In Vivo Regulation of Autoreactive B Cells by IL-6, CD40L and TNFα

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Abstract

Nikki J. Wagner: *In Vivo* Regulation of Autoreactive B Cells by IL-6, CD40L and TNFα
(Under the direction of Barbara Vilen)

Polyclonal B cell activation is essential to an effective innate immune response. However, autoreactive B cells must not be included in the polyclonal response to avoid autoimmunity. We have shown that IL-6 and CD40L secreted by TLR-stimulated dendritic cells (DCs) and macrophages (MΦs) selectively repress LPS-induced Ig secretion by autoreactive B cells. Here we introduce a third soluble factor involved in DC/MΦ-mediated B cell repression, TNFα. Like IL-6 and CD40L, DCs and MΦs derived from lupus-prone MRL/*lpr* mice secrete less TNFα in response to TLR stimulation than DCs and MΦs from C57BL/6 mice, suggesting secretion of TNFα by DCs/MΦs may have a role in autoimmune disease. We further demonstrate in an *in vivo* model that IL-6, CD40L and TNFα regulate LPS-stimulated autoreactive B cells, while mice lacking these factors do not. Our data indicate that IL-6, CD40L and TNFα mediate *in vitro* and *in vivo* autoreactive B cell repression during innate immune responses.

To God, my loving husband, and the many people who have contributed to my scientific and personal accomplishments.

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List of Abbreviations

3XKO- triple knockout mice lacking expression of IL-6, CD40L and TNFα

Ab- antibody

Ag- antigen

ANA-anti-nuclear antibody

APC- antigen presenting cell

APRIL- a proliferation-inducing ligand

ASC- antibody secreting cell

B6- C57BL/6

BCR- B cell receptor

BLyS- B lymphocyte stimulator (also called BAFF)

CM- conditioned media

DC- dendritic cell

DNA- deoxyribonucleic acid

dsDNA- double stranded deoxyribonucleic acid

dsRNA- double stranded ribonucleic acid

ELISA- enzyme-linked immunosorbent assay

ELISpot- enzyme-linked immunosorbent spot

FACS- fluorescent-activated cell sorting

FCS- fetal calf serum

FO- follicular

H&E- hematoxylin and eosin

HEL- hen egg lysozyme

HRP- horseradish peroxidase

i.p.- intraperitoneal

i.v.- intravenous

IC- immune complex

Ig- immunoglobulin

IL-6- interleukin 6

INF- interferon

INF-I- type I interferon (interferon α and b)

LPS- lipopolysaccharide

MΦ- macrophage

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

MyD88 myeloid differentiation factor 88

myDC- myeloid dendritic cell

MZ- marginal zone

PAMP- pathogen-associated molecular pattern

PBS- phosphate buffered saline

PCR- polymerase chain reaction

pDC- plasmacytoid dendritic cell

PRR- pattern-recognition receptor

rIL-6- recombinant IL-6

RNA- ribonucleic acid

RNP- ribonuclear protein

rsCD40L- recombinant, soluble CD40L

rTNFα- recombinant TNFα

sCD40L- soluble CD40L

SLE- Systemic Lupus Erythematosus

Sm- Smith antigen

snRNP small nuclear ribonucleoprotein

ssDNA- single stranded deoxyribonucleic acid

ssRNA- single stranded ribonucleic acid

Tg- transgenic

TLR- Toll-like receptor

TNFα- tumor necrosis factor α

VDJ- variable-diversity-joining

Chapter 1
Introduction

1.1 B Cell Tolerance

To protect from invading pathogens, the B cell receptor (BCR) is assembled by variable-(diversity)-joining (V(D)J) recombination to produce a repertoire of antibodies that recognize a wide range of antigens. This random process inadvertently results in the generation of autoreactive immunoglobulins (Ig). It is estimated that up to 50% of emergent B cells in the bone marrow are autoreactive [1-3]. However the number of autoreactive B cells in the periphery is much lower due to central tolerance mechanisms [4-13]. Autoreactive B cells that escape deletion or receptor editing in the bone marrow are subject to further mechanisms of tolerance, including peripheral deletion and receptor revision [12, 14-19]. Peripheral B cells that have avoided deletion or receptor rearrangement are maintained in an unresponsive state referred to as anergy. Anergic B cells do not secrete Ig in response to antigenic or mitogenic stimulus [20-23]. Lack of plasmablast formation and Ig secretion is common to all anergic B cells; however proliferation in response to either Ig or TLR stimulation and the ability of anergic B cells to transduce BCR signals vary depending antigen specificity [1, 24-29]. The avidity of the BCR for the autoantigen determines if an autoreactive B cell is deleted, its receptor is edited or it enters an anergic state. Stronger BCR signals invoke deletion or receptor editing, while weaker signals induce anergy [30]. The tolerization of autoreactive B cells is critical since their potential activation produces autoantibodies that can lead to autoimmune diseases.

1.2 Loss of B Cell Tolerance and Disease

Systemic Lupus Erythematosus (SLE) is an autoimmune disease where B cells play a critical pathogenic role [31]. In SLE, autoreactive B cells become activated and produce

autoantibody against nuclear components including DNA, RNA, ribonuclear proteins (RNPs) and histones [32-34]. While the exact etiology of SLE remains unclear, nucleic acids and antibodies form immune complexes (ICs) that contribute to disease pathogenesis [31, 35]. IC deposition in small blood vessels produces inflammation and tissue damage, leading to dermatitis, vasculitis, pneumonitis, nephritis, arthritis and/or cerebritis [36, 37]. ICs serve as potent immune stimuli since they coligate BCR and TLR, which can overcome B cell tolerance [38-46]. The loss of B cell tolerance in SLE results in autoreactive B cell activation and production of autoantibodies that leads to tissue damage.

Murine models of SLE have proven useful in studying diseases resulting from a loss of B cell tolerance. Mice of the autoimmune-prone strain MRL develop lupus-like symptoms and die by about 24 months of age [47]. Further, the spontaneous *lpr* mutation of Fas on the MRL background results in an accelerated disease process that produces SLE-like disease and death by 3-6 months [47, 48]. MRL/*lpr* mice have measurable serum autoantibody specific for single- and double-stranded DNA, and RNPs [32, 47, 49, 50]. These mice develop symptoms that mimic human SLE, including skin lesions, joint inflammation and glomerulonephritis [47], thus providing a model disease similar to human SLE. When autoreactive transgenic (Tg) B cells are expressed on the MRL/*lpr* background, they break tolerance, become activated and produce autoreactive antibodies [51-53]. Thus, in both mice and humans, loss of B cell tolerance leads to autoantibody production, and potentially autoimmune disease.

Deletion and receptor editing eliminate the possibility of autoreactive B cells becoming activated; however anergy has been shown to be reversible [54, 55]. The anergic state of autoreactive B cells is maintained by chronic antigen binding to the BCR [54]. Once

purified away from the source of antigen, anergic B cells regain the ability to respond to BCR/TLR stimulation [54, 55]. We have recently shown that dendritic cells (DCs) and macrophages (MΦs) are capable of regulating autoreactive B cells by secreting soluble factors, identified as IL-6, sCD40L [56, 57] and TNFα (Chapter 2). If DCs and MΦs are removed from autoreactive B cells, TLR stimulation results in Ig secretion [56, 57]. These data prove that regulation of anergic B cells via receptor occupancy or soluble factors must be constantly maintained to prevent autoantibody production.

1.3 2-12H Tg Model of B Cell Tolerance

Smith antigen (Sm) is an essential RNP involved in RNA splicing. Anti-Sm antibodies are present in the serum of 25-30% of SLE patients and correlate with more severe disease [32, 33, 58, 59]. To study tolerance mechanisms that regulate Sm-specific B cells, Ig-transgenic mice were developed that express a heavy chain (2-12H) isolated from MRL/*lpr* mice [24]. When the 2-12H Tg heavy chain pairs with endogenous light chains, the B cells produced have Sm-specific BCRs of varying affinities. It was initially reported that ~30% of the B cells in 2-12H Tg mice bound Sm; however recent functional analysis indicates the majority of B cells bind Sm, although some bind with low affinity [24, 60]. The 2-12H Tg mice have a B cell repertoire that is primarily autoreactive; however, on a non-autoimmune background they maintain tolerance [24].

To examine the role of different BCR affinities on tolerance, the 2-12H Tg mice were crossed with mice expressing restricted light chains [29, 60]. Pairing of 2-12H with the Vκ4 light chain produces Sm-specific B cells of moderate affinity [60]. In the presence of innate stimuli, B cells from these mice are repressed by IL-6 and sCD40L. The marginal zone (MZ)

population in 2-12H/Vκ4 mice exhibit a block in BCR-mediated signal transduction that may contribute to unresponsiveness [60]. Further, since this block is not present in 2-12H/Vκ4 follicular (FO) B cells, it appears that different affinities regulate MZ and FO B cell activation [60].

Low affinity autoreactive B cell can respond to stimuli differently than high and moderate affinity B cells. Therefore, the 2-12H Tg was paired with the Vκ8 light chain to produce 2-12H/Vκ8 B cells that bind Sm with low affinity [29]. Interestingly, 2-12H/Vκ8 mice have a limited MZ population with only ~1.5% of splenic B cells exhibiting MZ characteristics [29, 60]. Like 2-12H Tg B cells, 2-12H/Vκ8 B cells are repressed by IL-6 and sCD40L [56, 57], but Ig secretion by 2-12H/Vκ8 B cells is also repressed by TNFα (Chapter 2). The repression of 2-12H/Vκ8 B cells by TNFα may be due to the lower affinity of the BCR or to the lack of MZ B cells in the 2-12H/Vκ8 mice [29]. While the 2-12H Tg mice can be induced to break tolerance when injected with snRNPs [24], the low affinity 2-12Vκ8 mice do not produce antibody in response to snRNPs (M.A.Kilmon and B.J.Vilen, unpublished observations), indicating that the affinity of the BCR and/or B cell subsets play a critical role in maintaining B cell tolerance.

1.4 The Innate Immune Response

The innate immune system provides the first line of defense against invading pathogens through constitutively expressed, germline-encoded receptors called pattern-recognition receptors (PRRs). The innate system responds to conserved molecular motifs that are components of pathogens, called pathogen-associated molecular patterns (PAMPs). PAMPs are essential for the survival and/or function of the pathogen, therefore they make

excellent targets for innate immune recognition [61]. The conserved nature of PAMPs within a class of pathogens allows the innate immune system to recognize a wide variety of pathogens with a limited number of receptors. PAMPs include essential bacterial cell wall components, such as lipopeptides, peptidoglycan, glycolipids, flagellin, and lipopolysaccharide (LPS), and nucleic acids specific for bacteria and viruses [61].

PRRs are highly conserved between species (from plants to humans) [61]. The best studied PRRs are the Toll-like receptors (TLRs). Mammals have at least 12 TLRs to protect them from pathogens. TLRs 1-9 are expressed in both mice and humans; however TLR8 is non-functioning in mice [62-64]. TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface [64-66]. In general, TLR2 binds components of Gram positive bacteria and mycobacteria while TLR4 recognizes Gram negative bacteria. TLRs 1 and 6 dimerize with TLR2 for selective binding to different lipid portions of lipoproteins [67-71]. TLRs 3, 7, 8 and 9 are located intracellularly, and all the intracellular TLRs target nucleic acids, such as hypomethylated CpG DNA, and double and single stranded RNA [72-75]. TLRs 7 and 8 are highly homologous and both bind single stranded RNA [73]. TLRs 7 and 9 are sequestered in the endoplasmic reticulum (ER) until ligand binding upon which they move to endosomes [76-78]. Binding of PAMPs to TLRs induces a signaling pathways involving myeloid differentiation factor 88 (MyD88) dependent (for all TLRs except 3) and independent (for TLRs 3 and 4) pathways. MyD88-independent pathways induce activation of the transcription factor IRF3 and the expression of interferon-β [64, 79]. MyD88-dependant signaling activates the transcription factor NFκB and induces inflammatory cytokine secretion and the expression of co-stimulatory molecules [64]. The final outcome of TLR

ligand binding, however, depends on which TLR is bound and the type of cell on which the TLR is located.

1.5 Dendritic Cell and Macrophage Responses to TLR Stimuli

DCs and MΦs express the widest range of TLRs of any cell type [65], since their functions of clearing cellular debris and pathogens put them in constant contact with innate stimuli. LPS binding to TLR4 on MΦs induces NFκB activation that results in transcription of many genes, including those responsible for the production of the inflammatory cytokines IL-1, IL-6, IL-12 and TNFα and the chemokine IL-8 [61, 64]. The secretion of IL-1, IL-6, IL-12 and TNFα causes localized inflammation while IL-8 attracts neutrophils to the site. The binding of TLR ligands to DCs induces maturation and increases the ability of DCs to prime antigen-specific T cells. TLR-mediated NFκB activation in DCs and MΦs also induces the expression of molecules involved in T cell activation such as the costimulatory molecules CD80 and CD86 and upregulates the expression of MHCII [61], thus linking the innate and adaptive immune systems. Activated DCs and MΦ activate naïve B cells by secreting B lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL) [80-82]. Since BLyS and APRIL promote Ig class switching and plasma cell differentiation in naïve B cells [82], DCs and MΦs can induce T-independent B cell activation. TLR-mediated stimulation of DCs and MΦs results in the activation of both innate and adaptive immune responses.

Defects in DCs and MΦs are linked to SLE. MΦs from several strains of lupus-prone mice are defective in secreting inflammatory cytokines in response to TLR stimulation [83-85], suggesting that MΦ-mediated regulation of B cells is involved in disease development.

Monocyte-derived DCs from lupus patients may contribute to disease due to their overexpression of CD86 [86], potentially leading to excessive antigen presentation. DCs and MΦs from lupus-prone mice also display an excess of autoantigen on their surfaces, possibly providing a pool of high avidity antigen to activate autoreactive B cells (D.G.Carnathan, C.E.Hilliard, B.J.Vilen, unpublished observations). While myeloid DCs (myDCs) and MΦs are able to selectively repress autoreactive B cells through the soluble mediators IL-6, sCD40L [56, 57] and TNFα (Chapter 2), myDCs and MΦs from lupus-prone mice secrete less of these repressive factors [57, 87] (Chapter 2). Thus, in lupus-prone mice the response of MΦs and myDCs to innate stimuli is defective.

Plasmacytoid DCs (pDC) are specialized DCs that secrete large amounts of type I interferon (IFN-I) upon stimulation [88-90]. pDCs express TLRs 7 and 9 in humans and mice. Ligation of these receptors induces IFN-I production that promotes maturation of immature DCs (allowing priming and activation of antigen-specific T cells) and stimulation of B cells [91-94]. SLE patients have aberrantly high IFN-I levels and disruption of the IFN-I receptor reduces pathology in two lupus-prone mouse models [95, 96]. These data are likely due to the stimulatory effect type I IFN has on B cells. Therefore innate immune stimulation of pDCs is likely critical to the development and/or maintenance of autoreactive B cell activation and Ig production. Together these data indicate that DCs and MΦs regulate autoreactive antibody production and autoimmune disease process.

1.6 B Cell Responses to TLR Stimuli

B cells proliferate, differentiate to antibody secreting cells (ASCs), increase antigen presentation and secrete cytokines in response to TLR ligand binding [97, 98]. LPS binding

to TLR4 in murine B cells induces NFκB activation that upregulates transcription of B cell survival genes [99-101], leading to proliferation and differentiation to ASCs [102, 103]. Murine B cells express TLRs 4, 7 and 9 while human B cells express TLRs 7, 9 and 10 [104, 105]. Although human B cells do not respond to LPS, TLR9 signaling is functional in both human and murine B cells and both produce Ig in response to CpG DNA [104, 105]. While some effects of TLR stimulation on B cells may be indirect (the result of other colocalized cells responding to TLR stimulation), others are B cell-intrinsic [106, 107]. Further tying B cells to innate immune responses, a recent study [108] suggests that TLR-activated naïve B cells repress autoreactive T cells through the secretion of IL-10. This provides a mechanism by which naïve B cells prevent autoimmunity during an innate immune response. Thus, B cells' involvement in immune responses is not limited to the adaptive responses, but they also have a critical role in the innate immune responses.

Innate immune stimulation of B cells, however, can lead to autoantibody production. Concurrent BCR and TLR signaling causes some autoreactive B cells to break tolerance and become ASCs [38-46], linking TLR ligation to autoreactive B cell activation. In lupus-prone MRL/*lpr* mice, despite BCR stimulation by autoantigens, deletion of the TLR signaling protein MyD88 prevents the development of serum antinuclear antibodies or nephritis [109], implicating TLRs in murine lupus. Further, in rheumatoid arthritis models, anti-IgG antibodies combine with RNA and/or DNA in immune complexes (ICs) that bind the BCR and TLR of rheumatoid factor (RF)-specific B cells, resulting in their activation [38-46]. The requirement of either TLR binding or MyD88 signaling to generate anti-nuclear antibody production in the RF models [38-46] demonstrates the necessity of TLR signaling for autoantibody production. While the majority of work has focused on TLRs 7 and 9, LPS-

stimulation induces autoantibody production in an arthritis model [110] indicating that the TLR 4 also contributes to the break in tolerance. These data implicate TLR signaling as essential for the break in B cell tolerance and the development of autoreactive antibody that can lead to autoimmune disease.

1.7 Cytokine Regulation of Autoreactive B Cells

Autoreactive B cells continually exposed to self antigen lose the ability to respond to TLR stimulation due to constant BCR signaling and soluble factors produced by DCs/MΦs. However, these B cells regain the ability to respond to TLR ligands when antigen and soluble factors produced by myDCs and MΦs are removed [56]. The factors responsible for repressing Ig secretion have been identified as IL-6, sCD40L [56, 57] and TNFα (Chapter 2). The repressive action of IL-6, CD40L and TNFα on autoreactive B cells challenges our current understanding of the function of these cytokines. Previous studies show that IL-6, sCD40L and TNFα promote B cell activity [92, 111-117]; however these studies were performed in non-transgenic models, so the majority of the B cells were naïve. Since the same cytokines promote Ig secretion by naïve B cells and repress Ig secretion of autoreactive B cells, the history of BCR antigen exposure determines the response to IL-6, sCD40L and TNFα.

Chronic stimulation of the BCR reprograms the B cell to respond differently to cytokine and/or TLR signaling. Only B cells chronically exposed to antigen repress Ig secretion in response to IL-6, sCD40L [56, 57] and TNFα (Chapter 2). These cells exhibit constitutively elevated basal phospho-ERK [54, 118] and our studies show that inhibiting MEK abrogates the repressive affects of IL-6 and sCD40L on autoreactive B cells (J.A.Rutan

and B.J.Vilen, manuscript in preparation). Thus the ability of IL-6 and sCD40L to repress TLR4-mediated Ig secretion in autoreactive B cells is dependant on the MEK/ERK pathway, consistent with the idea that chronic antigen stimulation reprograms B cell signaling.

DCs and MΦs from lupus-prone mice show defects in the LPS-induced production of the cytokines that regulate autoreactive B cells [57, 87] (Chapter 2). Because of these defects, DCs and MΦs from lupus-prone MRL/*lpr* mice are unable to repress anti-Sm 2-12Vκ8 B cells [57, 87]. The decrease in IL-6, sCD40L and TNFα produced by MΦs and DCs in MRL/*lpr* mice together with our *in vitro* data suggests a role for cytokine-mediated regulation of autoreactive B cells in disease.

1.8 Model of B Cell Tolerance During Innate Immune Responses

In non-autoimmune mice, the presence of TLR ligands from viruses and bacteria stimulate DCs and MΦ to secrete IL-6, TNFα and sCD40L. While naïve B cells are initiating polyclonal Ig responses, Ig secretion by autoreactive B cells is repressed. This tolerance mechanism allows the host to attack infecting pathogens yet maintain unresponsiveness among the autoreactive B cell population. Mechanistically, autoreactive B cells repress Ig secretion by decreasing BLIMP-1 and XBP-1 levels in a MEK/ERK dependent manner (J.A.Rutan, and B.J.Vilen, manuscript in preparation). However, if genetic or environmental predisposition to autoimmune disease results in decreased production of IL-6, sCD40L and TNFα by DCs and MΦs, both naïve and chronically antigen-experienced B cells are activated by TLR ligand binding. This results in autoreactive antibody production that may lead to autoimmune disease. Alternatively (or additionally), genetic and/or environmental elements may alter the response of autoreactive B cells to

secreted repressive factors, such that they fail to be susceptible to IL-6, sCD40L and/or TNFα-mediated repression and are allowed to become activated like naïve cells. Preliminary data indicate that defective B cell responses are involved in murine MRL/*lpr* lupus (N.J.Wagner and B.J.Vilen, unpublished observations). This model suggests that chronic BCR stimulation reprograms receptors so that responses to IL-6R, CD40 and TNFR1/2 are altered, offering an explanation for the selective repression of autoreactive B during innate immune stimulation.

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Chapter 2
Dendritic Cell and Macrophage Regulation of Smith Antigen-Specific B Cells *In Vitro* and *In Vivo*

2.1 Introduction

Innate immune stimuli, such as bacterial LPS, viral nucleic acids or hypomethylated CpG DNA motifs, activate B cells through Toll-like receptors (TLRs) to generate polyclonal B cell responses that facilitate the clearance of pathogens. Innate immune responses induce the secretion of autoantibody, although these responses are normally transient [1-4]. However, if autoreactive B cells persist in producing antibody, autoimmune disease may develop [1-4]. We have previously shown that dendritic cells (DCs) and macrophages (MΦs) regulate autoreactive B cells during innate immune responses [5, 6]. TLR ligands stimulate DCs and MΦs to secrete cytokines, including TNFα, that have pleiotropic effects on immune responses.

The role of TNFα in autoimmunity is ambiguous. In human systemic lupus erythematosus (SLE) and murine models of lupus, TNFα has been implicated inflammation and organ damage [7-11]. However, other data suggest that TNFα may protect from SLE. Lupus-prone NZBxNZW mice have reduced disease and increased lifespan when treated with recombinant TNFα (rTNFα) at a young age [12]. Additionally, NZW mice, which are not lupus-prone, develop lupus-like disease when TNFα is reduced by genetic alteration [13]. In both cases, development of SLE is dependent on early TNFα levels. This suggests TNFα plays a role in establishing B cell tolerance. TNFα also influences anti-histone and anti-chromatin antibody production in humans and some patients receiving anti-TNFα therapy develop symptoms of SLE that resolve once anti-TNFα drugs are discontinued [14-19]. This implies that TNFα prevents SLE in mice and humans. The data indicate the timing and concentration of TNFα during disease progression determines the affect TNFα has on autoantibody production.

We have previously described IL-6 and soluble CD40L (sCD40L) as factors that selectively repress antibody secretion by autoreactive B cells [5, 6]. In this study we describe

TNFα as the third factor produced by DCs and MΦs that represses autoantibody secretion. TLR4-stimulated DCs and MΦs from lupus-prone MRL/*lpr* mice produce less TNFα than wildtype, suggesting that TNFα is important in B cell tolerance. Removal of IL-6, CD40L and TNFα affects the activation of TLR-stimulated autoreactive B cells *in vivo*, corroborating our *in vitro* data that these cytokines regulate autoantibody secretion. Further, Sm-specific B cells adoptively transferred into IL-$6^{-/-}$xCD40$L^{-/-}$xTNF$\alpha^{-/-}$ mice become activated following TLR4 stimulation. The data support previous *in vitro* findings that DCs, MΦs, and their secreted products regulate autoreactive B cells during innate immune responses.

2.2 Materials and Methods

Mice. C57BL/6 (B6) non-transgenic, MRL/MpJ-Fas^{lpr}/J (MRL/*lpr*), IL-$6^{-/-}$, CD40$L^{-/-}$ and TNF$\alpha^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-$6^{-/-}$, CD40$L^{-/-}$ and TNF$\alpha^{-/}$ mice were crossed to generate IL-$6^{-/-}$xCD40$L^{-/-}$xTNF$\alpha^{-/-}$ mice (3XKO). PCR was performed to determine if the mice inherited the disrupted genes, as previously described [20-22]. C57BL/Ly5.2 (B6/Ly5.2) mice were purchased from the National Cancer Institute (Frederick, MD). 2-12H Tg and 2-12H/Vκ8/$Ck^{-/-}$ (2-12H/Vκ8) have been described [23, 24]. 2-12H Tg mice were crossed to B6 10 generations to generate 2-12H/B6 Tg. Mice were housed in microisolater cages with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Bone marrow-derived DC (BMDC) and MΦ (BMMΦ) Culture. Bone marrow-derived DCs (BMDCs) (95% pure) and bone marrow-derived MΦs (BMMΦs) (98% pure) were generated as

previously described [5]. Conditioned medium (CM) was made from $1x10^4$ BMDCs and BMMΦs (0.2 ml) cultured 4 days with LPS (30 μg/ml).

B Cell Purification. Splenic B cells were negatively selected using the StemSep B cell enrichment kit (StemCell Technologies, Vancouver, BC). 2-12H/Vκ8 B cells were 85-97% as determined by flow cytometry. 2-12H/B6 Tg B cells isolated for adoptive transfer were 85-95 % pure and B cells isolated from 3XKO or B6 mice were 73-99 % pure.

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 (San Jose, CA), TEPC 183, rabbit IgG (isotype control for anti-TNFα) and *Escherichia coli* 055:B5 LPS from Sigma-Aldrich (St. Louis, MO), mouse GM-CSF, IL-4, and M-CSF from PeproTech (Rocky Hill, NJ), and CFSE from Invitrogen (Carlsbad, CA). 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 supernatants. Recombinant TNFα (rTNFα) and recombinant sCD40L (rsCD40L) were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-TNFα was obtained from Vic Johnson (CDC/NIOSH/HELD, West Virginia) and purified by Protein A affinity.

B Cell Culture. Purified B cells ($1x10^5$ per well in a 96-well plate) were cultured with 30 μg/ml LPS for 4 days. rIL-6, rsCD40L, rTNFα, BMDC or BMMΦ 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 rabbit IgG.

CFSE-based Proliferation Assay. Purified B cells ($1x10^6$ cells/ml) in pre-warmed 0.1% BSA/PBS were labeled with CFSE at a final concentration of 0.4 μM for 10 minutes at 37°C. The cells were washed with IMDM containing 5% FCS. CFSE-labeled cells were stimulated with LPS (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.

ELISAs. $IgM^a/κ$ (encoded by 2-12H/Vκ8) was captured with anti-κ (187.1) and detected with biotinylated anti-IgM^a (HB100) and Streptavidin-AP as previously described [24]. 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. TNFα was quantitated by capturing with anti-TNFα (clone TN3-19, eBioscience, San Diego, CA) and detecting with biotinylated polyclonal anti-TNFα (eBioscience) and Streptavidin-AP. rTNFα served as the standard control.

Antinuclear Antibody Test. Antinuclear antibodies in serum were diluted 1:50 in PBS with 2% FCS and detected by indirect immunofluorescence using Hep-2 substrate slides (Antibodies Inc., Davis, CA), anti-mouse IgG-Alexa647 and anti-mouse IgM-Alexa488 (Invitrogen).

Histology. Kidneys were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned and hematoxylin and eosin (H&E) stained. Sections were examined for glomerular and interstitial disease by pathologist Volker Nikeleit via light microscopy.

Bone Marrow Transplants. Six- to eight-week-old B6/Ly5.2 mice were lethally irradiated with 900 rads. Femurs and tibias were removed from donor mice and the marrow was flushed into PBS+0.5% BSA. Red blood cells were lysed with TAC. Bone marrow cells ($5\text{-}8x10^6$) in PBS were injected intravenously into the recipient mice 24 hours after irradiation. Recipient mice were checked after eight weeks for reconstitution by FACS analysis on blood cells, staining for Ly5.1 or Ly5.2 on B cells (B220), T cells (CD3) dendritic cells (CD11c) and macrophages (CD11b).

In vivo stimulation. An innate immune response was simulated by injecting 25 μg lipopolysaccharide (LPS) (*E.coli* O55:B5, Sigma-Aldrich) intraperitoneally (i.p.) once a week for the specified time(s).

ELISpots. ELISpots were preformed using plates (Millipore, Billerica, MA) coated with 10U/ml Sm (Immunovision, Springdale, AR), 5μg/ml ssDNA, (Sigma-Aldrich), anti-IgM^a (to detect antibody from transgenic B cells) or 40μg/ml histones (Immunovision) with 10μg/ml dsDNA (Sigma-Aldrich) (to detect anti-nucleosome antibody). Cells were plated at $2.5\text{-}5x10^6$cells per well, depending on the number of cells recovered from the preparation. After 24 hour incubation on coated plates, cells were washed off the plates and antibody remaining on the plates was detected using biotin-labeled anti-IgM, followed by Streptavidin -HPR (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).

Statistical analysis. Data was analyzed using the Student's *t* test. Significant *p* values (< 0.05) are denoted by *.

2.3 Results

TNFα represses Ig secretion from Sm-specific B cells

Maintaining B cell tolerance during innate immune responses is critical in preventing autoimmunity. We previously showed that IL-6 and sCD40L secreted by LPS-stimulated myeloid DCs (myDCs) and MΦs repress Ig secretion by autoreactive B cells, but not naïve B cells [5, 6]. However, when IL-6 is neutralized in B6 DC conditioned media (CM), 25% of Ig repression remains [5]. This suggests another repressive factor is secreted by DCs. To identify the third repressive factor, we neutralized CM from IL-$6^{-/-}$ DCs with a panel of antibodies to cytokines and chemokines. We found that anti-TNFα restored Ig secretion of LPS-stimulated 2-12H/Vκ8 B cells to 76% of control, while addition of an unrelated antibody had no effect on Ig secretion (Figure 2.1A). This indicates that TNFα is the second repressive factor secreted by DCs.

To corroborate the idea that TNFα regulates autoreactive B cells, we assessed if recombinant TNFα (rTNFα) repressed LPS-induced Ig secretion from 2-12H/Vκ8 B cells. We found that 50 ng/ml of rTNFα repressed 29% Ig secretion (Figure 2.1B, lane 4, p=.000008). In contrast, rIL-6 repressed 67% and rsCD40L repressed 70% (Figure 2.1B). Although rTNFα

repressed Ig secretion, it was considerably less than rIL-6 or rsCD40L (Figure 2.1B, [5, 6]). To assess if IL-6 enhanced the ability of TNFα to regulate Ig secretion, we cultured Sm-specific B cells with optimal levels of TNFα and suboptimal levels of IL-6 then assessed IgM^a/κ secretion. We chose to use IL-6 at 5 ng/ml because this amount repressed 32% of secretion, approximately the same amount as rTNFα. IL-6 did not significantly enhance the ability of TNFα to repress Ig secretion by 2-12H/Vκ8 B cells (data not shown). Thus, TNFα is capable of regulating autoreactive B cells however it is a less potent repressor than IL-6 and CD40L.

Neutralization of IL-6 and TNFα restores LPS-induced Ig secretion from Sm-specific B cells

Multiple factors secreted by DCs and MΦs repress autoreactive B cells. To determine if DCs produced additional factors, we neutralized CM from C57BL/6 DCs with anti-TNFα and anti-IL-6 (Figure 2.2). Removal of both IL-6 and TNFα restored Ig secretion (Figure 2.2, lane 6) indicating DCs utilize IL-6 and TNFα to repress autoantibody production. In addition to IL-6 and TNFα, MΦ secrete CD40L that is repressive to autoreactive B cells. We have previously shown that neutralization of IL-6 and CD40L from B6 MΦ CM restores Ig secretion of 2-12/Vκ8 B cells [6]. However the TNFα produced by activated MΦs also contributes to MΦ-mediated B cell repression, since IL-6 and CD40L-deficient MΦ CM neutralized with anti-TNFα is less repressive than $IL\text{-}6^{-/-}$ x $CD40L^{-/-}$ MΦ CM alone (data not shown). Therefore, DC/MΦ-secreted TNFα, CD40L and IL-6 are the repressive factors of chronically antigen-experienced B cells.

TNFα does not regulate Ig secretion by repressing B cell proliferation

Plasma cell differentiation requires B cell proliferation and the upregulation of BLIMP-1 and XBP-1. To determine whether TNFα regulates Ig secretion by diminishing proliferation, we

labeled cells with CFSE and measured proliferation by CFSE dilution. 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 (Figure 2.3). The data show that LPS-stimulated B cells incubated with or without rTNFα had overlapping CFSE histogram plots, indicating that similar numbers of cells existed in each generation, regardless of the presence of rTNFα (Figure 2.3). Thus, repression of Sm-specific B cells by TNFα is not due to decreased proliferation.

DCs and MΦs from lupus-prone mice are defective in secreting TNFα in response to TLR stimulation

Defects in DCs and MΦs are implicated in SLE, and we have shown 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 [5, 6, 25]. This suggests that repression of autoreactive B cells by DCs and MΦs is an important regulatory mechanism in maintaining tolerance. To determine if DCs and MΦs from lupus-prone mice were defective in secreting TNFα, we quantified TNFα by ELISA. LPS-stimulated bone marrow-derived DCs (BMDCs) from lupus-prone MRL/*lpr* mice secreted significantly less TNFα than B6 (p value = 0.00002, Figure 2.4A). Bone marrow-derived MΦs (BMMΦs) from MRL/*lpr* mice secreted less TNFα compared to B6 (Figure 2.4B), however two of the mice tested failed to show a defect. Thus, MRL/*lpr* mice exhibit defects in secretion of TNFα. Collectively, the data show that the regulation of autoantibody secretion by DCs and MΦs is defective in lupus-prone mice coincident with diminished secretion of three repressive factors.

Lack of IL-6, CD40L and TNFα expression does not result in spontaneous autoimmunity in vivo

Since DCs and MΦs from lupus-prone mice are defective in the secretion of soluble repressive factors [6, 25], we wanted to determine if loss of DC/MΦ-mediated tolerance leads to autoimmunity *in vivo*. Therefore we generated mice in which the IL-6, CD40L and TNFα genes were disrupted. Mice containing the single mutations [20-22] were mated to make double (IL-6$^{-/-}$xTNFα$^{-/-}$ and IL-6$^{-/-}$xCD40L$^{-/-}$) and triple mutant (IL-6$^{-/-}$xCD40L$^{-/-}$xTNFα$^{-/-}$) (3XKO) mice.

Knockout mice (double and triple knockouts) up to 12 months of age did not spontaneously produce serum antinuclear antibody (Table 2.1). Further, kidneys from knockout mice did not show significant pathology compared to wildtype B6 mice of similar age (Table 2.1). Similarly, the levels of anti-single stranded DNA (ssDNA), -nucleosome, or -Sm splenic antibody secreting cells (ASCs) were undetectable (Figure 2.5A). Together, these data suggest that in the absence of innate stimulation autoreactive B cells in 3XKO mice do not spontaneously develop into ASCs. This is consistent with our model that DCs, MΦs, and their secreted products regulate innate immune responses.

Defects intrinsic to the knockout mice could obscure antibody responses. CD40L knockout mice fail to form germinal centers and exhibit abnormal class switch recombination [20, 26]. TNFα knockout mice also lack a germinal center response and exhibit poorly defined splenic B and T cell regions [22, 27]. Thus the contributions of IL-6, CD40L and TNFα could be masked due to splenic abnormalities that preclude the production of antibody. To address this we generated chimeric mice by lethally irradiating B6/Ly5.2 mice and transplanting them with 3XKO (Ly5.1) bone marrow. Despite a more normal splenic architecture, chimeric 3XKO also

mice failed to spontaneously activate endogenous (Figure 2.5B) or adoptively transferred Sm-specific B cells (Figure 2.5D).

Autoreactive B cells overcome tolerance during innate immune responses

Our model proposes that during innate immune responses DCs and MΦs repress Ig secretion by autoreactive B cells [5, 6, 25]. Therefore, the lack of IL-6, CD40L and TNFα may be inconsequential to autoantibody production unless autoreactive B cells are challenged by innate stimuli. To test this, we injected B6 and 3XKO mice with LPS at doses reported to cause autoantibody production [28-30]. By 35 days after the first LPS injection, the ANA levels of B6 mice returned to baseline, but 50% (n=2) of the 3XKO mice remained ANA positive (Figure 2.6B). Further, the 3XKO mice had sustained serum antinuclear antibodies 42 post LPS injection (data not shown). Thus, while IL-6, CD40L and TNFα regulate tolerance of autoreactive B cells *in vitro*, they also play a role in restoring tolerance after innate stimuli.

To track a specific population of autoreactive B cells, we transferred Sm-specific B cells into chimeric B6 and 3XKO mice. The injected B cells were differentiated from native cells based on their Sm specificity and their IgM^a allotype. Since follicular (FO) and marginal zone (MZ) B cells become activated at different times post stimulation, we looked for activated Sm-specific B cells at days three and twelve post transfer. We found that 2-12H/B6 Tg B cells transferred into chimeric 3XKO mice became ASCs within three days, while cells transferred into mice with wildtype bone marrow did not produce antibody (Figure 2.7B and 2.C). However, twelve days post transfer the number of 2-12H/B6 ASCs were undetectable by Sm (Figure 2.7D) and anti-IgM^a ELISpot (Figure 2.7E). Additionally, there were no Sm-specific ASCs present in the bone marrow of the chimeric mice at any of the time points (data not shown), indicating

memory B cells had not developed and migrated to the bone marrow. Thus, transferred Sm-specific B cells become activated in LPS-stimulated chimeric 3XKO mice three days post transfer, but their activity has waned by day twelve post transfer. These data indicate that IL-6, CD40L and TNFα act on autoreactive B cells to prevent them from becoming activated during innate immune stimulation.

2.4 Discussion

During innate immune responses TLR ligands activate naïve B cells to promote polyclonal antibody secretion, while simultaneously repressing autoantibody secretion. TLR ligands also stimulate MΦs and DCs to regulate autoreactive B cells through their secretion of soluble factors [5, 6]. Here we identify a third factor, TNFα, which selectively represses autoreactive B cells. Neutralization studies show that loss of any single repressive factor has little impact on Ig secretion. However, neutralizing B6 DC CM with both anti-IL-6 and anti-TNFα restores Ig secretion following TLR4 ligation (Figure 2.2). This, combined with the repressive effects of MΦ-secreted CD40L [6], indicates that IL-6, CD40L and TNFα are the primary soluble factors that regulate autoreactive B cells. However, other factors may play minor role(s) in repressing innate immune responses. We propose this because CM from IL-$6^{-/-}$ DC neutralized with anti-TNFα remain repressive (Figure 2.1A), and DC/MΦ CM isolated from 3XKO mice represses about 40% of Ig secretion (data not shown). Our *in vivo* data support that we have identified the major repressive factors since lack of IL-6, CD40L and TNFα is sufficient to allow activation of autoreactive B cells (Figures 2.6 and 2.7).

The data show that although Ig secretion by 2-12H/Vκ8 B cells is repressed by TNFα, the magnitude of repression is less than IL-6 or CD40L. One possible explanation is that TNFα only represses a subset of B cells. We have found that TNFα is unable to repress 2-12H Tg B

cells (N.J.Wagner, S.-R. Lee, and B.J.Vilen, unpublished observations). 2-12H Tg mice have a fully populated marginal zone (MZ), while only ~1.5% of the splenic B cells of 2-12H/Vκ8 are MZ [23, 24, 31]. The difference in regulation by TNFα may be due to the presence or absence of MZ B cells. MZ B cells respond to antigen more quickly than FO cells; an IgM response from MZ cells occurs within 24 hours versus 2-3 days for follicular (FO) B cells [32]. TNFα may not be able to regulate the more reactive MZ B cells, while less reactive FO B cells respond to TNFα repression. Alternatively, TNFα may be able to repress autoreactive B cells of low affinity while those of higher affinity escape TNFα regulation. To test these possibilities, we are currently sorting FO and MZ B cells from 2-12H Tg mice to determine if TNFα represses various B cells subsets.

TNFα might contribute to the regulation of autoreactive B cells by influencing the amount of IL-6 produced by DCs and MΦs. We have shown that DCs from lupus-prone MRL/*lpr* mice fail to sustain IκBα phosphorylation following TLR ligand binding, leading to reduced activation of NFκB [25]. Sustained NFκB activation is required for IL-6 secretion [25]; along with LPS, TNFα also stimulates the NFκB pathway [33]. Therefore the reduced level of TNFα produced by lupus-prone DCs and MΦs may further contribute to diminished IL-6 production. Since IL-6 is a more repressive factor of autoreactive B cells (Figure 2.1), the role of TNFα may be to modulate IL-6 production by DCs and MΦs.

Our finding that IL-6, CD40L and TNFα are not involved in spontaneous differentiation of autoreactive B cells to ASCs supports our previous data that these factors regulate innate immune responses [5, 6, 25]. Without innate immune stimuli, peripheral autoreactive B cells are not activated; therefore DC/MΦ-mediated regulation is not required. We believe that IL-6, CD40L and TNFα are critical in regulating autoreactive B cells both *in vitro* and *in vivo* during

innate immune responses. Since it is proposed that TLR ligation of autoreactive B cells can overcome tolerance [34-43], DC/MΦ-mediated regulation of autoreactive B cells through secreted factors provides a mechanism by which tolerance is maintained in the presence of TLR ligands. In light of these data, we propose the regulation of autoreactive B cells by IL-6, CD40L and TNFα is relevant only during innate immune responses.

Transferred autoreactive 2-12H/B6 Tg B cells become activated with LPS stimulation in chimeric 3XKO mice, however the response is short lived (Figure 2.7). Since MZ B cells respond quickly, these data implicate a MZ response. MZ B cells are activated to become ASCs in 3-4 days when transferred into *lpr* mice, while FO B cells take 10-12 days to become ASCs (K.L.Conway and S.H.Clarke, personal communication). Additionally, the chimeric 3XKO mice may have residual follicular defects [27], that would lead to extrafollicular B cell responses that generally involve MZ B cells [44]. Therefore, it is likely that the response we see in the chimeric mice is due to transferred 2-12H/B6 MZ B cells.

Our data indicate that cytokines traditionally thought of as stimulatory to B cells may have an opposite effect, depending on the history of the BCR stimulation. We propose that chronic antigen stimulation through the BCR reprograms cytokine signaling. Thus, when TLR ligands bind antigen-experienced B cells, these cells do not respond by secreting Ig. This prevents an autoantibody response during infection. Conversely, removal of IL-6, CD40L or TNFα allows the activation of autoreactive B cells upon TLR4 ligation. In light of our data, we would urge caution in the use of cytokine-blocking treatments for autoimmune diseases. While short-term reduction in inflammation will be achieved, and may be sufficient for relief in some cases [14-18], the disease process may be initiated in patients with a predisposition to autoimmune disease. Data from our lab indicates that DCs and MΦs from lupus-prone mice

have defects in the production of IL-6, sCD40L and TNFα in response to LPS [25] (Figure 2.4). If DCs and MΦs from SLE patients show similar defects, an alternative to cytokine blocking treatments may be to restore B cell tolerance by transferring functional MΦs and DCs into patients to re-establish regulation of autoreactive B cells. This method of treatment would most likely be effective on emerging autoreactive B cells that have not become activated ACSs. Therefore the transfer of functional DCs and MΦs may need to be combined with B cell depletion therapy to start with tolerant, responsive B cells.

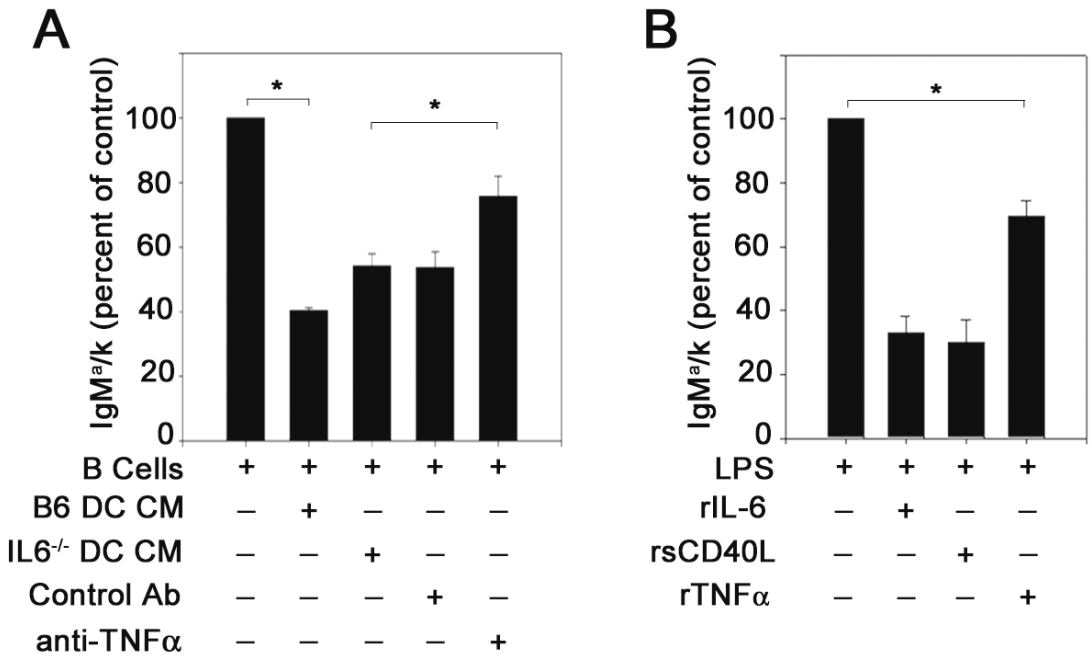

Figure 2.1. TNFα represses Ig secretion by Sm-specific B cells. Sm-specific (2-12H/Vκ8) B cells (1 x 10^5) were stimulated with 30 μg/ml LPS and co-cultured with (A) CM from LPS-activated B6 DCs, LPS-activated IL-$6^{-/-}$ DCs, IL-$6^{-/-}$ DC CM neutralized with anti-TNFα (324 μg) or isotype-matched control antibody or (B) rIL-6 (20ng/ml), rsCD40L (100ng/ml) or rTNFα (50ng/ml). IgM^a/κ levels were quantitated on day 4 by ELISA. LPS-stimulated B cells (100%) secreted 1-31 μg/ml. Data represent at least three independent experiments. Error bars represent SEM. (*$p \leq 0.05$)

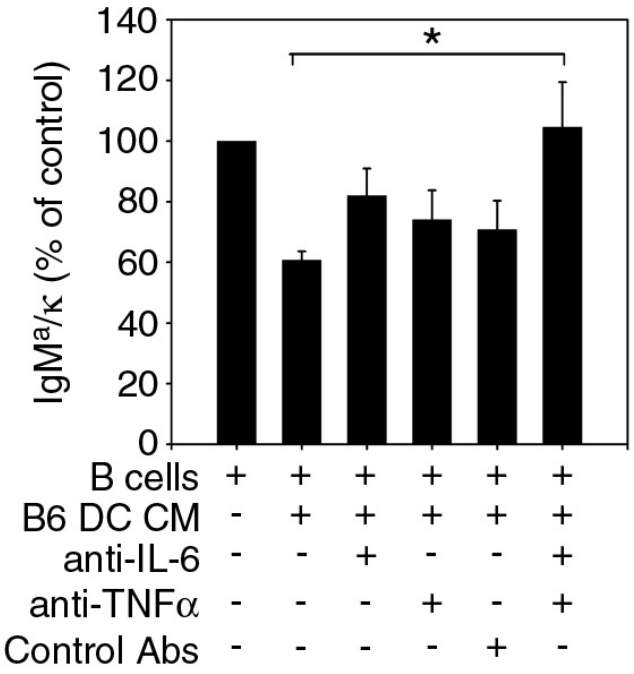

Figure 2.2. IL-6 and TNFα are the only factors secreted by DCs that repress anti-Sm Ig secretion. Purified anti-Sm (2-12H/Vκ8) B cells ($1x10^5$) from mice were stimulated with 30 μg/ml LPS in the presence LPS-activated B6 DC CM or B6 DC CM neutralized with anti-IL-6 (50 μg/ml), anti-TNFα (324 μg), both, or isotype-matched control antibodies. 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 independent experiments. Error bars represent SEM. (*$p \leq 0.05$)

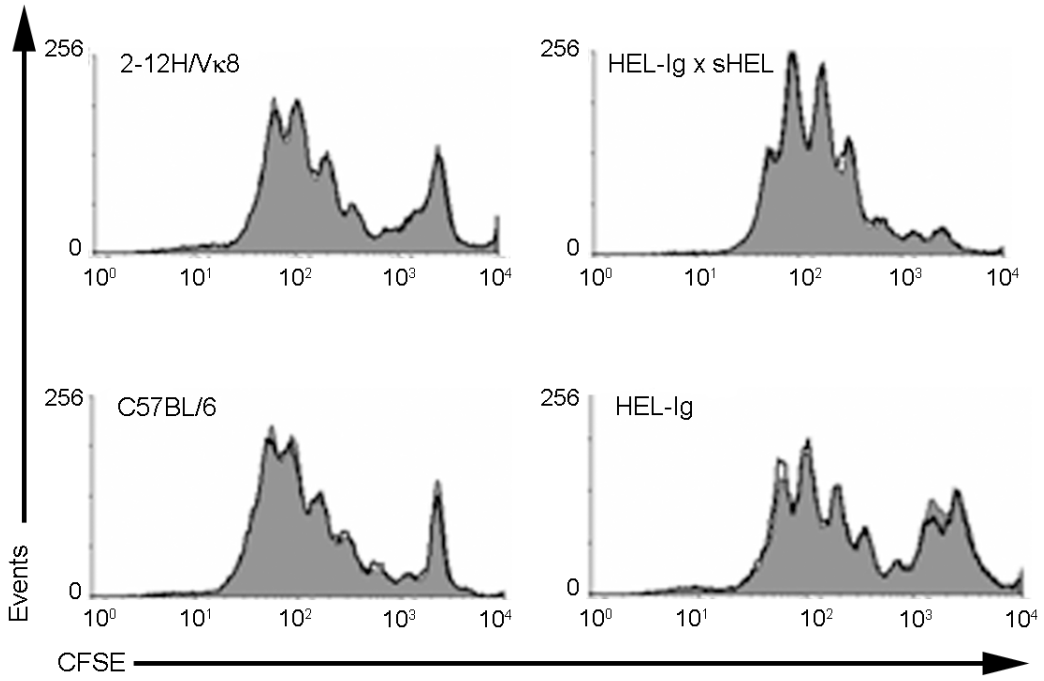

Figure 2.3. TNFα does not affect proliferation of B cells. Proliferation of LPS-stimulated (30μg/ml), CFSE-labeled B cells from 2-12H/Vκ8, HEL-Ig x sHEL, B6 and HEL-Ig mice was determined by FACS on day three. Cells were incubated in the presence (black line) or absence (gray fill) of rTNFα (50ng/ml). Data is representative of three experiments.

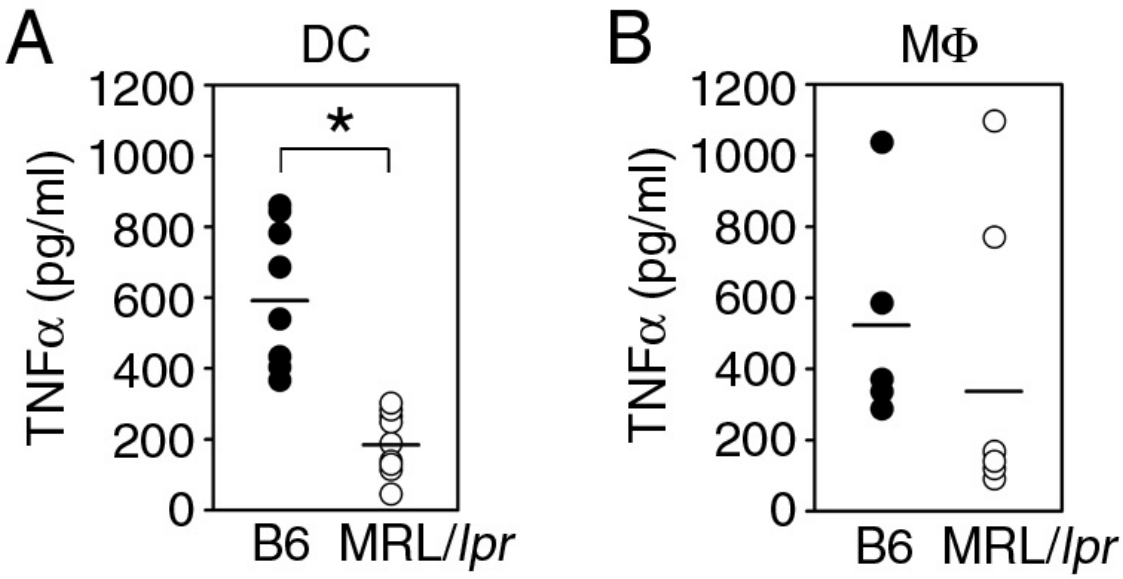

Figure 2.4. DCs and MΦs from lupus-prone MRL/*lpr* mice are defective in TNFα secretion. (A) BMDCs or (B) BMMΦs ($1x10^4$) from B6 (●) or MRL/*lpr* (○) were stimulated with LPS (30 μg/ml). TNFα was quantitated by ELISA from day 4 culture supernatants. Data represent at least 9 mice per group. (*$p \leq 0.05$)

Mouse Type	ANA	Histology
C57BL/6	0/5	0/4
IL-6$^{-/-}$	0	0/4
C40L$^{-/-}$	0	0/3
TNF$\alpha^{-/-}$	0	0/4
IL-6$^{-/-}$xCD40L$^{-/-}$	0/8	0/4
IL-6$^{-/-}$xTNF$\alpha^{-/-}$	0/8	0/4
3XKO	0/9	0/5

Table 2.1. Summary of mice monitored for spontaneous development of autoantibody or kidney pathology. Mice (6-13 of each type) were bled every 4 weeks to check serum antibody levels. Serum (1:50 dilution) ANA was detected using Hep-2 substrate slides, anti-mouse IgG and anti-mouse IgM. At 52 weeks kidneys were fixed, paraffin-embedded, sectioned, H&E stained and examined via light microscopy for pathology.

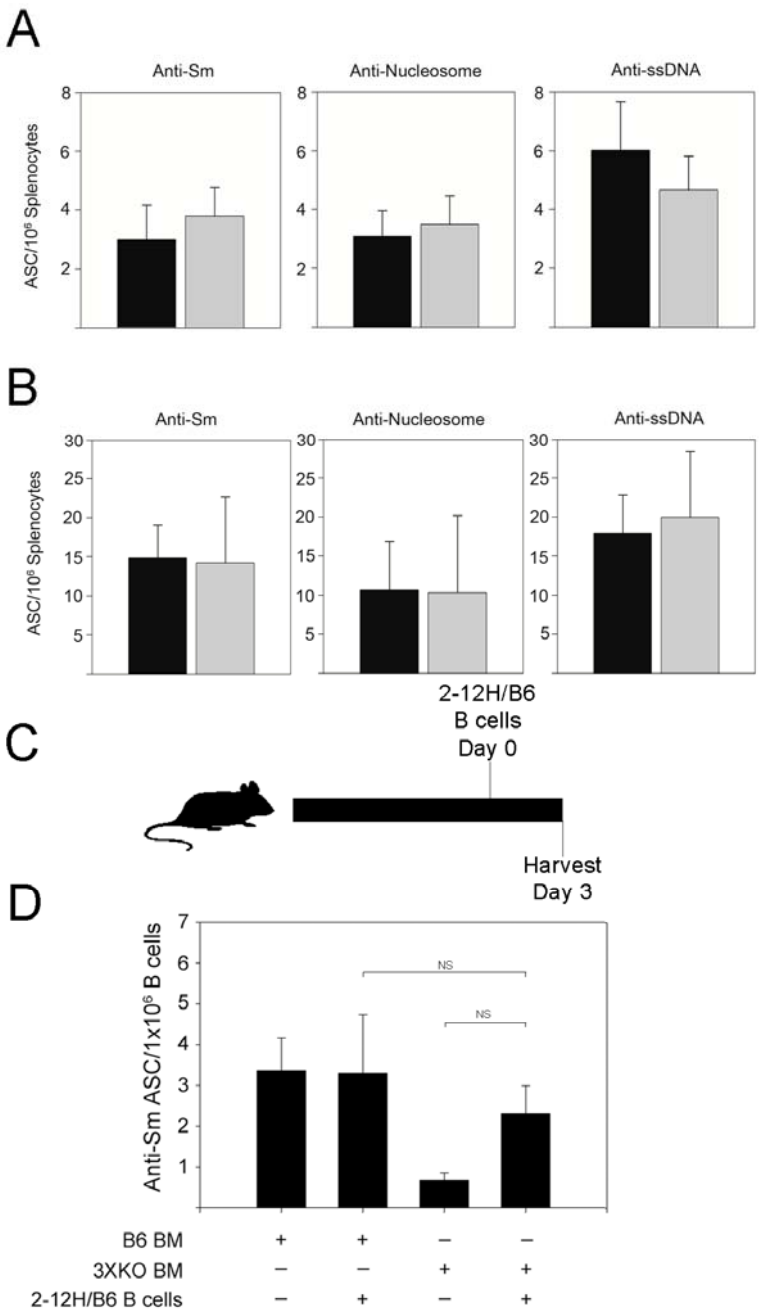

Figure 2.5. Mice lacking IL-6, CD40L and TNFα do not spontaneously develop autoreactive antibodies. Splenic cells from (A) non-chimeric or (B) chimeric B6 (black bars) and 3XKO (gray bars) mice were assayed for the presence of anti-Sm, -nucleosome, or –ssDNA ASCs by ELISpot. (C) Purified Sm-specific (2-12H/B6) B cells ($1x10^6$) were injected into chimeric mice reconstituted with B6 or 3XKO BM (that had not received LPS injections). Spleens were harvested three days post 2-12H/B6 B cell transfer. (D) Anti-Sm ASCs were detected by ELISpot three days post transfer. In panels A, B and D, secreted IgM was detected after cells were cultured on coated ELISpot plates 24 hours. Data represent at least three individual experiments. Error bars represent SEM. (NS= not significant, $p>0.05$)

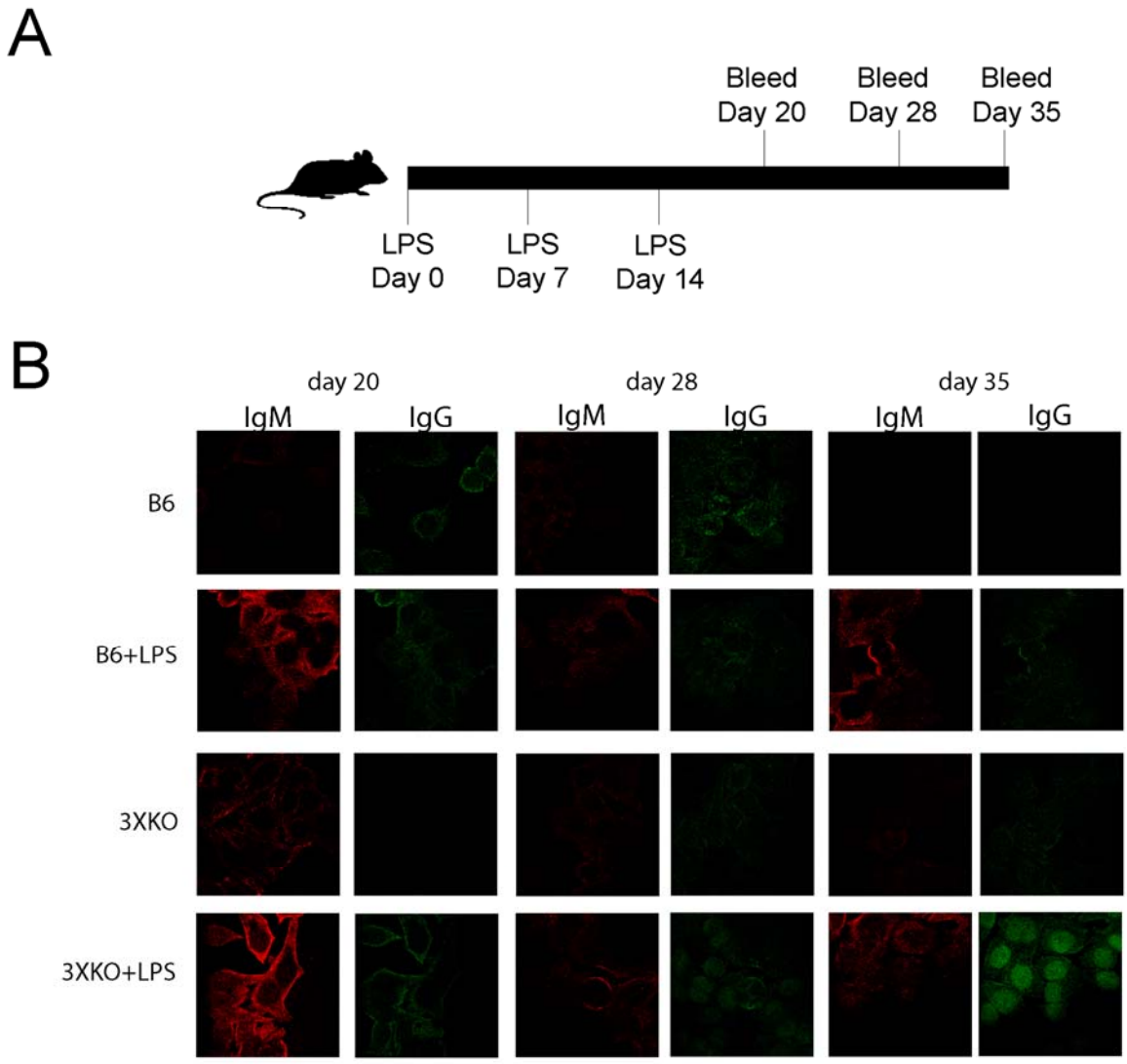

Figure 2.6. LPS-stimulated 3XKO mice generate autoreactive antibodies. (A) Non-chimeric 3XKO and B6 mice were injected with 25μg LPS/week for three weeks and bled to monitor serum autoantibodies. (B) Representative serum ANA (1:50 dilution) from B6 (top 2 rows) and 3XKO (bottom 2 rows), with and without LPS. Anti-nuclear IgM was detected with Hep-2 substrate slides, anti-mouse IgM-Alexa 488 (red) and anti-nuclear IgG was detected with anti-mouse IgG-Alexa 647 (green). Images were captured at a magnification of 60X.

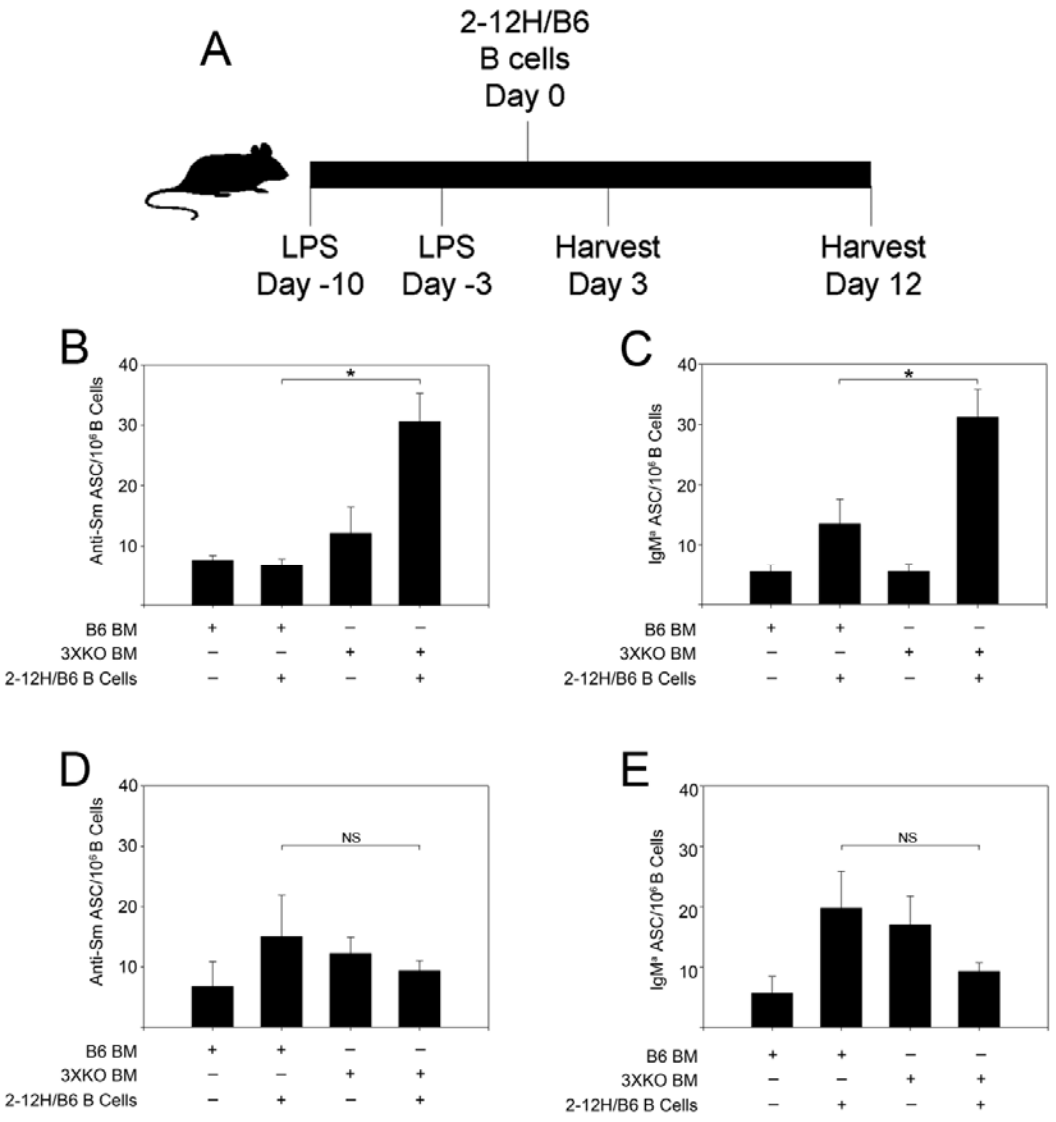

Figure 2.7. Sm-specific B cells transferred into LPS-stimulated mice lacking IL-6, CD40L and TNFα become short-lived ASCs. (A) Chimeric mice reconstituted with either B6 or 3XKO BM were injected with LPS to simulate an innate immune response. On day 0, $1.5x10^6$ purified Sm-specific B cells (2-12H/B6) were adoptively transferred into the mice. Three and twelve days later spleens were harvested to quantitate the number of transferred cells that had become ASCs. Three days post transfer (B) anti-Sm and (C) IgM^a ASCs were detected by ELISpot. Similarly, twelve days post transfer (D) anti-Sm and (E) IgM^a ASCs were detected. ASCs were detected following 24 hour incubation on coated ELISpot plates. Data is representative of at least three individual experiments. Error bars represent SEM. (*$p \leq 0.05$, NS= not significant, $p > 0.05$)

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Chapter 3
Discussion

During innate immune responses naïve B cells are activated to produce a polyclonal Ig response while autoreactive B cells remain quiescent. We have established that IL-6, CD40L and TNFα secreted by TLR-stimulated DCs and MΦs act on autoreactive B cells to repress Ig secretion [1-3] (Chapter 2). Importantly, our latest studies reinforce our *in vitro* data, proving *in vivo* that in the presence of innate immune stimuli, IL-6, CD40L and TNFα regulate autoreactive B cells (Chapter 2). We show that without TLR ligation, the absence of IL-6, CD40L and TNFα does not result in spontaneous autoantibody production (Figure 2.5). However, with LPS stimulation, autoreactive B cell break tolerance in mice lacking IL-6, CD40L and TNFα (3XKO mice) while they remain inactive in wildtype mice (Figures 2.6 and 2.7). Thus, IL-6, CD40L and TNFα play a role in regulating autoreactive B cells, both *in vitro* and *in vivo*.

The model system introduced in Chapter 2, using 3XKO mice, is valuable to confirm that IL-6, CD40L and TNFα regulate autoreactive B cells, but this model system does not address which of the factors play the primary role(s) or the order in which the factors act to repress B cell activation. Since MΦ-produced CD40L is able to repress marginal zone (MZ) B cells, while IL-6 is not [3], we predict that during an innate immune response CD40L would act first to repress autoreactive Ig secretion, followed by IL-6 that, with CD40L, represses follicular (FO) B cells [3]. TNFα appears to be a less potent repressive factor, compared to IL-6 and CD40L (Chapter 2), so its repressive function may be to reprogram an autoreactive B cell during development to be "repressible" [4, 5] or to affect IL-6 secretion by DCs and MΦs, and thereby repress Ig secretion (Chapter 2). The system of chimeric 3XKO mice can be adapted to use mice lacking one or two of the repressive factors to determine the individual roles each play in maintaining B cell tolerance.

This model system will be useful in determining how autoreactive B cell break tolerance, especially if future data reveal (as data in Chapter 2 suggest) that these mice develop autoreactive Ig, but no autoimmune disease. Thus, this model has a genetic predisposition to autoantibody production, but requires other genetic or environmental factors to develop disease. Such a model can be used to introduce other conditions known to induce to autoimmune disease, such as autoantigen burden, to determine what combination of circumstances push a mouse from autoantibody production to autoimmune disease. This is an exciting prospect since current model systems emulate systemic lupus erythematosus (SLE), but have not broken the process of disease development into discrete check points within a model system.

We have concentrated on repression of autoreactive B cells by DC/MΦ-secreted factors; however contact-dependant mechanisms of regulation are also implicated in B cell tolerance. When DCs and Sm-specific B cells are co-cultured, the level of Ig secretion is reduced compared to the repression by DC conditioned media (CM), suggesting that cell-cell mediated mechanisms, as well as soluble factors, are involved in regulation of autoreactive B cells [2]. Further, DC contact with B cells (autoreactive or not) seems to inhibit Ig synthesis (D.G.Carnathan and B.J.Vilen, unpublished observations). Since DCs repress Ig production in naïve and autoreactive B cells, selective regulation of autoreactive B cells may occur by some mechanism that keeps autoreactive B cell in contact with DCs. One possibility is that autoantigen expressed by DCs interacts with autoreactive BCRs, prolonging the DC/B cell interaction. This would inhibit B cell signaling in autoreactive B cells, while naïve B cells that do not recognize self antigen would not have prolonged DC colocalization, and therefore would avoid contact-mediated Ig repression. In fact, we have seen autoantigen on the surface

of DCs and MΦs (D.G.Carnathan, C.E.Hilliard, B.J.Vilen, unpublished observations). The location of the DC/B cell interaction may be critical, though, as DCs loaded with apoptotic cells can stimulate Sm-specific B cells with T cell help (K.L.Conway, S.H.Clarke, personal communication). Thus, B cells that come into contact with DCs may be repressed, unless they are in a location, such as a germinal center, where T cell help might overcome DC-mediated repression. Further, the data support our soluble mediator model since DCs pre-exposed to TNFα are unable to activate Sm-specific B cells, even with T cell help (K.L.Conway, S.H.Clarke, personal communication). This data indicates that under innate immune stimulation, when TNFα is secreted by DCs and MΦs, DC-mediated B cell activation would not occur. Therefore, DCs act to repress B cells by both soluble and contact-dependant mechanisms. T cell help can overcome contact-dependant repression, but, during inflammatory responses, such as innate immune responses, T cell help is overshadowed by the action of repressive soluble mediators.

Type I interferons (IFN-I) are another group of cytokines implicated in modulating autoimmune disease. Type I interferons include the closely related IFNα subtypes and the single IFNβ. SLE patients have abnormally high levels of IFNα in their serum [6, 7] and show a "signature" of IFN-induced gene expression [8-10]. Most cell types can secrete IFN-I, but the primary producer of INFα/β are plasmacytoid DCs (pDCs) [11-13]. In response to TLR stimulation, pDCs induce transcription, translation and secretion of IFN-I [11]. IFNα/β affect both innate and adaptive immune responses through activation of DCs, T cells and B cells [14-16]. IFN-I promote monocytes to become mature myeloid DCs (myDCs), capable of antigen presentation and T cell activation [16]. INFα, with concurrent TLR binding, promotes B cell plasmablast differentiation and Ig class switching, generating a

preferential IgG antibody response [17-19]. IFN-I significantly impacts immune responses and plays a role in autoimmune disease.

We have proposed a model of B cell regulation during innate immune responses such that soluble factors secreted by DCs and MΦs selectively repress autoreactive B cells while allowing a polyclonal Ig response by naïve B cells [2, 3, 20]. The data linking INF-I to autoimmunity suggests IFNα/β can be added to this model. Upon TLR stimulation, pDCs secrete IFN-I, inducing monocytes to develop into mature myDCs [16]. TLR ligand binding to myDCs results in their secretion of IL-6 and TNFα, factors shown to repress autoreactive B cells [2] (Chapter 2); since more DCs are present due to the action of IFN-I, a greater amount of IL-6 and TNFα is generated. Additionally, an IFN-I-mediated increase in DCs may prolong and/or increase DC/B cell contact, further inhibiting autoreactive B cell activation. In addition to DC-mediated repression, MΦs respond to TLR stimulation by secreting IL-6, CD40L and TNFα that selectively repress autoreactive B cells [2, 3] (Chapter 2). This system prevents autoreactive B cells from being activated and producing autoantibodies in response to innate immune stimuli.

B cell tolerance is not always maintained however, and autoimmunity does occur. Significant changes in the responses described above occur in an autoimmune response. TLR-stimulated lupus-prone DCs and MΦs secrete less IL-6, TNFα, and CD40L [1, 3] (Chapter 2). Once disease is established, high levels of inflammation lead to an increase in tissue damage, increasing the amount of self antigen available for presentation by antigen presenting cells (APCs). Additionally, in established SLE, aberrantly high serum levels of IFN-I are sustained [6, 7]. Therefore, the following is proposed when genetic and/or environmental factors lead to autoimmune disease. TLR stimulation of pDCs occurs, as it

does in non-autoimmune condition, resulting in the secretion of IFN-α/β. The increase in DCs from maturing monocytes however does not produce an increase in IL-6 and TNFα since lupus-prone DCs are defective in secreting these factors in response to TLR stimulation [1] (Chapter 2). The increased burden of autoantigen, caused either by the genetic and/or environmental elements leading to disease, or due to systemic inflammation caused by disease, is more efficiently presented by the IFN-activated DCs, compounding the adaptive autoimmune response. Since contact-dependent DC-mediated repression of B cells can be overcome with T cell help (K.L.Conway, S.H.Clarke, personal communication) the activation of autoreactive T cells through DCs contributes to autoantibody production. Additionally, IFN-I activated DCs produce the B cell survival factors BLyS and APRIL [21], allowing autoreactive B cells class switch to more pathogenic IgG via T-independent mechanisms. Excessive levels of BLyS are known to cause autoimmune symptoms [22-24], thus the increased number of activated DCs due to aberrantly high IFN-I could further contribute to autoimmune disease through BLyS production. Further impacting autoantibody secretion, lupus-prone MΦs stimulated with LPS do not secrete high levels of IL-6, CD40L or TNFα, and are therefore unable repress autoreactive B cells [1]. Finally, TLR stimulation of autoreactive B cells in conjunction with IFN-I binding (in the absence of DC/MΦ secreted IL-6, CD40L and TNFα) drives B cells to plasmablast differentiation [17-19]. Thus, in SLE, a combination of chronic high levels of serum IFN-I and defective DC/MΦ responses to TLR stimulation results in activation of autoreactive B cells and autoantibody production.

We have shown that IL-6, CD40L and TNFα are sufficient to regulate autoreactive B cells *in vivo* during innate immune stimulation (Chapter 2). 3XKO mice, while generating an autoantibody response, do not develop signs of autoimmune disease. This suggests that

defects in DC/MΦ-mediated B cell regulation contribute to autoimmunity, but alone do not induce disease. Adding INF-I to our model of autoreactive B cell regulation explains how elevated IFN-I contributes to established SLE. Since IFN-I functions upstream of the DC/MΦ defects that allow the break in B cell tolerance in the 3XKO model, exploring treatment options to restore functional DCs and MΦ to patients is a valid pursuit. The elevated levels of IFN-I in SLE patient serum would spur the transferred functional DC and MΦs to secrete repressive factors to prevent further loss of B cell tolerance.

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