The Role of Altered Antigen Receptor Signaling in Selection and Homeostasis of Peripheral B cells in Wiskott-Aldrich Syndrome

Nikita S. Kolhatkar

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2015

Reading Committee:

David J. Rawlings, Chair

Jessica A. Hamerman

Marion Pepper

Program Authorized to Offer Degree:

Department of Immunology

University of Washington

Abstract

The Role of Altered Antigen Receptor Signaling in the Selection and Homeostasis of Peripheral B cells in Wiskott-Aldrich Syndrome

Nikita S. Kolhatkar

Chair of the Supervisory Committee:
Professor David J. Rawlings
Department of Immunology

Humoral immunity and the ability to recognize and protect against a broad spectrum of pathogens is dependent on the range of receptor diversity within the B cell compartment. An inherent problem associated with generating a highly diverse B cell repertoire is the risk of selecting B cell receptors (BCRs) specific for self-antigens. To reduce the threat of autoimmunity, there are several tolerance checkpoints throughout B cell development aimed at purging self-reactive B cells from entering the mature repertoire. We have established a role for dual BCR and TLR signaling in modulating both B cell tolerance and homeostasis in autoimmune-prone settings using the murine model of Wiskott-Aldrich Syndrome.

Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency disorder frequently associated with systemic autoimmunity, including autoantibody-mediated cytopenias. WAS protein (WASp)-deficient B cells have increased B cell receptor (BCR) and Toll-like receptor (TLR) signaling suggesting that these pathways might impact both the establishment and homeostasis of the mature, naïve BCR repertoire. We evaluated the naïve B cell repertoire in both WASp-deficient mice and WAS subjects via high-throughput sequencing and single cell cloning analysis. We found altered heavy chain usage and enrichment for low affinity, self-reactive specificities in the naïve B cell repertoire. Alterations to the naïve repertoire were primarily due to enhanced proliferation of self-reactive transitional cells *in vivo* mediated by antigen- and Myd88-dependent signals.

In addition to modulating positive selection, we also found a role for dual BCR and TLR signals in regulating marginal zone (MZ) B cell homeostasis in WAS. WASp deficiency markedly reduces splenic MZ B cells without impairing MZ B cell development, implying that retention of WAS MZ B cells is abnormal. However, upon deletion of B-cell intrinsic TLR 7 signals or antigen-dependent BCR signals, MZ B cell retention is restored, indicating that dual TLR 7/BCR signals drive spontaneous activation and egress of MZ B cells. Our combined data support a model wherein modest alterations in B cell-intrinsic, BCR and TLR signals in WAS, and likely other autoimmune disorders, are sufficient to both alter B cell tolerance via positive selection of self-reactive transitional B cells as well as to disrupt MZ B cell homeostasis.

TABLE OF CONTENTS

List of Figures iii

Chapter I. Introduction 1

I.1 Generation of the innate and adaptive immune response 1

I.2 Bone Marrow B Cell Development and Selection 2

I.3 Peripheral B Cell Development and Negative Selection 3

I.4 Antigen-Dependent Positive Selection 5

I.5 Role of Mature B Cells in Immune Homeostasis and Autoimmunity 8

Chapter II. Role of Positive Selection in Wiskott-Aldrich Syndrome 11

II.1 Introduction 11

II.2 Results 12

II.2.1 WASp-deficient mice exhibit a skewed naïve B cell repertoire enriched for low affinity self-reactive BCRs 12

II.2.2 High-throughput BCR sequencing reveals an altered HC repertoire in $Was^{-/-}$ MZ B cells 15

II.2.3 WASp-deficiency does not perturb negative selection mechanisms 17

II.2.4 WASp-deficiency promotes antigen-mediated proliferation of transitional B cells 19

II.2.5 Myd88 signals contribute to enhanced selection and the altered naïve repertoire in $Was^{-/-}$ mice 22

II.2.6 Human WAS subjects also exhibit an altered naïve B cell repertoire 24

II.2.7 V_H4-34 expressing BCRs are positively selected in Wiskott-Aldrich Syndrome 26

II.3 Discussion 30

II.4 Methods 36

Chapter III. Role of TLR Signaling in Marginal Zone B Cell Homeostasis 40

III.1 Introduction 40

III.2 Results and Discussion 41

III.2.1 WASp-deficiency alters MZ B cell localization and retention within the MZ sinus. 41
III.2.2 B-cell intrinsic MyD88 signals activate and promote egress of $Was^{-/-}$ MZ B cells. 43
III.2.3 TLR7 is the dominant MyD88-dependent receptor promoting $Was^{-/-}$ MZ B cell activation. 45
III.2.4 Antigen-dependent BCR signals promote MZ B cell egress. 46
III.3 Methods 49
Chapter IV. Concluding Remarks 50
Chapter V. Glossary 53
Chapter VI. References 54

LIST OF FIGURES

Figure I.1. Role of dual TLR and BCR signals in modulating negative selection of transitional B cells. 5

Figure II.1. Altered specificity of the naïve B cell repertoire in WASp-deficient mice. 14

Figure II.2. High throughput BCR heavy chain sequencing of splenic B cells subsets from WT, *Was*$^{-/-}$, and *Was*$^{fl/fl}$x *Mb-1*^{cre} mice. 16

Figure II.3. *Was*$^{-/-}$ B cells exhibit intact negative selection. 18

Figure II.4. ***Was*$^{-/-}$ B cells exhibit high-levels of antigen-dependent clonal expansion**.21

Figure II.5. Antigen-specific selection of *Was*$^{-/-}$ transitional B cells requires Myd88 signals. 23

Figure II.6. WAS/XLT subjects exhibit an altered naïve B cell repertoire enriched for self-reactive specificities. 25

Figure II.7. WAS/XLT subjects exhibit enrichment for VH4-34 expressing BCRs in the naïve and IgM memory B cell compartments. 27

Figure II.8.WASp-deficiency promotes altered positive selection of self-reactive transitional B cells. 29

Figure II.9. Positive Selection of Autoreactive B Cells in Wiskott-Aldrich Syndrome.35

Figure III.1. **Abnormal retention of *Was*$^{-/-}$ B cells in MZ**. 42

Figure III.2. B-cell intrinsic MyD88 deletion restores MZ in WAS. 44

Figure III.3. Dual BCR/TLR7 signals promote WAS MZ depletion. 47

Chapter I. Introduction

I.1 Generation of the innate and adaptive immune response

Protection against an increasing number of foreign pathogens necessitates the co-evolution and diversification of the host's immune system. In order to maintain this process, the immune response is delineated into two branches that serve to promote both the rapid clearance of pathogens as well as the generation of high-affinity effector and memory responses to prevent recurrent infection. These two branches are commonly referred to as the innate and adaptive immune systems, respectively. The innate immune system is comprised of macrophages, neutrophils, dendritic cells and a variety of other myeloid cells that serve to initiate the primary response to pathogens via innate pattern recognition receptors and sensors. Innate immune cells are able to then trigger the adaptive immune system by both presenting antigenic peptides to effector B and T cells as well as secreting pro-inflammatory cytokines to amplify and fine tune these responses (1). The adaptive immune system is comprised of B and T lymphocytes that express randomly arranged antigen receptors capable of recognizing a vast array of both foreign and host antigens. Receptor specificity enables targeting of pathogens via secretion of antibodies and cytotoxins to mediate efficient pathogen clearance. Thus, repertoire diversification is crucial to mounting an effective immune response.

Due to the time required to generate an effective antigen-specific immune response, these processes are highly integrated in order to protect the host from foreign pathogens. To generate specific adaptive effector responses, the diversity of both B and T antigen receptor repertoires must span an exponential number of unique pathogens. Diversification of developing repertoire is a highly regulated process aimed at creating a dynamic, naïve compartment. While generating a broad-array of unique receptors, maximizing the diversity of the repertoire inevitably leads to the development of receptors that recognize self-antigens. Natural autoantibodies can aid in the clearance of apoptotic debris and detection of non-protein self-antigens expressed on bacterium (2), but an abundance of self-reactive B cell receptors (BCR) may predispose the host towards an inappropriate response resulting in collateral damage to the host and autoimmunity. Thus, it is crucial to purge the developing repertoire of self-reactive receptors with a series of tolerance checkpoints to reduce the potential for autoimmunity.

I.2 Bone Marrow B Cell Development and Selection

B cell development begins in the bone marrow with lineage commitment from the common lymphoid progenitor (CLP) to a pro-B cell as identified by up-regulation of surface IL-7Rα via transcription of *E2A*, *Pax5* and *Ebf1* genes (3). Following successful recombination of heavy chain variable genes, a pre-BCR complex, consisting of both Igα/β chains and surrogate light chain, is expressed on the cell surface (3). The ability of the pre-BCR heavy chain to transduce signal is tested and if productive, clonal expansion of the pre-B cell occurs (3-5); while, non-productive heavy chain BCRs are deleted (6). Rearrangements of variable genes in the light chain follow this process allowing the pre-B cell to further differentiate to an immature B cell which expresses a surface BCR, known as IgM (3). Inherent to this stochastic process is the danger that antigen receptors specific for self-antigen may form and thus, immature B cells must be closely screened to prevent self-reactive cells from entering the naïve compartment.

Central B cell tolerance is the first step in a series of stringent selection processes that is initiated in immature B cells via engagement of the BCR with circulating self-antigen within the bone marrow (7). The majority of early B cells have been previously characterized as self-reactive, implying that central tolerance is crucial to eradicating the bulk of autoreactive B cells from the developing repertoire (8). Negative selection mechanisms were revealed through the use of murine BCR transgenic models, such as the Hen Egg Lysozyme (HEL) BCR transgenic model, where the BCR recognizes a foreign (HEL) antigen (9). HEL-specific B cells are deleted when stimulated with membrane-bound HEL antigen mimicking the outcome of high-affinity BCR-self ligand interactions (9). Likewise, HEL-specific B cells down regulate their surface antigen receptor when stimulated with soluble HEL antigen; revealing that low-affinity/avidity interactions with self-ligand result in the induction of an anergic-like program (9). While autoreactive immature B cells can be deleted or rendered anergic, a tertiary mechanism of reducing self-reactivity exists, which is known as receptor editing. Receptor editing of the light chain allows for reduced autoreactivity of the receptor via expression of a new light chain in combination with the current heavy chain (10-12). Preferential selection of specific heavy chain variable genes have been observed from this point onwards during development indicating either structural or antigen-specific bias in the selection of functional, productive heavy chain B-cell receptors (13). Approximately 50-60% of immature B cells are deleted, edited or suppressed

during central tolerance; however, because this is a stochastic process, some moderately self-reactive B cells escape deletion via receptor editing or remain undetected via low-affinity interactions (7, 14). Once immature B cells have expressed and up-regulated a functional antigen receptor, these cells then egress into the splenic periphery to mature into naïve, follicular or marginal zone B cells.

I.3 Peripheral B Cell Development and Negative Selection

Recently emigrated splenic immature B cells are known as early transitional (T1) and late transitional (T2) B cells as identified by expression of the surface markers, AA4.1 (CD93), CD23 and CD21 (15). As central tolerance is a randomized process, negative selection mechanisms in addition to clonal deletion and anergy are utilized in the periphery for further removal of autoreactive B cells before entering the naïve compartment (9). These mechanisms are coordinated by a complex network of receptors such as the BCR (16), CD40 (17), B cell activating factor (BlySS/BAFF) receptor (18) and Toll Like Receptors (TLR) (19).

Transitional B cell maturation and survival is dependent on receiving pro-survival NF-κB and/or PI3-kinase (PI3K) signals via antigen-independent BCR signaling (18). Tonic BCR signals are required for maturation of transitional B cells into the naïve compartment as several studies have demonstrated that loss or impairment of BCR signaling results in a significantly reduced mature B cell compartment (20-22). For example, conditional ablation of the BCR (20) or reduced Btk activity in *xid* mice result in reduced B cell maturation of mature follicular and marginal zone B cells (22). Too strong of a BCR signal is equally detrimental as revealed by transitional cell sensitivity to BCR-induced apoptosis (23). Recent work in leukemia cell lines demonstrated that inducing proximal hyperactive pre-BCR signaling components also promotes cell death pathways reminiscent of that observed in self-reactive BCRs (24). These combined findings suggest that fluctuations in BCR signaling thresholds directly impacts B cell survival and thus impact transitional cell competition and survival into the mature niche.

In addition to tonic BCR signals, both transitional and mature B cells require access to several pro-survival signals to develop and persist (13, 18, 21). Competition for the pro-survival, Tumor Necrosis Factor (TNF) cytokine, BLySS/BAFF, via either the BAFF and/or TACI receptor, promotes transitional B cell survival via signaling through the alternative NF-κB and PI3-kinase pathways (18, 25). Deficiency in either BAFF or BAFFR results in a block in T2

(identified as $CD24^{hi}CD21^{lo}AA4.1^{mid}$) B cell development indicating that BAFFR is the main receptor by which this pro-survival message is generated (26). Furthermore, recent data implicates that signaling via CD40 and CD40 ligand (CD40L) expressed on CD4+ T cells can modulate transitional B cell survival and selection (17, 22). Signaling via CD40 drives activation of both alternative and canonical NF-κB pro-survival pathways in conjunction with the BCR (17, 27). Additionally, CD40 exhibits a compensatory role in the absence of BCR signals, as revealed by the severely reduced transitional B cell compartment size in Btk and CD40 doubly-deficient mice (22).

Furthermore, coordinate BCR and B-cell intrinsic, innate TLR signals play a critical role in immature B cell tolerance to nuclear self-antigens. Peripheral B cells express TLRs 4, 7, and 9, specific for LPS, ssRNA, and dsDNA, respectively (28, 29). Identification and processing of self-antigens requires synergistic activation by both BCR and TLR pathways, as shown in studies by Chaturvedi, et.al (30). These studies demonstrated that upon BCR engagement with DNA-containing antigens, the BCR is internalized and activates the MAPK pathway while trafficking to the autophagosome where TLR9 endosomes are recruited (30, 31). Synergy between the BCR and TLR pathways have been hypothesized to be important for the activation of B cells in autoimmune disorders or in response to viral infection, situations in which nucleic antigens are abundant; however, the role for B-cell intrinsic TLR signaling during development is unclear. Recent studies clearly demonstrate a role of DNA-specific TLRs (specifically, TLR9) in regulating central tolerance (19, 30, 31). Additionally, TLR9 ligation in conjunction with strong BCR cross-linking drives a pro-apoptotic pathway in peripheral B cells (19). Likewise, humans deficient in either IRAK-4 or MYD88 exhibit increased autoreactivity within the naïve B cell compartment, implying that innate signaling pathways are necessary for the elimination of autoreactive BCRs during development (32). Dual TLR and BCR signals can modulate peripheral tolerance by negatively selecting BCRs that do not exhibit high affinity responses towards self-nucleic antigens (Figure I.1). Taken together, these observations provide several mechanisms by which the peripheral B cell compartment is shaped to generate a diverse mature, naïve B cell repertoire.

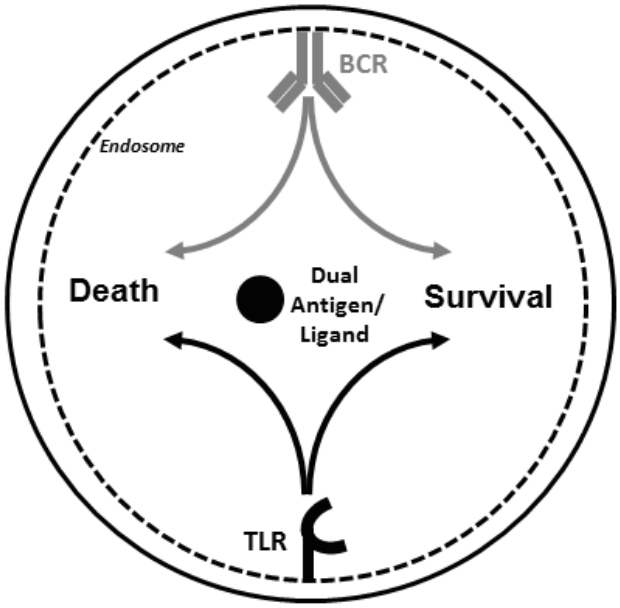

Figure I.1. Role of dual TLR and BCR signals in modulating negative selection of transitional B cells.
Schematic depicting how antigen engagement via both BCR and TLR pathways in transitional B cells can promote either death or survival dependent on affinity towards self-antigen. (Metzler et.al, in review).

I.4 Antigen-Dependent Positive Selection

While negative selection of developing B cells has been clearly demonstrated in the periphery, the potential role for antigen-dependent positive selection in this process has yet to be clearly defined. Several lines of evidence strongly imply a role for ligand-mediated selection in the naïve peripheral B cell compartment. First, previous studies indicate that V_H and V_L chain usage is skewed during B cell development and a limited subset of heavy and light chain genes first expressed within immature B cell subsets become enriched within the mature B cell population, consistent with BCR-specific shaping during development (33). Interestingly, both survival and expansion of antigen-specific BCRs have been shown to be partially dependent on the amount of endogenous self-antigen present indicating a role for antigen-mediated selection of specific BCR reactivity (34). Additionally, earlier reports indicated that tonic BCR signaling was antigen-independent (16); however, emerging data also imply that maturation occurs in conjunction with the tuning of the developing BCR repertoire via direct antigen-engagement (35). Specifically, work by Zikherman et.al, revealed that the vast majority of mature, naive B cells exhibit evidence of direct BCR engagement – presumably via self-antigens and/or possibly,

endogenous flora. Using Nur77-GFP reporter mice, this group showed that BCR-ligand engagement first occurs at the late transitional (T2) B cell stage, implying that entry into the naïve compartment is refined via antigen stimulation (35). Taken together these data demonstrate that antigen-dependent BCR signaling occurs in transitional or peripheral B cells to support a model by which receptor specificity serves to shape the naïve repertoire through BCR-antigen specific selection.

We hypothesized that positive selection driven by antigen-mediated selection occurs during the late transitional phase. Previous work in our laboratory identified a population of late-stage transitional B cells (T2 B cells) that is cycling *in vivo*, thereby suggesting that this population may be a target for antigen-dependent positive selection via clonal expansion (36). Our group and others identified evidence for expansion of specific BCR specificities during T2 B cell development based upon use of the M167 heavy chain transgenic mouse model. In this model, idiotype-specific, phosphorylcholine (PC) reactive cells are first enriched within the T2 B cell compartment and further expanded in the marginal zone (MZ) showing that at least this particular self-reactive specificity can be directly enriched beginning at the T2 B cell stage prior to differentiation into mature B cells (36, 37). Correspondingly, characterization of the Nur77-GFP transgenic reporter murine model demonstrated that T2 B cells exhibit BCR-mediated signaling within the periphery and suggests that this process serves to modulate the developing B cell repertoire (35). Based on these data, we hypothesize that in specific genetic settings, low-affinity self-reactive B cells may proliferate more robustly in response to self-antigen, particularly when other B cell tolerance mechanisms are relaxed; such environmental cues can then serve to significantly impact both the mature B cell repertoire and subsequent antigen-triggered activation of mature B cells.

Breaks in peripheral tolerance can be driven by several factors. *First*, excess soluble BAFF may limit negative selection of self-reactive B cells (7, 38, 39). Consistent with this observation, elevated serum BAFF levels have been described in human autoimmune disorders including Systemic Lupus Erythmatosus (SLE) and arthritis (38). BAFF transgenic mice that over-express serum BAFF exhibit splenomegaly, lupus-like disease and a significant increase in both peripheral B cell numbers and anti-nuclear autoantibodies (40, 41). Preliminary data from our laboratory and others indicates that excess BAFF also directly promotes alterations to the mature, naïve B cell repertoire by promoting the survival of self-reactive transitional B cells

(unpublished observations by S. Leach) (7, 14, 38, 42-44). While normally considered a limiting resource for transitional B cells, exogenous BAFF allows for auto-reactive B cells that may have been deleted in normal BAFF settings, to compete into the mature niche by providing alternative NFkB survival signals during peripheral tolerance (18, 41). *Second*, other survival signals, as observed in CD40/CD40L deficiency, can also alter the specificity of the mature B cell compartment in both humans and mice (22, 33, 45-47). Deficiency in either CD40 or CD40L results in either the accumulation of certain self-reactive BCRs or a reduction in the breadth of specificities recruited into the mature compartment; thus, indicating that CD40-CD40L interactions modulate both negative and positive selection of transitional B cells (17, 48).

In the context of TLR signaling and positive selection, few studies have thoroughly investigated the impact of TLR signals on altering peripheral tolerance. Giltiay et.al, demonstrated using the TLR7.1 transgenic murine model of SLE, that overexpression of TLR7 drives both the activation and proliferation of transitional B cells to form autoantibody producing extrafollicular B cells (49). In addition to this study, Walsh et.al, revealed that B-cell intrinsic TLR7 signals were required for induction of autoimmunity in the TLR7.1 transgenic model (50). Taken together, these data suggest that enhanced TLR signaling in transitional and/or mature B cells can potentiate autoimmunity via activation and expansion of autoantibody producing cells. How development is altered in the context of dual BCR and TLR signaling is unclear; however, given the strong correlation between TLR signals and humoral autoimmunity, it is important to understand the underlying mechanisms by which these signals impact peripheral tolerance, specifically positive selection of autoreactive B cells.

To further dissect how dual BCR and TLR signals modulate breaks in B cell tolerance via positive selection, we utilized a murine model of the primary immunodeficiency, Wiskott-Aldrich Syndrome (WAS), to evaluate the functional effects of altered antigen receptor signaling on B cell development and selection. Models of primary immunodeficiency provide an excellent system to understand the function of specific immune receptors and signaling pathways in both development and autoimmunity as several disorders are driven by loss-of-function mutations in genes encoding various components of antigen-receptor signaling cascades (51). Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency disease characterized by eczema, recurrent infections, thrombocytopenia, and a high prevalence of humoral autoimmunity (52,

53). WAS is caused by mutations in the gene encoding the Wiskott-Aldrich syndrome protein (WASp), a cytoplasmic protein expressed in hematopoietic cells involved in signal transduction from the cell surface to the actin cytoskeleton (52). Recent work from our lab and others indicate that WASp-deficient B cells exhibit hyper-responsive BCR and TLR signaling as well as demonstrate that enhanced B-intrinsic TLR signals, specifically TLR7, are sufficient to drive the induction of humoral autoimmunity (54, 55). While these studies were primarily focused on germinal center (GC) and class-switched autoantibody responses, these data prompted a thorough analysis of how altered BCR and TLR signals may impact B cell selection and tolerance.

Recent data characterizing the overall and class-switched B cell repertoires in human WAS subjects have indicated that WASp-deficiency results in alterations in various characteristics of both the mature and memory B cell repertoires such as increased CDR3 length and reduced rates of somatic hypermutation (SHM) (56, 57). While these reports represent novel insights into the B cell compartment in WAS, these studies failed to identify the mechanisms responsible for enriching the naïve compartment for autoreactive BCRs (ie. via breaks in negative and/or positive selection). To evaluate the effect of WASp-deficiency on transitional B cell selection, we utilized several approaches, including cell cycle analysis, various BCR transgenic models, high-throughput sequencing and single-cell cloning of both transitional and naïve B cell repertoires in both human and murine models of WASp-deficiency. These studies are described in detail in Chapter 2.

I.5 Role of Mature B Cells in Immune Homeostasis and Autoimmunity

Splenic murine transitional B cells mature into primarily two subsets, Marginal Zone (MZ) and Follicular Mature (FM) B cells. A third but much smaller B cell subset known as B1 B cells exists and are most prevalent within the peritoneal cavity and are important sources of antibodies during fetal and early development during which both the FM and MZ B cell niches are actively being filled. B1 B cells are thought to be a major source of natural IgM and IgA antibodies to gut microbial pathogens due to increased expression of innate pattern recognition receptors (2, 58). FM B cells constitute the majority of B2 B cells and reside within the splenic follicles as well as circulate through lymphoid follicles in the bone marrow and secondary

lymphoid organs (15). These cells are primarily responsible for T- cell dependent responses by presenting antigens to T-cell helper cells in order to initiate the germinal center (GC) response and production of high-affinity class-switched antibodies (15).

A much smaller subset of mature, naïve B cells are known as marginal zone (MZ) B cells. MZ B cells are characterized as non-recirculating, innate-like B cells that reside within the splenic marginal zone sinus and are primarily responsible for mounting T cell-independent humoral responses. Localization within the marginal zone sinus permits these cells to survey and rapidly respond to circulating blood-borne antigens as well as deposit or present antigen to follicular dendritic cells in order to generate the germinal center response (59). To successfully sample and deposit blood-borne antigens to the follicle, MZ B cells continuously shuttle between the red pulp and follicle (60). Migration to and from the follicle is dependent on Sphingosine-1 Phosphate (S1P) – mediated chemotaxis whereby S1P1-receptor expression on MZ B cells retains MZ B cells to the S1P ligand-rich red pulp; however, upon saturation of the receptor, these cells migrate towards CXCL13-rich follicles (61). In addition to S1P-CXCL13 mediated chemotaxis, MZ B cells also require integrins, Lymphocyte function-associated protein 1 (LFA-1) and α4β1 to maintain residence within the MZ sinus(62).

MZ B cells are characterized to be functionally pre-activated as well as up-regulate the expression of germline-encoded pathogen recognition receptors, such as Toll-like receptors (TLRs). Enhanced expression of TLRs permit MZ B cells to be well equipped to respond to antigens that express repeated carbohydrate epitopes or nucleic acid antigens, such as those found in apoptotic debris (59, 63). Dual signaling between BCR and TLR pathways in MZ B cells promote the activation and generation of low-affinity natural and class-switched antibodies to bridge the temporal gap between innate and adaptive humoral responses (59, 64). Interestingly, work by Lu et.al, showed that *in vivo* stimulation with the TLR 4 ligand, LPS, resulted in the relocalization of MZ B cells from the MZ sinus to the follicle indicating that TLR stimulation drives robust activation of MZ B cells causing the disruption of steady-state homeostasis (62). Given this heightened sensitivity towards TLR-ligands, commonly found in self-antigens, it is not surprising that MZ B cells are characterized to be enriched for autoreactive specificities (59, 65). Due to enhanced polyreactivity towards self-antigens, MZ B cells are attributed to producing natural IgM autoantibodies that can be both beneficial and pathogenic towards the host (59, 64).

The contribution of MZ B cells to humoral autoimmune disorders is of particular interest due to their innate-like features and the observed link between B-cell intrinsic TLR and BCR signals in driving the loss of B cell tolerance and autoimmunity (54, 55, 66). In murine models of lupus, MZ B cells enter the follicle and interact with CD4+ T cells (67). Interestingly, the presence of intrafollicular MZ B cells directly correlate with severity or onset of autoimmunity indicating that MZ B cells can directly contribute to the production of class-switched autoantibodies and augment disease pathogenesis (67). While some studies have implied that marginal zone B cells may initiate autoantibody production via spontaneous GC reactions, few studies thus far have definitively demonstrated a role for TLR signaling in MZ B cells in the context of humoral autoimmunity (68).

To test how TLR signals impact MZ B cell homeostasis and activation, we utilized the murine model of Wiskott-Aldrich Syndrome, previously described above. WASp-deficiency provides an excellent model for understanding the role of TLRs in MZ B cells as human WAS subjects often present clinically with impaired T-independent antibody responses and increased bacterial infections (52). Our group as well as others found that WASp-deficient mice had a striking decrease in the total number of marginal zone B cells despite no observed defects in MZ B cell development (69). Furthermore, this decrease was observed in a B-cell intrinsic WASp-deficient setting indicating that WASp is important for retaining MZ B cell within the marginal zone sinus (54). Given that B-cell intrinsic TLR signals are necessary for the induction of humoral autoimmunity in Wiskott-Aldrich Syndrome and TLR expression is most robust in MZ B cells, we assessed whether hyperresponsive TLR signals modulate MZ B cell homeostasis in Wiskott-Aldrich Syndrome. Previous work from our lab demonstrated that B-intrinsic TLR7 signals orchestrate both the loss of T cell tolerance and humoral autoimmunity; whereas TLR9 signals appear to ameliorate disease pathogenesis. How these individual TLR signals impact MZ B cell homeostasis is currently unknown. Using mixed bone marrow chimera models first described in studies by Becker-Herman et.al (54), we dissected the roles of individual TLRs in regulating MZ B cell localization and homeostasis in Wiskott-Adrich Syndrome. These studies are described in detail in Chapter 3.

Collectively, the results of our findings shed light on how modifications to antigen receptor signaling pathways can drastically alter tolerance mechanisms and B cell functionality. We show that fluctuations in receptor signal strength inadvertently skews peripheral tolerance

thresholds, thereby permitting auto-reactive transitional B cells to be positively selected into the naïve B cell niche. The functional consequences of these modifications are further amplified during activation of the mature B cell compartment; where heightened sensitivity to BCR and TLR stimuli can trigger self-reactive B cells to enter the GC to produce autoantibodies and directly contribute to the onset of humoral autoimmunity.

Chapter II. **Role of Positive Selection in Wiskott-Aldrich Syndrome**

II.1 **Introduction**

Development of the adaptive immune system requires selection of antigen receptors to establish a diverse but self-tolerant lymphocyte repertoire. Mechanisms to prevent selection of autoreactive B lymphocytes include clonal deletion, anergy and receptor editing (7, 18, 70). Alternatively, a growing body of literature also suggests that antigen-dependent positive selection of transitional B cells can occur via increased survival and/or clonal expansion (13, 34-36, 41, 71). These negative and positive selection mechanisms function in concert to shape the mature naïve B cell repertoire.

Positive selection of transitional B cells is regulated by tonic BCR signaling (17, 18), signaling via the cytokine, B-cell activating factor (BAFF) (2, 41) and T cell help via CD40L: CD40 signaling (17, 38, 72, 73) to promote cell survival. Positive selection may help to select BCR specificities that maintain key homeostatic functions, including apoptotic cell clearance or conserved pathogen recognition (2, 74). While positive selection can be beneficial for these important immune functions, enhanced positive selection of autoreactive BCRs, through incompletely defined mechanisms, is also predicted to occur in autoimmune-prone settings; and this process is likely to lead to an enrichment in BCR specificities that may facilitate detrimental immune responses.

In addition to BCR specificity, emerging data suggest a role(s) for TLR signals in modulation of B cell selection. Previous data have shown that TLR signaling adapters, including MyD88, IRAK-4 and UNC93b may operate in conjunction with the BCR to facilitate negative selection of autoreactive B cells (74, 75). In contrast to promoting negative selection in immature B cells, dual signals mediated via the BCR and TLR pathways in mature B cells (29, 40, 52, 53, 76-78) markedly enhance B cell activation and may directly initiate humoral autoimmunity. In

this latter setting, loss in B cell tolerance occurs via generation of self-reactive, germinal center responses leading, ultimately, to production of class-switched pathogenic autoantibodies (54, 75). Notably, while these combined data implicate TLR/MyD88 signals in both early and late B cell tolerance checkpoints, a potential role of BCR and/or TLR engagement in transitional B cell positive selection into the naïve mature B cell compartment has not been defined.

Wiskott-Aldrich Syndrome provides a model to understand the role of BCR and TLR signals in promoting autoimmunity as our previous work revealed that WASp-deficiency modestly enhances both BCR and TLR signaling in naïve B cells (54). Further, we and others have demonstrated that B cell intrinsic WASp-deficiency is sufficient to alter B cell tolerance and can promote production of class-switched autoantibodies and autoantibody-mediated autoimmune disease (54, 79). This break in tolerance is associated with spontaneous GC formation and requires both BCR and TLR/MyD88 signaling(7, 54, 55, 70).

In this study, we hypothesized that increased BCR and TLR signaling in WASp-deficient B cells may also impact establishment of the mature, naïve BCR repertoire. In partial support of this idea, previous studies have revealed evidence for skewing of heavy chain usage in both class-switched and bulk naive peripheral blood B cells isolated from WAS subjects (56, 57, 80). Here, we present a detailed analysis of the impact of WASp-deficiency on the selection of the naïve B cell repertoire in mice and humans. Our combined data support a model wherein altered BCR and TLR signaling orchestrates increased positive selection of transitional B cells expressing low-affinity, self-reactive BCRs leading to their enrichment within the naïve B cell compartment. As multiple human autoimmune disorders are associated with genetic changes potentially impacting B lineage signaling function, our findings have important implications with respect to understanding events that promote altered B cell selection in both WAS subjects and in other autoimmune prone individuals.

II.2 **Results**

II.2.1 *WASp-deficient mice exhibit a skewed naïve B cell repertoire enriched for low affinity self-reactive BCRs*

Increased λ-light chain (LC) usage in the mature B cell compartment of mice is correlated with increased autoreactivity(81-83). We therefore analyzed □- versus λ-LC usage in splenic B

cell subsets from young Was knock-out (*Was*$^{-/-}$) and control B6 mice. While there were no differences in λ-LC usage in immature bone marrow (BM) B cells (not shown) or early transitional (T1) splenic B cells, we observed an increase in the percentage of λ-LC expressing cells beginning in the late transitional (T2) subset and continuing within marginal zone precursor (MZp) and mature naïve [follicular mature, (FM) and marginal zone, (MZ)] B cell subsets (Figure II.1A). Consistent with a B cell-intrinsic process, equivalent findings were present in both *Was*$^{-/-}$ and B cell-specific WASp-deficient mice (*Was*$^{fl/fl}$*Mb-1*^{cre}), where the Was gene is deleted early in BM B cell development via lineage-specific Cre expression mediated by the Mb-1 promoter (79).

Based on these findings and previous work characterizing autoantibody production in Was$^{-/-}$ mice, we expanded our analysis to determine if WASp-deficiency impacts the spectrum of BCR specificities within the mature, naïve repertoire. We focused our analysis on MZ B cells as this compartment exhibited the greatest enrichment in λ-LC expressing B cells, cells predicted to be enriched for poly-reactive specificities (65, 67). Using established methods (8, 17), BCRs were cloned from single MZ B cells sorted from WT and *Was*$^{-/-}$ mice producing 74 WT and 64 *Was*$^{-/-}$ MZ recombinant mAbs. As anticipated for naïve B cells, BCR clones lacked evidence for somatic hypermutation (SHM; data not shown). Antibody specificity was evaluated with ELISA assays for a series of self-antigens including dsDNA, high and low affinity phosphorylcholine (PC-4 and PC-12), MDA-LDL and Sm-RNP (Figure II.1B). Antibodies were defined as reactive if the observed OD was greater than a threshold value set at 0.5 OD. The proportion of mAbs reactive with RNA antigens was increased in *Was*$^{-/-}$ (32% reactive) vs. WT (17%; Figure 1I.1B). Further, while we observed no significant differences in the percentage of reactive clones to other self-antigens, a much larger proportion of self-reactive *Was*$^{-/-}$ MZ clones exhibited low affinity responses (OD level of 0.5-1.5) than in WT (Figure II.1D). Consistent with this observation, calculation of the area under the curve (AUC) for clones reactive to each self-antigen revealed a trend towards decreased AUC for *Was*$^{-/-}$ clones (Figures II.1C-E). Thus, WASp-deficiency is associated with increased λ-LC usage in transitional and mature B cells and an increase in both RNA-reactive BCRs and other lower-affinity autoreactive clones within the MZ B cell compartment.

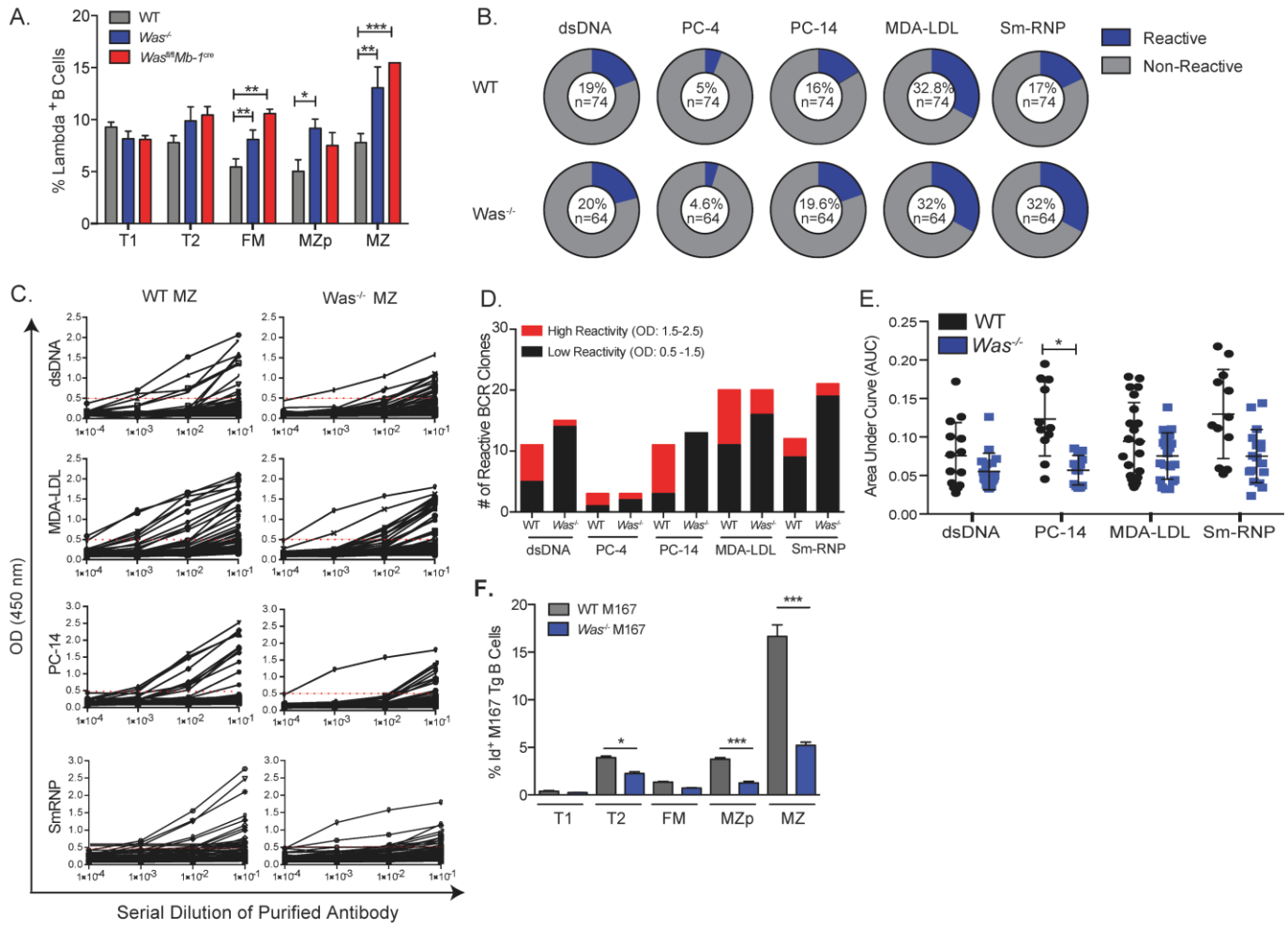

Figure II.1. Altered specificity of the naïve B cell repertoire in WASp-deficient mice.

(A) λ-LC usage in splenic B cell populations in 8-10 wk old C57/BL6 (n=6), *Was*$^{fl/fl}$ x *Mb-1*^{cre} (n=5) and *Was*$^{-/-}$ (n=7) mice assessed by flow cytometry. **(B-C)** Cloned WT and *Was*$^{-/-}$ MZ B cell mAb reactivities towards self-antigens (dsDNA, high (PC-4) and low (PC-14) affinity phosphorylcholine, MDA-LDL, and Sm-RNP) via ELISA depicted using a pie chart (blue = reactive clones identified based on threshold of 0.5 OD value; gray = nonreactive clones) with percentage of reactive clones and total number of clones tested noted. MZ B cells were FACS sorted and gated based on B220^{+}CD23^{lo}CD1d^{hi}CD24^{hi}CD21^{hi} surface marker expression from splenocytes pooled from 5-6 WT or *Was*$^{-/-}$ mice **(C)** ELISA OD values of serial dilution curves of WT and *Was*$^{-/-}$ MZ mAbs (100 ng/ul). **(D)** Proportion of low affinity (OD of 0.5-1.5) and high affinity (OD of 1.5-3) in reactive antibody clones to individual self-antigens. **(E)** Relative binding affinity displayed as area under the curve (AUC) of reactive antibodies. **(F)** Selection of idiotype (Id; M167)$^{+}$ B cells in peripheral B cell subsets in 10-12 wk old WT M167 Tg (n=9) and *Was*$^{-/-}$M167 Tg (n=9) mice. Error bars show SEM and statistical analysis using Student's t-test with, designated here and subsequent figures as *p< 0.05; **p <0.01; ***p<0.001; data are representative of at least 2 experiments.

Next, we evaluated whether WASp-deficiency impacted selection of B cells expressing a high affinity self-reactive BCR. We utilized the M167-transgenic (Tg) model, to assess positive selection of PC-specific idiotype $(Id)^+$ late-transitional and MZ B cells (36, 84). Strikingly, *Was*$^{-/-}$ M167-Tg mice showed significantly reduced percentage of M167 Id^+ B cells compared to WT mice. This change was evident in T2 B cells and retained in MZp and MZ cells, findings consistent with reduced selection for this high-affinity self-reactive BCR (Figure 1I.1F). Of note, serum low-affinity PC-specific IgM and IgG were markedly higher in *Was*$^{-/-}$ x M167 Tg mice compared to WT x M167 Tg mice (data not shown) indicating that while high affinity BCRs are not selected, there is enhanced production of low-affinity PC-specific autoantibodies in the absence of WASp. This trend towards increased selection to lower-affinity autoantibodies is further supported by the increase of low-affinity (PC-14) autoantibodies in the MZ compartment in *Was*$^{-/-}$ mice.

II.2.2 *High-throughput BCR sequencing reveals an altered HC repertoire in Was*$^{-/-}$ *MZ B cells*

To expand the scope of our repertoire analysis, we sequenced BCR heavy chain genes from peripheral B cell subsets. We utilized 5'-rapid amplification of cDNA ends (RACE) followed by 454-pyrosequncing of amplicons generated using IgD and IgM gene specific primers (Figure II.2A and II.2B). To limit any potential PCR amplification-induced bias, data analysis was restricted only to assessment of unique clonotypes. Over 80,000 unique sequences were identified from 3 independent experiments (each performed using pooled high-purity sorted B cell populations derived from at least 5 animals/genotype), resulting in 4,000-20,000 unique clonotypic sequences for each subset examined. Analysis of the total splenic B cell repertoire revealed altered VH gene family usage in *Was*$^{-/-}$ mice, with a significant decrease in VH9 and an increase in both VH10 and VH14 usage (Figure II.2A). Importantly, we confirmed that these changes were due to B-cell intrinsic WASp-deficiency as sequencing data in *Was*$^{fl/fl}$ *x Mb-1*^{cre} mice recapitulated these VH family alterations. Alternatively, analysis of *Was*$^{-/-}$ MZ B cells revealed a marked increase in VH10 expression as well as decreased VH7, VH9 and VH14 family usage (Figure II.2B). Detailed analysis of average HCDR3 lengths and JH usage did not reveal any statistically significant differences between WT and *Was*$^{-/-}$ B cell repertoires (data not shown). Together, these data demonstrate significant skewing of VH family gene usage in *Was*$^{-/-}$ naïve mature B cells including, in particular, a marked enrichment in VH10 and VH14 usage in

MZ and bulk splenic B cell compartments, respectively; VH families commonly associated with natural autoantibodies in mice (85-88).

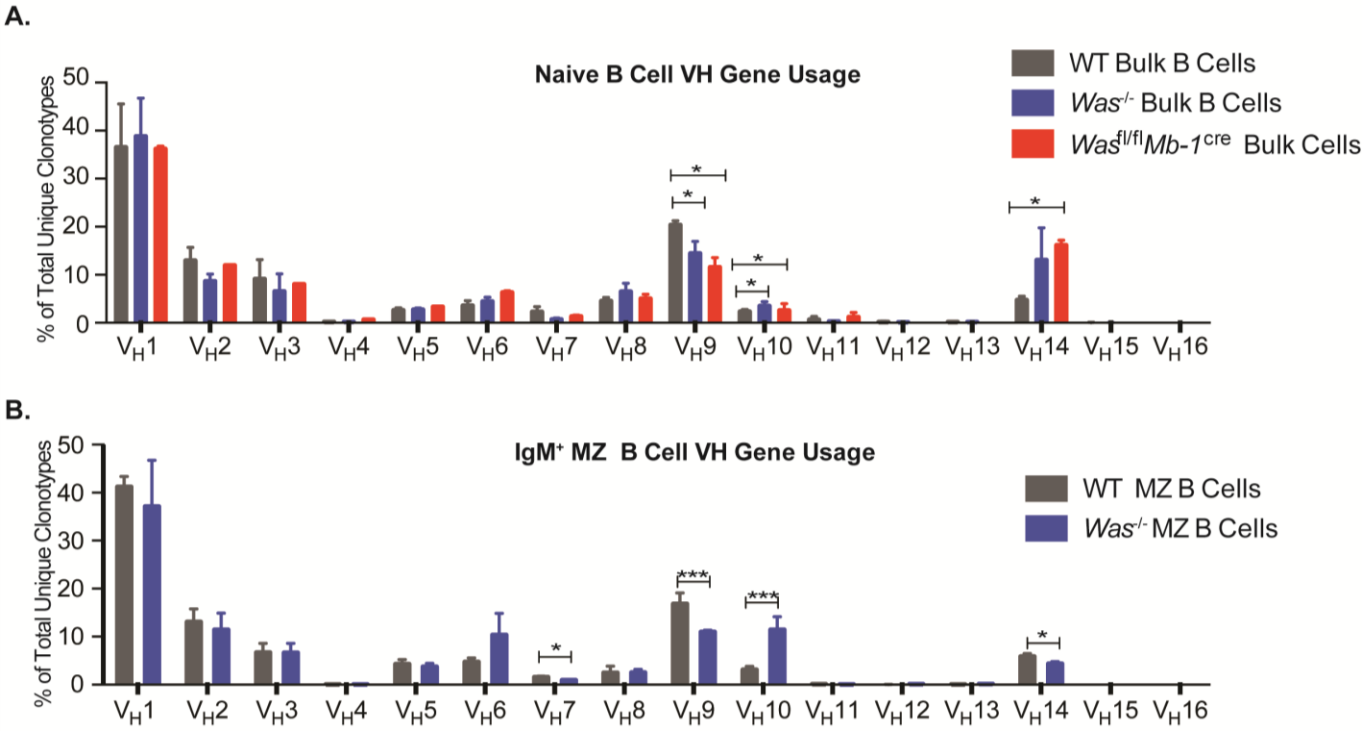

Figure II.2. High throughput BCR heavy chain sequencing of splenic B cells subsets from WT, *Was*$^{-/-}$, and *Was*$^{fl/fl}$x *Mb-1*^{cre} mice.

B cell populations were sorted (total of ~0.5-10x10^6 pooled cells/subset) from B6 WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$x *Mb-1*^{cre} mice using minimum of 5-6 mice/genotype per experiment (3 experiments total). RNA was isolated, sequenced and analyzed using a 5'-454 RACE platform (see methods). Data represents average of 3 experiments (15 mice per genotype) **(A)** Heavy chain variable (VH) gene family usage in bulk WT and *Was*$^{-/-}$ B cells (~10x10^6 cells/sample). **(B)** VH gene family usage in sorted WT and *Was*$^{-/-}$ B220$^+$CD21^{hi}CD24^{hi}CD23^{lo}CD1d^{hi} MZ B cells (~0.5 – 1x10^6 cells/sample). Statistical analysis included ANOVA and Student's t-test as appropriate; data representative of at least 3 experiments.

II.2.3 *WASp-deficiency does not perturb negative selection mechanisms*

We next utilized transgenic (Tg) models to determine whether the observed changes in the *Was*$^{-/-}$ naive BCR repertoire (identified via clonal analysis and sequencing studies) result from impaired negative selection. We evaluated both clonal deletion and anergy of WASp-deficient B cells using the Hen Egg Lysozyme (HEL): anti-HEL BCR (MD4) double Tg mouse model; where Tg B cells express a high affinity BCR specific for the foreign antigen, HEL (9, 89). Mixed BM chimeras were generated using WT or *Was*$^{-/-}$ MD4 Tg mice as donors. To emulate high vs. low affinity interactions with self-ligand, BM was transplanted into irradiated recipient mice expressing either membrane-bound HEL (mHEL) or soluble HEL (sHEL), respectively, or into controls lacking self-antigen (C57BL/6, B6) (Figure II. 3A-E). Both *Was*$^{-/-}$ and WT MD4 Tg B cells were deleted in mHEL recipient mice and HEL-specific serum antibodies were eliminated indicating that in this monoclonal setting, clonal deletion is unperturbed in *Was*$^{-/-}$ B cells (Figure II.3A-C). Similarly, we observed no defects in anergy induction in *Was*$^{-/-}$ B cells in sHEL expressing recipient mice with similar numbers of BM and splenic B cells (not shown), equivalent surface MD4 down-regulation (Figure II.3D and II.3E) and elimination of HEL-specific antibodies (Figure II.3F). In addition to deletion or anergy of autoreactive B cells, tolerance is also achieved via induction of antibody LC receptor editing. To evaluate whether this process is intact in *Was*$^{-/-}$ B cells, we used a quantitative assay for recombining sequence (RS) rearrangement. RS rearrangement occurs via recombination of a noncoding gene segment in the kappa LC locus (90). RS rearrangement levels were essentially identical in WT and *Was*$^{-/-}$ cells at each stage of BM B cell development (Fraction D and, kappa expressing Fractions E and F) as well as in splenic B cell subsets (kappa expressing T1, T2 and FM B cells; Figure II.3G). Collectively, these results indicate *Was*$^{-/-}$ mice manifest each of the key mechanisms responsible for negative selection of autoreactive immature B cells, suggesting that alterations in these events are unlikely to be primarily responsible for the observed changes in the BCR repertoire.

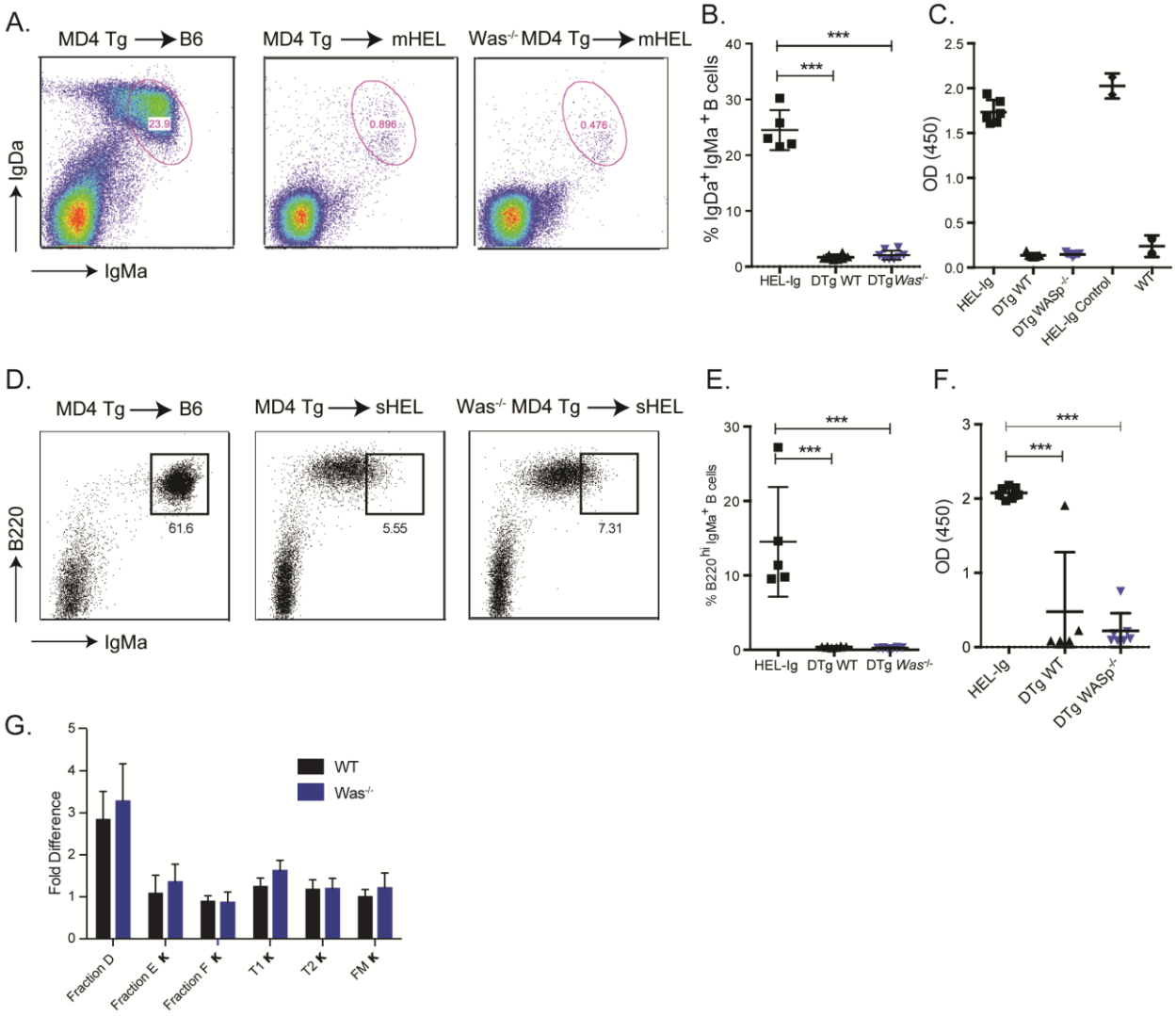

Figure II.3. *Was*$^{-/-}$ B cells exhibit intact negative selection.

(A-C) The HEL-Ig/membrane Hel (mHel) double transgenic (DTg) chimeric transplant model was used to determine whether self-reactive *Was*$^{-/-}$ B cells undergo deletion in the BM. Recipients were analyzed at 6wk post-transplant (n=6-7 per group; data representative of 2 independent experiments) **(A-B)** BM B cells were analyzed by FACS. **(C)** HEL-specific serum antibody levels. **(D)** The HEL-Ig/membrane Hel (sHel) double Transgenic (DTg) system was used to determine whether self-reactive *Was*$^{-/-}$ B cells undergo efficient induction of anergy (n=6-7 per group; data representative of 2 independent experiments) **(D-E)** BM B cells were analyzed by FACS; and **(F)** HEL-specific serum antibody levels. **(G)** Quantitative assay for recombining sequence (RS) rearrangement was used to estimate Kappa light chain rearrangement in BM and spleen B cell subsets of 6-8 wk old WT or *Was*$^{-/-}$ mice. Results are normalized to β-actin and presented as fold difference relative to WT kappa+ FM B cells. [n=7 in each group except for *Was*$^{-/-}$ Fractions E (n=6) and F (n=5)]. Data representative of at least two independent experiments.

II.2.4 *WASp-deficiency promotes antigen-mediated proliferation of transitional B cells*

Another hypothesis for altered repertoire is the expansion of positively selected clones. We first examined proliferation kinetics in transitional B and other B cell subsets as one potential surrogate measure of altered positive selection. Following 24 hr of *in vivo* BrdU labeling in *Was*$^{-/-}$, *Was*$^{fl/fl}$*Mb-1*^{cre} or WT mice, we estimated the percentage of cycling cells within BM and splenic B cell subsets. While there was no significant difference in BrdU incorporation within BM B cell subsets, we observed a significant increase in the percentage of BrdU^{+} transitional B cells in both *Was*$^{-/-}$ and *Was*$^{fl/fl}$ *Mb-1*^{cre} mice compared to WT control animals (Figure II.4A). To further evaluate cycling, we labeled B cell subsets with DAPI and Pyronin Y and analyzed the percentage of B cells in G0 vs. G2/S phase using flow cytometry (Figure II.4B). Transitional, MZp, and MZ subsets in *Was*$^{-/-}$ mice contained significantly higher proportions of cycling cells (Figure II.4B). Finally, we also observed evidence for enhanced cycling in Was$^{-/-}$ B cell subsets using Ki67 labeling (not shown; and Fig II.5A-B). Importantly, enhanced cycling was a BAFF independent phenotype. While *Was*$^{-/-}$ mice exhibit elevated serum BAFF, *Was*$^{fl/fl}$*Mb-1*^{cre} mice have normal BAFF levels yet manifest similar increased rates of peripheral B cell proliferation (Figure II.4C). Direct measurement of splenic BAFF protein and mRNA revealed no significant difference in WT vs. *Was*$^{-/-}$ genotypes (data not shown) additionally supporting the idea that local BAFF levels were unlikely to impact this cycling phenotype. Thus, using multiple approaches we identified a marked increase in the proportion of cycling transitional B cells in *Was*$^{-/-}$ and/or *Was*$^{fl/fl}$*Mb-1*^{cre} mice.

To begin to dissect the signals promoting increased transitional B cell proliferation in *Was*$^{-/-}$ mice, we utilized the Nur77-GFP transgenic model, where GFP expression driven by the Nur77 promoter permits assessment of BCR-mediated signaling (35). In this model, WT T1 B cells exhibit little or no GFP expression, whereas a proportion of T2 B cells become GFP^{hi}, consistent with self-antigen mediated BCR signaling initially occurring within the late transitional compartment (Figure II.4D, right vs. left panels). Strikingly, *Was*$^{-/-}$Nur77 Tg mice show roughly a 2-fold increase in the relative proportion of T2 GFP^{hi} B cells (Figure II.4D-E). Further, consistent with increased BCR signaling, *Was*$^{-/-}$ GFP^{+} T2 B cells exhibited increased GFP mean fluorescence intensity (MFI) compared to control T2 B cells (Figure II.4F). Next, we combined analyses using in vivo BrdU labeling with assessment using the Nur77-GFP reporter.

Consistent with the idea that BCR engagement promotes the cycling of T2 B cells, the majority of T2 $BrdU^+$ cells were located within the GFP^{hi} population (Figure II.4G).

To determine if self-reactive T2 B cells underwent altered clonal expansion in a WASp-deficient setting, we measured cell cycling using dual assessment of BrdU incorporation and Ki67 expression using the M167 Tg self-reactive BCR model. Interestingly, while the overall percentage of cycling T2 B cells was higher in WASp-deficient mice, *Was*$^{-/-}$ T2 M167 Id^+ B cells exhibited reduced cycling compared to WT M167 Id^+ cells (Figure II.4H and II.4I). This preferential expansion of $M167^-$ Id^- T2 B cells in *Was*$^{-/-}$ mice suggests that a larger proportion of lower affinity clones are able enter the mature, naïve repertoire.

Collectively, these findings suggest that the expanded, cycling T2 B cell population in *Was*$^{-/-}$ mice has engaged with self-antigen and that BCR engagement likely promotes and/or sustains cell cycling. Further, these data suggest that WASp-deficiency alters the breadth of self-reactive BCRs capable of competing for survival, limiting the competitive advantage of higher-affinity self-reactive clones, by permitting cells with lower-affinity self-reactive BCRs to more effectively compete and persist within the mature compartment.

Figure 4

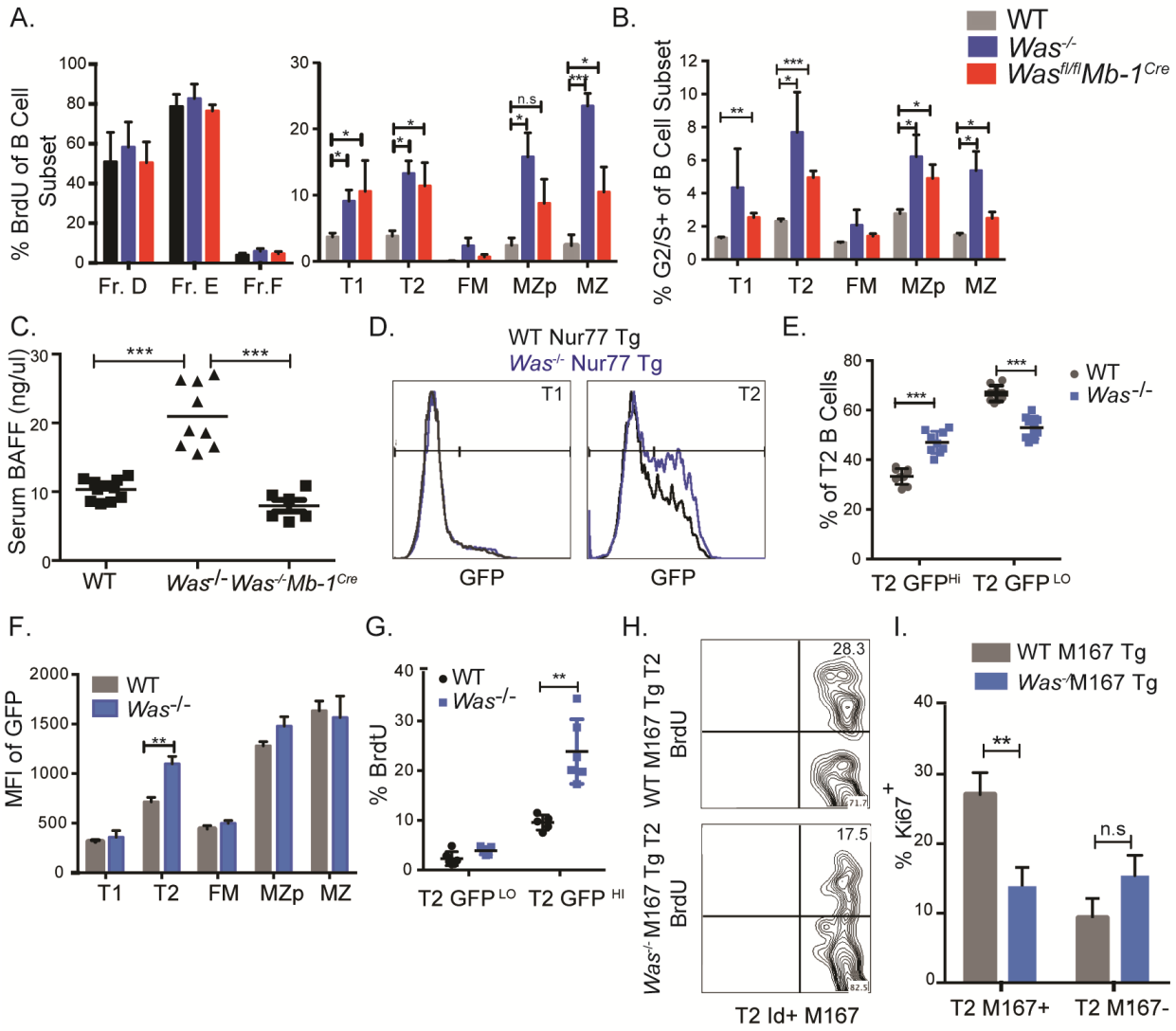

Figure II.4. ***Was*$^{-/-}$ B cells exhibit high-levels of antigen-dependent clonal expansion**.

(A) 8-10 wk old WT (n=7), *Was*$^{-/-}$ (n=9) and *Was*$^{fl/fl}$x *Mb*-1^{Cre}(n=6) mice were treated with BrdU *in vivo* for 24 hours. BM and splenic B cell subsets were analyzed for BrdU incorporation via FACS. **(B)** Cell cycle analysis of splenic B cell subsets via DAPI labeling in 8-10 wk old WT (n=7), *Was*$^{-/-}$ (n=9) and *Was*$^{fl/fl}$x *Mb*-1^{Cre}(n=6) mice **(C)** Serum BAFF levels in WT (n=11), *Was*$^{-/-}$ (n=9), and *Was*$^{fl/fl}$*xMb-1*^{cre} (n=6) mice. **(D)** Representative data showing GFP staining of splenic T1 (left panel) and T2 (right panel) B cells in WT and *Was*$^{-/-}$Nur77 Tg mice. **(E)** Percentage of GFP^{hi} and GFP^{lo} T2 B cells and **(F)** MFI of GFP in B cell subsets in WT and *Was*$^{-/-}$Nur77 Tg mice (n = 8/each). **(G)** Percentage of BrdU^{+} T2 GFP ^{hi} and ^{lo} cells in WT (n=7) and *Was*$^{-/-}$Nur77 Tg (n=6) mice. **(G-H)** 8-10 wk old WT M167 Tg (n=5) and *Was*$^{-/-}$M167 Tg mice (n=5) were treated with BrdU *in vivo* for 24 hours. **(H)** Representative contour FACS plot showing BrdU incorporation in WT (upper) vs. *Was*$^{-/-}$ (lower panel) M167 (Id)$^{+}$ T2 B cells. **(I)** Percentage of M167 Id^{+} Ki-67^{+} and Id^{-}Ki-67^{+} splenic T2 B cells in WT (n=5) vs. *Was*$^{-/-}$M167 (n=6) Tg mice. Data representative of at least two experiments.

II.2.5 *Myd88 signals contribute to enhanced selection and the altered naïve repertoire in Was$^{-/-}$ mice*

Our previous work has shown that B cell–intrinsic MyD88 signals are required to initiate germinal center responses and promote the generation of pathogenic autoantibodies in *Was*$^{-/-}$ B cell chimeric mice (54, 55). We therefore hypothesized that Myd88 signals might also impact naive B cell selection in WASp-deficient animals. We first determined whether Myd88 signaling facilitated transitional B cell proliferation. Using flow cytometry, we measured Ki67^{+} T1 and T2 B cells in splenic B cell subsets derived from WT, *Was*$^{-/-}$ and *Was*$^{-/-}$*Myd88*$^{-/-}$ mice (Figure II.5A). Strikingly, MyD88 deficiency markedly reduced the proliferation of WASp-deficient T1 and T2 B cells (Figure II.5A and II.5B). To further elucidate the impact of MyD88 signaling on the developing repertoire, *Was*$^{-/-}$*Myd88*$^{-/-}$ MZ B cells were sorted and used for deep-sequencing to evaluate BCR heavy chain gene usage. While most VH families were not impacted, Myd88 deficiency specifically abrogated enrichment of VH10 and VH14 family BCRs in the *Was*$^{-/-}$ MZ and bulk splenic B cell compartments, respectively (Figure II.5C and data not shown). Importantly, previous studies have correlated BCR VH10 and VH14 expression with nucleic acid-reactivity (85, 87, 88, 91). Thus, our findings suggest that in the setting of WASp-deficiency, Myd88 signals promote the selection of BCRs whose specificity correlates with anti-DNA and/or anti-RNA specificities leading to the observed expansion of VH10 and VH14 family gene usage. Taken together, these data indicate that Myd88 signals contribute to the altered naïve BCR repertoire in *Was*$^{-/-}$ mice.

Figure 5

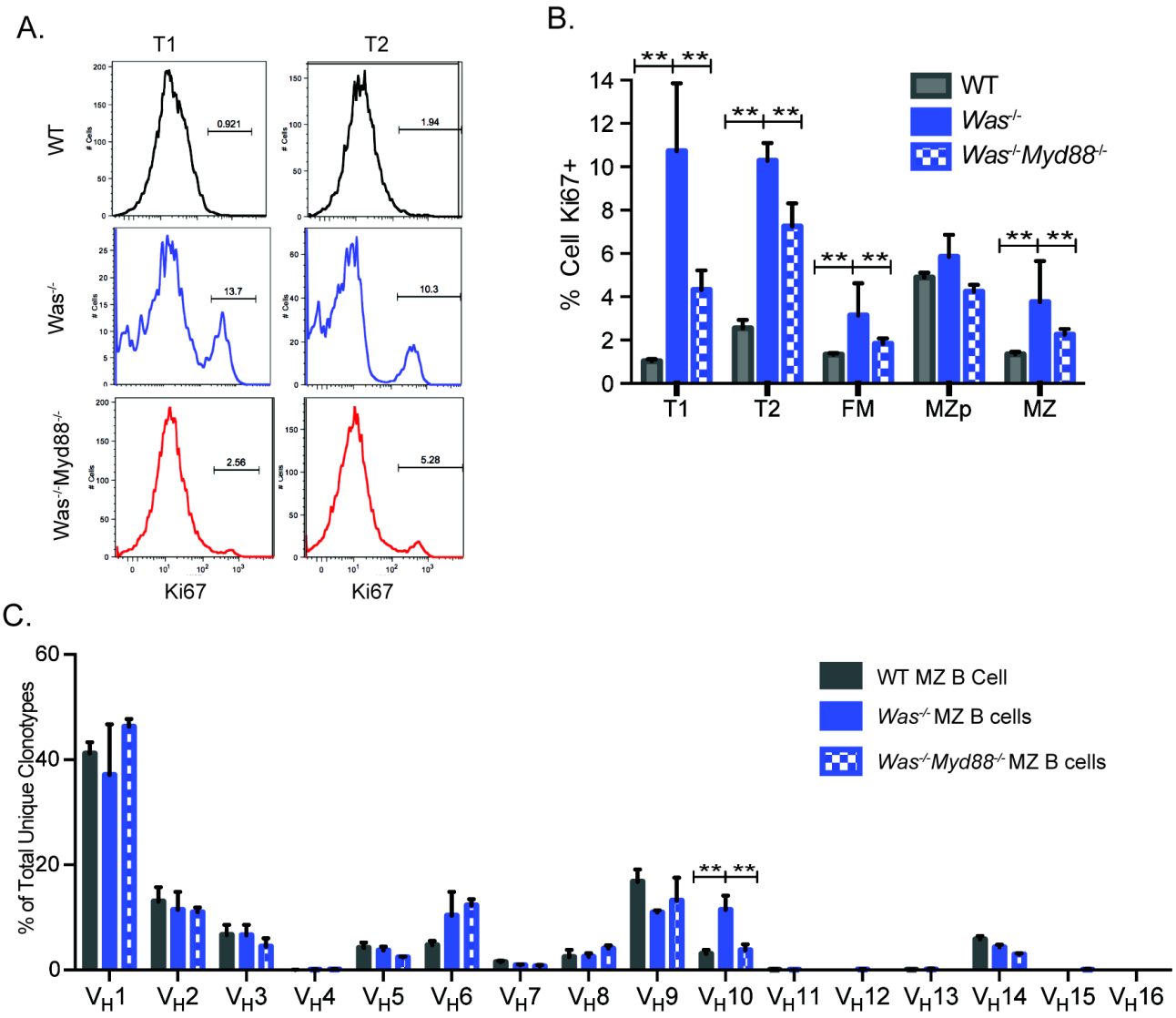

Figure II.5. Antigen-specific selection of *Was*$^{-/-}$ transitional B cells requires Myd88 signals.

(A) Representative FACS analysis of Ki67 staining of splenic T1 (B220^{+}CD24^{hi}CD21^{lo}) and T2 (B220^{+}CD24^{hi}CD21^{mid}) B cells in WT, *Was*$^{-/-}$, and *Was*$^{-/-}$*Myd88*$^{-/-}$ mice. **(B)** Percentage of Ki67^{+} B cells in WT (n=12), *Was*$^{-/-}$ (n =13), and *Was*$^{-/-}$*Myd88*$^{-/-}$ (n =12) mice. **(C)** VH gene family usage in sorted WT, *Was*$^{-/-}$, and *Was*$^{-/-}$*Myd88*$^{-/-}$ MZ B cells. Data representative of at least two experiments.

II.2.6 *Human WAS subjects also exhibit an altered naïve B cell repertoire*

To determine if BCR repertoire changes observed in the *Was*$^{-/-}$ murine model parallel findings in human subjects with WAS, we cloned BCRs from single mature naïve B cells isolated by cell sorting from the peripheral blood of one pediatric WAS and 2 pediatric and 1 adult healthy control subjects (Figure II.6C). Recombinant antibodies (54 WAS and 87 controls) were screened for reactivity to dsDNA, MDA-LDL, PC-12 and sm-RNP using ELISA assays. Compared to control BCRs, WAS BCRs were enriched for reactivity towards dsDNA, MDA-LDL, and PC-12 (Figure II.6A), and also demonstrated increased staining for nuclear antigens using a Hep2 cell-based IFA assay (Figure II.6B). These initial data suggest that human WASp-deficiency alters the naïve B cell repertoire leading to enrichment for B cells with self-reactive specificities.

To expand on these preliminary findings, and to obtain a more in depth assessment of the complete B cell repertoire in a larger number of subjects, we evaluated the transitional and naïve B cell repertoire using high-throughput sequencing. Highly purified B cell subsets were isolated by flow sorting from pediatric WAS subjects (n=3) and healthy controls (HC, n=4; Figure II.6C). Genomic DNA was extracted from each sample and analyzed for heavy chain gene usage using Illumina-based deep sequencing. Comparison of the heavy chain V gene usage in transitional B cells revealed no statistically significant differences in WAS vs. HC samples. In contrast, naïve WAS B cells exhibited increased usage of the VH4-34 gene family (Figure II.7A and II.7B). Notably, VH4-34 expressing BCRs have been previously characterized as highly enriched for self- and polyreactivity (92-94). While we observed no enrichment for VH4-34 sequences at the transitional to naïve B cell transition in HC samples (n=8), WAS subjects (n=9) exhibited an ~1.5 fold increase in VH4-34 expressing BCRs (Figure II.7B). Notably, earlier studies have shown that while VH4-34 BCRs are present in the naïve compartment in healthy subjects; they are nearly absent in the isotype switched memory compartment and secreted antibody repertoire of healthy individuals. In contrast, in lupus patients show a significant enrichment of VH4-34 in the memory compartment and in serum antibodies consistent with loss of tolerance in the GC reaction (92). Therefore, we also assessed the memory compartment of WAS patients and observed a significant increase VH4-34 expressing IgM memory cells (Figure II.7C). While VH4-34 BCRs were expanded in WAS, these naïve cells lacked SHM and IgM memory cells exhibited reduced rates of SHM compared to control (data not shown); consistent with the

observed T cell defect in WAS subjects and previous studies of the WAS memory compartment (56). Additional analysis of HDCR3 length and JH usage of HC and WAS subjects did not reveal significant differences (data not shown). Increased usage of VH4-34 heavy chain within the naïve repertoire was also present in non-transplanted adult WAS patients (n=4) indicating that skewing is evident as early as 1-3 years of age and comprises a developmental feature maintained into adulthood (Fig II.7D and II.7E).

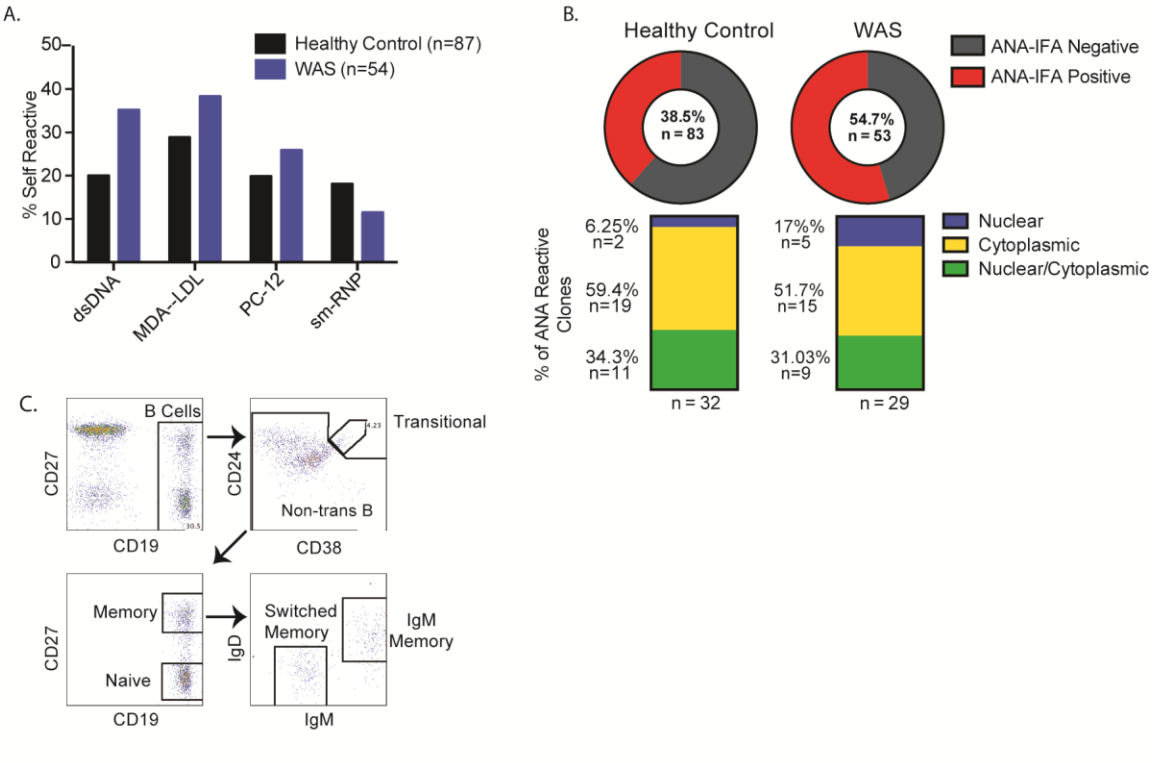

Figure II.6. WAS/XLT subjects exhibit an altered naïve B cell repertoire enriched for self-reactive specificities.
(A-B) BCRs were cloned from peripheral blood naïve B cells from a 10 mo. old WAS subject (n = 54 mAbs) and 2 pediatric and 1 adult HC subjects (n = 87 antibodies). Percentage of mAb clones reactive to self-antigens are shown. Data are representative of 1 of 2 experiments. **(B)** (upper panel) Pie charts display frequency of ANA-IFA reactive clones. (Lower panel) Staining pattern of each IFA-reactive clone, defined as nuclear, cytoplasmic or polyreactive (both nuclear and cytoplasmic), displayed according to overall percentages. **(C)** Human B cell subset gating and sorting strategy.

II.2.7 *V_H4-34 expressing BCRs are positively selected in Wiskott-Aldrich Syndrome*

Based on these BCR sequencing findings, we sought to further define the selection events operating upon VH4-34 HC expressing B cells in a larger number of subjects with altered WASp function. We sought to further define the selection events operating upon VH4-34 HC expressing B cells in a larger number of subjects with altered WASp function. We assessed the B cell compartment phenotype and tracked the selection of B cells in 6 pediatric WAS or X-linked thrombocytopenia (XLT) subjects and 5 age-matched HC subjects (Figure II.8A-C). Phenotypic analysis of WAS and HC subjects revealed no significant differences in either transitional B cell population size or surface IgM expression (data not shown). We used the anti-idiotypic monoclonal antibody, 9G4, specific for the VH4-34 heavy chain to track the selection of B cells throughout peripheral B cell development (92). Analysis of the transitional compartment revealed no significant difference in the relative proportion of $9G4^+$ B cells across all subjects; and there was no change in the proportion of $9G4^+$ cells in the naïve vs. transitional compartment in HCs In contrast, there was a significant enrichment of $9G4^+$ naïve mature B cells in WAS/XLT subjects with an average ~2.5-fold increase in the naïve vs. transitional compartment, consistent with our sequencing data (available for 3 of these 6 subjects). Consistent with these findings, we observed a similar trend in adult WAS subjects (Figure II.8D). To determine whether WAS/XLT $9G4^+$ B cells exhibit differences in VH4-34 BCR surface density, we assessed the MFI of 9G4 staining. Interestingly, transitional and naïve WAS B cells exhibited a trend for higher 9G4 expression compared to HC B cell subsets (Figure II.8E). Further, while the 9G4 MFI declined markedly between the transitional and naïve B cell stage in HCs, this change was less pronounced for WAS/XLT naïve B cells. These changes are consistent with the lower surface expression of IgM in HC 9G4+ B cells and the postulated anergic behavior of these autoreactive B cells (95).

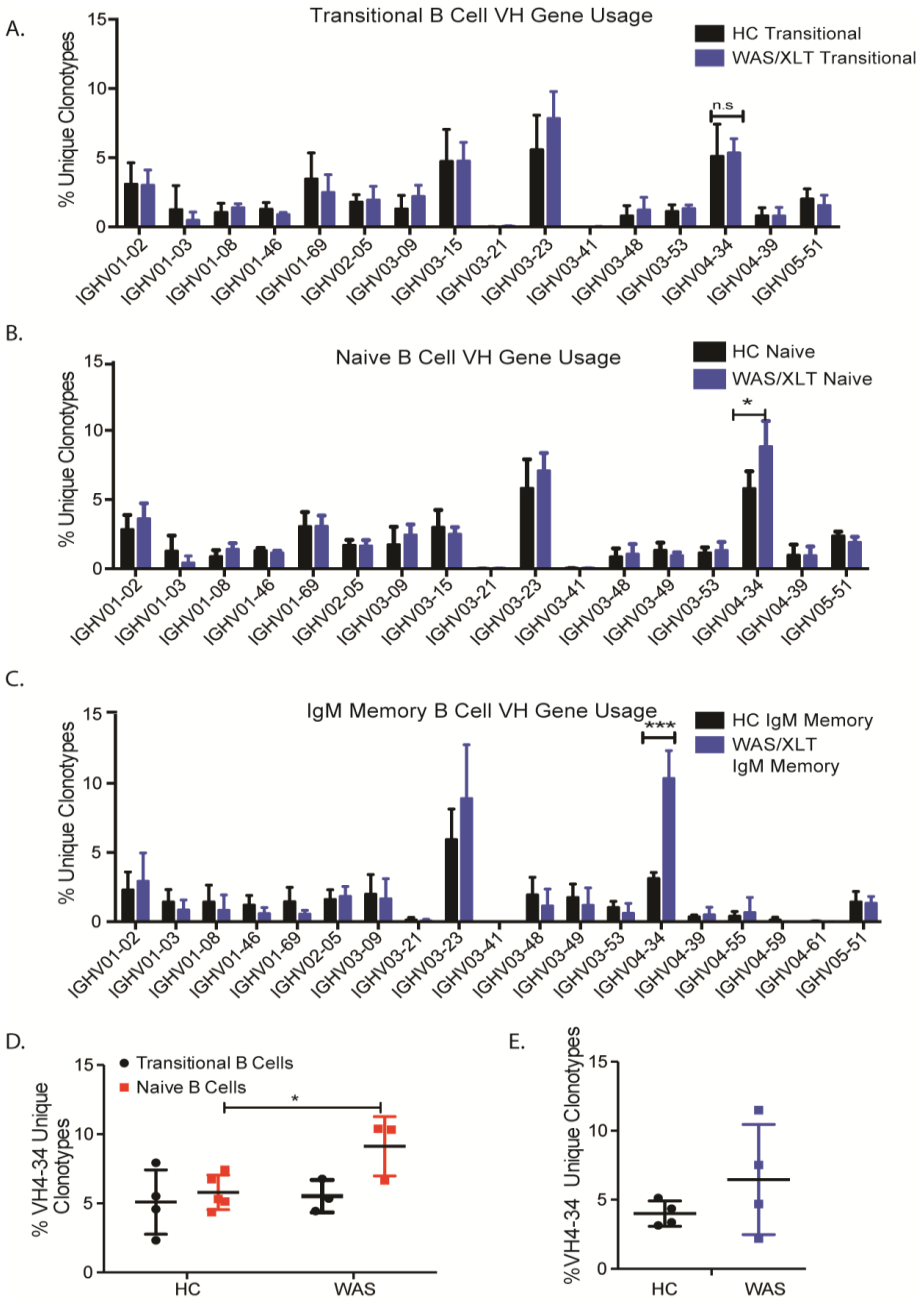

Figure II.7. WAS/XLT subjects exhibit enrichment for VH4-34 expressing BCRs in the naïve and IgM memory B cell compartments.

(A-C) Sorted subsets from 5 HC and 3 WAS pediatric subjects were analyzed by Illumina high-throughput sequencing of the BCR heavy chain and combined to show average VH family gene usage. **(A)** VH family usage of sorted transitional B cells ($CD19^{+}CD27^{+}CD24^{hi}CD38^{hi}$) displayed according to percentage of total unique clonotypes within the most abundant VH families; see Supplementary Table 1 for full VH family usage. **(B)** VH family usage of naive B cells ($CD19^{+}CD27^{-}$). **(C)** VH family usage of IgM memory B cells ($CD19^{+}CD27^{+}IgG^{-}$) **(D)** Percentage of VH4-34 within total unique, clonotypic sequences in naïve B cell compartment. **(E)** Percentage of VH4-34 sequences in naïve B cells in 4 HC (ages 23-29) and 4 WAS adult subjects (ages 18-28). **(D-H)** Data are representative of at least two experiments.

In addition to changes in 9G4+ representation in naïve B cell repertoire, we observed a significant enrichment for 9G4+ B cells in the total memory compartment (Figure II.8B). Consistent with our deep sequencing analysis in pediatric WAS subjects, this change was specific to the IgM memory population and not present in the switched memory B cells (Figure II.8B). In order to determine whether these alterations in the proportion of 9G4+ B cells predicted enhanced selection and activation of VH4-34 expressing B cells, we measured serum 9G4+ IgG levels by ELISA. We identified significantly increased levels of 9G4-specific IgG in WAS subjects compared to HCs (Figure II.8F), findings consistent with a loss of tolerance in germinal center responses that recruit B cells with these autoreactive BCR specificities.

Together, these observations demonstrate altered selection of self-reactive WAS/XLT B cells precisely at the transitional to naïve B cell transition; and suggest that VH4-34-specific self-antigen engagement likely promotes differential survival of WAS/XLT vs. HC $9G4^{+}$ B cells. Enrichment of these autoreactive specificities into the naïve compartment promotes increased selection and generation of autoreactive class-switched antibodies in WAS subjects.

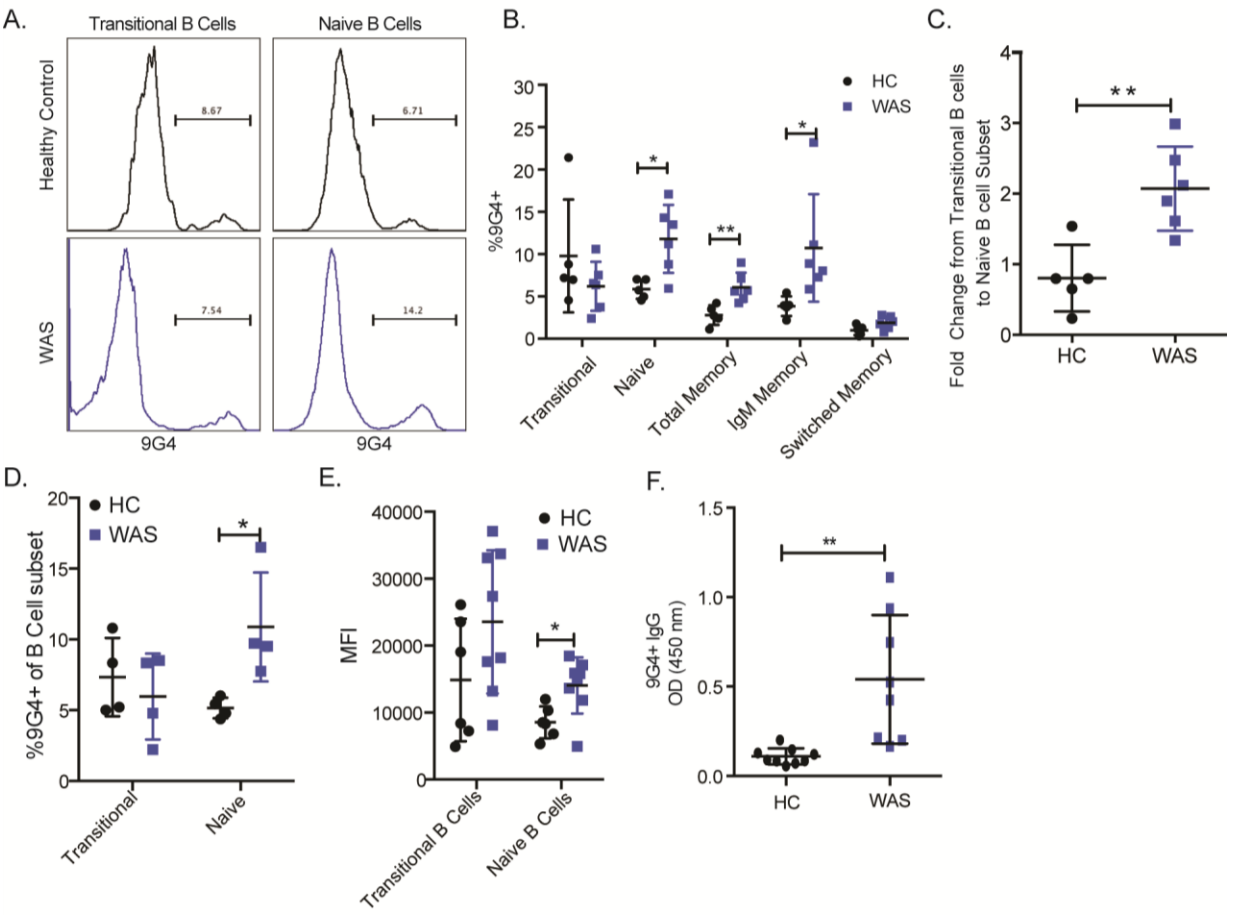

Figure II.8.WASp-deficiency promotes altered positive selection of self-reactive transitional B cells.

(A) Representative FACS histograms quantifying the percentage of $9G4^+$ peripheral blood transitional (left panels) and naïve B cells (right panels) in age-matched HC and WAS subjects. **(B)** $9G4^+$ percentages of B cell subsets in HC (n=5) and WAS subjects (n=6). **(C)** Relative fold change in percentage of $9G4^+$ transitional vs. naïve B cells in WAS (n=6) and HC subjects (n=5). **(D)** Percentage of 9G4+ transitional vs. naïve B cells in HC (n=4) and WAS adult subjects (n=4) **(E)** Cumulative data showing the MFI of 9G4 staining in B cell subsets in HC (n=6) and WAS pediatric subjects (n=8) **(F)** Serum 9G4-specific IgG levels were determined using plasma collected from pediatric WAS (n=8) and HC (n=9) subjects. Titers are displayed as relative absorbance values, OD (450 nm). Data is representative of at least two experiments.

II.3 **Discussion**

Expression of a sufficiently broad, naïve BCR repertoire is essential both for the response to pathogen challenge and to orchestrate B cell-mediated homeostatic functions. The naive BCR repertoire is established via the interplay of negative and positive selection mechanisms that operate within distinct developmental windows and microenvironments. Importantly, alterations in these events are predicted to increase the risk for autoimmune disease. In the current study, we utilized animal models and human subjects with the immunodeficiency disorder, WAS, to gain new insight regarding the pathways involved within transitional B cells that modulate peripheral B cell selection. Our studies establish a key role for combined BCR and TLR signals in orchestrating peripheral tolerance in the context of WASp-deficiency and have important broader implications with respect to human B cell tolerance.

Using a series of animal models, we show that WASp-deficiency significantly alters murine peripheral B cell tolerance as demonstrated by: a) increased λ-LC expression in T2, FM and MZ B cells; b) altered MZ B cell specificity with enrichment for lower-affinity, self-reactive BCRs; c) alteration in IgH family gene usage in the MZ compartment with, most notably, a striking increase in MZ B cells expressing VH10 family genes, previously shown to be associated with enhanced binding affinity for nucleic acids; and d) a heightened frequency of late transitional B cells undergoing homeostatic proliferation. Further, our data indicate that this proliferative program is mediated via both: i) self-antigen driven BCR engagement, as demonstrated using a transgenic Nur77-GFP reporter; and ii) TLR/Myd88 signaling, as shown by reduced proliferation in WASp-deficient B cells concurrently lacking Myd88. Finally, using deep sequencing we demonstrate a direct requirement for MyD88 signaling in driving the selection of VH10 and VH14 expressing BCRs into the MZ and splenic B cell compartment, respectively, in $Was^{-/-}$ mice. Importantly, while the majority of our data were derived using $Was^{-/-}$ mice, we performed a subset of key studies using $Was^{fl/fl}$ $Mb\text{-}1^{cre}$ mice and observed essentially identical findings including altered IGH family usage, enhanced transitional cell cycling and increased λ-LC expression. Also of note, while serum BAFF is modestly increased in $Was^{-/-}$ mice, the $Was^{fl/fl}$ $Mb\text{-}1^{cre}$ model exhibits normal levels. Thus, while potentially exaggerated by alterations in the BAFF signaling program, these changes occur independently of BAFF signaling. In combination, our murine model data support the conclusion that cell-intrinsic

signaling changes in WASp-deficient transitional B cells play a dominant role in altering B cell tolerance leading to a skewing of the naïve BCR repertoire.

Predicated upon our murine findings, we carried out studies using highly purified peripheral blood B cell subsets derived from subjects with WAS (or the related, but less severe disorder, XLT) and age-matched controls. Using single cell BCR cloning we observed evidence for altered naïve mature B cell specificity with enrichment for self-reactive BCRs. Based on this finding, we expanded our analysis using two alternative approaches that directly demonstrate altered tolerance in WAS subjects precisely at the late transitional to naïve mature B cell transition as shown by: a) deep-sequencing showing changes in VH family usage specifically within the mature, naïve repertoire including increased utilization of the self-reactive VH4-34 family; and b) flow cytometry using the monoclonal antibody, 9G4, specific to VH4-34, revealing preferential expansion of this inherently, autoreactive specificity within the naïve compartment. Together, these data provide a definitive demonstration of a human genetic disorder that specifically alters peripheral B cell tolerance at the transitional to naïve B cell stage.

Collectively, our dataset is most consistent with a model wherein WASp-deficiency promotes antigen-mediated positive selection of transitional B cells expressing low-affinity autoreactive BCRs into the naïve compartment. While we cannot eliminate the possibility that WASp-deficiency may subtly alter, or perhaps enhance, negative selection, we extensively tested the impact of WASp on negative selection using Tg models as well as analysis of receptor editing in a polyclonal setting. We observed no differences from WT controls indicating that WASp-deficiency does not impact either central or peripheral negative selection as assessed using these specific Tg models or within the sensitivity of the editing assay. Importantly, WASp-deficiency promotes rather than limits BCR signaling activity and, thus, altered negative selection is unlikely to explain the enrichment for specific self-reactive VH families at the transitional to naïve B cell stage in both mice and humans. In addition to key survival programs mediated via BAFF-R, tonic BCR, and CD40 signaling (17, 26, 41, 96) recent work suggests that antigen-driven, BCR signaling is a signature feature of B cells entering the late transitional (T2) compartment (35). Consistent with this idea, we previously identified a sub-population of T2 B cells in normal mice that undergo homeostatic expansion in response to antigen-mediated BCR signaling (36). Together, our new findings in *Was*$^{-/-}$ mice showing: i) heightened frequency of

transitional B cells undergoing homeostatic proliferation; and ii) an increased proportion of GFP^{hi} T2 B cells in Nur77-GFP-Tg reporter mice- suggest that antigen-mediated selection is enhanced in WASp- deficient T2 B cells. Further, the idea that WASp-deficiency preferentially promotes selection of B cells expressing lower-affinity autoreactive BCRs is supported by our single cell cloning analysis, as well as by data derived using the self-reactive, anti-PC heavy chain Tg model. In these latter studies, WASp-deficient T2 M167 Id^+ (high-affinity) B cells exhibited reduced proliferation compared to control cells; while, conversely, proliferation of M167 Id^- (lower affinity) *Was*$^{-/-}$ B cells is increased, consistent with a broadening of competitive specificities utilizing the PC-reactive heavy chain. Finally, our analysis in WAS subjects correlates directly with our murine data as demonstrated by the selective expansion of B cells with self-reactive VH genes precisely at the transitional B to naïve transition. Lastly, while we observed significant reduction in 9G4 expression at this transition in HC subjects, the change in expression was much less pronounced in WAS subjects consistent with the idea that self-antigen engagement of VH4-34-specific transitional B cells leads to enhanced survival in the absence of WASp.

Importantly, this study also demonstrates a novel role for TLR signaling in orchestrating peripheral tolerance and implicates dual TLR/BCR signaling in promoting selection of autoreactive BCRs into the naïve compartment. Previous work has linked TLR/Myd88 signaling to negative selection of immature B cells (74) and has extensively characterized dual TLR/BCR signals in activation of mature B cells and in triggering autoreactive GC responses (55, 66, 76, 77). The impact of TLR or dual TLR/BCR signals in shaping the naïve BCR repertoire, however, has not been investigated. Here, we show that Myd88 deficiency markedly reduced the frequency of cycling T2 B cells in *Was*$^{-/-}$ mice implying that Myd88 signals help to promote T2 B cell activation. Additionally, Myd88 deficiency abrogated the selection of VH10 expressing BCRs into the MZ; directly implicating Myd88 signals in modulating the naïve repertoire. Finally, while we cannot formally link our observations to dual BCR/TLR signaling, this model is supported by both: i) the previously described nucleic acid reactivity of the selecting BCRs in both mouse and humans; and ii) the finding that ablation of Myd88 eliminated VH10 enrichment. Surprisingly, investigation of individual TLRs (TLR 4, 7 and 9) revealed partial effects but suggest that no dominant TLR is responsible for the T2 proliferative response (data

not shown)- implying that a complex interplay of antigen specificity and TLR co-engagement likely orchestrates this dynamic process.

Previous studies have partially characterized the B cell repertoire in WAS subjects including cloning and sequencing of limited numbers of sequences generated using VH3 and VH4 specific heavy chain primers from sorted B cell subsets (56, 80) and next generation sequencing of total CD19+ peripheral blood B cells (57). Collectively, these studies have revealed that WAS subjects exhibit altered VH gene family usage (specifically VH3 and VH4 expressing BCRs), skewed CDR3 lengths, a reduction in SHM in class-switched antibodies, and a potential enrichment for memory cells expressing self-reactive clones including VH4-34 and VH3-30 (associated with anti-platelet specificities) (56, 57, 80). Although these studies reveal an altered memory B cell compartment likely impacted by the T cell functional deficits in WAS, we tested whether WASp-deficiency might directly impact the transitional and naïve B cell repertoires. Based on single cell studies of naïve mature BCR specificity that suggested enhanced self-reactivity, we used deep-sequencing of genomic DNA from 3 pediatric and 4 adult WAS subjects (and age-matched HCs) to obtain a quantitative assessment of the naïve repertoire. While we observed alterations in VH family gene usage within in the mature, naïve repertoire in WAS subjects, there were little or no differences in the transitional compartment. The most dramatic change observed was enrichment in VH4-34 usage, a VH family with germline, intrinsic self-reactivity, that is independent of both somatic mutation or associated light chains, and which exhibits affinity towards multiple self-antigens including RBC carbohydrate antigens associated with cold-agglutinins, dsDNA, and CD45/B220 isoforms expressed on immature B cells (93, 94, 97). This finding is clinically relevant as autoimmune RBC cytopenias, B cell lymphopenia and increased circulating dsDNA autoantibodies are each frequent features in WAS (53). Importantly, the enrichment in VH4-34 usage paralleled our findings showing VH10 and VH14 enrichment in the murine model, where selection is directly impacted by self-antigen and TLR-dependent signals. Finally, we utilized a well-characterized mAb, 9G4, to directly track selection of B cells expressing these autoreactive specificities (93). Our data clearly demonstrate preferential expansion of $9G4^{+}$ cells in the naïve repertoire of WAS but not HC subjects. Notably, our human repertoire findings are not likely related to disease associated inflammatory events as identical results were obtained in XLT subjects with no disease manifestations beyond thrombocytopenia. Interestingly, HC mature, naïve B cells exhibited decreased surface 9G4

staining, suggesting that BCR down-regulation is a mechanism whereby VH4-34 expressing cells might be tolerized during normal development. In contrast, WASp-deficient B cells exhibited higher 9G4 MFI, consistent with expansion of this population presumably via enhanced BCR and/or TLR signals. Finally, consistent with previous observations (56, 80), we observed additional enrichment for VH4-34 expression specifically within the IgM memory compartment in WAS subjects as well as significantly elevated serum 9G4+ IgG titers. Consistent with this finding, and similar to patients with systemic lupus erythematosus (SLE), a previous study has identified elevated levels of VH4-34 (previously referred to as VH4-21) IgM autoantibodies in WAS subjects (98).

Together, these multiple lines of evidence support a scenario in which enhanced VH4-34 positive selection in WAS leads to enrichment of self-reactive B cells within the naïve B cell compartment; and subsequently, as supported by our earlier work in murine models (54, 55), enhanced dual BCR/TLR signals orchestrate spontaneous activation of these self-reactive clones leading to GC formation and production of VH4-34 IgG autoantibodies (Figure II.9). This model is likely broadly relevant to other human autoimmune disorders including SLE, where waves of VH4-34-autoantibody-expressing antibody secreting cells are generated from activated 9G4+ naïve B cells accounting for a major proportion of disease relevant autoantibodies produced during lupus flares (99). Additionally, enhanced BCR/TLR signals may allow for autoreactive B cells to better compete into the naïve repertoire in other immunodeficiency disorders and conditions associated with significant B cell lymphopenia, leading to preferential selection for self-reactive specificities including receptors utilizing VH4-34 heavy chains.

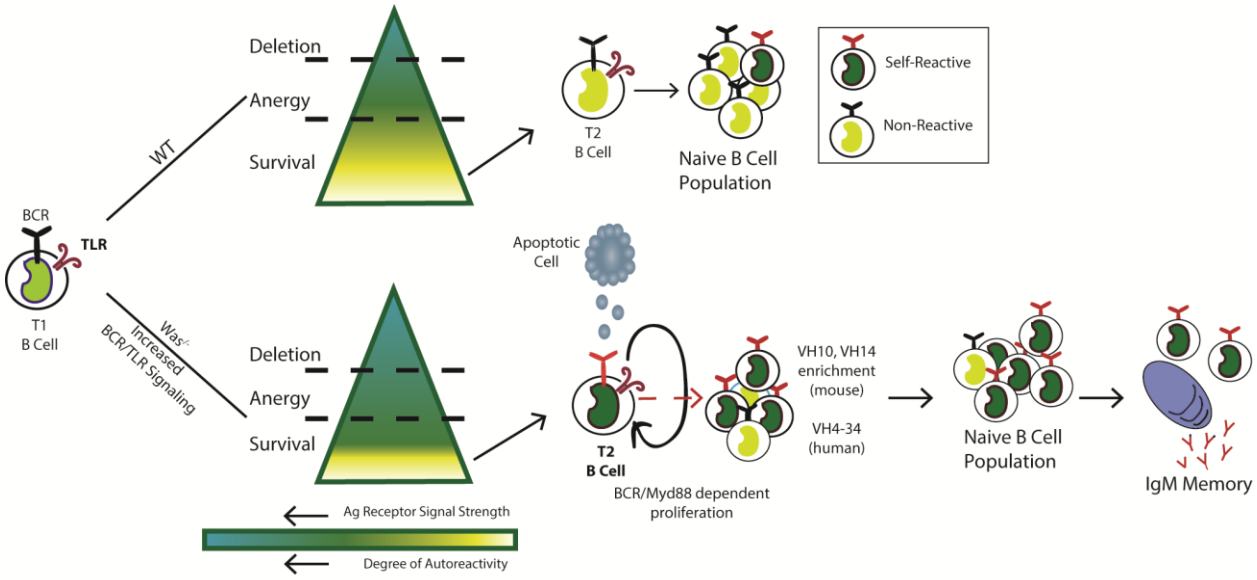

Figure II.9. Positive Selection of Autoreactive B Cells in Wiskott-Aldrich Syndrome. Schematic model illustrating the impact of WASp-deficiency on B cell selection in the context of cell-intrinsic increases BCR and TLR signaling. Moderately autoreactive T1/T2 B cells are able to compete and persist in the spleen via self-antigen triggered, dual BCR and MyD88 signaling. Upon engagement with self-antigens, including apoptotic debris, *Was*$^{-/-}$ B cells with distinct specificities are activated and positively selected via clonal expansion into both the MZ and FM compartments. This process results in an enrichment of self-reactive specificities in the naïve mature repertoire in both mice and humans setting (with specific enrichment for BCRs using VH10/14 and VH4-34 HCs, respectively). Self-reactive naïve B cells likely contribute to the production of natural poly- or self-reactive IgM; but these cells can also enter the GC and promote production of pathogenic class-switched autoantibodies.

In summary, this work provides new evidence supporting a model wherein modest alterations in BCR or TLR signal strength can alter the mature BCR repertoire and increases the risk for subsequent B cell mediated autoimmunity. Based on these studies, we anticipate that 9G4 mAb analysis may provide a biomarker for tracking B cell tolerance in subjects with WAS following transplantation or gene therapy, particularly in individuals with mixed chimerism where the risk for autoimmunity is increased. Finally, while WAS represents a rare genetic condition, there is mounting evidence that other human autoimmune risk alleles identified, for example, by genome wide association studies may impact these key signaling cascades, implying relevance to many more common immune disorders (75, 100).

II.4 Methods

Mice

C57BL/6, *Was*$^{-/-}$, Was$^{-/-}$ *Myd88*$^{-/-}$, *Was*$^{fl/fl}$ *Mb-1*^{cre}, *Was*$^{-/-}$MD4 Tg, MD4 Tg, M167 Tg, *Was*$^{-/-}$ M167 Tg, Nur77-GFP Tg, *Was*$^{-/-}$ Nur77-GFP Tg, sHEL, mHEL mice were bred and maintained in the specific pathogen–free animal facility of Seattle Children's Research Institute (Seattle, WA) and handled according to Institutional Animal Care and Use Committee approved protocols. M167 H chain mice (M167H Tg mice, line U243-4) were provided by J. Kenny and A. Lustig (National Institute on Aging, Bethesday, MD) and established as a M167H Tg/Tg homozygous breeding colony in the animal facility of the Albert Einstein College of Medicine (New York, NY) by S. Porcelli. *Was*$^{fl/fl}$ *Mb-1*^{cre} were provided by Luigi A. Notarangelo and Adrian Thrasher (Children's Hospital Boston). *Mb-1*^{cre} mice were provided by Michael Reth (Max Plank Institute of Immunobiology).

Human WAS and HC Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were obtained following obtaining informed consent using a Seattle Children's Research Institute Internal Review Board approved research protocol. PBMC were isolated from pediatric and adult WAS subjects or from healthy control subjects using Ficoll-paque density centrifugation (followed by red blood cell lysis in 15 mM NH_4Cl, 1mM $KHCO_3$, 10 μM EDTA). For sequencing and flow cytometry assays, single cell PBMC suspensions were enriched for B cells using $CD19^+$ B Cell Isolation II negative selection kit (Miltenyi).

Reagents and Antibodies

Anti-murine antibodies used in this study include AA4.1, CD24 (M1/69), CD21 (7G6), B220 (RA3-6B2), IgD (11-26C.2A) from BD Biosciences; and BP1 (FG35.4), CD23 (B3B4) from Caltag; IgM (1B4B1), Kappa (187.1), Lambda (JC5-1), and SA-HRP conjugated from Southern Biotechnology; CD19 (ID3) and IgM^a (DS-1) from BioLegend; and Cy5 anti-rabbit polyclonal IgG from Jackson ImmunoResearch, AlexaFluor647 anti-M167 (28-6-20) Rat IgG2a generously provided by S.Porcelli. Anti-human antibodies used in this study include CD19 (HIB19) and IgM (MHM-88) from Biolegend; CD10 ((HI10a), CD24 (ML5) and IgD (IAG-2) from BD

Pharmingen; CD27 (323) from eBioscience; and CD38 (HIT2) from BD Bioscience. Anti-human rat monoclonal, FITC-conjugated 9G4 antibody was generously provided by Dr. Ignacio Sanz (Emory University).

Flow cytometry and cell sorting

As previously described (17, 101), single-cell suspensions from BM, peripheral blood, and spleen were incubated with fluorescently labeled Abs and data were collected on a LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star). Cell sorting was done using an Aria II; sort purities were >90% in all studies. Murine naïve B cell subsets were gated as in (17) using B220, CD21, CD23, CD24, and CD1d surface markers. Human B cell subsets were gated as shown in Figure 6B.

BM Transplantations

For Hel-Ig studies, Hel-Ig Tg mice were crossed to *Was*$^{-/-}$ mice to generate HEL-Ig-*Was*$^{-/-}$ mice. BM was harvested from HEL-Ig *Was*$^{-/-}$ or HEL-Ig WT mice and 5 x 10^6 cells in PBS were injected i.v. into lethally (1050 cGy) irradiated mHEL or sHEL recipient mice.

V□-RS Quantitative PCR

Genomic DNA was isolated from sorted B cells using the Gentra PureGene Kit (Qiagen). Quantitative PCR was performed as described (Panigrahi et al. 2008). The amount of V□-RS product in each sample was normalized to the □-actin product and compared to the normalized target value in WT FM B220$^+$ CT [(DDCT) method].

ELISA and HEp-2 IFA Tests

Antibodies were tested for reactivity to MDA-LDL (Academy Bio-Medical; 20P-MD L-105), PC-4, PC-14, dsDNA (Sigma-Aldrich) and sm-RNP (Arotech Diagnostics Limited; ATRO1-10) at 100 ug/ml by ELISA with cloned monoclonal IgG antibodies at 10 ug/ml and serially diluted 1:5, 1:25, and 1:125. For ELISAs, 96 well Immuno plates (Nunc) were pre-coated (100 ug/ml) overnight at 4ºC with dsDNA, PC-4, PC-14, sm-RNP or MDA-LDL. After blocking with 0.5% BSA/PBS, recombinant antibodies were added, and plates were incubated with mouse anti-human IgG-HRP (Southern Biotechnology Associates) (1:2,000 dilution). BD IFA slides were

stained following manufacturer's instructions and were imaged following the same protocol described in (17). Serum BAFF levels were measured using BAFF/BlySS Quantikine ELISA kit (R&D Systems). Serum 9G4+ IgG levels were measured by coating ELISA plates with purified 9G4 IgG antibody (10 μg/ml) generously provided by Ignacio Sanz (Emory University). 9G4 IgG titers were detected using an HRP-anti-human IgG (JDC-10) secondary antibody.

High Throughput BCR Sequencing

Total RNA was obtained from purified murine B cells and cDNA was synthesized using a 5'-RACE kit (Ambion) and BCR heavy chain genes were amplified using a triple-nested primer strategy (as previously described in (17)). Total RNA was obtained from purified B cells (0.5-10 x 10^6 cells) using RNeasy reagents (Qiagen). Heavy chain cDNAs from each sample were synthesized using a 5′-RACE kit (Ambion), according to the manufacturer's protocol. For reverse transcriptase and PCR, Transcriptor High Fidelity (Roche) and Phusion Hot Start (New England Biolabs) was used, respectively. A triple nested primer strategy was used to amplify heavy chain IgM and IgD genes. An outer IgM and IgD constant region-specific primer was used for the RT reaction, a middle constant region IgM and IgD primer was used for the first round of PCR, and an innermost constant-region IgM and IgD primer (adjacent to the J segment of the variable region) was used for the second PCR round. 454 adapters were included in the primers during second round PCR, and a bar-code strategy was used to run multiple samples simultaneously. Amplicon libraries were used for high-throughput pyrosequencing on a GS Junior (Mycroarray). Barcoded sequence data was separated using Geneious software, and IMGT/HighVQuest was used for alignment to germline IgH VDJ regions. IgAT software was used to generate descriptive statistics and calculation of CDR3 characteristics.

For high throughput sequencing of human WAS and healthy control subjects, genomic DNA was extracted from sorted transitional, naive, and both IgM and switched memory B cell populations. Immunoglobulin heavy chain sequences were independently amplified using multiplex PCR with optimized primer sets. Following high-throughput sequencing using Adaptive Biotechnologies' ImmunoSEQ Illumina-based sequencing platform, sequences are then clustered into distinct clonotypes to determine overall frequencies. Clonotypes are identified based on their CDR3 (complementarity determining region 3) sequences. Clones at >5% of the total frequency within

each sample were identified as index trackable sequences. V, (D,) and J genes were identified for each clonotype.

Single-Cell BCR Cloning

Single murine MZ B cells ($B220^{hi}CD24^{hi}CD23^{lo}CD1d^{hi}$) were FACS sorted from 5-6, 10-12 week old WT and *Was*$^{-/-}$ mice into 96 well plates. BCRs were cloned from the cDNA of single cells and used to generate monoclonal antibodies using methods previously described in (17, 102). Human naïve B cells were sorted from HC (1 adult and 2 pediatric subjects) and WAS subjects (1 pediatric subject) into 96 well plates. cDNA was extracted from single cells and used to generate monoclonal antibodies using methods previously described in (103).

Cell Cycle Analysis using *in vivo* BrdU labeling

For *in vivo* labeling of cycling cells, mice were given water with 1 mg of BrdU and 20 g of sucrose for 24 hours. Both spleens and BM were collected and cells were surface stained, fixed and treated with DNase for 1 hours at 37° C and then stained for intracellular BrdU and Ki67 immediately before FACS analysis (as previously described in (17)).

Statistical analysis

Unpaired, 2-tailed, Student *t* tests were applied to determine the statistical significance of the differences between groups. The *p* values were considered significant when $p<0.05$ (*) and $p<0.01$(**) and $p<0.001$(***).

Chapter III. **Role of TLR Signaling in Marginal Zone B Cell Homeostasis**

III.1 **Introduction**

Marginal zone (MZ) B cells are a specialized B cell subset with a pivotal role in mounting antibody responses to blood-borne pathogens. MZ B cells reside within the splenic MZ where they are exposed to antigens within the peripheral circulation. Compared with follicular mature (FM) B cells, MZ B cells are rapidly activated by toll-like receptor (TLR) ligands resulting in robust T independent (T-I) antibody responses (104). MZ B cells are retained within the MZ by surface integrin and sphingosine 1-phosphate receptor 1 (S1PR1) signals (61, 62), and express a relatively restricted B cell receptor (BCR) repertoire targeting both conserved bacterial epitopes and self-antigens (105).Traditionally, MZ B cells are defined as MZ resident, non-recirculating and self-renewing (104). However, recent data demonstrates that MZ constantly shuttle between the splenic MZ and follicle (60). The signals promoting MZ B cell migration have not been completely identified, however *in vivo* LPS treatment promotes rapid relocation of MZ B cells to the follicle (106).

Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency disease characterized by eczema, recurrent infections, thrombocytopenia, and a high prevalence of humoral autoimmunity. The disease is caused by mutations in Wiskott-Aldrich syndrome protein (WASp), a cytoplasmic protein expressed in hematopoietic cells involved in signal transduction from the cell surface to the actin cytoskeleton (52). Both WAS patients and *Was*-deficient (*Was*$^{-/-}$) mice (69, 107) have a marked reduction in MZ B cells, with resulting decreased responses to T-I polysaccharide antigens and increased invasive *Streptococcus pneumonia* infections (69). While murine data has demonstrated cell intrinsic defects in integrin signaling and in CXCL13- or S1P-induced migration in WASp deficient B cells (69, 79, 107), how WASp deficiency directly impacts MZ B cells has not been determined. Our group and others have previously demonstrated that generation of MZ precursors (MZp; defined as CD1d^{lo}CD23^{hi}), the immediate developmental precursor of MZ B cells, is intact in WAS (79, 107), implying that dysregulated MZ B cell localization/retention, rather than MZ development, explains the MZ defect in WAS. We showed that *Was*$^{-/-}$ B cells exhibit defective integrin clustering following B cell receptor

(BCR) stimulation, and hypothesized that these integrin defects explained the WAS MZ defect (107).

In the current study, we made the surprising observation that integrin defects alone are not sufficient to explain the MZ defects in WAS. Rather, spontaneous activation of *Was*$^{-/-}$ MZ B cells via BCR and TLR7 signals promotes depletion of the marginal zone sinus. This observation suggests that enhanced BCR and TLR signaling contributes to MZ defects in WAS and provides new insight regarding how dysregulated B cell signals impact B cell homeostasis.

III.2 **Results and Discussion**

III.2.1 *WASp-deficiency alters MZ B cell localization and retention within the MZ sinus.*

Our previous findings, showing that development of *Was*$^{-/-}$ MZ B cells is intact, implied that altered B cell localization or retention likely contributes to the WAS MZ defect. After capturing circulating antigens in the MZ sinus, MZ B cells shuttle between the MZ and follicle, with ~50% MZ B cells localizing in the MZ sinus at any time (60, 108). To quantify whether MZ B cell localization was altered in WAS, we performed *in vivo* MZ B cell labeling using intravenous delivery of a PE-labeled anti-CD19 antibody 5 minutes prior to animal sacrifice. As previously demonstrated, ~50% of wild-type (WT) MZ B cells were PE labeled (and hence localized within the MZ sinus, not follicle) (108). In contrast, a smaller proportion of *Was*$^{-/-}$ MZ B cells were labeled (Fig. III.1A,C). We confirmed that this altered MZ localization was B cell-intrinsic, by performing *in vivo* labeling of *Was*$^{fl/fl}$ x *Mb-1*^{cre} mice, where *Was* deletion is limited to the B cell compartment (79) (Fig. III.1B,C). Failure to retain MZ B cells within the MZ sinus can result in MZ B cell release into the blood (62). Despite markedly fewer total splenic MZ B cells in WAS, there was a trend towards increased *Was*$^{-/-}$ MZ B cells within the peripheral circulation and a significant increase in the ratio of blood vs. splenic MZ B cells (Fig. III.1D) (108). Together, these data demonstrate that the MZ defect in WAS occurs because of a failure to retain appropriate positioning of *Was*$^{-/-}$ MZ B cells within the MZ sinus.

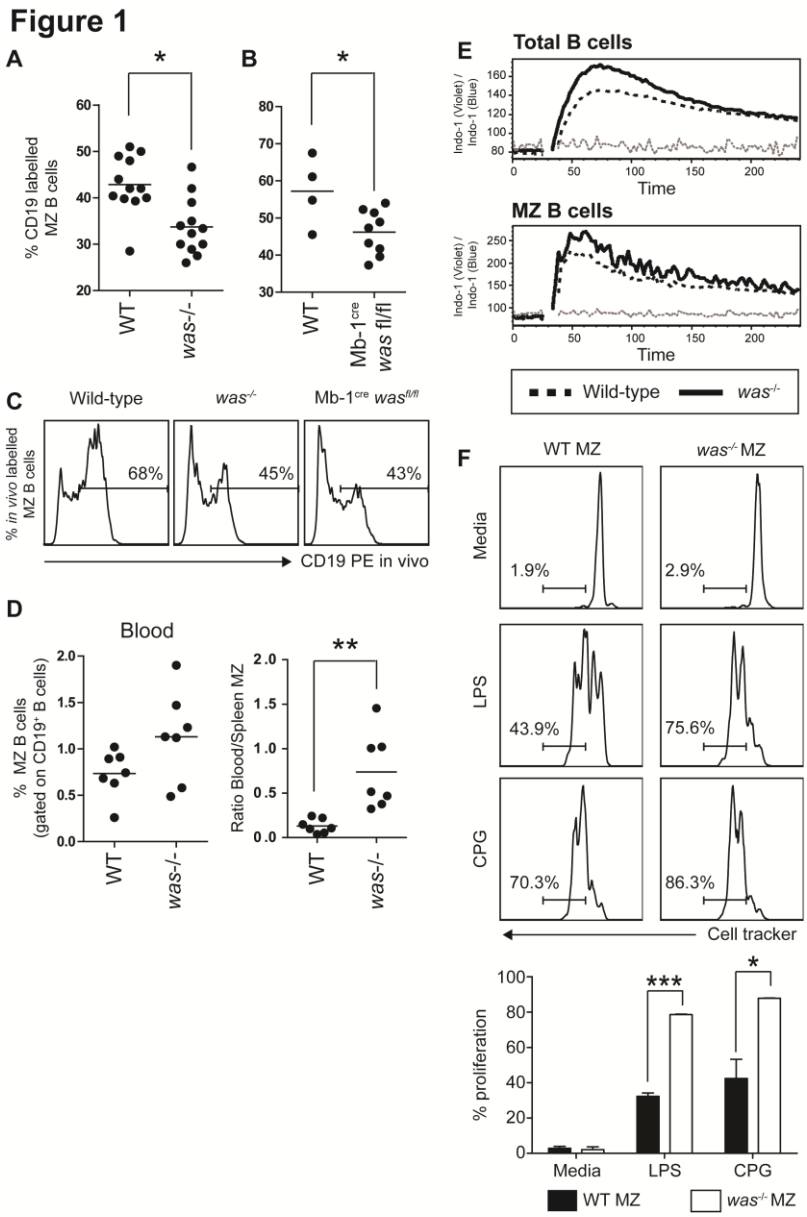

Figure III.1. **Abnormal retention of *Was*$^{-/-}$ B cells in MZ**.
(A, B) CD19-labeled MZ B cells (as % of MZ B cells) in WT vs. *Was*$^{-/-}$ (A) (n=12 per genotype), and *Was*$^{+/+}$ x Mb-1^{cre} vs. *Was*$^{fl/fl}$ x Mb-1^{cre} mice (B) (n=4,9). (C) Representative histograms of MZ CD19 PE-labeling. (D) Peripheral blood MZ B cell percentage (left), and ratio of blood MZ vs. splenic MZ B cells (MZ B cells as a % of B220^{+} B cells) (right) in WT and *Was*$^{-/-}$ mice. (n=7 per genotype) (A, B, D) Data representative of ≥2 experiments. (E) Ca^{2+} flux in total and MZ B cells from WT vs. *Was*$^{-/-}$ mice stimulated with 10μg/mL anti-IgM. (F) Proliferation of sorted WT vs. *Was*$^{-/-}$ MZ B cells 48 hours after stimulation with 5μg/mL LPS and 1μg/mL CpG. (E, F). Data representative of ≥3 experiments. Bar graphs show mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001, by unpaired Student's *t*-test.

III.2.2 *B-cell intrinsic MyD88 signals activate and promote egress of Was$^{-/-}$ MZ B cells.*

Integrin signals promote positioning and retention of MZ B cells within the MZ sinus (62). Previously, we observed altered integrin function in *Was*$^{-/-}$ B cells prompting our hypothesis that integrin defects explain the MZ defect in WAS (106). However, in addition to integrin defects, *Was*$^{-/-}$ FM B cells are modestly hyper-responsive to BCR and TLR stimuli *in vitro* (54). To evaluate whether this altered signaling in FM B cells is also evident in MZ B cells, we sorted MZ B cells from WT and *Was*$^{-/-}$ mice. As predicted, relative to WT MZ B cells, *Was*$^{-/-}$ MZ B cells exhibited enhanced calcium flux following BCR cross-linking (Fig. 1E). Further, *Was*$^{-/-}$ MZ B cells underwent accelerated proliferation following LPS and CpG stimulation (Fig. 1F).

Multiple TLR agonists (including TLR1/2, TLR3, TLR4 and TLR7) promote emigration of MZ B cells from the MZ (106). For this reason, we tested whether hyper-responsive TLR signals, in addition to integrin dysfunction, promote the spontaneous egress of *Was*$^{-/-}$ MZ B cells from the MZ. To test the B cell-intrinsic impact of TLR signaling on *Was*$^{-/-}$ MZ B cell positioning, we generated mixed bone marrow (BM) chimeras where B cells either lacked WASp alone or were deficient in both WASp and the TLR signaling adaptor, MyD88. Surprisingly, B cell MyD88 deletion markedly increased the percentage of *Was*$^{-/-}$ MZ B cells in BM chimeras (Fig. III.2A,B). Further, splenic immunohistochemistry confirmed that *Was*$^{-/-}$*.Myd88*$^{-/-}$ MZ B cells were appropriately positioned within CD1d^{hi} IgM^{hi} IgD^{lo} ring surrounding the B220^{+} B cell follicle (Fig. III.2C,D). Although increased relative to *Was*$^{-/-}$ chimeras, the percentage of *Was*$^{-/-}$*.Myd88*$^{-/-}$ MZ B cells did not reach WT levels implying potential additional contributions of defective BCR and/or chemokine-mediated integrin signaling in WAS MZ homeostasis. However, B cell-intrinsic MyD88 deletion prominently increased MZ B cell numbers and restores the localization of *Was*$^{-/-}$ MZ cells, implying that spontaneous MyD88-dependent activation and egress of *Was*$^{-/-}$ MZ cells largely explains the MZ defect in WAS.

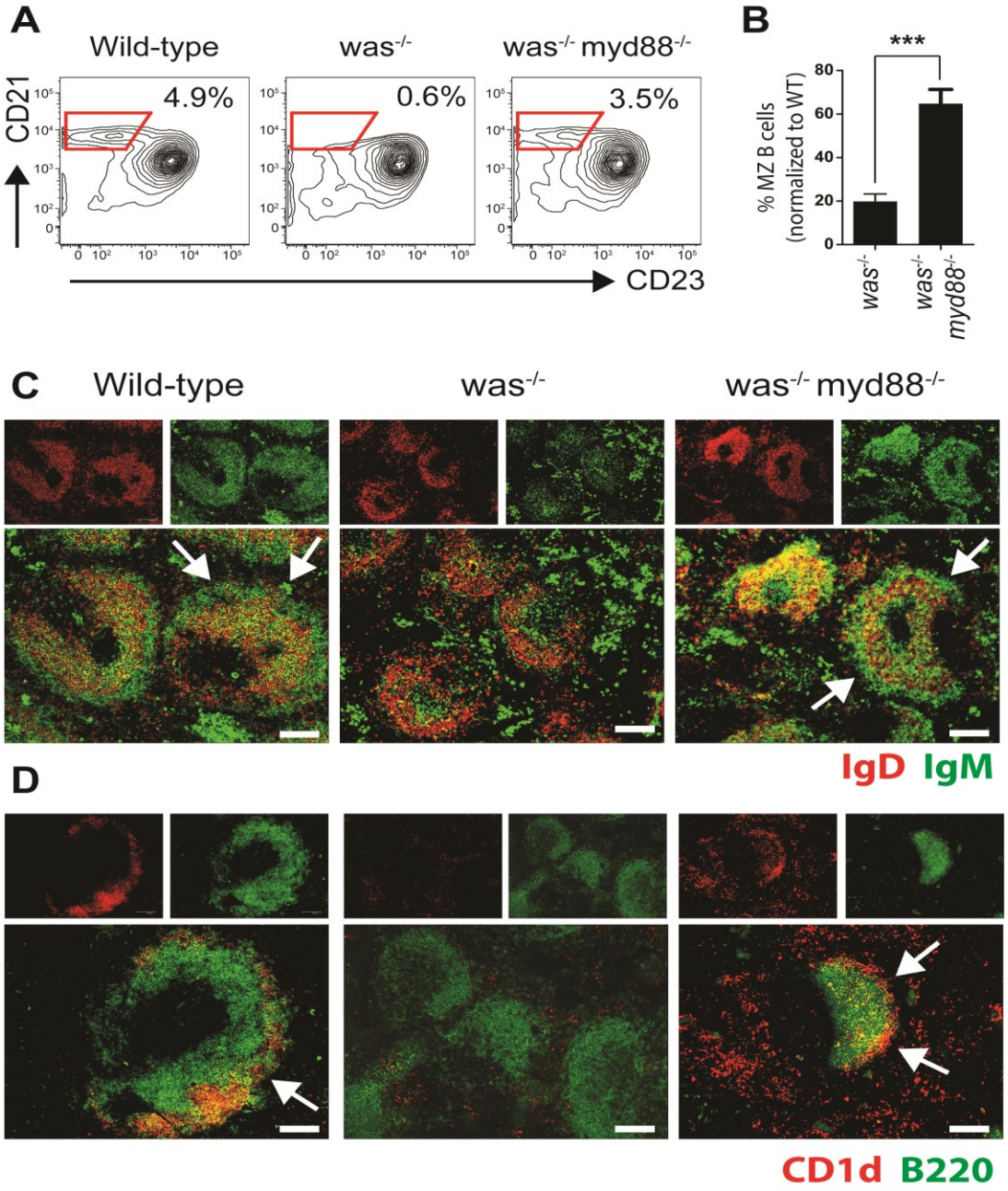

Figure III.2. B-cell intrinsic MyD88 deletion restores MZ in WAS.
(A) Representative flow plots (gated on $B220^{+}CD19^{+}$ B cells) showing restoration of $CD21^{hi}$ $CD23^{lo}$ MZ B cells in *Was*$^{-/-}$.*Myd88*$^{-/-}$ B cell chimeras. Numbers indicate percentage within MZ B cell gate. (B) Percentage of MZ B cells (normalized to WT chimera controls) in *Was*$^{-/-}$ and *Was*$^{-/-}$.*Myd88*$^{-/-}$ B cell chimeras. Bar graph shows mean ± SEM. ***P<0.001, by unpaired Student's t-test. (A,B) Data representative of 6 WT (n=17), 6 *Was*$^{-/-}$ (n=26) and 3 *Was*$^{-/-}$.*Myd88*$^{-/-}$ (n=14) independent chimeras. (C,D) Immunofluorescence staining showing restoration of $IgM^{hi}IgD^{lo}$ (C) and $CD1d^{+}B220^{+}$ cells (D) within the MZ in *Was*$^{-/-}$.*Myd88*$^{-/-}$ B cell chimeras. Arrows indicate MZ surrounding the B cell follicle. Bars 100μM. Data representative of 2 *Was*$^{-/-}$.*Myd88*$^{-/-}$ chimeras.

III.2.3 *TLR7 is the dominant MyD88-dependent receptor promoting Was$^{-/-}$ MZ B cell activation.*

We next evaluated which B-intrinsic, MyD88-dependent signals promoted the spontaneous activation and egress of *Was*$^{-/-}$ MZ cells from the MZ. As MyD88 co-ordinates a wide array of B cell-intrinsic signals, we focused our analysis on a subset of candidate receptors. First, we evaluated the role of B cell TLR4 signals, since LPS promotes rapid MZ B cell activation and exit from the MZ sinus (106) and bacterial products derived from intestinal microbiota are known to enter the peripheral circulation (109). Second, given the importance of the splenic MZ in clearance of circulating apoptotic material, we evaluated the impact of the autoimmunity-associated, endosomal TLRs, TLR7 and TLR9(29). Finally, we evaluated the impact of the deletion of the BAFF-family receptor transmembrane activator and CAML interactor (TACI), which also signals via MyD88 (110).

To test the B cell-intrinsic impact of these MyD88-dependent receptors on MZ biology, we generated mixed BM chimeras in which B cells were either WT, *Was*-deficient or *Was*$^{-/-}$*.Tlr4*$^{-/-}$, *Was*$^{-/-}$*.Tlr7*$^{-/-}$, *Was*$^{-/-}$*.Tlr9*$^{-/-}$, or *Was*$^{-/-}$*.Taci*$^{-/-}$ double-deficient. B cell-intrinsic deletion of TLR4 had no impact on MZ B cell numbers, suggesting that altered responses to bacterial-derived LPS does not impact WAS MZ architecture. In addition, although both MZ B cells and B cell TACI signals promote T-independent antibody responses(104, 111), lack of TACI expression did not impact *Was*$^{-/-}$ MZ B cell numbers. Strikingly, restoration of MZ B cell percentage and splenic architecture was dependent on B cell-intrinsic TLR7 signaling. In contrast, deletion of B cell TLR9 exerted no impact on MZ size in *Was*$^{-/-}$ B cell chimeras (Fig. III.3A,B). Importantly, TLR7 deletion exerted no additional impacts on altered splenic B cell development in WAS (data not shown), providing further support to our interpretation that changes in MZ B cell homeostasis, rather than MZ development, primarily explain the restoration of the MZ in *Was*$^{-/-}$*.Tlr7*$^{-/-}$ chimeras.

The endosomal receptors TLR7 and TLR9 promote anti-nuclear autoantibody formation in systemic lupus erythematosus (SLE), with TLR7 required for antibodies targeting RNA and RNA-associated proteins, and TLR9 promoting antibodies to dsDNA (29). Our combined finding support a model wherein nuclear self-antigens, likely derived from circulating apoptotic material, promote spontaneous MyD88-dependent egress of *Was*$^{-/-}$ B cell from the MZ. It was

initially surprising that B cell TLR7 signals promote MZ B cell egress in WAS, with no contribution from TLR9. However, similar opposing effects of TLR7 and TLR9 are observed in autoimmunity with TLR7 promoting, and TLR9 constraining, systemic inflammation (29). Directly consistent with this theme, we recently demonstrated that the impacts of TLR7 vs. TLR9 in spontaneous autoimmunity in WAS depend on B cell, and not myeloid, intrinsic signals (55). Interestingly, a similar role for MZ B cell TLR7 signals in regulating MZ homeostasis is demonstrated in TLR7 over-expressing transgenic (TLR7-Tg) mice, where MZ depletion is eliminated with normalization of TLR7 expression within B cells (112). In summary, these finding strongly suggest that, after recognition of RNA-containing self-antigens, B cell-intrinsic TLR7 signals are sufficient to promote spontaneous activation and exit of MZ B cells from the marginal sinus.

III.2.4 *Antigen-dependent BCR signals promote MZ B cell egress.*

The observation that B cell TLR7 signals promote both systemic autoimmunity and MZ defects in WAS prompted us to evaluate whether additional B cell-intrinsic signals modulate WAS MZ homeostasis. Following B cell antigen recognition and cross-linking of surface BCR, antigens are trafficked to endosomal compartments where they can activate intracellular TLRs. In this manner, B cells exhibit a unique propensity for activation via integration of dual BCR and TLR signals (29). For this reason, we hypothesized that the spontaneous activation and egress of *Was*$^{-/-}$ MZ B cells requires antigen-dependent BCR ligation. To test this hypothesis, we crossed *Was*$^{-/-}$ mice with MD4 transgenic mice expressing a BCR specific for the foreign antigen, Hen-Egg Lysozyme (HEL) (9). Mixed BM chimeras were generated in which B cells were either WT, MD4 transgenic, *Was*$^{-/-}$ or MD4.*Was*$^{-/-}$ transgenic. After splenic reconstitution, we analyzed splenic B cell subsets by flow cytometry. BCR signals also impact MZ B cell development and the percentage of MZ B cells is increased in MD4 transgenic mice (104). For this reason, we separately compared the relative percentages of MZ B cells in *Was*$^{-/-}$ vs. MD4.*Was*$^{-/-}$ B cell chimeras and in WT vs. MD4 transgenic chimeric controls. Surprisingly, there was a significant increase in MZ B cell numbers in MD4.*Was*$^{-/-}$ B cell chimeras relative to *Was*$^{-/-}$ chimeras (Fig. III.3C,D) suggesting that BCR dependent signals promote MZ B cell egress.

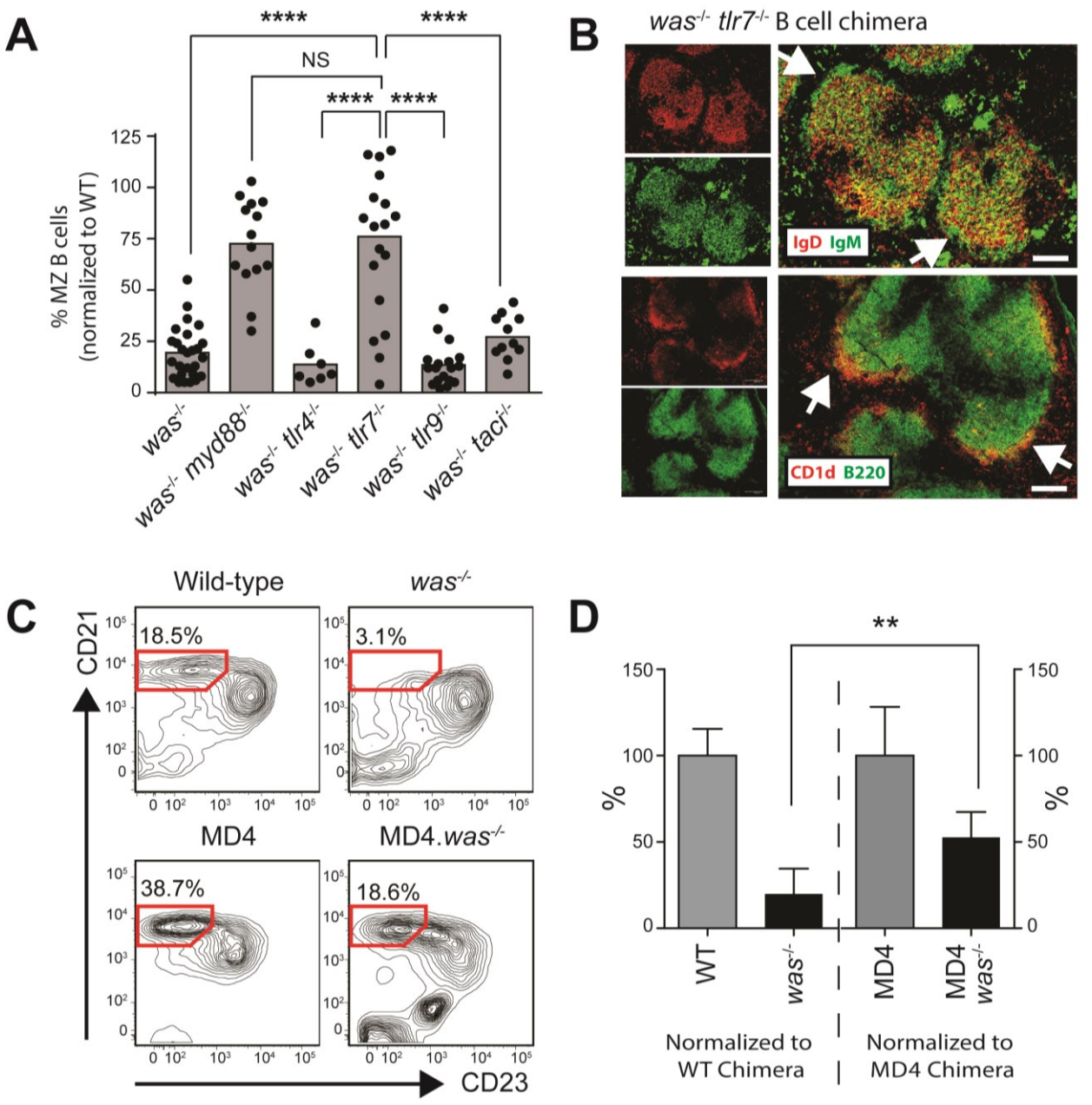

Figure III.3. Dual BCR/TLR7 signals promote WAS MZ depletion.
(A) Percentage MZ B cells (normalized to WT chimera controls) in indicated B cell-intrinsic chimeras. (B) Immunofluorescence staining showing restoration of $IgM^{hi}IgD^{lo}$ (upper panel) and $CD1d^{+}B220^{+}$ cells (lower panel) within the MZ in *was*$^{-/-}$.*tlr7*$^{-/-}$ B cell chimeras. Arrows indicate MZ surrounding the B cell follicle. Bars 100µM. (C) Representative flow plots (gated on $B220^{+}CD19^{+}$ B cells) showing increase in $CD21^{hi}$ $CD23^{lo}$ MZ B cells in MD4.*was*$^{-/-}$ B cell chimeras. (D) Percentage *was*$^{-/-}$ and MD4.*was*$^{-/-}$ MZ B cells (normalized to WT and MD4 chimera controls, respectively). Data representative of 6 WT (n=17), 6 *was*$^{-/-}$ (n=26), 3 *was*$^{-/-}$*.myd88*$^{-/-}$ (n=14), 4 *was*$^{-/-}$*.tlr7*$^{-/-}$ (n=19), 4 *was*$^{-/-}$*.tlr9*$^{-/-}$ (n=18), 2 *was*$^{-/-}$*.tlr4*$^{-/-}$ (n=7), 3 *was*$^{-/-}$*.taci*$^{-/-}$ (n=11), 2 MD4 (n=4) and 2 *was*$^{-/-}$.MD4 (n=3) independent chimeras. Bar graphs show mean ± SEM. $^{**}P<0.01$; $^{****}P<0.0001$; NS, not significant.

Importantly, emerging data demonstrate that dysregulated BCR signals are also sufficient to promote spontaneous, B cell-driven humoral autoimmunity (75). B cell-intrinsic deletion of *Was* (54, 112) or the Src family kinase *Lyn* (113), as well as B cell expression of *1858C→T* autoimmunity risk variant in protein tyrosine phosphatase nonreceptor 22 (*PTPN22*) (100), renders B cells hyper-responsive to BCR activation resulting in systemic autoimmunity. In contrast, decreasing BCR signaling in lupus-prone MRL.Mp$^{lpr/lpr}$ mice by crossing with the Bruton's tyrosine kinase (Btk) mutant Xid strain (114) or by treatment with specific Btk inhibitors (115) limits autoimmunity. In each of these murine models, B cell-intrinsic deletion of MyD88 (54, 116) or TLR7 (55) abrogates humoral autoimmunity.

Based on these observations, we propose that similar mechanisms may underlie humoral autoimmunity and MZ dysregulation in WAS. We hypothesize that antigen-specific BCR activation (likely via circulating apoptotic material or other self-antigens) integrates with B cell-intrinsic TLR7 signals to promote spontaneous $Was^{-/-}$ MZ B cell activation and exit from the MZ. In this manner, dual BCR/TLR activation may promote both depletion of the MZ and seeding of spontaneous autoimmune germinal centers by autoreactive MZ B cells.

In summary, we show that $Was^{-/-}$ B cells are able to appropriately localize within the marginal sinus when dysregulated TLR7 signals are abrogated. While previous work has implicated altered B cell integrin function in this process, these new findings indicate that TLR7 signals exert a dominant role in promoting MZ B cell egress; and these observations likely help to explain the defective T-I antibody responses and increased disseminated bacterial infections in WAS. More broadly, B cell-intrinsic signals may exert important impacts on the MZ in other settings. While the impact of BCR (and TLR) signals on FM vs. MZ fate decisions has been extensively studied, less attention has been paid to understanding how B cell signals promote MZ B cell activation and exit from the MZ. Since MZ B cells are enriched for autoreactive specificities and promote clearance of circulating apoptotic material, dysregulated MZ B cell TLR signals may impact both MZ homeostasis and autoimmune pathogenesis.

III.3 **Methods**

Animals: WT, *Was*$^{-/-}$, *Was* $^{-/-}$*.Myd88*$^{-/-}$, *Was*$^{-/-}$*.Tlr4*$^{-/-}$*, Was*$^{-/-}$*.Tlr7*$^{-/-}$, *Was*$^{-/-}$*.Tlr9*$^{-/-}$, *Was*$^{-/-}$*.Taci*$^{-/-}$, MD4 or *Was*$^{-/-}$.MD4 BM and B cell–deficient (μMT) BM (20:80 ratio, 5 x 10^6 total BM), was injected into lethally-irradiated (450cGy x 2 doses) μMT recipients. Chimeras were sacrificed at 12-24 weeks post BM transplantation. Mice were maintained in the specific pathogen-free (SPF) animal facility of Seattle Children's Research Institute (Seattle, WA). All animal and BM transplantation studies were conducted in accordance with Seattle Children's Research Institute IACUC approved protocols.

Flow cytometry and cell sorting: Single-cell splenocyte and blood suspensions were incubated with fluorescently-labeled antibodies prior to acquisition on LSR II (BD) and analysis using FlowJo software (Tree Star). B cells were sorted based on the cell surface markers $CD19^+$ $B220^+$ $CD21^{MID}$ $CD24^{MID}$ $CD23^{HI}$ $CD1d^-$ (FM) and $CD19^+$ $B220^+$ $CD21^{HI}$ $CD24^{HI}$ $CD23^{LO}$ $CD1d^+$ (MZ), using a FACSCalibur (BD) cell sorter with >90% sort purities in all studies. For *in vivo*-labeling of MZ B cells, 200 μl (5 μg) of PE-labeled CD19 antibody was injected i.v. 5 minutes prior to sacrifice, as described [12]. Anti-mouse antibodies used in this study include the following: CD24(M1/69) , CD21 (7G6), B220 (RA3-6B2), CD1d (1B1), CD19 (ID3), CD23 (B3B4), MOMA (MOMA-1), CD69 (H1.2F3), Cell Tracker Violet (CTV), CD3 (145.2C11), IgM, and IgD (11-26c.2a).

Immunofluorescence of spleen sections: Immunofluorescence staining of frozen splenic sections was performed, as described [13]. Images were acquired using a DM6000B microscope, DFL300 FX camera, and Application Suite Advanced Fluorescence software (Leica).

In vitro **cell stimulation:** Congenically marked, WT and *Was*$^{-/-}$ FACS-sorted MZ B cells were co-cultured together (~ 30,000-50,000 cells per genotype) in a round-bottomed 96-well plate and stimulated with LPS (5 μg/mL) or CpG (1μg/mL) for 48 hours. Proliferation was evaluated by Cell Trace Violet (Invitrogen) dilution, with proliferative response of each genotype distinguished by congenic marking (CD45.1 vs. CD45.2). Ca^{2+} flux was measured after 10μg anti-IgM $F(ab)_2$, stimulation as described [13].

Statistical analysis: P values were calculated using the unpaired Student's *t*-test (GraphPad Software, Inc.).

Chapter IV. **Concluding Remarks**

Collectively, the studies presented here demonstrate the consequences of altered antigen receptor signaling in peripheral B cell selection and homeostasis. Dissecting the interplay between antigen-driven BCR signals and competition for limited resources during transitional B cell development is imperative to understanding how tolerance is maintained in the periphery. In this work, we asked how hyper-responsive TLR and BCR signals skew tolerance and homeostatic functions to address how the tuning of these pathways modulates the loss of B cell tolerance in autoimmune-prone settings.

Comprehensive analysis of the mature, naïve B cell repertoire in WASp-deficient mice via single cell cloning and high-throughput sequencing revealed that heightened BCR and TLR signals drive enhanced positive selection and expansion of low-affinity autoreactive B cells into the MZ compartment. Correspondingly, in pediatric and adult human WAS subjects, analysis of the naive repertoire showed an enrichment in autoreactivity associated VH families, such as VH4-34, as well as increased reactivity to several self-antigens when compared to healthy controls. In order to specify where breaks in B cell tolerance occur in WASp-deficient mice, we utilized several BCR transgenic models to evaluate both negative and positive selection mechanisms. While negative selection remains largely intact, WASp-deficient transitional B cells exhibit heightened proliferation that is both antigen-specific and Myd88-dependent. Likewise, in human WAS subjects, we observed an increase in autoreactive BCRs between transitional and naïve B cells implying preferential expansion of these specificities during development. These findings provide the first definitive demonstration that preferential expansion of late transitional B cells is mediated by antigen-dependent BCR and TLR signaling in both murine and human settings.

While our studies suggest a novel pathway by which coordinate TLR and BCR signals orchestrate positive selection of autoreactive B cells in autoimmune-prone settings, it is also likely that this same mechanism is utilized under normal homeostatic conditions to mediate the

selection of natural autoantibodies into the naïve repertoire. Natural autoantibodies can be beneficial to the host such as the rapid clearance of apoptotic debris as well as response to both viral and bacterial pathogens. Components of apoptotic debris such as dsDNA and RNA can trigger several innate signaling pathways and so our group and others, hypothesize that B-intrinsic TLR signals may also promote positive selection of BCRs with these specificities during development (117). Early characterization of individual TLR knockouts did not uncover any statistically significant changes to B cell development as many of these outputs were characterized via quantitative analysis of peripheral B cell compartments (118). However, thorough investigation of the composition and specificity of the B cell repertoire may uncover how specific TLRs mediate tolerance and selection of natural autoantibodies during B cell development in a normal, non-autoimmune prone setting.

Interestingly, positive selection of specific self-reactive transitional B cells positively correlates with an enrichment of these same BCRs within the memory compartment, suggesting that altered selection can predispose germinal center or memory responses towards selecting for autoantibody producing cells. These trends were easily distinguished through the use of the 9G4 antibody. Use of idiotype antibodies, specific for certain VH families known to be expanded in humoral autoimmune settings, will serve as an excellent tool for future studies to monitor or diagnose subjects susceptible to developing these disorders. For example, WAS subjects that have undergone bone marrow transplantation often are at risk for developing split chimerism leading to increased production of autoantibodies (78). Based on our findings, we propose that the 9G4 antibody be utilized to monitor patients post-transplantation in order to detect expanded 9G4+ B cells in the naïve and/or memory B cell populations. These analyses may help to predict or identify loss of B cell tolerance during immune cell reconstitution. Additionally, by cloning 9G4-specific B cells, which despite expressing the same VH family exhibit a wide range of reactivities, throughout different stages of development, we may also be able to reveal a deeper understanding of how certain BCRs are preferentially selected during development and further in the germinal center response. Despite WAS being a rare immunodeficiency, our findings regarding positive selection via enhanced BCR and TLR signals are applicable to a broad range of autoimmune disorders that are associated with common signaling variants, such as the autoimmune risk variant PTPN22. Repertoire analysis of the naïve B cell repertoire in other autoimmune disorders may help to identify specific targets preferentially selected during

development as similar mechanisms of positive selection may be at work in autoimmune-prone settings.

Ultimately, our goal is to identify and understand the complex network of signals involved in directing positive selection during peripheral B cell development. Dysregulation of antigen receptor signaling cascades can result in collateral damage to the host as well as result in increased susceptibility to developing several autoimmune disorders. Hence, defining the mechanisms by which these signals integrate and regulate tolerance may provide valuable insights into therapeutic treatments for a wide array of autoimmune disorders associated with common signaling variants.

Chapter V. **Glossary**

BAFF: B Cell Activating Factor

BCR: B cell receptor

BM: Bone Marrow

BrDU: Bromodeoxyuridine

FM: Follicular Mature B Cells

HEL: Hen Egg Lysozyme

Hep-2: Human epithelial type 2

IFA: Indirect fluorescent antibody

LFA-1: Lymphocyte function-associated antigen-1

MDA-LDL: Malondialdehyde-modified low density lipoprotein

mHEL: membrane-bound HEL

MZ: Marginal Zone

MZP: Marginal Zone Precursor

PC: Phosphorycholine

S1P: Sphingosine 1 Phosphate

sHEL: soluble HEL

Sm-RNP: Smith-Ribonuclear Protein

TACI: Transmembrane activator and CAML Interactor

T1: Transitional Type 1

T2: Transitional Type 2

TLR: Toll Like Receptor

WAS: Wiskott-Aldrich Syndrome

WASp: Wiskott-Aldrich Syndrome protein

XLT: X-linked Thrombocytopenia

Chapter VI. References

1. Murphy, K., P. Travers, and M. Walport. 2008. *Janeway's Immunobiology*. Garland Science and Taylor & Francis Group, LLC.
2. Grönwall, C., and G. J. Silverman. 2014. Natural IgM: Beneficial Autoantibodies for the Control of Inflammatory and Autoimmune Disease. *J. Clin. Immunol.* 34: 12–21.
3. Hardy, R. R., and K. Hayakawa. 2001. B cell development pathways. *Annu. Rev. Immunol.* 19: 595–621.
4. Hardy, R. R., and K. Hayakawa. 1991. A developmental switch in B lymphopoiesis. *Proc. Natl. Acad. Sci. U.S.A.* 88: 11550–11554.
5. Jimi, E., R. J. Phillips, M. Rincon, R. Voll, H. Karasuyama, R. Flavell, and S. Ghosh. 2005. Activation of NF-kappaB promotes the transition of large, CD43+ pre-B cells to small, CD43- pre-B cells. *Int. Immunol.* 17: 815–825.
6. Pelanda, R., and R. M. Torres. 2006. Receptor editing for better or for worse. *Curr. Opin. Immunol.* 18: 184–190.
7. Meffre, E., and H. Wardemann. 2008. B-cell tolerance checkpoints in health and autoimmunity. *Curr. Opin. Immunol.* 20: 632–638.
8. Wardemann, H., S. Yurasov, A. Schaefer, J. W. Young, E. Meffre, and M. C. Nussenzweig. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301: 1374–1377.
9. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, and K. Raphael. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* 334: 676–682.
10. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *Journal of Experimental Medicine* 177: 999–1008.
11. Radic, M. Z., J. Erikson, S. Litwin, and M. Weigert. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *Journal of Experimental Medicine* 177: 1165–1173.
12. Tiegs, S. L., D. M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *Journal of Experimental Medicine* 177: 1009–1020.
13. Levine, M. H., A. M. Haberman, D. B. Sant'Angelo, L. G. Hannum, M. P. Cancro, C. A. Janeway, and M. J. Shlomchik. 2000. A B-cell receptor-specific selection step governs immature to mature B cell differentiation. *Proceedings of the National Academy of Sciences* 97: 2743–2748.
14. Cancro, M., and J. F. Kearney. 2004. J Immunol-2004-Cancro-15-9. *Journal of Immunology* 1–6.
15. Allman, D., and S. Pillai. 2008. Peripheral B cell subsets. *Curr. Opin. Immunol.* 20: 149–157.
16. Monroe, J. G. 2004. Ligand-independent tonic signaling in B-cell receptor function. *Curr. Opin. Immunol.* 16: 288–295.
17. Schwartz, M. A., N. S. Kolhatkar, C. Thouvenel, S. Khim, and D. J. Rawlings. 2014. CD4+ T cells and CD40 participate in selection and homeostasis of peripheral B cells. *The Journal of Immunology* 193: 3492–3502.
18. Stadanlick, J. E., M. Kaileh, F. G. Karnell, J. L. Scholz, J. P. Miller, W. J. Quinn, R. J. Brezski, L. S. Treml, K. A. Jordan, J. G. Monroe, R. Sen, and M. P. Cancro. 2008. Tonic B cell

antigen receptor signals supply an NF-kappaB substrate for prosurvival BLyS signaling. *Nat Immunol* 9: 1379–1387.
19. Miles, K., J. Heaney, Z. Sibinska, D. Salter, J. Savill, D. Gray, and M. Gray. 2012. A tolerogenic role for Toll-like receptor 9 is revealed by B-cell interaction with DNA complexes expressed on apoptotic cells. *Proceedings of the National Academy of Sciences* 109: 887–892.
20. Lam, K. P., R. Kühn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90: 1073–1083.
21. Srinivasan, L., Y. Sasaki, D. P. Calado, B. Zhang, J. H. Paik, R. A. DePinho, J. L. Kutok, J. F. Kearney, K. L. Otipoby, and K. Rajewsky. 2009. PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 139: 573–586.
22. Khan, W. N., A. Nilsson, E. Mizoguchi, E. Castigli, J. Forsell, A. K. Bhan, R. Geha, P. Sideras, and F. W. Alt. 1997. Impaired B cell maturation in mice lacking Bruton's tyrosine kinase (Btk) and CD40. *International ...* 9: 395–405.
23. Sater, R. A., P. C. Sandel, and J. G. Monroe. 1998. B cell receptor-induced apoptosis in primary transitional murine B cells: signaling requirements and modulation by T cell help. *Int. Immunol.* 10: 1673–1682.
24. Chen, Z., S. Shojaee, M. Buchner, H. Geng, J. W. Lee, L. Klemm, B. Titz, T. G. Graeber, E. Park, Y. X. Tan, A. Satterthwaite, E. Paietta, S. P. Hunger, C. L. Willman, A. Melnick, M. L. Loh, J. U. Jung, J. E. Coligan, S. Bolland, T. W. Mak, A. Limnander, H. Jumaa, M. Reth, A. Weiss, C. A. Lowell, and M. Müschen. 2015. Signalling thresholds and negative B-cell selection in acute lymphoblastic leukaemia. *Nature* 521: 357–361.
25. Schweighoffer, E., L. Vanes, J. Nys, D. Cantrell, S. McCleary, N. Smithers, and V. L. J. Tybulewicz. 2013. The BAFF receptor transduces survival signals by co-opting the B cell receptor signaling pathway. *Immunity* 38: 475–488.
26. Schiemann, B., J. L. Gommerman, K. Vora, T. G. Cachero, S. Shulga-Morskaya, M. Dobles, E. Frew, and M. L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293: 2111–2114.
27. Cabatingan, M. S., M. R. Schmidt, R. Sen, and R. T. Woodland. 2002. Naive B lymphocytes undergo homeostatic proliferation in response to B cell deficit. *Journal of Immunology* 169: 6795–6805.
28. Crampton, S. P., E. Voynova, and S. Bolland. 2010. Innate pathways to B-cell activation and tolerance. *Ann. N. Y. Acad. Sci.* 1183: 58–68.
29. Rawlings, D. J., M. A. Schwartz, S. W. Jackson, and A. Meyer-Bahlburg. 2012. Integration of B cell responses through Toll-like receptors and antigen receptors. *Nat. Rev. Immunol.* 12: 282–294.
30. Chaturvedi, A., D. Dorward, and S. K. Pierce. 2008. The B Cell Receptor Governs the Subcellular Location of Toll-like Receptor 9 Leading to Hyperresponses to DNA-Containing Antigens. *Immunity*.
31. Chaturvedi, A., and S. K. Pierce. 2009. How location governs toll-like receptor signaling. *Traffic* 10: 621–628.
32. Desnues, B., A. B. Macedo, A. Roussel-Queval, J. Bonnardel, S. Henri, O. Demaria, and L. Alexopoulou. 2014. TLR8 on dendritic cells and TLR9 on B cells restrain TLR7-mediated spontaneous autoimmunity in C57BL/6 mice. *Proceedings of the National Academy of Sciences* 111: 1497–1502.
33. Gu, H. 1991. Most peripheral B cells in mice are ligand selected. *Journal of Experimental Medicine* 173: 1357–1371.

34. Gaudin, E., Y. Hao, M. Rosado, R. Chaby, R. Girard, and A. Freitas. 2004. Positive selection of B cells expressing low densities of self-reactive BCRs. *Journal of Experimental Medicine* 199: 843–853.
35. Zikherman, J., R. Parameswaran, and A. Weiss. 2012. Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature* 489: 160–164.
36. Meyer-Bahlburg, A., S. F. Andrews, K. O. A. Yu, S. A. Porcelli, and D. J. Rawlings. 2008. Characterization of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic proliferation. *Journal of Experimental Medicine* 205: 155–168.
37. Kenny, J. J., A. M. Stall, D. G. Sieckmann, M. C. Lamers, F. D. Finkelman, L. Finch, and D. L. Longo. 1991. Receptor-mediated elimination of phosphocholine-specific B cells in x-linked immune-deficient mice. *Journal of Immunology* 146: 2568–2577.
38. Groom, J., S. L. Kalled, A. H. Cutler, C. Olson, S. A. Woodcock, P. Schneider, J. Tschopp, T. G. Cachero, M. Batten, J. Wheway, D. Mauri, D. Cavill, T. P. Gordon, C. R. Mackay, and F. Mackay. 2002. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjögren's syndrome. *J. Clin. Invest.* 109: 59–68.
39. Nemazee, D. 1996. Antigen receptor "capacity" and the sensitivity of self-tolerance. *Immunol. Today* 17: 25–29.
40. Groom, J. R., C. A. Fletcher, S. N. Walters, S. T. Grey, S. V. Watt, M. J. Sweet, M. J. Smyth, C. R. Mackay, and F. Mackay. 2007. BAFF and MyD88 signals promote a lupuslike disease independent of T cells. *Journal of Experimental Medicine* 204: 1959–1971.
41. Stadanlick, J. E., and M. P. Cancro. 2008. BAFF and the plasticity of peripheral B cell tolerance. *Curr. Opin. Immunol.* 20: 158–161.
42. Liu, Z., and A. Davidson. 2011. ScienceDirect.com - Trends in Immunology - BAFF and selection of autoreactive B cells. *Trends in immunology*.
43. Vincent, F. B., E. F. Morand, and F. Mackay. 2012. BAFF and innate immunity: new therapeutic targets for systemic lupus erythematosus. *Immunol Cell Biol*.
44. Ota, M., B. H. Duong, A. Torkamani, C. M. Doyle, A. L. Gavin, T. Ota, and D. Nemazee. 2010. Regulation of the B cell receptor repertoire and self-reactivity by BAFF. *The Journal of Immunology* 185: 4128–4136.
45. Carsetti, R., G. Köhler, and M. C. Lamers. 1995. Transitional B cells are the target of negative selection in the B cell compartment. *Journal of Experimental Medicine* 181: 2129–2140.
46. Kinnunen, T., N. Chamberlain, H. Morbach, J. Choi, S. Kim, J. Craft, L. Mayer, C. Cancrini, L. Passerini, R. Bacchetta, H. D. Ochs, T. R. Torgerson, and E. Meffre. 2012. Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood*.
47. Schwartz, M. A. 2013. Signals Controlling Peripheral B Cell Development, Selection, and Activation.
48. Kinnunen, T., N. Chamberlain, H. Morbach, T. Cantaert, M. Lynch, P. Preston-Hurlburt, K. C. Herold, D. A. Hafler, K. C. O'Connor, and E. Meffre. 2013. Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J. Clin. Invest.* 123: 2737–2741.
49. Giltiay, N. V., C. P. Chappell, X. Sun, N. Kolhatkar, T. H. Teal, A. E. Wiedeman, J. Kim, L. Tanaka, M. B. Buechler, J. A. Hamerman, T. Imanishi-Kari, E. A. Clark, and K. B. Elkon. 2013. Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells. *Journal of Experimental Medicine* 210: 2773–2789.
50. Walsh, E. R., P. Pisitkun, E. Voynova, J. A. Deane, B. L. Scott, R. R. Caspi, and S. Bolland. 2012. Dual signaling by innate and adaptive immune receptors is required for TLR7-induced B-

cell-mediated autoimmunity. *Proceedings of the National Academy of Sciences* 109: 16276–16281.
51. Pieper, K., B. Grimbacher, and H. Eibel. 2013. B-cell biology and development. *J. Allergy Clin. Immunol.* 131: 959–971.
52. Ochs, H. D., and A. J. Thrasher. 2006. The Wiskott-Aldrich syndrome. *J. Allergy Clin. Immunol.* 117: 725–38– quiz 739.
53. Notarangelo, L. D., and H. D. Ochs. 2003. Wiskott-Aldrich Syndrome: a model for defective actin reorganization, cell trafficking and synapse formation. *Curr. Opin. Immunol.* 15: 585–591.
54. Becker-Herman, S., A. Meyer-Bahlburg, M. A. Schwartz, S. W. Jackson, K. L. Hudkins, C. Liu, B. D. Sather, S. Khim, D. Liggitt, W. Song, G. J. Silverman, C. E. Alpers, and D. J. Rawlings. 2011. WASp-deficient B cells play a critical, cell-intrinsic role in triggering autoimmunity. *Journal of Experimental Medicine* 208: 2033–2042.
55. Jackson, S. W., N. E. Scharping, N. S. Kolhatkar, S. Khim, M. A. Schwartz, Q. Z. Li, K. L. Hudkins, C. E. Alpers, D. Liggitt, and D. J. Rawlings. 2014. Opposing Impact of B Cell-Intrinsic TLR7 and TLR9 Signals on Autoantibody Repertoire and Systemic Inflammation. *The Journal of Immunology* 192: 4525–4532.
56. Castiello, M. C., M. Bosticardo, F. Pala, M. Catucci, N. Chamberlain, M. C. van Zelm, G. J. Driessen, M. Pac, E. Bernatowska, S. Scaramuzza, A. Aiuti, A. V. Sauer, E. Traggiai, E. Meffre, A. Villa, and M. van der Burg. 2014. Wiskott-Aldrich Syndrome protein deficiency perturbs the homeostasis of B-cell compartment in humans. *J. Autoimmun.* 50: 42–50.
57. O'Connell, A. E., S. Volpi, K. Dobbs, C. Fiorini, E. Tsitsikov, H. de Boer, I. B. Barlan, J. M. Despotovic, F. J. Espinosa-Rosales, I. C. Hanson, M. G. Kanariou, R. Martínez-Beckerat, A. Mayorga-Sirera, C. Mejia-Carvajal, N. Radwan, A. R. Weiss, S.-Y. Pai, Y. N. Lee, and L. D. Notarangelo. 2014. Next generation sequencing reveals skewing of the T and B cell receptor repertoires in patients with wiskott-Aldrich syndrome. *Front Immunol* 5: 340.
58. Martin, F., and J. F. Kearney. 2000. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunol. Rev.* 175: 70–79.
59. Cerutti, A., M. Cols, and I. Puga. 2013. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat. Rev. Immunol.* 13: 118–132.
60. Arnon, T. I., R. M. Horton, I. L. Grigorova, and J. G. Cyster. 2013. Visualization of splenic marginal zone B-cell shuttling and follicular B-cell egress. *Nature* 493: 684–688.
61. Cinamon, G., M. Matloubian, M. J. Lesneski, Y. Xu, C. Low, T. Lu, R. L. Proia, and J. G. Cyster. 2004. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol* 5: 713–720.
62. Lu, T., and J. Cyster. 2002. Integrin-Mediated Long-Term B Cell Retention in the Splenic Marginal Zone -- Lu and Cyster 297 (5580): 409 -- Science Signaling. *Science Signalling*.
63. Martin, F., and J. F. Kearney. 1999. CD21high IgMhigh splenic B cells enriched in the marginal zone: distinct phenotypes and functions. *Curr. Top. Microbiol. Immunol.* 246: 45–50– discussion 51–2.
64. Puga, I., M. Cols, C. M. Barra, B. He, L. Cassis, M. Gentile, L. Comerma, A. Chorny, M. Shan, W. Xu, G. Magri, D. M. Knowles, W. Tam, A. Chiu, J. B. Bussel, S. Serrano, J. A. Lorente, B. Bellosillo, J. Lloreta, N. Juanpere, F. Alameda, T. Baró, C. D. de Heredia, N. Torán, A. Català, M. Torrebadell, C. Fortuny, V. Cusí, C. Carreras, G. A. Diaz, J. M. Blander, C.-M. Farber, G. Silvestri, C. Cunningham-Rundles, M. Calvillo, C. Dufour, L. D. Notarangelo, V. Lougaris, A. Plebani, J.-L. Casanova, S. C. Ganal, A. Diefenbach, J. I. Aróstegui, M. Juan, J. Yagüe, N. Mahlaoui, J. Donadieu, K. Chen, and A. Cerutti. 2011. B cell-helper neutrophils

stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol*.
65. Li, Y., H. Li, and M. Weigert. 2002. Autoreactive B cells in the marginal zone that express dual receptors. *Journal of Experimental Medicine* 195: 181–188.
66. Nickerson, K. M., S. R. Christensen, J. Shupe, M. Kashgarian, D. Kim, K. Elkon, and M. J. Shlomchik. 2010. TLR9 Regulates TLR7- and MyD88-Dependent Autoantibody Production and Disease in a Murine Model of Lupus. *The journal of …*.
67. Zhou, Z., H. Niu, Y.-Y. Zheng, and L. Morel. 2011. Autoreactive marginal zone B cells enter the follicles and interact with CD4+ T cells in lupus-prone mice. *BMC Immunol.* 12: 7.
68. Brummel, R., and P. Lenert. 2005. Activation of Marginal Zone B Cells from Lupus Mice with Type A(D) CpG-Oligodeoxynucleotides.
69. Westerberg, L. S., M. A. de la Fuente, F. Wermeling, H. D. Ochs, M. C. I. Karlsson, S. B. Snapper, and L. D. Notarangelo. 2008. WASP confers selective advantage for specific hematopoietic cell populations and serves a unique role in marginal zone B-cell homeostasis and function. *Blood* 112: 4139–4147.
70. Nemazee, D. 2006. Receptor editing in lymphocyte development and central tolerance. *Nat. Rev. Immunol.* 6: 728–740.
71. Hayakawa, K. 1999. Positive Selection of Natural Autoreactive B Cells. *Science* 285: 113–116.
72. Wang, H., and S. H. Clarke. 2003. Evidence for a ligand-mediated positive selection signal in differentiation to a mature B cell. *Journal of Immunology* 171: 6381–6388.
73. Eilat, D., and M. Wabl. 2012. B cell tolerance and positive selection in lupus. *The Journal of Immunology* 189: 503–509.
74. Isnardi, I., Y.-S. Ng, I. Srdanovic, R. Motaghedi, S. Rudchenko, H. von Bernuth, S.-Y. Zhang, A. Puel, E. Jouanguy, C. Picard, B.-Z. Garty, Y. Camcioglu, R. Döffinger, D. Kumararatne, G. Davies, J. I. Gallin, S. Haraguchi, N. K. Day, J.-L. Casanova, and E. Meffre. 2008. IRAK-4- and MyD88-dependent pathways are essential for the removal of developing autoreactive B cells in humans. *Immunity* 29: 746–757.
75. Jackson, S. W., N. S. Kolhatkar, and D. J. Rawlings. 2015. B cells take the front seat: dysregulated B cell signals orchestrate loss of tolerance and autoantibody production. *Curr. Opin. Immunol.* 33C: 70–77.
76. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin–IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607.
77. Silver, K. L., T. L. Crockford, T. Bouriez-Jones, S. Milling, T. Lambe, and R. J. Cornall. 2007. MyD88-dependent autoimmune disease in Lyn-deficient mice. *Eur. J. Immunol.* 37: 2734–2743.
78. Bosticardo, M., F. Marangoni, A. Aiuti, A. Villa, and M. Grazia Roncarolo. 2009. Recent advances in understanding the pathophysiology of Wiskott-Aldrich syndrome. *Blood* 113: 6288–6295.
79. Recher, M., S. O. Burns, M. A. de la Fuente, S. Volpi, C. Dahlberg, J. E. Walter, K. Moffitt, D. Mathew, N. Honke, P. A. Lang, L. Patrizi, H. Falet, M. Keszei, M. Mizui, E. Csizmadia, F. Candotti, K. Nadeau, G. Bouma, O. M. Delmonte, F. Frugoni, A. B. F. Fomin, D. Buchbinder, E. M. Lundequist, M. J. Massaad, G. C. Tsokos, J. Hartwig, J. Manis, C. Terhorst, R. S. Geha, S. Snapper, K. S. Lang, R. Malley, L. Westerberg, A. J. Thrasher, and L. D. Notarangelo. 2012. B cell-intrinsic deficiency of the Wiskott-Aldrich syndrome protein (WASp) causes severe

abnormalities of the peripheral B-cell compartment in mice. *Blood* 119: 2819–2828.
80. Simon, K. L., S. M. Anderson, E. K. Garabedian, D. Moratto, R. A. Sokolic, and F. Candotti. 2014. Molecular and phenotypic abnormalities of B lymphocytes in patients with Wiskott-Aldrich syndrome. *J. Allergy Clin. Immunol.* 133: 896–9.e4.
81. Doyle, C. M., J. Han, M. G. Weigert, and E. T. L. Prak. 2006. Consequences of receptor editing at the lambda locus: multireactivity and light chain secretion. *Proc. Natl. Acad. Sci. U.S.A.* 103: 11264–11269.
82. Witsch, E. J., and E. Bettelheim. 2008. Allelic and isotypic light chain inclusion in peripheral B cells from anti-DNA antibody transgenic C57BL/6 and BALB/c mice. *Journal of Immunology* 180: 3708–3718.
83. Fournier, E. M., M.-G. Velez, K. Leahy, C. L. Swanson, A. V. Rubtsov, R. M. Torres, and R. Pelanda. 2012. Dual-reactive B cells are autoreactive and highly enriched in the plasmablast and memory B cell subsets of autoimmune mice. *Journal of Experimental Medicine* 209: 1797–1812.
84. Kenny, J. J., C. O'Connell, D. G. Sieckmann, R. T. Fischer, and D. L. Longo. 1991. Selection of antigen-specific, idiotype-positive B cells in transgenic mice expressing a rearranged M167-mu heavy chain gene. *Journal of Experimental Medicine* 174: 1189–1201.
85. Brigido, M. M., M. Polymenis, and B. D. Stollar. 1993. Role of mouse VH10 and VL gene segments in the specific binding of antibody to Z-DNA, analyzed with recombinant single chain Fv molecules. *Journal of Immunology* 150: 469–479.
86. Matejuk, A., M. Beardall, Y. Xu, Q. Tian, D. Phillips, B. Alabyev, K. Mannoor, and C. Chen. 2009. Exclusion of Natural Autoantibody-Producing B Cells from IgG Memory B Cell Compartment during T Cell-Dependent Immune Responses. *The Journal of Immunology* 182: 7634–7643.
87. Jiang, C., M.-L. Zhao, R. M. Scearce, and M. Diaz. 2011. Activation-induced deaminase-deficient MRL/lpr mice secrete high levels of protective antibodies against lupus nephritis. *Arthritis Rheum.* 63: 1086–1096.
88. Maranhão, A. Q., M. B. W. Costa, L. Guedes, P. M. Moraes-Vieira, T. Raiol, and M. M. Brigido. 2013. A mouse variable gene fragment binds to DNA independently of the BCR context: a possible role for immature B-cell repertoire establishment. *PLoS ONE* 8: e72625.
89. Cyster, J. G., J. I. Healy, K. Kishihara, T. W. Mak, M. L. Thomas, and C. C. Goodnow. 1996. Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. *Nature* 381: 325–328.
90. Panigrahi, A. K., N. G. Goodman, R. A. Eisenberg, M. R. Rickels, A. naji, and E. T. L. Prak. 2008. RS rearrangement frequency as a marker of receptor editing in lupus and type 1 diabetes. *Journal of Experimental Medicine* 205: 2985–2994.
91. Whitcomb, E. A., B. B. Haines, A. P. Parmelee, A. M. Pearlman, and P. H. Brodeur. 1999. Germline structure and differential utilization of Igha and Ighb VH10 genes. *Journal of Immunology* 162: 1541–1550.
92. Pugh-Bernard, A. E., G. J. Silverman, A. J. Cappione, M. E. Villano, D. H. Ryan, R. A. Insel, and I. Sanz. 2001. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. *J. Clin. Invest.* 108: 1061–1070.
93. Richardson, C., A. S. Chida, D. Adlowitz, L. Silver, E. Fox, S. A. Jenks, E. Palmer, Y. Wang, J. Heimburg-Molinaro, Q.-Z. Li, C. Mohan, R. Cummings, C. Tipton, and I. Sanz. 2013. Molecular basis of 9G4 B cell autoreactivity in human systemic lupus erythematosus. *The Journal of Immunology* 191: 4926–4939.
94. Jenks, S. A., E. M. Palmer, E. Y. Marin, L. Hartson, A. S. Chida, C. Richardson, and I. Sanz.

2013. 9G4+ autoantibodies are an important source of apoptotic cell reactivity associated with high levels of disease activity in systemic lupus erythematosus. *Arthritis Rheum.* 65: 3165–3175.
95. Cappione, A., J. H. Anolik, A. Pugh-Bernard, J. Barnard, P. Dutcher, G. Silverman, and I. Sanz. 2005. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. *J. Clin. Invest.* 115: 3205–3216.
96. Lesley, R., Y. Xu, S. L. Kalled, D. M. Hess, S. R. Schwab, H.-B. Shu, and J. G. Cyster. 2004. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity* 20: 441–453.
97. Cappione, A. J., A. E. Pugh-Bernard, J. H. Anolik, and I. Sanz. 2004. Lupus IgG VH4.34 Antibodies Bind to a 220-kDa Glycoform of CD45/B220 on the Surface of Human B Lymphocytes. *The Journal of Immunology* 172: 4298–4307.
98. Grillot-Courvalin, C., J.-C. Brouet, S. Labaume, F. Piller, L. Z. Rassenti, G. J. Silverman, T. J. Kipps, and L. Silberstein. 1992. An anti-B cell autoantibody from Wiskott-Aldrich syndrome which recognizes i blood group specificity on normal human B cells. *Eur. J. Immunol.* 22: 1781–1788.
99. Tipton, C., C. F. Fucile, J. Darce, T. Ichikawa, I. Gregoretti, S. Schieferi, J. Horn, S. A. Jenks, R. J. Feldman, R. Mehr, C. Wei, F. E.-H. Lee, W. C. Cheung, A. F. Rosenberg, and I. Sanz. 2015. Diversity, cellular origin and autoreactivity of antibody-secreting cell expansions in acute SLE. *Nat Immunol*.
100. Dai, X., R. G. James, T. Habib, S. Singh, S. Jackson, S. Khim, R. T. Moon, D. Liggitt, A. Wolf-Yadlin, J. H. Buckner, and D. J. Rawlings. 2013. A disease-associated PTPN22 variant promotes systemic autoimmunity in murine models. *J. Clin. Invest.* 123: 2024–2036.
101. Meyer-Bahlburg, A., and D. J. Rawlings. 2008. B cell autonomous TLR signaling and autoimmunity. *Autoimmunity Reviews* 7: 313–316.
102. Tiller, T., C. E. Busse, and H. Wardemann. 2009. Cloning and expression of murine Ig genes from single B cells. *Journal of Immunological Methods* 350: 183–193.
103. Tiller, T., E. Meffre, S. Yurasov, M. Tsuiji, M. C. Nussenzweig, and H. Wardemann. 2008. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *Journal of Immunological Methods* 329: 112–124.
104. Martin, F., and J. F. Kearney. 2002. Marginal-zone B cells. *Nat. Rev. Immunol.* 2: 323–335.
105. Lopes-Carvalho, T., and J. F. Kearney. 2004. Development and selection of marginal zone B cells. *Immunol. Rev.* 197: 192–205.
106. Rubtsov, A. V., C. L. Swanson, S. Troy, P. Strauch, R. Pelanda, and R. M. Torres. 2008. TLR agonists promote marginal zone B cell activation and facilitate T-dependent IgM responses. *Journal of Immunology* 180: 3882–3888.
107. Meyer-Bahlburg, A., S. Becker-Herman, S. Humblet-Baron, S. Khim, M. Weber, G. Bouma, A. J. Thrasher, F. D. Batista, and D. J. Rawlings. 2008. Wiskott-Aldrich syndrome protein deficiency in B cells results in impaired peripheral homeostasis. *Blood* 112: 4158–4169.
108. Muppidi, J. R., T. I. Arnon, Y. Bronevetsky, N. Veerapen, M. Tanaka, G. S. Besra, and J. G. Cyster. 2011. Cannabinoid receptor 2 positions and retains marginal zone B cells within the splenic marginal zone. *Journal of Experimental Medicine* 208: 1941–1948.
109. Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu, and J. N. Weiser. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat Med* 16: 228–231.
110. He, B., R. Santamaria, W. Xu, M. Cols, K. Chen, I. Puga, M. Shan, H. Xiong, J. B. Bussel, A. Chiu, A. Puel, J. Reichenbach, L. Marodi, R. Döffinger, J. Vasconcelos, A. Issekutz, J.

Krause, G. Davies, X. Li, B. Grimbacher, A. Plebani, E. Meffre, C. Picard, C. Cunningham-Rundles, J.-L. Casanova, and A. Cerutti. 2010. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nat Immunol* 11: 836–845.
111. Mackay, F., and P. Schneider. 2008. TACI, an enigmatic BAFF/APRIL receptor, with new unappreciated biochemical and biological properties. *Cytokine Growth Factor Rev.* 19: 263–276.
112. Hwang, S.-H., H. Lee, M. Yamamoto, L. A. Jones, J. Dayalan, R. Hopkins, X. J. Zhou, F. Yarovinsky, J. E. Connolly, M. A. Curotto de Lafaille, E. K. Wakeland, and A.-M. Fairhurst. 2012. B cell TLR7 expression drives anti-RNA autoantibody production and exacerbates disease in systemic lupus erythematosus-prone mice. *The Journal of Immunology* 189: 5786–5796.
113. Lamagna, C., Y. Hu, A. L. DeFranco, and C. A. Lowell. 2014. B cell-specific loss of Lyn kinase leads to autoimmunity. *The Journal of Immunology* 192: 919–928.
114. Steinberg, E. B., T. J. Santoro, T. M. Chused, P. A. Smathers, and A. D. Steinberg. 1983. Studies of congenic MRL-Ipr/Ipr.xid mice. *Journal of Immunology* 131: 2789–2795.
115. Honigberg, L. A., A. M. Smith, M. Sirisawad, E. Verner, D. Loury, B. Chang, S. Li, Z. Pan, D. H. Thamm, R. A. Miller, and J. J. Buggy. 2010. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proceedings of the National Academy of Sciences* 107: 13075–13080.
116. Hua, Z., A. J. Gross, C. Lamagna, N. Ramos-Hernández, P. Scapini, M. Ji, H. Shao, C. A. Lowell, B. Hou, and A. L. DeFranco. 2014. Requirement for MyD88 signaling in B cells and dendritic cells for germinal center anti-nuclear antibody production in Lyn-deficient mice. *The Journal of Immunology* 192: 875–885.
117. Suurmond, J., and B. Diamond. 2015. Autoantibodies in systemic autoimmune diseases: specificity and pathogenicity. *J. Clin. Invest.* 125: 2194–2202.
118. Silver, K., H. Ferry, T. Crockford, and R. J. Cornall. 2006. TLR4, TLR9 and MyD88 are not required for the positive selection of autoreactive B cells into the primary repertoire. *Eur. J. Immunol.* 36: 1404–1412.

Curriculum Vitae
Nikita Kolhatkar, PhD

Education:

University of Washington, Seattle WA 9/10-8/15
PhD, Department of Immunology

Mount Holyoke College, South Hadley, MA 9/05-5/09
B.A. Biological Sciences

Research Experience:

Graduate Student, University of Washington 3/11-8/15
Thesis Advisor: David J. Rawlings

Research Scientist, University of California, San Francisco 6/09-8/10
Principal Investigators: Lisa M. Coussens and V. Courtney Broaddus

Undergraduate Honors Thesis Student, Mount Holyoke College 9/08-5/09
Thesis Advisor: Sharon Stranford

Summer Research Intern, University of California, San Francisco 6/08-8/08
Principal Investigators: Lisa M. Coussens

Undergraduate Student Researcher, Mount Holyoke College 9/07-5/08
Principal Investigators: Craig Woodard

Undergraduate Summer Research Intern, University of Connecticut, Farmington, CT 6/07-8/07
Principal Investigator: Pramod K. Srivastava

Publications:

1. Altered BCR and TLR signals promote enhanced positive selection of autoreactive transitional B cells in Wiskott-Aldrich Syndrome. Kolhatkar NS, Brahmandam A, Thouvenel C, Becker-Herman S, Jacobs HM, Schwartz MA, Khim S, Panigrahi AK, Luning Prak ET, Thrasher AJ, Notarangelo LD, Candotti F, Torgerson TR, Sanz IA and Rawlings DJ. 2015 (In Press, *Journal of Experimental Medicine).*
2. BAFF promotes systemic autoimmunity via TACI-dependent activation of transitional B cells. Jacobs H, Thouvenel C, Leach S, Arkatkar T, Metlzer G, Scharping N, Kolhatkar NS, Rawlings DJ, and Jackson SW. 2015. (In review at *Journal of Experimental Medicine*)
3. B cell-intrinsic TLR7 signals promotes depletion of the marginal zone in a murine model of Wiskott-Aldrich Syndrome. Kolhatkar NS, Scharping NE, Sullivan JM, Jacobs HM, Schwartz MA, Khim S, Notarangelo LD, Thrasher AJ, Rawlings DJ and Jackson SW. 2015. (In press, *European Journal of Immunology*).

4. B cell take the front seat: dysregulated B cell signals orchestrate loss of tolerance and autoantibody production. *Current Opinion in Immunology*. April 2015; 33: 70-77. Jackson SW, Kolhatkar NS and Rawlings DJ.

5. CD4+ T cells and CD40 participate in selection and homeostasis of peripheral B cells. *The Journal of Immunology* 2014, 193:3492–3502. Schwartz MA, Kolhatkar NS, Thouvenel C, Khim S, Rawlings DJ.

6. Opposing Impact of B Cell-Intrinsic TLR7 and TLR9 Signals on Autoantibody Repertoire and Systemic Inflammation. *The Journal of Immunology* 2014, 192:4525–4532. Jackson SW, Scharping NE, Kolhatkar NS, Khim S, Schwartz MA, Li QZ, Hudkins KL, Alpers CE, Liggitt D, Rawlings DJ.

7. Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells. *Journal of Experimental Medicine* 2013, 210:2773–2789. Giltiay NV, Chappell CP, Sun X, Kolhatkar N, Teal TH, Wiedeman AE, Kim J, Tanaka L, Buechler MB, Hamerman JA, Imanishi-Kari T, Clark EA and Elkon KB.

8. The Bcl-2 repertoire of mesothelioma spheroids underlies acquired apoptotic multicellular resistance. *Cell Death and Disease*. June 2011; 174 (2). Barbone D, Ryan JA, Kolhatkar N, Chacko AD, Jablons DM, Sugarbaker DJ, Bueno R, Letai AG, Coussens LM, Fennell DA, Broaddus VC.

9. T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell*. August 2009; 16 (2): 91-102. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM.