ABSTRACT

Title of Document: ACTIN-BINDING PROTEIN 1 AND DYNAMIN-2 MODULATE ATTENUATION OF B-CELL SIGNALING AND REGULATE B-CELL FUNCTION.

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B-cells mediate humoral immune responses with production of an amazingly diverse repertoire of antigen specific antibodies. Antigen binding to the B-cell receptor (BCR) induces coordinated BCR signaling and BCR-mediated antigen internalization, processing, and presentation to helper T-cells, necessary for generation of humoral memory. Prolonged or uncontrolled BCR signaling has been linked to development of autoimmunity; therefore controlled attenuation of B-cell signaling is required for maintenance of B-cell tolerance. This study investigates the role of an actin adaptor protein, actin-binding protein 1 (Abp1/HIP-55/SH3P7), in BCR-mediated signal transduction and subsequent B-cell function. I found that in both *Abp1*$^{-/-}$ and *Abp1*$^{-/-}$-

bone marrow chimeric mice, in which only B-cells lack Abp1 expression, the number of spontaneous germinal center and marginal zone B-cells, and levels of autoantibody are significantly increased. Serum levels of T-independent antibody and total IgM and IgG antibodies are elevated in *Abp1*$^{-/-}$ mice, whereas T-dependent IgG responses are reduced and fail to undergo affinity maturation. Upon binding membrane-associated antigen, surface BCR clustering is enhanced and B-cell contraction delayed in *Abp1*$^{-/-}$ B cells, concurrent with slow but persistent increases in F-actin at BCR signalosomes. Furthermore, BCR signaling is enhanced in *Abp1*$^{-/-}$ B cells, including Ca^{2+} flux and phosphorylation of B-cell linker protein (BLNK), mitogen-activated protein kinase kinase MEK1/2, and extracellular signal-regulated kinase (ERK), coinciding with reductions in recruitment of the inhibitory signaling molecules, hematopoietic progenitor kinase 1 (HPK1) and SH2-containing inositol 5-phosphatase (SHIP-1) to BCR signalosomes. Our previous studies demonstrated a role for Abp1 in BCR internalization by linking the actin cytoskeleton to the endocytic machinery protein dynamin2. This study shows that dyanmin2 is recruited to the BCR upon antigen binding, and its recruitment is dependent on its proline rich domain (PRD). BCR internalization and trafficking to late endosomes requires the PRD, GTPase function, and tyrosine phosphorylation sites of dynamin2. B-cells expressing a GTPase dead mutant of dynamin2 exhibit enhanced BCR clustering and signaling. These results indicate Abp1 negatively regulates BCR signaling by coupling actin remodeling to B-cell contraction, activation of inhibitory signaling molecules, and the endocytic machinery protein dynamin2, revealing a novel regulatory mechanism for peripheral B-cell development and antibody response.

ACTIN-BINDING PROTEIN 1 AND DYNAMIN-II MODULATE ATTENUATION OF B-CELL SIGNALING AND REGULATE B-CELL FUNCTION.

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Dedication

This work is dedicated to my husband, James Fallen. None of this would have been possible without your unconditional love and support. You are my best friend, and the best husband and father to Mia and Mac I could have ever wished for. I love you, and thank you.

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Table of Contents

Dedication ii
Acknowledgements iii
Table of Contents v
List of Figures vii
List of Abbreviations ix

Chapter 1: Introduction 1
1.1 B-lymphocytes and humoral immunity 1
1.2 B-cell development 4
1.3 B-cell effector mechanisms and immunological memory 9
1.4 B-cell dysregulation: immunodeficiency, autoimmunity, and B-cell cancers 15
1.5 The BCR and its signaling pathways 19
1.6 BCR mediated antigen processing and presentation 28
1.7 BCR dynamics during B-cell activation 33
1.8 Actin dynamics during B-cell activation 36
1.9 Abp1 in lymphocyte activation and endocytosis 45
1.10 The role of dynamin 2 in moderating signal transduction and actin dependent endocytosis 50
1.11 Rationale/Aims 55
1.11.1 Aim 1. The role of Abp1 in regulation of BCR signaling, B-cell development, and immune response. 56
1.11.2 Aim 2. The role of Dyn2 in regulating BCR signal transduction and internalization. 57

Chapter 2: Actin binding protein 1 links B-cell antigen receptor to negative signaling pathways 59
2.1 Abstract 59
2.2 Introduction 60
2.3 Materials and Methods 64
2.3.1 Mice and cell culture 64
2.3.2 Flow cytometry 65
2.3.3 Immunohistochemistry 67
2.3.4 Bone marrow chimera mice 67
2.3.5 Serological analysis 68
2.3.6 Immunizations 68
2.3.7 Preparation of mono-biotinylated Fab' antibody 69
2.3.8 Preparation of antigen tethered planar lipid bilayers 69
2.3.9 Total internal reflection microscopic analysis 70
2.3.10 Confocal microscopic analysis 71
2.3.11 Calcium analysis 71
2.3.12 Statistical analysis 72

2.4 Results 72
2.4.1 B-cell-specific Abp1 deficiency is sufficient to increase the development of marginal zone and germinal center B-cells. 72
2.4.2 Production of autoAb, T-independent Ab responses, and total Ab increase while T-dependent high affinity Ab responses decrease in Abp1$^{-/-}$ mice. 87
2.4.3 Abp1 deficiency augments B-cell spread and BCR clustering. 90
2.4.4 Abp1 is recruited to the leading edge of the spreading B-cells upon BCR activation. 95
2.4.5 BCR signaling is enhanced in Abp1$^{-/-}$ B-cells. 95
2.4.6 Abp1 is required for the recruitment of the inhibitory signaling molecules HPK1 and SHIP-1 to the B-cell contact zone. 101
2.4.7 Abp1 regulates BCR-induced actin reorganization. 105
2.5 Discussion 107

Chapter 3: Dynamin-2 regulates BCR internalization, and signal transduction. 117
3.1 Abstract 117
3.2 Introduction 118
3.3 Materials and Methods 122
3.3.1 DNA constructs and transfection. 122
3.3.2 Confocal analysis. 122
3.3.3 Flow cytometry. 123
3.3.4 TIRF analysis 124
3.4 Results 125
3.4.1 The PRD of Dyn2 is necessary for its recruitment to the BCR upon activation. 125
3.4.2 Dyn2's phosphorylation, PRD domain, and GTPase activity are necessary for BCR trafficking to late endosomes. 130
3.4.3 Dyn2 PRD domain is necessary for Abp1 recruitment. 135
3.4.4 Dyn2 GTPase deficiency augments BCR clustering, but not B-cell spreading. 138
3.4.5 Dyn2 GTPase function is involved in the regulation of BCR signal transduction. 140
3.5 Discussion 144

Chapter 4: General Discussion and Future Work 151
4.1 General summary and working model 151
4.2 Future Work 157
4.2.1 Abp1 Project 157
4.2.2 Dynamin Project 160
Bibliography 164

List of Figures

Fig 1.1	Antibody/BCR structure and isotypes
Fig 1.2	B-cell developmental stages
Fig 1.3	Germinal center formation
Fig 1.4	The BCR and its signaling pathways
Fig 1.5	Antigen internalization and processing
Fig 1.6	Coordination of actin remodeling with reorganization of BCRs at the cell surface
Fig 1.7	Abp1 structure and function
Fig 1.8	Dynamin-2 structure and function
Fig 2.1	Abp1 deficiency does not affect B-cell maturation in the bone marrow
Fig 2.2	Abp1 deficiency affects the development of splenic MZ B-cells
Fig 2.3	Abp1 deficiency affects the expansion of PerC B1a B-cells
Fig 2.4	Abp1 differentially expressed in B-cell subsets
Fig. 2.5	Abp1 deficiency promotes spontaneous differentiation of GC B-cells, and initiation of splenomegaly
Fig. 2.6	Reconstitution of bone marrow chimera mice
Fig. 2.7	Only B-cells in *Abp1*$^{-/-}$ bone marrow chimeric mice lack the expression of Abp1
Fig. 2.8	B-cell-specific Abp1 deficiency is sufficient to increase the differentiation of MZ and spontaneous GC B-cells
Fig. 2.9	AutoAb production increased in *Abp1*$^{-/-}$ and *Abp1*$^{-/-}$-Ch mice
Fig. 2.10	Production of T-independent Ab responses, and total Ab increases while T-dependent high affinity Ab responses decrease in *Abp1*$^{-/-}$ mice
Fig. 2.11	Abp1 knockout augments B-cell spread and BCR clustering

Fig. 2.12 Earlier and greater percentage of capping in *Abp1*$^{-/-}$ B-cells

Fig. 2.13 BCR activation induces recruitment of Abp1 to the outer edge of the spreading B-cell

Fig. 2.14 BCR signaling is enhanced in *Abp1*$^{-/-}$ B-cells

Fig. 2.15 The expression levels of membrane IgM and signaling molecules in unstimulated splenic B-cells

Fig. 2.16 Abp1 is required for recruitment of the inhibitory signaling molecule HPK1 to the B-cell contact zone

Fig. 2.17 Abp1 is required for recruitment of the inhibitory signaling molecule SHIP-1 to the B-cell contact zone

Fig. 2.18 Abp1 is not required for recruitment of the pSyk to the B-cell contact zone

Fig. 2.19 Abp1 regulates actin remodeling

Fig. 2.20 Abp1 regulates actin remodeling by modulating activation of the actin nucleation promoting factor WASP

Fig. 2.21 Abp1 regulates actin remodeling by modulating activation of the actin nucleation promoting factor N-WASP

Fig. 3.1 Dyn2 PRD domain necessary for its recruitment to the B-cell membrane

Fig. 3.2 GFP-DynK44A and GFP-DynY231/597F mislocalized to cell surface at later time points of B-cell activation

Fig. 3.3 Percent of labeled surface BCRs

Fig. 3.4 Dyn's phosphorylation, PRD domain, and GTPase activity are necessary for BCR translocation to LAMP1^{+} compartments

Fig. 3.5 Dynasore reduces BCR internalization to late endosome

Fig. 3.6 Dyn2 PRD domain necessary for recruitment of actin adaptor Abp1

Fig. 3.7 Dyn2 GTPase deficiency augments BCR clustering but does not affect B-cell spread

Fig. 3.8 Dyn2 GTPase function necessary for BCR signal transduction

Fig. 4.1 Working model

List of Abbreviations

Ab	Antibody
Abp1	Actin-binding protein one
ADF	Actin depolymerizing factor homology domain
Ag	Antigen
AID	Activation-induced cytidine deaminase
ANA	Anti-nuclear antibody
APC	Antigen presenting cell
AutoAb	Auto-antibody
BAD	Bcl-associated death promoter
BAFF-R	B cell-activating factor belonging to the TNF family (BAFF)-receptor
BCAP	B-cell adaptor molecule for PI3K
B-cells	B lymphocytes
BCR	B-cell receptor
BLNK	B-cell linker protein
BM	Bone marrow
B_{reg}	B-regulatory cell
Btk	Bruton's tyrosine kinase
CME	Clathrin mediated endocytosis
Co-IP	Co-immunoprecipitation
CRAC	Calcium-release-activated channels
C-region	Constant region

DAG	Diacylglycerol
DC	Dendritic cell
dsDNA	Double stranded DNA
Dyn2	Dynamin2
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ERM	Ezrin/radixin/moesin
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcγRIIB	Fc gamma receptor IIB
F_{DC}	Follicular Dendritic Cell
FO B-cell	Follicular B-cell
GC	Germinal center
GC B-cell	Germinal center B-cell
GED	GTPase effector domain
GFP	Green fluorescence protein
GTP	Guanosine-5'-triphosphate
Grb2	Growth factor receptor-bound protein 2
Hip-55	HPK1-interacting protein of 55 kDa (Abp1)
HPK1	Hematopoietic progenitor kinase-1
HRP	Horseradish peroxidase

HSC	Hematopoietic stem cell
Ig	Immunoglobulin
IKK	IκB Kinase
IL-7	Interleukin-7
IL-10	Interleukin-10
IP_3	Inositol triphosphate
IS	Immunological synapse
IRM	Interference reflection microscopy
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	c-Jun NH2-terminal kinase
KLH	Keyhole limpet hemocyanin
LAB	Linker for activation of B-cells
LAMP-1	Lysosomal associated protein one
LAT	Linker for activation of T-cells
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MFI	Mean fluorescence intensity
mIg	Membrane bound immunoglobulin
MHC-II	Major histocompatibility complex-II
MIIC	MHC-class II containing compartments
μMT	B-cell null mouse
MZ	Marginal zone

NFκB	Nuclear factor kappa-B
NFAT	Nuclear factor of activated T cells
NP	(4-hydroxy-3-nitrophenyl)acetyl
N-WASP	Neural Wiskott–Aldrich syndrome protein
PBS	Phosphate buffered saline
PC	Plasma cell
PerC	Peritoneal cavity
PH	Pleckstrin homology
PI	Primary Immunodeficiency
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PI3K	Phosphoinositide 3 kinase
PKC-β	Protein kinase C-β
PLCγ2	Phospholipase Cγ2
PNA	Peanut agglutinin
PRD	Proline rich domain
RasGRP	Ras guanyl nucleotide-releasing protein
SFK	Src family kinase
SH2	Src homology domain 2
SH3	Src homology domain2
SHIP-1	SH2-domain-containing inositol 5-phosphatase
SHM	Somatic hyper-mutation
SHP1	SH2-domain containing protein tyrosine phosphatase

Syk	Src-homology 2 (SH2) domain containing tyrosine kinase
SMAC	Supramolecular activation complex
T1 B-cell	Transitional 1 B-cell
T2 B-cell	Transitional 2 B-cell
T-cells	T lymphocytes
TCR	T-cell receptor
T_{FH}	T-follicular helper cell
TFI	Total fluorescence intensity
TIRF	Total internal reflection microscopy
T_{reg}	T-regulatory cell
V-region	Variable region
WASP	Wiskott–Aldrich syndrome protein
XHM	X-linked hyper-IgM
XLA	X-linked agammaglobulinemia
Y/pY	Tyrosine residue/phosphorylated tyrosine residue

Chapter 1: Introduction

1.1 B-lymphocytes and humoral immunity

Protection against invading pathogens is mediated by two major branches of immunity; first, the more rapid innate immune response, followed by an adaptive immune response (1). The innate immune response provides immediate protection against infection utilizing a limited number of receptors and secreted proteins that are encoded in the germline, and recognize molecular patterns common to many pathogens. While innate immunity does not confer long-lasting immunity to the host, it does contribute to the induction of the adaptive immune response. If pathogens breach the initial protective defenses of the innate immune response, the adaptive response will be engaged, leading to the expansion of antigen specific lymphocytes that precisely target pathogens and provide immunological memory to stave off subsequent infection with the same pathogen.

The adaptive immune response can be further divided into two major branches; cell-mediated and humoral immunity. While the cell-mediated response is primarily mediated by T lymphocytes (T-cells), humoral immunity is primarily driven by B lymphocytes (B-cells). The hallmark of humoral immunity is antigen induced production of antibodies (immunoglobulin, Ig) by the B-cells, which are generated with increasing specificity for the antigen throughout the course of an infection. Antibody (Ab) is a protein complex composed of two identical heavy chains joined to two identical light chains (Fig. 1.1) (2-5). The N-terminus of both light and heavy chains contains a region

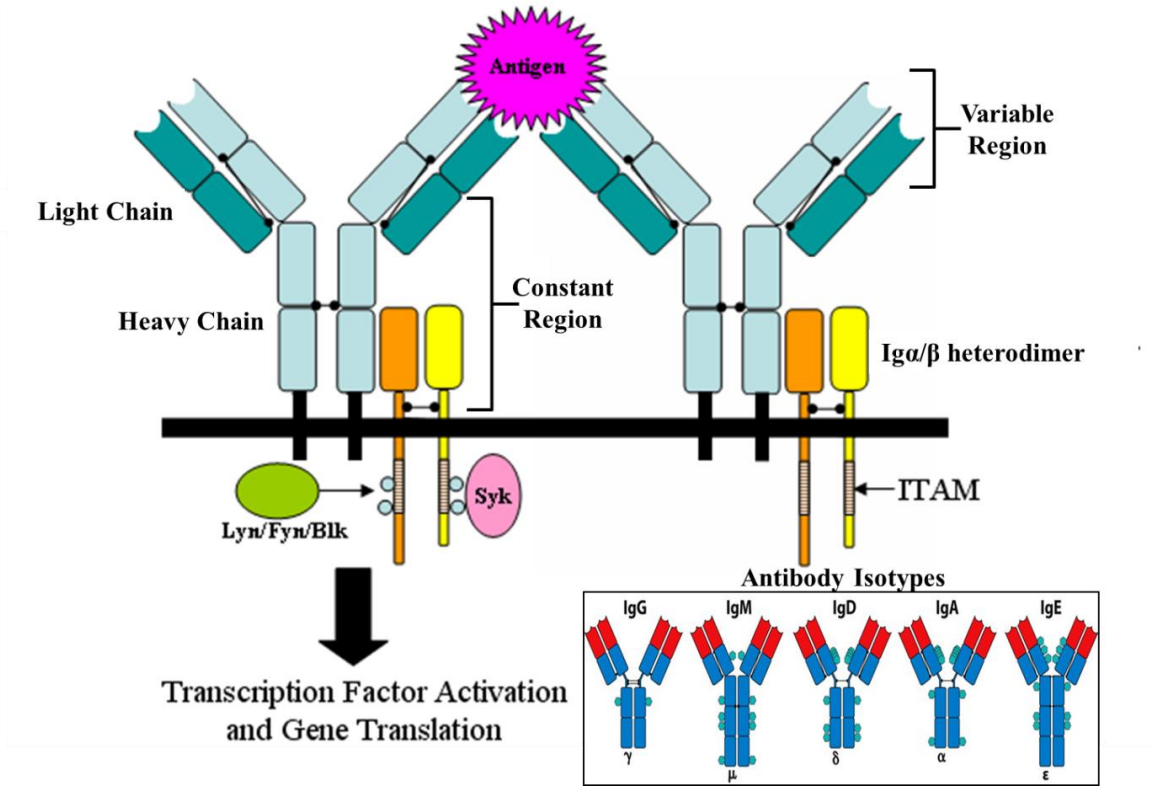


Figure 1.1 Antibody Structure and isotypes. Antibody, also known as immunoglobulin, is composed to two identical heavy chains (light blue – top image) associated by disulfide bonds to two identical light chains (dark blue – top image). The N-terminus of the heavy and light chains, comprising the variable region, forms the antigen binding region of the antibody or the membrane bound immunoglobulin. There are five major C-terminal isotypes of immunoglobulin, including IgG, IgM, IgD, IgA, and IgE (inset image). At the surface of B-cells, membrane bound immunoglobulin is non-covalently associated with the signal transduction unit of the BCR, the Igα/β heterodimer. Upon BCR crosslinking by antigen, tyrosines within the ITAMs of the Igα/β heterodimer become phosphorylated, and serve to recruit signaling molecules necessary for B-cell signal transduction. - Inset image modified from Janeway's Immunobiology, 8th edition (2011).

of high sequence variation (the variable region, or V-region), with which the antibody binds antigens of immense and changing diversity encountered throughout the lifetime of a host (6). In addition to variability within the V-region of the Ig, there are five major isotypes of Ig determined by the conserved regions in the C-terminus (constant region or C-region). The Ig isotype determines its effector mechanism. Mature naïve B-cells predominantly express IgM (μ) and IgD (δ) isotypes, whereas antigenic encounter drives isotype switching to IgG (γ), IgA (α), or IgE (ε) isotypes. B-cells express a surface bound form of Ig that contains a transmembrane domain as well as a short cytoplasmic domain at its C-terminus. Membrane Ig (mIg) is non-covalently associated Igα/Igβ heterodimer, forming the B-cell receptor (BCR, Fig. 1.1)(5). As B-cells develop from hematopoietic precursors to fully activated effector cells, they rely upon signals generated from the BCR to determine their fate. Signaling through a pre-BCR and later the mature BCR can in some instances lead to cell survival and proliferation, and in others to cell death. In the immunological periphery, mature B-cells sense and respond to antigenic encounter through their BCR. Once the BCR binds antigen it transduces this encounter into intracellular signals via its associated Igα/Igβ heterodimer. These intracellular signals eventuate in up-regulation of genes required for B-cell survival, proliferation, and differentiation into antibody secreting plasma cells. Furthermore, the B-cell internalizes the BCR bound antigen for processing and presentation to helper T-cells, which is required for generation of immunological memory. Signals received from helper T-cells are also critical for somatic hypermutation (SHM) and class-switch recombination (CSR) for isotype switching leading to BCR/antibody affinity maturation. The affinity maturation process enables enhanced antigen specific B-cell activation as well as

production of secreted antibodies with much higher affinity for antigen. Secreted antibody acts to neutralize pathogens, by opsonizing them for clearance by other cells of the immune system, or by complement mediated cytolysis of the pathogen.

1.2 B-cell development

Conventional B-cell development begins in the bone marrow (BM), where hematopoietic stem cell (HSC) precursors develop into progenitor B-cells (pro-B-cells, $B220^{+}/CD43^{+}/CD24^{low}/BP\text{-}1^{low}$) (Fig. 1.2) (7, 8). Stimulatory contact with interleukin-7 (IL-7) producing bone marrow stromal cells aids in the initiation of Ig heavy chain gene rearrangement within the pro-B-cell (9). IL-7 supports both the generation and expansion of progenitors, as well as immunoglobulin gene recombination. Joining of diversity (D_H) and joining (J_H) heavy chain gene segments of Ig occurs at the early pro-B-cell stage while joining of variable (V_H) and DJ segments occurs in the late pro-B-cell stage. Upon successful completion of heavy chain VDJ gene rearrangement, the pro-B-cell transitions to a pre-B-cell ($B220^{+}/CD43^{-}/IgM^{-}$, Fig. 1.2). The rearranged heavy chain of IgM is expressed at the surface of the pre-B-cell in a complex with a surrogate light chain; this complex is termed the pre-BCR (Fig. 1.2) (10). Unsuccessful VDJ recombination can lead to a blockage in B-cell development at this stage, and in some cases lead to cell death, however, successful completion of the pre-BCR in association with Igα/Igβ sends a signal for proliferation and discontinuation of heavy chain VDJ recombination before the cells then proceed to the next stage in development. Cytoplasmic Bruton's tyrosine kinase (Btk), the membrane phosphatase CD45R/B220, CD24 (heat shock antigen; HSA), CD25 (IL-2 receptor), and CD19 (BCR co-receptor) are also expressed at the pre-

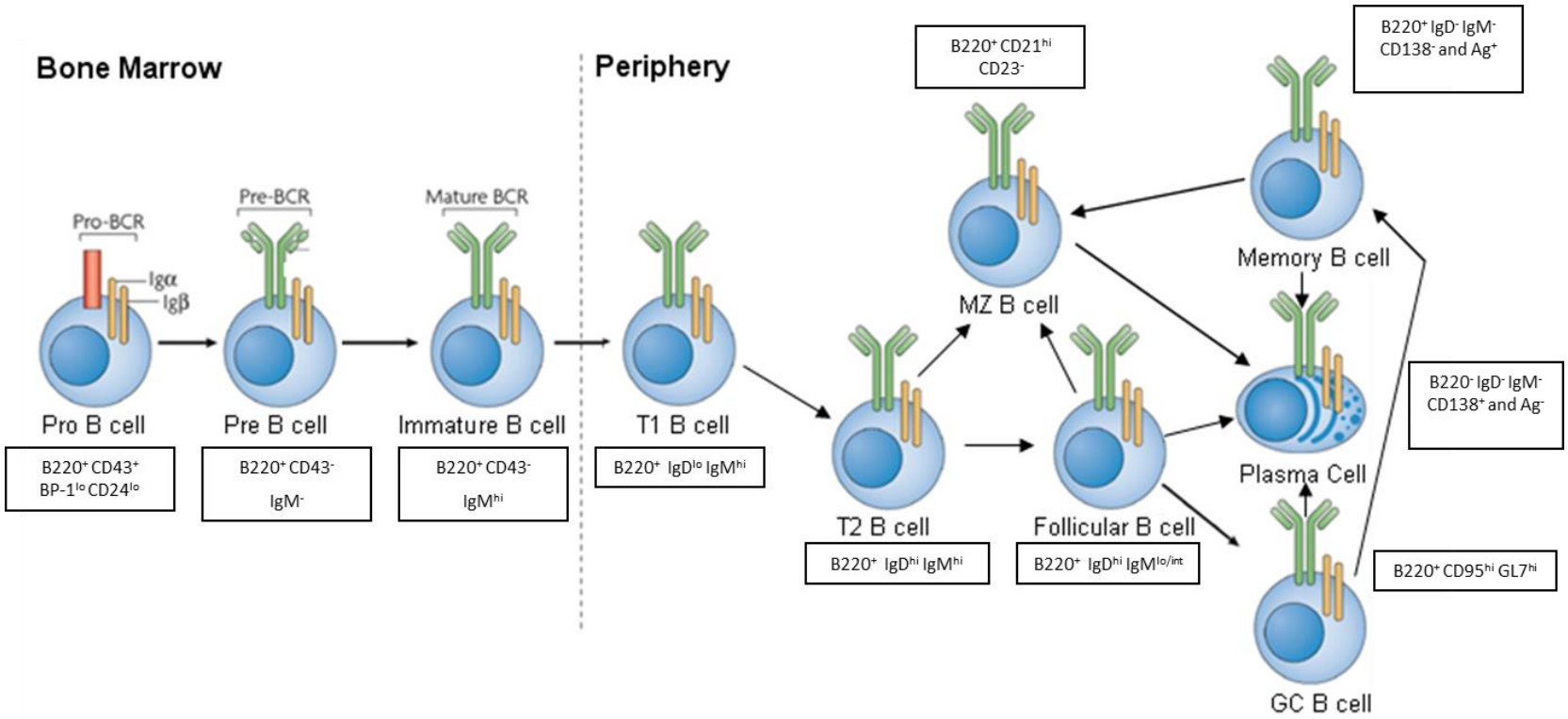

Bone Marrow
Periphery
Pro-BCR
Igα
Igβ
Pre-BCR
Mature BCR
Pro B cell
Pre B cell
Immature B cell
T1 B cell
B220+ CD43+ BP-1lo CD24lo
B220+ CD43- IgM-
B220+ CD43- IgMhi
B220+ IgDlo IgMhi
B220+ CD21hi CD23-
MZ B cell
T2 B cell
Follicular B cell
B220+ IgDhi IgMhi
B220+ IgDhi IgMlo/int
B220+ IgD- IgM- CD138- and Ag+
Memory B cell
Plasma Cell
GC B cell
B220- IgD- IgM- CD138+ and Ag-
B220+ CD95hi GL7hi

Figure 1.2 B-cell development. B-cell development begins in the BM, where HSCs develop into pro-B-cells. Interactions with IL-7 producing BM stromal cells initiates Ig heavy chain gene rearrangement. The rearranged heavy chain associates with a surrogate light chain, forming the Pre-BCR. Successful expression of the Pre-BCR at the surface of B-cells marks the transition to the pre-B-cell stage. The next stage is highlighted by successful rearrangement of the Ig light chain gene. When the rearranged Ig light chain is expressed at the cell surface with the Ig heavy chain, it is termed the mature BCR, and Pre B-cells transition to the Immature B-cell stage. At this stage the cells undergo a process of negative selection, to ensure the B-cells do not bind to self-molecules, and then the B-cells leave the BM, and continue to mature in secondary lymphoid tissues from Transitional 1 (T1) to Transitional 2 (T2) B-cells. Conventional mature naïve B-cells in the immunological periphery consist of marginal zone B-cells (MZ B-cells), and Follicular B-cells (FO B-cells). As mature naïve B-cells encounter antigen in the presence co-stimulatory signals, they proliferate and differentiate into antibody secreting plasma cells. When FO B-cells process and present antigenic peptide to receive help signals from helper T-cells, they migrate and differentiate into germinal center B-cells, which undergo isotype switching and affinity maturation, and can differentiate into long-lived high affinity plasma or memory B-cells. – Image modified from Cambier et al. (2007). Nature Reviews Immunology: PMID 17641666.

B-cell stage, and are necessary for transition to the next B-cell stage (11). The next stage is highlighted by the successful rearrangement of a light chain gene, which leads to cell surface expression of a fully formed IgM-based BCR. At this point the cell is considered an immature B-cell ($B220^{+}/CD43^{-}/IgM^{high}$, Fig. 1.2) (2-4, 12). The immature B-cells in the bone marrow now undergo a process of negative selection which enables the deletion of B-cells which strongly bind to self-antigen. During this process, the fate of the immature B-cell depends upon the strength of the signal originating from the surface Ig. Generally, there are four major outcomes for B-cells which bind to host/self-molecules (self-reactive B-cells): cell death by apoptosis, rearrangement of heavy and light chains to produce a new receptor (receptor editing), initiation of anergy (a permanent state of unresponsiveness to antigens), or clonal ignorance as the antigen is either in low concentration, sequestered, or absent (13, 14). This process thereby ensures what is known as central B-cell tolerance, and B-cells which escape negative selection, either by not binding to self-molecules or binding only minimally then migrate to the immunological periphery.

Immature B-cells leave the bone marrow and migrate to secondary lymphoid tissues where they continue to mature. B-cells newly arriving in the secondary lymphoid tissues have high surface expression of IgM, and low surface expression of IgD and are termed Transitional 1 B-cells (T1, $B220^{+}/IgM^{high}/IgD^{low}$, Fig. 1.2) (12, 15). The cells continue to mature into Transitional 2 B-cells (T2, $B220^{+}/IgM^{high}/IgD^{high}$) that express high levels of both surface IgM and IgD (Fig. 1.2). The development and maintenance of the T1 and T2 cells depends on signals from the BCR and through the BAFF receptor (B-cell activating factor belonging to the tumor-necrosis factor family) (16, 17). These cells

undergo a second round of negative selection in the periphery to ensure tolerance to self-antigens which are not available in the bone marrow; this process ensures what is known as peripheral tolerance. If the cells survive the second round of selection, they will progress to a more mature phenotype based on the expression and activation levels of key proteins such as Btk, CD45/B220, Syk and the Igα chain (18-22). Conventional mature naïve B-cells consist of non-recirculating marginal zone B-cells (MZ, $B220^{+}/CD21^{high}/CD23^{-}$, Fig. 1.2) enriched in the marginal zones of the spleen and recirculating follicular B-cells (FO, $B220^{+}/IgM^{low}/IgD^{high}$) which pass through the peripheral lymphatic system and the follicles of the lymph nodes and spleen where it is speculated they receive signals for survival (23-25). FO B-cells constitute the vast majority of recirculating B-cells (26). MZ B-cells have a more restricted BCR repertoire than their FO counterparts, and the receptors have been shown to be biased toward bacterial cell-wall components. Additionally, MZ B-cells have been show to engage in immune responses as early as four hours after antigenic encounter, which is in part due to a lower threshold than FO B-cells for activation, proliferation, and differentiation into antibody secreting cells (24). MZ B-cells are of indispensable importance in responding to T-cell independent antigens, i.e. antigens which generally do not elicit T-cell help. (27). Once the mature FO and MZ B-cells encounter their cognate antigen and co-stimulatory signals, they undergo a series of transcriptional modifications which instruct the cells to undergo clonal expansion and isotype switching. If FO B-cells receive T-cell help, they form germinal centers (GC), where the GC B-cells ($B220^{+}/CD95^{high}/GL7^{high}$) proliferate rapidly, undergo affinity maturation, isotype switching, and finally become memory B-cells and long lived plasma cells (Fig. 1.2) (26, 28-30).

In addition to the conventional B-cells mentioned above, commonly referred to as B2 cells, another subset of B-cells with antigen receptors of limited diversity exists, termed B1 B-cells (31, 32). B1 B-cells arise from the fetal liver early in embryonic development, and are self-renewing in the periphery. B1 B-cells do not require T cell help, and they possess cell-cycle and activation properties distinct from conventional B2 B-cells. They reside predominantly in the peritoneal (PerC) and pleural cavities, and are a major source of "natural", "innate", or "T-cell independent" antibody, or antibody specific for polymeric antigens, such as polysaccharides and lipids (33-35). These cells can be further divided into B1a and B1b subsets based on the expression of cell surface markers CD5 or Mac1 (CD11b) respectively (B1b - $IgM^+/IgD^-/CD5^+/CD11b^+$ and B1b - $IgM^+/IgD^-/CD5^-/CD11b^+$).

1.3 B-cell effector mechanisms and immunological memory

B-cells encounter and recognize both T-cell independent and T-cell dependent antigens in the immunological periphery, which may be either soluble or presented on the surface of antigen presenting cells (APCs). T-cell independent antigens include predominantly polysaccharide antigens or mitogenic stimuli, which stimulate humoral antibody responses in the absence of T-cell help. T-cell independent antibody response is predominantly secreted IgM, however, recent evidence suggest that a low level of IgG antibody is also produced in the absence of T-cell help. T-cell dependent antigens, on the other hand are usually protein antigens, and once bound by the membrane BCR, induce a series of cellular modifications which allow the B-cells to generate a greatly sustained and more precise response to the invading pathogen.

Upon binding cognate T-cell independent antigen, the Igα/Igβ of the BCR signal for B-cell activation, including up-regulation of chemokine receptors for homing to T-cell rich areas of secondary lymphoid tissues (36, 37), as well as BCR/antigen complex internalization. Once internalized, the antigens are cleaved and loaded onto major histocompatibility complex class II molecules (MHCII) within specialized compartments termed MHC-class II containing compartments (MIIC), and delivered to the cell surface in preparation for presentation to $CD4^+$ T-cells. Within the T-cell rich zones, the B-cells receive additional signals for activation and proliferation from $CD4^+$ T-cells that have been activated by antigenic peptide presented by dendritic cells (DCs), which alters T-cell response to chemokines necessary for directing the T-cells to the B-cell follicle (37). B-cells interact with their cognate T-cells by binding one another via multiple cell surface molecules, and in turn promote further activation. In addition to MHCII loaded with antigenic peptide, which binds the T-cell receptor (TCR) on the surface of the CD4+ T-cells, antigenic stimulation of the B-cells drives the up-regulation of the co-stimulatory molecules B7.1 and B7.2, which bind CD28 on the T-cell surface. CD40, which is constitutively expressed on B-cells, interacts with CD40 ligand (CD40L/CD154) on activated T-cells (38). In addition to cell surface molecule-mediated interaction between the B and T-cells, activated $CD4^+$ T-cells secrete IL-2, IL-4 and IL-5, which promotes production of short lived terminally differentiated antibody secreting cells (plasma cells) and stimulates GC precursors to migrate into primary follicles which contain IgM^+/IgD^+ B-cells, and densely packed follicular dendritic cells (36). Here the GC precursors undergo intense proliferation, and after several days, a defined GC can be observed (Fig.1.3).

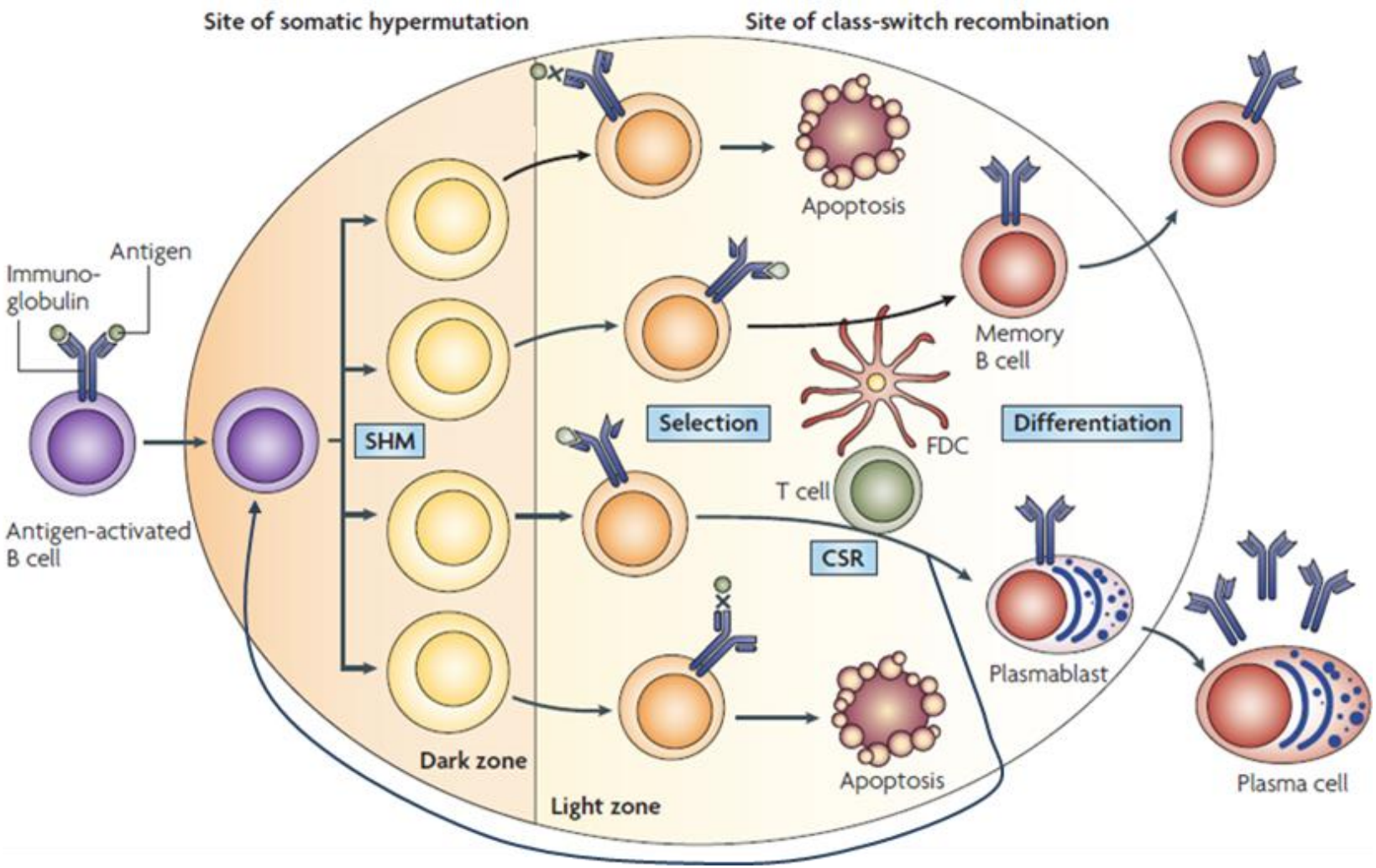

Site of somatic hypermutation
Site of class-switch recombination
Antigen
Immuno-
globulin
Antigen-activated
B cell
SHM
Dark zone
Light zone
Selection
Apoptosis
Memory
B cell
FDC
T cell
CSR
Differentiation
Plasmablast
Plasma cell
Apoptosis

Fig. 1.3 Germinal center formation. Upon antigenic encounter, B-cells undergo clonal expansion within the dark zone of the germinal center. The B-cells undergo somatic hypermutation (SHM), which introduces point mutations into genes within the V-region of Ig heavy and light chains. The B-cells migrate to a follicular dendritic cell (FDC) and T-follicular helper cell (T_{FH}) rich zone, termed the light zone. As the B-cells encounter antigen again on FDCs, the B-cells with higher affinity BCRs (as a result of the SHM) internalize, process, and present antigen to the T_{FH}-cells faster and to a greater extent than clones with lower affinity BCRs. These clones preferentially receive help from the T_{FH}-cells in the form of signals for survival and class switch recombination (CSR) to express different mIg isotypes. 10% of the highest affinity B-cell clones undergo iterative cycles between the dark and light zones, promoting clonal expansion of high affinity GC B-cell, which terminally differentiate into long lived high affinity memory B-cells and plasma cells. Image modified from Klein et al. (2008). Nature Reviews Immunology: PMID 18097447.

Within the germinal centers, the B-cells undergo further proliferation, forming a B-cell dense area termed the dark zone (Fig. 1.3) (39). The rapidly dividing B-cells down regulate cell-surface expression of immunoglobulin, and initiate a phase of affinity maturation. Affinity maturation occurs in activated B-cells, when activation-induced cytidine deaminase (AID) introduces point mutations into genes within the V-region of Ig heavy and light chains, by a process known as somatic hypermutation (SHM). This process acts to diversify the antibody repertoire by altering the affinity of Ig for antigen, and requires additional stimulatory signals from activated T-cells. The B-cells then begin to re-express Ig at their cell surface, and migrate in a chemokine dependent manner to a follicular dendritic cell (FDC), T-follicular helper cell (T_{FH}) and macrophage rich zone, termed the light zone (Fig. 1.3). As the B-cells encounter antigen again on FDCs in the light zone, the B-cells with higher affinity BCRs (as a result of SHM) are capable of internalizing and processing and presenting antigen to the T_{FH}-cells faster and to a greater extent than clones with lower affinity BCRs (30). The higher affinity clones will then preferentially receive help from the activated T_{FH}-cells, and therefore preferentially be selected for survival and receive signals to undergo CSR (class switch recombination, also known as isotype switching) to express a different mIg isotype. Recent research has discovered that approximately 10% of the highest affinity B-cell clones, which as a result capturing and presenting antigen the fastest and to the greatest extent, will undergo iterative cycles between the dark and light zones, thereby promoting selective clonal expansion of GC B-cells with the strongest mIg/antigen binding capacity (30). The high affinity and class switched GC B-cells, with T-cell help, can finally differentiate into long lived high affinity memory B-cells and plasma cells (Fig. 1.2) (26, 28, 29). While B-cells

can be activated withoutT-cell help, affinity maturation and memory B cell differentiation require T-cell help.

Isotype switching is another mechanism whereby activated B-cells adapt to respond more specifically to invading pathogens. During CSR, the rearranged V-region gene segments of the Ig μ (IgM) and δ (IgD) heavy chains recombine with downstream γ, α or ε constant regions, resulting in the production of IgG, IgA or IgE antibodies. While primary and T-independent Ab responses are dominated by low affinity secreted IgM, secondary antibody responses to T-cell dependent antigens that elicit T-cell help are primarily composed of higher affinity IgG, as well as IgA and IgE. While the V-region of the mIg or secreted antibody confers its antigen specificity, the C-region of the antibody is responsible for aiding in the recruitment of effector cells or molecules necessary for clearance of specific pathogens. The Fc (Fragment crystallizable) portions of different isotypes are recognized by isotype specific Fc receptors, activating various phagocytic cells, such as macrophages and neutrophils, which aid in clearance of pathogens opsonized with antibodies, and activate immune responses. Additionally, the Fc portion of secreted IgM and IgG, when bound to the surface of pathogens, recruit complement cascade proteins, and activate complement-mediate lysis of the pathogen. IgE binds to the Fcε receptor I on mast cells, basophils, and eosinophils, which triggers the release of inflammatory mediators. The Fc portion of specific secreted antibody isotypes can also aid in delivering antibody to sites where antibody is not generated, through a process of active transport. In these cases, a group of Fc receptors are responsible for transport of IgA or IgG across epithelial cells to mucous secretions such as tears and milk (IgA), as well as bile and fetal blood circulation (IgG). Furthermore, Fc

receptor-mediated endocytosis of Ig, in certain cases, protects antibodies from degradation, extending their lifetime (40).

B cells differentiate into long lived plasma cells or memory B-cells via affinity maturation and isotype switching in the germinal center, before entering the circulation. The long lived plasma cells migrate to the bone marrow which provides a survival niche. In the bone marrow, the long-lived plasma cells continuously secret high affinity antibodies which act to protect the host, in some cases for the lifetime of the animal. Long lived high affinity memory B-cells circulate within the host, or migrate to the marginal zone of the spleen, where they are poised to rapidly differentiate into high affinity antibody secreting plasma cells in the event of antigenic re-exposure [25]. Generation of long lived high affinity antibody secreting plasma cells and antigen experienced memory B-cells are thus a hallmark of the adaptive immune response and the basis of the long-term protection induced by immunization.

1.4 B-cell dysregulation: immunodeficiency, autoimmunity, and B-cell cancers

In healthy individuals, B-cells work with other branches of the immune system to generate a robust immune response towards invading pathogens, with the outcome of pathogen clearance and procurement of long lived antigenic memory. After a pathogen is cleared from the body, B cell-mediated antibody responses are down-regulated, and B-cell populations return to a resting state. Within a healthy individual, there is a delicate balance between too few B-cells and too many B-cells, as well as an ability to respond to antigens versus an over- or under-reactive response to perceived antigenic threats. The

best studied cases in which B-cells contribute to a disease state include immunodeficiency, autoimmunity, and B-cell cancers (leukemia and lymphoma).

Primary immunodeficiency (PI) is caused by genetic mutations, and results in impairment or complete lack of some aspect of the immune system. Consequently, the main symptom of PIs is an inability to fight off infections. PIs have been associated with defects in almost every cell type of the immune system, and while some PI's are caused by defects in one cell type, others may have more broad defects observed in multiple cell types. With regards to B-cells, there are two major categories of immunodeficiency: those in which all or individual B-cell subsets are absent or reduced, and those in which B-cells (either because of inherent defects or defects in other "helper" populations) are incapable of generating a robust humoral immune response. An example of one of the best known PI's includes X-linked agammaglobulinemia (XLA), also known as Bruton agammaglobulinemia. XLA is the result of a mutation of the *BTK* gene, and while Pro-B-cells with *BTK* mutation are present in normal numbers, they are unable to mature to pre-B-cells (41). Patients with XLA are especially susceptible to recurrent bacterial infections, usually of the respiratory and gastrointestinal tract. Another example of B-cell immunodeficiency is X-linked hyper-IgM (XHM), which is due to a mutation in the gene for CD40 ligand (CD40L/CD154). While B-cells in patients with XHM can initiate an IgM response, they are incapable of undergoing class-switch recombination, and therefore over produce IgM, while having little or no other antibody isotype production (42). People who suffer from XHM have increased susceptibility to infection with bacteria, viruses, fungi, and parasites. In addition, they are at increased risk for developing autoimmune disorders and malignancies (43).

Autoimmune disease is speculated to arise as a result of genetic mutations (whether from environmental/viral/bacterial/toxin exposure and/or inheritance), and in many cases results in overactive B-cell signaling, or impaired negative signaling, which promotes selective survival and proliferation of B-cell clones with "auto-reactive" BCRs, which are normally eliminated by negative selection during B-cell development. The plasma cells that differentiate from these auto-reactive B cells produce self-reactive antibodies, also known as auto-antibodies (autoAb), that can have specificity for a vast number of host molecules (e.g. anti-ds-DNA, anti-histone, etc). AutoAbs have been detected in many autoimmune disease models, including Type I diabetes, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, psoriasis, thyroiditis, heart disease and myasthenia gravis (44). In many cases, the autoAbs target host molecules or cells for destruction, whether by complement mediated cytolysis or phagocytosis by other cell types, thereby being a critical immunopathological factor.

The fundamental problem in treatment of autoimmune disease lies in the fact that the precise molecular mechanisms that promote the shift in B-cell tolerance towards a pathogenic antibody response remains elusive, therefore targeted treatments remain stalled or impractical. However, recent studies have revealed a role for a subset of T and B-cells, called T- and B-regulatory cells (Tregs/Bregs), in preventing autoimmunity by means of suppressing the activation of a various immune cells. While both Tregs and Bregs employ multiple means by which they aid in maintenance of T/B-cell tolerance to prevent autoimmunity, both cell types rely heavily on the production of the anti-inflammatory cytokine IL-10 to moderate their suppressive activities (45, 46). Production of IL-10, whether by Tregs or Bregs, primarily aids in preventing activation

of effector T-cells and DC's, which subsequently prevents activation of auto-reactive effector T/B-cells, and the induction of autoimmunity (45, 46). Furthermore, production of IL-10 by Bregs has also been shown to promote the differentiation of Tregs (47). Current research utilizing T/Bregs has attempted to adoptively transfer either natural or induced T/Bregs to achieve targeted suppression of inflammatory or autoimmune disease progression, with promising results (48-50).

Additional research aimed at preventing or suppressing autoimmune disease has focused on the role of aberrant cell signaling within auto-reactive prone cell subsets. Many of these studies have again focused on aberrant B-cell signaling, as production of autoAbs in many instances is a hallmark of autoimmune disease development. Signaling molecules within B-cells discovered to aid in tolerance towards self-antigens play diverse roles in within B-cells. These molecules include cell cycle modulators, actin mediators, negative regulatory kinases and phosphatases, as well as adaptor molecules (51-55). Our recent research, as well as others, has highlighted a role for the actin regulating proteins Wiskott–Aldrich Syndrome protein (WASP) and Neural Wiskott–Aldrich Syndrome protein (N-WASP), as well as the negative regulatory signaling molecules Hematopoietic progenitor kinase 1 (HPK1) and SH2-domain-containing inositol 5-phosphatase (SHIP-1) in loss of B-cells tolerance (52, 54, 56, 57). Briefly, B-cell specific loss of WASP, N-WASP, SHIP-1, or HPK1 has been shown to promote induction of an autoimmune phenotype (52, 54, 56, 58, 59).

B-cell cancers usually arise as a result of genetic mutations caused by viral infection, environmental or inherited factors (60). In many cases, these B-cell modifications can cause constitutive activation, or deactivation of key regulatory

elements of B-cell singling (60), which in turn results in enhanced B-cell survival and proliferation, which are hallmarks of B-cell cancer. These B-cell mutations can occur within almost any development subset, and give rise to distinct B-cell cancers (60). Uncontrolled proliferation of the cancerous B-cells can inhibit the growth/differentiation of normal B cells and other cells, which in turn leads to a wide variety of pathologies. Additionally, cancerous B-cells have also been shown to metastasize to areas of the body they are not normally found, where they then develop into tumors. Many newly developed drugs to treat B-cell cancers deviate from traditional chemotherapeutic treatments (which are not cell specific, and in many cases simply target rapidly dividing cells), by specifically targeting B-cels for destruction (i.e. monoclonal Abs such as anti-CD20 (Rituxan®)), or by disrupting B-cell specific signaling pathways, such as those therapies recently enlisted to regulate Btk, Phosphoinositide 3 kinase δ (PI3Kδ), BCR-Abl (Gleevec®), and Syk signaling (60, 61).

As the diversity of B cell-mediated immune disorders and cancers continuously increases and targeted treatments are still lacking, the full elucidation of the cellular and molecular mechanisms by which BCR signaling is regulated for B-cell development, survival, activation, and attenuation becomes critical for diagnosis and treatment of B-cell immunodeficiency, autoimmunity, and B-cell cancer.

1.5 The BCR and its signaling pathways

The BCR is the antigen sensor of B cells, consisting of mIg associated with the Igα/Igβ heterodimer. The membrane Ig of the BCR is responsible for antigen binding, while the Igα/Igβ heterodimer propagates intracellular signals (62). The mIg of the BCR

is composed of two identical heavy and light chains with which each BCR is able to bind two identical antigenic epitopes simultaneously (Fig. 1.1). Each heavy chain is bound covalently by a disulfide bond to a single light chain, and the two heavy chains are bound together by one or more disulfide bonds. The transmembrane domain anchors the mIg at the B cell surface, exposing two antigen binding variable regions for antigen binding. The non-covalent interaction between the mIg and the Igα/Igβ heterodimer involves their trans-membrane domains, and this association is required for expression of the BCR at the surface of B-cells (63). Igα and Igβ are transmembrane proteins linked by disulfide bonds in the extracellular domains. The cytoplasmic domains of Igα and Igβ each contain one immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic tails with the sequence D/E XX-YXXφ-X_{6-8}-YXXφ, where X is any amino acid and φ is a bulky hydrophobic amino acid (64, 65). ITAMs are conserved signal transduction motifs shared by many signaling receptors within the immune system, including the signaling apparatus of T-cells, the TCR (65). Upon B-cell activation by antigen binding, the ITAMs are phosphorylated at their tyrosine residues (Y), and phosphorylated ITAMs serve as docking sites for spleen tyrosine kinase (Syk), thereby initiating formation of a signaling platform known as a "signalosome" (64, 66, 67).

BCR signal activation requires receptor oligomerization into two dimensional clusters of different sizes depending on the valency and physical configuration of the antigen. BCR clusters become preferentially associated with "lipid rafts" which are rich in cholesterol and glycosphingolipids, thereby forming relatively stable regions on the outer leaflet of the plasma membrane. The ordered lipid environment acts to sequester or exclude molecules based on their biochemical properties (68-70). Lipid rafts which are

enriched with signaling and cytoskeletal elements, have been shown to be important signaling and endocytosis sites (68-70). Studies by Tolar et al. suggest that binding of antigen induces a conformational shift in the cytoplasmic domain of the Igα/β heterodimer, from a "closed" to an "open" conformation. This conformational change has been proposed to allow phosphorylation of ITAMs within Igα/β by raft resident Src-family kinases (SFK): particularly Lyn, which is the predominant SFK expressed in B cells (22, 71, 72). Dual phosphorylation of ITAM tyrosines leads to the binding of the Src-homology 2 (SH2) domain containing Syk via its tandem SH2 domains, recruiting it to surface signaling microdomains (Fig. 1.4) (73, 74). Syk is then activated by Src kinases as well as auto-phosphorylation (75). Syk acts as a key player in propagating a multitude of downstream signaling events, and disruption of Syk signaling blocks most downstream BCR signaling events (76). Activated Syk subsequently phosphorylates the major B-cell associated adaptor protein, B-cell Linker protein (BLNK/SLP-65), and these phosphorylated tyrosines act as docking sites for multiple SH2 domain containing proteins such as Btk, Phospholipase Cγ2 (PLCγ2), Vav, and the adaptor protein Grb-2 (Fig. 1.4) (77-79). Scaffolding/adaptor proteins such as BLNK are essential for linking BCRs to downstream kinases by recruiting them and their substrates into the proximity of clustered BCRs. The importance of such adaptor molecules is underscored by studies that showed that BLNK deficient mice display profound defects in B-cell signaling and development (77, 80, 81).

PI3K, which upon recruitment by the BCR co-receptor CD19 or the adaptor molecule B-cell adaptor molecule for PI3K (BCAP), phosphorylates phosphatidylinositol

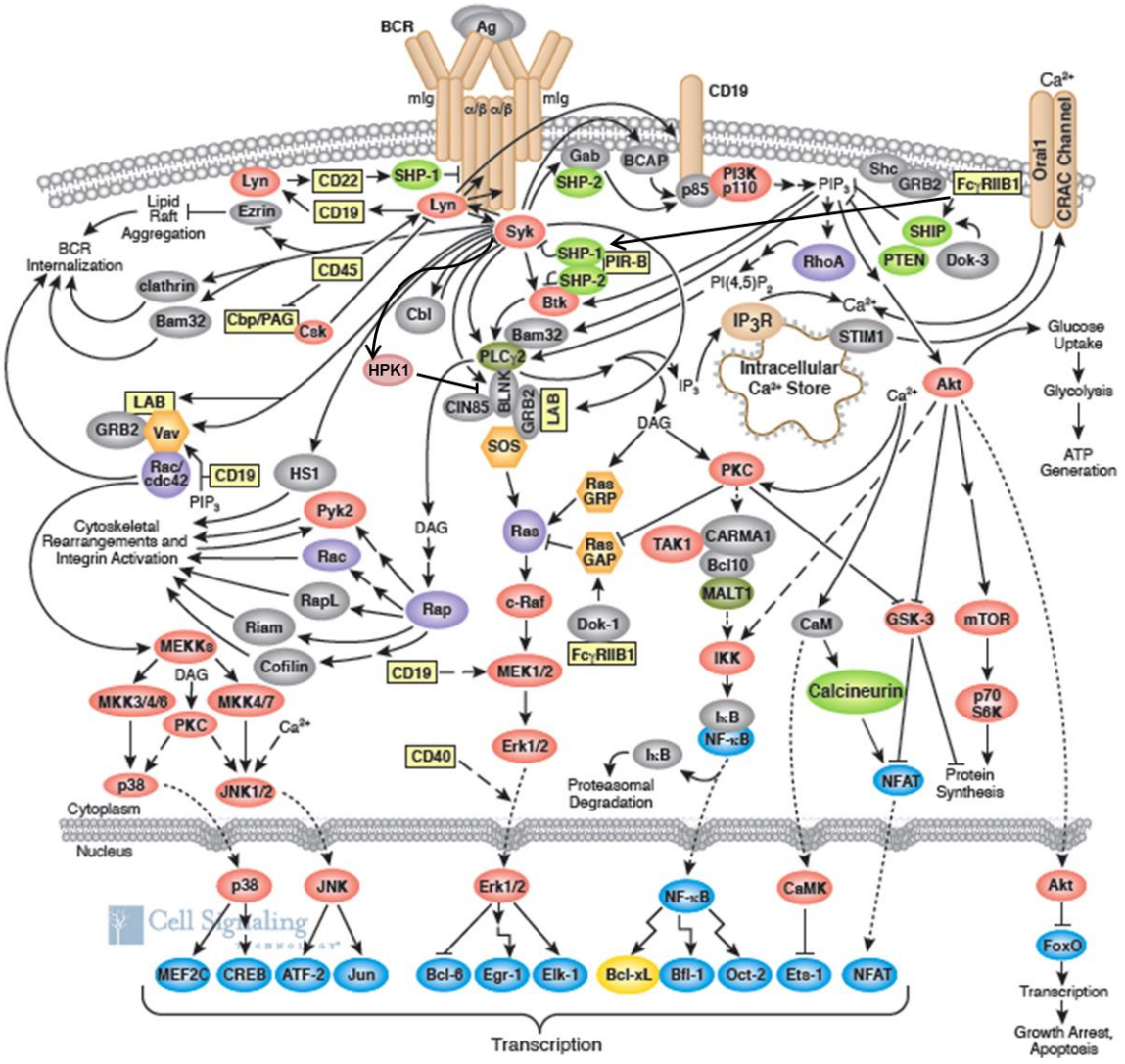

BCR
Ag
mIg
α/β α/β
mIg
CD19
Ca2+
Orai1
CRAC Channel
Gab
BCAP
SHP-2
p85
PI3K p110
PIP3
Shc
GRB2
FcγRIIB1
Lyn
CD22
SHP-1
Lyn
Lipid Raft Aggregation
Ezrin
CD19
Syk
SHP-1
PIR-B
SHP-2
SHIP
PTEN
Dok-3
RhoA
PI(4,5)P2
BCR Internalization
clathrin
Bam32
CD45
Cbp/PAG
Csk
Cbl
Btk
Bam32
PLCγ2
IP3R
STIM1
Ca2+
Intracellular Ca2+ Store
IP3
HPK1
CIN85
BLNK
GRB2
LAB
SOS
Glucose Uptake
Glycolysis
ATP Generation
Akt
LAB
GRB2
Vav
Rac/ cdc42
CD19
PIP3
HS1
DAG
Ras GRP
PKC
Cytoskeletal Rearrangements and Integrin Activation
Pyk2
Rac
RapL
Rap
Riam
Cofilin
DAG
Ras
Ras GAP
TAK1
CARMA1
Bcl10
MALT1
c-Raf
Dok-1
FcγRIIB1
IKK
CaM
GSK-3
mTOR
MEKKs
DAG
MKK3/4/6
MKK4/7
PKC
Ca2+
CD19
MEK1/2
Calcineurin
p70 S6K
IκB
NF-κB
CD40
Erk1/2
IκB
Proteasomal Degradation
NFAT
Protein Synthesis
p38
JNK1/2
Cytoplasm
Nucleus
p38
JNK
Erk1/2
NF-κB
CaMK
Akt
FoxO
Cell Signaling Technology
MEF2C
CREB
ATF-2
Jun
Bcl-6
Egr-1
Elk-1
Bcl-xL
Bfl-1
Oct-2
Ets-1
NFAT
Transcription
Transcription
Growth Arrest, Apoptosis

Figure 1.4 The BCR and its signaling pathways. B-cell signaling is initiated when mIg binds antigen. Immediately following antigen engagement, the BCRs translocate to lipid rafts, where the signaling apartus of the BCR (the Igα/β heterodimer) comes into contact with raft resident Src kinases. The Src kinases phosphoryalte tyrosine residues within the ITAM sequence of Igα/β, which then serve as docking sites for the key B-cell signaling kinase Syk. Syk inturn, phosphorylates and activates a multitude of downstream kinases, GTPases, and adaptor proteins, which act to propagate downstream signals necessary for actin cytoskeltal modificaiton, and transicriptional regulation of cellular differentiation, activation, proliferation, and survival. The complex nature of BCR signaling allows a mulititude of cellular responses, which also depend upon the maturational state of the B-cell, the nature of the antigen bound, the duration and magnitude of the signal, as well as engagement of co-receptors. The duration and magnitude of BCR mediated signaling are limited by a variety signaling elements inclding co-ligation of the BCR with FcγRIIB by immune complex which activates negative regulatory SHIP-1 and SHP1, by activation of HPK1 (with subsequent down-regulation of the major adaptor protein BLNK), as well as BCR internalization. Image modified from Cell Signaling Technologies, http://www.cellsignal.com/common/content/content.jsp?id=pathways-bcell

4,5-bisphosphate (PIP_2), to form PIP_3 (82, 83). PIP_3 then severs as a docking site for molecules containing pleckstrin homology (PH) domains, thereby recruiting them to signalosomes (84, 85). Recruitment of Btk to the membrane occurs by its interaction with BLNK as well as the interactions of its (PH) domain with phosphatidylinositol 3,4,5-trisphosphate (PIP_3) in lipid rafts. Once recruited to the plasma membrane, Btk becomes a target for phosphorylation by Src family kinases and Syk within a kinase activation loop, which enables Btk to auto-phosphorylate and become fully activated (86-89). Once bound to BLNK and fully activated, Btk acts in concert with Syk to phosphorylate PLCγ2, which is recruited by BLNK (90, 91). The activation of both Btk and PLCγ2 in turn positively feeds back into PI3K activation and activation of the Ras-Raf-MEK1/2- Extracellular signal regulated kinase (ERK) pathways (Fig. 1.4) (92, 93). Furthermore, Btk is responsible for activating the actin nucleation promoting factor WASP, which is necessary for dynamic actin reorganization required for B-cell antigenic responses (89). Additionally, Syk, Btk and PI3K work in concert to activate the serine/threonine kinase Akt (Fig. 1.4) (94, 95), which then acts to induce B-cell proliferation by inhibiting the pro-apoptotic protein BAD (Bcl-associated death promoter) (96). Mutational studies of Btk have revealed that the PH domain of Btk is necessary for its activation, and that Btk activation is critical for B-cell development in humans. A point mutation in the PH domain of Btk, which blocks the recruitment of Btk to the plasma membrane leads to humoral immunity defects (97).

PLCγ2, activated by Btk and Syk (90, 98, 99), cleaves PIP_2, generating inositol trisphosphate (IP3) and diacylglycerol (DAG, Fig. 1.4). The binding of IP3 to its specific receptor on the endoplasmic reticulum (ER) causes the release of intracellular calcium

stores. The depletion of ER Ca^{2+} promotes the activation of calcium-release-activated channels (CRAC) at the plasma membrane and allows for the influx of extracellular Ca^{2+} ions (Fig.1.4) (100, 101). DAG, in concert with calcium, activates protein kinase C-β (PKC-β), which eventuates in activation of the transcription factor nuclear factor kappa B (NF-κB) by phosphorylating IκB Kinase (IKK, Fig. 1.4) (102). NF-κB then up-regulates anti-apoptotic proteins such as Bcl-xl and cyclin D2 (103, 104). DAG also activates Ras guanyl nucleotide-releasing protein (RasGRP), a potent activator of Ras. Ras is upstream of the Raf/MEK1/2 pathway, and Ras activation results in the activation of the mitogen activated protein kinase (MAPK) ERK (105, 106). Ca^{2+} also activates calcineurin, which in turn activates the transcription factor NFAT (nuclear factor of activated T cells, Fig. 1.4). Both NFAT and NF-κB are essential for transcriptional activation of genes required for B-cell proliferation, survival and differentiation (107).

The interaction of Vav and Growth factor receptor-bound protein 2 (Grb2)/ Son-of-sevenless (SOS, a guanine exchange factor) with phosphorylated tyrosines on both CD19 and BLNK activate the Ras and Rho family GTPases respectively. SOS and PKC-β activated RasGRP work in concert to activate Ras followed by subsequent ERK activation (Fig. 1.4). Activation of the Rho-family GTPase Rac leads to activation of the MAP kinases c-Jun NH2-terminal kinase (JNK) and p38 (Fig. 1.4) (76, 108-110). The MAP kinase JNK then activates the transcription factors c-jun and ATF-2 (110), while ERK activates Elk-1 and c-myc (111), and p38 activates ATF-2 (112), all of which cooperate with NF-κB and NFAT to determine the cellular response to antigen engagement (103, 110, 111, 113).

The BCR also utilizes co-receptors, such as CD19, to control and amplify BCR initiated signals. CD19 is a B-cell specific co-receptor, which is expressed early in B-cell development. On the B-cell plasma membrane, CD19 is predominantly expressed in a complex with CD21 (a complement receptor), and tetraspanin CD81 (114). When CD21 binds antigens tagged with complement, it acts to crosslink the BCR and CD19 complex, and effectively lowers the threshold for B-cell activation (115). Co-ligation of the BCR and the CD19 containing complex enables recruitment of the signaling molecules Vav, PI3K, and Lyn to CD19, and subsequent activation of PLCγ2 and MAP kinase pathways (115). B-cells deficient in CD19 are defective in initiating BCR stimulated signaling, as well as recruitment of downstream effector, and cell spreading, when incubated with antigen presenting membranes (116). Mice deficient in CD19 or CD21 show reduced humoral immune responses towards T-cell dependent antigens, reduced germinal center formation, and defects in antibody secretion as well as defective affinity maturation (117-119).

BCR signaling is relatively transient and tightly controlled by various inhibitory molecules, including phosphatases and kinases. Down regulation of BCR signaling has been shown to be important, as lack of signal attenuation has been associated with a number of autoimmune disorders (53, 120). One of the best studied mechanisms of BCR signal attenuation occurs when antibody-antigen immune complexes co-ligate the BCR with the low affinity Fc-gamma receptor IIB (FcγRIIB) co-receptor. This receptor is composed of an extracellular Fc-binding domain and an intracellular domain which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) (53). As its name suggests, the FcγRIIB receptor binds the Fc Region of IgG. When IgG is produced to

sufficient levels during an immune response, and most of the remaining antigen is bound with IgG, the BCR and FcγRIIB bind the antigen and antibody portions of the immune complex, and this co-ligation brings the ITIM of the FcγRIIB into close proximity of the lipid raft resident kinase Lyn. Lyn subsequently phosphorylates the tyrosine residue within the ITIM, thereby providing a docking site for the inhibitory phosphatases SH2-domain containing protein tyrosine phosphatase (SHP1), and SH2-domain-containing inositol 5-phosphatase (SHIP-1 – Fig.1.4) (121). SHP1 is a protein tyrosine phosphatase which blocks signal activation by dephosphorylation, while SHIP-1 is an inositol phosphatase that specifically hydrolyzes PIP_3 to PIP_2, thereby removing the docking site for PH domain containing signaling molecules, e.g. Btk, Akt, and PLCγ2 (59). Of note, BCR activation by antigen alone also induces SHIP-1 activation. Additionally, SHIP-1 has been shown to bind BCR ITAMs with only one of the two tyrosines phosphorylated in anergic B-cells, which is critical for maintenance of the anergic state (122). As a result of the action of these two phosphatases, BCR mediated cell survival, cellular proliferation, antibody production and cytokine release are disrupted (123-125). Thus, these two inhibitory molecules work in concert to attenuate BCR signaling, which is necessary to maintain B-cell tolerance (44).

Another known mechanism of B-cell signal attenuation involves the protein HPK1, a serine/threonine kinase. BCR signaling activates HPK1 by preferentially linking HPK1 with the adaptor molecule BLNK (Fig. 1.4). Once bound, HPK1 phosphorylates BLNK at threonine 152, which induces BLNK/14-3-3 binding (55). Once bound to Thr152, 14-3-3 is suggested to recruit a currently unknown E3 ligase, which ubiquitinates BLNK at the Lysine residues Lys-37, Lys-38, and Lys-42 (55). This

ubiquitination then promotes proteasomal degradation of BLNK, and subsequent down regulation of BCR signaling (55). HPK1 deficiency has been shown to be associated with up-regulated MAPK activation, specifically, ERK, JNK, and p38, as well as IKK activation, B-cell proliferation, and up-regulation of several activation markers (55). Dysregulated B-cell signaling profiles, resulting from HPK1 deficiency, have been shown to be associated with the increased antibody production and experimental autoimmune encephalomyelitis (56, 126).

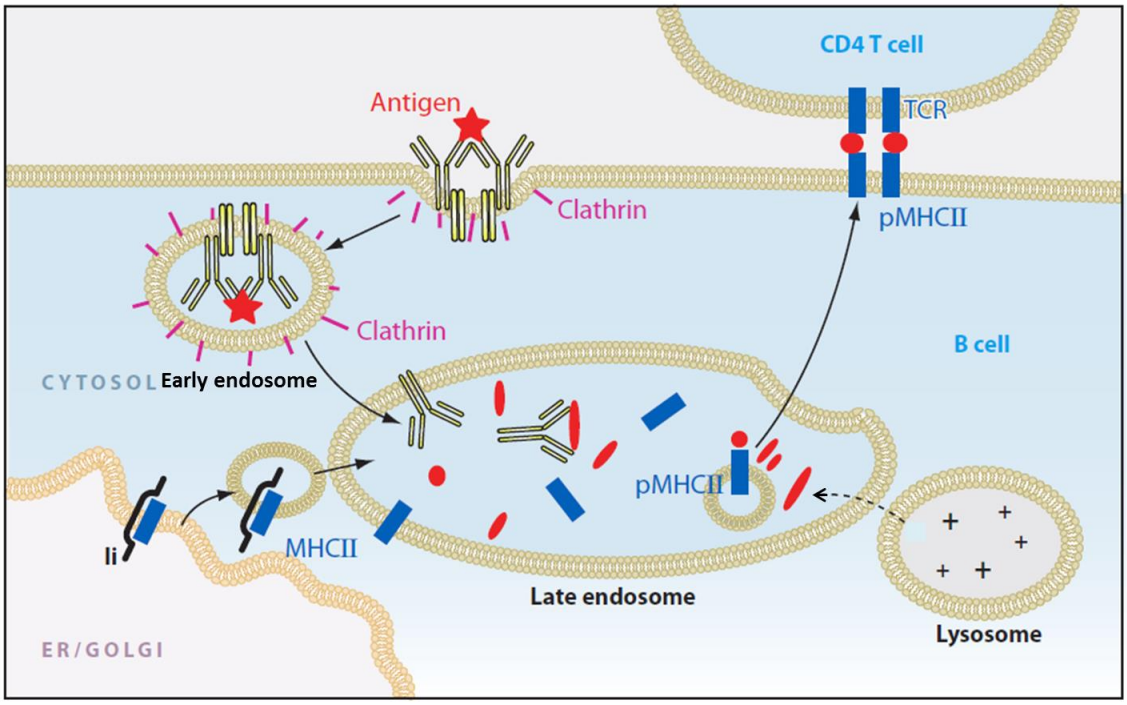
1.6 BCR mediated antigen processing and presentation

In addition to induction of signaling from the BCR, necessary for the implementation of genetic modifications for a multitude of cellular responses, antigen binding also promotes the internalization of the BCR/antigen complex, which in turn serves to down-regulate signaling at the B-cell surface (127, 128). The BCR/antigen complex is internalized constitutively at low levels by fluid-phase pinocytosis into early endosomes, after which many BCRs are recycled back to the cell surface (129). However, it has been demonstrated that BCR cross-linking by multivalent antigens can substantially enhance the rate of antigen up-take and processing for presentation to $CD4^+$ T-cells (130, 131). As such, antigens at very low levels can still stimulate an adaptive immune response. The major mechanism for BCR internalization is clathrin-mediated endocytosis (CME) (128). Upon binding its cognate antigen, the BCR is rapidly translocated to lipid rafts rich in cholesterol, glycosphingolipid, phosphatidylinositides, and signaling molecules. Recognition of the specific internalization motif on the cytoplasmic tail of Igβ, and cis regulatory elements in Igα, leads to the recruitment and

binding of the adaptor protein AP-2 (132, 133). AP-2 then aids in the recruitment of clathrin (134). A study from our lab, in collaboration with Dr. Francis Brodsky, showed BCR induced recruitment of clathrin to the plasma membrane and BCR-containing vesicles, and demonstrated that the Src-kinase inhibitor PP2 blocks this recruitment (128). The phosphorylation of clathrin in lipid rafts by Src-kinases following BCR crosslinking is required for BCR internalization, which was demonstrated by inhibition of clathrin phosphorylation and subsequent BCR internalization by the disruption of lipid rafts (135). Once clathrin has been recruited to the plasma membrane and phosphorylated, the membrane begins to invaginate to form a coated-pit. Coated pit invagination is driven by local membrane changes in lipid composition, natural curvature of the clathrin triskelion, recruitment of BAR domain containing proteins, and forces applied by the actin cytoskeleton (136-139). This process initiates the formation of the actin associated endocytic platform. The Bar-domain containing proteins recruited to the clathrin pit include endophilin and amphiphysin. Endophilin acts to alter local lipid compositions at the plasma membrane via its acyl transferase activity, and binds dynamin, the large GTPase "mechanoenzyme" which catalyzes the pinching off of the newly formed vesicles (140). Amphiphysin, which also plays a major role in recruiting dynamin to endocytic vesicles during CME, also binds AP-2 and clathrin (132, 141-144). After the internalizing vesicle has grown into a deeply invaginated pit, proteins such as dynamin, amphiphysin, and endophilin interact with filamentous actin (F-actin) to promote vesicle scission (139). The actual scission step occurs when dynamin Guanosine-5'-triphosphate (GTP) is hydrolyzed, and the prompt for this hydrolysis may include interactions with PIP_2, sorting nexin-9, amphiphysin, and dynamin GED domains

(145). Dynamin's interaction with F-actin and the nascent endocytic vesicle most likely occurs via interactions with syndapin oligomers, profilin, cortactin, and actin-binding protein 1(Abp1) (145-150). Syndapin oligomers themselves recruit the actin regulating protein N-WASP to the plasma membrane where (like WASP) they cause localized F-actin rearrangement via Arp2/3 (146). Once the vesicles have pinched off, the ATPase Hsc70 is recruited to the clathrin coated vesicle, where it aids in the release of the clathrin coat (151). Then, the internalizing vesicles fuse with one another, and form early endosomes (152).

According the maturation model of endosomal compartment trafficking, the early endosome undergoes acidification by acquiring proton pumps from Golgi. As it progresses to the interior of the cell, endosome membrane buds inward via the endosomal sorting complex required for transport (ESCRT) system, forming multivesicular bodies, while continuously acquiring lysosomal proteins from Golgi, such as lysosomal-associated membrane protein 1 (LAMP-1) and protein hydrolases (153). At this stage the multivesicular body is generally termed the late endosome (Fig. 1.5). The reduced pH activates proteases, which in turn cleave the antigen into peptide fragments. During this process newly synthesized MHC-II molecules from the ER and Golgi are delivered to late endosomes by vesicular transport, after which this compartment is termed MIIC in antigen presenting cells (154). This is where the antigenic peptide is loaded onto MHCII molecules, and then shuttled to the surface of the cell for presentation to $CD4^+$ T-cells (Fig.1.5). $CD4^+$ T-cells recognize the antigenic peptide-MHCII complex via their TCR (Fig. 1.5). The TCR is structured in such a manner that it can only relay



1.5 BCR mediated antigen processing and presentation. Upon BCR ligation, the signaling apparatus of the BCR initiates signals to direct BCR/antigen complex internalization into early endosomes via CME. As the early endosomes internalize they begin to decrease in pH. The enocytosing vesicles acquire LAMP-1 and protein hydrolases. Reduced pH within the vesicles serves to activate acid proteases, which cleave the internalized antigen into fragments. MHCII containing vesicles from the ER fuse with the late endosome, now termed the MIIC, where antigenic peptide is loaded on to the newly made MHCII, then shuttled to the surface of the B-cell for presentation to $CD4^+$ T_H cells, which in turn provide signals for enhanced B-cell activation and clearance of T-dependent antigens. Image modified from Kurosaki et al. (2010). Annual Reviews of Immunology. PMID 19827951.

intracellular signals sufficient for T-cells activation when the antigen-derived peptide is complexed with a MHC molecule (155).

It has become apparent in recent years that BCR-mediated signaling and BCR/antigen trafficking for processing and presentation are closely linked . While it has been shown that phosphorylation of the ITAMs within the Igα/β heterodimer is ultimately not necessary for BCR internalization, stable co-expression of mIg with the Igα/β heterodimer is required (156). As mentioned previously, while BCR crosslinking is not necessary for BCR/mono-valent antigen internalization, crosslinking by multivalent antigens greatly enhances the speed of antigen processing and presentation (130). It was shown that the nature of the BCR/antigen interaction affects both BCR mediated signaling and endocytosis. Antigens that only minimally crosslink BCRs are rapidly internalized for processing and presentation, and generate relatively transient BCR signal activation (157). On the other hand, large multivalent antigens that highly cross link BCRs at the surface of the cells are internalized much more slowly, and generate elevated and prolonged signaling profiles (157). B-cells deficient in Lyn were unable to internalize their BCRs (158), while another study demonstrated the inability of Syk deficient B-cells to fuse BCR/antigen containing endosomal compartments with compartments containing MHCII, thereby disabling their ability to form peptide-MHCII complexes for antigenic presentation (159). A role for these early kinases was further defined by the finding that treatment of B-cells with the Src-family kinase inhibitor PP2 prevented both the recruitment and phosphorylation of clathrin, thereby severely reducing BCR mediated internalization (128).

Early studies speculated that BCR/antigen internalization served to extinguish BCR mediated signaling, however, recent studies have suggested that while some aspects of the signaling are extinguished upon BCR internalization, sequential phosphorylation of downstream kinases continue to take place as the BCR/antigen complex traffic through endosomal compartments for antigenic processing (160). It was shown that upon blocking BCR/antigen internalization with the dynamin I and II disrupting agent Dynasore, BCR proximal and distal MAP kinases were recruited to the plasma membrane, where they became hyperphosphorylated, which led to dysregulation of B-cell gene transcription (160). As such, these data suggest that the subcellular localization of the BCR regulates or "compartmentalizes" sequential aspects of BCR mediated signaling, thereby regulating B-cell activation (160). This and the aforementioned studies strongly suggest that BCR signaling and BCR endocytosis regulate each other.

1.7 BCR dynamics during B-cell activation

Recent studies have indicated that the mobility and organization of BCRs at the surface of B-cells influence the activation kinetics and magnitude of the B-cell signaling (161-163). Advances in high resolution and live-cell imaging techniques have allowed better real time analysis of BCR dynamics in both resting cells and upon receptor engagement. While BCR clustering has been shown to be essential for the BCR to initiate signal transduction, it has been suggested, through direct stochastic optical reconstruction microscopy (114) and quantitative bifluorescence complementation assays (164), that the BCRs at the surface of the unstimulated B-cell may be present in nanoclusters that are not visible using conventional microscopy techniques. However,

this work remains controversial. It was suggested that the BCRs in these nanoclusters are likely in an inhibitory conformation (164, 165), but that their presence is probably required for mediating some level of tonic signaling required for B-cell survival (114).

Tolar et al. showed that the BCR itself maintains an intrinsic ability to cluster into oligomers, which is dependent upon the Cμ4 domain of mIgM, as well as a short N-terminal sequence within the transmembrane domain of mIgM (166). And while expression of the full length wild-type (wt) BCR at the surface of the B-cell requires antigenic ligation for clustering and signal induction, expression of a truncated BCR, where the extracellular domain only contains the Cμ4 segment, is sufficient to induce clustering and signal initiation in the absence of extracellular signals (166). These results suggest that elements within the Fc portion of mIgM may be hidden or "sterically blocked" in the absence of antigenic binding, and binding unveils these regions necessary for receptor clustering (166).

Upon antigen binding, BCRs increase lateral mobility, and they begin to migrate to form microclusters (167, 168). As the microclusters form, they become preferentially associated with lipid rafts, which enables raft resident kinases to phosphorylate Igα/Igβ, initiating signaling (116, 166, 169). BCRs within the signaling active microclusters, also referred to as signalosomes, become relatively immobile (166), but continue to grow by recruiting more BCRs into the microcluster over the course of several minutes. Concurrent with microcluster growth, B-cells interacting with antigen presenting surfaces, whether on APCs in vivo, or on artificial antigen presenting surfaces in vitro, spread along the presenting surface. The spreading allows the cell to accumulate more antigen, thereby activating more BCRs, all while simultaneously recruiting more BCRs

into the immobilized microclusters. As the microclusters grow, they migrate towards one pole of the cell where they coalesce into a central cluster, also called a supramolecular activation complex (SMAC). While many of the studies showing formation of the B-cell SMAC are in response to antigen presented by antigen presenting surfaces, recent data from our lab showed that the SMAC also forms upon B-cell stimulation by soluble multivalent antigen. However, these SMACs are more transient than those formed between B-cells and antigen presenting surfaces (170). In cells interacting with antigen presenting surfaces, the centripetal movement of microclusters into a central cluster takes place at the B-cell surface directly contacting the antigen presenting surface, which is termed the "contact zone". The SMAC, which forms in the contact zone, is similar in nature to the originally identified T-cell immunological synapse (IS) that is formed between a T-cell and an APC. However the two differ in their requirements for formation. The T-cell immunological synapse requires co-engagement of adhesion molecules between the T-cell and the APC, while the B-cell SMAC does not, although adhesion molecule ligation can enhance BCR mediated singling, especially when antigens are presented at a low density (171). Additionally, the B-cell SMAC forms upon stimulation with soluble multivalent antigen, while the T-cell IS does not. These unique characteristics of B-cell SMAC formation with subsequent B-cell activation allow the B-cell to respond to a much wider variety of antigens compared to T-cells. Finally, the SMAC formed in B-cells is much more transient than the IS formed in T-cells, which is likely due to the rapid internalization of the BCR/antigen complex necessary for antigen processing and presentation in the B-cells.

It has also been shown that receptor clustering is a target for negative regulation, as co-ligation of the BCR and FcγRIIB by immune complex inhibits BCR interactions with lipid rafts, microcluster formation, as well as BCR central cluster formation (172, 173). Taken together with the above studies, recent evidence further support the unfolding paradigm that BCR reorganization at the surface of B-cells is necessary for appropriate B-cell response to soluble and membrane associated antigen.

1.8 Actin dynamics during B-cell activation

Early studies suggested that B-cell activation by antigen ligation induced actin remodeling within the cell (174). Recent studies have also indicated that the actin cytoskeleton can act to moderate the levels and duration of BCR mediated signaling in both resting B-cells, also known as "tonic" signaling, and BCR stimulated cells (161, 175). The cortical actin cytoskeleton, which lies directly beneath the plasma membrane, is the primary actin structure in resting B-cells. The cytoskeleton is linked to the plasma membrane by the ERM (ezrin/radixin/moesin) family proteins (176, 177). These proteins are capable of binding both F-actin and plasma membrane proteins. In unstimulated cells, the cortical actin cytoskeleton has been suggested to act as a "barrier" to the movement of BCRs at the cell surface, thereby preventing oligomerization and spontaneous activation of unligated surface BCRs. By linking to membrane anchor proteins via its interaction with ERM proteins, the cortical actin cytoskeleton is suggested to act like a picket fence, limiting lateral movement of membrane proteins which have tails that extend into the cytoplasm. A study by Treanor et al. showed that unligated BCRs in actin- and ezrin-poor regions had enhanced lateral mobility, when compared to

BCRs in actin- and ezrin-rich regions (178). The "barrier" hypothesis was further supported when cells treated with actin disrupting agents, in the absence of antigenic stimulation, showed Ca^{2+} flux, signaling molecule activation, and activation marker up-regulation, similar to those of cells treated with anti-IgM (178). This study further showed that the cytoplasmic domain of Igβ is the domain which influences the ability of the BCR complex to diffuse through the cortical cytoskeleton network (178).

In response to antigenic stimulation, BCR mediated signaling promotes a rapid and transient disassembly of the cortical actin cytoskeleton, which is necessary for the increased lateral mobility of the BCRs, and initiation of BCR microcluster formation (Fig. 1.6) (167, 168). The disassembly requires BCR-mediated activation of Rap-GTPases necessary for the dephosphorylation and activation of the actin severing protein cofilin (167), as well as a transient dephosphorylation of the ERM family of proteins. ERM dephosphorylation enables disassociation of the underlying cytoskeleton from the plasma membrane (168), leading to the disassembly of the cortical actin cytoskeleton. In B-cells the ERM family protein ezrin has been shown to link the actin cytoskeleton to the plasma membrane via its interaction with the lipid raft resident Csk-binding protein, and its dephosphorylation upon B-cell activation disrupts this interaction (179). Following the initial depolymerization of the cortical actin cytoskeleton upon BCR engagement, the cytoskeleton undergoes rapid reassembly, which appears to take place predominantly at sites of actively signaling BCR microclusters (Fig. 1.6), suggesting the signals emanating from these clusters generate signals for de-novo actin polymerization (170). Recent work by Ketchum et al. showed that treatment of B-cells with actin cytoskeleton disrupting agents inhibits BCR cluster growth and mobility in response to mobile ligands (163),

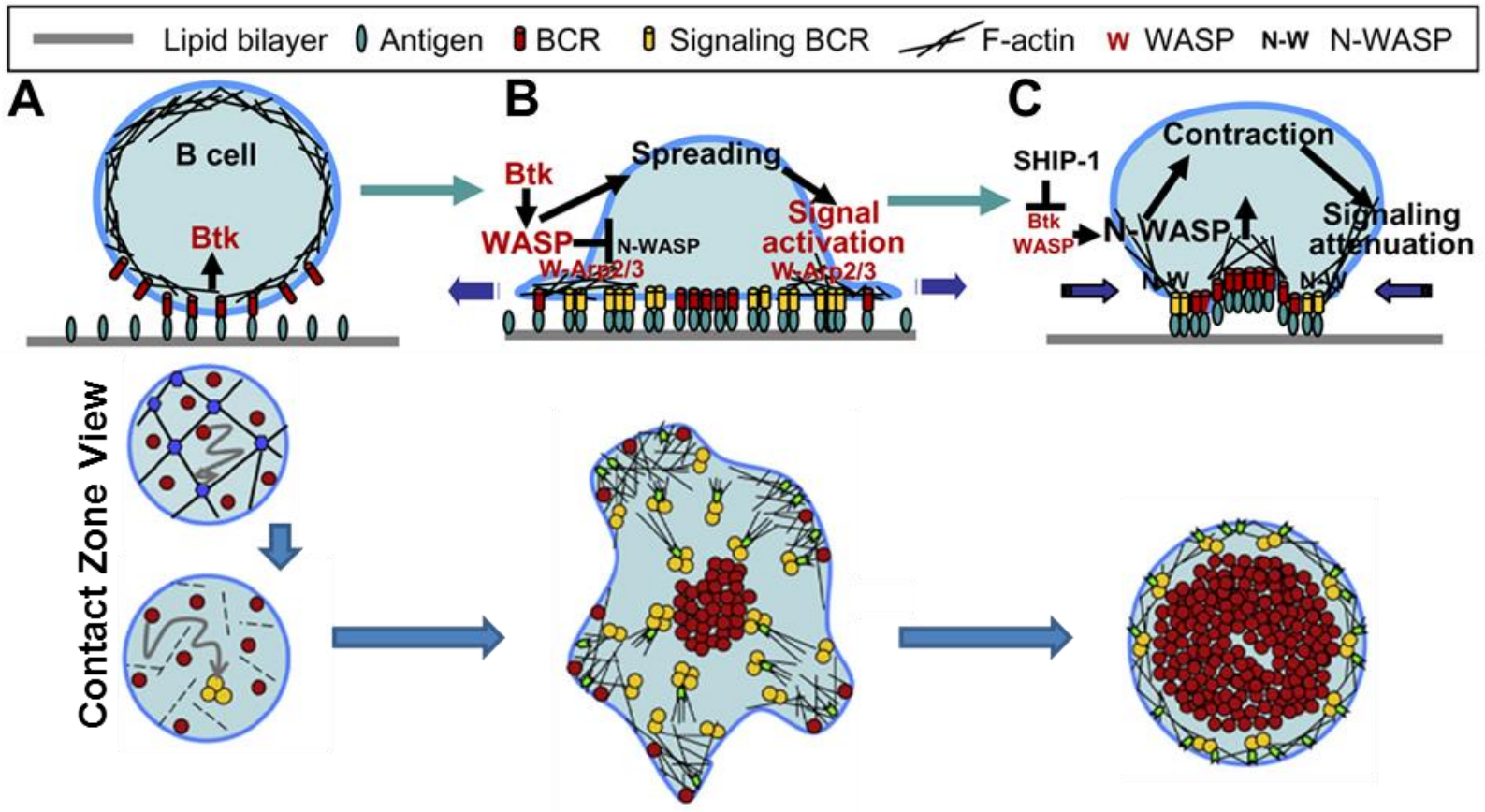

Lipid bilayer
Antigen
BCR
Signaling BCR
F-actin
W WASP
N-W N-WASP
A
B cell
Btk
B
Btk
Spreading
WASP
N-WASP
W-Arp2/3
Signal activation
W-Arp2/3
C
SHIP-1
Contraction
Btk
WASP
N-WASP
Signaling attenuation
N-W
N-W
Contact Zone View

Fig. 1.6 Coordination of actin remodeling with reorganization of BCRs at the cell surface. (A) Upon antigen binding to the BCR, the cortical actin network undergoes rapid and transient depolymerization and detachment from the plasma membrane. The actin depolymerization releases mobility barriers, and enables the BCRs and BCR nanoclusters to interact. Antigen binding also induces early activation of Btk. (B) Activated Btk induces the activation and recruitment of WASP (W) to the cell surface, where it acts to stimulate directed actin polymerization by its ability to bind and activate Arp2/3, and suppress the activation of N-WASP (N-W). The directed actin polymerization drives B-cell spreading and facilitates BCR clustering and signaling. Extension of filopodia and lamellipodia allows the cell to gather more antigen, and their contraction transports BCR-antigen towards the center of the contact zone, thereby forming larger clusters. (C) Later SHIP-1 activation acts to inhibit Btk, and thus WASP activation, thereby releasing the suppressive control of WASP on N-WASP. Activated N-WASP aids in the clearance of actin from the B-cell contact zone, which stimulates the contraction and coalescence of BCR microclusters into a central cluster and subsequent BCR internalization, leading to BCR signal attenuation at the cell surface. Image modified from Liu et al. (2013). PLoS Biology, PMID 24223520.

suggesting a necessity for an actively polymerizing cytoskeleton. The data generated from our lab demonstrate that BCR signaling activates actin nucleation**.** promoting proteins such as WASP (hematopoietic specific) and N-WASP (ubiquitously expressed), which results in membrane dynamics associated with the formation of filopodia and lamellipodia required for cell motility and cell shape changes (Fig. 1.6) (180). In B-cells interacting with antigen presenting surfaces, the initial B-cell contact occurs by filopodia, which are supported by actin bundles, which occurs before BCR microclusters become detectable. As the B-cells come to rest on the antigenpresenting surface, F-actin rapidly accumulates in the contact zone, which promotes multiple rounds of extensions and retractions of filopodia and lamellipodia, which aid in both gathering more antigen to activate more BCRs, as well as B-cell spreading on the antigen presenting surface (Fig. 1.6) (170, 181). Newly activated BCR microclusters often form at the edge of the actin rich structures, and as they retract, the signaling BCR microclusters move towards the center of the B-cell/antigen-presenting surface contact zone. As F-actin continues to accumulate at the contact zone, the B-cells continue to spread along the antigen presenting surface over the course of several minutes. After the B-cell has reached maximal spread, the levels of F-actin in the B-cell contact zone begin to diminish. An actin ring at the outer edge of the spreading membrane is retained, while the level of F-actin begins to reduce in the center of the contact zone, where BCR microclusters aggregate to form the B-cell central cluster (Fig. 1.6). At the same time, dynamic extensions of filopodia and lamellipodia decrease, the B-cell membrane in total begins to retract, resulting in a reduction in the total contact zone area, and the B-cell central cluster continues to sequester BCR microclusters (Fig. 1.6)(170, 181). These studies

suggest that as the B-cell switches from actively spreading upon the antigen presenting surface to contracting, which facilitates BCR central cluster formation, that the B-cell is likely switching from actively undergoing actin polymerization to depolymerization. However, the direct mechanistic links for this switch have yet to be uncovered.

Reorganization of the actin cytoskeleton, which initiates B-cell morphological changes associated with initial BCR microcluster formation, B-cell spreading, B-cell contraction, and BCR central cluster formation require signals from the stimulated BCR. Signaling molecules such as Syk, CD19, PLCγ2, Vav, Btk, and Rap have been shown to be required for appropriate actin cytoskeleton rearrangement during B-cell activation (116, 159, 169, 182). Our lab has shown that the Tec kinase family member Btk is necessary for BCR-induced de-novo actin polymerization, as well as BCR microcluster formation, B-cell spreading, and central cluster formation upon encounter with antigen presenting membranes (Fig. 1.6) (181). Defects in Btk deficient cells are so profound, that the deficient B-cells are unable to establish stable contact with the antigen presenting surfaces. Additionally, other Tec kinases have been shown to be important for formation of the T-cell immunological synapse (183). Our lab discovered that defects in actin regulation caused by mutations in the PH domain of Btk, which prevents its recruitment to the plasma membrane, are more severe than gene knockout of individual actin regulators such as WASP, which suggests that Btk may play a role in activating multiple actin cytoskeleton regulatory proteins (52, 181). As Btk is the major downstream signaling component of the BCR co-receptor CD19, is comes as no surprise that co-stimulation of CD19 induces enhanced actin polymerization, which leads to enhanced antigen stimulated BCR micro-cluster formation as well as B-cell spreading (116, 184).

Our lab has also identified SHIP-1 as a negative regulator of actin polymerization and F-actin accumulation upon BCR ligation, as SHIP-1-deficient cells show enhanced F-actin accumulation in the B-cell contact zone, increased B-cell spreading, and an inability to form BCR central clusters, which is associated with greatly reduced B-cell contraction (Fig. 1.6) (181). SHIP-1 had previously been identified as a negative regulator of BCR mediated signaling, and is known to moderate the activation levels of key B-cell signaling molecules such as Btk, PLCγ2, and Akt (59, 124). However, the observed actin related defects in SHIP-1-deficient cells are likely primarily mediated by SHIP-1 inhibitory actions on Btk, as treatment of the *SHIP-1*$^{-/-}$ cells with the Btk inhibitor (LFM-A13) reverse the observed defects (181). Accordingly, a model for actin mediated dynamics upon BCR ligation includes BCR-initiated activation of Btk, which induces actin polymerization to promote BCR microcluster formation and cell spreading (Fig. 1.6). As SHIP-1 becomes activated, it exerts is negative regulatory effects by reducing the activation of Btk, thereby disabling actin polymerization, driving inhibition of B-cell spreading and promoting B-cell contraction and subsequent accumulation of BCR microclusters into a central cluster (Fig. 1.6). The actin dynamics observed upon BCR ligation are therefore a result of the balance between Btk and SHIP activation.

In addition to BCR-antigen binding initiating signaling cascades which promote mobilization of actin cytoskeletal factors necessary for optimal B-cell response and BCR internalization, it has been discovered in recent years that actin cytoskeleton dynamics both positively and negatively feed back into BCR mediated signaling (185, 186). The primary steps in which actin works to positively feed into enhanced BCR mediated signaling include initial BCR microcluster formation, microcluster growth and eventual

coalescence, as well as signaling molecule recruitment. In Jasplakinolide treated B-cells, which acts to stabilize the actin network, neither soluble nor membrane bound antigen were able to induce significant levels of BCR mediated signaling. However, as mentioned previously, disruption of the actin network by treatment with latrunculin caused microcluster formation of BCRs, and BCR mediated signaling similar in nature to IgM crosslinking, in the absence of antigenic stimulation. As such, it is noted that the transient disassociation of the cortical actin cytoskeleton is necessary for proper BCR transduced signaling, and microcluster formation. In addition, recent studies have suggested that the actin cytoskeleton may sever to partition other positively regulating signaling molecules away from the BCR in resting cells. One such example is CD19, which in complex with CD21/CD81 appears to be partitioned away from BCRs by the cytoskeleton in resting B-cells (114). Moreover, additional downstream signaling molecules necessary for B-cell response are indirectly transported to signaling active BCR microclusters by interactions with a multitude of actin binding proteins. One such example includes the recruitment of the signaling adaptor molecule Grb2 by its interaction with WASP. Additionally, actin polymerization is necessary for enhanced BCR clustering consequently amplifying BCR signaling. One mechanism by which actin amplifies BCR signaling is by promoting B-cell spreading on antigen presenting surfaces, which allows the capture of more antigen and therefore the activation of more BCRs (187). Another mechanism by which the actin cytoskeleton aids in amplifying BCR clustering and signaling is by shuttling both BCRs and BCR microclusters. Actin polymerization at BCR microclusters, as well as retrograde actin flow which takes place at the periphery of the B-cell contact zone, directionally moves BCRs and BCR

microclusters to merge, and eventually form a central cluster. This is supported by published data from our lab which shows that treatment of cells with latrunculin prevents the coalescence of BCR microclusters into central clusters.

Conversely, actin has been implicated in negatively regulating BCR signaling as well. Evidence for a link between actin dynamics and negative regulation of BCR mediated signaling began coming to light when studies showed that disruption of function or knockout out of proteins associated with actin regulation were connected to diverse autoimmune phenotypes, which generally occur as a consequence of overactive B-cell signaling. One example identified includes the deficiency of the actin nucleation factor WASP, which results in reduced humoral immune responses to pathogens, but also wide spread systemic autoimmunity (52, 58). Recent studies from our lab have focused on the mechanisms whereby actin dynamics control negative regulation of BCR signaling. Our studies and those of others have shown that when BCR microclusters coalesce with one another, their interactions with early singling molecules, such as Syk, and lipid raft association begin to diminish. This is concurrent with decreasing levels of F-actin in the vicinity of the central cluster, likely due to local actin depolymerization, which is suggested to reduce membrane dynamics and cellular protrusions, consequently aiding in the coalescence of BCR microclusters to form a central cluster. As the central cluster is formed, the B-cell transitions from an active signaling to an attenuation phase, which occurs simultaneously as the B-cell switches from actively spreading to contracting on antigen presenting membranes. Inhibition or delay of B-cell contraction has been shown by our lab to result in persistent BCR microclusters, F-actin accumulation within the contact zone, and prolonged and enhanced signal activation, as

well as failure to form a central cluster. Our lab has specifically shown that B-cell specific gene knockout of the actin nucleation promoting factor N-WASP (54), and the negative regulatory signaling molecule SHIP-1(181), result in an impaired transition from spreading to contracting . This defect is concurrent with an inability to transition to the signal attenuation phase, which leads to enhanced and prolonged cell signaling and BCR microcluster formation. Moreover, the serum levels of autoAbs are significantly elevated in B-cell specific *SHIP1*$^{-/-}$ mice and *N-WASP*$^{-/-}$ mice, providing strong evidence that actin dynamics are essential in negatively regulating BCR mediated signaling.

Another mechanism whereby dynamically reorganizing actin may act to promote BCR signal down-regulation is by the recruitment of negative regulatory elements to BCR signalosomes. For instance, actin reorganization is necessary for recruitment of Abp1 to both the T-cell and B-cell surface, and Abp1 has been shown to be necessary for the recruitment of HPK1 to the T-cell immunological synapse. HPK1 in turn has been shown to be a negative regulator of both B and T-cell singling (55, 126). Our lab has shown that N-WASP is recruited to the B-cell contact site upon BCR/antigen ligation, and B-cell conditional knockout of N-WASP resulted in decreased activation of SHIP1, suggesting that N-WASP may be responsible for bringing SHIP1 to BCR microsignalosomes, thereby mediating BCR signal down-regulation (54).

1.9 Abp1 in lymphocyte activation and endocytosis

Abp1, also known as SH3P7 and HPK1-interacting protein of 55 kDa (HIP-55), was identified in a screen for Src homology-3 (SH3) domain containing molecules in yeast (188), and was later shown to be involved in actin-dependent endocytosis in yeast

(189). Kessels et. al cloned the mammalian homologue of Abp1, and he and others discovered that Abp1 is ubiquitously expressed in the mouse, although it is expressed at low levels in the ovaries and skeletal muscles (190-192). Abp1, a member of the debrin family of actin-binding proteins, contains multiple protein-protein interaction domains. The 55 kDa phospho-protein contains two N-terminal actin binding domains (actin-depolymerizing factor homology domain (ADF) and Helical domain), a centrally located proline rich domain (PRD) which contains two Src-tyrosine phosphorylation consensus sites, and finally a C-terminal SH3 domain (Fig. 1.7). Early studies showed that Abp1 binds to F-actin, but not monomeric G-actin, via its two independent actin binding domains with a 1:5M saturation stoichiometry, however, its binding does not alter filament dynamics (191, 192). Abp1 is recruited to the leading edge of migrating cells, fibroblast lamellipodia, and to the cell membrane upon BCR and TCR ligation, where it colocalizes with actin, and its recruitment appears to depend upon de novo actin polymerization (150, 191, 192). In yeast, Abp1 can directly influence actin polymerization by activating the actin nucleator Arp2/3. However, while mammalian Abp1 also binds F-actin, it lacks the additional acidic domains necessary for Arp2/3 activation (189, 193). However, Pinyol et al. have shown that Abp1 can directly bind to and activate N-WASP (an activator of Arp2/3) with the help of Cdc42 in neuronal cells (Fig. 1.7) (194). The two tyrosine phosphorylation (YxxP) motifs at position 334 and 344 have been shown to be phosphorylated by Src family, Syk, and Zap-70 kinases in vitro (191, 195, 196). Finally, the SH3 domain of Abp1 has been implicated in directly binding or influencing the recruitment of a number of molecules necessary for signal

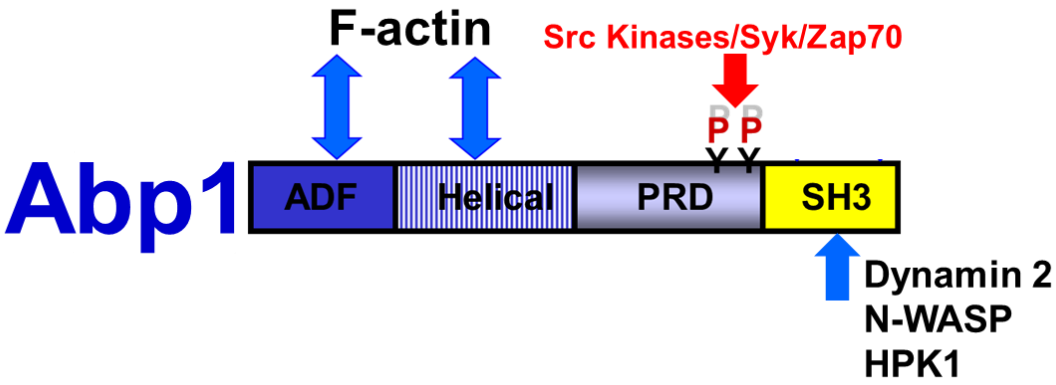


Figure 1.7 Structure and known binding partners of Abp1. Abp1 is a 55 kDa phospho-protein that has been implicated in modulating cell signaling and internalization events. It contains two N-terminal F-acting binding domains (ADF and Helical), a centrally located PRD domain, which contains two consensus tyrosine phosphorylation sites which are substrates for Src/Syk/Zap70 kinases, and a C-terminal SH3 domain. The SH3 domain of Abp1 has shown to bind the GTPase Dyn2, where the interaction is necessary for Tf and BCR internalization, as well as HPK1, an upstream kinase essential for B and T-cell signal propagation. Abp1 has also been show to bind the F-actin regulating protein N-WASP in neuronal cells.

transduction, actin polymerization, endocytosis, cellular adhesion, as well as cell migration (Fig. 1.7) (149, 190, 194, 196).

While Abp1 itself does not have any intrinsic enzymatic activity, its combined protein interaction domains implicate Abp1 as an important scaffolding molecule for coordinating signal transduction with actin-mediated events such as cell surface receptor dynamics and endocytosis. As such, early reports showed that Abp1 was phosphorylated after BCR or TCR ligation, suggesting that it may represent a common effector of antigen receptor-signaling pathways in lymphocytes (191, 197). Our lab showed that this phosphorylation is required for Abp1 recruitment to the plasma membrane upon BCR crosslinking, as a phosphorylation mutant of Abp1 showed defective recruitment (150). A report by Ensenat et al. discovered that Abp1 specifically bound to the MAP4K HPK1 via its SH3 domain and HPK1's PRD in 293T cells. HPK1 is a MAP4K that is able to activate the JNK pathway by activating multiple MAP3Ks, which then activate multiple MAP2Ks (126). Ensenat et al. discovered that HPK1 and JNK activity were enhanced by Abp1 binding (190). Activated JNK in turn phosphorylates and activates the transcription factor c-jun, which is a component of the AP-1 transcription factor that regulates cell proliferation and apoptosis (110). Recent studies in B and T-cells show that HPK1 acts as a negative regulator of multiple aspects of BCR and TCR singling respectively. In T-cells HPK1 acts to negatively regulate NFAT activation, T-cell proliferation, T-cell-dependent antibody responses, cytokine production, and induction of autoimmunity (55, 56, 126), while in B-cells, HPK1 has been shown to negatively regulate activation of BLNK, MAPK's (p38, ERK, and JNK), PLCγ2, and IκB kinase (55). HPK1-deficient B-cells are hyper-proliferative, and show enhanced Ca^{2+} flux upon

BCR stimulation (55). Recent studies utilizing T-cells derived from *Abp1*$^{-/-}$ mice showed that these T-cells cells appeared to have decreased activation of several key T-cell signaling/adaptor molecules including the p38, linker for activation of T-cells (LAT), and PLCγ1, as well as defective up-regulation of the activation marker CD69 (195). As a likely consequence of the signaling defects observed in the *Abp1*$^{-/-}$ T-cells, the Abp1 knockout mice display reduced T-cell proliferation, cytokine production, and reduced humoral immune response to T-dependent antigens (195). In B-cells, in addition to tyrosine phosphorylation, the recruitment of Abp1 to the plasma membrane also requires actin reorganization, as the recruitment is abolished upon treatment with the actin disrupting agent Latrunculin (150). A study by Kessels et al. discovered that the constitutively active form of Rac, a GTPase that acts to moderate actin polymerization, caused a rapid accumulation of Abp1 to sites of cell growth such as fibroblast lamellipodia or the leading edge of migrating cells (192). The findings that BCR signaling induces the phosphorylation of Abp1 and that Abp1 is necessary for optimal activation of signaling molecules downstream of the TCR raises the possibility that Abp1 may serve as an adaptor protein that links upstream to downstream signaling events of the BCR for B-cell activation. The ability of Abp1 to interact with the actin cytoskeleton directly and actin moderating elements also suggests Abp1 as a possible link between BCR mediated signaling and actin cytoskeleton dynamics upon BCR engagement.

Abp1 has long been recognized as important for endocytosis within many different cell types. The earliest studies of Abp1 in yeast indicated that Abp1 was essential for actin-dependent endocytosis. As mentioned previously, yeast Abp1 binds to F-actin, while having the ability to directly activate the actin nucleator Arp2/3, thereby

directly influencing actin polymerization dynamics. However, mammalian Abp1 does not retain this capability. Abp1 was shown to function in receptor-mediated endocytosis of transferrin, by its interaction with the GTPase dynamin 2 (Dyn2), which acts to pinch off endocytosing vesicles from the cell surface (149, 198). Our lab published research showing that Abp1 can interact with Dyn2 in B-cells, and this interaction requires the SH3 domain of Abp1 and the PRD of Dyn2 (Fig. 1.7) (150). Importantly, Abp1 is necessary for high efficiency BCR-mediated antigen processing and presentation (150).

1.10 The role of dynamin 2 in moderating signal transduction and actin dependent endocytosis

Dynamin is a 100 kDa GTPase which acts as a molecular motor necessary for pinching off nascent endocytosing vesicles from a variety of intracellular membranes (143, 199-202), and has more recently been implicated in moderating actin dynamics and cell signaling. Dynamin has three major isoforms, dynamin 1 which is neuron specific, dynamin 2 which is ubiquitously expressed, and dynamin 3 which is predominantly expressed in the testis, brain, and lung (202). Dynamin is composed of five domains: an N-terminal GTPase domain, a middle domain, a PH domain, a GTPase effector domain (GED), and a C-terminal PRD (Fig. 1.8) (203). Dynamin is recruited to sites of endocytosis, where it oligomerizes to form spiral collar-like structures which constrict to pinch off the nascent vesicles (Fig. 1.8) (204). The GTPase activity of dynamin is greatly enhanced by self oligomerization (>100-fold) into spiral-like structures. The oligomerization is mediated via the interaction of the GED of one molecule with the GTPase domain of another, which subsequently activates dynamin's intrinsic GTPase

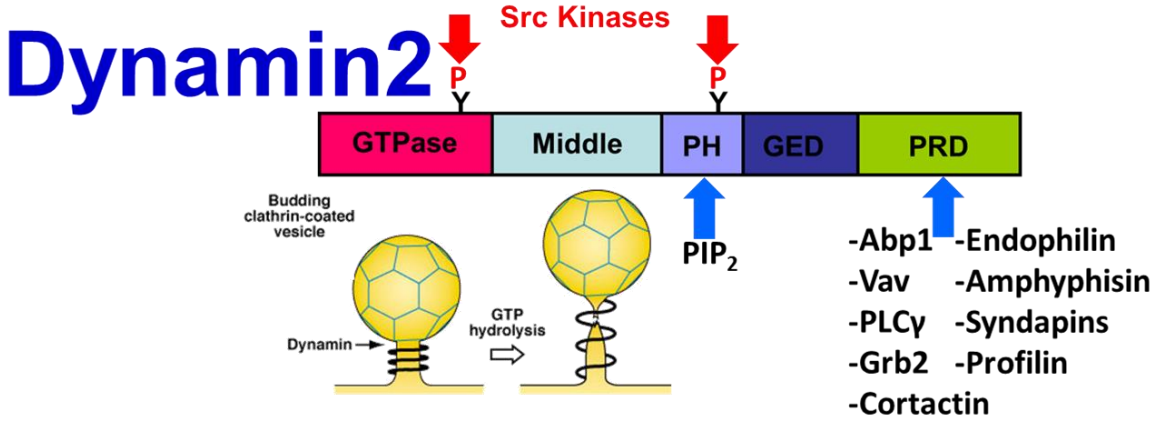

Dynamin2
Src Kinases
P
Y
P
Y
GTPase
Middle
PH
GED
PRD
Budding clathrin-coated vesicle
Dynamin
GTP hydrolysis
PIP2
-Abp1
-Vav
-PLCγ
-Grb2
-Cortactin
-Endophilin
-Amphyphisin
-Syndapins
-Profilin

Figure 1.8 Structure and known binding partners of Dyn2. Dyn2 is a 100 kDa GTPase, which has been shown to be necessary for scission of newly formed budding vesicles from a variety of membranes. Dyn2 contains an N-terminal GTP-ase domain, followed by a "middle" domain, a PH domain, a GED, and a C-terminal PRD. The GTPase domain of Dyn is necessary for its pinchase activity, which releases budding vesicles from membranes. The "middle" domain of Dyn is proposed to predominantly serve as a structural motif, which aids in Dyn-Dyn interactions. With its PH domain, Dyn is binds membrane lipids. The GED, while also serving as a structural component in Dyn-Dyn binding, predominantly acts as a GTPase effector for Dyn, serving to greatly increase Dyn's intrinsic GTPase function. The C-terminal PRD of dynamin has been implicated in binding a vast number of SH3 domain containing proteins which have broad ranging functions within cells including signal propagation and actin cytoskeleton modification. Dyn also has two consensus tyrosine phosphorylation sites which are substrates for Src family kinases. Inset image depicts collars formed by Dyn-Dyn interactions, and GED initiated Dyn GTP hydrolysis resulting in scission of a membrane vesicle. Inset image modified from http://www2.mrc-lmb.cam.ac.uk/groups/hmm/Dynamin/index.html

activity (205, 206). The middle domain of Dyn has also been proposed to aid in self-oligomerization (207). The PH domain of dynamin binds to the lipid $PI(4,5)P_2$ at the plasma membrane, and the binding is speculated to increase membrane curvature. It is not surprising that deletion mutants of this domain show reduced receptor-mediated endocytosis (208, 209). Preliminary data has shown that the PRD of dynamin is necessary for its recruitment to clathrin-coated pits (150, 210). Additionally, the PRD of dynamin has been shown to bind the SH3 domain of numerous proteins involved in a variety of cellular processes, including clathrin-mediated endocytosis and actin modulation (211). Some of these proteins include amphiphysin (210), endophilin (210), PLCγ (212), Grb (213), Cortactin (148), Vav (214), and Abp1(150).

As early as 1999, Ahn et al. showed that Dyn2 can be tyrosine phosphorylated at positions 231 and 597 by Src kinases in response to β adrenergic receptor stimulation, and this phosphorylation was shown to be important for the internalization of the β2 adrenergic receptor (215). Later, Sajahn et al. showed that phosphorylation of Dyn2 was necessary for caveolae-mediated endocytosis in endothelial cells (216). However, in mast cells, FcεRI ligation induces the de-phosphorylation of Dyn2, which was shown to hasten CME of the FcεRI (217). As such, it remains unclear whether phosphorylation of dynamin is necessary for its recruitment or function during CME of signaling BCRs. While it is unknown whether dynamin itself must be phosphorylated, our lab has shown that BCR-mediated signaling is necessary for the recruitment of dynamin to the B-cell surface and the BCR, as B-cells treated with the Src kinase inhibitor PP2 display defective recruitment of dynamin upon BCR engagement (Bruce K. Brown, Ph.D., un-published data). Recent work by Malhotra et al. has also indicated that upon BCR

stimulation, Dyn2 is recruited to endocytosing BCRs in a complex with Grb2 and Vav, which is dependent upon phosphorylation and activation of LAB (linker for activation of B-cells) (218, 219). Moreover, small interfering RNA–mediated gene silencing of Dyn2 in Jurkat T cells reduced levels of activated pERK, pJNK, and pPLCγ1, with the eventual outcome of reducing IL-2 promoter activity as well as CD69 up-regulation upon TCR stimulation (214). Further investigation revealed that the PRD of dynamin was necessary for IL-2 promoter activation, and more specifically a constitutive interaction with Vav1 was required for TCR signals resulting in IL-2 promoter activation (214). These results suggest that dynamin, while essential for the physical removal of nascent vesicles from the plasma membrane, may also contribute, by proxy, to receptor signal transduction.

In addition to providing physical force to remove endocytosing vesicles from the plasma membrane and possible roles in signal transduction from the BCR, recent studies have suggested dynamin as a link between cellular endocytosis machinery and the actin cytoskeleton. Our lab has previously shown that actin remodeling is critical for signal enhanced BCR endocytosis but is not required for constitutive endocytosis of BCRs at the cell surface (137), which suggests that BCR signaling can up-regulate receptor internalization by inducing actin remodeling. While our lab has shown that Dyn2 recruitment upon BCR crosslinking is independent of F-actin (Bruce K. Brown, Ph.D., un-published data), dynamin has been shown to be linked to actin in a variety of ways. Dynamin directly interacts with Vav (214, 218) which serves as a guanine nucleotide exchange factor (GEF) for Rho family GTPases, cortactin which activates the Arp2/3 complex (220), syndapins which aid in activation of N-WASP (221), profilin which promotes ATP exchange on monomeric actin for actin filament elongation (222), and

Abp1 (149, 150). In rat fibroblasts, siRNA knockdown or dominant negative transfection of a GTPase mutant of dynamin (dynamin-K44A), induced mislocalization of Rac, consequently interfering with Rac induced actin polymerization at the leading edge of lamellipodia (223). Moreover, Gomez et.al defined Dyn2 as a critical regulator of the actin cytoskeleton in response to TCR engagement, as Jurkat T-cells with shRNA-Dyn2 knockdown showed diminished actin reorganization to the immunological synapse (214). As mentioned above, Abp1 has been shown to directly bind F-actin and is necessary for BCR internalization and subsequent antigen processing and presentation (150). Abp1 and Dyn2 constitutively interact, and they are both recruited to the plasma membrane upon BCR crosslinking (150). In light of the preceding studies, it is becoming clearer that Dyn2 plays an indirect role in recruitment of actin to sites of signaling receptors/sites of endocytosis. How Dyn2 works to coordinate this recruitment has yet to be fully fleshed out.

1.11 Rationale/Aims

B-cells, as the key mediators of humoral immunity, rely upon tight regulation of BCR-mediated signaling in response to pathogenic encounter to ensure an appropriate response. The absence or breakdown of regulated signaling can lead to diverse complications ranging from immunodeficiency to autoimmunity. BCR-initiated signaling and BCR/antigen internalization for processing and presentation are essential for the production of high affinity and long-lasting antibodies that can effectively neutralize pathogens. The major question that will be addressed in this dissertation is how the processes of BCR-mediated signal transduction, dynamic actin rearrangements,

and subsequent BCR internalization for antigen processing are linked to generate a robust humoral immune response. More specifically, I have examined the role of Abp1 and Dyn2 in coordinating these processes, and my research has discovered that both Abp1 and Dyn2 are involved in modulating BCR signaling and subsequent BCR internalization for antigen processing and presentation, by linking actin to signaling and the endocytic apparatus. My studies focused primarily upon the role of Abp1 as a negative regulator of BCR-mediated signaling and dynamic actin re-organization, as well as its contribution to regulating peripheral B-cell development, response to immunization, and controlling B-cell autoimmunity. I also examined the role of Dyn2 in regulation of BCR-mediated signals, by modulating BCR clustering and subsequent BCR internalization. Furthermore, I have examined the role of individual domains of Dyn2 necessary for governing these processes.

1.11.1 Aim 1. The role of Abp1 in regulation of BCR signaling, B-cell development, and immune response.

This aim was designed to examine the hypothesis that the actin adaptor molecule Abp1 acts to link actin dynamics to BCR signaling and internalization, thereby aiding in B-cell immune response. To address this hypothesis, I examined whether Abp1 was recruited to signaling active BCR microclusters in B-cells stimulated with antigen presenting surfaces, and the effect of Abp1 knockout on 1) the morphology of B-cells, and BCR clustering, 2) F-actin accumulation at surface BCRs, and the activation of other known actin-regulating proteins, 3) activation of BCR proximal and distal signaling molecules, 4) the development of B-cell subsets, 5) spontaneous GC B-cell development

in the absence of immunization, and autoAb production, 6) and BCR affinity maturation, and T-dependent and T-independent antibody responses. To investigate this hypothesis, we utilized both germline *Abp1* knockout and *Abp1*$^{-/-}$ -chimeric mice which specifically lack Abp1expression in only B-cells. This work will shed light upon the dynamic and interwoven processes of BCR signaling and actin reorganization as they relate to B-cell signal regulation, activation, development, and B-cell-mediated immune responses.

1.11.2 Aim 2. The role of Dyn2 in regulating BCR signal transduction and internalization.

This aim was designed to examine the hypothesis that BCR-initiated signaling regulates the cellular location of Dyn2, that Dyn2 plays a role in further propagating BCR mediated signaing and BCR internalization. More specifically, I sought to examine the domains or function of Dyn2 necessary for its recruitment to the plasma membrane, as well as those necessary for BCR internalization upon BCR crosslinking, and the effect of a Dyn2 GTPase mutation on BCR clustering and signal transduction. To investigate this hypothesis, I transfected B-cells with dominant negative mutants of Dyn2 conjugated to green fluorescent protein (GFP), including GFP-Dyn-K44A (a GTPase mutant), GFP-DynΔPRD (Dyn2 lacking its PRD), GFP-Dyn2-Y231/597F (a phosphorylation mutant of Dyn2), as well as full length Dyn2, and examined 1) their recruitment to the BCR upon receptor crosslinking, 2) internalization of the BCRs in the differentially transfected cells, 3) their ability to recruit the actin adaptor molecule Abp1, and 4) BCR clustering kinetics and signal propagation in cells transfected with the GTPase dead mutant of Dyn2. Furthermore, I sought to examine BCR internalization in B-cells treated with the small

molecule inhibitor of dynamin GTPase function, Dynasore. This work will shed light upon the coordination between BCR signal propagation, actin dynamics, and BCR internalization, and B-cell morphological response to antigen exposure.

Chapter 2: Actin binding protein 1 links B-cell antigen receptor to negative signaling pathways

2.1 Abstract

Prolonged or uncontrolled B-cell receptor signaling has been associated with autoimmunity. We previously demonstrated a role for actin in BCR signal attenuation. This study reveals that Actin-binding protein 1 (Abp1/HIP-55/SH3P7) is a critical negative regulator of BCR signaling, and links actin remodeling with negative regulatory pathways of the BCR. In both *Abp1*$^{-/-}$ and bone marrow chimeric mice where only B-cells lack Abp1 expression, the number of spontaneous germinal center and marginal zone B-cells in the spleen and levels of autoAb are significantly increased, while B-cell development in the bone marrow is unaltered. Serum levels of T-independent antibody and total IgM and IgG are elevated, while T-dependent antibody responses are markedly reduced and fail to undergo affinity maturation. Upon activation, surface BCR clustering is enhanced and B-cell contraction delayed in *Abp1*$^{-/-}$ B-cells; concurrent with slow but persistent increases in F-actin at BCR signalosomes. Furthermore, BCR signaling is enhanced in *Abp1*$^{-/-}$ B-cells compared to wild-type B-cells, including increased levels of Cat^{2+} flux and phosphorylated BLNK, MEK1/2 and ERK, coinciding with reductions in the recruitment of the inhibitory signaling molecules HPK1 and SHIP-1 to BCR signalosomes. My results indicate that Abp1 contributes to maintenance of B-cell tolerance via negatively regulating BCR signaling by coupling actin remodeling to B-cell contraction and the activation of inhibitory signaling molecules, which contributes to the

regulation of peripheral B-cell development and antibody responses. This study reveals a novel mechanism for negative regulation of BCR signaling.

2.2 Introduction

B-lymphocytes are responsible for mounting antibody responses as well as regulating other branches of immune responses against infectious agents. B-cells are activated by signal transduction mediated by their BCRs (161). Recent studies using high resolution live cell imaging have enabled a better understanding of the molecular dynamics of the early events of BCR signaling (114, 168, 172, 224). Upon binding antigen, surface BCRs rapidly reorganize from monomers and nanoclusters into microclusters (161). BCR microclusters recruit and stabilize surrounding lipid rafts, which brings lipid raft-resident Src kinases into physical proximity of receptors and induces the phosphorylation of the ITAMs in the cytoplasmic tails of the BCR (113, 225). Phosphorylated ITAMs provide docking and activation sites for the tyrosine kinase Syk. These events lead to the recruitment and phosphorylation of proximal signaling molecules to BCR microclusters, activating signal cascades. Ultimately BCR signaling induces transcriptional activation of genes required for B-cell survival and proliferation (226, 227).

The level and duration of BCR signaling is tightly regulated. Prolonged or uncontrollable BCR signaling is associated with B-cell-mediated autoimmunity (44, 120). Downregulation of BCR signaling is mediated by various phosphatases and kinases, including SHIP-1 (59, 124) and HPK1(55). While SHIP-1 is the primary downstream component of the inhibitory co-receptor FcγRIIB, BCR activation also activates SHIP-1

through Lyn-mediated phosphorylation (59, 228). In anergic B-cells, SHIP-1 binds to partially phosphorylated ITAMs of the BCR, thereby maintaining their anergic state (122). SHIP-1 inhibits the activation of PLCγ2, Btk, and Akt by converting their membrane docking sites, PtdIns(3,4,5)P_3, into PtdIn(3,4)P_2, consequently blocking their signal transduction (59, 124). B-cell-specific SHIP-1 deficiency causes hyper-responsiveness and impaired positive selection of B-cells in germinal centers (57). HPK1, a Ste20 serine/threonine kinase, inhibits BCR signaling by inducing the threonine phosphorylation and subsequent ubiquitination and degredation of BLNK (55), the key adaptor protein of necessary for BCR signal transduction. HPK1 deficiency leads to increases in the activation levels of BLNK, MAP kinases, NF-κB, and B-cell proliferation (55). Furthermore, HPK1-deficient mice are more susceptible to experimentally induced autoimmune encephalomyelitis (126).

In addition to inhibitory phosphatase and kinases, one of the earliest events of BCR activation, receptor clustering, is also a target for negative regulation. BCR-FcγRIIB colligation by immune complexes inhibits BCR clustering (173). We have recently demonstrated that the coalescence of BCR microclusters into a central cluster facilitates BCR signal attenuation (181). This coalescence process requires actin-mediated B-cell contraction and SHIP-1 activation (181).

Actin is critical for both amplification and downregulation of BCR signaling. The earliest signals from the BCR induce transient disassociation and disassembly of cortical actin beneath the plasma membrane, which increases the lateral mobility of surface BCRs (167, 168, 178). Actin disassembly frees surface BCRs from confinement generated by the cortical actin to form microclusters, facilitating signal initiation. Following actin

disassembly, actin reassembles in a polarized fashion in the vicinity of BCR microclusters, driving surface BCRs to one pole of cells (170, 175). The microclusters recruit more BCRs and coalesce with one another. When engaging antigen presented on a cell surface, such as those of follicular dendritic cells, actin reassembly expands the contact of B-cells with the presenting surface. B-cell spreading increases the number of BCRs engaged, thereby enhancing BCR signaling, which further promotes BCR microcluster formation (187). Upon maximimal spread, the level of F-actin starts to decrease in the region of the B-cell membrane contacting the antigen presenting surface, and the B-cell membrane contracts, which facilitates the coalescence of BCR microclusters, promoting signal downregulation (170, 181, 187, 229). Persistent actin accumulation at the B-cell contact zone and delayed B-cell contraction, caused by B-cell-specific N-WASP knockout, lead to increased and prolonged BCR signaling, and results in elevated numbers of spontaneously formed germinal center B-cells and autoantibody production (54). These data indicate that actin remodeling is closely coordinated with the reorganization and signaling of surface BCRs. However, the molecular mechanisms underlying the communication between actin and BCR signaling has not been fully elucidated.

Abp1 is an adaptor protein that contains multiple interaction domains, including an ADF domain at its N-terminus, a highly conserved SH3 domain at its C-terminus, and a helical domain and PRD in the center (190-192). Its ability to simultaneously bind to F-actin and a PRD-containing molecule implicates a role for Abp1 in mediating communications between actin and BCR signaling. While the yeast homolog of Abp1 binds to and directly activates Arp2/3 (193), mammalian Abp1 does not retain this

capability, but can indirectly regulate actin polymerization by binding to N-WASP in neurons (194). Additionally, Abp1 has been shown to be involved in synaptic vesicle recycling in neuronal cells (230), and neutrophil phagocytosis and migration by linking actin with these processes (196, 231). A regulatory role for Abp1 in signaling was first demonstrated in T-cells. While T-cell-development is normal in *Abp1*$^{-/-}$ mice, T-cells from *Abp1*$^{-/-}$ mice display decreases in signal transduction from the TCR with subsequent reductions in T-cell proliferation and cytokine production in vitro (195). Furthermore, T-cell-dependent antibody responses are reduced in *Abp1*$^{-/-}$ mice (195). In T-cells, Abp1 is recruited to the IS and responsible for recruiting the kinase HPK1 (232). However, the molecular mechanism by which Abp1 contributes to TCR signaling and T-cell activation remains elusive. In B-cells, BCR activation induces the phosphorylation of two consensus tyrosine motifs within the PRD of Abp1 (150, 191). Our lab has shown that Abp1 phosphorylation and BCR-induced actin remodeling are required for Abp1 recruitment to the cell surface (150). We also previously demonstrated that Abp1 is required for BCR-mediated antigen internalization and processing (150), and that antigen-induced BCR internalization requires actin remodeling (170, 181). Abp1 binds to both F-actin and Dyn2 via its ADF-H/Helical and SH3 domains, respectively (149, 150, 191). However, how Abp1 contributes to BCR signaling remains elusive.

In this study, I demonstrate that Abp1 is a negative regulator of BCR signaling and B-cell activation. *Abp1*$^{-/-}$ B-cells display greater levels of BCR signaling than wt B-cells, which correlates with increased numbers of spontaneously formed GC B-cells and autoAb production in *Abp1*$^{-/-}$ mice and *Abp1*$^{-/-}$ bone marrow chimeric mice. *Abp1*$^{-/-}$ mice display decreased T-dependent Ab responses, and defective affinity maturation, however

conversely show increased total serum levels of IgM and IgG, and an enhanced T-independent Ab response. My mechanistic examination suggests that Abp1 attenuates BCR signaling by promoting BCR microcluster coalescence and B-cell contraction, and by recruiting the inhibitory molecules SHIP-1 and HPK1 to BCR microclusters. Thus, my results reveal Abp1 as a novel mechanistic link between actin remodeling and negative signaling, exerting a B-cell-intrinsic inhibition on B-cell activation.

2.3 Materials and Methods

2.3.1 Mice and cell culture

Wild-type C57BL/6 mice expressing either CD 45.1 or CD 45.2, and μMT (B-cell null) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Abp1 knockout mice (*Abp1*$^{-/-}$) were generated and crossed onto a C57BL/6 background, as previously described (195). To isolate splenic B-cells, single-cell suspensions of splenocytes were subjected to density-gradient centrifugation in Ficoll (Sigma-Aldrich, St Louse, MO) to obtain mononuclear cells, treated with anti-Thy1.2 monoclonal Ab (mAb) (BD Biosciences, Franklin Lakes, NJ) and guinea pig complement (Rockland Immunochemicals, Gilbertsville, PA) to remove T cells, and panned for 2 h to remove monocytes. BM was flushed from mouse femurs with PBS supplemented with 3% fetal bovine serum (FBS), and subjected to density-gradient centrifugation in Ficoll (233). Peritoneal cells were flushed from the peritoneal cavity with phosphate buffered saline (PBS) supplemented with 3% FBS (234). All animal work was reviewed and approved by the Institutional Animal Care and Usage Committee of the University of Maryland.

2.3.2 Flow cytometry

For flow cytometric analyses, cell suspensions from BM, spleen, or peritoneal cavity were incubated with Fcγ Receptor blocking Abs (anti-mouse CD16/CD32, BD Bioscience) on ice, and stained at optimal dilutions of conjugated Abs in PBS supplemented with 1% FBS. Anti-mouse Abs and reagents used to stain BM cells included Pacific Blue-anti-CD24 (BioLegend, San Diego, CA), biotinylated anti-Ly-51 (BP-1), streptavidin-PE, FITC-anti-CD43, PerCP-Cy5.5-anti-B220, and APC-anti-IgM (BD Biosciences) (7, 235). Anti-mouse Abs and reagents used to stain transitional 1 (T1), transitional 2 (T2), follicular (FO), and isotype switched (IS) splenic B-cells included biotinylated-anti-IgD (Southern Biotech, Birmingham, AL), Alexa Fluor (AF) 405-streptavidin (Life Technologies, Carlsbad, CA), PerCP-Cy5.5-anti-B220, and FITC-anti-IgM (BD Biosciences) (236). Anti-mouse Abs and reagents used to stain splenic marginal zone B-cells (MZ) included Pacific Blue-anti-CD21 (BioLegend), PerCP-Cy5.5-anti-B220, and PE-anti-CD23 (BD Biosciences) (236). Anti-mouse Abs and reagents to stain B1a and B1B-cells from the peritoneal cavity included biotinylated-anti-IgD (Southern Biotech), PE-Cy5- streptavidin, FITC-anti-IgM, and APC-anti-CD5 (BD Biosciences), and eFlour 450-anti-CD11b (eBiosciences, San Diego, CA) (237). Anti-mouse Abs and reagents to stain splenic GC B-cells included biotinylated-anti-CD95 (BD Biosciences) AF488-streptavidin (Invitrogen, Carlsbad, CA), PE-anti-GL7, and PerCP-Cy5.5-anti-B220 (BD Biosciences) (238).

To determine the level of Abp1 expression in each B-cell subset, the preceding samples were fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin, and stained with goat-anti-Abp1 Ab, followed by fluorescently conjugated secondary Ab.

Goat-anti-mouse Abp1 Ab was generated by immunization of a goat with GST-Abp1 fusion proteins by Alpha Diagnostics International (San Antonio, TX) and purified using protein G-Sepharose column (150).

To analyze intracellular signaling molecules, splenic B-cells were incubated with FcγR blocking mAb at 4^{o}C, stained with APC anti-mouse CD19 Ab (BD Biosciences) to label B-cells, washed, activated with 10 μg/ml F(ab')2-goat-anti-mouse IgG+M (Jackson ImmunoResearch), fixed, and stained with Abs specific for phosphotyrosine mAb 4G10 (pY, EMD Millipore, Billerica, MA), pBLNK (Santa Cruz Biotechnology, Santa Cruz, CA), pMEK1/2, pERK, pp38, or pJNK (Cell Signaling Technology, Danvers, MA) followed by fluorescently conjugated secondary Abs. Cells were pretreated and treated during stimulation with the MEK1/2 (U0126, 10 μM, InvivoGen, San Diego, CA) or JNK (SP600125, 10 μM, A.G. Scientific Inc., San Diego, CA) as controls to confirm specificity of pERK and pJNK Ab labeling.

To determine the expression levels of mIgM, signaling, and actin-related molecules in unstimulated splenic B-cells, B-cells were treated with FcγR–block Ab, labeled with PerCP Cy5.5 anti-mouse B220 Ab, fixed, permeabilized, and labeled with Abs or reagents, including anti-mouse IgM (BD Biosciences), anti-BLNK, anti-SHIP-1, anti-WASP, anti-N-WASP (Santa Cruz Biotechnology), anti-MEK, anti-ERK, anti-JNK, and anti-HPK1 (Cells Signaling Technologies), and AF488 Phalloidin for F-actin (Life Technologies), followed by fluorescently conjugated secondary Abs where necessary.

Cells were analyzed with a FACSCanto II flow cytometer and FACSDiva Software (BD Biosciences) or FlowJo (Tree Star, Ashland, OR).

2.3.3 Immunohistochemistry

Spleens from wt and *Abp1*$^{-/-}$ mice were embedded in O.C.T Compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Sections (10 μm) were collected using a cryostat. Samples were fixed in acetone, blocked with goat serum (Jackson ImmunoResearch, West Grove, PA) and avidin/biotin (Sigma Aldrich), and then stained with optimal concentrations of the following Abs/reagents: biotinylated-anti-IgD (Southern Biotech), AF405-streptavidin (Life Technologies), AF 488-anti-Thy1.2 (eBioscience), and AF 647-peanut agglutinin (PNA) (Life Technologies). Cells were imaged using a Zeiss LSM 710 Confocal Microscope (Carl Zeiss Microscopy, Jenna, Germany). GC size was calculated using Zen Lite 2011 Software (Carl Zeiss Microcopy).

2.3.4 Bone marrow chimera mice

BM chimeric mice were generated using a previously published method (10). C57BL/6 wt recipient mice expressing CD45.1 were lethally irradiated (1000 cGy). BM from *Abp1*$^{-/-}$ or wt mice (CD45.2^{+}) was mixed at a 20:80 ratio with B-cell null μMT BM (1×10^7 total BM cells in 150 μl of PBS) and transplanted via retro-orbital injection into the lethally irradiated mice (52). To eliminate previously activated B-cell populations in transfered BM cells, BM from young *Abp1*$^{-/-}$ and wt donors (4–5 wk) depleted of plasma B-cells was used. Plasma cells were depleted by incubating cells with biotin-conjugated rat anti-CD138 (BD Biosciences) followed by EasySep Streptavidin RapidSpheres and EasySep Magnet (Stem Cell Technologies, Vancouver, BC). Donor sera were also pre-screened to verify absence of anti-double stranded DNA (dsDNA) antibodies. Irradiated mice without BM transplant died within 14 days. Four months post adoptive transfer, the

percentages of B-cells in the BM ($B220^{+}/CD43^{+/-}$), and B-cells ($Thy1.2^{-}/CD19^{+}$) and T-cells ($Thy1.2^{+}/CD19^{-}$) in the spleen, as well as their expression of CD45.1, CD45.2, and Abp1 were analyzed by flow cytometry and analyzed with FlowJo, (PE anti-mouse Thy1.2 [BD Biosciences], AF488 anti-mouse CD19 [Biolegend], PerCP Cy5.5 anti-mouse CD45.1 and APC anti-mouse CD45.2 [BD Biosciences]). After reconstitution, B-cells in lethally irradiated recipients ($CD45.1^{+}$) were entirely donor derived ($CD45.2^{+}$).

2.3.5 Serological analysis

The presence of anti-nuclear Ab (ANA) in sera was tested using ANA (HEp-2) antigen substrate slides as per manufacturer instructions (MBL International Corporation, Des Plaines, IL). The serum levels of anti-dsDNA in wt, *Abp1*$^{-/-}$, wt-Ch, and *Abp1*$^{-/-}$-Ch were quantified by Enzyme-Linked Immunosorbent Assay (ELISA) using a previously published protocol (52). Briefly, ELISA plates were pre-coated at 4°C with dsDNA (10 mg/ml, Sigma-Aldrich). After blocking, the plates were incubated with diluted triplicate serum samples and goat anti–mouse IgG-horseradish peroxidase (HRP) (Jackson ImmunoResearch). HRP was detected using OptEIA TMB substrate (BD Bioscience).

2.3.6 Immunizations

To analyze T cell–independent and T-cell dependent antigen responses, mice were immunized by intraperitoneal (i.p.) injection of (4-hydroxy-3-nitrophenyl)acetyl (NP) conjugated to either Ficoll (NP-Ficoll, T-cell independent antigen) or keyhole limpet hemocyanin (NP-KLH, T-cell dependent antigen) (Biosearch Technologies, Petaluma, CA) in PBS or Sigma Adjuvant System (Sigma Aldrich) respectively (40 µg/mouse in 200 µl volume). For T-cell dependent experiments, mice were boosted on

day 28 with a second injection of NP-KLH. To determine anti-NP Ab concentration in response to NP-Ficoll or NP-KLH immunization, NP_{30}-BSA (Biosearch Technologies) coated ELISA plates were incubated with diluted serum, followed by HRP-conjugated anti-mouse Abs including anti-IgM, or a cocktail of anti-IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotech) (239). HRP was detected using OptEIA TMB substrate. The samples were run in triplicate and corrected for background binding. Total serum Abs were analyzed by coating ELISA plates directly with diluted serum, followed by HRP conjugated anti-mouse Abs. Relative IgG affinity from the serum of NP-KLH immunized mice was assessed as the concentration ratio of IgG bound to NP_4-BSA versus NP_{30}-BSA on day 7 and day 35 after the first immunization (57).

2.3.7 Preparation of mono-biotinylated Fab' antibody

Mono-biotinylated Fab' fragment of anti-mouse Ab (mB-Fab'-anti-IgG+M) was generated from the F(ab')2 fragment (Jackson ImmunoResearch) using a published protocol (240). The disulfide bond that links the two Fab fragments was reduced using 2-mercaptoethylamine, and the reduced cysteine was biotinylated by maleimide activated biotin (Thermo Scientific, Waltham, MA). Fab' was further purified using Amicon Ultra centrifugal filters (Millipore). One biotin per Fab' was confirmed using a biotin quantification kit (Thermo Scientific). Fab' was labeled with AF546 (AF546-mB-Fab'-anti-IgG+M) based on the manufacturer-recommended protocol (Invitrogen).

2.3.8 Preparation of antigen tethered planar lipid bilayers

Liposomes were made by combining 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-cap-biotin (Avanti Polar Lipids,

Alabaster, AL) in a 100:1 molar ratio, using the Avanti Polar Lipids Extruder. Coverslip chambers (Nunc/Thermo Scientific) were coated with the planar lipid bilayer by incubating with the liposomes (0.05 mM) for 10 min (72). After extensive washes, the coated coverslip chamber was incubated with 1 μg/ml streptavidin (Jackson ImmunoResearch Laboratories), followed by 1 μg/ml AF546-mB-Fab'–anti-IgG+M mixed with 4 μg/ml mB-Fab'–anti-IgG+M Ab. For a non-antigen control, surface BCRs were labeled by incubating with AF546-Fab'–anti-IgG+M (5 μg/ml) on ice for 30 min. The labeled B-cells were then incubated with biotinylated holo-transferrin (Tf; 8 μg/ml, an equal molar concentration of 5 μg/ml mB-Fab′–anti-Ig+M; Sigma-Aldrich) tethered to lipid bilayers by streptavidin.

2.3.9 Total internal reflection microscopic analysis

Images were acquired using a Nikon laser TIRF system on an inverted microscope (Nikon TE2000-PFS, Nikon Instruments Inc., Melville, NY) equipped with a 60X, NA 1.49 Apochromat TIRF objective (Nikon), a Coolsnap HQ2 CCD camera (Roper Scientific, Trenton, NJ), and two solid-state lasers of wavelength 491 and 561 nm. Interference refection images (IRM), AF488, and AF546 were acquired sequentially. To image intracellular molecules, B-cells were incubated with mB-Fab'-anti-Ig-tethered lipid bilayers at 37ºC for varying lengths of time. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin, and stained for Abp1, HPK1 (Cell Singling Technology), pSHIP-1 (Y1020, Cell Singling Technology), pSyk (Cell Signaling Technologies), pWASP (S483/S484, Bethyl Laboratory, Montgomery, TX), and pN-WASP (Y256, Millipore). F-actin was stained using AF488-phalloidin. The B-

cell contact area was determined based on IRM images using MATLAB software (The MathWorks, Natick, MA). The total and mean fluorescence intensity of each staining in the B-cell contact zone was determined using Andor iQ software (Andor Technology, Belfast, UK). Relative intensity of IRM and fluorescence intensity of BCRs, or intracellular molecules along lines drawn across cells were measured using Andor iQ software (Andor Technology). Background fluorescence generated by mB-Fab'-anti-Ig tethered to lipid bilayers in the absence of B-cells or secondary antibody controls, was subtracted. For each set of data, more than 50 individual cells from three independent experiments were analyzed.

2.3.10 Confocal microscopic analysis

Splenic B-cells were incubated with FcR blocking mAb for 10 min on ice, stained with Cy3-Fab-goat-anti-mouse IgM (Jackson ImmunoResearch) to label BCRs, washed, and activated with 10 μg/ml F(ab')2-goat-anti-mouse IgG+M (Jackson ImmunoResearch) at 37^{o}C. Cells were fixed at designated time point with 4% paraformaldehyde, and imaged with a Zeiss LSM 710 confocal Microscope (Carl Zeiss Microscopy). For each set of data, more than 50 individual cells from three independent experiments were analyzed.

2.3.11 Calcium analysis

The intracellular calcium flux was measured by flow cytometry using the calcium-sensitive dyes Fluo4 AM and Fura Red (Molecular Probes, Eugene, OR) and manufacturer-recommended protocols. Splenic B-cells were stained with 2 μg/ml Fluo4 and 5 μg/ml Fura Red for 30 min at 37°C, washed, and analyzed using a FACSAria flow

cytometer (BD Biosciences). The basal fluorescence intensity of Fluo4 AM and Fura Red were measured for 60 s before stimulation with 10 μg/ml F(ab')2-goat anti-mouse IgG+M (Jackson ImmunoResearch) at 37°C. The fluorescence intensity of Fluo4 AM and Fura Red in fluorescence levels were then measured for 300 s. The relative levels of intracellular calcium were determined by a ratio of Fluo4 AM to Fura Red emission values using FlowJo software (Tree Star, Ashland, OR).

2.3.12 Statistical analysis

Data analysis was performed using Excel software (Microsoft, Redmond, WA) and analyzed using the Student's t test. The statistical differences between groups are indicated with p-values in the related graphs as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.4 Results

2.4.1 B-cell-specific Abp1 deficiency is sufficient to increase the development of marginal zone and germinal center B-cells.

Previous studies have shown that there is no significant defect in T cell development in *Abp1*$^{-/-}$ mice (195), but whether Abp1 contributes to B-cell development and maturation has not been fully examined. To determine whether and at which stage Abp1 may play a role in B-cell development, B-cell subsets in the bone marrow, spleen, and PerC in *Abp1*$^{-/-}$ mice were analyzed, in comparison with those in wt mice. The Abp1 knockout mouse model was developed and characterized by Han et al. (195), and we previously confirmed the absence of the Abp1 gene and protein expression in the Abp1 knockout mice (150). Bone marrow cells, splenocytes and peritoneal cells were isolated

from 6-8 week old wt and *Abp1*$^{-/-}$ mice, labeled with antibodies specific to cell surface markers indicative of pre-determined maturational stage, and examined by flow cytometry. I utilized Hardy Classification to identify B-cells at different maturation stages in the bone marrow, including the pre-pro (A - B220^{+}/CD43^{+}/CD24^{low}/BP-1^{low}) , pro (B - B220^{+}/CD43^{+}/CD24^{high}/BP-1^{low}), early pre (C - B220^{+}/CD43^{+}/CD24^{high}/BP-1^{high}), late pre (D - B220^{+}/CD43^{-}/IgM^{low}), immature (E - B220^{+}/CD43^{-}/IgM^{int}), and mature recirculating (F - B220^{+}/CD43^{-}/IgM^{high}) B-cell populations (Fig. 2.1A). Cells were quantified as a percentage of total bone marrow isolated, and I observed no significant differences in the isolated BM B-cell subsets (Fig. 2.1B). Splenocytes were stained to identify transitional 1 (T1, B220^{+}/IgM^{high}/IgD^{low}), transitional 2 (T2, B220^{+}/IgM^{high}/IgD^{high}), follicular (FO, B220^{+}/IgM^{low}/IgD^{high}), isotype switched (IS, B220^{+}/IgM^{-}/IgD^{-}), and marginal zone (MZ, B220^{+}/CD21^{high}/CD23^{low}) B-cells (Fig. 2.2A). B-cell subsets in the spleen were quantified as the average number of cell type isolated per spleen (Fig. 2.2B). Analysis showed that the *Abp1*$^{-/-}$ mice had significantly greater numbers of MZ B-cells compared to their wt counterparts (Fig. 2.2B). Peritoneal cells were stained to identify B1a (IgM^{+}/IgD^{-}/CD5^{+}/Cd11b^{+}) and B1b (IgM^{+}/IgD^{-}/CD5^{-}/Cd11b^{+}) cell populations (Fig. 2.3A). B-cell subsets in PerC were quantified as the average number of cell type isolated per peritoneal cavity (Fig. 2.3B). The results from flow cytometry analysis showed that the numbers of B1a B-cells in the peritoneal cavity were significantly increased, compared to those of wt mice (Fig.2.3). Taken together, these results suggest that Abp1 is involved in the negative regulation of marginal zone and B1a B-cell development in the spleen and peritoneal cavity, but not in early B-cell development in bone marrow.

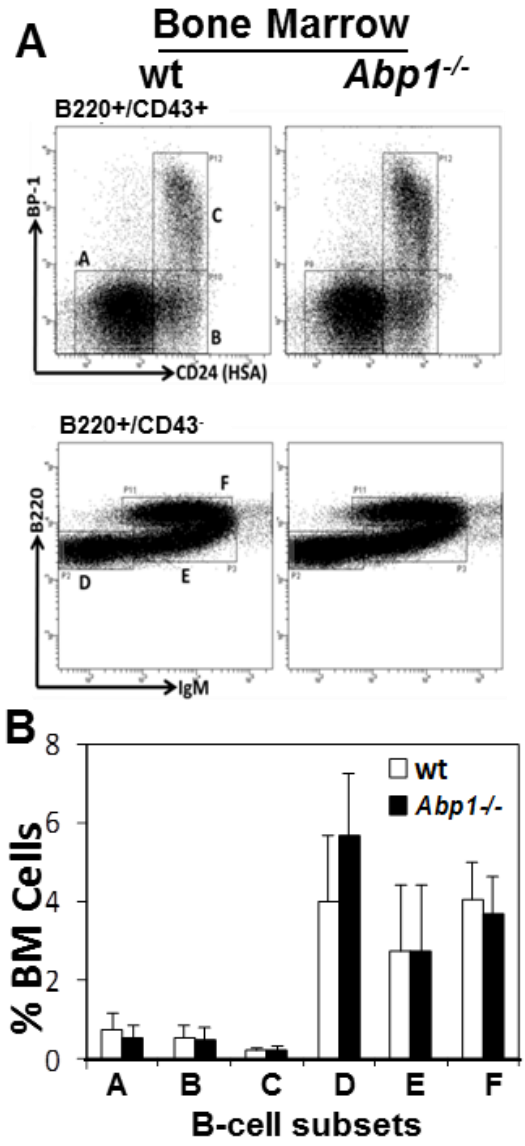


Fig. 2.1 Abp1 deficiency does not affect B-cell maturation in the bone marrow. (A-B) Cells from BM of wt and *Abp1*$^{-/-}$ mice were labeled with Abs specific for surface markers of pre-pro- (A), pro- (B), early pre- (C), late pre- (D), immature (E) and re-circulating mature B-cells (F) in the BM, and analyzed using flow cytometry. Shown are representative dot plots **(A)**, and the average percentage (+S.D.) of total cells extracted from BM **(B)** (n=5).

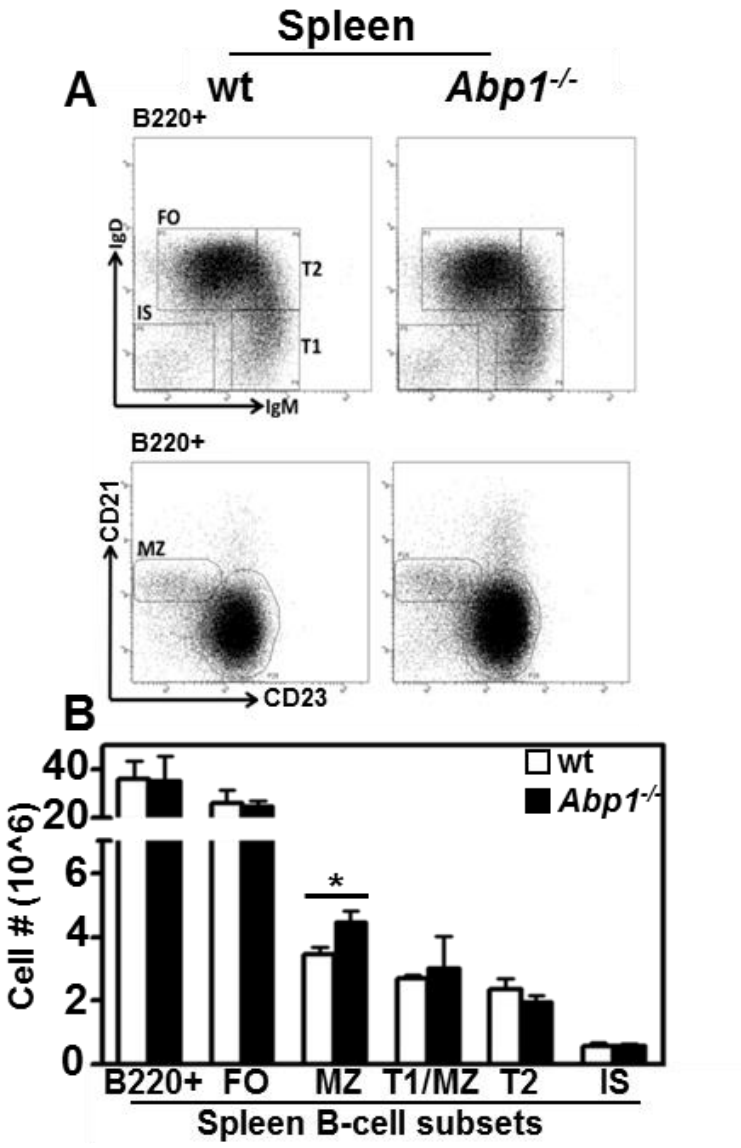


Fig. 2.2 Abp1 deficiency affects the development of splenic MZ B-cells. (A-B) Cells from spleens of wt and *Abp1*$^{-/-}$ mice were labeled with Abs specific for surface markers of transitional 1 (T1), transitional 2 (T2), follicular (FO), isotype switched (IS) and marginal zone (MZ) B-cells in the spleen, and analyzed using flow cytometry. Shown are representative dot plots **(A)**, and the average number (+S.D.) of total cells extracted from splenn **(B)** (n=5). $p<0.05$

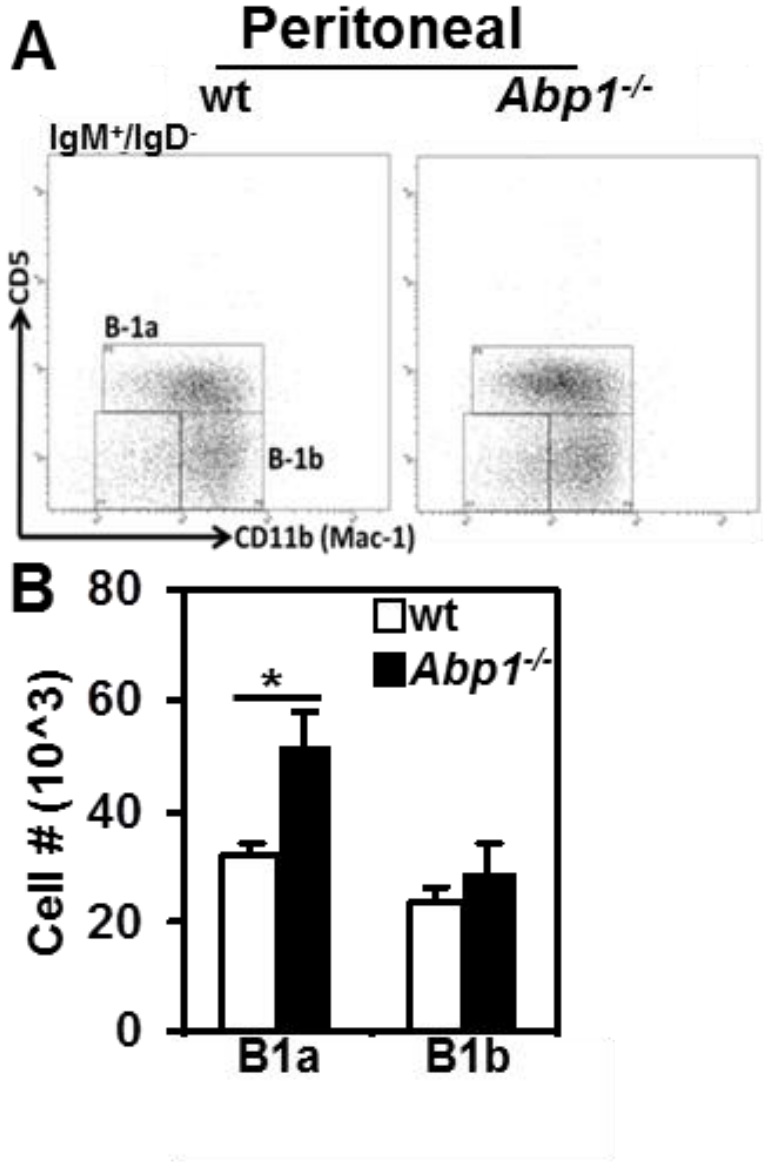


Fig. 2.3 Abp1 deficiency affects the expansion of PerC B1a B-cells. (A-B) Cells from PerC of wt and *Abp1*$^{-/-}$ mice were labeled with Abs specific for surface markers of B1a and B1b B-cell subsets, and analyzed using flow cytometry. Shown are representative dot plots **(A)**, the average number (+S.D.) of cells extracted from PerC **(B)** (n=4). $p<0.05$

The isolated effect of Abp1 deficiency on the development of peripheral B-cells led to the question of whether Apb1 is differentially expressed in the various B-cell subsets. To address this question, I determined the levels of Abp1 protein expression in each B-cell subset by intracellular staining of Abp1 by flow cytometry (Fig. 2.4). Results were normalized to the mean fluorescent intensity (MFI) of Abp1 in isotype switched (IS) B-cells, which displayed the highest expression level among all B-cell subsets. Indeed, the expression level of Abp1 protein increased as the B-cells matured, and the mature B-cells in the spleen expressed a significantly higher level of Abp1 than pro- and pre-B-cells in the bone marrow. Together with the previous experiments, the data indicate that Abp1 deficiency appears to only affect the maturation of specific subsets of peripheral B-cells, and this may be due to differential expression levels of Abp1 within the various subsets.

Since peripheral mature B-cells are activated in B-cell follicles to form germinal centers, I determined the number and distribution of germinal center B-cells in the spleen of non-immunized mice using flow cytometry and immunohistochemistry. Germinal center B-cells in spleens were labeled with antibodies specific for B220, CD95 and GL7 (GC, $B220^{+}/CD95^{high}/GL7^{high}$) (Fig. 2.5A). In the spleen of non-immunized *Abp1*$^{-/-}$ mice, the number of GC B-cells was significantly higher than that in the wt mice (Fig. 2.5B). Consistent with the results of flow cytometry analysis, immunohistological examination showed more frequent and larger PNA^{+} germinal centers in the spleens of *Abp1*$^{-/-}$ than that in wt mice (Fig. 2.5C-D). Additionally, *Abp1*$^{-/-}$ mice also exhibit splenomegaly (Fig. 2.5E), as previously reported (230). These results indicate a role for Abp1 in suppressing the formation of spontaneous germinal center B-cells.

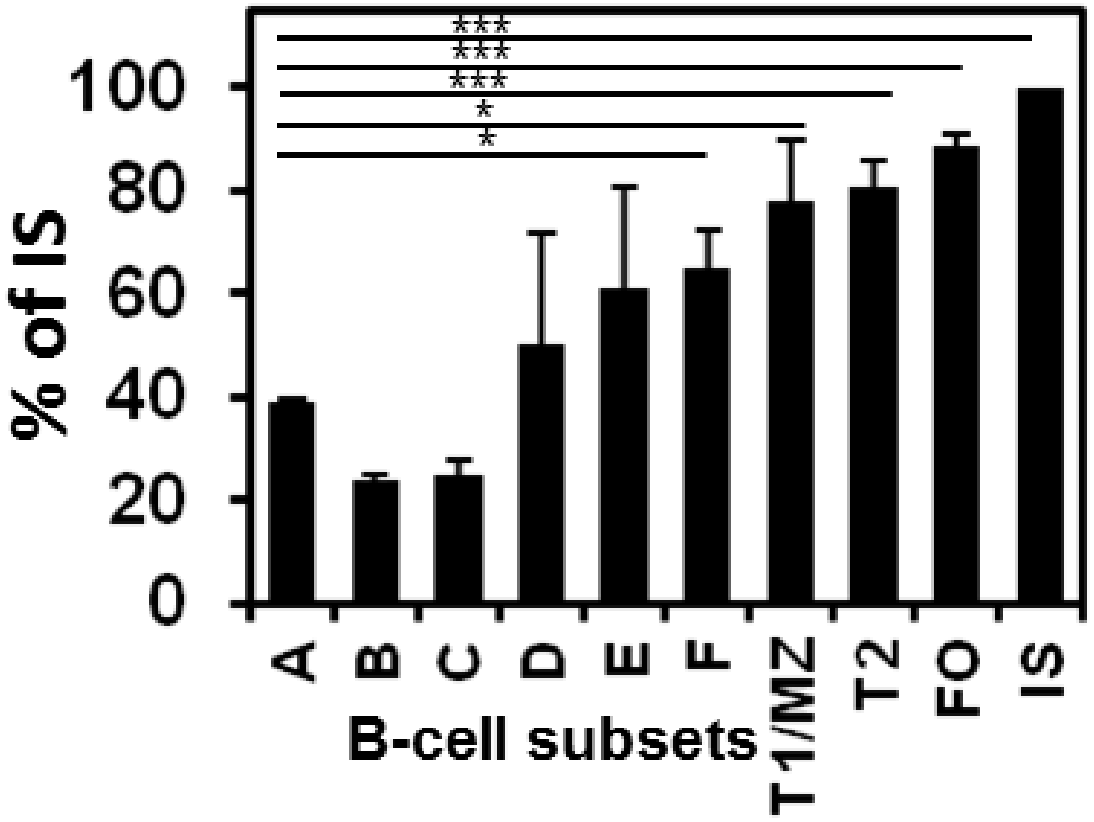
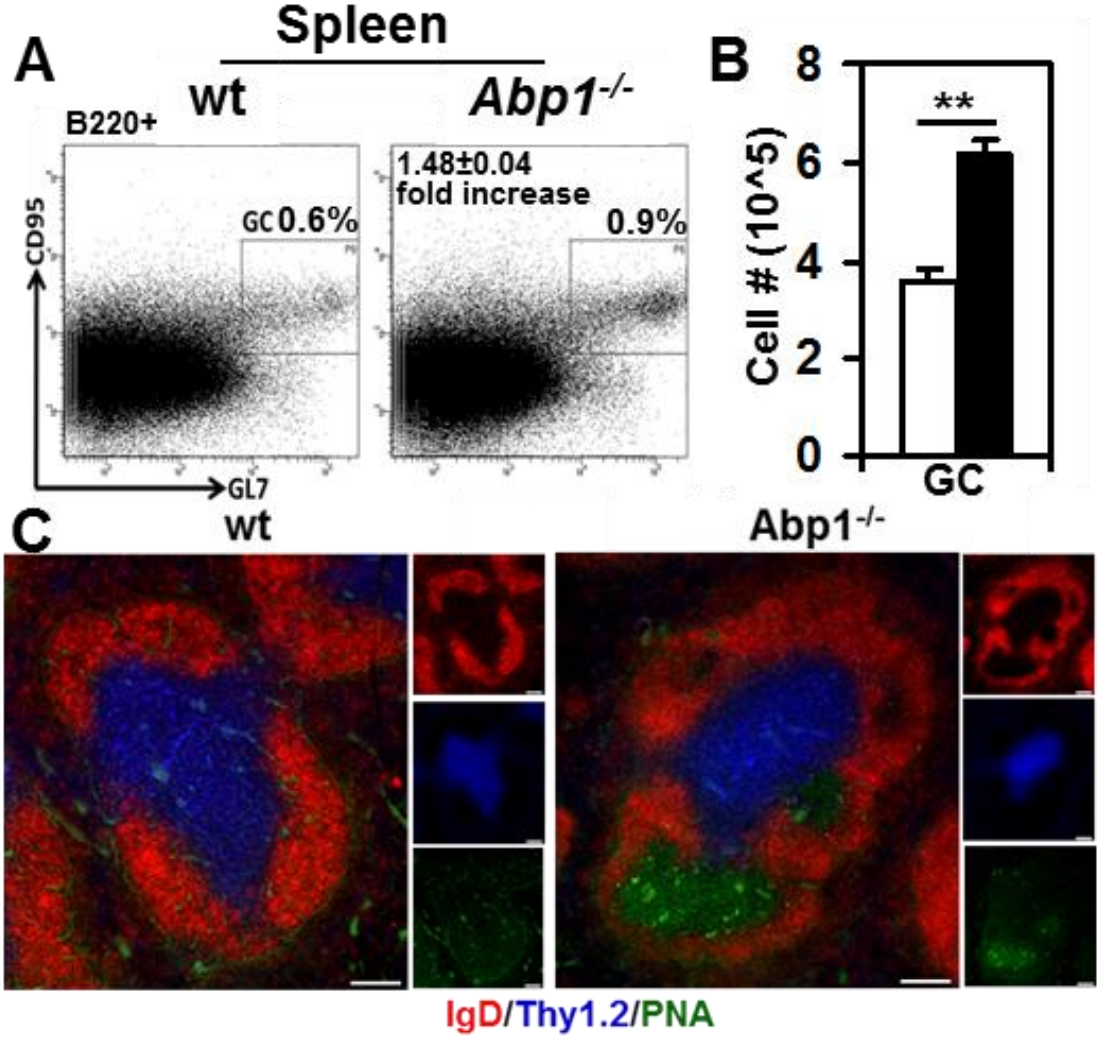


Fig. 2.4 Abp1 differentially expressed in B-cell subsets. Abp1 expression levels in different B-cell subsets. BM cells and splenocytes from wt mice labeled as in Fig 2.1-2.3 were stained with anti-Abp1 Ab. Shown are the average MFIs of Abp1 as a percent of the IS population (+SD, n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$


A
Spleen
wt
Abp1-/-
B220+
CD95
GL7
GC 0.6%
1.48±0.04
fold increase
0.9%
B
Cell # (10^5)
8
6
4
2
0
**
GC
C
wt
Abp1-/-
IgD/Thy1.2/PNA

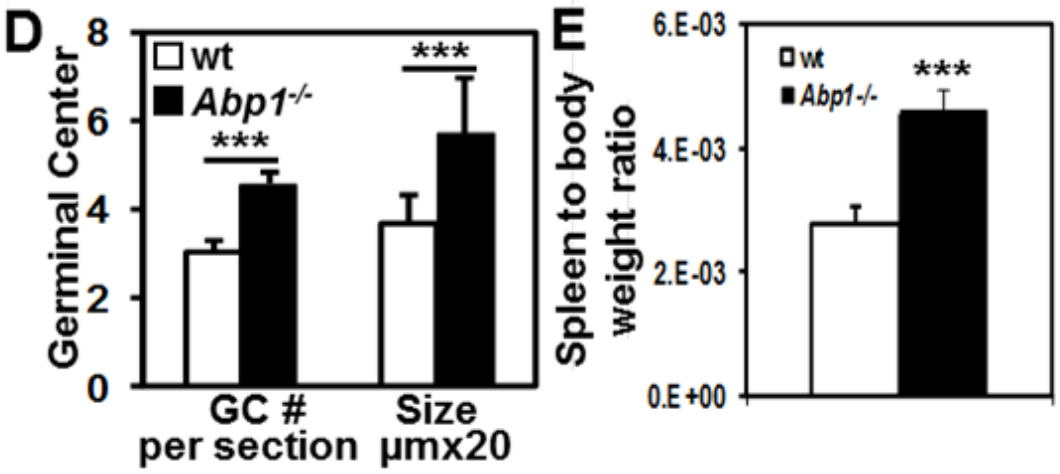


Fig. 2.5 Abp1 deficiency promotes spontaneous differentiation of GC B-cells, and initiation of splenomegaly. (A-B) GC B-cell development in *Abp1*$^{-/-}$ mice. Cells from spleens of wt and *Abp1*$^{-/-}$ mice were labeled with Abs specific for surface markers of GC B-cells, and analyzed using flow cytometry. Shown are representative dot plots **(A)**, and the average number (+S.D.) of GC-B-cells extracted from spleen **(B)** (n=4). **(C)** Immunofluorescent stained images of spleen sections from mice (6 months old). Bars, 100 µm (n=12 sections/4 wt or *Abp1*$^{-/-}$ mice). **(D)** Average numbers of PNA^{+} GCs per spleen section and average length of GC (µmx20) (+S.D., n=12 sections/4 wt or *Abp1*$^{-/-}$ mice). **(E)** Spleen weight of 6-8 weeks old mice as a percentage of body mass (+S.D.) (n=25). ** $p<0.01$, ***, $p<0.001$

To examine whether the phenotype observed in *Abp1*$^{-/-}$ mice is B-cell intrinsic, I generated bone marrow chimeric mice in which only B-cell lack Abp1 expression (52). Cells from bone marrow (Fig. 2.6 A-C), and spleens (Fig. 2.6 D-F) of lethally irradiated CD45.1^{+} wt C57BL/6 transplanted with BM from *Apb1*$^{-/-}$ or wt mice (both CD45.2^{+}) mixed at a 20:80 ratio with μMT BM, were labeled with Abs specific for surface markers of B-cells in the bone marrow (A), B and T-cells in the spleen (D), and analyzed by flow cytometry for both expression CD45.1 and CD45.2 and cell number to determine re-constitution efficiency (Fig. 2.6C and F). Both wt chimera (wt-Ch) and *Abp1*$^{-/-}$-chimera (*Abp1*$^{-/-}$-Ch) BM (Fig. 2.6C), and splenic cells (Fig. 2.6F) stained positive for CD45.2 expression (donor cells), and the percentage of B-cells extracted from the bone marrow (Fig. 2.6B) and B and T-cells from the spleen (Fig. 2.6E) were comparable those found in wt C57Bl6 mice. To confirm B-cell chimerism, B-cells from the BM and spleen were further labeled with anti-Abp1 Ab to determine Abp1 protein expression within various cell subsets from wt-Ch and *Abp1*$^{-/-}$-Ch (Fig. 2.7). Flow cytometry confirmed that only B-cells lacked Abp1 expression in fully reconstituted *Abp1*$^{-/-}$-Ch (Fig. 2.7). Finally, GC and MZ numbers were analyzed in both wt-Ch and *Abp1*$^{-/-}$-Ch mice, and similar to *Abp1*$^{-/-}$ mice, *Abp1*$^{-/-}$-Ch mice displayed elevated numbers of spontaneous GC and MZ B-cells in the spleen, compared to those in wt-Ch (Fig. 2.8).

Taken together, these results suggest that Abp1 exerts a B-cell intrinsic inhibition on the spontaneous development of GC B-cells and differentiation of MZ and B1a B-cells, but is not involved in early B-cell maturation in the bone marrow.

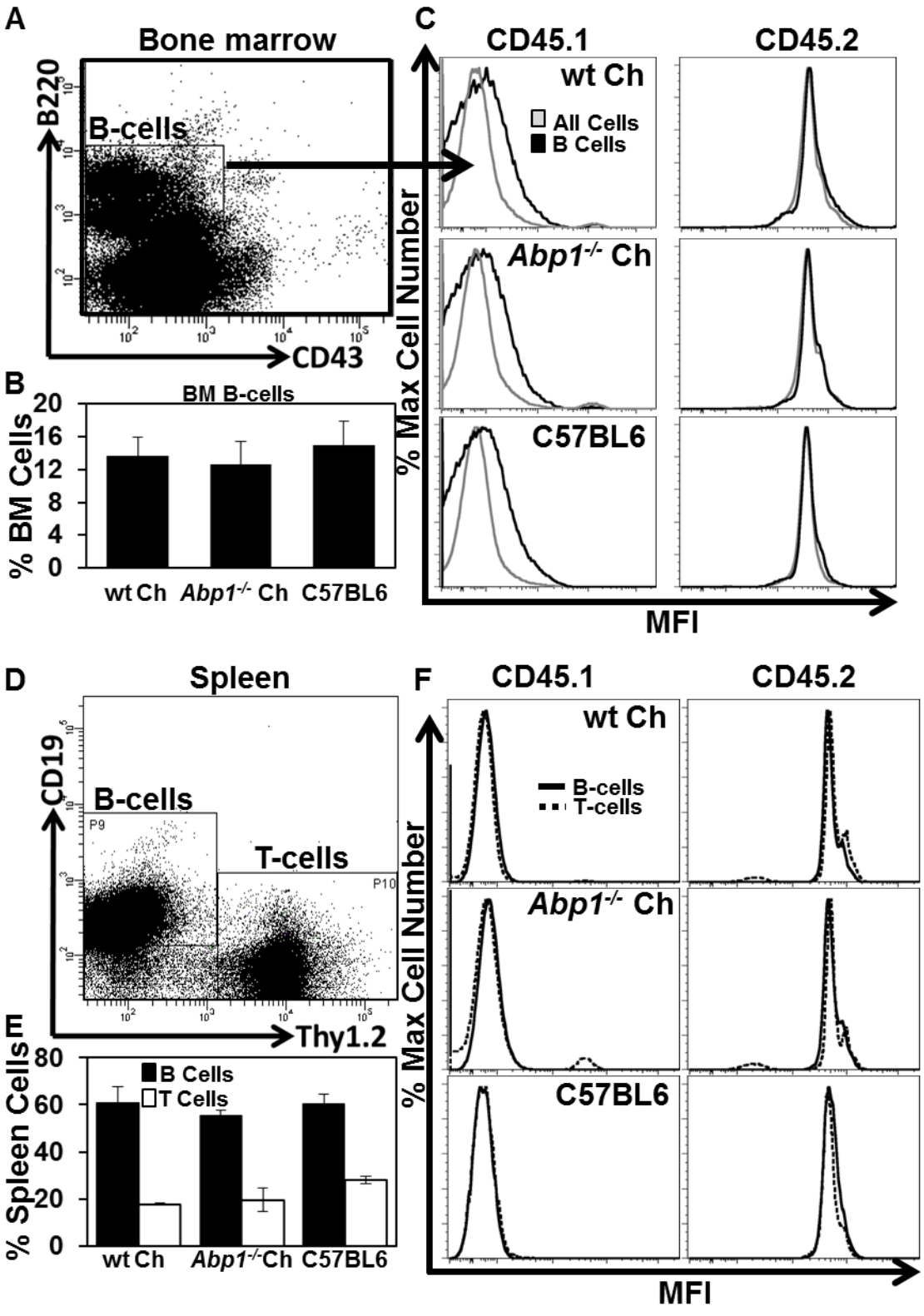

A
Bone marrow
B220
B-cells
CD43
B
BM B-cells
% BM Cells
20
16
12
8
4
0
wt Ch
Abp1-/- Ch
C57BL6
C
CD45.1
CD45.2
wt Ch
All Cells
B Cells
Abp1-/- Ch
C57BL6
% Max Cell Number
MFI
D
Spleen
CD19
B-cells
P9
T-cells
P10
Thy1.2
E
% Spleen Cells
80
60
40
20
0
B Cells
T Cells
wt Ch
Abp1-/-Ch
C57BL6
F
CD45.1
CD45.2
wt Ch
B-cells
T-cells
Abp1-/- Ch
C57BL6
% Max Cell Number
MFI

Fig. 2.6 Reconstitution of bone marrow chimera mice. Lethally irradiated wt C57BL/6 ($CD45.1^+$) were transplanted with BM from *Apb1*$^{-/-}$ or wt mice ($CD45.2^+$) mixed at a 20:80 ratio with BM from B-cell null μMT mice. Four months post transplantation, cells from BM **(A-C)** and spleens **(D-F)** of wt (wt-Ch) and *Abp1*$^{-/-}$ (*Abp1*$^{-/-}$-Ch) BM chimera mice were labeled with Abs specific for surface markers of B-cells **(A)** in the BM, for B and T-cells in the spleen **(D)** and for CD45.1 and CD45.2, and analyzed using flow cytometry. Shown are representative dot plots **(A and D)**, the average percentage (+S.D.) of total cells extracted from MB **(B)** and B and T-cells from the spleen **(E)**, as well as the relative expression of CD45.1 and CD45.2 in total BM cells, BM B-cells **(C)**, and splenic B and T-cells **(F).** n=3.

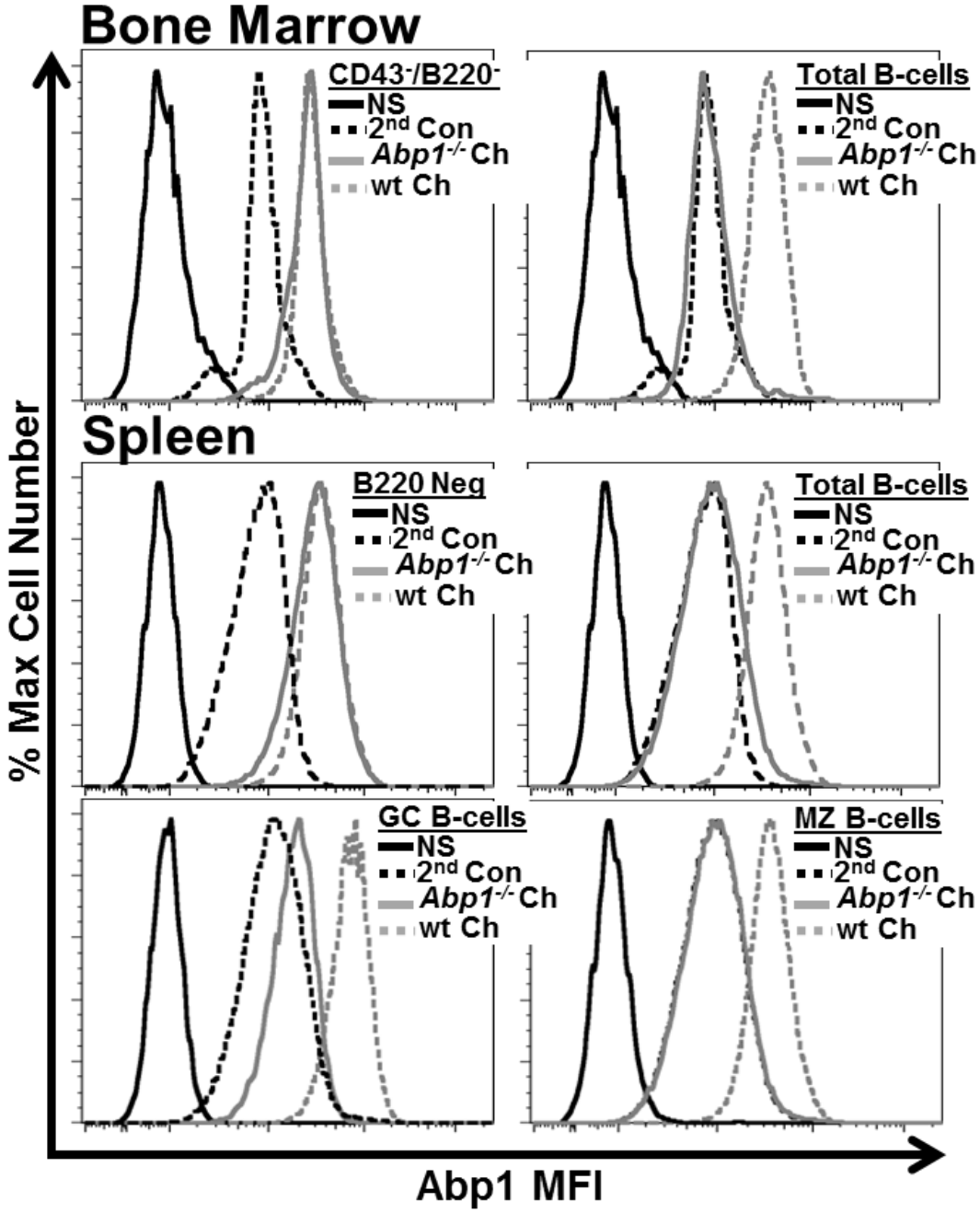

Bone Marrow
CD43-/B220-
NS
2nd Con
Abp1-/- Ch
wt Ch
Total B-cells
NS
2nd Con
Abp1-/- Ch
wt Ch
Spleen
B220 Neg
NS
2nd Con
Abp1-/- Ch
wt Ch
Total B-cells
NS
2nd Con
Abp1-/- Ch
wt Ch
GC B-cells
NS
2nd Con
Abp1-/- Ch
wt Ch
MZ B-cells
NS
2nd Con
Abp1-/- Ch
wt Ch
% Max Cell Number
Abp1 MFI

Fig. 2.7 Only B-cells in *Abp1*$^{-/-}$ bone marrow chimeric mice lack the expression of Abp1. BM cells and splenocytes from *Abp1*$^{-/-}$ and wt BM chimeric mice were labeled with Abs specific for the surface markers of total B-cells in the BM and spleen, and MZ and GC B-cells in the spleen. The cells were fixed, permeabilized, and stained for Abp1. Cells were analyzed by flow cytometry. Shown are representative histograms (n=3).

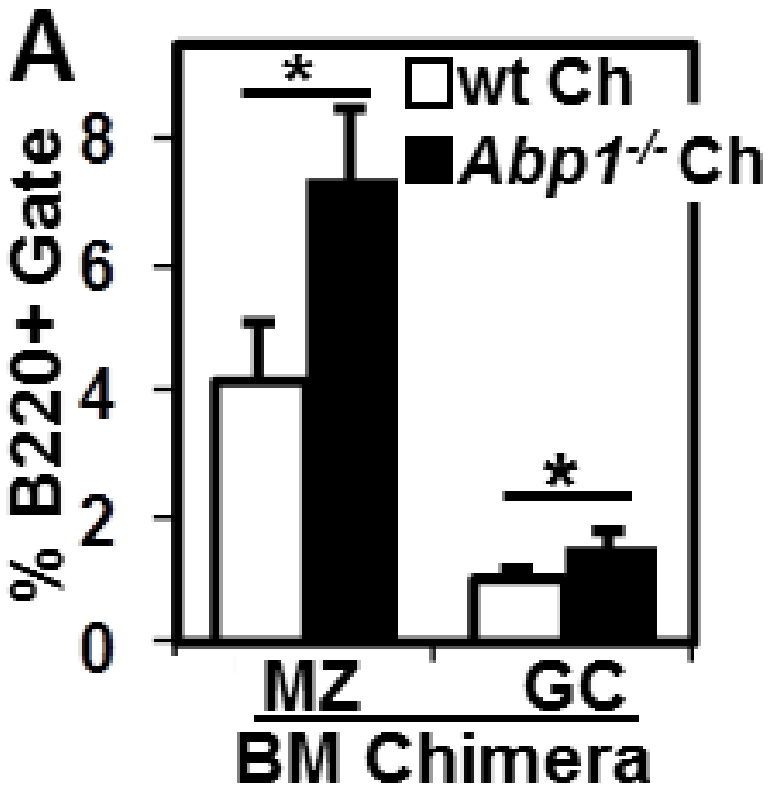


Fig. 2.8 B-cell-specific Abp1 deficiency is sufficient to increase the differentiation of MZ and development of spontaneous GC B-cells. (A) MZ and GC B-cell subsets in *Abp1*$^{-/-}$-Ch and wt-Ch mice. Cells from spleens were labeled for surface markers of MZ and GC- B-cells, and analyzed by flow cytometry. Shown are average percentages of MZ and GC B-cells per B220^{+} splenic B-cells (+S.D., n=3). *, $p<0.05$

2.4.2 Production of autoAb, T-independent Ab responses, and total Ab increase while T-dependent high affinity Ab responses decrease in Abp1$^{-/-}$ mice.

Spontaneous GC formation has been described as a characteristic of autoimmune susceptible mouse strains (241). To determine whether the formation of spontaneous GC B-cells in *Abp1*$^{-/-}$ mice is associated with B-cell-mediated autoimmunity, I monitored the production of autoAbs in the sera of non-immunized wt and *Abp1*$^{-/-}$ mice. Using an anti-nuclear antibody slide test (ANA), I found that while wt mice did eventually become positive for the presence of anti-nuclear antibodies, *Abp1*$^{-/-}$ mice were positive with autoAb much earlier, with 17% positive within 1.5 months and 50% positive by 6 months, compared to 0% of the wt mice (Fig. 2.9A). I further quantified anti-double stranded DNA (dsDNA) antibodies in the sera of non-immunized wt and *Abp1*$^{-/-}$ mice at 1.5, or 6 months of age, using ELISA (Fig. 2.9B). In agreements with the results of the ANA test, *Abp1*$^{-/-}$ mice had significantly higher levels of anti-dsDNA antibody production than wt mice by 6 months of age. Furthermore, *Abp1*$^{-/-}$-Ch mice also showed elevated levels of anti-dsDNA Ab compared to wt-Ch mice, 4 months after bone marrow transplant (Fig. 2.9B).

To determine whether the increased numbers of marginal zone, GC, and B1a B-cells in *Abp1*$^{-/-}$ mice have any impact on antibody responses, I immunized wt and *Abp1*$^{-/-}$ mice with the T-independent antigen NP-Ficoll once or the T-dependent antigen NP-KLH twice, 28 days apart, and quantified NP-specific and total IgM and/or IgG in the serum using ELISA. In response to NP-Ficoll, *Abp1*$^{-/-}$ mice exhibited significant increases in both NP-specific (Fig. 2.10A) and total IgM (Fig. 2.10B) throughout the time course compared to wt mice. In response to NP-KLH, *Abp1*$^{-/-}$ mice exhibited only

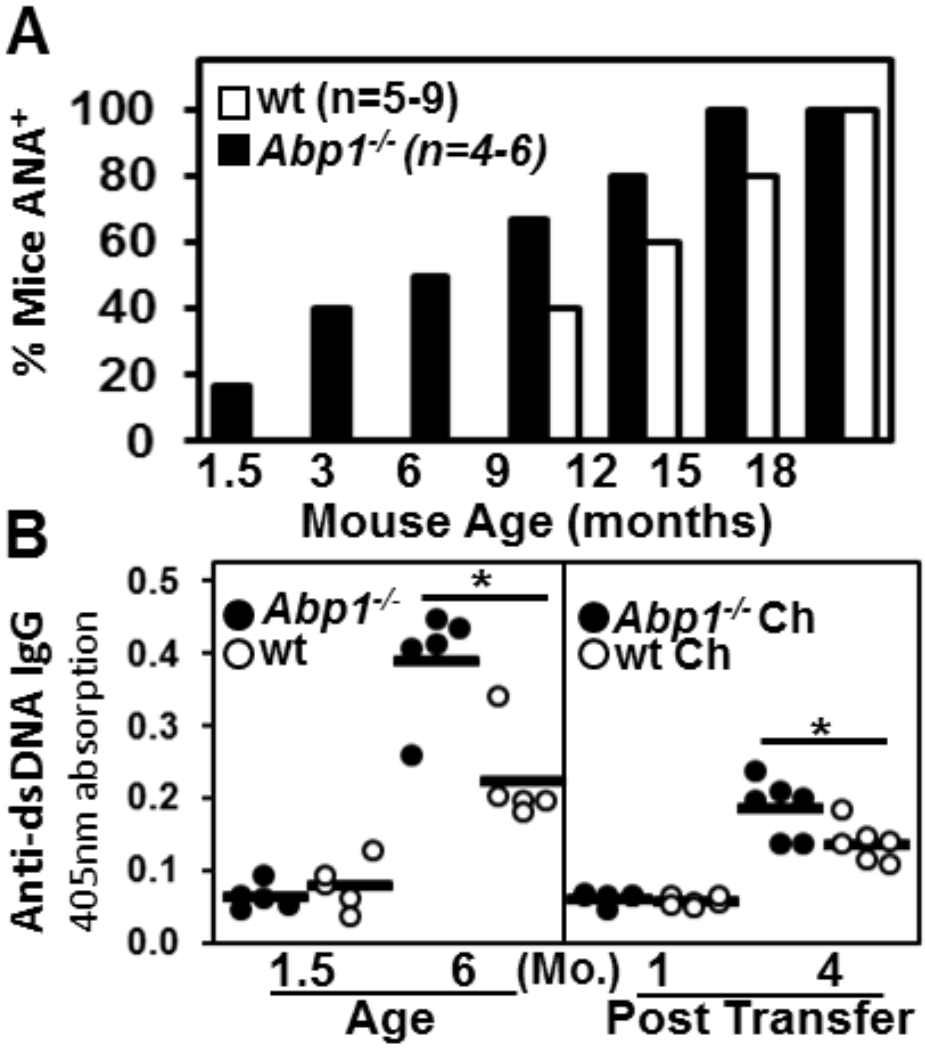


Fig. 2.9 AutoAb production increased in *Abp1*$^{-/-}$ and *Abp1*$^{-/-}$-Ch mice. (A) Immunofluorescence microscopy analysis of anti-nuclear Ab in the serum of non-immunized wt and *Abp1*$^{-/-}$ mice (n=4~9) at different ages. **(B)** ELISA quantification of anti-dsDNA Ab in the serum of wt and *Abp1*$^{-/-}$ mice of 1.5 and 6 months of age, and wt-Ch and *Abp1*$^{-/-}$-Ch mice at 1 and 4 months post transplant. Dots represent individual mice. *, $p<0.05$

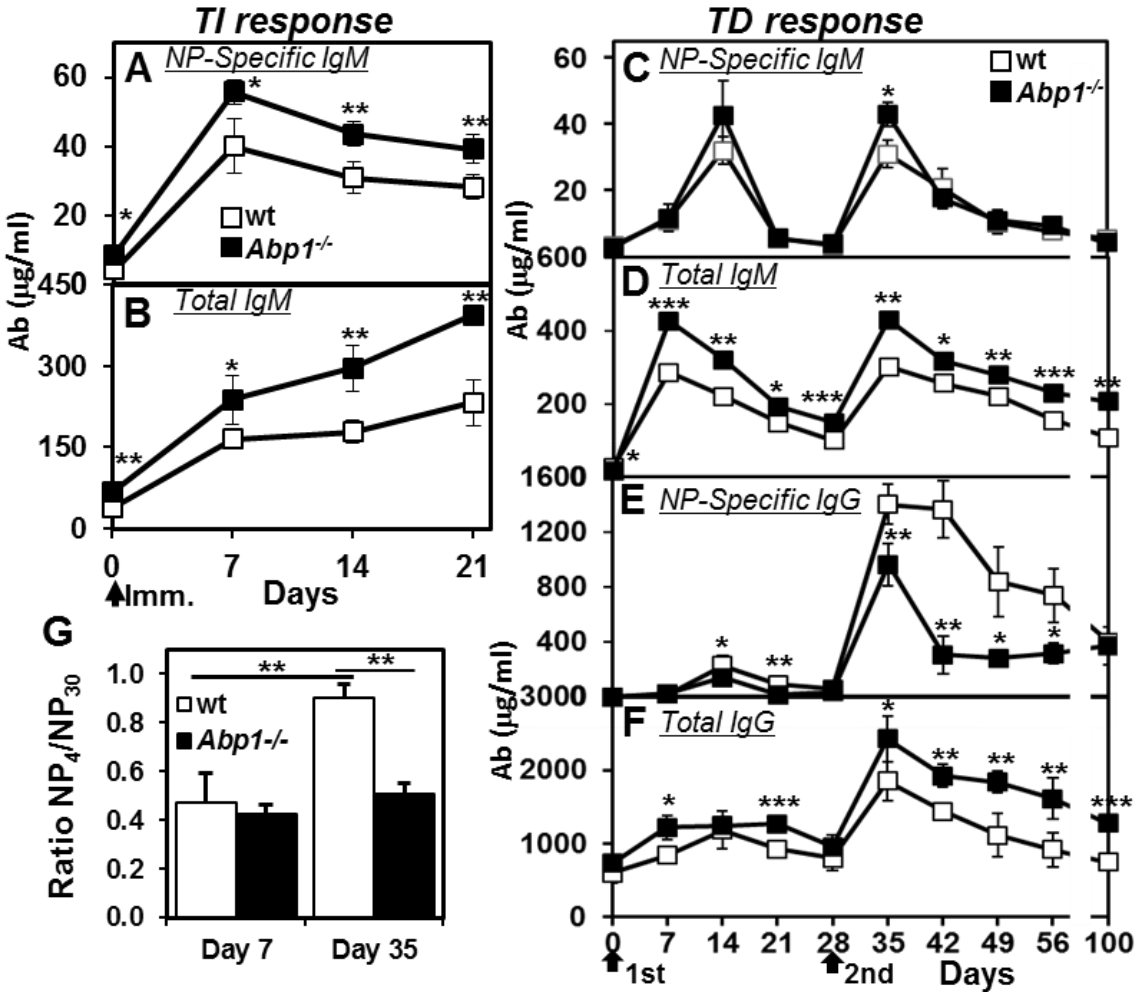


Fig. 2.10 Production of T-independent Ab responses, and total Ab increases while T-dependent high affinity Ab responses decrease in *Abp1*$^{-/-}$ mice. (A-F) 6~8 weeks old mice (n=4~5) were immunized with NP-Ficoll **(A-B)** or NP-KLH **(C-F)**. NP-specific IgM **(A, C)**, total IgM **(B, D)**, NP-specific IgG **(E)**, or total IgG **(F)** in the serum (µg/ml) determined by ELISA. **(G)** Relative affinity of NP-specific IgG in NP-KLH- immunized mice assessed as ratios of IgG bound to NP_4 versus NP_{30} by ELISA (+SD, n=4~5). *, $p<0.05$, **, $p>0.01$, ***, $P<0.001$

slightly elevated antigen-specific IgM at day 35, 7 days after the boost, but the level of total IgM was significantly increased throughout the time course compared to those in wt mice (Fig 2.10C-D). Similar to a previous report (195), the serum level of antigen-specific IgG was decreased in *Abp1*$^{-/-}$ knockout mice, compared to that of wt mice (Fig. 2.10E). This decrease was especially dramatic after the boost with NP-KLH (Fig. 2.10E), indicating that the secondary antibody response is more severely affected by Abp1 deficiency than the primary antibody response. However, the level of total IgG in the serum was significantly increased in *Abp1*$^{-/-}$ mice during both the primary and secondary antibody responses (Fig. 2.10F), similar to the total IgM. I further evaluated Ab affinity in NP-KLH immunized mice by determining the ratio of Ab binding to low (NP_4) and high valent (NP_{30}) antigen by ELISA. The ratio was significantly increased in wt mice but not in *Abp1*$^{-/-}$ mice post boost (Fig. 2.10G). Thus, while *Abp1*$^{-/-}$ mice display impaired affinity maturation, and are defective in mounting T-dependent Ab responses, they conversely have elevated T-independent, non-specific, and self-reactive Ab responses.

2.4.3 Abp1 deficiency augments B-cell spread and BCR clustering.

As the increased production of spontaneous GC B-cells and autoAb is a B-cell-intrinsic effect, I further examined the impact of Abp1 deficiency on early events of BCR-mediated B-cell activation, including BCR clustering and B-cell morphological changes. I labeled and cross-linked surface BCRs using AF546 labeled, mono-biotinylated Fab' fragment of anti-mouse IgG+M antibody (AF546-mB-Fab'–anti-Ig) that was tethered to planar lipid bilayers by streptavidin (Fab'-anti-Ig-tethered lipid bilayer). This activation system allow us to use interference reflection microscopy (IRM)

to analyze the area of the B-cell membrane region that contacts the Fab'-anti-Ig-tethered surface (B-cell contact zone), which reflects B-cell spreading and contraction, as well as total internal reflection fluorescence microscopy (TIRF) to analyze and quantify relative amounts of fluorescently labeled BCRs and Abp1 at the B-cell contact zone. I also analyzed BCR clustering in B-cells stimulated by AF546-mB-Fab'-anti IgG+M, cross-linked by soluble streptavidin using confocal microscopy. I found that the contact area of *Abp1*$^{-/-}$ B-cells did not reduce after 3 min of activation as with wt B-cells, consequently being significantly more spread than that of wt B-cells at 7 and 9 min, indicating an inhibition of cell contraction (Fig. 2.11A-B). The total fluorescence intensity (TFI) of labeled BCRs in the contact zone of *Abp1*$^{-/-}$ B-cells continuously increased over time, compared to wt B-cells that reached a plateau at 3 min and decreased by 9 min (Fig. 2.11A and C). However, the labeled BCRs remained punctuate throughout the contact zone of *Abp1*$^{-/-}$ B-cells, and did not coalesce into a central cluster by 7 min as in wt B-cells (Fig. 2.11A). Furthermore, mean fluorescence intensity (MFI) of BCR staining in the contact zone of *Abp1*$^{-/-}$ B-cells was lower than that of wt B-cells (Fig. 2.11D), even though the TFI of BCRs was higher in *Abp1*$^{-/-}$ than wt B-cells (Fig. 2.11C). In response to cross-linking with mB-Fab'-anti-Ig plus soluble streptavidin, a significantly higher percentage of *Abp1*$^{-/-}$ B-cells showed polarized BCR caps than wt B-cells as early as 1 min of activation (Fig. 2.12 A-B). Together, these results indicate that *Abp1*$^{-/-}$ B-cells sequester greater amounts of BCRs in the contact zone or polarized BCR caps upon encountering membrane-associated or soluble antigen, but are defective in the contraction process and formation of BCR central clusters.

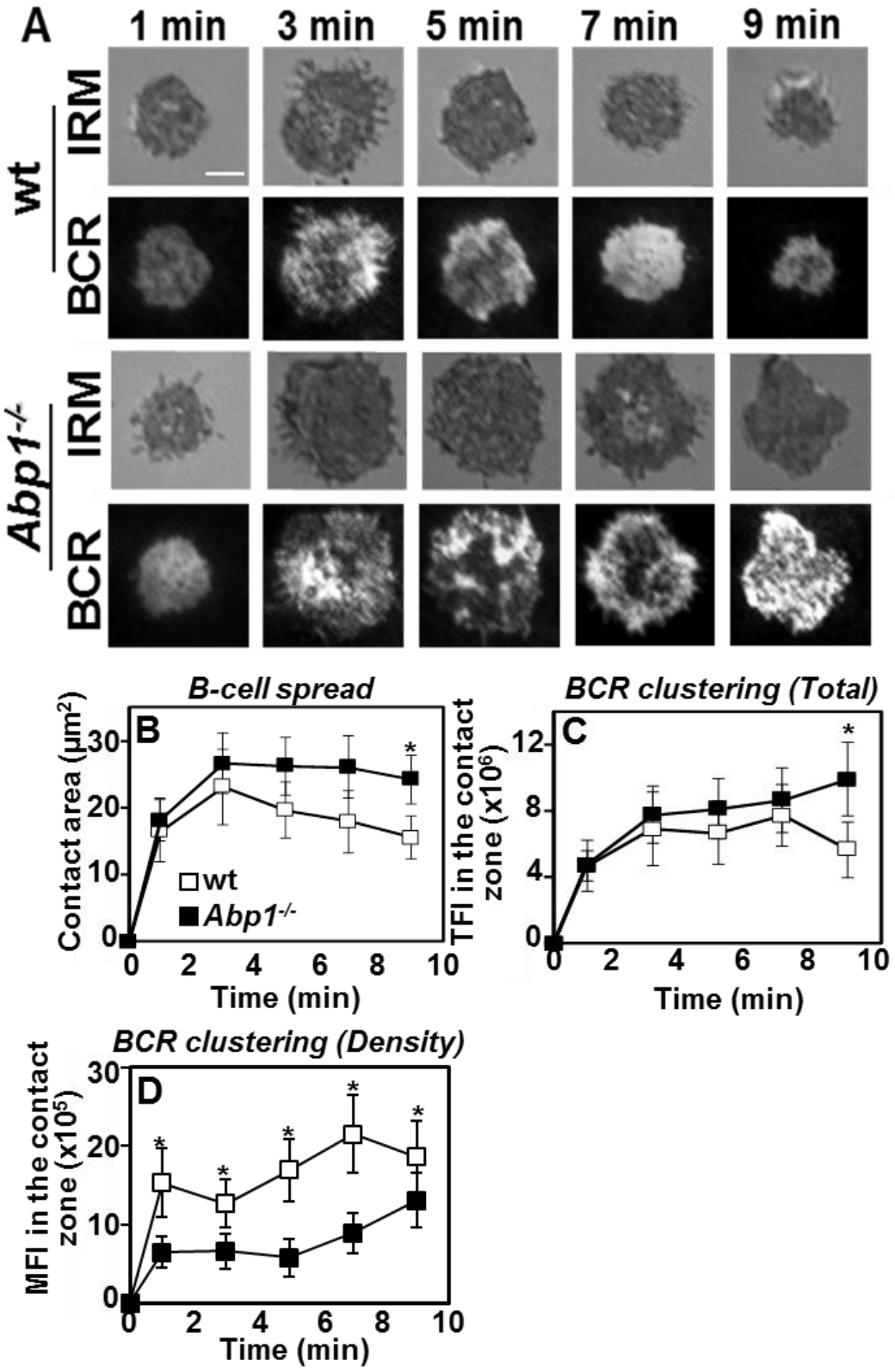

A
1 min
3 min
5 min
7 min
9 min
wt
IRM
BCR
Abp1-/-
IRM
BCR
B-cell spread
B
Contact area (μm2)
wt
Abp1-/-
Time (min)
BCR clustering (Total)
C
TFI in the contact zone (x10^6)
Time (min)
BCR clustering (Density)
D
MFI in the contact zone (x10^5)
Time (min)

Fig. 2.11 Abp1 knockout augments B-cell spread and BCR clustering. (A-D) IRM and TIRF analysis of wt and *Abp1*$^{-/-}$ splenic B-cells incubated with Fab'-anti-Ig-tethered lipid bilayers. Representative images, bar 5 μm, (n=3) **(A)**. Average contact area **(B),** TFI **(C),** and MFI **(D)** of labeled BCRs from > 50 cells per time point (n=3), quantified using Andor iQ software. *, $p<0.05$.

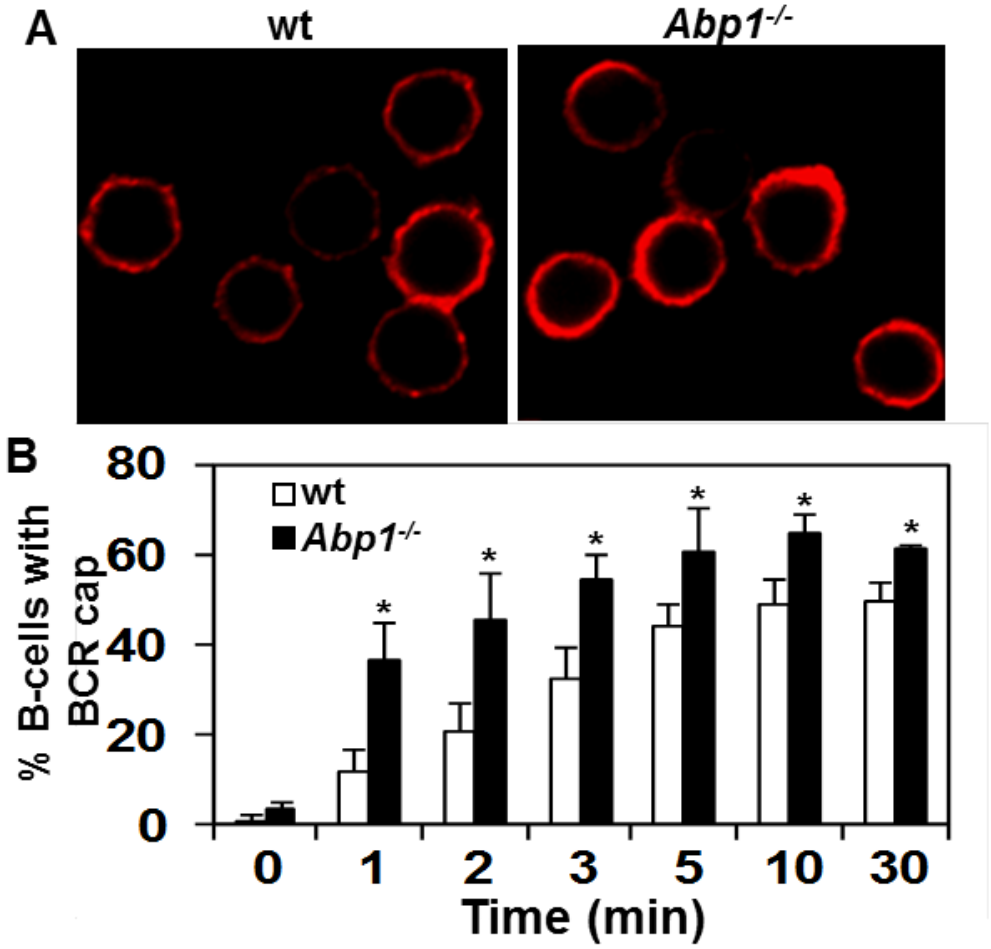


Fig. 2.12 Earlier and greater percentage of capping in *Abp1*$^{-/-}$ B-cells. (A-B) Confocal analysis of splenic B-cells stained with AF546-mB-Fab'–anti-IgG+M then streptavidin to activate. Representative images at 1 min **(A)**, and average percentage (+S.D.) of cells showing polarized BCR caps **(B)** quantified from >100 cells per time point (n=3), bar 5 µm. *, $p<0.05$.

2.4.4 Abp1 is recruited to the leading edge of the spreading B-cells upon BCR activation.

We have previously shown Abp1 is recruited to the cell surface upon B-cell treatment with soluble antigen, and that its tyrosine phosphorylation is necessary for this recruitment (150). To better understand the mechanism by which Abp1 contributes to BCR signaling, I examined the spatial relationship of Abp1 with BCRs at the cell surface. Upon incubation with Fab'-anti-Ig-tethered lipid bilayer, Abp1 was readily recruited to the B-cell contact zone as early as 1 min of activation, as BCR microclusters just became visible (Fig. 2.13A). While the contact zone area and size of BCR clusters change over time, Abp1 predominantly accumulated at the outer edge of the contact zone, and did not significantly colocalize with surface BCRs throughout the activation process (Fig. 2.13A-B). The MFI of Abp1 in the contact zone rose over time, with a dramatic peak observed at 7 min of activation (Fig. 2.13C), concurrent with B-cell contraction, but long after peak BCR TFI and maximal B-cell spread (Fig. 2.11B-C). These results indicate Abp1 is specifically recruited to the leading edge of the B-cell membrane, predominantly during B-cell contraction, in response to BCR activation.

2.4.5 BCR signaling is enhanced in Abp1$^{-/-}$ B-cells.

Increased numbers of spontaneous germinal center B-cells, autoAb, and total antibody suggest enhanced B-cell activation. To investigate this possibility, I examined BCR-induced activation of proximal and distal signaling molecules in *Abp1*$^{-/-}$ B-cells. Isolated splenic B-cells were activated by BCR cross-linking for various times, labeled with antibodies specific for the activated forms of signaling molecules downstream of the

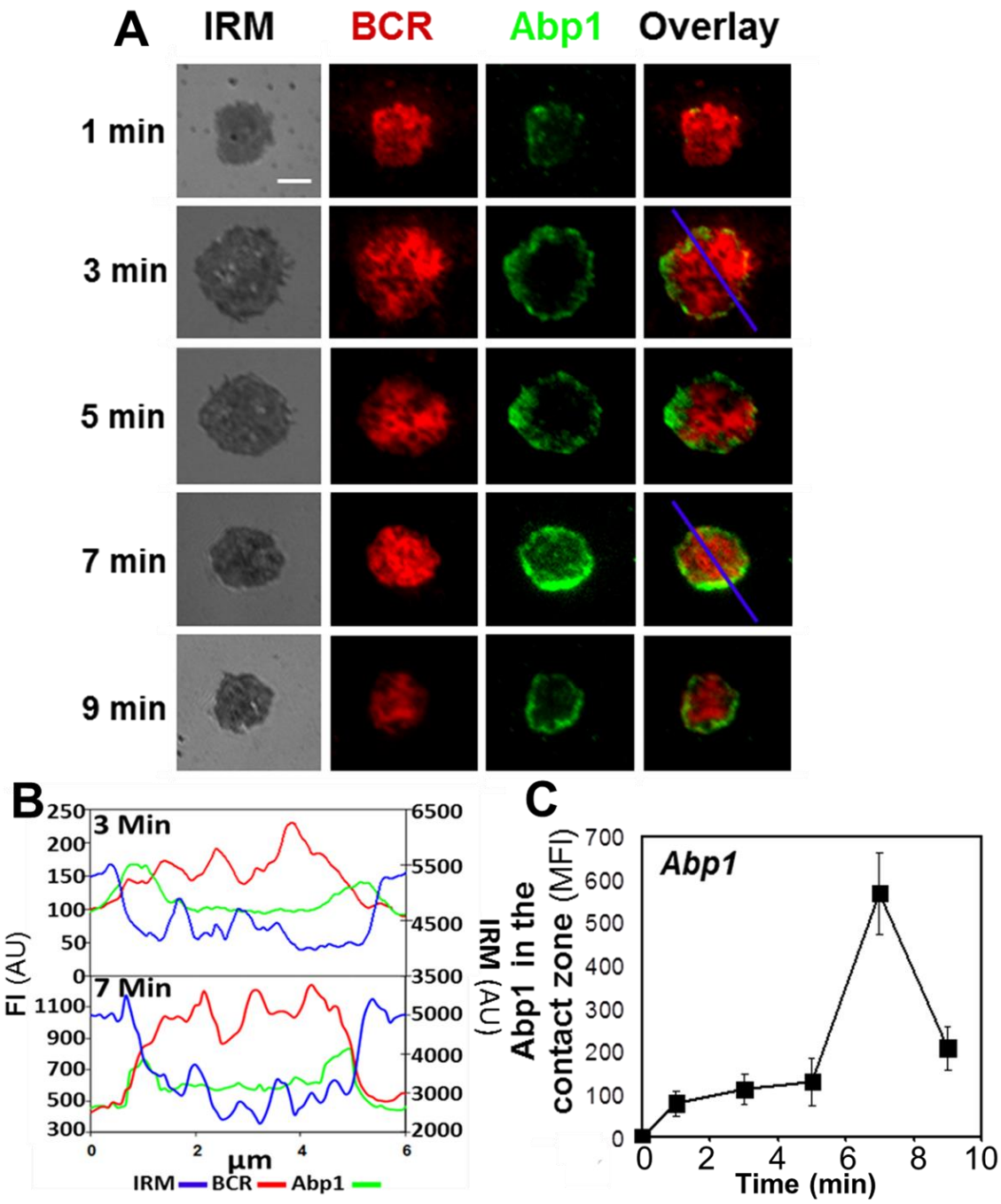

A
IRM
BCR
Abp1
Overlay
1 min
3 min
5 min
7 min
9 min
B
3 Min
7 Min
FI (AU)
IRM (AU)
µm
IRM BCR Abp1
C
Abp1
Abp1 in the contact zone (MFI)
Time (min)

Fig. 2.13 BCR activation induces recruitment of Abp1 to the outer edge of the spreading B-cell. **(A)** TIRF and IRM analysis of Abp1 in the contact zone of splenic B-cells incubated with Fab'-anti-Ig-tethered lipid bilayers. Representative images (n=3), bar 5 μm. **(B)** Relative intensity of IRM, and fluorescence intensity of BCRs and Abp1 across the blue line in cells at 3 and 7 min from **(A)**. **(C)** Average MFI of Abp1 (±S.D.) from >50 cells for each time point quantified using Andor iQ (n=3).

BCR, and analyzed using flow cytometry. *Abp1*$^{-/-}$ B-cells exhibited increased levels of tyrosine phosphorylation (pY) (Fig. 2.14A) and phosphorylated BLNK (pBLNK) (Fig. 2.14B), the key B-cell adaptor molecule, upon BCR cross-linking. However, the activation of downstream MAP kinases was differentially impacted by Abp1 deficiency. The levels of phosphorylated MEK1/2 (pMEK1/2) (Fig. 2.14C) and its downstream target the MAP kinase ERK (pERK) (Fig. 2.14D) were significantly increased upon BCR activation in *Abp1*$^{-/-}$ B-cells, compared to wt B-cells. Not only were the peak levels of the phosphorylated forms of these signaling molecules higher in *Abp1*$^{-/-}$ B-cells, but the levels of these phosphorylated proteins also took much longer to return to basal levels (Fig. 2.14A-D). In contrast, the level of phosphorylated JNK (pJNK) was significantly reduced (Fig. 2.14E), while the level of phosphorylated p38 (pp38) was not affected in *Abp1*$^{-/-}$ B-cells (Fig. 2.14F). Treatment with ERK or JNK inhibitors reduced the levels of pERK and pJNK to basal levels (Fig. 2.14D-E), confirming staining specificity. Consistent with enhanced proximal signaling, *Abp1*$^{-/-}$ B-cells displayed a higher level of calcium flux in response to BCR stimulation than their wt counterpart. There was no significant difference in the expression levels of membrane IgM, BLNK, MEK1/2, ERK and JNK between resting *Abp1*$^{-/-}$ and wt B-cells (Fig. 2.15). This result indicates that the enhanced and prolonged signaling in *Abp1*$^{-/-}$ B-cells is not due to increased expression of BCRs or signaling molecules.

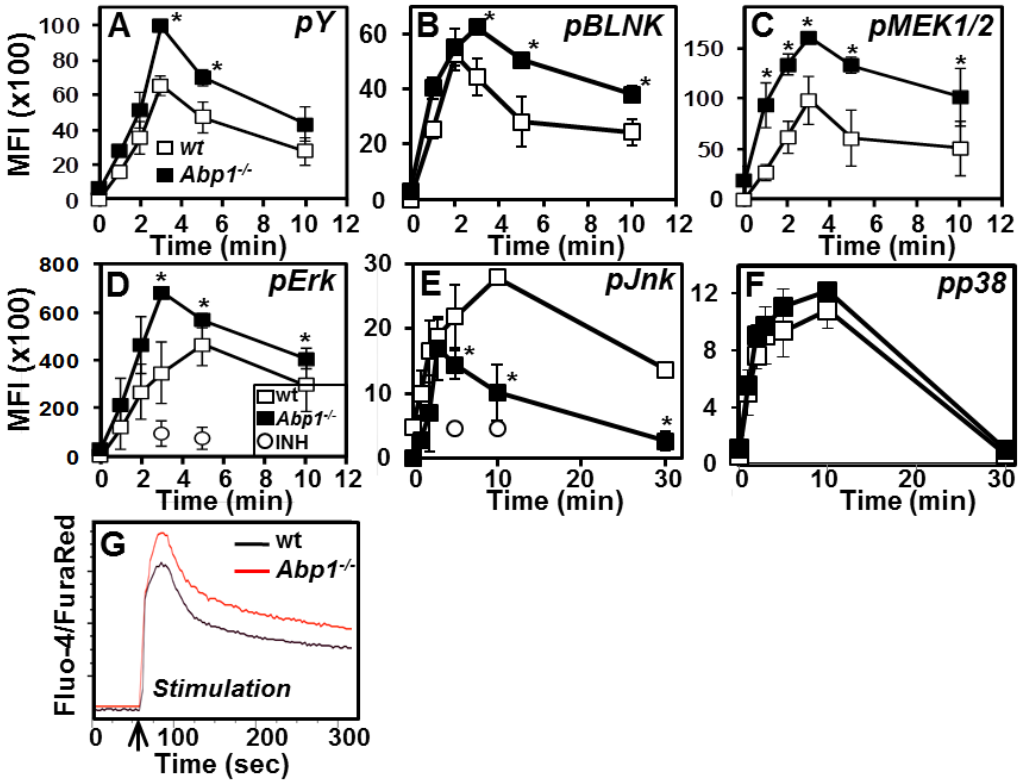


Fig. 2.14 BCR signaling is enhanced in *Abp1*$^{-/-}$ B-cells. (A-F) Splenic B-cells from wt and *Abp1*$^{-/-}$ mice were activated with F(ab')$_2$-goat anti-mouse IgG+M, fixed, permeabilized, labeled for phosphotyrosine (pY) **(A)**, pBLNK **(B)**, pMEK1/2 **(C)**, pERK **(D)**, pJNK **(E)**, and pp38 **(F)**, and analyzed by flow cytometry (average MFI ±S.D., n=3). **(D-E)** Upstream ERK and JNK inhibitor controls at indicated times. **(G)** Ca^{2+} flux in splenic B-cells activated with F(ab')$_2$-goat anti-mouse IgG+M using flow cytometry (n=3). *, $p<0.05$.

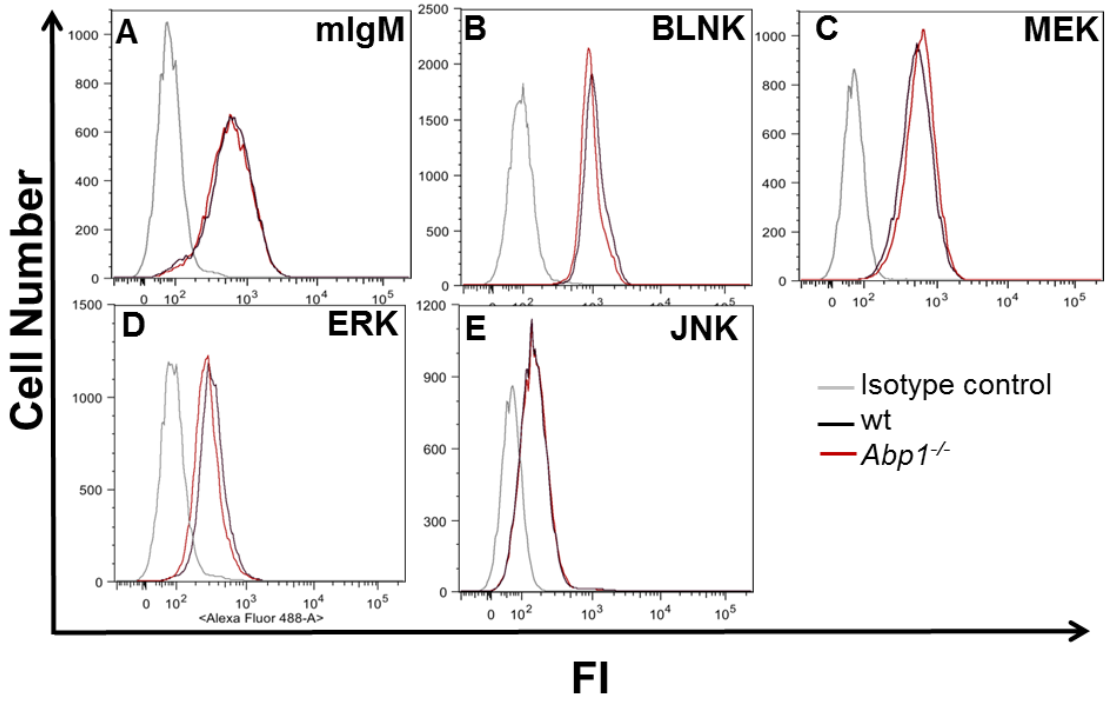


Fig. 2.15 The expression levels of membrane IgM and signaling molecules in unstimulated splenic B-cells. Splenic B-cells from resting wt and *Abp1*$^{-/-}$ mice were labeled with anti-B220 Ab, fixed, permeabilized, and labeled for IgM **(A)**, BLNK **(B)**, MEK1/2 **(C)**, ERK **(D)**, and JNK **(E)**. Cells were analyzed by flow cytometry. Shown are representative histograms (n=3).

2.4.6 Abp1 is required for the recruitment of the inhibitory signaling molecules HPK1 and SHIP-1 to the B-cell contact zone.

Enhanced signaling in *Abp1*$^{-/-}$ B-cells suggests a negative regulatory role for Abp1 in BCR signaling. One possible mechanism by which Abp1 may negatively regulate signaling is by modulating inhibitory signaling molecules downstream the BCR. To test this hypothesis, I examined the effects of Abp1 deficiency on the recruitment of two known inhibitory molecules, HPK1 and SHIP-1, to the BCR using TIRF. After incubation with Fab'-anti-Ig tethered-lipid bilayer, I stained intracellular HPK1 or phosphorylated (activated) SHIP-1 (pSHIP-1) (Fig. 2.16 and 2.17), and quantified the relative levels of HPK1 or pSHIP1 in the B-cell contact zone (Fig. 2.16B and 2.17B) using TIRF. In wt cells, HPK1 was detected as soon as 1 min of activation, peaked at 7 min of activation, and reduced by 9 min (Fig. 2.16A-B). While the *Abp1*$^{-/-}$ B-cells showed the same trend of HPK1 recruitment, the MFI of HPK1 in the contact zone was significantly reduced throughout the time course (Fig. 2.16A-B). Similar to the recruitment of HPK1, pSHIP-1 was detected in the contact zone of wt B-cells at 1 min; however significant accumulation of pSHIP did not occur until roughtly 7 min of activation (Fig. 2.17A-B). Compared to wt B-cells, the peak MFI of pSHIP in the contact zone of *Abp1*$^{-/-}$ B-cells at 7 min was significantly reduced (Fig. 2.17A-B). However, Abp1 deficiency had no significant effect on the recruitment of phosphorylated Syk (pSyk) to BCR clusters (Fig. 2.18A-B), as well as the expression levels of HPK1 and SHIP (Fig. 2.16C and 2.17C). These results indicate that Abp1 is required for the recruitment and activation of the key inhibitory molecules HPK1 and SHIP-1 downstream of BCR stimulation.

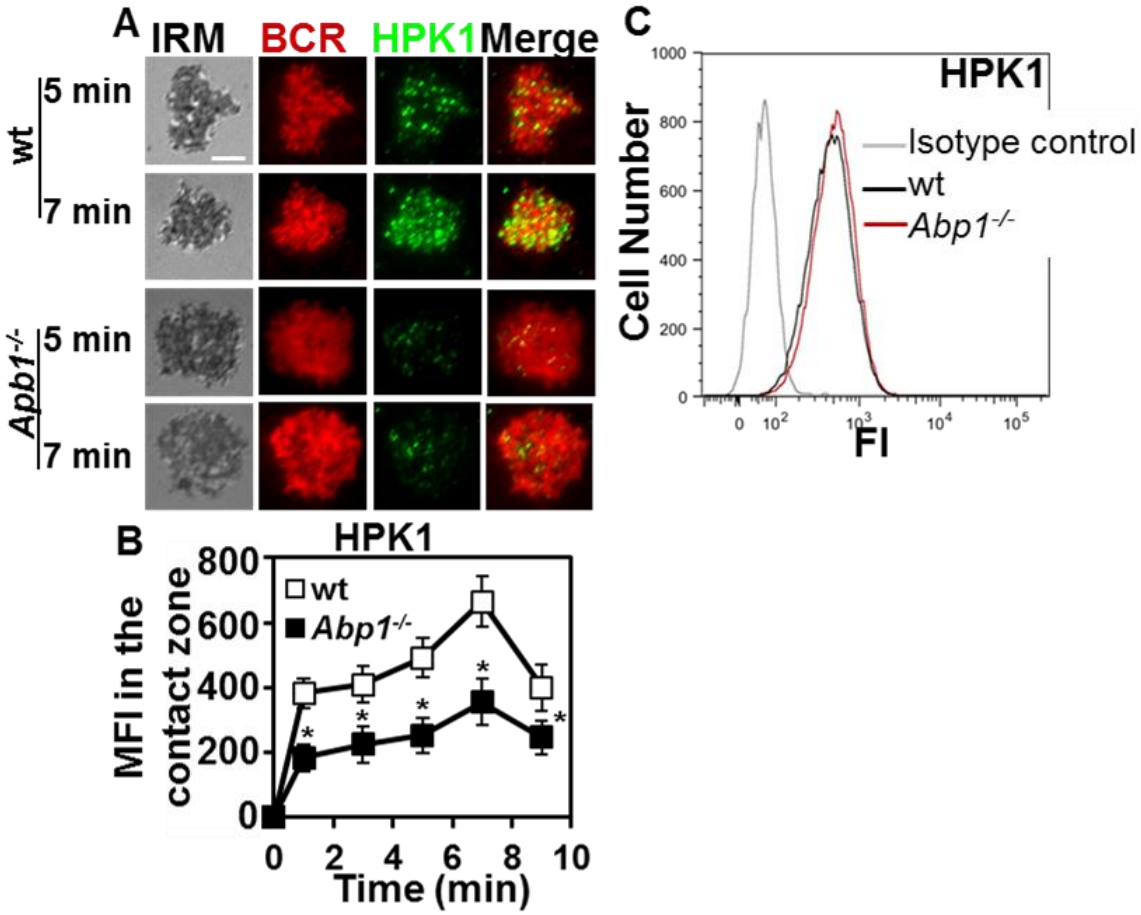


Fig. 2.16 Abp1 is required for recruitment of the inhibitory signaling molecule HPK1 to the B-cell contact zone. (A-B) TIRF analysis of HPK1 in the contact zone of wt and *Abp1*$^{-/-}$ B-cells incubated with Fab'-anti-Ig-tethered lipid bilayers. Shown are representative images (Bar, 5 µm). **(B)** MFI of HPK1 (±S.D.) in the contact zone (50 cells per time point, n=3). **(C)** Resting splenic B-cells from wt and *Abp1*$^{-/-}$ mice were labeled with anti-B220 Ab, fixed, permeabilized, and labeled for HPK1. Cells were analyzed by flow cytometry. Shown are representative histograms (n=3). *, $p<0.01$.

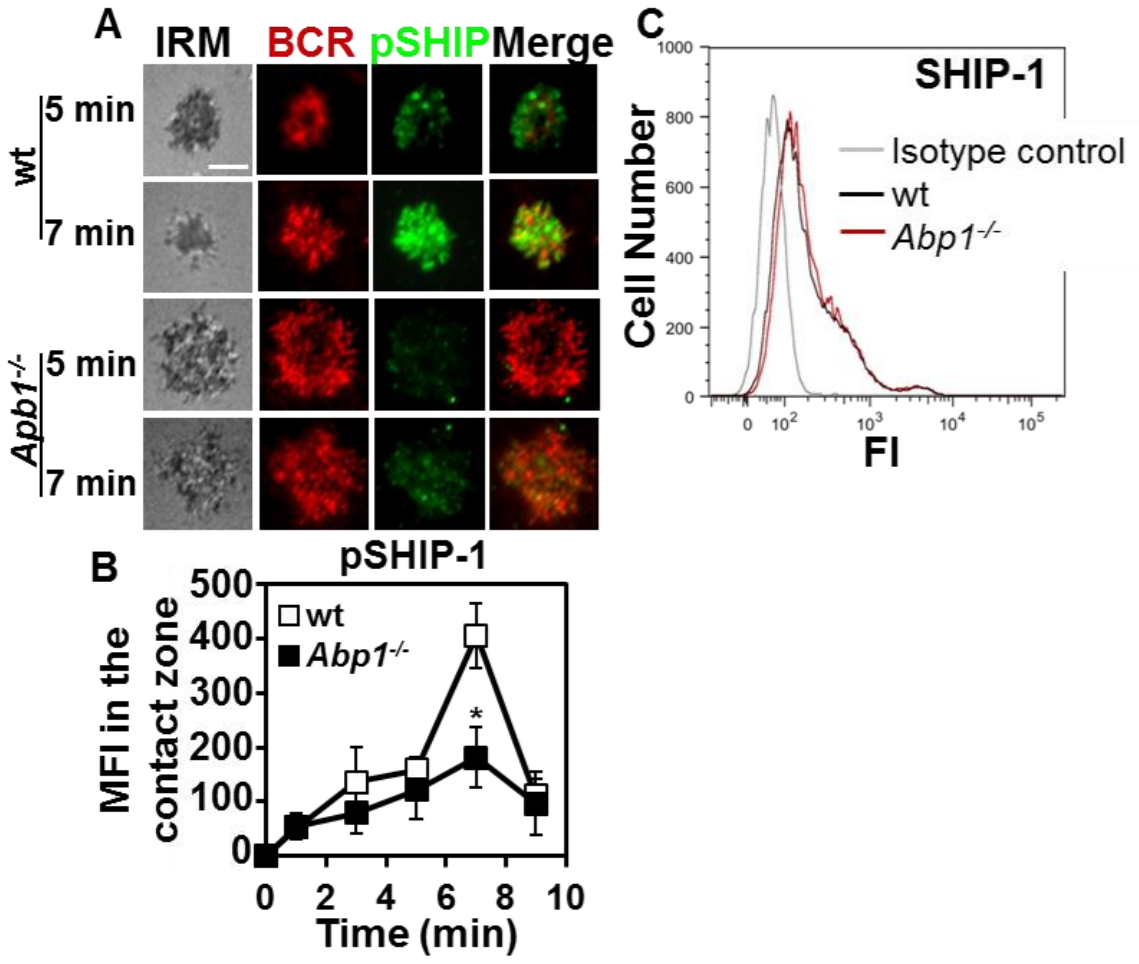


Fig. 2.17 Abp1 is required for recruitment of the inhibitory signaling molecule SHIP-1 to the B-cell contact zone. (A-B) TIRF analysis of pSHIP-1 in the contact zone of wt and *Abp1*$^{-/-}$ B-cells incubated with Fab'-anti-Ig-tethered lipid bilayers. Shown are representative images (Bar, 5 µm). **(B)** MFI of pSHIP-1 (±S.D.) in the contact zone (50 cells per time point, n=3). **(C)** Resting splenic B-cells from wt and *Abp1*$^{-/-}$ mice were labeled with anti-B220 Ab, fixed, permeabilized, and labeled for SHIP-1. Cells were analyzed by flow cytometry. Shown are representative histograms (n=3). *, $p<0.01$.

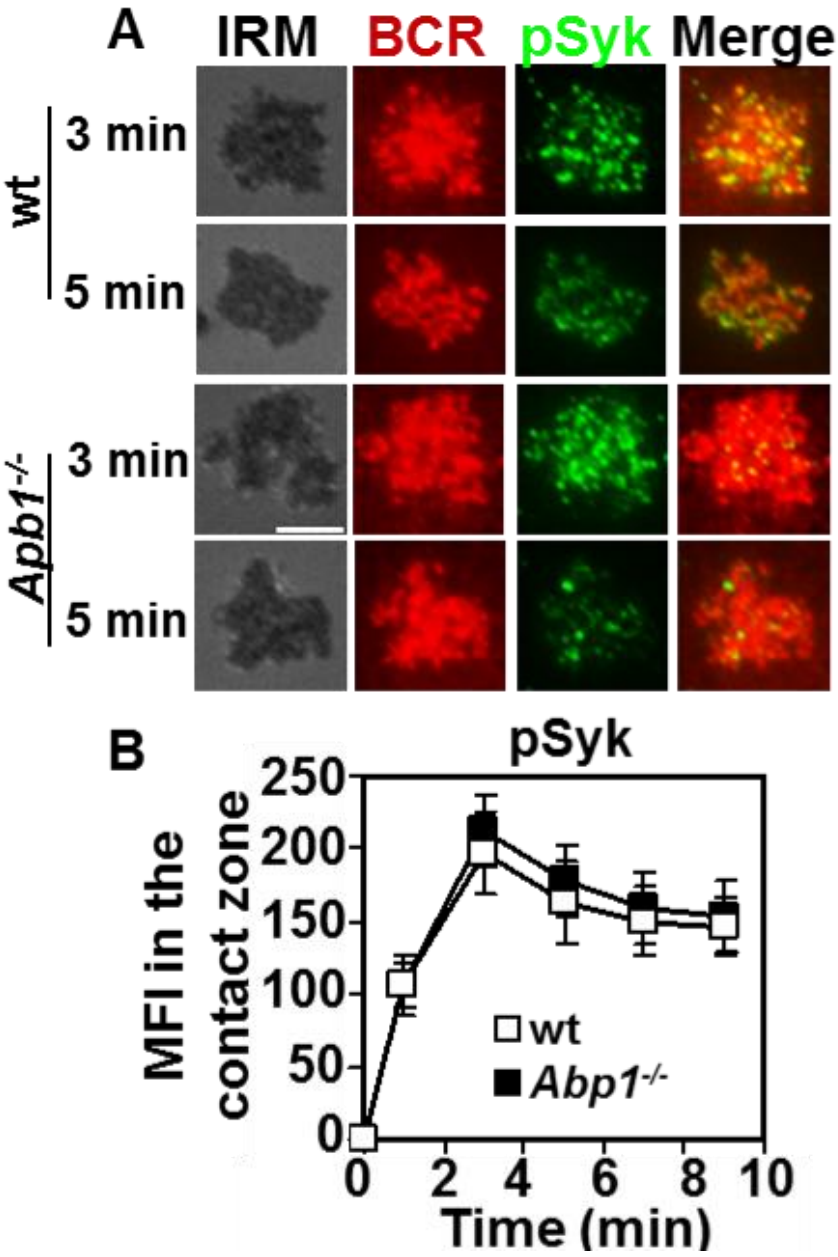


Fig. 2.18 Abp1 is not required for recruitment of the pSyk to the B-cell contact zone. (A-B) TIRF analysis of pSyk in the contact zone of wt and *Abp1*$^{-/-}$ B-cells incubated with Fab'-anti-Ig-tethered lipid bilayers. Shown are representative images (Bar, 5 µm). **(B)** MFI of pSyk (±S.D.) in the contact zone (50 cells per time point, n=3).

2.4.7 Abp1 regulates BCR-induced actin reorganization.

Increased B-cell spreading and BCR clustering at the surface of *Abp1*$^{-/-}$ B-cells, which are known to be actin dependent, and the ability of Abp1 to bind to F-actin (191), suggest a role for Abp1 in BCR-induced actin remodeling. To investigate this hypothesis, I examined the effects of Abp1 deficiency on F-actin accumulation at the B-cell contact zone upon BCR activation. F-actin was stained with fluorescently labeled phalloidin, and the distribution and relative levels of F-actin at the B-cell contact zone were imaged and quantified using TIRF. In wt B-cells, the MFI of F-actin in the contact zone rapidly increased during the first 3 min of incubation with Fab'-anti-Ig-tethered lipid bilayer, peaked at ~3 min when B-cell spreading is maximal, and rapidly reduced as the B-cells contracted (Fig. 2.19A-B). In contrast, the MFI of F-actin in the contact zone of *Abp1*$^{-/-}$ B-cells rose much more slowly than that of wt B-cells, did not peak until 7 min, and remained at the peak level up to 9 min (Fig. 2.19A-B). Consequently, the F-actin level in the contact zone of *Abp1*$^{-/-}$ B-cells was significantly lower at 3 min but significantly higher at 7 and 9 min than that of wt B-cells. As the levels of F-actin in resting B-cells was not altered by Abp1 deficiency (Fig. 2.19C), my data suggest that Abp1 may primarily contribute to signal-induced actin remodeling, more specifically, both the early accumulation and later clearance of F-actin from the B-cell contact zone.

Abp1 has been shown to regulate actin dynamics by interacting with the actin nucleation promoting factor N-WASP in neurons (194). Our recent studies have demonstrated that WASP is required for actin assembly during B-cell spreading, however, N-WASP plays a unique role in actin clearance during B -cell contraction (54). To investigate whether Abp1 regulates BCR-induced actin remodeling via WASP and N-

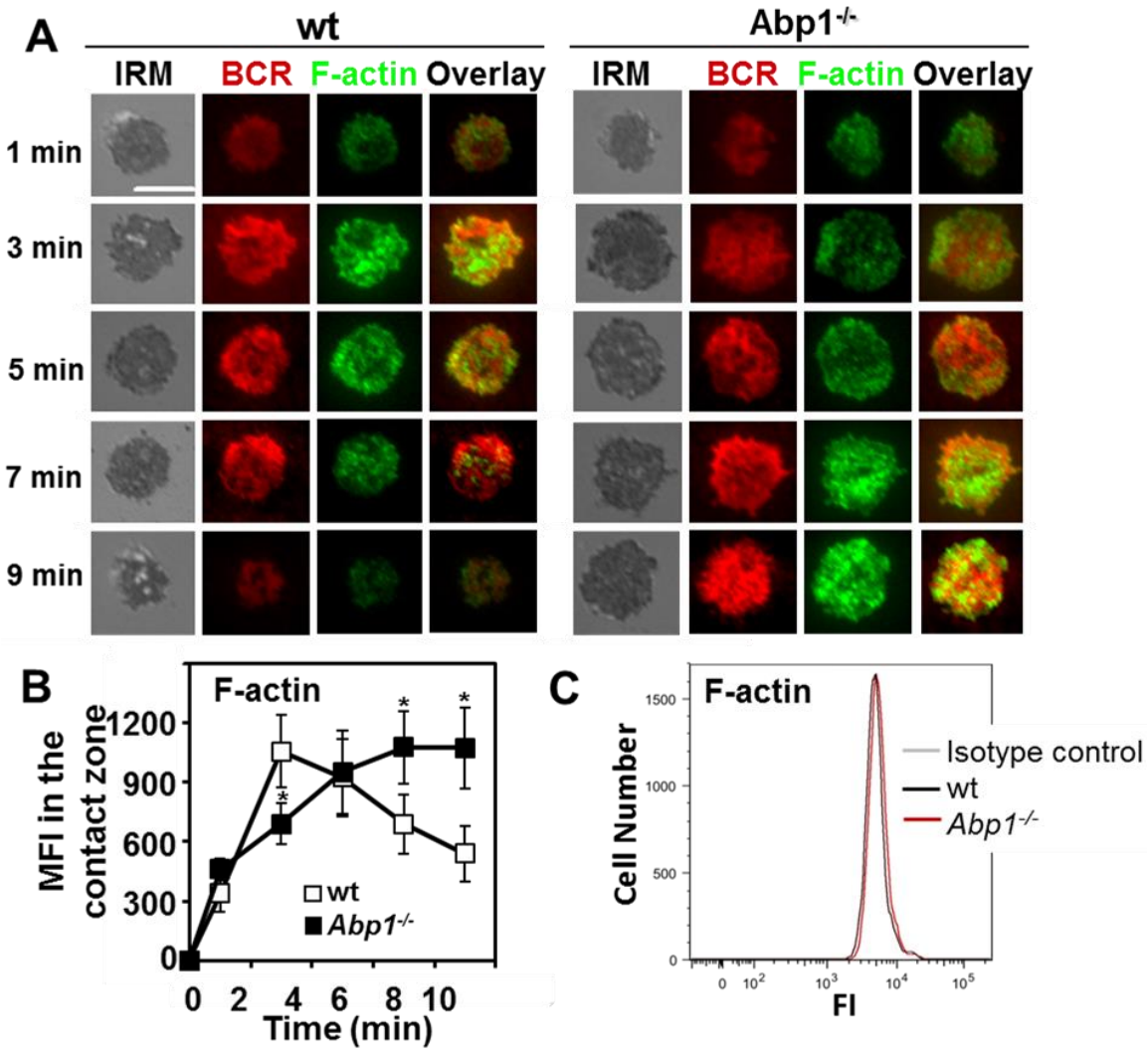


Fig. 2.19 Abp1 regulates actin remodeling. **(A-B)** Splenic B-cells from wt and *Abp1*$^{-/-}$ mice were activated with Fab'-anti-Ig-tethered lipid bilayers, fixed, permeabilized, and stained with AF488 phalloidin for F-actin **(A)**. Shown are representative images (n=3), bar 5 µm. The MFI (±S.D.) of F-actin **(B)** in the contact zone quantified in > 50 cells per time point (n=3). **(C)** Resting splenic B-cells from wt and *Abp1*$^{-/-}$ mice were labeled with anti-B220 Ab, fixed, permeabilized, and labeled for F-actin. Cells were analyzed by flow cytometry. Shown are representative histograms (n=3). *, $p<0.05$.

WASP, I analyzed the impact of Abp1 deficiency on the recruitment and activation of these two actin regulators upon BCR activation. Activated WASP and N-WASP were detected by TIRF, using antibodies specific for their phosphorylated form. Similar to our previously published results, pWASP MFI peaked first at ~5 min of activation with Fab'-anti-Ig-tethered lipid bilayer, followed by pN-WASP (pN-WA), which peaked at ~7 min (Fig. 2.20A-B and 2.21A-B) (54). In *Abp1*$^{-/-}$ B-cells, pWASP MFI increased with the same kinetics and to similar levels as those of wt B-cells, but reduced more slowly after reaching maximal levels compared to wt (Fig. 2.20B). In contrast, pN-WASP MFI in the contact zone of *Abp1*$^{-/-}$ B-cells was significantly lower than wt B-cells, even though wt and *Abp1*$^{-/-}$ B-cells showed similar kinetics of recruitment (Fig. 2.21B). Flow cytometry analysis further confirmed the reduced levels of pN-WASP in *Abp1*$^{-/-}$ B-cells (Fig. 2.21C). Abp1 deficiency has no significant effect on basal expression levels of WASP and N-WASP (Fig. 2.20C and 2.21D). These results suggest that Abp1 regulates BCR-induced actin remodeling by suppressing WASP activation, which induces F-actin accumulation and B-cell spreading, and by enhancing N-WASP activation, which triggers actin clearance and B-cell contraction.

2.5 Discussion

Abp1 is an actin-binding adaptor protein that is expressed widely from yeast to mammalian cells. Previous studies have revealed a multitude of cellular funtions for Abp1 including: endocytosis in a variety of cell types (149, 150, 198, 230, 232, 242), synaptic vesicle recycling in neurons (149, 230), phagocytosis (196, 243), cell migration (231, 244, 245), and TCR-mediated signaling and T-cell activation (195, 197, 232). This

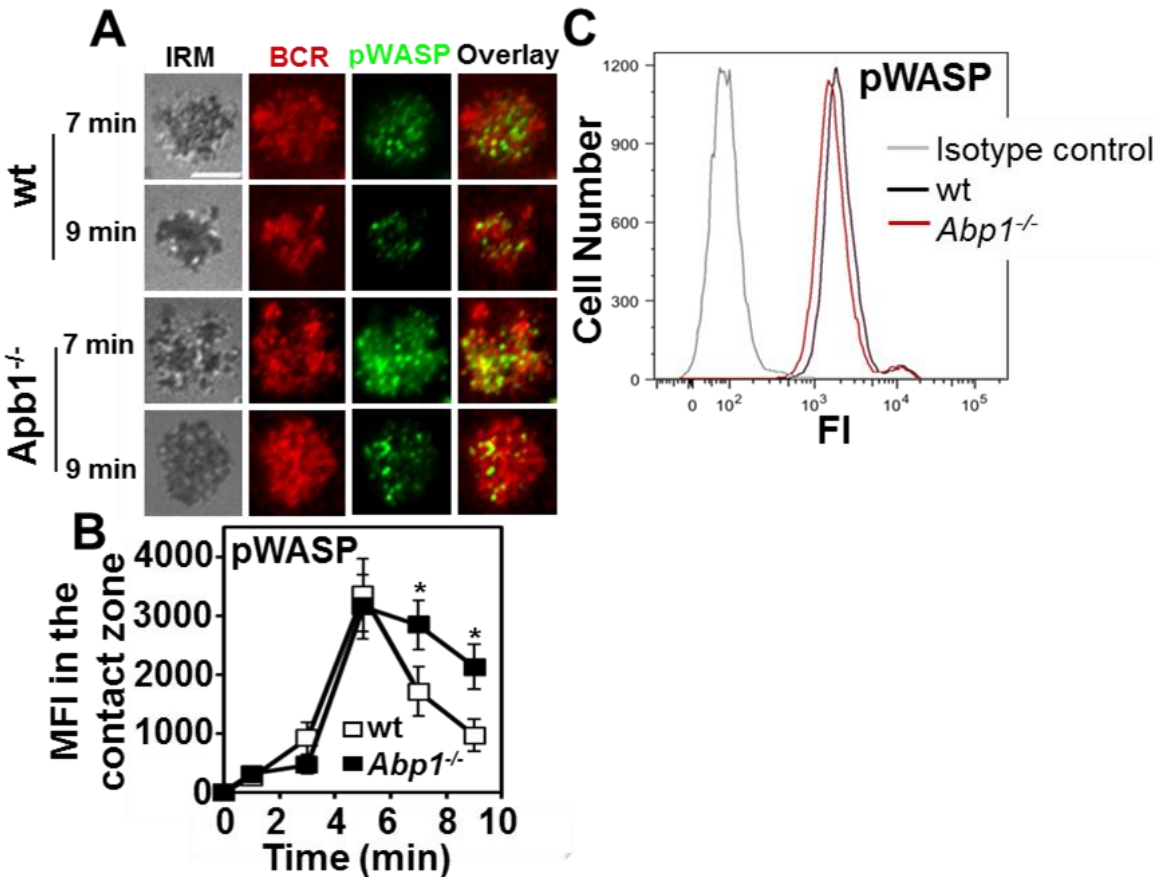


Fig. 2.20. Abp1 regulates actin remodeling by modulating activation of the actin nucleation promoting factor WASP. (A-B) Splenic B-cells from wt and *Abp1*$^{-/-}$ mice were activated with Fab'-anti-Ig-tethered lipid bilayers, fixed, permeabilized, and stained for p-WASP **(A)**. Representative images at 7 and 9 min (n=3), bar 5 µm. The MFI (±S.D.) of p-WASP **(B)** in the contact zone quantified in > 50 cells per time point (n=3). **(C)** Resting splenic B-cells from wt and *Abp1*$^{-/-}$ mice were labeled with anti-B220 Ab, fixed, permeabilized, and labeled for pWASP. Cells were analyzed by flow cytometry. Shown are representative histograms (n=3). *, $p<0.05$.

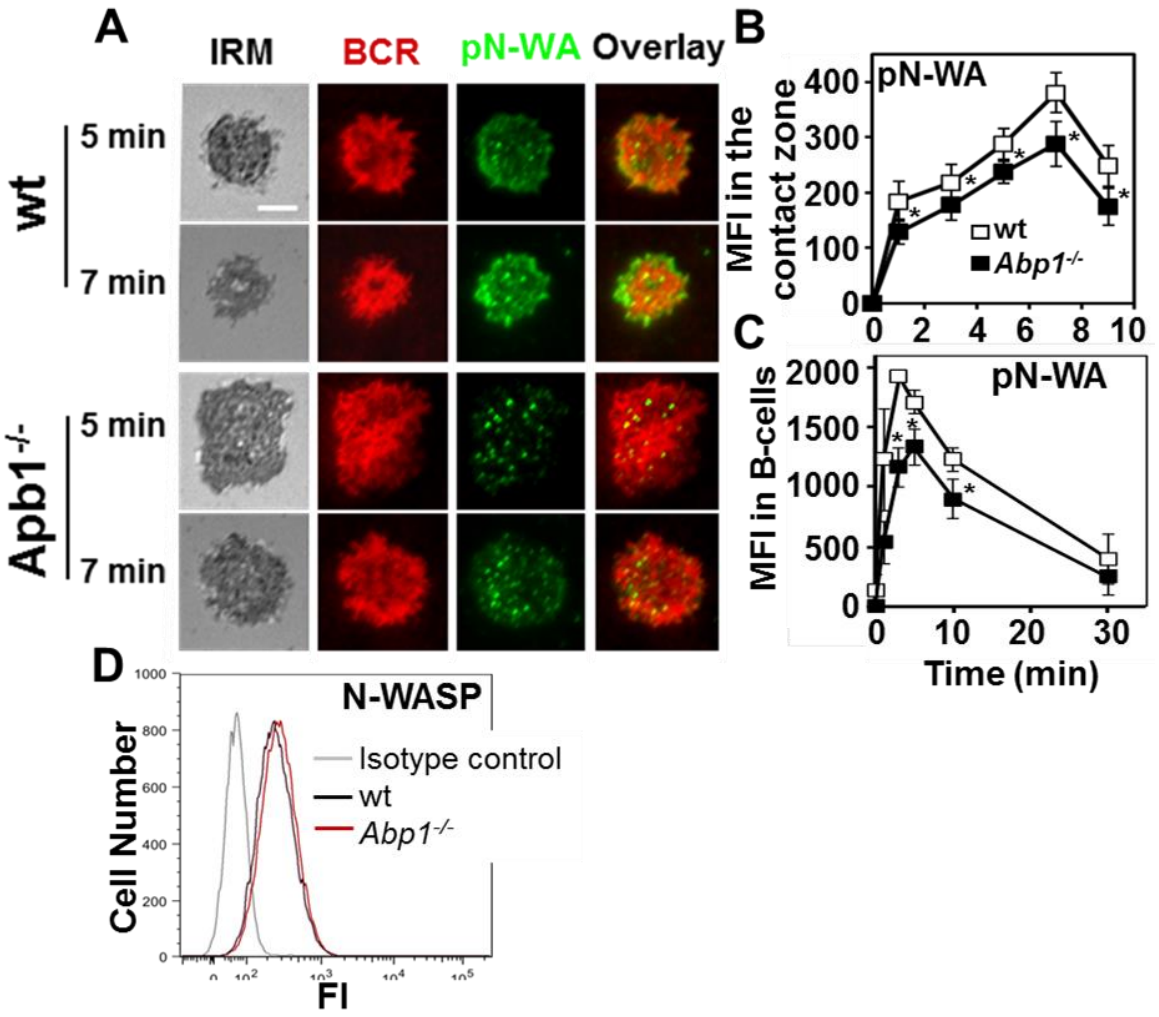

A
IRM
BCR
pN-WA
Overlay
wt
5 min
7 min
Apb1-/-
5 min
7 min
B
pN-WA
MFI in the contact zone
400
300
200
100
0
0 2 4 6 8 10
wt
Abp1-/-
C
pN-WA
MFI in B-cells
2000
1500
1000
500
0
0 10 20 30
Time (min)
D
N-WASP
Isotype control
wt
Abp1-/-
Cell Number
1000
800
600
400
200
0
FI

Fig. 2.21. **Abp1 regulates actin remodeling by modulating activation of the actin nucleation promoting factor N-WASP**. **(A-B)** Splenic B-cells from wt and *Abp1*$^{-/-}$ mice were activated with Fab'-anti-Ig-tethered lipid bilayers, fixed, permeabilized, and stained for pN-WASP **(A)**. Representative images at 7 min (n=3), bar 5 μm. The MFI (±S.D.) of pN-WASP **(B)** in the contact zone quantified in > 50 cells per time point (n=3). **(C)** Resting splenic B-cells from wt and *Abp1*$^{-/-}$ mice were labeled with anti-B220 Ab, and either activated with F(ab')$_2$-goat anti-mouse IgG+M **(C)**, or left un-activated **(D)**, fixed, permeabilized, stained for pN-WASP **(C)** or total N-WASP **(D)**, and analyzed by flow cytometry (n=3). Shown are average MFI ±S.D **(C)**, or representative histogram **(D)**. *, $p<0.05$.

study reveals a novel function for Abp1: attenuation of BCR signaling, which contributes to the regulation of peripheral B-cell differentiation and Ab responses. I demonstrate that Abp1 exerts a B-cell intrinsic inhibition on spontaneous activation and differentiation of mature follicular and self-reactive B-cells into GC B-cells and Ab-secreting cells, and on the generation of MZ and B1a B-cells. Abp1 is required for B-cell affinity maturation and mounting effective T-dependent Ab responses. In addition to its critical role in BCR uptake of antigen for processing (150), this study shows that Abp1 primarily contributes to the attenuation stage of BCR signaling by promoting B-cell contraction and coalescence of BCR microclusters into polarized central clusters, and by the recruitment of the inhibitory signaling molecules SHIP-1 and HPK1 to BCR microsignalosomes.

This study shows that the effects of Abp1 deficiency on the differentiation of MZ B-cells and spontaneous GC B-cells are B-cell intrinsic. The increased numbers of MZ, B1a, and GC B-cells provide an explanation for the enhanced T-independent response and elevated autoAb levels in *Abp1*$^{-/-}$ mice. As expression levels of the BCR and signaling molecules are not altered in *Abp1*$^{-/-}$ B-cells, my results support the notion that Abp1-mediated down-regulation of BCR signaling directly influences the differentiation of these peripheral B-cell subsets. These data also suggest that Abp1 is potentially involved in suppressing Ag-independent signals required for the differentiation of GC, MZ and B1a B-cells. Actin dynamics are known to regulate tonic BCR signaling via modulating BCR-BCR and BCR-CD19 interactions (114). Thus, it is possible that Abp1 regulates tonic signaling in resting B-cells by modulating actin dynamics and receptor interactions. Additional research is required to address these questions.

Failure of *Abp1*$^{-/-}$ mice to generate high affinity Ab, while simultaneously producing high levels of non-specific IgG during T-dependent Ab responses, indicates defective affinity maturation. In GCs, B-cells with higher affinity BCRs are selected to survive and differentiate, due to a greater ability to signal and capture/present antigen to T-cells than low affinity B-cells (36). Enhanced/prolonged BCR signaling in *Abp1*$^{-/-}$ B-cells may interfere with affinity-dependent signal regulation. Reductions in antigen uptake and presentation (150) likely ablate the ability of *Abp1*$^{-/-}$ B-cells to acquire T-cell help. Impairment in the activation of *Abp1*$^{-/-}$ T-cells may further exacerbate the affinity maturation defect observed in *Abp1*$^{-/-}$ mice. The affinity maturation defect provides another possible explanation for increased levels of autoAb in *Abp1*$^{-/-}$ mice, as antigen-specific B-cells are not preferentially selected from B-cell pools that also contain auto-reactive B-cells.

The negative regulatory function of Abp1 in BCR signaling shown in this study is in contrast to its positive regulatory role in TCR activation reported previously(195). Han et al. showed that Abp1 is required for the optimal activation of TCR signaling. While T-cell development and maturation was largely unchanged in *Abp1*$^{-/-}$ mice, TCR-induced phosphorylation of the adaptor protein LAT, and the key proximal signaling molecule PLCγ1, and activation of the MAP kinases Jnk and p38 were partially defective (195). The defective TCR signaling was associated with reduced T-cell proliferation, cytokine production, and T-cell-dependent antibody responses in *Abp1*$^{-/-}$ mice (195). Similar to T-cell development, Abp1 deficiency does not have a significant effect on B-cell development in the bone marrow, possibly due to its relatively low expression level in B-cells of the bone marrow. However, BCR signaling is enhanced in *Abp1*$^{-/-}$ B-cells in

response to in vitro stimulation, exhibiting higher levels of tyrosine and BLNK phosphorylation, a key adaptor protein for proximal signaling from the BCR (246). While the phosphorylation of Jnk also decreases, as observed in *Abp1*$^{-/-}$ T-cells, *Abp1*$^{-/-}$ B-cells display faster kinetics and higher levels of Erk phosphorylation than wt B-cells. As the *Abp1*$^{-/-}$ mice used in this study were generated by the same group that reported the T-cell study (195), and because I observed a similar defect in T-cell-dependent antibody responses in the *Abp1*$^{-/-}$ mice, I suggest that the opposing functions of Abp1 in T and B-cells are not due to differences in the mouse model. While the molecular mechanisms underlying differential regulation of BCR and TCR signaling remain unknown, my results demonstrate cell type-specific functions for Abp1 in signal regulation.

Based on its domain structure, it is logical to hypothesize that Abp1 would mediate signal regulation by interacting with signaling molecules via its SH3 domain and PRD. However, the molecular details underlying its regulatory function remain unknown. The results present here, and in previously published studies (149, 150, 190, 194, 195) suggest three mechanisms by which Abp1 may promote signal attenuation, including: 1) modulating actin dynamics and actin-dependent early BCR activation events, 2) regulating the recruitment of inhibitory signaling molecules, and 3) by BCR internalization. Recent studies using high resolution live cell imaging have revealed self-clustering of BCR monomers and nano-sized clusters on the cell surface into microclusters as a critical event for BCR activation. The microclusters serve as signaling platforms for BCRs to recruit and interact with signaling molecules (114, 168, 172, 224). We have previously demonstrated that while BCR clustering initiates signaling, the ensuing coalescence of BCR microclusters into polarized central clusters acts to attenuate

BCR signaling activities (54, 170, 181). The formation of BCR microclusters depends on actin remodeling, which not only directs the lateral movement of BCRs, but also mediates B-cell morphological changes induced by BCR activation including cell spreading, which amplifies BCR clustering, and cell contraction which drives coalescence of BCR microclusters (170, 181, 187). The results presented here show that Abp1 is not critical for the formation of BCR microclusters and B-cell spreading. Rather, it is required for B-cell contraction and coalescence of BCR microclusters. The impact of Abp1 deficiency on the cellular events leading to signal attenuation may be attributed to its role in BCR-induced actin remodeling, where Abp1 plays a more prominent role in the clearance, rather than the early accumulation of F-actin in the B-cell contact zone. In support of this hypothesis, failure to clear F-actin in the contact zone of *N-WASP*$^{-/-}$ B-cells has been linked to enhanced and prolonged BCR signaling (54).

How Abp1 regulates BCR-induced actin remodeling is not fully understood. While the yeast homolog Abp1p can induce actin polymerization by activating the actin nucleating factor Arp2/3 (193), mammalian Abp1 does not retain this capability. However, mammalian Abp1 has been shown to promote actin polymerization indirectly in neurons by direct interactions with N-WASP, which enhances the actin nucleation promoting activity of N-WASP (194). Unlike neurons, B-cells and other types of immune cells express both N-WASP and its hematopoietic specific homolog WASP. Our recently published data show distinct functions for WASP in actin assembly and B-cell spreading, and of N-WASP in the clearance of F-actin for B-cell contraction (54). Furthermore, the study revealed a negative relationship between the two (54). Here I show here that levels of active WASP and N-WASP are increased and decreased in *Abp1*$^{-}$

$^{-/-}$ B-cells respectively. This suggests Abp1 preferentially promotes N-WASP activation, which suppresses WASP activation. Similar recruitment kinetics for N-WASP (54) and Abp1 to the B-cell contact zone and similar phenotypes observed in *Abp1*$^{-/-}$ and *N-WASP*$^{-/-}$ mice further support a functional relationship between the two and the notion that Abp1 modifies actin remodeling by regulating N-WASP.

Abp1 has been shown to be responsible for bringing HPK1 to the IS formed between T-cells and antigen presenting cells by direct interaction via its SH3 domain and HPK1's PRD domain (232). If HPK1, recruited to the B-cell surface by Abp1, is activated by Src family or Syk kinases, it can bind and phosphorylate BLNK, which leads to the ubiquitination and down regulation of this key adaptor protein, and its downstream signaling pathways (55). While I did not directly examine HPK1 activation in this study, the enhanced levels of phosphorylated BLNK and Erk, and elevated calcium influx in *Abp1*$^{-/-}$ B-cells support the notion that Abp1 is involved in HPK1 activation. To our surprise, BCR-induced activation and recruitment of the inhibitory phosphatase SHIP-1 also requires Abp1. This represents a new mechanism by which Abp1 mediates its negative regulatory function in B-cell activation. SHIP-1 is well known for its function in inhibiting major signaling molecules downstream of the BCR, including PLCγ2, Btk, and Akt (59, 124). B-cell-specific gene deletion of SHIP-1 causes hyper-responsiveness and defective affinity maturation of B-cells (57), which is associated with enhanced BCR signaling observed at the B-cell contact zone, and inhibition of BCR microcluster coalescence and B-cell contraction (57, 59, 181). *Abp1*$^{-/-}$ mice and B-cells share many of the phenotypes observed in B-cell specific *SHIP-1*$^{-/-}$ and *HPK1*$^{-/-}$ mice and B-cells , which indicates that recruitment of SHIP-1 and HPK1 to surface BCRs for activation is

likely the primary mechanism underlying Abp1 negative regulation of BCR signaling and B-cell activation. The question remains, however, how does Abp1 bring SHIP-1 to the BCR signalosome. Possible mechanisms, including direct or indirect interaction of Abp1 with SHIP-1 via other proteins, remain to be explored.

Abp1 can also promote signal attenuation by mediating BCR internalization. We have previously demonstrated that the expression of Abp1 is critical for efficient internalization and intracellular processing of BCR-bound antigen (150). While BCR internalization itself does not turn off receptor signaling activity, instead changing the nature and location of BCR signaling (160), inhibition of BCR endocytosis by perturbing the actin cytoskeleton, or by N-WASP gene deletion is associated with enhanced signaling (54, 170, 247). While Abp1 likely utilizes multiple mechanisms to attenuate BCR signaling, it should be noted that these mechanisms are likely interrelated, as Abp1-associated actin remodeling may be important for the recruitment of inhibitory signaling molecules and BCR internalization, and recruited inhibitory signaling molecules can in turn regulate actin remodeling and actin-mediated cellular events. The interrelationship among these mechanisms is the subject of my future studies.

This study further demonstrates the importance of the actin cytoskeleton in the attenuation of BCR signaling and B-cell activation for the maintenance of B-cell tolerance. I identified Abp1 as a missing link between actin remodeling, and signal down-regulation mechanisms, which expand our understanding of the molecular mechanism underlying the negative regulation of B-cell activation.

Chapter 3: Dynamin-2 regulates BCR internalization, and signal transduction.

3.1 Abstract

B-cell activation is initiated by the binding of antigen to BCRs. Optimal activation of B-cells depends on coordinated BCR signaling, antigen internalization and processing, as well as actin cytoskeletal rearrangement; however, the molecular nature of this coordination has yet to be fully illuminated. The GTPase Dynamin is involved in endocytosis, actin remodeling, and signaling, thereby suggesting a role in coupling these cellular apparatuses. This study examined the domains or function of Dynamin2 (Dyn2) necessary for recruitment of Dyn2 to the plasma membrane, endocytosis of signaling active BCRs, regulation of the actin-mediated events of B-cell spreading and BCR clustering, and propagation of BCR signals. This study utilized A20 B-cells transfected with green fluorescent protein (GFP) fusion proteins of wt full length Dyn2 (GFP-Dyn), truncated Dyn2 missing its PRD (GFP-DynΔPRD), a GTPase dead mutant of Dyn2 (GFP-DynK44A), or a phosphorylation mutant of Dyn2 (GFP-DynY231/597F). My results show that upon BCR crosslinking, Dyn2 is recruited to the plasma membrane, where it colocalizes with the BCR, and that the PRD of Dyn2 is necessary for this recruitment. The tyrosine phosphorylation sites, GTPase function, and the PRD of Dyn2 are required for efficient BCR transport to the late endosome. Using the Dyn specific GTPase inhibitor Dynasore, I further confirmed the requirement for Dyn's GTPase function for BCR transport to the late endosome. In addition to Dyn2 recruitment and

BCR trafficking, the PRD of Dyn2, but not its GTPase and tyrosine phosphorylation sites, is necessary for recruitment of the actin adaptor protein Abp1 to the B-cell surface. Furthermore, in B-cells transfected with the GTPase dead mutant of Dyn2, actin-dependent BCR clustering but not B-cell spreading is enhanced, which is associated with elevated global tyrosine phosphorylation and downstream JNK activation, compared to GFP-Dyn transfected and control cells. These results suggest that Dyn2 is mobilized by BCR signaling to coordinate BCR internalization and signal propagation.

3.2 Introduction

B-cell signal transduction events are initiated when antigen triggers clustering of BCRs in lipid rafts, which induces phosphorylation and therefore activation of BCR proximal Src family kinases, which in turn phosphorylate the tyrosine residues in the cytoplasmic domain of Igα/β, the signal transducing units of the BCR. The phosphorylated tyrosines (pY) serve as a docking site for the kinase Syk. Syk, once bound, and activated itself, initiates the formation of signalosomes, recruiting various signaling molecules via linker proteins. One consequence of antigenic stimulation is the activation of MAPK signaling pathways (248), leading to the activation of the MAPK's p38, JNK, and ERK, which in turn activate transcription factors necessary for B-cell functions, including up-regulation of activation markers, proliferation, and survival.

Another consequence of antigenic stimulation is enhanced endocytosis and trafficking of the BCR/antigen complex through the endosomal pathway, which in turn enhances B-cell antigen presentation capability (130, 131). B cell antigen presentation to T cells is required for the differentiation of B cells to memory B cells, thereby generating

high affinity and long-lasting antibody responses. BCR endocytosis is a predominantly CME event. The endocytosing BCR/antigen complex transitions from early endosomes to $LAMP1^{+}$ late endosomes. Decreasing pH activates proteases in endosomes which act to fragment the antigen. Within the late endosome, proteolytically cleaved antigenic peptides are loaded onto MHCII molecules, and the compartment is then termed a MIIC. Antigenic peptide-loaded MHC class II complexes are then shuttled to the surface of the B-cell for presentation to T-cells to acquire T-cell help (154).

Previous work from our lab and others showed that actin remodeling is required for BCR internalization (137), B-cell signal propagation, as well as signal attenuation (168, 175). In resting B-cells, the cortical actin cytoskeleton has been proposed to act as a barrier against BCR clustering and activation, which was demonstrated by the finding that treatment with an actin destabilizing drug lead to the initiation of BCR activation in the absence of BCR stimulation (178). Upon antigenic encounter, the cortical actin cytoskeleton disassembles, allowing enhanced lateral mobility of BCRs, and BCR microcluster formation. Actin then reassembles in polarized fashion, which serves to drive BCR microcluster formation, and direct the signaling active microclusters towards one pole of the cell. When interacting with antigen presenting membranes, actin re-polymerization drives B-cell spreading at the contact site between the B-cell and the presenting surface. Spreading results in engagement of more BCRs, and therefore enhanced signaling. After the cells reach maximal spread, the B-cell membrane then begins to contract, and F-actin begins to clear from the contact zone between the B-cell and antigen presenting surface. Contraction drives BCR microclusters coalescence and subsequent central cluster formation. The contraction of the B-cell membrane with

concurrent BCR microcluster convergence to form a central cluster has been shown to promote BCR signal down-regulation. As such, our lab has demonstrated that increased F-actin accumulation at the B-cell contact zone, or delayed B-cell contraction, due to either B-cell specific N-WASP or germline Abp1 knockout, is associated with impaired formation of BCR central clusters, reduced BCR internalization, and amplified and persistent B-cell signaling, as well as elevated autoAb production and spontaneous GC B-cell formation (54, 249). These data further strengthen the idea that actin remodeling upon BCR stimulation is coordinated with BCR signal transduction and endocytosis. However, the precise molecular mechanism has yet to be defined.

Dyn is a 100 kDa GTPase that has been most well-studied for its role in clathrin-mediated endocytosis, where it is critical for scission of endocytic vesicles from the plasma membrane (203, 250-252). There are three major isoforms of Dyn (Dyn1, 2, and 3), and all Dyn family members contain a GTPase domain, a "middle'' domain, and a GTPase effector domain (GED). Intra-molecular interactions between the middle domain and GED are known to aid in Dyn self-assembly into ring-like and tubular structures at the base of endocytosing vesicles, where GTP hydrolysis causes the Dyn structure to constrict, acting to "pinch off" or release the vesicle from the donor membrane. Dyn also contains a pleckstrin homology (PH) domain that binds the phosphoinositide $PI(4,5)P_2$ at the plasma membrane (253), and a C-terminal proline rich domain (PRD) that is important for targeting to coated pits and mediating interactions with SH3 domain containing proteins (203, 250, 251). Recent studies have also begun to implicate a role for Dyn in receptor mediated signal transduction, as well as actin modulation within cells. Chaturvedi et al. showed that B-cells treated with an inhibitor of Dyn GTPase function

(dynasore), caused differential regulation of proximal and distal BCR signaling molecules compared to untreated cells (160). Malhotra et al. discovered that the B-cell adaptor protein LAB recruited a complex of Dyn2, Vav, and Grb2 in activated B-cells, and that this interaction was required for BCR internalization (218, 219). In another study, the interaction between Dyn2 and Vav was shown to be necessary for proper actin accumulation at the immunological synapse (214). Additionally, our lab has previously shown that Dyn2 interacts with the actin modulating protein Abp1 via its PRD domain (150).

Here I further investigated the domains or functions of Dyn2 necessary for its recruitment to the BCR upon BCR stimulation, and for BCR internalization and signaling. I also examined the role of the GTPase domain of Dyn2 in moderating the actin-dependent events of B-cell spreading and BCR clustering in cells activated with antigen presenting surfaces. My results show that the PRD of Dyn2 was necessary for its recruitment to the cell surface upon BCR stimulation. Additionally, I found that the PRD, GTPase function, and tyrosine phosphorylation of Dyn2 are all necessary for the translocation of BCRs from the cell surface to late endosomes. Furthermore, I discovered that the GTPase function of Dyn2 is necessary for the actin dependent event of BCR clustering, but not B-cell spreading. Finally I discovered that the GTPase domain of Dyn2 is required for appropriate BCR mediated signal propagation. My results further support the notion that the Dyn2 not only aids in BCR endocytosis and signaling, but that the individual domains of Dyn2 likely play unique roles in aiding in endocytosis and BCR signal propagation.

3.3 Materials and Methods

3.3.1 DNA constructs and transfection.

B-cell lymphoma A20 IIA1.6 cells were cultured at 37^{o} C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Plasmid DNA constructs coding for GFP fusion proteins of Dyn 2 (GFP-Dyn), Dyn 2 with its PRD deleted (GFP-Dyn ΔPRD), a Dyn 2 phosphorylation mutant (GFP-DynY321/597F), and a Dyn 2 GTPase mutant (GFP-DynK44A) were kindly provided by Dr. Mark McNiven, (Mayo Clinic, Rochester). DNA constructs were introduced into A20 B-cells by electroporation using an A20 specific Lonza Nucleofection kit (Lonza, Cologne, Germany).

3.3.2 Confocal analysis.

A20 B-cells untransfected or transfected with GFP-Dyn, GFP-Dyn ΔPRD, GFP-DynY231/597F, or GFP-DynK44A were incubated with AF 546-conjugated F(ab')$_2$-goat anti-mouse IgG (Life Technologies) for 20 min at 4^{o}C to label the surface BCR. Cells were washed and then adhered to poly-lysine-coated slides (Sigma-Aldrich) for 20 min at 4^{o}C and then chased at 37^{o}C for varying lengths of time to allow for the internalization of the BCR. For some experiments, cells at different time points were fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin in DMEM supplemented with 10% FBS, 10mM Hepes, and 10mM glycine, and incubated with a monoclonal Ab specific for lysosome-associated membrane protein 1 (LAMP-1) (ID4B, ATCC, Manassas, VA) or goat-anti-Abp1, followed by fluorescently conjugated secondary Abs. Cells were analyzed using a confocal laser scanning microscope (LSM 510 or LSM710; Carl Zeiss Microscopy). The correlation coefficients of Dyn2 constructs with the BCR, or the BCR

with LAMP-1 in the A20 B-cells before and after BCR crosslinking was determined using the LSM Zen software, from over 100 cells per time point from three independent experiments. Abp1 recruitment in A20 cells transfected with the various Dyn2 mutants was assessed visually.

A20 B-cells were also treated with the small molecule inhibitor specific for Dyn GTPase function, Dynasore (254). Cells were pre-treated, as well as continuously treated throughout the experiment with 50 µM Dynasore. After pre-treatment with Dynasore, surface BCRs were labeled with AF 546-conjugated $F(ab')_2$-goat anti-mouse IgG for 20 min at 4^{o}C, washed, adhered to poly-lysine-coated slides for 20 min at 4^{o}C, and then chased at 37^{o}C for 30 min to allow internalization of the BCR. Cells were fixed, permeabilized, and labeled with rat anti-LAMP-1 Ab, followed by AF633 anti-rat IgG (Life Technoliges). Cells were analyzed using a LSM 510 or LSM710 confocal microscope. Distribution patterns of the BCR with LAMP-1 at 30 min were quantified by visual quantification in greater than 100 cells per time point per experiment.

3.3.3 Flow cytometry.

A20 B-cells untransfected and transfected with GFP-Dyn, GFP-Dyn ΔPRD, GFP-DynY231/597F, or GFP-DynK44A were incubated with biotinylated $F(ab')_2$-goat anti-mouse IgG (20 µg/ml; Jackson ImmunoResearch) for 30 min at 4^{o}C to label surface BCRs. After washing off unbound antibodies, cells were chased at 37^{o}C for varying lengths of time. The chase was terminated by adding ice-cold DMEM containing 6 mg/ml BSA (DMEM-BSA). The biotinylated antibodies remaining on the cell surface after the chase were stained with PE-streptavidin (5 µg/ml; BD Bioscience) at 4^{o}C. The

cells were then fixed with 4% paraformaldehyde and analyzed using a flow cytometer (FACSCalibur or FACSCanto II, BD Bioscience). GFP-expressing cells were gated for analysis. The data were plotted as a percentage of the mean fluorescence intensity of cell-surface PE-streptavidin at time 0.

To analyze B-cell signaling in A20 B-cells untransfected and transfected with GFP-Dyn or GFP-DynK44A, cells were activated with 10 μg/ml F(ab')2-goat-anti-mouse IgG (Jackson ImmunoResearch), fixed, permeabilized, and stained with Abs specific for phosphotyrosine (pY) mAb 4G10 (EMD Millipore), or pJNK (Cell Signaling Technology) followed by fluorescently conjugated secondary Abs. Cells were gated into GFP^+ populations, and analyzed for up-regulation of pY or pJNK using a FACSCanto II (BD Biosciences).

3.3.4 TIRF analysis

Images were acquired using a Nikon laser TIRF system on an inverted microscope (Nikon TE2000-PFS, Nikon Instruments Inc.) equipped with a 60X, NA 1.49 Apochromat TIRF objective (Nikon), a Coolsnap HQ2 CCD camera (Roper Scientific), and two solid-state lasers of wavelength 491 and 561 nm. Interference refection images (IRM), AF488, and AF546 were acquired sequentially. A20 B-cells untransfected and transfected with GFP-Dyn or GFP-DynK44A were incubated with mB-Fab'-anti-Ig-tethered lipid bilayers at 37ºC for varying lengths of time. Cells were then fixed with 4% paraformaldehyde, and imaged for the recruitment of GFP-Dyn, GFP-K44A, or BCRs. ,. The B-cell contact area was determined based on IRM images using MATLAB software (The MathWorks). The total and mean fluorescence intensity (TFI and MFI) of the BCRs

or GFP-constructs in the B-cell contact zone was determined using Andor iQ software (Andor Technology). Background fluorescence generated by mB-Fab'-anti-Ig tethered to lipid bilayers in the absence of B-cells or secondary antibody controls, was subtracted. For each set of data, more than 50 individual cells from three independent experiments were analyzed.

3.4 Results

3.4.1 The PRD of Dyn2 is necessary for its recruitment to the BCR upon activation.

Previous studies have shown that BCR crosslinking results in the recruitment of Dyn2 to the cell surface, where it binds to the plasma membrane via its PH domain, and participates in receptor mediated endocytosis. To determine the domain or functions of Dyn2 necessary for its recruitment to the plasma membrane, I examined the effect of various mutations, including a deletion of the PRD, mutation of two tyrosine phosphorylation sites, and a GTPase-dead mutation on Dyn2 recruitment to the plasma membrane upon BCR crosslinking. A20 B-cells were transiently transfected with GFP-Dyn, GFP-Dyn ΔPRD, GFP-DynY231/597F, or GFP-DynK44A, and the cells were incubated with AF 546-conjugated $F(ab')_2$-goat anti-mouse IgG at 4^oC to label the surface BCR and warmed to 37^oC for 2 min to activate BCR signal transduction. Calculation of the Pearson's colocalization coefficient between the GFP-Dyn proteins and the BCR, used to indicate Dyn2 recruitment to the B-cell surface, was assessed after imaging by confocal fluorescence microscopy. A negative correlation coefficient indicates that the two proteins mutually exclude one another, while a score of 0 implies a random pattern of colocalization, and a score of 1 implies 100% colocalization of two

proteins. After 2 min of activation, the cellular distribution of the full length GFP-Dyn changed from a cytoplasmic distribution at zero minutes, to a cell surface distribution pattern, where it colocalized with the BCR (Fig. 3.1A). Similarly, GFP-DynY231/597F and GFP-DynK44A appeared at the plasma membrane after 2 min of stimulation (Fig.3.1A). In contrast, GFP-Dyn ΔPRD remained in a cytoplasmic distribution (Fig. 3.1A). The data (Fig 3.1B), showed significant increases in the correlation coefficient of full length GFP-Dyn, the Dyn GTPase mutant (GFP-DynK44A), and the Dyn phosphorylation mutant (GFP-DynY231/597F) after the 2 min stimulation. However, the colocalization between the BCR and the truncated Dyn mutant lacking its PRD (GFP-DynΔPRD) did not significantly increase (Fig. 3.1B). Further examination of the distribution patterns of the various Dyn2 constructs after 30 min of BCR stimulation shows that while the GFP-DynΔPRD remained predominantly cytoplasmic, GPF-DynK44A appeared to accumulate at vesicles intimately associated with the plasma membrane (3.2A-B). We hypothesize these may be partially endocytosed vesicles which are stalled at the pinching off phase of internalization. Also, the GFP-DynY231/597F transfected cells showed greater percentages of cells with GFP-DynY321/597F localized to plasma membrane vesicles as well as evenly distributed at the plasma membrane after 30 min of activation, compared to GFP-Dyn and GFP-DynΔPRD transfected cells (Fig. 3.2A-B). The results show that the PRD, but neither Dyn2 phosphorylation nor its GTPase function, is required for recruitment to the plasma membrane upon BCR activation, and that the GTPase function and phosphorylation of Dyn2 are required for its release from the plasma membrane.

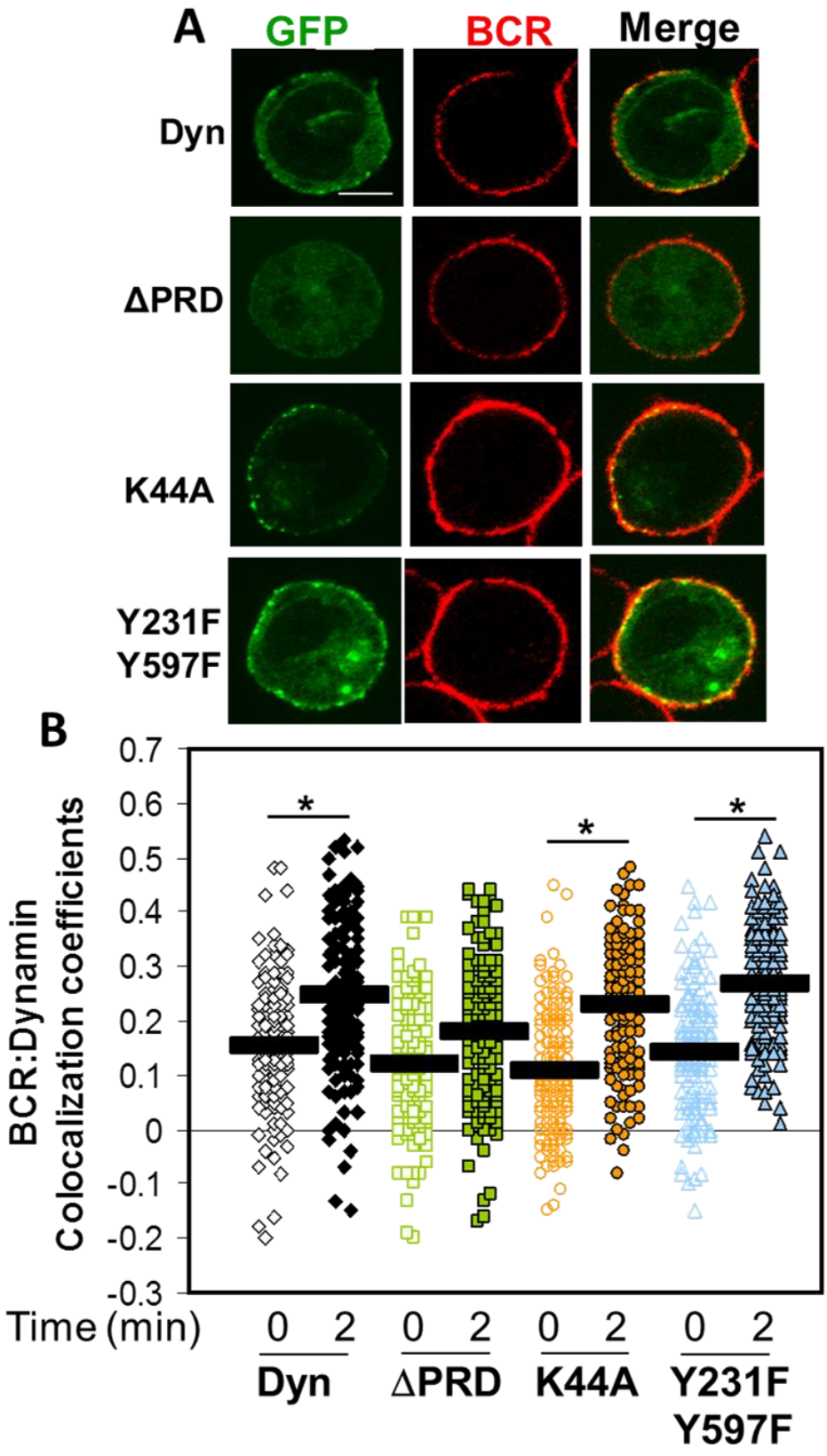

A
GFP
BCR
Merge
Dyn
ΔPRD
K44A
Y231F
Y597F
B
BCR:Dynamin
Colocalization coefficients
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
*
*
*
Time (min)
0 2 0 2 0 2 0 2
Dyn
ΔPRD
K44A
Y231F
Y597F

Fig. 3.1 Dyn2 PRD domain necessary for its recruitment to the B-cell membrane. **(A-B)** A20 cells transfected with different GFP-dynamin mutants were activated with AF 546-conjugated $F(ab')_2$-goat anti-mouse IgG for 2 min. Cells were fixed, and imaged by laser scanning confocal microscopy. Shown are representative images **(A)** (n=3, scale bar = 5 μm). Shown are the correlation coefficients of GFP-dynamin with the BCR at time zero, and 2 min **(B)**. Data generated from >100 cells per time point (n=3). * $p<0.05$

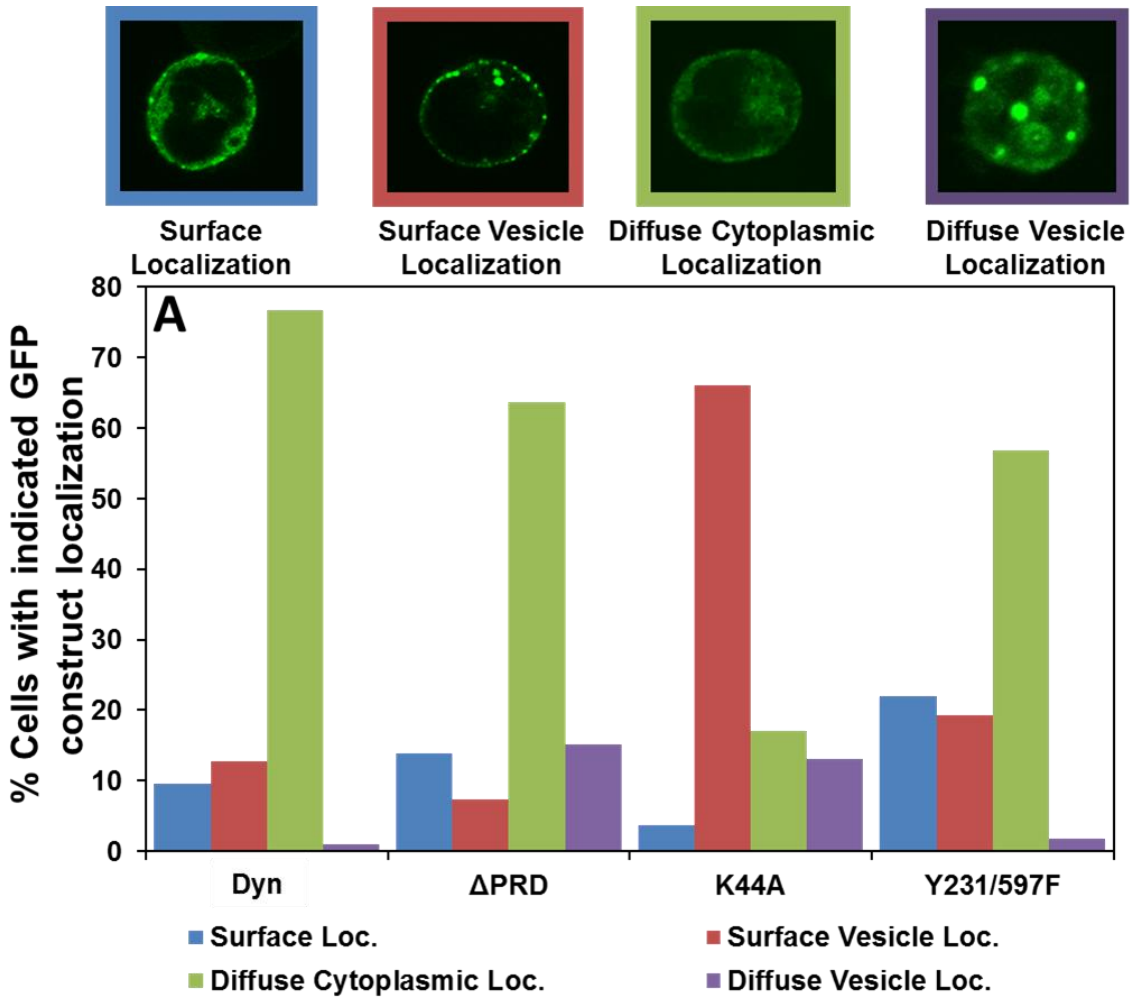


Fig. 3.2 GFP-DynK44A and GFP-DynY231/597F mislocalized to cell surface at later time points of B-cell activation. A20 cells transfected with different GFP-dynamin mutants were activated with AF546-conjugated F(ab')$_2$-goat anti-mouse IgG for 30 min, fixed, and imaged by confocal microscopy. GFP$^+$ cells were visually quantified into four categories. Shown are representative images of the major categories of Dyn distribution after 30 min of BCR stimulation. (n=3)

3.4.2 Dyn2's phosphorylation, PRD domain, and GTPase activity are necessary for BCR trafficking to late endosomes.

To examine the domains or function of Dyn2 necessary for internalization of the BCR from the cell surface and its movement to late endosomes, a process necessary for antigen processing, I analyzed the ability of A20s transfected with GFP-Dyn, GFP-Dyn ΔPRD, GFP-DynY231/597F, or GFP-DynK44A to internalize cross-linked BCRs and to transport the BCR to late endosomal compartments in these B-cells, using flow cytometry and immunofluorescence microscopy. To analyze BCR internalization, I labeled the surface BCRs on the transfected cells with biotinylated goat anti-mouse IgG at 4^{o}C. The cells were washed, and then chased at 37^{o}C for varying lengths of time. After the chase, the biotinylated antibodies remaining at the cell surface after the chase were stained with PE-streptavidin. The cells were fixed and analyzed by flow cytometry. Cells were gated as GFP^{+} (experimental), or GFP^{-} (control) cells. The data were plotted as a percentage of the mean fluorescence intensity of cell-surface PE-streptavidin at time 0. Full length GFP-Dyn and GFP-DynY231/597F transfected cells showed similar levels of BCR remaining at the cell surface compared to control cells (Fig3.3 A and D), while GFP-DynK44A transfected cells showed slightly reduced, but non-significantly altered levels of BCR remaining at their surface compared to control cells (Fig. 3.3C). Cells transfected with GFP-DynΔPRD exhibited significantly sustained levels of BCRs remaining at the cell surface at all time points compared to control cells (Fig. 3.3D).

As the streptavidin used in the endocytosis analysis described above may not have access to biotin labeled BCRs within clathrin-coated pits that fail to detach from the plasma membrane, I further evaluated the domains or function of Dyn2 necessary for

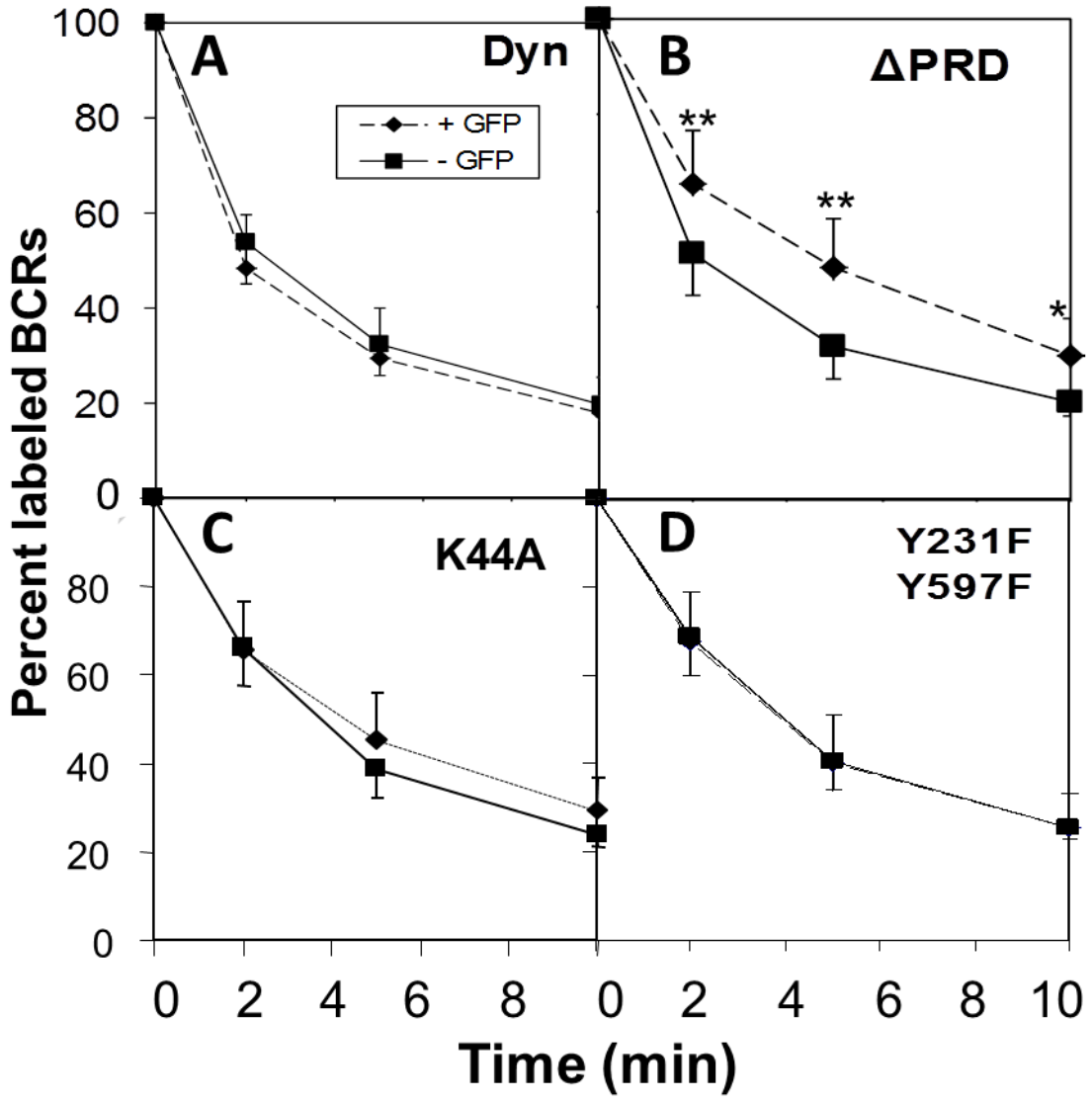
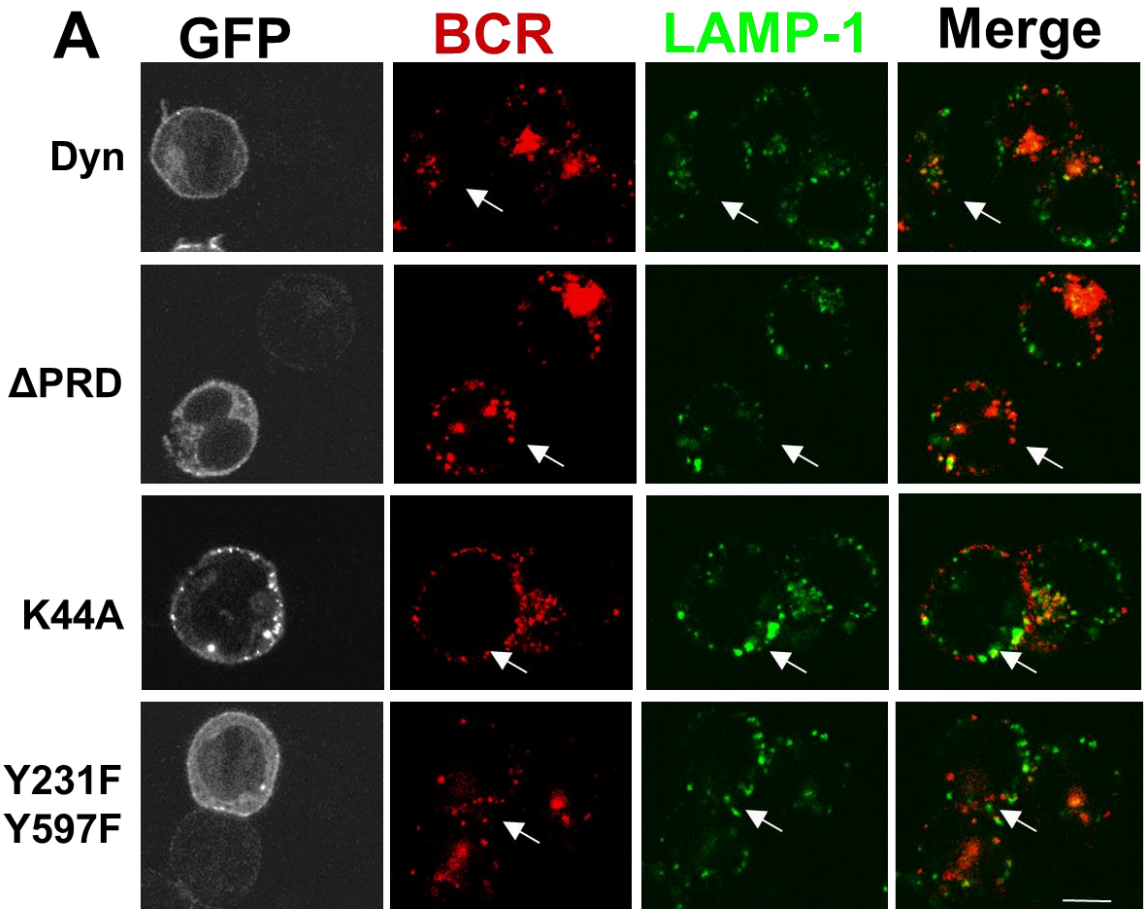


Fig. 3.3 Percent of labeled surface BCRs. (A-D) A20 cells transfected with different GFP-dynamin mutants were labeled and activated with biotin conjugated $F(ab')_2$-goat anti-mouse IgG for various times. Biotin-anti-mouse IgG left at the cell surface was detected by PE-streptavidin and quantified by flow cytometer. Cells were gated into GPP^- and GFP^+ populations, and the data were plotted as the percentage of the surface-labeled BCR at time 0. Shown are the averages (±S.D., n=3). *, $p<0.05$, **, $p<0.01$.

BCR internalization by examination of the colocalization of the BCR with the late endocomal marker LAMP-1 using immunofluorescence microscopy. Cells were incubated with AF546-conjugated $F(ab')_2$-goat anti-mouse IgG at 4^oC to label and activate the surface BCR, and then chased at 37^oC for 30 min to allow BCR internalization. After 30 min, the cells were fixed, permeabilized, and labeled with an Ab specific for LAMP-1. The cells were analyzed with a confocal laser scanning microscope. I again utilized software to calculate the Pearson's correlation coefficient, this time between the BCR and LAMP-1 staining compared between GFP^+ and GFP^- control cells. In line with the flow cytometry analysis, full length GFP-Dyn showed similar colocalization between the BCR and LAMP-1^+ compartments after the 30 min chase when compared to GFP^- control cells (Fig. 3.4A and B), while the GFP-DynΔPRD B-cells showed significantly reduced BCR/LAMP-1 colocalization compared to control cells (Fig 3.4A and C). Unlike the results from the flow cytometry analysis, cells expressing the GPF-DynY231/597F and GFP-DynK44A showed significantly reduced BCR/LAMP-1 colocalization when compared to control cells (Fig. 3.4A, D and E). Similar to what we observed at 2 and 30 min, the distribution of GPF-DynK44A and the GFP-DynY231/597F remained on vesicles intimately associated with the plasma membrane (Fig. 3.1A, 3.2, and 3.4). These results suggest that the PRD as well as the GTPase function and phosphorylation of Dyn2 are necessary for internalization and trafficking of the BCR to the late endosome.

To further confirm the requirement of the Dyn2 GTPase for BCR internalization, we examined the effect of the small molecule inhibitor of Dyn2 GTPase activity, Dynasore, on BCR/LAMP-1 colocalization. Briefly, cells were pre-treated with


A
GFP
BCR
LAMP-1
Merge
Dyn
ΔPRD
K44A
Y231F
Y597F

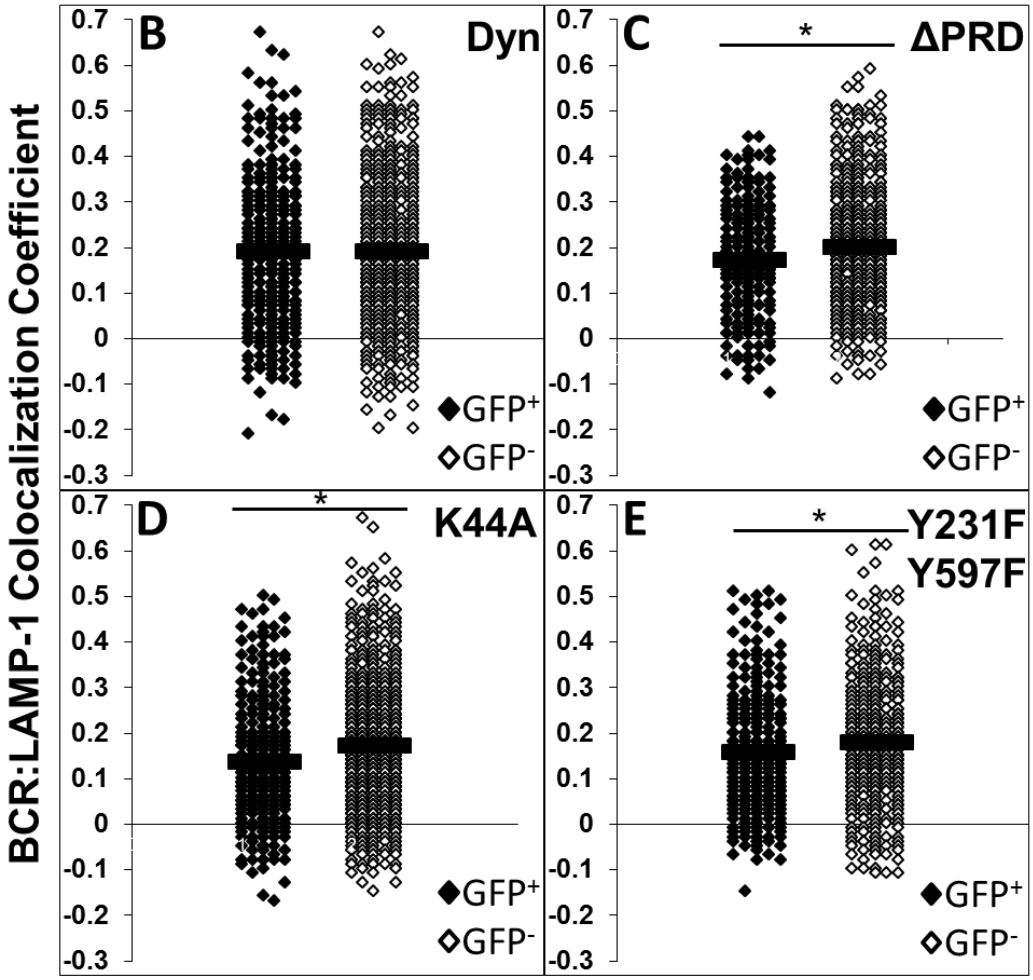
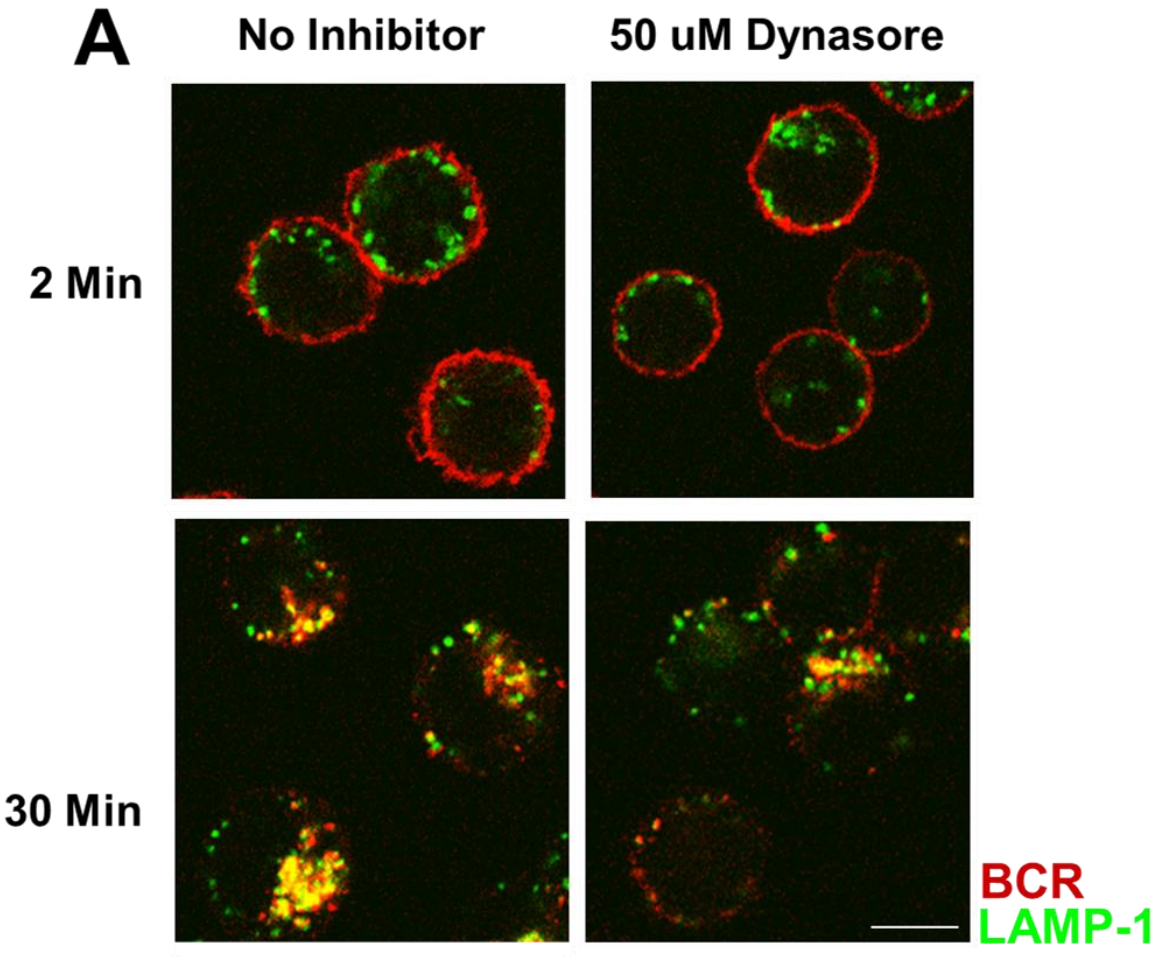


Fig. 3.4 Dyn2's PRD domain, GTPase activity, and phosphorylation are necessary for BCR translocation to LAMP1$^+$ compartments. A20 cells transfected with different GFP-dynamin mutants were activated with AF 546-conjugated F(ab')$_2$-goat anti-mouse IgG for 30 min to allow internalization of the BCR. The cells were fixed, permeabilized, and labeled with an Ab specific for LAMP-1 then imaged with a confocal laser scanning microscope. Shown are representative images, arrows indicate GFP$^+$ cells **(A)** (n=3). Shown are the correlation coefficients of the BCR with LAMP-1 in untransfected (GFP$^-$) control cells and GFP$^+$ cells at 30 min **(B-E)**. Data generated from >200 cells per condition (n=3). *, $p<0.05$

Dynasore before BCR labeling and crosslinking at 4ºC and throughout the duration of a 30-min chase at 37ºC to allow BCR internalization. Using immunofluorescence microscopy, we quantified the percentage of cells showing either no BCR/LAMP-1 colocalization, colocalization at surface vesicles, scattered vesicle colocalization, or extensive colocalization. Our results show that while most of untreated cells display extensive BCR/LAMP-1 colocalization at the perinuclear region, BCRs in most of the Dynasore-treated cells localized at surface vesicles after 30 min of activation (Fig. 3.5A-C). Collectively these results indicated that the PRD and GTPase function, as well as the tyrosine phosphorylation of Dyn2, are all involved in BCR internalization and transport of actively signaling BCRs to the late endosome.

3.4.3 Dyn2 PRD domain is necessary for Abp1 recruitment.

Our lab previously showed the interaction between Abp1 and Dyn2, mediated by the PRD and SH3 of Dyn2 and Abp1 respectively, to be necessary for BCR internalization after BCR crosslinking (150). To further investigate which domain or function of Dyn2 is necessary for Abp1 recruitment to the plasma membrane upon BCR crosslinking, we transfected A20 B-cells with GFP-Dyn2 constructs, including GFP-Dyn, GFP-Dyn ΔPRD, GFP-DynY231/597F, or GFP-DynK44A, and monitored the recruitment of Abp1 to the plasma membrane upon BCR crosslinking. Abp1 was labeled with a goat-anti-Abp1 Ab after permeabilization and analyzed by confocal microscopy. Abp1 recruitment to the plasma membrane was visually assessed, and quantified. My preliminary analysis showed that while GFP-Dyn, GFP-DynK44A, and GFP-DynY231/597F appeared to have no effect on Abp1 recruitment, A20 B-cells transfected


A
No Inhibitor
50 uM Dynasore
2 Min
30 Min
BCR
LAMP-1

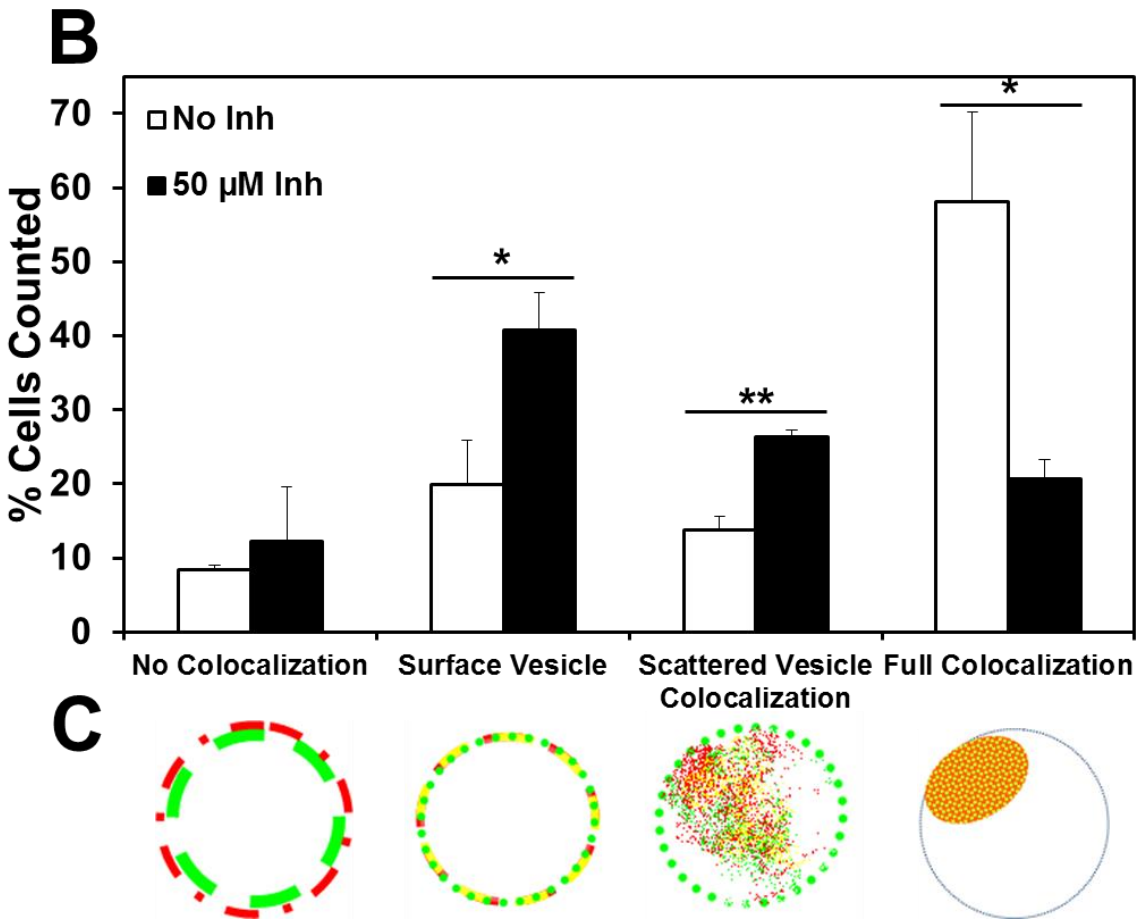


Fig. 3.5 Dynasore reduces BCR internalization to late endosome. (A-B) A20 cells were activated with AF 546-conjugated F(ab')$_2$-goat anti-mouse IgG for 30 min to allow internalization of the BCR either with our without 50 µM Dynasore. The cells were fixed, permeabilized, and labeled with an Ab specific for LAMP-1 then imaged with a confocal laser scanning microscope. Shown are representative images from 0 and 30 min **(A).** Bar, 5 µm (n=3). Visual quantification of BCR colocalization with LAMP-1 at 30 min in greater than 225 cells per condition **(B)**. (n=3). Schematic representation of different colocalization stages shown below **(C)**. *, $p<0.05$, **, $p<0.01$

with the GFP-DynΔPRD were defective in the recruitment of Abp1 to the cell periphery (Fig 3.6A-B). The preliminary results suggest that the interaction between Abp1 and Dyn2, via their SH3 domain and PRD respectively, is necessary for BCR induced recruitment of Abp1 to the plasma membrane.

3.4.4 Dyn2 GTPase deficiency augments BCR clustering, but not B-cell spreading.

As Dyn2 has been implicated to function in both actin dynamics and receptor internalization, both of which influence signaling (149, 150, 220-222), we examined the impact of Dyn2 on the actin-mediated events of BCR clustering and B-cell spreading, using IRM and TIRF. A20 B-cells transfected with the either GFP-Dyn or GFP-DynK44A, the GTPase defective mutant of Dyn2, were activated with Fab'-anti-Ig tethered-lipid bilayer for various times, and imaged by IRM to analyze the the area of the B-cell membrane contacting antigen-presenting surface (B-cell contact area) and TIRF to monitor BCR clustering and GFP-Dyn or GFP-DynK44A recruitment. My results show that B-cell spreading on the antigen presenting surfaces was not affected by the expression of Dyn-GFP or Dyn-GFPK44A when compared to control non-transfected (NT) cells (Fig. 3.7A-B). Similar to non-transfected controls, GFP-Dyn (Dyn) expressing cells showed a gradual increase in BCR clustering at the B-cell contact site over time (Fig. 3.7A and C). However, the cells transfected with the GTPase mutant of Dyn2 (K44A) exhibited elevated levels of BCR clustering throughout the time course of activation compared to GFP-Dyn transfected cells (Fig. 3.7A and C). Both GFP-Dyn and GFP-DynK44A were recruited to the contact zone with similar kinetics (Fig. 3.7A and D), suggesting the alteration in BCR clustering is not likely due to differences in

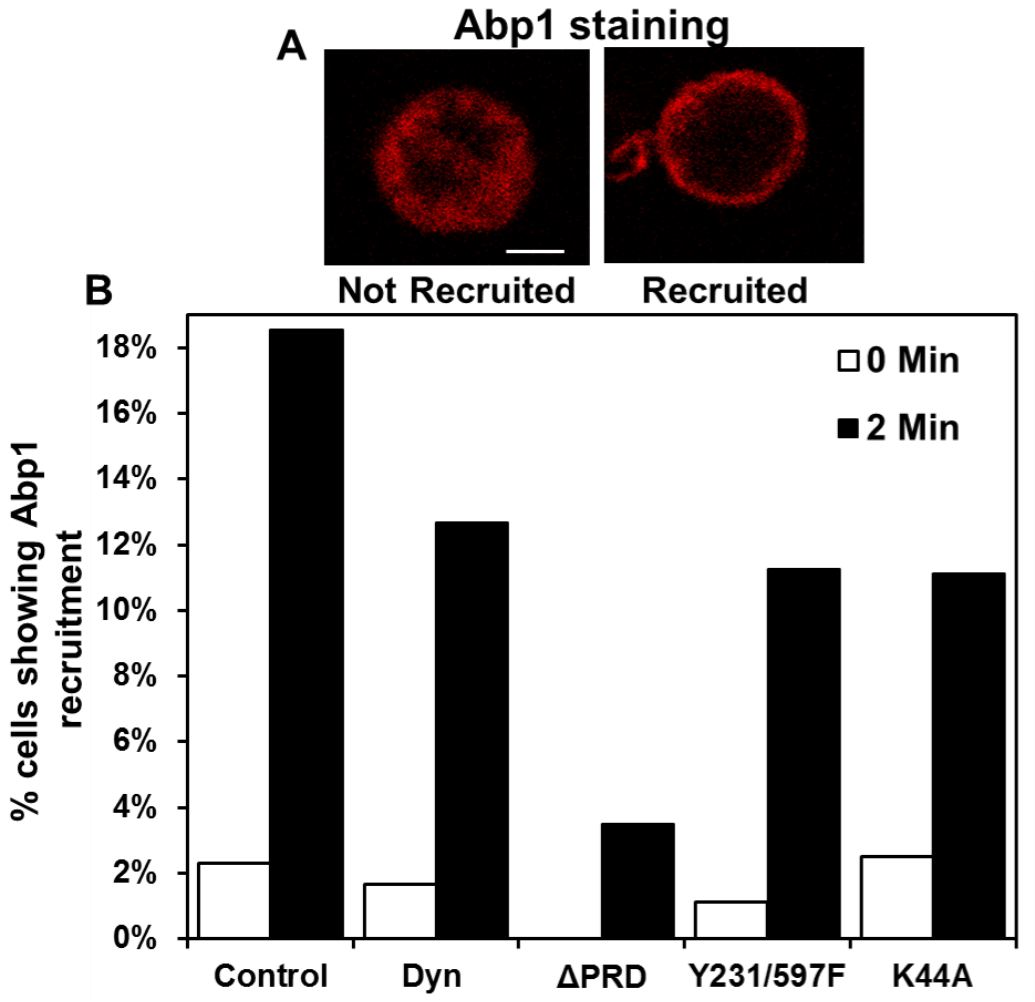


Fig. 3.6 Dyn2 PRD domain necessary for recruitment of actin adaptor Abp1. (A-B) A20 cells transfected with different GFP-dynamin mutants were activated with AF 546-conjugated F(ab')$_2$-goat anti-mouse IgG for 2 min. Cells were fixed, permeabilized, and labeled with anti-Abp1 and fluorescent secondary antibody. Cells were imaged by laser scanning confocal microscopy. Shown are representative images defining recruited or non-recruited Abp1 **(A)** (n=2, scale bar 5 μm). GFP$^+$ cells were visually quantified as non-recruited or recruited at 0 and 2 min **(B)**. Data generated from >50 cells per time point.

recruitment of Dyn-GFP or GFP-DynK44A. These results suggest that Dyn2 contributes to the negative regulation of BCR clustering, but not to B-cell spreading in GTPase-dependent manner.

3.4.5 Dyn2 GTPase function is involved in the regulation of BCR signal transduction.

Our finding on the regulatory roles for Dyn2 in BCR clustering, BCR internalization, and BCR-induced Abp1 recruitment suggests that Dyn2 can modulate BCR signaling indirectly through the above activities. To test this hypothesis, A20 B-cells transfected with either GFP-Dyn or GFP-DynK44A were activated with soluble $F(ab')_2$-goat-anti-mouse IgG for varying lengths of time. The overall signaling activity of the cells was measured by the levels of tyrosine phosphorylation using phosphotyrosine mAb (pY) and flow cytometry. Examination of pY up-regulation in BCR stimulated GFP^+ populations of GFP-Dyn or GFP-DynK44A transfected A20 B-cells showed an increase in pY in the GFP-DynK44A transfected cells throughout the time course when compared to the GFP-Dyn transfected cells (Fig 3.8A). Furthermore, BCR stimulated GFP-DynK44A transfected cells showed enhanced activation of the MAPK JNK throughout the time course when compared to GFP-Dyn transfected A20 cells (Fig. 3.8B). These results suggest that the GTPase function of Dyn2 is necessary for appropriate signal transduction from the BCR, and that elimination of Dyn2 GTPase function acts to inhibit negative signal induction from the BCR.

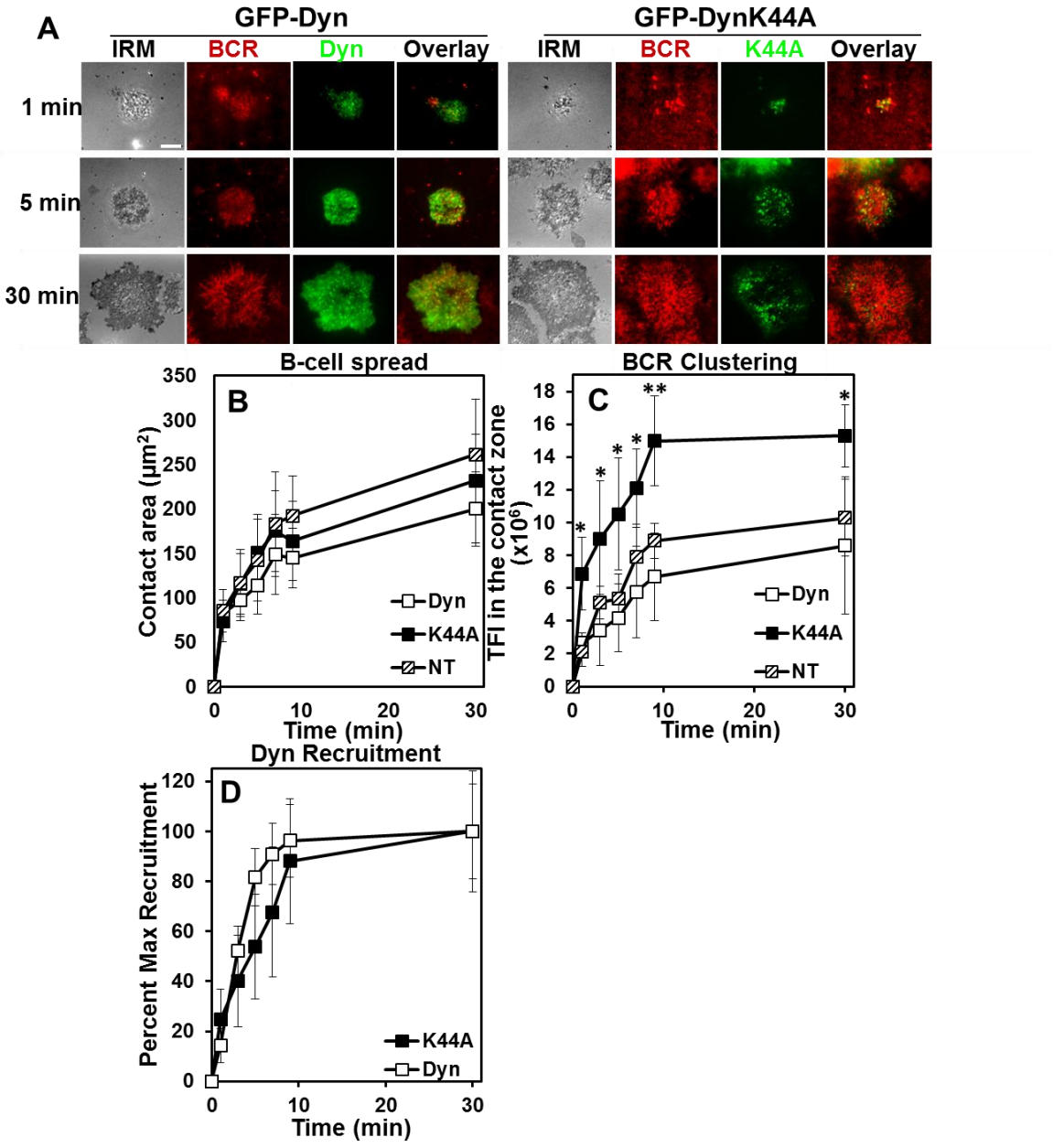

A
GFP-Dyn
IRM
BCR
Dyn
Overlay
GFP-DynK44A
IRM
BCR
K44A
Overlay
1 min
5 min
30 min
B-cell spread
B
Contact area (μm²)
Time (min)
Dyn
K44A
NT
BCR Clustering
C
TFI in the contact zone (x10⁶)
Time (min)
Dyn
K44A
NT
Dyn Recruitment
D
Percent Max Recruitment
Time (min)
K44A
Dyn

Fig. 3.7 Dyn2 GTPase deficiency augments BCR clustering but does not affect B-cell spread. (A-D) IRM and TIRF analysis of GFP-Dyn and GFP-DynK44A transfected A20 B-cells incubated with Fab'-anti-Ig-tethered lipid bilayers. Representative images, bar **5** μm, (n=3) **(A)**. Average contact area **(B),** TFI of labeled BCRs **(C)**, and MFI of GFP-Dyn or GFP-DynK44A in contact zone **(D)** from > 50 cells per time point (n=3), quantified using Andor iQ software. *, $p<0.05$, **, $p<0.01$.

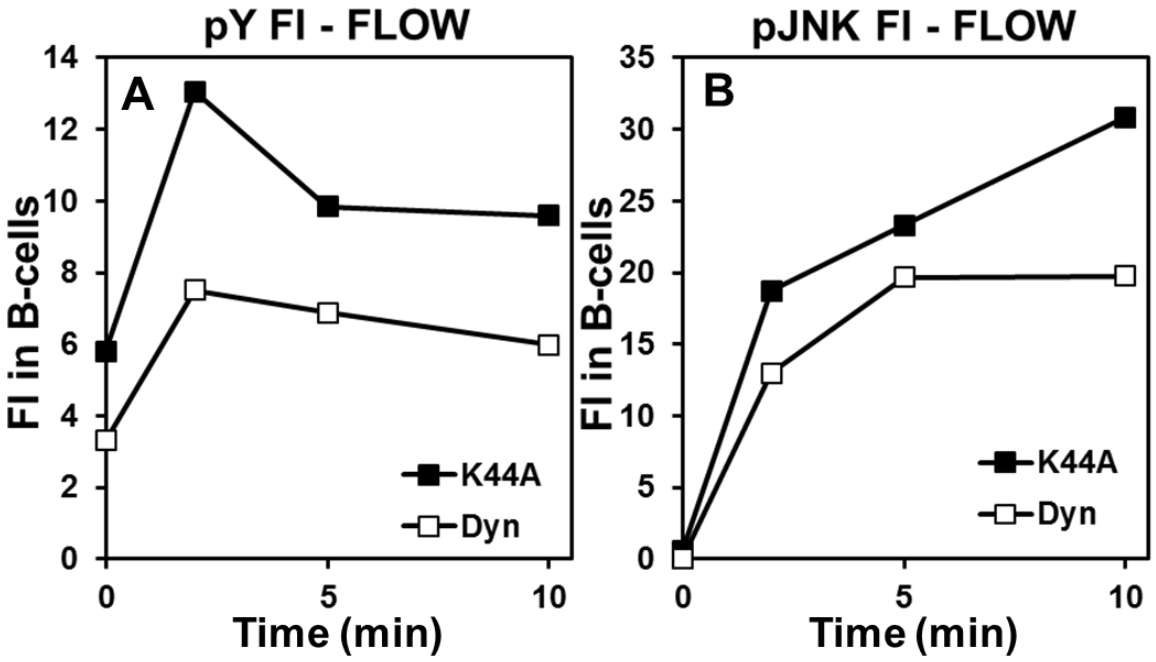


Fig. 3.8 Dyn2 GTPase function necessary for BCR signal transduction. (A-B) Flow cytometry analysis of GFP-Dyn (Dyn) and GFP-DynK44A (K44A) transfected A20 B-cells activated with AF 546-conjugated $F(ab')_2$-goat anti-mouse IgG, fixed, permeabilized, and labeled with anti-phospho-tyrosine (pY - **A**) or anti-phospho-JNK (pJNK - **B**) (n=2).

3.5 Discussion

Dyn is a large mechano-enzyme necessary for membrane scission of newly budding vesicles from membranes, including removal of endocytosed BCR containing vesicles from the B-cell surface for antigen processing and presentation (160, 218, 255, 256). Recent research has implicated a role for Dyn in both actin modulation as well as signal propagation via interaction with signaling intermediates (150, 218, 219, 256). This study reveals that the different domains of Dyn2 are necessary for BCR/antigen uptake and signaling. My results show that the PRD of Dyn2 is required for its recruitment to the plasma membrane upon BCR crosslinking, while the PRD, GTPase domain, and tyrosine phosphorylation are all required for efficient BCR internalization and trafficking to LAMP-1^{+} compartments. Only the PRD of Dyn2 appears to be necessary for the recruitment of Abp1, an actin adaptor protein that is involved in both BCR endocytosis and signaling, to the cell surface upon B-cell activation. This study also shows that the GTPase activity of Dyn2 contributes to the negative regulation of BCR clustering but not B-cell spreading along antigen presenting surfaces. Consequently, A20 B-cells transfected with the GTPase dead mutant of Dyn2 showed elevated levels of phosphorylated tyrosine and the MAPK pJNK upon BCR stimulation.

This study revealed that the PRD of Dyn2 is required for its recruitment to the plasma membrane, and colocalization with the BCR at the early times of BCR mediated activation, which is in accordance with studies in other cell types. Shpetner et al. previously showed that while full length Dyn2 bound to clathrin coated pits on Cos-7 cell plasma membranes, mutations of the Dyn2 PRD disrupted this interaction (200). Furthermore, a study by McNiven et al. showed that Dyn2 recruitment to membrane

ruffles and lamellipodia of fibroblast cells treated with platelet-derived growth factor (PDGF) was dependent upon its interaction with the actin modulating protein cortactin, and disrupted by deletion of the cortactin SH3 domain or Dyn2 PRD (257). However, the exact molecular framework necessary for Dyn2 recruitment to the plasma membrane of B-cells remains to be dissected. Our lab has previously shown that B-cells treated with the Src kinase inhibitor PP2 showed reduced recruitment of Dyn2 upon BCR crosslinking (Bruce K. Brown, PhD, unpublished data); however, we show here that the tyrosine phosphorylation mutant of Dyn2 (GFP-DynY231/597F) was recruited normally to the plasma membrane where it colocalized with the BCR. This finding suggest that while phosphorylation of Dyn2 at its conserved pY sequences is not required for its recruitment, Src family kinases can trigger the recruitment of Dyn2 to signaling active BCRs at the cell surface by phosphorylation/activation of other molecules. Collectively, the data indicate that Dyn2 recruitment relies upon interaction with an as of yet unknown SH3 domain containing protein(s), and Src-mediated phosphorylation/activation of an unknown binding partner(s). While Dyn has been shown to interact with a wide variety of SH3 domain containing molecules, such as Grb2, amphiphysin, cortactin, etc. (141, 144, 210, 214, 218, 219, 257, 258) the identification of the precise protein interaction necessary for Dyn recruitment to signaling active BCRs will be the focus of our future studies. One approach to address this question is overexpression of SH3 domains of suspected Dyn2 interacting partners, after which we could examine BCR mediated Dyn2 recruitment to the signaling active BCRs.

One of the new findings of this study is that BCR internalization and trafficking to late endosomes requires multiple domains of Dyn2, including the PRD, GTPase function,

and its tyrosine phosphorylation sites. However, the function of each Dyn2 domain in the process has not been fully characterized. My results show that the GFP-DynΔPRD construct fails even the initial step of recruitment to signaling active BCRs, indicating a role for the PRD in the surface recruitment of Dyn2. My finding that GFP-DynK44A expression leads to the accumulation of BCRs stuck in membrane associated vesicles further suggests a role for Dyn2 GTPase function in membrane scission for BCR internalization. The role of phosphorylation in Dyn2 function is inconsistent in the literature (215-217). Here we show that the phosphorylation, while unnecessary for Dyn2 recruitment, is required for BCR endocytosis and trafficking to the late endosomal compartments. As such, it is likely that Src kinase phosphorylation of another molecule is necessary for Dyn2 recruitment to the B-cell membrane, which would explain reduced Dyn2 recruitment in cells treated with PP2. My data also suggest that Dyn2 phosphorylation after recruitment to signaling active BCRs may be required for interaction with a SH2 domain containing protein, and that this interaction is crucial at later stages of BCR internalization.

Previous reports utilizing other cell types and model membranes suggest that Dyn2 GTP hydrolysis is the driving force for the final pinching off phase of the endocytic process (206, 209). Consistent with these reports, my data show both labeled BCR as well as GFP-DynK44A stuck at surface localized vesicles at both early (2 min) and late time points (30 min) of activation. However, my flow cytometry-based BCR internalization analysis did not corroborate my confocal immunofluorescence analysis, showing no significant differences in the percentage of surface-labeled BCRs available for PE-streptavidin binding. It is possible that the flow cytometry-based method is

unable to differentiate between BCRs in budding vesicles and in fully endocytosed vesicles, due to inaccessibility of PE-streptavidin, a relatively large protein complex, to nascent vesicles budding from the plasma membrane which are moderately constricted by Dyn2 oligomer formation. As such, different techniques are required to support our results from the immunofluorescence analysis.

This study also revealed that the GTPase function of Dyn2 is necessary for the negative regulation of the actin-dependent event of BCR clustering, but does not play a role in B-cell spreading upon interaction with membrane-bound antigens. There are two possible mechanism by which Dyn2 may aid in down regulation of BCR clustering; by promoting BCR internalization and by modifying actin remodeling. Dyn2 has been shown to be linked to the actin cytoskeleton in a variety of cells types, via interaction with a growing list of actin modulating proteins (148, 150, 219, 256). Our lab has previously shown that Dyn2 constitutively interacts with the actin adaptor protein Abp1 via the Dyn2 PRD and Abp1 SH3 domains (150). This interaction links the actin cytoskeleton to Dyn2, enabling actin to facilitate the Dyn2-mediated membrane scission step of BCR internalization (150). The data presented here further suggest that this interaction occurs via interaction between the Dyn2 PRD, and Abp1 SH3 domain. My results here show that this interaction is responsible for recruitment of Abp1 to the plasma membrane upon BCR stimulation. These data further suggest that Dyn2 can indirectly interact with the actin cytoskeleton via Abp1.

While B-cell spread was unaffected in B-cells transfected with GFP-DynK44A, it is important to note that A20 B-cells (a cancer cell line) show different spread kinetics compared to primary B-cells. Primary B-cells in contact with antigen presenting surfaces

spread optimally by 3 min of activation, and gradually, as F-actin within the contact site begins to decrease, the cells contract to aid in the coalescence of BCRs for signal attenuation. In contrast, A20 B-cells do not appear to significantly contract even after 30 min of activation with membrane bound antigen (lab unpublished data, (163)), which may be a common characteristic of cancerous B-cells (259). As such, unlike primary B-cells, A20 B-cells may have a modified mechanism by which they signal B-cell spread, or they may have an overriding signal which prevents their contraction. In this case, we are unable to determine whether Dyn2 has a regulatory function in B cell contraction and signal attenuation using A20 cells. To examine whether Dyn2 plays a role in B-cell spread kinetics, we could attempt to transfect primary B-cells with the Dyn2 mutants. However, primary lymphocyte transfection remains very difficult, with very low resultant transfection efficiencies, and exacerbated cell death.

BCR activation requires receptor clustering at the cell surface (114, 161, 162). As such, it is no surprise that the increased BCR clustering observed in cells expressing the GTPase dead mutant of Dyn2 is associated with higher levels of pY, as well as the MAPK pJNK. In agreement with this data, Chaturvedi et al. showed that cells treated with GTPase inhibitor of Dyn2 (dynasore) have dysregulated (and in many cases elevated) phosphorylation of key proximal and distal signaling elements of the BCR (160). Dynasore-mediated signal dysregulation was suggested to be caused by mislocalization of the BCR and signaling molecules. Signaling molecules which would normally be localized and activated within the interior of cells are erroneously recruited to the plasma membrane-associated vesicles where BCR endocytosis is stalled. Chaturvedi et al. postulated that BCRs signal continuously even after BCRs enter

endosomal compartments, after which, they are exposed to different kinases, phosphatases, or lipid environments from those found at the plasma membrane. However, this hypothesis has yet to be tested.

It should be mentioned that Dyn2 also functions in membrane dynamics and vesicle budding at various intracellular organelles, thereby possibly resulting in disruption of B-cell function at sites other than endocytosing BCRs at the plasma membrane. One previous study showed that Dyn2, in conjunction with cortactin, links the actin cytoskeleton to transport of newly synthesized proteins from the trans-Golgi network (260). In addition, Dyn has been implicated in caveolae-mediated endocytosis and late endosome to Golgi transport (203, 261). Dyn function has also been shown to be important for mitochondrial fusion and fission, as well as cell division (207). Therefore, the defects observed in the B-cells transfected with GFP-DynΔPRD, GFP-DynK44A, and GFP-DynY231/597F constructs may arise due to defects in multiple aspects of B-cell function that are necessary for cell survival or antigen response.

The studies presented in this chapter further substantiate both the necessity of Dyn2 for BCR internalization and its proposed function as a modulator of actin and signal transduction during B-cell activation. However, much more work will be necessary to fully outline the role Dyn2 plays in these activities. Future work would include identifying protein(s) necessary for Dyn2 recruitment, further characterizations of the role of Dyn2 phosphorylation in the interaction of Dyn2 with its partners, and greater characterization of signaling defects in B-cells expressing different Dyn2 mutants. These additional studies will help further our understanding of not only Dyn2's role in BCR

internalization and signal propagation, but will generally better define the somewhat contested link between BCR signaling and internalization.

Chapter 4: General Discussion and Future Work

4.1 General summary and working model

B-cell function, namely B-cell generated humoral immune response towards invading pathogens, is critical to host defense against infectious pathogens. Key to this protective function is regulated B-cell signaling of pathogen encounter, as well as processing and presentation of antigenic peptides to garner T-cell help for optimal B-cell activation. More and more research suggests that these processes are linked, and that the nature, magnitude, and duration of the signals emanating from the BCR determine B-cell fate, and therefore necessitate tight control. In this dissertation, I sought to better understand how the processes of BCR-mediated signal transduction, dynamic BCR and actin rearrangements, and subsequent BCR internalization for antigen processing are linked, with the goal of gaining further insight into how these processes work to collectively regulate host humoral immune response. Critical examination of the molecular interactions and physiological responses which take place during B-cell signaling, BCR/antigen complex internalization, and subsequent processing and presentation, as well as how these processes are interrelated will help us better understand which processes are subverted, leading to B-cell mediated disease. With a clearer understanding of these processes and how they are related we will be capable of generating enhanced targeted treatments to prevent or overcome the dysregulated cellular functions associated with B-cell related disease, and aid in discovery of novel vaccination strategies to enhance B-cell function.

The goals of this study were twofold: (1) to dissect the molecular mechanisms by which Abp1 links actin remodeling to BCR signal transduction, and examine the impact of Abp1 deficiency on B-cell development and immune response, and (2) to examine the domains or functions of Dyn2 necessary for its recruitment, BCR internalization, and BCR mediated signal transduction. While these research topics were pursued independently, the topics are intimately linked, as Abp1 and Dyn2 constitutively interact, and this interaction is necessary for the precise coordination of BCR signal transduction, dynamic actin rearrangement, and BCR/antigen internalization and trafficking. As such, the molecular interplay between Abp1 and Dyn2 are necessary for optimal B-cell survival and initiation of humoral immune responses.

My Ph.D. dissertation research demonstrates a novel function for the actin regulating protein Abp1 in BCR signal attenuation, which is critical for the regulation of peripheral B-cell differentiation and Ab responses. My research was initiated based on the previous finding from our lab that Abp1 was required for efficient BCR-mediated antigen internalization, processing, and presentation, in part due to its interaction with the GTPase Dyn2 (150). Here I showed that the actin regulating protein Abp1 is recruited to the surface of the B-cell in a BCR dependent manner. Moreover, I showed that *Abp1*$^{-/-}$ B-cells displayed elevated levels of BCR triggered signaling, such as elevated pY, pBLNK, pMEK1/2, pERK, as well as increased levels of Cat^{2+} flux. The elevated level of signaling was concurrent with decreased recruitment and/or activation of the two known negative regulatory signaling molecules pSHIP1 and HPK1. Examination of the early actin-dependent events of B-cell activation revealed that *Abp1*$^{-/-}$ B-cells displayed enhanced spreading when activated with membrane-associated antigen, and enhanced

BCR clustering when activated with soluble or membrane-associated antigen, compared to wt B-cells. The *Abp1*$^{-/-}$ B-cells also exhibited defects in clearance of F-actin from the B-cell contact zone with membrane associated antigen, which was simultaneous with dysregulation in the recruitment/activation of the actin regulating proteins WASP and N-WASP. My work here also indicates a critical role for Abp1 in negative regulation of the development of MZ and B1a B-cells, as Abp1 deficient and *Abp1*$^{-/-}$-Ch mice show elevated numbers of MZ and B1a B cells. My results further show that Abp1 is involved in regulating the generation of spontaneous germinal center B-cells, as Abp1 deficient and *Abp1*$^{-/-}$-Ch mice have increased numbers of spontaneous germinal center B-cells, as well as greater numbers and size of germinal centers in the spleen of un-immunized mice. In agreement with this data, the Abp1 deficient mice generate an enhanced non-specific antibody response as well as increased T-cell independent antibody response, while they are simultaneously defective in mounting antigen specific responses towards T-cell dependent antigens or undergoing affinity maturation. Finally, the *Abp1*$^{-/-}$ and *Abp1*$^{-/-}$-Ch mice generate autoantibodies much earlier, and to much higher levels than control animals. Collectively, the data have led us to propose the following working model (Fig. 4.1), in which we suggest that Abp1 mediates its negative regulatory capacity by three major mechanisms: 1) by controlling B-cell spreading and subsequent contraction via modulation of the actin nucleating proteins WASP and N-WASP, 2) by recruitment and activation of the negative regulatory proteins HPK1 and SHIP1, and 3) finally by regulating BCR endocytosis (Fig. 4.1). The absence of this regulatory capacity results in *Abp1*$^{-/-}$ B-cells that display hyper-signaling profiles, and an inability to transition from the actively spreading to contracting phase normally associated with BCR signal attenuation,

and finally, an inability to clear F-actin and coalesce and internalize their actively signaling BCRs. We postulate that these defects cumulatively result in the generation of an inappropriate germinal center response, where the hyper-activated B-cells migrate to the secondary lymphoid follicles, where they expand in the absence of T-cell signals. Consequently, the expanded B-cell populations produce elevated levels of non-specific and self-reactive antibodies, while simultaneously displaying defective T-cell dependent antibodies or undergoing affinity maturation (both of which require T-cells help).

Work related to my second aim; examining the domains or functions of Dyn2 necessary for its appropriate recruitment, BCR internalization, and BCR mediated signal transduction, revealed that the PRD of Dyn2 is necessary for its recruitment and colocalization with the BCR at the B-cell surface. Examination of the transfected A20 cells at later times of BCR stimulated activation revealed that the GFP-DynK44A construct appeared to be predominantly localized to intracellular vesicles intimately associated with the plasma membrane. Additionally, the cells transfected with the GFP-DynY321/597F have a larger proportion of cells showing a somewhat smooth cell surface distribution or cells surface vesicle distribution. We suspect that these Dyn2 constructs are likely stuck on the necks of the endocytosing vesicles, unable to release from the membrane and return to a cytosolic distribution. Flow cytometry evaluation of the percent of BCR remaining at the surface of A20 B-cells transfected with the various Dyn2 constructs revealed that only the cells transfected with the GFP-Dyn ΔPRD construct had a significantly higher percentage of BCRs remaining at the cell surface after BCR stimulated activation. However, confocal microscopic examination followed by colocalization coefficient calculations in B-cell transfected with the various Dyn2

constructs, revealed that the PRD domain, as well as the GTPase function, and tyrosine phosphorylation sites all displayed reduced colocalization between the BCR and LAMP-1, suggesting that they do in fact have reduced BCR internalization to LAMP-1^{+} compartments after BCR crosslinking. As mentioned in chapter 3, we suspect that the flow cytometry evaluation of percent BCR remaining at the B-cell surface, may not be sensitive enough to detect the difference between fully endocytosed BCR's, and those stuck in partially endocytosed vesicles, unable to undergo the scission step necessary for vesicle release. Therefore, the B-cells transfected with the GFP-DynK44A and GFP-DynY231/597F may erroneously appear to have similar levels of endocytosed BCRs compared to control cells. The role of the GTPase domain in moderating BCR internalization after crosslinking was further supported by the experiment utilizing the small molecule inhibitor of Dyn2 (Dynasore), which revealed that inhibition of the GTPase activity of endogenous Dyn2 results in reduced BCR/LAMP-1, and therefore reduced BCR internalization. This study further confirmed that the PRD domain of Dyn2 is required for recruitment of Abp1 to the B-cell plasma membrane. Furthermore, I show that Dyn2 GTPase deficiency augments BCR clustering, but not B-cell spreading, and its GTPase function is involved in the regulation of BCR signal transduction. Collectivly, results from previous studies, and those presented here, lead us to propose a model in which Dyn2 is necessary for BCR trafficking to late endosomes, and acts as a negative regulatory element in BCR mediated signaling via its capacity to 1) regulate BCR endocytosis, 2) and via interaction with known signaling intermediates as well as adaptor molecules such as Abp1 (Fig.4.1).

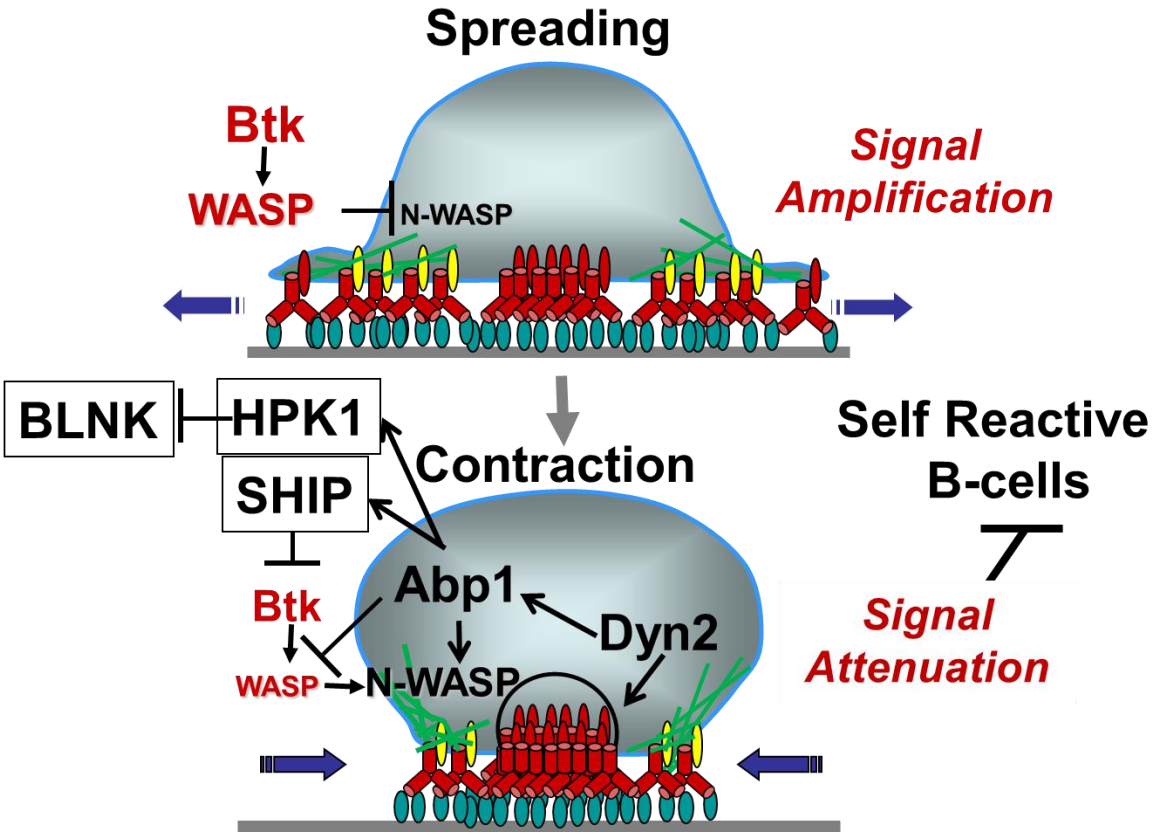


Fig. 4.1 Working model. Antigen binding to the BCR promotes activation of Btk, which induces recruitment and activation of WASP. WASP stimulates actin polymerization and suppresses activation of N-WASP. Actin polymerization drives B-cell spreading and facilitates BCR clustering and amplified signaling. Later recruitment of Abp1 results in recruitment and activation of HPK1, and SHIP-1. HPK1 drives signal attenuation by inducing phosphorylation and degradation of BLNK, while SHIP-1 inhibits Btk, and thus WASP activation, thereby inducing activation of N-WASP. Activated N-WASP promotes clearance of actin from the contact zone, which stimulates the contraction and coalescence of BCR microclusters into a central cluster. Dyn2 and Abp1 aid in subsequent BCR internalization all of which promotes BCR signal attenuation. Image from Dr. Wenxia Song

4.2 Future Work

4.2.1 Abp1 Project

While this research has revealed novel roles for Abp1 in B-cell regulation, there is much that remains to be examined. As both *Abp1*$^{-/-}$ and *Abp1*$^{-/-}$-Ch mice display elevated levels of autoAbs, characteristic of a B-cell intrinsic autoimmune phenotype, future work should include further characterization of the autoimmune phenotype and its disease presentation. As a result of elevated levels of autoAbs produced in many autoimmune diseases, immune complexes deposit in the glomeruli of the kidneys, causing kidney disorders (262-266). As such, our future work will include a pre-screen of wt, *Abp1*$^{-/-}$, wt-Ch, and *Abp1*$^{-/-}$-Ch mice for proteinuria and immune complex deposition in the glomeruli, which can indicate kidney damage (265, 266). If there is immune complex deposition in the glomeruli, we will use immunohistochemistry to examine the kidneys for the presence of mesangial thickening and hypercellularity, which are indicative of sclerotic glomerulonephritis, which may be caused by immune complex deposition (266, 267).

In 2006 Connert et al. reported that *Abp1*$^{-/-}$ mice displayed splenomegaly, lung emphysema, as well as four chamber dilation of the heart (230). This group also noticed that by 60 to 290 days after birth, a large proportion of the Abp1 deficient mice had partial paralysis of the hind limbs, ruffled fur, muscle trembling, convulsions, as well as reduced body weight (230). While several of these findings are likely linked to the reduced synaptic vesicle recycling observed in the *Abp1*$^{-/-}$ mice (230), many of these symptoms may also arise as a result of autoimmune related inflammation. Indeed,

autoimmunity has been linked to heart and lung disease, as well as overall poor health with resultant weight loss (268, 269). Similar to previous studies, we found that *Abp1*$^{-/-}$ mice display splenomegaly (Fig.2.5E), and reduced body weight compared to their wt counterparts ((195, 249), and data not shown). In the future we propose to determine if the enlarged lung and heart phenotypes have arisen as a consequence of autoimmunity within the *Abp1*$^{-/-}$ mice. We will weigh and examine the histology of lung and heart tissues from the wt, *Abp1*$^{-/-}$, wt-Ch, and *Abp1*$^{-/-}$-Ch mice to determine if there are internal tissue/structural abnormalities including mononuclear or polynuclear cell infiltration, heart chamber dilation or fibrosis, as well as lung emphysema. To more specifically examine whether the observed tissue enlargement observed in the *Abp1*$^{-/-}$ animals was due in part to autoimmunity, we will assay wt, *Abp1*$^{-/-}$, wt-Ch, and *Abp1*$^{-/-}$-Ch mice for the presence of anti-cardiac myosin heavy chain and anti-Type-IV collagen/decorin/or elastin autoAbs by ELISA (270, 271), and for general immune complex deposition within the heart and lung tissues by immunofluorescence. Furthermore, the frequency of plasma cells that secret heart or lung specific autoAbs could be assessed by ELISPOT using our previously published protocol (239). In addition, we have backcrossed our *Abp1*$^{-/-}$ genotype onto a Balb/c background through greater than six generations, and future work will investigate possible development of autoimmunity and related disease state within this breed.

As the spontaneous development of MZ, B1a, and GC B-cells, as well as autoAb production is likely the result of dysregulated negative BCR signing in the absence of Abp1, a better understanding of the role of Abp1 in this negative regulation is necessary. The data presented in chapter two demonstrate that Abp1 is necessary for the recruitment

and/or activation of the negative regulatory proteins HPK1, and SHIP1, however, it remains unknown how Abp1 influences these processes. Abp1 has no enzymatic function, and therefore is not proposed to directly activate either HPK1 or SHIP-1. As mentioned, Abp1 has been shown to directly bind to HPK1 in T-cells, where it is necessary for recruitment of HPK1 to the T-cell IS (232). Therefore we hypothesize that Abp1 also directly binds HPK1 in B-cells, via their SH3 and PRD respectively, which we can experimentally test by co-immunoprecipitation (co-IP) or by a Duolink assay (272). The requirement of Abp1 protein expression for the recruitment of activated pSHIP-1 to the B-cell IS is a novel finding of this study, however, how Abp1 acts to regulate pSHIP-1 localization remains to be elucidated. Abp1 could possibly bind pSHIP-1 directly via their SH3 and PRD respectively or possibly through adaptor proteins, such as the scaffolding protein Grb2 (known to bind HPK1 and SHIP-1) (273, 274). These possible interactions can also be tested with co-IP, and/or Duolink assays.

Previously published studies from our lab showed that N-WASP was also involved in the recruitment of activated pSHIP-1(54), and we show here that Abp1 facilitates the recruitment of activated N-WASP (pN-WASP) (249) to BCR signalosomes. As Abp1 has been shown to directly bind to N-WASP in neurons (194), it is also a possibility that Abp1 regulates pSHIP-1 recruitment via forming a complex with N-WASP bound Grb2 (275). While positively regulating N-WASP, Abp1 appears to selectively inhibit the activation of hematopoietic specific WASP, suggesting that Abp1 regulates both WASP and N-WASP, but in different manners. As such, it will be important to further characterize not only the link between Abp1 and pSHIP-1, but also Abp1 and WASP and N-WASP. It is a possibility that Abp1 may differentially interact

with WASP and N-WASP or prefer interacting with one of the two. These complicated interactions could again be tested by co-IP, or Duolink assay, as well as colocalization studies utilizing confocal microscopy or TIRF, in combination with various knockout mice.

4.2.2 Dynamin Project

While the research outlined in chapter three defines a novel role for the different domains or functions of Dyn2 in BCR signal transduction and subsequent BCR trafficking to late endosomes, how precisely the domains or functions moderate these outcomes remains to be fully explored. First, how Dyn2 is recruited to the plasma membrane has not been fully resolved. Here I show that the truncated form of Dyn2 missing its PRD is not recruited to the plasma membrane upon BCR crosslinking, as such, it is likely that a SH3 domain containing protein is necessary for Dyn2 recruitment upon BCR activation. To identify the protein that is responsible for recruiting Dyn2 to BCR-containing clathrin coated pits, possible studies include confocal microscopic examination of cells over expressing SH3 domains from known Dyn2 interacting proteins to determine if the over expression inhibits Dyn2 recruitment to the plasma membrane.

The question of whether Dyn2 phosphorylation is required for endocytosis is somewhat contested (215-217). Previously our lab showed that treatment with the Src kinase inhibitor PP2 resulted in defective Dyn2 recruitment to the plasma membrane upon BCR crosslinking (Bruce K. Brown PhD., unpublished data). Here I show that the Y231/597F phosphorylation site mutations do not prevent Dyn2 from being recruited to the plasma membrane, however, the expression of the mutated Dyn2 does inhibit BCR

internalization. These data implicate the possibility that Dyn2 is phosphorylated after recruitment to signaling active BCRs, which may be required for interaction with a SH2 domain containing protein, and that this interaction is crucial for BCR internalization. To test this theory, future studies might include an electron microscopic analysis of cells transfected with GFP-Dyn vs GFP-DynY231/597F to examine 1) the recruitment of transfected Dyn2 to the plasma membrane, and 2) the internalization of gold labeled BCRs, to better understand the stage in endocytosis in which the phosphorylation mutant is stalled. Furthermore, to identify proteins interacting with phosphorylated Dyn2, we can compare proteins co-immuneprecipitated with GFP-Dyn vs GFP-DynY231/597F, in an attempt to probe membranes for known SH2 domain molecules. Possible candidates include Grb2, BLNK, and PLCγ2.

While multiple studies have shown that the large GTPase Dyn2 acts as a "pinchase" to aid in scission of nascent endocytosing vesicle from the plasma membrane (206, 209, 210), how each domain of Dyn2 specifically contributes to BCR endocytosis remains to be fully outlined. Liu et al. recently showed that while GTPase defective Dyn2 can still form collars around artificial lipid membranes, they are defective in releasing buds from the membrane (206). Here I showed by confocal microscopy that the PRD, GTPase function, and tyrosine phosphorylation of Dyn2 are necessary for BCR/LAMP-1 colocalization after 30 min of B-cell activation. However, my flow cytometry assay showed that there were similar levels of biotinylated BCRs left at the cell surface after BCR internalization in B-cells expressing the GTPase and phosphorylation mutants of Dyn2 compared to control B-cells. Thus we suspect that these mutants are recruited normally and form oligomers to constrict the necks of the

nascent vesicles, however, while these vesicles fail to be released from the plasma membrane, the membrane constriction acts to prevent PE-streptavidin from reaching the surface labeled BCRs. To examine this possibility, future studies will include immunoelectron microscopy examination of BCR internalization in B-cells transfected with the Dyn2 mutants. We could also use cryoEM to examine localization of Dyn2 mutants stained with a gold labeled antibody towards Dyn2. Observing the gold-labeled Dyn2 will allow determination of whether the molecule is being mislocalized or recruited to the plasma membrane, but possibly stuck at the membrane, or on the necks of endocytosing vesicles. Observing the gold labeled BCRs will allow a much higher resolution view of the specific stage in which the Dyn2 mutants act to stall BCR trafficking to late endosomal compartments.

As mentioned, Dyn2 has been implicated in signal transduction in multiple cells types, including B-cells, where Dyn2 has been shown to form a complex with Vav and Grb2 via the adaptor molecule LAB (218, 219). Here we show that Dyn2 is required for appropriate BCR clustering kinetics, and our lab has previously shown that F-actin dependent BCR clustering is intrinsically tied to regulation of BCR signaling (54, 170, 181, 249). Furthermore, we further confirm here that Dyn2 is required for recruitment of the actin modulating protein Abp1, which is also known to aid in BCR signal attenuation. Finally, we did preliminary experiments with B-cells transfected with GFP-Dyn and GFP-DynK44A, and discovered that activation induced levels of pY and the MAPK pJNK was increased in the cells transfected with the GTPase dead mutant. As we speculate that the Dyn2 mutations alter BCR transduced signaling, because they influence the location of the BCR (by blocking the BCRs in clathrin-coated pits), we will further

examine the activation of major proximal and distal signaling molecules in BCR signaling pathway by TIRF and flow cytometry in cells transfected with GFP-DynPRD, GFP-Dyn K44A, and GFP-DynY231/597F constructs.

In total, that data from this dissertation provides insight into novel regulatory mechanisms within B-cells necessary for activation, survival, and humoral immune responses. Collectively, a better understating of the mechanisms by which B-cells signal, internalize their antigen receptors for antigen processing and presentation, and how these processes are related will shed light on regulatory mechanisms which might be manipulated to either enhance humoral immune responses, or dampen B-cell mediated autoimmune responses.

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