not when cultured with rsCD40L (Figure 2.7B). Total IgM production by B cells was unaffected in all mice (Figure 2.7A). Our results suggest that autoreactive B cells from B6/*lpr* mice treated with DCs and MΦs have restored susceptibility to IL-6. This indicates that DC/MΦ treatment can restore DC/MΦ-mediated regulation in autoreactive B cells from non-Tg mice.

We utilized sero-positive and sero-negative B6/*lpr* mice. Apoptotic cell burden was determined by TUNEL-staining of spleen sections from DC/MΦ treated B6/*lpr* mice compared to C57BL/6 and PBS-treated B6/*lpr* mice. Both sero-negative and sero-positive DC/MΦ-treated B6/*lpr* mice showed similar trends to the DC/MΦ 2-12H/*lpr* mice where apoptotic cells were not reduced as compared to PBS injected and C57BL/6 mice controls (Figure 2.7C). These results indicate that clearance of apoptotic cells by injected DCs and MΦs are not improved in non-Tg mice.

Since the 2-12H/*lpr* mice are only capable of making anti-Sm IgM, we tested B6/*lpr* mice that could make all isotypes of Ig and antibodies specific for additional nuclear

antigens. Antibodies against dsDNA and nucleosomes are of particular interest due to their correlation with disease flares and lupus nephritis and as the initial antibodies produced in SLE patients due to the release of nucleosomes from uncleared apoptotic cells, respectively [38, 39, 62, 66, 111, 136, 143]. We used sero-positive and sero-negative mice to determine whether autoantibody production could be terminated or prevented. Anti-dsDNA and anti-nucleosome antibodies were quantified by ELISA for both IgM and IgG isotypes. Autoantibody production in sero-positive B6/*lpr* mice treated with DCs and MΦs were unaffected except for one mouse that showed an increased production in both anti-dsDNA and anti-nucleosome IgM (Figure 2.8A). In sero-negative mice, autoantibody production was not prevented or halted in DC/MΦ treated B6/*lpr* mice compared to PBS injected B6/*lpr* mice (Figure 2.8B). These results indicate that the injection of DCs and MΦs are unable to affect the autoantibody production in either sero-positive or sero-negative B6/*lpr* mice.

Discussion

During an innate immune response, autoreactive B cells are regulated by soluble repressive factors (Wagner et al. in preparation)[33, 34]. However, we show that autoreactive B cells from mice harboring an increased burden of apoptotic cells are not susceptible to the repressive factors and thus secrete Ig in response to TLR4 stimulation. There is a correlation between the inability to repress Ig secretion of autoreactive B cells with an increased burden of apoptotic cells. In addition, the elevated basal pERK levels associated with B cell anergy is lost coincident with loss of susceptibility to the repressive

factors. Treatment of these mice with C57BL/6 DCs and MΦs restored susceptibility to the soluble repressive factors and increased basal pERK levels to levels comparable to those seen in anergic B cells. However, the apoptotic cell burden was unaffected in the DC/MΦ treated mice. The autoantibody production in DC/MΦ treated mice was reduced in sero-positive Tg mice, but unaffected in sero-negative Tg and all non-Tg mice.

Mice that displayed an increased burden of apoptotic cells had autoreactive B cells that were not repressed by IL-6, sCD40L, or TNFα. We induced tolerance to IL-6, sCD40L, and TNFα in autoreactive B cells of DC/MΦ injected *lpr* mice, even though the apoptotic cell burden was not reduced. These results indicate that our hypothesis that susceptibility to the repressive factors would be restored through the removal of the apoptotic cell burden was not supported due to an inability to reduce the apoptotic cell burden although DC/MΦ-mediated susceptibility was restored in autoreactive B cells from DC/MΦ-treated mice. There are several reasons that could explain why the apoptotic cell burden was not reduced. First, the injected DCs and MΦs may not have homed to the spleen. MΦs enter many different tissues, while DCs home mainly to lymph nodes [63, 163]. Since the cells are being injected through the tail vein, many of them will be cleared in the liver and some may be impeded in the lungs and prevent them from reaching the spleen. Although there is not a net reduction of apoptotic cells seen in the spleen, there could be clearance occurring system wide.

Immune complexes in lupus patients and lupus-prone mice are formed from autoantibody binding to apoptotic debris. MRL/*lpr* mice that were unable to make IgG antibodies no longer displayed kidney inflammation, cellular infiltration, and nephritis [121].

In addition, the IgM autoantibodies produced in these mice appeared to be similar to natural antibodies through a lack of somatic mutation [121]. Therefore, the pathogenic forms of autoantibodies in SLE patients and lupus-prone mice are those of the IgG isotype. There was an increase in the IgM autoantibody levels of the Tg mice that were sero-negative at the start of the injections, which could be to promote clearance of apoptotic cells. However, sero-positive 2-12H/*lpr* mice receiving DCs and MΦs exhibited decreased autoantibody levels. This could indicate that enough clearance of apoptotic cells occurred to reduce IgM production. Alternatively, it might reflect a role for DCs and MΦs on plasmablast formation and survival, although the exact mechanism is unknown. Unfortunately, the majority of B6/*lpr* mice injected with cells showed no change in the autoantibody levels. One caveat is that IgG autoantibodies tend to be produced by long-lived plasma cells, have a 6-8 day half-life in mice and 7-21 day half-life in humans, and are difficult to deplete [164, 165]. Thus the sero-positive mice may not be able to become sero-negative. We also saw that autoantibody production was not delayed in DC/MΦ treated sero-negative mice, indicating that this treatment is unable to prevent plasma cell formation.

We utilized the spleen to determine apoptotic cell burden, since it has previously been shown to harbor the burden and because we obtained the B cells from here for the susceptibility experiments. There seems to be enough cells making it to the spleen to induce susceptibility, but not enough to be clearing the apoptotic cells or having an impact on the autoantibody levels. Therefore, additional studies should be conducted to determine the localization of the injected cells, which will allow for identification of areas

with the highest concentration of injected cells to observe for changes in apoptotic cell burden. By identifying the localization of the cells injected through the tail vein, we will be able to determine to what organs they localize, such as the kidneys or spleen. Additionally, cells could be directly injected into the spleen to see if they have an impact on clearance of apoptotic cells. This would remove the requirement of cells having to home to the spleen after injection through the tail vein. These studies would allow for the development of improved approaches to the DC/MΦ treatment.

The length of survival of the injected cells is unknown, which could explain why there is no difference in the amount of apoptotic cells in the dendritic cell and macrophage injected mice. One reason is because the injected cells could be dying at about the same rate as the apoptotic cells are being cleared by the live injected cells. Also, knowing the length of survival of the injected cells will be beneficial to determine how many are dying, at what rate, and if there is anything that could be done to improve their survival and ultimately their impacts on the system. The use of the previously mentioned labeling of injected cells would also allow for the determination of survival. This would be an area where the use of bone marrow chimeras would be beneficial, since the healthy dendritic cells and macrophages would be continually produced and the rate of new live cells injected would be irrelevant. DC/MΦ injection may be a good therapeutic for lupus if optimization improves the removal of the apoptotic cell burden and reduction of serum autoantibody levels.

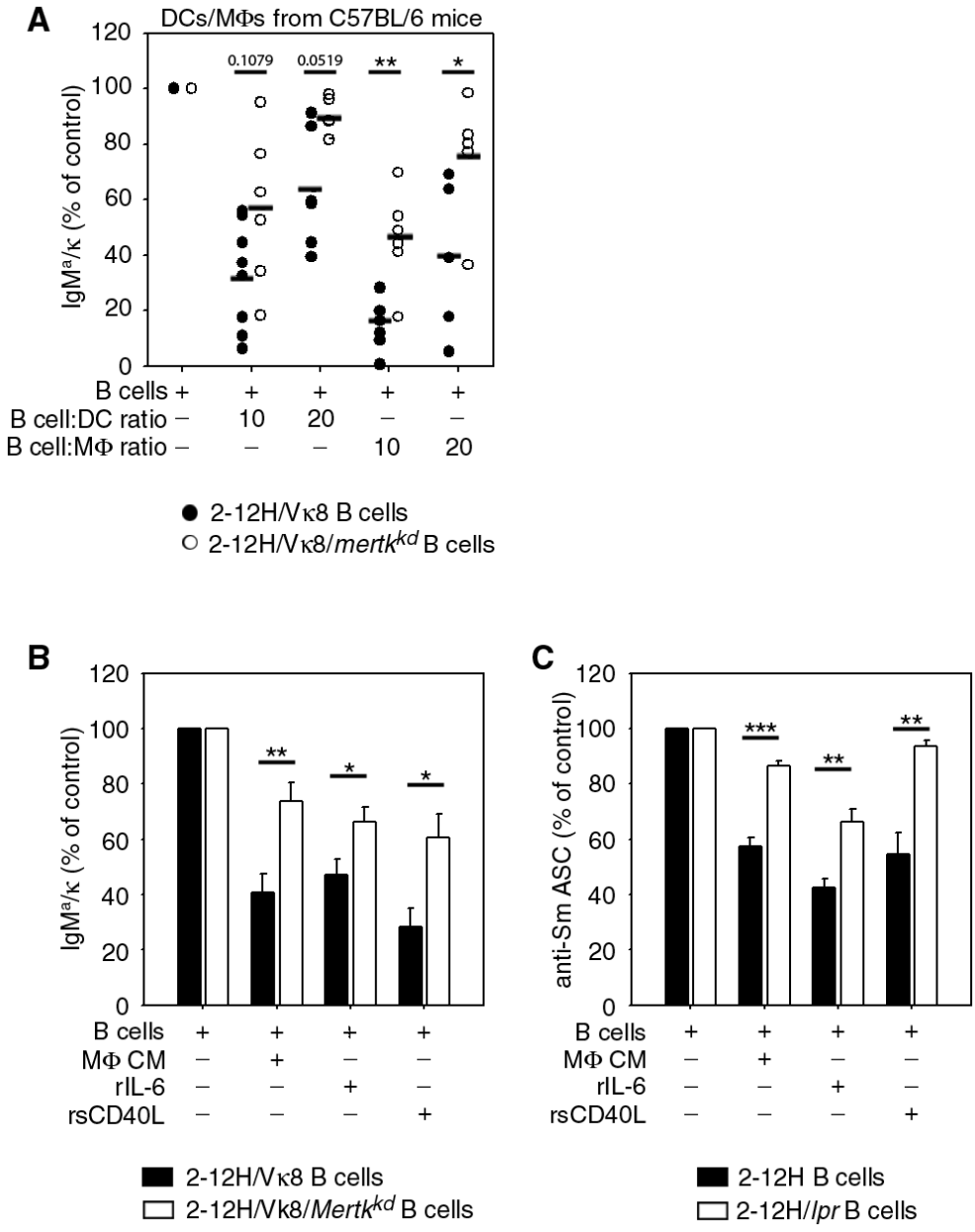

Figure 2.1. B cells from mice harboring clearance defects are not susceptible to DC/MΦ-mediated regulation. B cells ($1x10^5$) from 2-12H/Vκ8 and 2-12H/Vκ8 *MerTK*^{kd} mice were stimulated with LPS (30 μg/mL) and cultured in the presence or absence of DCs or MΦs from C57BL/6 mice at ratios of 10:1 or 20:1 B cell:DC/MΦ (A) or MΦCM, rIL-6 (20 ng/mL) or rsCD40L (100 ng/mL) (B) for 4 days. IgMa/k was quantified by ELISA. (C) B cells ($1x10^5$) from 2-12H and 2-12H/*lpr* were stimulated with LPS (30 μg/mL) and cultured in the presence or absence of MΦCM, rIL-6 (20 ng/mL) or rsCD40L (100 ng/mL) for 3 days when B cells were harvested and plated on an Sm-specific enzyme-linked immunosorbent spot (ELISpot) plate for 8 hours. Data represent at least 3 experiments. (*P < 0.05, **P < 0.01, ***P < 0.001)

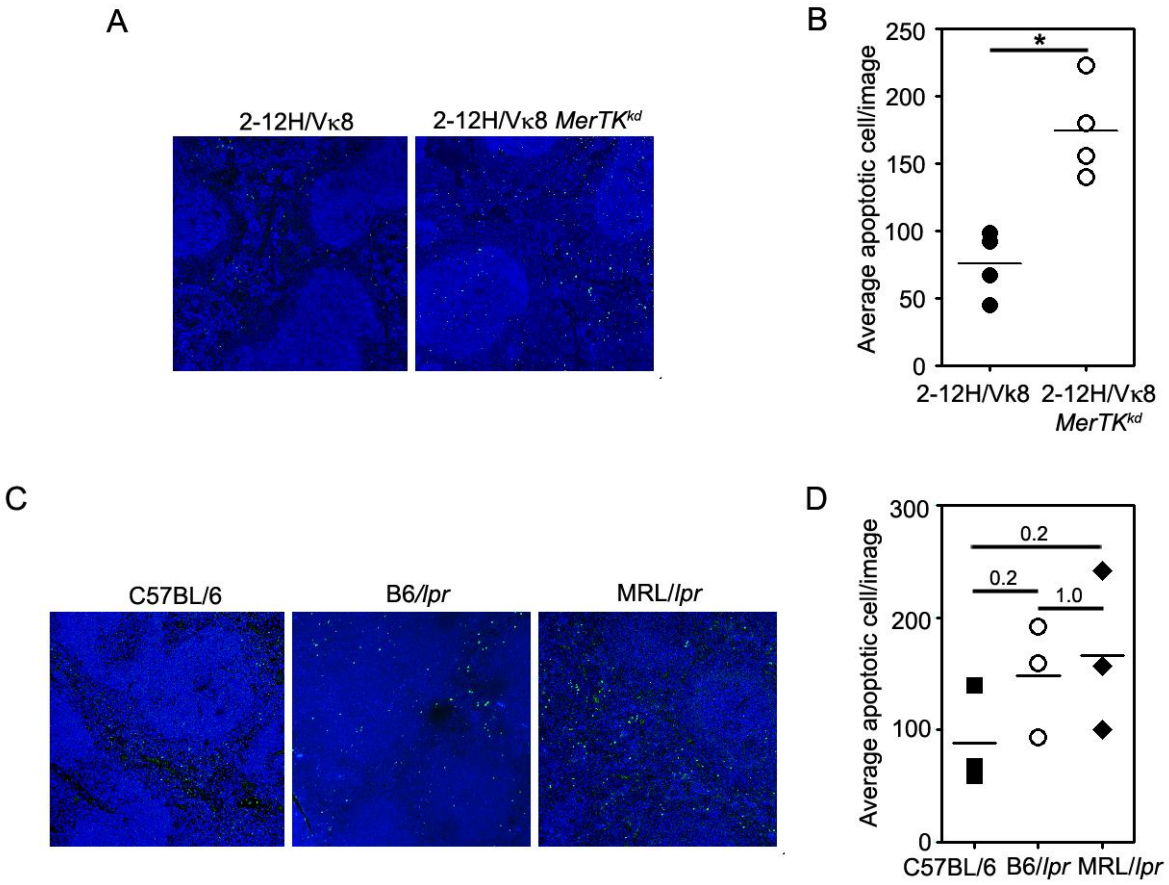

Figure 2.2. Lupus-prone mice harbor an increased burden of apoptotic cells. Apoptotic cells in spleens were detected by TUNEL (green) for 2-12H/Vκ8 and 2-12H/Vκ8 *MerTK*^{kd} (A) and for C57BL/6, B6/*lpr*, and MRL/*lpr* (C). B and D are the quantification of apoptotic cells in images. Images representative of at least 2 experiments with an n of 3. (*P < 0.05)

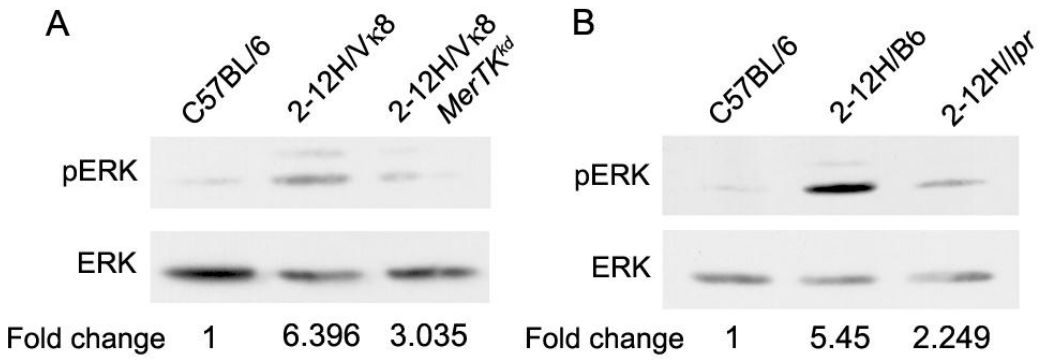

Figure 2.3. Reduced pERK levels in B cells from *MerTK^{kd}* and *lpr* mice. B cells ($3x10^6$) isolated from C57BL/6, 2-12H/Vκ8, and 2-12H/Vκ8 *MerTK^{kd}* (A) and C57BL/6, 2-12H/B6, and 2-12H/*lpr* (B) were lysed without stimulation and immunoblotted for pERK and total ERK. Normalized pERK to Total ERK then compared mouse strains. Representative of at least 3 experiments.

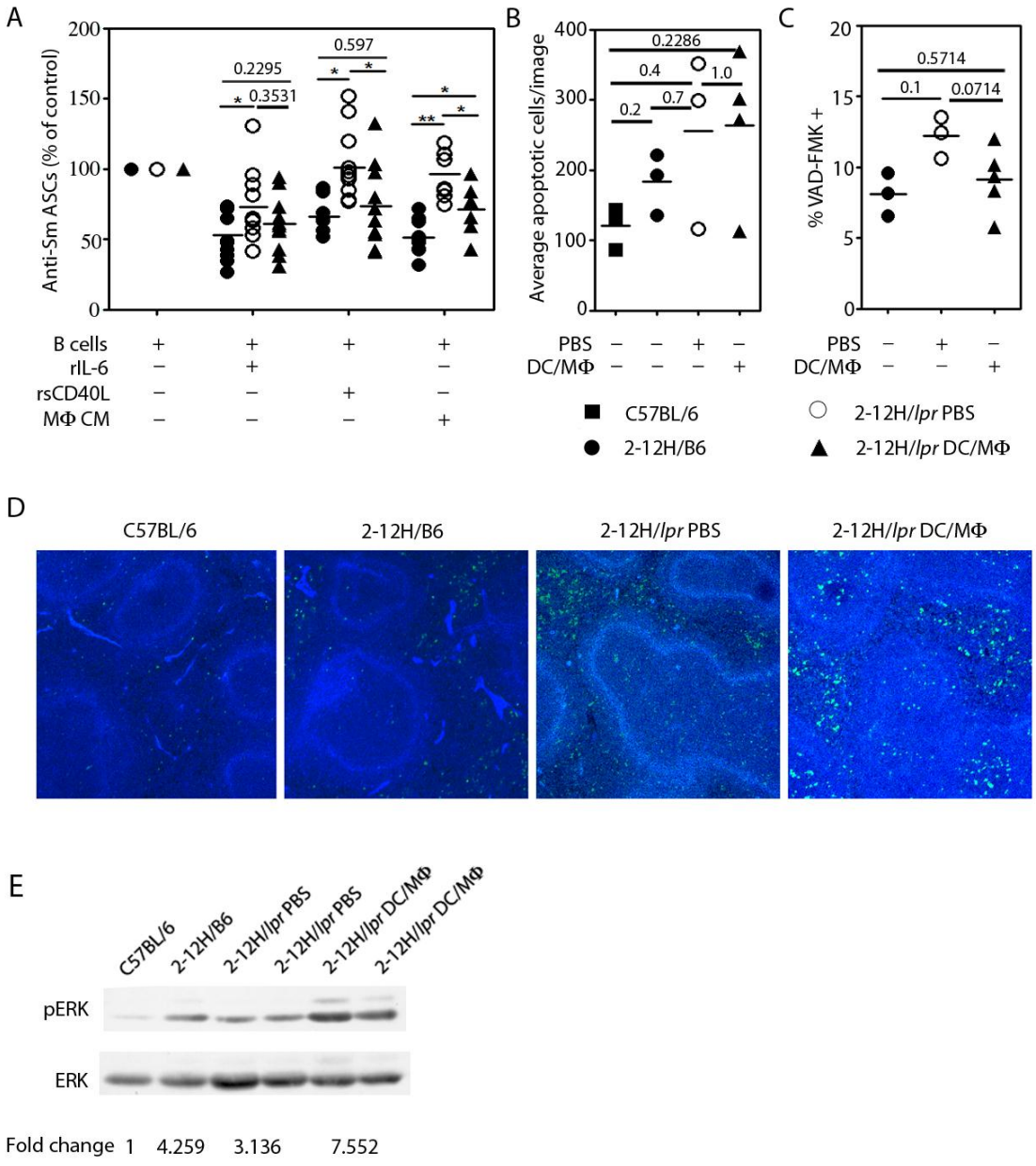

Figure 2.4. Injecting DC/MΦ into 2-12H/*lpr* mice restores susceptibility to CD40L. 2-12H/*lpr* mice were injected with PBS (200 μL) or DCs ($6x10^6$) and MΦs ($2x10^6$) every 5 days for 6 weeks. (A) At six weeks, B cells ($1x10^5$) were isolated and stimulated with LPS (30 μg/mL) in the presence or absence of rIL-6 (20 ng/mL), rsCD40L (100 ng/mL), or MΦCM for 3 days and were then harvested and plated on Sm-specific ELISpot plates for 8 hours to determine the number of antibody secreting cells (ASCs). Spleen sections from PBS and DC/MΦ treated 2-12H/*lpr* and control mice were stained with TUNEL (D) and quantified (B). (C) VAD-FMK staining of splenocytes ($5x10^5$) was utilized as another measure of apoptotic cells. (E) B cells ($1x10^5$) were isolated and lysed without stimulation and immunoblotted for pERK and Total ERK. Data represent at least 2 experiments with an n of 3. (*P < 0.05, **P < 0.01)

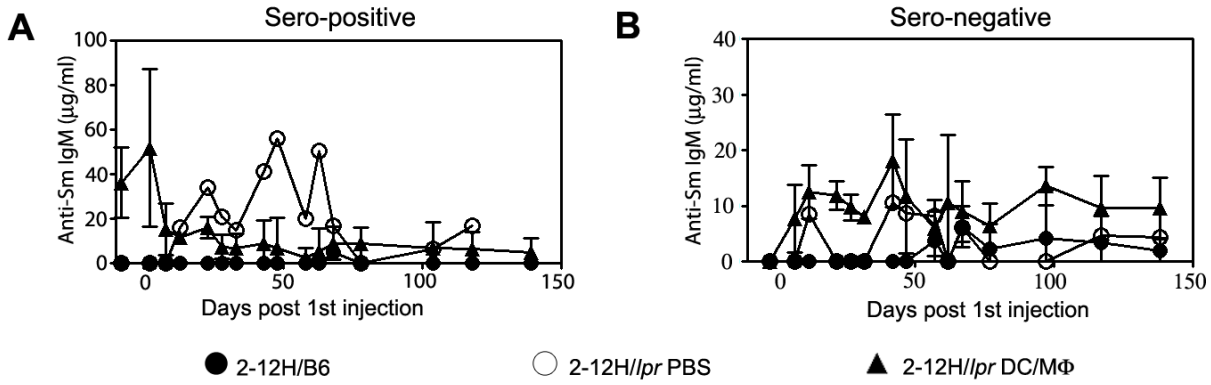

Figure 2.5 Serum autoantibody levels are reduced in 2-12H/*lpr* DC/MΦ injected mice. Sero-positive (A) or sero-negative (B) 2-12H/*lpr* mice were injected with PBS (200 μL) or DCs ($6x10^6$) and MΦs ($2x10^6$) every 5 days for 20 weeks and bled every 1-2 weeks. Serum autoantibody levels were quantified by Sm ELISA. Data represent at least 3 mice except for PBS treated mice in A and B where n = 1.

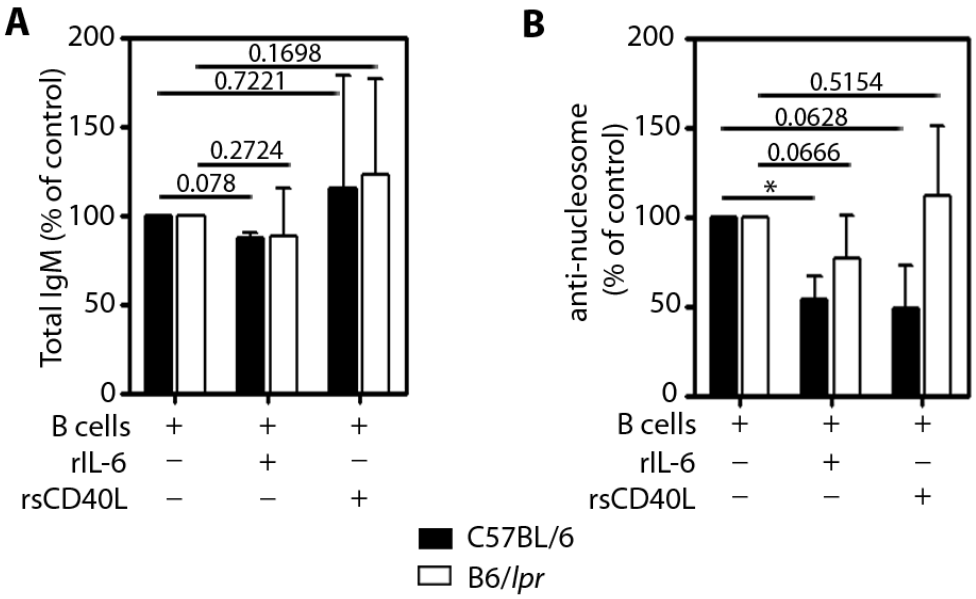

Figure 2.6 Autoreactive B cells from B6/*lpr* mice are not susceptible to Ig repression by IL-6 and sCD40L. B cells ($1x10^5$) from non-age matched C57BL/6 (6-7weeks) and B6/*lpr* (12-16 weeks)mice were stimulated with LPS (15 μg/mL) and cultured in the presence or absence of rIL-6 (5 ng/mL) or rsCD40L (25 ng/mL) for 4 days. Total IgM and anti-nucleosome Ig was quantified by ELISA. Data represents an n=3. (*$p < 0.05$)

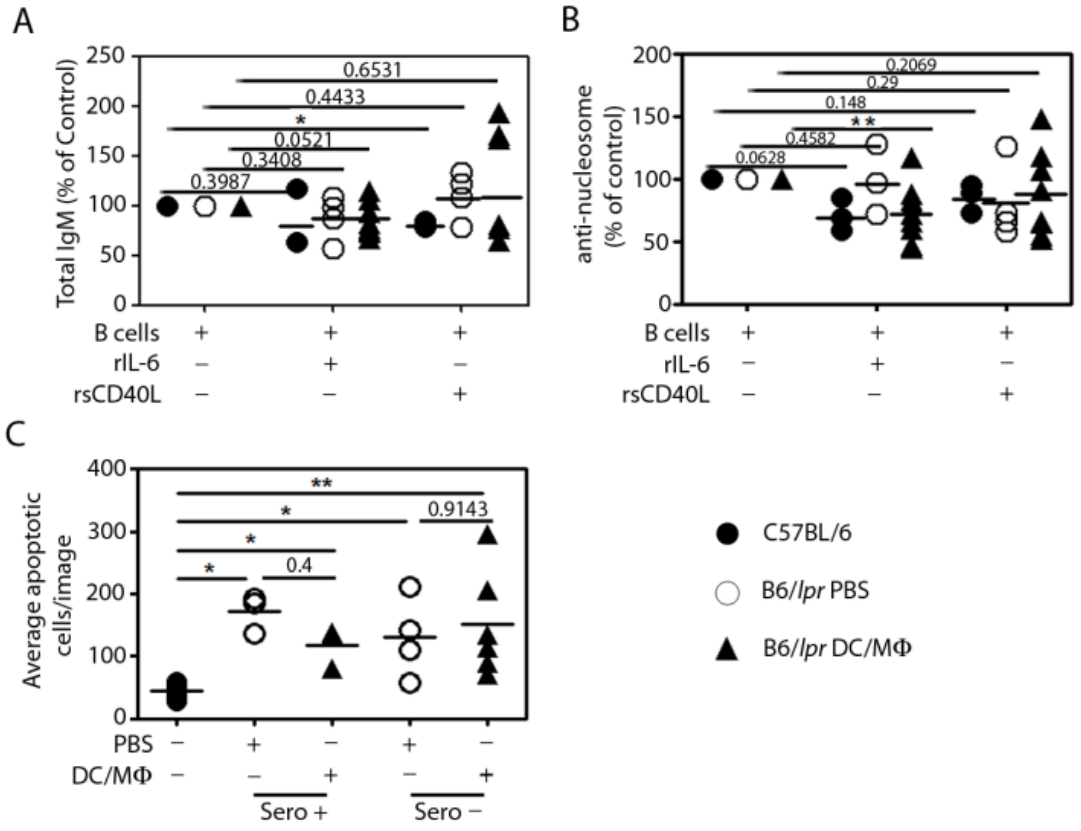

Figure 2.7. Susceptibility regained to rIL-6 and apoptotic cell burden of DC/MΦ injected B6/*lpr* mice are unchanged. Sero-negative B6/*lpr* mice were injected with DCs ($6x10^6$) and MΦs ($2x10^6$) or PBS (200 μL)every 5 days for 10 weeks. At 10 weeks, B cells ($1x10^5$) were isolated and stimulated with LPS (15 μg/mL) in the presence or absence of rIL-6 (5 ng/mL) or rsCD40L (25 ng/mL) and cultured for 4 days. Total IgM (A) and anti-nucleosome Ig (B) were quantified by ELISA. (B) Spleen sections from PBS and DC/MΦ treated sero+ and sero- B6/*lpr* and control mice were stained with TUNEL and quantified. Data represents at least an n=3. (*$p < 0.05$)

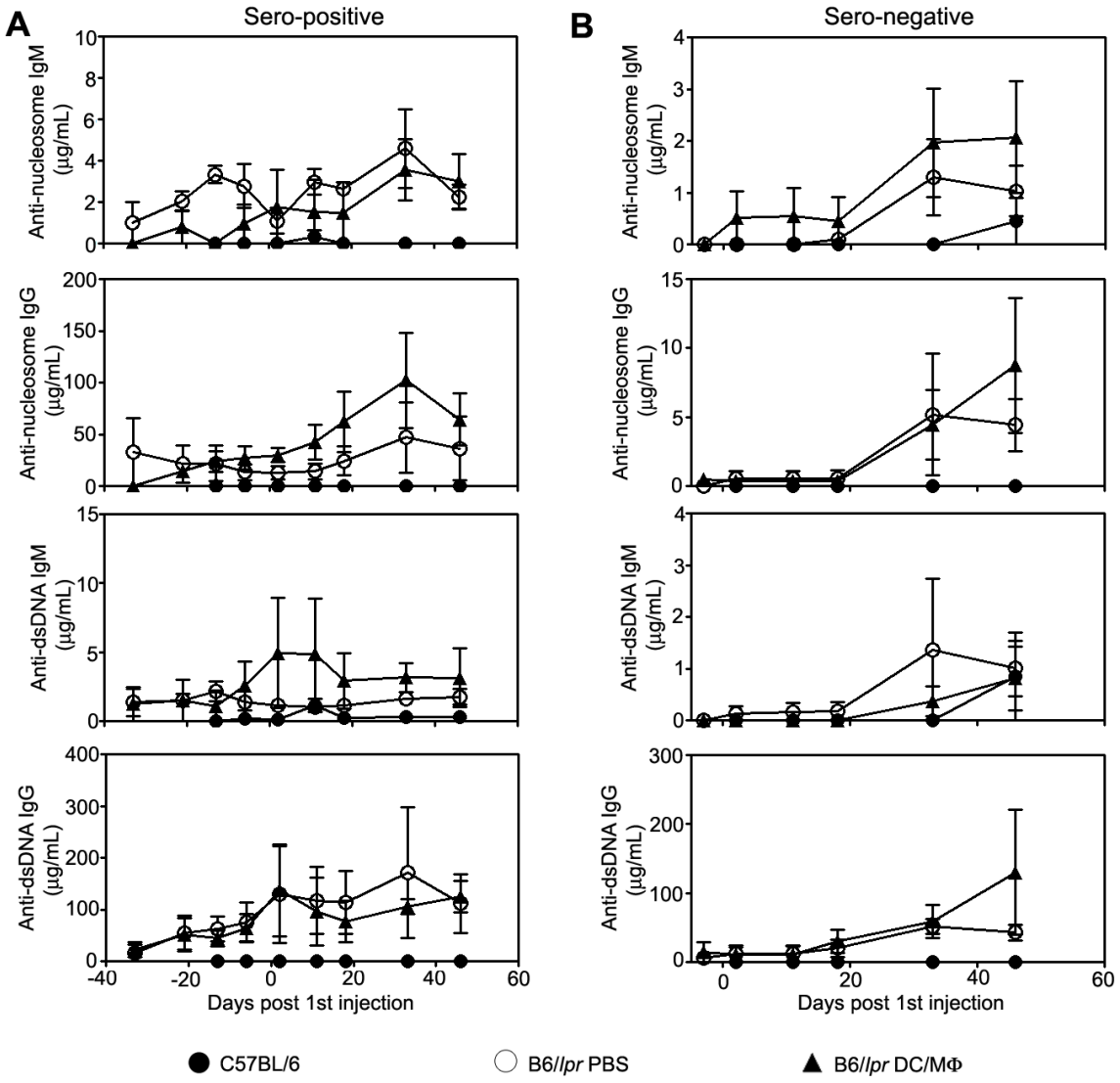

Figure 2.8. Serum autoantibody levels are unchanged in B6/*lpr* DC/MΦ injected mice. B6/*lpr* sero-positive (A) and sero-negative (B) mice were injected with PBS (200ml) or DCs ($6x10^6$) and MΦs ($2x10^6$) every 5 days for 7 weeks and bled every 1-2 weeks. Serum anti-nucleosome IgM and IgG and anti-dsDNA IgM and IgG were quantified by ELISA. Data represent at least 3 mice except for the DC/MΦ injected in sero-positive which represents 2 mice.

CHAPTER 3

DISCUSSION

SLE is a chronic multisystemic autoimmune disease characterized by the production of autoantibodies. The autoantibodies recognize nuclear components that are presented on the surface of apoptotic cells and debris allowing for formation of immune complexes. In SLE patients, the phagocytes do not function correctly, allowing for a build-up of apoptotic cells [36, 66, 67, 72, 75, 80, 81, 83, 96, 100, 136, 143]. This increases the presence of self-antigen in SLE patients that contributes to the dysregulation of autoreactive B cells and promotes their production of autoantibodies [14, 61, 62, 66, 70, 95, 136, 137]. Once formed, the immune complexes are deposited in tissues throughout the body causing inflammation and ultimately tissue damage [38, 91]. Some of the tissues the immune complexes are deposited in are the skin, kidneys, blood vessels, joints, and the nervous system [37-39, 74, 75, 91]. This leads to a variety of symptoms that patients can present with making diagnosis and treatment more challenging.

There are currently many therapeutics in use to treat SLE, however, there are no cures. In addition, many of the therapies rely on immunosuppression, which can have negative impacts on the immune system allowing for an increased risk of infection. The drugs themselves can also have negative impacts on SLE patients with prolonged use. New treatments are focused on neutralization of cytokines overproduced in SLE patients, such as anti-IL-10, anti-TNFα, and most recently anti-BAFF antibodies [124, 126, 134, 135]. In addition, there has been utilization of anti-CD20 antibody treatment which promotes the removal of B cells, including autoreactive and memory B cells, but plasma cells are unaffected since they do not express CD20 [124, 166]. Autoantibodies are made by plasma cells and since they are not being depleted by this therapy, it is not as potent and has been

found to be ineffective in relieving symptoms if complete $CD20^+$ B cell depletion does not occur [124].

Interestingly, SLE treatments have never been pursued toward the removal of apoptotic cells and immune complexes. Apoptotic cells and immune complexes contribute to activation of autoreactive B cells, autoantibody production, and tissue inflammation [61, 64, 96, 142]. Therefore, we developed a novel approach to address this component. The use of healthy dendritic cells and macrophages injected into the patient is a therapy that was tested in Chapter 2. The goal of this therapy is to remove the apoptotic cell burden that contributes to immune complex deposition in tissues and activation of autoreactive cells along with clearance of the immune complexes themselves. Removal of apoptotic cells and immune complexes should decrease inflammation and prevent further tissue damage (Figure 3.1). In the process, the reduction of autoantibodies may be possible through removal of plasma cell survival niches in inflamed tissues (Figure 3.1). Finally, autoreactive cells should become susceptible to regulation mechanisms through reduction of the dysregulating antigens. While the preliminary results did not show everything that was expected, optimization could allow for these outcomes.

One of the key organs subjected to damage by immune complexes is the kidney. Through deposition of immune complexes in glomeruli of the kidney, inflammation occurs and promotes the production of cytokines [49, 111]. These cytokines induce homing of immune cells into the kidney to clear immune complexes and remove inflammation [49]. However, phagocytes from SLE patients are unable to clear the immune complexes further exacerbating the inflammation and tissue damage through production of additional

cytokines [64, 96, 102]. In addition, plasma cells have been found in the inflamed kidneys of lupus-prone NZB/W F1 mice in inflammatory cytokine produced niches [87]. Their production of antibodies in the kidney can contribute to the already present problem of immune complexes [86, 91]. The goal with the DC/MΦ injection treatment is that some of the cells will migrate to the kidney and help clear the apoptotic cells and immune complexes being deposited there. Through this process, the apoptotic cells and immune complexes will be cleared, inflammation will be reduced in the kidneys, and the infiltrating immune cells will leave. There is also the benefit of stopping the production of all the pro-inflammatory cytokines that contribute to the survival of plasma cells in the niches. Ultimately, this could prevent further damage to the kidney and organ failure and potentially restore tissues that have been damaged by inflammation.

Autoantibodies are a contributing factor to pathogenesis of lupus and the ability to prevent their production would be extremely beneficial. We saw that production was reduced in mice that were initially making IgM autoantibodies. However, in mice that made all isotypes of autoantibodies we did not see any change, except for an increase in IgM autoantibodies in one mouse receiving cell injections (Chapter 2). This result indicates that it may be difficult to prevent autoantibody production. Previous treatments with anti-CD20 antibody, anti-BAFF antibody, and allogeneic mesenchymal stem cells have been found to have little to no impact on autoantibody production [123, 124, 130, 134]. This may be due to the reduction of surface receptors on and location of plasma cells. On the other hand, if the injection of DCs and MΦs could increase the production of autoantibodies of the IgM isotype, there could be a decrease in disease symptoms. Reduced disease symptoms were

seen in MRL/*lpr* mice that were capable of making only IgM antibodies and in SLE patients that produced more IgM than IgG autoantibodies indicating a protective role [117-119, 121]. One of the main producers of IgM that recognizes apoptotic cells are B-1 B cells [104, 107]. The expansion of this B cell population or stimulation by BCR or TLR to produce increased amounts of natural IgM through DC/MΦ injection would promote regulation of symptoms [104, 116]. This regulation is due to their ability to promote clearance of apoptotic cells in a non-inflammatory process through complement receptors and not by Fc receptors that promote inflammation, as would be seen by IgG autoantibodies [61, 76, 77, 107, 109, 110]. Therefore, IgM autoantibodies would help enhance clearance of apoptotic cells and potentially reduce inflammation. In addition, even if this treatment is unable to affect the autoantibody levels, if the apoptotic cell burden and apoptotic cell debris is removed, then the autoantibodies would have no antigen to bind and even though present in the serum would have no impacts on the patient's health.

Another idea of a therapeutic is the use of bone marrow chimeras, where the patient would undergo a nonmyeloablative transplant. The goal of this technique is the same as the cell injection, but the healthy dendritic cells and macrophages would be produced by the bone marrow received by the patient. This therapy would allow for continued production of healthy dendritic cells and macrophages throughout the remainder of the patient's life, rather than potentially having to get injections throughout their life with the proposed injection treatment. Currently, no effect on the production of IgG autoantibodies have been seen in MRL/*lpr* mice treated with wild-type bone marrow, which is similar to the DC/MΦ injection results indicating that both treatments appear to be

incapable of impacting autoantibody producing plasma cells. The kidneys have yet to be examined for inflammation and infiltration of immune cells. However, absent or reduced inflammation and infiltration compared to control mice, would indicate that introducing healthy DCs and MΦs into lupus-prone mice will reduce disease symptoms. However, these treatments require allograft transplants requiring little to no MHC mismatch between the donor and recipient to reduce the chance and severity of the recipient developing graft-versus-host disease. This may make the treatments more of a challenge, but positive results would make them beneficial to pursue [127, 133].

Based on our results and the potential benefits that could be obtained with improvement of the current therapy protocol, there could be increased improvements through the use of this therapy in combination with others. The main goal of our therapy is to remove the apoptotic cells, which is the basis of the immune complexes. However, the new anti-BAFF therapy can impact B cell survival, including plasma cells [89]. Therefore, if we used our therapy along with the anti-BAFF therapy, then we could eliminate both components of the immune complexes and potentially prevent the production of autoantibodies in the future if the apoptotic burden remained low and if all IgG isotype autoantibody-producing plasma cells were removed. In addition, there may be other therapies that could be utilized to help improve our therapy, such as anti-IL-10. Since IL-10 levels are increased in lupus patients, its ability to prevent inflammation becomes dysregulated along with being involved in increased apoptosis and disease activity [82, 100, 131]. By including the anti-IL-10 therapy along with either the DC/MΦ cell injection alone or with the anti-BAFF therapy, we could block the additional IL-10 that is likely to be

produced by the injected cells on contact with apoptotic cells that would contribute to increased disease activity. Ultimately, even if some goals of our therapy were not possible, combining it with other therapies could synergistically improve the overall benefits of all the therapies utilized.

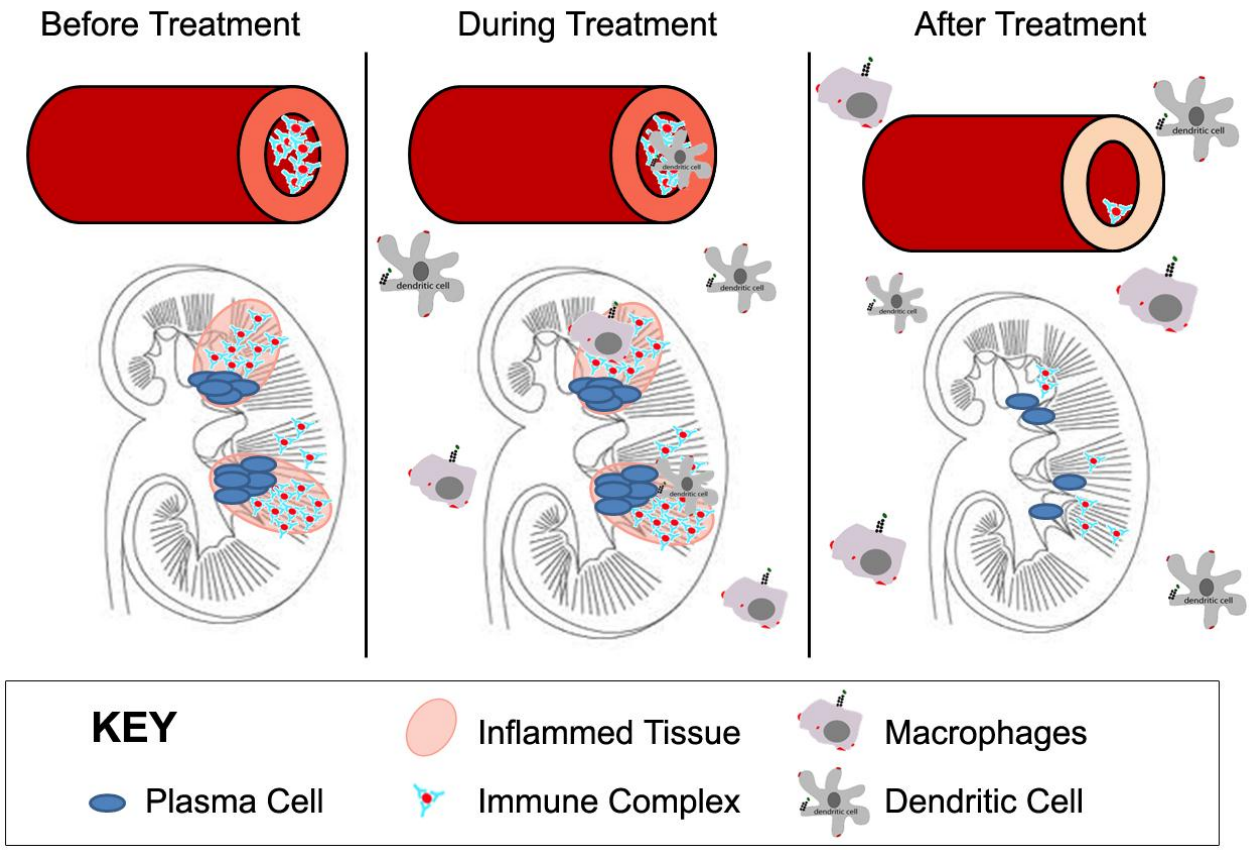

Figure 3.1 DC/MΦ injection to reduce apoptotic cells, inflammation, and disease pathogenesis. Lupus patients would have immune complexes being deposited into tissues throughout their body, which would promote inflammation. Through injection of DCs and MΦs, the immune complexes and apoptotic cells would be cleared. This would then lead to a reduction in inflammation and potentially autoantibody production through the removal of plasma survival niches, which have been found in the kidney [87, 91].

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