Regulation of autoreactive B cells during innate immune responses

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ABSTRACT

Jennifer Ann Rutan: Regulation of autoreactive B cells during innate immune responses

(Under the direction of Dr. Barbara J. Vilen)

A diverse repertoire of B cell specificities is critical to combat pathogenic threats, but this diversity is tinged with the threat of autoimmunity.   Autoimmune diseases such as systemic lupus erythematosus (SLE) underscore the need for strict regulation of B cell activation during immune responses.   Regulation of the adaptive immune response is dependent on B cell receptor (BCR) ligation, affinity of the BCR-antigen interaction, and the presence of costimulatory molecules. Mechanisms of central tolerance and peripheral tolerance eradicate B cells that inappropriately bind self-antigens. Autoreactive B cells that escape these mechanisms of tolerance and encounter antigen in the absence of costimulatory signals enter an unresponsive state known as anergy. Anergic B cells do not differentiate into plasma cells, activate transcription factors such as Blimp-1 and XBP-1, or secrete immunoglobulin (Ig). B cells can also be activated by toll-like receptor (TLR) ligands and contribute to the innate immune response, the first line of defense against pathogens. Bacterial and viral components are the archetypical TLR ligands, but endogenous DNA and RNA can also bind TLRs and activate the innate immune system.   Therefore, it is critical to regulate the innate immune response to prevent autoimmunity, and this regulation requires unique mechanisms that are not necessarily dependent on BCR ligation. Ideally, these mechanisms would also

discriminate between naïve and chronically antigen-experienced B cells, allowing naïve B cells to respond to threats while potentially autoreactive B cells remain quiescent. We have recently described a mechanism where antigen-experienced B cells are regulated by IL-6 and sCD40L released by dendritic cells (DCs) and macrophages (MΦs) during innate immune responses. Naïve B cells are unaffected by these factors, permitting a robust immune response in the absence of autoimmunity. IL-6/sCD40L-mediated repression is dependent on ERK activation and "repressed" antigen-experienced cells exhibit a decrease in activated ERK in the nucleus. Identifying new targets for SLE therapies could enable specific regulation of antigen-experienced B cells instead of non-specific B cell depletion and immunosuppression.

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with gratitude for their unending love, support, and patience.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

BCR	B cell receptor
BLIMP-1	B lymphocyte induced maturation protein 1
BLyS	B lymphocyte stimulator
BMT	nonmyeloablative bone marrow transplant
DC	dendritic cell
dsDNA	double-stranded DNA
DTT	dithiothreitol
ERK	extracellular signal-related kinase
FcR	Fc receptor
FO	follicular
HEL	hen egg lysozyme
HSCT	nonmyeloablative hematopoietic stem cell transplant
IC	immune complex
Ig	immunoglobulin
IL-4	interleukin-4
IL-6	interleukin-6
IL-6R	interleukin-6 receptor (CD126)
KSR	kinase suppressor of Ras
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
mRNA	messenger RNA

MΦ	macrophage
MZ	marginal zone
NES	nuclear export signal
NLS	nuclear localization signal
PAMP	pathogen-associated molecular pattern
PI3-K	phosphatidylinositol 3-kinase
PMSF	phenylmethanesulfonyl fluoride
RF	rheumatoid factor
sCD40L	soluble CD40L
SLE	systemic lupus erythematosus
Sm	Smith antigen
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoproteins
ssRNA	single-stranded RNA
Tg	transgenic
TLR	Toll-like receptor
XBP-1	X-box protein 1

CHAPTER I

Introduction

This review was originally published in *Immunologic Research*.

1.1 Learning tolerance: a B cell's story

A diverse B cell repertoire is critical in combating pathogens, but inherent in generating diversity is the threat of autoimmunity. In the bone marrow, central tolerance mechanisms such as deletion or receptor editing remove high affinity autoreactive B cells before they exit to the periphery (1-10). Those that escape are subject to receptor revision (8, 11-14), peripheral deletion (15, 16), or a shortened lifespan because they fail to enter B cell follicles (17-22). In rare cases, autoreactive B cells are fully functional but indifferent to their specific antigen (23-25). Finally, many low affinity autoreactive B cells are maintained in an unresponsive state known as anergy. Anergic B cells do not receive sufficient activation signals to differentiate into plasma cells or secrete immunoglobulin (Ig) in response to antigenic or mitogenic stimulation (26-29). Their proliferative responses to B cell receptor (BCR) or toll-like receptor (TLR) signaling as well as their lifespans vary in different models (18, 30-35). Some anergic B cells transduce BCR-derived signals (30, 32, 36-40), while others exhibit desensitized BCRs (30, 33, 41). Quiescence is dependent on chronic exposure to self-antigen and occupancy of the BCR (42, 43). Furthermore, ERK activation is critical in sustaining anergy during polyclonal stimulation by TLR ligands (27, 28). Recently, it was demonstrated that anergy can be mediated by DCs and MΦs. In the presence of TLR ligands, DCs and MΦs release IL-6 and sCD40L that selectively represses Ig secretion from autoreactive B cells (44, 45). In the absence of DCs/MΦs and their soluble factors, autoreactive B cells regain the ability to secrete Ig, indicating that this mechanism of tolerance is reversible. These data suggest that autoreactive B cells must be maintained in close proximity to DCs/MΦs during innate immune responses to prevent autoimmunity.

The affinity and avidity of the antigen-BCR interaction determines whether developing B cells will be deleted, edited, anergized, or ignored (46). Self-reactive B cells that survive these developmental checkpoints tend to bind self-antigens with low affinity and they remain anergic in the absence of costimulation by cognate T cells. Many of the early transgenic (Tg) models expressed BCRs with high affinities. However, concerns were raised that these models may not adequately reflect how bona fide low affinity self-antigens are regulated (1, 26). To study the tolerance mechanisms regulating low-affinity B cells, new Ig Tg models were generated that expressed BCRs specific for double-stranded (ds) DNA, single-stranded (ss) DNA, rheumatoid factor (RF), insulin, and Smith antigen (Sm).

1.2 A short history of Sm

Sm antigens are conserved proteins that are indispensable in RNA splicing. In 1966, Sm was identified as a unique autoantigen, the first non-histone target of autoantibodies in systemic lupus erythematosus (SLE) patients (47). Named for 15-year-old Stephanie Smith, antibodies to Sm became one of the diagnostic criteria for SLE (48), detectable in 5-30% of SLE patients (48, 49). Later studies correlated anti-Sm titers with kidney disease (50-53).

The autoantibodies isolated from patient sera proved invaluable in the initial studies of the Sm proteins (54). The small nuclear RNA (snRNA U1, U2, U4, or U5) in each small nuclear ribonucleic particle (snRNP) is predicted to thread through the center of a heptameric ring of Sm proteins (E, F, G, D1, D2, D3, and B/B') (55). Three of the Sm proteins (D1, B, and D3) have long, positively charged tails that contact the pre-

mRNA in the 5' splice site region (56). This interaction stabilizes the commitment

complex formed when U1 snRNP binds the pre-mRNA substrate (57). The remaining

snRNPs, U2 and U4/U5/U6 (the triple snRNP) bind to pre-mRNA, rearrange to form the

splicesome, and remove introns from the transcript (58).

Self-antigens composed of protein and DNA or RNA can co-ligate the BCR with

TLRs, potentially overcoming tolerance mechanisms and activating autoreactive B cells.

For example, concomitant ligation of the BCR and TLR9 by chromatin:IgG immune

complexes activates RF-specific B cells in the absence of T cell help or overt TLR

stimulation (38, 59, 60). Signaling through both the BCR and TLR9 is necessary to

activate NF-κB in these B cells (37). Recently, RNA-IgG immune complexes were

shown to activate RF-specific B cells via TLR7 (39). TLR7 binds ssRNA, a viral antigen

that acts as a sensor of infection as well as a component of the U1 snRNP splicing

complex, a known autoantigen in SLE (61, 62). Aberrantly high expression of TLR7, or

an increased burden of immune complexes and/or apoptotic cells, aggravates disease in

lupus-prone mice. For instance, the gene duplication of TLR7 in *Yaa* mice results in

hyperactive B cells, exacerbation of disease in lupus-prone models, and shifts

autoantibody specificities to RNA (63, 64). Reducing TLR7 gene expression ameliorates

disease and increases survival (63). Hence, the combination of self-proteins and TLR

ligands within immune complexes and on the surface of apoptotic cells can mistakenly

activate autoreactive B cells and result in autoimmunity.

1.3 Arresting and silencing Sm-specific B cells

Sm-specific autoantibodies are a hallmark of both human and murine lupus. To identify the mechanisms that regulate Sm-specific B cells, the 2-12H Tg mice were generated (35, 65). In this model, an Ig heavy chain, 2-12H, was identified from an Sm-specific hybridoma derived from an MRL/*lpr* mouse. The 2-12H chain pairs with a variety of light chains, giving rise to B cells specific for Sm and/or ss-DNA. B cells from the 2-12H model express BCRs of multiple affinities that develop and are regulated on a non-autoimmune background.

Tolerance to Sm is dependent on several cell types. B cells are the most obvious suspects in SLE since disease pathology is mediated by autoantibodies. *In vivo*, Sm-specific B cells are regulated since 2-12H Tg mice have low titers of anti-Sm antibodies (35, 66). However, *ex vivo* non-subsetted 2-12H B cells (uncontaminated by DCs and MΦs) are activated by TLR stimulation (LPS, CpG, dsRNA) *in vitro* but their Ig secretion is lower than that of C57BL/6 controls (35, 67). The follicular (FO) B cell subset is repressed by DCs and MΦs secreting IL-6 and sCD40L, while secretion by the MZ B cell subset is partially repressed, but only by MΦs and sCD40L (45). Some MZ B cells and peritoneal B-1 cells ignore endogenous levels of Sm, but an increase in the number of apoptotic cells can activate peritoneal and MZ B cells (66, 68, 69). Sm-specific B cells arrested at the pre-plasma cell stage, interrupting plasma cell differentiation and preventing Ig secretion (32)

Restricting the light chain that pairs with 2-12H allowed for the analysis of Sm-specific B cells of moderate and low affinity (32, 70). The 2-12H/Vκ4 Tg mouse was generated to examine regulation of higher affinity anti-Sm responses (70). B cells from

this mouse are distributed among splenic transitional, FO, and MZ subsets, as well as the peritoneal B-1 subset (70). 2-12H/Vκ4 B cells are anergic and all subsets are hyporesponsive to LPS *in vitro*. Additionally, MZ B cells exhibit a block in BCR signaling (70). LPS-stimulated 2-12H/Vκ4 B cells are repressed by IL-6 and sCD40L (unpublished data). To study low-affinity anti-Sm responses, the 2-12H/Vκ8 Tg mouse was created (32). In this model, only transitional and FO B cells are present and these cells are regulated by anergy (32). As in the previous anti-Sm models, 2-12H/Vκ8 B cells are susceptible to IL-6- and sCD40L-mediated repression (44, 45).

T cells are implicated in SLE and Sm-specific T cells are present in the repertoires of both normal and autoimmune mice (71). Sm-specific T cells in 2-12H Tg mice are anergic and do not proliferate in response to B cells presenting Sm (71). Anergic T cells are also unable to upregulate CD40L and provide costimulation to their cognate B cells (72). Anti-Sm B cells do not secrete Ig *in vivo* (35, 65), perhaps because they are deprived of T cell costimulation. However, in autoimmune situations, autoreactive T cells induce class-switching and somatic hypermutation of anti-Sm B cells, resulting in high levels of pathogenic high-affinity IgG autoantibodies (73, 74). Paradoxically, anti-Sm B cells are required to tolerize Sm-specific T cells from C57BL/6 mice, but they activate Sm-specific T cells from MRL/*lpr* mice (71, 75, 76). This indicates that although T cells are necessary for the development of autoantibodies and disease, they are also regulated by autoreactive B cells in normal individuals.

DCs and MΦs regulate innate and adaptive immune responses by tolerizing or activating T and B cells. The continued ingestion and presentation of self-antigens or the acute presentation of foreign antigen by DCs/MΦs either tolerizes or activates T cells to

drive adaptive immune response. The activation of DCs during innate immune responses

induces the secretion of IL-6 that promotes immunity by releasing CD4$^+$ T-helper cells

from their inhibitory functions (77). This promotes polyclonal activation of naive B

cells, the production of neutralizing antibody and the clearance of the invading pathogen.

  In addition to regulating T cells, DCs and MΦs affect the fate of B cells. They

activate naïve B cells by secreting type I interferon, IL-6, and B lymphocyte stimulator

(BLyS) (78-80). They also repress Ig secretion by B cells that have been chronically

exposed to antigen. Our laboratory showed that DCs and MΦs regulate autoantibody

production, in part through their secretion of IL-6 and sCD40L (44, 45). Repression is

selective in that naïve B cells (not chronically exposed to antigen) are unaffected by the

presence of IL-6 and sCD40L while Ig secretion by autoreactive B cells is repressed

(Figure 1A). Coupled with the data showing that IL-6 de-represses regulatory T cells, a

mechanism emerges explaining how the pleiotropic affects of IL-6 produced by DCs and

MΦs simultaneously promotes immunity and represses autoimmunity during innate

immune responses.

  Signal transduction through many cell surface receptors influences neighboring

receptors. For example, crosstalk between the IFN-αβ and IL-6 receptor (IL-6R)

signaling pathways augment transcription factor binding and gene expression in mouse

embryonic fibroblasts (MEFs) (81). Similarly, stimulation of B cells with sCD40L, IL-4,

and LPS reprograms the BCR signaling pathway, enhancing ERK activation and

bypassing the requirement for phosphatidylinositol 3-kinase (PI3-K) (82-86). The

findings that only B cells chronically exposed to self-antigen are susceptible to repression

by IL-6 and sCD40L suggests that chronic BCR-derived signals "reprogram" the

outcome of IL-6R and CD40 signal transduction. On a molecular level, the ability of IL-6 and sCD40L to repress Ig secretion reflects diminished BLIMP-1 and XBP-1 mRNA and protein levels. These data indicate that regulation occurs upstream of transcriptional activation. In support of this, pharmacologically inhibiting MEK restores LPS-induced Ig secretion. This suggests that the ability of IL-6/sCD40L to repress TLR4-induced Ig secretion is MEK/ERK-dependent (unpublished data).

Susceptibility of B cells to IL-6/sCD40L requires that B cells be chronically exposed to antigen, consistent with a central role for the BCR in tolerance. High affinity neo-self-antigens direct a unique tolerance scheme compared to low-affinity self-antigens. Anergic B cells from high affinity models are characterized by elevated phospho-ERK (42, 87). In addition, the binding of high affinity antigen to the BCR and constitutive MEK/ERK activation is sufficient to repress TLR4 and TLR9-induced Ig secretion (27, 28). In the low affinity Sm model (2-12H/Vκ8) basal phospho-ERK levels are comparable to those in the HEL model. However, unlike the HEL model, the binding of soluble SmD or snRNPs coupled with elevated phospho-ERK levels does not repress TLR-induced Ig secretion (unpublished data). This indicates that ERK is only part of the "BCR-derived" signals that regulate innate immune responses *in vivo*. Although B cells expressing high affinity receptors can be repressed by IL-6 and sCD40L, antigen stimulation is sufficient. In contrast, B cells expressing low affinity receptors for bona fide self-antigens (Sm and ssDNA) do not achieve sufficient BCR-derived signals to influence TLR4-induced Ig secretion and depend on additional signals from IL-6/sCD40L (unpublished data).

The finding that DCs secrete IL-6 while MΦs secrete IL-6 and sCD40L suggests that the anatomical location within the secondary lymphoid organs might dictate how autoreactive B cells are regulated. Marginal zone B cells are solely repressed by sCD40L while FO B cells are repressed by IL-6 and sCD40L (45). This specificity may result from the anatomic location, since different subsets of DCs and MΦs localize to specific regions of the spleen. For example, B cells are retained in the marginal zone by MΦs (88) and they are regulated by MΦ-derived sCD40L. However, upon activation and differentiation into pre-plasma cells, they may become susceptible to repression by IL-6 secreted by DCs within the periarteriolar lymphoid sheath (PALS). Thus, depending on the anatomical location of the autoreactive B cell, DCs and/or MΦs can repress autoantibody production during innate immune responses.

1.4 Failed tolerance: Sm-specific autoantibodies in a murine model of SLE

MRL/*lpr* mice are a well-characterized murine model of SLE, with adult-onset disease mediated by autoantibody deposition and tissue destruction. The prevalence of anti-Sm autoantibodies in human SLE patients and MRL/*lpr* mice is approximately 25% (89, 90). BCR Tg models have been bred onto the MRL background, allowing B cells of known specificities to be followed throughout development. Developmental arrest, follicular exclusion, and receptor editing are defective in MRL/*lpr* mice (91, 92). Expression of the 2-12H transgene in MRL/*lpr* mice increases the prevalence of the anti-Sm response, accelerates disease, and leads to higher serum anti-Sm levels (65). Sm-specific B cells from 2-12H mice are arrested at the pre-plasma cell stage, while B cells from the 2-12H/MRL/*lpr* mice bypass this checkpoint and become activated (93).

9

The presence of class-switched autoantibodies in MRL/*lpr* mice suggests a breakdown in tolerance within the adaptive immune response. In MRL/*lpr* mice where somatic hypermutation and isotype switch recombination are blocked (AID$^{-/-}$), lupus-like symptoms such as glomerulonephritis, proteinuria, and immune complex deposition are ameliorated (94). Early studies showed a critical role for T cells in disease because thymectomized MRL/*lpr* mice failed to develop lupus-like disease (95). Subsequently, it was shown that defects in central deletion and the number and function of T-regulatory cells allow CD4$^+$ T-helper cells to activate autoreactive B cells, induce terminal differentiation and autoantibody production (96-101).

Dysregulation of the innate immune system is apparent in MRL/*lpr* mice (39). Immune complexes containing RNA or chromatin stimulate RF-specific B cells through TLR7 and TLR9 to secrete anti-Sm and anti-chromatin (38, 39). Consistent with a role for TLRs, autoantibody responses were reduced in MyD88$^{-/-}$/ MRL/*lpr* mice (39) and disease was ameliorated (102). TLR7$^{-/-}$/ MRL/*lpr* mice exhibit reduced autoantibody titer and gene duplication of TLR7 shifts autoantibody specificities toward RNA, exacerbating disease (63, 64). This reveals TLR7 as a key receptor in promoting autoimmunity when tolerance is overcome (63, 103). Like TLR7$^{-/-}$ mice, TLR9$^{-/-}$/ MRL/*lpr* mice exhibited lower titers of anti-DNA autoantibodies. However unlike TLR7$^{-/-}$ mice, they remain plagued by accelerated kidney disease and increased mortality (103). These data suggest that TLR9 induces anti-DNA responses but also has an anti-inflammatory effect, possibly through its induction of regulatory T cells (104, 105). The function of regulatory T cells in TLR9$^{-/-}$/MRL/*lpr* mice is impaired, potentially allowing autoreactive cells to exacerbate disease (106). These data indicate that innate immune responses have

tremendous and opposing effects on autoantibody production and disease in MRL/*lpr* mice.

Defects in DC/MΦ-mediated tolerance are evident in lupus-prone mice. Our studies indicate that secretion of IL-6 and sCD40L by DCs/MΦs, as well as reprogramming of IL-6R and CD40 in autoreactive B cells, promote tolerance during innate immune responses. This implies that defects in either the secretion of IL-6/sCD40L or the selective response of autoreactive B cells to these factors regulate autoantibody production. Our studies indicate that DCs and MΦs from MRL/*lpr* mice are unable to repress autoreactive B cells (Figure 1B). Defects in DC/MΦ-mediated repression are coincident with diminished secretion of IL-6 and sCD40L and failure to sustain IL-6 mRNA production and activation of the IκB/NFκB pathways (107). Similarly, we found that IL-6 and sCD40L fail to repress B cells from lupus-prone mice (unpublished data). The finding that lupus-prone mice harbor defects in DCs/MΦs and B cells suggests the influence of an environmental stimulus.

1.5 Apoptotic cells: dangerous in death

Apoptotic cells pose a unique challenge to the immune system since they are a rich source of nuclear antigens, including DNA, immune complexes, nucleosomes, histones, snRNPs and snRNAs, Ro, La, and Sm. These self-antigens can be modified by phosphorylation and/or alternate protein cleavage, revealing novel epitopes that were not available during the induction of B and T cell tolerance (108-111). During cell death, self-antigens are distributed in large and small blebs on the cell surface and act as targets for autoreactive B cells in a membrane-bound, multimeric form (112-114). The display

11

of self-antigens on apoptotic cells is necessary to develop autoantibodies. For instance, autoantibodies to cytoplasmic but not nuclear antigens develop when nuclear fragmentation is blocked in apoptotic cells (115). Therefore, it is imperative that scavenger cells such as MΦs and DCs quickly clear apoptotic cells and their associated self-antigens to minimize inflammatory and autoimmune responses.

Apoptotic cells are bound and cleared by a variety of receptors on DCs and MΦs. Ingestion of apoptotic cells reduces the secretion of proinflammatory cytokines by DCs and MΦs, inhibiting their maturation and controlling the activation of T cells and autoimmune responses to apoptotic cells (116-121). Decreased clearance of apoptotic cells and defects in phagocytosis by MΦs in SLE patients and lupus-prone mice may lead to autoimmunity (69, 108, 122-124). The increase in apoptotic cells and antigens could dysregulate DCs and MΦs, triggering a chronic anti-inflammatory response that downregulates the production of cytokines important for B cell tolerance (45, 107).

If apoptotic cells fail to be cleared efficiently, they may become necrotic and expel self-antigens and TLR ligands that are opsonized by autoantibodies, forming immune complexes (125, 126). Like apoptotic cells, immune complexes (ICs) present self-antigens in a multimeric form and enhance phagocytosis by DCs and MΦs (127, 128). While apoptotic cells stimulate anti-inflammatory responses, ICs bind to Fc receptors (FcRs) and elicit inflammatory responses from MΦs and DCs (128). Individual FcRs have distinct activating and inhibitory functions to ensure balance in a normal immune system. Activating FcRs include FcγRI and FcγRIII, which phagocytose ICs and provoke inflammatory responses from DCs and MΦs (129-131). These receptors are necessary and sufficient for glomerulonephritis in (NZB/NZW) F1 mice (132-134). Co-

ligation of TLR9 and FcγRIII by chromatin:IC induces more DC activation than ligation of FcγRIII alone, indicating that TLRs synergize with FcR signaling (135). In contrast to the activating FcRs, FcγRIIB is an inhibitory FcR with various functions depending on cell type. It modulates MΦ phagocytosis, DC maturation, BCR signaling, IgG secretion, and the expansion of autoreactive IgG-producing B cells (134, 136-145). Autoantibodies and glomerulonephritis develop in certain strains of mice deficient in FcγRIIB, affirming its role in preventing autoreactive B cell activation (146, 147). Additionally, antigen internalized by DCs through FcγRIIB, but not FcγRI or FcγRIII, is presented in its native form and activates antigen-specific B cells (148). Immune complexes and FcRs have important roles in the immune system, but their dysregulation can result in autoantibody secretion and nephritis.

Clearance of apoptotic cells and their associated autoantigens is crucial in preventing activation of B cells, DCs, MΦs, and T cells. When mice are immunized with apoptotic cells or exhibit a defect in apoptotic cell clearance, Sm-specific antibodies are detectable in the serum and MZ and B-1 B cells are inappropriately activated (66, 68, 69). This suggests that recognition of autoantigens on apoptotic cells can drive B cell terminal differentiation (66, 68). Impaired clearance of apoptotic cells in Fas^{lpr} (69) and mer^{kd} (149, 150) mice prevents BCR-mediated reprogramming of IL-6R and CD40 (unpublished data). These data suggest that exposure of autoreactive B cells to apoptotic cells or immune complexes can impact DC/MΦ-mediated tolerance. In summary, apoptotic cells and the self-antigens they display can play both autoimmune and anti-inflammatory roles. Imbalances in apoptotic cell clearance or cellular responses result in inflammatory responses and autoimmune disease.

1.6 Selective repression: new treatments for SLE?

Systemic lupus erythematosus (SLE) is primarily a B cell-mediated autoimmune disease, with symptoms arising from autoantibody deposition and inflammation in target organs, such as the kidneys, skin, and brain. Until recently, treatments for SLE were dependent on immunosuppression, which depresses immune function and causes dangerous side effects such as opportunistic infections (151). Therapies that target specific cell types or biological processes are now being developed, with the hopes that they will be more powerful and have less harmful side effects. For instance, chimeric anti-CD20 (rituximab), depletes peripheral B cells and reduces the severity of SLE symptoms in many patients (152). However, a subset of patients is resistant to rituximab treatment (151). Additionally, rituximab recently failed a late-stage study when its efficacy in achieving a clinical response was no greater than a placebo (153). In murine studies of human CD20, B cells in autoimmune-prone strains were refractory to depletion by rituximab (154), compared to non-autoimmune-prone strains. Other biological agents being studied or developed target complement activation, B cell-T cell interactions, cytokines, TLRs, interferon, or direct removal of antibodies from circulation. However, developing therapies to target each lupus-related autoantigen would be cumbersome and slow. A more efficient approach would be a therapy that selectively targeted autoreactive B cells through a common trait to restore tolerance. In previous studies, we determined that DC- and MΦ-mediated repression via IL-6 and sCD40L is effective on B cells of multiple specificities. These data suggest that any B cell chronically exposed to antigen would be susceptible to DC/MΦ-mediated tolerance. We are currently determining whether the lack of these soluble factors promotes autoimmunity in normal mice during

innate immune responses. Preliminary data indicate that Sm-specific B cells adoptively

transferred into chimeric mice lacking IL-6 and sCD40L become activated and secrete

autoantibodies (unpublished data). This is consistent with our model indicating that

DCs/MΦs and their secreted products regulate autoreactive B cells during innate immune

responses. Future experiments will examine if tolerance can be restored in lupus-prone

mice reconstituted with a mix of autoimmune and non-autoimmune hematopoietic stem

cells. If DC/MΦ-mediated tolerance is found to be defective in SLE patients, future

therapies could target their *in vivo* activation or introduce normal DCs/MΦs to reinstate B

cell tolerance of newly emerging B cells following B cell depletion therapy. One

approach would be nonmyeloablative bone marrow transplant (BMT) or

nonmyeloablative hematopoietic stem cell transplant (HSCT) to promote mixed

chimerism. This may reinstate tolerance by providing a pool of DCs/MΦs that repress

autoreactive B cells. Such an approach has shown promise in controlling B cell mediated

autoimmune disease in humans and mouse models (155-160), resulting in remission of

rheumatoid arthritis in a human patient (158) and reduction in lupus-like disease in mice

(155, 157).

1.7 Summary

The autoreactive B cells that incite multi-organ damage in SLE patients become

dysregulated long before clinical symptoms appear. Autoantigens and TLR ligands

released from apoptotic cells can overcome the tolerance of autoreactive B cells.

Autoreactive T cells provide costimulation and promote the secretion of high-affinity,

class-switched autoantibodies. These autoantibodies can bind nuclear self-antigens, form

immune complexes, and provoke inflammatory responses from DCs and MΦs.

Fortunately, DCs and MΦs specifically repress autoreactive B cells during TLR

stimulation by secreting IL-6 and sCD40L. Restoring normal DC and MΦ function in

SLE patients might repress autoreactive B cells and re-establish balance among the

complex components of the immune system.

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1.9 Figures

Figure 1.1. DCs and MΦs repress antibody secretion from autoreactive B cells via IL-6 and sCD40L. (A) In non-autoimmune mice, B cell tolerance is maintained during innate immune responses by DCs and MΦs. Although TLR stimulation promotes Ig secretion by B cells, it simultaneously induces DCs and MΦs to secrete IL-6 and sCD40L. These soluble factors repress Ig secretion from autoreactive B cells while allowing innate stimuli to produce a polyclonal response by naïve B cells. **(B)** In lupus-prone mice, DCs and MΦs are defective in the production of IL-6 and sCD40L, allowing both autoreactive and naïve B cells to produce Ig in response to TLR stimulation.
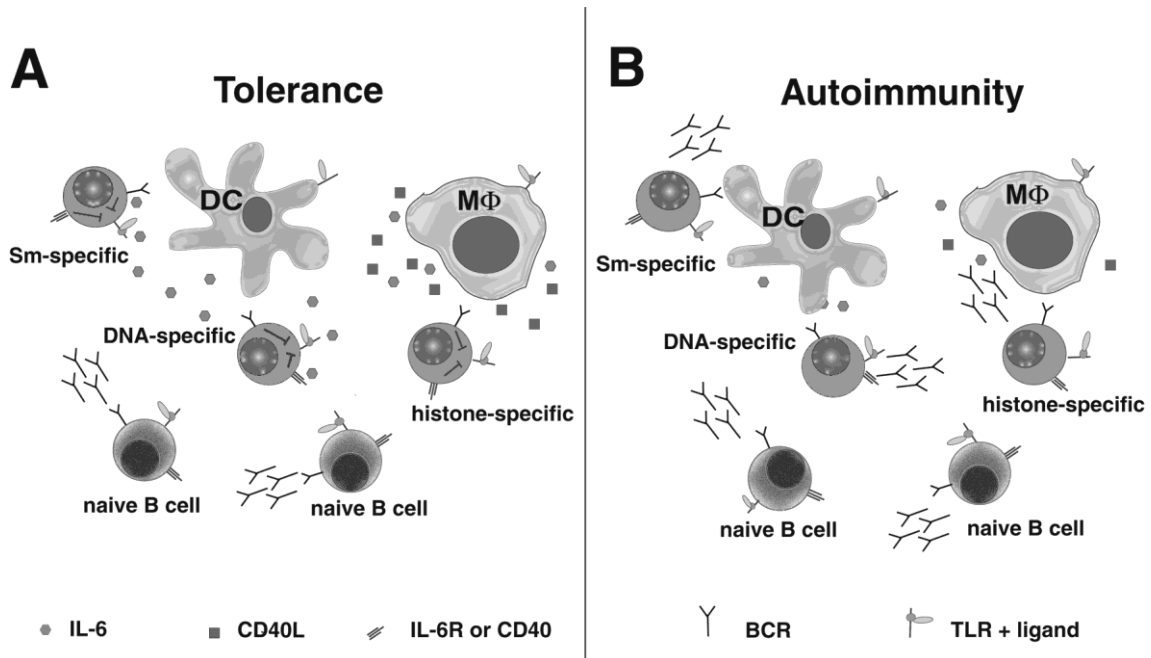
CHAPTER II

Tolerogenic ERK signaling prevents differentiation of autoreactive B cells

during innate immune responses

2.1 Abstract

Systemic lupus erythematosus (SLE) highlights the dangers of dysregulated B cells and the importance of initiating and maintaining tolerance. In addition to central deletion, receptor editing, peripheral deletion, receptor revision, anergy, and indifference, we have described a mechanism of B cell tolerance wherein dendritic cells (DCs) and macrophages (MΦs) regulate chronically antigen-experienced B cells during innate immune responses. In part, DCs and MΦs repress antigen-experienced B cells by releasing interleukin-6 (IL-6) and soluble CD40 ligand (sCD40L). This mechanism is selective in that IL-6 and sCD40L do not affect Ig secretion by naïve cells during innate immune responses. In this manuscript, we show that repression by IL-6 and sCD40L is mediated by ERK activation and distinctive subcellular localization. This elaborate mechanism allows differential regulation of naïve and antigen-experienced B cells, permitting vigorous immune responses in the absence of autoimmunity

2.2 Introduction

The innate immune system pioneers the response to pathogens, promoting an inflammatory response and activation of a variety of immune cells. B cell activation and immunoglobulin (Ig) secretion are crucial for an effective early response and efficient clearance of pathogens. A broad swath of B cell specificities is critical in combating an array of pathogens during both the innate and adaptive immune responses, but the threat of autoimmunity is inherent in this diversity. Breaches in B cell tolerance can result in autoimmune diseases such as systemic lupus erythematosus (SLE). Since innate immune responses are inherently non-specific, it seems that these responses must incorporate tolerance mechanisms that distinguish between naïve and chronically antigen-experienced B cells. We recently identified a tolerance mechanism that selectively represses Ig secretion by autoreactive B cells during innate immune responses. We find that chronically antigen-experienced B cells are maintained in an unresponsive state by dendritic cells and macrophages that secrete IL-6 and/or sCD40L in response to innate stimuli binding Toll-like receptors (TLRs). DC/MΦ-mediated tolerance is reversible *in vitro*; removal of dendritic cells and macrophages allow previously unresponsive B cells to secrete Ig in response to TLR ligands. Treating de-repressed B cells with dendritic cells, macrophages, recombinant IL-6 (rIL-6) or recombinant soluble sCD40L (rsCD40L) restores the repressed phenotype. The repressive effect of IL-6 and sCD40L is limited to chronically antigen-experienced B cells and naïve B cells remain competent to secrete Ig (1-3). This suggests that chronic B cell receptor (BCR) ligation *in vivo* affects the outcome of IL-6 receptor (IL-6R) and CD40 signal transduction. In effect, these receptors are "reprogrammed." In similar situations, IFN-αβ signaling and sCD40L, IL-

4, and LPS reprogram the IL-6R and the BCR, respectively (4-9). Our finding that DCs and MΦs selectively regulate autoreactive B cells provides a cellular explanation for the ability of the immune system to mount polyclonal responses to pathogens without promoting a break in tolerance.

The molecular basis for the selective repression of antigen-experienced B cells during innate immune responses remains unclear. However, others have shown that BCR signaling opposes TLR-induced Ig secretion by antigen-experienced B cells and that this repression is mediated by ERK activation. In this report, we show that inhibiting extracellular signal-regulated kinase (ERK) activation during rIL-6 or rsCD40L stimulation prevents their repression of LPS-induced Ig secretion. The repression mediated by IL-6 and sCD40L is targeted upstream of plasma cell transcription factors such as BLIMP-1 and XBP-1. Analysis of ERK phosphorylation showed that neither IL-6 nor sCD40L drastically changed the levels of activated ERK. However, chronically antigen-experienced B cells maintained higher basal phosphorylation of ERK. Finally, we determined that the subcellular localization of activated ERK is differentially regulated in naïve versus antigen-experienced B cells. These findings reveal an additional layer of antigen-experienced B cell regulation by DCs/MΦs during innate immune stimulation. Identifying the molecular basis of IL-6/sCD40L-mediated repression may afford unique opportunities for therapeutics that obviate the need for immunosuppression in B cell-mediated autoimmune diseases.

2.3 Materials and Methods

Mice

2-12H/Vκ8 Tg and 2-12H Tg mice were created as previously described (10, 11). 2-12H/Vκ4 mice were created and provided by Stephen Clarke (manuscript in press). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were 8 to 16 weeks old at the time of analysis. Animals were maintained in an accredited animal facility at University of North Carolina (Chapel Hill, NC).

Reagents

Recombinant IL-6 (rIL-6) was purchased from BD Biosciences (San Diego, CA) and Peprotech (Rocky Hill, NJ). Recombinant soluble CD40L (rsCD40L) was from R&D Systems (Minneapolis, MN). Fluorochrome- or biotin- labeled antibodies specific for CD9, CD19, CD3, CD11b, and CD11c were purchased from BD Biosciences. Antibodies specific for phospho-ERK and total ERK1/2 were purchased from Cell Signaling Technologies. Anti-BLIMP-1 was purchased from Novus Biologicals (Littleton, CO). Antibodies specific for XBP-1, lamin A, and beta-tubulin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies for immunoblotting were purchased from Promega (anti-rabbit-HRP, Madison, WI) or Invitrogen (anti-mouse IgG1-HRP, Carlsbad, CA). 187.1 (anti-κ), HB100 (anti-IgM[a]), RS3.1 (anti-IgM[a]), 33-60 (anti-IgM), B7.6 (anti-IgM), and 2.4G2 (CD16/32) were purified from hybridoma culture supernatant using Protein G Sepharose (GE Healthcare, Piscataway, NJ) or MEP HyperCel (BioSepra, Marlborough, MA) and biotinylated if necessary (Pierce, Rockford, IL). Sodium orthovanadate was

obtained from Millipore (Billerica, MA). Protease inhibitors (phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), aprotinin, alpha-1-antitrypsin, and leupeptin), and LPS from *E. coli* 055:B5 was purchased from Sigma (St. Louis, MO), and LPS from *E. coli* 0111:B4 was purchased from Invivogen (San Diego, CA). The MEK1 inhibitor U0126 was purchased from Calbiochem (San Diego, CA) and resuspended in dimethyl sulfoxide (DMSO, Sigma).

B cell purification

Splenic B cells were isolated by negative selection (StemCell Technologies, Vancouver, Canada). B cells were 90-95% pure (with fewer than 10% dendritic cells and macrophages), as determined by flow cytometry. Marginal zone (MZ) B cells were depleted from C57BL/6 splenocytes by supplementing the EasySep antibody mixture with biotinylated anti-CD9.

LPS stimulation

Purified B cells (1 x 10^5/per well) were cultured in the presence or absence of Sigma LPS (30ug/mL) for four days. Optimal amounts of rIL-6 (20 ng/mL), rsCD40L (25-75 ng/mL), and U0126 (0.5 μM) were added at the initiation of the cultures. Cell-free culture supernatants were harvested and IgM^a/κ or total IgM secretion was measured by ELISA.

ELISA

IgM^a/κ from 2-12H/Vκ8 and 2-12H/Vκ4 B cell culture supernatants was captured with rat anti-mouse κ (187.1) and detected with anti-IgM^a-biotin (HB100-biotin), and streptavidin-alkaline phosphatase (Sigma) as described (11). Total IgM from C57Bl/6 B cell culture supernatants was captured with rat anti-mouse IgM (33-60) and detected with rat anti-mouse IgM-biotin (B7.6) and streptavidin-alkaline phosphatase. IgM^a from 2-12H cell culture supernatants was captured with rat anti-mouse IgM^a (RS3.1) and detected with anti-IgM^a-biotin (HB100-biotin) and streptavidin-alkaline phosphatase. The standard for all ELISAs consisted of purified mouse IgM^a/κ (TEPC 183, Sigma). Data were plotted as percent of control, which was calculated as the percent secretion relative to cultures of LPS-stimulated purified B cells.

Flow cytometry

Single-cell suspensions of splenocytes were prepared and red blood cells were lysed using TAC lysis solution (1 M Tris, 0.15 M ammonium chloride, and 0.1 M EDTA). All staining was done in phosphate-buffered saline with 2% Fetal Clone II (Hyclone, Logan, UT). Fc receptors were blocked with anti-CD16/32 (2.4G2) for 5 min at room temperature. Surface markers were stained for 30 minutes at 4ºC in the dark. Cells were analyzed at the University of North Carolina Flow Cytometry Facility (Chapel Hill, NC) using a Cyan flow cytometer (Dako, Carpinteria, CA). Data were analyzed using Summit software (Dako). All data represent cells that fell within the lymphocyte gate determined by forward and side scatter. One to 5 x 10^5 cells per sample were analyzed.

Real-time PCR

Total RNA was extracted from 1-2 x 10^6 B cells using TRIZOL (Invitrogen) as recommended by the manufacturer. cDNA was prepared with random hexamers and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and analyzed in triplicate real-time PCR reactions. For relative quantification of message levels of Xbp-1, and secreted IgM, primers were designed using Primer3 (http://frodo.wi.mit.edu) and verified by dissociation analysis as well as gel electrophoresis and cloning and sequencing of PCR products (Table 2.1). Primers for Blimp-1 and 18s RNA have been described ((12, 13). ABsolute™ QPCR SYBR® Green ROX (500nM) Mix (ABgene, Epsom, UK) was used with the following conditions: 2 min at 50ºC, 10 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 15 s and 62 °C for 1 min, ending with a dissociation step of 95ºC for 15 s, 62ºC for 20 s, and 97ºC for 15 s to ensure that single products were amplified. Reactions were performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Quantification of transcripts relative to 18s RNA transcripts was determined using the $\Delta\Delta C_t$ method (14) and expressed as fold-induction relative to 18s RNA.

Table 2.1 Real-time PCR primers and their sequences.

Primer	Sequence
Blimp-1 forward	5'-TGTTGGATCTTCTCTTGGAAAA-3'
Blimp-1 reverse	5'-GTGTAAAGTAGACTGCCTTGA-3'
Xbp-1 (total) forward	5'-ACACGCTTGGGAATGGACAC'-3'
Xbp-1 (total) reverse	5'-CCATGGGAAGATGTTCTGGG-3'
18s forward	5'-TCAAGAACGAAAGTCGGAGGTT-3'
18s reverse	5'-GGACATCTAAGGGCATCACAG-3'

Message levels of IL-6 receptor and GAPDH RNA were measured by real-time PCR using ABI On-Demand TaqMan primers and FAM-labeled probes (Applied Biosystems) according to the manufacturer's directions. Fluorescence was measured in triplicate real-time reactions by an ABI Prism 7000 SDS thermocycler and relative C_T values were obtained from ABI Prism 7000 SDS software. Using the $\Delta\Delta C_T$ method, fold-induction values of IL-6 receptor were calculated relative to 18s RNA.

Immunoblotting

Purified splenic B cells were either lysed *ex vivo* or cultured with LPS alone (30 μg/mL) or in combination with rIL-6 (20 ng/mL) or rsCD40L (optimal dose of 25 or 75 ng/mL) for three days. 3×10^6 cells were lysed in buffer containing 1% Igepal CA-630 (formerly known as NP-40, Sigma) and protease and phosphatase inhibitors (phenylmethanesulfonyl fluoride (PMSF), aprotinin, alpha-1-antitrypsin, leupeptin, and sodium orthovanadate). Whole-cell lysates were separated by SDS-PAGE, transferred to

PVDF membrane in a semi-dry blotting apparatus, and immunoblotted for phospho-ERK and total ERK or BLIMP-1 and XBP-1. HRP-conjugated antibodies and ECL or ECL+ (GE Healthcare) were used to detect proteins.

Nuclear and cytoplasmic extraction

Purified splenic B cells were stimulated with LPS alone or in combination with rIL-6 or rsCD40L for 30 minutes. 15×10^6 cells were disrupted by hypotonic lysis and cytoplasmic and nuclear fractions were prepared as described previously (15, 16) with modifications. Protease and phosphatase inhibitors (described above, plus DTT) were added to both cytoplasmic and nuclear extract buffers. Protein concentrations were determined by the Bradford method (Bio-Rad) (17). Cytoplasmic (5μg) and nuclear (10μg) extracts were separated by SDS-PAGE and immunoblotted as above with phospho-ERK, total ERK, lamin A, and beta-tubulin.

Statistical analysis

When comparing antibody secretion in treated and untreated cultures, the one-sample *t* test was used. The unpaired *t* test with Welch's correction was used to test for differences in antibody secretion between experimental groups. Statistical analyses were performed with GraphPad Prism (La Jolla, CA).

2.4 Results

IL-6 and sCD40L regulate immunoglobulin secretion by antigen-experienced B cells

B cells expressing the 2-12H transgene recognize the self antigen Sm with a range of affinities depending on the light chain that is expressed. 2-12H/Vκ8 B cells bind Sm with low affinity and fail to respond to LPS stimulation in *ex vivo* splenic cultures. These anergic B cells comprise an excellent system to study the regulation of B cells specific for a bona fide self-antigen. We previously reported that splenic 2-12H/Vκ8 B cells are repressed by IL-6 and sCD40L produced by dendritic cells and macrophages in response to LPS (2, 3). In LPS-stimulated cultures of low-affinity 2-12H/Vκ8 B cells, rIL-6 and rsCD40L repressed 47% and 63% of Ig secretion, respectively (Figure 1A). This mechanism of regulation is not limited to low-affinity Sm-specific B cells. A range of BCR affinities for Sm is apparent in 2-12H mice due to their unrestricted light chain (10). LPS-induced Ig secretion from 2-12H B cells is repressed 34% by rIL-6 and 54% by rsCD40L (Figure 1B). 2-12H/Vκ4 B cells bind Sm with a moderate affinity and are also susceptible to rIL-6- and rsCD40L-mediated repression (47% and 35%, respectively, Figure 1C). In contrast, naïve C57BL/6 B cells (Figure 1D) are not significantly repressed by these cytokines, indicating that continuous exposure to self-antigen *in vivo* is necessary to "reprogram" the IL-6 receptor and CD40 to repress Ig secretion. The level of IL-6 receptor (CD126) message is not significantly different between antigen-experienced and naïve B cells (Figure 1E), suggesting that the specificity of this repression is determined by a difference in signaling or downstream gene expression. In contrast, CD40 expression is increased on 2-12H/Vκ8 B cells compared to C57BL/6 B cells (Figure 1F). However, it has been observed that CD40 is upregulated by BCR

signaling and is increased on other anergic B cells that have presumably encountered antigen and are primed for T cell costimulation (18, 19). Therefore, higher expression of CD40 on 2-12H/Vκ8 B cells could be due to antigen experience *in vivo* and this increase in expression, along with receptor reprogramming, could potentially explain the increased susceptibility of 2-12H/Vκ8 cells to sCD40L-mediated repression.

IL-6 and sCD40L regulate transcription factors involved in plasma cell differentiation

Since IL-6 and sCD40L repress Ig secretion, we examined several transcription factors involved in plasma cell differentiation. BLIMP-1 is often referred to as the "master regulator" of plasma cell differentiation and immunoglobulin secretion (20). This transcription factor is responsible for extinguishing the mature B cell program by repressing Bcl-6, Pax5, and c-myc, while promoting immunoglobulin production and secretion through other factors such as XBP-1 (20-22). Levels of Blimp-1 message (Figure 2A) were reduced in 2-12/Vk8 cells treated with rIL-6 (by 49%) or rsCD40L (by 47%). We expected that BLIMP-1 protein levels would be similarly repressed. BLIMP-1 was induced by LPS but reduced 50% with rIL-6 treatment and 60% with rsCD40L treatment. XBP-1 also plays an important role in Ig secretion, coordinating the unfolded protein response (21, 23). Because XBP-1 is induced by BLIMP-1, we predicted that rIL-6 and rsCD40L would repress Xbp-1 message and proteins levels as well. Message levels of Xbp-1 in cultures treated with LPS and IL-6 decreased by 69% (Figure 2A). rsCD40L repressed Xbp-1 message levels by 58%. As expected, XBP-1 protein levels reflected the decrease in message levels. Both rIL-6 and rsCD40L repressed XBP-1 protein levels by more than 60%. These results indicate that IL-6 and sCD40L exert their

44

repressive influence upstream of key transcriptional factors that control Ig secretion by chronically antigen-experienced B cells.

IL-6- and sCD40L-mediated repression is rooted in tolerogenic ERK activation

Antigen-experienced B cells are subject to chronic, tolerogenic BCR ligation *in vivo* and constant BCR occupancy is required to maintain anergy (24, 25). Others have shown that chronic ERK activation represses LPS- and CpG-induced Ig secretion in hen egg lysozyme-specific (HEL-Ig) B cells and that this repression can be reversed by pharmacologically inhibiting MEK (26, 27). Interestingly, both IL-6R and CD40 signaling induce ERK activation (28-30). Therefore, we predicted that IL-6- and sCD40L-mediated repression might be dependent on ERK activation resulting from IL-6R or CD40 signaling. We cultured 2-12H/Vκ8 B cells with LPS, rIL-6 or rsCD40L, and the MEK inhibitor, U0126. When ERK activation was inhibited in 2-12H/Vκ8 B cells by U0126 treatment, rIL-6 and rsCD40L were no longer able to repress LPS-induced Ig secretion (Figure 3). These data indicate that rIL-6- and rsCD40L-mediated repression of antigen-experienced B cells is dependent on ERK activation.

Basal ERK phosphorylation is increased in anti-Sm B cells

Increases in basal ERK phosphorylation have been observed in antigen-experienced B cells versus their naïve counterparts (31, 32). This increase has been ascribed to chronic ligation of the BCR by self antigen. However, HEL-specific B cells have a very high affinity for HEL. Low-affinity interactions with self antigens such as Sm are more physiologically relevant, but it is not known whether these interactions

result in increased basal ERK phosphorylation. We examined phosphorylation of ERK in freshly isolated naïve (C57BL/6) and antigen-experienced (2-12H/Vκ8) B cells and observed that 2-12H/Vκ8 B cells had higher basal ERK phosphorylation than C57BL/6 B cells (Figure 4). It is conceivable that *in vivo*, both high- and low-affinity self antigens confer tolerance by chronically ligating the BCR and activating ERK.

Unique subcellular localization of phosphorylated ERK in chronically antigen-experienced B cells

The ERK MAP kinase signaling cascade is quantitatively, temporally, and spatially regulated to prevent inappropriate activation or duration of receptor-mediated signaling (33). We have examined the amplitude and temporal patterns of ERK activation by rIL-6 and rsCD40L in naïve (C57BL/6) and antigen-experienced (2-12H/Vκ8) B cells. rIL-6 and rsCD40L induced equivalent levels of ERK phosphorylation in naïve and antigen-experienced B cells within 30 minutes and duration of phosphorylation was not consistently longer in either C57BL/6 or 2-12H/Vκ8 B cells (data not shown). Since the magnitude and duration of ERK phosphorylation did not appear to vary with chronic antigen experience, we theorized that the subcellular localization of phosphorylated ERK may be differentially regulated. ERK is anchored in the cytoplasm by its association with MEK, which contains a nuclear export signal (33). When ERK is activated, it generally dissociates from MEK and translocates to the nucleus, but certain stimuli can induce phosphorylation without translocation (33). We stimulated naïve and antigen-experienced B cells with LPS alone or in combination with rIL-6 or rsCD40L and isolated cytoplasmic and nuclear fractions. These extracts were

immunoblotted for phosphorylated and total ERK as well as cytoplasmic and nuclear proteins. When we compared the distribution of phosphorylated ERK, differences between naïve and antigen-experienced B cells were evident. It was evident that in naïve B cells, LPS stimulation caused an increase in phosphorylated ERK in the nucleus regardless of rIL-6 and rsCD40L stimulation (Figure 5A). In contrast, when antigen-experienced B cells were stimulated with LPS, more phosphorylated ERK was evident in the nuclear fraction than in the cells stimulated with LPS combined with either rIL-6 or rsCD40L (Figure 5B). It appears that rIL-6 and rsCD40L alter the localization of activated ERK in antigen-experienced B cells compared to naïve B cells. However, it is unclear how phosphorylated ERK is anchored in the cytoplasm during IL-6- and sCD40L-mediated repression.

2.5 Discussion

The innate immune system mounts a vigorous defense against pathogens by recognizing and responding to pathogen-associated molecular patterns (PAMPs) such as LPS and CpG. This polyclonal response is swift and robust, but inherently nonspecific. This lack of specificity is potentially hazardous because cells specific for autoantigens can trigger a bystander autoimmune response. For instance, there is a strong association between EBV seroconversion and SLE (34), and SLE patients have elevated Epstein-Barr virus loads in their blood (35). Additionally, during viral immunization of transgenic mice expressing a viral neo-self antigen, autoreactive B cells can evade negative selection in the periphery and become memory B cells (36). These autoreactive B cells can be reactivated by a second immunization; perhaps the presence of PAMPSs in subsequent infections would be sufficient to reactivate this pool of autoreactive B cells. A mechanism to discriminate between naïve and autoreactive B cells is crucial for a safe, effective immune response to pathogens. Ideally, the same polyclonal activators that activate naïve B cells would induce tolerance in autoreactive, antigen-experienced B cells to prevent unintentional autoimmunity. Our studies have shown that IL-6 and sCD40L, via ERK activation, repress LPS-induced Ig secretion in chronically antigen-experienced transgenic B cells but do not significantly affect naïve B cells. These soluble factors effectively repress B cells from several transgenic mouse models with low or intermediate BCR affinity, including 2-12H, 2-12H/Vκ4, 2-12H/Vκ8, as well as high affinity B cells from HEL-Ig x sHEL mice (2, 3). This mechanism specifically targets chronically antigen-experienced B cells and therefore would be an extremely valuable tool in silencing autoreactive B cells in human diseases such as SLE.

IL-6- and sCD40L-mediated repression of Ig secretion in chronically antigen-experienced B cells is mediated by ERK and reversible with pharmacological inhibitors of MEK. Basal ERK phosphorylation is increased in chronically antigen-experienced 2-12/Vκ8 B cells compared to naïve C57BL/6 B cells, similar to antigen-experienced B cells from HEL-Ig x sHEL mice (31, 32). Since LPS, IL-6, and sCD40L all activate ERK, we initially predicted that ERK activation would be greater in repressible 2-12H/Vκ8 cells compared to naïve C57BL/6 B cells. However, we observed no quantitative differences in the magnitude of ERK phosphorylation between antigen-experienced and naïve B cells. Additionally, ERK activation was sustained for similar periods of time in both antigen-experienced and naïve B cells. However, the effects of activated ERK can be regulated by its subcellular localization (33, 37, 38). After ERK is activated, it translocates to the nucleus where it phosphorylates a variety of transcription factors (39, 40). ERK lacks any obvious nuclear export signals (NES) or nuclear localization signals (NLS). Therefore, it is reliant on MEK to either retain it in the cytoplasm or release it to allow nuclear translocation (33, 41, 42). Other proteins such as kinase suppressor of Ras (KSR) can act as scaffolding that binds MEK and ERK and facilitates MAPK signaling (43). We observed reduced nuclear localization of activated ERK in repressed antigen-experienced B cells compared to naïve B cells. Therefore, it appears that the subcellular localization, rather than the magnitude and duration of ERK activation, may regulate the differential effects of IL-6- and sCD40L-mediated repression in naïve and chronically antigen-experienced B cells.

Although differential localization of activated ERK is coincident with IL-6/sCD40L-mediated repression, additional studies are necessary to determine if exclusion

of activated ERK from the nucleus is necessary or sufficient for repression of antigen-experienced B cells. To determine if the exclusion of activated ERK from the nucleus is sufficient for repression, antigen-experienced B cells could be retrovirally transduced with a MEK1/ERK2 fusion construct that is constitutively active and localized to the cytoplasm and assess if the B cells fail to secrete Ig (44). We predict expression of this construct would constitutively repress Ig secretion because ERK would be maintained in the cytoplasm. To determine if exclusion of activated ERK from the nucleus is necessary for repression, a second MEK1/ERK fusion protein could be transduced into antigen-experienced B cells. In this construct, the four leucines of the MEK1 NES are mutated to alanine, blocking nuclear export (LAMEK1/ERK2) (44). We predict expression of this construct would prevent IL-6/sCD40L-mediated repression of antigen-experienced B cells since activated ERK would be constitutively localized in the nucleus.

Activated ERK translocates to the nucleus and phosphorylates transcription factors such as ETS-1. ETS-1 negatively regulates BLIMP-1 and can repress plasma cell differentiation (45, 46). Decreased nuclear levels of activated ERK could result in decreased ETS-1 phosphorylation and impact its regulation of BLIMP-1 and plasma cell differentiation. Phosphorylation of ETS-1 and other ERK targets could be examined to determine the impact of decreased nuclear localization of activated ERK. These data could identify potential links between ERK and transcription factors such as BLIMP-1 and XBP-1. This information could provide additional targets for regulating antigen-experienced B cells during innate immune responses.

. The autoreactive B cells that wreak so much damage in SLE patients become dysregulated long before any clinical symptoms of disease. Genetic and environmental

factors come together to unbalance all the normal mechanisms of tolerance. Other than

immunosuppressive and B cell-depleting therapies, there is currently no way to silence

these autoreactive cells while preserving the ability to mount a vigorous immune

response against pathogens. Treatment options would be vastly improved if a therapy

could incorporate a selective mechanism of tolerance such as the ERK-mediated

repression described in this study.

2.6 References

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2.7 Figures

Figure 2.1. IL-6 and sCD40L selectively repress Ig secretion by chronically antigen-experienced B cells. B cells from 2-12H/Vκ8 (*A*), 2-12H (*B*), 2-12H/Vκ4 (*C*), or C57BL/6 (*D*) mice were stimulated with LPS (30 μg/mL) or LPS combined with recombinant IL-6 (rIL-6, 20 ng/mL) or recombinant soluble CD40L (rsCD40L, optimal dose of 25 or 75 ng/mL) for four days. IgM^a/κ (2-12H/Vκ8 and 2-12H/Vκ4), IgM^a (2-12H), or total IgM (C57Bl/6) were quantitated by ELISA. LPS-stimulated B cells (100%) secreted 1.73-18.01 μg/ml Ig (*A*), 12.93-78.49 μg/ml (*B*), 7.06-26.75 μg/ml (*C*), and 16.81-27.50 μg/ml (*D*) from at least three experiments. (*E*), *Ex vivo* B cells were isolated from C57BL/6 or 2-12H/Vκ8 mice, total RNA was extracted, reverse-transcribed to cDNA, and levels of IL-6 receptor (IL-6R) relative to GAPDH were determined by real-time PCR in 3 experiments. The levels of IL-6R were not significantly different between C57BL/6 and 2-12H/Vκ8 B cells. (*F*), Splenocytes from C57BL/6 or 2-12H/Vκ8 mice were stained for expression of CD19 and CD40 and analyzed by flow cytometry. The relative median fluorescence intensity (MFI) of CD40 on CD19+ cells is shown. The MFI of CD40 on 2-12H/Vκ8 B cells was 1.6-fold higher than that of C57BL/6 B cells. Statistical analysis was performed using 1-sample *t* test by comparing treated and untreated cultures (*A-D*) or C57BL/6 and 2-12H/Vκ8 B cells (*E-F*). Data represent at least 3 experiments. Error bars represent plus or minus standard error (SEM). (*** $P<0.001$, **$P<0.01$.)
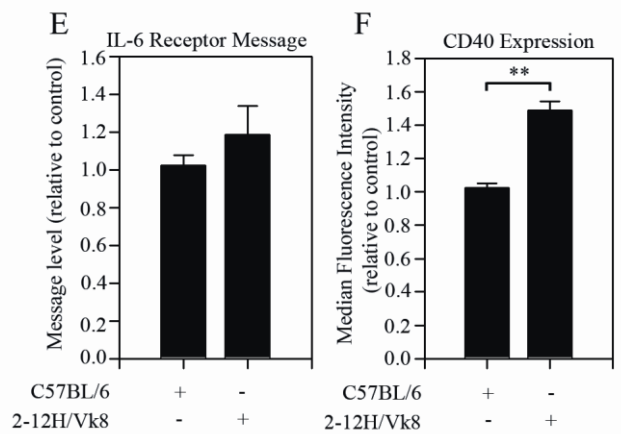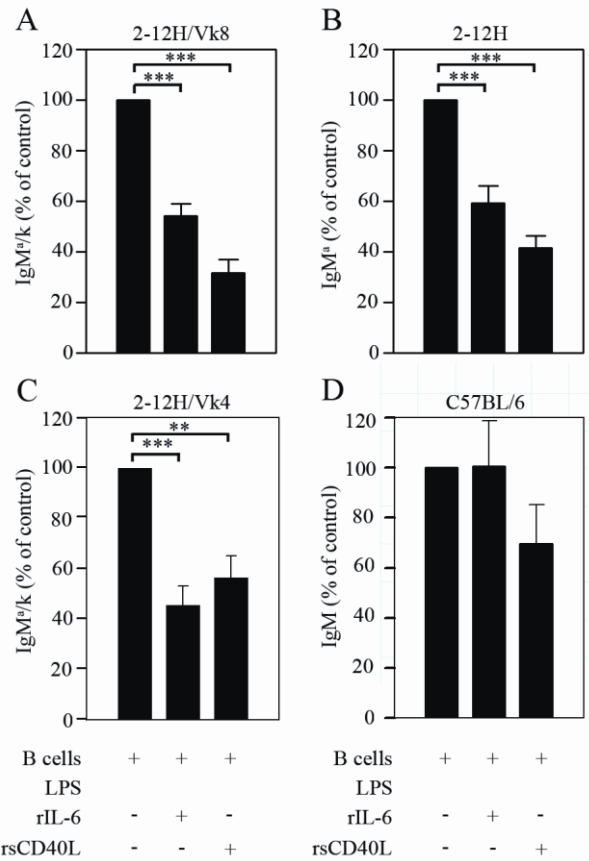
Figure 2.2. IL-6 and sCD40L treatment causes a decrease in levels of transcription factors involved in plasma cell differentiation. (*A-B*)*,* 2-12H/Vκ8 B cells were stimulated for four days with LPS or LPS combined with rIL-6 or rsCD40L. Total RNA was extracted, reverse-transcribed to cDNA, and relative levels of Blimp-1 (*A*) and XBP-1 (*B*) message were measured by real-time PCR in four experiments. Statistical analysis was performed using 1-sample *t* test by comparing treated and untreated cultures (*A-B*). Error bars represent plus or minus SEM. (***P<0.01, *P<0.05.) (*C-D*)*,* 2-12H/Vκ8 B cells were cultured for three days with LPS or LPS combined with rIL-6 or rsCD40L. Lysates from 3 x 10^6 cells were separated by SDS-PAGE and immunoblotted for BLIMP-1 (*C*) and XBP-1 (*D*). The density of each band was quantitated with ImageJ and the fold changes were calculated. The data shown represent at least 3 experiments.
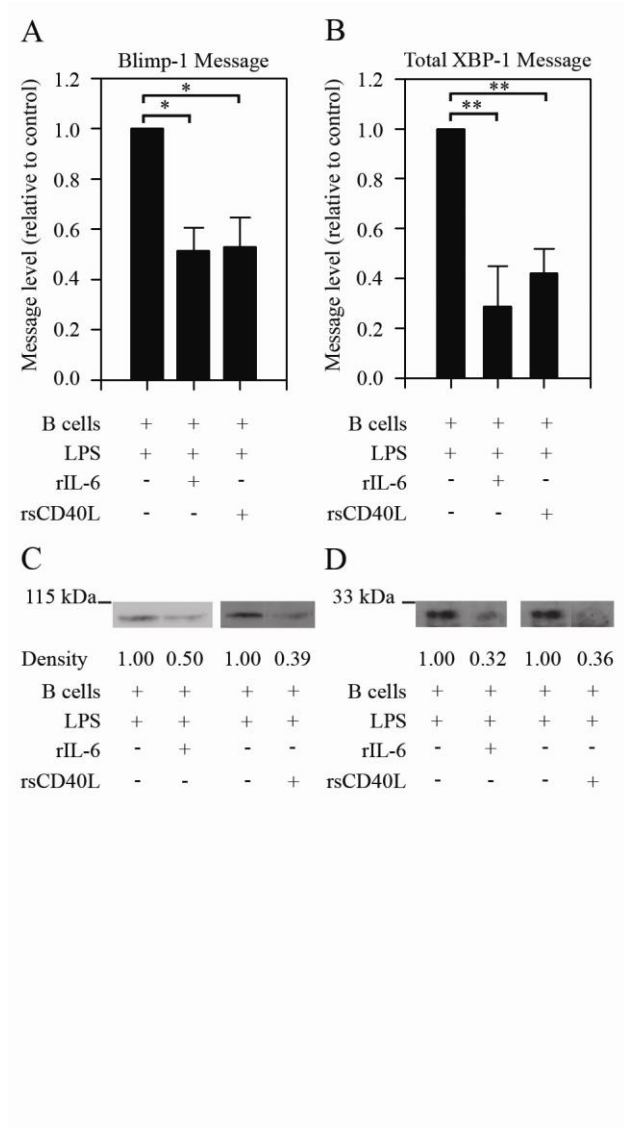
Figure 2.3. IL-6- and sCD40L-mediated repression is dependent on ERK activation.
2-12H/Vk8 B cells were stimulated for four days with LPS or LPS combined with rIL-6,
rsCD40L, or U0126. IgM[a]/κ levels in the culture supernatant were assayed by ELISA.
LPS-stimulated B cells (100%) secreted 1.35-18.01 μg/ml in at least four experiments.
Statistical analysis was performed using 1-sample *t* test by comparing treated and
untreated cultures or Welch's unpaired *t* test for comparisons between treatments. Data
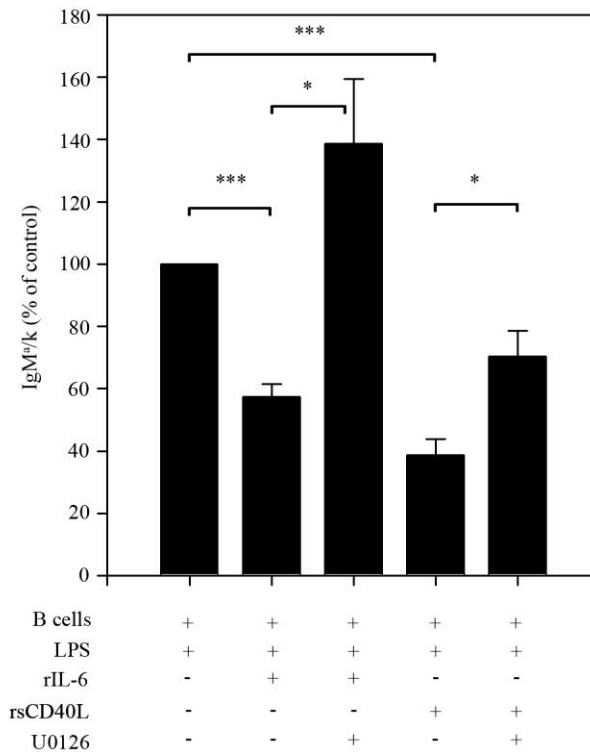represent at least 3 experiments. Error bars represent plus or minus SEM. (*** $P<0.001$,
*$P<0.05$.)

B cells	+	+	+	+	+
LPS	+	+	+	+	+
rIL-6	-	+	+	-	-
rsCD40L	-	-	-	+	+
U0126	-	-	+	-	+

Figure 2.4. Chronically antigen-experienced 2-12H/Vκ8 B cells exhibit higher basal phosphorylation of ERK than naïve C57BL/6 B cells. 3×10^6 purified *ex vivo* C57BL/6 and 2-12H/Vκ8 B cells were lysed and the proteins were separated on an SDS-PAGE gel, transferred to PVDF membrane, and immunoblotted with antibodies specific for phosphorylated ERK and total ERK1/2. The density of each band was quantitated with ImageJ and the ratio of p-ERK2 to ERK 2 was calculated. The data shown represent at least three experiments.
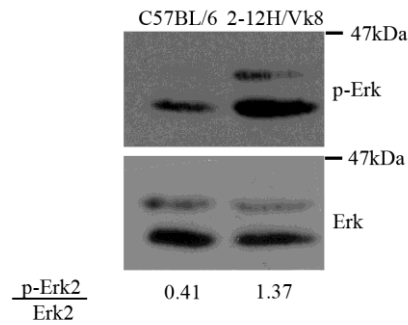
Figure 2.5. IL-6 and sCD40L alter the subcellular localization of activated ERK in chronically antigen-experienced B cells. 15×10^6 purified *ex vivo* C57BL/6 (*A*) and 2-12H/Vκ8 (*B*) B cells were stimulated with LPS alone or in combination with rIL-6 and rsCD40L for 30 minutes. Cytoplasmic and nuclear fractions were isolated and the proteins were separated on an SDS-PAGE gel, transferred to PVDF membrane, and immunoblotted with antibodies specific for phosphorylated ERK, total ERK1/2, lamin A, and beta tubulin. The density of each band was quantitated with ImageJ and the ratio of p-ERK2 to ERK 2 was calculated. Phosphorylated ERK in the nuclear fractions of LPS-stimulated 2-12H/Vκ8 B cells decreased 38% and 62% with rIL-6 or rsCD40L treatment, compared with 27% and 0% reduction in the corresponding C57BL/6 samples. The data shown represent one experiment.
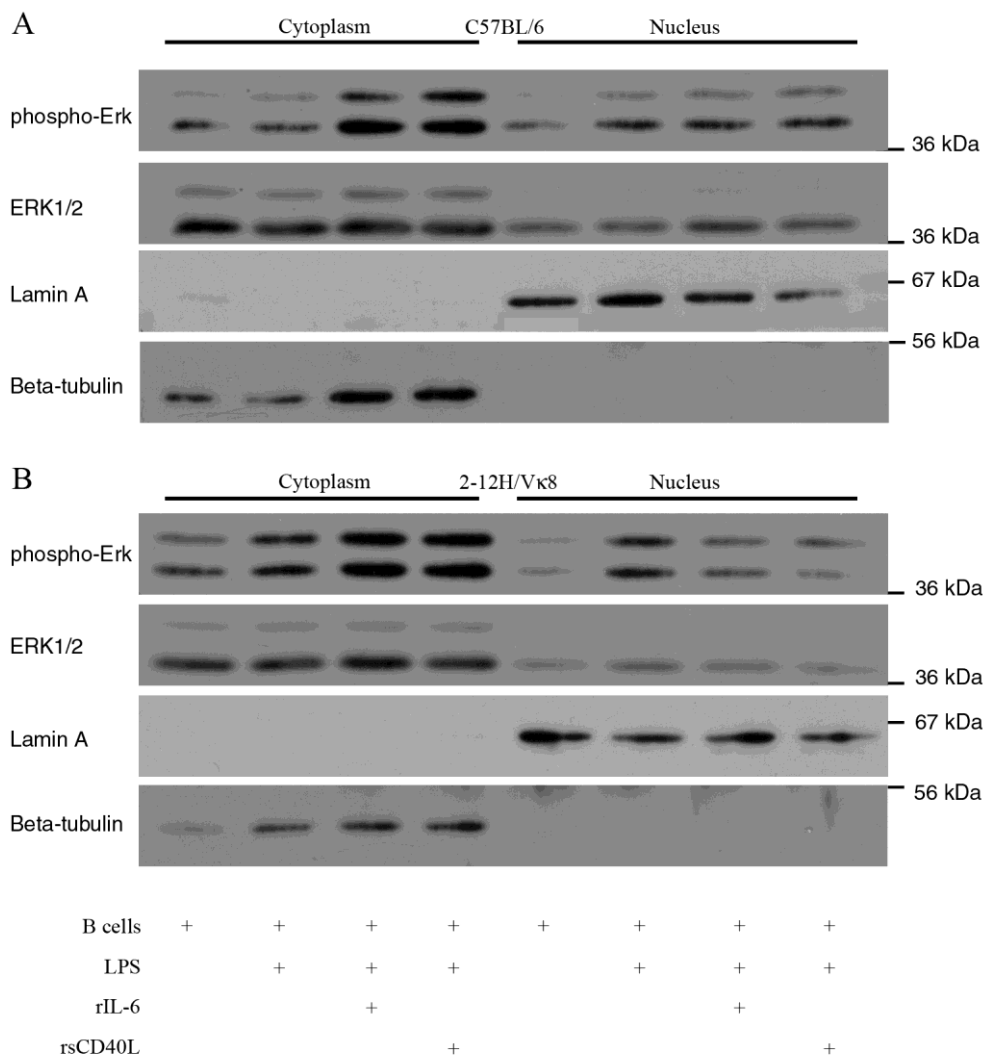
CHAPTER III

Discussion

Innate immunity is an ancient defense system based upon the simplest principles of pattern recognition. The adaptive immune system mounts highly specific responses to clear and destroy pathogenic targets. The intersection of the innate and adaptive immune systems can lead to uncontrolled inflammation and autoimmunity if cross-reactive self-proteins are recognized by the innate immune system and in turn trigger a powerful adaptive immune response. In addition to tolerance mechanisms based upon antigen recognition by the BCR and TCR, we are uncovering mechanisms that govern innate activation of naïve and antigen-experienced B cells.

The path to B cell activation and Ig secretion is lined with checkpoints to prevent inappropriate activation of autoreactive B cells. From the earliest stages of development, B cells are exquisitely sensitive to the affinities of their BCRs for autoantigens. In the bone marrow, central deletion and receptor editing remove autoreactive B cells from the repertoire before they exit to the periphery (1, 2). Additional trials await new B cell emigrants as they enter the spleen and are stimulated by a new set of autoantigens. Peripheral deletion, receptor revision, and follicular exclusion eliminate another swath of B cells with inappropriate specificities (3-5). Finally, low-affinity autoreactive B cells that bypass the previous mechanisms are held in an unresponsive state upon antigen ligation. Without appropriate costimulatory signals and cytokines to accompany antigen ligation, these B cells are suspended in an anergic state (6). Anergic B cells are unable to differentiate into plasma cells and secrete Ig, but may be receptive to BCR or TLR signals for proliferation or survival (7). Although these cells remain quiescent in normal individuals, the wide array of autoreactive specificities can be dangerous if tolerance is breached by a non-specific innate immune response.

Autoreactive B cells risk polyclonal activation during infection and inflammatory responses. TLRs are sensors of pathogens, transducing signals from a variety of bacterial and viral ligands, including LPS, methylated CpG DNA, and ssRNA (8, 9). However, endogenous ligands such as non-methylated CpG DNA and ssRNA are present on the surface of apoptotic cells and by DCs and MΦs (Carnathan et al, manuscript in preparation). These TLR ligands are often presented in the context of protein complexes such as snRNPs or opsonized apoptotic cells. Thus, rich arrays of autoantigens have the potential to co-ligate the BCR, TLRs, and Fc receptors, creating complex signals capable of activating autoreactive B cells (10, 11).

Receptor cross-talk allows ligands binding one receptor to regulate the response of another receptor to its ligand. In chronically antigen-experienced cells, it appears that the BCR, IL-6R, and CD40 form a tangled web of signaling pathways that alter the normal outcomes of TLR signaling. Elevated basal ERK phosphorylation is observed in 2-12H/Vκ8, Ars/A1, and HEL-Ig x sHEL B cells, (12, 13), suggesting that the specific autoantigens are chronically signaling through the BCR. High-affinity antigens such as HEL are sufficient to repress LPS or CpG-induced Ig secretion via ERK activation (14, 15). However, the 2-12H/Vκ8 BCR has a low affinity for its specific antigen (Sm), and recombinant Sm is not able to repress LPS-induced Ig secretion in these B cells (data not shown). It is unknown whether a higher valency antigen, such as Sm in the context of small nuclear ribonucleic particles (snRNPs) or apoptotic cells, would be able to repress 2-12H/Vκ8 B cells. Therefore, an additional mechanism is required to maintain tolerance in low-affinity B cells during innate immune stimulation.

We have previously described a mechanism to regulate antigen-experienced B cells during a polyclonal, innate immune response (16, 17). DCs and MΦs are activated by TLR ligands and secrete IL-6 and sCD40L, preventing polyclonal activation of antigen-experienced B cells in the vicinity. However, naïve B cells are activated by TLR ligands and are not susceptible to DC/MΦ-mediated repression. In contrast, IL-6 and sCD40L can promote activation and proliferation in naïve B cells, leading to plasma cell differentiation or germinal center formation (18, 19). DC/MΦ-mediated tolerance appears to be a fail-safe mechanism of repressing antigen-experienced B cells during polyclonal stimulation.

IL-6R or CD40 signaling provides a source of tolerogenic ERK in low-affinity autoreactive B cells. These soluble factors repressIg production in antigen-experienced but not naïve B cells (Figure 1). It is interesting to consider if the differential effects of IL-6R and CD40 signaling in chronically antigen-experienced B cells is a consequence of the chronic BCR signaling these cells experience *in vivo*. We hypothesize that chronic BCR signaling reprograms the IL-6R and CD40 to produce "tolerogenic" instead of "activating" phosphorylated ERK. In naïve B cells or in autoreactive B cells that have overcome tolerance, such as 2-12H/MRL/*lpr*, IL-6 and sCD40L fail to repress Ig secretion (data not shown). The inability of IL-6 or sCD40L to repress autoimmune B cells may be due to differences in BCR signaling or a defect in crosstalk between the BCR and IL-6R and CD40. In these mice, we would expect that IL-6- and sCD40L-induced ERK would behave in an activating manner, similar to naïve B cells, rather than its tolerogenic actions in chronically antigen-experienced cells. Similarly, SLE patients exhibit elevated levels of IL-6 and sCD40L in their plasma, but these systemic levels are

much lower than the amounts of IL-6 and sCD40L we find necessary to repress Ig secretion *ex vivo* (20, 21). Increased sCD40L actually correlates with anti-dsDNA titers and disease activity (20, 21). In these patients, increased IL-6 and sCD40L may actually enhance Ig secretion from B cells that have already bypassed all tolerance checkpoints.

With these data, we link two unlikely repressive factors to a molecular mechanism of anergy that is dependent on ERK activation. Both IL-6R and CD40 are capable of activating ERK in addition to other, more well-known signaling pathways (22-25). When it was revealed that chronic BCR signaling induced tolerogenic ERK activation (14, 15), we predicted that IL-6R and CD40 signaling also resulted in tolerizing ERK activation. We established that IL-6- and sCD40L-mediated repression was dependent on ERK activation and only antigen-experienced B cells were susceptible to this mechanism. With this information, we commenced our search for the molecular basis of ERK-mediated repression.

Plasma cell differentiation and Ig secretion is a complex process involving many signaling molecules and transcription factors. Since we had previously determined that IL-6 and sCD40L repressed secreted Ig protein levels in 2-12H/Vκ8 B cells, we systematically worked backwards from Ig secretion to transcription factor levels and the ERK/MAPK signaling pathway. We confirmed that IL-6 and sCD40L also repressed secreted Ig message levels (unpublished observations) and concluded that the target of repression was upstream of Ig transcription. We examined the message and protein levels of two key transcription factors in plasma cell differentiation, BLIMP-1 and XBP-1. These transcription factors were significantly repressed by IL-6 and sCD40L, so we focused our search on the regulation of ERK itself. Like antigen-experienced B cells in

other models, 2-12H/Vκ8 B cells exhibit higher basal levels of phosphorylated ERK than C57BL/6 B cells. However, we did not find significant differences in the magnitude of IL-6/sCD40L-induced ERK activation in antigen-experienced versus naïve B cells. In both antigen-experienced and naïve B cells, IL-6/sCD40L augmented LPS-mediated ERK activation. Similar levels of phosphorylated ERK were observed in both cell types when we stimulated with IL-6 or sCD40L alone. Therefore, we did not observe significant differences in ERK activation by IL-6R and CD40 signaling, despite the increased expression of CD40 on 2-12H/Vκ8 B cells. Next we examined the duration of IL-6/sCD40L-induced ERK activation in antigen-experienced and naïve B cells and did not observe consistent differences between the two cell types. Therefore, we eliminated differences in quantitative and temporal regulation of ERK activation as a mechanism for differential susceptibility of 2-12H/Vκ8 and C57BL/6 B cells. Finally, we investigated the subcellular localization of phosphorylated ERK in antigen-experienced and naïve B cells stimulated with LPS and IL-6/sCD40L. Interestingly, we detected a reduction in phosphorylated ERK in nuclear fractions of antigen-experienced B cells stimulated with IL-6 or sCD40L. However, total protein levels of ERK1/2 were not visibly decreased. These results suggest that phosphorylated ERK is retained in the cytoplasm in repressed B cells, preventing its activation of downstream targets in the nucleus. After confirming these results, our next step would be to determine if nuclear exclusion of ERK is necessary and sufficient for repression and if IL-6/sCD40L-stimulation of antigen-experienced B cells reduces phosphorylation of ERK targets in the nucleus. Changes in phosphorylation of targets such as Ets-1 could impact its regulation of other transcription factors and repress plasma cell differentiation (26, 27). Identifying links between ERK

and transcription factors such as BLIMP-1 and XBP-1 could further delineate the mechanism of IL-6/sCD40L-mediated repression.

In summary, DCs and MΦs differentially regulate antigen-experienced and naïve B cell activation during innate immune responses by secreting IL-6 and sCD40L. IL-6 and sCD40L induce tolerogenic ERK, which is activated but unable to accumulate in the nucleus in antigen-experienced B cells. In contrast, these soluble factors induce activating ERK in naïve B cells, which is phosphorylated and accumulates in the nucleus, where it can potentially phosphorylate nuclear targets such as Ets-1. Knowing the molecular intricacies of ERK-mediated repression of autoreactive B cells could yield additional targets for therapies that selectively repress autoreactive B cells. New therapies could potentially reprogram the IL-6R or CD40, retain activated ERK in the cytoplasm, or prevent activation of transcription factors that link ERK signaling and BLIMP-1. Selective, targeted therapies like these would be a vast improvement over current options, which are limited to immunosuppression and global B cell depletion. Enhancing DC/MΦ-mediated repression and restoring differential responses to IL-6/sCD40L in autoreactive B cells in SLE patients and could reset the adaptive immune system and allow it to function safely and effectively during innate immune responses.

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