THE ROLE OF CONNEXIN 43 IN B CELL RECEPTOR SIGNALING AND THE HIGH THROUGHPUT SCREEN FOR NOVEL B CELL RECEPTOR SIGNALING INHIBITORS

by

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Abstract

B cells migrate throughout the body, following chemokine gradients, to search for foreign antigens. When a B cell encounters an antigen-presenting cell (APC) bearing antigens that bind its B cell receptors (BCRs), it spreads across the surface of the APC to scan for additional antigens. This spreading is followed by contraction in which BCR microclusters recruit signaling enzymes and form an immune synapse. The resulting BCR signaling initiates an activation process in which B cells differentiate into antibody-producing plasma cells, which are directed to the bone marrow by chemokines, where they survive and produce antibodies for long periods of time. Both chemokine-induced B cell migration and BCR-induced cell spreading involve changes in the cytoskeleton.

The gap junction protein connexin 43 (Cx43) regulates the migration of neuronal cells during brain development and interacts with many cytoskeletal-regulating partners. Previous work in our lab showed that Cx43 is important for BCR-induced spreading and chemokine-induced B cell migration, and for sustained activation of Rap1 GTPases, master regulators of cytoskeletal organization. However, the mechanism by which Cx43 enhances BCR- and chemokine-induced Rap1 activation was not known. The goal of my project was to determine whether Cx43 modulates all BCR signaling reactions, perhaps by acting on BCR-proximal signaling components, or whether it selectively regulates the activation of Rap1. I found that Cx43 influences the activation of certain BCR signaling pathways, specifically the PLCγ pathway, while having less effect on other targets of BCR signaling. This is the first evidence that Cx43 modulates BCR signaling and provides new insights into how Cx43 contributes to BCR-induced cytoskeletal regulation.

BCR signaling is also important for B lymphoma cell survival and for the negative selection processes that eliminate autoreactive B cells. Although B cell depleting drugs have been used to treat B cell malignancies and B cell-mediated autoimmune diseases, there is variable efficacy. Small molecule inhibitors might be useful alternatives or additions to B cell depletion. Although several inhibitors of B cell signaling enzymes are being clinically tested, I have developed and optimized assay conditions to enable a large-scale screen for additional inhibitors of key components of BCR signaling pathways.

Preface

I. Published Work

A version of Figure 2-3 was published and reproduced/adapted with permission: Steven Machtaler, May Dang-Lawson, Kate Choi, Caren Jang, Christian C. Naus and Linda Matsuuchi. The gap junction protein Cx43 regulates B lymphocyte spreading and adhesion. The Journal of Cell Science. (2011) vol 124: 2611-2521. I was responsible for the experiments in Figure 2-3A, whereas both Steven Machtaler and I were responsible for the experiments in Figure 2-3B.

II. Collaboration

Chapter 3 was done in collaboration with the Centre for Drug Research and Development under the supervision of the Head of Drug Screening, Tom Pfeifer. I designed and optimized all the protocols prior to the screen. Data from Figures 3-9, 3-10 and 3-11 were collected by Nicolette Honson, who modified my protocols to accommodate the conditions at their facility.

III. UBC Research Ethics Board certificates

Rodent Biology and Husbandry certification number: RBH-972-10

Canadian Council on Animal Care (CCAC) number: 4197-10

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7-aminoactinomycin D	7-AAD
Actin-related protein	Arp
Activation-induced cytidine deaminase	AID
Adenosine diphosphate	ADP
Adenosine monophosphate	ADP
Adenosine triphosphate	ATP
American Type Culture Collection	ATCC
Antigen presenting cell	APC
β-mercaptoethanol	2-ME
B cell adaptor for PI3K	BCAP
B cell linker	BLNK
B cell lymphoma 10	BCL10
B cell receptor	BCR
B cell-specific activator protein	BSAP
Bicinchoninic acid	BCA
B lymphocyte chemoattractant	BLC
B-lymphocyte-induced maturation protein 1	BLIMP1
Bruton's tyrosine kinase	Btk
C57BL/6 mice	B6 mice
Calcium	Ca^{2+}
Calcium and DAG guanine exchange factor	CalDAG-GEF
Carboxyfluorescein diacetate succinimidyl ester	CFDA-SE
Carboxyfluorescein succinimidyl ester	CFSE
CARD-containing MAGUK	CARMA
Casein kinase 1	CSK1
Caspase-associated recruitment domain	CARD
CC chemokine ligand	CCL
CC chemokine receptor	CCR
Central supramolecular activation cluster	c-SMAC
Centre for Drug Research and Development	CDRD
c-Jun N-terminal kinase	JNK
Class switch recombination	CSR
Cluster of differentiation	CD
Common lymphoid progenitor	CLP
Connexin	Cx
CXC chemokine ligand	CXCL
CXC chemokine receptor	CXCR
CXCL12-abundant reticular cell	CAR cell
Cyclic adenosine monophosphate	cAMP
Daltons	Da
Dendritic cell	DC
Deoxycytidine	dC
Deoxyribonucleic acid	DNA
Deoxyuridine	dU

Diacylglycerol	DAG
Diffuse large B cell lymphoma	DLBCL
Early B cell factor	EBF
Endoplasmic reticulum	ER
Ethylenediaminetetraacetic acid	EDTA
Extracellular signal-regulated kinase	Erk
Ezrin, radixin and moesin	ERM
Filamentous actin	F-actin
Fluorescein isothiocyanat	FITC
Fluorescence-activated cell sorting	FACS
Fluorescence resonance energy transfer	FRET
Focal adhesion kinase	FAK
Follicular B cell	FO B cell
Follicular dendritic cell	FDC
Forward Scatter	FSC
Globular actin	G-actin
Glycosylation-dependent cell-adhesion molecule 1	GLYCAM
G protein-coupled receptor	GPCR
Granulocyte colony-stimulating factor	G-CSF
Granulocyte macrophage colony-stimulation factor	GM-CSF
Growth factor receptor binding protein 2	Grb2
GTPase-activating protein	GAP
Guanine exchange factor	GEF
Guanosine triphosphate	GTP
Hank's Buffered Saline Solution	HBSS
Heat inactivated fetal bovine serum	HI-FBS
Hematopoietic lineage cell-specific protein 1	HS1
Hematopoietic stem cell	HSC
High endothelial venule	HEV
Hour(s)	hr
IκB kinase	IKK
Immunoglobulin	Ig
Immunoglobulin superfamily cell adhesion molecule	IgSF CAM
Immunoreceptor Tyrosine-based Activation Motif	ITAM
Inhibitor of kappa B	IκB
Inositol-1,4,5-trisphosphate	IP_3
Intercellular adhesion molecule	ICAM
Interferon	IFN
Interleukin	IL
Kilodaltons	kDa
Known Drug 2	KD2
Library of Pharmacologically Active Compounds	LOPAC
Linker of activated B cells	LAB
Lipopolysaccharide	LPS
Lymphocyte function-associated antigen	LFA
Lymphocyte Peyer's patch adhesion molecule	LPAM

Lymphoid-primed multipotent progenitor	LMPP
Major histocompatibility class II	MHC II
Mammalian target of rapamycin complex	mTORC
Marginal zone B cell	MZ B cell
Mean fluorescence intensity	MFI
Membrane-associated guanylate kinase	MAGUK
Membrane-bound immunoglobulin	mIg
Mesenchymal stem cell	MSC
Minute(s)	min
Mismatch repair	MMR
Mitogen-activated protein kinase	MAPK
Mitogen-activated protein kinase kinase	MAPKK
Mitogen-activated protein kinase kinase kinase	MAPKKK
Mucosa-associated lymphoid tissue lymphoma translocation protein	MALT1
Multipotent progenitor	MPP
N-acetyl-L-Cysteine	NAC
Neural precursor cell-expressed developmentally down-regulated protein 4	Nedd4
Nuclear factor kappa light chain enhancer of activated B cells	NF-κB
Nuclear factor of activated T-cells	NFAT
Paired box protein 5	Pax5
Peridinin chlorophyll	PerCP
Peridinin chlorophyll-cyanin-5.5	PerCP-Cy5.5
Peripheral node addressin	PNAD
Peripheral supramolecular activation cluster	p-SMAC
Phenylmethylsulfonyl fluoride	PMSF
Phosphate-buffered saline	PBS
Phosphatidylinositol 3-kinase	PI3K
Phosphatidylinositol-3,4,5-trisphosphate	$PI(3,4,5)P_3$
Phosphatidylinositol-4,5-bisphosphate	$PI(4,5)P_2$
Phosphoinositide dependent kinase 1	PDK1
Phospholipase C	PLC
Phycoerythrin	PE
Pleckstrin Homology	PH
Polyacrylamide gel electrophoresis	PAGE
Positive-regulatory-domain containing 1	PRDM1
Proline-rich tyrosine kinase 2	Pyk2
Protein kinase A	PKA
Protein kinase C	PKC
P-selectin glycoprotein ligand-1	PSGL-1
Radioimmunoprecipitation assay	RIPA
Ras guanyl nucleotide-releasing proteins	RasGRPs
Recombination activating genes	Rag
Regulators of G protein signaling	RGS
Ribonucleic acid	RNA
Room temperature	RT
Roswell Park Memorial Institute medium	RPMI

Rotations per minute	RPM
Second(s)	sec
Serine	Ser
SH2 containing transforming protein	Shc
Short hairpin RNA	shRNA
Side Scatter	SSC
Small interfering RNA	siRNA
Sodium dodecylsulfate	SDS
Sodium Vanadate	Na_3VO_4
Son of sevenless	SOS
Sphingosine 1-phosphate	S1P
Spleen tyrosine kinase	Syk
Src homology 2 domain	SH2
Standard Deviation	SD
Standard Error of Means	SEM
Stem cell factor	SCF
Stromal cell-derived factor-1	SDF-1
Stromal interaction molecule 1	STIM1
Subcapsular sinus	SCS
Terminal deoxynucleotidyl transferase	TdT
Threonine	Thr
Toll-like receptor	TLR
Tris-buffered saline	TBS
Tris-buffered saline with 0.1% Tween	TBST
Tumor necrosis factor	TNF
Tumor-necrosis factor receptor-associated factor 6	TRAF6
Tyrosine	Tyr
Uracil-DNA glycosylase	URG
Vascular adhesion molecule	VCAM
Very late antigen	VLA
Wiscott Aldrich syndrome protein	WASp
X-box-binding protein 1	XBP1
Zeta-chain-associated protein kinase	Zap
Zonula occludens-1	ZO-1

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Thank you to my family for their support and hopefully, I've received some good thumb muscle alleles to not suffer from "pipetter's hand syndrome." A very special thank you to my loving boyfriend, Jason Sena, who has given me the comfort and care that I needed. I'd also like to thank anyone else whom I have not mentioned as memories are not forgotten, but are momentarily inaccessible due to a malfunction in the retrieval process.

Lastly, I would like to thank God again because He is the beginning and the end.

Dedication

This thesis is dedicated to whoever clicked on the link to my project and needs information from any part of it. Props to you!

1. Introduction

1.1 The immune system

1.1.1 Innate immunity and adaptive immunity

Humans are exposed to and are attacked by pathogens including bacteria, viruses, fungi, and parasites through contact, ingestion and inhalation. Our ability to avoid infection and disease depends on our evolutionarily conserved immune system, which includes the innate immune system and the adaptive immune system.

The innate immune system consists of initial barriers as well as specialized cells and molecules, which provide very rapid responses to infection. The first barriers to infections include the skin, lysozyme that is abundant in tears, saliva, and mucus, cilia in the respiratory tract, acid and proteolytic enzymes in the stomach, and anti-microbial peptides such as defensins that are secreted by epithelial cells in the tongue, cornea, salivary glands, esophagus and respiratory tract (Murphy, 2011). These initial barriers are broad and relatively non-specific, and if they are breached, the specialized cells and molecules of the innate system will be present to launch an immune response to eliminate the invading pathogen.

Cells of the innate system including, mast cells, eosinophils, basophils, neutrophils, macrophages, dendritic cells and natural killer cells, function by identifying and eliminating pathogens that could cause infection. These cells express pattern recognition receptors, including Toll-like receptors (TLRs) and C-type lectin receptors that recognize conserved patterns on microbes called microbe-associated molecular patterns (Akira et al., 2006; Janeway and Medzhitov, 2002; Medzhitov, 2007).

If the innate immune response is overwhelmed and does not rapidly eliminate the pathogen, the adaptive immune response is triggered, providing the last, but most potent layer of immunological defense. This system consists of highly specialized cells, including B cells and T cells, which are able to recognize and remember specific pathogens, and mount much stronger attacks each time the same pathogen is encountered. During the generation of B and T cells, the recombination of their B cell receptor (BCR) and T cell receptor genes, respectively, generates a vast number of different antigen receptors, each expressed on different cells, allowing recognition of many different pathogens. B cells produce antibodies that can prevent infection via complement activation, neutralization, and opsonization. T cells can direct a number of other processes that help eliminate pathogens. Cytotoxic T cells can directly kill infected cells, pro-inflammatory Th1 cells can recruit and activate macrophages that phagocytose pathogens, and Th2 cells produce cytokines that activate B cells and mast cells, thereby promoting antibody-mediated elimination of pathogens.

The adaptive immune system is highly adaptable because over the course of an infection, the B cell immunoglobulin receptor gene undergoes somatic hypermutation, increasing specificity and affinity, in order to increase the efficacy of pathogen elimination. B and T cells are capable of remembering the particular pathogens to which it mounted an immune response. During the activation of B and T cells, some will become memory cells, which can survive for years. When the body encounters the same pathogen again, these memory cells recognize the pathogens and are induced to proliferate and secrete antibodies, bypassing the lag phase required for the activation of naïve B and T cells and the generation of high affinity antibodies. This allows for rapid antibody production and clearance of the pathogens.

1.1.2 B lymphocytes and the B cell receptor

B lymphocytes play an important role in the adaptive immune system. B cells recognize foreign antigens using their BCRs. Clustering of the BCR by multivalent antigens, or by arrays of monomeric antigens displayed on the surface of other cells, induces the activation of intracellular signaling pathways, resulting in activation, proliferation and differentiation of the B cells into plasma cells or memory B cells. Plasma cells continuously secrete antibodies against the specific antigens presented, whereas memory B cells become activated, proliferate and differentiate into plasma cells upon future encounters with the same antigens.

The BCR is comprised of a membrane-bound immunoglobulin (mIg) heavy and light chain subunit that is non covalently associated with a disulfide-linked heterodimer, Igα (CD79a) and Igβ (CD79b) (Fig. 1-1) (Hombach et al., 1990). The mIg serves as the antigen binding subunit whereas the Igα/β subunit is responsible for the propagation of intracellular signals (Reth, 1992). The interaction between the mIg and the signaling subunits is critical for the assembled BCR to exit the endoplasmic reticulum (ER), as the absence of any of these four BCR polypeptides results in retention within the ER (LeBien, 1998). Surface expression of the BCR is necessary for B cells to develop and differentiate into antibody-producing plasma cells (Matsuuchi and Gold, 2001).

The Igα and Igβ polypeptides each possess an Immunoreceptor Tyrosine-based Activation Motif (ITAM) (Fig. 1-1) with the consensus sequence $(D/E)x_{0-2}Yxx(L/I)x_{6-8}Yxx(L/I)$, with x denoting any amino acid. The tyrosine residues within the ITAMs are phosphorylated after antigen binding to the mIg, creating SH2 domain binding motifs that recruit signaling

proteins to the BCR (Grande et al., 2007; Reth, 1989) (Section 1.4). This is an essential step in initiating BCR signaling pathways.

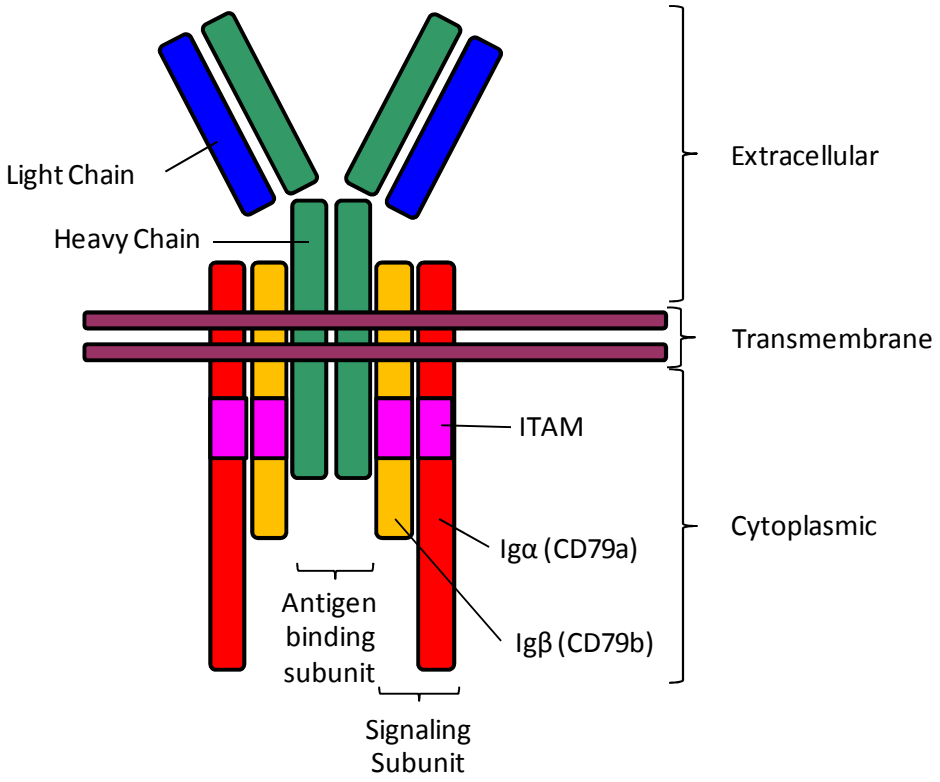


Figure 1-1. Schematic representation of the BCR

The BCR consists of two subunits, the mIg, which binds antigen, and the Igα/β signaling subunit. mIg is comprised of two identical membrane-bound Ig heavy chains (green) and two identical Ig light chains (blue). The disulphide-linked Igα/β subunit consists of one Igα (red) and Igβ (yellow) heterodimer, each with a single ITAM (pink) within their cytoplasmic domains.

B cells require co-stimulatory help from T helper cells to produce maximal antibody responses, including affinity maturation, class switching, and differentiation into plasma and memory cells. B cells internalize and degrade antigens into short peptides, which are then loaded onto major histocompatibility class II (MHC II) proteins, and subsequently displayed on the surface to T helper cells. After recognition of this MHC II-peptide complex, T helper cells

upregulate and express on their surface co-stimulatory receptors, and secrete cytokines to help B cell activation (Cannons et al., 2006; Fazilleau et al., 2009). This is described in further detail in Section 1.2.2.

Besides producing antibodies and activating T cells by presenting antigenic peptides, B cells have other important roles in the immune system. Depleting B cells during mice development results in a decrease in thymocyte numbers and diversity, defects in lymphoid tissue organogenesis, and an absence of marginal zone macrophages (LeBien and Tedder, 2008). B cells also produce a number of regulatory cytokines including pro-inflammatory mediators such as interleukin-6 (IL-6) (Barr et al., 2012). Conversely, regulatory B cells oppose inflammation by secreting IL-10 (Noh and Lee, 2011) (Section 1.2.3).

1.1.3 Antibody function

Antibodies protect against invading pathogens in several ways. They can neutralize pathogens and their toxins by binding to the surface of pathogens, inhibiting them from binding to cells in the body and preventing invasion and infection (Burton, 2002; Michetti et al., 1992; Peachman et al., 2006; Pirro et al., 1995; Winner et al., 1991). Some bacteria express polysaccharide coats that are not easily phagocytosed; therefore, antibodies can coat these pathogens, rendering the pathogen more readily taken up by phagocytic cells, which express Fc receptors that bind to the constant region of antibodies (McKinlay et al., 1993; Raff et al., 1988; Ravetch and Bolland, 2001). Finally, when antibodies bind to antigens on pathogens, complement proteins can bind to these clustered IgM or IgG antibodies and trigger the complement pathway, leading to the formation of the membrane attack complex that forms a

transmembrane channel that promotes lysis of the bacteria (Frank and Fries, 1991; Zlatarova et al., 2006).

1.1.4 B cell-mediated diseases

Antibody production by B cells is a normal mechanism for pathogen clearance. However, when antibodies recognize a self-antigen, it can result in chronic inflammation and organ damage. Auto-reactive B cells can present self-antigen to auto-reactive T cells, which can initiate inflammatory reactions via the production of cytokines that recruit and activate cells of the innate immune system. T cells and innate immune cells that infiltrate organs can cause cell death and tissue damage that can impair the function of the organ. Examples of such B cell-mediated autoimmune diseases are type 1 diabetes, lupus, autoimmune thyroiditis, Sjögren's disease, and rheumatoid arthritis (Yanaba et al., 2008). In patients with these diseases, a subset of B cells expressing membrane lymphotoxin-α/β contributes to the formation of ectopic germinal centers that form an environment that promote further B cell activation (Browning, 2006).

Besides its involvement in autoimmune diseases, B cell lymphomas are one of the most common cancers. The hallmark of many B cell lymphomas is the abnormal translocation of a proto-oncogene into an active Ig locus, leading to constitutive expression of the oncogene. Also, non-Ig genes (e.g. the Pim-1 kinase) can accumulate mutations during the process of somatic hypermutation and become activated oncogenes (Pasqualucci et al., 2001). Three molecular processes contribute to chromosome translocations and mutations: somatic recombination, somatic hypermutation and class switch recombination (Section 1.2). This explains why B cell

cancers are more common than T cell lymphomas as T cells undergo somatic VDJ recombination but do not undergo somatic hypermutation or class switch recombination (Kuppers, 2005). Most B cell lymphomas still express the BCR and the loss of BCR expression during translocation-mediated oncogenic events leads to cell death. Low level antigen-independent BCR signaling is required to sustain levels of pro-survival factors in both normal and transformed B cells (Kuppers, 2005) (Section 1.4.5).

B cell depletion is one of the most widespread therapies used for treating B cell malignancies and autoimmunue diseases. Rituximab and Belimumab are the only two B cell depletion drugs that are currently administered. Rituximab is an anti-CD20 antibody that is approved by the Food and Drug Administration (FDA) for rheumatoid arthritis and various B cell non-Hodgkin lymphomas (Selewski et al., 2010). Belimumab is an anti-BAFF (B cell activating factor) antibody and is approved for use in lupus and is in Phase II for rheumatoid arthritis (Stohl and Hilbert, 2012). BAFF is required for B cell development and survival. Another drug, Epratuzumab (anti-CD22 antibody) is in Phase III for lupus. B cell depletion drug therapy often results in incomplete elimination of autoreactive B cells or B cell lymphomas, and relapse rates still remain high (Engel et al., 2011).

Because BCR signaling is needed for proper B cell development, and the survival of B cells and B cell lymphomas, drugs targeting BCR signaling are also in use. Syk is needed for the survival of mature B cells, and is constitutively active in some B cell lymphomas. The Syk inhibitor Fostamatinib disodium leads to apoptosis in B cell lymphomas and has shown promise in a Phase II trial (Reeder and Ansell, 2011). Also, Btk is required for B cell maturation and is

also overexpressed in multiple B cell lymphomas. The Btk inhibitor PCI-32765 prevents B cell activation the growth of malignant B cells that overexpress Btk, and is in Phase I/II trials (Reeder and Ansell, 2011).

Novel therapies that provide a more complete B cell depletion could improve treatments for B cell-mediated autoimmune diseases and B cell lymphomas. Additional BCR signaling inhibitors may also be useful to limit B cell activation and survival, and could be used in combination with B cell depleting drugs.

1.2 B cell development

1.2.1 Development within the bone marrow

Hematopoiesis, blood cell production that replenishes more than 7×10^9 blood cells per kilogram body weight per day, occurs in the fetal liver of embryos and the red bone marrow in children (Purton and Scadden, 2008). The human spleen does not support hematopoiesis after birth, except during hematopoietic stress (O'Malley et al., 2005). As we age, adipose-containing yellow marrow displaces red marrow in the shafts of long bones (Purton and Scadden, 2008). In adults, red marrow remains mainly in the ribs, pelvic bones, cranium, sternum and vertebrae (Purton and Scadden, 2008). In mice, the spleen remains hematopoietically active throughout life, and all bones support hematopoiesis (Weiss, 1974).

The earliest blood cell progenitors are the hematopoietic stem cells (HSCs) that reside in red bone marrow. The maintenance and self-renewal of HSCs and differentiation of these cells into B cells depend on their ability to interact with adhesion molecules, chemokines, and

cytokines expressed and secreted by bone marrow stromal cells as well as the activation of transcription factors such as the early B cell factor (EBF), E2A, and Pax5 (Bartholdy and Matthias, 2004; Hardy and Hayakawa, 2001; Hardy et al., 2007; Nutt and Kee, 2007; Pillai et al., 2004). There are two different niches in the bone marrow which contribute to HSC self-renewal and differentiation: the endosteal niche, which is important for the maintenance of long term quiescence of HSCs, and the vascular niche, which is important for the proliferation and differentiation of HSCs, and for the delivery of these cells to the circulation. The endosteal niche is mainly comprised of osteoblasts, specialized cells responsible for synthesis and deposition of extracellular matrix (ECM) of the bone, important in the development of bone. Osteoblasts secrete cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF) and IL-6, and express factors including angiopoietin, thrombopoietin, WNT, Notch, N-cadherin and osteopontin, which support HSC numbers, survival, and differentiation (Wang and Wagers, 2011).

HSCs detach from the bone niche and associate with vascular structures where they interact with perivascular nestin^{+} mesenchymal stem cells (MSCs) and CXC chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells of the vascular niche. Nestin^{+} MSCs are tightly associated with nerve fibers of the sympathetic nervous system that regulate HSC mobilization and they express HSC maintenance factors such as CXCL12, stem cell factor (SCF), angiopoietin-1, IL-7, vascular cell adhesion molecule 1 (VCAM) and osteopontin (Ehninger and Trumpp, 2011). CAR cells are much more abundant than nestin^{+} MSCs, and also express CXCL12 and SCF (Ehninger and Trumpp, 2011).

When HSCs, characterized as lineage (Lin)$^-$CD45R$^-$KIT$^+$SCA1$^+$ fms-related tyrosine kinase 3 (FLT3)$^-$CD34$^-$, adopt a lymphoid fate, they differentiate into Lin$^-$B220$^-$KIT$^+$SCA1$^+$ FLT3$^-$CD34$^+$ multipotent progenitors (MPPs) which have lost the self-renewal ability but remain multipotent. MPPs with T cell potential migrate to the thymus and progenitors with B cell potential remain in the bone marrow. MPPs differentiate into Lin$^-$CD45R$^-$KIT$^+$SCA1$^+$FLT3$^+$ CD34$^+$ lymphoid-primed multipotent progenitors (LMPPs) which lack erythroid cell and megakaryocyte potential but retain myeloid and lymphoid cell potential (Adolfsson et al., 2005). LMPPs can then differentiate into Lin$^-$KIT^{low}SCA1^{low} IL-7 receptor (IL-7R)$^+$ or Lin$^-$AA4.1$^+$ SCA1^{low}IL-7R$^+$ common lymphoid progenitors (CLPs) which can generate B- and T-lineage cells but not myeloid-lineage cells (Izon et al., 2001; Kondo et al., 1997).

CLPs can give rise to the earliest B cell progenitor cells, the CD45R$^+$CD43$^+$AA4.1$^+$ CD19$^-$Ly6C$^-$ pre-pro-B cells, which have low expression of the recombination activating genes (Rag1 and Rag2). These cells have not begun to rearrange their IgH and IgL genes, which are still in the germline configuration. Most pre-pro-B cells are in contact with CAR cells, and B cell commitment and repression of alternative linage differentiation are regulated by transcription factors, including IKAROS (Reynaud et al., 2008), PU.1 (DeKoter et al., 2002; Scott et al., 1997), c-Myb (Lieu and Reddy, 2009), the early B cell factor (Pongubala et al., 2008), E2A (Greenbaum and Zhuang, 2002) and the paired domain factor B cell-specific activator protein (BSAP) known as Pax5. Once pre-pro-B cells differentiate into CD45R$^+$CD19$^+$ CD43$^+$AA4.1$^+$ pro-B cells, they migrate to different regions of the bone marrow and associate with IL-7-secreting stromal cells (Kurosaki et al., 2010).

Early pro-B cells express CD19 on their surface and are the first B lineage cells to express a precursor form of the BCR, the pro-BCR, composed of Igα, Igβ and calnexin (Nagata et al., 1997). To generate a diverse repertoire of antigen receptors, B cells undergo somatic recombination, involving gene recombination of the heavy and light chains. Pro-B cells start increasing the expression of Rag1, Rag2 and the terminal deoxynucleotidyl transferase (TdT), allowing the rearrangement of the immunoglobulin heavy chain genes. The immunoglobulin heavy chain region contains 65 Variable (V_H) genes, 27 Diversity (D) genes, 6 Joining (J_H) genes and 9 Constant (C) genes, with introns between each gene (Li et al., 2004). In pro-B cells, one random D gene segment is joined to a random J_H gene segment on both alleles of the heavy chain, and any DNA between these two genes are deleted. This leads to the generation of late pro-B cells. After formation of the DJ_H rearrangements, the V_H genes become accessible to the VDJ recombinase. Each V_H gene segment is paired with a leader (L) gene segment, separated by an intron, and each pair has its own promoter sequence. The L region directs the receptor to the ER (Murphy, 2011). On only one allele, there is a joining of a random V_H gene segment to the newly recombined DJ_H segment, and any DNA between these two genes are removed from the genome. V_HDJ_H occurs in only one allele, a process called allelic exclusion, in order to generate monospecific B cells that express one unique antigen receptor (Jung et al., 2006). If an unsuccessful rearrangement occurs (one that cannot result in a heavy chain polypeptide being synthesized), the second allele will undergo somatic recombination. The joining of V_H to DJ_H brings the promoter associated with the V_H gene to an enhancer located in the intron between the J_H and C regions, thus enabling transcription (Guo et al., 2011; Subrahmanyam and Sen, 2010).

The Cμ region lies closest to the J_H region; therefore, all late pro-B cells express μ heavy chains. The mRNAs are translated in the cytoplasm and the L sequence is removed as the protein is transported to the ER. Late pro-B cells also express surface λ5 (Sakaguchi and Melchers, 1986) and VpreB (Kudo and Melchers, 1987) in association with a glycoprotein BILL-cadherin (Karasuyama et al., 1993; Ohnishi et al., 2000) to form surrogate light chains. This can pair with the membrane μ heavy chain to form the pre-BCR that signals to the late pro-B cell that a productive rearrangement has been made and marks the transition to the pre-B cell (Karasuyama et al., 1990).

Early large pre-B cells lose c-kit and CD43 (Rolink et al., 1994), while upregulating the surface marker CD25. Rag1 and Rag2 genes are downregulated upon μ heavy chain expression and signaling. This is followed by several rounds of proliferation (Grawunder et al., 1995). Large pre-B cells then stop dividing and become small resting pre-B cells, where they cease expression of the surrogate light chains and express the μ heavy chain alone in the cytoplasm. They re-express the RAG proteins and start to rearrange the light chain genes, which only contain V and J genes. On one allele, the light chain Igκ is rearranged first and if no productive rearrangement occurs, the Igλ starts to rearrange instead, which results in 60% of all B cells carrying Igκ, and 40% carrying Igλ in humans (Chen et al., 1993; Durdik et al., 1984; Engel et al., 1999). In mouse, 95% of all B cells express Igκ, and 5% express Igλ. Upon successful assembly of the light chain gene, a fully functional BCR can be formed, composing of complete IgM molecules (rearranged heavy and light chain) that associates with the Igα and Igβ chains, and the cell becomes an immature B cell that has the ability to recognize antigens (Cambier et al., 2007).

At this stage, immature B cells are checked for auto-reactivity before leaving the bone marrow to mature. Since the bone marrow is an environment where only components of the host (self-antigens) are present, any BCR binding will be to self-antigens (Grimaldi et al., 2005). If the maturation of these auto-reactive immature B cells occurs, autoimmunity will develop (Grimaldi et al., 2005). B cell-mediated autoimmune diseases are defined as when auto-reactive mature B cells secrete antibodies that bind to self components, mounting an immune response to the body (Grimaldi et al., 2005). Therefore, BCR crosslinking of immature B cells leads to negative selection through anergy, receptor editing or clonal deletion, to prevent the generation of auto-reactive B cells (Healy and Goodnow, 1998; Nemazee, 2006). The remaining immature B cells that do not recognize self-antigens in the bone marrow migrate to the periphery as transitional B cells (Carsetti et al., 1995), where they can sample self-antigens that are not present in the bone marrow (Allman et al., 1992; Allman et al., 1993; Nicholson et al., 1995).

Two negative selection mechanisms eliminate auto-reactive B cells in the bone marrow. The first is receptor editing, where the Rag genes are re-expressed, restarting the rearrangement of the light chain genes of the BCR. This changes the antigen specificity so the receptor will no longer recognize that particular self-antigen (Gay et al., 1993; Tiegs et al., 1993). If receptor editing results in a BCR that is still self-reactive, the second mechanism, deletion (through apoptosis), proceeds (Hartley et al., 1991; Nemazee and Burki, 1989; Sandel and Monroe, 1999). If receptor editing produces a BCR that is not self-reactive, the immature B cells may leave the bone marrow to encounter peripheral self-antigens and proceed through negative selection again (Aplin et al., 2003; Qian et al., 2001). The mechanisms of how BCR signaling triggers receptor

editing or cell death have yet to be determined. A summary of the developmental stages of B cells in the bone marrow is shown in Figure 1-2.

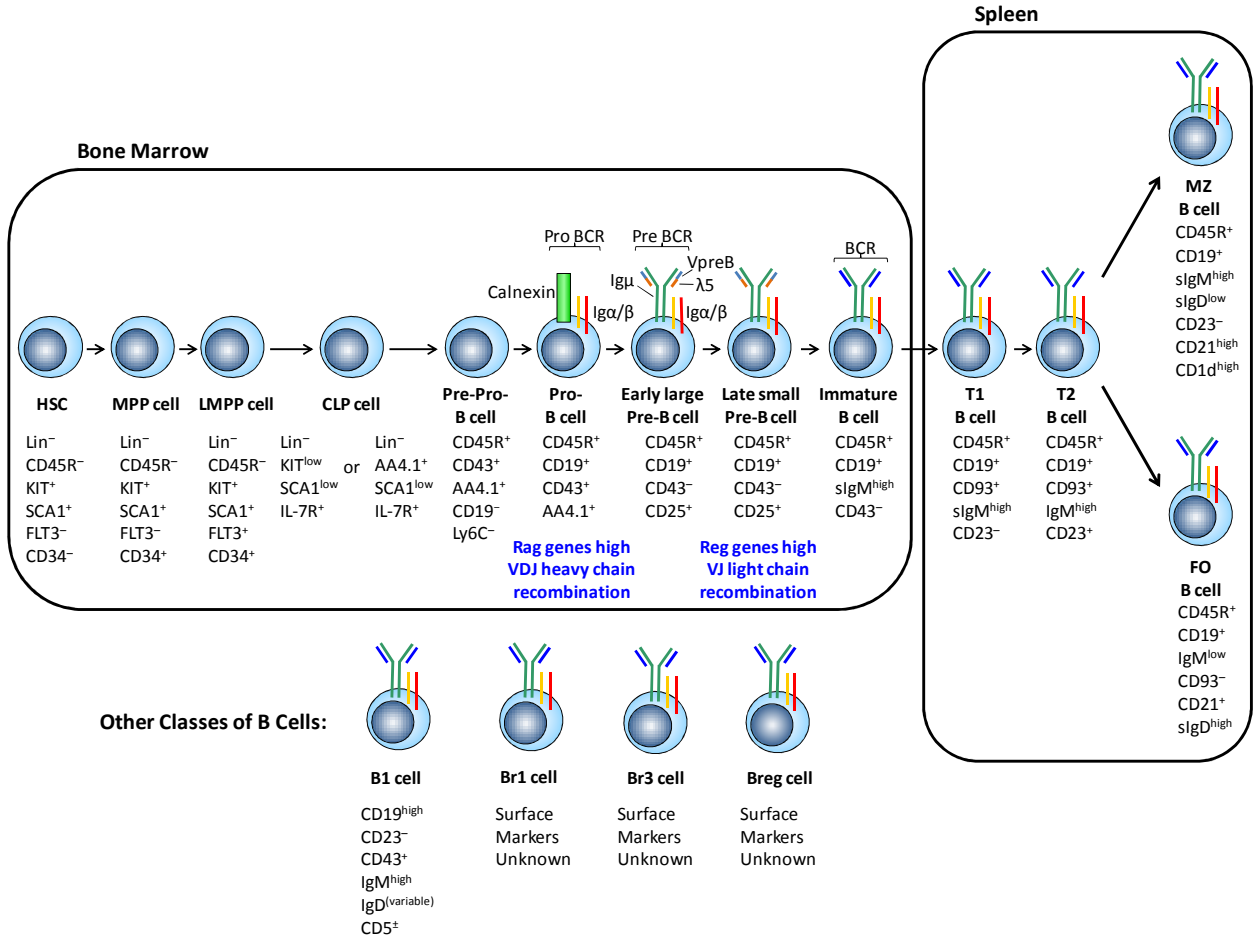


Figure 1-2. The developmental stages of B cells and B cell subsets

A schematic representation of the stages of B cell development starting from the multipotent stem cell that gives rise to all the blood cells, to mature B cells. Surface markers and the forms of BCR are also included. HSC: hematopoietic stem cell; MPP: multipotent progenitor; LMPP: lymphoid-primed multipotent progenitor; CLP; common lymphoid progenitor; T: transitional; MZ: marginal zone; FO: follicular; Breg: B regulatory cells.

1.2.2 Development outside the bone marrow

Approximately 10% of the immature B cell population exit the bone marrow and only around 30% of that will develop into mature B cells (Allman et al., 1992; Allman et al., 1993; Carsetti et al., 1995; Sater et al., 1998). The cells that leave the bone marrow are called $CD93^{+}sIgM^{high}CD23^{-}$ transitional (T)1 cells (Siggs et al., 2011), which migrate to the periarteriolar lymphoid sheath of the spleen in response to CXCL13, CC chemokine ligand 19 (CCL19), and CCL21 chemokines secreted by the follicular dendritic cells (FDCs) (Gunn et al., 1998; Lo et al., 2003). Blood-borne self-antigens are filtered in this area, allowing negative selection to occur (Su and Rawlings, 2002). The remaining T1 cells migrate into primary follicles where they upregulate CD23, becoming $CD93^{+}IgM^{high}CD23^{+}$ T2 B cells (Carsetti et al., 2004; Pillai et al., 2005). Within the follicles of the spleen, T2 cells undergo positive selection where they sample more soluble self-antigens to check for the generation of a functional BCR. Also, the strength of the self-antigen-induced BCR signaling regulates mature lineage commitment into either follicular B cells or marginal zone B cells. Weak BCR signaling favors the development of marginal zone B cells, relatively strong signaling favors the development of follicular B cells, and even stronger signaling can induce apoptotic death or anergy (Pillai and Cariappa, 2009; Pillai et al., 2004; Su and Rawlings, 2002). A summary of the developmental stages of B cells in the spleen is shown in Figure 1-2.

When naïve (has not encountered cognate antigen) FO B cells bind antigens, they traffic to the border between the follicle and the T cell zone, called the B-T boundary (Okada and Cyster, 2006); otherwise they exit the spleen and travel to other secondary lymphoid organs to sample antigens. B cells internalize and degrade antigens, which are then presented on MHC II

proteins as short peptides for the follicular helper T (T_{FH}) cells (Shekhar and Yang, 2012). At the B-T boundary, they receive help from the T_{FH} cells, which express the costimulatory molecules in response to MHC II-peptide recognition, CD40L, ICOS, and OX40, that bind to B cell CD40, ICOS-L, OX40L, respectively. T_{FH} cells also secrete cytokines IL-4, IL-5, IL-6, IL-21, and interferon- γ (IFNγ). These co-stimulatory molecules and cytokines are required for Ig class switching, germinal center formation, plasma cell differentiation and memory B cell formation (Cannons et al., 2006; Fazilleau et al., 2009). Activated B cells may continue to migrate and form an extrafollicular focus where they differentiate into short-lived plasma cells or re-enter follicles to form germinal centers where they differentiate into antibody-secreting plasma cells or memory cells (Oracki et al., 2010). A more detailed discussion on antigen encounter will be presented in Section 1.3.

The antibodies secreted by plasma cells have the same antigen specificity as their naïve B cell BCR counterpart and the isotype of the heavy chain is usually switched to another class for specific effector functions. This is done through class switch recombination (CSR). Also, the affinity is much greater, due to a process called affinity maturation, resulting in a preferential outgrowth of cells expressing an immunoglobulin with high affinity. This is done through somatic hypermutation, which introduces point mutations in the immunoglobulin heavy chain, creating a variety of affinity for a particular antigen. Insertions and deletions have been observed as well (Odegard and Schatz, 2006).

Somatic hypermutation occurs at a rate of approximately 10^{-3} mutations per base pair per cell division, which is a million fold higher than the rate of spontaneous mutations. Mutations

start approximately 100-200 base pairs downstream of the V_H promoter, continues within the V_HDJ_H, and ends 1.5-2kb downstream of the promoter. Transitions (C to T; G to A) occur twice as frequently as transversions (C to A/G; G to C/T). The enzyme, activation-induced cytidine deaminase (AID), deaminates the deoxycytidines (dC) in single stranded DNA mainly in WRC hotspots (W=A/T; R=A/G), which changes the dC to a deoxyuridine (dU). If the U-G mismatch is not repaired before DNA replication, DNA polymerases will insert an adenosine opposite to uracil, giving rise to C>T and G>A transition mutations. If the uracil is removed by uracil-DNA glycosylase (URG), creating an abasic site, base excision repair can occur involving error-prone polymerases, which can fill in the gap by inserting any nucleotide in place of the uracil. This will give rise to both transition and transversion mutations. Lastly, the patch-repair process can also occur at the U-G mismatch, where the mismatch repair (MMR) heterodimer enables gap formation and the error-prone polymerase can generate mutations outside the initial mismatch (Odegard and Schatz, 2006; Peled et al., 2008; Teng and Papavasiliou, 2007).

Naïve mature B cells express heavy chain Cμ and/or Cδ, and activation of these cells can lead to CSR, where the C region on the heavy chain is switched to Cγ3, Cγ1, Cγ2b, Cγ2a, Cε or Cα, changing the effector function of the antibody. There is a transcriptional promoter, followed by a non-coding exon (I-exon), and a switch (S) sequence, consisting of GC-rich tandem repeats (20-80 base pairs), located upstream of each C region, except Cδ. AID-induced deamination of dC to dU also occurs in CSR and because S regions contain many WRC hotspots, URG and the MMR complex produce double strand breaks in transcriptionally active S regions. It is thought that the unique transcriptional properties of Sμ (ex. proximity to the Eμ enhancer) renders it a preferred AID substrate; therefore CSR is rarely observed between two downstream S regions

(Chaudhuri and Alt, 2004). This results in a change from IgM or IgD expression to the expression of either IgA, IgE, IgG1, IgG2a, IgG2b, or IgG3 isotypes. IgD expression occurs by alternative splicing of the Cμ-Cδ genes (Stavnezer et al., 2008).

Upon exposure to antigens, B cells increase in size and start proliferating. Some will secrete low amounts of antibody, which are then referred to as plasmablasts and have decreased expression of CD45R, FLt3, and MHC II. Plasmablasts no longer express the chemokine receptors CXC chemokine receptor 5 (CXCR5) and CC chemokine receptor 7 (CCR7), which control trafficking in the germinal center. However, they still express CXCR4, which is the receptor for the chemokine CXCL12. This is thought to allow them to leave and migrate to the $CXCL12^{high}$ survival niches of the bone marrow, a crucial step for the generation of long-lived plasma cells. A small proportion of long-lived plasma cells are in the spleen of both human and mice, and under chronic inflammation, plasma cells can be found in inflamed tissues. If plasmablasts are generated in the presence of IFNγ, they will also express CXCR3. CXCR3 ligands are secreted by cells in inflamed tissues, such that plasmablasts are recruited to inflamed tissues. Also, during a mucosal immune response, the expression of CCR9 and CCR10, can be induced in plasmablasts. Their ligands, CCL25 and CCL28, respectively, are produced mainly in mucosal tissue, allowing plasmablasts to migrate to mucosal sites. The development into plasma cells depends on the activation of the PRDM1 (positive-regulatory-domain containing 1) gene, which encodes the transcription factor, BLIMP1 (B-lymphocyte-induced maturation protein 1), termed the master regulator of plasma cell development. Plasmablast differentiation into plasma cells also depends on the presence of IL-21, suppression of PAX5 (paired box protein 5), and the expression of XBP1 (X-box-binding protein 1). Plasma cells are non-

migratory, do not express surface Ig, CD45R, nor MHC II, and their main role is to continuously secrete antibodies. The long-term survival of plasma cells in the bone marrow is dependent on the expression of CXCL12, tumor necrosis factors (TNFs), IL-5, IL-6 and ligands for CD44 in the survival niches. However, all the signals that allow long-term plasma survival in the bone marrow are not completely understood as there is no known cocktail to maintain plasma cells *in vitro* (Radbruch et al., 2006; Tarlinton et al., 2008).

1.2.3 Mature B cell subsets

The $sIgM^{low}CD93^{-}CD21^{+}sIgD^{high}$ FO B cells, which reside in splenic B cell follicles, make up around 70% of total splenic B cells and can migrate to other secondary lymphoid organs, such as the follicles of lymph nodes, or to the vascular sinusoids of the bone marrow (Allman and Pillai, 2008; Cariappa et al., 2005). Their main function is to recognize antigen and mount a T cell-dependent immune response, leading to activation, proliferation and differentiation of the B cells into plasma cells or memory B cells.

$sIgM^{high}sIgD^{low}CD23^{-}CD21^{high}CD1d^{high}$ MZ B cells do not recirculate throughout the body but remain localized in the marginal zone of the spleen, between the red and white pulps. Most of the blood that enters the spleen exit the circulation through the follicular arteriole into the marginal sinus, flow through the marginal zone, and finally into the venous sinuses before returning to the circulation; therefore, the marginal zone is where major antigen filtering and scavenging occurs. MZ B cells respond, through T cell-independent immune responses, to blood-borne pathogens that are released into the marginal sinus from the follicular arteriole (Pillai et al., 2005). These cells are innate-like due to their limited but polyreactive IgM antigen

receptor repertoire, which recognizes microbial polysaccharides and self-antigens (Viau and Zouali, 2005). They also have a lower activation threshold than FO B cells, allowing rapid responses to pathogen, and a high tendency to differentiate into short-lived plasma cells (Lopes-Carvalho et al., 2005).

In addition to FO B cells and MZ B cells, which constitute the B2 cell population, other types of B cells exist, including the B1 and Breg subsets. $CD19^{high}CD23^{-}CD43^{+}IgM^{high}$ $IgD^{(variable)}CD5^{\pm}$ B1 cells make up 5% of the total B cell pool and are found in the pleural and peritoneal cavities, and intestines. In contrast to the continuous generation of B2 cells, B1 cells undergo self-renewal, where there is limited proliferation to replace dying cells. They are the main producers of natural antibodies, help maintain tissue homeostasis, and participate in immune defense against mucosal pathogens. Like MZ B cells, B1 cells express a highly polyspecific antigen receptor repertoire that binds both self-antigens and microbial antigens. B1 cells expressing CD5 are called B1a cells and those lacking CD5 are called B1b. B1a cells are generated before birth and during the first few weeks after birth in the fetal liver, while B1b cells are generated from the fetal liver and from precursors in the adult bone marrow. During infection at mucosal surfaces, B1a cells can redistribute to regional lymph nodes, differentiate into IgM-secreting plasma cells without clonal expansion. Systemic infections cause both B1a and B1b cells migrate to the spleen of the intestinal lamina propria, where they differentiate into IgM- or IgA-secreting cells. Lastly, during repeated antigen stimulation, B1b cells undergo clonal expansion and differentiate into memory-like B cells (Baumgarth, 2011).

Regulatory B cells have been identified in blood, bone marrow, lymph node, spleen and the peritoneum of adult mice. Regulatory B cells are important in suppressing autoimmunity and inflammation. Recently, three types of regulatory B cells have been discovered and seem to be a mirror image of the three types of regulatory T cells: Type I IL-10-producing regulatory T cells (Tr1), Type III TGF-β-producing helper T cells (Th3), and $Foxp3^+$ regulatory T cells (Treg). Similarly, the IL-10 producing B cells are termed "Br1" or "B10" cells, the TGF-β-producing regulatory B cells are termed "Br3" cells and the $Foxp3^+$ regulatory B cells are termed "Breg" cells (Noh and Lee, 2011). However, not much is known about these B cell subsets.

1.3 B cell antigen encounter *in vivo*

B cells encounter antigen mostly in secondary lymphoid organs, including lymph nodes, which collects antigen from lymph fluids coming from the afferent lymph vessel, and the spleen which receives antigen from the blood through the follicular arterioles (spleens lack afferent lymph vessels). Migrating B cells enter the lymph nodes through high endothelial venules (HEVs), which are specialized postcapillary venules found in lymphoid tissues that support lymphocyte-only extravasation into lymphoid tissues due to the expression of specific chemokines and adhesion molecules (Girard and Springer, 1995; Lopez-Giral et al., 2004; Okada et al., 2002). HEV-like vessels are also found at chronic inflammatory sites where they support lymphocyte recruitment (Kobayashi et al., 2009). Naïve B cells first encounter antigen via antigen presenting cells (APCs) near the HEVs. B cells with BCRs that recognize the antigens can then travel to the T cell zone near the follicle for activation, whereas naïve B cells continue to the B cell follicle, which are microenvironments containing dense populations of naïve B cells, and can encounter antigen within follicles. B cells that do not recognize any antigen will exit the

lymphoid tissue and migrate to another secondary lymphoid organ (Cyster, 2010) (Fig. 1-3). Activated B cells also enter follicles and develop germinal centers within the follicles. Germinal centers are sites of rapid B cells proliferation and differentiation into plasma cells (Victora and Nussenzweig, 2012).

Unlike T cells that recognize processed or partially degraded proteins presented on MHC molecules, B cells recognize intact antigens. The mechanism by which antigens reach B cells is dependent on the characteristics of the antigen, especially its size. Antigens may occur in one of the following three forms: small soluble antigens, particulate or opsonized antigens, and large, cell-borne antigens (Cyster, 2010).

Antigens that enter the tissues (e.g. through a wound) follow the flow of interstitial fluid, which drains into the surrounding lymph vessels. Through the afferent lymphatic vessel, lymph fluid drains into the lymph node subcapsular sinus (SCS), which is located between the capsule and cortex (Fig. 1-3). The lymph node contains a conduit system, which is a network of fibroblastic reticular cells that ensheath collagen fibers. This conduit network filters large antigens and allows for the direct entry of small soluble antigen (<70 kDa) into the B cell follicles (Batista and Harwood, 2009; Roozendaal et al., 2008).

In contrast, larger antigens, such as immune complexes and particles, are excluded from the conduit network and must be transported to the B cell follicle in other ways. In the SCS, as antigen-containing lymph fluid passes through, $CD169^+$ SCS macrophages can pick up particulate antigens, which can be shuttled across the membrane, or transcytosed, such that it is

displayed on membrane processes that extend into the cortex (Fig. 1-3). This is ideal for capturing particulate material and making it accessible to B cells that come in contact with SCS macrophage processes (Cyster, 2010; Harwood and Batista, 2010a). Although classical macrophages are specialized phagocytes, SCS macrophages have low rates of antigen internalization and degradation and can therefore present intact antigen (Phan et al., 2009). B cells transiently accumulate near the SCS, and decrease their velocity once they encounter antigens displayed by SCS macrophages. Lymph fluid contains many self-proteins and dietary molecules (especially lymph traveling from the intestine) including carbohydrates and lipids, which are thought pass freely through the lymph node. Therefore, specific receptors on the SCS macrophages are required for the capture of foreign particles (Cyster, 2010).

Antigens that are too large to enter passively into lymphatic vessels are carried to the lymph node by dendritic cells (DCs), which are localized near the HEVs (Fig. 1-3). DCs are specialized cell types that internalize antigens at peripheral sites, travel to lymph nodes and present processed antigens to T cells. However, DCs have lower amounts of lysosomal proteases than classical macrophages, degrade internalized antigens more slowly and can also present intact antigens to B cells (Delamarre et al., 2005).

The most prominent cell that captures and displays antigens in lymph nodes are the FDCs, which are found in the B cell follicles (Fig. 1-3). They mainly capture opsonized antigens as they express complement receptors 1 and 2, and the Fc receptor, FcγRIIb. However, because FDCs are non-migratory cells that are resident in the follicles, antigens must be brought to them. FDCs can display antigens on their surface for long periods of time and FDC-displayed antigens

are protected from proteolysis since follicular stromal cells secrete the protease inhibitor, serpin-a1 (α1-antitrypsin) (Cyster, 2010). In the spleen, MZ B cells can transfer opsonized antigens that they acquire from the blood to FDCs. In lymph nodes, which lack marginal zones, B cells specific for any antigen can capture immune complexes using complement receptors, migrate into the follicle and transfer these immune complexes to FDCs (Cinamon et al., 2008).

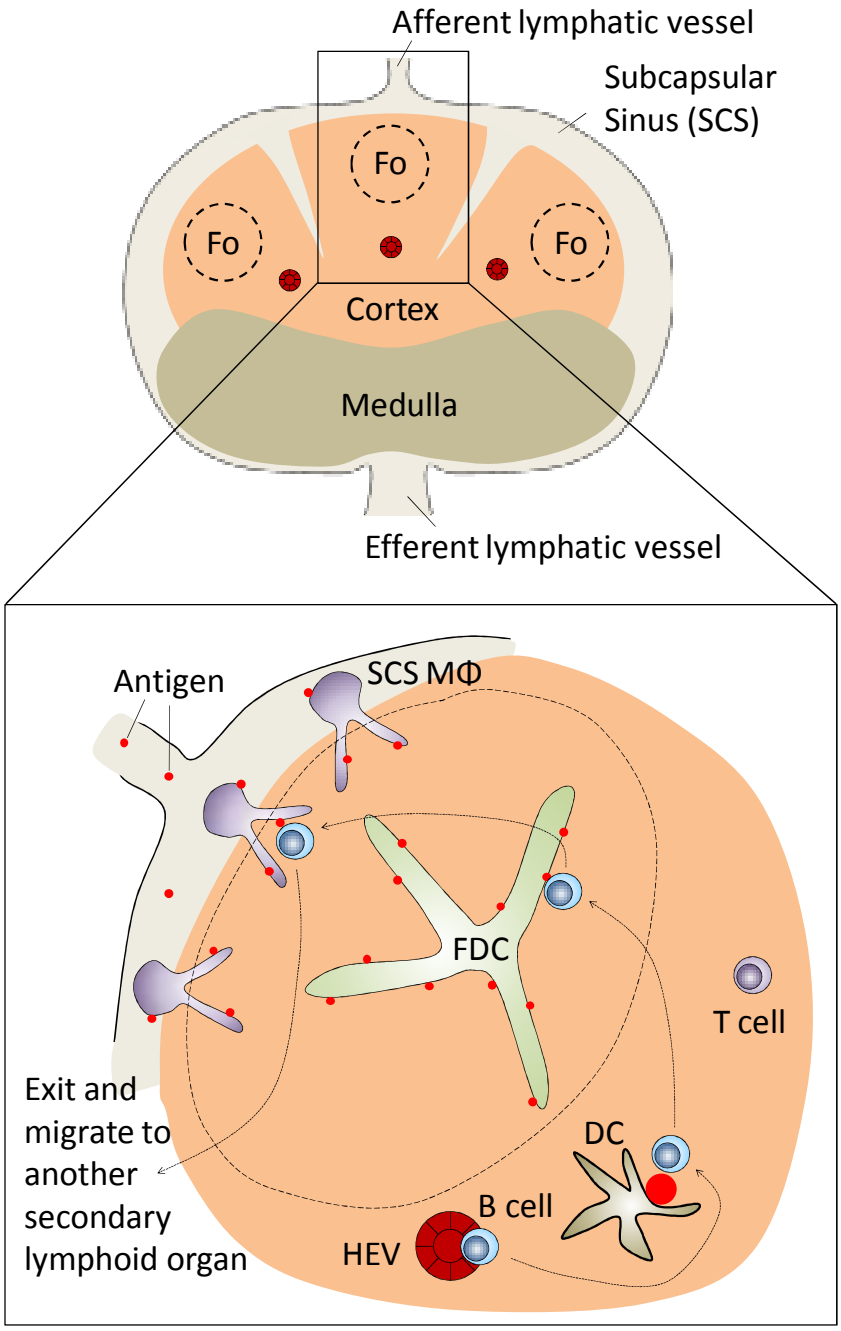


Figure 1-3. Antigen encounter in lymph nodes

A schematic diagram of B cell antigen encounter within a lymph node. B cells enter the lymphoid tissue through HEVs, and they first encounter large cell-borne antigen on presented DCs. If B cells don't recognize the antigen, they move into follicles where they encounter either soluble antigens, or antigens presented on SCS macrophages (MΦ) and FDCs. B cells that don't recognize anything will exit the current lymphoid tissue and migrate to a different secondary lymphoid organ.

The BCR can recognize and initiate signaling in response to both soluble and membrane-bound antigens. However, *in vivo*, the majority of B cell activation is thought to be initiated by membrane-bound antigens, including antigens that have been captured by APCs via Fc receptors, complement receptors, or other cell surface molecules such as lectins that bind carbohydrates on viral glycoproteins (Carrasco and Batista, 2006a; Carrasco and Batista, 2006b; Harwood and Batista, 2010b). This is because interactions between integrins such as very late antigen-4 (VLA-4; $\alpha_4\beta_1$ integrin) and leukocyte function-associated antigen-1 (LFA-1; $\alpha_L\beta_2$ integrin) on the B cell with their ligands on APCs, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), respectively, promote adhesion and facilitate antigen recognition by lowering the threshold for B cell activation (Carrasco and Batista, 2006a). In addition, the B cell surface protein CD19, which acts as a co-receptor for the BCR and serves as a scaffolding protein for signaling enzymes, is essential for optimal B cell responses to membrane-bound antigens but not soluble antigens (Depoil et al., 2008). Therefore, it seems favorable for B cells to be activated by membrane-bound antigens.

1.4 BCR signaling

1.4.1 Igα/β signaling in pro-B cells and pre-BCR signaling

Signaling by the BCR is important for B cell development, for the survival of mature B cells and B cell lymphomas, and for mounting a humoral immune response. During early B cell development, Igα and Igβ are first expressed in pro-B cells (Keren and Melamed, 2005). The ITAMs in the Igα/β cytoplasmic domains are critical for further B cell development, as B cell development in mice with mutations in the cytoplasmic domains of Igα or Igβ is blocked at the pro-B cell stage. When the μ heavy chain is synthesized, it pairs with the surrogate light chains,

VpreB and λ5, and associates with the Igα/β subunit to form a complete pre-BCR. Signaling by the pre-BCR promotes the transition from the pro-B cell stage to the pre-B cell stage (Keren and Melamed, 2005). Once the pre-BCR is assembled on the cell surface, antigen-independent tyrosine kinase-dependent signaling begins. Signals from the pre-BCR suppress further V_HDJ_H recombination, thereby establishing allelic exclusion at the Ig heavy chain locus, and induce proliferation (Geier and Schlissel, 2006).

Whether there is a ligand for the pre-BCR is still under debate. Although a soluble recombinant form of the pre-BCR can bind both galectin-1 (Gauthier et al., 2002) and heparin sulfate (Bradl et al., 2003) on stromal cells, isolated pre-B cells can proliferate in the absence of stromal cells or other potential exogenous ligands for the pre-BCR. Moreover, when the extracellular domains of the pre-BCR are truncated, this leads to spontaneous Igα/β aggregation and signaling. This suggests that the pre-BCR signals in a ligand-independent manner (Geier and Schlissel, 2006).

The signaling pathway of the pre-BCR is not completely characterized. However, the Syk family kinases, Syk and Zap70, are important for pre-BCR-dependent development. Normally, B cell development in Rag-deficient mice is arrested at the pro-B cell stage and anti-Igβ antibodies can induce differentiation into pre-B cells. However, $Syk^{-/-}Zap70^{-/-}$ pro-B cells, although they have normal synthesis of the pre-BCR components, fail to respond to anti-Igβ antibodies, and fail to undergo allelic exclusion, showing that pre-BCR signaling is dependent on Syk and Zap70 (Schweighoffer et al., 2003).

1.4.2 BCR signaling in immature B cells

When the Ig light chain gene is rearranged and Ig light chains are synthesized, it can pair with newly synthesized μ chains and displace the pre-BCR. This forms a mature BCR with the μ heavy chain and the Igα/β chains. This change marks the transition of the pre-B cell into an immature B cell. As mentioned in Section 1.2.1, *in vivo* BCR crosslinking on immature B cells leads to negative selection through anergy, receptor editing or deletion in order to limit the generation of auto-reactive B cells. *In vitro*, evidence indicate that BCR crosslinking on immature B cells leads to maturation arrest, followed by cell death (Koncz et al., 2002). This maturation arrest period is thought to give time for immature B cells to undergo receptor editing. Because the bone marrow is not present to provide proper signals *in vitro*, receptor editing will not occur and the immature B cells die. Within 18 hours following BCR crosslinking, most are irreversibly committed to maturation arrest, which is nearly complete after 24 hours. These experiments were done with the WEHI-231 mouse immature B cell line (Jakway et al., 1986). Prior to this irreversible commitment, treating immature B cells with lipopolysaccharide (LPS) (Jakway et al., 1986) or CD40 ligand (Santos-Argumendo et al., 1994; Tsubata et al., 1993) can prevent anti-IgM-induced cell death. It is thought that *in vivo,* since the bone marrow, where immature B cells reside, is a sterile environment, any microbial component that reaches the bone marrow must mean that the immune system was incapable of clearing such powerful infections (Azulay-Debby et al., 2007). Immature B cells that recognize microbial components, such as LPS, will develop into mature B cells, regardless of whether their BCRs recognize self or foreign antigens, so that an immune response can be mounted against the pathogen. This polyclonal activation caused by infections could therefore lead to the production of self-reactive antibodies (Azulay-Debby et al., 2007).

Immature B cells are very sensitive to low concentrations of antigen, which can induce tolerance to self. In contrast, mature B cells require a higher antigen concentration for activation (Gross et al., 2009). Although BCR engagement leads to anergy or apoptosis in immature B cells but activation in mature B cells, the early BCR signaling events are similar in both cell types. This is discussed in Section 1.4.3 and 1.4.4.

1.4.3 BCR signaling in mature B cells

During an immune response, mature naïve B cells in lymphoid organs come in contact with antigens that are soluble in lymph fluid or on the surface of APCs, resulting in the activation of BCR signaling pathways and the activation of B cells (Allen et al., 2007a). Antigen binding induces the clustering of BCRs, which is a critical for the initiation of BCR signaling. *In vitro*, only multivalent soluble antigens containing more than one copy of the same epitope can activate B cells through the BCR; monovalent antigens in solution cannot. It was thought that the physical crosslinking of the BCRs by multivalent antigens is needed for the initiation of signaling (Fleire et al., 2006; Sohn et al., 2008; Weber et al., 2008). However, the requirement for crosslinking does not explain how B cells respond to monovalent antigens *in vivo*, such as toxins. Recent studies have shown that monovalent membrane-bound antigens can also induce BCR signaling via conformational changes in the BCR, exposing a clustering interface in the ectodomain of the membrane Ig (Tolar et al., 2009). The ability of soluble monovalent antigens to stimulate the BCR is still unknown.

APC-mediated B cell activation causes BCR-induced reorganization of the cytoskeleton, allowing B cells to spread across APCs. This enhances antigen scanning and gathering such that

a level of BCR signaling required for B cell activation can be achieved (Harwood and Batista, 2010b). This spreading process is followed by a contraction phase, which ultimately results in reorganization of membrane-associated proteins, including the BCR and integrins, into an immunological synapse (Fig. 1-4). This structure is characterized by a central aggregation of antigen-bound BCRs, termed the central supramolecular activation cluster (c-SMAC), surrounded by a ring of adhesion molecules, including integrins, called the peripheral SMAC (p-SMAC) (Kurosaki et al., 2010).

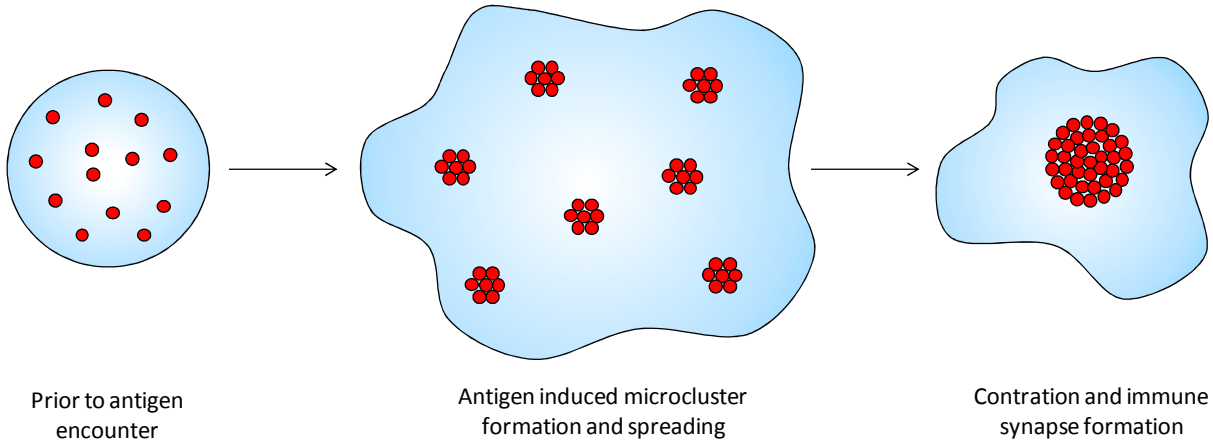


Figure 1-4. Antigen-induced spreading and contraction
A schematic representation of the early events of antigen binding. B cells extend their membranes across APCs to scan for more antigens. Antigen binding induces the formation of BCR microclusters, which are sites of active BCR signaling. B cell membranes then contract and concentrate the BCR in a central aggregate, forming an part of the immune synapse.

The binding of antigen leads to the formation of BCR microclusters, containing 50 -500 BCR molecules. BCR microcluster formation requires cytoskeletal rearrangements, as actin polymerization inhibitors prevent microcluster formation (Harwood and Batista, 2010b). However, another study has shown that BCRs signaling leads to the disassembly of actin

filaments at the membrane, allowing BCRs to freely flow in these regions to form microclusters (Treanor et al., 2010). This apparent paradox was resolved in a recent article (Freeman et al., 2011), where they show that actin depolymerization is a pre-requisite for the actin reorganization required for BCR microcluster formation.

After binding multivalent antigens, the BCR aggregates in cholesterol- and sphingolipid-rich plasma membrane microdomains termed lipid rafts. The tyrosine residues (Tyrs) within the ITAMs in the cytoplasmic domains of Igα and Igβ are then phosphorylated by the Src family kinases Lyn, Fyn, Blk, and Lck, some of which are enriched in these lipid rafts (Cheng et al., 2001; Fleire et al., 2006; Kurosaki, 2002). The phosphorylated ITAMs act as recruitment centers for SH2 domain-containing signaling proteins such as Syk and additional Src family kinases (Campbell et al., 1991; Gold et al., 1990; Jiang et al., 1998; Pawson and Nash, 2000). Once Src family kinases bind to these phosphorylated tyrosine residues, they undergo conformational changes that allow autophosphorylation on positive regulatory sites (Kurosaki, 2002; Niiro and Clark, 2002). Recruitment and activation of tyrosine kinases result in the activation of multiple downstream signaling pathways including the phospholipase Cγ (PLCγ) pathway, the Ras/mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (Gold et al., 2000). A summary of BCR signaling is shown in Figure 1-5.

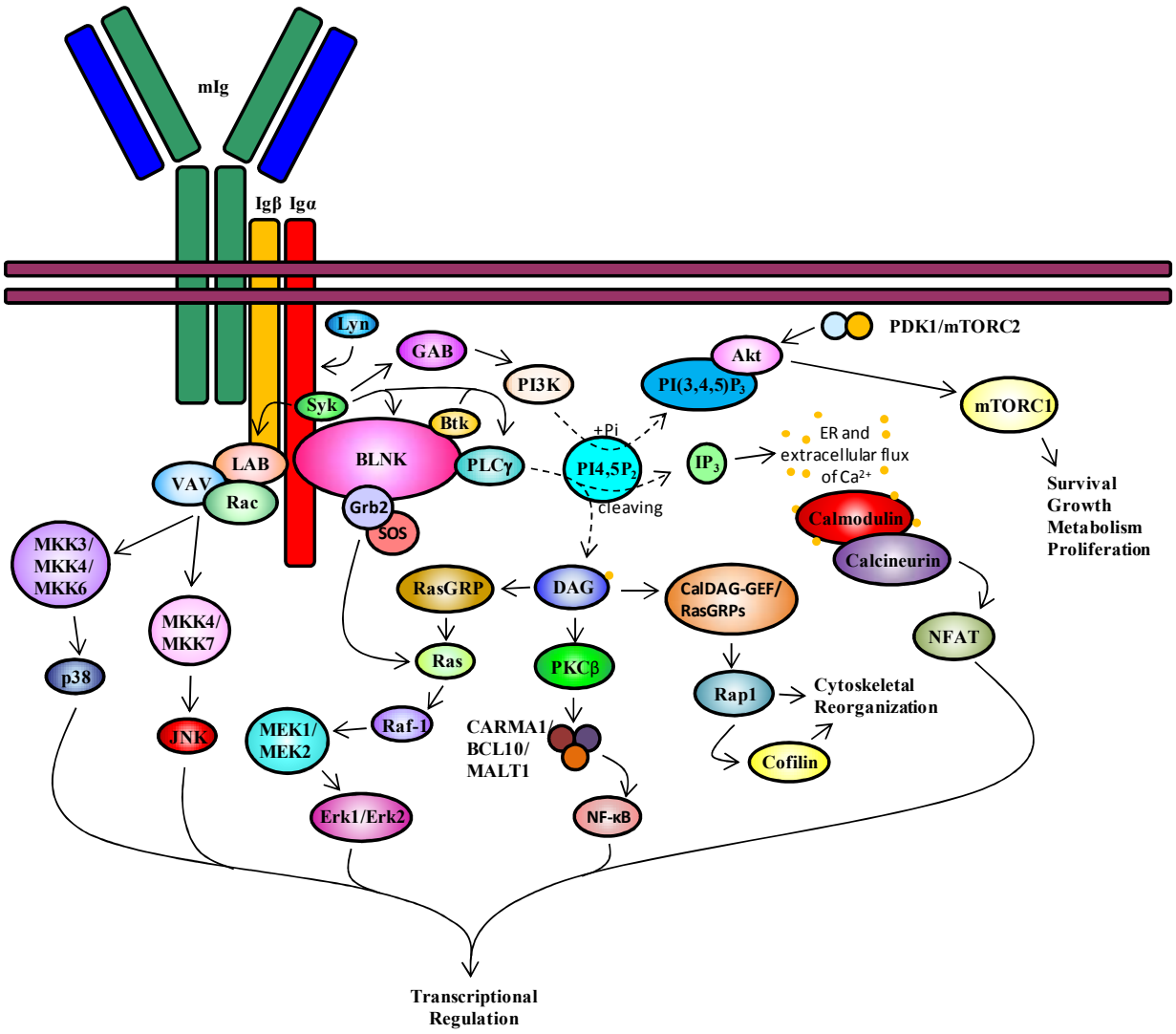


Figure 1-5. BCR signaling pathways

Schematic representation of the signaling pathways that are activated upon BCR crosslinking. The major pathways are shown: the PLCγ pathway, the Ras/MAPK pathway, and the PI3K pathway. Solid arrows indicate direct or indirect activation. The functions of dashed arrows are indicated in the figure.

In addition to the ITAM Tyrs, Igα also has two flanking non-ITAM Tyrs at positions 176 and 204, which are also phosphorylated by Src family kinases (Fu et al., 1998; Kabak et al., 2002). Phosphorylated Tyr 204 acts a recruitment site for the adaptor protein, B cell linker

(BLNK), also called SLP-65, which is then phosphorylated by Syk (Fu et al., 1998; Kabak et al., 2002). BLNK has an SH2 domain and at least five sites for tyrosine phosphorylation (Dal Porto et al., 2004). Phosphorylated Tyr 176 has also been shown to be important for BLNK-dependent signaling as BLNK is not phosphorylated when either of these Igα non-ITAM Tyrs are mutated to phenylalanine (Kabak et al., 2002; Patterson et al., 2006; Siemasko et al., 2002). The additional non-ITAM Tyr phosphorylation sites in Igα may promote the activation of different signaling pathways than Igβ, which has only ITAM Tyrs. (Clark et al., 1992; Kim et al., 1993; Pao et al., 1998; Sanchez et al., 1993; Williams et al., 1994).

1.4.3.1 PLCγ-dependent signaling

After BLNK is phosphorylated by Syk, it recruits PLCγ and Bruton's Tyr kinase (Btk) through their SH2 domains (Kurosaki, 2002). Btk, together with Syk, phosphorylates and activates PLCγ, which then binds the membrane phospholipid phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) via its Pleckstrin Homology (PH) domain that binds phosphatidylinositols with high affinity (Dal Porto et al., 2004). PLCγ then cleaves $PI(4,5)P_2$ into the second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Kurosaki, 2002).

IP_3 binds to IP_3 receptors in the ER membrane to trigger the release of intracellular calcium (Ca^{2+}) from ER Ca^{2+} stores into the cytosol. The loss of luminal ER Ca^{2+} triggers a sustained influx of extracellular Ca^{2+} across the plasma membrane. The proposed mechanism is that the decrease in Ca^{2+} within the ER results in the dissociation of Ca^{2+} from the EF-hand motif of the stromal interaction molecule 1 (STIM1), an ER membrane protein, which then clusters

and relocates in the ER in close proximity to the plasma membrane. Here, it interacts with Ca^{2+} release-activated Ca^{2+} channel modulator 1 (CRACM1, also called ORAI1) in the plasma membrane, which then induces Ca^{2+} influx (Kurosaki et al., 2010). The ER and extracellular Ca^{2+} flux combine to dramatically but transiently increase intracellular Ca^{2+} concentration.

Calmodulin binds cytosolic Ca^{2+} with its four EF hands (Grabarek, 2005) and activates several molecules, including the serine (Ser)/threonine (Thr) phosphatase, calcineurin. In resting lymphocytes, the transcriptional activator, nuclear factor of activated T-cells (NFAT), is located in the cytoplasm as a hyperphosphorylated, inactive form. Calcineurin dephosphorylates multiple phosphoserines on NFAT, unmasking its nuclear localization sequence, allowing its nuclear translocation and activation (Hogan et al., 2003; Medyouf and Ghysdael, 2008). In T cells, nuclear NFAT promotes the transcription of genes encoding multiple cytokines including IL-2, IL-3, IL-4, IL-5, GM-CSF, IFNγ, TNFα, as well as the cell surface proteins CD40L, CTLA-4 and FasL (Masuda et al., 1998).

DAG, on the other hand, along with Ca^{2+} activates protein kinase Cβ (PKCβ). Activated PKCβ phosphorylates caspase-associated recruitment domain (CARD)-containing membrane-associated guanylate kinase (MAGUK) 1 (CARMA1) on Ser668. This results in the binding of the adaptor protein, B cell lymphoma 10 (BCL10), which subsequently recruits mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) that binds to BCL10 via its Ig-like domain and to CARMA1 via its caspase-like domain (Bertoni and Zucca, 2006; Kurosaki et al., 2010). MALT1 interacts with TNF receptor (TNFR)-associated factor 6 (TRAF6), which activates auto-K63-linked ubiquitination, and trans-K63-linked ubiquitination

of MALT1 and BCL10. This recruits the inhibitor of kappa B (IκB) kinase (IKK) via the ubiquitin-binding IKKγ subunit (Thome et al., 2010). The IKKβ subunit phosphorylates CARMA1 on S578, which stabilizes its association with BCL10/MALT1 (Kurosaki et al., 2010). IKK also phosphorylates IκB, marking it for ubiquitination and subsequent proteasomal degradation. In resting cells, IκB proteins bind to NF-κB (nuclear factor kappa light chain enhancer of activated B cells) dimers to sequester them in the cytoplasm as inactive complexes (Dal Porto et al., 2004). The dissociation of IκB allows NF-κB to translocate into the nucleus where it targets genes that are important for B cell development, survival, proliferation, and immunoglobulin class switching (Dal Porto et al., 2004).

DAG and Ca^{2+} contribute to the activation of CalDAG-GEFs (Ca^{2+} and DAG guanine exchange factors) and RasGRPs (Ras guanyl nucleotide-releasing proteins), which are a family of GEFs for the Ras family GTPases, including Ras and Rap1 (Stone, 2011). This GEF family contains a Ras exchange motif, a DAG-binding C1 domain, 2 EF hands that bind Ca^{2+}, and a GEF domain. GTPases can switch signal transduction chains on and off by cycling between an active GTP-bound form and an inactive GDP-bound form (Vigil et al., 2010). This is done by the hydrolysis of GTP by the intrinsic GTPase activity, the activity of which is accelerated by GTPase-activating proteins (GAPs). GEFs activate GTPases by promoting the dissociation of GDP, allowing the more abundant GTP to bind to the GTPase and convert it to the active conformation (Vigil et al., 2010).

Rap1 regulates actin polymerization, cell polarity and integrin activation, all of which are important for cell adhesion, migration, and spreading (Bos, 2005). In addition, actin-dependent

spreading of B cells on surfaces coated with antibodies specific for the BCR is inhibited by blocking the activation of Rap1 by overexpressing Rap1-specific GAPs (Lin et al., 2007). Rap1 activation is also important for the activation of Cofilin, which severs actin filaments, a process that is critical for actin reorganization (Section 1.5.4).

1.4.3.2 Ras/Erk pathway and other MAPK pathways

The GEF RasGRP3, when bound to DAG and Ca^{2+}, is phosphorylated on Thr133 by the DAG-activated PKCβ. This allows the Ras GTPase to become activated and bind directly to the Ser/Thr-specific mitogen-activated protein kinase kinase kinase (MAPKKK), Raf-1, which activates its protein kinase activity. Raf-1 phosphorylates the Tyr/Thr-specific MAPKKs, MEK1 and MEK2, which in turn phosphorylate the Ser/Thr-specific MAPK, Erk1 and Erk2 (Gardner et al., 1994; Kurosaki et al., 2010; Roberts and Der, 2007). Erk can also be activated through a PLCγ-independent pathway in which the adaptor proteins BLNK or Shc (Src homology 2 domain containing transforming protein) bind to the phosphorylated Igα/β ITAMs and recruit Grb2 (growth factor receptor binding protein 2) and the Ras GEF SOS (son of sevenless). This results in the activation of Ras and the initiation of the Ras/Erk pathway (Roose et al., 2007). Erk has multiple substrates that regulate many cellular processes. Erk can form dimers that are transported into the nucleus, where they phosphorylate transcriptional factors including Fos, Jun, and Ets family members, which are involved in B cell development and the proliferation of mature B cells (Dal Porto et al., 2004; Gold, 2002).

The other two major MAPKs, JNK and p38, are activated through a different pathway. During BCR signaling, another adapter protein, linker of activated B cells (LAB), is recruited to

the BCR and is phosphorylated by Syk (Ahn et al., 2006). The GEF, Vav, then binds to phosphorylated LAB, which promotes its tyrosine phosphorylation (Malhotra et al., 2009). Rac GTPases are activated by phosphorylated Vav, and Rac subsequently activates MAPKKs that are upstream of JNK and p38. JNK is dually phosphorylated by the MAPKKs MKK4 and MKK7, whereas p38 can be phosphorylated by MKK3, MKK4 and MKK6 (Ip and Davis, 1998; Zarubin and Han, 2005). Both JNK and p38 have many substrates, including protein kinases, transcription factors, and are involved in B cell development and survival (Cook et al., 2007; Ip and Davis, 1998).

1.4.3.3 PI3K/Akt pathway

Syk also phosphorylates the adaptor proteins, Gab1, Gab2, and B cell adaptor for PI3K (BCAP) (Maus et al., 2009; Okada et al., 2000), which then recruit the p85 regulatory unit of PI3K to the plasma membrane (Dal Porto et al., 2004). PI3K can phosphorylate $PI(4,5)P_2$ to generate phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$). The serine/threonine kinase Akt possesses a PH domain, which binds PIP_3 with high affinity, and brings itself to the plasma membrane. Once at the membrane, Akt is phosphorylated by phosphoinositide dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) on residues Thr308 and Ser473, respectively. Activated Akt has many downstream effectors that are involved in B cell survival, growth, metabolism, proliferation (Limon and Fruman, 2012; Maira et al., 2009).

The mTOR kinases is composed of two multi-protein complexes, mTORC1, which has the raptor subunit, and mTORC2, which has the rictor subunit. The main function of mTORC1 is to sense nutrients and mitogenic signals, whereas mTORC2 responds to growth factors. Akt,

which is activated by mTORC2, can promote mTORC1 activation. When conditions are favorable, mTORC1 triggers biosynthetic pathways that are essential for the uptake of nutrients, amino acid, protein and lipid biosynthesis, cell cycle progression, proliferation and survival. mTORC2 is important for B cell cycle progression, proliferation, size, and survival (Argyriou et al., 2012; Fruman and Limon, 2012).

1.4.4 Differences in BCR signaling between immature and mature B cells

Although BCR signaling pathways are similar in immature and mature B cells, the outcomes of BCR engagement are different. Stimulation through the BCR in immature B cells results in receptor editing, anergy, or cell death, whereas in mature B cells, it results in activation and proliferation. The signaling pathways responsible for this differential response, i.e. death versus survival, are not fully characterized. It is possible that this is due to differences in the expression levels of the same signaling effectors. The levels of proximal signaling molecules, BLNK, PLCγ2, and Btk, are higher in immature B cells than in mature B cells whereas Syk expression is decreased (Benschop et al., 2001). Less Ca^{2+} is released from intracellular stores in immature B cells than in mature B cells but immature B cells exhibit greater maximal Ca^{2+} influx compared to mature B cells (Benschop et al., 2001). Also, gene chip analysis showed that the expression of downstream signaling molecules (NF-κB, JAKs [Janus Kinase], MAPK), anti-apoptotic molecules (Bcl-2, A1), and inhibitory co-receptors (CD22, FCγRIIB) is higher in mature B cells than in immature B cells (Hoffmann et al., 2002).

Differences in signaling can also be due to differences in the duration of the activation or expression of specific signaling proteins. Erk phosphorylates the proapoptotic protein, Bim,

marking it for ubiquitination and subsequent degradation via the proteasome. In proliferating WEHI-231 murine immature B lymphoma cells, there is a cyclic pattern of Erk activation. BCR crosslinking in these cells induces transient activation of ERK, which falls below basal levels within 16 hours, and no longer cycles. This may suggest that the cycling of Erk activation is required for the sustained cell progression (Craxton et al., 2005; Gauld et al., 2002). Apoptosis in immature B cells also correlates with a rapid increase in the expression c-myc, a transcription factor involved in cell growth and proliferation, followed by a rapid decline below basal levels (Harnett, 2004). Similarly, levels of Pim-1, a Ser/Thr kinase involved with the survival and proliferation of hematopoietic cells, increase following BCR stimulation, which then decline below basal levels, correlating with growth arrest. TLR ligands, LPS and CpG DNA can reverse this growth arrest by sustaining the levels of Pim-1 and c-myc, respectively (Yi et al., 1996; Zhu et al., 2002a).

Lastly, mature B cells express approximately five times less IgM on their surface compared to immature B cells and respond well to high doses of anti-IgM, whereas in immature B cells, maximal BCR-induced tyrosine phosphorylation is achieved at lower concentrations of stimulating antibody (Koncz et al., 2002).

1.4.5 Tonic BCR signaling

Antigen-independent BCR signaling, termed 'tonic' or 'basal' signaling, is required to promote survival. Lam et al. first demonstrated the idea of tonic signaling when the inducible ablation of BCR resulted in the death of peripheral B cells (Lam et al., 1997). In a follow up study, they showed that the induced loss of the Igα ITAM led to the loss of mature B cells

(Kraus et al., 2004). In mice with a conditional deletion of IgM, expression of a constitutively active PI3K rescues mature B cell survival; however, expression of a constitutively active form of IKKβ (constitutive NF-κB signaling) is unable to rescue B cell survival (Srinivasan et al., 2009). Also, the balance between BCR-mediated Syk activation and protein tyrosine phosphatase-mediated Syk inhibition plays a role in tonic BCR signaling (Chen et al., 2008). Despite the important of these signaling molecules in regulating tonic BCR signaling, the molecular mechanisms remain to be defined. It has been shown that on resting mature B cells, surface BCRs exist mainly as monomers, with less mobile dimers or oligomers (Tolar et al., 2005). There is also an emerging idea of actin cytoskeleton "picket fences" which form confined domains in the plasma membrane to limit lateral diffusion of the BCRs. Each domain consists of several BCRs that constantly interact with each other within these domains to generate tonic signals (Treanor and Batista, 2010).

Tonic BCR signaling-mediated survival is also important in B cell lymphoma as most non-Hodgkin's B cell lymphomas, which accounts for the majority of lymphoma-related cases, express surface BCR. Constitutive BCR signaling is critical for the basal growth of B lymphoma cell lines as Igα- or Igβ-specific small interfering RNA (siRNA) or the inhibition of Syk induced growth arrest (Gururajan et al., 2006). In a separate study, Chen et al. found that a subset of diffuse large B cell lymphoma (DLBCL), the most common non-Hodgkin's lymphoma, relies upon tonic BCR signaling for survival, and are highly sensitive to Syk inhibitors (Chen et al., 2008). Much is still unknown as this field is relatively new, and targets for treatment are unresolved; however the Syk inhibitor, Fostamatinib disodium, has shown promising results in treating lymphoma patients (Reeder and Ansell, 2011).

1.5 B cell migration

B cell development, activation, and effector functions occur in different organs including the bone marrow, spleen, and other secondary lymph organs. B cell migration within one tissue or from one to another is highly mediated by secreted or cell surface factors, as well as components associated with the ECM. These factors include chemical attractants, cytokines, hormones and adhesion molecules. B cells migrate towards increasing concentrations of chemoattractants with adhesion molecules on both the lymphocyte and the interacting cells, as well as components of the ECM providing traction.

1.5.1 Chemokine and chemokine receptors

Chemokines are small chemotactic cytokines and there are approximately 50 peptide ligands and 20 receptors in humans. Most chemokines have four characteristic cysteines, and are separated into different subgroups based on the motif displayed by the first two cysteines: C, CC, CXC and CXXXC. Chemokines can mediate the migration of immune cells to sites of inflammation (inflammatory chemokines) as well as mediate trafficking within primary and secondary lymph organs (homeostatic/lymphoid chemokines).

Chemokine receptors are members of the seven transmembrane domain G protein-coupled receptors (GPCRs) family. A chemokine monomer is sufficient to bind and activate a monomeric chemokine receptor; however, at high concentrations, chemokines can spontaneously form homo- or hetero-oligomers through their amino termini. These aggregates exhibit synergy and enhance leukocyte migration and activation. Chemokine receptors can also form aggregates, although the functional consequences are not known (Thelen and Stein, 2008).

GPCRs binds G-proteins, which are trimers of α, β and γ subunits. The α subunit has the GTPase activity, important for signal transduction, and the β and γ subunits normally inhibit the GDP-bound α-subunit and localize it to the plasma membrane. Most chemokine receptors couple to the inhibitory G-protein, G_i, family, which has an inhibitory effect on adenylyl cyclase isoforms, ACV and ACVI (Mahadeo et al., 2007; Mellado et al., 2001). The reason why chemokine receptors generally couple to G_i is undefined, and whether they couple to other G subclasses is unknown.

The GPCR serves as a GEF for the Gα, and the binding of chemokine to its receptor results in exchanging the GDP for GTP on the $G\alpha_i$, dissociating both itself and the Gβγ from the receptor. GTP-$G\alpha_i$ and Gβγ subunits activate PLCβ, leading to the formation of IP_3 and DAG, which can induce Ca^{2+} mobilization, and the activation of MAPKs and Rap1 GTPases, respectively (Badr, 2010; Badr et al., 2008; Thelen and Stein, 2008). Rap1 GTPases then mediates integrin activation and cell polarization, which are important for cell migration. Ras and Rho GTPases are activated as well; however, Rap1 is the only GTPase that is sufficient to induce a polarized phenotype in lymphoid cell lines (Thelen and Stein, 2008). Phosphorylated PI3K can mediate the activation of PKC, Akt, FAK (focal adhesion kinase) and Pyk2 (proline-rich tyrosine kinase 2); the latter two are critical regulators of cell migration and adhesion (Mellado et al., 2001; Tse et al., 2012). A schematic diagram of CXCR4 signaling is provided in Figure 1-6. Chemokine receptor signaling is turned off through the slow intrinsic GTP hydrolysis on $G\alpha_i$ that promotes dissociation from effectors and re-association with the Gβγ. Also, a family of GAPs, known as regulators of G protein signaling (RGS), bind to $G\alpha_i$ prohibit its interaction with effectors or with Gβγ (Mellado et al., 2001).

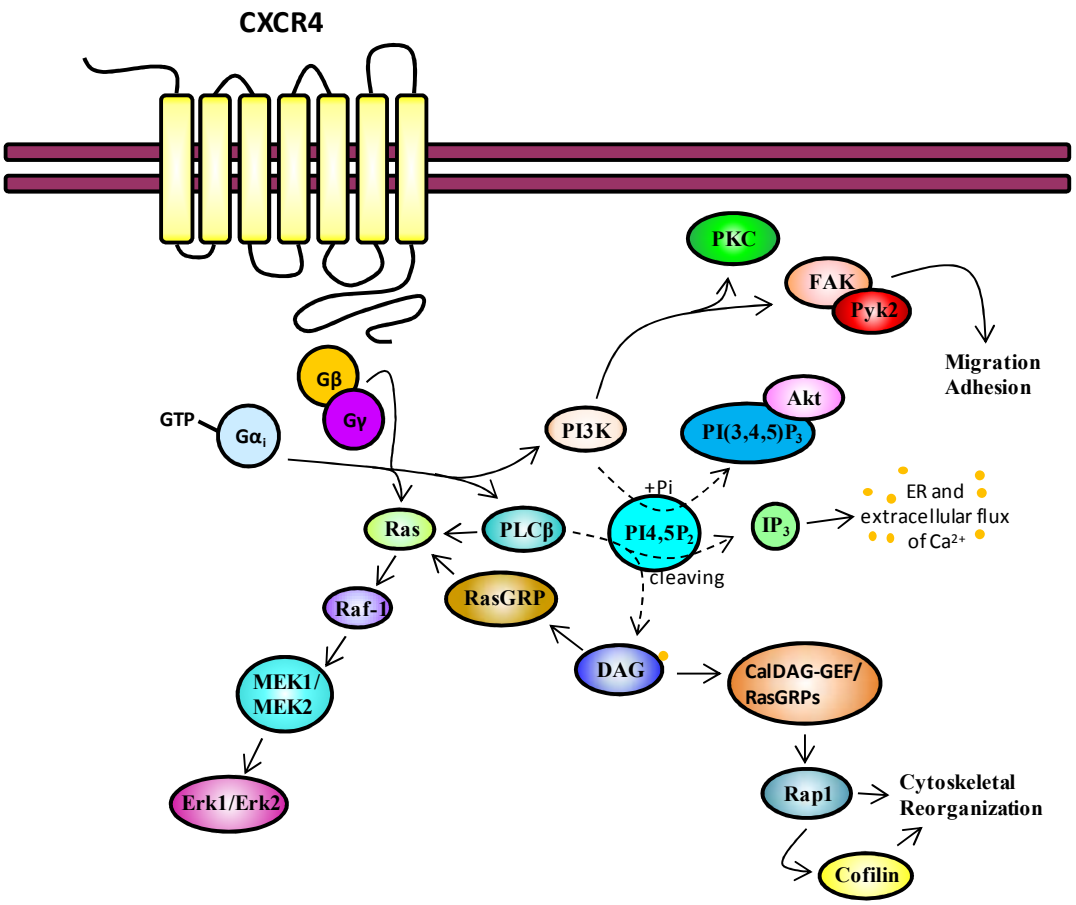


Figure 1-6. CXCR4 signaling pathway

A schematic representation the chemokine receptor, CXCR4, signaling pathway upon binding to the chemokine, CXCL12. The major pathways are shown: PLCβ pathway, Ras/MAP kinase pathway, and PI3K pathway. Solid arrows indicate direct or indirect activation and the function of dashed arrows are indicated in the figure.

1.5.2 Chemokine-mediated B cell migration

The bone marrow contains stromal cells that create special microenvironment, niches, that maintain hematopoietic cell viability and supply factors for their development. The spatial organization of hematopoietic cells and their interaction with these niches are important for the regulation of hematopoiesis.

The chemokine CXC12 (also known as stromal cell-derived factor-1 [SDF-1]) and its receptor CXCR4 is the first chemokine-chemokine receptor set that has been shown to be important for hematopoiesis. It was found that a small population of stromal cells, CAR cells, had very strong CXCL12 expression, and could aid B cell development (Nagasawa, 2007). The current model of B cell movement within the bone marrow suggests that HSCs, which are located near the endosteum or endothelial cells, would move towards the CAR cells and develop into pre-pro B cells. Once these cells differentiate into pro-B cells, they migrate towards IL-7 expressing stromal cells. As pro-B cells develop into pre-B cells, they leave these IL-7 expressing cells, and when they express a fully functional IgM, they exit the bone marrow as immature B cells (Nagasawa, 2006).

Immature B cells express the chemokine receptor CXCR5, which allow them to follow chemokine CXCL13 (also called B lymphocyte chemoattractant [BLC]) gradients secreted by FDCs of the spleen (Gunn et al., 1998; Lo et al., 2003). Most B cells will become FO B cells, and increase expression of CXCR5 and CCR7 chemokine receptors, which allow them to move between secondary lymphoid organs via homeostatic chemokines, CXCL13, CCL19 and CCL21 (Okada and Cyster, 2006). Also, CXCL12, CCL19 and CCL21 are expressed on the lumenal surface HEVs. These chemokines activate integrins, allowing B cells to migrate into lymphoid organs. When B cells exit the blood through the HEV and enter into the lymphoid tissue, they follow CCL19 and CCL21 gradients secreted by the T cell zone stromal cells, and scan for antigen in the cortex of the lymphoid organ. If they do not recognize any antigen, they continue to the B cell follicle, following the gradient of CXCL13 secreted by follicular stromal cells (Ansel et al., 2000; Nolte et al., 2003). CXCL13 is bound to the surface of FDCs, which allows

B cells to scan the surface of the FDC for antigens (Okada and Cyster, 2006). After migrating within the follicle for half a day to a day, non-cognate B cells egress from the lymphoid tissue and into circulation via the lipid chemoattractant, sphingosine 1-phosphate (S1P), produced by endothelial cells (Rivera et al., 2008; Rivera and Chun, 2008). When B cells reach the blood, they downregulate the expression of S1P receptors in response to the high levels of S1P, which allows them to follow chemokine gradients to other lymphoid organs (Rivera et al., 2008).

Antigen-engaged B cells upregulate CCR7 expression 2-3 fold, allowing their migration to the B-T boundary where they receive help from T_{FH} cells (Okada and Cyster, 2006). CCL21, the chemokine recognized by CCR7, extends as a gradient into the B cell follicle. These fully activated B cells will either differentiate into short-lived plasma cells or re-enter follicles to form germinal centers to undergo proliferation, affinity maturation, class switch recombination and subsequent differentiation into plasma cells or memory B cells. Plasma cells reduce their expression of CXCR5 and CCR7, which decreases their responsiveness to the follicular and T cell zone chemokines. Their expression of CXCR4 is crucial for the migration of plasma cells to regions where the ligand, CXCL12, is expressed, including the splenic red pulp, lymph node medullary cords, and bone marrow. They reside in niches that support their long term survival and production of antibodies (Shapiro-Shelef and Calame, 2005). Some plasma cells can also home to mucosal sites including the colon, respiratory/urogenital tract and mammary glands via CCR9 and CCR10 ligands, whereas others can home to sites of inflammation via CXCR3 ligands (Radbruch et al., 2006; Stein and Nombela-Arrieta, 2005; Tarlinton et al., 2008).

1.5.3 Leukocyte adhesion cascade

Leukocyte migration into tissues is regulated by a series of adhesive steps that allow them to attach to the postcapillary venule endothelial wall, travel along the wall to endothelial borders, pass through the endothelium and subendothelial basement membrane, and migrate through the interstitium. This multistep leukocyte adhesion cascade occurs during homeostatic migration to secondary lymph organs, or as an inflammatory response to recruit leukocytes to specific sites (Ley et al., 2007; Muller, 2011) (Fig. 1-7).

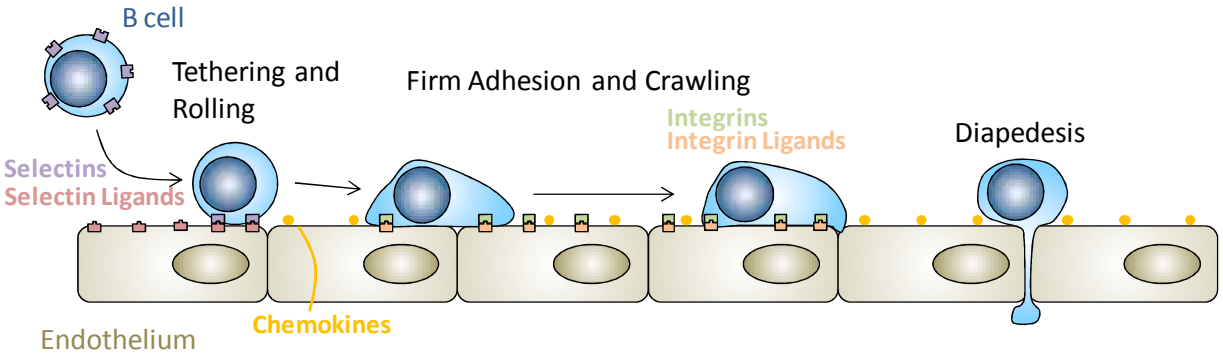


Figure 1-7. Leukocyte adhesion cascade

A schematic representation the leukocyte adhesion cascade. B cells form transient interactions with the endothelium via selectins and selectin ligands. When they sense chemokines bound to the endothelial membranes, they upregulate integrins, which bind to integrin ligands on the endothelial wall, mediating firm adhesion and crawling. Once a suitable site for diapedesis is found, B cells exit the vasculature.

Blood flow within these venules generate shear forces ranging from 1 to 4 dyn/cm2 on and along the endothelium (Sackstein, 2005), which is too strong for leukocytes to immediately attach to the endothelium. However, leukocytes do form transient loose interactions with the endothelium, allowing them to tether and roll along the endothelial wall. This is mediated by

selectins, a family of C-type lectin domain-containing glycoproteins that bind carbohydrates. This family consists of E-selectin (CD62E), expressed by inflamed endothelial cells, P-selectin (CD62P), expressed by activated platelets and inflamed endothelial cells, and L-selectin (CD62L), expressed by most leukocytes (Ley et al., 2007). Upon stimulation, by agents such as histamine and thrombin, P-selectin is released rapidly within minutes from Weibel-Palade bodies contained within endothelial cells. Stimulation with cytokine results in the slower (within four hours) expression of E-selectin, which is regulated at the transcriptional level. L-selectin is constitutively expressed by naïve lymphocytes. During inflammation, P-selectins mainly regulate the capturing of leukocytes in the blood whereas E-selectins stabilize rolling and decrease the speed of leukocytes (Ley and Kansas, 2004; Ley et al., 2007). L-selectin mediates lymphocyte homing to peripheral lymph nodes (Subramanian et al., 2012).

L-selectin binds peripheral node addressins (PNADs), which are unique sugar structures on highly glycosylated and sulphated forms of sialomucins, including CD34, glycosylation-dependent cell-adhesion molecule 1 (GLYCAM1), podocalyxin, endoglycan and endomucin. These are expressed abundantly on peripheral lymph node HEVs, allowing lymphocyte to traffic to the lymph nodes (Miyasaka and Tanaka, 2004). P-selectin glycoprotein ligand-1 (PSGL-1) can also serve as a ligand for L-selectin. Leukocytes express both L-selectin and PSGL-1, allowing them to roll on leukocytes that are already bound to the endothelium, called secondary capture or tethering. This is important for tethering leukocytes that do not express ligands for P- and E-selectins (Ley and Kansas, 2004; Ley et al., 2007). PSGL-1, depending on its glycosylation, can bind all three selectins. Fucosyltransferase-VII, core 2 β1,6-glucosaminyltransferase, β1,4-galactosyltransferase, and sialyl 3-galactosyltransferase are

important for PSGL-1 binding to P- and L-selectins. Fucosyltransferase-VII and sialyl 3-galactosyltransferase are required for PSGL-1 binding to E-selectins (Ley and Kansas, 2004). Increasing tensile force applied to these bonds increases their strength and longevity, which are called catch bonds. At even higher shear forces, these bonds become slip bonds, which become weaker and shorter lived (McEver and Zhu, 2010).

Leukocytes cannot firmly adhere to the endothelium through relatively weak bonds generated by selectins and their ligands. However, rolling along the endothelial wall brings the leukocyte in close proximity to chemokines and adhesion molecules on the endothelium that aid in leukocyte activation and firm adhesion to the endothelium. Numerous cell types are activated by inflammatory cytokines during inflammation to synthesize chemokines and lipid chemoattractants, which are then secreted. Endothelial cells are induced to express adhesion molecules, and express proteoglycans that immobilize chemokines on their cell surface (Allen et al., 2007b; Bromley et al., 2008). On the contrary, HEV endothelial cells constitutively express adhesion molecules (Miyasaka and Tanaka, 2004). The binding to these chemokines induces GPCR signaling on the leukocyte, leading to the activation of its integrins.

Integrins are a family of adhesion receptors that bind ECM components, serum proteins and immunoglobulin superfamily cell adhesion molecules (IgSF CAMs), and their cytoplasmic tail interacts with the cytoskeleton, thereby mediating cell-cell and cell-ECM adhesion. This is important for providing attachment to the ECM, providing traction during movement, and inducing signaling pathways, all important for the regulation of cell proliferation, survival, differentiation, migration, tumor invasion and metastasis (Niu and Chen, 2011). These

heterodimeric transmembrane receptors are composed of an α and a β subunit, and from 18 different α and 8 different β subunits, 24 different forms of integrin receptors can be made. The integrins involved in B cell trafficking are LFA-1 ($\alpha_L\beta_2$), VLA-4 ($\alpha_4\beta_1$), and lymphocyte Peyer's patch adhesion molecules-1 (LPAM-1, $\alpha_4\beta_7$). The respective ligands expressed on the surface of endothelial cells are IgSF CAMS: ICAM-1 and ICAM2, VCAM-1, and MAdCAM-1 (Luo et al., 2007; Niu and Chen, 2011). Not only are integrins involved with leukocyte:endothelial cell adhesion, rolling and arrest, they mediate transendothelial migration as well.

Once the leukocyte has attached, it crawls laterally along the endothelium, searching for a site for diapedesis, the migration of blood cells through intact endothelial walls. Leukocytes make podosome-like protrusions that probe the endothelial surface for a suitable site (Carman and Springer, 2008). All the preceding steps, including rolling, activation, adhesion, and crawling, are reversible, but once the leukocyte commits to diapedesis, it does not go back (Muller, 2011). Diapedesis can occur paracellularly or transcellularly (Carman and Springer, 2008). Once the leukocyte has attached, the endothelium forms a "transmigratory cup" around the migrating cell to ensure that the vasculature is maintained. This cup contains ICAM-1- and VCAM-1-rich projections with cytoplasmic molecules such as ERM (ezrin, radixin and moesin) proteins, which connects actin filaments with the plasma membrane, and cytoskeletal components (vinculin, α-actinin and talin-1) (Carman and Springer, 2008; Ley et al., 2007; Phillipson et al., 2008). During paracellular migration, inflamed endothelial cells may redistribute their junctional molecules to facilitate diapedesis. Signaling through the endothelial ICAM-1 molecules induces the opening of endothelial cell-cell junctions. As the leukocyte moves through, it can interact with these junction molecules that further promotes the loosening

of adhesive contacts (Ley et al., 2007). During transcellular migration, ICAM-1 is moved to caveolae-containing structures, and leukocyte protrusions can enter into the endothelial cell cytoplasm. The ICAM-1 and caveolin then cluster around this protrusion, forming a channel through which the migrating leukocyte can squeeze (Ley et al., 2007; Millan et al., 2006).

1.5.4 Cell motility and cytoskeletal remodeling

Cell migration is a complex process that starts with cell polarization, and subsequent actin-dependent protrusion of the membrane at the leading edge. The cell extends and forms new adhesions with the substratum at the leading edge, followed by forward translocation of the cell body via actomyosin-based contraction forces. Disassembly of adhesions occurs at the trailing edge, followed by retraction at the cell rear (Ridley et al., 2003). Actin is the most abundant cytoskeletal component and exists in two forms: monomeric, globular actin (G-actin) and filamentous actin (F-actin), which is a double helix polymer of G-actin (Pollard, 2007; Pollard and Borisy, 2003). G-actin bound with ATP polymerizes into F-actin. Actin filaments are polarized, consisting of a faster-growing barbed end and a slow-growing pointed end (Welch and Mullins, 2002).

Actin-branching is helped by the actin-related protein Arp2/3 complex. In response to extracellular stimuli, such as chemokines, Rho GTPases are activated, leading to the activation of the Wiscott Aldrich syndrome protein (WASp), which then activates the Arp2/3 complex (Korobova and Svitkina, 2008; Pollard and Borisy, 2003). The elongating filament pushes the plasma membrane until the filament is capped by actin capping proteins, terminating the polymerization. Disassembly of the actin filament is accomplished through Cofilin, which

catalyzes the hydrolysis of ATP and releases a phosphate and G-actin. Conversely, the protein profilin exchanges the ADP for ATP and binds these ATP-actin monomers to maintain a pool of polymerizable G-actin within the cell (Pollard and Borisy, 2003).

1.6 Connexins

Connexins are the building blocks of gap junctions and hemichannels that aid in cell-cell or cell-environment communication, respectively, through the exchange of small metabolites (glucose, amino acids, nucleotides), ions (Na^+, K^+) and second messengers (Ca^{2+}, cAMP, IP_3) (Goodenough and Paul, 2009; Laird, 2010). Larger molecules such as siRNAs, have been shown to pass through connexin channels (Valiunas et al., 2005). Connexin family members assemble to form a hexameric channel, called connexons, and are usually homomeric, but can intermix to form heteromeric connexons (Gemel et al., 2008). Connexons at the cell surface are called hemichannels and some cells can pass small molecules between the cell and the environment. When two hemichannels come together from adjacent cells, a gap junction is formed, mediated by the interactions among the cysteine residues on the connexin extracellular loops (Foote et al., 1998; Tong et al., 2007). Gap junction channels can be homotypic (two identical connexons), or heterotypic (two different connexons) (Willecke et al., 2002; Yeager et al., 1998). There are 21 different connexins expressed in humans, and 20 in mice, each designated with number suffixes corresponding to their approximate molecular weight in kilodaltons (eg. Cx43 is around 43 kDa in size) (Willecke et al., 2002). Their proposed structure includes 4 transmembrane domains, forming two extracellular and one intracellular loop, with intracellular amino- and carboxyl-termini. The general structure is identical among connexins; however, the loops and carboxyl-termini can differ in length.

Gap junction channels concentrate at cell:cell interfaces into very high density clusters referred to as gap junctional plaques (Laird, 2006; Loewenstein, 1981; Yamasaki, 1990). They have also been characterized in terms of specific tissue distribution, channel conductance, transjunctional voltage dependence of gating, and modulation by physiological factors (Beyer et al., 1990).

1.6.1 Connexin 43 (Cx43)

One of the most widely expressed connexins is Cx43, which has very high sequence homology between different species in vertebrates (Solan and Lampe, 2009). It is responsible for the synchronization of myocardial contractions in the ventricular myocardium, where it is highly expressed (Christ et al., 1993; Gros and Jongsma, 1996; Severs, 1994). The half-life of Cx43 in cells that contain gap junctional plaques is 1-3 hours, which is very short compared to typical plasma membrane proteins. These kinetics could serve as a mechanism to regulate communication through the connexon in response to stimuli or changes in the environment (Laird, 2006). The knockout of Cx43 in mice results in death due to heart malformations caused by a swelling of the right ventricular outflow tract, and blockage of the blood flow into the lungs (Reaume et al., 1995). Overexpression of Cx43 in neural crest cells causes heart malformations due to hypertrophy of the right ventricle, and narrowing of the pulmonary outflow region (Ewart et al., 1997).

1.6.2 Cx43 regulation

Gap junction communication is regulated by changes in voltage, ion concentrations, pH, and phosphorylation states of the Cx43 cytoplasmic tail (Herve et al., 2007). Phosphorylation of

Cx43 can regulate trafficking, assembly and disassembly, degradation, and gating of gap junction channels (Lampe and Lau, 2004). The carboxyl-terminus of Cx43 contains many serine, threonine and tyrosine residues that can be phosphorylated, and these seem to be the only location for phosphorylation as the intracellular loop and the amino-terminus do not contain any known sequences and phosphorylation of these regions have not been reported (Lampe and Lau, 2000; Lampe and Lau, 2004). Cx43 is translated as a 42 kDa non-phosphorylated protein (Cx43-NP) and could then be phosphorylated to give a 44 kDa protein (Cx43-P1) and further phosphorylated to give a 46 kDa protein (Cx43-P2), which is predominantly in gap junction plaques (Musil et al., 1990; Musil and Goodenough, 1991). Because the mass of a phosphate group is 80 Da, the higher shift in mass could result from conformational changes (Lampe et al., 2006; Solan et al., 2007).

Many protein kinases are suggested to phosphorylate the carboxyl tail of Cx43, including PKC, MAPKs, casein kinase 1 (CK1), v-Src, and PKA. PKC can phosphorylates Cx43 on Ser262 and Ser368, which can decrease gap junction coupling, ATP release and dye uptake (De Vuyst et al., 2007). Phosphorylation of Ser368 does not affect Cx43 migration in SDS-PAGE gels, but the phosphorylation of Ser262 gives a Cx43-P2 form (Solan and Lampe, 2009). Phosphorylation of Ser255, Ser279 and Ser282 by MAPKs is important in down-regulating gap junction communication (Lampe and Lau, 2004; Solan and Lampe, 2009). CK1 phosphorylates Cx43 *in vitro* on Ser325, Ser328, and Ser330, shifting it to the P2 form, and the phosphorylation of these sites are important for gap junction assembly (Lampe and Lau, 2004; Solan and Lampe, 2009). Tyr247 and Tyr265 are phosphorylated by v-Src, and they have roles in channel closure. v-Src also phosphorylates Ser262, Ser279, Ser282 and Ser368, although the functional outcome

is unknown (Lampe and Lau, 2004; Solan and Lampe, 2009). Several tandem serines can be phosphorylated (Ser364, Ser365, Ser368, Ser369, and Ser373) through PKA, which promotes cell coupling (Imanaga et al., 2004; Lampe and Lau, 2004). Phosphorylation of Ser365 is necessary for the shift to the P1 isoform (Solan and Lampe, 2009). Although many target sites and protein kinases are known, the biological roles of specific connexin phosphorylation events still need to be elucidated.

Among all the connexins, Cx26 has one of the shortest cytoplasmic tails that is not phosphorylated, but it still forms functional channels. Also, Cx43 with this region truncated will still form functional channels despite the absence of the phosphorylatable longer cytoplasmic tail (Cooper et al., 2000; Dunham et al., 1992). This indicates that phosphorylation is not absolutely required for the formation of functional gap junctions channels, but may serve to regulate the opening and closing of gap junction channels.

The phosphorylation of certain residues of Cx43 can create potential binding sites for proteins. Phosphorylation of Tyr247 and Tyr265 results in potential SH2 binding domains, which contain phosphorylated tyrosine residues. Moreover, the proline-rich region (amino acids 274-283) could be a potential SH3 binding domain, which has a consensus sequence X-proline-X-X-proline. The carboxyl-terminal tail of Cx43 also has a proposed microtubule binding domain within amino acids 234-243, a Nedd4 binding site involved in ubiquitination at amino acids 282-286, and a PDZ binding domain at the end of the tail (amino acids 379-382) (Giepmans, 2004; Palatinus et al., 2011). These binding sites could serve to facilitate the

interaction of Cx43 with many different proteins and suggest potential roles for Cx43. A summary of the phosphorylation and binding sites are illustrated in Figure 1-8.

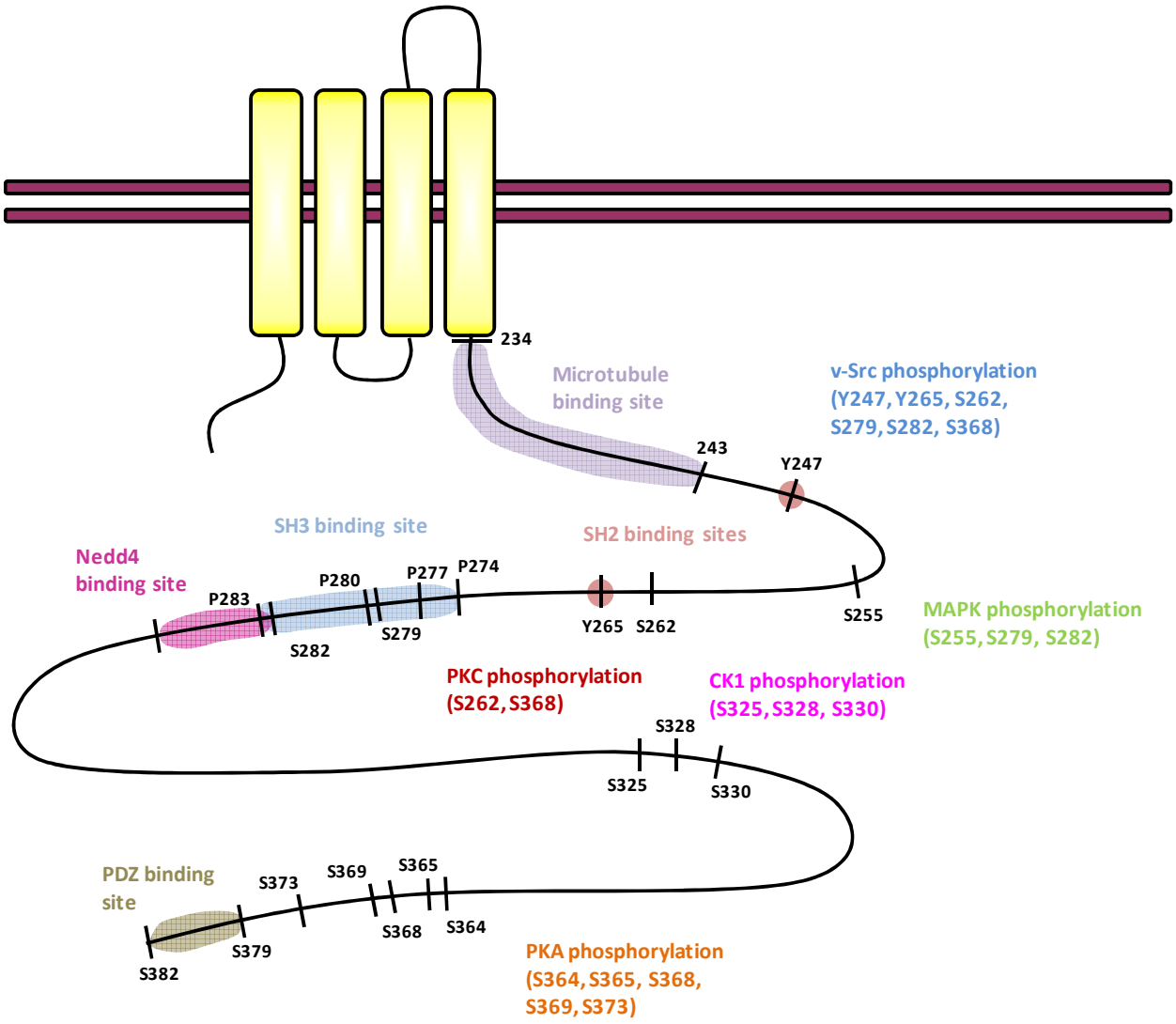


Figure 1-8. Proposed phosphorylation and protein binding sites in Cx43

Amino acid locations of protein binding sites as well as phosphorylation sites by known kinases (v-Src, MAPK, PKC, CK1 and PKA) are indicated.

1.6.3 Proteins interacting with the Cx43 carboxyl-terminal tail

Recently, Cx43 is emerging as an adaptor protein via its carboxyl-terminal tail, which is nearly 150 amino acids long, and contains many protein binding sites. Several proteins have been shown to possibly interact directly or indirectly with the carboxyl-terminus of Cx43, including cytoskeletal proteins, such as actin and α-/β-tubulin, and actin-binding proteins, such as cortactin, and myosin II (Olk et al., 2009). These interactions have been found through many techniques, including co-immunoprecipitation, co-localization studies, immuno-electron microscopy, and fluorescence resonance energy transfer (FRET).

Co-localization of actin and Cx43 increases with cyclic mechanical strain between actin filament bundles and gap junctions in mechano-sensitive human tenocytes (Wall et al., 2007). Additionally, the phosphorylation of Cx43 also plays a role in its interaction with actin. In mouse astrocytes, the interaction between Cx43 and actin, shown by co-immunoprecipitation, is reduced with hypoxia-induced dephosphorylation of Cx43, and their co-localization with actin is also reduced in mouse myoblasts, when a MAPK inhibitor is used (Li et al., 2005; Squecco et al., 2006). Lastly, during neuronal migration along radial glia, Cx43 frequently co-localizes with the actin puncta, and the knockdown of Cx43 decreases the number of actin puncta (Elias et al., 2007). Although actin has been shown to co-localize or co-immunoprecipitate with Cx43, it is more likely that Cx43 is connected to the actin cytoskeleton through other cytoskeleton interaction proteins rather than binding directly.

Cortactin, an actin-binding protein involved in adhesion, spreading, endocytosis, and migration in many cell types (Bryce et al., 2005; Lai et al., 2009), has a p38 MAPK-dependent

interaction with Cx43 suggested by co-immunoprecipitation and co-localization studies using myoblasts (Squecco et al., 2006). The hematopoietic lineage cell-specific homolog of cortactin, HS1, has not yet been shown to interact with Cx43, but it is an intriguing possibility that it too could have similar Cx43 interactions as cortactin.

Myosin II is responsible for the contraction of muscle cells, generating tension, and has a role in cell division, and adhesion and migration by moving the body of the cell forward and retracting the rear end (Conti and Adelstein, 2008). Myosin II is associated with annular gap junctions that are being internalized for degradation and may provide contractile forces for Cx43 endocytosis. The inhibition of myosin II with blebbistatin reduces the co-localization of actin with Cx43 in tenocytes (Wall et al., 2007). This suggests that both actin and myosin II may act together to stabilize gap junctions to maintain cell coupling.

Tubulin was the first cytoskeletal protein that has been shown to directly bind Cx43 via pull-down experiments using the carboxyl-terminal tail of Cx43 (Giepmans et al., 2001). This tubulin binding domain in the carboxyl-terminal tail is only found in Cx43 and not in the tails of other members of the Cx family. Microtubules mediate the trafficking of connexons to the plasma membrane by delivering Cx43-containing vesicles to plasma membrane locations rich in N-cadherin complexes (Shaw et al., 2007). The trafficking of Cx43 is not well studied in B cells, but it may be mediated by microtubules, facilitating the high turnover rates of Cx43.

Cx43 also recruits a scaffold protein, zonula occludens-1 (ZO-1), which is one of the most widely studied binding partners of the Cx43 carboxyl-terminal tail. It has a PDZ domain

that binds Cx43, and an actin binding domain to links Cx43 to the cytoskeleton. ZO-1 regulates the internalization of gap junctions, as well as the size and the transition of uncoupled connexons into gap junctions (Palatinus et al., 2011).

1.6.4 Cx43 role in cell migration

Because Cx43 is associated with many proteins that bind or regulate the cytoskeleton, its role in cell migration has been an emerging topic of interest. Cx43 is highly expressed in migrating neural crest cells during embryogenic development. The downregulation of Cx43 decreases cell motility in these cells, as well as in 3T3 mouse embryonic fibroblasts, and in C6 glioma cells (Bates et al., 2007; Wei et al., 2005; Xu and Ma, 2006). Conversely, Cx43 has also been shown to negatively regulate cell migration. In two separate wound-healing studies, the downregulation of Cx43 accelerated wound closure (Qiu et al., 2003; Simpson et al., 2008). In addition, knockdown of Cx43 increased the migration of Hs578T breast cancer cells (Shao et al., 2005). Thus, Cx43 can positively or negatively influence cell migration depending on the cell type and the levels of Cx43 expression.

Whether Cx43 modulation of cell migration is dependent on the formation of a functional gap junction channel or independent of channel function remains unclear. The expression of Cx43 lacking the carboxyl-terminal tail results in decreased cell migration in C6 glioma cells (Bates et al., 2007), 3T3 fibroblasts (Moorby, 2000) and in neurons during normal brain development in mice (Cina et al., 2009). These studies demonstrate that the carboxyl-terminus, but not the channel domain nor the extracellular domain, is important for the modulation of cell migration. Conversely, other studies show that gap junction formation can be important for cell

migration. Expression of Cx43 with a mutated channel region that blocked channel function rescued neuronal migration in cells that had endogenous Cx43 levels knocked down. Also, the expression of a Cx43 with one of the extracellular cysteines mutated could not rescue migration in these cells lacking Cx43. It was proposed that neuronal migration is mediated by the adhesive properties of the Cx43 extracellular regions (Elias et al., 2007). Blocking the channel with carbenoxolone decreases the migration of glioblastoma cells (Oliveira et al., 2005). These conflicting results have not been clearly explained and the molecular mechanism by which Cx43 effects cell migration remains to be resolved.

1.6.5 Cx43 in lymphocytes

Cx43 is the predominant connexin isoform expressed in the bone marrow, thymus, spleen and other lymphoid tissue (Alves et al., 1998; Krenacs and Rosendaal, 1995; Krenacs and Rosendaal, 1998). Cx43 heterozygous mouse embryos have reduced frequencies of mature T and B cells, but the hematopoietic defects in heterozygotes disappear after 4 weeks. In these adult mice, the regeneration of hematopoietic cells was impaired after myeloablation. Also, induced Cx43 gene deletion in adult mice stromal cells does not alter cell numbers in peripheral blood. However, after depletion of blood cells, hematopoiesis recovery was impaired. These studies indicate the importance of Cx43 during the formation of blood cells during embryogenesis or during hematopoietic stress, but not for steady-state hematopoiesis (Montecino-Rodriguez et al., 2000; Presley et al., 2005). Cx43 is also important for the coordinated secretion of CXCL12 in bone marrow stromal cells which may maintain HSC adhesion and survival (Schajnovitz et al., 2011).

Gap junctions are involved during immune responses, as stimulation with LPS increased Cx43 expression in T and B lymphocytes. They also mediate communication between T and B cells as mimetic peptides and gap junction inhibitors abrogated dye transfer between these cells, significantly reduced the synthesis of IgM, IgG and IgA by B cells, and decreased cytokine production (Oviedo-Orta et al., 2001; Oviedo-Orta et al., 2000). Gap junction communication is also important in peptide transfer and cross presentation in activated monocytes and dendritic cells (Mendoza-Naranjo et al., 2007; Neijssen et al., 2005), and in forming junctions between dendritic cells to aid in their activation (Matsue et al., 2006). Gap junctions can also help in the transfer of cAMP, an inhibitor of T cell growth, differentiation, and proliferation, from T regulatory (Treg) cells to $CD4^+$ T cells to mediate the suppressive activity of Treg cells (Bopp et al., 2007). Also, Cx43 accumulates at the immunological synapse between T cells and dendritic cells and requires the actin cytoskeleton, but not microtubules (Mendoza-Naranjo et al., 2011).

Gap junctions are formed between immune cells and endothelial cells during leukocyte extravasation. The inhibition of gap junction communication decreased transendothelial migration in monocytes (Eugenin et al., 2003), increased that of neutrophils (Zahler et al., 2003), and had little influence on T and B cells (Oviedo-Orta et al., 2002). An explanation of how connexin mediates cell migration could be through the adhesive properties of the cysteine residues that are in the extracellular loops (Elias et al., 2007; Lin et al., 2002). Conversely, others suggest that Cx43 decreases adhesion to the endothelium through the extracellular release of ATP by hemichannels (Eltzschig et al., 2006). ATP will undergo hydrolysis into AMP and then is further hydrolysed into adenosine. This results in the activation of adenosine receptors

that decrease the adhesion of leukocytes through the cAMP-PKA pathway (Eltzschig et al., 2006; Lorenowicz et al., 2007).

Cx43 is highly expressed in HSCs, and its level decreases as it differentiates into MPPs (Forsberg et al., 2005). Previous findings from our lab showed that the expression of Cx43, which is high in immature and mature B cell lines, is lost in terminally differentiated plasmacytomas (Machtaler et al., 2011). These results suggests that Cx43 is highly expressed in B cells that are more mobile (eg. immature and mature B cells) and is downregulated in more sessile B cells (eg. plasma cells). Also, the carboxyl-terminal tail is important for BCR-, chemokine-, and integrin-mediated cell spreading and adhesion to endothelial cell layers (Machtaler et al., 2011). Lastly, we found that Cx43 expression increases B cell random motility and B cell migration towards chemokine (Machtaler, unpublished data). Because Cx43 expression positively regulates B cell spreading and migration, these results suggest that Cx43 may play a role in regulating cytoskeletal dynamics in B cells. However, the molecular mechanisms of how Cx43 expression affects B cell spreading and migration are not known. B cell spreading and migration are critical for B cell development and function, and molecules that regulate these processes could be key regulators of B cell function in both health and disease.

1.7 Rationale, aims and hypotheses

Rationales:

1) During B cell activation, B cells adhere, and spread across APCs, which are processes that are dependent on the cytoskeleton. Cx43 is required for the BCR to cause sustained or optimal adhesion and spreading. How Cx43 enhances the activation of key cytoskeletal regulators that are regulated by the BCR is not known. Also, it not known whether Cx43 enhances all BCR signaling by enhancing specific proximal signaling reactions or whether it selectively enhances specific BCR signaling pathways. The role of Cx43 in BCR signaling could be useful in understanding B cell functions.

2) The dysregulation of B cell development can cause autoimmune diseases (e.g. lupus, rheumatoid arthritis), and additionally, B cell cancers are very common (e.g. B cell chronic lymphocytic leukemia). There is a lack of effective treatments for these diseases. B cell depletion with Rituximab is currently in use but depletion is not complete and relapse rates are high (Engel et al., 2011; Foon et al., 2012). Also, inhibitors of key BCR signaling proteins such as Syk and Btk, which depletes BCR-dependent lymphomas, are in clinical trials, but response rates are low (Reeder and Ansell, 2011). Additional inhibitors of BCR signaling are needed in order to better treat B cell-mediated autoimmune diseases and B cell malignancies.

Aims:

1) To identify key signaling proteins whose activation by the BCR is modulated by Cx43

2) To identify small molecule inhibitors of this process that could be used therapeutically to inhibit B cell activation and survival

Hypotheses:

1) Cx43 regulates specific BCR signaling pathways that are involved in cytoskeletal reorganization.

2) Compounds that prevent antigen-induced cell death in immature B cells will represent novel probes for identifying, and perhaps regulating, key signaling proteins that control B cell survival and Ag-induced B cell activation.

2 The role of Cx43 in BCR signaling

2.1 Introduction

Cytoskeletal reorganization is important during B cell development, as they move from different niches, guided by chemokines, to receive growth signals for proper development. It is also important during B cell activation where the B cell receptor (BCR) binds to membrane-bound antigens on antigen presenting cells (APCs). The B cell then spreads across the APC to gather antigen and contract to form an immune synapse, a driving force for B cell activation. Recently, connexin 43 (Cx43), a gap junction protein important for the cell-cell or cell-environment passage of molecules, was shown to mediate neuronal migration during murine brain development (Cina et al., 2009; Elias et al., 2007). Additionally, the cytoplasmic tail of Cx43 contains many putative sites for phosphorylation and protein binding, and has been shown to potentially bind to regulators of cytoskeletal dynamics (Olk et al., 2009). We have shown that Cx43 is expressed in B cells and that it is important in B cell spreading and migration as well as in the activation of the master regulator of cytoskeletal dynamics, Rap1 GTPase (Machtaler et al., 2011). The aim of this chapter is to study the molecular mechanism of how Cx43 could regulate both BCR-induced spreading and chemokine receptor-induced migration in B cells via Rap1 GTPase with the majority of the focus on BCR signaling.

During BCR signaling, three major pathways are activated: the PLCγ pathway, the Ras/Erk pathway, and the PI3K/Akt pathway. Using shRNA knockdown of Cx43 in the WEHI-231 murine B lymphoma cell line, I found that Cx43 was important for the activation of the PLCγ pathway, which leads to the activation of Rap1. Moreover, Cx43 was also important for the phosphorylation of HS1, which regulates the accumulation of F-actin at the immune synapse.

These data suggest that Cx43 mediates certain pathways during BCR signaling, possibly via the carboxyl-terminal tail acting as a signaling adaptor to recruit signaling proteins.

2.2 Materials and methods

2.2.1 Antibodies

Polyclonal goat anti-mouse IgM (μ-chain specific, #115-005-020) and polyclonal anti-mouse IgG (Fc Fragment specific, #115-005-008) antibodies used for stimulating B cells were purchased from Jackson ImmunoResearch Labs (West Grove, PA). Antibodies used for western blotting are shown in Table 2-1.

The anti-phosphotyrosine monoclonal antibody in Table 2-1 (clone 4G10), which recognizes tyrosine phosphorylated proteins from all species, was prepared in our laboratories (Richards et al., 1996). 4G10 hybridomas were grown to confluency in cell culture media containing 1% final concentration of glucose, 25 mM final concentration of Hepes, and 5 mM final concentration of NaOH. Cells were left until they die, and culture supernatants was spun at 2000 RPM and filter sterilized.

Antibody recognizes	Concentration/Dilution Used	Host (Formulation)	Company	Catalog #
Phospho-Akt (Ser 473)	1:1000	Polyclonal rabbit	CST	9271
Akt	1:1000	Polyclonal rabbit	CST	9272
Phospho-Btk (Tyr223)	1:1000	Polyclonal rabbit	CST	3531
Btk	1:1000	Polyclonal rabbit	CST	3532
Phospho-Cofilin (Ser3)	1:1000	Polyclonal rabbit	CST	3311
Cofilin	1:1000	Polyclonal rabbit	CST	3312
Cx43	1:1000	Polyclonal rabbit (amino acids 363-382 specific)	Sigma-Aldrich	C6219
Phospho-Erk (p44/42 MAP kinase) (Thr202/Tyr204)	1:1000	Polyclonal rabbit	CST	9101
Erk (p44/42 MAP kinase)	1:1000	Polyclonal rabbit	CST	9102
Phospho-HS1 (Tyr397)	1:1000	Polyclonal rabbit	CST	4507
HS1 (rodent specific)	1:1000	Polyclonal rabbit	CST	4557
Phospho-Lyn (Tyr507)	1:1000	Polyclonal rabbit	CST	2731
Lyn	1:1000	Polyclonal rabbit	CST	2732
Phospho-p38 MAP kinase (Thr180/Tyr182)	1:1000	Polyclonal rabbit	CST	9211
p38 MAP kinase	1:1000	Polyclonal rabbit	CST	9251
Phospho-PLCγ2 (Tyr1217)	1:1000	Polyclonal rabbit	CST	3871
PLCγ2	1:1000	Polyclonal rabbit	CST	3872
Phospho-SAPK/JNK (Thr183/Tyr185)	1:1000	Polyclonal rabbit	CST	9251
SAPK/JNK	1:1000	Polyclonal rabbit	CST	9252
Phospho-Syk (Tyr519/520)	1:1000	Polyclonal rabbit	CST	2711
Syk	1:1000	Polyclonal rabbit	CST	2712
Phoshotyrosine 4G10 (Morrison et al., 1989)	0.5 μg/mL	Monoclonal mouse	In-house	-

Table 2-1. List of antibodies used for western blotting
CST: Cell Signaling Technologies (Danvers, MA). Sigma-Aldrich (Saint Louis, MO).

Goat anti-rabbit IgG (H+L) conjugated to horseradish peroxidase (HRP) and goat anti-mouse IgG (H+L) conjugated to HRP antibodies were purchased from Bio-Rad (#170-6515 and #170-6516, respectively, Mississauga, Ontario). The Fc receptor blocking reagent, anti-mouse CD16/CD32 (clone 2.4G2, American Type Culture Collection (ATCC), Manassas, VA) was prepared in-house.

The fluorophore-conjugated antibodies, rat anti-mouse IgM-FITC (clone eB121-15F9, #11-5890-85), rat anti-mouse CD184/CXCR4-FITC (clone 2B11, #53-9991-80), rat anti-mouse CD11a/LFA1-FITC (clone M17/4, #11-0111-82), and rat anti-mouse CD49d/VLA4-FITC (clone R1-2, #12-0492-81) were purchased from eBiosciences (San Diego, CA). The goat anti-mouse IgG-Alexa 488 (#A11029) was purchased from Invitrogen Life Technologies (Burlington, Ontario). Purified rat IgG2a κ isotype control (#14-4321-81) and rat IgG2b κ isotype control (#14-4031-81) were purchased from eBiosciences.

2.2.2 Cells and cell culture

The A20 IgG^+ mature murine B cell line and the WEHI-231 IgM^+ immature murine B cell line were obtained from the ATCC. Cells were maintained in RPMI-1640 medium (Invitrogen Life Technologies, #21870-092) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (Invitrogen Life Technologies, #12483-020), 2 mM L-glutamine (Sigma-Aldrich, #G8540), 1 mM sodium pyruvate (Sigma-Aldrich, #P5280), 50 units/ml penicillin (Invitrogen Life Technologies, #15140122), 50 μg/ml streptomycin (Invitrogen Life Technologies, #15140122), and 50 μM β-mercaptoethanol (2-ME) (Sigma-Aldrich, #M7154). Cells were incubated at 37^{o}C in an atmosphere 5% CO_2. Cells stably transduced with Cx43

shRNA constructs were cultured in supplemented RPMI containing 1 mg/ml Hygromycin B (Invitrogen Life Technologies, #10687010).

2.2.3 Constructs and transduction

Cx43 siRNA-1 (intracellular loop) 5′-GAAGTTCAAGTACGGGATT-3′, rat Cx43 from 398 to 416 and siRNA-2 (second extracellular loop to the third transmembrane domain) 5′-CCATCTTCATCATCTTCAT-3′, rat Cx43 from 617 to 637, in the pH1.1-QCXIH retroviral vectors [GenScript Corporation (Scotch Plains, NJ)] were a gift from the Naus Lab (University of British Columbia, Vancouver, British Columbia) (Shao et al., 2005). As a control, the nonsense sequence 5'-AATTCTCCGAACGTGTCACGT-3' was used. WEHI-231 and A20 cells were retrovirally transduced with Cx43 siRNA-1, Cx43 siRNA-2, Cx43 siRNA-1+2, and nonsense constructs, as described (Krebs et al., 1999).

2.2.4 Cell stimulation and preparation of samples

Cells were washed with phosphate-buffered saline (PBS), resuspended to10^7 cells/ml of modified Hepes-buffered saline (25 mM sodium Hepes pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME) and 500 μl aliquots were added to each 1.7 ml microfuge tube. Cells were then incubated on ice for 10 min and in a water bath at 37^{o}C for 20 min. WEHI-231 cells were stimulated with 20 μg/ml goat anti-mouse IgM or 100 nM recombinant mouse chemokine, CXCL12/SDF-1α (R&D Systems, # 60-SD/CF, Minneapolis, MN). A20 cells were stimulated with 20 μg/ml polyclonal anti-mouse IgG. Reactions were stopped by adding 500 μl of cold PBS supplemented with 1 mM Na_3VO_4, and then spun at 1,500 RPM for 2 min at 4^{o}C.

Supernatants were removed and the cells were lysed with cold radioimmunoprecipitation assay (RIPA) lysis buffer (30 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Igepal (Sigma-Aldrich), 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with protease inhibitors (1 mM pepstatin A, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM Na_3VO_4, 25 mM β-glycerophosphate). Samples were then centrifuged at 14,000 RPM at 4°C for 15 min to remove cellular debris and the protein concentration of each sample was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnologies, Rockford, IL). A_{562} was determined using an Ultrospec 2011 pro spectrophotometer (GE Healthcare, #80-2112-21). Lysates were diluted 1:5 with 5x dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer (62.5 mM Tris-HCl pH 6.8, 4% glycerol, 2.5% SDS, 0.02% bromophenol blue, 100 mM dithiothreitol), boiled for 5 min and used immediately or were stored at -20°C for later use.

2.2.5 Lysis method to extract Connexin 43

To confirm the knockdown of Cx43, cells were lysed with cold modified K buffer (50 mM $CaCl_2$, 1% Triton-X 100, 1% Igepal, in PBS) (Troxell et al., 1999) containing protease inhibitors (1 mM pepstatin A, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1mM Na_3VO_4, 25 mM β-glycerophosphate). Samples were sonicated for 10 seconds using the Misonix XL Sonicator Ultrasonic Cell Processor (Misonix Incorporated, Farmingdale, NY), and centrifuged at 14,000 RPM at 4°C for 15 min to remove cellular debris before determining the protein concentration of each sample using the BCA protein assay kit. Lysates were then diluted 1:5 with 5x SDS-PAGE reducing sample buffer and incubated at 37°C for 30 min.

2.2.6 Protein dephosphorylation assay

WEHI-231 cells were stimulated with 20 μg/ml goat anti-mouse IgM as in Section 2.2.4 and cell lysates were prepared as in Section 2.2.5. One 30 μg aliquot of each cell lysate was subjected to dephosphorylation and an identical 30 μg aliquot of the lysate was used as an untreated control. Dephosphorylation of the lysates was carried out by the addition of 30 units of calf intestinal alkaline phosphatase (New England BioLabs, #M0290S, Ipswich, MA) and then diluted in 1x NEBuffer (New England BioLabs). Control lysates were diluted in 1x NEBuffer only. Lysates were incubated at 37^{o}C for 60 min and were then adjusted to 1x SDS-PAGE reducing sample buffer and further incubated for 30 min at 37^{o}C.

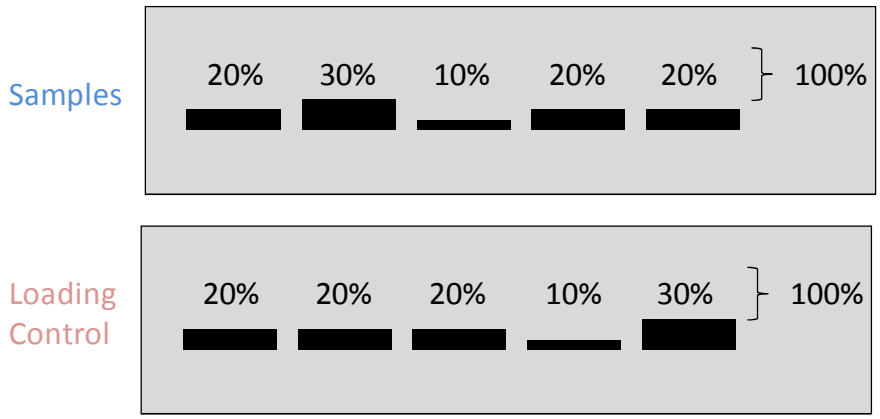
2.2.7 Western blotting and analysis

Samples were immediately loaded onto a 10% SDS polyacrylamide gel (Bio-Rad) or if they were stored at -20^{o}C, they were re-boiled for 5 min prior to loading onto the gel. Samples were loaded at 30 μg/lane and were run along with Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad) in a dual vertical mini-gel apparatus with a water cooling system (CBS Scientific, Del Mar, CA) in running buffer (50 mM Tris, 0.4 M glycine, 0.1% SDS) at a constant voltage of 150-195 V for 1-2 hr.

Separated proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (Bio-Rad) using a Transblotter Transfer Apparatus (Bio-Rad) filled with transfer buffer (20 mM Tris-HC1, 150 mM glycine, 20% methanol) at a constant voltage of 100 V for 1-1.5 hr with an ice pack to prevent overheating. Nitrocellulose filters were blocked for 30 min in a solution of Tris-buffered saline, pH 8.0 (TBS) with 5% skim milk powder or bovine serum

albumin (BSA) (Fisher Scientific, Ottawa, Ontario) while shaking at room temperature (RT). The blocked filters were then incubated with the appropriate primary antibody in TBS, pH 8.0, with 0.1% Tween-20 (TBST) and 5% BSA, while shaking gently at 4^{o}C overnight. Removal of excess primary antibody was followed by 3 successive 10 min washes of the filter with TBST. Filters were then incubated with the appropriate HRP-conjugated secondary antibody in TBS with 5% skim milk powder while shaking gently for 1 hr at RT. Excess secondary antibody was removed with 3 successive 10 min washes of the filter with TBST. Filters were then incubated with Amersham™ ECL™ Western Blotting Detection Reagent (GE Healthcare, #RPN2106, Baie-D'Urfe, Quebec) or Amersham™ ECL™ Prime (GE Healthcare, #RPN2232) and exposed to Classic Blue Autoradiography Film BX (Mandel Scientific #MSF-BX810, Guelph, Ontario). Films were developed using a Kodak X-OMAT 1000A Processor (MedTec Marketing Group, Burnaby, British Columbia). If necessary, the filters were incubated with antibody stripping buffer (TBS, pH 2.0) while shaking gently for more than 20 min at RT, washed with TBS and reprobed with another primary antibody.

Densitometry was performed using the ImageJ software (National Institutes of Health, Bethesda, MD). The density of each band was expressed as a percentage of the total density of all the bands in one western blot. These percentages were then normalized to the highest percentage value within each western blot (relative density), which was the 5 min time point in all experiments, except Cofilin, which was the 60 min time point. The relative densities of the phosphorylated bands was then divided by the relative densities of the loading controls (adjusted relative densities) (Fig. 2-1). The adjusted relative densities from three experiments were averaged.



Samples		Relative Densities	Loading Control		Relative Densities	Adjusted Relative Densities
Lane 1	20%	0.66	Lane 1	20%	1	0.66
Lane 2	30%	1	Lane 2	20%	1	1
Lane 3	10%	0.33	Lane 3	20%	1	0.33
Lane 4	20%	0.66	Lane 4	10%	0.5	1.33
Lane 5	20%	0.66	Lane 5	30%	1.5	0.44

Figure 2-1. A schematic representation of the calculation of adjusted relative densities

2.2.8 Preparation of cells for flow cytometry

To assess expression of proteins on the cell surface, cells (10^6) were resuspended in 50 µl of FACS (fluorescence-activated cell sorting) buffer (PBS, 2% HI-FBS) in a 96-well plate (BD Biosciences flat bottom #351172 or preferably, round bottom #353917, Mississauga, Ontario). These cells were incubated with 1.25 µg/ml of rat anti-mouse IgM-FITC, 5 µg/ml of rat anti-mouse IgG-Alexa 488, 1.25 µg/ml of rat anti-mouse CD184/CXCR4-FITC, 1.25 µg/ml of rat anti-mouse CD11a/LFA1-FITC, 0.5 µg/ml of rat anti-mouse CD49d/VLA4-PE, or 1.25 µg/ml of the isotype control antibodies, rat IgG2a κ or rat IgG2b κ, for 30 min on ice, protected from light. Prior to staining A20 cells, 25 µg/ml of anti-mouse CD16/CD32 antibody was added to each well. FACS buffer (100 µl) was added to each well, and the plates were spun at 1,500 RPM for

5 min at 4^{o}C. Cells were then washed twice further with 150 µl FACS buffer prior to resuspension in 350 µl FACS buffer. Samples were kept on ice in round bottom polystyrene tubes (BD Biosciences, #352054), protected from light.

To quantify BCR internalization, WEHI-231 cells (10^6 cells) were resuspended in 100 µl of FACS buffer in a 96-well plate. Cells were incubated on ice for 10 min and in a 37^{o}C incubator for 20 min. They were then stimulated with 20 µg/ml goat anti-mouse IgM for the appropriate times and reactions were stopped with 100 µl of 8% paraformaldehyde (Cedarlane, Burlington , Ontario) on ice for 10 min. Cells were spun at 1,500 RPM at 4^{o}C and were resuspended in 100 µl FACS buffer. Rat anti-mouse IgM-FITC (1.25 µg/ml) was added to each well for 30 min, on ice, protected from light. 100 µl of FACS buffer was added to each well, and the plates were spun at 1,500 RPM for 5 min at 4^{o}C. The cells were then washed twice further with 150 µl FACS buffer prior to resuspension in 350 µl FACS buffer. Samples were kept in round bottom polystyrene tubes on ice, protected from light. This assay is represented graphically in Figure 2-2 and was modified from (Jang et al., 2010).

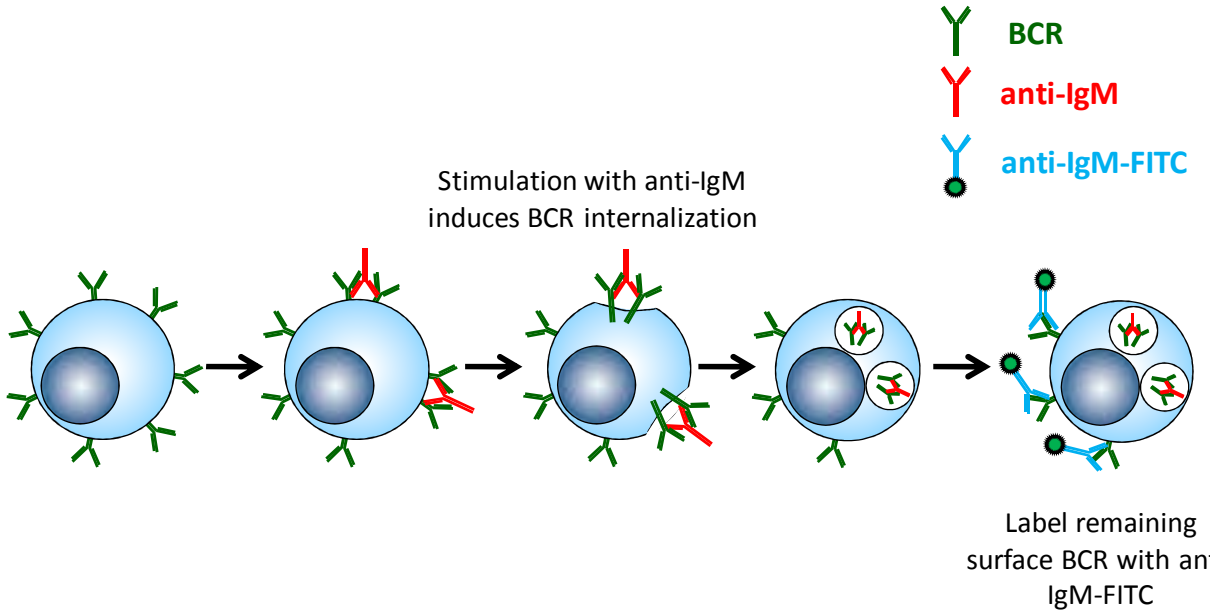


Figure 2-2. Overview of the BCR internalization assay

2.2.9 Calcium flux assay

Cells were washed twice with Hank's Buffered Saline Solution (HBSS) lacking phenol red, calcium and magnesium (Invitrogen Life Technologies, #14175095) that was supplemented with 2.5% HI-FBS, and 10 mM Hepes (Sigma-Aldrich, #H3375) pH 7.4 (adjusted with potassium hydroxide). After resuspending the cells to 10^7 cells/ml, 500 µl of cells were used per experimental sample; 200 µl were used for the unstained and single-stained control samples. The calcium sensitive dyes, Fluo-4 (Invitrogen Life Technologies, #F-14201) and Fura Red (Invitrogen Life Technologies, #F-3021) were prepared as a 1 mM stock, and final concentrations of 1 µM and 2 µM were used, respectively. Pluronic acid F-127 (Invitrogen Life Technologies, #P-3000MP) was used at a final of 0.02% to facilitate dye entry by eliminating possible hydrolysis by external esterases, and to facilitate more efficient cell loading while maintaining cell integrity. A 2x solution of the Fluo-4, Fura Red and Pluronic acid in supplemented HBSS was added to an equal volume of cells. For the single stain controls,

pluronic acid and either Fluo-4 or Fura Red were added. Cells were then incubated at 37^{o}C for 45 min, protected from light. Supplemented HBSS was used to wash the cells once and to resuspend the cells to 1 ml. The cells were then allowed to rest for 20 min at RT, protected from light. Cells were then resuspended in 3 ml of supplemented HBSS and transferred to round bottom polystyrene tubes, each with 500 µl, such that each sample has approximately 1-2 x 10^6 cells. Samples were kept on ice, protected from light until assayed by flow cytometry.

2.2.10 Flow cytometry data collection and analysis

Samples were acquired on a BD LSRII Flow Cytometer (BD Biosciences) using the BD FACS Diva software (BD Biosciences). The appropriate lasers were chosen according to the fluorophore-conjugated secondary antibodies used. Data was analyzed using FlowJo Flow Cytometry analysis software (Tree Star Inc., Ashland, OR).

For surface staining and the BCR internalization experiments, cells were gated on forward scatter/side scatter (FSC/SSC) to exclude debris, and the mean fluorescence intensity (MFI) was determined using FlowJo. FSC is roughly proportional to the diameter of the cell, and SSC is proportional to the granularity. For the surface staining experiments, values were normalized to the MFI of membrane IgM for WEHI-231, and membrane IgG for A20. For the BCR internalization experiments, values were normalized to the MFI of IgM in the unstimulated samples.

For the calcium flux experiments, cells were warmed to RT before acquiring. Samples were acquired for 1 min to generate a baseline, and then 20 µg/ml goat anti-mouse IgM was

added to the sample which was then further analyzed for 5 min. A second stimulus, ionomycin (Invitrogen Life Technologies, #I-24222), was used as a positive control to saturate the Ca^{2+}-sensing dyes in the cells. Cells were gated on FSC/SSC, and a parameter was created for the ratio of Fluo-4 to Fura Red, using FITC:PerCP or FITC:PerCPCy5.5. The kinetics of the calcium flux was analyzed using this parameter. Peaks and slopes were analyzed and compared.

2.2.11 Anti-Ig dose response curves and growth curves

To assess the sensitivity of WEHI-231 cells to anti-IgM, decreasing amounts of goat anti-mouse IgM, diluted in 30 µl supplemented RPMI were then added to black with clear bottom 384-well plates (BD Biosciences, #353962). WEHI-231 cells were resuspended to 1000 cells in 20 µl of supplemented RPMI and added to each well. A 0.2 µg/ml concentration of puromycin dihydrochloride (Calbiochem/EMD Millipore, #540222, Etobicoke, Ontario) was used as a control to induce cell death. Plates were incubated at 37^{o}C for 2 days. A 1:10 dilution of a 0.01% w/v stock solution of Alamar Blue (Resazurin sodium salt) (Sigma-Aldrich, #R7017) was added to the wells 4 hr prior to reading the fluorescence (A_{590}).

To assess cell growth, WEHI-231 cells were resuspended to 1000 cells in 50 µl of supplemented RPMI and added to each well of clear bottom 384-well plates. Plates were incubated at 37^{o}C for 3 days and a 1:10 dilution of a 0.01% w/v stock solution of Alamar Blue was added to the wells 4 hr prior to reading the fluorescence (A_{590}).

A590 was determined using a SPECTRAmax® GEMINI-XS spectrophotometer (Molecular Devices, Sunnyvale, CA) and values were expressed as arbitrary fluorescence units.

2.2.12 Statistics

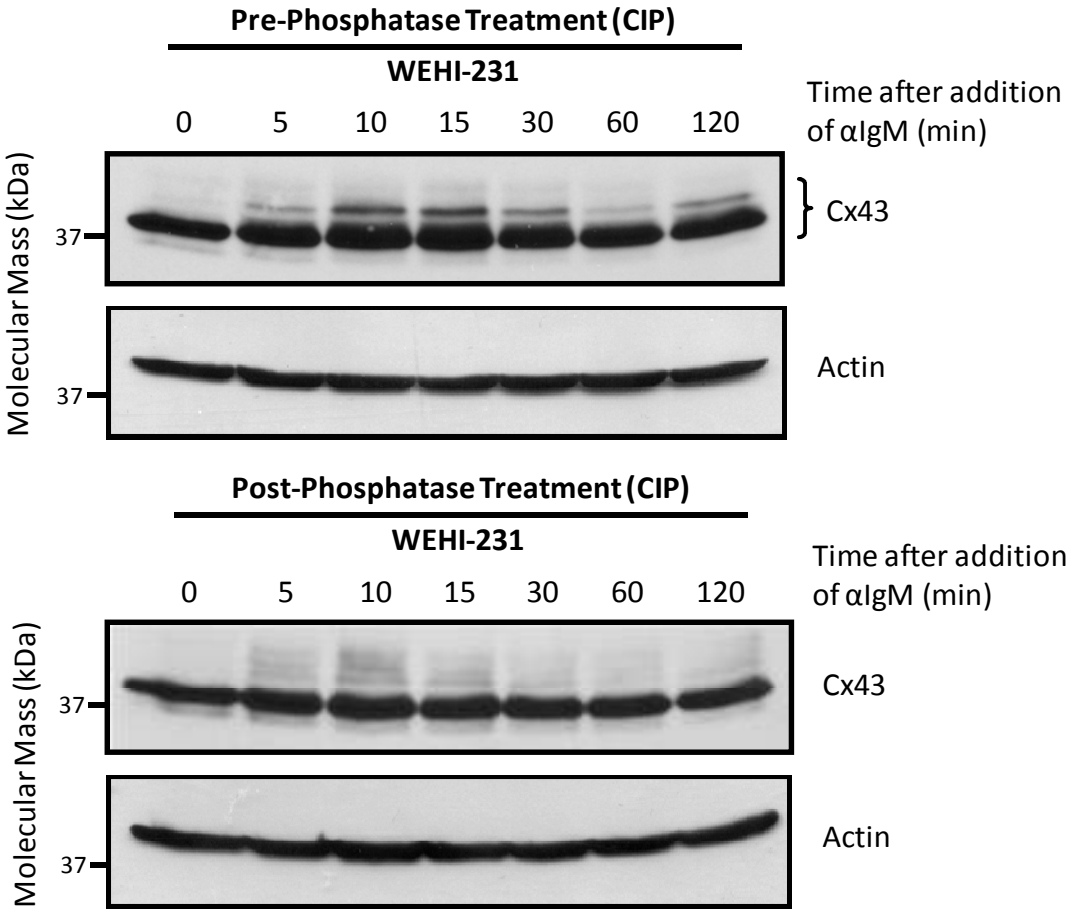
Student's paired two-tailed t-test was used to compare sets of matched samples using Microsoft Excel.

2.3 Results

2.3.1 Cx43 is a target of BCR and CXCR4 signaling

Cx43 is expressed in immature and mature B cell lines, but not in plasmacytoma cell lines (Machtaler et al., 2011). The broad bands observed on Cx43 western blots in these studies are consistent with previous reports of differential phosphorylation of Cx43 in mammalian cells, including normal rat kidney cells, rat mammary tumor cells and mouse fibroblasts (Lampe and Lau, 2000). To determine whether Cx43 is phosphorylated in B cells and whether such phosphorylation is altered by receptor signaling, the WEHI-231 IgM^+ immature murine B cell line was stimulated with either anti-IgM or the chemokine receptor CXCR4 ligand CXCL12/SDF-1. A substantial bandshift was first observed at 5 min after addition of these stimuli. This response was maximal at 15 min and began to decline after 30 min (Fig. 2-3). To test the idea that the anti-IgM- and CXCL12-induced bandshift to a higher molecular weight was due to phosphorylation of Cx43, lysates were treated with calf intestinal alkaline phosphatase (CIP) prior to SDS-PAGE separation. This resulted in a disappearance of the bandshifts, suggesting that the receptor-mediated band shifts were due to the phosphorylation of Cx43. Thus, Cx43 is a downstream target of both BCR and CXCR4 signaling. Stimulating with CXCL12 resulted in phosphorylation that was less sustained than stimulating with anti-IgM. This may be because B cells need to bind chemokines to direct their migration, and shorter chemokine receptor signaling may allow cells to keep sensing new chemokine signals.

A


Pre-Phosphatase Treatment (CIP)
WEHI-231
Time after addition of αIgM (min)
0 5 10 15 30 60 120
Molecular Mass (kDa)
37
Cx43
37
Actin
Post-Phosphatase Treatment (CIP)
WEHI-231
Time after addition of αIgM (min)
0 5 10 15 30 60 120
Molecular Mass (kDa)
37
Cx43
37
Actin

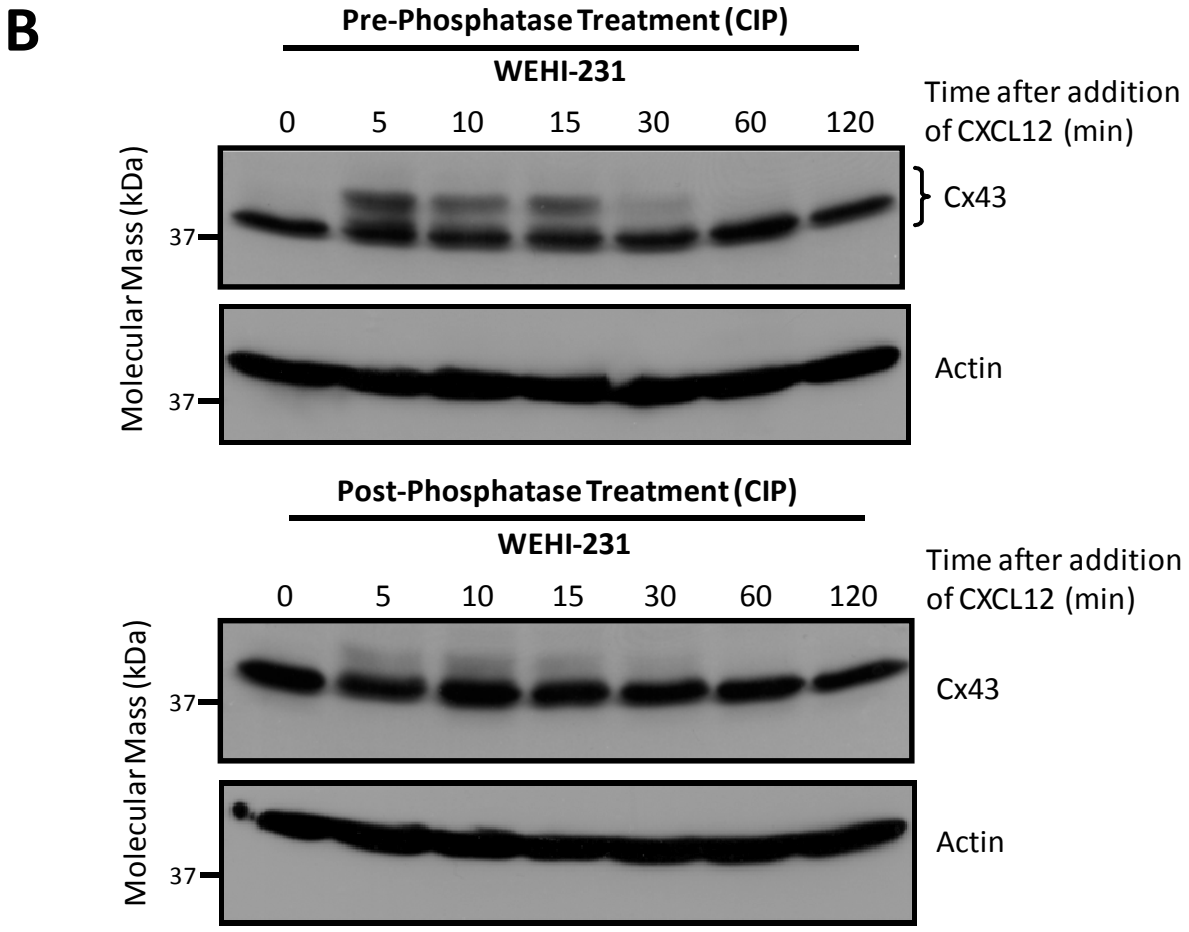


Figure 2-3 Cx43 is phosphorylated in response to BCR and CXCR4 stimulation
WEHI-231 cells were stimulated with either 20 μg/ml anti-IgM **(A)** or 100 nM CXCR4 ligand, CXCL12 **(B)**, and lysates were divided into two equal aliquots, one of which was treated with calf intestinal alkaline phosphatase (CIP) and the other with buffer alone. Proteins were separated by SDS-PAGE and Cx43 was detected by immunoblotting with an antibody against the cytoplasmic tail (Sigma-Aldrich, see Section 2.3.1). Blots were stripped and reprobed for actin to assess equal protein loading. For each panel, similar results were obtained in three independent experiments. Reprinted with permission (Machtaler et al., 2011).

2.3.2 Characterization of B cell lines where Cx43 has been knocked down using shRNA

Our goal was to assess the effect of the knockdown in expression of the Cx43 protein on BCR-induced spreading and chemokine-mediated migration, adhesion and extravasation. As a

loss of function approach for studying the function of Cx43 in B cells, our lab used shRNA-encoding vectors to generate stable populations of the WEHI-231 B lymphoma cell line in which Cx43 expression is knocked down. (Fig. 2-4A). The WEHI-231 immature murine B cell line has been commonly used to study BCR signaling (Gold et al., 1999; Grandoch et al., 2009) as well as B cell migration and adhesion (Durand et al., 2006; McLeod et al., 2002; McLeod et al., 2004). Previous work showed that the knockdown of Cx43 using shRNA 2 in WEHI-231 cells resulted in decreased BCR-induced spreading, as indicated by a smaller contact area, than using shRNA 1+2 (Machtaler et al., 2011). Therefore, in subsequent studies, I used only shRNA 2. In order for these knockdown cell lines to be a useful tool for studying the role of Cx43 in B cells, we must first ensure that they do not have irrelevant alterations that would complicate the interpretation of these studies. Therefore, I asked whether the cell surface expression of relevant signaling receptors (BCR, CXCR4) and integrins (LFA-1, VLA-4) was similar in control and knockdown cells (Fig. 2-4B). I also asked whether the rate of anti-IgM-induced BCR internalization (Fig. 2-4C), through flow cytometry, was identical in the knockdown cells and cells transduced with a scrambled shRNA. The expression of BCR, CXCR4, LFA-1 and VLA-4 as well as the rate of anti-IgM-induced BCR internalization were similar in the Cx43 knockdown (Cx43KD) cells. I also showed that there were no changes in the growth rate of the Cx43KD WEHI-231 cells (Fig. 2-5A) or in the ability of anti-IgM antibodies to induce growth arrest and death in these cells (Fig. 2-5B), as measured by Alamar Blue (Resazurin sodium salt), an indicator of metabolic activity. These results show that the knockdown of Cx43 does not affect cell growth, expression of signaling receptors and integrins, or the sensitivity to anti-IgM.

Resazurin is a non-fluorescent, blue colored, redox indicator dye, which is converted into the red-fluorescent, pink colored, resorufin via reduction reactions of metabolically active (live) cells. The amount of fluorescence and color produced is proportional to the number of living cells. It has a peak excitation wavelength of 535 nm and a peak emission wavelength of 590 nm.

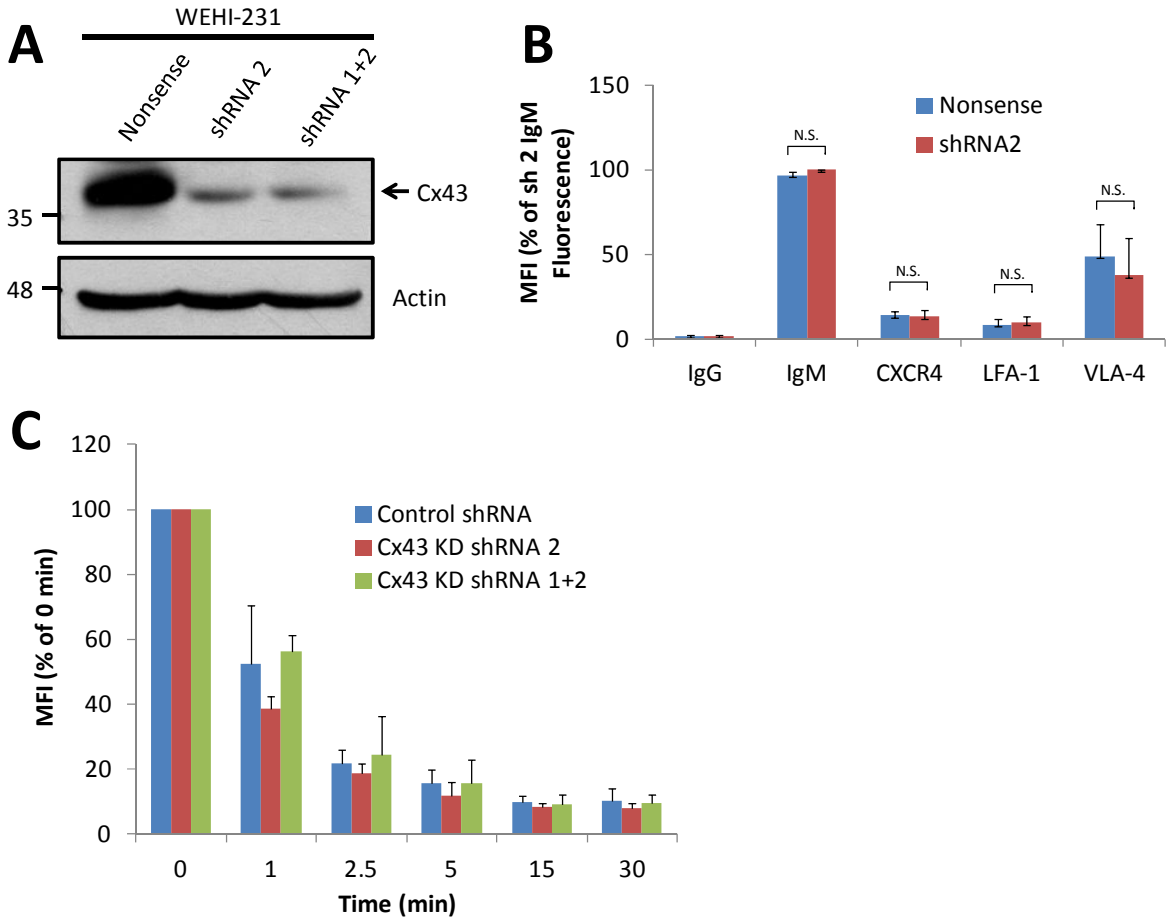


Figure 2-4. Decreased Cx43 expression does not alter surface receptor levels or anti-IgM-induced BCR internalization

(A) Cx43 protein levels in WEHI-231 cells that were transduced with retroviruses encoding either nonsense shRNA (control) or Cx43 shRNA 2 (knockdown) were assessed by immunoblotting. Actin was used as a loading control. This was done prior to each sigaling experiment that used the same cell lysates.

(B) Surface expression of key cell surface proteins in WEHI-231 control (nonsense shRNA) and Cx43KD cells were assessed by flow cytometry using fluorophore-conjugated antibodies. The graph shows data from three independent experiments (mean + SEM). N.S., not significant.

(C) To measure the rate of anti-IgM-induced BCR internalization, WEHI-231 cells were exposed to goat anti-mouse IgM antibodies at 37^{o}C for the indicated times before being fixed and stained with rat anti-mouse IgM-FITC. The graph shows data from three independent experiments (mean + SEM).

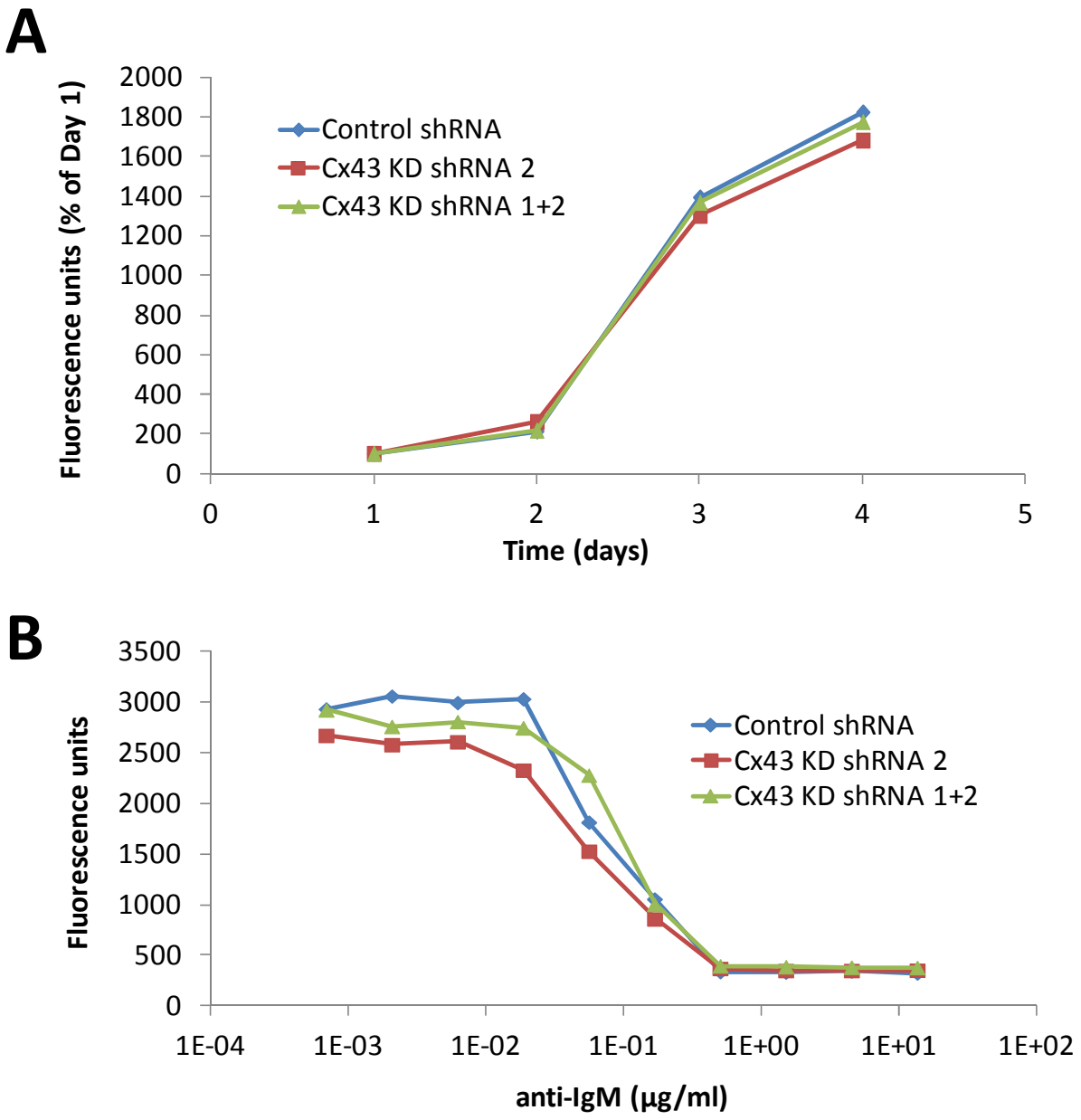


Figure 2-5. Decreased Cx43 expression does not alter growth rate or anti-IgM-induced cell death

(A) WEHI-231 cells were cultured in supplemented media and relative cell numbers were measured daily using the metabolic indicator Alamar Blue.

(B) WEHI-231 cells were cultured with various concentrations of 20 µg/ml anti-IgM. After 48 hours, cell viability was measured using the metabolic indicator Alamar Blue.

2.3.3 Cx43 is involved with the BCR-induced phosphorylation of PLCγ and Erk but not in Akt activation in WEHI-231 Cells

Others in the lab have shown that the WEHI-231 Cx43KD cells exhibit a decreased ability to sustain the activation of Rap1 GTPase induced by stimulating either the BCR or CXCR4 (Machtaler et al., 2011). Rap activation is essential for B cell spreading (Lin et al., 2008) and we found that the Cx43KD cells exhibit an impaired ability to spread on immobilized anti-Ig or migrate in response to CXCL12 (Machtaler et al., 2011). The BCR activates the Rap1 GTPase via activation of phospholipase Cγ2 (PLCγ2), which leads to the production of diacylglycerol (DAG). DAG, as well as the synthetic analog, phorbol myristate acetate (PMA), binds to DAG-dependent guanine exchange factors that activate Rap1 GTPases such as CalDAG-GEFs and RasGRPs (Ghandour et al., 2007; Stone, 2011). In contrast to BCR-induced Rap1 activation, PMA-induced Rap1 activation is sustained in Cx43KD cells, comparable to the response seen in control cells (Machtaler et al., 2011). This suggests that Cx43 acts upstream of DAG generation by PLCγ2 and either enhances BCR-induced activation of the PLCγ2→DAG pathway or globally enhances BCR signaling.

To distinguish these possibilities, I looked at activation of the major BCR signaling pathways (PLCγ, Ras/Erk, PI3K/Ak) in Cx43KD versus control WEHI-231 cells. During BCR signaling, PLCγ2, Ras/Erk and PI3K/Akt pathways regulate many cellular responses including gene expression, cell cycle progression and cytoskeletal reorganization (Fig. 1-4). All of these pathways are critical for B cell growth, survival, proliferation and differentiation. I found that loss of Cx43 caused a trend towards lower phosphorylation of PLCγ2 (Fig. 2-6A,B), compared to the response seen in the control cells. These results indicate that Cx43 is important in the

sustained activation of PLCγ2, which coincides with the previous finding that Cx43 is important in sustained Rap1 activation.

The second major BCR signaling pathway involves Erk. Because Erk is downstream of PLCγ2 via Ras GTPases, I asked whether Cx43 is important for Erk activation as well. I found that Cx43KD cells had impaired phosphorylation of Erk (p42 and p44 isoforms) compared to control cells (Fig. 2-7A). Anti-IgM stimulation of control cells induced high levels of Erk phosphorylation at 5 min, which slowly decreased over 60 min. However, Erk phosphorylation was at much lower levels throughout the time course in Cx43KD cells. In contrast, the phosphorylation of Akt, the third major signaling pathway, was unaffected by the loss of Cx43 (Fig. 2-7B). This suggests that Cx43 selectively modulates BCR-induced activation of PLCγ2, as opposed to being a global regulator of early BCR signaling events.

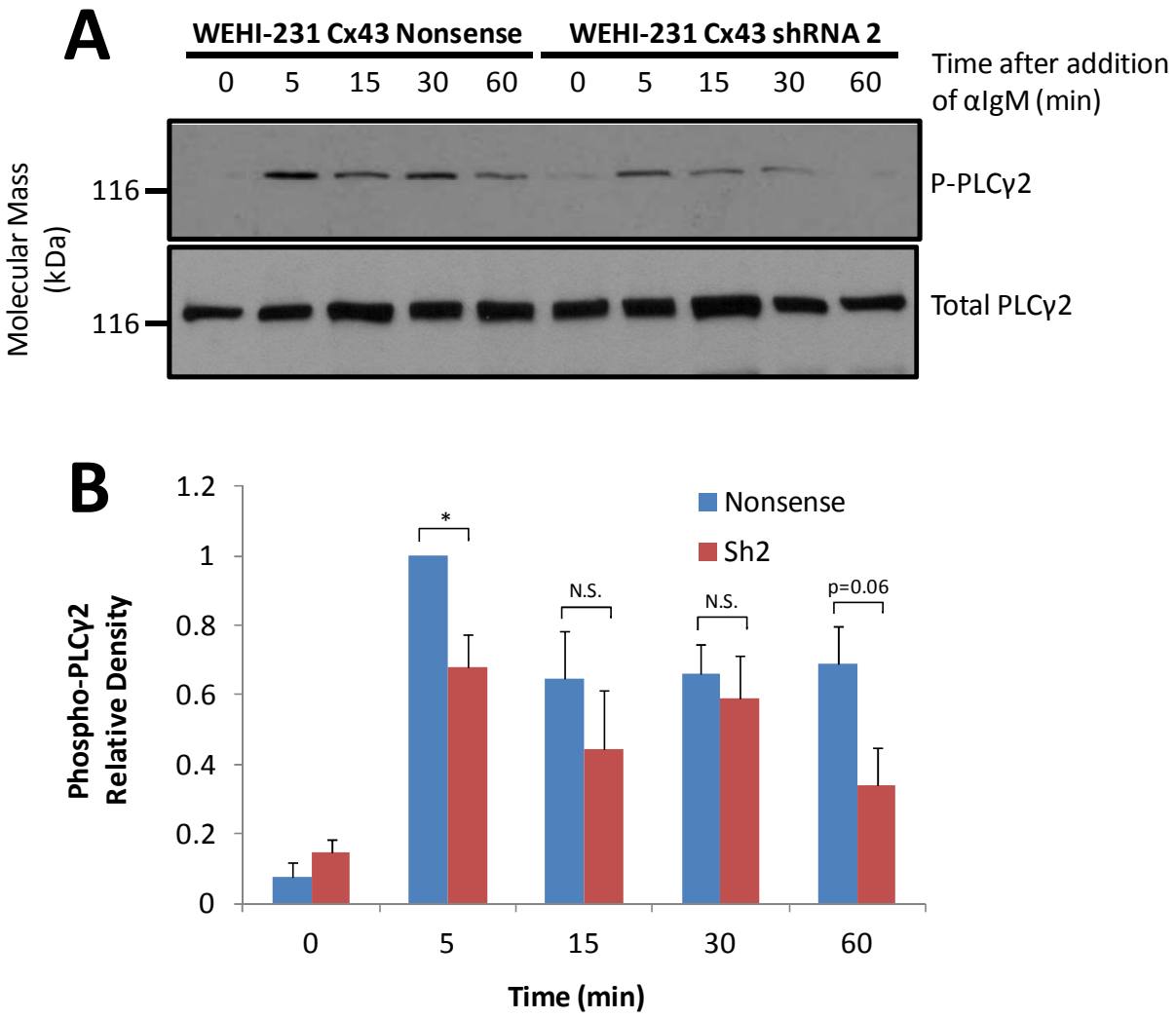
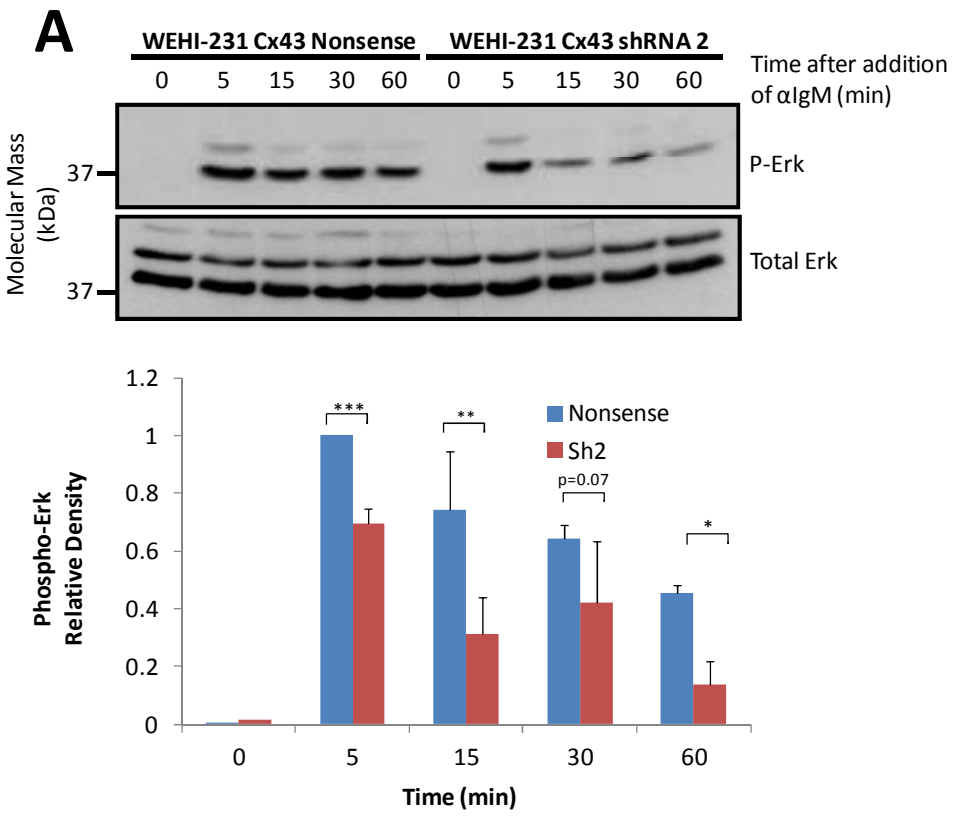


Figure 2-6. Cx43 is important for the activation of PLCγ2

WEHI-231 cells were retrovirally transduced with either control shRNA (blue) or Cx43 shRNA 2 (red). The cells were stimulated with 20 µg/ml anti-IgM for the times indicated. Lysates were probed for phosphorylated PLCγ2 (Tyr1217). Western blot is the representative data from four independent experiments. Graphs are presented as average adjusted relative densities + SEM (n = four independent experiments), relative to the highest density time point, 5 min. N.S. = not significant; * = $p<0.05$.


A
WEHI-231 Cx43 Nonsense
WEHI-231 Cx43 shRNA 2
0 5 15 30 60 0 5 15 30 60
Time after addition of αIgM (min)
Molecular Mass (kDa)
37
37
P-Erk
Total Erk
Phospho-Erk Relative Density
1.2
1
0.8
0.6
0.4
0.2
0
***
**
p=0.07
*
Nonsense
Sh2
0 5 15 30 60
Time (min)

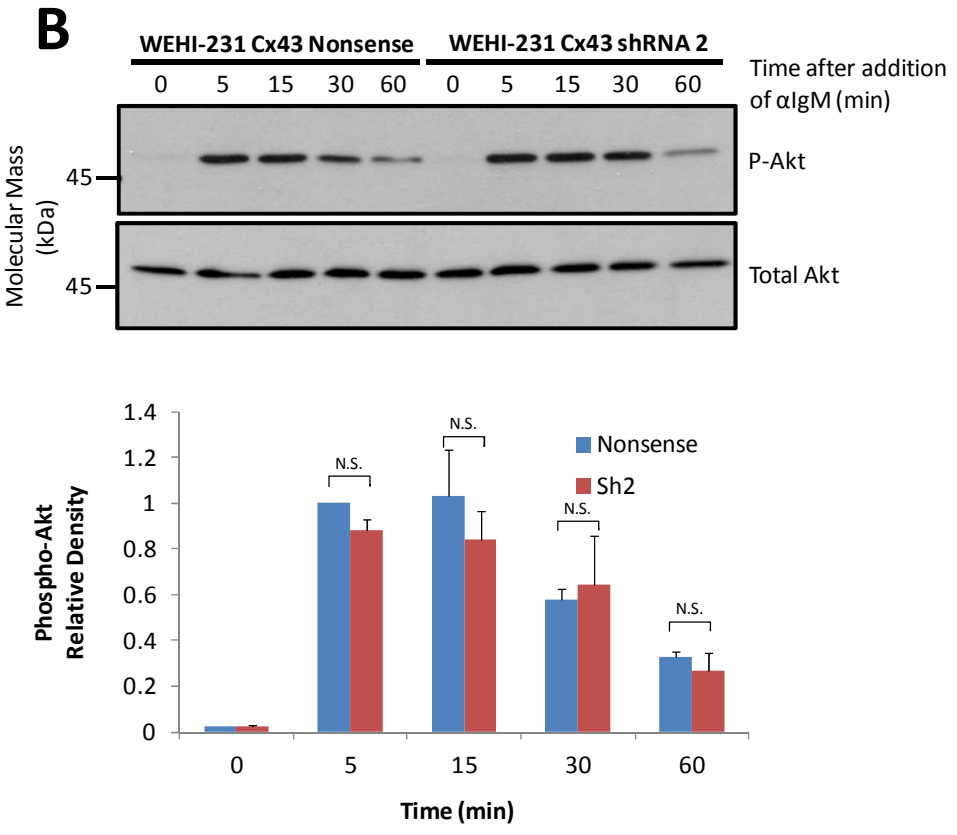
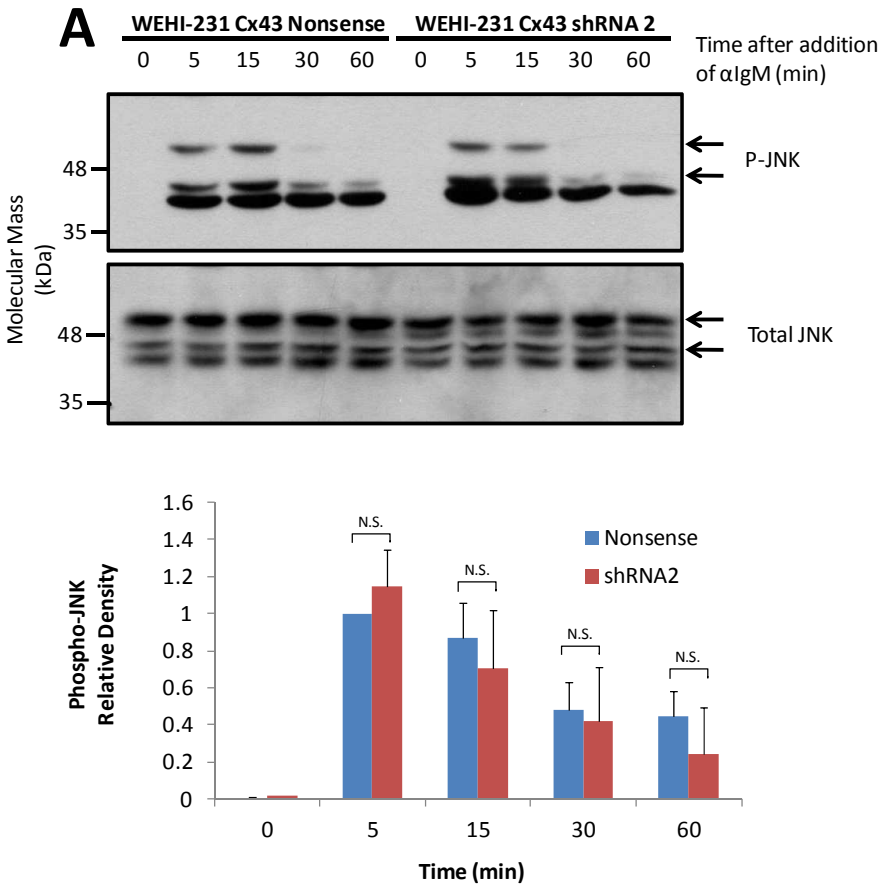

B
WEHI-231 Cx43 Nonsense
WEHI-231 Cx43 shRNA 2
0 5 15 30 60 0 5 15 30 60
Time after addition of αIgM (min)
Molecular Mass (kDa)
45
45
P-Akt
Total Akt
Phospho-Akt Relative Density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
N.S.
N.S.
N.S.
Nonsense
Sh2
0 5 15 30 60
Time (min)

Figure 2-7. Knockdown of Cx43 does not alter the BCR-induced phosphorylation of Akt but decreases phosphorylation of Erk

WEHI-231 cells that were retrovirally transduced with either control shRNA (blue) or Cx43 shRNA 2 (red) were stimulated with 20 μg/ml anti-IgM for the indicated times. Lysates were probed for **(A)** phosphorylated Erk (Thr202/Tyr204) or **(B)** phosphorylated Akt (Ser473). Western blots are the representative data from three independent experiments. Graphs are presented as average adjusted relative densities + SEM (n = three independent experiments), relative to the highest density time point, 5 min. N.S. = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

2.3.4 Cx43 is not involved with the BCR-induced phosphorylation of the JNK and p38 MAP kinases in WEHI-231 Cells

Given that the knockdown of Cx43 did not impair Akt activation, I examined the phosphorylation of JNK/SAPK and p38 MAPK, which are also involved in the regulation of B cell growth, survival, proliferation and differentiation. Both the activation of JNK/SAPK and p38 MAPK in response to BCR stimulation with anti-IgM were assessed by western blot. The phosphorylation of the two isoforms of JNK/SAPK, p46 and p54, named according to their molecular weights, was unaffected by the loss of Cx43 (Fig. 2-8A). Stimulation of both nonsense and Cx43 shRNA 2 transduced WEHI-231 cells through the BCR induced high levels of JNK/SAPK phosphorylation at 5 and 15 min, which decreased at 30 min. Similarly, BCR-induced phosphorylation of p38 MAPK was not impaired by the knockdown of Cx43 (Fig. 2-8B). Phosphorylation of p38 MAPK increased at 5 min and persisted until 60 min. Therefore, given that BCR-induced phosphorylation of PLCγ and ERK were affected by Cx43 expression and Akt, JNK, and p38 were not, Cx43 expression selectively affects some signaling responses as opposed to or more so than others.


A
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WEHI-231 Cx43 shRNA 2
0 5 15 30 60 0 5 15 30 60
Time after addition of αIgM (min)
Molecular Mass (kDa)
48
35
P-JNK
48
35
Total JNK
Phospho-JNK Relative Density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
Nonsense
shRNA2
0 5 15 30 60
Time (min)

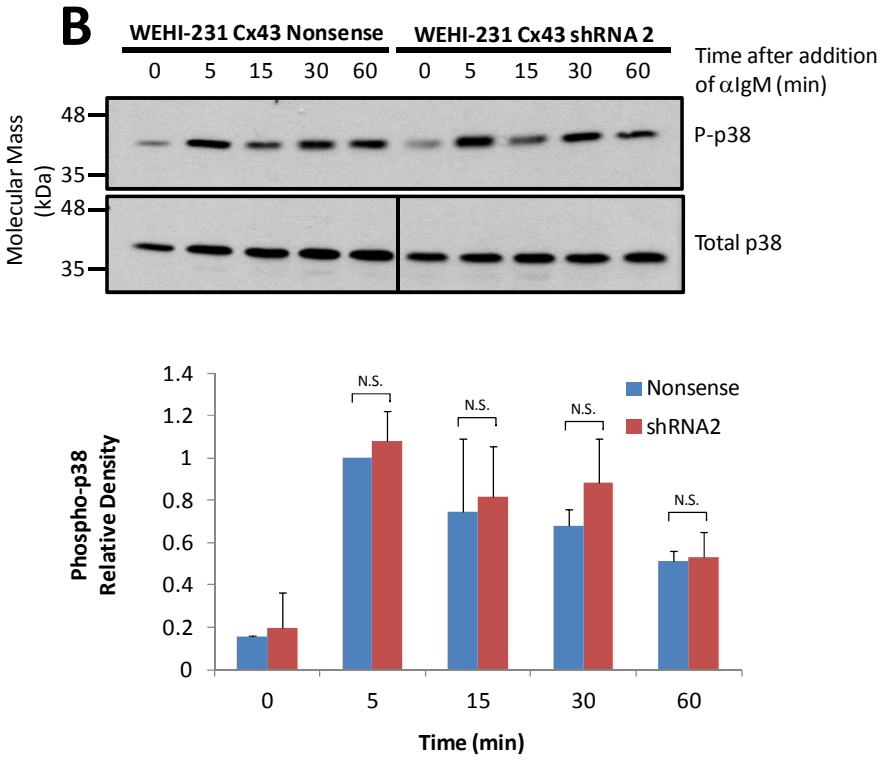

B
WEHI-231 Cx43 Nonsense
WEHI-231 Cx43 shRNA 2
0 5 15 30 60 0 5 15 30 60
Time after addition of αIgM (min)
Molecular Mass (kDa)
48
35
P-p38
48
35
Total p38
Phospho-p38 Relative Density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
Nonsense
shRNA2
0 5 15 30 60
Time (min)

Figure 2-8. Knockdown of Cx43 does not alter the BCR-induced phosphorylation of JNK and p38

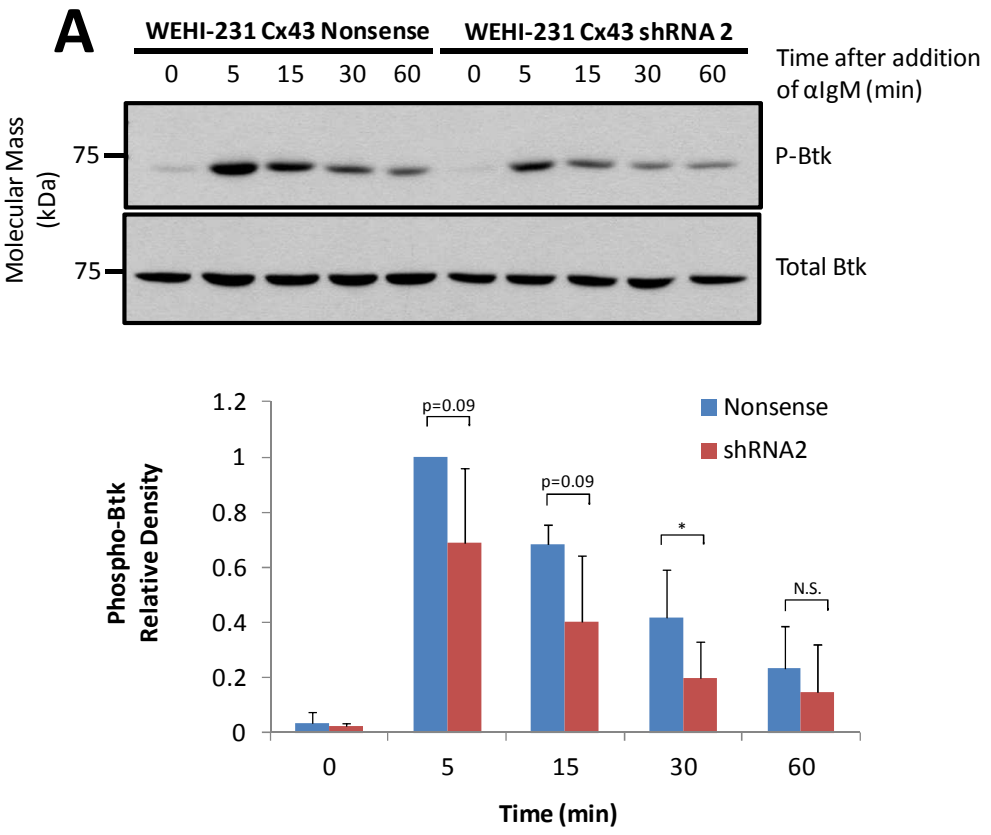
WEHI-231 cells were retrovirally transduced with either control shRNA (blue) or Cx43 shRNA 2 (red). The cells were stimulated with 20 μg/ml anti-IgM for the indicated times. Lysates were probed for **(A)** phosphorylated JNK (Thr183/Tyr185) or **(B)** phosphorylated p38 (Thr180/Tyr182). Western blots are the representative data from three independent experiments. Graphs are presented as average adjusted relative densities + SEM (n = three independent experiments), relative to the highest density time point, 5 min. N.S. = not significant.

2.3.5 Cx43 is involved with the BCR-induced phosphorylation of Lyn, Syk, and Btk

Because the loss of Cx43 interferes with PLCγ2 activation, I investigated BCR signaling upstream of PLCγ2, including the activation of proximal BCR signaling molecules such as Lyn, Syk and Btk (Fig. 1-4). PLCγ2 is phosphorylated by both Btk and Syk (Section 1.4.3) so I asked whether the phosphorylation of these enzymes is affected when Cx43 is knocked down. Clustering the BCR with anti-IgM induced phosphorylation of Btk at 5 min in both WEHI-231 transfected with nonsense shRNA and WEHI-231 transfected with Cx43 shRNA, and began to decline in the later time points. However, in Cx43KD cells, the phosphorylation of Btk was less at all time points compared to the control cells (Fig. 2-9A). Syk phosphorylation was also impaired when Cx43 was knocked down. In both control and Cx43KD cells, there was a similar strong increase in the phosphorylation of Syk at 5 min, which decreased thereafter. However, the phosphorylation of Syk in Cx43KD cells decreased much faster than the control cells (Fig. 2-9B).

Although Lyn is one of the Src family kinases that is responsible for the phosphorylation of Syk and Btk (Section 1.4.3), Lyn plays a bigger role in the negative regulation of BCR

signaling (Xu et al., 2005). The phosphorylation of the Y508 residue causes Lyn to become inactive. I found that in WEHI-231 cells with Cx43 knocked down, Lyn phosphorylation was impaired during BCR signaling although BCR-induced Lyn phosphorylation was comparable to that in control cells at 5 min (Fig. 2-9C). This means that the expression of Cx43 enhances Lyn phosphorylation on the inactivation site, preventing its negative effects on BCR signaling. Altogether, the loss of Cx43 impairs the activation of multiple BCR signaling molecules that are upstream of PLCγ2 activation.


A
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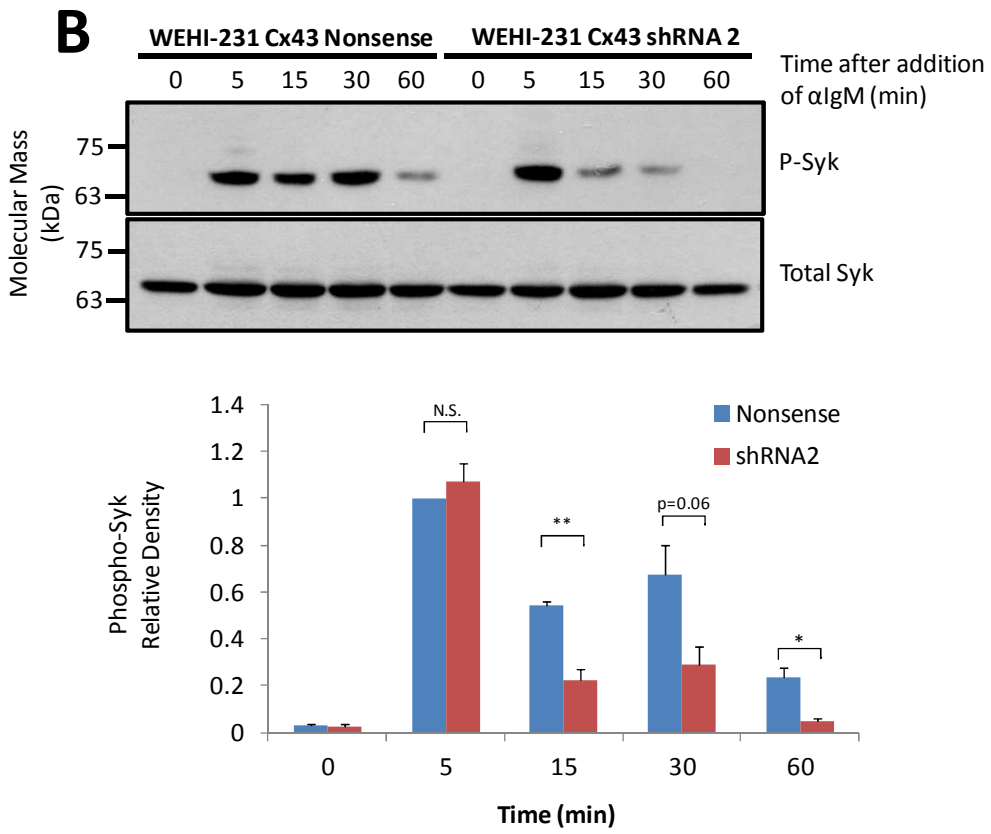
WEHI-231 Cx43 shRNA 2
0 5 15 30 60 0 5 15 30 60
Time after addition of αIgM (min)
Molecular Mass (kDa)
75
P-Btk
75
Total Btk
Phospho-Btk Relative Density
1.2
1
0.8
0.6
0.4
0.2
0
p=0.09
p=0.09
*
N.S.
Nonsense
shRNA2
0 5 15 30 60
Time (min)


B
WEHI-231 Cx43 Nonsense
WEHI-231 Cx43 shRNA 2
0 5 15 30 60 0 5 15 30 60
Time after addition of αIgM (min)
Molecular Mass (kDa)
75
63
P-Syk
75
63
Total Syk
Phospho-Syk Relative Density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
**
p=0.06
*
Nonsense
shRNA2
0 5 15 30 60
Time (min)

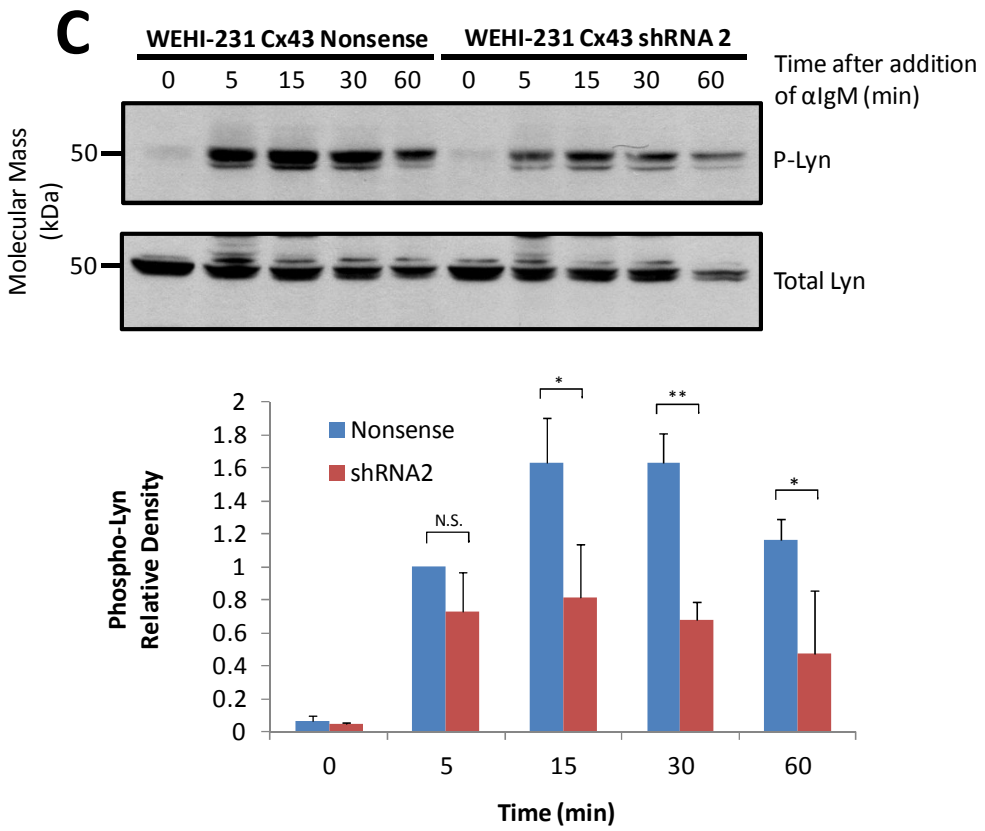


Figure 2-9. Cx43 is important for BCR-induced activation of Btk, Syk, and Lyn

WEHI-231 cells were retrovirally transduced with either control shRNA (blue) or Cx43 shRNA 2 (red) and then stimulated with 20 µg/ml anti-IgM for the indicated times. The same lysates were probed for **(A)** phosphorylated Btk (Tyr223), **(B)** phosphorylated Syk (Tyr519/520) or **(C)** phosphorylated Lyn (Tyr507). Western blots are the representative data from three independent experiments. Graphs are presented as average adjusted relative densities + SEM (n = three independent experiments), relative to the highest density time point, 5 min. N.S. = not significant; * = $p<0.05$; ** = $p<0.01$.

2.3.6 Cx43 is involved with the BCR-induced activation of Cofilin, but not calcium release

I have shown that when WEHI-231 cells with Cx43 knocked down are stimulated through the BCR using anti-IgM, PLCγ2 phosphorylation is impaired. This is consistent with the activation of Rap1 GTPase, one of the downstream effectors of PLCγ2, being impaired

(Machtaler et al., 2011). Therefore, I hypothesize that BCR-induced activation of other signaling events that are downstream of PLCγ2 and Rap would be also impaired, specifically calcium signaling and Cofilin activation. The activation of PLCγ2 leads to the production of DAG and inositol trisphosphate (IP_3). IP_3 binds to receptors on the ER and initiates the release of calcium into the cytoplasm. On the other hand, as described in Section 1.4.3, DAG is involved in the activation of the Rap1 GTPases, and activated Rap1 promotes the activation of Cofilin, and actin-severing protein (Freeman et al., 2011).

Using real time flow cytometry and calcium sensitive dyes (Fluo-4 and Fura Red), I found that there is no difference in cytosolic calcium release between WEHI-231 cells transduced with either nonsense shRNA or Cx43 shRNA. (Fig. 2-10A,B). Cells were collected using the flow cytometer for 1 min to collect the base line. Anti-IgM was then added to the cells, which induced a small calcium peak that lasted approximately 2 min and then returned to approximately basal levels. Ionomycin was then added to establish maximum calcium flux as a positive control for dye loading. Ca^{2+} peaks induced by anti-IgM are not as important as the subsequent very low sustained levels of Ca^{2+}. Because the sustained levels of activated PLCγ2 are impaired in WEHI-231 Cx43KD, we must look at longer time points of the Ca^{2+} readout, NF-AT nuclear localization, as discussed in the Section 4.2.1.

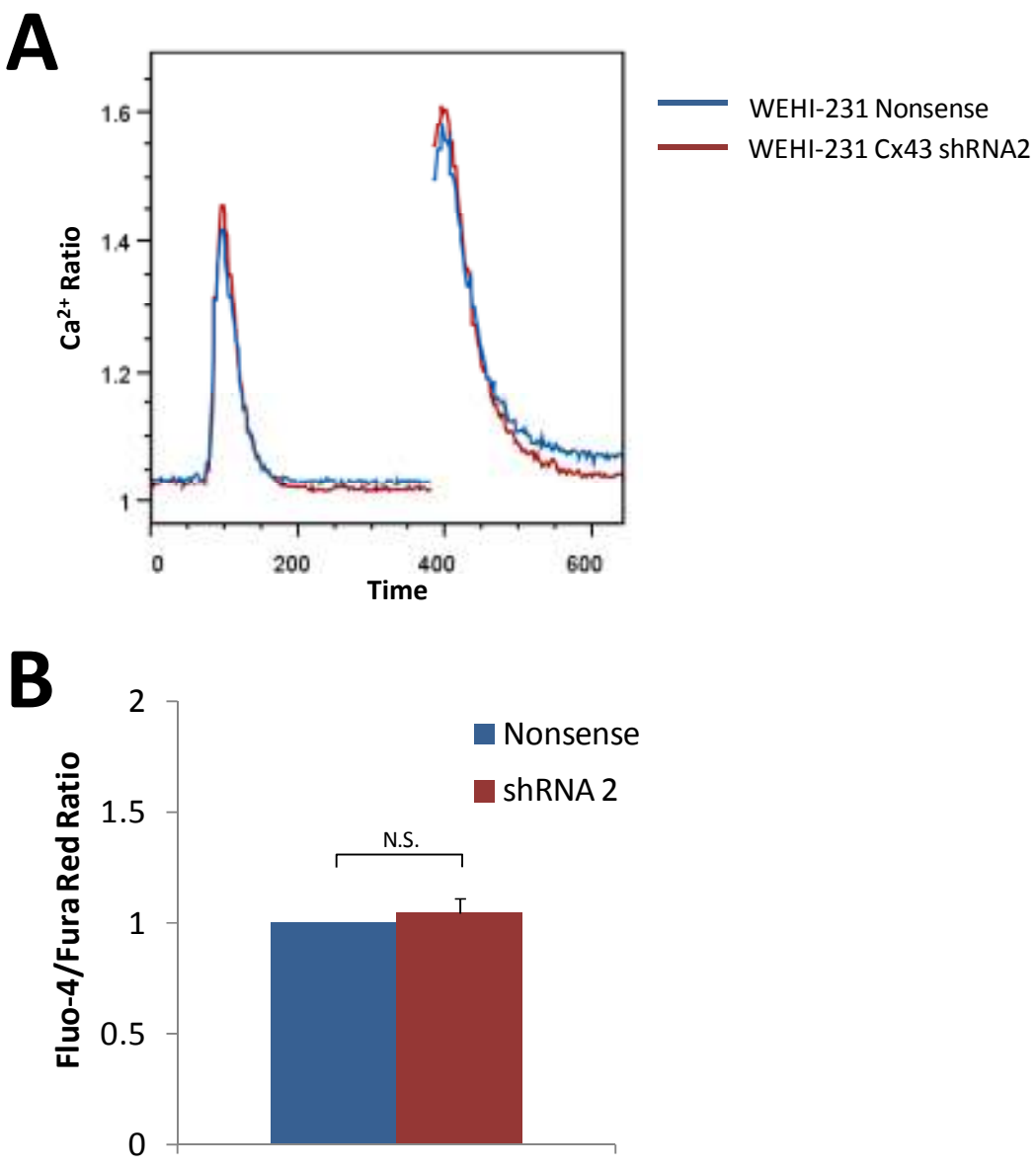


Figure 2-10. Cx43 is not involved in BCR-induced calcium flux

WEHI-231 cells were retrovirally transduced with either control shRNA (blue) or Cx43 shRNA 2 (red) and loaded with Fluo-4 and Fura Red calcium-sensing dyes. Samples were acquired for 1 min and then stimulated with 20 µg/ml anti-IgM for 5 min. Ionomycin (1 µg/ml) was then added for 4 min as a dye control to achieve maximum flux. Kinetics are based on the Fluo-4/Fura Red ratio. **(A)** The histogram is the representative data from three independent experiments. **(B)** The graph is presented as average relative value of the anti-IgM-induced Ca^{2+} peak + SEM (n = three independent experiments), relative to WEHI-231 nonsense. N.S. = not significant.

The activated form of Cofilin is dephosphorylated, which can act on actin filaments by severing them, allowing new actin networks to be assembled (Pollard and Borisy, 2003), which is important for B cell spreading and migration (Freeman et al., 2011). I found that when WEHI-231 cells with Cx43 knocked down were stimulated with soluble anti-IgM, the phosphorylation pattern of Cofilin was different compared to the control cells. In control WEHI-231 nonsense shRNA cells, Cofilin was mildly phosphorylated at 0 min and this phosphorylation decreased until 15 min. Cofilin was then rephosphorylated by 30 min (Fig. 2-11). In the Cx43 shRNA 2 cells, Cofilin was also mildly phosphorylated at 0 min and was slowly dephosphorylated until 15 min. However, the levels of Cofilin rephosphorylation did not reach the same levels as the control cells at 30 min and 60 min. This suggests that the rephosphorylation of Cofilin is impaired in cells that lack Cx43, and subsequently, the regulation of actin severing may be impaired as well.

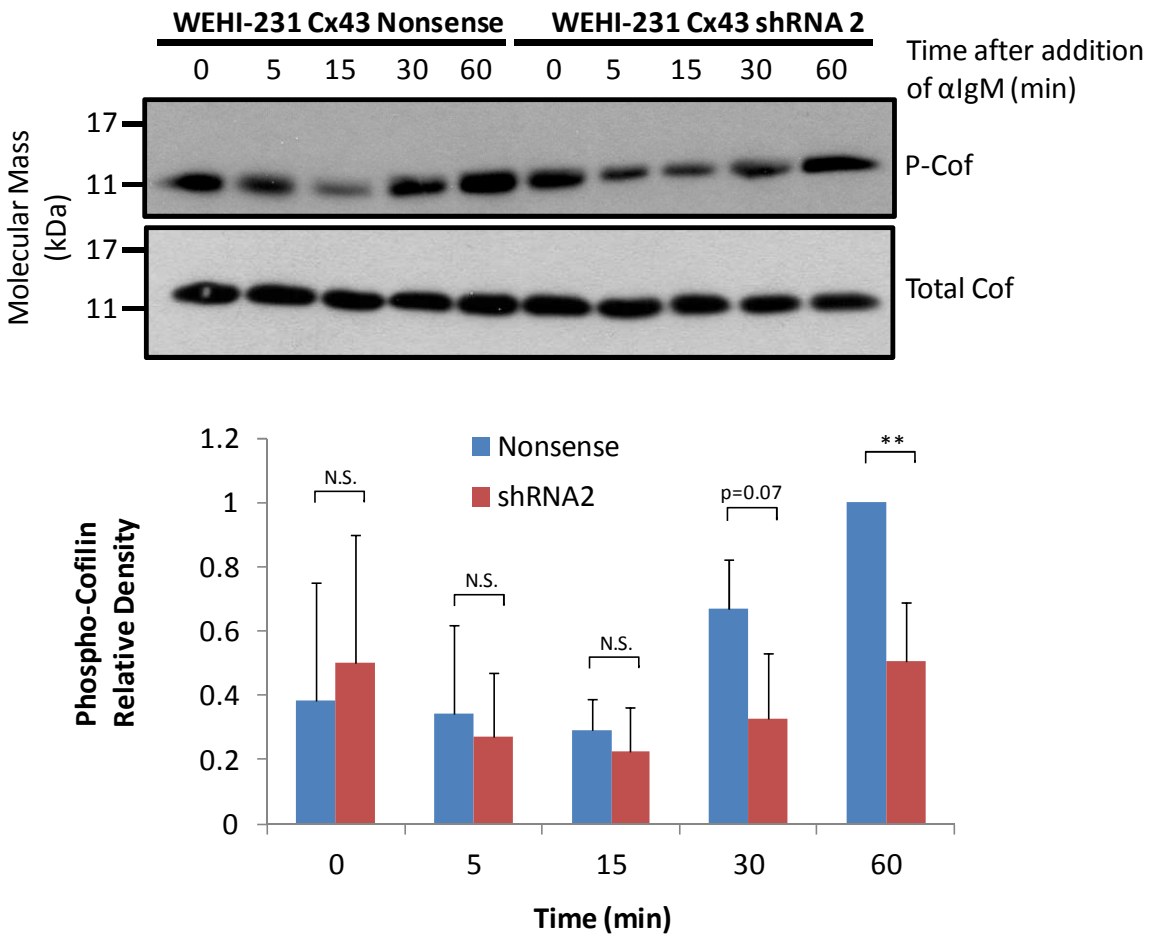


Figure 2-11. Cx43 is involved in BCR-induced activation of Cofilin

WEHI-231 cells were retrovirally transduced with either control shRNA (blue) or Cx43 shRNA 2 (red) and then stimulated with 20 µg/ml anti-IgM for the times indicated. Lysates were probed for phosphorylated Cofilin (Ser3). Western blots are the representative data from three independent experiments. Graphs are presented as average adjusted relative densities + SEM (n = three independent experiments), relative to the highest density time point, 60 min. N.S. = not significant; ** = $p<0.01$.

2.3.7 Cx43 is involved with the BCR-induced phosphorylation of a key cytoskeletal regulator, HS1

Because we have shown that Cx43 is involved with BCR-mediated cell spreading and Rap1 GTPase activation (Machtaler et al., 2011), I asked whether Cx43 modulates other key

targets of BCR signaling that control cytoskeletal organization, in particular, the HS1 scaffolding protein, Pyk2 and FAK tyrosine kinases, and myosin light chain II (MLCII). HS1 is a hematopoietic lineage cell-specific homolog of cortactin, a protein involved in adhesion, spreading, endocytosis, and migration in many cell types (Bryce et al., 2005; Lai et al., 2009). Both cortactin and HS1 stabilize F-actin filaments by binding to the Arp2/3 complex and actin filaments (Uruno et al., 2003). HS1 stabilizes lamellipodial protrusions during TCR-induced T cell spreading (Gomez et al., 2006) and also regulates adhesion and migration in NK cells and B cells (Butler et al., 2008; Scielzo et al., 2010). HS1, having two SH2 domains, can also act as a scaffolding protein, which may increase specific BCR signaling pathways by recruiting PLCγ to the membrane (Gomez et al., 2006). Moreover, cortactin binds to Cx43 in a p38 MAPK-dependent manner, as demonstrated by co-immunoprecipitation and co-localization in myoblasts. HS1 may undergo the same interaction with Cx43 in hematopoietic cells (Olk et al., 2009). I found that the loss of Cx43 impaired the ability of anti-IgM-induced HS1 phosphorylation in WEHI-231 cells, at all time points (Fig. 2-12). Because HS1 is phosphorylated by Syk, the defect in HS1 signaling coincides with the defect in Syk phosphorylation in Cx43KD cells.

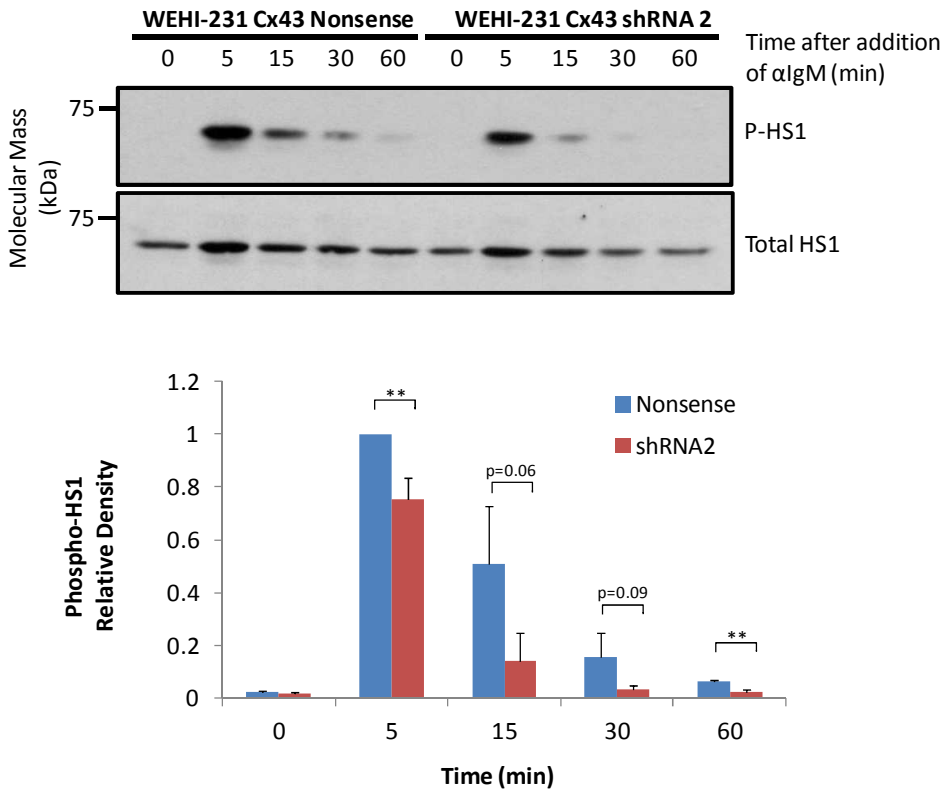


Figure 2-12. Cx43 is involved in BCR-induced activation of HS1

WEHI-231 cells were retrovirally transduced with either control shRNA (blue) or Cx43 shRNA 2 (red) and then stimulated with 20 µg/ml anti-IgM for the times indicated. Lysates were probed for phosphorylated HS1 (Tyr397). Western blots are the representative data from three independent experiments. Graphs are presented as average adjusted relative densities + SEM (n = three independent experiments), relative to the highest density time point, 5 min. N.S. = not significant; ** = $p<0.01$.

Pyk2 and FAK are non-receptor tyrosine kinases that regulate cell migration, adhesion, spreading, and morphology in many cell types (Mitra et al., 2005). Both Pyk2 and FAK are important for BCR-induced B cell spreading and their phosphorylation on critical activating residues is dependent on Rap1 activation (Tse et al., 2009). My preliminary results have shown that BCR-induced Pyk2 or FAK phosphorylation is not affected when Cx43 is knocked down (data not shown).

Similarly, MLCII is involved in cytoskeletal reorganization and its activity is regulated by phosphorylation on key residues by the myosin light chain kinase, which is activated via the Ca^{2+}/calmodulin pathway. My preliminary results have shown that BCR-induced phosphorylation of MLCII was of shorter duration in Cx43KD WEHI-231 cells than in control cells (data not shown).

Altogether, under the conditions that I tested, this suggests that Cx43 expression influences certain specific signaling pathways activated by the BCR in WEHI-231 immature B cells.

2.3.8 Cx43 does not alter BCR-induced Akt and Erk phosphorylation in A20 mature B cells

All the previous data shown were done using the murine immature IgM^+ B cell line, WEHI-231. However, knocking down Cx43 also shows an effect in A20 cells, as Rap activation is not sustained (Machtaler et al., 2011). Therefore, I asked whether the effects of Cx43 were similar in the murine mature IgG^+ B cell line, A20. Using a similar system to the WEHI-231, others in the lab have generated stable populations of the A20 B lymphoma cell line in which Cx43 expression was knocked down using shRNA-encoding vectors (Fig. 2-13A). The knockdown of Cx43 was more effective in A20 cells that are transduced with shRNA 1+2 than with shRNA 2 only. Therefore in subsequent studies, I used only shRNA 1+2. The surface expression of major signaling receptors (BCR, CXCR4, LFA-1, VLA-4) was identical on the cells transduced with the control shRNA and with the Cx43 shRNA knockdown cells (Fig 2-13B). Next, I asked whether Cx43 was important for the activation of BCR signaling pathways

after stimulation with anti-IgG. In contrast to what I found in WEHI-231 cells, I found that the phosphorylation of PLCγ, Akt, Erk and Cofilin was not affected by the loss of Cx43 (Fig. 2-13C,D,E,F).

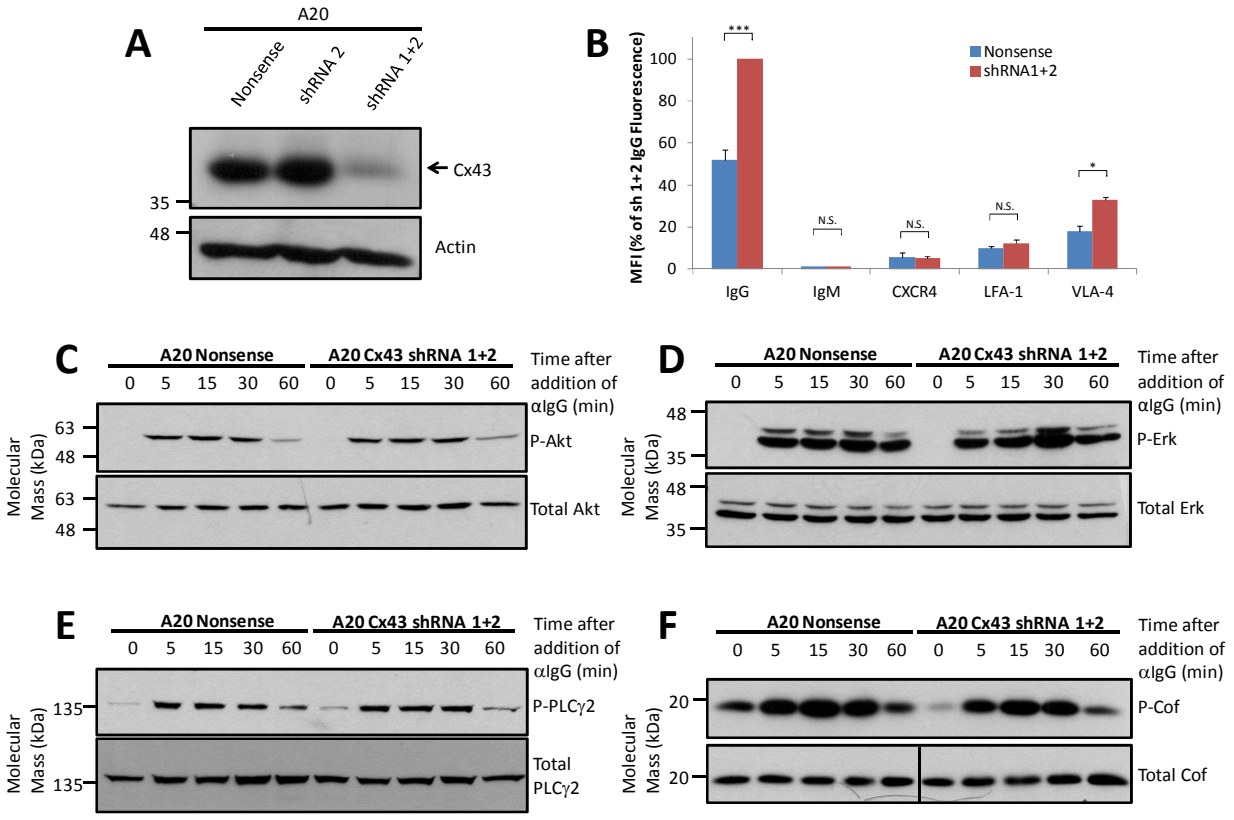


Figure 2-13. Decreased Cx43 expression in A20 cells does not alter key surface protein levels or the phosphorylation of key BCR signaling molecules

(A) Cx43 protein levels in A20 cells that were transduced with retroviruses encoding either nonsense shRNA (control) or Cx43 shRNA 2 (knockdown) were assessed by immunoblotting. Actin was used as a loading control. This was done prior to each signaling experiment that used the same cell lysates.

(B) Surface expression of key cell surface proteins in A20 control (blue) and Cx43KD cells (red) were assessed by flow cytometry using fluorophore-conjugated antibodies. The graph shows data from three independent experiments (mean + SEM). N.S., not significant; *** = $p<0.001$; * = $p<0.05$.

(C-F) A20 cells were then stimulated with 10 μg/ml anti-IgG for the times indicated. Lysates were probed for **(C)** phosphorylated Akt (Ser473), **(D)** phosphorylated Erk (Thr202/Tyr204), **(E)** phosphorylated PLCγ (Tyr1217), or **(F)** phosphorylated Cofilin (Ser3). Western blots are the representative data from three independent experiments.

2.3.9 The effect of Cx43 on chemokine signaling

As described in Section 2.3.3, others in the lab have shown that the knockdown of Cx43 in WEHI-231 decreases the ability of these cells to migrate towards the chemokine, CXCL12. In addition, as for BCR signaling, the activation of Rap1 is not sustained in response to CXCL12 in the Cx43KD cells in contrast to control cells. CXCR4 presumably activates Rap1 via the activation of phospholipase Cβ3 (PLCβ3), which also generates DAG that can activate Rap GEFs (Badr, 2010; Badr et al., 2008; Thelen and Stein, 2008). Because CXCR4 is a major chemokine receptor for B cell migration, and Cx43 influences CXCL12-induced Rap1 activation, I wanted to know if Cx43 influences the major signaling pathways of CXCR4, which are the PLCβ3, Akt, Erk, and the Pyk2/FAK complex. CXCL12-induced activation of Akt and Erk were similar between WEHI-231 control cells and WEHI-231 Cx43KD cells (Fig. 2-14A,B). Assessing the phosphorylation of PLCβ3 and Pyk2/FAK could not be done during the scope of this thesis and assays will need to be optimized. Because the knockdown of Cx43 impairs B cell migration towards chemokine, I asked whether Cx43 modulates other CXCR4 signaling pathways that control cytoskeletal organization, including the activation of HS1. However, HS1 was not phosphorylated in response to CXCL12 (data not shown). The effect of Cx43 on chemokine signaling in B cells still needs to be elucidated.

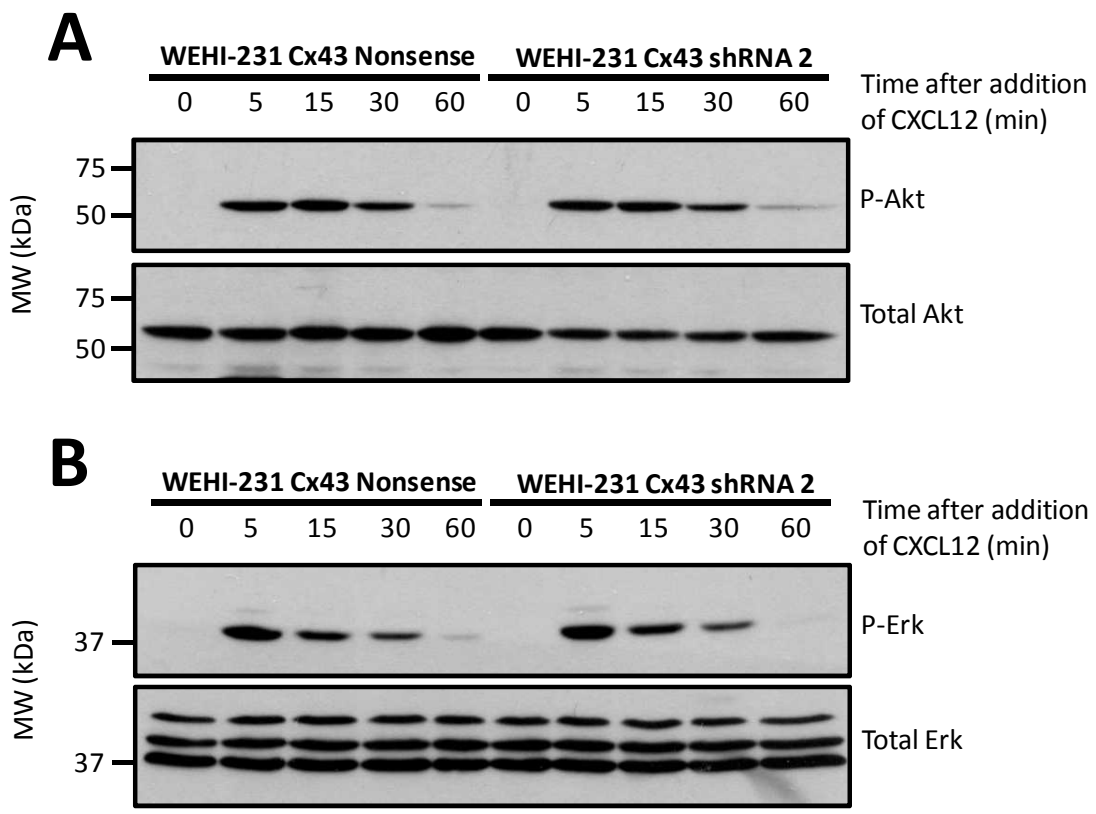


Figure 2-14. Cx43 is not important for CXCL12-induced phosphorylation of Akt or Erk

WEHI-231 cells that were retrovirally transduced with either control shRNA or Cx43 shRNA 2 were stimulated with 100 ng/ml CXCL12 for the times indicated. Lysates were probed for **(A)** phosphorylated Akt (Ser473) or **(B)** phosphorylated Erk (Thr202/Tyr204**)**. Representative data from three independent experiments are shown.

3 High throughput screen for novel inhibitors of BCR signaling

3.1 Introduction

Aberrant B cell activation can lead to autoimmune diseases or to B cell malignancies. B cell depletion with the anti-CD20 antibody Rituximab is now being used to treat many of these diseases, but total B cell depletion renders people susceptible to infections and its effects are only temporary (Clark and Ledbetter, 2005; Engel et al., 2011; Foon et al., 2012; Hu et al., 2009; Leandro and de la Torre, 2009; Levesque, 2009). Therefore the development of effective drugs that limit B cell activation could be a useful adjunct to B cell depletion. Small molecule chemical inhibitors of key enzymes involved in BCR signaling (e.g. Syk, Btk) are already in clinical trials for such diseases (Chen et al., 2008; Reeder and Ansell, 2011) but additional inhibitors that target other signaling molecules may also be useful. Given my project's focus on B cell signaling pathways, we embarked on a screening project, together with the Centre for Drug Research and Development (CDRD) (2259 Lower Mall, Vancouver, BC), to identify novel inhibitors of BCR signaling.

To facilitate high throughput screening of small molecule and natural compound libraries, I optimized the assay conditions for the primary screen using the immature murine B cell lines CH31 and WEHI-231 as model systems. BCR signaling in immature B cells is quite similar to that in mature B cells, but leads to apoptosis as opposed to cell activation and proliferation. The use of CH31 cells, which undergo cell death after BCR signaling, allowed for testing of the assay conditions as well as for identifying and eliminating toxic compounds that would have contributed as false positives if screened using mature B cell lines. I went on to optimize the assay conditions for a secondary screen using mature murine splenic B cells, which proliferate in

response to BCR signaling. In this assay putative inhibitors of BCR signaling pathways would oppose anti-Ig-induced B cell activation and proliferation. Lastly, to establish human relevance, I optimized the assay conditions for a parallel screen that employed the B104 human B lymphoma cell line, which, like CH31 and WEHI-231 cells, undergoes growth arrest and apoptosis in response to BCR signaling.

The goal of this part of my thesis project is to identify small molecule inhibitors of the BCR signaling pathway that could potentially be used as research tools to study B cell signaling, and potentially as therapeutic agents to limit the activation of autoimmune B cells or the survival of B lymphoma cells. Such inhibitors could be the parent compounds for developing novel drugs to treat B cell tumors or reduce the suffering associated with B cell-mediated autoimmune diseases.

3.2 Materials and methods

3.2.1 Antibodies and inhibitors

Polyclonal goat anti-mouse IgM (μ-chain specific, #115-005-020) antibodies and polyclonal goat anti-human IgM (Fc5μ fragment specific, #109-005-043) antibodies that were used to stimulate cells were purchased from Jackson ImmunoResearch Labs (West Grove, PA). The Pyk2 inhibitor PF-719 (Tse et al., 2012) and the FAK inhibitor PF-573228 (Tse et al., 2012) were gifts from Dr. Leonard Buckbinder (Pfizer Global Research and Development, Groton, CT). The reactive oxygen species scavenger N-acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich (#A8199, Saint Louis, MO). The Src family kinase inhibitor PP2 (#529573), and the

Syk inhibitor piceatannol (#527948) were purchased from Calbiochem/EMD Millipore (Etobicoke, Ontario, Canada).

3.2.2 Cells and cell culture

The CH31 IgM^+ immature murine B cell line (Haughton et al., 1986; Pennell et al., 1984) and the B104 IgM^+ immature human B cell line (Kim et al., 1991) were gifts from Dr. A. DeFranco (University of California, San Francisco, CA) and Dr. E. Clark (University of Washington, Seattle, WA), respectively. Cells were maintained in supplemented RPMI as described in Section 2.2.2.

3.2.3 Anti-Ig dose response curves for immature B cell lines

To determine dose response relationships for anti-IgM-induced cell death in CH31 and B104 cells, the cells were cultured with varying concentrations of goat anti-mouse IgM or goat anti-human IgM, respectively. The antibodies were diluted in 30 µl supplemented RPMI and added to black 384-well plates with clear bottoms (BD Biosciences, #353962, Mississauga, Ontario, Canada). CH31 cells were resuspended to 5×10^4 cells/ml in supplemented RPMI and 20 µl (1000 cells) was added to each well. B104 cells were resuspended to 3.5×10^5 cells/ml supplemented RPMI and 20 µl (7500 cells) was added to each well. The cytotoxic agent puromycin dihydrochloride (0.2 µg/ml) (Calbiochem/EMD Millipore, #540222) was used as a positive control to induce cell death. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 2 days. A 1:10 dilution of a 0.01% w/v stock solution of Alamar Blue (Resazurin sodium salt) (Sigma-Aldrich, #R7017) was added to the wells 4 hr prior to reading the fluorescence (A_{590}). An overview diagram is shown in Figure 3-1.

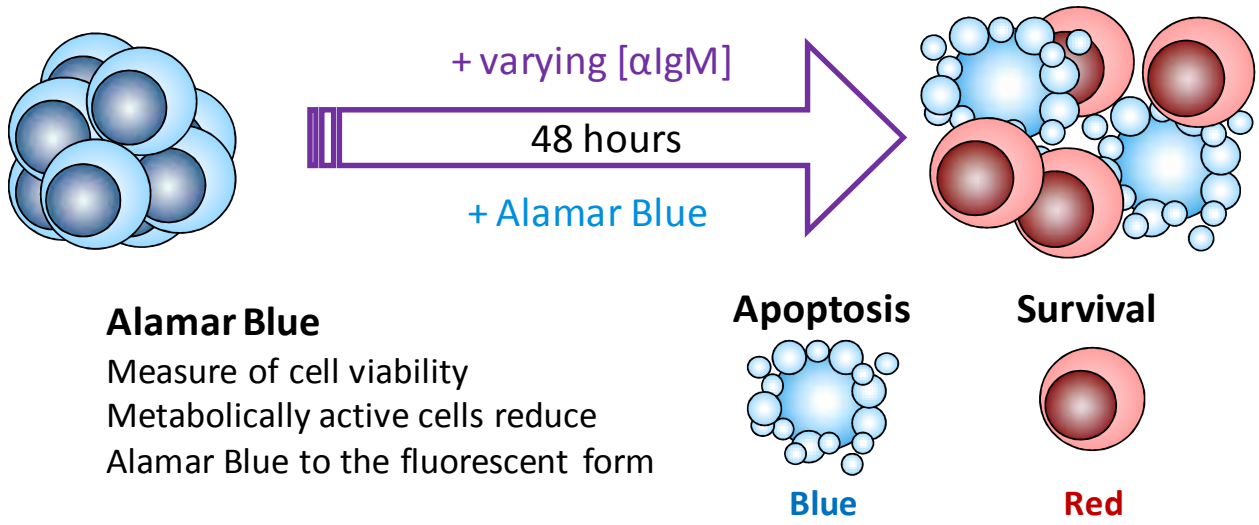


Figure 3-1. Overview diagram for generating dose response relationships for anti-IgM-induced cell death in the CH31 and B104 cells lines

3.2.4 B cell line non-automated compound screen

CH31 cells were cultured with suboptimal concentrations of anti-mouse IgM (10 ng/ml, 15 ng/ml, 20 ng/ml) and different concentrations of either the Src family kinase PP2 (10 µM, 1 µM, 0.1 µM), the Syk inhibitor piceatannol (40 µM, 20 µM, 4 µM), the Pyk2 inhibitor PF-719 (5 µM, 1 µM, 0.5 µM, 0.05 µM), the FAK inhibitor PF-573228 (5 µM, 1 µM, 0.25 µM, 0.05 µM), or the reactive oxygen species inhibitor NAC (20 mM, 10 mM, 5 mM). Cells were treated with compound only, anti-IgM only, compound and anti-IgM, LPS and anti-IgM, or media only.

B104 cells were cultured with 80 ng/ml anti-human IgM and different concentrations of either Compounds 1, 2, 3, or 4 obtained from KD2 screen in Section 3.3.3 (10 µM, 1 µM). Cells were treated with compound only, anti-IgM only, compound and anti-IgM, or media only.

Different combinations of anti-IgM, compounds, or LPS, depending on the treatment, were added to black with clear bottom 384-well plated and were topped up to 30 µl. The appropriate amount of the cells, CH31 (1000 cells/well) or B104 (7000 cells/well), were added (20 µl) to the appropriate wells. The final culture volume was 50 µl. Plates were incubated at 37^{o}C in a 5% CO_2 atmosphere for 2 days. A 1:10 dilution of a 0.01% w/v stock solution of Alamar Blue was added to the wells 4 hr prior to reading the fluorescence. Fluorescence (A_{590}) was determined using a SPECTRAmax® GEMINI-XS spectrophotometer and values were expressed as arbitrary fluorescence units.

3.2.5 Automated compound screen with B cell lines

The screening of compounds with the CH31 and B104 cell lines was done in collaboration with the CDRD at UBC. The CDRD screened libraries of known compounds including the Library of Pharmacologically Active Compounds (LOPAC) and the Known Drug 2 (KD2) library. Using an automated pinning robot, compounds were pinned into wells containing the cells and these 384 well plates were incubated for 30 min prior to the addition of an appropriate suboptimal concentration (IC_{80} or IC_{70}) of anti-IgM specific for either the CH31 or B104 cell line. The plates were then incubated for an additional 48 hr at 37^{o}C in a 5% CO_2 atmosphere. Alamar Blue was added to the wells 4 hr prior to reading the fluorescence (A_{590}).

3.2.6 Mice

C57BL/6 (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were used at 6–12 weeks of age. All animal experiments followed protocols approved by

the Animal Care Committee of the University of British Columbia (Vancouver, British Columbia) for Dr. Michael R. Gold.

3.2.7 Isolation of primary B lymphocytes

Spleens were isolated from B6 mice, placed into cold FACS buffer (PBS, 2% HI-FBS), and crushed through a 70 μm nylon mesh cell strainer (BD Biosciences, #352350). The cells were pelleted by centrifuging at 1,500 RPM for 5 min at 4^{o}C, then resuspended at 10^8 cells/ml in RoboSep buffer (StemCell Technologies, #20104, Vancouver, British Columbia, Canada) with 5% rat serum (StemCell Technologies, #19700). Primary splenic B cells were then isolated via negative selection using the automated RoboSep system (StemCell Technologies) according to the manufacturer's instructions (protocol #19754).

3.2.8 Carboxyfluorescein succinimidyl ester (CFSE) labeling of cells

Primary B cells were washed twice with PBS to get rid of excess FBS in the FACS buffer, and then resuspended to 10^7 cells/ml in 2 μl PBS with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Invitrogen Life Technologies, #C1157). Cells were incubated for 8 min at RT, protected from light, in order to allow the non-fluorescent and highly cell permeable molecule CFDA-SE to enter the cell, where intracellular esterases remove acetate groups from CFDA-SE. This converts CFDA-SE into the fluorescent compound carboxyfluorescein succinimidyl ester (CFSE), which is retained within cells via its covalent coupling to intracellular molecules (Parish, 1999). The CFSE labeling reaction was stopped by adding an equal volume of HI-FBS and then washing the cells twice with PBS and resuspending

them in supplemented RPMI. CFSE has a peak excitation wavelength of 492 nm and a peak emission wavelength of 517 nm.

3.2.9 Anti-Ig dose-response curves for primary murine splenic B cells

Isolated primary splenic B cells from B6 mice were labeled with CFDA-SE or left unstained. The cells were then resuspended to 10^6 cells/ml and 100 µl (10^5 cells) was added to each well of flat bottom 96 well plates (BD Biosciences #351172). The cells were cultured with varying concentrations of goat anti-mouse IgM. Where indicated, IL-4 or LPS were added at final concentrations of 10 ng/ml and 10 µg/ml, respectively. Extra wells containing unstained cells or CFDA-SE labeled cells were made for staining controls. The final culture volume was 300 µl. Quadruplicate plates were incubated at 37^{o}C in a 5% CO_2 atmosphere for 1-4 days. An overview diagram is shown in Figure 3-2. At 24, 48, 72 and 96 hr, one of the quadruplicate plates was spun at 1,500 RPM for 5 min. After aspirating the medium, the cells were then resuspended in 350 µl of FACS buffer containing 1 µg/ml 7-aminoactinomycin D (7-AAD) (Calbiochem/EMD Millipore, #129935). As controls, unstained cells and cells stained with CFSE only were resuspended in 350 µl of FACS buffer without 7-AAD. Cells were then transferred to round bottom polystyrene tubes (BD Biosciences, #352054) and incubated for 10 min on ice in the dark. 7-AAD is a membrane-impermeable dye that is taken up only by dead or dying cells, and undergoes a spectral shift upon binding to DNA. The 7-AAD/DNA:complex has a peak excitation wavelength of 500 nm and a peak emission wavelength of 692 nm.

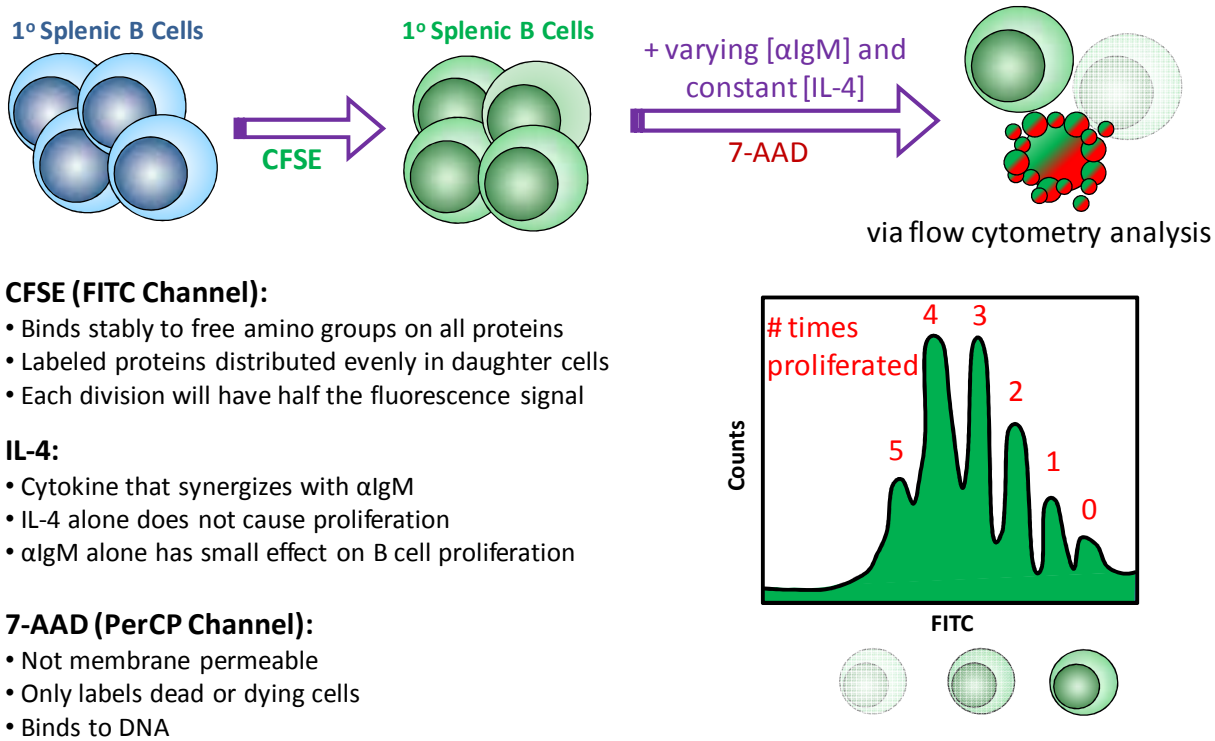


Figure 3-2. Overview diagram of CFSE proliferation assay for primary splenic B cells

3.2.10 Primary murine B cell secondary screen

Primary splenic B cells from B6 mice, isolated as in Section 3.2.7, were labeled with CFSE as in Section 3.2.8 and resuspended in supplemented RPMI. Three different suboptimal concentrations of anti-mouse IgM (27 µg/ml, 9 µg/ml, 3 µg/ml) were added to flat bottom 96 well plates. IL-4 and LPS were used at 10 ng/ml and 10 µg/ml, respectively. Compounds 1, 2, 3, and 4 identified in the screen in Section 3.3.3 were used at three different concentrations (100 µM, 10 µM, 1 µM). The final culture volume was 300 µl. Duplicate plates were incubated at 37°C in a 5% CO_2 atmosphere for 2 days. After 24 or 48 hr, one plate was taken out and spun at 1,500 RPM for 5 min. The cells were resuspended in 350 µl of FACS buffer containing 1 µg/ml 7-AAD. Unstained cells and cells to be labeled with CFSE only were resupended in 350 µl of

FACS buffer without 7-AAD. Cells were then transferred to round bottom polystyrene tubes and incubated for 10 min on ice in the dark before being analyzed by flow cytometry.

3.2.11 Flow cytometry data collection and analysis

Samples were acquired on a BD LSRII Flow Cytometer (BD Biosciences) using the BD FACS Diva software (BD Biosciences). FITC and PerCP (or PerCP-Cy.5.5) channels were used to detect the CFSE and 7-AAD fluorescence, respectively. The APC channel was used to compensate for autofluorescence. Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR). As shown in Figure 3-3, debris (forward scatter (FSC) values < ~60) and 7-AAD-positive (dead) cells were gated out before assessing CFSE fluorescence on 7-AAD-negative (live) cells. The CFSE peak from day 0 was overlaid with the CFSE peak from each subsequent day. A gate was drawn from the left edge of the day 0 CFSE peak, as indicated in Figure 3-3, to determine the percent of cells that had dilute CFSE (i.e. had proliferated).

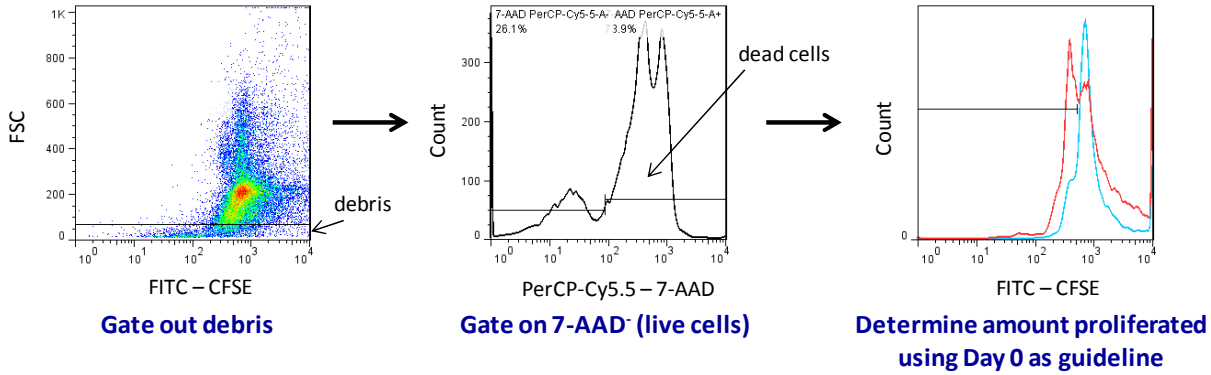


Figure 3-3. Overview of CFSE dilution analysis using FlowJo software

To determine the "% inhibition of proliferation", first, the percentage of cells that had proliferated in compound-treated samples was divided by the percentage of cells that had

proliferated in the anti-IgM/IL-4-only samples. Since we are looking at inhibition, this normalized percentage is subtracted from 1 (which is then called "% inhibition"). Similarly, the percentage of live cells within the proliferated population in compound-treated sample was divided by the percentage of live cells within the proliferated population in the anti-IgM/IL-4 only samples. Since we are interested in the number of dead cells, this normalized percentage is subtracted from 1 (which is then called "% dead"). The" % dead" is subtracted from the "% divided" to yield the "% inhibition of proliferation" in live cells only.

3.3 Results

3.3.1 Screening approach using CH31 cells

BCR signaling in mature B cells leads to cell activation and proliferation. We want to screen for inhibitors of BCR signaling; however, inhibition of BCR signaling leads to death in mature B cells, which would make it difficult to distinguish between inhibitors of BCR signaling and compounds that are merely cytotoxic. In contrast, BCR signaling in immature B cells and immature B cell lines is quite similar mechanistically to that in mature B cells but leads to apoptosis. In this case, inhibition of BCR signaling would promote the survival of immature B cells. Thus, the use of the CH31 IgM^+ immature murine B cell line would allow a screen for inhibitors of BCR signaling while ruling out toxic compounds that would have been false positives if screened using mature B cells (Fig. 3-4).

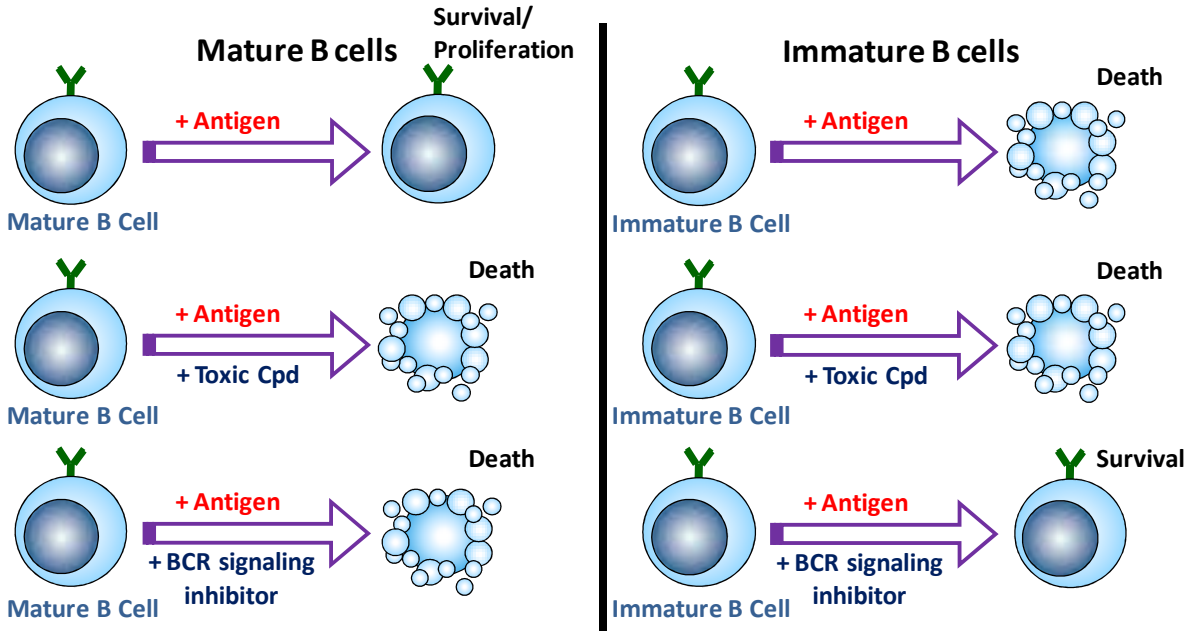


Figure 3-4. Differences in BCR signaling output in mature and immature B cells

Antigen or anti-Ig stimulation of mature B cells promotes survival and proliferation whereas in immature B cells, it leads to cell death by apoptosis. Toxic compounds cause death regardless of the stage of B cell development. Addition of BCR signaling inhibitors with antigen or anti-Ig would prevent anti-Ig-induced death in immature B cells but would induce death in mature B cells, which would be indistinguishable from the effects of a toxic compound, which would represent a false positive.

CH31 is thought to be a transformed version of an immature B cell, and has been used as a model for antigen-induced growth arrest and cell death (Scott, 1993). Eighteen hours following BCR crosslinking by anti-IgM antibodies, most CH31 cells are irreversibly committed to apoptotic programmed cell death. During this time, the BCR signals that commit the cell to growth arrest and apoptosis can be prevented by ligands for TLRs such as LPS and CpG DNA (Zhong et al., 2004) as well as by the cytokine IL-4 (Carey et al., 2007), and the T cell surface protein, CD40 ligand (Zhu et al., 2002b).

We wanted to choose a suboptimal concentration of anti-IgM that is capable of inducing death, but not high enough to induce death regardless of the addition of BCR signaling inhibitors. To do this, we identified IC_{85} through the titration of anti-IgM. CH31 cells were cultured with different concentrations of goat anti-mouse IgM (Fig. 3-5A,B) for 48 hr. This allows enough time to ensure that cells have initiated apoptosis. The cell viability dye, Alamar Blue, was used to assess the relative amount of live cells. From the dose response curve, the IC_{85} for anti-IgM was 20 ng/ml anti-IgM. This concentration was used for future compound screening and testing.

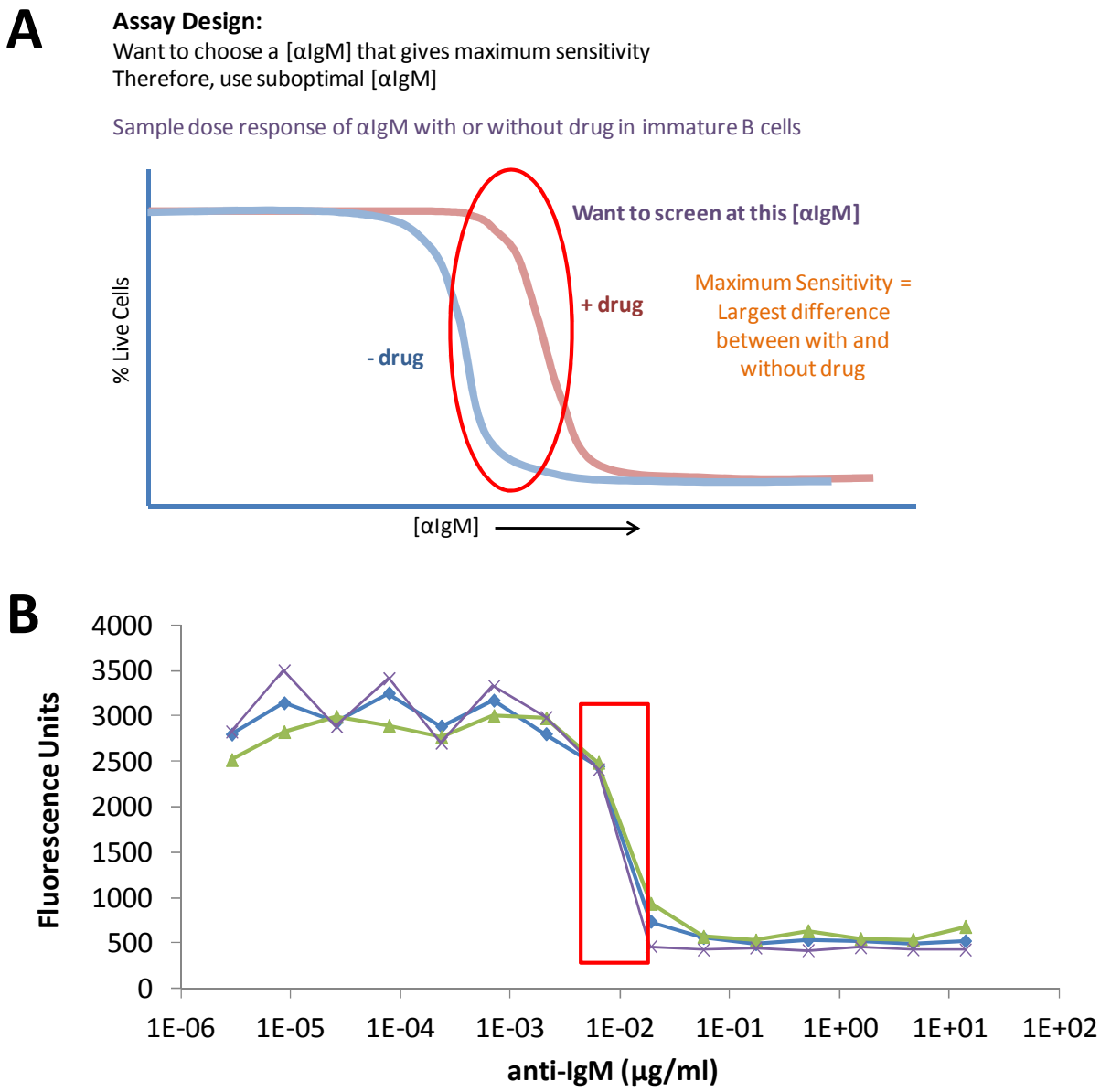


Figure 3-5. Dose response curve for anti-IgM-induced growth arrest in CH31 cells

(A) Schematic of the assay design for finding the appropriate concentration of anti-IgM to use for the primary screen with CH31 cells.

(B) CH31 cells were cultured with different concentrations of goat anti-mouse IgM for 48 hr. After 48 hr, cell viability was measured using the metabolic indicator, Alamar Blue. The portion of the curve indicated by the red box represents the concentrations of anti-IgM that give the maximum sensitivity. Triplicate wells were pooled together for analysis in each experiment. Each curve represents the data from one of three independent experiments.

3.3.2 Assay optimization: testing known inhibitors of BCR signaling on CH31 cells

As positive controls for the screening assay, I used the TLR4 ligand LPS, which has been shown to prevent BCR-induced growth arrest in immature B cells. Also, I have tested several known inhibitors of key that are critical for BCR signaling as Src family kinases, Syk, Btk, Pyk2, and FAK.

CH31 cells were cultured with either 0, 10, 15 or 20 ng/ml anti-IgM in the presence of media only or LPS, and with varying concentration of BCR signaling inhibitors, where indicated. Cells were always added last to the wells. LPS is a component of the outer membrane of Gram-negative bacteria that binds to TLR4, induces the polyclonal activation and proliferation of mature B cells, and prevents anti-IgM-induced cell death in immature B cells and immature B cell lines such as CH31 and WEHI-231 (Gottschalk and Quintans, 1995; Wechsler-Reya and Monroe, 1996). LPS is thought to sustain the activation of proteins involved in survival and growth whose BCR-induced activation in immature B cells is short lived but then falls below basal levels, associating with cell death (Gottschalk and Quintans, 1995; Wechsler-Reya and Monroe, 1996).

When BCRs are clustered at the surface by anti-Ig antibodies, Src family kinases phosphorylate Igα and Igβ, which act as recruitment sites for SH2 domain-containing signaling molecules such as Src family kinases and the Syk tyrosine kinase (Campbell et al., 1991; Gold et al., 1990; Jiang et al., 1998; Pawson and Nash, 2000). Recruitment and activation of these kinases result in the activation of multiple downstream signaling pathways (Gold et al., 2000). The Src family kinase inhibitor (PP2) and the Syk inhibitor (piceatannol) have been used

extensively (Liu et al., 2010; Robak and Robak, 2012). I found that when cells were cultured with suboptimal concentrations of anti-IgM and varying concentrations of PP2 or piceatannol, anti-IgM-induced growth arrest/cell death was not blocked (Fig. 3-6A, B, C). The inhibitors also did not block the ability of LPS to prevent anti-Ig-induced growth arrest.

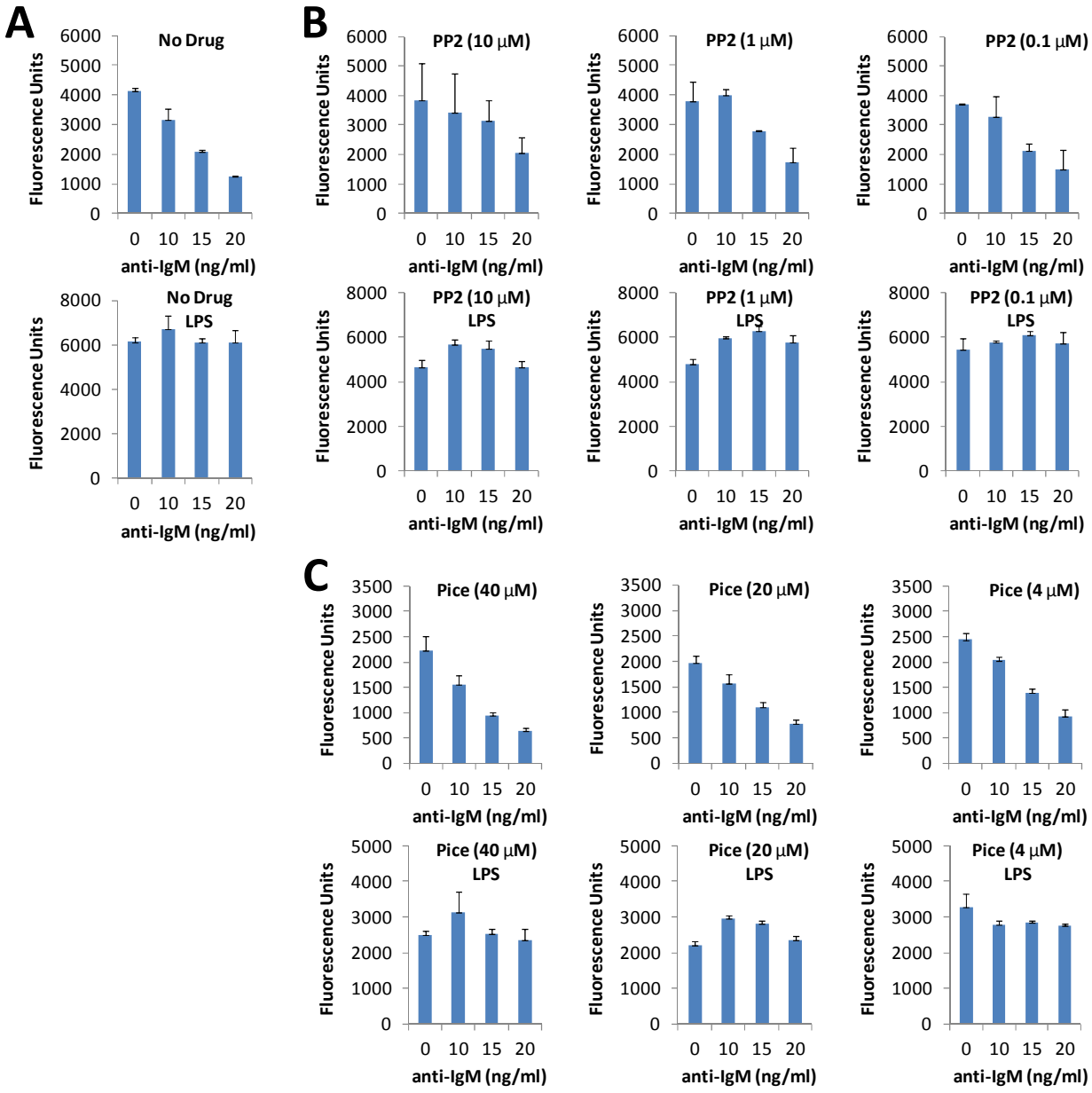


Figure 3-6. The Src family kinase and Syk inhibitors did not inhibit anti-IgM-induced growth arrest

Cells were cultured for 48 hr with suboptimal concentrations of anti-IgM and either **(A)** no compound or the indicated concentrations of **(B)** PP2 or **(C)** piceatannol. LPS (10 μg/ml) was used as a positive control for inhibition of anti-IgM-induced apoptosis. Each bar is the mean + SD for three replicate wells. Representative data from one of three independent experiments are shown.

Pyk2 and FAK, both downstream targets of BCR signaling, play an important role in regulating cell adhesion, morphology and migration (Okigaki et al., 2003; Rovida et al., 2005; van Buul et al., 2005). Therefore I tested whether the Pyk2-selective inhibitor (PF-719) and the FAK-selective inhibitor (PF-228) (Tse et al., 2009; Tse et al., 2012) would be able to block anti-IgM-induced cell death in CH31 cells. At higher concentrations, both compounds were toxic as the cells underwent growth arrest/cell death even in the absence of anti-IgM, and at lower concentrations, they did not prevent anti-IgM-induced growth arrest/cell death (Fig. 3-7A, B, C).

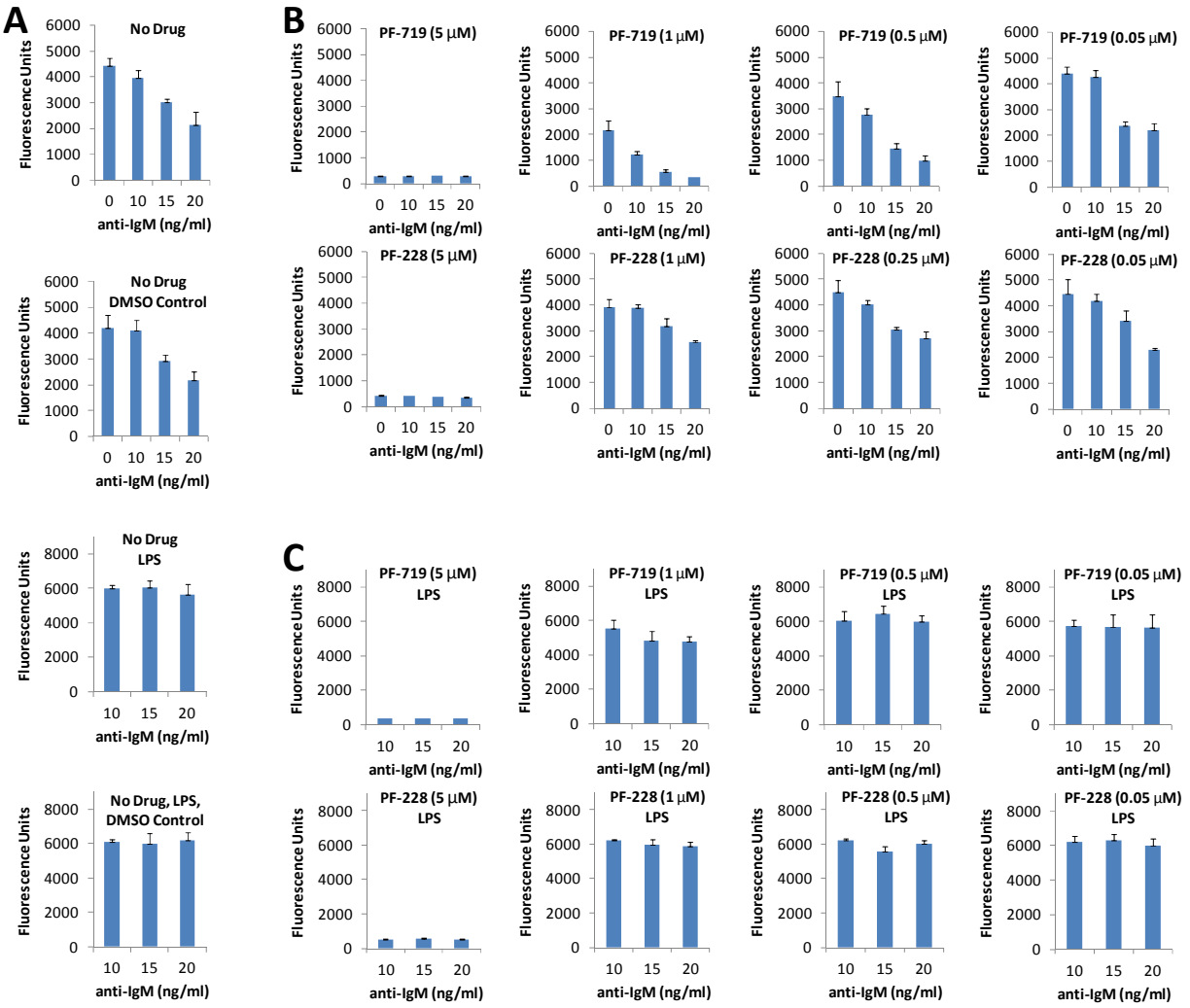


Figure 3-7. The Pyk2 and FAK inhibitors did not inhibit anti-IgM-induced growth arrest
Cells were cultured for 48 hr with suboptimal concentrations of anti-IgM and either **(A)** no compound or the indicated concentrations of **(B)** PF-719 or **(C)** PF-573228. LPS (10 μg/ml) was used as a positive control for inhibition of anti-IgM-induced apoptosis. Each bar is the mean + SD for three replicate wells. Representative data from one of three independent experiments are shown.

BCR signaling results in the generation of intracellular reactive oxygen species such as hydrogen peroxide and superoxide (Devadas et al., 2002), which can function as second messengers by regulating the activity of redox-sensitive kinases and phosphatases such as Lyn,

Erk, and JNK (Adler et al., 1999; Reth, 2002; Tonks, 2005). Therefore I asked whether the membrane permeable anti-oxidant N-acetyl L-cysteine (NAC) (Lee et al., 2007) could prevent anti-IgM-induced growth arrest/cell death in response to suboptimal concentrations of anti-IgM. I found that NAC resulted in an increase in Alamar Blue fluorescence (Fig 3-8). However, this was not due to NAC preventing anti-IgM-induced growth arrest/cell death, but was a false positive due to NAC chemically reducing the Alamar Blue dye into its fluorescent form. Indeed, NAC increased Alamar Blue fluorescence in cells incubated in medium only (data not shown). Visual inspection of brightfield images revealed that anti-IgM caused the same amount of cell death in the presence or absence of NAC (data not shown). Dead cells were identified as cells that were not round and had increased intracellular granules. Therefore, NAC did not prevent anti-IgM-induced growth arrest/cell death.

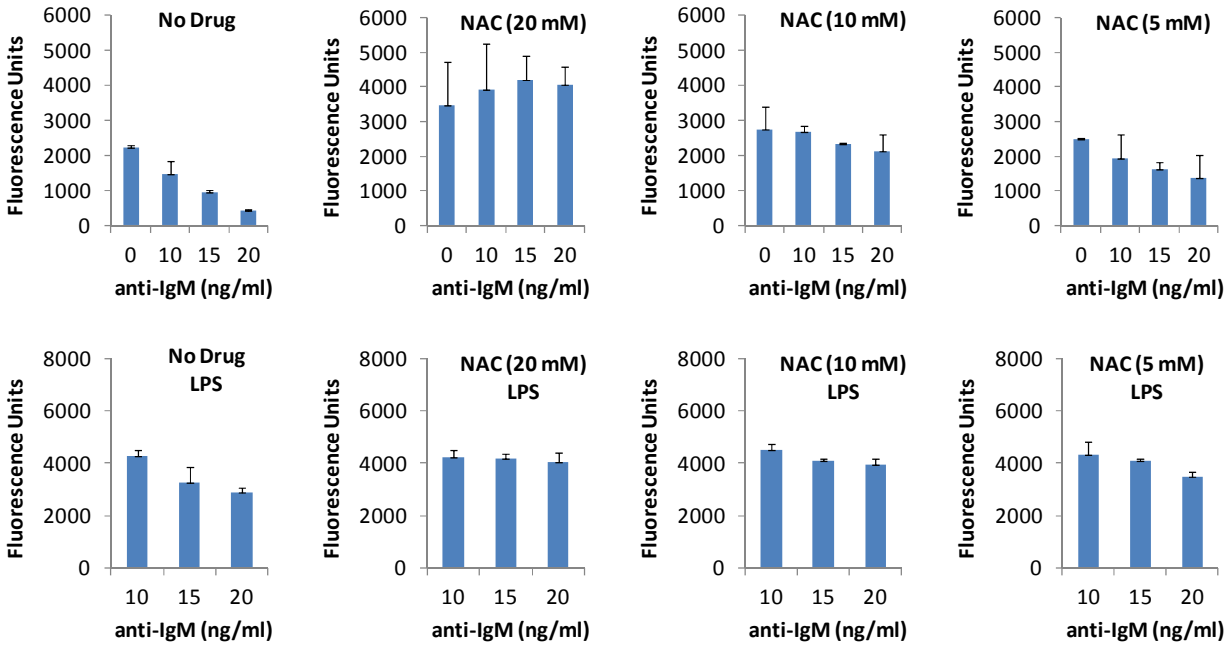


Figure 3-8. The anti-oxidant, NAC, did not inhibit anti-IgM-induced growth arrest
Cells were cultured for 48 hr with suboptimal concentrations of anti-IgM and the indicated concentrations of NAC. LPS (10 μg/ml) was used as a positive control for inhibition of anti-IgM-induced apoptosis. Each bar is the mean + SD for three replicate wells. Representative data from one of three independent experiments are shown.

Unfortunately, positive control experiments showing that these inhibitors were in fact working were not done (i.e. whether they blocked their respective targets). These experiments were merely done to test out assay conditions to ensure that the number of cells used were still viable and quantifiable after a 48 hr incubation, the concentrations of anti-IgM used killed the cells around 50-85%, and LPS could serve as a positive control for the prevention of anti-IgM-induced growth arrest/cell death.

3.3.3 Primary screen with CH31 cells

Because we optimized assay conditions for screening with the CH31 cells, we proceeded to screen various compound libraries with the hope of discovering interesting inhibitors. To ensure that the experimental setup and assay conditions are robust, an initial screen was performed in partnership with the CDRD using the Library of Pharmacologically Active Compounds (LOPAC), consisting of 1276 compounds. This pilot screen was performed using the optimized Alamar Blue assay described above with 1000 CH31 cells/well being incubated in the presence of compounds for 30 min, followed by adding 20 ng/ml anti-IgM and culturing the cells for 2 days. Compounds that caused significant inhibition of anti-Ig-induced growth arrest/cell death (from now on, called "actives") identified from this screen exhibited only modest inhibition (up to 22%) of BCR-induced growth arrest/cell death (Fig. 3-9).

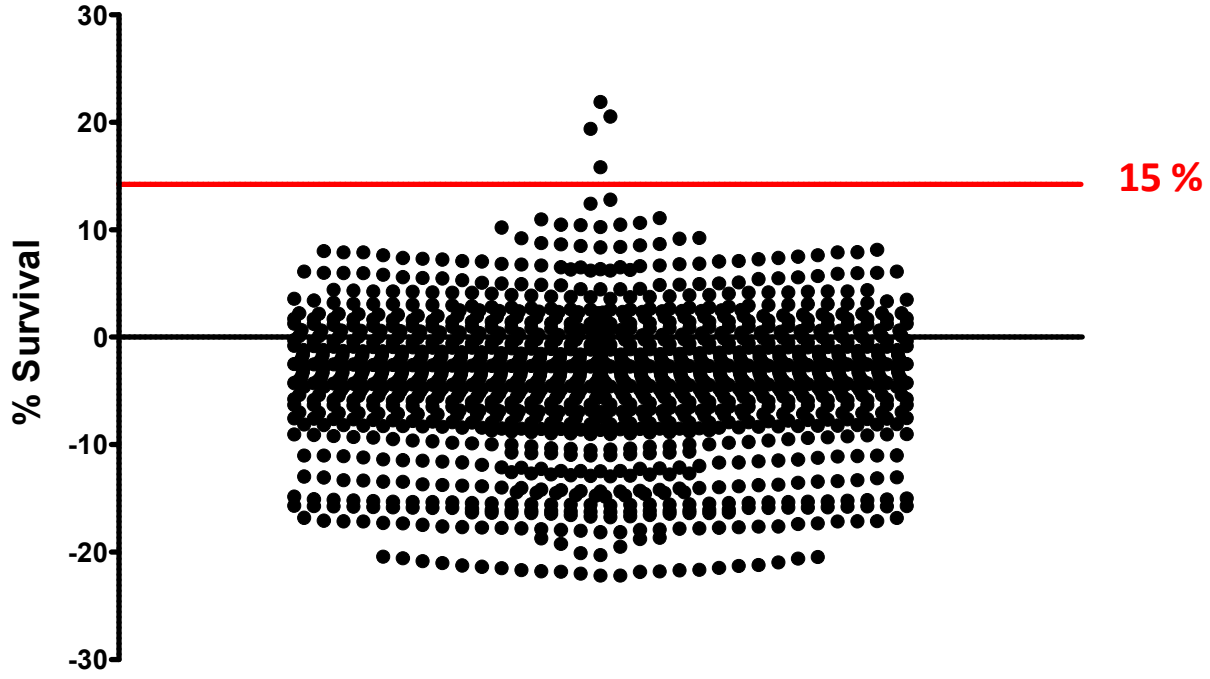


Figure 3-9. Primary screen of the LOPAC library using CH31 cells

Cells were incubated with compounds from the LOPAC library for 30 min at 37^{o}C before adding 20 ng/ml anti-IgM. Plates were incubated for 48 hr and Alamar Blue was added for the final 4 hr. Four compounds caused >15% increase in survival relative to the anti-IgM only treatment. The percent survival indicates the percent increase of live cells compared to treatment with anti-IgM only. The compounds causing 'negative survival' were toxic. This data and figure were supplied by the CDRD.

Since the assay proved to be robust and the LOPAC screen results were encouraging, we moved on to screen the full library, called the Known Drugs 2 Library (KD2), which contains 4761 compounds including the LOPAC library. As with the LOPAC screen, the most active inhibitors of anti-Ig-induced growth arrest/cell death had only modest effects, causing up to 27% cell survival (Fig. 3-10). Nine compounds from this screen increased the survival of anti-IgM-treated CH31 cells by >18% and four of these compounds were chosen for validation using dose response curves. Dose responses were done because any compound that is acting in a real

biochemical manner should have a dose response, and because lower or higher concentrations could show greater activity with less toxicity. Three of the four compounds were dose responsive (Fig. 3-11).

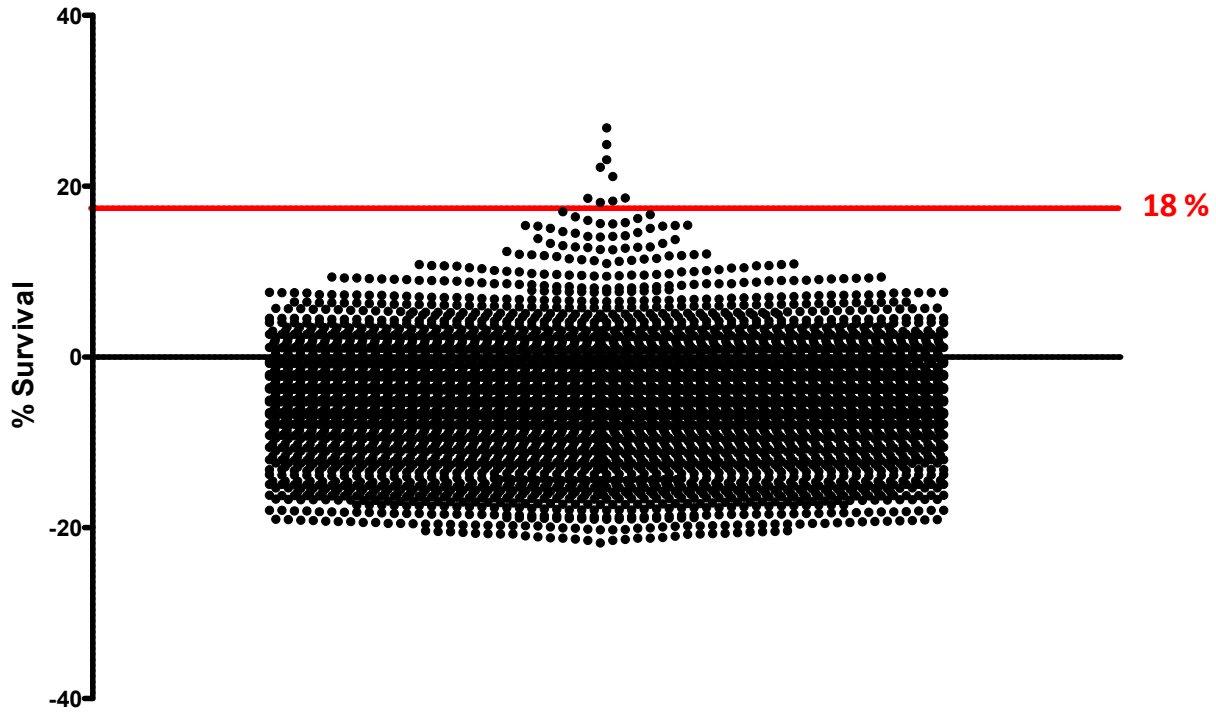


Figure 3-10. Primary screen of the KD2 library using CH31 cells

Cells were incubated with compounds from the KD2 library for 30 min at 37^{o}C before adding 20 ng/ml anti-IgM. Plates were incubated for 48 hr and Alamar Blue was added for the final 4 hr. Nine compounds caused >18% increase in survival relative to the anti-IgM only treatment. The percent survival indicates the percent increase of live cells compared to treatment with anti-IgM only. The compounds causing 'negative survival' were toxic. This data and figure were supplied by the CDRD.

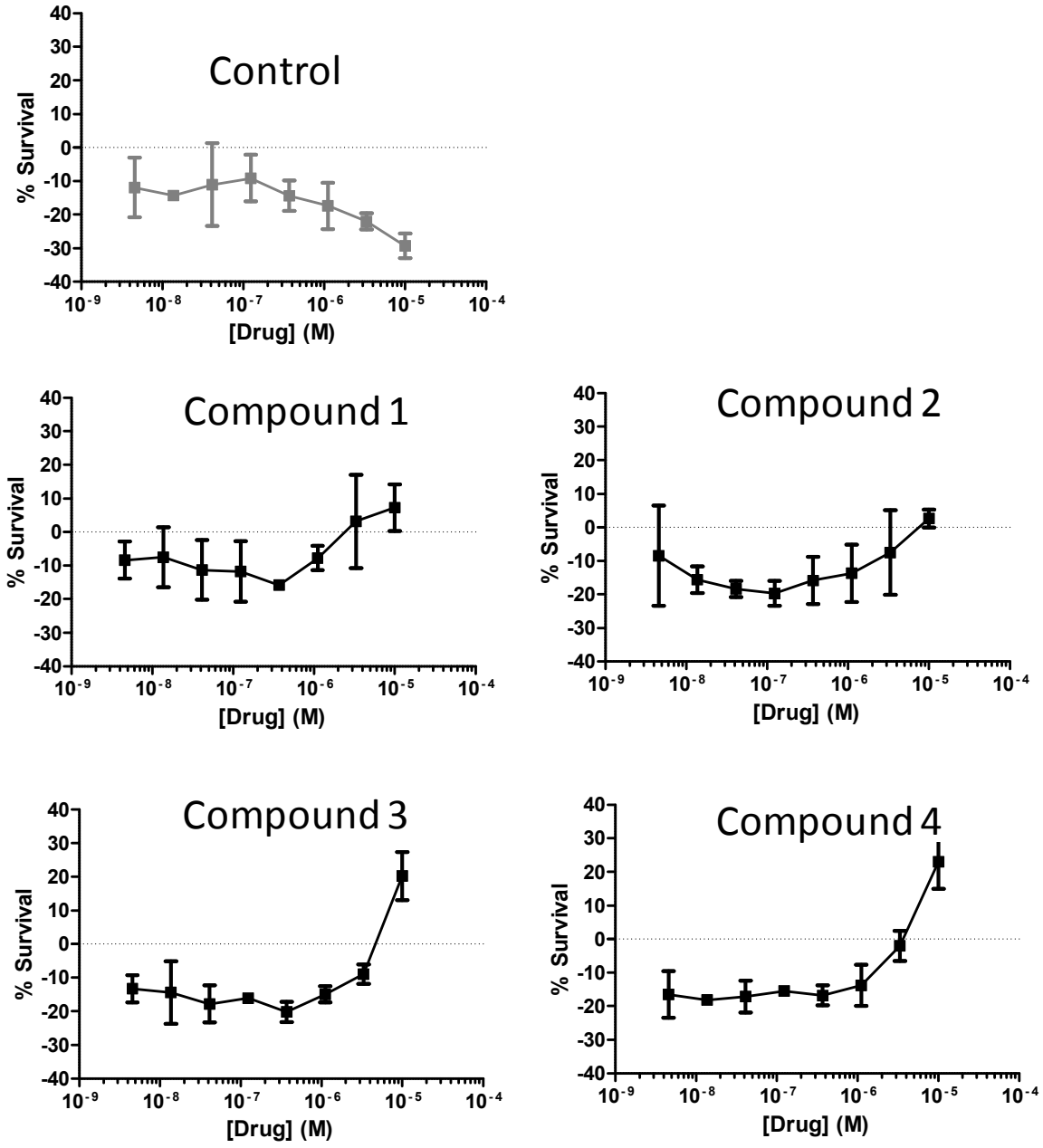


Figure 3-11. Dose response curves for the active compounds from the KD2 screen

CH31 cells were cultured with increasing concentration of four of the nine active compounds from the KD2 screen for 30 min and 20 ng/ml anti-IgM was added and cells were further cultured for 2 days. Alamar Blue dye was added 4 hours prior to reading for determining cell viability. The percent survival indicates the percent increase of live cells compared to treatment with anti-IgM only. Data shown is the average of three independent experiments + SEM. This data and figure were supplied by the CDRD.

3.3.4 Secondary screen with primary B cells

Our ultimate goal is to find compounds that inhibit BCR signaling in mature B cells and inhibit their survival or activation. Our initial screen used the immature B cell line, CH31, in order to rule out toxic compounds and reduce the number of false positives. The active compounds from the primary screen would be used in a secondary screen with mature B cells. Splenic B cells from C57BL/6 mice were isolated, labeled with CFSE and were cultured with different concentrations of anti-IgM in the presence of IL-4. IL-4, which is a cytokine produced by activated T cells during normal immune responses, has little or no ability to induce B cell proliferation by itself, but instead synergizes with BCR-induced survival and proliferative signals (Hodgkin et al., 1991; Howard et al., 1982; Rabin et al., 1986). From the dose response curve, I chose three suboptimal concentrations of anti-IgM (3 μg/ml, 9 μg/ml, 27 μg/ml) to test the active compounds that had been confirmed in the dose response assays from the primary screen (Fig. 3-12). The positive control used was a potent PI3K inhibitor, LY294002 (So and Fruman, 2012). All four compounds tested exhibited a modest (5-15%) ability to reduce anti-IgM-induced B cell proliferation (Fig. 3-13A,B). Although the biggest inhibition seemed to occur when compounds were used at 100 μM, they were toxic (data not shown). Therefore, the biggest inhibition occurred when using the median concentrations of the compound (10 μM). Thus, the active compounds identified in the primary screen with an immature B cell line were capable of attenuating BCR-induced proliferation in mature B cells. The extent of inhibition was of similar magnitude in the immature B cell line and the mature B cells.

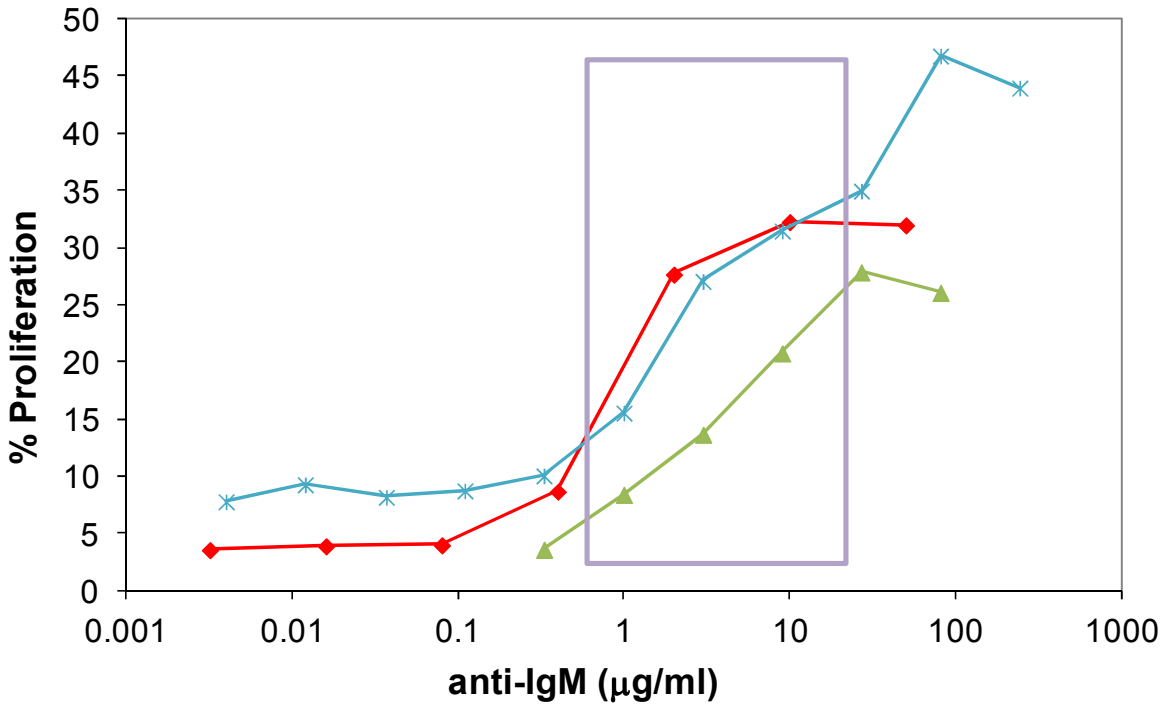


Figure 3-12. Dose response curve for anti-IgM-induced proliferation of murine splenic B cells

Primary murine splenic B cells were labeled with CFSE and cultured with the indicated concentrations of anti-IgM for 2 days. The cells were labeled with 7-AAD to label dead and dying cells prior to being analyzed by flow cytometry. The percent of cells that had proliferated was determined as in Figure 3-3. Triplicate wells were pooled together for analysis in each experiment. Each curve represents one of three independent experiments. The portion of the curve indicated by the purple box depicts the concentration of anti-IgM that would be optimal for compound screening (~IC_5 - IC_{95}).

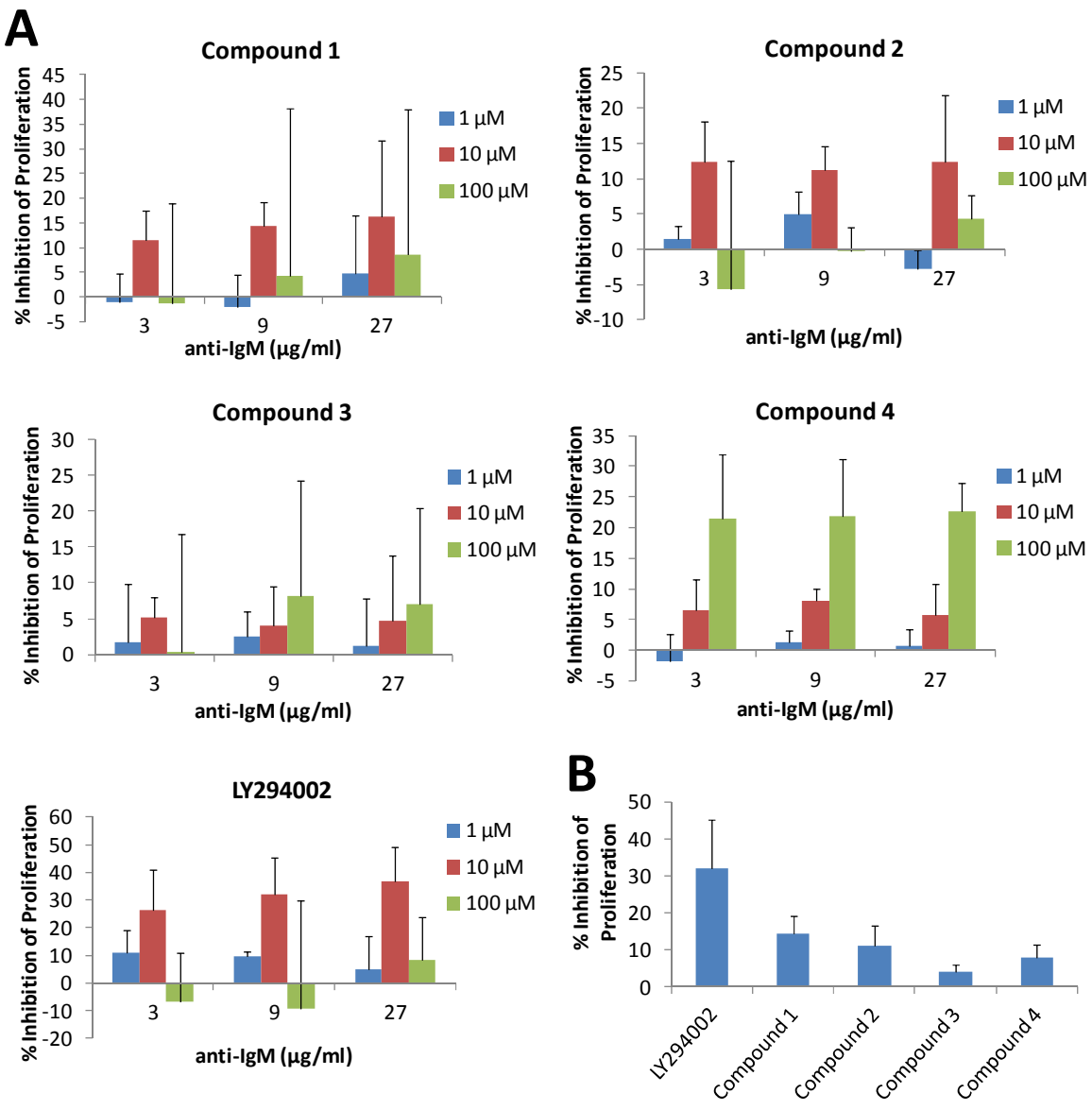


Figure 3-13. Inhibition of anti-IgM-induced murine splenic B cell proliferation by active compounds from the KD2 screen

(A) Primary murine splenic B cells were labeled with CFSE and cultured with anti-IgM in the presence or absence of different concentrations of the active compounds from the primary screen or with the PI3K inhibitor LY294002 for 2 days. The cells were labeled with 7-AAD before being analyzed by flow cytometry. The percent inhibition of proliferation indicates the percent increase of live cells compared to treatment with anti-IgM+IL-4 only (Section 3.2.11). Triplicate wells were pooled together for analysis in each experiment. Each bar is the mean + SEM for three independent experiments. A summary of the largest inhibition (10 µM compound) at the median concentration of anti-IgM (9 µg/ml) is shown in **(B)**.

3.3.5 Reconfirming compounds in a human B cell line

To establish the relevance of these screening assays for human B cells, I tested the four active compounds from the primary screens for their ability to inhibit the growth arrest of the human immature B cell line, B104. This cell line has been used to study anti-Ig-induced growth arrest/cell death (Graves et al., 1996). First, I established an anti-IgM dose response curve with the B104 cells and found that the IC_{70} for anti-IgM-induced growth arrest was ~80 ng/ml (Fig. 3-14). Using this concentration of anti-IgM, I incubated the B104 cells with either 1 or 10 μM of the active compounds from the primary screen and assessed their ability to prevent anti-IgM-induced growth arrest using the Alamar Blue assay. All 4 active compounds from the primary screen except Compound 1 also reduced anti-IgM-induced growth arrest in the human B104 cell line (Fig. 3-15A). Compound 1 appeared to induce growth arrest/cell death more than anti-IgM alone. Because no compounds were toxic from culturing these cells with the compounds alone (Fig. 3-15B), Compound 1 probably acts synergistically with anti-IgM to induce growth arrest/cell death. Because the compounds from the primary screen using mouse immature B cells also prevented growth arrest/cell death in human immature B cells, I have confidence that the mouse B cell lines used for screening are good models for human B cells.

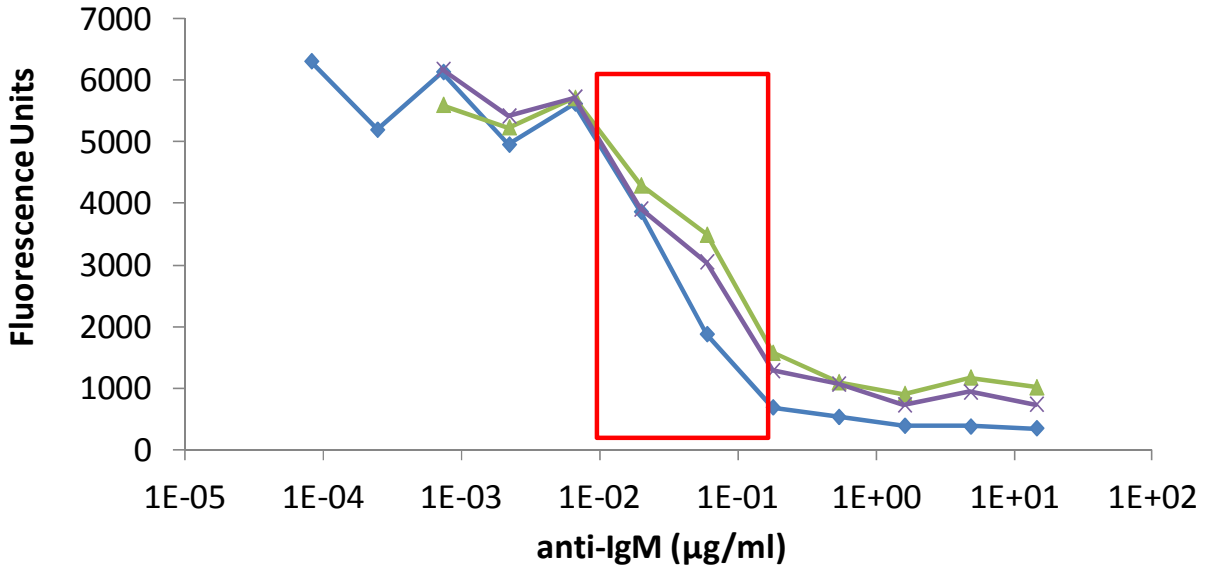


Figure 3-14. Dose response curve for anti-IgM-induced growth arrest in B104 immature human B cells

B104 cells were cultured with different concentrations of goat anti-human IgM for 48 hr before cell viability was measured using the Alamar Blue assay. The portion indicated by the red box represents the IC_5 to IC_{95} for anti-IgM-induced growth arrest. Each curve represents one of three independent experiments.

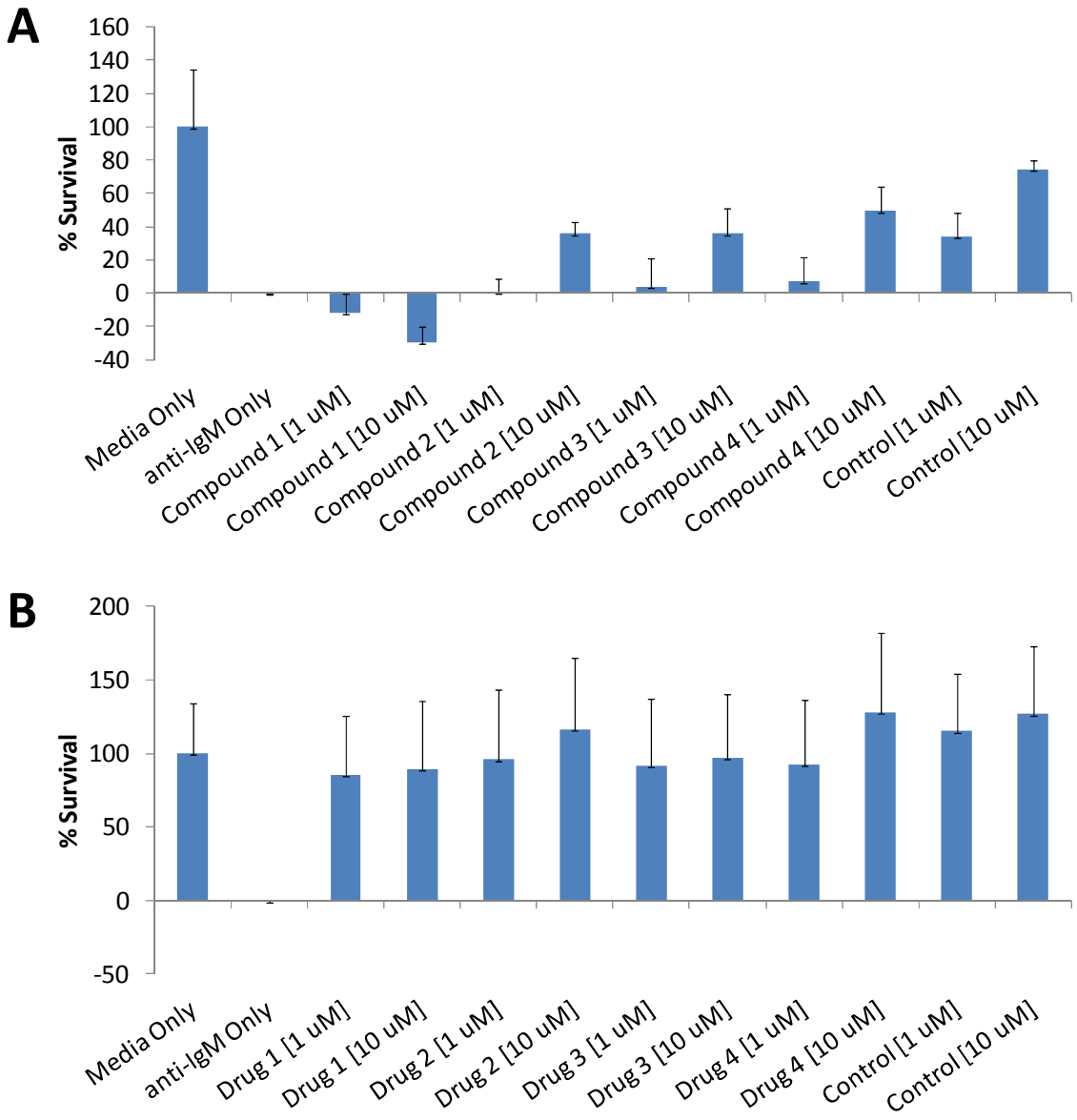


Figure 3-15. Inhibition of anti-IgM-induced growth arrest in B104 cells by active compounds from the KD2 screen

(A) Cells were cultured with 80 ng/ml of anti-IgM with two different concentrations of the actives found from the primary screen (1 μM, 10 μM) for 48 hr. Alamar Blue was used to assess cell viability. Each bar is the mean + SEM for three independent experiments.

(B) Cells were cultured with only the active compounds to assess their cytotoxicity. None of the compounds exhibited significant cytotoxicity. Each bar is the mean + SEM for three independent experiments. Values were relative to anti-IgM only, normalized to 0% survival, and media only, normalized to 100% survival.

The main accomplishments of this chapter were the optimization of assay conditions for several B cell lines (mouse immature B cell lines, CH31 and WEHI-231 [data not shown] and the human immature B cell line, B104) as well as primary splenic B cells, and finding compounds that could be reconfirmed in different model systems from screening compound libraries using these optimized conditions. In this chapter, I found the concentrations of anti-Ig that gives a sensitive assay, which were given to the CDRD to use for screening compound libraries. We found 4 compounds that were able to prevent anti-Ig-induced growth arrest/cell death in both mouse and human immature B cell lines, as well as to prevent anti-Ig-induced proliferation in mature primary splenic B cells. We are now using these assay conditions to screen compounds from much larger compound libraries that could potentially be inhibitors of BCR signaling and be developed as novel drugs.

4 Discussion

4.1 Overall summary of Chapter 2

In this thesis, I showed that Cx43 is a novel regulator of BCR signaling. This finding is consistent with the recent report from our lab showing that Cx43 enhances BCR-induced cell spreading (Machtaler et al., 2011). Using loss-of-function approaches I showed that Cx43 selectively enhances the activation of specific downstream targets of BCR signaling. The presence of Cx43 prolongs BCR-induced activation of the Btk/PLCγ signaling pathway, which controls the Rap GTPase, a master regulator of cytoskeletal organization. Consistent with the role of Cx43 in promoting B cell spreading, the presence of Cx43 enhances the activation not only of Rap but also of another cytoskeletal regulator, the HS1 adaptor protein. Interestingly, other BCR signaling pathways were unaffected by the presence of Cx43, at least under the conditions tested. These results, which are described in Chapter 2, are summarized in Figure 4-1. Although I did not completely describe the role of Cx43 in chemokine receptor signaling, my initial findings suggest that Cx43 also selectively modulates the activation of specific downstream targets of chemokine receptor signaling. Thus, my work provides the basis for further studies of the role of Cx43 in BCR and chemokine receptor signaling.

Chapter 2 summary: The role of Cx43 in BCR Signaling

Our lab has recently been shown that Cx43 mediates BCR-induced cell spreading and chemokine receptor CXCR4-induced B cell migration (Machtaler et al., 2011). To extend these findings, I showed that Cx43 is a target of both BCR and CXCR4 signaling, undergoing a dramatic bandshift on SDS-PAGE gels, which phosphatase treatment suggests is due to phosphorylation (Fig. 2-3). Cx43 phosphorylation of the cytoplasmic tail may create putative

binding sites for signaling proteins (Giepmans, 2004; Lampe and Lau, 2004; Solan and Lampe, 2009). Although binding to Cx43 in a phosphorylation-dependent manner has not been shown, several cytoskeleton-regulating proteins bind Cx43, including myosin II (Olk et al., 2009). Cx43 enhances Rap activation (Machtaler et al., 2011), and Rap activation is important for B cell spreading and migration (Lin et al., 2008; Lin et al., 2010). Therefore, my hypothesis was that Cx43 affects these processes via its ability to enhance Rap activation. My goal was to determine whether Cx43 specifically enhances Rap activation in B cells or modulates multiple BCR and chemokine signaling pathways. By using shRNA to prevent the expression of Cx43, I showed that Cx43 is important for the BCR-induced phosphorylation of multiple components of BCR signaling pathways, specifically Lyn, Syk, Btk, PLCγ2, Erk, Cofilin, HS1 (see summary in Figure 4-1; also Figures 2-6, 2-7, 2-9, 2-11, 2-12). Cofilin and HS1 are important for cytoskeletal reorganization and can potentially link Cx43 to B cell spreading and migration (Freeman et al., 2011; Scielzo et al., 2010). In contrast, the expression of Cx43 did not affect BCR-induced phosphorylation of Akt, JNK, or p38, or impair the initial BCR-induced Ca^{2+} influx (see summary in Figure 4-1; also Figures 2-7, 2-8, 2-10). These results suggest that Cx43 has an important but selective role in BCR signaling, at least under the conditions tested, specifically, stimulating the cells with saturating concentrations of anti-IgM (i.e. much more than what would be needed to cluster all BCR's on the surface of a cell) for 1-60 minutes. The mechanism by which Cx43 selectively enhances specific aspects of BCR signaling remains to be determined. I discuss possible models in Section 4.2

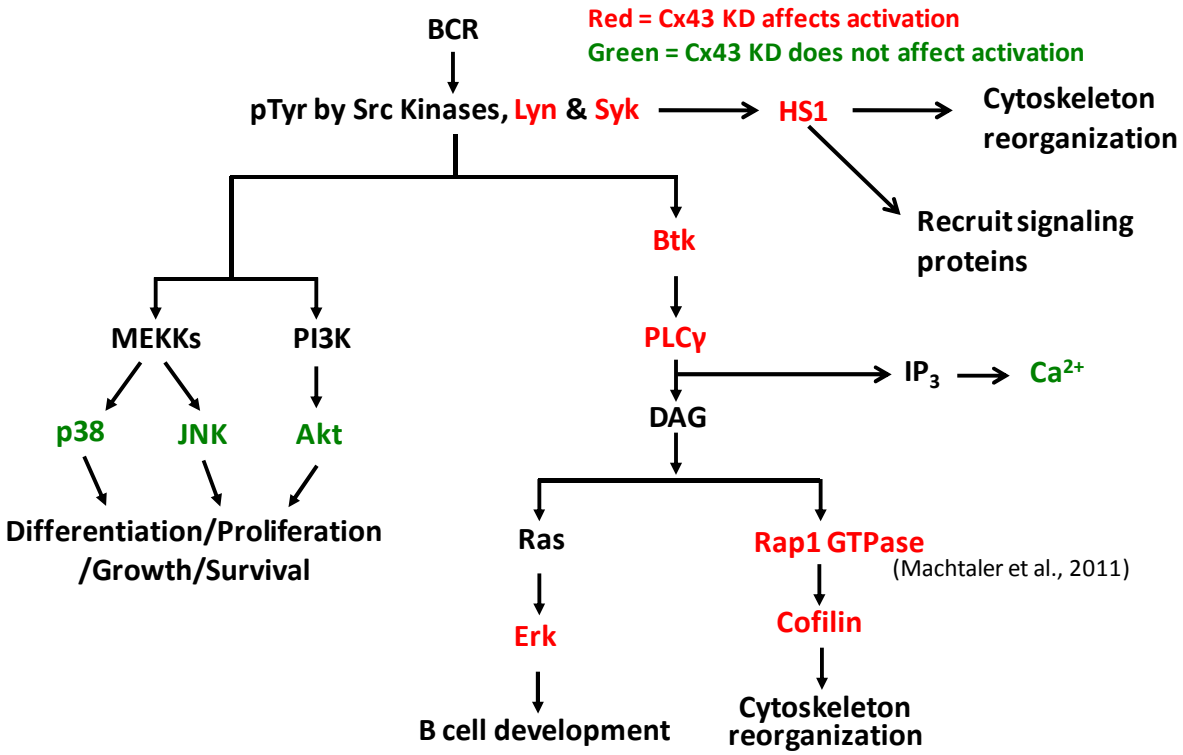


Figure 4-1. Summary of the results in Chapter 2

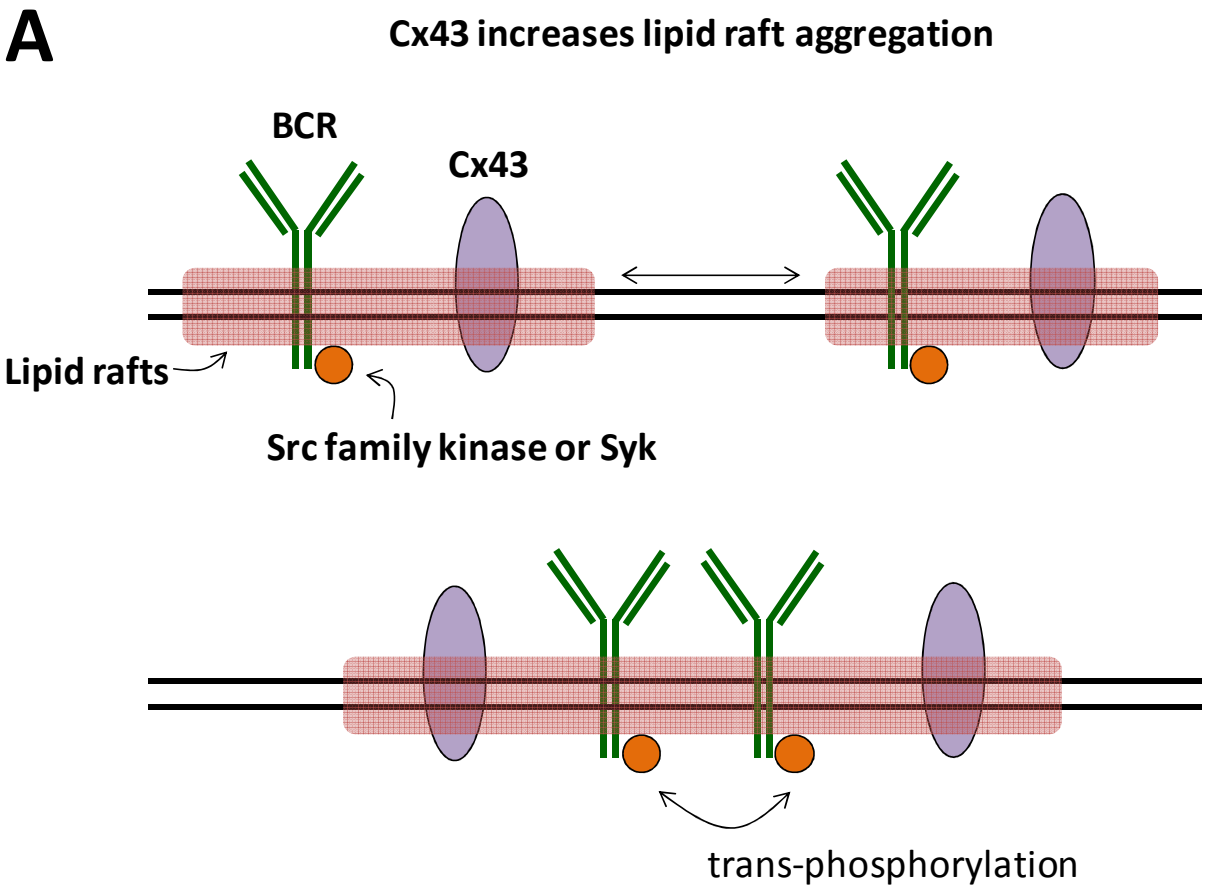
Summary of BCR signaling pathways and how they are affected by the presence of Cx43. Phosphorylation of the targets labeled in red are enhanced and prolonged by the expression of Cx43 whereas activation of the targets labeled in green are not affected by the expression of Cx43. How Cx43 modulates the activation of the Rap1 GTPase has already been published (Machtaler et al., 2011).

4.2 Proposed Models for Cx43's role in BCR signaling

4.2.1 Model 1: Cx43 is a global modulator of BCR signaling that targets proximal BCR signaling reactions

In this model, Cx43 would enhance BCR-induced activation of the most proximal effectors of BCR signaling, Src family kinases and the Syk kinase, leading to increased activation of all BCR signaling pathways. One possible way could be enhancing lipid raft aggregation (Fig. 4-2A). During BCR signaling after ligand binding, BCRs are thought to

associate with lipid rafts, which are membrane microdomains rich in cholesterol and sphingolipids, and can compartmentalize cellular processes. These lipid rafts may serve to separate proteins involving positive BCR signaling (eg. Lyn) from those involved in negative signaling (eg. CD45) (Gupta and DeFranco, 2007; Pierce, 2002). Given that Cx43 may be recruited to lipid rafts upon phosphorylation (Lin et al., 2003; Schubert et al., 2002), it is possible that Cx43 may serve to enhance aggregation of lipid rafts, thereby facilitating BCR clustering. Another possibility could be that Cx43 may physically co-localize with the BCR and perhaps recruit Src kinases or Syk in a phosphorylation-dependent manner (Fig. 4-2B). These models predict that all BCR signaling reactions would be enhanced by the presence of Cx43.



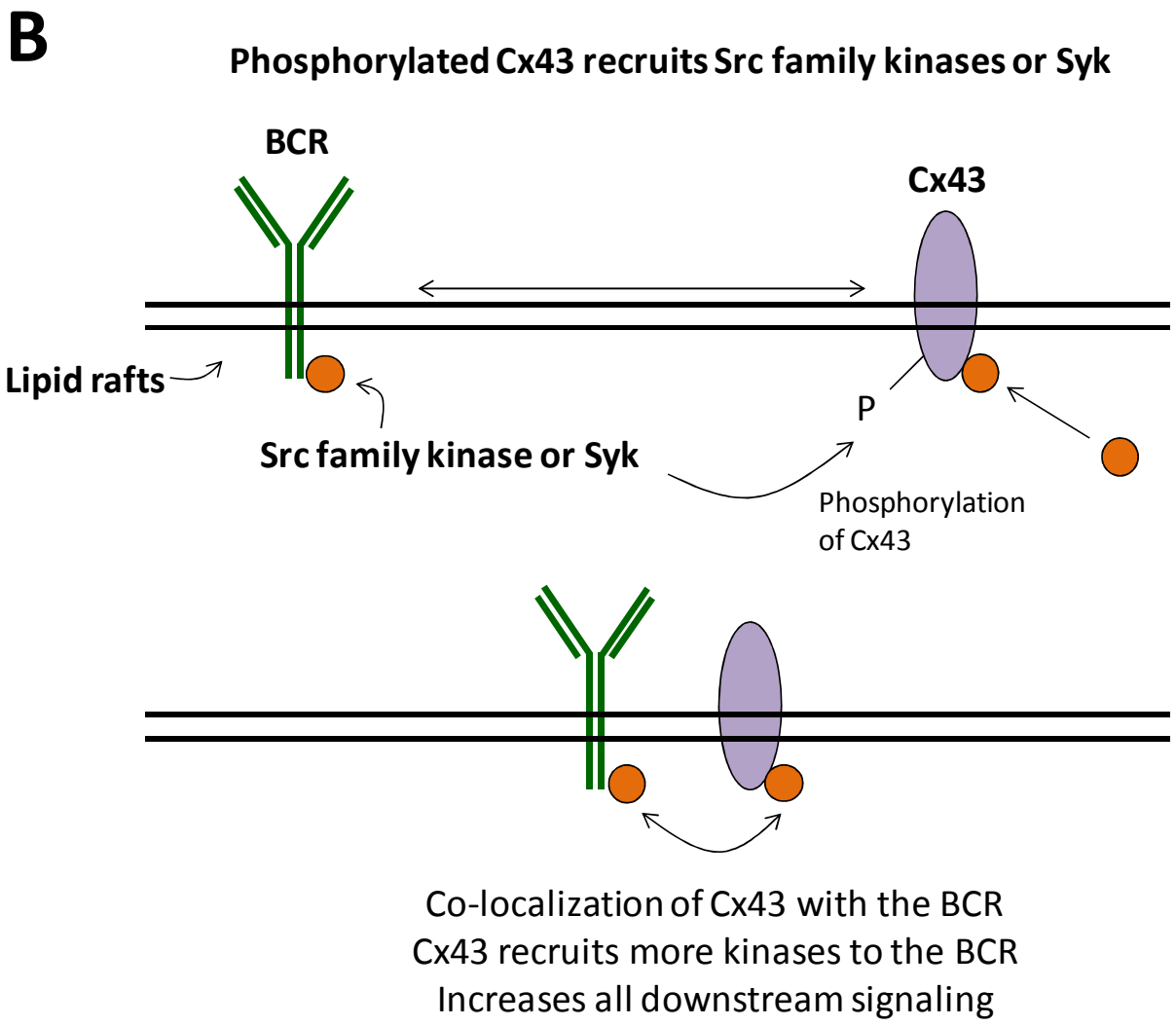


Figure 4-2. Proposed models for how Cx43 is a global modulator of BCR signaling

(A) The phosphorylation of the BCR and Cx43 causes recruitment to lipid rafts. Cx43 may enhance lipid raft aggregation, facilitating BCR aggregation and Src family kinase or Syk (orange circle) trans-phosphorylation.

(B) BCR-induced phosphorylation of Cx43 may create SH2 binding domains on the cytoplasmic tail of Cx43. These domains may recruit SH2 domain-containing Src family kinases or Syk (orange circle). Physical co-localization of Cx43 with the BCR allows increased recruitment of Src family kinases and Syk to the BCR, thereby increasing all downstream signaling reactions.

However, this was not the case at the concentration of anti-IgM I used in Chapter 2. I found that the phosphorylation of Akt, JNK, and p38, as well as Ca^{2+} fluxes were unaffected by Cx43 expression. Because the signaling experiments in Chapter 2 were done using super-maximal amounts, one possible explanation is that some these downstream signaling pathways are maximally activated by low levels of BCR-induced Lyn and Syk activation. It is estimated that there are approximately 10^5 BCRs on the surface of each B cell. Given that I used 10^6 cells per time point in each experiment, and one anti-IgM clusters two BCRs together, the saturating amount of anti-IgM that would engage all BCRs are equilibrium conditions would be approximately 12 ng. However, because I was looking at short time courses, I wanted to synchronize BCR signaling in order to induce large measurable signaling events, hence, I used 20 μg. At this concentration of anti-IgM, I may have missed differences that would have been observed at sub-optimal concentrations of the stimulating ligand. Performing anti-IgM dose-response curves would address this problem. In addition, to dose-response curves, for some signaling reactions that appeared to be unaffected by Cx43 expression, more complete time courses may also reveal differences. For example, BCR-induced Ca^{2+} fluxes were assayed over a time course of 5 min whereas PLCγ2 phosphorylation was not sustained over longer periods of time in Cx43 KD cells. Moreover, the initial spike in intracellular Ca^{2+} levels that is due to the release of intracellular Ca^{2+} stores may not be important for responses such as anti-Ig-induced growth arrest in WEHI-231 cells (Page and DeFranco, 1988). Instead, it appears to be the very small, sustained Ca^{2+} increases that are important for BCR-induced effects on cell growth. One way to assess Ca^{2+}-dependent BCR signaling over a longer time scale would be to look at NFAT nuclear localization over a period of minutes to 6-8 hours. NFAT is a transcription factor that is localized in the cytosol in resting cells. Upon Ca^{2+} binding, calmodulin, the calcium sensor

protein, activates the phosphatase calcineurin, which dephosphorylates NFAT proteins, resulting in a conformational change that reveals a nuclear localization signal. The nuclear localization of NFAT could be assessed by immunofluorescence microscopy.

Potential outcomes of the anti-IgM dose response experiments and the longer time courses experiments could be:

(A) The phosphorylation of Akt, JNK and p38, or NFAT nuclear localization is reduced in Cx43 KD cells, especially at sub-optimal doses of anti-Ig or at longer time points, consistent with the requirement for Cx43 to sustain specific BCR signaling reaction. Such results would lead me to conclude that Cx43 enhances all BCR signaling pathways (at least enhances all that I have examined), and may not selectively modulate specific pathways. The most likely mechanistic basis for this is that Cx43 enhances BCR-proximal signaling events such as tyrosine kinase activation, which in turn regulate all other BCR signaling events.

(B) The phosphorylation of Akt, JNK and p38, as well as NFAT nuclear localization, are not dependent on Cx43 expression. In this case, I would conclude that Cx43 selectively enhances specific BCR signaling pathways.

4.2.2 Model 2: Cx43 is a selective modulator of specific BCR signaling pathways

If Cx43 acts to selectively enhance the activation specific signaling pathways of the BCR, one way in which it may do so is to act as a transmembrane scaffolding protein that binds specific signaling molecules. This may involve putative interaction motifs in the C-terminal cytoplasmic domain of Cx43 (Fig. 1-8), or in its other cytoplasmic domains, and could be dependent on BCR-induced phosphorylation within one or more of the Cx43 cytoplasmic

domains. This would be analogous to the BCR co-receptor, CD19, which amplifies BCR signaling by recruiting PI3K to the plasma membrane, thereby promoting activation of the PI3K/Akt pathway by the BCR (Fruman and Limon, 2012; Kurosaki et al., 2010). Because the cytoplasmic tail of Cx43 has potential binding motifs for SH2 domain-containing proteins, it is possible that upon phosphorylation of these motifs, signaling molecules such as BLNK bind to Cx43 (Fig. 4-3). BLNK could then recruit PLCγ and Btk, which could lead to the phosphorylation of PLCγ. It is possible that assembly of the BLNK/Btk/PLCγ signalosome nucleated by the binding of BLNK to phosphorylated Igα represents an initial wave of BCR signaling whereas assembly of BLNK/Btk/PLCγ signalosomes by Cx43 is important for sustained PLCγ phosphorylation, as well as sustained downstream signaling events including the activation of Rap1 and Cofilin, both of which regulate actin reorganization. In addition, Cx43 could bind the scaffolding protein HS1 through its SH2-binding domain to the putative SH2 domain on the Cx43 cytoplasmic tail, bringing it in proximity to the BCR signalosome that includes Syk, which has been shown to phosphorylate HS1 (Scielzo et al., 2010; Yamanashi et al., 1997). HS1 could then recruit PLCγ to the membrane near the BCR signalosome (Gomez et al., 2006), facilitating the phosphorylation of PLCγ. HS1 is also important in regulating cytoskeletal dynamics via activating the Arp2/3 complex (Hao et al., 2005). Another possibility could be that BCR-induced phosphorylation of Cx43 has a more direct effect on the cytoskeleton through HS1. We could test this model using phospho-tyrosine-specific antibodies against Cx43 and co-immunprecipitation to see if HS1 and/or BLNK binds phosphorylated Cx43.

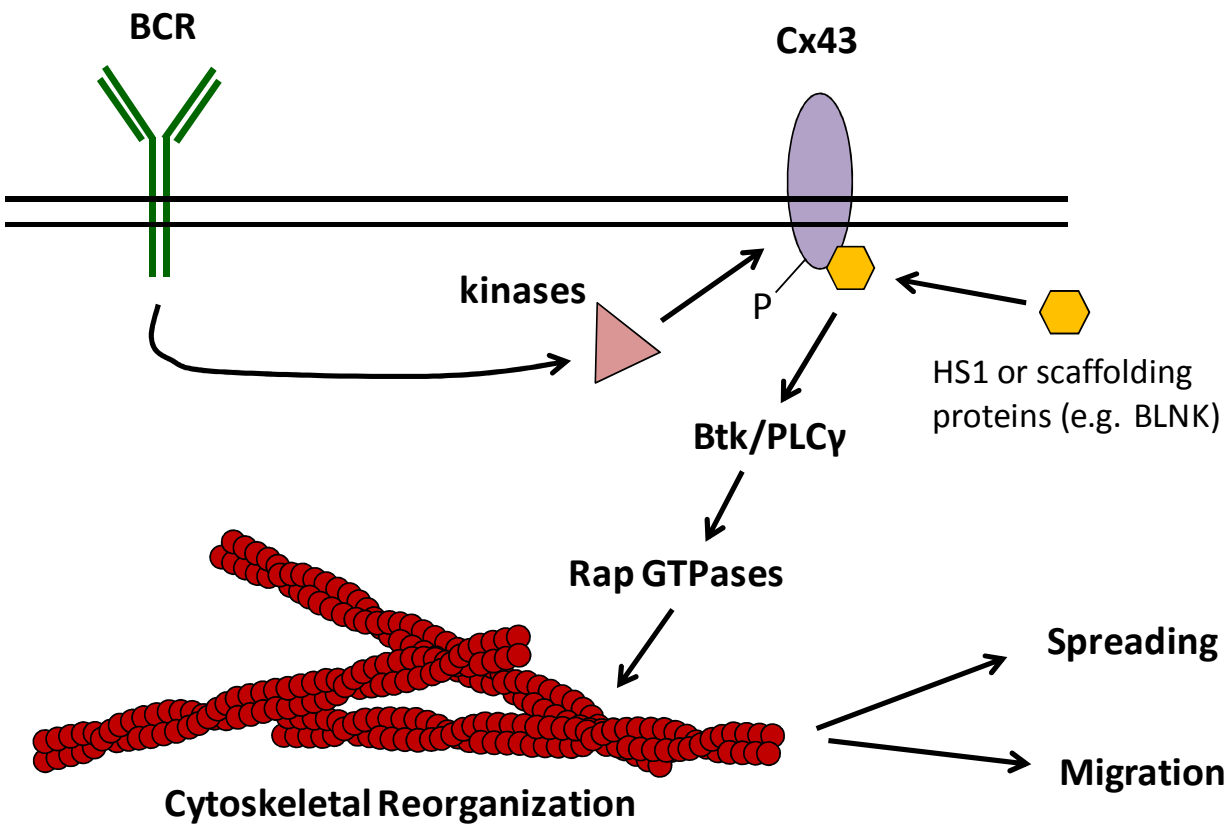


Figure 4-3. A proposed model for how Cx43 influences BCR- and chemokine receptor-induced cytoskeletal rearrangements

Signaling by the BCR activates kinases (pink triangle) that phosphorylate the Cx43 carboxyl-terminal tail. Phosphorylated Cx43 can act as a scaffold protein, recruiting other scaffolding molecules including HS1 or possibly BLNK (yellow hexagon). Binding of these molecules recruits and promotes the activation of the PLCγ pathway, which subsequently leads to the activation of Rap1 GTPases via exchange factors that are controlled by DAG. Rap1 controls the localization and activity of multiple proteins that regulate cytoskeletal reorganization, enabling the breakdown and reorganization of the actin cytoskeleton that enables B cell spreading and B cell migration. HS1 also has a role in cytoskeletal reorganization, and BCR-induced recruitment of HS1 to the phosphorylated Cx43 may increase actin reorganization more directly.

4.3 Overall summary of Chapter 3

BCR signaling is important not only for normal B cell activation during protective immune responses but also underlies aberrant B cell activation during autoimmune diseases and the survival of malignant B cells. Thus, drugs that inhibit BCR signaling may be useful for a variety of disease states. As part of this thesis, I also optimized assays for screening compound libraries for novel inhibitors of BCR signaling. The results of preliminary screens that were performed in collaboration with the CDRD are summarized in Chapter 3. Work on this project is still ongoing.

Chapter 3 summary: High throughput screen for novel inhibitors of BCR signaling

Drugs that deplete B cell populations are currently being used or being investigated for the treatment of B cell-mediated autoimmune diseases such as rheumatoid arthritis and lupus, and various B cell malignancies including non-Hodgkin lymphomas (Engel et al., 2011; Selewski et al., 2010; Stohl and Hilbert, 2012). However, such drugs render patients susceptible to infections and are associated with high relapse rates due to incomplete B cell depletion, many of which are tissue-resident MZ B cells (Gong et al., 2005). Also, some inhibitors of BCR signaling proteins are currently in clinical trials but more are needed. Therefore, additional drugs, especially orally available small molecule inhibitors of B cell activation that can inhibit or deplete harmful B cells while maintaining some degree of normal B cell function, could be useful.

Given my project's focus on B cell signaling pathways, I participated in a screening project in collaboration with the CDRD to identify novel inhibitors of BCR signaling. I optimized several assays that would be used for screening for such inhibitors. These assays

made use of both mouse (CH31, WEHI-231) and human (B104) immature B cell lines. Immature B cell lines are an advantage for the primary screen because they can be used to rule out cytotoxic compounds. Mature B cells proliferate in response to BCR stimulation but die when treated with BCR signaling inhibitors, which makes it difficult to differentiate with cytotoxic compounds. On the other hand, immature B cells, which have similar BCR signaling mechanisms as mature B cells, die when stimulated through their BCR. Thus inhibitors of BCR signaling would prevent cell death, resulting in a screening assay that would ignore the large number of potentially cytotoxic compounds, a common problem in cell-based screening assays. These assays proved to be robust, in that there were large differences between positive and negative control treatments, and they have facilitated preliminary screens. In order to further analyze the potential utility of compounds identified in these initial screens, I also developed a secondary screening assay that could be used to assess the ability of these compounds to prevent anti-Ig-induced proliferation of primary B cells from mouse spleen. I am currently working on developing tertiary screens in which compounds identified in these screens can be tested whether they prevent anti-Ig-induced proliferation in primary human B cells as well as human B cell lymphomas. Assays to do this will be similar to assays developed in Section 3.2.9 and 3.2.10.

Successful compounds might be pursued by the CDRD and moved towards the clinic. Pre-clinical trials will involve mice to test absorption, distribution, metabolism, toxicity and excretion of the drug both *in vitro* and *in vivo*. These trials will allow us to determine whether the drug candidate meets the criteria to be an investigational new drug, allowing clinical phase trials to begin. Clinical phases trials include: Phase I (Is the drug safe?), Phase II (Does the drug

work?), Phase III (Is it better than the drugs we have now?), and Phase IV (Are there long term side-effects?).

4.4 Future directions

4.4.1 Is Cx43 a global or selective modulator of BCR/chemokine receptor signaling and does Cx43 contribute to early BCR/chemokine receptor signaling or just to sustaining it?

As mentioned previously, I was using a super-maximal concentration of anti-IgM and chemokine CXCL12 and looked at protein phosphorylation for up to 1 hour. Doing anti-IgM and CXCL12 dose responses may give us a better view of how proteins that are sensitive to low concentrations of stimulant are being phosphorylated and whether their activation is actually modulated by Cx43. Each protein has a different EC50 (half maximal efficacy concentration) to anti-IgM and stimulating the cells at the EC50 value of anti-IgM corresponding to each protein will reveal whether Cx43 modulates that particular protein. Also, certain proteins such as Ca^{2+}-mediated NFAT activation occurs at time points beyond the 1 hour that I examined in Chapter 2. Looking at longer stimulation time points would allow me to determine if Cx43 influences early signaling or helps sustain signaling.

4.4.2 How does Cx43 enhance BCR or chemokine receptor signaling?

a) Identify proteins that bind to Cx43 in response to BCR and chemokine receptor signaling: Whether proteins bind to Cx43 in B cells has not been shown. Given that cortactin binds to Cx43 in many cell types (Bryce et al., 2005; Lai et al., 2009), I propose that the hematopoietic-specific homolog of cortactin, HS1, will bind to Cx43 in B cells. There are many proteins that bind Cx43 either directly or indirectly in other cell lines (Section 1.6.3), including

myosin II and ZO-1. These candidate proteins that interact with the Cx43 carboxyl tail can be identified through co-immunoprecipitation studies in B cells. An unbiased approach would utilize chemical crosslinking to preserve native protein-protein interactions. Reversal of the crosslinking process and subsequent mass spectrometry could determine which proteins bind Cx43.

b) Identify how these proteins connect Cx43 to BCR signaling pathways: Once the proteins that bind to Cx43 are identified, it will allow us to gain mechanistic insights into how Cx43 modulates BCR signaling pathways. Because both BCRs and Cx43 molecules may accumulate onto lipid rafts after phosphorylation (Schubert et al., 2002), and Cx43 may act as a scaffolding protein due to its numerous putative protein binding domains in the cytoplasmic tail (Palatinus et al., 2011), the close proximity of Cx43 to the BCR may aid in the activation of these proteins bound to Cx43 by bringing them near the BCR-activated kinases that phosphorylate and activate them. Co-localization experiments would be useful to determine whether Cx43 accumulates near the antigen-bound BCR.

c) Identify how these proteins bind to Cx43: The carboxyl-terminal cytoplasmic tail contains many phosphorylation sites that can potentially be protein-binding sites. Mutation of specific phosphorylation sites through site-specific mutagenesis could address if these sites link Cx43 to proteins that enhance BCR signaling.

4.4.3 Does Cx43's effects on BCR and chemokine receptor signaling have a role in B cell biology *in vivo*?

One of the caveats of Chapter 2 is that the signaling was done with immortalized B cell lines, which may have altered expression of various signaling proteins. To overcome this obstacle, we would need to examine whether Cx43 influences BCR and CXCR4 signaling during normal B cell development and *in vivo* B cell activation. Because homozygous Cx43 knockout mice exhibit perinatal lethality, B cell-specific Cx43 knockout mice could be generated by crossing $Cx43^{flox/flox}$ mice (Liao et al., 2001) to mb-*1*/*Cre* mice (Hobeika et al., 2006). The mb-1 gene, which encodes Igα is expressed only in B cells and hence, in these mice, the Cre recombinase would be expressed only in B cells. $Cx43^{flox/flox}$ mice that are crossed with mice that are heterozygous for the Cre insertion into the mb-1 locus would yield mice in which the Cx43 gene is deleted only in B cells. These mice could then be used to address the B cell-intrinsic role of Cx43 in B cell development and function.

a) B cell development: B cell-specific Cx43 knockout mice will be compared to wild-type mice, as well as to the two parental mouse strains to determine whether loss of Cx43 leads to a defect in B cell development. Flow cytometry would be used to determine the numbers and relative frequencies of different B cell progenitors in the bone marrow, the number of peripheral B cells in the spleen and lymph nodes, the number of innate-like B-1 and marginal zone B cells in the spleen and the number of B-1 cells in the peritoneal cavity .

b) B cell trafficking *in vitro* and *in vivo*: Migration assays *in vitro* will need to be done to examine chemokine-stimulated adhesion, migration and extravasation across endothelial cell

monolayers. To assess the role of Cx43 in B cell trafficking *in vivo*, wild type and Cx43-deficient B cells will be labeled with different fluorescent dyes, mixed in equal numbers, injected into the tail vein of mice, and their relative ability to home to lymph nodes would be assessed by flow cytometry.

c) B cell activation *in vitro* and *in vivo*: Whether Cx43-deficient primary B cells exhibit impaired activation and proliferation in response to anti-Ig antibodies or TLR ligands will first be examined *in vitro*. In addition to looking at BCR signaling (to see if the failure to sustain BCR signaling is reproduced in the Cx43 knockout B cells), it would good to look at anti-Ig-induced upregulation of activation markers (eg. CD69/CD80) via flow cytometry, activation of key transcription factors (eg. AP-1, NFAT, and NF-κB) via mobility shift assays involving non-denaturing polyacrylamide gels, and changes in gene expression via microarrays. It would be interesting to examine levels of each Ig isotype in serum *in vivo*, and then look at levels of specific Abs such as the levels of circulating natural antibodies (eg. anti-cardiac myosin, which is relevant for heart transplants; anti-oxidized LDL, which is relevant for the clearance of oxidized LDLs that cause atherosclerosis) produced by marginal zone B cells B-1 cells. Also, antibody responses to both T-dependent (i.e. contains a protein component) and T-independent antigens (eg. bacterial polysaccharides) will be measured (El Shikh et al., 2009; Kalache et al., 2011; Pawlak et al., 2012).

4.4.4 Targeting BCR signaling as potential treatments for B cell-mediated diseases

a) Autoimmune diseases: Inappropriate BCR-induced activation of self-reactive B cells can lead to autoimmune disease that can result in chronic inflammation and organ damage.

Normally, self-reactive B cells are not deleted but remain anergic, and inappropriate activation of such circulating cells can lead to autoimmunity (Cambier et al., 2007). Additionally, B cells play a major role in transplant rejection by generating antibodies that target the transplanted tissue for complement- and cell-mediated damage. B cells also promote chronic antibody-independent graft rejection by presenting donor antigens to T cells, producing cytokines, and developing ectopic lymphoid structures that promote inflammatory response (Platt et al., 2011). Because inappropriate BCR signaling can lead to the activation of autoimmune B cells, the inhibition of BCR signaling could be useful for treating B cell-mediated autoimmune diseases by aiding in B cell depletion.

b) B cell lymphomas: Antigen-independent BCR signaling is also important for the survival of some B cell cancers, especially non-Hodgkin's B cell lymphomas as most still express the BCR and require BCR signaling (Gururajan et al., 2006). The inhibition of BCR signaling could be a useful strategy for eliminating these malignant cells. Also, B cell dissemination and metastasis rely on cytoskeletal dynamics. Our lab has previously shown that Rap1 is important for B cell dissemination and tumor formation (Lin et al., 2010). Inhibition of BCR signaling or cytoskeletal dynamics could therefore prevent tumor growth or dissemination.

B cell depletion drugs and inhibitors of specific BCR signaling proteins such as Syk and Btk are currently in use to treat B cell-mediated autoimmune diseases and B cell lymphomas. These drugs work well initially, but relapse rates are high due to incomplete depletion of B cells (Engel et al., 2011; Reeder and Ansell, 2011). Additional drugs that can inhibit B cell function or deplete B cells more efficiently are needed.

Because Cx43 is important for Rap1 activation and is a positive regulator of BCR signaling (Chapter 2), downregulating its expression may be useful for treating B cell-mediated autoimmune diseases or BCR-expressing B cell lymphomas. Importantly, our screen in Chapter 3 for novel inhibitors of BCR signaling has the potential to identify small molecule inhibitors of BCR signaling, which could then be developed as drugs. This screen may also identify novel BCR signaling proteins that are targeted by these drugs and are important for B cell survival and activation.

4.5 Significance

This thesis provides the first evidence that Cx43 modulates specific aspects of BCR signaling, especially pathways that influence cytoskeletal dynamics. This work identifies a new regulator of B cell cytoskeletal reorganization, a process that is important for many aspects of normal B cell function including the migration of B cells into and within lymphoid organs during development and circulation as well as B cell activation. Similar cytoskeletal dynamics are important in B cell-mediated autoimmune diseases and the dissemination of cancerous B cells. Further understanding of cytoskeletal reorganization and the role of Cx43, may facilitate the development of better ways to prevent or treat these disease processes. At the same time a screen for novel inhibitors of BCR signaling could identify novel small molecule inhibitors that could be used therapeutically to inhibit B cell activation and B cell survival in patients suffering from B cell-mediated autoimmune diseases or B cell cancers.

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