

ABSTRACT

Title of Thesis: MECHANISMS BY WHICH THE ACTIN
CYTOSKELETON SWITCHES B CELL
RECEPTOR SIGNALING FROM THE
ACTIVATION TO THE ATTENUATION MODE

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Molecular Genetics

The B cell-mediated humoral immune response is critical in fighting off invading pathogens and potentially harmful foreign substances. B cells detect antigens through the B cell receptor (BCR). The binding of cognate antigen to the BCR induces a signaling response, a critical initiation and regulatory step for B cell activation and differentiation. The actin cytoskeleton has been shown to play essential roles in BCR signaling. When encountering membrane-associated antigens, actin amplifies signaling by driving B cell spreading and BCR clustering, while promoting signal attenuation by causing B cell contraction. This signal attenuation is essential for curtailing the activation of autoreactive B cells. However, the mechanism by which the actin cytoskeleton switches BCR signaling from amplification to attenuation was unknown. My thesis research examined the mechanisms by which actin reorganization transitions B cells from spreading to contracting and B cell contraction switches BCR signaling from amplification to attenuation, using mouse splenic B cells, a functionalized planar lipid bilayer system, and total internal reflection fluorescence microscopy. Our results show that branched actin polymerized by Arp2/3 is required for B cell transition from spreading to contraction after driving B cell spreading. Ubiquitously expressed Neuronal Wiskott-Aldrich Syndrome Protein (N-WASP), but not the haematopoietically specific WASP, activates the branched actin polymerization and generates inner actin foci from

lamellipodial actin networks, by sustaining their lifetime and centripetal movement. N-WASP-dependent inner actin foci are necessary for recruiting non-muscle myosin II, creating an actomyosin ring-like structure at the periphery of the membrane contact region to drive B cell contraction. B cell contraction primarily increases the BCR molecular density in individual BCR-antigen clusters, measured by the peak fluorescence intensity. Inhibition of B cell contraction by Arp2/3 inhibitor and B cell-specific N-WASP knockout (cNKO) reduced the increasing rates of BCR molecular density. Increased molecular density caused by B cell contraction leads to decreases in the levels of phosphorylated BCR, the stimulatory kinase Syk, the inhibitory phosphatase SHIP-1, and their phosphorylated forms in individual BCR clusters. However, the levels of total Syk and SHIP-1 have a different relationship with BCR density in individual clusters: Syk does not decrease until a high threshold of BCR density, which can be achieved only by contracting B cells, but SHIP-1 consistently reduces with the increase in BCR molecular density. Inhibiting B cell contraction by cNKO reduces the molecular density of BCR clusters but does not affect the relationship of the Syk and SHIP-1 levels with BCR molecular density in clusters. Taken together, our results suggest that the actin cytoskeleton reorganizes from the lamellipodial branched actin networks to centripetally moving actin foci, enabling actomyosin ring-like structure formation, through N-WASP-activated Arp2/3. Actomyosin-mediated B cell contraction attenuates BCR signaling by increasing receptor molecular density in individual BCR clusters, which causes the dissociation of both stimulatory and inhibitory signaling molecules. My thesis research results reveal a novel negative regulatory mechanism for BCR signaling, an essential checkpoint for generating pathogen-specific and suppressing self-reactive antibody responses.

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SIGNALING FROM THE ACTIVATION TO THE ATTENUATION MODE

by

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Dedication

I dedicate this thesis to my family and friends for their guidance and continued support. My parents, Debashis and Sumana Bhanja, for being supportive and patient through every step of my life and career, and for always being there whenever I needed them. My grandparents Dr. Pradyot and Dr. Arati Bhanja, for inspiring me to pursue research, and my other grandparents Subhash and Bela Khashnobish, for instilling in me a love for adventure and art. Last, but not least, my friends, for their encouragement and camaraderie.

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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 The role of B cells in immune responses.....	1
1.1.1 Innate Immunity.....	1
1.1.2 Adaptive Immunity	2
1.2 B cell effector functions	4
1.2.1 Antigen-induced B cell activation.....	4
1.2.2 Immunoglobulins and their function.....	5
1.3 B cell development and activation.....	8
1.3.1 Early B cell development	8
1.3.2 B cell affinity maturation in the germinal center	10
1.3.3 The effects of mis-regulation of B cell activation.....	14
1.4 BCR signaling	17
1.4.1 BCR signaling in response to antigen	17
1.4.2 BCR signaling initiates actin cytoskeletal rearrangement.....	21
1.5 The role of actin in regulating BCR-mediated B cell activation	25
1.5.1 Initiation of BCR signaling	25
1.5.2 BCR signaling amplification.....	29
1.5.2 BCR signaling down regulation	32
1.5.2 Antigen uptake	34
1.6 How the properties of antigen-presenting surfaces affect B cell activation	36
1.6.1 Chemical and physical properties of antigen-presenting surfaces affect BCR and actin dynamics in B cells.....	36
1.7 Rationale and Aims	38
1.7.1 Aim 1: To determine how B cells transition from spreading to contracting.....	39
1.7.2 Aim 2: To determine how B cell contraction drives the transition of BCR signaling from amplification to attenuation.....	40
Chapter 2: N-WASP-dependent branched actin polymerization promotes B cell receptor signaling attenuation by increasing the molecular density of receptor clusters.....	42
2.1 Abstract.....	42
2.2 Introduction	44
2.3 Results	49
2.3.1 Arp2/3, activated by N-WASP but not WASP, is required for B cell contraction	49
2.3.2 Arp2/3, downstream of N-WASP, generates inner F-actin foci, driving B cell contraction.....	53
2.3.3 Inner F-actin foci are derived from lamellipodia behind the spreading membrane	57
2.3.4 N-WASP-activated Arp2/3 generates inner actin foci by sustaining the lifetime and the centripetal movement of lamellipodia actin	60
2.3.5 Inner actin foci, generated by N-WASP-activated Arp2/3, facilitate NMII recruitment and reorganization into ring-like structures	63
2.3.6 B cell contraction increases the BCR molecular density in individual clusters	66

2.3.7 Increased BCR molecular density by B cell contraction reduces BCR phosphorylation levels in individual microclusters	70
2.3.8 Increases in BCR molecular density by B cell contraction promote the disassociation of the stimulatory kinase Syk from BCR microclusters	74
2.3.9 Increases in BCR molecular density by B cell contraction also promote the disassociation of the inhibitory phosphatase SHIP-1 from BCR microclusters.....	80
2.4 Discussion.....	85
2.5 Materials and Methods	92
2.5.1 Key Reagents Table	92
2.5.2 Mice and B cell isolation.....	93
2.5.3 Pseudo-antigen-coated planar lipid bilayers (PLBs)	94
2.5.4 Total internal fluorescence (TIRF) microscopy	95
2.5.5 Inhibitors	96
2.5.6 Analysis of the actin cytoskeleton.....	96
2.5.7 Analysis of BCR-Fab' clusters.....	97
2.5.8 BCR signaling	98
2.5.9 Statistical analysis	99
2.5.10 MATLAB scripts.....	99
Chapter 3: Discussion	100
3.1 General summary.....	100
3.2 Working model.....	109
3.3 Limitations of the study and future directions.....	113
Supplementary figures and videos	117
Supplementary Figure S1	117
Supplementary Figure S2	119
Supplementary Figure S3	121
Supplementary Figure S4	123
Supplementary Figure S5	125
Supplementary Figure S6	127
Video Legends.....	129
Appendices.....	132
Bibliography	167

List of Figures

Fig. 1.1	Antibody structure and its isotypes
Fig. 1.2	The germinal center response
Fig. 1.3	BCR clustering induced by multivalent antigen initiates BCR signaling
Fig. 1.4	BCR engagement by multivalent antigen activates actin remodeling in B cells
Fig. 1.5	Actin influences BCR signaling at multiple stages of early B cell activation by using distinct mechanisms
Fig. 2.1	Arp2/3, activated by N-WASP but not WASP, is required for B cell contraction
Fig. 2.2	Arp2/3, downstream of N-WASP, generates inner F-actin foci, driving B cell contraction
Fig. 2.3	Inner F-actin foci are originated from lamellipodia behind the spreading membrane
Fig. 2.4	N-WASP-activated Arp2/3 sustains the lifetime and the centripetal movement of inner F-actin foci
Fig. 2.5	Inner F-actin foci, generated by N-WASP-activated Arp2/3, facilitate NMII recruitment and ring-like structure formation
Fig. 2.6	B cell contraction increases the molecular density within BCR clusters
Fig. 2.7	Increased molecular density in BCR clusters leads to reductions in BCR phosphorylation
Fig. 2.8	The effects of BCR-Fab' density on the association of Syk with BCR-Fab' clusters and its phosphorylation

Fig. 2.9	The effects of BCR-Fab' density on the association of SHIP-1 with BCR-Fab' clusters and its phosphorylation
Fig. 3.1	Working Model
Sup. Fig. S1	B cells spread for around 1 minute, which is followed by contraction for around 1.5 minutes, on Fab'-coated-planar lipid bilayers
Sup. Fig. S2	Treatment with CK-666 significantly decreases Arp2/3 recruitment within two minutes of its addition
Sup. Fig. S3	Perturbation of Arp2/3 with CK-666 before maximal B cell spreading decreased the spreading kinetics, but not when CK-666 was introduced after maximal spreading
Sup. Fig. S4	Wiskostatin treatment inhibits N-WASP activation while enhancing WASP activation in B cells
Sup. Fig. S5	Inner F-actin foci are originated from lamellipodia behind the spreading membrane
Sup. Fig. S6	AF546-Fab' clusters, tracked and analyzed over time using a custom MATLAB code, indicates that B cell contraction increases the molecular density within BCR clusters

List of Abbreviations

Abp1	actin-binding protein 1
Ag	antigen
APC	antigen presenting cell
Arp2/3	actin related protein complex
BCAP	B cell adaptor for phosphatidylinositol 3-kinase
BCR	B cell receptor
BLNK	B cell linker
Btk	Bruton's tyrosine kinase
C3d	complement component 3d
CaM	calmodulin
Cbl	Casitas B-lineage lymphoma
Cdc42	cell division control protein 42 homolog
C _H	constant region of heavy chain
C _L	constant region of light chain
CSR	class switching recombination
CTL	cytotoxic T lymphocyte
DAG	diacyl glycerol
DAMP	damage-associated molecular pattern
DC	dendritic cell
DEL	duck egg lysozyme
D _H	diversity region of heavy chain
Dok-3	docking protein 3
dSTORM	direct stochastic optical reconstruction microscopy

ERM	ezrin/radixin/Moesin
F _{ab}	fragment, antigen-binding
F _c	fragment, crystallizable
FcγR	Fc-gamma-receptor
FDC	follicular dendritic cell
FI	fluorescence intensity
FRET	fluorescence resonance energy transfer
GC	germinal center
GEF	guanidine exchange factor
GFP	green fluorescence protein
Grb2	growth factor receptor-bound protein 2
GTP	guanidine triphosphate
HEL	hen egg lysozyme
HPK1	hematopoietic progenitor kinase 1
HSC	hematopoietic stem cell
Ig	immunoglobulin
IL	interleukin
IP ₃	inositol triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
J _H	joining region of heavy chain
J _L	joining region of light chain
LIMK	LIM domain kinase
MAP	mitogen activated protein
MFI	mean fluorescence intensity
MHC-I	major histocompatibility complex I

MHC-II	major histocompatibility complex II
mIg	membrane-bound immunoglobulin
MLCK	myosin light chain kinase
MZ	marginal zone
NF- κ B	nuclear factor κ B
NLR	nod-like receptor
NMII	non-muscle myosin II
NPF	nucleation promoting factor
N-WASP	neuronal Wiskott-Aldrich syndrome protein
PAMP	pathogen-associated molecular patterns
PI(4,5)P ₂	phosphatidylinositol-4,5-bisphosphate
PI3K	phosphatidylinositol 3-kinase
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PIP ₃	phosphatidylinositol-3,4,5-triphosphate
PLB	planar lipid bilayer
PLC γ 2	phospholipase C- γ 2
PMS	plasma membrane sheet
PRR	pattern recognition receptor
pWASP	phosphorylated neuronal Wiskott-Aldrich syndrome protein
pSHIP-1	phosphorylated SHIP-1
pSyk	phosphorylated Syk
pN-WASP	phosphorylated Wiskott-Aldrich syndrome protein
PTEN	phosphatase and tensin homolog
pY	phosphorylated tyrosine
Rag	recombination-activating gene
RFP	red fluorescence protein

Rho	ras homology family
RhoA	ras homology family member A
ROCK	Rho-associated protein kinase
SCID	severe combined immuno-deficiency
SH2	Src homology 2
SHIP-1	Src homology 2 domain-containing inositol 5-phosphatase-1
SHP-1	Src homology 2 domain tyrosine phosphatase-1
SIM	structured illumination microscopy
SLC	surrogate light chain
SLE	systemic lupus erythematosus
SMIFH2	small molecule inhibitor of formin homology-2 domain
SPT	single particle tracking
STED	stimulated emission depletion
STICS	spatiotemporal image correlation spectroscopy
Syk	spleen tyrosine kinase
TCR	T cell receptor
T _{cyt} cell	cytotoxic T cell
TIRF	total internal reflection fluorescence
Tf	transferrin
T _{fh}	T follicular helper cell
T _H cell	helper T cell
TLR	toll-like receptor
V _H	variable region of heavy chain
V _L	variable region of light chain
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein

WAVE WASP and WASP-family verprolin-homologous protein.

Chapter 1: Introduction

1.1 The role of B cells in immune responses

1.1.1 Innate Immunity

The immune system is responsible for protecting the human body against foreign substances by identifying and neutralizing pathogens and toxins. The human immune system consists of two branches: innate immunity and adaptive immunity. The innate immune system serves as the first line of defense against invading pathogens and is a faster response with broad specificity as compared to the adaptive immune system. The innate immune system protects us by recognizing molecules commonly expressed by pathogens, enabling it to detect and eliminate a wide variety of pathogens. The innate immune response is activated through pattern recognition receptors (PRRs) like toll-like receptors (TLRs), nod-like receptors (NLRs), and retinoic acid-inducible gene (RIG-1) like receptors, which can bind pathogen associated molecular patterns (PAMPs), like flagella, lipopolysaccharide (LPS), and nucleic acid variants like dsRNA, which are typically found associated with pathogens¹. The innate immune system can also detect damage associated molecular patterns (DAMPs) which are found on damaged host cells^{2,3}. The innate immune system also includes the complement pathway, which can be activated either through antibodies bound to pathogens, or directly through PAMPs like mannose expressed on the surface of pathogens⁴. Although the innate immune response does not target specific pathogens and does not provide long-lasting protection against them, it serves to initiate the adaptive immune response. For example, pathogens phagocytosed by professional antigen-presenting cells (APCs) like macrophages are degraded in phago-lysosomes and their fragments are presented as complexes with major histocompatibility complex-II (MHC-II) molecules expressed exclusively on professional

APCs, to activate immune cells of the adaptive immune system. The innate immune system also communicates with the adaptive immune system through cytokine secretion^{3,5,6}.

1.1.2 Adaptive Immunity

The adaptive immune system provides highly specific and long-lasting protection against a wide variety of pathogens^{3,7,8}. It can be further divided into two sub-branches: cell-mediated and humoral immunity⁹. Cell mediated immunity is mainly provided by T lymphocytes (or T cells), including CD4⁺ T cells (T helper or T_h cells) and CD8⁺ T cells (cytotoxic T or T_{cyt} cells or CTLs). T helper cells are activated by antigen (Ag) presented on MHC-II receptors expressed on the professional APCs: macrophages, dendritic cells and B cells. APCs identify and internalize pathogens, degrade them, and present their fragments complexed with MHC-II to T_h cells. The T_h cells are activated by recognizing Ag-MHC-II complexes using their T cell receptors (TCRs). Activated T_h cells secrete cytokines and growth factors, providing stimulating signals to the APC^{3,10-13}. CTLs are activated by Ag-MHC-I complexes. MHC-I molecules are expressed by all cells and is responsible for presenting Ag from intracellular pathogens like viruses. Mis-expressed proteins associated with cancerous cells can also be presented on MHC-I, which activate CTLs. Upon activation, CTLs release cytotoxic proteins such as perforin and granzymes, consequently killing its target cell^{14,15}. Both CD4⁺ and CD8⁺ T cells are able to differentiate into memory T cells, which provide long-lasting T cell-mediated protection during the next exposure to the same Ag^{16,17}.

Humoral immunity is mainly provided through the secretion of Ag-specific antibodies (also called immunoglobulins, Ig) by plasma cells, which are differentiated from B cells^{11,18}.

Antibodies serve two major roles: neutralization and opsonization. Antibodies that are specific to Ag, like endotoxins released by bacteria or associated the bacterial surface. The binding neutralizes pathogens and pathogen-associated Ag by preventing them from attacking

host cells. Antibodies that are specific to Ag expressed on the surface of pathogens can bind to them, which is also called opsonization, effectively “tagging” them. This makes it easier for phagocytes to eliminate pathogens by recognizing the antibodies bound to them.

Additionally, antibody-opsonized pathogens can also activate the complement cascade through the classical pathway, which subsequently kills the pathogen by generating pores in its cell membrane. In addition to plasma cells, B cells can also differentiate into long-lived memory B cells, which allow the immune system to retain information about past infections, and provide long-lasting antibody-mediated protection against invading pathogens.

1.2 B cell effector functions

1.2.1 Antigen-induced B cell activation

The research in this dissertation will focus on the role of B cells in the humoral immune response. B cells patrol the circulatory and lymphatic systems, particularly the secondary lymphoid organs like lymph nodes and the spleen. Here, B cells survey Ags drained into the secondary lymphoid organs and collected and presented by APCs like macrophages and dendritic cells. APCs can significantly increase the likelihood of Ag-specific B cell activation by concentrating Ag and providing co-stimulatory signals¹⁹.

B cells detect Ag by the B cell receptor (BCR) expressed on their surface. The BCR consists of a membrane-associated Ig (mIg), which is non-covalently associated with a Ig α and Ig β (also called CD79a and CD79b respectively) heterodimer^{20–24}. Membrane Ig is responsible for detecting Ag. CD79a and CD79b have intracellular signaling domains, which relay signals in response to external Ag to the B cell cytoplasm and nucleus²⁵. Ag-induced BCR signaling primes the B cell to internalize, process, and present Ag on MHC-II for T_h cell recognition¹¹. Upon recognition of Ag presented by B cells, T_h cells are activated to provide a secondary signal to the B cell for activation and differentiation^{13,26}. The T cell help activated by B cell antigen presentation is essential for B cells to differentiate into long-lived antibody-secreting plasma cells and memory B cells. Antibody-secreting cells secrete Ag-specific Igs, while Ag-specific memory B cells enter into circulation, and will be activated in response to the next exposure to the same Ag. Activated B cells enter into germinal centers reactions in secondary lymphoid organs, where they undergo affinity maturation through somatic hyper-mutation, and class-switching recombination to increase the affinity of their mIgs to the specific Ag^{7,8,11}. This allows B cells to provide Ag-specific, high affinity, and long-lasting protection against invading pathogens in the form of secreted Igs.

1.2.2 Immunoglobulins and their function

The primary function of B cells is to generate Ag-specific antibodies. Antibodies function by binding their cognate Ag (the Ag that the antibodies are specific to), which can neutralize pathogens and attract other immune cells and complement to clear pathogens

The antibody molecule is a Y-shaped glycoprotein complex consisting of two identical heavy chains and two identical light chains, linked by disulfide bonds^{20,21,27,28} (Fig. 1.1). Each light chain consists of two Ig domains, while each heavy chain consists of four to five Ig domains (depending on the isotype). The N-terminus of the heavy and light chains form the variable regions, which bind Ag, while the remainder of the heavy and light chains together make up the constant region of the Ig. Random rearrangement of the genes encoding the variable regions ensures that individual B cells have unique binding sites on their BCR and subsequently on their secreted Ig. This process of random rearrangement of Ig-encoding genes is called VDJ recombination, which occurs during B cell maturation in the bone marrow^{29,30}. VDJ recombination depends on recombination activating genes (RAG1 and RAG2), which mediate the random recombination of the variable (V), diversity (D), and joining (J) gene segments of the Ig heavy chain, and the V and J gene segments of the Ig light chain. This randomized variable region on the mIgs in different B cells provides a wide range of Ag-specificity for different antibodies produced by the entire population of B cells in the immune system, known as the B cell repertoire. Each antibody molecule has two identical Fab regions, consisting of two pairs of the heavy and the light chains and forms the two arms of the Y-shaped antibody molecule. The rest of the antibody molecules contains several crystallizable regions (Fc region) of the two heavy chains. Each of the two Fab regions has a variable region, which binds its cognate Ag at a specific site known as the epitope. The Fc region is used to interact with complement proteins and Fc receptors expressed on the surface

of various immune cells. The isotype of the antibody is determined by the heavy chains and their Fc regions and can be of five types: IgG (γ), IgM (μ), IgA (α), IgD (δ), or IgE (ϵ).

All the five Ig isotypes are expressed as membrane forms on the surface of B cells. Naïve (Ag-inexperienced) B cells express mIgM and mIgD BCR. Activation by Ag and T_h cells can induce class switching recombination (CSR) to change the expression of mIgM/mIgD to mIgG, mIgE, or mIgA. Isotype switch B cells are the source of plasma cells secreting antibody with various isoform. However, only four isotypes, IgM, IgG, IgE, and IgA, are expressed as secreted forms. The four different isotypes of Ig have different effector functions within the immune system, through isoform-specific Fc receptors expressed by different immune cells. For example, IgG binds to a family of Fc γ receptors (Fc γ R)³¹. Binding of IgG opsonized bacteria to Fc γ RI expressed on macrophages enhances their phagocytotic activity³² and to Fc γ RIIB on the B cell surface inhibits B cell activation^{33,34}. The most abundant secreted antibody is IgG. IgM, secreted as pentamer, and IgA, as a dimer, are essential for the mucosal immunity. IgE is involved in allergic responses and activates allergic reactions by binding to the Fc ϵ R on basophils, mast cells and eosinophils. Thus, B cells play a wide variety of roles in the immune response by secreting antibodies. B cells differentiate into isotype switched long-lived memory B cells and plasma cells is essential for maintaining antibody levels in the circulatory and lymphatic system³¹ and mount a rapid and robust recall response. The generation of long-lived memory B cells and plasma cells is the fundamental basis of vaccines, which are the most effective preventatives against infectious diseases.

Figure 1.1

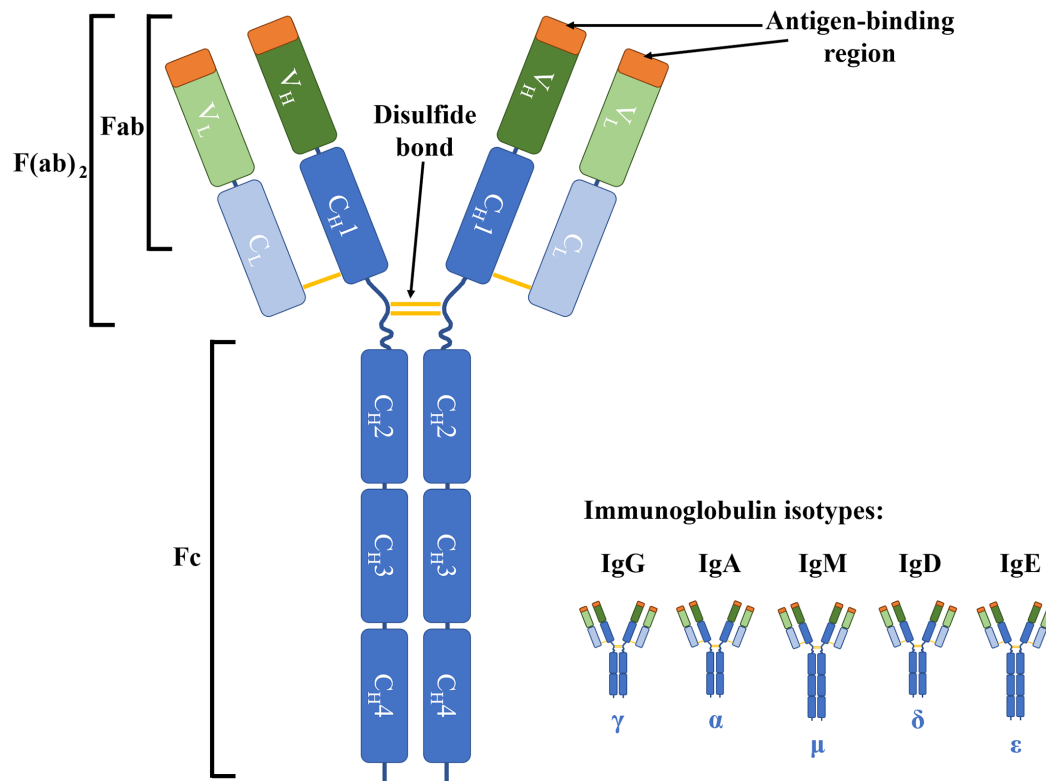


Figure 1.1: Antibody structure and its isotypes. Antibodies are made up of two identical heavy chains and two identical light chains linked by disulfide bonds. Each heavy and light chain consists of a variable region at their N-terminus, while the rest of the chain is constant. Each light chain consists of two Ig domains, while each heavy chain consists of four to five Ig domains (depending on the isotype), with the Ig domain closest to the N-terminus being called the variable region. The Fc region can bind Fc receptors, while the Fab regions bind antigen. The Fab and Fc region are connected by a hinge region that imparts flexibility to the antibody molecule.

1.3 B cell development and activation

1.3.1 Early B cell development

B cells originate from hematopoietic stem cells (HSCs) in the bone marrow. B cells undergo multiple developmental stages involving gene rearrangement and expressing the BCR to mature into B cells. As the bone marrow is sterile, B cells that just mature are called naïve B cells. During their development in the bone marrow, B cell clones that express self-reactive BCR are eliminated to establish self-tolerance. HSCs differentiate into lymphoid progenitor cells, which then differentiate into pro-B cells, which express CD45R/B220 and the pro-BCR consisting of calnexin and CD79a/CD79b before VDJ recombination of Ig genes³⁵. IL-7 secretion by bone marrow stromal cells induces Rag1/2-mediated rearrangement of Ig heavy chain genes in pro-B cells²⁹. The diversity region of heavy chain (D_H) and joining region of heavy chain (J_H) recombine first, followed by the addition of the variable region of heavy chain (V_H). The translated Ig heavy chain is joined by a surrogate light chain (SLC) during the pre-B cell stage, to make up the pre-BCR³⁵, leading to the differentiation of pre-B cells. Humans and mice with Rag1/2 deficiency causes severe combined immunodeficiency disease (SCID), failing to produce mature B cells^{35,36}. Furthermore, introduction of genetically rearranged Ig molecules induced the differentiation of mature B cells in SCID mice³⁷. Successful assembly of the pre-BCR, consisting of genetically rearranged Ig heavy chains, SLCs and $Ig\alpha/\beta$, is essential for providing pre-B cells with an activation signal, for inducing gene rearrangement of Ig light chain and differentiate into immature B cells that express the BCR with rearranged Ig heavy chains and light chains. In the absence of pre-BCR signal, cells undergo apoptosis and are eliminated. After expressing the complete BCR, immature B cells that have high affinity to self-Ag are eliminated through apoptosis, while those having low affinity to self-Ag can be rescued by Ig gene editing to modify their Ag-specificity^{35,38}. After this negative selection step, B cells leave the bone marrow and enter secondary

lymphoid organs for further development into transitional 1 (T1, IgD^{hi}/IgM^{low}), transitional 2 (T2, IgD^{hi}/IgM^{hi}), marginal zone (MZ) B cells and mature follicular B cells, forming B cell follicles.

Mature, Ag-inexperienced B cells survive and survey the secondary lymphoid organs, such as spleen and lymph nodes, and the circulatory system, looking for Ag. Follicular B cells make up the majority of B cells found in lymph nodes and the spleen, while MZ B cells are found in the marginal zones of B cell follicles. MZ B cells primarily respond to T cell-independent Ag, such as LPS, through their BCRs, and respond rapidly³⁹, leading to the generation of short-lived, relatively low affinity IgM-secreting cells. Follicular B cells can be activated by both soluble Ag and Ag presented by professional APCs. The differentiation of follicular B cells into high affinity memory B cells and plasma cells require a secondary stimulation signal from T_h cells. Only polypeptides or polypeptide containing molecules can be loaded onto MHC-II to be presented to T_h cells. B cells capture Ag through the BCR for processing and presentation^{26,40}. T cells recognize Ag-MHC-II complexes through their TCRs and are activated upon interacting with Ag-presenting B cells. Besides the TCR engaging Ag-MHC-II complexes, B cells also express the co-stimulatory receptors CD40 and B7-1, which interact with CD40L and CD28 respectively, expressed on T cells^{41,42}. Integrin ligands such as intracellular adhesion molecules (ICAM1 and ICAM2) expressed on B cells bind integrins like lymphocyte function antigen 1 (LFA-1) expressed on T cells, promoting B-T cell interaction^{43,44}.

1.3.2 B cell affinity maturation in the germinal center

B cell affinity maturation is a process by which mature B cells activated in response to an Ag increase the affinity of their BCR to the same Ag, consequently increasing the affinity of their secreted Igs to the same Ag. This is essential for generating antibodies that have high Ag-specificity and Ag-binding affinity, and effective humoral responses. In order to accomplish this, B cells undergo multiple rounds of somatic hypermutation and antigen-based selection, to select B cell clones that express a BCR with high specificity and affinity to the Ag for clonal expansion and differentiation. The process of affinity maturation occurs in germinal centers and is called the germinal center response.

The germinal center (GC) is a specially organized cellular environment (Fig. 1.2) found in secondary lymphoid organs⁴⁵⁻⁴⁷. GCs are generated several days following exposure to Ag, reach a peak after one to two weeks, and can last for several weeks⁴⁵. GC reactions are initiated upon antigen stimulation of follicular B cells in the secondary lymphoid organs. Activated B cells rapidly proliferate while undergoing somatic hypermutation (SHM) in the variable regions of their Ig genes^{45,46}. Proliferating B cells form the dark zone (DZ) of GCs due to the high cell density⁴⁸. Guided by the chemokine CXCL13, germinal center B (GCB) cells migrate to the light zone of GCs, where follicular dendritic cells (FDCs) and follicular T helper (T_{fh}) cells are located⁴⁹. FDCs capture Ag drained to the secondary lymphoid organs and display Ag on their surface for B cell recognition^{7,40,46,50}. Only B cells expressing BCRs that have high binding affinity to Ag are competitive enough to engage Ag and effectively capture Ag on the surface of FDCs for processing and presentation. T_{fh} cells activated by Ag-MHC-II presented by high affinity B cells in turn provide B cells with stimulating signals through direct interactions of surface molecules and paracrine secretion of cytokines⁵⁰⁻⁵². These signals drive Ig isotype switching and the differentiation of GCB cells to high affinity antibody-secreting plasma cells and memory B cells^{46,53}. Non-specific B cells that fail to

engage and capture Ag undergo apoptosis in the absence of T cell help⁵⁴. Survived low affinity B cells can return to the DZ for another round of Ig somatic hypermutation^{7,46}. Through this highly competitive process, B cells expressing high affinity BCRs to Ag are selected to generate antibody responses.

Figure 1.2

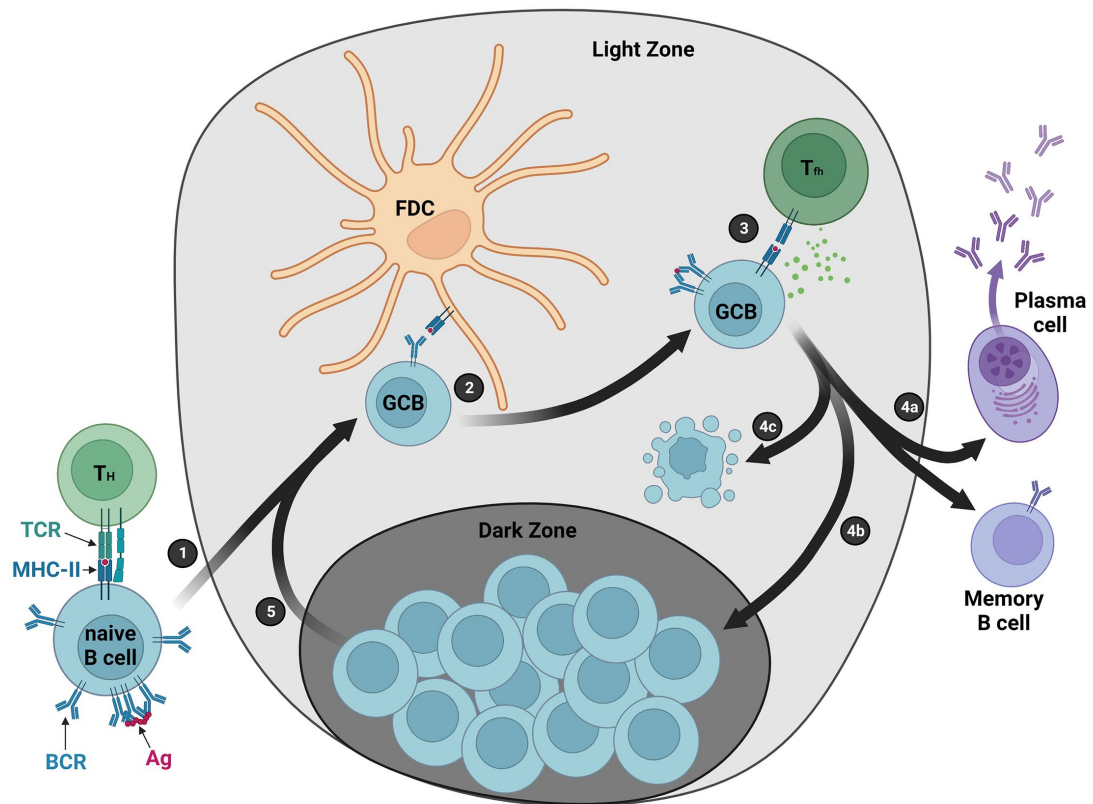


Figure 1.2: The germinal center response. (1) Ag-inexperienced (naïve) B cells, upon encountering their cognate Ag in secondary lymphoid organs will internalize, process and present the Ag to T helper cells, which are consequently activated. The T helper cells secrete cytokines to prime the B cell to enter the germinal center. (2) Following this, the B cell migrates to the light zone (LZ), where it encounters Ag presented by follicular dendritic cells (FDCs). (3) B cells that express high affinity Ag-specific BCR are able to acquire the most Ag from the FDCs and present them to T_h cells found in the LZ, and are selected by the T_h cells through secretion of cytokines which serve as an activating signal for the B cell. (4a) A strong BCR affinity to Ag results in strong signal from T_h cells, leading to class-switching recombination (CSR) and terminal differentiation of the B cell, which yields high affinity memory B cells and plasma cells. (4b) Moderate BCR affinity to Ag results in moderate signal from T_h cells, which causes the B cell to enter the dark zone (DZ), where it undergoes clonal proliferation and somatic hypermutation (SHM) to modulate its BCRs' specificity and affinity. (4c) Weak BCR affinity to Ag results in weak signal from T_h cells, which causes the B cell to undergo apoptosis or leaves it in a state of anergy. (5) B cell clones in the DZ migrate back to the LZ to survey Ag on FDCs again. Several rounds of proliferation and SHM in the DZ followed by selection in the LZ leads to the generation of high affinity memory B cells and plasma cells. Created with *BioRender.com*.

1.3.3 The effects of mis-regulation of B cell activation

In healthy individuals, B cells work with other immune cells to ensure a robust immune response against invading pathogens, which eliminates said pathogen and confers long-lasting immune memory. After a pathogen or toxin is cleared from the body, B cell populations and B cell-mediated antibody responses are downregulated. In healthy individuals, there exists a delicate balance between insufficient B cell activation and un-regulated excessive B cell activation in response to perceived antigenic threats. The most well studied cases in which defects in B cells lead to disease are in immunodeficiency diseases, auto-immune diseases, and B cell cancers (B cell leukemia and lymphomas).

Primary immunodeficiency (PI) is caused due to genetic mutations, and results in complete or partial impairment of some aspect of the immune system, resulting in an inability of the host to fight off infections. In the context of B cells, PIs might affect B cell function either by affecting the B cells themselves or through affecting other immune cells responsible for activating and regulating B cell responses. A well-known example of a PI affecting B cells directly to result in immunodeficiency is X-linked agammaglobulinemia (XLA), also known as Bruton's agammaglobulinemia, results from X-linked gene deletion of the BTK gene. This leads to impairment of B cell development, wherein they are unable to mature into pre-B cells from pro-B cell stage⁵⁵. Patients with XLA are especially susceptible to recurrent bacterial infections due to an absence of mature B cells. Another example of B cell immunodeficiency is X-linked hyper-IgM syndrome (XHM syndrome), which occurs as a result of a mutation in the gene encoding CD40 ligand (CD40L/CD154) expressed on T_H cells. In XHM patients, B cells are able to mount low affinity IgM responses, but are unable to receive T cell help and enter germinal centers, leading to an absence of high affinity isotype-switched antibody responses. XHM patients typically suffer from increased susceptibility to viral, fungal and

parasitic infections. They also exhibit increased risks of auto-immune disorders and malignancies⁵⁶.

Current knowledge about auto-immune disease suggests that they arise from genetic mutations in genes involved in regulating immune cell activity, and the impairment of these genes in B cells results in either over-reactive B cell signaling or lack of signaling attenuation. This leads to the selection of B cells that might activate even in response to self-Ag, and generate either low affinity non-specific antibodies, or in some cases high affinity antibody against self-Ags, known as auto-antibodies. Auto-antibody production has been observed in several auto-immune diseases including Type 1 diabetes, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus (SLE), psoriasis and myasthenia gravis⁵⁷. A well-known example of how mis-regulation of B cell activation can lead to immune dysfunction is Wiskott-Aldrich syndrome (WAS). This disease is caused due to an x-linked mutation in the gene encoding the Wiskott-Aldrich syndrome protein (WASP), which is expressed in hematopoietic cell lineages, and is a nucleation promoting factor (NPF) of Arp2/3 complex, which nucleates branched actin^{58,59}. The clinical manifestations of WAS include thrombocytopenia, increased susceptibility to bacterial and viral infections, and auto-antibody production⁶⁰⁻⁶². In B cells, WAS causes a marked reduction in MZ B cell populations and Ag-specific B cell responses, while increasing the population of self-reactive B cells indicating a breakdown of self-tolerance⁶³. Interestingly, these symptoms include both immune deficiencies and auto-immune complications.

In B cells, several signaling molecules have been identified as potential causes for various auto-immune phenotypes. These include cell cycle modulators, inhibitory kinases and phosphatases, as well as adaptor molecules⁶⁴⁻⁶⁷. Research previously conducted in our lab and other groups has highlighted the role for actin regulatory proteins WASP, neuronal Wiskott-Aldrich syndrome protein (N-WASP) and non-muscle myosin II (NMII), as well as inhibitory

signaling molecules SH2-domain containing inositol-5-phosphatase-1 (SHIP-1) and hematopoietic progenitor kinase 1 (HPK1) in the loss of B cell self-tolerance and the generation of auto-immune phenotypes⁶⁵⁻⁷¹.

B cell cancers typically arise as a result of genetic mutations acquired through inheritance or caused due to environmental factors or viral infection. In many cases, these mutations can lead to constitutive activation or deactivation of key regulatory machinery in B cells, which in turn leads to enhanced B cell proliferation and survival, which are key hallmarks of cancer⁷². Uncontrolled proliferation of cancerous B cells can lead to impairment in the development of normal B cells or other immune cells, leading to a diverse range of pathologies. Furthermore, metastatic B cells are known to migrate to niches where B cells aren't usually found, decreasing the likelihood of finding metastatic tumors. Newly developed therapeutics against B cell cancers specifically target B cells, for example the monoclonal anti-CD20 antibody (Rituximab), or specifically disrupt B cell signaling pathways, such as through the use of Btk inhibitors (Ibrutinib) and PI3K δ inhibitors (Idelalisib)⁷³.

As B cell-related immune disorders become more diverse, targeted treatment options remain limited due to insufficient knowledge of B cell signaling pathways. It is therefore becoming increasingly important to fully understand the molecular pathways governing B cell development, selection, BCR signaling activation, and attenuation, to diagnose and treat immunodeficiencies, auto-immune diseases and B cell cancers.

1.4 BCR signaling

1.4.1 BCR signaling in response to antigen

The BCR initiates B cell activation upon binding antigen that it can recognize using its BCRs (cognate antigen). The BCR consists of an immunoglobulin with transmembrane anchors, non-covalently associated with Ig α /Ig β heterodimer. The Ig α / β chains, also known as CD79a/CD79b, have Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) in their cytoplasmic domains. The ITAM is a signal transduction motif consisting of two tyrosine residues, each followed by two residues, separated by a 6-8 residue spacer: YxxL/I X₍₆₋₈₎ YxxL/I²³. These tyrosine residues on each ITAM are phosphorylated upon receptor activation⁷⁴. Multivalent soluble antigen, or surface-bound (insoluble) antigen, but not soluble monovalent antigen, can induce a BCR signaling response⁷⁵⁻⁷⁷.

Super-resolution imaging studies have shown that BCR resides as monomers or nanometer-sized clusters in the unstimulated cell⁷⁸⁻⁸⁰. Multiple identical epitopes on multivalent antigens or membranes cause surface BCRs to physically cluster to form BCR-antigen microclusters⁸¹. These BCR microclusters become associated with lipid rafts on the plasma membrane, which contain raft-resident Src kinases like Lyn, which phosphorylate the BCR's ITAMs⁸²⁻⁸⁴.

Phosphorylation is followed by the recruitment of downstream kinases like Spleen tyrosine kinase (Syk) and Bruton's tyrosine kinase (Btk), and adaptor proteins like B cell Linker (BLNK) to these receptor clusters, forming signalosomes⁸⁵⁻⁸⁷ (Fig. 1.3). Syk is a 72 kDa protein consisting of two SH2 domains, with which it binds dual phosphorylated ITAMs, and a kinase domain which activates phospholipase C- γ 2 (PLC γ 2), leading to the amplification of BCR signaling and early signaling events such as Ca²⁺ flux⁸⁸⁻⁹¹. Syk also enables the recruitment of BLNK by phosphorylating a non-ITAM tyrosine residue in the Ig α chain, enabling the recruitment of other downstream signaling molecules⁹². Btk is recruited to

phosphatidylinositol-3,4,5-triphosphate (PIP₃), found in lipid rafts, and activates PLCγ2 and phosphatidylinositol-5-kinase PI5K⁹³. Phosphatidylinositol-4,5-bisphosphate (PIP₂) and PIP₃ in lipid rafts plays an important role in the recruitment of downstream signaling molecules^{94,95}. PI5K activation via Btk converts phosphatidylinositol-5-phosphate (PIP) to PIP₂, ensuring its maintenance in lipid rafts⁹⁶. The accumulation of signaling molecules near the ITAMs of BCR present in lipid rafts leads to signal activation at BCR microclusters and allows propagation of signaling cascades to the cytoplasm and nucleus through calcium signaling, Rho family kinases, Mitogen Activated Protein (MAP) kinases, as well as transcription factors, like Nuclear Factor κB (NF-κB) (Fig. 1.4).

When exposed to membrane-bound antigen, B cells spread on the surface, generating more BCR microclusters, which then coalesce into larger clusters by moving centripetally towards the center of the contact zone between the B cell and the antigen-presenting surface^{77,97,98}. When activated by soluble multivalent antigen (or anti-BCR antibodies which can also crosslink BCR), BCR microclusters polarize to form a BCR “cap” on one pole of the B cell^{98,99}. Cluster coalescence and cap formation both lead to a dramatic reduction in BCR lateral mobility. The formation of such BCR caps has been shown to depend on the actin cytoskeleton¹⁰⁰. During this stage, phosphatases like Src Homology 2 domain tyrosine Phosphatase-1 (SHP-1) and Src Homology 2 domain-containing Inositol 5-Phosphatase (SHIP-1) are recruited to the larger BCR clusters, dephosphorylating activated signaling molecules, leading to a downregulation of BCR signaling, which reverses the initial signaling amplification. Such a signaling balance has been illustrated by the interplay between the stimulatory kinase Btk and inhibitory phosphatase SHIP-1, wherein SHIP-1 knockout B cells displayed enhanced Btk phosphorylation and enhanced BCR signaling. Inhibition of Btk using a small molecule inhibitor in SHIP-1 knockout B cells rescued this phenotype, bringing BCR signaling back down to normal levels^{101,102}. Thus, Btk and SHIP-1 fine tune the timing

and levels of BCR signaling, with amplification of BCR signaling first, followed by BCR signaling attenuation.

In resting B cells, tonic BCR signaling, a relatively low-level of signaling, is necessary for naïve B cells to survive¹⁰³. However, exposure to cognate antigen primes the B cell for proliferation and affinity maturation. The entry of B cells into germinal centers to competitively acquire antigen for affinity maturation appears to require strong signaling^{104–106}. The BCR detects extracellular signals in the form of antigen, soluble or tethered, and forms signalosomes, leading to a cellular response. However, the mechanism underlying BCR activation upon antigen binding is not completely understood. A few models have been proposed, describing the possible mechanisms¹⁰⁷. One of these models is the BCR clustering-activation model, according to which monomeric surface BCRs are clustered into oligomers upon antigen binding, triggering ITAM phosphorylation by raft-resident Src kinases⁷⁵. The binding to surface antigen induces a conformational change in the BCR molecule, which enhances BCR oligomerization^{77,108}. BCRs with different isotype of mIgs, IgM and IgG, undergo distinct conformational changes, leading to different force-sensitivity between IgM-expressing naïve B cells and mIgG expressing effector B cells, which would explain why IgG BCR have a lower threshold for activation than IgM BCR^{95,109,110}. A different model states that surface BCRs are present as oligomers in resting B cells, and dissociate from the oligomers when spaced out by antigen binding, making the ITAMs accessible for phosphorylation^{111,112}. Evidence for both these models exists and the exact mechanism for BCR activation is yet to be objectively defined.

Figure 1.3

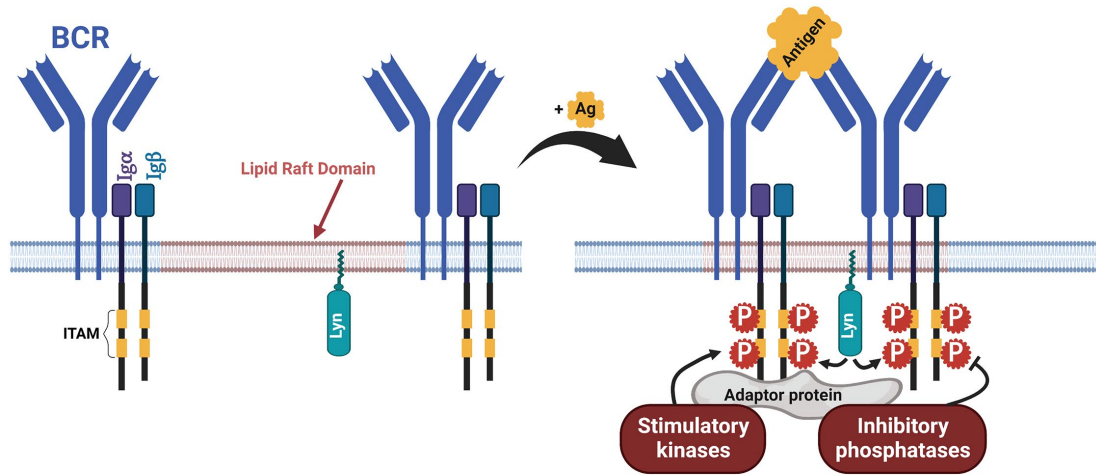


Figure 1.3: BCR clustering induced by multivalent antigen initiates BCR signaling.

BCR, typically existing as monomers on the B cell membrane, are physically clustered upon exposure to multivalent soluble Ag or surface-tethered Ag. This causes BCR-Ag clusters to enter into lipid raft domains within the B cell membrane. Raft-resident Src kinases like Lyn are then able to phosphorylate BCR ITAMs, leading to the recruitment of adaptor protein BLNK and signaling molecules. These signaling molecules can be either stimulatory or inhibitory towards BCR signaling, and can thus fine-tune the BCR signaling in response to Ag. Created with *BioRender.com*.

1.4.2 BCR signaling initiates actin cytoskeletal rearrangement

Upon BCR engagement, downstream signaling through the adaptor protein BLNK and Btk recruits and activates the guanine nucleotide exchange factors (GEFs) like Vav, which activate Rho family GTPases like RhoA, Rac1/2, and Cdc42^{87,113,114} (Fig. 1.4). These GTPases and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂] activate actin nucleation promoters like Wiskott-Aldrich Syndrome Protein (WASP) expressed exclusively in immune cells and Neuronal-WASP (N-WASP), a ubiquitously expressed homolog of WASP. Proteins of the WASP family are well known actin regulators that activate the Actin-Related Protein complex (Arp2/3) and its nucleation of branched actin filaments^{115,116}. Wiskott-Aldrich Syndrome (WAS), caused by WASP-deficiency, is a severe immune disorder, characterized by immune deficiency and high incidences of autoimmune diseases and lymphoid cancers¹¹⁷. Another Arp2/3 activator, WASP-family verprolin-homologous protein (WAVE), may also function downstream of the BCR. Knocking out the HEM-1 protein, an essential component of the WAVE regulatory complex (WRC), resulted in defects in B cell activation in response to antigen^{118,119}. In addition to the Arp2/3 complex that nucleates branched actin sheets and lamellae, B cells express another type of actin nucleators, formins that nucleate bundled actin filaments^{120–122}. A polarized actin response and morphological changes in B cells predominantly occur in response to a polarized array of antigens, as opposed to soluble antigen. This is likely due to the activation of specific actin remodeling proteins near sites of BCR signaling. The activation of these actin nucleators, triggered by antigen-induced BCR signaling at lipid rafts, directs actin polymerization and remodeling at sites of BCR signaling, resulting in a cellular response polarized in the direction of antigenic stimulation¹²³.

Btk has been shown to be essential for activating actin remodeling by BCR signaling. Btk induces actin reorganization by activating WASP and N-WASP through phosphorylating Vav and phosphatidylinositol-5 kinase¹²⁴. Btk also recruits Phosphatidylinositol-4-phosphate 5-

kinase (PIP5K) to lipid rafts, generating phosphatidylinositol-4,5-bisphosphate (PIP₂), which recruits WASP and N-WASP^{96,124}. WASP and N-WASP activate Arp2/3 mediated actin polymerization at BCR-antigen interaction sites. This leads to polarized actin accumulation, causing the B cell to spread over the antigen-coated surface⁹⁷. Interestingly, besides its involvement in B cell spreading, N-WASP was found to have a unique role in B cell contraction. In B cells stimulated with antigen tethered to lipid bilayers, both WASP and N-WASP are required for F-actin accumulation at the B cell-antigen contact zone and cell spreading, while activated N-WASP curtails F-actin accumulation and promotes B cell contraction⁶⁶. Furthermore, WASP and N-WASP negatively regulate each other's activation and likely compete for the available activation machineries and Arp2/3 in the cell. They are also differentially regulated by BCR signaling. Btk promotes WASP activation and inhibits N-WASP activation, while SHIP-1 promotes N-WASP activation and inhibits WASP activation by inhibiting Btk¹⁰¹. The mechanisms by which WASP and N-WASP are differentially regulated by BCR signaling and influence the activation of each other remain unknown. The activation mechanism, timing, and localization of formins in B cells is also not well understood. While formins have been shown to generate actomyosin arcs in T cells, propelling TCR clusters centripetally¹²⁵, its role in BCR activation remain to be explored.

BCR activation also recruits cofilin and gelsolin, which are actin remodeling proteins involved in severing F-actin. F-actin severing can induce a transient depolymerization of the cortical F-actin network, freeing up BCR, allowing them to move laterally and form BCR microclusters^{98,126}. The adaptor protein BLNK recruits Phospholipase C- γ 2 (PLC γ 2), which cleaves phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate inositol triphosphate (IP₃) and diacylglycerol (DAG)⁸⁶. IP₃ triggers intracellular Ca²⁺ flux, which activates calmodulin and Myosin Light Chain Kinase (MLCK), which in turn phosphorylates and activates non-muscle myosin II (NMII)¹²⁷. NMII has been shown to regulate actin polymerization and

generate contractile forces by pulling actin filaments together¹²⁷. In B cells, NMII was shown to affect actin remodeling, generate contractile forces and enhance antigen extraction^{128,129}.

Figure 1.4

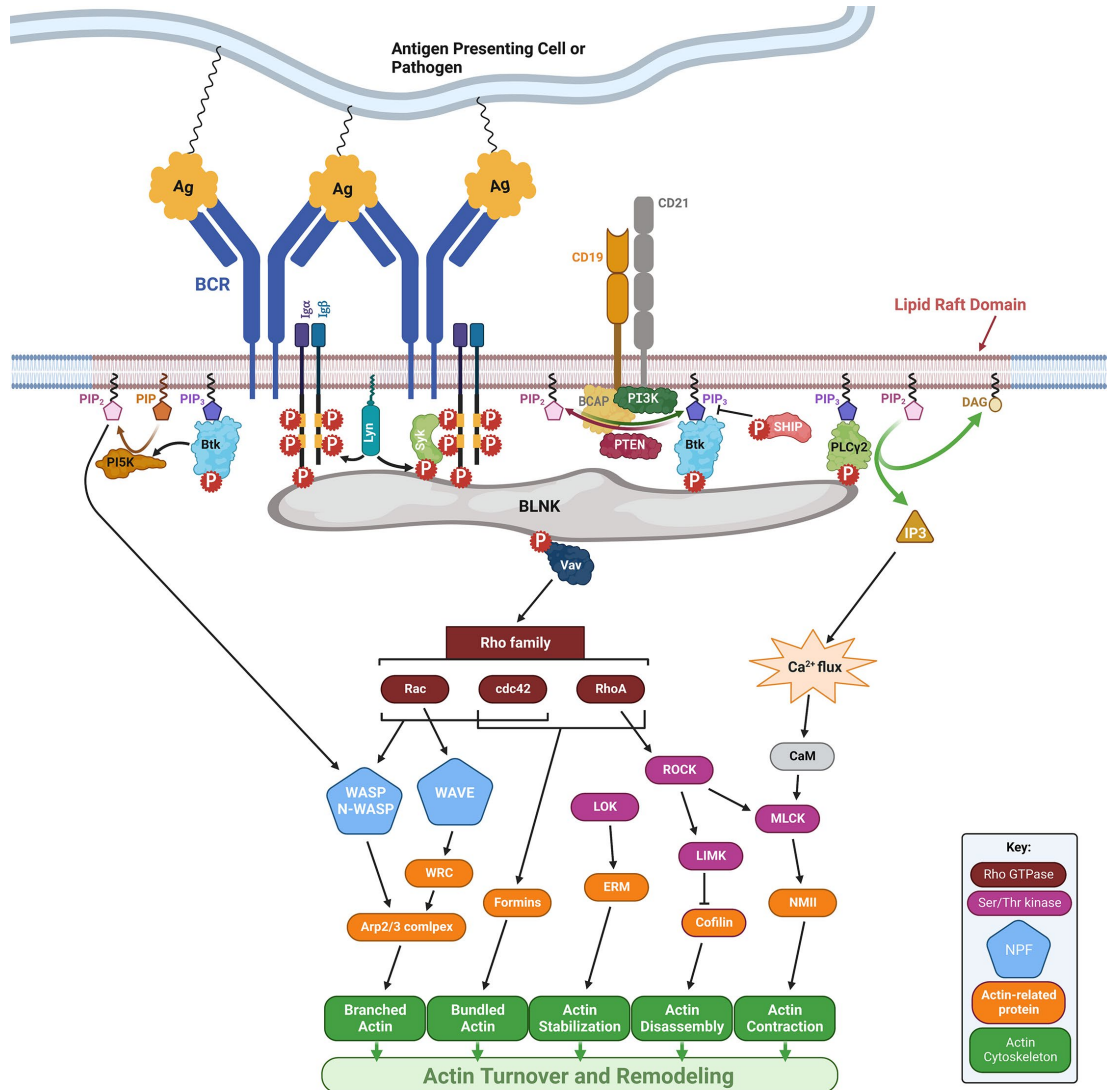


Figure 1.4: BCR engagement by multivalent antigen activates actin remodeling in B cells. BCR signaling is initiated by BCR binding to antigen, which activates actin turnover and remodeling through Rho family GTPases, nucleation promoting factors, Ser/Thr kinases and motor proteins. Coreceptor molecules like CD19 also influence key steps in BCR signaling. Created with *BioRender.com*.

1.5 The role of actin in regulating BCR-mediated B cell activation

While actin turnover and remodeling are initiated by Ag-induced BCR signaling, BCR signaling can also be regulated by actin and actin-related proteins. This suggests the existence of a closed feedback loop between Ag-induced BCR signaling and actin remodeling, which helps to fine-tune the B cell response to Ag^{117,130}. The following sections discuss the role of actin and actin-related proteins in the regulation of Ag-induced BCR signaling during various stages of B cell activation.

1.5.1 Initiation of BCR signaling

In resting B cells, IgM and IgG BCRs are predominantly present as monomers or nanoclusters, with some highly heterogeneous protein islands, up to 500 BCR molecules per island, shown using direct Stochastic Optical Reconstruction Microscopy (dSTORM)¹³¹. These BCR typically have low lateral mobility due to their lateral containment by the cortical F-actin network beneath the plasma membrane¹³². This cortical actin network is tethered to the plasma membrane by Ezrin/Radixin/Moesin (ERM) family of proteins, which can bind both F-actin and transmembrane proteins¹³³, and are phosphorylated by lymphocyte-oriented kinase (LOK) in B cells¹³⁴. Cortical actin can thus regulate the spatial distribution and dynamics of membrane receptors via ERM proteins¹³⁵. BCR lateral mobility is enhanced in actin and ezrin poor regions, and the deletion of BCR cytoplasmic tails also increases its lateral mobility^{132,136}. Disruption of cortical actin can induce BCR signaling⁹⁸. Thus, the cortical actin network forms a physical barrier around BCRs, constraining their motion^{78,132} (Fig. 1.5 A).

Upon BCR engagement and activation by multivalent or membrane antigen, ERM proteins are rapidly dephosphorylated and detach from the cortical actin network, followed by a rapid depolymerization of F-actin mediated by cofilin and gelsolin^{98,126}. Toll-like receptor

stimulation also induces cofilin activation, and cofilin-mediated actin severing reduces the spatial confinement of BCR, sensitizing B cells and enhances signaling¹³⁷. The release of these constraints increases BCR lateral mobility, leading to the reorganization of BCR-antigen complexes into microclusters, with up to ~5000 BCR molecules per cluster¹³¹. Transient depolymerization of cortical actin was shown to be necessary and sufficient for initiating a BCR signaling response, that typically occurs as a result of exposure to cognate antigen. Depolymerization of actin in resting B cells using Latrunculin led to slow but spontaneous BCR clustering and signaling activation⁹⁸. In contrast, stabilization of cortical F-actin in resting B cells using Jasplakinolide prohibited BCR clustering and signaling activation even in the presence of multivalent antigen and membrane-bound antigen⁹⁸. These studies showed that the cortical actin cytoskeleton plays a pivotal role in the initiation of BCR signaling by forming a mechanical barrier and thereby impeding BCR lateral mobility prior to antigenic stimulation. Upon exposure to cognate antigen, the increase in BCR lateral mobility and intermixing with stimulatory co-receptors enhances antigen-induced BCR signaling, which drives the priming of naïve B cells. This actin-mediated increase in BCR mobility is absent in resting B cells and differentiates tonic BCR signaling from B cell-priming BCR signaling in response to antigen.

Figure 1.5

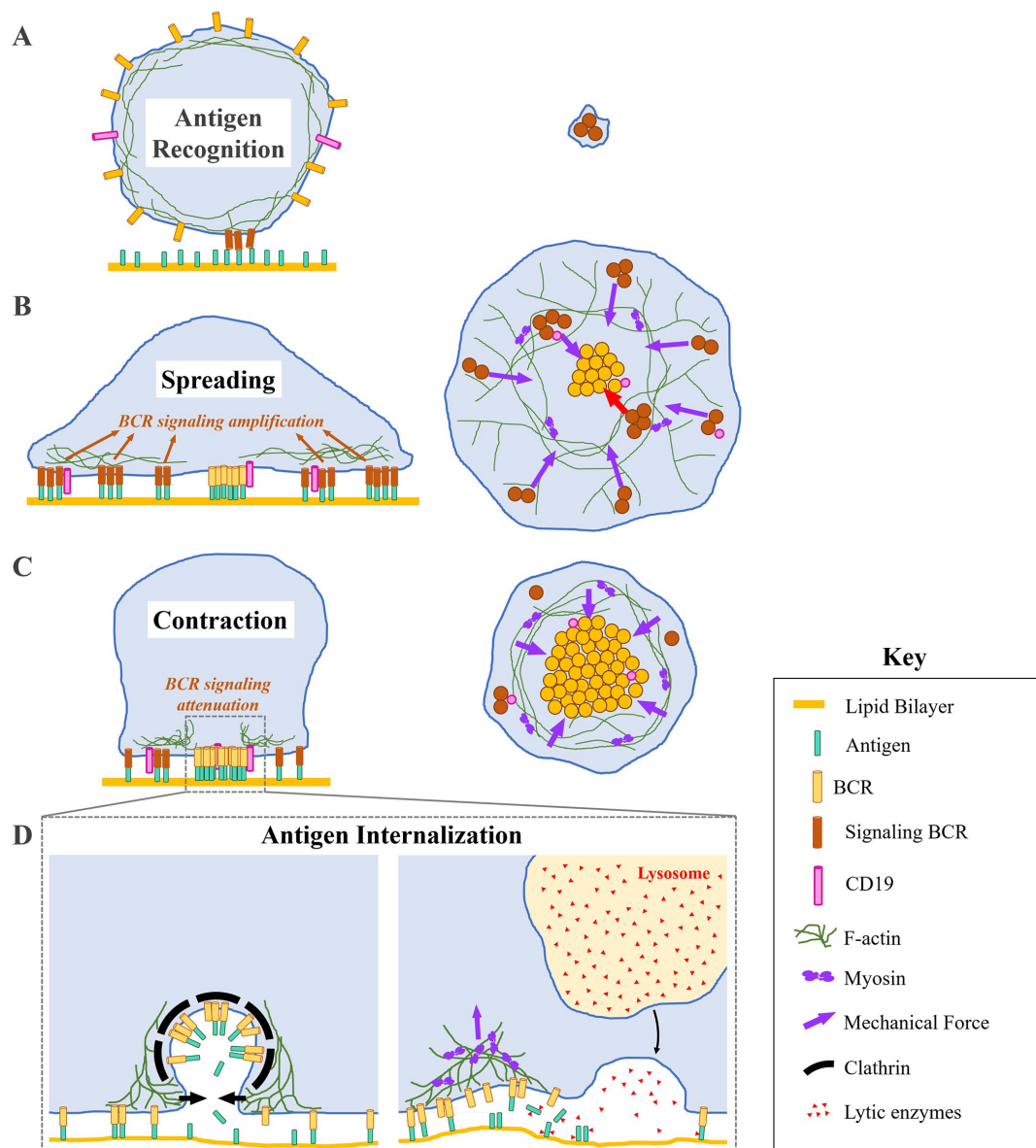


Figure 1.5: Actin influences BCR signaling at multiple stages of early B cell activation by using distinct mechanisms. (A) Transient depolymerization of cortical actin induced by BCR-antigen interaction releases BCR from actin-mediated confinement. (B) Actin drives B cell spreading and enhances the mobility and inter-mixing of BCR and CD19, leading to amplification of BCR signaling. (C) Actin drives B cell contraction, merging BCR microclusters and reducing their mobility, which downregulates BCR signaling. (D) BCR internalization of antigen via the clathrin-dependent pathway requires F-actin at the membrane fission step. Clathrin-independent antigen uptake requires actomyosin mediated mechanical force and extracellular enzymatic cleavage.

1.5.2 BCR signaling amplification

Subsequent to its role as a mechanical barrier in the absence of BCR stimulation, the actin cytoskeleton plays an important role in the amplification of BCR signaling. One mechanism by which the actin cytoskeleton influences BCR signaling is controlling cell morphology to determine the size and shape of the contact zone between the B cell and antigen-presenting surface. Following the transient depolymerization of cortical actin upon antigen recognition, F-actin is rapidly polymerized at the BCR-antigen interaction sites¹³⁸, driving B cell spreading over the antigen presenting surface. Remodeling of F-actin at the periphery of the contact zone enables continued B cell spreading and antigen engagement via the BCR. The enhanced contact increases the number of BCR-antigen microclusters which recruit more signaling molecules, thus amplifying BCR signaling^{97,117} (Fig. 1.5 B). Actin polymerization is not uniform but forms dynamic filaments and foci in the contact zone, which is likely initiated at and associated with signaling BCR microclusters¹³⁹, further amplifying signaling. The reduced cell spreading and signaling in mice with B cell specific gene knockout of WASP and/or N-WASP confirms the involvement of Arp2/3 and branched actin in signaling amplification⁶⁶. Similarly, the reduced B cell spreading caused by HEM-1 mutations that impair WAVE activity implicates the WAVE regulatory complex, Arp2/3 complex, and branched actin in BCR signaling amplification¹¹⁹.

Actin's role in amplifying BCR signaling is not limited to its effect on B cell morphology. Ketchum et al. have demonstrated that actin dynamics directly increases BCR cluster mobility on the B cell membrane interacting with PLBs, where the antigen are laterally mobile, but not on glass, where the antigens are laterally immobile¹⁴⁰. This is in contrast to the role of actin before antigenic stimulation, when it acts as a mechanical barrier constraining BCR mobility. Single molecule tracking of individual BCRs in B cells activated on PLBs showed that inhibition of the actin nucleating factors Arp2/3 and formins

dramatically reduces BCR diffusivity¹⁴¹. Furthermore, single particle tracking (SPT) of surface BCRs on WASP and N-WASP knockout B cells revealed that the loss of both these Arp2/3 activators reduced BCR diffusivity, but N-WASP knockout has a much larger effect than WASP. N-WASP was also found to modulate the diffusivity of the coreceptor CD19¹⁴¹. The reduction in diffusivity was correlated with disorganized actin dynamics and reduced actin flow speeds, indicative of a direct link between actin dynamics and nano-scale mobility of BCRs.

While Arp2/3 nucleates branched actin, formins are known to nucleate actin bundles¹²¹. Accordingly, the foci in the B cell contact zone were found to predominantly contain Arp2/3 nucleated branched actin, while formins were found to nucleate filaments that interconnect the foci¹³⁹. TIRF-Structural Illumination Microscopy (TIRF-SIM) was used to show that dynamic arc-like actin structures control the spatial patterning of BCR microclusters and drive their centralization¹⁴². These arc-like structures were disrupted upon Arp2/3 inhibition. Stimulated Emission Depletion (STED) Microscopy was used to show that the arc-like structures were associated with myosin IIA. The above studies show that dynamic branched as well as bundled actin structures enhance receptor diffusivity, spatially re-organize BCR-antigen clusters, increase their signaling, and enhance transcriptional activation in B cells. Although BCR diffusivities have been shown to be dependent on actin accumulation, the nanoscale architecture and dynamics of actin responsible for controlling BCR lateral mobility and spatial patterning are yet to be visualized.

BCR signaling has also been shown to be influenced by the presence of co-receptor molecules like CD19, CD21 and CD81 in BCR-antigen clusters. Actin regulators downstream of the BCR also modulate BCR interaction with these coreceptors. In resting cells, the actin cortex has been found to control the activation of the BCR and its interaction with coreceptors like CD19 by defining barriers that limit receptor mobility^{78,80}. The presence of

CD19 in BCR microclusters reduces the threshold for BCR signaling by recruiting phosphatidylinositol 3-kinase (PI3K)^{143–145}. PI3K-generated phosphatidylinositides around BCR clusters form the major scaffold for downstream signaling proteins, enhancing BCR signaling. Thus, the composition and dynamics of BCR clusters affects BCR signaling. Arp2/3-generated branched actin has been shown to affect the lateral mobility of the co-receptor CD19 and its interaction with BCR, wherein Arp2/3 inhibition decreased the colocalization of CD19 with BCR as well as CD19 phosphorylation¹⁴².

BCR signaling is tightly regulated by the local balance of signaling kinases and phosphatases recruited to BCR signalosomes. While kinases like Syk and Btk are phosphorylated earlier during the B cell response and amplify BCR signaling, phosphatases like SHP-1 and SHIP-1 are phosphorylated later and attenuate BCR signaling by dephosphorylating the BCR ITAMs, downstream signaling molecules, and phosphatidylinositol phosphates that are the plasma membrane docking sites of many signaling molecules^{66,101}. The signaling state of BCR clusters was found to be related to their extent of clustering. Smaller BCR microclusters generated soon after antigen engagement showed higher levels of tyrosine phosphorylation (pY), while larger BCR clusters formed later from the merging of BCR microclusters had lower levels of pY but higher levels of phosphatases like SHIP-1^{101,146}. Therefore, the activation/attenuation state of a given BCR in a cluster depends upon the number of BCRs in the cluster to a certain extent. Even though it remains to be proven, the retrograde flow of actin likely drives the centripetal flow and merging of BCR microclusters, regulating the BCR signaling state.

1.5.2 BCR signaling down regulation

Regulation of BCR signaling is of paramount importance for proper immune responses.

During naïve B cell activation, BCR proximal signaling was found to rapidly increase until it reaches a maximum and then drops steadily. Notably, the maximal BCR signaling is achieved in primary B cells at the time of maximum spreading. Following maximal spreading and BCR microcluster formation, the cell morphology switches to contraction, which together with retrograde actin flows, promotes BCR microclusters to move inwards towards the center of the contact zone. The mechanism by which the actin cytoskeleton switches B cells' behavior from spreading to contraction remains unexplored, but it is likely caused by a combination of actin turnover and dynamic redistribution of F-actin structures at the end of B cell spreading. Patients having a mutation in the cofilin cofactor protein WD repeat-containing protein 1 (WDR1) displayed enhanced B cell spreading and increased F-actin accumulation (reduced actin turnover), as well as increased apoptosis of stimulated B cells, which shows that actin turnover is important for B cell contraction and subsequent B cell activation¹⁴⁷.

As B cells contract, BCR signaling goes down^{83,117,148} (Fig. 1.5 C). This reduction in protein tyrosine phosphorylation is concurrent with the recruitment of phosphatases like SHIP-1¹⁰¹ (Fig. 1.5 E). Knocking out these negative regulators of BCR signaling leads to impairment in the selection of antigen-specific B cells, leading to the generation of autoreactive B cells and auto-antibody¹⁴⁹. Interestingly, knocking out actin regulatory proteins (N-WASP and NMII) involved in B cell contraction not only impaired contraction, but also delayed BCR signaling downregulation and led to auto-antibody production and elevated levels of surface activation markers, respectively^{128,150}. Furthermore, knocking out phosphatases, like SHIP-1, delays B cell contraction¹⁰¹. These results suggest direct links between B cell contraction and BCR signaling downregulation. Lack of B cell contraction and BCR signaling downregulation leads to continued BCR signaling, also referred to as chronic active BCR signaling, a typical

characteristic of B lymphomas and B cells from autoimmune patients^{151–153}. However, the underlying biochemical interactions that trigger and control contraction and signaling downregulation are unknown.

There are multiple pathways linking BCR signaling and actin dynamics. The ubiquitin ligase Cbl and adaptor proteins Grb2 and Dok-3 are involved in the centripetal movement of BCR clusters in the contact zone¹⁵⁴. The adaptor molecule Actin-binding protein 1 (Abp1) links the actin cytoskeleton and BCR signal attenuation by binding to both F-actin and Hematopoietic Progenitor Kinase 1 (HPK1) that inhibits BLNK, which promotes B cell contraction, BCR microcluster merger, and SHIP-1 recruitment to BCR microclusters¹⁵⁵. Activated RhoA in response to BCR signaling leads to the activation of Rho-associated protein kinase (ROCK), which then activates LIM domain kinase (LIMK)^{156,157}. LIMK has been shown to reduce cofilin-mediated actin disassembly and actin turnover¹⁵⁸. This ROCK-mediated LIMK activation likely stabilizes centripetally moving actin structures at the B cell contact zone during B cell contraction.

During B cell spreading, activated WASP co-localizes with BCR microclusters and at the leading edge of the cell^{100,124}, promoting actin polymerization associated with cell spreading and BCR microcluster motility. WASP-deficiency also reduces BCR internalization, one of the mechanisms for signal attenuation, suggesting that WASP can contribute to positive and negative regulation of BCR signaling. On the other hand, B cells from B cell-specific N-WASP knockout mice display delayed contraction, impaired formation of BCR central clusters, and a drastic reduction in BCR internalization¹⁵⁰, indicating a dominant role of N-WASP in BCR signaling attenuation. As WASP and N-WASP negatively regulate each other, a balance between WASP and N-WASP's branched actin nucleating activity appears to support the balance of B cell spreading and contraction. Distinct roles of WASP and N-WASP in B cell spreading and contraction is surprising because the two proteins share

greater than 50% sequence homology and activate the same actin nucleation factor, Arp2/3 complex. While the underlying cause for the different functions of WASP- and N-WASP-activated branched actin nucleation remains unknown, these studies suggest that sustained actin dynamics are necessary for effective signal downregulation.

1.5.2 Antigen uptake

B cells expressing high-affinity BCRs are competitive for antigen engagement, capture, endocytosis, process, and presentation to acquire T cell help, which is essential for the selection of high affinity B cell clones to expand and differentiate. In addition to antigen presentation, antigen-induced BCR internalization may also help clear antigen. The actin reorganization is required for BCR internalization of both soluble and surface-bound antigen¹⁰⁰ (Fig. 1.5 D). The kinetics of BCR-mediated antigen internalization appear to depend on BCR-antigen binding affinity and the type of antigen: faster for internalization of soluble multivalent antigen than surface-bound antigen^{124,159}. Upon binding to soluble multivalent antigen, B cells gather antigen-bound BCRs to “caps” in an actin-dependent manner, and subsequently, BCRs endocytose antigen through a clathrin-mediated pathway^{100,160}. Actin has been shown to be required for the fission of clathrin-coated pits that contain BCR-antigen complexes^{22,160}. FCH and double SH3 domain-containing protein 2 (FCHSD2), a protein that generates F-actin at sites of clathrin-mediated endocytosis, selectively co-localizes with relatively larger nanoscale BCR-Ag clusters, generating F-actin around them, which enhances their endocytosis. In contrast, relatively smaller nanoscale BCR clusters (induced by lowering the concentration of soluble antigen) were only associated with classical clathrin-mediated endocytic pits¹⁶¹. The BCR can internalize antigen through clathrin independent pathways (Fig. 1.5 D). An actin-dependent clathrin-independent BCR internalization pathway has been suggested¹⁶². Upon exposure to antigen-coated plasma membrane sheets (PMS), B cells generate dynamic actin foci that co-localize with clathrin at

sites of antigen uptake. Inhibiting actin polymerization and specifically inhibiting Arp2/3 that nucleates branched actin by an inhibitor, CK-666, resulted in a significant reduction in antigen uptake, but inhibiting formin that nucleates bundled actin by an inhibitor, SMIFH2, only had a minor effect¹³⁹. Extraction of membrane-associated antigen relies on NMII-mediated contractile forces¹⁶³. B cells extract antigen either by applying mechanical forces through the BCR or by releasing lytic enzymes into the site of interaction between the B cell and the antigen presenting surface to enzymatically cleave antigen from the presenting surface^{164–166}. Our recent data show that BCR engaging antigen tightly associated to surfaces in high-affinity permeabilizes the B cell plasma membrane at antigen binding sites in a NMII-dependent manner. The membrane permeabilization triggers a repair response, including lysosomal exocytosis, which provides lytic enzymes for antigen cleavage from presenting surfaces¹⁵⁹. Therefore, the actin cytoskeleton is involved in multiple antigen capture pathways in an affinity dependent manner.

1.6 How the properties of antigen-presenting surfaces affect B cell activation

1.6.1 Chemical and physical properties of antigen-presenting surfaces affect BCR and actin dynamics in B cells

The physical properties of antigen that B cells encounter *in vivo* are much more complex than the model antigen used for research so far. The plasma membrane of antigen presenting cells is a two-dimensional fluid with heterogeneous lateral mobility and various morphologies, forming a complex nanotopography. The lateral mobility of antigen on the presenting surface has been shown to influence B cell actin dynamics and surface BCR clustering¹⁴⁰, similar to what was reported for T cells¹⁶⁷. In the lymph nodes and spleen, B cells encounter antigens mainly on APCs, such as marginal zone macrophages and follicular dendritic cells (FDCs). The surfaces of dendritic cells are highly convoluted with folds that have a radius of curvature on the order of 200 to 300 nm. The dendrites of FDCs found in B cell follicles form intricate three-dimensional networks that are several microns in length and 100-300 nm in diameter¹⁶⁸. Recent studies have used nanofabrication to study the influence of topography on B cell activation. Antigen-coated parallel nanoridges, with dimensions similar to *in vivo* structures, were shown to induce different B cell morphological changes from flat surfaces, with enrichment of actin and BCRs near the nanoridges. The spacing between nanoridges also modulate actin dynamics and calcium signaling in B cells¹⁶⁹. The presence of the integrin ligand intercellular cell adhesion molecule-1 (ICAM-1) on antigen presenting substrates leads to improved B cell responses through LFA-1/ICAM-1 interaction, which has been shown to reduce the threshold for B cell activation by reducing the level of antigen required to activate B cells⁴³. Furthermore, integrins, like LFA-1, are sensitive to mechanical forces and actomyosin-generated force-dependent conformational changes in LFA-1 may influence the transduction of costimulatory signals in T cells^{170,171}. In B cells, actomyosin-generated force-dependent conformational changes in integrins might be involved in BCR affinity maturation.

The antigen presenting surfaces encountered by B cells *in vivo* are much softer (with stiffness in the range of 0.5-5 kPa) than surfaces commonly used in most studies. Recent studies have examined how the mechanical compliance of the antigen presenting substrate affects B cell signaling activation using hydrogels with tunable stiffness to activate B cells. These studies show that stiffer hydrogels, having an elastic modulus of around 1100 kPa, are more effective in triggering BCR clustering and phosphorylation than softer hydrogels¹⁷². Such hydrogel substrates also enable the measurement of forces exerted by B cells using a technique called traction force microscopy. Cellular forces have been shown to be critical for the BCR to discriminate antigen binding affinity and antigen extraction. Antigen-coated hydrogels of known stiffness are embedded with fluorescent beads as fiducial markers. As B cells exert forces on the surface during antigen engagement, the gel deforms. The displacement field of the embedded beads can be used to determine the magnitude and direction of cellular forces exerted by B cells during activation. Using this technique, studies have shown that B cells generate forces on the order of 10-20 nN on substrates with stiffness similar to APCs (0.5 to 1kPa)¹⁷³. These forces increase during the first 5 minutes following B cell interaction with the antigen presenting surface and then plateaued¹²⁹. Cellular forces are known to be generated by actomyosin and dynein-mediated contractility. Forces have been detected at myosin and Arp2/3 dependent protrusive actin patches^{128,129,163}. Such forces have been shown to be required for BCR conformational changes, which facilitate BCR-BCR interaction and signaling activation, and the internalization of surface-associated antigen by the BCR, pulling antigen from presenting surfaces. The activation of IgM-BCR expressed on naïve B cells requires a much higher force threshold than the activation of isotype-switched IgG- or IgE-BCR expressed on germinal center and memory B cells¹⁰⁹. This threshold difference at the molecular level may explain the differential morphological changes, force generation, and BCR signaling between germinal center B cells and naïve B cells^{105,173}.

1.7 Rationale and Aims

Successful B cell activation requires BCR signaling amplification in response to Ag, followed by BCR signaling attenuation. B cells are known to exhibit an actin cytoskeleton-mediated spreading and contraction response, concurrent with amplification followed by attenuation of signals transmitted through the BCR^{117,130,174}. Our lab has previously shown that actin regulatory proteins WASP and N-WASP play distinct roles in this spreading-contraction response with N-WASP playing a critical role in B cell contraction and BCR signaling attenuation⁶⁶. Importantly, knocking out N-WASP leads to autoimmune phenotypes similar to those resulting from knocking out inhibitory phosphatase signaling molecule SHIP-1^{66,101}. This suggests an interplay between the actin cytoskeletal response and the BCR signaling response. Furthermore, it is well established that BCR signaling can induce actin rearrangement and actomyosin-mediated forces, but it is also becoming increasingly evident that the actin cytoskeleton also in turn regulates BCR signaling at various stages of Ag-induced B cell activation. However, the mechanisms by which the actin cytoskeleton regulates BCR signaling as B cells switch from spreading to contracting are not well studied.

I hypothesize that N-WASP-mediated, but not WASP-mediated branched actin nucleation is required for B cell contraction. Furthermore, B cell contraction causes BCR signaling attenuation by increasing the molecular density of BCR in BCR-Ag clusters, which selectively recruit more inhibitory phosphatases than stimulatory kinases. Based on what we know about the effects of knocking out or inhibiting WASP, N-WASP and NMII, I predict that N-WASP and Arp2/3 drive the generation of F-actin structures that are responsible for B cell contraction. This contraction decreases the surface area of contact between the B cell and the Ag-presenting surface (B cell contact zone), consequently increasing the density of BCR-Ag clusters in the contact zone. This increase in molecular density might result in selective recruitment of inhibitory phosphatases, but not stimulatory kinases at denser BCR-Ag

clusters, either due to space constraints or conformational changes within the BCR molecule, explaining the observed phenomenon of BCR signaling attenuation in response to B cell contraction.

This dissertation addresses the question of whether N-WASP generates distinct branched actin architecture as compared to WASP, if the N-WASP-mediated actin structures are required for B cell contraction, if B cell contraction increases BCR density in BCR-Ag clusters, and how increasing BCR affects the recruitment and phosphorylation of stimulatory and inhibitory signaling molecules. Specifically, this research shows, for the first time, how N-WASP-mediated dynamic F-actin structures induce B cell contraction, which can in turn affect BCR signaling through the dissociation of both stimulatory and inhibitory signaling molecules from BCR-Ag clusters. Furthermore, this research also shows that the increasing BCR density does not completely explain BCR signaling downregulation in response to B cell contraction, raising interesting questions about other possible mechanisms such as through mechanical force-induced conformational changes to the BCR.

1.7.1 Aim 1: To determine how B cells transition from spreading to contracting

This aim was designed to determine which actin structures are associated with B cell contraction, and how they induce B cell contraction. To determine whether B cell contraction requires specific actin-related proteins, we used an experimental model of surface bound antigen tethered to planar lipid bilayers (PLBs) to stimulate mouse B cells. To test the involvement of Arp2/3-mediated branched actin nucleation in B cell contraction, we stimulated primary splenic B cells from mice in the presence or absence of a small molecule inhibitor (SMI) against Arp2/3, and compared the population of contracting B cells between control and inhibitor-treated B cells. The B cell contact zones were imaged using interference reflection microscopy (IRM) and time-lapse microscopy. Whether or not WASP and N-

WASP are required for B cell contraction were elucidated by using WASP knockout (WKO) mice, B cell-specific N-WASP knockout (cNKO) mice, and an SMI against N-WASP. To determine which actin structures are associated with B cell contraction, we imaged the B cell actin cytoskeleton proximal to the Ag-PLBs, comparing WT or untreated B cells with various SMI-treatments or B cells from WKO mice or B cells from cNKO mice. Actin structures were observed using LifeAct-GFP-, or LifeAct-RFP-expressing mice, or by using phalloidin-staining, and imaged using total internal reflection fluorescence (TIRF) microscopy. To study how N-WASP-mediated branched actin structures induce B cell contraction, we compared the F-actin structures and their dynamics during B cell contraction in WT or untreated B cells, to those in B cells treated with an Arp2/3 inhibitor, or an N-WASP inhibitor or cNKO B cells (the same treatments/knockouts that perturb B cell contraction). Finally, we determined whether N-WASP-mediated branched actin structures are required for NMII recruitment by imaging B cells from GFP-NMII-expressing mice using TIRF microscopy, and perturbing contraction in these cells using an SMI against N-WASP.

1.7.2 Aim 2: To determine how B cell contraction drives the transition of BCR signaling from amplification to attenuation

This aim was designed to determine whether B cell contraction leads to an increase in BCR density in BCR-Ag clusters, and how increasing BCR density affects the recruitment and phosphorylation of stimulatory and inhibitory signaling molecules at these BCR clusters. To observe BCR-Ag clusters we used fluorescent-labeled pseudo-antigen-coated PLBs, and identified BCR-Ag clusters using the fluorescence intensity of the BCR-clustered fluorescent Ag. We studied the effect of B cell contraction on BCR density by perturbing B cell contraction using SMI against Arp2/3, or an SMI against N-WASP, or cNKO mice, and imaging the B cell contact zone using TIRF time-lapse microscopy. To study the relationship between BCR phosphorylation and BCR density, we fixed the B cells stimulated on

fluorescent-Ag-PLBs, and stained phosphorylated CD79a (Ig α ITAM), and compared the BCR phosphorylation among BCR-Ag clusters with varying BCR density. To analyze the recruitment and phosphorylation levels of signaling molecules at BCR clusters, we fixed the B cells stimulated on fluorescent-Ag-PLBs, and stained Syk, a stimulatory kinase, and SHIP-1, an inhibitory phosphatase. We also stained phosphorylated Syk (pSyk) and phosphorylated SHIP-1 (pSHIP-1), and measured the amount of signaling molecule relative to BCR present in individual BCR clusters with varying molecule density. This allowed us to establish a relationship between BCR density in clusters, and the recruitment and phosphorylation levels of Syk and SHIP-1.

Chapter 2: N-WASP-dependent branched actin polymerization promotes B cell receptor signaling attenuation by increasing the molecular density of receptor clusters

2.1 Abstract

B cell receptor (BCR) signaling responses to antigenic stimulation is a critical initiation and regulatory step for B cell activation and differentiation. The actin cytoskeleton plays essential roles in modulating BCR signaling. When encountering cell-surface antigens, actin-driven B cell spreading on the antigen-presenting surface amplifies signaling, while B cell contraction following the spreading leads to signal attenuation, which curtails autoreactive B cell activation. However, the mechanism by which actin switches BCR signaling from amplification to attenuation was unknown. Here, we show that branched actin polymerized by Arp2/3 is required for B cell contraction besides its role in B cell spreading. Contracting B cells exhibit centripetally moving inner actin foci, originated from lamellipodial actin networks, in the B cell membrane region contacting antigen-presenting surfaces. N-WASP, but not WASP, activated branched polymerization form these actin foci by sustaining their lifetime and centripetal movement. N-WASP-dependent inner actin foci are necessary for recruiting non-muscle myosin II to create an actomyosin ring-like structure at the periphery of the membrane contact region. B cell contraction particularly increases the BCR molecular density of individual clusters. Increased molecular density by B cell contraction leads to decreases in phosphorylated BCR, the stimulatory kinase Syk, the inhibitory phosphatase SHIP-1, and their phosphorylated forms in individual BCR clusters. Taken together, our results suggest that the actin reorganizes from the lamellipodial actin networks to centripetally moving inner actin foci, enabling actomyosin ring-like structure formation,

through N-WASP-activated Arp2/3. Actomyosin-mediated B cell contraction attenuates BCR signaling by inducing disassociation of both stimulatory kinases and inhibitory phosphatase from BCR clusters, revealing novel insights into actin-facilitated signal attenuation.

2.2 Introduction

B cells are responsible for generating antibody responses essential for eliminating invading pathogens. B cell receptors (BCRs) expressed on the B cell surface detect the presence of cognate antigens. The binding of antigen to the BCR leads to the activation of signaling cascades^{11,175–178}. The signaling cascades can induce transcriptional programs that prepare B cells for proliferation and differentiation^{179–181}. BCR signaling also induces rapid antigen internalization, processing, and presentation for T cell recognition, which provides the second signal required for B cell clonal expansion and differentiation to high-affinity antibody-secreting cells and memory B cells^{182–185}. BCR signaling is tightly regulated by various external and internal factors to activate antibody responses that are specific and also qualitatively and quantitatively measured to encountered antigen.

Antigen-induced receptor reorganization activates BCRs at the B cell surface. Antigen binding leads to BCR clustering at lipid rafts^{186,187}, which enables raft-resident kinases, like the Src kinase Lyn and Fyn, to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the BCR signaling subunit CD79a/b heterodimer^{89,188,189}. Double phosphorylated ITAMs recruit and activate spleen tyrosine kinase Syk, which in turn activates multiple downstream signaling pathways, including phospholipase C γ 2 (PLC γ 2), Bruton's tyrosine kinase (Btk), Ras-GTPase, and phosphatidylinositol-3 kinase (PI3K), initiating signaling cascades^{89,190–193}. Signaling activation also induces the recruitment of inhibitory phosphatases, such as SH2-containing tyrosine phosphatase-1 (SHP-1) and phosphatidylinositol-5 phosphatase-1 (SHIP-1), to BCR signaling complexes. Such recruitment occurs even in the absence of ligands that can colligate surface BCRs with the inhibitory co-receptors upstream of these phosphatases, such as CD22 and Fc γ RIIB^{194–196}. SHIP-1 and SHP-1 have been shown to bind singly or doubly phosphorylated ITAMs through their SH2 domain^{197,198}. SHP-1 can dephosphorylate the BCR

and its downstream kinases^{199,200}, while SHIP-1 removes the plasma membrane docking lipids for stimulatory kinases, like Btk and Akt^{201–203}, which lead to BCR signal attenuation. The interplay between the stimulatory kinases and the inhibitory phosphatases controls the balance of antibody responses against pathogens and self. Deficiencies in stimulatory kinases by mutations, such as Btk, cause X-linked agammaglobulinemia (XLA)⁵⁵, while deficiencies in the inhibitory phosphatases SHP-1 or SHIP-1 cause autoimmune diseases^{203–206}. However, the mechanisms by which BCR signaling attenuation is initiated and regulated remain elusive.

B cells encounter both soluble and membrane-associated antigens *in vivo*. Membrane-associated antigens include antigens on antigen-presenting cells, like follicular dendritic cells (FDCs) in B cell follicles or germinal centers, and pathogenic cells, like bacteria, parasites, and cancer cells^{207–209}. The binding of multi-valent soluble antigen and membrane-associated antigen with any valency induces dynamic reorganization of surface BCRs into microclusters, triggering BCR signaling^{76,210–212}. Subsequently, surface BCRs continue moving to the plasma membrane region contacting antigen-presenting surfaces or one pole of a B cell for soluble antigen, which leads to the growth and the merger of BCR microclusters, forming super-molecular activation complexes^{213,214}. When interacting with antigens on the surface of professional antigen-presenting cells that provide adhesion molecules for B cells to interact, BCR clustering leads to the formation of the immunological synapse^{44,215}. Multiple mechanisms have been proposed for signaling induction by antigen-induced surface BCR reorganization. The binding of surface BCRs to membrane-associated antigen has been shown to induce a conformational change in the BCR extracellular domain, exposing a proximal membrane region of membrane IgM that promotes receptor clustering²¹⁶. Antigen-binding also changes the conformation of the BCR cytoplasmic domains from a closed to an open form, facilitating the recruitment of signaling molecules^{217–219}. Alternatively, antigen-

BCR interaction increases the molecular spacing of BCRs in clusters, facilitating BCR interaction with signaling molecules^{220,221}. The inhibitory phosphatases SHP-1 and SHIP-1 are also recruited to BCR clusters^{222,223}. However, the mechanisms underlying the phosphatase recruitment and its relationship with the BCR interaction with signaling activation complexes are unknown.

The actin cytoskeleton is essential for antigen-induced BCR reorganization on the B cell surface in response to both soluble and membrane-associated antigen²¹⁴. The early signaling of the BCR triggers transient actin depolymerization first via Rap GTPase and its downstream cofilin²²⁴, which disassembles the existing cortical actin network that confines the lateral movement of surface BCRs^{225,226}. Following the transient depolymerization, BCR signaling activates rapid actin polymerization through the actin nucleation promoting factors Wiskott-Aldrich Syndrome Protein (WASP) and neuronal-WASP (N-WASP), leading to F-actin accumulation at BCR-antigen interact sites. Actin polymerization and treadmilling in B cells stimulated by soluble antigen drive surface BCRs to cluster and move to one pole of a B cell, forming a BCR cap^{214,227}. In B cells interacting with membrane-associated antigen, actin organizes into two dynamic fronts. One treadmills outwards, driving B cell membrane spreading, which expands the B cell membrane region contacting the antigen-presenting surface (contact zone), enabling more BCRs to engage antigen. The other creates retrograde flow towards the center of the B cell contact zone, driving the centripetal movement and growth of BCR microclusters^{214,228}. These together amplify BCR signaling^{174,229}. The extent of B cell spreading and the amount of BCR-antigen complexes gathered in the contact zone depend on the affinity and density of antigen on the membrane^{213,215,230}. Following the spreading, B cells contract, reducing the contact zone and driving BCR microcluster to merge into center clusters to form immunological synapses^{203,213,231}.

We have previously shown that mouse primary B cells with Btk deficiency^{203,232} or double knockout of the actin nucleation promoting factors WASP and N-WASP²³¹ fail to spread on the antigen-presenting surface and establish stable interactions with membrane-associated antigen, drastically reducing BCR signaling. Btk can activate WASP and N-WASP through Vav, the guanine nucleotide exchange factor of Cdc42 and Rac and phosphatidylinositol-5 kinases that generates phosphatidylinositol-4,5-bisphosphate (PI4,5P₂)^{176,233–235}. Surprisingly, B cell-specific knockout of N-WASP (cNKO) but not WASP knockout (WKO) enhances B cell spreading, delays B cell contraction, and inhibits the centralization of BCR clusters at the contact zone²³¹. Consistent with these findings, Wang et al. have shown that knockdown of the branched actin nucleation factor Arp2/3 downstream of WASP and N-WASP^{236,237} disrupts the dynamic actin reorganization induced by membrane-associated antigen that drive BCR microcluster growth and merger^{238,239}. Furthermore, the F-actin severing protein cofilin and its upstream regulators are responsible to provide G-actin for Arp2/3, keeping actin treadmilling^{224,240,241}. Using B cell-specific and germline knockout, we later found that in addition to N-WASP, the actin motor non-muscle myosin IIA (NMIIA)²⁴² and the actin-binding adaptor protein Abp1²⁴³ are required for B cell contraction and BCR central cluster formation. Wang et al. recently shown that in the presence of the adhesion molecule ICAM on the antigen-presenting surface, which facilitates the synapse formation, B cells form contractile actomyosin arcs in a formin (bundle actin nucleation factors)-dependent manner, driving centripetal movement of BCR clusters in the B cell contact zone²⁴⁴. However, how the actin cytoskeleton transitions B cells from the spreading to contraction phase is unclear.

We have previously shown that B cell contraction is critical for BCR signaling attenuation. Delaying and inhibiting B cell contraction by deficiency of actin regulators, N-WASP, Abp1, or NMIIA, prolongs and/or increases BCR signaling by enhancing the activation of stimulatory kinases and suppressing the activation of inhibitory phosphatases, which elevates

the production levels of autoantibodies in mice^{231,242,243}. Besides the enhancement effect of Abp1 on the activation of the BCR signaling adaptor protein BLNK through recruiting hematopoietic protein kinase (HPK1)⁶⁷, N-WASP and NMIIA are not known to interact with kinases and phosphatases directly. The growth, merger, and centralization of antigen-BCR complexes at the BCR contact zone during the B cell contraction phase are associated with the reduced signaling capability of BCRs^{203,231,242}. These findings revealed that B cell contraction is one of the mechanisms for inducing BCR signaling attenuation. How B cell contraction transitions BCR signaling from amplification to attenuation is unknown.

This study explored the mechanisms by which the actin cytoskeleton remodels from spreading lamellipodia to contractile structures and by which B cell contraction suppresses BCR signaling using planar lipid bilayer (PLB)-based stimulatory surfaces and total internal fluorescence microscopy (TIRF). Here, we show that the contractile actomyosin structures responsible for B cell contraction originate from branched actin at spreading lamellipodia. N-WASP/Arp2/3- mediated actin polymerization prolongs the lifetime of the lamellipodial actin structures, enables NMIIA recruitment, and drives their movement to the center of the B cell contact zone. B cell contraction increases the molecular density within individual BCR-antigen clusters, which induces the disassociation of both the stimulatory kinase Syk and the inhibitory phosphatases SHIP-1 from BCR clusters, leading to signal attenuation. Our study reveals a new mechanism underlying BCR signal downregulation, controlling autoantibody production.

2.3 Results

2.3.1 Arp2/3, activated by N-WASP but not WASP, is required for B cell contraction

Arp2/3-generated branched actin is known to drive lamellipodia for B cell spreading^{239,245}. However, whether branched actin is involved in B cell contraction following spreading was unknown. To address this question, we perturbed the polymerization of branched actin using the Arp2/3 inhibitor CK-666 (50 μ M) and its non-functional derivative CK-689 as a control. Pre-warmed splenic B cells from WT C57BL/6 mice were incubated with monobiotinylated Fab' fragment of anti-mouse IgM+G attached to planar lipid bilayers (Fab'-PLB) by biotin-streptavidin interaction at 37°C and imaged live using interference reflection microscopy (IRM). The contact area of B cells treated with CK-689 rapidly increased upon landing on Fab'-PLB and reached a maximal at ~0.5 min, indicating spreading (Supplementary Figure S1A and video 1A). Following the maximal spreading, most B cells reduced their contact zone, indicating contraction (Supplementary Figure S1A and Video 1A). Based on the morphological dynamics, we treated B cells with CK-666 at the beginning of the incubation (Time 0) before B cell spreading or at 2 min when B cells had already spread. The effectiveness of CK-666 was detected by reductions in Arp2/3 staining in the contact zone (Supplementary Figure S2). As expected, CK-666 treatment at 0 but not 2 min reduced the kinetics of B cell spreading (Supplementary Figure S3). Importantly, CK-666 treatment at 0 and 2 min both significantly reduced the percentage of B cells undergoing contraction (Figure 2.1A-C and Supplementary Video 1A). The inhibitory effect of CK-666 on B cell contraction, particularly CK-666 treatment after B cell spreading, suggests that Arp2/3-mediated branched actin polymerization is required for B cell contraction.

WASP and N-WASP are two actin nucleation promoting factors upstream of Arp2/3 expressed in B cells^{237,246}. To determine if both or one of the two are responsible for

activating Arp2/3 for B cell contraction, we utilized an N-WASP inhibitor, Wiskostatin (Wisko; 10 μ M), and splenic B cells from B cell-specific N-WASP knockout mice (cNKO) and WASP knockout (WKO) mice^{247,248}. Wisko has been shown to inhibit N-WASP activation (Supplementary Figure S4A) while enhancing WASP activation in B cells (Supplementary Figure S4B). We found that both Wisko (Figure 2.1D, E, and Supplementary Video 1B) and cNKO (Figure 2.1F, G, and Supplementary Video 1C), but not WKO (Figure 2.1H, I, and Supplementary Video 1D), significantly reduced the percentage of B cells undergoing contraction, compared to the vehicle, flox, or WT control. These results suggest that N-WASP- but not WASP-activated Arp2/3 mediates branched actin polymerization for B cell contraction.

Figure 2.1

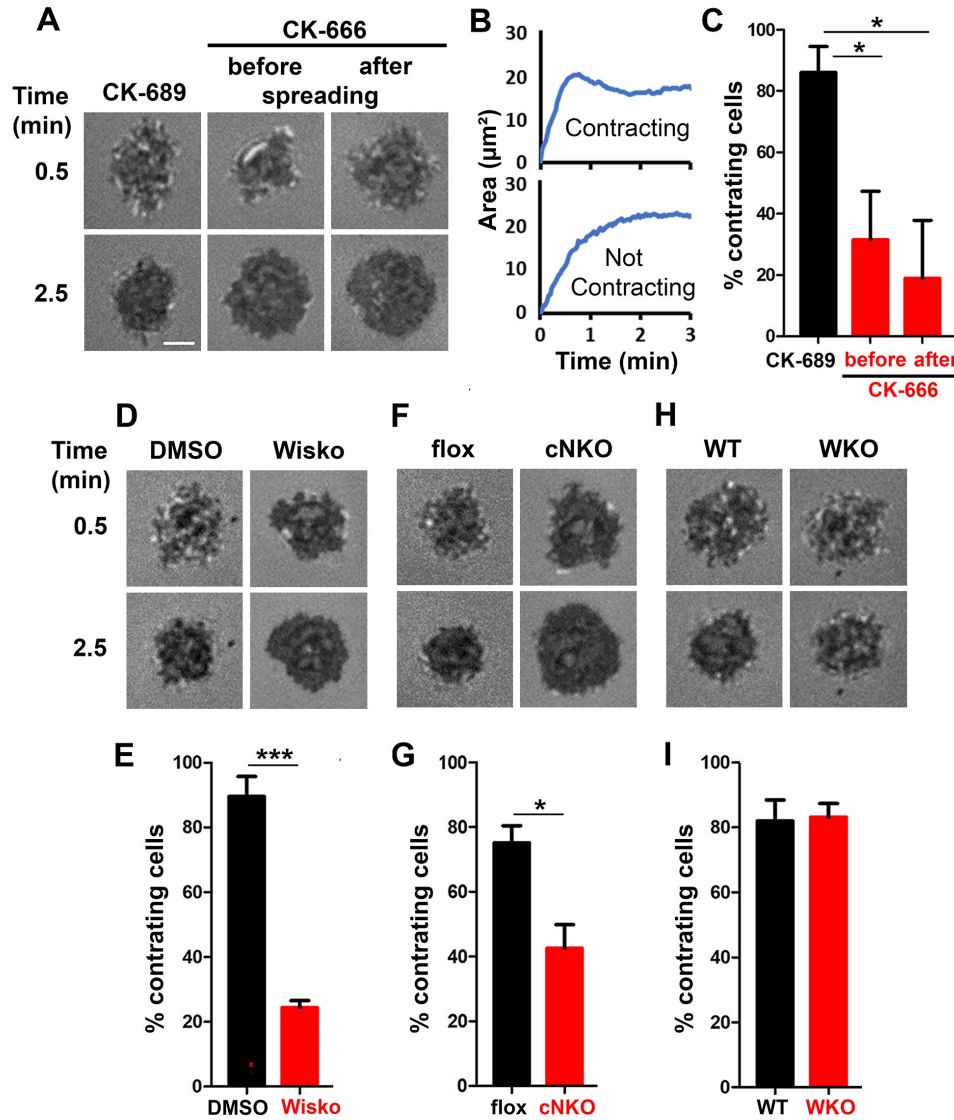


Figure 2.1. Arp2/3, activated by N-WASP but not WASP, is required for B cell contraction. Splenic B cells were incubated with planar lipid bilayers coated with monobiotinylated Fab' fragment of goat anti-mouse IgG+M (Fab'-PLB) in the absence and presence of various inhibitors and imaged live at 37°C by interference reflection microscopy (IRM). The B cell plasma membrane area contacting with Fab'-PLB (B cell contact zone) was measured using IRM images and custom codes made in MATLAB. **(A)** Representative

IRM images of splenic B cells from C57BL/6 mice treated with CK-689 or CK-666 (50 μ M) before (0 min) and after maximal spreading (2 min). **(B)** Representative plots of the B cell contact area versus time from one contracting cell and one non-contracting cell. **(C)** Percentages ($\pm$ SEM) of CK-666-treated B cells that underwent contraction compared to those of CK-689-treated cells. **(D)** Representative IRM images of splenic B cells from C57BL/6 mice treated with DMSO or Wiskostatin (Wisko, 10 μ M) 10 min before and during incubation with Fab'-PLB. **(E)** Percentages ($\pm$ SEM) of Wisko-treated B cells that underwent contraction compared to those of DMSO-treated cells. **(F)** Representative IRM images of splenic B cells from flox control and B cell-specific N-WASP knockout (cNKO) mice. **(G)** Percentages ($\pm$ SEM) of cNKO B cells that underwent contraction compared to flox control cells. **(H)** Representative IRM images of splenic B cells from WT or WASP knockout mice (WKO). **(I)** Percentages ($\pm$ SEM) of WKO B cells that underwent contraction compared to those of WT cells. Data were generated from three independent experiments, with $\sim$ 25 cells per condition, per experiment. Scale bar, 2 μ m. * p < 0.05, *** p < 0.001, by paired student's t -test.

2.3.2 Arp2/3, downstream of N-WASP, generates inner F-actin foci, driving B cell contraction

To understand how Arp2/3 transitions B cells from spreading to contraction, we identified actin structures associated with contracting B cells that were sensitive to CK-666 treatment. We visualized F-actin by phalloidin staining and compared F-actin organization in the contact zone of B cells at the spreading (2 min) and contraction (4 min) phases using TIRF. While B cells in both spreading and contraction phases exhibited phalloidin staining outlining the contact zone, only B cells at the contracting phase showed phalloidin patches brighter than phalloidin staining outlining the contact zone. These actin patches were organized into a ring-like structure, resided $\sim 1 \mu\text{m}$ behind the spreading front, and surrounded an actin-poor center (Figure 2.2A). Here, we named these actin patches inner actin foci. We identified inner actin foci based on their peak fluorescence intensity (FI), ≥ 2 folds of the mean fluorescence intensity (MFI) of phalloidin in the no foci area, diameters, $\geq 250 \text{ nm}$, and locations, $1 \mu\text{m}$ away from the outline of the contact zone. We found that such actin inner foci were detected in $>60\%$ B cells in the contracting phase (4 min) but only in $<20\%$ B cells in the spreading phase (2 min) (Figure 2.2B). CK-666 treatment at 0 min, which inhibited B cell contraction, significantly reduced the percentage of B cells showing actin inner foci at 4 min but not at 2 min (Figure 2.2B). However, the phalloidin staining outlining the contact zone remained prominent in B cells that lost actin inner foci (Figure 2.2A). Similarly, cNKO drastically reduced the percentage of B cells showing actin inner foci at 4 min but not at 2 min (Figure 2.2C). These results suggest that the formation of these inner actin foci is associated with B cell contraction.

We further quantified the number of inner actin foci in individual B cells utilizing B cells from mice expressing the LifeAct-GFP transgene²⁴⁹ (Figure 2.2D, E, and G), which allowed us to image F-actin reorganization live (Supplementary Video 2), and phalloidin staining for

flox control and cNKO B cells (Figure 2.2F). Consistent with what we had seen with phalloidin staining, CK-666 treatment at time 0 significantly reduced the number of inner actin foci in the contact zone (Figure 2.2D and Supplementary Video 2A, B). When we followed the same B cells before and after CK-666 treatment at 2 min, many of the inner actin foci formed at 1 min 50 sec before the treatment disappeared after the treatment (2 min 20 sec), significantly reducing the number of inner actin foci (Figure 2.2E). Similar to CK-666 treatment, cNKO significantly reduced the number of inner actin foci in the contact zone, compared to flox controls (Figure 2.2F). In contrast, WKO, which does not affect B cell contraction, also did not significantly change the number of inner actin foci formed, compared to WT control B cells (Figure 2.2G and Supplementary Video 2C, D). Thus, N-WASP- but not WASP-activated Arp2/3 drives the formation and the maintenance of contraction-associated inner actin foci.

Figure 2.2

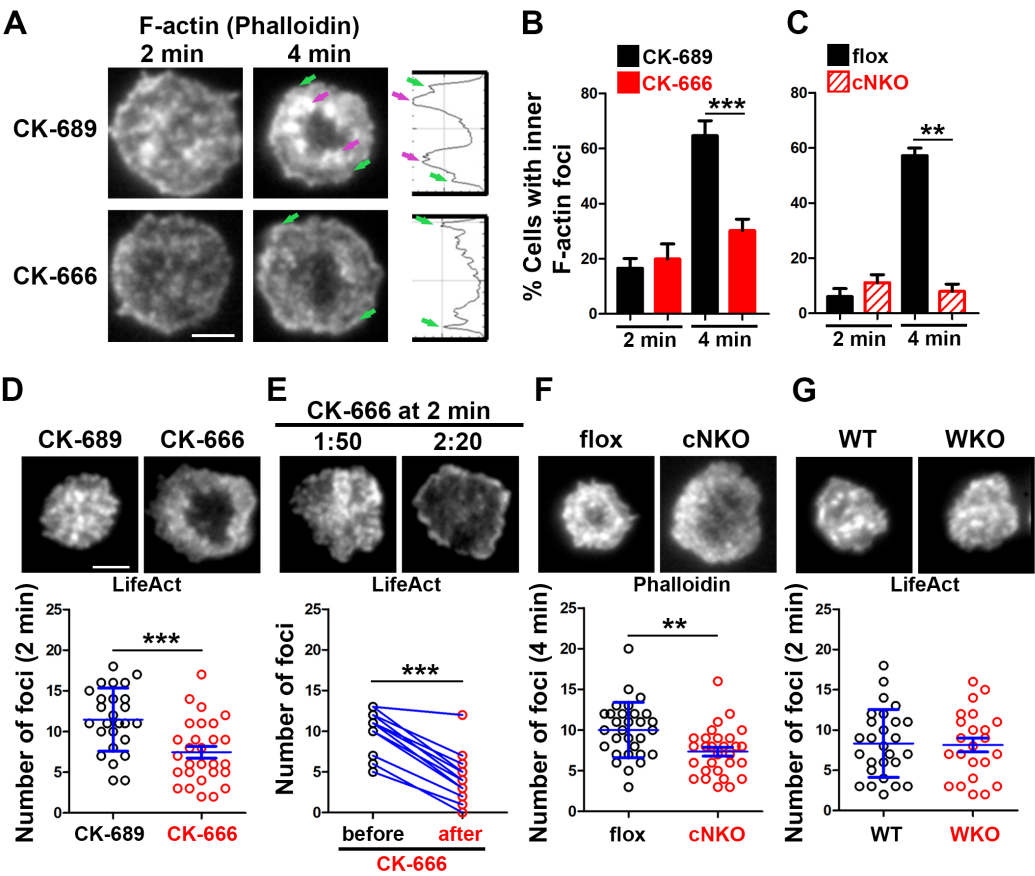


Figure 2.2. Arp2/3, downstream of N-WASP, generates inner F-actin foci, driving B cell contraction. (A-C) WT splenic B cells were treated with CK-689 or CK-666 (50 μ M) before and after maximal spreading during incubation with Fab'-PLB (A-B), and flox control and cNKO B cells were incubated with Fab'-PLB at 37°C (C). Cells were fixed at 2 and 4 min, permeabilized, stained for F-actin with phalloidin, and analyzed using TIRF. Shown are representative TIRF images of phalloidin staining in the contact zone of CK-689 and CK-

666-treated B cells and fluorescence intensity (FI) profiles of phalloidin staining along arrow crossing cells **(A)**. Green arrows indicate lamellipodial F-actin, and purple arrows indicate inner actin foci forming a ring-like structure. Percentages of cells (per image) ($\pm$ SEM) with inner F-actin foci forming ring-like distribution among CK-689 versus CK-666 treated cells **(B)** and flox control versus cNKO B cells **(C)** before (2 min) and after (4 min) contraction were determined by visual inspection of phalloidin FI line-profiles across the B cell contact zones. Data were generated from 3 independent experiments with 5 images per condition per experiment and $\sim$ 15 cells per image. **(D-G)** Inner F-actin foci were identified by their diameter ($\geq$ 250 nm), peak FI ($\geq$ 2 fold of no foci area, and location (1 μ m away from the outer edge) and quantified as the number per cell using TIRF images. **(D)** Shown are representative images of splenic B cells from LifeAct-GFP-expressing mice treated with CK-689 or CK-666 from 0 min during incubation with Fab'-PLB at 37°C and the average number ($\pm$ SEM) of inner LifeAct-GFP foci per cell at 2 min. **(E)** LifeAct-GFP B cells were treated with CK-666 at 2 min. Shown are representative TIRF images of LifeAct-GFP in the contact zone of B cells (top) and the average number ($\pm$ SEM) of inner LifeAct-GFP foci (bottom) in the same cell 10s before and 20s after CK-666 treatment. **(F)** Shown are representative TIRF images of phalloidin-stained in the contact zone of flox control and cNKO B cells after incubating with Fab'-PLB for 2 min (top) and the average number ($\pm$ SEM) of inner F-actin foci per cell (bottom). **(G)** Shown are representative TIRF images of WT and WKO B cells that express LifeAct-GFP incubated with Fab'-PLB for 2 min and the average number ($\pm$ SEM) of inner F-actin foci per cell (bottom). Data points represent individual cells from three independent experiments with 10 **(D, F, and G)** or 6 **(E)** cells per condition per experiment. Scale bar, 2 μ m. ** $p < 0.01$, *** $p < 0.001$, by non-parametric student's t -test.

2.3.3 Inner F-actin foci are derived from lamellipodia behind the spreading membrane

Utilizing live-cell TIRF imaging of B cells from mice expressing LifeAct-GFP, we examine the formation process of contraction-associated inner actin foci. We randomly generated eight kymographs from time-lapse images of LifeAct-GFP from each cell (Figure 2.3A and Supplementary Figure S5). From kymographs, we found that most actin foci were first detected closely behind lamellipodia. Upon maximal spreading, these actin foci moved centripetally while gaining intensity, becoming inner actin foci, in cells undergoing spreading to contracting transition (Figure 2.3B, top panels). In contrast, actin foci behind lamellipodia quickly disappeared and regenerated after the maximal spreading in B cells not undergoing contraction (Figure 2.3B, bottom panels). To confirm this observation, we quantified the percentage of the eight kymographs from each cell exhibiting lamellipodia-derived inner actin foci and found six of the eight kymographs from contracting cells showed lamellipodia-derived inner actin foci, compared to one or two kymographs from non-contracting cells (Figure 2.3C). To examine the temporal relationship between the generation of lamellipodia-derived inner actin foci and contraction, we plotted the percentage of kymographs with lamellipodia-derived inner actin foci over time with the spreading to contracting transition time as 0 (Figure 2.3D). We found that the percentage of kymographs showing lamellipodia-derived inner actin foci peaked at almost the exact time for the spreading to contracting transition (Figure 2.3D), suggesting a close temporal relationship between the two events. Furthermore, the N-WASP inhibitor Wisko, but not WKO, significantly inhibited the formation of lamellipodia-derived inner actin foci (Figure 2.3E). Thus, inner actin foci originate from branched actin driving lamellipodia and form simultaneously with the B cell spreading to contracting transition.

Figure 2.3

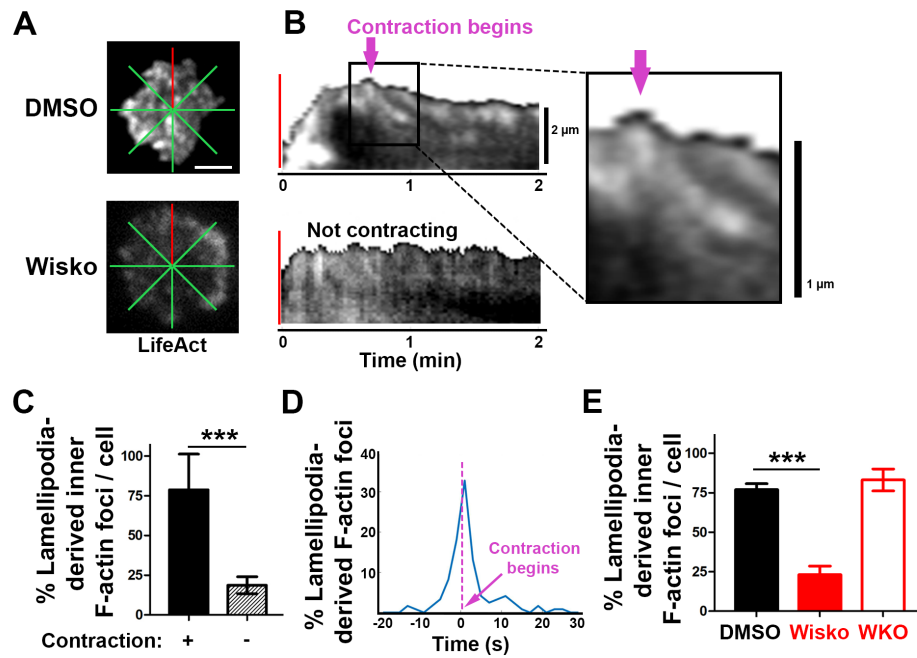


Figure 2.3. Inner F-actin foci are originated from lamellipodia behind the spreading membrane. Mouse splenic B cells from LifeAct-GFP transgenic mice were treated with DMSO or Wisko (10 μ M), imaged live using TIRF during incubation with Fab'-PLB at 37°C, and analyzed using kymographs generated by NIH ImageJ. **(A)** One frame of TIRF time-lapse images of LifeAct-GFP in the contact zone of B cells treated with DMSO or Wisko. Lines indicate eight kymographs that were randomly generated from each cell. **(B)**

Representative kymographs were generated from TIRF time-lapse images of LifeAct-GFP at the red line in (A). Top panel, a contracting cell. Arrows indicate the start of contraction with inner F-actin foci originating from lamellipodia. Bottom panel, a non-contracting cell. Lamellipodia-derived inner F-actin foci were identified by their LifeAct-GFP FI >2 fold of their nearby region, inside location in the contact zone, migrating out the lamellipodial F-actin, and trackable for >8 sec. **(C)** Percentages ($\pm$ SEM) of kymographs showing inner F-actin foci originating from lamellipodia per cell that did and did not undergo contraction. Data were generated from 3 independent experiments with ~10 cells per condition per experiment. **(D)** A histogram of inner F-actin foci emerging (expressed as percentages of the total events, blue line) over time relative to the time of B cell contraction (defined as 0 sec, indicated by a purple dash line and arrow). Data were generated from 5 independent experiments with ~9 cells per condition per experiment. **(E)** Percentage ($\pm$ SEM) of inner F-actin foci originated from lamellipodia observed in 8 randomly positioned kymographs of each DMSO- or Wisko-treated WT or untreated WKO B cell. Data were generated from 3 independent experiments with ~10 cells per condition per experiment. Scale bars, 2 μ m. *** $p < 0.001$, by non-parametric student's t -test.

2.3.4 N-WASP-activated Arp2/3 generates inner actin foci by sustaining the lifetime and the centripetal movement of lamellipodia actin

We examined the mechanism by which N-WASP and Arp2/3 generate inner actin foci by measuring the relative lifetime and mobility of inner actin foci using kymographs generated from TIRF time-lapse images of B cells expressing LifeAct-GFP. Inner actin foci were identified as described above, and their moving tracks were manually determined (Figure 2.4A, black dashed lines). The time window that an inner actin patch could be detected in a kymograph was measured as the relative lifetime, as actin foci could move away from the kymograph line (Figure 2.4A). The distance each actin foci moved during its lifetime was used to calculate its moving speed (Figure 2.4A). Compared to CK-689-treated B cells, CK-666 treatment at time 0 significantly reduced the relative lifetime and the centripetal moving speed of inner actin foci (Figure 2.4B-D). When treating with CK-666 at 2 min when B cells had spread, the relative lifetime and the centripetal moving speed of inner actin foci were significantly lower after than before the treatment in the same cell and the same kymograph (Figure 4E-G, line-linked dots). Similarly, the N-WASP inhibitor Wisko significantly reduced the relative lifetime and the centripetal moving speed of inner actin foci (Figure 2.4H-J); however, WKO had no significant effect (Figure 2.4K-M). These results show that N-WASP and Arp2/3 mediated branched actin polymerization prolongs the lifetime of lamellipodia-derived actin foci and drives them to move inward in the B cell contact zone.

Figure 2.4

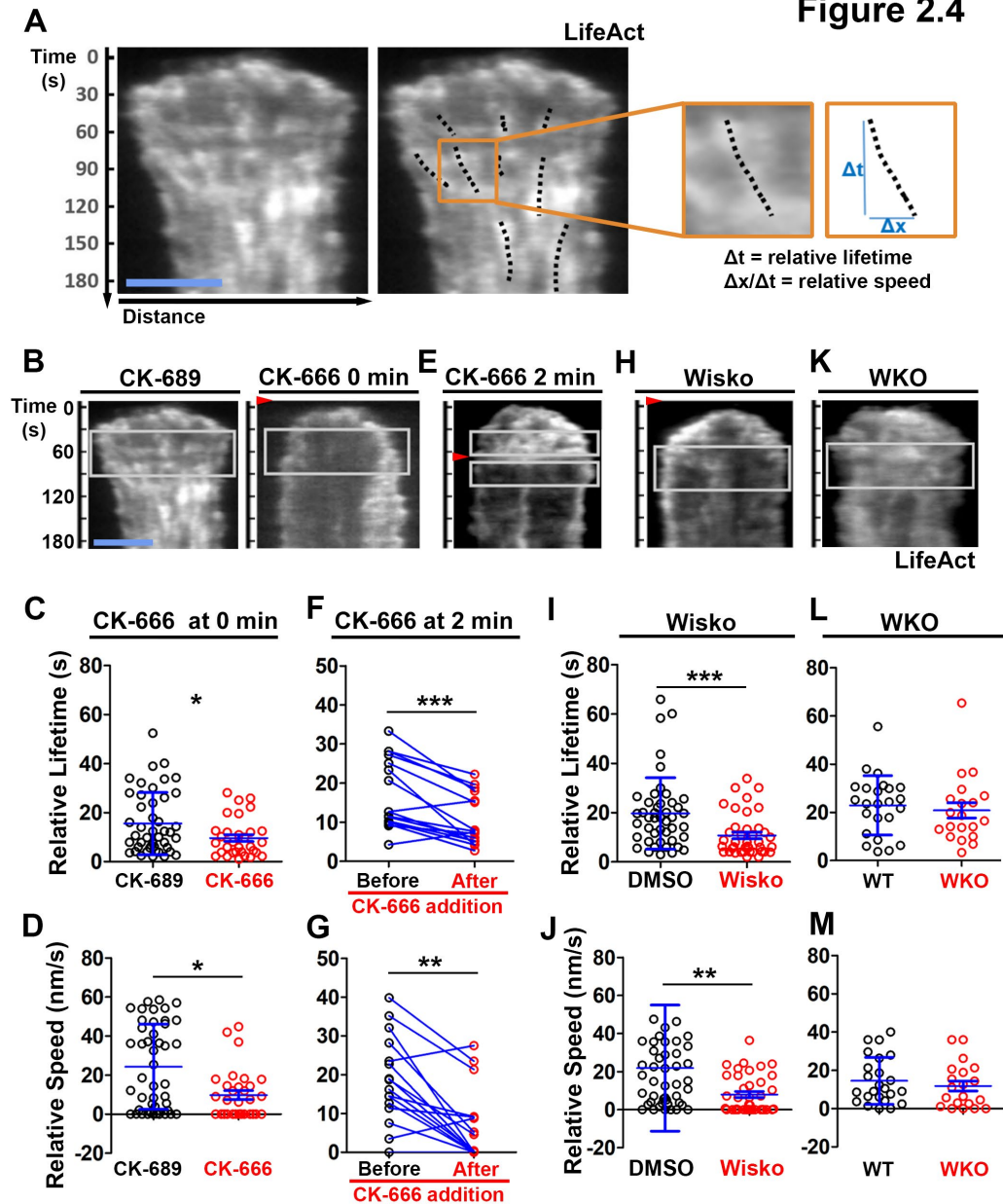


Figure 2.4. N-WASP-activated Arp2/3 sustains the lifetime and the centripetal movement of inner F-actin foci. LifeAct-GFP-expressing B cells were treated with or without various inhibitors and imaged live by TIRF during interaction with Fab'-PLB at 37°C. Three randomly positioned kymographs were generated for each cell using time-lapse images. **(A)** A representative kymograph from TIRF time-lapse images of a DMSO-treated B cell (Left panels). Inner F-actin foci were identified as described in Figure 3, and those that

can be tracked for at least 4 seconds (dashed lines) during the 60 sec after maximal spreading (white rectangles in B) in individual kymographs were analyzed. Relative lifetimes of inner F-actin foci were measured using the duration each focus could be detected in a kymograph. The relative distances traveled by the foci were measured using the displacement of each focus in a kymograph. Relative speed was calculated for each inner F-actin focus by dividing its relative distance by its relative lifetime (right panels). **(B-D)** B cells were treated with CK-689 or CK-666 (50 μ M) from the beginning of the incubation with Fab'-PLB (0 min). Shown are representative kymographs of a CK-689- and a CK-666-treated B cell **(B)**, relative lifetimes **(C)**, and relative speed **(D)** of inner F-actin foci in CK-689- versus CK-666-treated B cells. **(E-G)** B cells were treated with CK-666 at the time of maximal spreading (2 min). Shown are a representative kymograph of a CK-666-treated cell **(E)**, relative lifetimes **(F)**, and relative speeds **(G)** of inner F-actin foci in 30-sec windows before the inhibition and 10 sec after the inhibition in the same cells (linked by blue lines). **(H-J)** B cells were treated with DMSO or Wisko (10 μ M) 10 min before and during interaction with Fab'-PLB. Shown are a representative kymograph of a Wisko-treated B cell **(H)**, relative lifetimes **(I)**, and relative speed **(J)** of inner F-actin foci in DMSO versus Wisko-treated B cells. **(K-M)** LifeAct-GFP-expressing WT and WKO B cells were incubated with Fab'-PLB. Shown are a representative kymograph of a WKO B cell **(K)**, relative lifetimes **(L)**, and relative speeds **(M)** of WT versus WKO B cells. Data points represent the averaged values from inner F-actin foci in individual cells, with 3 kymographs per cell and ~12 cells per condition per experiment from 3 independent experiments. Scale bar, 2 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by non-parametric and paired student's *t*-test.

2.3.5 Inner actin foci, generated by N-WASP-activated Arp2/3, facilitate NMII recruitment and reorganization into ring-like structures

As non-muscle myosin II (NMII) is required for B cell contraction²⁴², we examined the relationship between the formation of inner actin foci and the recruitment and reorganization of NMII using B cells from mice expressing GFP-NMIIA transgene and TIRF. Upon interacting with Fab'-PLB, the GFP-NMIIA MFI in the contact zone of untreated WT B cells increased rapidly in the first minute and slowly afterward (Figure 2.5A, B and Supplementary Video 3). Treatment of the N-WASP inhibitor Wiskin significantly reduced the GFP-NMIIA MFI in the contact zone and its initial increase rate (determined by the slope of the GFP-NMIIA MFI increase) (Figure 2.5A-C). Kymographs generated from time-lapse TIRF images of B cells from mice expressing GFP-NMIIA and RFP-LifeAct showed that recruited NMIIA was accumulated between lamellipodia and inner actin foci when the foci moved from lamellipodia centripetally (Figure 2.5D and Supplementary Video 3). Recruited NMIIA reorganized with inner actin foci to form a ring-like structure in the contact zone (Figure 2.5A, D, E, and Supplementary Video 3). The percentage of B cells with NMIIA ring-like structures increased over time as more B cell underwent contraction (Figure 2.5E, F). The percentage of cNKO B cells with NMIIA ring-like structures, visualized by immunostaining, was significantly lower than that of flox control B cells (Figure 2.5D, E). The N-WASP inhibitor Wiskin also reduced NMIIA recruitment and ring-like structure formation (Supplementary Video 3). Surprisingly, the percentage of WKO B cells with NMIIA ring-like structure was higher than that of flox control B cells (Figure 2.5D, E). Thus, N-WASP and Arp2/3 mediated branched actin polymerization promotes the recruitment and the reorganization of NMII ring-like structures by generating inner actin foci.

Figure 2.5

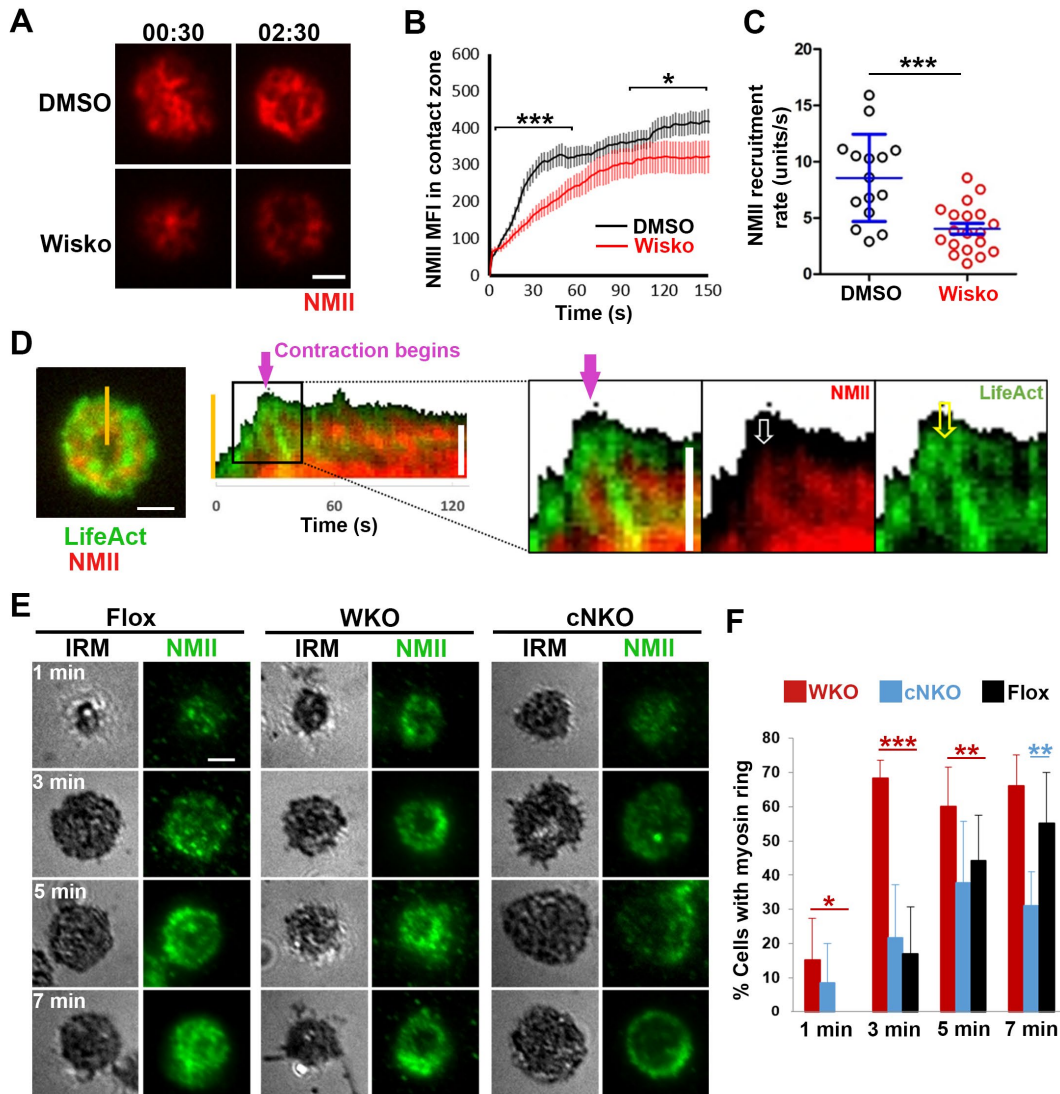


Figure 2.5. Inner F-actin foci, generated by N-WASP-activated Arp2/3, facilitate NMII recruitment and ring-like structure formation. (A-C) B cells from mice expressing the GFP fusion of non-muscle myosin IIA (GFP-NMIIA) transgene were treated with DMSO or Wisko (10 μ M) 10 min before and during incubation with Fab'-PLB. The B cell contact zones were imaged live using TIRF. Shown are representative TIRF images of DMSO- and Wisko-treated B cells at 30 sec (during spreading) and 2 min 30 sec (after maximal

spreading) post landing **(A)**, Scale bars, 2 μm), the averaged GFP-NMIIA MFI ($\pm\text{SEM}$) **(B)**, and the initial increasing rates ($\pm\text{SEM}$) of GFP-NMIIA in the contact zone (the slope of the initial GFP-NMIIA MFI versus time curves of individual cells) **(C)** of DMSO- and Wisko-treated B cells. Data points represent individual cells from 3 independent experiments with ~ 6 cells per condition per experiment. * $p < 0.05$, *** $p < 0.001$, by Kolmogorov-Smirnov test **(B)** or non-parametric student's t -test **(C)**. **(D)** Primary B cells from mice expressing both GFP-NMIIA and LifeAct-RFP transgenes were incubated with Fab'-PLB at 37°C and imaged live by TIRF. Shown are a representative TIRF image of a cell and a kymograph generated from time-lapse TIRF images at the yellow line. The purple arrow indicates the starting point of contraction, the white arrow GFP-NMIIA recruitment proximal to the spreading membrane, and the yellow arrow an F-actin (LifeAct-RFP) focus originating at the lamellipodia and moving away from the spreading membrane. **(E, F)** Primary B cells from flox control, WKO, and cNKO mice were incubated with Fab'-PLB for indicated times. Cells were fixed, permeabilized, stained for NMII light chain, and imaged by IRM and TIRF. Shown are representative TIRF images **(E)** and percentages ($\pm\text{SD}$) of B cells with the NMII ring-like structure in individual images **(F)**, identified by visual inspection. The data were generated from 3 independent experiments with 5 images per condition per experiment. Scale bars, 2 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by non-parametric student's t -test.

2.3.6 B cell contraction increases the BCR molecular density in individual clusters

To understand how B cell contraction promotes BCR signaling attenuation, we examined the impact of B cell contraction on the properties of BCR clusters. We first measure the MFI of AF546-Fab' attached to PLB gathered by interacting B cells into the contact zone as an indication of the overall BCR molecular density (Figure 2.6A and Supplementary Video 4). The clustering of AF546-Fab' on PLB by B cell binding directly reflected surface BCR clustering, as B cell binding to transferrin (Tf)-tethered PLB did not cause surface BCRs to cluster²⁴². The MFI of AF546-Fab' in the contact zone of untreated WT B cells increased over time (Figure 2.6A-G and Supplementary Video 4). Notably, the increasing rates of AF546-Fab' MFI were significantly higher during B cell contraction than before B cell contraction in all control cells and conditions (Figure 2.6D-K). Significantly, inhibiting B cell contraction by CK-666 treatment at 0 (Figure 2.6A, D, H, and Supplementary Video 4A, B) or 2 min (Figure 6A, E, I), the N-WASP inhibitor Wisko (Figure 2.6B, F, J, and Supplementary Video 4C, D), and cNKO (Figure 2.6C, G, K, and Supplementary Video 4E, F) all abolished the increases in AF546-Fab' gathering rates. We further examined the peak FI of AF546-Fab' in individual microclusters to reflect the BCR molecular density in individual clusters. AF546 clusters were identified based on their diameters ≥ 250 nm, peak FI ≥ 1.1 fold outside the B cell contact zone, and trackable for ≥ 20 sec (Figure 2.6L and Supplementary Figure S6). Most of AF546-Fab' microclusters could be identified only after B cell spreading. Time-lapse imaging by TIRF enabled us to measure the increasing rate of AF546-Fab' peak FI in individual clusters (Figure 2.6M and Supplementary Figure S6). Consistent with our finding with the AF546-Fab' MFI in the contact zone, the peak FI of individual clusters increased at faster rates during contraction than after contraction (Figure 2.6N). Furthermore, CK-666 (Figure 2.6O), Wisko (Figure 2.6P), and cNKO (Figure 2.6Q) all significantly reduced the increasing rate of the AF546-Fab' peak FI in individual clusters.

These results show that B cell contraction significantly increased the molecular density of BCRs in BCR clusters.

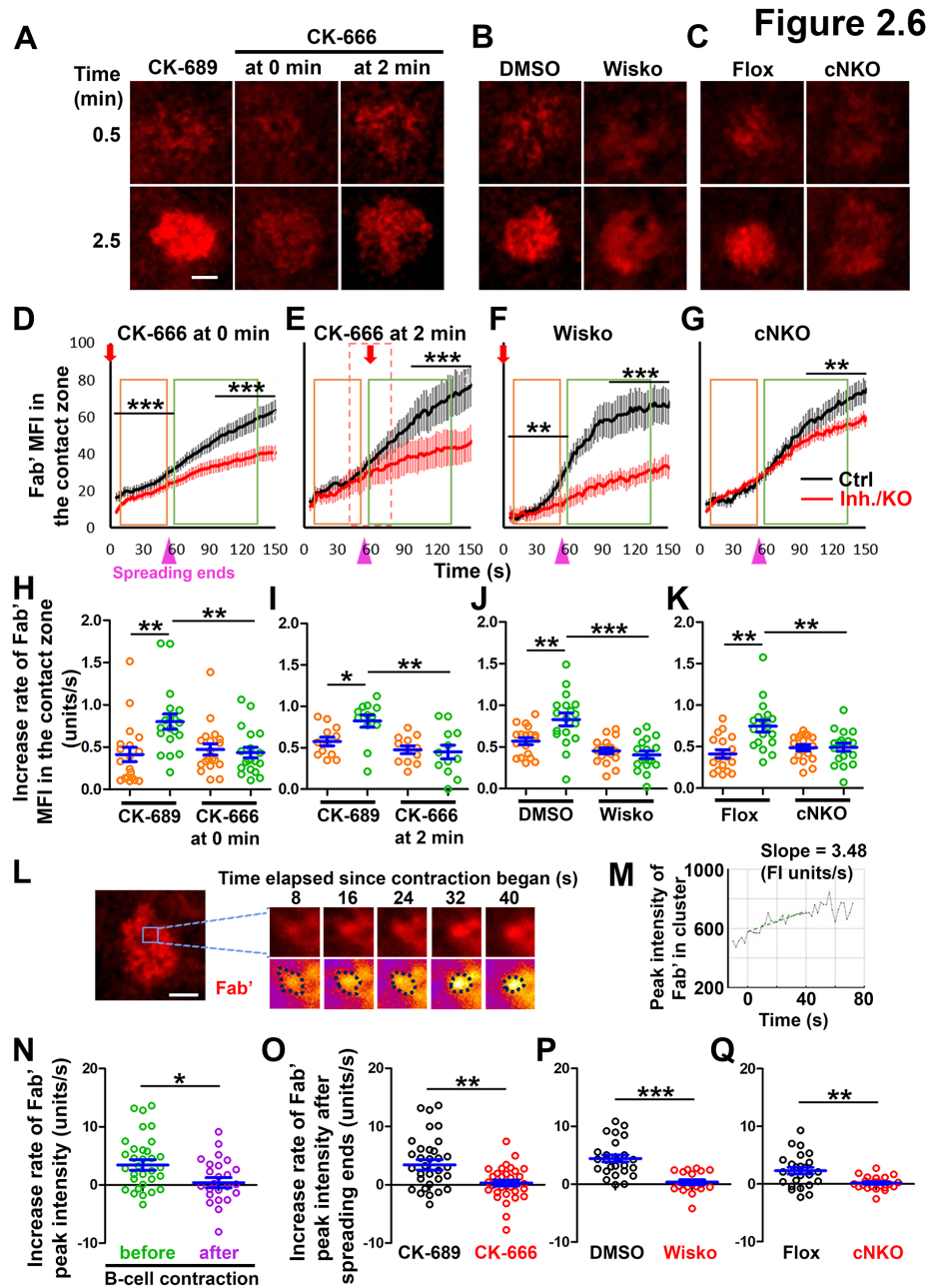


Figure 2.6. B cell contraction increases the molecular density within BCR clusters.

Primary B cells from WT mice were treated with CK-689 or CK-666 from the beginning of the incubation with AF546-Fab'-PLB (0 min) or at the maximal B cell spreading (2 min) (A, D, E, H, I, N, O). WT B cells were treated with DMSO or Wisko (10 μ M) 10 min before and during the incubation with AF546-Fab'-PLB (B, F, J, P). B cells from flox control and cNKO mice were incubated with AF546-Fab'-PLB (C, G, K, Q). The B cell contact zones

were imaged live by TIRF. **(A-C)** Representative frames from time-lapse images at 30 sec (during B cell spreading) and 2 min 30 sec (after maximal spreading). Scale bars, 2 μm . **(D-G)** The MFI of AF546-Fab' in the contact zone was plotted over time. **(H-K)** Rates of AF546-Fab' MFI increases in the B cell contact zone during spreading (orange rectangles in D-G) versus during contraction (green rectangles in D-G) were determined by the slope of AF546-Fab' MFI versus time plots. Data were generated from 3 independent experiments with 6~12 cells per experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by Kolmogorov-Smirnov test **(D-G)** or paired student's t -test **(H-K)**. **(L)** Representative frame from a time-lapse of a CK-689 treated B cell (left) showing AF546-Fab' clusters 30 seconds after spreading ended and contraction began, and enlarged time-lapse images (right) show a single AF546-Fab' cluster over a 40-sec time window since contraction began. AF546 clusters were identified using the criteria: ≥ 250 nm in diameter, ≥ 1.1 fold of FI outside the B cell contact zone, and trackable for ≥ 20 sec. Scale bars, 2 μm . **(M)** The peak FI of AF546-Fab' cluster was measured over time, and the increasing rates of AF546-Fab' peak FI of individual clusters were determined by the slope of the plot. **(N-Q)** The increasing rates ($\pm$ SEM) of AF546-Fab' in individual clusters were compared between a time window during contraction and a time window after contraction ends, between B cells treated with CK-689 and CK-666 from 0 min **(O)**, between DMSO- and Wisko-treated B cells **(P)**, and between flox control and cNKO B cells **(Q)** during contraction or the same time window in non-contracting B cells. Data points represent individual cells, the averaged slopes of clusters detected in one B cell, from 3 independent experiments with 6~12 cells per condition per experiment. * $p < 0.05$, * $p < 0.01$, *** $p < 0.001$, by non-parametric student's t -test.

2.3.7 Increased BCR molecular density by B cell contraction reduces BCR phosphorylation levels in individual microclusters

To understand how BCR molecular density influenced BCR signaling capability, we examined the relationship between the MFI ratio of phosphorylated CD79a (pCD79a, Y182) to AF546-Fab' (reflecting the relative level of BCR phosphorylation) and AF546-Fab' peak FI (reflecting BCR molecular density) in individual clusters. We randomly collected equal numbers of AF546-Fab' clusters from B cells interacting with Fab'-PLB for 1, 3, 5, and 7 min, using a gradient threshold of their AF546-Fab' MFI, 1.1 to 4.1 fold higher than the background, and with diameter ≥ 250 nm (≥ 150 clusters per time-point, per experiment from 3 independent experiments). The dot plot showed that when the AF546-Fab' peak FI of individual clusters was relatively low, the MFI ratios of pCD79a to AF546-Fab' increased with the AF546-Fab' peak FI in individual clusters of WT C57BL/6 and flox control B cells (Figure 2.7 A-D). When the AF546-Fab' peak FI reached a certain level, the MFI ratios of pCD79a to AF546-Fab' decreased as the AF546-Fab' peak FI in individual clusters further increased (Figure 2.7 A-D). Inhibiting B cell contraction by cNKO kept the AF546-Fab' peak intensity in individual clusters within a relatively low range, where the MFI ratios of pCD79a to AF546-Fab' increased with the AF546-Fab' peak FI (Figure 2.7B, E). Additionally, the average pCD79a to Fab' MFI ratios were much higher in cNKO B cells than flox control B cells, when comparing AF546-Fab' clusters with the same range of peak intensity, (Figure 2.7D, E). Similarly, inhibiting B cell contraction using the Arp2/3 inhibitor CK-666 treatment at 0 min reduced AF546-Fab' MFI (Figure 2.7F) but increased pCD79a MFI (Figure 2.7G) and the MFI ratios of pCD79a relative to AF546-Fab' (Figure 2.7H) in individual clusters. When comparing spreading stage (1 and 3 min), flox control B cells increased AF546-Fab' MFI (Figure 2.7I) but decreased pCD79a MFI (Figure 2.7J) and the MFI ratios of pCD79a relative to AF54-Fab' (Figure 2.7K) in individual clusters at the contraction stage (5 and 7

min). In contrast, non-contractive cNKO B cells only slightly increased AF546-Fab' MFI (Figure 2.7I) but significantly increased pCD79a MFI (Figure 2.7J) and the pCD79a to AF546-Fab' MFI ratios (Figure 2.7K) in individual clusters at the contraction stage compared to the spreading stage. Consequently, individual clusters in non-contractive cNKO B cells had significantly lower AF546-Fab' MFI but significantly higher pCD79a MFI and pCD79a to AF546-Fab' FIRs than contractive flox control B cells at 5 and 7 min but not at 1 and 3 min (Figure 2.7I-K). These data suggest that increases in BCR molecular density in BCR clusters by B cell contraction inhibit BCR phosphorylation.

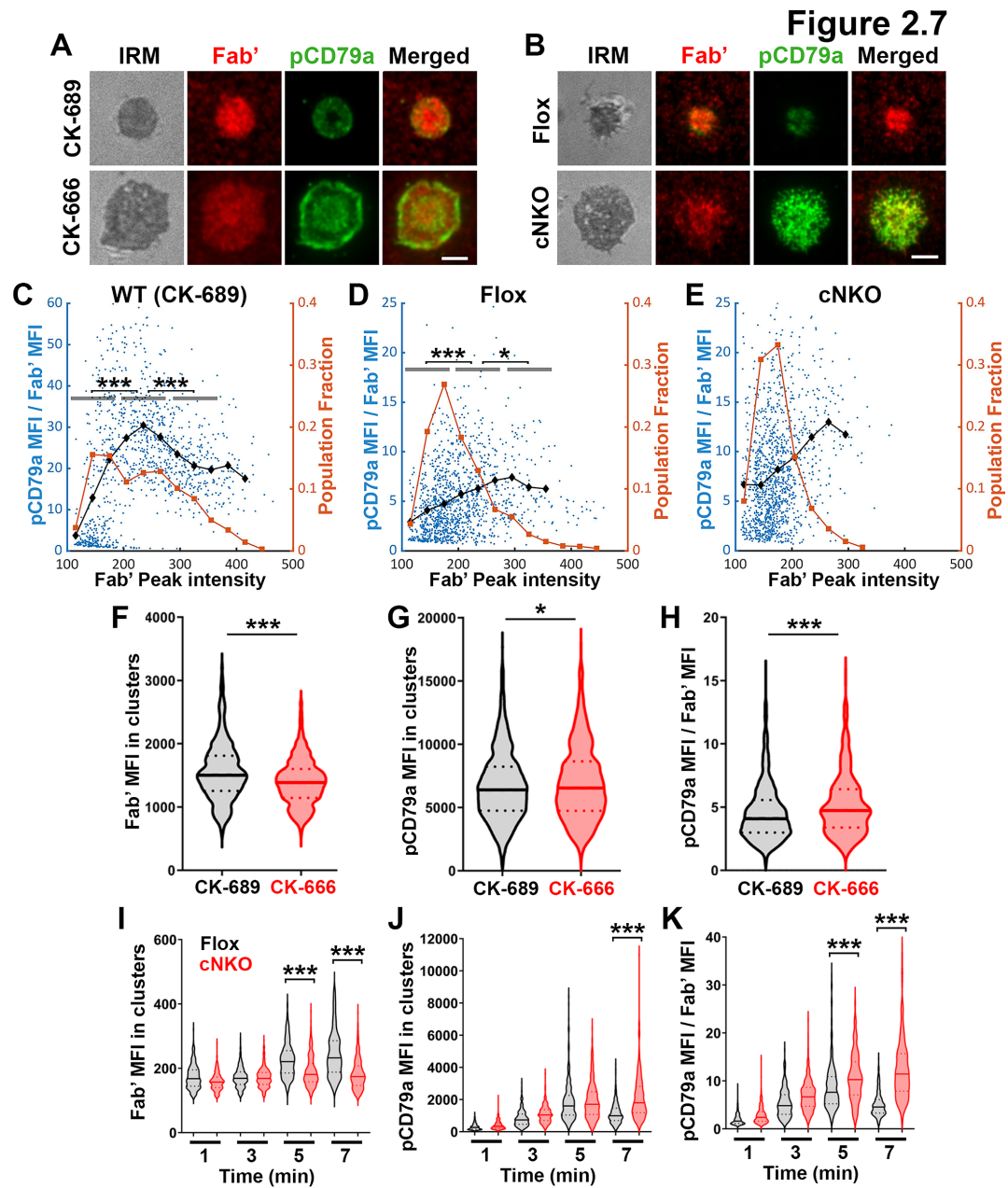


Figure 2.7. Increased molecular density in BCR clusters leads to reductions in BCR phosphorylation. WT B cells treated with CK-689 and CK-666 at 2 min post incubation with AF546-Fab'-PLB (**A, C, F-H**) and flox control and cNKO B cells incubating with AF546-Fab'-PLB (**B, D, E, I-K**) were fixed at 1, 3, 5, and 7 min, permeabilized, and stained for phosphorylated CD79a (pCD79a, Tyr182), and imaged using TIRF and IRM. (**A, B**) Representative IRM and TIRF images of a CK-689- versus a CK-666-treated cell (**A**) and a

flox control versus a cNKO B cell **(B)** at 7 min. Scale bars, 2 μ m. **(C-E)** Ratios of pCD79a MFI relative to AF546-Fab' MFI were plotted against AF546-Fab' peak IF in individual AF546-Fab' clusters in the contact zone of CK-689-treated WT B cells **(C)** or flox control **(D)** or cNKO B cells **(E)**. AF546-Fab' clusters were identified as described in Figure 6 and supplementary Figure S6 from an equal number of cells of the 4 time points. Data points represent individual AF546-Fab' clusters with an equal number of clusters from each time-point. The black line represents the average ratios of pCD79a MFI to Fab' MFI in individual BCR-Fab' clusters with indicated Fab' peak intensity ranges. The red line represents the fraction of the BCR-Fab' clusters of the total with indicated Fab' peak intensity ranges. Data were generated from 3 independent experiments with ~20 cells per condition per experiment. * $p < 0.05$, *** $p < 0.001$, by non-parametric student's t -test, between BCR-Fab' cluster groups with 100~200, 200~300, and 300~400 Fab' peak intensity. **(F-H)** The MFI ($\pm$ SEM) of AF546-Fab' **(F)** and pCD79a **(G)**, and the MFI ratio ($\pm$ SEM) of pCD79a relative to AF546-Fab' **(H)** in individual AF546-Fab' clusters were compared between CK-689- and CK-666-treated B cells at 7 min post-stimulation. **(I-K)** The MFI ($\pm$ SEM) of AF546-Fab' **(I)** and pCD79a **(J)**, and the MFI ratio ($\pm$ SEM) of pCD79a relative to AF546-Fab' **(K)** in individual AF546-Fab' clusters at indicated times were compared between flox control and cNKO B cells. Data points represent individual clusters; in violin plots, the solid horizontal line represents the mean and the dotted lines represent the quartiles of the distribution. Data were generated from 3 independent experiments with ~20 cells per condition per experiment. * $p < 0.05$, *** $p < 0.001$, by non-parametric student's t -test.

2.3.8 Increases in BCR molecular density by B cell contraction promote the disassociation of the stimulatory kinase Syk from BCR microclusters

The increased BCR molecular density may promote signaling attenuation by inducing the disassociation and/or the dephosphorylation of stimulatory kinases from and at BCR clusters. To test this hypothesis, we analyzed the relative amounts of Syk, a major stimulatory kinase in the BCR signaling pathway, and its phosphorylated form pSyk in individual contact zones and BCR clusters, in relation to the molecular density of BCRs. Splenic B cells were incubated with Fab'-PLB for 3 and 7 min (when most cells were at the spreading and contraction phase, respectively), fixed, permeabilized, stained for Syk or pSyk, and imaged using TIRF. We first measured the MFI of total Syk (Figure 2.8A) and the MFI ratio of Syk relative to AF546-Fab' (Figure 2.8B) in individual B cell contact zones to reflect the overall level of Syk recruitment to BCR activation sites. While Syk MFI and MFI ratios of Syk relative to AF546-Fab' in the contact zone of flox control B cells were reduced at the contraction stage (7 min), these parameters persisted at similar levels at the contraction stage in cNKO B cells, compared to those at the spreading stage (3 min) (Figure 2.8A, B). We next measured MFI ratios of Syk relative to AF546-Fab' in individual BCR clusters to reflect the relative amount of Syk associated with individual BCR clusters and analyzed their relationship with AF546-Fab' peak intensity (reflecting the molecular density within BCR clusters. AF546-Fab' clusters were analyzed as described above. In flox control B cells, the highest fractions of Fab' clusters had an AF546-Fab' peak intensity at the 150~200 range (Figure 2.8C, red line and symbol). The average MFI ratios of Syk to Fab' in individual clusters increased at a low peak intensity range (120~140) and did not significantly decrease until Fab' peak intensity reached a relatively high range (>280) (Figure 2.8C, black line and symbol). Inhibition of B cell contraction by cNKO reduced the Fab' peak intensity of clusters (Figure 2.8D, E, red line and symbol) but increased the average Syk to Fab' MFI ratios in

clusters in a wide range of Fab' peak intensity (Figure 2.8D, E, black lines and symbols). However, the average Syk to Fab' MFI ratios of cNKO B cells started decreasing in clusters with Fab' peak intensity ≥ 220 and reduced to levels similar to those in flox control B cells in clusters with Fab' peak intensity ≥ 240 (Figure 2.8D, E). These data suggest that increases in the molecular density of BCR clusters, reflected by Fab' peak intensity, induce a disassociation of Syk from BCR clusters in both flox control and cNKO B cells. Our results also show that BCR clusters in cNKO B cells have significantly higher levels of Syk association than those in flox control B cells, even though they have similar Fab' peak intensity.

We next analyzed the relationship of the phosphorylated Syk (pSyk) level with BCR molecular density using the method described above. The pSyk MFI and pSyk to Fab' MFI ratios in the contact zone of individual flox control B cells were reduced at the contraction stage (7 min), compared to those at the spreading stage (3 min) (Figure 2.8F, G). In contrast, the pSyk MFI in the contract zone of individual cNKO B cells was increased and the pSyk to Fab' MFI ratios remained the same at the contraction stage, compared to those at the spreading stage (Figure 2.8F, G). These data were consistent with our previously published data that inhibition of B cell contraction by cNKO enhances BCR signaling and delays signaling attenuation²³¹. When examining individual BCR-Fab' clusters, we found that the MFI ratios of pSyk relative to AF546-Fab' gradually decreased with increases in Fab' peak intensity in both flox control and cNKO B cells (Figure 2.8H, J), even though we did not observe an increases in pSyk to Fab' MFI ratio at the low Fab' peak intensity ranges. Similar to the Syk to Fab' MFI ratio, the average pSyk to Fab' MFI ratios of individual clusters in cNKO B cells were much higher than those in flox control B cells, except for those at the high Fab' peak intensity range (Figure 2.8H-J). To confirm this result, we collected equal numbers of pSyk clusters in the same cells for analyzing, based on the criteria of 1.3 folds

higher than the background outside the contact zone with a diameter ≥ 250 nm. Similar to clusters identified by AF546-Fab', the average MFI ratios of pSyk to Fab' in these pSyk clusters decreased with the increases in their Fab' peak intensity in both flox control and cNKO B cells (Figure 2.8K-M, black lines and symbols). Again, the average pSyk to Fab' MFI ratios of pSyk clusters were much higher in cNKO than flox control B cells, but were reduced to similar levels in clusters with relatively high Fab' peak intensity (Figure 2.8M, black line and symbol). Similar reductions of both total Syk and pSyk with increasing molecular density of BCR clusters suggest that a disassociation of Syk from BCR clusters, caused by cell contraction-induced increases in molecular density, contributes to BCR signaling attenuation.

Figure 2.8

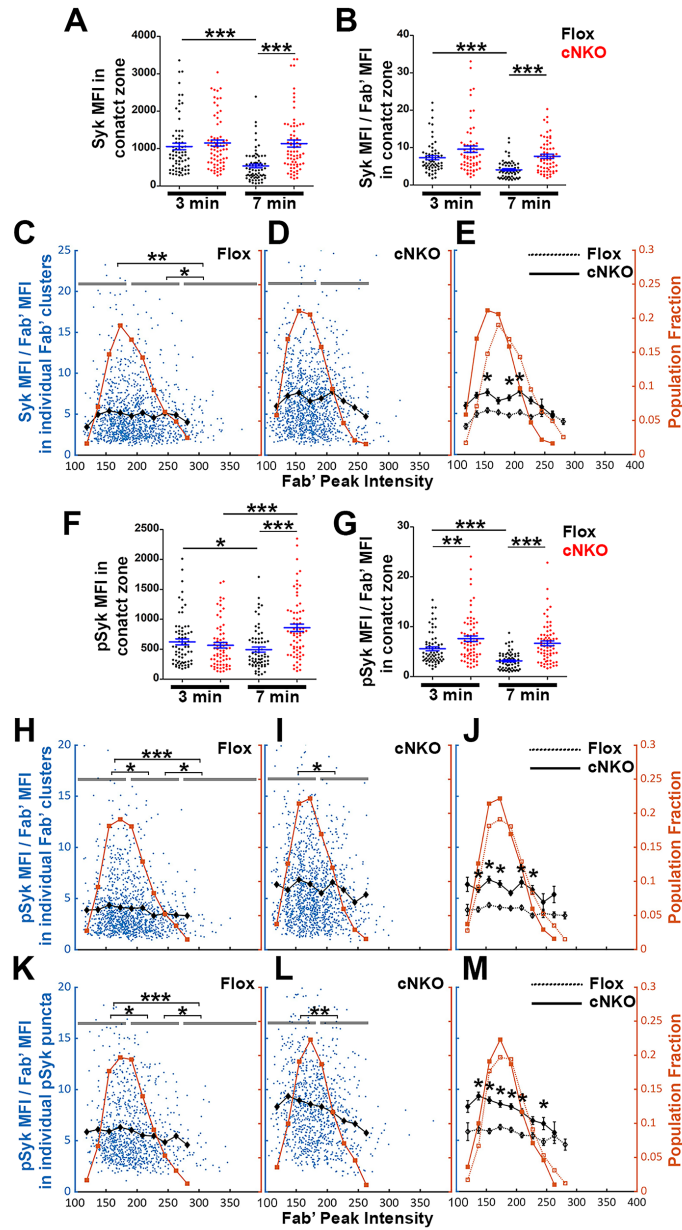


Figure 2.8. The effects of BCR-Fab' density on the association of Syk with BCR-Fab' clusters and its phosphorylation. Primary B cells from flox control and cNKO mice were incubated with AF546-Fab'-PLB at 37°C, fixed at 3 or 7 min, stained for total Syk and phosphorylated Syk (pSyk Tyr519/520), and imaged by TIRF. **(A, B)** The MFI ($\pm$ SEM) of Syk **(A)**, and the MFI ratio of Syk relative to Fab' ($\pm$ SEM) **(B)** in the B cell contact zone were compared between flox control and cNKO B cells at 3 and 7 min. Data points represent

individual cells from 3 independent experiments with ~23 cells per condition per experiment. **(C-E)** Ratios of Syk MFI relative to AF546-Fab' MFI were plotted against AF546-Fab' peak IF in individual AF546-Fab' clusters in the contact zone of Flox control **(C)** and cNKO B cells **(D)**. An overlay of flox control and cNKO plots is also shown **(E)**. AF546-Fab' clusters were identified as described in Figure 6 and supplementary Figure S6 from an equal number of cells at 3- and 7-min post-stimulation. Data points represent individual AF546-Fab' clusters with equal number of clusters from each time-point. The black lines represent the average ratios of Syk MFI to Fab' MFI in individual AF546-Fab' clusters with indicated Fab' peak intensity ranges. The brown lines represent the fraction of the AF546-Fab' clusters out of the total at indicated Fab' peak intensity ranges. **(F,G)** The MFI ($\pm$ SEM) of pSyk **(F)**, and the MFI ratio of pSyk relative to Fab' ($\pm$ SEM) **(G)** in the B cell contact zone were compared between flox control and cNKO B cells. Data points represent individual cells from 3 independent experiments with ~23 cells per condition per experiment. **(H-J)** Ratios of Syk MFI relative to AF546-Fab' MFI were plotted against AF546-Fab' peak IF in individual AF546-Fab' clusters in the contact zone of flox control **(H)** and cNKO B cells **(I)**. An overlay of flox control and cNKO plots is also shown **(J)**. **(K-M)** MFI ratios of pSyk relative to AF546-Fab' were plotted against AF546-Fab' peak IF in individual pSyk puncta in the contact zone of Flox control **(K)** and cNKO B cells **(L)**. An overlay of flox control and cNKO B cells is also shown **(M)**. The black lines represent the average ratios of pSyk MFI to Fab' MFI in individual AF546-Fab' clusters **(H-J)** or individual pSyk puncta **(K-M)** at indicated Fab' peak intensity ranges. The brown lines represent the fraction of the total clusters at indicated Fab' peak intensity ranges. pSyk puncta were identified using the criteria: intensity ≥ 1.5 times the background noise outside B cells, and size ≥ 250 nm from cells. Data points represent individual pSyk puncta **(K, L)**, collected from an equal number of cells from 3- and 7-min time-points. Data were generated from 3 independent experiments with ~23 cells per condition per experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by non-

parametric student's *t*-test, between flox control and cNKO (**A, B, F, G**) and between AF546-Fab' cluster group with different Fab' peak intensity ranges (**C, H, K**).

2.3.9 Increases in BCR molecular density by B cell contraction also promote the disassociation of the inhibitory phosphatase SHIP-1 from BCR microclusters

The inhibitory phosphatase, such as SHIP-1, is known to be essential for B cell signaling attenuation²⁰³, which leads to a hypothesis that increases in the molecular density of BCR clusters by B cell contraction may promote SHIP-1 recruitment. We used the methods described above to address this hypothesis, staining cells for total SHIP-1 and phosphorylated SHIP-1 (pSHIP-1). Both flox control and cNKO B cells increased the MFI of total SHIP-1 staining in individual contract zones at the contraction stage (7 min) compared to the spreading stage (3 min), but the increase in cNKO B cells was significantly less than flox control B cells (Figure 2.9A), consistent with delayed signaling attenuation. The MFI ratios of SHIP-1 relative to AF546-Fab' in flox control B cells were reduced at the contraction stage compared to the spreading stage (Figure 2.9B) due to an increased accumulation of BCRs in the contract zone by the B cell contraction stage. However, the SHIP-1 to Fab' MFI ratios in the contact zone of cNKO B cells were not significantly changed when cells transitioned from the spreading to the contraction stage (Figure 2.9B). When examining individual Fab' clusters, we found that the average SHIP-1 to Fab' MFI ratios in both flox control and cNKO B cells reduced with Fab' peak intensity at similar rates, even though contraction inhibition by cNKO reduced the Fab' peak intensity of BCR clusters (Figure 2.9C-D). Notably, the reduction of the SHIP-1 to Fab' MFI ratios with increasing Fab' peak intensity occurred at the lowest detectable Fab' peak intensity, when the Syk to Fab' MFI ratios increased and sustained (Figure 2.9C-D). Furthermore, this reciprocal relationship between the SHIP-1 to Fab' MFI ratio and Fab' peak intensity continued in the entire Fab' peak intensity range. It also remained the same in both flox control and cNKO B cells (Figure 2.9C-D). These results suggest that SHIP-1 disassociates from BCR clusters when their molecular density increases

and the SHIP-1 disassociation is more sensitive to the molecular density of BCR clusters than Syk disassociation.

Similar to the relationship of total SHIP-1 with BCR molecular density, the pSHIP-1 MFI in the individual contact zones were increased at the contraction stage (7 min), compared to those at the spreading stage (3 min), in both flox control and cNKO B cells (Figure 2.9F, G). The pSHIP-1 to Fab' MFI ratios reduced in individual contact zone of flox control B cells, while remaining at a similar level in cNKO B cells at the contraction stage, compared to those at the spreading stage (Figure 2.9F, G). Furthermore, the average MFI ratios of pSHIP-1 relative to AF546-Fab' in individual Fab' clusters decreased with increases in Fab' peak intensity at similar rates in flox control and cNKO B cells (Figure 2.9H-J, black lines and symbols), even though contraction inhibition by cNKO reduced Fab' peak intensity of BCR clusters (Figure 2.9J, red lines and symbols), similar to the SHIP-1 to Fab' MFI ratio. The average pSHIP-1 to Fab' MFI ratios in individual pSHIP-1 clusters, collected in the same way as pSyk clusters, showed the same decreases with the increases in their Fab' peak intensity in both flox control and cNKO B cells (Figure 2.9K-M, black line and symbol). Notably, the average pSHIP-1 to Fab' MFI ratios in individual Fab' or pSHIP-1 clusters in flox control and cNKO B cells were at similar levels at the same Fab' peak intensity ranges (Figure 2.9H-M). These results indicate that contraction-caused molecular density increases within individual BCR clusters do not induce preferential recruitment of SHIP-1; instead, it promotes the disassociation of SHIP-1, reducing pSHIP-1 in BCR clusters.

Figure 2.9

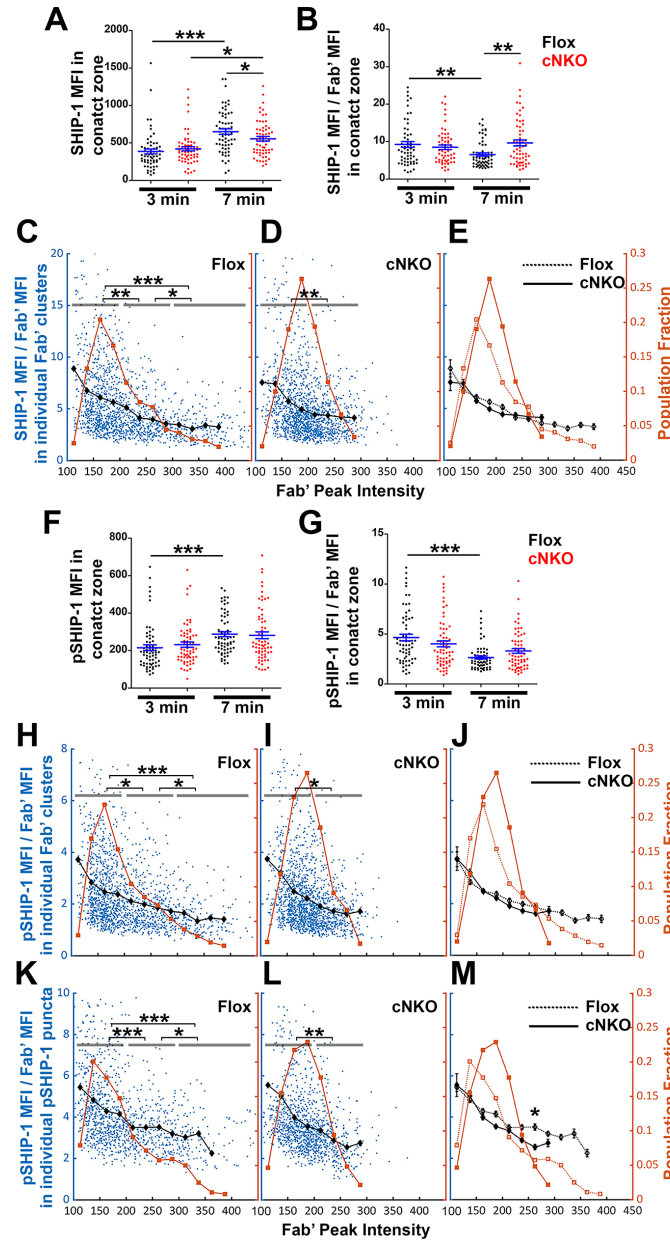


Figure 2.9. The effects of BCR-Fab' density on the association of SHIP-1 with BCR-Fab' clusters and its phosphorylation. Primary B cells from flox control and cNKO mice were incubated with AF546-Fab'-PLB at 37°C, fixed at 3 or 7 min, stained for total SHIP-1 and phosphorylated SHIP-1 (pSHIP-1 Tyr1020), and imaged by TIRF. **(A, B)** The MFI ($\pm$ SEM) of SHIP-1 **(A)**, and the MFI ratio of SHIP-1 relative to AF546-Fab' ($\pm$ SEM) **(B)** in the B cell contact zone were compared between flox control and cNKO B cells at 3- and 7-

min. Data points represent individual cells from 3 independent experiments with ~23 cells per condition per experiment. **(C-E)** Ratios of SHIP-1 MFI relative to AF546-Fab' MFI were plotted against AF546-Fab' peak IF in individual AF546-Fab' clusters in the contact zone of Flox control **(C)** and cNKO B cells **(D)**. An overlay of flox control and cNKO plots is also shown **(E)**. AF546-Fab' clusters were identified as described in Figure 6 and supplementary Figure S6 from an equal number of cells at 3- and 7 min post-stimulation. Data points represent individual AF546-Fab' clusters with equal number of clusters from each time-point. The black lines represent the average ratios of SHIP-1 MFI to Fab' MFI in individual AF546-Fab' clusters with indicated Fab' peak intensity ranges. The brown lines represent the fraction of the AF546-Fab' clusters out of the total at indicated Fab' peak intensity ranges. **(F,G)** The MFI ($\pm$ SEM) of pSHIP-1 **(F)**, and the MFI ratio of pSHIP-1 relative to Fab' ($\pm$ SEM) **(G)** in the B cell contact zone were compared between flox control and cNKO B cells. Data points represent individual cells from 3 independent experiments with ~23 cells per condition per experiment. **(H-J)** Ratios of pSHIP-1 MFI relative to AF546-Fab' MFI were plotted against AF546-Fab' peak IF in individual AF546-Fab' clusters in the contact zone of flox control **(H)** and cNKO B cells **(I)**. An overlay of flox control and cNKO plots is also shown **(J)**. **(K-M)** MFI ratios of pSHIP-1 relative to AF546-Fab' were plotted against AF546-Fab' peak IF in individual pSHIP-1 puncta in the contact zone of Flox control **(K)** and cNKO B cells **(L)**. An overlay of flox control and cNKO B cells is also shown **(M)**. The black lines represent the average ratios of pSHIP-1 MFI to Fab' MFI in individual AF546-Fab' clusters **(H-J)** or individual pSHIP-1 puncta **(K-M)** at indicated Fab' peak intensity ranges. The brown lines represent the fraction of the total clusters at indicated Fab' peak intensity ranges. pSHIP-1 puncta were identified using the criteria: intensity ≥ 1.5 times the background noise outside B cells, and size ≥ 250 nm from cells. Data points represent individual pSHIP-1 puncta **(K, L)**, collected from an equal number of cells from 3- and 7-min time-points. Data were generated from 3 independent experiments with ~23 cells per condition per experiment. * $p < 0.05$, ** p

<0.01, *** $p < 0.001$, by non-parametric student's t -test, between flox control and cNKO (**A**, **B**, **F**, **G**) and between AF546-Fab' cluster group with different Fab' peak intensity ranges (**C**, **H**, **K**).

2.4 Discussion

B cells undergo actin-mediated spreading followed by a contraction in response to membrane-associated antigen, which is essential for amplifying BCR signaling and forming immunological synapses²¹³. We have previously shown that B cell contraction on antigen-presenting surfaces after spreading promotes BCR signaling attenuation, which is critical for controlling autoreactive B cells^{203,231,242}. However, how the actin cytoskeleton reorganizes from structures pushing the plasma membrane outwards into those contracting the plasma membrane and how actin-mediated B cell contraction downregulates BCR signaling remain elusive. This study reveals that the formation of inner actin foci at the contact membrane distantly behind the lamellipodial actin network drives B cell switching from spreading to contraction. These inner foci are derived from the lamellipodial actin network that drives spreading and are generated by N-WASP- but not WASP-activated Arp2/3-mediated branched actin polymerization. N-WASP-activated actin polymerization prolongs the lifetime and drives the centripetal movement of these actin foci. Such inner actin foci are required for recruiting NMII, enabling B cell contraction. B cell contraction primarily increases BCR molecular density in existing clusters, which promotes the disassociation of both the stimulatory kinase Syk and the inhibitory phosphatase SHIP-1, leading to signaling attenuation.

Previous studies have shown that Arp2/3-mediated branched actin polymerization is essential for B cell spreading and the actin centripetal flow at the B cell plasma membrane contacting antigen-presenting membrane²³⁹. The actin structure that supports B and T cell spreading to form the immunological synapse with antigen-presenting cells is similar to the lamellipodial actin networks^{244,250,251}. The lamellipodial actin network consists primarily of branched actin filaments polymerizing against the plasma membrane with bundled actin filaments as frames^{250,252}. However, contractile actin structures are typically generated from bundled actin

filaments in the most well-studied cases of cell focal adhesion and migration^{253–255}. Here, we have shown that the formation of inner actin foci, which are required for B cell contraction, are highly dependent on the branched actin nucleation factor Arp2/3, which is demonstrated by the Arp2/3-specific inhibitor CK-666, the N-WASP inhibitor Wisko, and cNKO. Furthermore, these inner actin foci are directly derived from lamellipodial actin networks; however, instead of polymerizing against the plasma membrane, Arp2/3 appears to polymerize actin in the opposite direction, sustaining actin foci and driving them away from lamellipodial actin networks. Our results suggest that inner actin foci are not typical contractile actin structures described previously, even though they recruit NMII and mediate membrane contraction. However, this study does not exclude the involvement of bundle actin filaments. Based on the requirement of formin-activated bundle actin filament for lamellipodial actin networks and B cell spreading, we can speculate possible roles for bundle actin filaments in the formation and the localization of these inner actin foci. Wang et al.²⁴⁴ have recently shown that B cells generate actomyosin arcs when interacting with membrane-associated antigen in the presence of adhesion molecules, such as in the case of interacting with the professional antigen-presenting cells like FDCs. Differing from T cells, B cells also respond to membrane-associated antigen without the help of adhesion molecules, when engaging antigen on pathogenic cells, like bacteria, parasites, and cancer cells. Even though the relationship between actomyosin arcs and inner actin foci remains to be explored, the inner actin foci revealed here in the absence of adhesion molecules may be early versions of the actomyosin arcs that can be potentially enhanced and stabilized by adhesion molecule interactions between B cells and antigen-presenting membrane, consequently increasing the sensitivity of B cells to antigen.

Immune cells, including B cells, express both hematopoietic-specific WASP and a ubiquitous homolog of WASP, N-WASP. These two share high sequence homology, the activation

mechanism, and the Arp2/3 activation function^{236,237,256,257}. We have previously identified distinct functions of the two. While both of them are required for B cell spreading, N-WASP plays a unique role in B cell contraction. While WASP and N-WASP double knockout B cells fail to spread on antigen-presenting surfaces, B cell-specific N-WASP knockout delays B cell spreading²³¹. Furthermore, the activation of the two peaks at different times (phosphorylated WASP peaks during B cell spreading and phosphorylated N-WASP peaks during contraction) and is detected at distinct location (most WASP at the periphery and N-WASP scattering across the B cell contact zone)²³¹. Here, we identified the exact role of N-WASP in B cell contraction – generating inner actin foci from the lamellipodial actin networks by activating Arp2/3-mediated actin polymerization. N-WASP- but not WASP-activated actin polymerization prolongs the relative lifetime of actin foci and enables them to treadmill inwards. The distinct activation time window and location at the B cell contact zone probably give N-WASP a unique opportunity to generate inner actin foci. However, how WASP and N-WASP are activated at different locations and times remains to be explored. As cNKO only reduces but does not block B cell contraction and inner actin foci formation, it suggests that other actin factors are involved, such as WASP-family verprolin-homologous protein (WAVE)^{258–260}.

Our recently published data show that NMII is required for B cell contraction. Activated NMII is recruited to the B cell contact zone to form a peripheral ring surrounding the contact zone during B cell contraction. This NMII recruitment is SHIP-1-dependent²⁴². Here, we further show that in addition to SHIP-1, N-WASP, not WASP, is also involved in the NMII recruitment and NMII ring formation in the B cell contact zone. These findings together suggest that SHIP-1 coordinates with N-WASP to recruit NMII. Our previous findings that SHIP-1 promotes B cell contraction by facilitating N-WASP activation²⁰³ support this notion. However, it is surprising that the recruitment of NMII activated by BCR signaling to the

contact zone is associated with inhibitory signaling molecules. BCR downstream signaling, including Ca^{2+} flux and Rho-family GTPase activation, likely induces the activation of the NMII motor activity in B cells^{261–264}. The activation switches NMII from the incompetent folded conformation to the competent extended conformation, enabling NMII molecules to bind to F-actin and assemble into contractible bipolar filaments and stacks^{265,266}. The denser F-actin organization has been shown to increase NMII filament stacking²⁶⁷. Here, we have shown that inner F-actin foci generated by N-WASP-activated Arp2/3 are more stable and also appear to be denser and thus likely promote NMII binding and stacking more efficiently than F-actin generated by WASP-activated Arp2/3. Indeed, our kymograph analysis detected the accumulation of NMII at a similar time window and spatial location when and where inner F-actin foci were deriving from lamellipodial F-actin, supporting our hypothesis. Contrastingly, Kumari et al showed that in primary T cells, F-actin foci were generated by Arp2/3 and WASP, but not N-WASP, and help maintain T cell immune synapses through myosin-generated traction forces^{268,269}. These studies also demonstrated a distinct role for WASP and N-WASP in the generation of dynamic branched actin foci that facilitate myosin recruitment in T cells, however interestingly, these foci are activated by distinct branched actin nucleation promoting factors in B and T cells.

We have previously shown that inhibiting B cell contraction on antigen-presenting surfaces by B cell-specific knockout of N-WASP or NMIIA enhances and/or prolongs BCR signaling, leading to autoantibody production^{231,242}. This study shows that increasing molecular density within BCR clusters or B cell synapses is a mechanism by which B cell contraction promotes BCR signaling attenuation. Surprisingly, molecular density increases induce the disassociation of both the stimulatory kinase Syk and the inhibitory phosphatase SHIP-1, which contradicts the existing dogma of sequential association of signaling activation and inhibitory molecules^{203,213,231,232}. Interestingly, the disassociation of SHIP-1 is much more

sensitive to the BCR molecular density than the disassociation of Syk, as Syk does not disassociate until the molecular density of BCR clusters reaches the top 10%, while SHIP-1 disassociates from BCR clusters with increases in the entire detectable range of the molecular density. When B cell contraction is inhibited, the molecular density of BCR clusters is reduced to a range with limited Syk disassociation and normal SHIP-1 disassociation, resulting in increased BCR signaling levels. While the resolution of our TIRF microscope limited our ability to identify nascent and merging BCR clusters, the consistency of the data from AF546-Fab' and pSyk or pSHIP-1 clusters confirms our results. How the molecular density within BCR clusters promotes signaling molecule disassociation is unknown. We speculate that the molecular crowdedness may push signaling molecules out of BCR clusters. The disassociation of SHIP-1 with 145 kDa molecular weight is more sensitive to the molecular density of BCR clusters than the disassociation of Syk with 72 kDa, half of the SHIP-1 molecular weight, supporting these hypotheses. However, these hypotheses remain to be further examined.

Here, we found that the pCD79a and pSyk levels were significantly higher in individual BCR clusters of cNKO B cells than those of flox control B cells, even when they have the same molecular density. These data suggest that there are additional mechanisms for B cell contraction to promote BCR signaling attenuation. In addition to increasing molecular density within BCR clusters, the contractive forces generated by actomyosin rings on the B cell membrane and BCR clusters, particularly those with high molecular density, may cause changes to the conformations of both BCRs and associated signaling molecules and their lateral interactions in the membrane. These changes may favor the disassociation rather than the association of signaling molecules with BCR clusters. Recently solved molecular assembly structures of both human and mouse BCRs^{20,21} support the possibilities of conformational and lateral molecular interaction changes as mechanisms for BCR signaling

regulation. Actomyosin-mediated contraction forces have also been shown to be required for BCR endocytosis of antigen¹²⁹, which removes the BCR from the cell surface, downregulating the BCR surface signaling. Whether the inner actin foci identified here play any role in BCR endocytosis remains an interesting question. Our early finding that N-WASP is required for BCR endocytosis²³¹ supports this notion. Similar to the differential impact of the molecular density on the disassociation of Syk and SHIP-1 from BCR clusters, B cell contraction may differentially affect the interaction of surface BCRs with cytoplasmic and membrane-anchored signaling molecules, such as the lipidated and lipid raft-resided Src kinase Lyn that is responsible for phosphorylating both the immunoreceptor tyrosine-based activation motif (ITAM) of CD79a and the immunoreceptor tyrosine-based inhibitory motifs (ITIM) of CD22 and FcγRIIb^{89,270–273}. As Syk binds to phosphorylated ITAM of the BCR, increased CD79a phosphorylation enhances the Syk association with the BCR and its phosphorylation²⁷⁴.

The results presented here have revealed novel insights into the mechanisms underlying actin-facilitated signaling attenuation of the BCR. Taking our previous and current data together, we propose a new working model for this actin-facilitated signaling attenuation. Upon maximal spreading, N-WASP behind lamellipodial networks activates Arp2/3-mediated branched actin polymerization, which prolongs the lifetime of lamellipodial actin and drives their movement away from the lamellipodia to the center of the B cell contact zone, generating inner actin foci. NMII is preferably recruited to these relatively stable inner foci, enabling the formation of actomyosin structures and leading to B cell contraction. B cell contraction drives the centripetal movement and the merger of BCR microclusters formed during B cell spreading and also particularly increases the molecular density within BCR clusters. Increased molecular density promotes the disassociation of signaling molecules from BCR clusters, probably due to crowdedness and conformational changes, leading to signal

downregulation. We should also note that the actin reorganization that drives B cell contraction is likely to be activated by inhibitory signal molecules, as the activation of N-WASP and the recruitment of NMII to the B cell contact zone all depend on SHIP-1^{203,231,242}. Thus, actin reorganization downstream of inhibitory phosphatases reinforces signaling attenuation by driving B cell contraction.

2.5 Materials and Methods

2.5.1 Key Reagents Table

Reagent type	Designation	Source	Identifiers	Additional Information
Chemical compound	1,2-dioleoyl-sn-glycero-3-phosphocholine	Avanti Polar Lipids	850375 P	5 mM
Chemical compound	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-cap-biotin	Avanti Polar Lipids	870273 C	50 μ M
Purified protein	Streptavidin	Jackson Laboratories	016-000-084	1 μ g/ml
Antibody fragment	F(ab') ₂ fragment of goat IgG anti-mouse Ig(G+M) (polyclonal)	Jackson Laboratories	115-006-068	-
Antibody fragment	Cy3-Fab fragment of goat IgG anti-mouse IgM (polyclonal)	Jackson Laboratories	115-167-020	1 μ g per 1 x 10 ⁶ cells
Chemical compound	2-Mercaptoethylamine HCL	Thermo Fisher Scientific	20408	50 mM
Chemical compound	EZ-Link™ Maleimide-PEG ₂ -biotin	Thermo Fisher Scientific	A39261	20 mM per mM of protein
Antibody Labeling Kit	Alexa Fluor 546 antibody labeling kit	Thermo Fisher Scientific	A20183	-
Purified protein	Biotinylated holo-transferrin	Millipore Sigma	T0665-50MG	-
Antibody	Rat IgG _{2b} anti-mouse CD90.2 (Thy1.2) (monoclonal)	Biolegend	105351	1 μ l per 2 x 10 ⁶ cells
Chemical compound	CK-689	Millipore Sigma	182517-25MG	50 μ M
Chemical compound	CK-666	Millipore Sigma	SML0006-5MG	50 μ M
Chemical compound	Wiskostatin	Millipore Sigma	W2270-5MG	10 μ M
Antibody	Rabbit IgG anti-mouse Arp2 antibody (polyclonal)	Abcam	ab47654	1:100
Antibody	Rabbit IgG anti-mouse pWASP antibody (polyclonal)	Thermo Fisher Scientific	PA5-105572	1:100
Antibody	Rabbit IgG anti-mouse pN-WASP antibody (polyclonal)	Thermo Fisher Scientific	PA5-105307	1:100
Antibody	AF488-goat IgG anti-rabbit IgG antibody (polyclonal)	Thermo Fisher Scientific	A-11034	1:200
Antibody	AF546-goat IgG anti-rabbit IgG antibody (polyclonal)	Thermo Fisher Scientific	A-11035	1:200
Chemical compound	Acti-stain-488 Phalloidin	Cytoskeleton	PHDG1-A	200 nM
Chemical compound	Acti-stain-555 Phalloidin	Cytoskeleton	PHDH1-A	200 nM

Antibody	Rabbit IgG anti-mouse NMIIA antibody (polyclonal)	Abcam	ab75590	1:100
Antibody	Rabbit IgG anti-mouse pCD79a (Tyr182) (monoclonal)	Cell Signaling Technology	14732S	1:100
Antibody	Rabbit IgG anti-mouse pSyk (Tyr519/520) (monoclonal)	Cell Signaling Technology	2710S	1:100
Antibody	Rabbit IgG anti-mouse pSHIP-1 (Tyr1020) (polyclonal)	Cell Signaling Technology	3941S	1:100
Antibody	Rabbit IgG anti-mouse Syk (polyclonal)	Thermo Fisher Scientific	PA5-17812	1:100
Antibody	Rabbit IgG anti-mouse SHIP-1 (polyclonal)	Thermo Fisher Scientific	PA5-115894	1:100

2.5.2 Mice and B cell isolation

Wild type (WT) C57BL/6 mice and WASP knockout (WKO) mice on a C57BL/6 background were purchased from Jackson Laboratories. C56BL/6 mice expressing LifeAct-GFP transgene²⁴⁹ were kindly provided by Dr. Roberto Weigert at National Cancer Institute, USA. C57BL/6 mice expressing LifeAct-RFP transgene were kindly provided by Dr. Klaus Ley at La Jolla Institute for Allergy & Immunology. C57BL/6 mice expressing GFP-non-muscle myosin IIA (NMIIA) transgene²⁷⁵ were kindly provided by Dr. Robert Adelstein at the National Heart, Lung, and Blood Institute, USA. WKO mice were bred with LifeAct-GFP mice to obtain LifeAct-GFP-expressing WKO mice. GFP-NMIIA mice were crossed with LifeAct-RFP mice to generate mice expressing both GFP-NMIIA and transgenes. N-WASP^{flox/flox} on a 129Sv background²⁴⁸ were kindly provided by Dr. Lisa Westerberg at Karolinska Institute, Sweden. B cell-specific N-WASP knockout mice (cNKO, CD19^{Cre/+}N-WASP^{flox/flox}) and floxed littermate controls (N-WASP^{flox/flox}) were obtained by breeding N-WASP^{flox/flox} mice with CD19^{Cre/+} mice on a C57BL/6 background²⁴⁸.

Primary B cells were isolated from the spleens of 6 to 18 weeks old male or female mice, using a previously published protocol²³². Briefly, mononuclear cells were isolated by Ficoll density-gradient centrifugation (Millipore Sigma), and T cells were eliminated by complement-mediated cytolysis with anti-mouse CD90.2 monoclonal antibody (BD

Biosciences) and guinea pig complement (Innovative Research Inc). Monocytes and dendritic cells were eliminated by panning at 37 °C and 5% CO₂. Isolated B cells were kept on ice in DMEM (Lonza) supplemented with 0.6% BSA (Thermo Fisher Scientific) and pre-warmed at 37 °C in PBS for up to 9 min during all experiments. All work involving mice was approved by the Institutional Animal Care and Usage Committee of the University of Maryland.

2.5.3 Pseudo-antigen-coated planar lipid bilayers (PLBs)

PLBs were prepared using a previously described method^{214,276}. Briefly, liposomes were generated from a mixture of 5 mM (total concentration) 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-cap-biotin (Avanti Polar Lipids) at a 100:1 molar ratio by sonication. Glass coverslips, cleaned overnight with Piranha solution (KMG chemicals), were attached to 8-well chambers (Lab-Tek) and incubated with liposomes (0.05 mM) in PBS for 20 min at room temperature and washed with PBS. The chambers were then incubated with 1 µg/ml streptavidin (Jackson ImmunoResearch Laboratories) for 10 min, washed with PBS, and then incubated with 10 µg/ml mono-biotinylated Fab' fragment of goat anti-mouse Ig(G+M) (pseudo-antigen) (Fab'-PLB) or 16 µg/ml biotinylated-transferrin (Tf-PLB) as a non-stimulatory control for 10 min, followed by PBS wash. To visualize pseudo-antigen clustering, a mixture of 0.5 µg/ml Alexa Fluor (AF) 546-labeled and 9.5 µg/ml unlabeled mono-biotinylated Fab' fragment of goat anti-mouse Ig(G+M) was used. Mono-biotinylated Fab' fragments were generated as previously described²⁷⁷. Briefly, the disulfide bond linking the two Fab molecules of F(ab')₂ fragment of goat anti-mouse Ig(G+M) was reduced using mercaptoethylamine HCL (Thermo Fisher Scientific) and biotinylated by using maleimide-PEG₂-biotin (Thermo Fisher Scientific). Mono-biotinylated Fab' fragments of goat anti-mouse Ig(G+M) were labeled with AF546 using an Alexa Fluor 546 antibody labeling kit (Thermo Fisher Scientific). The molar

ratio of AF546 to Fab' in the AF546-labeled Fab' was about 2 determined by a Nanodrop spectrophotometer (Nanodrop Technologies).

2.5.4 Total internal fluorescence (TIRF) microscopy

To visualize molecules proximal to interacting sites between B cells and Fab'- or Tf-PLB, we utilized total internal reflection fluorescence (TIRF) microscopy. Interference reflection microscopy (IRM) was used to detect the contact zone between the B cell and the PLBs. Images were acquired using a Nikon 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 or a DeltaVision Elite Deconvolution TIRF system on an inverted microscope (Olympus IX71 Inverted, Olympus Corporation of the Americas) equipped with a 63X, NA 1.49 TIRF objective, a sCMOS camera (Andor/Oxford Instruments), and 488 and 561 nm laser lines. IRM, AF488, and AF546 images were acquired sequentially. The plasma membrane area of B cells contacting PLB was determined using IRM images and custom codes by MATLAB. Whether a B cell is contracting or not was determined using the area versus time plots, wherein if a B cell's contact zone area reduces consistently for at least 10 seconds after reaching a maximum value, then it is classified as contracting. To image intracellular molecules, B cells were incubated with Fab'-PLBs for varying lengths of time in PBS at 37°C, fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin, and stained for various molecules. For live cell imaging, images were acquired as soon as B cells were dropped into coverslip chambers coated with Fab'- or Tf-PLBs containing PBS in a humidity chamber at 37 °C, at 2 sec per frame, up to 7 min.

2.5.5 Inhibitors

CK-666 (50 μ M) was used to perturb Arp2/3 activity in B cells²⁷⁸ and its non-functional derivative CK-689 (50 μ M) as a control. CK-666 or CK-689 was added at either 0 min, the start of stimulation, or 2 min when most B cells reached maximum spreading. Notably, B cells take approximately 1 to 1.5 min to land on PLB. The time when B cells were added to Fab'-PLB is referred to as the start of stimulation, and the time when B cells landed on PLB as the start of spreading. The effectiveness of CK-666 was determined by its inhibitory effects on the recruitment of Arp2/3, stained by an anti-Arp2 antibody, to the B cell contact zone using TIRF. Wiskostatin (10 μ M) was used to perturb N-WASP activity in B cells²⁴⁷, with DMSO used as vehicle control. Splenic B cells were pretreated with Wiskostatin for 10 min at 37°C before incubating with Fab'-PLB in the presence of Wiskostatin, as the pretreatment is necessary and sufficient to reduce the level of phosphorylated N-WASP in the B cell contact zone and B cell contraction on Fab'-PLB. Possible effects of Wiskostatin on WASP activation were evaluated by measuring the mean fluorescence intensity (MFI) of phosphorylated WASP in the contact zone of B cells treated with or without Wiskostatin (10 μ M) for 10 min using immunostaining and TIRF.

2.5.6 Analysis of the actin cytoskeleton

F-actin was visualized by phalloidin staining in fixed cells and by LifeAct-GFP or LifeAct-RFP expressed by primary B cells from transgenic mice in live cells. Inner F-actin foci, visualized by both phalloidin staining and LifeAct-GFP, were manually identified using NIH ImageJ based on the following three criteria: 1) ≥ 250 nm in diameter, 2) its peak fluorescence intensity (FI) was ≥ 2 fold higher than the FI containing no foci, and 3) at least 1 μ m away from the outer edge of the B cell contact zone. To analyze the spatiotemporal relationship between inner F-actin foci and the lamellipodial F-actin network, we generated 8 radially and equally spaced kymographs from each cell using time-lapse images and MATLAB. Each

kymograph was either classified as contracting or not contracting based on the movement of the leading edge of the B cell contact zone. The percentage of 8 kymographs from each cell exhibiting inner F-actin foci that could be traced back to lamellipodial F-actin when the B cell switched from spreading to contraction was determined.

The relative speeds and lifetimes of F-actin foci were determined using three randomly positioned kymographs from each B cell at a 60-sec window right after B cell maximal spreading. Inner F-actin foci were identified as described above. Only those remaining visible in individual kymographs for at least 4 sec were analyzed. The time duration of each actin foci detected in a kymograph was used to determine the relative lifetime. The distances individual F-actin foci moved during their lifetimes were used to determine the relative speed. The term ‘relative’ has been used because the disappearance of an F-actin focus could be due to its movement outside of the region used for the kymograph.

To determine the rate of myosin recruitment, GFP-NMIIA MFI on the B cell contact zone was plotted over time, and the slope for the initial segment of the MFI versus the time plot was determined using linear regression. The percentage of B cells with the NMIIA ring was determined by visual inspection.

2.5.7 Analysis of BCR-Fab’ clusters

BCR clusters were identified by clustering of AF546-Fab’ on PLBs using custom codes made in MATLAB. We utilized the MFI of the Fab’-PLB region outside of the B cell contact zone as a threshold. To ensure that individual clusters of varying FI are detected as distinct objects, we used 16 graded thresholds from 1.1 to 4.1 fold of the background (0.2 fold apart) to acquire 16 sets of binary masks for each frame of time lapse images from each cell. When clusters were detected by multiple thresholds at the same location, only the one identified by the highest threshold was retained. Objects that were smaller than 250 nm in diameter or objects that could not be tracked for at least 20 seconds were eliminated. This allowed us to

detect the position of BCR-Fab' clusters and track them over time until they become too dim to track or merge with other clusters. We chose the peak intensity as a metric for the extent of Fab' clustering by the BCR, because it does not rely on the area occupied by each cluster. The rate of peak intensity increase was determined by linear regression of peak intensity versus time curves for each cluster for a given window of time.

2.5.8 BCR signaling

Splenic B cells were incubated with AF546-Fab'-PLB at 37°C and fixed at 1, 3, 5, 7, and 9 min. After fixation, cells were stained for phosphorylated CD79a (pCD79a) at Y183, Syk (pSyk) at Y519/520, SHIP (pSHIP) at Y1020, or total Syk and SHIP proteins. We identified BCR-Fab' clusters as described above using, 16 graded thresholds from 1.1 to 4.1 fold of the background (0.2 fold apart) to acquire 16 sets of binary masks for each frame of time lapse images from each cell. When clusters were detected by multiple thresholds at the same location, only the one identified by the highest threshold was retained. Objects that were smaller than 250 nm in diameter were eliminated. We determined the peak intensity of AF546-Fab', the MFI of AF546-Fab', and the MFI of pCD79a or pSyk or pSHIP-1 or Syk or SHIP-1 within each cluster. The ratio of pCD79a MFI to AF546-Fab' MFI in the same cluster was used to estimate the relative phosphorylation level of BCR in that cluster. This MFI ratio was plotted against AF546-Fab' peak intensity to analyze the relationship between relative phosphorylation levels of BCR and BCR density across several clusters. To estimate the relative recruitment level of Syk in a cluster, we calculated the ratio of Syk MFI to Fab' MFI in the same cluster, and this MFI ratio was plotted against Fab' peak intensity to determine the relationship between relative recruitment level of Syk and BCR density across several clusters. To estimate the amount of pSyk relative to BCR in a cluster, we calculated the ratio of pSyk MFI to Fab' MFI in the same cluster, and this MFI ratio was plotted against Fab' peak intensity to determine the relationship between phosphorylation of Syk and BCR density

across several clusters. SHIP-1 and pSHIP-1 in Fab' clusters were analyzed in a similar manner. To detect pSyk or pSHIP-1 puncta, we used a threshold of 1.3 fold of the background staining outside the B cell contact zone to isolate fluorescently-labeled puncta. Detected puncta that were smaller than 250 nm in diameter were eliminated. To estimate the amount of pSyk relative to BCR in a puncta, we calculated the ratio of pSyk MFI to Fab' MFI in the same puncta, and this MFI ratio was plotted against Fab' peak intensity to determine the relationship between phosphorylation of Syk, and BCR density across several puncta. pSHIP-1 puncta were detected and analyzed in the same way as pSyk puncta.

2.5.9 Statistical analysis

Statistical analysis was performed using the Mann-Whitney U non-parametric test for unpaired groups having different sample sizes or the Student's *t*-test for paired groups with the same sample size. To compare curves, Kolmogorov-Smirnov test was used. Statistical analyses were performed in Microsoft Excel, GraphPad Prism, and MATLAB. All data are presented as mean $\pm$ SEM (standard error of the mean).

2.5.10 MATLAB scripts

All MATLAB scripts used for this study can be found in the Appendices.

Chapter 3: Discussion

3.1 General summary

B cell-mediated humoral immune responses are critical to host defense against infectious diseases. The binding of the BCR on the B cell surface to Ag is the initiation step of antibody responses, which induces intracellular signaling. Research accumulated up to now shows that BCR signaling undergoes a state of amplification followed by attenuation. BCR signaling attenuation is critical for preventing autoimmunity^{101,150,242}. It has been shown that B cell spreading over the Ag-presenting surface contributes to BCR signaling amplification, while BCR signaling attenuation requires B cell contraction, which follows B cell spreading. My research aims to better understand the mechanisms by which B cells switch from spreading to contraction, and how this morphological switch results in downregulation of BCR signaling. Delineation of the molecular mechanisms underlying BCR signaling downregulation will help us better understand the underlying causes of auto-antibody production, leading to autoimmunity. With a clearer understanding of the relationship between actin cytoskeleton-driven morphological changes in B cells and BCR signaling, we will be able to design better targeted therapies for life-threatening illnesses associated with mis-regulation of B cell activation, as well as aid in the discovery of more efficient vaccine-design strategies to enhance B cell-mediated antibody responses.

The goals of this study were twofold: (1) to determine how B cells transition from spreading to contraction, and (2) to determine how B cell contraction drives the transition of BCR signaling from amplification to attenuation. Together, these aims explore how B cells downregulate BCR signaling triggered by membrane-associated Ag. We have identified

specific elements of the actin cytoskeletal machinery that can drive B cell contraction, and consequently BCR signaling downregulation.

The research in this dissertation reveals a novel mechanism through which N-WASP-mediated branched actin foci originating from the lamellipodia induces B cell contraction at the end of B cell spreading, which is critical for decreasing BCR phosphorylation levels in BCR-Ag clusters, an essential step for preventing auto-immunity. This research was based on the previous observations that WASP and N-WASP, both branched actin nucleation promoting factors (NPFs), play distinct roles during the B cell spreading and contraction response. In addition to contributing to B cell spreading, N-WASP is essential for B cell contraction and the downregulation of BCR signaling. This was demonstrated by the failure of splenic B cells from B cell-specific N-WASP knockout mice (conditional N-WASP KO, cNKO) in contraction after spreading on Ag-coated PLBs and elevated auto-antibody levels in cNKO mice⁶⁶. This suggested an interplay between WASP and N-WASP, with the latter being critical for switching B cell spreading to contraction.

A novel finding of this study is that Arp2/3-nucleated branched actin, which is known to be essential for B cell spreading, also plays a critical role in B cell contraction. We observed membrane-proximal F-actin structures nucleated at the contact zone between B cells and Ag-coated PLBs, using both phalloidin staining of fixed cells and time-lapse imaging of LifeAct-GFP or LifeAct-RFP-expressing mice. TIRF microscopy was used to ensure visualization of F-actin proximal to the B cell contact zone with no background signals from the B cell's interior. We found that dynamic F-actin foci populated the inner region of the B cell contact zone during contraction. These foci appeared behind the lamellar region, approximately in the shape of a ring. Importantly, when perturbing branched actin nucleation using a small

molecule inhibitor (SMI) of Arp2/3 complex, CK-666, both the percentage of B cells undergoing contraction and the number of the inner F-actin foci were reduced. This result suggests that these inner F-actin foci are mainly composed of branched actin. Since we didn't test the role of Formins due to unavailability of a specific SMI against it, we cannot rule out bundled actin in the generation of these foci. Interestingly, the inhibition of Arp2/3 didn't abolish the inner F-actin foci, but only decreased their relative lifetime and relative centripetal speed. This might suggest that the loci of nucleation, where Arp2/3 initiates branched actin foci nucleation, still exist, and might be composed of actin bundles nucleated by formins. This alludes to the possibility of actin bundles serving as a framework for the nucleation and propagation of branched actin foci in B cells. A similar mechanism of dynamic branched actin foci interspersed with F-actin filaments has been proposed to maintain T cell immune synapses in response to antigen on supported lipid bilayers, however, these branched actin foci in T cells were found to be dependent on WASP, not N-WASP, which is in contrast to our findings in B cells²⁶⁸.

My thesis research examined the distinct roles of WASP and N-WASP in generating these branched actin foci. Comparing splenic B cells from WT and WASP KO (WKO) mice expressing LifeAct-GFP, we found that WKO B cells were able to contract as well as generate dynamic inner F-actin foci. In contrast, the N-WASP SMI, Wiskostatin, and cNKO reduced B cell contraction and inner F-actin foci formation. This result suggests that N-WASP, but not WASP, activates Arp2/3 to nucleate inner F-actin foci. The relative lifetime and centripetal speed of the foci were significantly reduced in CK-666- or Wiskostatin-treated B cells, suggesting that N-WASP helps to maintain these dynamic actin structures. Taken together, these results suggest that WASP and N-WASP drive the nucleation of distinct branched F-actin structures: WASP and N-WASP activated branched F-actin

generates lamellipodia for B cell spreading while the N-WASP activated branched F-actin generates inner actin foci for B cell contraction.

This study also addressed the question how and when the inner F-actin foci were generated. We observed F-actin structures in the contact zone of LifeAct-GFP-expressing B cells at the peak of spreading, when B cells switch from spreading to contraction. Interestingly, we found that retraction of the leading edge of the B cell contact zone was spatiotemporally correlated with the emergence of F-actin foci behind the lamellipodium. Additionally, perturbation of N-WASP, but not WASP significantly decreased the presence of lamellipodia-derived F-actin foci, further reinforcing the role of these foci in switching B cell spreading to contraction.

Previous research conducted by our lab has shown that non-muscle myosin II (NMII) is required for B cell contraction and the downregulation of BCR signaling in response to Ag-coated PLBs²⁴². Since the perturbation of N-WASP decreased the emergence and relative centripetal speed of the inner F-actin foci, we hypothesized that N-WASP-mediated branched actin structures recruit NMII, leading to their re-organization into F-actin foci that are distinct from the lamellipodia, followed by acto-myosin treadmilling, which moves these foci centripetally towards the rear of the lamella. This is supported by the role of myosin in the generation of transverse arcs and actin treadmilling in motile cells²⁵⁰. To test this, we looked at myosin recruitment to F-actin structures using LifeAct-RFP and GFP-NMIIA-expressing mice. First, we observed that perturbation of N-WASP using Wiskostatin drastically reduced NMII recruitment to the B cell contact zone, suggesting that N-WASP-mediated structures recruit NMII. More specifically, we observed NMII begin to appear at the rear of the leading edge of the lamellipodial actin at the peak of spreading, just before the emergence of an F-actin focus. Additionally, as the F-actin foci move centripetally inward, NMII forms a ring-

like distribution between the other lamellipodial distribution of F-actin and the inner ring-like distribution of inner F-actin foci. Based on earlier evidence for the role of Arp2/3 and myosin in actin treadmilling and the leading-edge advance of migrating cells, we speculate that the inner F-actin foci might be composed of transverse arcs, which are known to be rich in myosin²⁵⁰. In support of our observations, acto-myosin ring formation, studied by a previous graduate student in our lab, showed a dependence on N-WASP, but not WASP (Michelle Lazzaro, unpublished data). Briefly, NMII was stained in WKO and cNKO B cells and compared to WT B cells. While in WT B cells, NMII was observed to form a ring at the rear of the lamella, cNKO B cells did not generate this NMII ring, and WKO B cells displayed premature NMII ring formation. Taken together, these results show that N-WASP, but not WASP nucleates F-actin foci, that recruit myosin, emerge from the lamellipodia, and move centripetally inwards away from the leading front of the cell membrane at the peak of B cell spreading. These F-actin foci are associated with B cell contraction, as perturbation of N-WASP and Arp2/3, which are required for their formation and maintenance, also inhibits B cell contraction.

Work relating to my second aim, examining the effect of B cell contraction of BCR-Ag clusters and BCR signaling, showed that the molecular density of BCR in BCR-Ag clusters increases more rapidly during B cell contraction as compared to during B cell spreading or after B cell contraction. To observe BCR-Ag clusters, we utilized a 1:10 mixture of AF546-labeled:unlabeled F_{ab} fragment of anti-mouse Ig(G+M) coated onto PLBs to stimulate B cells. Our lab previously analyzed this Ag-presentation model and found that it successfully mimics “real” Ag presented on the cell membranes of APCs¹⁵⁹. Upon BCR-mediated clustering of the fluorescently-labeled Ag on the PLB, its local fluorescence intensity increases, which we detected using TIRF time-lapse microscopy, and used as a metric for BCR density in BCR-

Ag clusters. Perturbation of N-WASP or Arp2/3 using SMIs or cNKO mice, which were previously shown to inhibit B cell contraction, also showed reduced BCR density as compared to contracting control B cells, confirming that B cell contraction is necessary for increasing the density of BCR in BCR-Ag clusters.

To understand how BCR density affects BCR signaling, we studied the relationship between BCR density and the relative level of BCR phosphorylation within individual BCR-Ag clusters, measured using pCD79a staining. We found that beyond an initial rise in BCR phosphorylation, it is inversely related to increasing BCR density in BCR-Ag clusters.

Additionally, perturbing B cell contraction through inhibition of Arp2/3 or N-WASP caused B cells to retain high levels of BCR phosphorylation as compared to contracting control B cells, suggesting that the increased molecular density of BCR in BCR-Ag clusters plays a role in downregulating BCR signaling.

Previous research on BCR signaling has shown that the signaling kinase Btk is essential for BCR signaling activation, while the phosphatase SHIP-1 is essential for downregulation of BCR signaling. Knocking out SHIP-1 in mouse splenic B cells resulted in a lack of B cell contraction and auto-immune phenotypes. Additionally, inhibition of Btk using an SMI against it in SHIP-1 KO B cells rescued the effect of knocking out SHIP-1¹⁰¹. This suggested an antagonistic interplay between the stimulatory kinase Btk and inhibitory phosphatase SHIP-1 in fine-tuning BCR signaling in response to Ag on PLBs. Based on these findings, I hypothesized that denser BCR-Ag clusters selectively recruit more inhibitory phosphatases than stimulatory kinases, which then downregulates BCR phosphorylation specifically in denser BCR clusters, leading to overall BCR signaling attenuation in the B cell. To test this hypothesis, we analyzed the recruitment and phosphorylation levels of stimulatory kinase Syk and inhibitory phosphatase SHIP-1 in BCR clusters of varying density. Syk was selected due

to its direct association with dual-phosphorylated BCR ITAMs and its known role in activating BCR signaling in response to Ag²⁷⁹. Upon being phosphorylated, pSyk activates PLC γ 2, leading to the amplification of BCR signaling and early signaling events such as Ca²⁺ flux⁸⁹⁻⁹¹. SHIP-1 was selected for its well characterized role in the downregulation of BCR signaling^{101,280}. SHIP-1 is widely known to be recruited to the ITIM in the intracellular domain of Fc γ RIIb in B cells, but recent studies have also shown its Fc γ RIIb-independent recruitment to PIP₂ through Dok3/Grb2¹⁹⁶. It has also been suggested that SHIP-1 may directly bind to partially phosphorylated ITAMs¹⁹⁷. Upon being phosphorylated, pSHIP-1 antagonizes BCR signaling indirectly by depleting PI(3,4,5)P₃ on the inner leaflet of lipid raft domains, which serves as a docking site for Btk and PLC γ 2. Therefore, pSHIP-1 indirectly affects BCR phosphorylation through antagonizing lipid raft components⁹⁴.

To examine the recruitment and phosphorylation levels of Syk and SHIP-1 in BCR-Ag clusters, we stained pSyk, Syk, pSHIP-1 and SHIP-1 in B cells (in separate experiments), and imaged them using TIRF microscopy. We found that both the recruitment and phosphorylation levels of Syk, relative to BCR, decreased with increasing BCR density. Unexpectedly, the recruitment and phosphorylation levels of SHIP-1, relative to BCR, also decreased with increasing BCR density. Even though the overall recruitment and phosphorylation levels of SHIP-1 in the B cell contact zone increase during B cell contraction, their levels relative to the BCR decrease as BCR-Ag clusters get denser. Therefore, with increasing BCR density, seemingly less and less pSyk and pSHIP-1 is available per BCR molecule, within each BCR cluster. cNKO B cells, which lack B cell contraction and are unable to increase BCR density to the same extent as contracting B cells, displayed elevated levels of both pSyk and pSHIP-1. A closer comparison of the sensitivities of Syk and SHIP-1 recruitment to BCR density revealed that while the recruitment of Syk

decreased after a certain threshold of BCR density, the recruitment of SHIP-1 decreased over a relatively greater range of BCR densities. Additionally, the threshold of BCR density at which Syk recruitment decreases coincides with the maximum BCR density achieved by non-contracting B cells, suggesting that B cell contraction is required to achieve the optimal BCR density for dissociation of Syk. On the other hand, SHIP-1 recruitment decreases more rapidly with increasing BCR density at lower ranges of BCR density as compared to Syk. As a result, cNKO B cells, which lack B cell contraction, displayed similar levels of SHIP-1 recruitment relative to BCR as compared to control B cells. This is in contrast to our previous understanding of the role of SHIP-1 in downregulating BCR signaling. Firstly, with increasing BCR density, both stimulatory and inhibitory signaling molecules are dissociated from BCR clusters. Secondly, the lack of BCR signaling downregulation in non-contracting B cells is mainly due to enhanced Syk signaling, with similar SHIP-1 levels relative to BCR in contracting and non-contracting B cells.

Taken together, these results bring into focus a previously undescribed mechanism through which B cell contraction, mediated by N-WASP and Arp2/3, but not WASP, downregulates BCR signaling by increasing the molecular density of BCR in BCR-Ag clusters. This increase in molecular density leads to a decrease in BCR signaling through the disassociation of both stimulatory and inhibitory signaling molecules, wherein the stimulatory kinase Syk disassociates from BCR clusters upon reaching a threshold of BCR density, achievable through B cell contraction. Although this only partially explains the lack of BCR signaling downregulation in cNKO B cells, which lack B cell contraction, it exposes a novel mechanism by which B cells use the density of BCR to modulate their signaling. The disassociation of signaling molecules from denser BCR-Ag clusters might be due to space constraints near the ITAMs of BCR that are very densely packed. The observation that Syk

recruitment reduces at higher ranges of BCR density while SHIP-1 recruitment reduces more drastically at lower ranges of BCR density, combined with the fact that Syk has roughly half the molecular weight of SHIP-1, supports this theory^{88,281}. Alternatively, increased BCR density might change the magnitude or direction or both, of B cell-generated forces applied through the B cell receptor. It has been shown that B cells are able to generate piconewton level forces that are transmitted through the BCR, and studies have also shown that these forces might induce a conformational change in the BCR molecule which affects their accumulation in BCR-Ag clusters^{75,218}. Such conformational changes might be responsible for increasing the density of BCR-Ag clusters, leading to the inaccessibility of BCR ITAMs from stimulatory and inhibitory signaling molecules.

3.2 Working model

Based on our findings we propose the following working model (Fig. 3.1). At the peak of B cell spreading, N-WASP, but not WASP, activates Arp2/3 to nucleate dynamic branched actin foci distinct from lamellipodial F-actin. These N-WASP-mediated branched actin foci, upon originating from the lamellipodia, recruit NMII, which drives their centripetally inward movement away from the leading front of the B cell membrane towards the central part of the B cell contact zone. N-WASP-activated Arp2/3 help maintain these dynamic inner F-actin foci by extending their lifetime, which allows NMII to be recruited to them. These F-actin foci likely generate the acto-myosin ring observed in B cell contact zones during B cell contraction, driving B cell contraction. B cell contraction downregulates BCR signaling by increasing the molecular density of BCR-Ag clusters. At very low BCR density, relatively high levels of both Syk and SHIP-1 are recruited to BCR clusters. At medium BCR density, the level of Syk associated with BCR clusters remains consistent, possibly due to the direct binding of Syk to the dual-phosphorylated ITAMs of the BCR, while SHIP-1 recruitment decreases. This results in an overall increase in the signaling capacity of BCR clusters go from low to medium density. At very high BCR density, both Syk and SHIP-1 disassociate from BCR clusters, leading to an overall reduction in BCR signaling as BCR clusters go from medium to high density. The dependence of Syk and SHIP-1 disassociation with BCR-Ag clusters with their molecular density is likely due to a spatial limitation among densely packed BCRs, but could also be due to differences in B cell-generated mechanical forces applied through the BCR, resulting in conformational changes within BCR signaling domains.

When the actin-related proteins like Arp2/3, N-WASP, and NMII are inhibited or knockout, B cells fail or delay the contraction process, as the branched actin foci that are critical for B

cell contraction are not effectively generated, maintained, and propagated. Without B cell contraction, the molecular density of BCR clusters likely favor stimulatory kinases, like Syk, which prolongs and elevates BCR signaling. Similar to cNKO B cells, SHIP-1 KO B cells display delayed B cell contraction and overall elevated phosphorylation levels^{66,101}. In both cases, the lack of BCR signaling downregulation leads to elevated BCR signaling even in B cells that might have low affinity to Ag in germinal centers, which allow these low affinity B cells to escape from Ag-bind affinity-based selection and differentiate into antibody-secreting plasma cells, thereby leading to low affinity antibody and auto-antibody production.

Figure 3.1

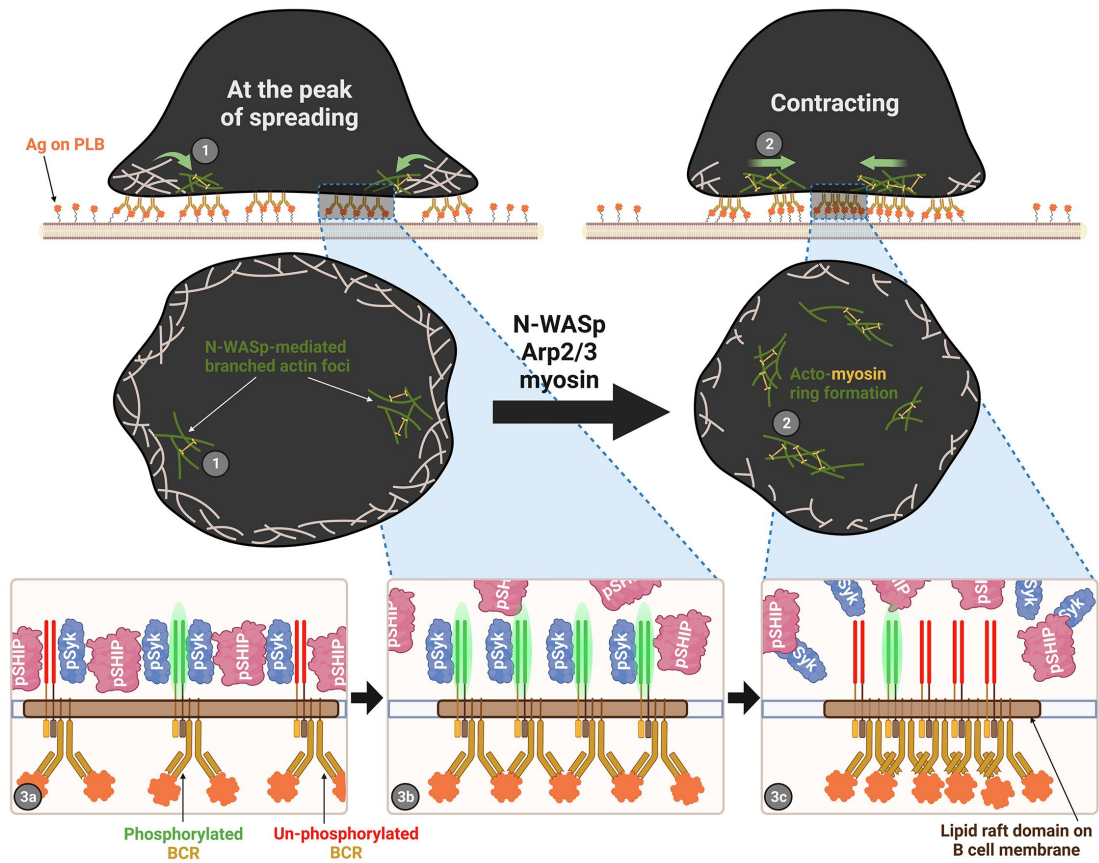


Figure 3.1: Working model. At the peak of B cell spreading, N-WASP, but not WASP, activates Arp2/3 to nucleate dynamic branched actin foci distinct from lamellipodial F-actin (1). These N-WASP-mediated branched actin foci, upon originating from the lamellipodia, recruit NMII, which drives their centripetally inward movement away from the leading front of the B cell membrane towards the central part of the B cell contact zone (2). N-WASP and Arp2/3 help maintain these dynamic inner F-actin foci by extending their lifetime, which allows NMII to be recruited to them. These F-actin foci likely generate the acto-myosin ring typically observed in B cell contact zones during B cell contraction, driving B cell contraction. B cell contraction downregulates BCR signaling by increasing the molecular density of BCR in BCR-Ag clusters. At very low BCR density, relatively high levels of both Syk and SHIP-1 are recruited to BCR clusters (3a). At medium BCR density, Syk recruitment

remains consistent, possibly due to the combined effect of Syk being directly recruited to dual-phosphorylated ITAMs and its dissociation from relatively higher density BCR-Ag clusters, while SHIP-1 recruitment decreases (3b). This results in an overall increase in ITAM phosphorylation as BCR clusters go from low to medium density. At very high BCR density, even Syk disassociates from BCR clusters (3c), leading to an overall reduction in phosphorylated ITAMs, which downregulates BCR signaling as BCR clusters go from medium to high density. Created with *BioRender.com*.

3.3 Limitations of the study and future directions

While this study answers some of the questions we set out to answer, it raises several new ones. The branched actin foci originating from the lamellipodia at the peak of B cell spreading, are yet to be characterized. This could be potentially achieved through super-resolution imaging using techniques such as direct stochastic optical reconstruction microscopy (dSTORM) and structured illumination microscopy (SIM)^{139,282}. These technologies would enable visualization of detailed organizations of both denser F-actin fibers and less dense actin filament networks. The dynamics of these actin structure can be examined using B cells expressing fluorescence protein fusion of F-tractin, an F-actin binding peptide similar to LifeAct. F-tractin is currently considered the best among live actin probes as it is not excluded from any major F-actin structures unlike LifeAct²⁸³. A major drawback of using F-tractin is the need for transfection, as B cells must be provided with stimulatory signals in the form of LPS or IL-2 to induce successful transfection. These stimulatory signals will induce B cell proliferation and differentiation, changing B cells from the naïve to stimulated state and influencing their response to Ag stimulation.

Although we showed that dynamic F-actin foci generated by N-WASP- but not WASP-activated Arp2/3 enables the transition from B cell spreading to contraction, we did not explore how WASP and N-WASP nucleate branched actin structures with distinct properties and behavior. WASP and N-WASP share more than 50% sequence homology, and the structural differences between them might explain the difference between their roles in B cells²⁵⁷. Both WASP and N-WASP are known to be activated by PI(4,5)P₂ (PIP₂) and CDC42, but N-WASP has also been shown to be activated by Rac1 in a more potent manner, which might explain why N-WASP-mediated branched actin structures dominate over the WASP-mediated branched actin structures during B cell contraction²⁸⁴. More in-depth studies

of WASP and N-WASP might help elucidate these functional differences between them and the branched actin structures they nucleate, and might also help understand the molecular mechanisms underlying Wiskott-Aldrich syndrome.

Surprisingly, we observed little to no colocalization between the inner F-actin foci visualized by LifeAct-RFP and NMII by GFP-NMIIA. This might be due to the known limitation of LifeAct peptide in binding denser F-actin fibers, which are most likely the same structures that recruit NMII²⁸³. Since LifeAct readily binds more dynamic F-actin filaments (but not denser fibers), it is possible that F-actin foci observed using LifeAct-GFP or LifeAct-RFP are only partial representations of the actual actin cytoskeletal network involved in switching B cell spreading to contraction. To counteract this, we used phalloidin-staining to visualize F-actin structures in more detail, but due to the need for fixation, we were unable to observe actin dynamics. Phalloidin staining of contracting B cells revealed F-actin foci similar to LifeAct, but were more obviously organized in a ring-like distribution around the center of the contact zone. Co-staining NMIIA by antibody and F-actin by phalloidin may reveal the acto-myosin structures better.

The experiments described in this dissertation mostly utilized artificial Ag-presenting surfaces to stimulate primary splenic B cells from mice. While PLB preserves the lateral motility of the cell membrane, the membrane composition and the stiffness are very different from *in vivo*. Song lab has previously established neuronal cell line transfected with transmembrane hen egg lysozyme (mHEL) and show that primary B cells expressing the Ig transgene that is specific for HEL binds to mHEL on the cell surface, which induces surface BCR clustering¹⁵⁹. As the mHEL-expressing cells mimic APCs *in vivo* better than Ag-coated PLB, it would be interesting to test if B cells undergo similar spreading and contraction when

engaging mHEL-expressing cells. Since the density of BCR in BCR-Ag clusters affect BCR phosphorylation levels, it stands to reason that the density of Ag on the Ag-presenting surface and the binding affinity of the BCR to Ag likely affect BCR signaling via modulating the molecular density within individual BCR-Ag clusters. I hypothesize that B cells expressing high affinity BCRs will spread and contract more effectively, clustering more BCRs and eventually with higher molecular density, than those engaging Ag with lower affinity, which leads to high levels of signaling followed by effective attenuation. This can be tested by comparing the responses of Ag-specific B cells, such as those from MD4 mice, on mHEL-expressing or membrane duck egg lysozyme (mDEL)-expressing epithelial cell lines, where mDEL serves as a low-affinity version of mHEL against HEL-specific MD4 B cells²⁸⁵. Additionally, since all measurements of BCR molecular density were made using the peak intensity of AF546-Fab' in this study, further validation using dSTORM might be more convincing.

Even though cNKO inhibited B cell contraction and reduced the molecular density within BCR cluster, cNKO did not affect the disassociation of both stimulatory and inhibitory signaling molecules with the increase in the molecular density of BCR clusters. However, the levels of both total Syk and phosphorylated Syk, but not total SHIP-1 and phosphorylated SHIP-1, in individual BCR clusters were higher in cNKO than WT B within BCR clusters of similar molecular densities. Therefore, the elevated Syk signaling in cNKO B cells is only partially explained by its hypothesized dependence on the molecular density within BCR clusters. The cause for this elevated Syk recruitment and phosphorylation in cNKO cells remains an interesting avenue for scientific exploration.

Lastly, how increasing molecular density of BCR in BCR-Ag clusters causes the dissociation of Syk and SHIP-1 remains unexplored. This could be either due to a lack of space between densely packed BCRs, or it could be due to conformational changes induced by B cell-generated forces for B cell contraction and forces transmitted through the BCR²¹⁸. The involvement of forces can be examined using DNA-based force probes to tether Ag to PLBs and traction force microscopy. Using these methods, we can characterize the overall forces that B cells exert on the Ag-presenting surface and forces applied through individual BCR-Ag clusters with varying BCR density. Conformational changes in the BCR can be analyzed using fluorescence resonance energy transfer (FRET)-based assays²⁸⁶⁻²⁸⁸.

In total, the data in this dissertation reveal a novel mechanism by which B cells utilize actin-cytoskeletal remodeling to negative regulate BCR signaling in response to Ag presented on APCs, a key step in the early stages of B cell activation. Collectively, a better understanding of the factors regulating the duration and extent of BCR signaling response to Ag, consequently the selection of Ag-specific B cells in germinal centers, will shed critical light on regulatory mechanisms which control the quality and quantity of humoral responses.

Supplementary figures and videos

Supplementary Figure S1

Figure S1

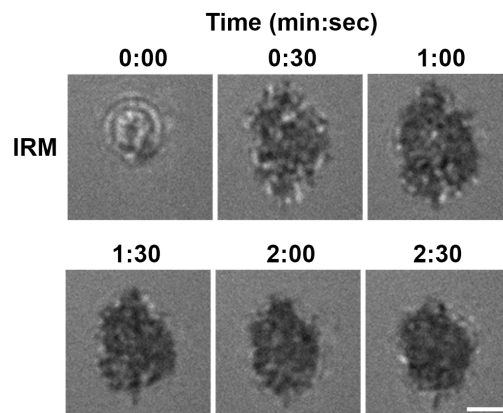


Figure S1. B cells spread for around 1 minute, which is followed by contraction for around 1.5 minutes, on Fab'-coated-planar lipid bilayers. Splenic B cells were incubated with planar lipid bilayers coated with monobiotinylated Fab' fragment of goat anti-mouse IgG+M (Fab'-PLB) and imaged live at 37°C by interference reflection microscopy (IRM). The B cell plasma membrane area contacting with Fab'-PLB (B cell contact zone) was visualized using IRM. Shown are individual frames from a time-lapse IRM image of one B cell. Scale bar, 2 μm .

Figure S2

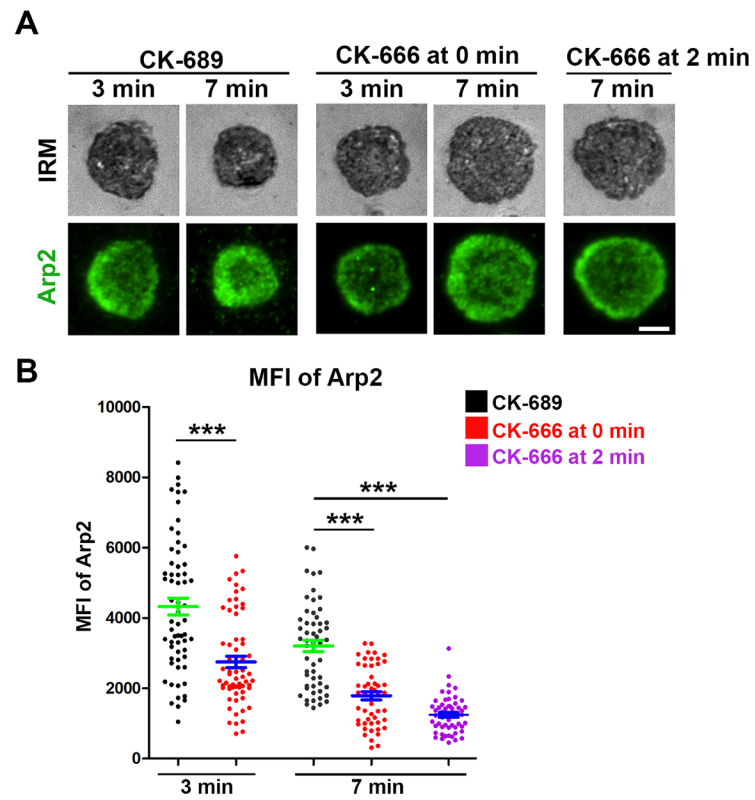


Figure S2. Treatment with CK-666 significantly decreases Arp2/3 recruitment within two minutes of its addition. (A-B) WT splenic B cells were treated with CK-689 or CK-666 (50 μ M) before and after maximal spreading during incubation with Fab'-PLB at 37°C. Cells were fixed at 3 and 7 min, permeabilized, stained for Arp2, and analyzed using IRM and TIRF. **(A)** Shown are representative images of WT splenic B cells treated with CK-689 or CK-666 from 0 min (before maximal spreading), or from 2 min (after maximal spreading), during incubation with Fab'-PLB at 37°C. **(B)** The MFI of Arp2 in the contact zone at 3 min and 7 min were compared between B cell treated with CK-689 (black dots), CK-666 from 0 min (red dots), and CK-666 from 2 min (purple dots). Data points represent individual cells from 3 independent experiments with ~20 cells per condition per experiment. Scale bar, 2 μ m. *** $p < 0.001$, by non-parametric student's t -test.

Figure S3

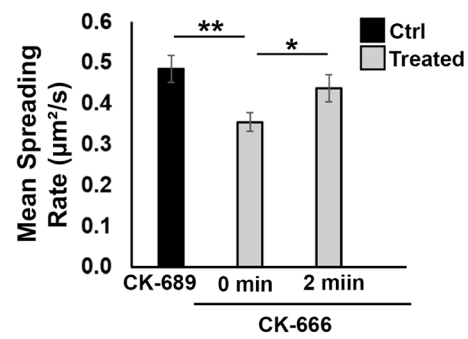


Figure S3. Perturbation of Arp2/3 with CK-666 before maximal B cell spreading decreased the spreading kinetics, but not when CK-666 was introduced after maximal spreading. WT splenic B cells were treated with CK-689 or CK-666 (50 μ M) before and after maximal spreading during incubation with Fab'-PLB, and imaged live at 37°C by IRM. The area occupied by the B cell contact zone was measured using IRM images and custom codes made in MATLAB. Mean spreading rate of each cell was quantified using linear regression on the contact zone-area vs time curve of that cell, during its early spreading phase. The averaged mean spreading rates ($\pm$ SEM) of WT splenic B cells treated with CK-689 or CK-666 from 0 min (before maximal spreading), or from 2 min (after maximal spreading), during incubation with Fab'-PLB at 37°C were compared.

Figure S4

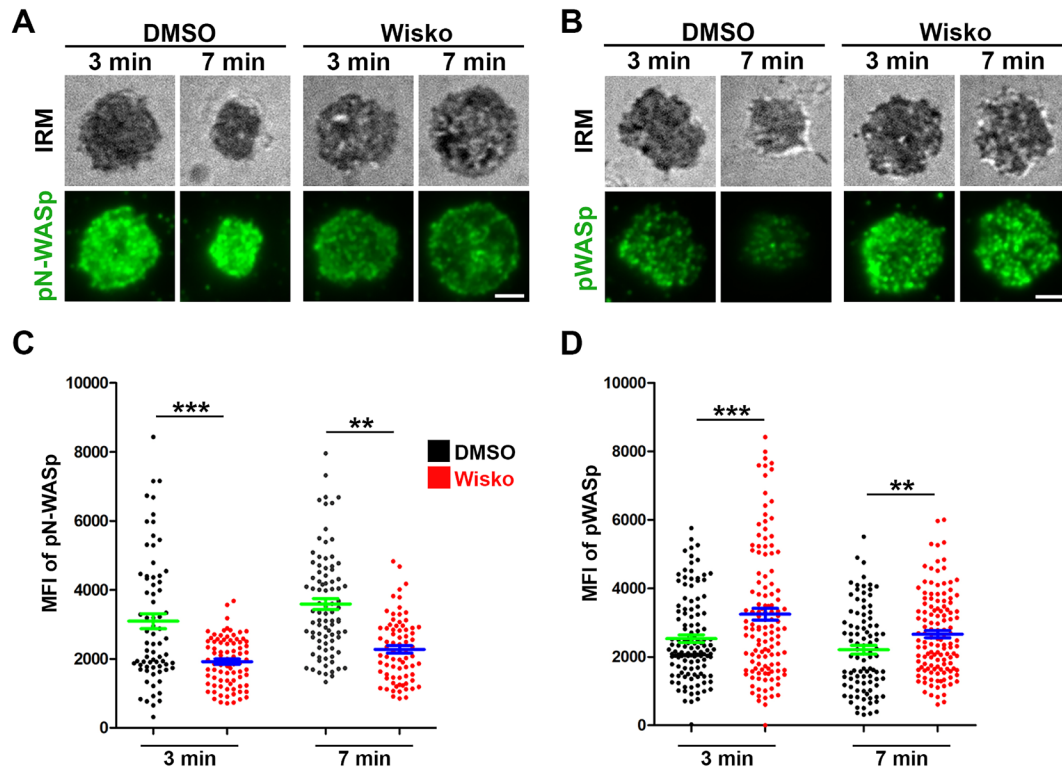


Figure S4. Wiskostatin treatment inhibits N-WASP activation while enhancing WASP activation in B cells. WT splenic B cells were pre-treated with Wiskostatin (Wisko; 10 μ M) or DMSO (control) for 10 min at 37°C before incubation with Fab'-PLB in the presence of 10 μ M Wisko (or DMSO). Cells were fixed at 3 and 7 min, permeabilized, stained for pN-WASP or pWASP, and analyzed using IRM and TIRF. Shown are representative IRM and TIRF images of DMSO and Wisko-treated B cells at 3 min and 7min, showing pN-WASP **(A)** and pWASP **(B)** at the B cell contact zone. The MFI ($\pm$ SEM) of pN-WASP in the B cell contact zone was compared between DMSO and Wisko-treated B cells **(C)**, and the MFI ($\pm$ SEM) of pWASP in the B cell contact zone was compared between DMSO and Wisko-treated B cells **(D)**. Data points represent individual cells from 3 independent experiments with ~25 cells per condition per experiment. Scale bar, 2 μ m. ** $p < 0.01$, *** $p < 0.001$, by non-parametric student's *t*-test.

Figure S5

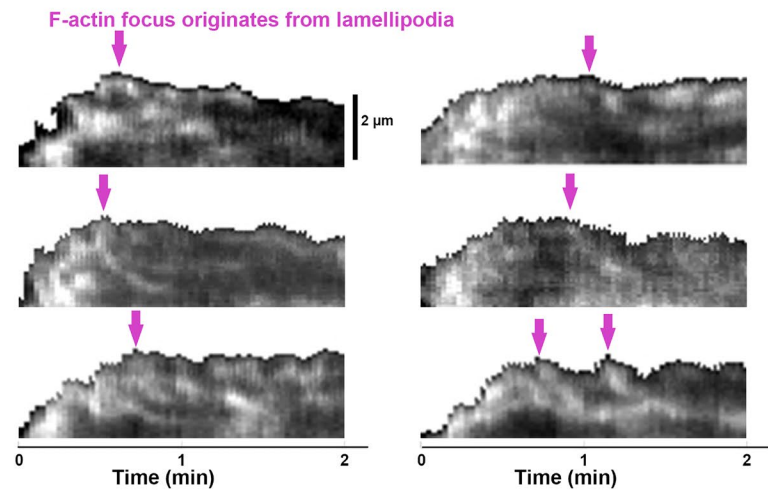


Figure S5. Inner F-actin foci are originated from lamellipodia behind the spreading membrane. Mouse splenic B cells from LifeAct-GFP transgenic mice were treated with DMSO or Wisko (10 μ M), imaged live using TIRF during incubation with Fab'-PLB at 37°C, and analyzed using kymographs that were randomly generated from each cell. Arrows indicate the emergence of inner F-actin foci near the lamellipodia. Lamellipodia-derived inner F-actin foci were identified by their LifeAct-GFP FI >2 fold of their nearby region, inside location in the contact zone, migrating out the lamellipodial F-actin, and trackable for >8 sec. Shown are examples of kymographs from separate DMSO-treated (control) B cells with visible lamellipodia-derived inner F-actin foci.

Supplementary Figure S6

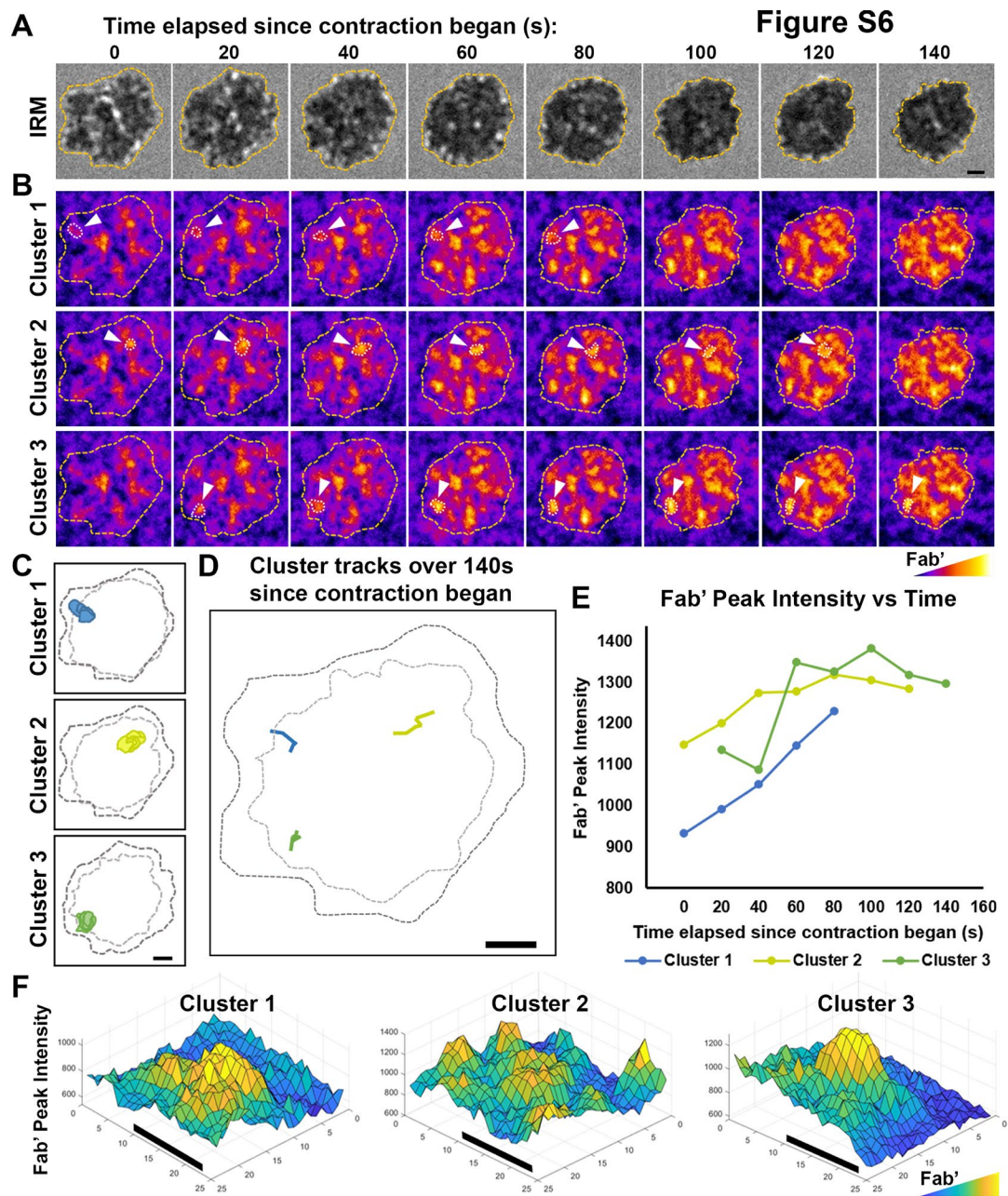


Figure S6. AF546-Fab' clusters, tracked and analyzed over time using a custom MATLAB code, indicates that B cell contraction increases the molecular density within BCR clusters. Primary B cells from WT mice were incubated with AF546-Fab'-PLB at 37°C, and imaged live using IRM and TIRF. Shown are individual frames from time lapse images of IRM (**A**) and TIRF (**B**) showing AF546-Fab' clusters within the contact zone of one DMSO-treated (vehicle control for Wisko) B cell, for 140 sec since the beginning of contraction (immediately after maximal spreading). Fab' FI has been represented as a heat-map using NIH ImageJ. The boundary of the contact zone, detected by a custom MATLAB script, is shown in yellow dashed lines. Arrows point to three representative clusters among the several clusters detected in the contact zone using custom MATLAB codes. Cluster detection masks for the three representative clusters have been shown, indicating their frame-by-frame segmentation (**C**). Cluster tracks for the three AF546-Fab' clusters are shown alongside the initial and final state of the contact zone shown in gray dashed lines (**D**). Tracks were generated by following the peak of AF546 FI in each cluster as it moved. AF546-Fab' peak intensity versus time curves for the three representative clusters are shown over the time-duration that each cluster could be detected (**E**). Surface-plots (also called 2.5-D plots) of AF546-Fab' intensity showing a zoomed-in region consisting of each of the three Fab' clusters (**F**). Colors in (B) and (F) are scaled to AF546-Fab' intensity values. Scale bars, 1 μm .

Video Legends

Video 1. Arp2/3, activated by N-WASP but not WASP, is required for B cell

contraction. Splenic B cells were incubated with planar lipid bilayers coated with monobiotinylated Fab' fragment of goat anti-mouse IgG+M (Fab'-PLB) in the absence and presence of various inhibitors and imaged live at 37°C by interference reflection microscopy (IRM). The B cell plasma membrane area contacting with Fab'-PLB (B cell contact zone) was measured using IRM images and custom codes made in MATLAB. **(A)** Representative IRM time-lapse images of splenic B cells from C57BL/6 mice treated with CK-689 or CK-666 (50 μ M) before and after maximal spreading. The frame in which the contact zone first appears, viewed using IRM, was considered time 0. A "+" sign at the bottom right of an image denotes the presence of inhibitor. **(B)** Representative IRM time-lapse images of splenic B cells from C57BL/6 mice treated with DMSO or Wiskostatin (Wisko, 10 μ M) 10 min before and during incubation with Fab'-PLB. **(C)** Representative IRM time-lapse images of splenic B cells from flox control and B cell-specific N-WASP knockout (cNKO) mice. **(D)** Representative IRM time-lapse images of splenic B cells from WT or WASP knockout mice (WKO). The video is sped up by 20x compared to real time. Original IRM and TIRF time-lapse videos were acquired at 0.5 frames per second. Scale bar, 2 μ m.

Video 2. Arp2/3, downstream of N-WASP, generates inner F-actin foci, driving B cell

contraction. Mouse splenic B cells from LifeAct-GFP transgenic mice were incubated with Fab'-PLB in the absence and presence of various inhibitors and imaged live at 37°C by IRM and total internal reflection fluorescence (TIRF) microscopy. Representative IRM **(A)** and TIRF **(B)** time-lapse images of splenic B cells from C57BL/6 mice treated with CK-689 or CK-666 (50 μ M) before and after maximal spreading. A "+" sign at the bottom right of an image denotes the presence of inhibitor. Representative IRM **(C)** and TIRF **(D)** time-lapse

images of splenic B cells from C57BL/6 mice treated with DMSO or Wisko (10 μ M) 10 min before and during incubation with Fab'-PLB. Representative IRM (C) and TIRF (F) time-lapse images of splenic B cells from LifeAct-GFP-expressing (WT) or WASP knockout mice expressing LifeAct-GFP (WKO X LifeAct-GFP). The video is sped up by 20x compared to real time. Original IRM and TIRF time-lapse videos were acquired at 0.5 frames per second. Scale bar, 2 μ m.

Video 3. Inner F-actin foci, generated by N-WASP-activated Arp2/3, facilitate NMII recruitment and ring-like structure formation. B cells from mice expressing GFP fusion of non-muscle myosin IIA (GFP-NMIIA) transgene as well as LifeAct-RFP (GFP-NMIIA X LifeAct-GFP) were treated with DMSO or Wisko (10 μ M) 10 min before and during incubation with Fab'-PLB. The B cell contact zones were imaged live using IRM and TIRF. Shown are representative IRM and TIRF time-lapse images of DMSO-versus Wisko-treated B cells. The video is sped up by 20x compared to real time. Original IRM and TIRF time-lapse videos were acquired at 0.5 frames per second. Scale bar, 2 μ m.

Video 4. B cell contraction promotes a rapid increase in the molecular density within BCR-Fab' clusters. Mouse splenic B cells were incubated with planar lipid bilayers coated with a 1:10 molar ratio mixture of AF546-labelled and unlabeled Fab' fragment of goat anti-mouse IgG+M (AF546-Fab'-PLB) in the absence and presence of various inhibitors and imaged live at 37°C by IRM and total internal reflection fluorescence (TIRF) microscopy. Representative IRM (A) and TIRF (B) time-lapse images of splenic B cells from C57BL/6 mice treated with CK-689 or CK-666 (50 μ M) before and after maximal spreading. A "+" sign at the bottom right of an image denotes the presence of inhibitor. Representative IRM (C) and TIRF (D) time-lapse images of splenic B cells from C57BL/6 mice treated with DMSO or Wisko (10 μ M) 10 min before and during incubation with Fab'-PLB.

Representative IRM (**E**) and TIRF (**F**) time-lapse images of splenic B cells from Flox (ctrl) or cNKO mice. Colors in (B), (D), and (F) are scaled to AF546-Fab' intensity values. The video is sped up by 20x compared to real time. Original IRM and TIRF time-lapse videos were acquired at 0.5 frames per second. Scale bar, 2 μm .

Appendices

MATLAB scripts

Script 1: To detect B cell contact zones in IRM images. This script inputs raw 16-bit .nd2 images and outputs a binary mask of all detected contact zones for further analysis.

```
function [MAP] = TIRF_findCells_nd2(MAP,dir_loc)

%Designed for New NIKON Microscope Images (.nd2)

%Inputs a struct of masks (MAP) and directory location of an IRM image.
%Stores the newly obtained logical (binary) mask, well ID and the image
name in the struct MAP and returns it.

%Circles objects and asks user to remove if necessary. Also has two
different modes: "z" and "x".
%"z" enables user to select objects instead of removing them. The code
removes all other unselected objects (reversemode).
%"x" enables user to manually draw objects.

locL = length(dir_loc);
bksl = locL;
count = 1;
for i = locL:-1:1
    if dir_loc(i)=='\ '
        bksl(count) = i;
        count = count+1;
    else
        continue;
    end
end
bksl_1 = bksl(1);
bksl_2 = bksl(2);
well = dir_loc(bksl_2+1:bksl_1-1);%records the time-point or well

disp(' ');
disp('*Looking for objects...')

IP = openND2_still([dir_loc, '.nd2']);
im = IP(:,:,1);%inputs IRM image
im = imadjust(im);
im1 = (imadjust(uint16(bpass(65535-im,1,150,20))));%inverts and band-passes
IRM image
t = 40000;%set threshold value
imbw = im1>t;%apply threshold
se = strel('disk',1,0);
se2 = strel('disk',2,0);
imbw1 = bwareaopen((imerode((imdilate(imbw,se2)),se)),750);%clean up noise
Rd = 1000;%drop-off radius
[imlb, n] = bwlabel(imbw1);%label objects in logical matrix
```

```

for k = 1:1:n
    I = (imlb==k);%isolate each cell object
    if beyond_dropoff_v1(I,Rd) == 1%check if the centroid of the cell is
too far
        imbw1 = (imbw1 - I);%omit cell if too far
    else
        continue;
    end
end%excludes cells beyond the drop-off radius
imbw1 = imfill(imbw1,'holes');%fills holes

f = 1;%set flag
while f==1

    [imlb, n] = bwlabel(imbw1);%label objects in logical matrix
    B = cell(1,n);

    subplot(1,2,1);
    h1 = imshow(imadjust(im));

    subplot(1,2,2);
    imshow(imadjust(im));
    hold on;

    for i = 1:n
        I = (imlb==i);%isolate each cell object
        Bound = bwboundaries(I);
        B(1,i) = Bound(1,1);%get boundaries of each cell
        l = size(B{1,i});

        for j = 1:l
            plot(B{1,i}(:,2), B{1,i}(:,1), 'r.', 'MarkerSize', 2);
        end%plots boundaries
        [cm_x, cm_y] = Centroid(I);
        text((cm_y-
20),cm_x,num2str(i),'Color','green','FontSize',10);%labels cells on image
    end

    x = 1024;
    y = 1024;
    ang=0:0.01:2*pi;
    xp=Rd*cos(ang);
    yp=Rd*sin(ang);
    plot(x+xp,y+yp,'m.');//plots 'drop-off circle'
    hold off;

    %now check if user is happy with labelled objects...
    InfoBox = uicontrol('style','text');
    set(InfoBox,'String','To remove objects, enter their corresponding
numbers separated by spaces. If everything looks good, enter 0. ');
    set(InfoBox,'Position',[20,20,700,50]);%puts a sentence in the figure
with instructions for the user

    disp('To remove objects, enter their corresponding numbers separated by
spaces. ');

```

```

disp('You can also press "z" or "x" to enter one of two add-on
modes. ');
disp('"z" = Keep selected objects (reverse mode). "x" = Draw cells
manually. ');
prompt = 'If everything looks good, enter 0. ';%also prompts user for
input(s)
omit = input(prompt,'s');
if (strcmp(omit,'0')==1)
    if ((strcmp(omit,'x')==1)&&(strcmp(omit,'z')==1))%For default mode:
"Remove selected."
        omit = ([omit ' ']);
        omit_num = 1;
        omit_val = ['0'];
        omit_List = [];
        omit_str_len = length(omit);
        for i = 1:1:omit_str_len%acquires list from string input
            if (omit(i) ~= ' ')
                omit_val = ([omit_val omit(i)]);
            elseif (omit(i) == ' ')
                omit_List(omit_num) = str2num(omit_val);
                omit_num = omit_num + 1;
                omit_val = ['0'];
            end
        end

        for i = 1:1:size(omit_List,2)%omits objects
            omit_obj = omit_List(i);
            if (omit_obj == 0)
                continue;
            elseif ((omit_obj > n)|| (omit_obj <=0))
                error(['ERROR! Could not find object number '
num2str(omit_obj)]);%throws error for out-of-bound input
            end
            imbw1 = imbw1 -
bwselect(imbw1,B{1,omit_obj}(1,2),B{1,omit_obj}(1,1));%remove unwanted
object and loop...
            disp([num2str(omit_obj) ' removed.']);
        end

        if omit_List(size(omit_List,2))==0
            f = 0;
        end

    elseif (strcmp(omit,'z')==1)%For 'z' mode: "Keep objects, remove
all else."

        prompt = 'Enter the objects you want to keep, separated by
spaces. ';
        omit = input(prompt,'s');
        omit = ([omit ' ']);
        omit_num = 1;
        omit_val = ['0'];
        omit_List = [];
        omit_str_len = length(omit);
        for i = 1:1:omit_str_len%acquires list from string input

```

```

        if (omit(i) ~= ' ')
            omit_val = ([omit_val omit(i)]);
        elseif (omit(i) == ' ')
            omit_List(omit_num) = str2num(omit_val);
            omit_num = omit_num + 1;
            omit_val = ['0'];
        end
    end
    imbw2 = logical(zeros(size(imbw1,1),size(imbw1,2)));
    for i = 1:1:size(omit_List,2)%selects objects
        omit_obj = omit_List(i);
        if (omit_obj == 0)
            continue;
        elseif ((omit_obj > n)|| (omit_obj <=0))
            error(['ERROR! Could not find object number '
num2str(omit_obj)]);%throws error for out-of-bound input
        end
        imbw2 = imbw2 +
bwselect(imbw1,B{1,omit_obj}(1,2),B{1,omit_obj}(1,1));%add object to new
logical and loop...
        disp([num2str(omit_obj) ' saved.']);
    end
    imbw1 = imbw2;
    if omit_List(size(omit_List,2))==0
        f = 0;
    end

elseif (strcmp(omit,'x')==1)%For 'x' mode: "Draw objects manually."
    add_Fg = 1;
    add_count = 1;
    add_mask = zeros(size(im,1),size(im,2));
    add_IM = double(im);
    add_sillh = imbw1.*(max(add_IM));
    add_IM = (add_IM.*(1-imbw1))+add_sillh;
    imshow(imadjust(uint16(add_IM)));
    hold on;

    while(add_Fg == 1)
        Mobj = imfreehand;
        new_add = createMask(Mobj,h1);
        add_mask(:, :, add_count) = new_add;
        add_count = add_count + 1;
        prompt = 'If you want to circle more cells, enter "x". If
not, enter anything else.';
        rep = input(prompt,'s');
        if strcmp(rep,'x')~=1
            add_Fg = 0;
        end
    end
    hold off;

    add_mask_all = sum(add_mask,3);
    check_ovr1p = (imbw1.*add_mask_all);
    if (sum(check_ovr1p(:))>0)

```

```

        error('ERROR! Some of your circled cells overlap with
existing objects on this image...');
    end
    imbw1 = imbw1 + add_mask_all;
    disp(['Additional ' num2str(add_count-1) ' objects were added
to the mask.']);
    end

    elseif size(omit) == 0
        disp('You did not enter any text...');%throws error for blank input

    elseif strcmp(omit,'0')
        f = 0;%set flag to exit while loop and save stencil/mask
        break;
    end
end
end

M = logical(imbw1);

[imlb2, nf] = bwlabel(M);

ST = struct('Well',well,'fname',dir_loc,'stencil',M);%temporary data
element to be stored in a struct

flag = 0;%checks for repetition before saving data
sz = size(MAP,2);
for i = sz:-1:2
    if strcmp(MAP(i).fname,dir_loc)==1
        flag = 1;
        MAP(i) = ST;
        disp('Mask overwritten!');
        break;
    end
end
if flag==0
    MAP = ([MAP,ST]);
end%stores well, filename and stencil in the MAP struct

disp('*****COMPLETED!!!');
disp(['Number of objects saved = ' num2str(nf)]);

end

```

Script 2: To quantify all MFI and TFI data from images. This script inputs a list of binary masks of B cell contact zones, quantifies data using a sub-function (Script 2.1), and returns a table of data for each contact zone analyzed in the form of a table.

```
function [Q1] = TIRF_quantifyALL_nd2_3ch_v2(MAP)

%Designed for New NIKON Microscope Images (.nd2)

%Inputs a struct MAP of stencils and the directory location of the data
%folder for a whole TIRF experiment. Accesses fluorescent images for each
%stencil, quantifies data, removes outliers and stores it in an output
%struct

Q1 = struct([]);
Q = MAP;

WL = { ' ' };
count = 1;
for i = 2:length(Q)
    flag = 0;
    for j = 1:size(WL,2)
        if strcmp(WL{1,j},Q(i).Well)
            flag = 1;
        end
    end
    if flag == 0
        WL{1,count} = Q(i).Well;
        count = count+1;
    end
end
%generates a list of 'wells' from the struct input

for i = 1:size(WL,2)
    well = WL{1,i};
    ST = getWells(Q,well);%gets all stencils from one well

    W = struct([]);
    for j = 1:size(ST,2)
        dir_loc = ST(j).fname;%gets directory location from input struct
        M = ST(j).stencil;%gets stencil from input struct
        if max(M(:))==0
            continue;
        else
            R = quantifyMap_nd2_3ch_v2(M,dir_loc);%computes and stores
values as a Struct R
            W = [W, R];%appends data from one set of images to the data for
the whole timepoint (well)
        end
    end
    Q1 = [Q1,W];%appends data from one well to the main data Struct
(output)
end
```

```
viewStruct(Q1);
```

```
end
```

Script 2.1: To quantify contact zone area (μm^2), MFI and TFI from one 3-channel 16-bit .nd2 image. This script inputs a 3-channel image consisting of one IRM channel and two fluorescent channels (labeled ‘red’ and ‘green’), as well as a mask of all B cell contact zones in the image. It calculates and outputs the contact zone area (μm^2), MFI and TFI of the ‘red’ channel, and MFI and TFI of the ‘green’ channel.

```
function [RES] = quantifyMap_nd2_3ch_v2(M, dir_loc)

%Designed for New NIKON Microscope Images (.nd2)

%Inputs a stencil and its directory location to quantify the corresponding
%fluorescent images. Produces a struct array of data from each cell object
%in the stencil

locL = length(dir_loc);
bksl = locL;
count = 1;
for i = locL:-1:1
    if dir_loc(i)=='\ '
        bksl(count) = i;
        count = count+1;
    else
        continue;
    end
end
bksl_1 = bksl(1);
bksl_2 = bksl(2);
well = dir_loc(bksl_2+1:bksl_1-1);%records the time-point or well

imbw1 = M;
pix_size = 0.072; %pixel size (physical length of one pixel) in micron
%For 60X lens and 1.0X mag, it is 0.108.
%For 60X lens and 1.5X mag, it is 0.072.
%For 100X lens and 1.0X mag, it is 0.065.
pix_area = 1/(pix_size*pix_size); %pixel to area conversion ratio

[imlb, n] = bwlabel(imbw1);%label objects in final logical matrix

IP = open2Dmc_img([dir_loc, '.nd2']);
imr0 = IP(:,:,2);%inputs red image (channel 1)
img0 = IP(:,:,3);%inputs green image (channel 2)

img = double(img0);
imr = double(imr0);
```

```

RES = struct();%creates a struct to store results

for i = 1:n
    I = (imlb==i);%isolate each cell object

    se1 = strel('disk',10,0);
    se2 = strel('disk',100,0);
    ibin_ex1 = imdilate(I,se1);
    ibin_ex2 = imdilate(I,se2);
    ibin_bg = ibin_ex2 - ibin_ex1;
    I_bg = ibin_bg-(ibin_bg.*(imdilate(imbw1,se1)));

    imgi = img.*I;
    imri = imr.*I;
    imgibg = img.*I_bg;
    imribg = imr.*I_bg;
    Parea = bwarea(I);
    Pbg = bwarea(I_bg);

    gMean = sum(imgi(:))/Parea - sum(imgibg(:))/Pbg;
    gTotal = gMean*Parea;

    rMean = sum(imri(:))/Parea - sum(imribg(:))/Pbg;
    rTotal = rMean*Parea;

    RES(i).Well = well;%stores well (time-point) info

    RES(i).Area = (Parea/pix_area);%stores IRM area

    if gMean < 0
        RES(i).G_TFI= 0;
        RES(i).G_MFI = 0;
    else
        RES(i).G_TFI=gTotal;%stores TFI of Green
        RES(i).G_MFI=gMean;%stores MFI of Green
    end

    if rMean < 0
        RES(i).R_TFI= 0;
        RES(i).R_MFI = 0;
    else
        RES(i).R_TFI=rTotal;%stores TFI of Red
        RES(i).R_MFI=rMean;%stores MFI of Red
    end

end%quantified individual cell objects and stored values in the struct

end

```

Script 3: To analyze time-lapse images and acquire Area, MFI and TFI of two fluorescent channels, in a frame-by-frame manner, for each B cell. This MATLAB liveScript is composed of several sections with some sub-functions (Scripts 3.1-3.4) It inputs several time-lapse 16-bit .tiff images consisting of 3 channels: IRM, and two fluorescent channels, each containing one B cell (cropped out). It outputs contact zone area (μm^2), MFI and TFI in the form of an excel file with each sheet displaying data for multiple cells.

TIRFvideo_v6

Use this LiveScript code to quantify video crops. First, create an Experiment File called 'Vdata' to store all the info. Then create masks from the IRM channel or actin channel if any. View the mask files (binary/ies) in ImageJ and select the 'contact_frame' and 'start_frame' values. You cannot make further adjustments to the mask in this version. After repeating this for each crop, quantify all the data and store it in an excel file.

This version saves the 'frameRATE', 'pixLEN', 'num_channels', 'actin_channel' and the 'channels' struct in 'Vmeta' (in Mask_IRM of the 1st dummy element of Vdata). Also saves 'cellID', 'fName', 'CFR' (contact frame), 'SFR' (start frame), 'Mask_IRM' and 'Mask_act' (if there's an actin channel) for each crop. After quantification, saves IRM Area, IRM MFI, dA/dt from IRM channel data, and MFI, TFI and dX/dt for each fluorescent channel that follows, into a new Experiment File called 'Qdata'. Upon exporting Qdata to an excel file, all the data quantified for each cell is pasted column-wise and different fields of data are pasted in different sheets of the excel file (in order).

[1] Create a new Experiment (MATLAB) File.

```
% Create a new Experiment (MATLAB) File
clear;
Vdata =
struct('cellID',[], 'fName',[], 'CFR',[], 'SFR',[], 'Mask_IRM',[], 'Mask_act',
[],[]);
% DO NOT EDIT BEFORE THIS POINT!
frameRATE = 0.5;% in fps
pixLEN = 72;% size of pixel in nm
num_channels = 3;
actin_channel = 2;% Enter -1 if no actin channel
channels = struct('name','IRM','color','n');
channels(2).name = 'F-actin';
channels(2).color = 'g';
channels(3).name = 'NMII';
channels(3).color = 'r';
% DO NOT EDIT PAST THIS POINT!
Vmeta =
struct('frameRATE',frameRATE,'pixLEN',pixLEN,'num_channels',num_channels,
'actin_channel',actin_channel,'channels',channels);
```

```

Vdata(1).Mask_IRM = Vmeta;
disp('Vdata created!');

[2] Make binary and save them as a .tiff file.
% Make binary(ies) from a cropped video file and save it as a .tiff
video in the same folder as the input video
filename = ['E:\KOX008-live-WKO-LA_12-3-20\Vid2_WKO\KOX008_vid2_cell-
08.tif'];
IRM_thresh = 1900;% Enter a negative number for auto-threshhold
Actin_thresh = -1;% Enter a negative number for auto-threshhold
IRM_sizeCUToff = 60;
Actin_sizeCUToff = 60;
% DO NOT EDIT PAST THIS POINT!
Vmeta = Vdata(1).Mask_IRM;
frameRATE = Vmeta.frameRATE;
pixLEN = Vmeta.pixLEN;
num_channels = Vmeta.num_channels;
actin_channel = Vmeta.actin_channel;
if actin_channel<0
    Mask_IRM =
TIRFvid_getBinaries_v2(filename,num_channels,IRM_thresh,IRM_sizeCUToff)
;
    Mask_act = [];
    disp('Binary generated from IRM!');
else
    Mask_IRM =
TIRFvid_getBinaries_v3(filename,num_channels,actin_channel,IRM_thresh,I
RM_sizeCUToff,Actin_sizeCUToff);
    Mask_act = [];
    disp('Binary generated from actin!');
end
IP = openTIF_vid(filename,num_channels);
V_c1 = IP{1,1};
V_c1 = uint16(V_c1);
N_slices = size(V_c1,3);
out_file = [filename(1:(length(filename)-4)) '_binary.tiff'];
TIRFvid_saveIMstack_v1(Mask_IRM,out_file);
disp('Binary saved!');

```

```

[3] Set 'contact_frame' and 'start_frame', and update binary. Then save it to Vdata.
% Set 'contact_frame' and 'start_frame' values and adjust the
binary(ies) (everything before the 'touch_frame' is blacked out)
%contact_frame = 63;% This is the frame when the cell appears to have
contacted the PLB
start_frame = 64;% This is the frame when the cell just begins to
spread on the PLB
contact_frame = start_frame;%To default start_lags to 0...
% DO NOT EDIT PAST THIS POINT!

```

```

for i = 1:start_frame-1
    Mask_IRM(:, :, i) = zeros(size(Mask_IRM, 1), size(Mask_IRM, 2));
end
disp(['Contact frame set : ' num2str(contact_frame)]);
disp(['Start frame set : ' num2str(start_frame)]);
% Save the binary again to update it with the start frame.
out_file = [filename(1:(length(filename)-4)) '_binary.tiff'];
TIRFvid_saveIMstack_v1(Mask_IRM, out_file);
disp('Binary saved!');
% Save the data for this crop, including the mask(s), to Vdata.
Vmask_N = size(Vdata, 2);
VMN = Vmask_N + 1;
for i = 1:Vmask_N
    fname = Vdata(i).fName;
    if strcmp(fname, filename)
        VMN = i;
        break;
    end
end
fnl = size(filename, 2);
Vdata(VMN).cellID = [filename(fnl-12) '_' filename(fnl-5 : fnl-4)];
Vdata(VMN).fName = filename;
Vdata(VMN).CFR = contact_frame;
Vdata(VMN).SFR = start_frame;
Vdata(VMN).Mask_IRM = Mask_IRM;
Vdata(VMN).Mask_act = Mask_act;
viewStruct(Vdata);

```

[4] Quantify the whole Experiment to acquire a 'Qdata' file.

% Quantify a 'Vdata' file to yeild a 'Qdata' file.

% DO NOT EDIT THIS SECTION!

Qdata = TIRFvid_quantifyExpt_v1(Vdata);

disp('Data quantification completed!');

[5] Export 'Qdata' to an excel file.

% Export the data saved in Qdata to an excel file (out_excel) beginning at a selected

% cell location (out_cell).

out_excel = 'E:\K0X008-live-WK0-LA_12-3-20\K0X008_data_raw.xlsx';

out_cell = 'C2';

% DO NOT EDIT PAST THIS POINT!

OP = TIRFvid_export2excel_v1(Qdata, out_excel, out_cell);

disp('Data successfully exported!');

disp('The order of the sheets is as follows:');

for i = 1:size(OP, 1)

disp(['Sheet ' num2str(i) ' = ' OP{i, 1}]);

end

Script 3.1: To acquire a mask of a B cell contact zone from an IRM time-lapse image.

```
function [M3] =
TIRFvid_getBinaries_v3(filename,num_channels,actin_channel,IRM_thresh,Actin_
_thresh,IRM_sizeCUTOff,Actin_sizeCUTOff)

IP = openTIF_vid(filename,num_channels);
Vi = IP{1,1};
Vf = IP{1,actin_channel};
Vi = uint16(Vi);
Vf = uint16(Vf);
tn = size(Vf,3);
manual_thresh = Actin_thresh;

%Detect footprints in last frame:

FRi = Vi(:,:,tn);% (IRM)
FRf = Vf(:,:,tn);% (actin)
THi = IRM_thresh;
%THf = Actin_thresh;
ISCO = IRM_sizeCUTOff;
ASCO = Actin_sizeCUTOff;

imbw1 = FRi<THi;%apply threshold (IRM)
se = strel('disk',3,0);%for smoothing
se3 = strel('disk',3,0);%for dilation of prev_fr
imbw1 = imerode((imdilate(imbw1,se)),se);%dilation/erosion=smoothing (IRM)
imbw1 = bwareaopen(imbw1,ISCO);%cleans noise (IRM)
imbw1 = imfill(imbw1,'holes');%fills holes (IRM)
prev_fr1 = imdilate(imbw1,se3);

[counts,x] = imhist(uint16(FRf),65535);
THf = otsuthresh(counts)*65535;

if manual_thresh>0
    THf = manual_thresh;
end

imbw2 = FRf>THf;%apply threshold (actin)
imbw2 = imerode((imdilate(imbw2,se)),se);%dilation/erosion=smoothing
(actin)
imbw2 = bwareaopen(imbw2,ASCO);%cleans noise (actin)
imbw2 = imfill(imbw2,'holes');%fills holes (actin)
prev_fr2 = imbw2;

imbw3 = (imbw1+imbw2)>0;
imbw3 = imerode((imdilate(imbw3,se)),se);
imbw3 = imfill(imbw3,'holes');%fills holes

%[imlb,N] = bwlabel(imbw3);
%imbw3 = imbw3 - (imlb==2);

% Detect footprints in remaining frames until the first:
```

```

M1 = zeros(size(Vf));
M2 = M1;
M3 = M1;
M1(:,:,tn) = imbw1;
M2(:,:,tn) = imbw2;
M3(:,:,tn) = imbw3;
for i = tn-1:-1:1
    FRi = Vi(:,:,i);
    %   THi = 65535*graythresh(FRi);%set threshold (IRM)
    imbw1 = FRi<THi;%apply threshold (IRM)
    imbw1 = imerode((imdilate(imbw1,se)),se);%dilation/erosion=smoothing
    (IRM)
    imbw1 = bwareaopen(imbw1,ISCO);%cleans noise (IRM)
    imbw1 = imfill(imbw1,'holes');%fills holes (IRM)
    [imlb1,n] = bwlabel(imbw1);
    if n>1
        for j = 1:n
            obj = imlb1==j;
            [cm_x, cm_y] = Centroid(obj);
            if prev_fr1(round(cm_x),round(cm_y))==0
                imbw1 = imbw1 - obj;
            end
        end
    end
    M1(:,:,i) = imbw1;
    prev_fr1 = imdilate(imbw1,se3);

    FRf = Vf(:,:,i);
    [counts,x] = imhist(uint16(FRf),65535);
    THf = otsuthresh(counts)*65535;

    if manual_thresh>0
        THf = manual_thresh;
    end

    imbw2 = FRf>THf;%apply threshold (actin)
    imbw2 = imerode((imdilate(imbw2,se)),se);%dilation/erosion=smoothing
    (actin)
    imbw2 = bwareaopen(imbw2,ASCO);%cleans noise (actin)
    imbw2 = imfill(imbw2,'holes');%fills holes (actin)
    [imlb2,n] = bwlabel(imbw2);
    if n>1
        for j = 1:n
            obj = imlb2==j;
            [cm_x, cm_y] = Centroid(obj);
            if prev_fr2(round(cm_x),round(cm_y))==0
                imbw2 = imbw2 - obj;
            end
        end
    end
    M2(:,:,i) = imbw2;
    prev_fr2 = imbw2;

    imbw3 = (imbw1+imbw2)>0;
    imbw3 = imerode((imdilate(imbw3,se)),se);

```

```

    imbw3 = imfill(imbw3,'holes');%fills holes

    %if i>80
    %    [imlb,N] = bwlabel(imbw3);
    %    imbw3 = imbw3 - (imlb==2);
    %end

    M3(:,:,i) = imbw3;

end

end

```

Script 3.2: To save an image stack (time-lapse image) to a specified directory location

```

function [] = TIRFvid_saveIMstack_v1(im,filename)

len = length(filename);
if strcmp(filename(len-4:len),'.tiff') == 0
    filename = [filename '.tiff'];
end

if exist(filename) == 2
    resp = questdlg('There is already a file by that name. Would you like to overwrite it?','Overwrite Existing File?','Yes','No, cancel SAVE','Yes');
    if strcmp(resp,'Yes')==1
        delete(filename);
        imwrite(im(:,:,1),filename,'WriteMode','append');
        for i = 2:1:size(im,3)
            imwrite(im(:,:,i),filename,'WriteMode','append');
        end
    end
else
    imwrite(im(:,:,1),filename,'WriteMode','append');
    for i = 2:1:size(im,3)
        imwrite(im(:,:,i),filename,'WriteMode','append');
    end
end

end

end

```

Script 3.3: To analyze fluorescent channel data in time-lapse images consisting of 3-channels: IRM and two fluorescent channels

```

function [Qdata] = TIRFvid_quantifyExpt_v1(Vdata)

Qdata = Vdata;

Vmeta = Vdata(1).Mask_IRM;
frameRATE = Vmeta.frameRATE;

```

```

pixLEN = Vmeta.pixLEN;
num_channels = Vmeta.num_channels;
actin_channel = Vmeta.actin_channel;
channels = Vmeta.channels;

for k = 2:size(Vdata,2)
    disp(['Quantifying cell ' num2str(k-1) ' of ' num2str(size(Vdata,2)-1)
    '...']);
    filename = Vdata(k).fName;
    IP1 = openTIF_vid(filename,num_channels);
    V_IRM = IP1{1,1};
    V_FLU1 = IP1{1,2};
    if length(channels)>2
        V_FLU2 = IP1{1,3};
    end
    tn = size(V_IRM,3);
    Mask_IRM = Vdata(k).Mask_IRM;
    Mask_act = Vdata(k).Mask_act;
    CFR = Vdata(k).CFR;
    SFR = Vdata(k).SFR;
    Q_sl = Vdata(k);

    % Calculate Area data:
    Area = zeros(1,size(Mask_IRM,3));
    for i = 1:tn
        bin = Mask_IRM(:,:,i);
        Area(i) = sum(bin(:));
    end
    Area = Area*(pixLEN*pixLEN/1000000);%correct pixel area to area in um
    squared
    Area = Area(SFR:length(Area));
    Area = [0 Area];
    if(k==2)
        Qdata(1).Area = 'Area';
    end
    Q_sl.Area = Area;%records Area

    VcI = ones(size(V_IRM));
    VcI = VcI - double(V_IRM);
    TFI1 = zeros(1,size(Mask_IRM,3));
    MFI1 = zeros(1,size(Mask_IRM,3));
    for i = SFR:tn
        bin = Mask_IRM(:,:,i);
        bin_prod = bin.*VcI(:,:,i);
        tfi1 = sum(bin_prod(:));
        TFI1(i) = tfi1;
        mfi1 = tfi1/sum(bin(:));
        MFI1(i) = mfi1;
    end
    TFIbg1 = zeros(1,size(Mask_IRM,3));
    MFIbg1 = zeros(1,size(Mask_IRM,3));
    for i = SFR:tn
        bin = Mask_IRM(:,:,i);
        binBG = 1-bin;
        bin_prod_bg = binBG.*VcI(:,:,i);

```

```

        tfibg1 = sum(bin_prod_bg(:));
        TFibg1(i) = tfibg1;
        mfibg1 = tfibg1/sum(binBG(:));
        MFibg1(i) = mfibg1;
    end
    MFI1_corr = MFI1 - MFibg1;%Background correction of MFI
    MFI1_corr = MFI1_corr(SFR:length(MFI1_corr));
    MFI1_corr = [0 MFI1_corr];
    if(k==2)
        Qdata(1).IRM_MFI = 'IRM_MFI';
    end
    Q_sl.IRM_MFI = MFI1_corr;%records IRM MFI (density)

    dAdt = diff(Area);
    dAdt(1) = 0;
    dAdt = [0 dAdt];
    dAdt = dAdt*frameRATE;
    if(k==2)
        Qdata(1).dAdt = 'dAdt';
    end
    Q_sl.dAdt = dAdt;%records dA/dt

    %Calculate data from other fluorescent channel(s)
    for j = 2:length(channels)
        Mask = Mask_IRM;
        ch_N = channels(j).name;
        TFI1 = zeros(1,size(Mask,3));
        MFI1 = zeros(1,size(Mask,3));
        if j==2
            V_FLU = V_FLU1;
        elseif j==3
            V_FLU = V_FLU2;
        end
        for i = SFR:tn
            bin = Mask(:, :, i);
            bin_prod = bin.*V_FLU(:, :, i);
            tfi1 = sum(bin_prod(:));
            TFI1(i) = tfi1;
            mfi1 = tfi1/sum(bin(:));
            MFI1(i) = mfi1;
        end
        TFibg1 = zeros(1,size(Mask,3));
        MFibg1 = zeros(1,size(Mask,3));
        TFibg1_total = TFibg1;
        for i = SFR:tn
            bin = Mask(:, :, i);
            binBG = 1-bin;
            bin_prod_bg = binBG.*V_FLU(:, :, i);
            tfibg1 = sum(bin_prod_bg(:));
            TFibg1(i) = tfibg1;
            mfibg1 = tfibg1/sum(binBG(:));
            MFibg1(i) = mfibg1;
            TFibg1_total(i) = mfibg1*(sum(bin(:)));
        end
        MFI1_corr = MFI1 - MFibg1;%Background correction of MFI

```

```

MFI1_corr = MFI1_corr(SFR:length(MFI1_corr));
MFI1_corr = [0 MFI1_corr];
if(k==2)
    if j==2
        Qdata(1).C2_MFI = [ch_N '_MFI'];
    elseif j==3
        Qdata(1).C3_MFI = [ch_N '_MFI'];
    end
end
if j==2
    Q_s1.C2_MFI = MFI1_corr;
elseif j==3
    Q_s1.C3_MFI = MFI1_corr;
end%records MFI

TFI1_corr = TFI1 - TFIbg1_total;
TFI1_corr = TFI1_corr(SFR:length(TFI1_corr));
TFI1_corr = [0 TFI1_corr];
ch_N = channels(j).name;
if(k==2)
    if j==2
        Qdata(1).C2_TFI = [ch_N '_TFI'];
    elseif j==3
        Qdata(1).C3_TFI = [ch_N '_TFI'];
    end
end
if j==2
    Q_s1.C2_TFI = TFI1_corr;
elseif j==3
    Q_s1.C3_TFI = TFI1_corr;
end%records TFI

TFI1 = [0 TFI1];
TFI1 = TFI1(SFR:length(TFI1));
dXdt = diff(TFI1);
dXdt(1) = 0;
dXdt = [0 dXdt];
dXdt = dXdt*frameRATE;
if(k==2)
    if j==2
        Qdata(1).C2_dXdt = [ch_N '_dXdt'];
    elseif j==3
        Qdata(1).C3_dXdt = [ch_N '_dXdt'];
    end
end
if j==2
    Q_s1.C2_dXdt = dXdt;
elseif j==3
    Q_s1.C3_dXdt = dXdt;
end%records dX/dt
end
S_lag = (SFR-CFR)/frameRATE;
Qdata(1).S_lag = 'S_lag';
Q_s1.S_lag = S_lag;%records S_lag
Qdata(k) = Q_s1;

```

end

end

Script 3.4: To export time-lapse data for one B cell into a specified Excel file.

```
function [OP] = TIRFvid_export2excel_v1(Qdata,out_excel,out_cell)

Vmeta = Qdata(1).Mask_IRM;
frameRATE = Vmeta.frameRATE;
FN = fieldnames(Qdata);
div = 0;
for i = 1:size(FN,1)
    if (strcmp(FN{i,1}, 'Area') || strcmp(FN{i,1}, 'MFI_out'))
        div = i;
        break;
    end
end
OP = FN(div:(length(FN)-1),1);

cell_prop = isstrprop(out_cell, 'alpha');
cell_X = out_cell(1:sum(cell_prop));
cell_Y = out_cell(sum(cell_prop)+1:length(out_cell));
if length(cell_X)==1
    cXn = double(cell_X)-64+1;
else
    cXn = ((double(cell_X)-64)*[26; 1])+1;
end
Y_loc = num2str(str2num(cell_Y)+1);
max_fr = 0;

for k = 2:size(Qdata,2)
    cellID = {Qdata(k).cellID};
    Mask_IRM = Qdata(k).Mask_IRM;
    len_fr = size(Mask_IRM,3);
    if len_fr>max_fr
        max_fr = len_fr;
    end
    active_sheet = 1;
    for j = div:size(FN,1)
        Djk = getfield(Qdata(k),FN{j,1});
        if cXn<=26
            X_loc = char(cXn+64);
        else
            X_loc = [char(floor(cXn/26)+64) char(rem(cXn,26)+64)];
        end
        cell = [X_loc cell_Y];
        record_excel(cellID,out_excel,active_sheet,cell);
        cell = [X_loc Y_loc];
        record_excel(Djk',out_excel,active_sheet,cell);
        active_sheet = active_sheet + 1;%next sheet/field
    end
end
```

```

        cXn = cXn + 1;%next crop/shift right
    end

    Time = [1:1:max_fr];
    Time = Time/frameRATE;
    Time = [0 Time];
    if length(cell_X)==1
        cXn = double(cell_X)-64;
    else
        cXn = ((double(cell_X)-64)*[26; 1]);
    end
    active_sheet = 1;
    for j = div:(size(FN,1)-1)
        if cXn<=26
            X_loc = char(cXn+64);
        else
            X_loc = [char(floor(cXn/26)+64) char(rem(cXn,26)+64)];
        end
        cell = [X_loc cell_Y];
        record_excel({'Time(s)'},out_excel,active_sheet,cell);
        cell = [X_loc Y_loc];
        record_excel(Time',out_excel,active_sheet,cell);
        active_sheet = active_sheet + 1;%next sheet/field
    end
    cell = [X_loc Y_loc];
    key_other = {'Start lag(s)'};
    record_excel(key_other,out_excel,active_sheet,cell);

end

```

Script 3.5: To generate 8 kymographs from time-lapse images of a B cell consisting of 3 channels: IRM, and two fluorescent channels. The 8 kymographs are radially equally spaced. Inputs ‘Vdata’ acquired from Scripts 3.1, 3.2 and 3.3, and writes a .tiff image consisting of the 8 kymographs to the same location as the original raw data (time-lapse multi-channel image).

```

%Load Vdata first!

for mapN = 2%Which cell in Vdata to analyze?

    manual_R = -1;%Enter -1 for auto radius...

    filename = Vdata(mapN).fName;
    savename = [filename(1:length(filename)-4) '_kymos.tif'];

    IP = openTIF_vid(filename,3);
    T = [Vdata(mapN).SFR (Vdata(mapN).SFR-1+90)];
    Mask = Vdata(mapN).Mask_IRM;

    CMX = [];
    CMY = [];

```

```

B = [];
for t = T(1):1:T(2)
    Mask_fr = Mask(:,:,t);
    [cmx,cmy] = Centroid(Mask_fr);
    CMX = [CMX cmx];
    CMY = [CMY cmy];
    Bound = bwboundaries(Mask_fr);
    bound = Bound{1,1};
    Bdist = [];
    for i = 1:1:size(bound,2)
        bdist = sqrt(((bound(i,1)-cmx)*(bound(i,1)-cmx))+((bound(i,2)-
cmy)*(bound(i,2)-cmy)));
        Bdist = [Bdist bdist];
    end
    B = [B max(Bdist)];
end
cmx = round(mean(CMX));
cmy = round(mean(CMY));
if manual_R>0
    R = manual_R;
else
    R = 5 + ceil(max(B));
end
ang_divs = 180;
ang_sect = (360/ang_divs);
con = pi/180;

for ch = 1:3
    if ch==1
        vid = Mask;
    else
        vid = IP{1,ch};
    end
    res = zeros((R*ang_divs)+1,1);

    for t = T(1):1:T(2)
        fr = vid(:,:,t);
        Tsect = zeros(1,ang_sect);
        for div = 1:1:ang_divs
            sect = zeros(R,ang_sect);
            for r = 1:1:R
                for sect_ang = 1:1:ang_sect
                    ang = sect_ang + ((div-1)*30);
                    x = cmx + (r*cos(ang*con));
                    y = cmy + (r*sin(ang*con));
                    sect(R+1-r,sect_ang) = fr(round(x),round(y));
                end
            end
            Tsect = vertcat(Tsect,sect);
        end
        res = horzcat(res,Tsect);
    end

    res = res(2:size(res,1),2:size(res,2));

```

```

res2 = zeros(1,size(res,2));

for i = [1 24 46 68 91 113 136 158]
    res_kymo = res(((i-1)*R)+1:((i-1)*R)+R,:);
    res2 = vertcat(res2,res_kymo);
end

res2 = res(2:size(res2,1),:);

if ch==1
    res2 = 65535*(1-res2);
end

imwrite(uint16(res2),savename,'WriteMode','append');
end
end

```

Script 4: To identify BCR clusters and signaling molecule puncta from 16-bit .nd2 images consisting of 3 channels: IRM, AF546-Fab', and (stained) signaling molecule. Inputs B cell contact zone masks in the form of 'MAP' (output of Script 1). Utilizes a sub-function (Script 4.1) to quantify various parameters including Fab peak intensity, Fab MFI, and signaling molecule MFI in each detected cluster, and returns the data as a table.

**function [Q1,Q2,Qcells,cMAP] =
TIRF_quantifyClusters_nd2_3ch_v3(MAP)**

%Designed for New NIKON Microscope Images (.nd2)

*%Inputs a struct MAP of stencils and the directory location of the data
%folder for a whole TIRF experiment. Accesses fluorescent images for each
%stencil, quantifies data, removes outliers and stores it in an output
%struct*

```

Q1 = struct([]);
Q2 = struct([]);
Qcells = struct([]);
M = MAP;

cMAP = MAP(1);

WL = {' '};
count = 1;
for i = 2:length(M)
    flag = 0;
    for j = 1:size(WL,2)
        if strcmp(WL{1,j},M(i).Well)
            flag = 1;
        end
    end
end

```

```

    if flag == 0
        WL{1,count} = M(i).Well;
        count = count+1;
    end
end
%generates a list of 'wells' from the struct input

for i = 1:size(WL,2)
    well = WL{1,i};
    ST = getWells(M,well);%gets all stencils from one well
    cm = ST;
    W1 = struct([]);
    W2 = struct([]);
    CW = struct([]);
    Ccount = 1;
    for j = 1:size(ST,2)
        dir_loc = ST(j).fname;%gets directory location from input struct
        map = ST(j).stencil;%gets stencil from input struct
        if max(map(:))==0
            continue;
        else
            [R1,R2,CR,c_sten,CcountI] =
quantifyClustersFOV_nd2_3ch_v9(map,dir_loc,Ccount);%computes and stores
values as a Struct R
            if (~isempty(fieldnames(R1)))||(~isempty(fieldnames(R2)))
                if (~isempty(fieldnames(R1)))
                    W1 = [W1, R1];%appends data from one set of images to the
data for the whole timepoint (well)
                end
                if (~isempty(fieldnames(R2)))
                    W2 = [W2, R2];%appends data from one set of images to
the data for the whole timepoint (well)
                end
                if (~isempty(fieldnames(CR)))
                    CW = [CW, CR];
                end
                Ccount = Ccount + CcountI;
            end
            cm(j).stencil = c_sten;
        end
    end
    %W = processData_v3(W);
    if (~isempty(fieldnames(W1)))
        Q1 = [Q1,W1];%appends data from one well to the main data Struct
(output)
    end
    if (~isempty(fieldnames(W2)))
        Q2 = [Q2,W2];%appends data from one well to the main data Struct
(output)
    end
    if (~isempty(fieldnames(CW)))
        Qcells = [Qcells,CW];%appends data from one well to the main data
Struct (output)
    end
    cMAP = [cMAP,cm];

```

end

end

Script 4.1: To identify BCR clusters and signaling molecule puncta from one 16-bit .nd2 image consisting of 3 channels: IRM, AF546-Fab', and (stained) signaling molecule.

```
function [RES1,RES2,CRES,c_sten,n] = quantifyClustersFOV_nd2_3ch_v9(map,
dir_loc, CC)

%Designed for New NIKON Microscope Images (.nd2)

%Inputs a stencil and its directory location to quantify the corresponding
%fluorescent images. Produces a struct array of data from each cell object
%in the stencil

locL = length(dir_loc);
bksl = locL;
COUNT = 1;
for i = locL:-1:1
    if dir_loc(i)=='\ '
        bksl(COUNT) = i;
        COUNT = COUNT+1;
    else
        continue;
    end
end
bksl_1 = bksl(1);
bksl_2 = bksl(2);
well = dir_loc(bksl_2+1:bksl_1-1);%records the time-point or well

%disp(["Well = " well]);

imbw = map;
pix_size = 0.072; %pixel size (physical length of one pixel) in micron
%For 60X lens and 1.0X mag, it is 0.108.
%For 60X lens and 1.0X mag, it is 0.072.
pix_area = 1/(pix_size*pix_size); %pixel to area conversion ratio

c_sten = cell(2,1);
[imlb, n] = bwlabel(imbw);%label objects in final logical matrix

IP = open2Dmc_img([dir_loc, '.nd2']);
imr = IP(:,:,2);%inputs red image (BCR)
img = IP(:,:,3);%inputs green image (signaling molecule)

RES1 = struct();
RES2 = struct();
CRES = struct();%creates a struct to store results
count_1 = 1;
count_2 = 1;
```

```

cnt = 1;

D = 140;
for i = 1:n
    I = (imlb==i);%isolate each cell object
    Parea = bwarea(I);
    [cmx,cmy] = Centroid(I);
    cmx = round(cmx);
    cmy = round(cmy);
    imI = I((cmx-(D/2)) : (cmx+(D/2)-1),(cmy-(D/2)) : (cmy+(D/2)-1));
    imR = imr((cmx-(D/2)) : (cmx+(D/2)-1),(cmy-(D/2)) : (cmy+(D/2)-1));
    imR = double(imR);
    imG = img((cmx-(D/2)) : (cmx+(D/2)-1),(cmy-(D/2)) : (cmy+(D/2)-1));
    imG = double(imG);

    R_bg = [];
    G_cz = [];
    for j = 1:D
        for k = 1:D
            if imI(j,k)==1
                G_cz = [G_cz imG(j,k)];
            else
                R_bg = [R_bg imR(j,k)];
            end
        end
    end
    th_R = [4.1 3.9 3.7 3.5 3.3 3.1 2.9 2.7 2.5 2.3 2.1 1.9 1.7 1.5 1.3
1.1]*median(R_bg);% <-----EDIT BCR THRESHOLDS!!!
    %th_R = [1.5 1.3]*median(R_bg);% <-----EDIT BCR THRESHOLDS!!!
    th_G = 1.3*median(G_cz);% <-----EDIT Green ("p-sig-mol") THRESHOLD!!!
    BCR_norm_frac = median(R_bg)/100;

    RMask = cell(1,size(th_R,2));
    th_R1 = th_R(1);
    imRi1 = double(imR>th_R1);
    imRi1 = imRi1.*imI;
    imRi1 = imfill(imRi1,'holes');
    imRi1 = bwareaopen(imRi1,3,4);
    se = strel('disk',1,0);

    [imlb1,n1] = bwlabel(imRi1);
    for j = 1:n1
        obj = imlb1==j;
        if sum(obj(:))<10
            imRi1 = imRi1 - obj;
            imRi1 = imRi1 + imdilate(obj,se);
        end
    end
    Mask1 = imerode(imRi1,se);
    Mask1 = imdilate(Mask1,se);
    RMask{1,1} = Mask1;
    R_Mask = Mask1*size(th_R,2);

    for k = 2:size(th_R,2)
        th_R2 = th_R(k);

```

```

imRi2 = double(imR>th_R2);
imRi2 = imRi2.*imI;
imRi2 = imfill(imRi2,'holes');
imRi2 = bwareaopen(imRi2,3,4);
[imlb2,n1] = bwlabel(imRi2);
for j = 1:n1
    obj = imlb2==j;
    overlap = obj.*imRi1;
    objArea = sum(obj(:));
    if sum(overlap(:)>0)|| (objArea>1000)%Area upper-cutoff
        imRi2 = imRi2 - obj;
    else
        if sum(obj(:))<10
            imRi2 = imRi2 - obj;
            imRi2 = imRi2 + imdilate(obj,se);
        end
    end
end
imRi1 = imRi1 + imRi2;
Mask2 = imerode(imRi2,se);
Mask2 = imdilate(Mask2,se);
RMask{1,k} = Mask2;
R_Mask = (R_Mask + (Mask2*(size(th_R,2)+1-k)));
end

imGi = double(imG>th_G);
imGi = imGi.*imI;
imGi = imfill(imGi,'holes');
imGi = bwareaopen(imGi,3,4);
imGi = imerode(imGi,se);
imGi = imdilate(imGi,se);

GMask = imGi;

RGMask = R_Mask+GMask;

if (sum(RGMask(:))>0)
    for j = 1:size(th_R,2)
        Mask_i = RMask{1,j};
        [imRlb,n2] = bwlabel(Mask_i);%checking clusters...
        for k = 1:n2
            Ci = double(imRlb==k);
            Cr = imR.*Ci;
            Cg = imG.*Ci;
            Area = sum(Ci(:));
            TFI = sum(Cr(:))/BCR_norm_frac;
            MFI = TFI/Area;
            Peak = max(Cr(:))/BCR_norm_frac;
            PbM = Peak/MFI;
            pMFI = sum(Cg(:))/Area;
            pMFIR = pMFI/MFI;

            RES1(count_1).Well = well;%stores well (time-point) info
            RES1(count_1).Cell = CC-1+i;%stores cell number

```

```

        RES1(count_1).Type = j;%stores BCR cluster type: 1 = High;
2 = Med; 3 = Low...
        RES1(count_1).Area = Area;%stores BCR cluster size in pix
        RES1(count_1).TFI = TFI;
        RES1(count_1).MFI = MFI;
        RES1(count_1).Peak = Peak;
        RES1(count_1).PbM = PbM;
        RES1(count_1).pMFI = pMFI;
        RES1(count_1).pMFIR = pMFIR;
        count_1 = count_1+1;
    end
end

[imGlb,n2] = bwlabel(GMask);%checking p-sig-mol puncta...
for k = 1:n2
    Ci = double(imGlb==k);
    Cr = imR.*Ci;
    Cg = imG.*Ci;
    Area = sum(Ci(:));
    TFI = sum(Cg(:));
    MFI = TFI/Area;
    Peak = max(Cg(:));
    PbM = Peak/MFI;
    rPeak = max(Cr(:))/BCR_norm_frac;
    rTFI = sum(Cr(:))/BCR_norm_frac;
    rMFI = sum(Cr(:))/(Area*BCR_norm_frac);
    rPbM = rPeak/rMFI;
    pMFIR = MFI/rMFI;
    RES2(count_2).Well = well;%stores well (time-point) info
    RES2(count_2).Cell = CC-1+i;%stores cell number
    RES2(count_2).Type = 0;%stores BCR cluster type: 0 = p-sig-
mol...
    RES2(count_2).Area = Area;%stores puncta size in pix
    RES2(count_2).TFI = rTFI;
    RES2(count_2).MFI = rMFI;
    RES2(count_2).Peak = rPeak;
    RES2(count_2).PbM = rPbM;
    RES2(count_2).pTFI = TFI;
    RES2(count_2).pMFI = MFI;
    RES2(count_2).pPeak = Peak;
    RES2(count_2).pPbM = PbM;
    RES2(count_2).pMFIR = pMFIR;

    count_2 = count_2+1;
end

c_sten{1,i} = R_Mask;
c_sten{2,i} = GMask;

CRES(cnt).Well = well;%stores well (time-point) info
CRES(cnt).Cell = CC-1+i;%stores cell number
CRES(cnt).Area = (Parea/pix_area);%stores IRM area
cnt = cnt+1;
else
    c_sten{1,i} = 0*imRi1;

```

```

        c_sten{2,i} = 0*imRi1;
    end
end
end

```

Script 5: To analyze individual BCR clusters in time-lapse images. This MATLAB script is composed of several sections with some sub-functions (Scripts 5.1 and 5.2). It inputs 'Vdata' acquired using Script 3, identifies AF546-Fab clusters in one cropped B cell for every frame in the original 16-bit .tiff time-lapse image, generates tracks connecting the detected clusters, analyzes the peak intensity and MFI of AF546 in each cluster, and returns the output as a table.

```

%% TIRFvid_vTrack_v1

%Detect BCR clusters and save detection binary to file.
%Acquire BCR tracks and view tracks and track-plots.
%Quantify BCR cluster data and view plots vs time.

%% DETECT OBJECTS (LOAD VDATA FIRST!!!)

VdataN = 32;% Which cell in Vdata??
%----DO NOT EDIT PAST THIS POINT!-----
cellData = Vdata(VdataN);
IP = openTIF_vid(cellData.fName,3);
vidR = IP{1,2};%inputs red image (BCR)
vidMask = cellData.Mask_IRM;
SFR = cellData.SFR;
D1 = size(vidMask,1);
D2 = size(vidMask,2);
sem = strel('disk',2);
cMask = 0*vidMask;
czA = zeros(1,size(cMask,3));
tn = size(vidMask,3);
MBG = zeros(1,tn);
for fr = SFR:tn
    imR = vidR(:,:,fr);
    imI = vidMask(:,:,fr);
    czA(fr) = bwarea(imI);
    R_bg = [];
    for i = 1:D1
        for j = 1:D2
            if imI(i,j)==0
                R_bg = [R_bg imR(i,j)];
            end
        end
    end
    MBG(1,fr) = median(R_bg);
    th_R = [4.1 3.9 3.7 3.5 3.3 3.1 2.9 2.7 2.5 2.3 2.1 1.9 1.7 1.5 1.3
1.1]*median(R_bg);% <-----EDIT BCR THRESHOLDS!!!
    RMask = cell(1,size(th_R,2));

```

```

th_R1 = th_R(1);
imRi1 = double(imR>th_R1);
imRi1 = imRi1.*imI;
imRi1 = imfill(imRi1,'holes');
imRi1 = bwareaopen(imRi1,3,4);
se = strel('disk',1,0);
[imlb1,n1] = bwlabel(imRi1);
for j = 1:n1
    obj = imlb1==j;
    if sum(obj(:))<10
        imRi1 = imRi1 - obj;
        imRi1 = imRi1 + imdilate(obj,se);
    end
end
Mask1 = imerode(imRi1,se);
Mask1 = imdilate(Mask1,se);
RMask{1,1} = Mask1;
R_Mask = Mask1*size(th_R,2);
for i = 2:size(th_R,2)
    th_R2 = th_R(i);
    imRi2 = double(imR>th_R2);
    imRi2 = imRi2.*imI;
    imRi2 = imfill(imRi2,'holes');
    imRi2 = bwareaopen(imRi2,3,4);
    [imlb2,n1] = bwlabel(imRi2);
    for j = 1:n1
        obj = imlb2==j;
        overlap = obj.*imRi1;
        objArea = sum(obj(:));
        if sum(overlap(:)>0)||(objArea>1000)%Area upper-cutoff
            imRi2 = imRi2 - obj;
        else
            if sum(obj(:))<10
                imRi2 = imRi2 - obj;
                imRi2 = imRi2 + imdilate(obj,se);
            end
        end
    end
    imRi1 = imRi1 + imRi2;
    Mask2 = imerode(imRi2,se);
    Mask2 = imdilate(Mask2,se);
    RMask{1,i} = Mask2;
    R_Mask = (R_Mask + (Mask2*(size(th_R,2)+1-i)));
end
cMask(:, :, fr) = R_Mask>0;
end

%% SAVE RAW CLUSTER DETECTION MASK (VIDEO).

filename = cellData.fName;
filename = [filename(1:(length(filename)-4)) '_clusters-mask.tiff'];
TIRFvid_saveIMstack_v1(cMask,filename);

%% SAVE RAW CLUSTER BOUNDARIES (VIDEO).
seb = strel('disk',1);

```

```

bMask = 0*cMask;
for i = 1:1:size(cMask,3)
    bMask(:,:,i) = imdilate(cMask(:,:,i),seb) - cMask(:,:,i);
end
filename = [filename(1:(length(filename)-4)) '_cluster boundaries.tiff'];
TIRFvid_saveIMstack_v1(bMask,filename);

%% CHECK AREA VS TIME PLOT TO DETERMINE 'SWFR': THE FRAME WHEN CONTRACTION
BEGINS.

figure;
plot([1:1:size(czA,2)],czA,'b.-');
xlabel('frame #');
ylabel('Area (pix)');

%% CREATE FRAME-BY-FRAME CLUSTER ASSOCIATIONS (BASICALLY TRACK THEM).

SWFR = 115;% EDIT = the frame when contraction begins....

%---DO NOT EDIT PAST THIS POINT!-----
DET = cell(1,1);
DETP = DET;
stop = SWFR + 61;% EDIT range (after contraction begins)
start = SWFR - 11;% EDIT range (before spreading ends)
disp(['start FR = ' num2str(start+1)]);
disp(['stop FR = ' num2str(stop-1)]);
count = 1;
for fr = stop:-1:start
    maskFRi = uint16(cMask(:,:,fr));
    [imlbi,nci] = bwlabel(maskFRi);
    Pi = [];
    PiP = [];
    for ob = 1:1:nci
        obj_i = imlbi==ob;
        Pi(ob) = count;
        PiP(ob) = ob;
        count = count+1;
    end
    DET{fr-start+1,1} = Pi';
    DETP{fr-start+1,1} = PiP';
end
for fr = stop-1:-1:start
    maskFRj = uint16(cMask(:,:,fr));
    [imlbj,ncj] = bwlabel(maskFRj);
    Pj = [];
    PjP = [];
    for ob = 1:1:ncj
        obj_j = imlbj==ob;
        tracked = trackObj_v1(obj_j,imlbi,1,1,3);%-----EDIT
        if tracked==0
            Pj(ob) = count;
            PjP(ob) = ob;
            count = count+1;
        else
            Pj(ob) = Pi(tracked);

```

```

        PjP(ob) = ob;
    end
end
DET{fr-start+1,1} = Pj';
DETP{fr-start+1,1} = PjP';
end
for fr = stop-2:-1:start
    maskFRk = uint16(cMask(:, :, fr));
    [imlbk, nck] = bwlabel(maskFRk);
    Pk = [];
    PkP = [];
    for ob = 1:1:nck
        obj_k = imlbk==ob;
        tracked = trackObj_v1(obj_k, imlbi, 1, 1, 3); %-----EDIT
        if tracked==0
            tracked = trackObj_v1(obj_k, imlbi, 1, 1, 3); %-----EDIT
            if tracked==0
                Pk(ob) = count;
                PkP(ob) = ob;
                count = count+1;
            else
                Pk(ob) = Pi(tracked);
                PkP(ob) = ob;
            end
        else
            Pk(ob) = Pj(tracked);
            PkP(ob) = ob;
        end
    end
    DET{fr-start+1,1} = Pk';
    DETP{fr-start+1,1} = PkP';
    Pi = Pj;
    Pj = Pk;
    imlbi = imlbi;
    imlbi = imlbi;
end
RES1 = zeros(count-1, size(DET, 1));
RES2 = RES1;
for i = 1:size(DET, 1)
    mat = DET{i, 1};
    matP = DETP{i, 1};
    for j = 1:size(mat, 1)
        RES1(mat(j, 1), i) = mat(j, 1);
        RES2(mat(j, 1), i) = matP(j, 1);
    end
end
end

%% DELETE TRACKS SMALLER THAN A CERTAIN THRESHOLD LENGTH (IN FRAMES).

fr_thresh = 10; %---EDIT---

%---DO NOT EDIT PAST THIS POINT!-----
RES_bin = RES1>0;
for i = 1:size(RES_bin, 1)
    if sum(RES_bin(i, :))<fr_thresh

```

```

        RES2(i,:) = 0*RES2(i,:);
    end
end
figure;
hold on;
for i = 1:size(RES1,1)
    for j = 1:size(RES1,2)
        if RES1(i,j)>0
            plot(j,RES1(i,j),'r.');
        end
        if RES2(i,j)>0
            plot(j,RES1(i,j),'b.');
        end
    end
end
hold off;
xlabel(['Frame # (contraction begins at #' num2str(SWFR-start+1) ')']);
ylabel('Cluster ID');

%% Prepare corrected video Mask of clusters (dMask).

dMask = 0*cMask;
for i = 1:1:size(RES2,1)
    for j = size(RES2,2):-1:1
        ob = RES2(i,j);
        if ob>0
            fr = cMask(:,:,start+j-1);
            mlb = bwlabel(fr);
            obj = mlb==ob;
            dMask(:,:,start+j-1) = [dMask(:,:,start+j-1) + obj];
        end
    end
end
for i = 1:1:size(RES2,1)
    for j = size(RES2,2)-1:-1:2
        ob = RES2(i,j);
        if ob>0
            fr = cMask(:,:,start+j-1);
            mlb = bwlabel(fr);
            obj = mlb==ob;
            dMask(:,:,start+j-1) = [dMask(:,:,start+j-1) + obj];
        else
            if RES2(i,j-1)>0
                fr = cMask(:,:,start+j-2);
                mlb = bwlabel(fr);
                obj = mlb==RES2(i,j-1);
                dMask(:,:,start+j-1) = [dMask(:,:,start+j-1) + obj];
            end
        end
    end
end
for i = 1:1:size(RES2,1)
    for j = 1
        ob = RES2(i,j);
        if ob>0

```

```

        fr = cMask(:,:start+j-1);
        mlb = bwlabel(fr);
        obj = mlb==ob;
        dMask(:,:start+j-1) = [dMask(:,:start+j-1) + obj];
    end
end
end
dMask = dMask(:,:start:stop);

%% Save dMask.

OutFolder = 'C:\Users\anbha\Desktop\Automatic tracking\DEM0\';
CellName = 'INH043_vid1_cell-03';

%----DO NOT EDIT PAST THIS POINT!-----
filename = [OutFolder CellName '_clusters-tracked_v1.tiff'];
TIRFvid_saveIMstack_v1(uint16(dMask),filename);

%% Quantify.

QC = cell(size(RES2,1),size(RES2,2));
mbg_mat = MBG(1,start:stop);
for i = 1:1:size(RES2,1)
    for j = size(RES2,2):-1:1
        ob = RES2(i,j);
        bg = mbg_mat(1,j);
        if ob>0
            fr = cMask(:,:start+j-1);
            mlb = bwlabel(fr);
            bin = mlb==ob;
            RFR = vidR(:,:start+j-1);
            obj_ID = [CellName '__obj-' num2str(i,'%04.f')];
            Q = analObj_v1(RFR,bin,bg,obj_ID,(start+j-1),(start-SWFR-
1+j));%frame correction....
            QC{i,j} = Q;
        end
    end
end
for i = 1:1:size(RES2,1)
    for j = size(RES2,2):-1:2
        ob = RES2(i,j);
        bg = mbg_mat(1,j);
        if ob>0
            fr = cMask(:,:start+j-1);
            mlb = bwlabel(fr);
            bin = mlb==ob;
            RFR = vidR(:,:start+j-1);
            obj_ID = [CellName '__obj-' num2str(i,'%04.f')];
            Q = analObj_v1(RFR,bin,bg,obj_ID,(start+j-1),(start-SWFR-
1+j));%frame correction....
            QC{i,j} = Q;
        else
            if RES2(i,j-1)>0
                fr = cMask(:,:start+j-2);
                mlb = bwlabel(fr);

```

```

        bin = mlb==RES2(i,j-1);
        RFR = vidR(:,:,start+j-1);
        obj_ID = [CellName '__obj-' num2str(i,'%04.f')];
        Q = analObj_v1(RFR,bin,bg,obj_ID,(start+j-1),(start-SWFR-
1+j));%frame correction....
        QC{i,j} = Q;
    end
end
end
for i = 1:1:size(RES2,1)
    for j = 1
        ob = RES2(i,j);
        bg = mbg_mat(1,j);
        if ob>0
            fr = cMask(:,:,start+j-1);
            mlb = bwlabel(fr);
            bin = mlb==ob;
            RFR = vidR(:,:,start+j-1);
            obj_ID = [CellName '__obj-' num2str(i,'%04.f')];
            Q = analObj_v1(RFR,bin,bg,obj_ID,(start+j-1),(start-SWFR-
1+j));%frame correction....
            QC{i,j} = Q;
        end
    end
end
for i = size(RES2,1):-1:1
    RES_row = RES2(i,:);
    if sum(RES_row)==0
        QC(i,:) = [];
    end
end
QC(:,size(QC,2)) = [];
QC(:,1) = [];

QCN = QC;
for i = 1:size(QC,1)
    for j = 1:size(QC,2)
        if ~isempty(QC{i,j})
            Q = QCN{i,j};
            bg = Q.BG;
            MFI_n = Q.MFI/bg;
            Peak_n = Q.Peak/bg;
            Q.MFIN = MFI_n;
            Q.PeakN = Peak_n;
            QCN{i,j} = Q;
        end
    end
end
end

```

Script 5.1: To track detected clusters by creating frame-by-frame association between two consecutive frames.

```
function [REF_OBJ] = trackObj_v1(obj,ref,obj_dil,ref_dil,min_ovrlp)

if obj_dil>0
    DOB = imdilate(obj,strel('disk',obj_dil));
else
    DOB = obj;
end

List = [];
Lcount = 1;
[reflb,N] = bwlabel(ref);
for i = 1:N
    P = reflb==i;
    if ref_dil>0
        DP = imdilate(P,strel('disk',ref_dil));
    else
        DP = P;
    end
    OL = DP.*DOB;
    if sum(OL(:))>=min_ovrlp
        List(Lcount,1) = i;
        List(Lcount,2) = sum(OL(:));
        Lcount = Lcount + 1;
    end
end

if isempty(List)
    REF_OBJ = 0;
else
    List = sortrows(List,2,'descend');
    REF_OBJ = List(1,1);
end
end
```

Script 5.2: To analyze detected clusters and output several measured parameters including AF546 peak intensity and MFI in that cluster.

```
function [Q] = analObj_v1(RFR,bin,BG,obj_ID,FR,cFR)

cut = RFR.*bin;
Area = sum(bin(:));
[CX,CY] = Centroid(bin);
TFI = sum(cut(:));
MFI = TFI/Area;
Peak = max(cut(:));
PbM = Peak/MFI;
```

```
Q =
struct('ID',obj_ID,'FR',FR,'cFR',cFR,'Area',Area,'CX',CX,'CY',CY,'TFI',TFI,
'MFI',MFI,'Peak',Peak,'PbM',PbM,'BG',BG);
```

Script 6: To input multi-channel 16-bit .nd2 images (used as a sub-function in some of the above scripts)

```
function [RES] = openND2_still (file_loc)

%Auto-detects number of channels in a (non-Zstack & non-video) image and
%returns as a 3-dim matrix (x,y,c).

C = b fopen_silent(file_loc);
C1 = C{1,1};
CL = size(C1,1);
RES = C1{1,1};
if CL>1
    for i = 2:1:CL
        RES(:, :, i) = C1{i,1};
    end
end
end
```

Script 7: To input multi-channel 16-bit .tiff time-lapse images (used as a sub-function in some of the above scripts)

```
function [RES] = openTIF_vid (file_loc,num_channels)

%Returns [1xN] cell containing 'N' 3-dim matrices (x,y,t), where 'N' is the
%number of channels.

C = b fopen_silent(file_loc);
C1 = C{1,1};
CL = size(C1,1);

if rem(CL,num_channels)>0
    error('ERROR! Check your input.');
```

```
else
    T = CL/num_channels;
    RES = cell(1,num_channels);
    samp = C1{1,1};
    for j = 1:num_channels
        RESch = zeros(size(samp,1),size(samp,2),T);
        for i = 1:T
            RESch(:, :, i) = C1{(num_channels*(i-1)+j),1};
        end
        RES{1,j} = RESch;
    end
end
end
```

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