ABSTRACT

Title of Document: THE CROSSTALK BETWEEN B-CELL RECEPTOR MEDIATED SIGNALING AND THE ACTIN CYTOSKELETON

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Oligomerization of the B-cell receptor (BCR) by antigen leads to both signal transduction and antigen internalization for processing and presentation. Previous studies indicate that these processes intersect at the actin cytoskeleton to coordinate the two cellular processes for the optimal activation of B-cells. The exact mechanism by which signals are transduced via the actin cytoskeleton into the efficient internalization and transport of BCR-antigen complexes is not well delineated. In this thesis, I demonstrate that Bruton's tyrosine kinase (Btk), a Tec kinase in the early signaling pathway of the BCR, is able to transduce signals from the BCR to actin regulatory proteins such as WASP and N-WASP. Upon BCR activation, Btk modulates actin dynamics by increasing the levels of phosphorylated, active WASP and N-WASP in B-cells. Btk regulates the activity of WASP and N-WASP by increasing the levels of PtdIns-4,5-P_2 and phosphorylated Vav, both of which are

required for WASP and N-WASP activation. Inhibition of Btk activity by a point mutation or a specific inhibitor prevents BCR-induced increases in PtdIns-4,5-P_2 as well as in phosphorylated WASP, N-WASP and Vav. Furthermore, Btk deficiency or inhibition leads to a severe reduction in BCR-mediated antigen internalization, processing, and presentation to cognate T-cells. Further studies on the role of WASP show no significant effect of WASP deficiency on BCR internalization, while WASP deficiency affects B-cell development, decreasing the numbers of T1/T2 immature B-cells and marginal zone B-cells. Intriguingly, the protein expression levels of N-WASP and WAVE-2, homologues of WASP, increase in $WASP^{-/-}$ B-cells, implicating a compensatory role for WASP homologues in the absence of WASP. Over-expression of N-WASP's proline-rich domain inhibits BCR-mediated antigen uptake and intracellular transport. All of these data indicate that Btk, which is activated upon BCR binding to antigen, regulates actin dynamics and consequently antigen uptake and transport, by activating WASP and N-WASP via Vav and phosphatidylinositides. This presents a novel mechanism by which BCR-mediated signaling regulates BCR-mediated antigen processing and presentation.

THE CROSSTALK BETWEEN B-CELL RECEPTOR MEDIATED SIGNALING AND THE ACTIN CYTOSKELETON

By

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Dedication

To Science,

*f*or being a *tour de force*

&

To Endless Curiosity,

*f*or what lies behind the watchface is an unexplored world filled with wondrous truths!

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I was born not knowing and have had only a little time to change that here and there.

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*f*or teaching me about the Doppler Effect when I was but 3 watching a sunset

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*f*or teaching me to love all things living

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AP-2	Adaptor protein 2
APC	Antigen presenting cells
Arp2/3	Actin related proteins 2 and 3
BAFF	B-cell activating factor of the tumor-necrosis-factor family
Bam32	B cell adaptor molecule of 32 KDa
Bcl-xl	Bcl2-like 1
BCR	B cell antigen receptor
BLNK	B cell linker protein
Btk	Bruton's tyrosine kinase
CD	cluster of differentiation
CTX-B	Cholera toxin subunit B
CRIB	Cdc42/Rac1 interactive binding
DAG	Diacylglycerol
DAMP	Danger associated molecular patterns
DC	Dendritic cell
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FO	Follicular B-cells
GEF	GTPase exchange factor
GFP	Green fluorescent protein
HA	Hemagglutinin
HEL	Hen egg lysozyme
Ig	Immunoglobulin
IP_3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine based activation motif
JNK	c-Jun NH_2-terminal kinase

KZH	I-A^k/LacZ-inducible/ HEL specific T-cell hybridoma
LAMP-1	Lysosomal associated membrane protein 1
LAT B	Latrunculin B
LFM A-13	Leflunomide metabolite analog 13
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mIg	Membrane immunoglobulin
MIIC	MHC class II containing compartment
c-Myc	cytoplasmic myelocytomatosis oncogene
MZB	Marginal Zone B-cells
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κ B
NK	Natural killer cell
N-WASP	Neural Wiskott-Aldrich syndrome protein
PAMP	Pathogen associated molecular patterns
PH	Pleckstrin homology
PI	Phophoinositol
PI-3-K	Phosphatidyinositol-3-kinase
PtdIns-4,5-P_2	Phosphatidyinositol 4,5-bisphosphate
PtdIns-3,4,5-P_3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PLCγ2	Phospholipase C-γ
PRD	Proline rich domain
PRR	Pathogen recognition receptors
SH2	Src homology 2
SH3	Src homology 3
SHIP	(SH2)-domain-containing inositol 5-phosphatase
SHP-1	(SH2)-domain-containing protein tyrosine phosphatase 1
siRNA	short interfering RNA
TCR	T cell antigen receptor
Tf	Transferrin

TfR	Transferrin receptor
TH	Tec homology
T_H	T helper cells
TLR	Toll-like receptor
TK	Tyrosine kinase
VCA	Verprolin/Cofilin homology/Acidic
WA	WH2/Acidic domain
WAS	Wiskott Aldrich Syndrome
WASP	Wiskott Aldrich Syndrome Protein
WIP	WASP-Interacting Protein
WT/Wt	wild type
XID	X-linked Immunodeficiency
XLA	X-linked Agammaglobulinemia
XL	Cross-linking surface BCR

Chapter 1: General Introduction

The immune system is constantly in a state of homeostatic flux, balancing the acts of protection and self-destruction. Any deliberate shift in this homeostasis, caused by an invading foreign body or the host itself, elicits a complex immune response comprised of *innate* and *adaptive* facets. The *innate* immune response is a pan-species, evolutionarily conserved, first line of defense against pathogens. Originally thought to lack specificity, the vertebrate innate immune response has emerged as being highly capable of discriminating self from non-self [1-3]. It plays an increasingly appreciated role in conventional immune defense against invading pathogens, as well as in cancer, autoimmunity and immunodeficiency [4-8]. The innate immune response is broadly triggered by highly conserved pathogen associated molecular patterns (PAMPs) which are shared by large groups of microorganisms or endogenous stress signals termed danger associated molecular patterns (DAMPs). These patterns are recognized by cognate pattern recognition receptors (PRRs). PRRs are germline encoded, non-clonally specific receptors that rapidly activate the effector cells which express them. Cells of the skin, epithelia of the gut and lung and leukocytes like macrophages, neutrophils, eosinophils, mast cells, dendritic cells (DCs),express PRRs and are involved in initiating an innate immune response [9-15]. Classically, the main functions of this response are the phagocytosis and killing of microbes and the activation of complement and pro-inflammatory pathways [16-18]. However, innate involvement in determining the outcome of an adaptive immune response is now widely accepted [19-21]. In contrast to the primeval nature of the innate immune system, the *adaptive* or a*cquired* branch has

been a recent and dramatic acquisition of the vertebrate phylum. It is identified by the presentation of the four archetypal features of an immune response, namely: specificity, memory, diversity, and self-nonself discrimination. The development and progression of the adaptive immune response is dependent on the tightly regulated activation of T and B-lymphocytes, which possess clonally-distinct antigen receptors on their cell surface. These receptors, are germline encoded and generated from numerous gene fragments undergoing rearrangement, mutagenesis, expression and selection in order to recognize and discriminate pathogen-specific features from self [22, 23]. Such clonally specific B/T-cells are responsible for responding to the invading pathogen and later in the immune response generate a 'memory' of the pathogenic antigen [24-26]. The outcome of the adaptive immune response is the neutralization and elimination of antigen, accompanied by the acquisition of long-lasting protection from secondary challenge with the same pathogen [27], referred to as immunological *memory*. Thus, the *adaptive* arm is responsible for the bulk of specific and memory responses to diverse antigenic epitopes.

The *adaptive* immune response is intrinsically, exceedingly collaborative in nature and requires the cooperation of cellular components other than T and B lymphocytes, which are all essential in determining the direction and intensity of the response. An assortment of cells such as Macrophages, Natural Killer (NK) cells and DCs are recruited by the adaptive arm. T cells neither recognize *whole* antigens nor self –activate, thus necessitating the help of cells designated antigen presenting cells (APC). APCs capture whole antigens and present them in a recognizable form and context to T-cells, i.e. cleaved and processed antigenic peptides on major histocompatibility complexes (MHC).

APCs also provide secondary/co-stimulatory signals, required for sustained T-cell activation and presence during the immune response. A variety of cells can function as antigen presenting cells with varying efficiencies of associated T-cell activation. DCs, macrophages and B-cells are all able to fulfill the role of a professional APC, satisfactorily. Interplay between the molecular and cellular entities of both *innate* and *adaptive* arms is now considered a requirement for a competent immune response. The *innate* immune response, through the ligation of PRRs like Toll-like receptors (TLRs) by their specific ligands, provides signals that initialize and prime the cellular systems responsible for the late-stage activation of T and B lymphocytes [20, 21]. In fact, DCs, macrophages and B-cells are qualitatively, heavily influenced by PRR stimulation. Their antigen presenting functions are specifically enhanced as they upregulate cell surface co-stimulatory molecules, peptide:MHC complexes and secrete specific sets of cytokines and chemokines in response to the TLR stimulation [18, 20]. The cytokine and chemokine profile elicited by these cells is specific for each TLR ligand and is further responsible for recruiting and activating cognate T- and B-cells, eliciting the hallmarks of the *adaptive* immune system.

1.1 B lymphocytes

The humoral response, dominion of B-lymphocytes, is a chief constituent of the *adaptive* immune response. B-cells, among the most proficient cells in the immune system in terms of whole antigen recognition, capture, processing and presentation to T-cells are thus, inextricably part of the sustained adaptive response to antigens. The ensuing antibody (or humoral) response is not only robust but exceedingly specific towards the

antigen. In order to ensure the specificity and efficiency of these responses, B-cell activation is tightly regulated by many components. Antigen nature as well as B-cell milieu in the form of TLR stimulation, cytokine profile and co-receptor ligation, determine the potency of the response generated. Antigen recognition and capture are essentially restricted by the specificity of the clonotypic antigen receptor expressed on each mature naïve B-cell. The B-cell receptor (BCR) on a mature B-cell can sense unique, non-conserved epitopes on antigens in their native forms. When a B-cell encounters an antigen with multiple identical epitopes, the antigen crosslinks or oligomerizes several BCRs on the B cell surface. This leads to two key events: the first and most widely studied is the initiation of signaling cascades originating from the receptor itself, resulting in the up-regulation of various proteins that are required for B-cell gene regulation and progression through the cell cycle. Antigen-binding secondly results in the reorganization of the cell cytoskeleton and enhanced BCR-mediated internalization of antigen complexes. Antigen internalized through the BCR is subsequently processed within specific endosomal compartments into smaller antigenic peptides and presented in complex with MHC class II receptors to recruit specific T cell help [28, 29]. The return of these peptide: MHC II complexes to the B-cell surface and recruitment of cognate T-cell help, triggers the T-cell dependent phase of B-cell activation and antibody response. Crucial cues provided by armed T-helper cells (T_H) along with constant antigen exposure shape the strength and specificity of the subsequent humoral response [25, 30]. B-cell activation and antigen-specific antibody induction require not only T_H cells but also TLR activation in B cells [31]. This TLR dependence of the humoral immune response can be described in two ways. The initial maturation of

dendritic cells and T_H cell activation, which is required to provide T-cell help to B-cells, is TLR dependent. Secondly, TLRs expressed on B-cells have a direct role in B-cell activation and antibody production. Thus, B lymphocytes must integrate two distinct signals before they proliferate and make antibodies; the first coming from antigens and the second coming from either helper T cells or microbial products such as lipopolysaccharide (LPS) or CpG DNA [31]. TLR and BCR signaling pathways converge and this cooperation results in optimal signal transduction and B-cell activation [32]. B-cell activation eventually leads to their differentiation into terminal, effector plasma cells which secrete antigen-specific antibodies. The secreted antibodies are similar to the membrane bound receptor and reside in most of the body fluids. They bind microorganisms and foreign antigens with specificity, neutralize their toxicity and target them for destruction by other arms of the immune system.

B-cell activation is thus thought to classically proceed in a dual–stage manner. The first stage of signals is provided at the instance of antigen encounter, by the initiation of signaling cascades at the BCR on the cell surface and subsequent antigen internalization. Second stage signals arise from the recognition of processed antigen on MHC II by the TCR, cytokines secreted by the armed T_H cell and interaction of B cells with TLR-ligands and T cells through co-stimulatory molecules such as CD40-CD40L [30, 33]. Naïve B lymphocytes integrate signals from the two cascades for successful expansion, class switching and Ig secretion. The ‘two signal’ model proposed by Bretscher and Cohn is the currently accepted model in naïve B cell activation [34].

1.2 B lymphocyte development and maturation

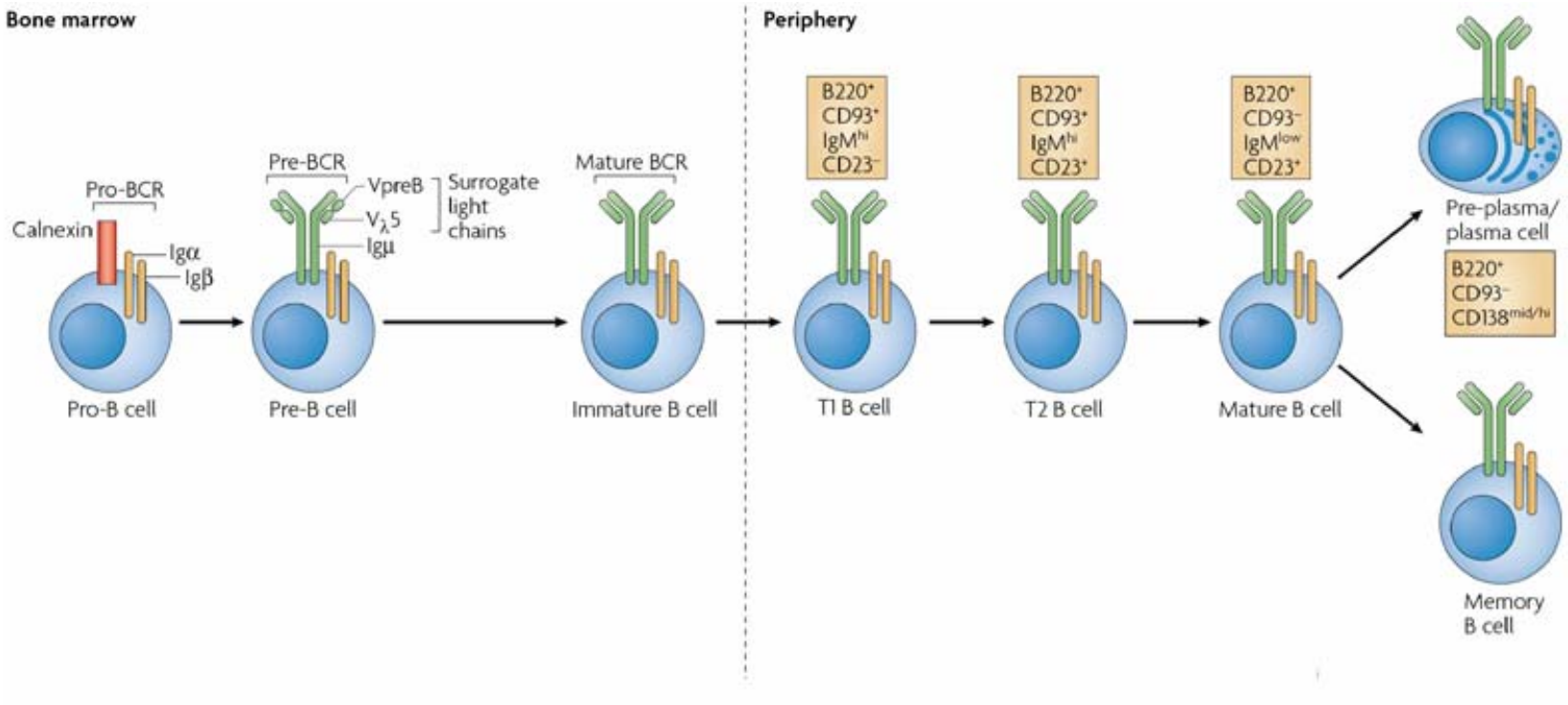
B-cells, by means of a series of developmental programmes and checkpoints, form a diverse, and flexible repertoire that is reactive to nearly all potential pathogens (Fig *1.1*). Initial B-lineage cells arise from common pluripotent hematopoietic stem cell (HSC) progenitors in the fetal liver and in the bone marrow after birth. Lymphoid progenitors are scattered throughout the bone marrow, and the earliest ones preferentially interact with vascular cell adhesion molecule -1^+ ($VCAM1^+$) CXC-chemokine ligand 12 (CXCL12)- IL-7 producing stromal cells [35, 36]. CXCL12 is an important chemokine for attracting and retaining lymphoid progenitors in the bone marrow. IL-7 is needed to support the generation and expansion of progenitors, as well as for immunoglobulin gene recombination. VCAM-1 binds very late antigen - 4 (VLA-4) on committed lymphoid progenitors and helps establish intimate adhesive contacts between stromal cells and early lineage progenitors, thereby ensuring paracrine effects of the soluble factors (stromal cell factor (SCF) and cytokine FLT3 ligand (FLT3L)) they later secrete [37, 38].

The development of B-lineage cells through their several stages correlates well with immunoglobulin gene segment rearrangement and the expression of several cell-surface and cytoplasmic proteins. The earliest B-lineage cells are known as the progenitor or pro-B cells, as they are progenitor cells committed to the B-cell lineage. Rearrangement of the immunoglobulin heavy chain locus takes place in pro-B cells; joining of D_H and J_H heavy chain gene segments occurs in the early pro-B cell stage and joining of V_H and DJ_H segments in the late pro-B cell stage. The rearrangement and expression of the heavy chain is sufficient for their progression into the next stage. The subsequent pre-B cell

phase is marked by the expression of the *pre-BCR*, where productive gene-rearrangements in the heavy chain V, D and J loci are now associated with the invariant surrogate light chain (SLC) components λ5 and VpreB. Btk (a member of the cytoplasmic Tec kinases), CD45R/B220 (membrane tyrosine phosphatase), CD24 (heat shock antigen; HSA), CD25 (IL-2 receptor), and CD19 (B-cell lineage marker and co-receptor) are also expressed in pre-B cells and are important for their progression to the successive stages [39]. The presence of the pre-BCR molecule complete with the invariant Igα/Igβ heterodimer on the cell surface plays a critical role in cell differentiation. If the heavy chain locus rearrangement is defective, the absence of a heavy chain protein leads to a block in development at this stage and induces cell death. The expression of a productive pre-BCR on the B-cell surface causes a proliferative burst at this point and light chain rearrangements are induced. Once light-chain rearrangement is concluded and a complete IgM molecule is expressed on the cell-surface, the cell is called an immature B-cell. The ensuing fate of an immature B-cell with an IgM on its surface is guided by the nature of signals received through the membrane/surface bound IgM (mIgM or sIgM). There are four possible fates for self-reactive immature B cells, depending on the nature of the ligand they bind: cell death by apoptosis, production of a new receptor by receptor editing, the induction of a permanent state of unresponsiveness to antigens also known as anergy and clonal ignorance because the antigen is either in low concentration, sequestered or absent [40, 41]. The above process of clonally eliminating self reactive B-cells is often referred to as negative selection. The immature B-cells which escape negative selection, by minimally reacting or not reacting to self antigen, migrate to peripheral lymphoid organs like the spleen.

FIGURE 1.1. B cell development.

B-cell development occurs in both the bone marrow and peripheral lymphoid tissues such as the spleen. In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-B-cell receptor (pre-BCR, which is comprised of an Ig_{μ} heavy chain and surrogate light chains (VpreB or $V_{\lambda}5$)) and finally a mature BCR (comprised of rearranged heavy- and light-chain genes) that is capable of binding antigen. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells. Cells successfully completing this checkpoint leave the bone marrow as transitional B cells, eventually maturing into mature follicular B cells (or marginal-zone B cells). Following an immune response, antigen-specific B cells develop into either plasma (antibody-secreting) cells or memory B cells. This figure is adapted from [42].


Bone marrow
Periphery
Pro-BCR
Calnexin
Igα
Igβ
Pro-B cell
Pre-BCR
VpreB
$V_\lambda 5$
Igμ
Surrogate light chains
Pre-B cell
Mature BCR
Immature B cell
B220+ CD93+ IgMhi CD23−
T1 B cell
B220+ CD93+ IgMhi CD23+
T2 B cell
B220+ CD93− IgMlow CD23+
Mature B cell
Pre-plasma/ plasma cell
B220+ CD93− CD138mid/hi
Memory B cell

Immature B cells complete the rest of their maturation after migrating to the periphery where they differentiate from newly emigrant transitional type 1 (T1) B-cells to T2 cells and then to mature B cells which express a high level of IgD and a low level of IgM on their cell surface [43, 44]. Transitional T1/T2 B-cells are so referred to as they are in the process of transitioning from the immature phenotype to the mature. The maintenance and development of the T1 and T2 cells depends on signals from the BCR and B-cell activating factor of the tumor-necrosis-factor family (BAFF) [45, 46]. The transitional B-cells undergo more rounds of selection for self-tolerance and survival, the fate of self-reactive B-cell clones in the periphery is three-fold: deletion (apoptosis), anergy or survival. Thus, ideally, competent B-cells that are un-reactive to self-antigen or deleted from the repertoire. Differentiation of the transitional B-cells into mature B-cells is then characterized on the basis of signaling thresholds and the expression levels of key proteins such as Btk, CD45, Syk and the Igα chain [47-51]. Two main subsets of mature B-cells have been described and each subset of B cells has different requirements for their generation and maintenance [52]. Mature B cells can be classified as B1 cells which are enriched in the pleural and peritoneal cavities and the conventional B2 cells, otherwise known as re-circulating or follicular B-cells. B1cells are self-renewing cells that are further divided into B1a and B1b subsets based on the expression of cell surface markers CD5 or Mac1 (CD11b) respectively. They possess cell-cycle and activation properties distinct from the bulk of re-circulating B2 B-cells. B1 cells are hypothesized to form the bulk of the "innate" or "T-independent" IgM-biased humoral response to certain antigens [53]. B1 cells are generated by distinct precursors and are preselected to express a BCR repertoire that is restricted. The most distinctive feature of $CD5^{+}$ B-1 B cells is

their elaboration of autoantibodies for certain self-specificities that include branched carbohydrates, glycolipids and glycoproteins [54]. The mature B2 population is itself heterogeneous and is comprised of non-recirculating marginal zone B cells (MZB) enriched in the marginal zones of the spleen and recirculating follicular B cells which pas through the peripheral lymphatic system and surveil the follicles of the lymph nodes and spleen [52]. It appears that MZB, like B-1 B cells, are preselected to express a restricted BCR repertoire that is biased toward bacterial cell-wall constituents and senescent self-components. MZB however are generated via differing selective pressures which include the rate of clonal enrichment, CD19 and Btk expression [52, 55, 56]. Also similar to B1 cells, MZB participate very early in the immune response (as early as 4h after antigenic challenge) and are especially important in the response to T-independent antigens [57]. These properties are due in part to the fact that marginal zone B cells have a lower threshold than recirculating or immature B cells for activation, proliferation and differentiation into antibody-secreting cells [52]. Mature, naïve, resting B cells express functional, clonotypic antigen receptors on their cell surface and are at the crux of the primary humoral response.

1.3 B cell effector responses and memory

In the peripheral lymphoid organs and blood, mature B-cells are engaged in constant surveillance of their environment for foreign antigens. B cells expressing a BCR that binds antigen with higher affinity (determined by inter-clonal competition [58, 59]) are activated and migrate to the interface between the B-cell follicle and the T-cell area of the secondary lymphoid organ in a chemokine-dependent manner [60]. Here, the B cells meet

their cognate T cells, which have been activated by exposure to DCs presenting antigen-derived peptides and have moved towards the B-cell follicles as a result of changes in their responsiveness to chemokines [60].

Initiation of the T-dependent phase of humoral responses requires the activation of the B-cell by conjugate formation with the activated cognate T-helper cell. CD40, which is constitutively expressed on B cells, binds CD154 or CD40 ligand (CD40L) on activated T cells. CD40L and cytokines secreted by activated $CD4^{+}$T cells (IL-2, IL-4 and IL-5) promote B cell proliferation, isotype switching, Ig secretion and germinal center (GC) formation [61, 62]. The GC is a prototypical follicular structure comprised primarily of rapidly dividing B-cells formed in the secondary lymphoid organs and is described in greater detail below. Class or isotype switching occurs in the germinal center and refers to the process of switching the heavy chain from μ and δ to γ, α or ε. This occurs by a mechanism called class switch recombination (CSR). During this process the rearranged VDJ gene segment of the heavy chain recombines with downstream γ, α or ε constant regions leading to the production of IgG, IgA or IgE antibodies respectively. Other T-cell surface proteins important in B-cell activation are Inducible costimulator (ICOS) and CD28 which bind ICOSL and B7.1/ B7.2 respectively on B cells [63, 64]. OX40-OX40L and CD27-CD70 interactions have also been implicated in the co-stimulation provided by B: T cell interaction [28]. Upon B- T conjugate formation, cell-bound and secreted effector molecules synthesized by T-helper cells activate B cells to undergo clonal expansion and leads to a bifurcation of B-cell differentiation [65]. Some activated B cells migrate to the bridging channels at the edges of the lymphoid areas of the spleen or to the

medullary cords in the lymph nodes, and these B cells differentiate into short-lived end-stage effector cells called plasma cells [65, 66]. Plasma cells secrete antigen-specific antibody. Other activated B cells follow an alternative pathway that leads back into the B-cell follicle and results in the formation of a germinal centre [65]. The first phase of germinal-centre development involves extensive B-cell proliferation [67]. The dividing B cells down regulate cell-surface expression of immunoglobulin and initiate diversification of their immunoglobulin molecules by introducing mutations into the V-region-encoding gene segments of the immunoglobulin heavy chain (VH segments), a process known as somatic hypermutation (SHM) [67-70]. These cells then re-express immunoglobulin molecules on their cell surface and are tested for their ability to bind antigen in the form of immune complexes on the surface of organizational cells of stromal origin that are known as follicular dendritic cells (FDCs). This testing is competitive, and cells vie among themselves and with circulating antigen-specific antibody for access to a finite and possibly very small amount of antigen [71]. Competition for antigen is considered to be the driving force of *affinity maturation*, the process by which the average affinity of the BCRs expressed by the responding B cells improves over time (which reflects the preferential survival of cells that express BCRs with greater affinity for antigen) [67, 72]. Upon exiting the GC the affinity matured, isotype switched cells can differentiate to form long-lived memory B cells or long-lived plasma cells. The long-lived plasma cells home to the bone marrow and secrete high affinity antibodies, which may serve to maintain baseline antibody levels for the life of the animal. Affinity-matured memory B cells, are defined as long-lived, quiescent antigen-experienced lymphocytes, which are non-secreting cells that circulate or home to the marginal zone of the spleen and can rapidly

proliferate and differentiate to plasma cells upon antigen reencounter [25]. Thus, B cells reacting to T-dependent antigens give rise to antibody secreting cells and memory cells which are considered cardinal features of a successful adaptive immune response.

1.4 The BCR and its signaling pathways

The multi-subunit B-cell antigen receptor (BCR) contains antigen recognition and signaling components. The clonotypic, membrane associated form of immunoglobulin (mIg) is associated non-covalently with a disulfide linked signaling heterodimer – Igα/Igβ. Thus, the BCR uses different membrane spanning subunits for the antigen binding (mIg) and signal transduction (Igα/Igβ). The mIg of the BCR is composed of two identical heavy and light chains with which each BCR is able to bind two identical antigenic epitopes simultaneously. Differing from the secreted Ig (sIg), the mIg contains a short spacer sequence at the normal heavy chain C-terminus, a single transmembrane spanning region and a short (3-28 residues) cytoplasmic tail. The cytoplasmic tails of the Igα/Igβ function as the receptor's primary signal transducers. The Igα/Igβ heterodimer associates non-covalently with the mIg and this association is required for the surface expression of the mIg [73]. Igα (CD79a) and Igβ (CD79b) are covalently linked by disulfide bonds and each spans the membrane once. Their cytoplasmic tails are 61 and 48 amino acids respectively, and they contain an immunoreceptor tyrosine-based activation motif (ITAM) on each chain [74]. The ITAM is a consensus motif (D/E XX-YXXϕ-X_{6-8}-YXXϕ, where X is any amino acid and ϕ is a bulky hydrophobic amino acid) that is involved in signal transduction and is found in the cytoplasmic tails of many signaling molecules including CD3 and ζ chains of the T cell antigen receptor (TCR) complex and

several Fc receptors [75]. These highly conserved motifs form the primary foci of phosphorylation following antigenic stimulation and thus serve as the receptor's interface with cytoplasmic effectors responsible for signaling transduction [74, 76, 77]. Biochemical analyses by Schamel and Reth showed that every mIg associates with only one Igα/Igβ heterodimer [78]. More recently, fluorescence resonance energy transfer (FRET) studies in live cells have confirmed the 1:1 stoichiometry of mIg and Igα/Igβ heterodimer [79]. Even though the exact arrangement of the mIg and the Igα /Igβ heterodimer remains unclear, the importance of the Igα/Igβ heterodimer in B-cell function is very significant [73].

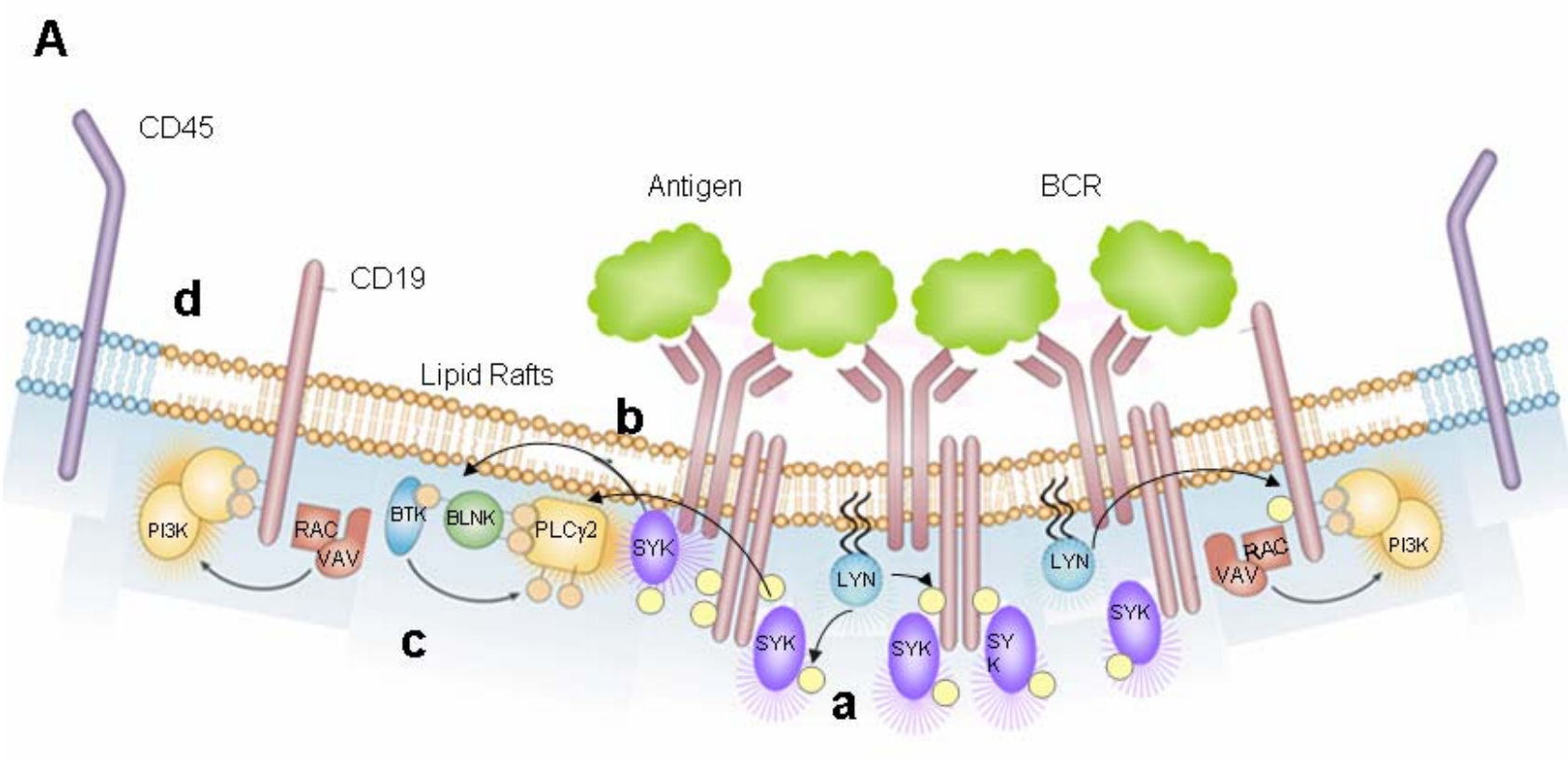
BCR engagement with multivalent antigens leads to their aggregation and translocation into *lipid rafts* rich in signaling and cytoskeletal elements [80]. Tolar and Pierce [79] provide evidence that suggests that receptor engagement leads to a conformational change in the cytoplasmic domains of the Igα/Igβ heterodimer of the BCR from a 'closed' to an 'open' form which facilitates phosphorylation of the ITAMs by Src-family kinases like Lyn. In recent years the idea of ordered membrane microdomains as signaling platforms has gained theoretical precedence with the discovery of 'lipid rafts': cholesterol and glycosphingolipid rich regions on the outer leaflet of the plasma membrane. Lipid rafts provide for a relatively ordered lipid environment which sequesters or excludes signaling molecules based on their biochemical properties potentially providing the BCR with a platform for signaling and trafficking [80-82]. In the resting, mature B cell the monomeric BCR is excluded from the rafts while the Src kinase Lyn is constitutively within lipid rafts [80]. The resting BCRs non-phosphorylated

ITAMs indulge in a low affinity interaction with the unique N-terminal Src-homology 2 (SH2) domain of the Src family tyrosine kinases, Lyn, Fyn, Lck and Blk [83, 84]. Oligomeric BCR clusters that move into the rafts associate almost exclusively with Lyn, which phosphorylates the ITAMs of Igα/Igβ [85].

Signal initiation in the B-cell results from the aggregation of BCR by the antigen (Fig *1.2*). BCR oligomerization and translocation into lipid rafts leads to concomitant conformational changes in it structure. These changes are coupled to conformational changes in the *catalytic* domain of the associated Src kinases. Rapid phosphorylation of a conserved tyrosine in this catalytic domain activates Src kinases. The close apposition of the receptor-associated Src kinases during aggregation allows for their efficient trans-phosphorylation and activation. Once fully activated, the Src kinases then cross-phosphorylate the ITAMs of two closely apposed BCR molecules [79, 86]. The phosphorylated ITAMs provide a binding site for the SH2-domain containing tyrosine kinase Syk, recruiting it to surface signaling microdomains [87]. Syk is activated by Src kinases and also by autophosphorylation [88] and is a crucial player in BCR signaling as disruption of Syk impairs most downstream signaling events [86]. The adaptor protein BLNK (B-cell linker protein) also called SLP-65, is phosphorylated on multiple tyrosines by Syk, these in turn become docking sites for SH2 -domain containing proteins like Bruton's tyrosine kinase (Btk), Phospholipase-C γ2 (PLC-γ2), and adaptor proteins, Grb-2, Vav and Nck [89-91]. Accordingly, BLNK is a central element in BCR (and pre-BCR) signaling and BLNK-deficient mice show severe defects in BCR signaling and B-cell development [92-94]. Docked adaptor molecules, BLNK, Bam32, Grb2, serve as

FIGURE 1.2. BCR signaling pathways

Upon antigen binding BCRs cluster and translocate into lipid rafts where they initiates the signaling cascade. Shown is a bi-partite, simplified schematic of the BCR signaling pathway. (A) *Early signaling*. (a) Upon antigen binding, Src kinase Lyn phosphorylates the ITAMs of Igα/Igβ and key tyrosine residues on the Syk tyrosine kinase. (b) Syk recruits and activates the adaptor protein, BLNK, PI-3Kinase and Btk. (c) BLNK provides docking sites for various proteins which amplify the antigen-induced signaling. (d) In addition, accessory molecules such as CD19/CD21 co-receptor complex enhance signaling by recruiting and activating Rac, PI-3k and Vav, while CD45 is excluded from these signaling microdomains. (B) *Transcription factor activation.* Early BCR signaling culminates in the activation of the MAP Kinase Kinase (MKK) pathway and Ca^{2+} influx. Eventually this leads to the activation of transcription factors like NF-κB, NFAT, AP-1, and c-myc, which control gene expression and cell survival. Figures have been adapted and modified from [95].


A
CD45
CD19
d
Lipid Rafts
Antigen
BCR
b
PI3K
RAC
VAV
BTK
BLNK
PLCγ2
SYK
c
LYN
SYK
SYK
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a
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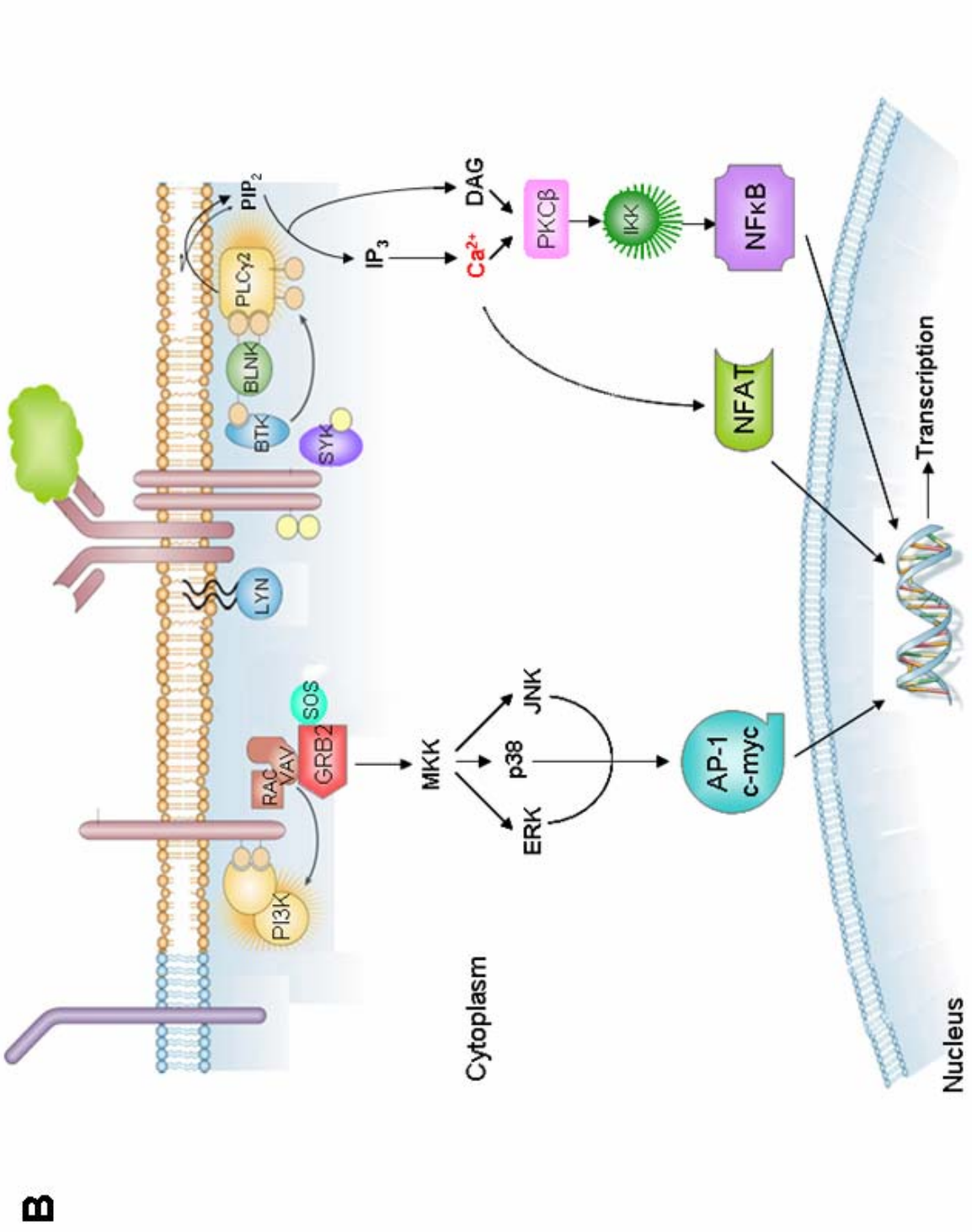
B
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SOS
GRB2
VAV
RAC
MKK
JNK
p38
ERK
AP-1
c-myc
PI3K
Cytoplasm
Nucleus

scaffolds for key kinases/phosphatases and their substrate proteins and thus, successfully amplify the signal in the vicinity of the BCR. Recruitment of Btk to the membrane probably occurs by its interaction with BLNK as well as the interactions of its pleckstrin homology (PH) domain with PtdIns-3,4,5-P_3. These interactions bring Btk into the proximity of Syk, making it a target for phosphorylation by Syk and Lyn and increases its activity [96-98]. Btk and Syk, in turn, phosphorylate and activate (PLC-γ2) [99, 100] and also cyclically mobilize phosphoinositide -3- kinase (PI3K) and the Rho-Ras-Raf-ERK pathways [101-105]. Hence, antigen binding to the BCR leads to a sequential activation of Lyn, Syk and Btk which amplifies the signal and initiates a number of downstream pathways (Fig. *1.2*). PI-3K and PLCγ2 are both crucial effector enzymes that generate key second messengers in BCR signaling [78].

In addition to the local enrichment of kinases, BCR stimulation also leads to the local biogenesis and enrichment of lipid moieties like phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2) [106-108]. Both PI3-K and PLCγ2 use PtdIns-4,5-P_2 as a substrate. PI3-K phosphorylates PtdIns-4,5-P_2, producing phosphatidylinositol 3,4,5-triphosphate (PtdIns-3,4,5-P_3) which recruits molecules with PH domains (like Btk) to the BCR signalosome [109, 110]. Syk, Btk and PI3-kinase also activates Akt, a serine-threonine kinase, [111, 112] which promotes B-cell proliferation by inhibiting a pro-apoptotic protein –BAD (Bcl-associated death promoter) [113]. PLCγ2 hydrolyzes PtdIns-4,5-P_2 to inositol trisphosphate ($InsP_3$), and diacyl glycerol (DAG). $InsP_3$ triggers the release of calcium from the endoplasmic reticulum (ER) stores, and consequently increases calcium influx at the plasma membrane. A decisive outcome of signal initiation is the generation of the

Ca^{2+} flux, which serves to transduce early signaling to downstream epigenetic modifications, determining cell fate. The generation of the Ca^{2+} flux in B-cells requires the activation of PLCγ2 directly by Btk and Syk on the adaptor BLNK [90, 99, 114, 115]. DAG and calcium together activate protein kinase C β (PKC-β). PKC-β activates nuclear factor-κB (NF-κB), which upregulates the anti-apoptotic protein Bcl-xl and cyclin D2 [116, 117]. PKC-β and Rac activate mitogen activated protein kinases (MAPKs) p38 and c-Jun NH2 terminal kinase (JNK) [118]. The interaction of Vav and Grb2/SOS (guanine exchange factors) with phosphorylated residues on both CD19 and BLNK, activate the Rho and Ras family GTPases respectively. Activated Ras initiates the Raf/MEKK pathway which results in the activation of MAP kinase ERK (extracellular signal regulated kinase) [119]. PKCs can also activate the Ras family of GTPases directly subverting the involvement of Vav/Grb2 and Sos, thus, initiating the MAP kinase pathway and subsequent activation of the B-cell. The MAP kinases activate specific transcription factors, like c-jun, c-fos, Elk and c-Myc which cooperate with NF-κB and NFAT to determine the cellular response to antigen engagement [116, 120-122]. It is credible that the profile of these activated transcription factors determines B-cell fate, and the synergistic or opposed effects of these events can thus, lead to B-cell activation, anergy or apoptosis.

BCR signaling is also significantly influenced by receptor-associated accessory molecules. BCR aggregation also results in the co-aggregation and phosphorylation of the BCR co-receptor molecule CD19 by Lyn [123]. The CD19/CD21 complex enhances signaling by the BCR by recruiting and activating Rac, PI-3k and Vav [123], and is

essential for B cell response to thymus-dependent protein antigens [124]. Co-aggregation of the CD19/CD21 complex may enhance the immune response to antigen as much as 10,000 fold; increased Vav phosphorylation, calcium mobilization and activation of the MAP kinase pathway are underlying reasons for this synergy [119, 125, 126]. CD22, the paired immunoglobulin-like receptor B (PIRB) and FcγRIIB are negative regulators of BCR signaling. These molecules have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on their cytoplasmic tails. ITIMs recruit phosphatase like Src homology- 2 (SH2)-domain-containing inositol 5-phosphatase (SHIP) and (SH2)-domain containing protein tyrosine phosphatase 1 (SHP1) which serve to inhibit BCR signaling [127, 128]. Notably the tyrosine phosphatases CD45 and CD148 in addition to SHP1 and SHIP are paramount in regulating the extent of signal amplification. CD45 is postulated to function as a rheostat, and thus sets the threshold for BCR signaling. Although, CD148 has been identified only recently, it is thought to perform the same function as CD45 [129].The role of CD45 in B-cells is complex as it prevents the hyper-phosphorylation of Lyn at its inhibitory tyrosine residue (Y508). The phosphorylation of this particular residue on Lyn represses the kinase [130]. Thus, it would seem that CD45 primarily helps initiate signaling by the Src kinases. However, as CD45 is generally excluded from the lipid rafts signaling microdomains formed after BCR crosslinking, its subsequent activity is restricted [131] and the activity of cytoplasmic inhibitory kinases like Csk on Src kinases balances out the influence of CD45. There is no present evidence for the spatiotemporal location of CD148 on BCR stimulation, however, studies from T-cells hint that it may also be excluded from ligand induced BCR clusters [132]. Interestingly, CD22 and SHP1 are also excluded from the lipid rafts [80]. It is important to note that most inhibitory

receptors and molecules do not play unilateral roles in B-cells but instead they have complex roles in the signaling pathways, often up-regulating pro-survival signals whilst down-regulating BCR-proximal signals.

A theoretical model for the initiation of BCR signaling has surfaced with the current understanding of early events in B-cell activation. In the resting B cell membrane, BCRs are free to diffuse along with Lyn and large receptor tyrosine phosphatases CD45 and CD148. The ligand aggregated BCRs, however, change conformation and become heavily phosphorylated, actively assembling a signaling complex or 'signalosome' of positive signaling mediators in the lipid raft. This signalosome is kinetically and spatially segregated from the bulky phosphatases and other negative regulators. It is assumed that localized cytoskeletal reorganization would help sustain signalosomes. Thus, the B cell receptor uses a number of effector molecules, adaptor proteins and the lateral heterogeneity of the plasma membrane to regulate the precision of its initial signal transduction and influence later cell fate decisions.

1.5 Btk in B cell signaling

Bruton's tyrosine kinase (Btk) belongs to the Tec family protein-tyrosine kinases (PTKs), which is the second largest family of cytoplasmic (non-receptor) PTKs [133]. The significance of the Tec family of non-receptor kinases came with the discovery that mutations in Btk were the cause of inherited immunodeficiency diseases in both humans (XLA, for X-linked agammaglobulinemia) and a less severe version in mice (*Xid*, for X-linked immunodeficiency) [134-136].These mutations are responsible for compromised

B-cell ontogeny. Mutations causing XLA have been found in all the domains of Btk; however, the mutation resulting in the *Xid* phenotype in mice is caused by a point mutation of arginine to cysteine (R28C) in the Pleckstrin Homology (PH) domain of Btk and a comparable mutation resulting in XLA has been found [137-140]. In humans, a Btk deficiency causes B-cell developmental defects with a partial block between pro- and pre-B cell stage, resulting in the virtual absence of B-cells and a pronounced reduction in serum immunoglobulins (Igs) of all classes [47, 138, 141]. *Xid* mice show poor antibody responses to T-independent type II (TI-II) antigens but normal responses to T-dependent (TD) antigens. Naïve animals also have greatly reduced serum titers of certain Ig isotypes – IgM and IgG_3 particularly [47]. This corresponds with a near complete absence of the $CD5^+$ (B-1) subset of B-cells, the number of mature splenic B-cells (B-2 subset) is reduced to 60% of its wt numbers especially the mature $IgM^{lo}IgD^{hi}$ B population and increases in $IgM^{hi}IgD^{lo}$ B cells that resemble immature transitional B-cells [47, 142].The role of Btk in B-cell development is now understood to be dependent on its role in signaling pathways emanating from the pre-BCR [143]. The expression of this protein and its attendant signaling activities in pre- and immature B-cells is considered crucial for the progression of the B-cell developmental programme [137, 144]. This has been confirmed by the severe developmental defects seen in the Btk deficient (xid), Btk null and Btk/Tec double knockout mouse models [140, 142, 145]. Btk is a 76 kDa multi-domain protein with multiple sequence motifs for protein-lipid and protein-protein interactions, including a PH, Tec homology (TH), Src homology 3 (SH3), SH2, and the kinase/catalytic/SH1 domains. Both the kinase and PH domains are indispensable for Btk

activity in mature B-cells [133, 135, 138] (Fig. *1.3*), although it is thought to function primarily as an adaptor protein during B-cell development [146].

Btk activation is initiated at the plasma membrane [110]. The binding of it PH domain to PtdIns-3,4,5-P_3 and its interactions with the phosphorylated residues of the adaptor protein BLNK, recruit Btk to the plasma membrane [98, 109, 110, 147]. Here, a Src family kinase, Lyn, phosphorylates Btk at Tyr^{551} in the activation loop/kinase domain [96]. This initial activation of Btk can be modulated by Syk as well in the presence of BLNK [96-98]. Subsequent auto-phosphorylation at Tyr^{223} in the SH3 domain leads to complete kinase activation [97]. The binding and activation of Btk on BLNK/SLP65 along with PLCγ2 helps to bring PLCγ2 in close proximity to Btk and Syk [148]. An essential function of Btk is the phosphorylation of PLCγ2 on key tyrosine residues, which is necessary for the full activity of PLCγ2 [100]. The phosphorylation of PLC γ2 sets in motion the hydrolysis of PtdIns-4,5-P_2 eventually leading to calcium mobilization and PKC activation [105, 114, 149-152]. These events lead to the downstream activation of various transcription factors (NF-κB, NFAT, AP-1, etc.) that are anti-apoptotic and aid the B-cell response. PtdIns-3,4,5-P_3, which is important for Btk activation, is a product of PI-3-kinase (PI3K) [147]. Although PI3-K is upstream in the Btk activation cycle, PI-3K activation is also amplified in part by the relocation and activation of Btk [147, 149]. As a molecular shuttle, Btk transports PtdInsP-5-Kinase (PIP5-K) to the plasma membrane where PIP5-K is responsible for the synthesis of PtdIns-4,5-P_2 [149]. The activation of PIP-5-Kinases enhances the activation of PI-3-kinases in a Btk dependent manner by increasing the substrate for PI-3-Kinase (PtdIns-4,5-P_2) at the membrane [107, 149].

FIGURE 1.3. Schematic model of Btk and interacting molecules.

Btk is a prominent member of the Tec family in the B-cells. Being multi-modular its several domains are involved in protein-protein and protein-lipid interactions. Btk's Pleckstrin Homology (PH) domain can bind the PtdIns3,4,5-P_3 lipid moiety which serves a role in its redistribution and activation. The point mutation of an arginine at residue 28 to a cysteine is responsible for the xid phenotype and is highlighted (R28C). The conserved Tec homology (TH) domain is divided into Bruton's tyrosine kinase (BTK; Btk motif) and PRR (proline-rich region). The TH can bind several protein partners, most significantly the Src kinases. Btk interacts with WASP and Vav (*in vitro)* with its SH3 domain and binds phosphorylated residues on BLNK via its SH2 domain. Btk is activated by sequential phosphorylation, initial phosphorylation by the Src kinases occurs at Tyr^{551} and autophosphorylation by its own effector domain occurs at site at Tyr^{223}. Btk's effector/SH1/kinase domain is important in the phosphorylation and activation of PLCγ2 in B-cells.

Btk serves as an adaptor for various signaling proteins, such as BLNK, Vav, BAM11 and PKCθ [134, 153-155] and likely participates in the formation of a stable signalosome near the BCR. Moreover, it plays an important role in propagating pro-survival signals by initiating the transcription of the Bcl-xL gene, a direct downstream effect of its various functions [156] and inhibiting apoptic pathways in mature B-cells [157]. The interacting partners of Btk vary from cytosolic proteins to nuclear transcription factors [148, 154, 158], thus equipping Btk with a diverse array of functions and making it a critical component of BCR signaling pathways.

Similar to Btk, mutations affecting the T cell Tec kinases, Itk and/or Rlk, result in defective T-cell development, TCR-induced PLC-γ1 activation, Ca^{2+} mobilization, and ERK activation [159-161]**.** In addition, Itk has recently been shown to regulate TCR-mediated actin polymerization and polarization as well as the localized activation of Cdc42 at the T-cell APC immune-synapse (IS) [162]**.** Thus, Tec kinases play key roles in regulating TCR-mediated polarization of actin, integrins, and signaling molecules to the immune synapse [163]. Lymphocyte signaling events share many common aspects, thus, it is reasonable to hypothesize that the role of Btk in B-cells maybe analogous to the role of Itk and Rlk in T-cells. By this understanding, hypothetically, Btk may be able to integrate signaling pathways and actin dependent cellular changes upon encountering antigen in B-cells, just as Itk/Rlk are able to do at the IS in T-cells. It will most likely transduce early signals into cytoskeletal reorganization in the B-cell by modulating the activity of actin-regulatory proteins including WASP and N-WASP, as is seen with Itk activity in T-cells [164]. This regulation of the actin cytoskeleton in B-cells may be

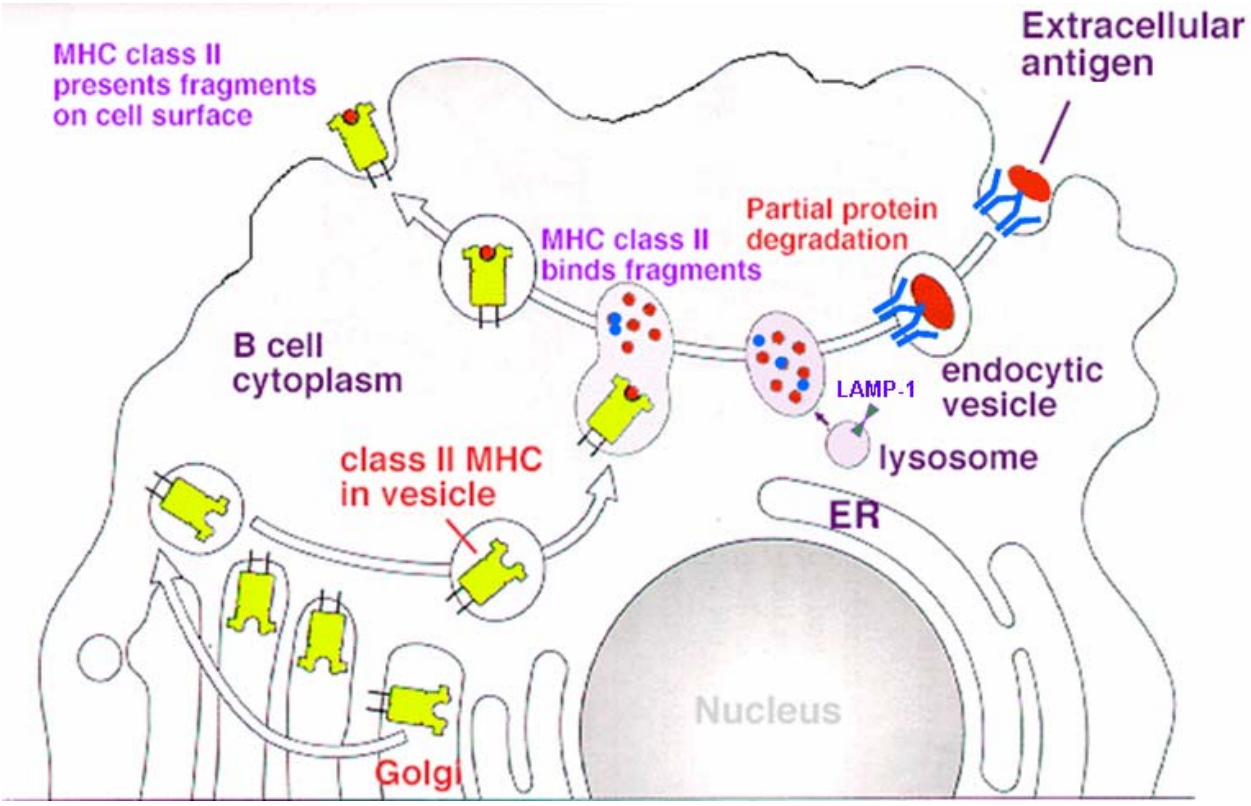
important in antigen internalization and subsequent presentation to T-cells and thus in determining the ultimate fate of the activated B-cell.

1.6 BCR-mediated antigen uptake and processing pathways

In the absence of specific antigen, the BCR is constitutively internalized at a low rate and recycled rapidly through the early endosomes, returning to the surface for antigen surveillance [165-167] (Fig. *1.4*). Cross-linking the BCR by a cognate antigen, even at concentrations 1000-fold less than those required for pinocytosis, greatly enhances receptor internalization. The main mode of BCR internalization is via clathrin-mediated endocytosis (CME) [168], although pinocytic sampling by the B-cell is also observed [169]. Receptor ligation is associated with its rapid translocation into cholesterol rich lipid rafts, the phosphorylation of clathrin in lipid rafts, the re-organization of the actin cytoskeleton and increased BCR internalization [80, 168, 170-172]. BCR engagement accelerates the transience of BCR-antigen complexes through the endocytic pathway to specialized late endosomal compartments rich in MHC class II (MIIC) [173]. In B cells, most studies indicate that compartments derived from late endosomes, collectively referred to as MIIC, are the primary sites in which antigen-processing and MHC class II peptide loading occurs [174]. The MIIC vesicles resemble late endosomes (LE)and pre-lysosomes in that they are LAMP-1^+ (lysosomal associated membrane protein-1), acidic and contain proteolytic enzymes for protein fragmentation, such as cathespins and thiol reductases [175]. Morphologically, they appear multivesicular or multilaminar. After antigen proteolysis and peptide loading in the MIIC, the peptide: MHC II complexes are transported to the plasma membrane for antigen presentation to T-cells.

FIGURE 1.4. BCR mediated antigen presentation.

BCRs cross-linked by antigen are rapidly internalized from the cell membrane into early endosomes. They are then trafficked to MHC class II containing $LAMP1^{+}$ late endosomes or lysosomes. MHCII molecules are assembled in the endoplasmic reticulum and are transported to $LAMP1^{+}$ vesicles, where processed antigen is loaded into the peptide groove. The MHC-II: peptide complexes are then transported to the cell surface to be presented to T cells. (Adapted from Developmental Biology, 8th Edition, 2006)



1.7 The role of signaling in the intracellular trafficking of the BCR

Recent evidence has shown that the signaling and antigen processing functions of the BCR may be linked. BCR crosslinking by multivalent antigens accelerates the transport of the BCR to the MIIC [167]. Internalization is dependent upon the phosphorylation of the conserved ITAM motif on Igα/Igβ [165, 166]. Tyrosine kinase inhibitors that block BCR-induced signaling inhibit antigen-induced BCR internalization [176]. Additionally, the kinases, Lyn and Syk, which are activated upon BCR crosslinking, are also essential for BCR internalization [177, 178]. The exact role of these signaling kinases in endocytic events was further clarified when it was observed that crosslinking the BCR routinely induces the phosphorylation and recruitment of clathrin into lipid rafts on the plasma membrane and BCR-containing vesicles. Furthermore, this process was susceptible to treatment with the Src-kinase inhibitor, PP2, which fully abolishes the phosphorylation and recruitment of clathrin to the vicinity of the BCR [168]. Thus, the coordination of endocytic molecules and adaptors relies on signals emanating directly from the BCR. The nature of antigen and mIg interactions also greatly affect the residency of the BCR on the cell surface and greatly modifies the internalization of the antigen-receptor complex[172]. Relatively low valency antigens induce a rapid internalization of the BCR and a transient protein tyrosine phosphorylation, whereas high valency antigens reduce BCR internalization, extend the residency of the BCR on the cell surface signaling microdomains, and increase the level of BCR signaling [179]. These results show that BCR induced signals and endocytic events regulate each other.

BCR signaling also coordinates the transience of the BCR-antigen complex through the endocytic pathway [180, 181] and does not merely control internalization events. The targeting of the BCR to late endosomes is disrupted by Src kinase inhibitors, emphasizing the need of BCR signaling in targeting antigen-BCR complexes to the late endosomes [181]. Using BCR-chimeric proteins a number of studies have also shown the importance of Igα/Igβ complex in BCR trafficking to the late endosomes following BCR crosslinking [182-184]. Mutations of the tyrosine's in the ITAMs of Igα/Igβ lead to defects in BCR trafficking to late endosomes and it appears that Igβ is primarily involved in dictating the intracellular route of the BCR [184]. These studies indicate that BCR signaling is important for BCR trafficking to the late endosomes.

1.8 The actin cytoskeleton and early B-cell responses

As a major, ubiquitous protein in all metazoan cells, actin serves central roles in shape determination, cytokinesis and cell motility as well as in the establishment of cell-cell and cell-matrix interactions [185]. Actin is a 43 kDa protein which is both highly abundant and extremely well-conserved throughout evolution. Within the cell, actin monomers (*G or Globular-actin*) polymerize into polar filaments (*F or Filamentous-actin*), with fast growing (barbed or *plus*) and slower growing (pointed or *minus*) ends in an ATP dependent fashion. Since nucleation is kinetically unfavorable, proteins like the Arp2/3 complex and nucleation promoting proteins help accelerate the formation of actin dimers and trimers and stabilize them.

Filamentous or F-actin controls membrane plasticity (including cytoskeleton-propelled deformation and protrusion) in addition to many other structural modulations in cells. Thus, the majority of nucleation of branched actin filament network occurs on the cytoplasmic surface of membranes. Immunocytochemical studies in B-cells show that microfilaments, composed primarily of actin filaments, are linked to the inner surface of the plasma membrane and in this immediate vicinity form a complex meshwork, the membrane skeleton [170, 186, 187]. These networks of actin filaments, most prominently localized to the cell periphery in the vicinity of the BCR, are especially sensitive to signals generated at the plasma membrane-cytoplasm interface [188]. In B-cells, the actin cytoskeleton undergoes rapid and dramatic alterations in conformation and arrangement in response to cell stimulation [170, 189, 190]. In addition to causing obvious structural changes (membrane ruffling and capping), the actin cytoskeleton is involved in signal transduction leading to the eventual activation and proliferation of the B-cell [191]. A recent study showed that regulated depolymerization of actin in response to antigenic stimulus is important for downstream signaling events like transcription factor activation [192]. Moreover, cell spreading and antigen accumulation models that are predicted for B-cells encountering membrane-bound/immobilized antigen, also rely highly on the plasticity of the cytoskeleton to accomplish B-cell activation [193, 194].

The engagement of antigen to the BCR induces the association of several signaling molecules in proximity to the BCR with the actin cytoskeleton, including, but not limited to Lyn, Syk, GTP binding proteins and adaptor proteins, such as actin binding protein 1 (Abp1 or SH3P7) [191, 195, 196]. This accumulation of signaling molecules in the

cytoskeletal fraction supports the hypothesis that the cytoskeleton is involved in ligand-induced signal transduction. Yet, even though signaling molecules are shown to accumulate in the cytoskeletal fraction of B-cells, disrupting the actin cytoskeleton does not inhibit BCR proximal signaling [171]. The rapid translocation of the BCR into detergent insoluble lipid rafts in response to antigen cross-linking has also been shown to be actin cytoskeleton independent, as disruption of the actin cytoskeleton with cytochalasin D does not prevent this translocation [131]. Cytochalasins disrupt the formation of F-actin by capping the plus ends of polymerizing filaments and preventing the addition of G-actin monomers, thereby actively preventing actin polymerization. These studies suggest that the role of actin after BCR crosslinking is downstream of early membrane signaling. The basis for cytoskeletal reorganization after receptor ligation and its interaction with signaling elements has not been precisely defined. Findings such as the ability of tyrosine kinase inhibitors to block BCR-induced polymerization of G-actin to F-actin [197] and also block BCR trafficking to LAMP-1^{+} endosomes [198, 199] indicate that tyrosine kinase(s) may act as an essential link between BCR signaling and cytoskeletal reorganization, leading to optimal rates of BCR-antigen internalization. The B-cell specifically harnesses the clathrin-mediated endocytic pathway for the internalization of antigen-BCR complexes, as it lacks caveolin [168], and the actin cytoskeleton has been shown to play an important role in BCR internalization and trafficking. Recent evidence shows that altering the elasticity of the actin cytoskeleton, by using pharmacological inhibitors that either disrupt or stabilize the cytoskeleton, is detrimental to the ability of the B-cell to internalize the BCR-antigen complex. Thus when inhibitors, cytochalasin D, latrunculin B (sequesters G-actin monomers and thus

causes actin depolymerization), and jasplakinolide (stabilizes F-actin and prevents depolymerization of filaments), were added to B-cells activated by antigen, all three inhibited the antigen-enhanced internalization of the BCR [171]. Thus, not only is BCR internalization dependent on the actin cytoskeleton but these studies highlight the need for a plastic and flexible cortical actin cytoskeleton for the correctly modulation of internalization events. In Cytochalasin D treated cells, cortical actin is observable as patches that colocalize with the BCR at the plasma membrane, implying that the BCR is trapped in vesicles that are associated with actin [171]. Electron microscopic observations show that in Cytochalasin D-treated cells, BCRs accumulate in elongated clathrin-coated pits, indicating that BCR-containing clathrin-coated pits fail to pinch off from the plasma membrane. In contrast, constitutive BCR internalization was not affected by Cytochalasin D treatment, implying that specific involvement of actin cytoskeleton in BCR internalization is upregulated by signals generated by BCR crosslinking [171]. There is emerging evidence which indicates that actin is involved in specifying sites of coated pit formation on the plasma membrane. It was shown that AP-2, an essential adaptor protein required in endocytosis, is organized in linear arrays that co-localize with actin stress fibers, and disrupting actin with cytochalasin D disturbed this arrangement of AP-2 [200]. To reinforce the involvement of actin in clathrin-mediated endocytosis, Latrunculin B treatment leads to an increased *lateral* but not inward movement of clathrin coated pits on the plasma membrane [201]. These findings implicate actin in the assembly of endocytic machinery and rapid internalization of the BCR following antigen crosslinking; however, the exact mechanism and component proteins remain to be elucidated.

A functional actin cytoskeleton has been suggested to also be important for antigen presentation in B cells. Soreng *et al.* [202] showed that presentation of soluble antigens by antigen-specific B cells is strongly inhibited when the actin cytoskeleton is disrupted. Actin disruption may lead to loss of either antigen transport, degradation, loading or some combination of these activities. Barois *et al.* [203] demonstrated that MHC II maturation is dependent on actin filaments. Disruption of actin filaments leads to incompletely degraded Ii, faulty transport of MHC II molecules, and a decrease in the amount of peptide –loaded MHC class II [203]. Moreover, Brown *et al.* show that disrupting actin prevents transport of the BCR-antigen complex into $LAMP^+$ endosomes [171]. These studies suggests that actin filaments are necessary for transporting both antigen and MHC class II into the MIIC. Taken together they imply that dynamic changes in the actin cytoskeleton are critical regulatory points for both BCR-antigen transport and B-cell signaling events. These changes in the actin cytoskeleton may be modulated by actin regulatory proteins and signaling tyrosine kinase(s) to ensure effective BCR internalization and transport and this is indicated by actin's role in both BCR signaling and trafficking.

1.9 The Role of WASP in B-cells

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive disease initially described as a clinical triad of immunodeficiency, idiopathic thrombocytopenia and eczema. The immunodeficiency is more completely characterized by recurrent pyogenic, viral and opportunistic infections, neutropenia and acute hematopoietic malignancies, with a tendency to develop a range of autoimmune phenomena and lympho-proliferative

disease. A variety of hematopoietic cells are affected by the genetic defect, including cells from both lymphoid and myeloid lineages such as lymphocytes, neutrophils, monocytes and platelets [204, 205]. Early studies noted both signaling and cytoskeletal abnormalities in lymphocytes from WAS patients [206, 207]. The gene was identified by positional cloning (Xp11.23) and correlated with the corresponding gene product mutated in these patients –WASP (*WAS Protein*) [208, 209]. With the discovery of more universally expressed WASP homologues in mammals, namely, *Neural* or N-WASP and WAVE/Scar proteins (WASP-family verprolin homology domain containing protein/Suppressor of cyclic-AMP receptor), it was recognized that these proteins are widely conserved in a variety of other species as well (human, mouse, rat, bovine, *Drosophila*, yeasts). Mammals are known to express at least five WASP family members: namely WASP (founding member), N-WASP, Scar/WAVE-1, Scar/WAVE-2 and Scar/WAVE-3 [206, 210].

WASP is a 501-amino acid, 62-65kDa, proline-rich protein, expressed exclusively in hematopoietic cells [210]. WASP family proteins are typically organized into several conserved modular domains (Fig. *1.5*). These domains mediate interactions with a number of cytoplasmic effectors involved in signal transduction and reorganization of the cytoskeleton by activating Arp2/3-mediated actin nucleation. At their carboxyl terminus is a polyproline-rich region 'P', one or two WASP-homology 2 (WH2) regions, a central basic connecting region 'C' (or cofilin-homology region) and a terminal acidic region 'A'. This carboxy-terminal region, variously referred to as 'WA', 'WCA' or 'VCA' is crucial for the binding of both actin monomers and the Arp2/3 complex. The poly-proline

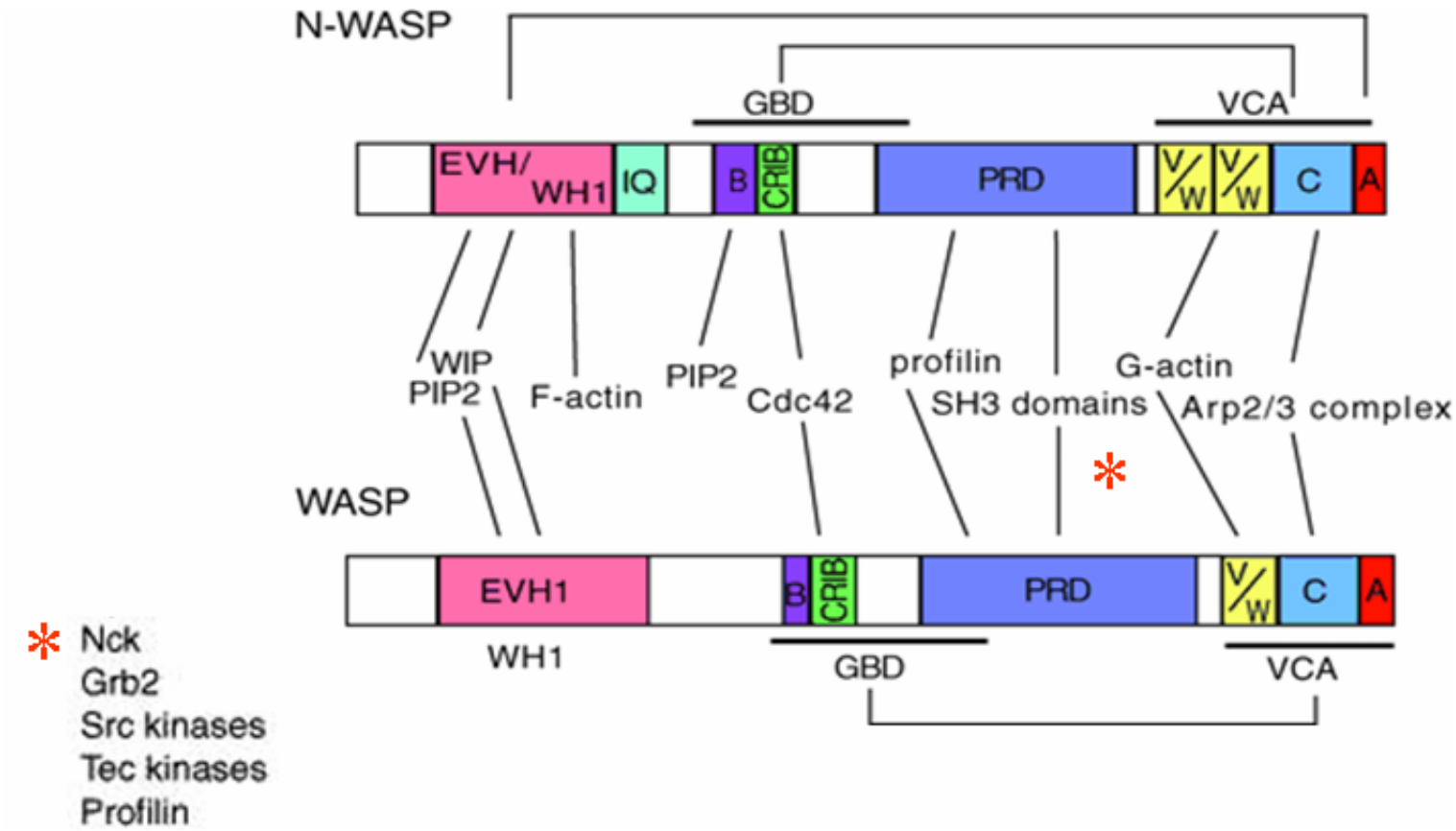
stretch likely mediates interaction with a variety of SH3 domain containing proteins. A central GTP-ase binding domain (GBD) or Cdc42 and Rac interactive binding (CRIB) domain is important for the activation of WASP, and is found adjacent to a lysine-rich basic region 'B' which is implicated in binding membrane-bound poly-phosphoinositide, specifically PtdIns-4,5-P_2. The amino-terminal WH1 domains of WASP family proteins are more divergent and are implicated in cytoskeletal regulation, although their exact roles is unclear [210, 211].

In their unactivated state, WASP and N-WASP exist in an autoinhibited state in which the WA domain interacts with the hydrophobic core of the CRIB domain, resulting in the physical occlusion of Arp2/3 binding sites. Thus, in their autoinhibited conformation, neither WASP nor N-WASP can initiate Arp2/3 binding or actin nucleation [207]. Prenylated GTP-Cdc42 and PtdIns-4,5-P_2 binding disrupts this intra-molecular interaction thereby exposing the Arp2/3-complex binding site [212]. There is some evidence that the effect of concerted binding of GTP-Cdc42 and PtdIns-4,5-P_2 synergistically enhances the activity of WASP and N-WASP [212, 213]. Moreover, the interaction with PtdIns-4,5-P_2 may relocate the proteins to membrane compartments which are rich in poly-phosphoinositides. The simultaneous interaction of SH3 domain containing proteins such as WISH (WASP-interacting SH3 protein), Tec kinases, Vav and SH2 domain containing proteins such as Nck and Grb2 as well tyrosine phosphorylated cell-surface receptors with WASP, might contribute not only to the activation of WASP and N-WASP but also their redistribution to the cell membrane [207, 210, 214]. Recent studies have established that phosphorylation of WASP itself plays a

FIGURE 1.5. Domain Structures of WASP, N-WASP, WAVE and various binding partners.

WASP, N-WASP and WAVE-2 are multi-modular proteins possessing several protein-protein interaction motifs. In particular, the VCA or WA domain is mainly responsible for their binding to actin, while the GBD binds small GTPases like Cdc42 or Rac1. The VCA and GBD are expected to be involved in intermolecular interactions which stabilize an autoinhibitory state of these proteins. The PRD participates in multi-dimension interactions with an assortment of proteins (red asterisk); of importance is the fact that this domain is responsible for WASP/N-WASP's interaction with members of the Src and Tec kinase families. Sites of tyrosine phosphorylation lie adjacent to the CRIB domain, while serine phosphorylation sites, responsible for activation are located within the VCA domain. WAVE proteins lack a central CRIB or GBD domain and posses a conserved N-terminal Scar homology domain.

A, acidic domain; B, basic region; CRIB/GBD: Cdc42-binding domain or GTPase-binding domain; PRD, proline-rich domain; IQ, calmodulin-binding domain (only in N-WASP); WH1,WASp-homology domain 1.; WH2, WASP-homology domain 2; VCA or WA, verprolin-homology, cofilin-homology, acidic domain or the region encompassing the WH2 domain(s), cofilin binding domain and the acidic domain.


N-WASP
GBD
VCA
EVH/ WH1
IQ
B
CRIB
PRD
V/W
V/W
C
A
WIP
PIP2
F-actin
PIP2
Cdc42
profilin
SH3 domains
G-actin
Arp2/3 complex
WASP
EVH1
B
CRIB
PRD
V/W
C
A
WH1
GBD
VCA
Nck
Grb2
Src kinases
Tec kinases
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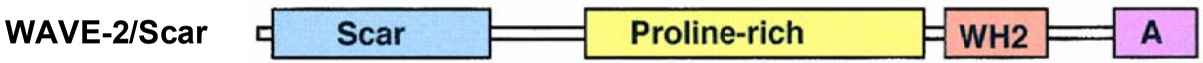

WAVE-2/Scar
Scar
Proline-rich
WH2
A

key role in its activation. WASP is not only tyrosine phosphorylated in response to BCR crosslinking, but is also phosphorylated at conserved serine residues in the WA domain. Tyr^{291} of WASP has been shown to be an *in vitro* substrate for Abl, Btk, and Src family kinases [215, 216]. Tyr^{291} lies adjacent to the CRIB domain of WASP, and the phosphorylation of this residue destabilizes the interaction of the CRIB domain with the WA region and thus increases the ability of WASP to initiate actin nucleation. Interestingly, phosphorylation and dephosphorylation of this residue can only occur efficiently when active Cdc42 is bound to WASP [216], implying that the regulation of WASP is stratified, meaning that unless certain activating components upstream in WASP activation are not already bound, the effect of other activating influences is limited. Phosphorylation of an equivalent tyrosine in N-WASP Tyr^{256} also increases basal activity of N-WASP and there is evidence that Src kinases are responsible for this phosphorylation in vivo [217]. There is data that suggests that the phosphorylation of N-WASP by Src kinases triggers ubiquitination and eventual degradation of N-WASP, as ubiquitination of N-WASP is not detectable if Src kinases are inhibited [218]. Thus, proteolysis could serve to ultimately inactivate WASP and N-WASP after a cycle of activation by Cdc42, PtdIns-4, 5-P_2 and phosphorylation. The phosphorylation of the serines 483/484 which lie within the WA domain of both WASP and N-WASP was shown to be caused by Casein Kinase 2 (CK2), and this event increases the affinity of WASP for the Arp2/3 complex thereby increasing the rate of actin nucleation[215]. The pathway linking B-cell activation to serine phosphorylation of WASP via CK2 is poorly understood and it is important to establish what BCR proximal components are important in known mechanisms of WASP activation. Other serine and tyrosine residues have been

discovered to play an important role in the ability of WASP to stimulate actin polymerization, of these Tyr^{256} and Ser^{242} on WASP are important for enhanced actin nucleation and are phosphorylated by the activated Cdc42-associated kinase-1 (ACK1) [219].

Patient samples and the development of WASP-null mice have made it possible to dissect the precise role of WASP in lymphocytic signaling. The abnormalities associated with WAS show various defects in cell motility of monocytes in response to microbial chemoattractants like formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe or fMLP),and chemokines like monocytes chemotactic protein-1 (MCP-1) and macrophage inflammatory protein 1-α (MIP1α) [207]. Moreover, macrophages and dendritic cells show an impaired ability to express podosomes as well decreased adhesion capacity [207]. Phagocytic defects are also seen WASP-deficient macrophages and neutrophils. Moreover, WASP seems to be required for NK cell cytotoxicity [205]. Lymphocytes from WAS patients are morphologically abnormal; they have reduced number of surface microvilli, show variably impaired transduction of early activation signals through antigen receptors on both B and T cells, show abnormal levels and capping of actin as well faulty TCR capping [204, 205, 207]. The phenotype associated with WASP deficiency has been thoroughly characterized in T-cells. T-cell trafficking in response to well-established chemoattractants (SDF-1/CXCL12) is greatly reduced and TCR-induced T-cell proliferation (in part due to defective IL-2 production), T-cell: APC synapse formation and intracellular Ca2+ fluxes in anti-CD3 activated T-cells are severely depressed. In contrast, T-cell responses to non-specific mitogens are often normal,

suggesting a role for WASP in TCR proximal signaling events [204, 205, 220]. Importantly, thymocyte development is impaired in WASP-negative mice, presenting with lower numbers of single positive (SP) $CD4^+$ and $CD8^+$ T-cells, presumably associated with restricted progression to the $CD4^+CD8^+$ double-positive (DP) stage during thymocyte development [207, 221-223].

The role of WASP in B-cell function and BCR signaling has been more difficult to determine. WAS patients show deficient antibody responses to polysaccharide antigens in particular, and low or absent levels of isohemagglutinins (antibodies against blood group antigens) indicate that there are also intrinsic abnormalities of B-cell function [204, 205, 224]. Quantitative abnormalities of total immunoglobulin levels develop in many patients, manifesting typically as low levels of IgM and increased levels of IgA, IgD and IgE [207]. Further examination of antibody responses revealed that immune responses to protein antigens are frequently blunted and often not associated with isotype switching [207, 210, 224]. In B-cells from WAS patients, the pattern of responses to antigen-receptor stimulation are unclear as both normal and abnormal proliferative responses have been described [225, 226]. Studies with mouse WASP-null (or $WASP^{-/-}$) B-cells show normal proliferation and normal BCR capping in response to B-cell stimulation [223]. It was initially noted that $WASP^{-/-}$B-cells presented soluble but not particulate antigens. However, this defect is overcome by providing LPS and IL-4 to $WASP^{-/-}$ B-cells [227]. A delayed and slightly reduced humoral response (class switching is normal), reduction/absence of the marginal zone subset of B-cells (MZB), delayed germinal center reaction and an abnormal splenic architecture are defects which standout in the mouse

model [224, 228]. Some of the humoral defects in these mice might be related to morphological (shortened filopodia), aggregation and homing defects of B-cells and secondary organizational abnormalities of lymphoid architecture and germinal center formation, rather than to antigen-induced signaling deficiency [102]. Additionally the absence of MZBs perhaps explains the lack of an antibody response to polysaccharide antigen [52, 57, 220, 228]. It seems probable that intrinsic defects in B-cell function, rather than a failure of T-cell help, produce many of these defects as transfer of wt T-cells in to $WASP^{-/-}$ mice does not rescue or modify the B-cell deficiencies, additionally the transfer of $WASP^{-/-}$ B-cells into irradiated wt mice (lacking endogenous lymphocytic components) fails to induce adequate repopulation of the normal B-cell niches upon antigenic challenge [228].This indicates that the problem largely lies with the ability of $WASP^{-/-}$ B-cells to home or migrate in response to antigenic stimulus. Yet, altogether, these results add up to a relatively mild effect of WASP deficiency on B-cell function and implicate functional degeneracy or redundancy which complements the lack of WASP. The redundancy hypothesis is supported by the relatively high level of conserved homology between WASP family proteins. N-WASP, which has been shown to be expressed in B-cells [102]**,** shares 50% length-wise homology overall with WASP [229] and 68% homology in the domains responsible for actin nucleation [230]. The finding that the role of N-WASP in vesicular movement can be fully replaced by WASP [231] provides additional evidence for the functional redundancy of these two proteins, especially in case of deficiency. While WASP and N-WASP share functional and structural similarity, they are regulated by several other constraints that have varying effects on their activation. These differences may contribute to the preferential activation

of one protein over another under certain scenarios. For instance, N-WASP possesses an additional C-terminal WH2 domains which is thought to enhance actin polymerization, although the importance of the second WH2 domain of N-WASP is disputed, with one study claiming enhanced ability to stimulate Arp2/3-induced filament nucleation and another finding no effect [232-234]. Comparisons between the rates of actin polymerization between N-WASP, WASP and Scar/WAVE proteins show that Scar/WAVE proteins stimulate actin polymerization the least while actin polymerization rates for WASP and N-WASP are 16-fold and 70-fold higher, respectively [234]. The exceptionally enhanced ability of N-WASP to stimulate actin polymerization has been reported to be dependent on key amino acids in the C-terminal acidic domain [234], which may determine the stability of the protein. This further indicates intrinsic differences between WASP and N-WASP biochemistry. It is known that the WH1 domains of WASP and N-WASP bind WIP (WASP-interacting protein), which forms hetero-complexes with them and is possibly important in stabilizing their structures and function [204, 207, 235, 236]. Recent evidence indicates that WIP and N-WASP form a functional complex during the formation of filopodia and actin tails [237], whilst WIP binding to WASP inhibits WASP activity [238]. In WIP deficient mice, T-cells show similar but more profound defects in activation compared to WASP null mice, whilst B-cells show enhanced proliferation and CD69 expression after BCR ligation, indicating that WIP operates as a negative regulator of B-cell activation [239]. The mechanism by which WIP exerts this control on B-cell physiology is currently not known. However, the differential impact of WIP on WASP and N-WASP, both of which are expressed in B-cells, could very well determine the functional balance between these two proteins in B-

cells. There is some dispute as to which of the two proteins, WASP and N-WASP is preferentially used in different cellular processes. Tomasevic *et al.* found that WASP preferentially activates in the presence of Cdc42, while N-WASP prefers Rac1 for activation, while both proteins depend on the adaptor protein Nck [240]. Furthermore, PtdIns-4, 5-P_2 preferentially activates N-WASP either alone or in a synergistic manner in the presence of Rac1/Cdc42, and that PtdIns-4,5-P_2 had either no effect or inhibitory effect on WASP activity in the presence of Cdc42/Rac1 in the *in vitro* model used by Tomasevic *et al*. Conversely, other studies [212, 231] show that PtdIns-4,5-P_2 is required for WASP activity in cellular or physiological context. Benesch *et al.*, show that intracellular actin-based rocketing of PtdIns-4,5-P_2 rich vesicles is normally dependent on N-WASP activity, but in case of N-WASP deficiency, this activity can be fully restored by WASP [231]. These conflicting results may reflect the different affinity with which WASP and N-WASP bind PtdIns-4,5-P_2 and the cellular context provided by different cell types and *in vitro* models. While the basic domain of N-WASP has been shown to bind PtdIns-4,5-P_2, an analogous PtdIns-4,5-P_2 binding site for WASP has not been definitively described. Although the basic domain of WASP is implicated in PtdIns-4,5-P_2 binding, it is much smaller than that of N-WASP, consequently binding to PtdIns-4,5-P_2 with an affinity lower than N-WASP [212]. The disparate affinities of WASP and N-WASP for PtdIns-4, 5-P_2 provides another strategy for their differential regulation. Moreover, the presence of a unique calmodulin binding region on N-WASP (called the IQ domain) [229] and the phosphorylation state of Tyr^{256} residue on N-WASP have been implicated in nucleo-cytoplasmic shuttling of N-WASP [217] but not WASP. These provide other examples of domain/activation dependent sub-cellular location and

subsequent segregation of function. Thus, intrinsic differences in the molecular biology of these proteins may play an important role in determining their cellular distribution and level of activity in B-cells.

As mentioned above, it is known that both WASP and N-WASP activation is mediated by way of concerted phosphorylation of key residues and interactions with a myriad of binding partners. However the dependency of these events on the integrity of upstream signaling proteins, specifically in B-cells, is not well established. Moreover, though certain kinases are implicated in the activation of WASP by *in vitro* studies and in T-cells, no rigorous mechanism of activation tying upstream signaling events and downstream events of antigen-transport and presentation has been presented in B-lymphocytes. Presently, while both WASP and N-WASP have been shown to be expressed in B cells [102], their individual roles in early B cell activation remain elusive. I propose that these proteins are dynamically regulated by several components of the signaling pathway upstream of their activation and moreover, they play both unique and compensatory roles in the modulation of actin for productive B-cell activation upon antigen experience.

1.10 Hypothesis:

Previous studies show that the regulation and reorganization of the actin cytoskeleton after antigenic stimulation is necessary for BCR internalization and trafficking and is highly dependent on signals emanating from the BCR. Here, I hypothesize that actin reorganization events are coupled to the BCR signaling pathway by Btk, which controls

the degree of activation of actin regulatory proteins like WASP and N-WASP. Although it is likely that under normal conditions only one of these proteins is fully activated and involved in BCR-induced actin reorganization, it is equally possible that they are invested in unique roles in the B-cell by being spatially and functionally segregated. However, in conditions of WASP deficiency, we speculate that other WASP family proteins step in and complement the deficiency. Even though both WASP and N-WASP share several common regulatory factors, it is likely that the availability of activation factors determines which protein is activated, to what degree and where it is then redistributed. It is also possible that these proteins are regulated by signals that do not overlap. The work presented here was proposed to test these hypotheses.

1.11 Significance

Effective signal transduction and antigen internalization, following BCR crosslinking by an antigen, are ultimately important in controlling downstream cellular responses to the antigen. Abnormal regulation of BCR function leads to serious immunodeficiencies and autoimmune diseases [241]. The regulation of BCR function is dependent on many cellular components, their activities, and regulated interaction between these cellular components, particularly, signaling, cytoskeletal and endocytic components. Understanding the cross-talk between the actin cytoskeleton and BCR signaling will enable us to devise strategies to drive or control a defective or overactive B-cell response to diverse antigens and broaden our perspective on the general cooperation of distinct cellular entities to achieve a proficient immune response.

Chapter 2: Btk regulates BCR-mediated antigen processing and presentation by controlling actin cytoskeletal dynamics in B cells

2.1 Abstract

The high efficiency of antigen processing and presentation by B cells requires antigen-induced BCR signaling and actin cytoskeleton reorganization, although the underlying mechanism for such requirements remains elusive. Here, we identify Bruton's tyrosine kinase (Btk) as a linker connecting BCR signaling to actin dynamics and the antigen transport pathway. Using xid mice and a Btk inhibitor, we show that BCR engagement increases actin polymerization and Wiskott-Aldrich syndrome protein (WASP) activation in a Btk-dependent manner. Concurrently, we observe Btk-dependent increases in the levels of phosphatidylinositide-4,5-bisphosphate and phosphorylated Vav upon BCR engagement. The rate of BCR internalization, its movement to late endosomes, and efficiency of BCR-mediated antigen processing and presentation are significantly reduced in both xid and Btk inhibitor-treated B cells. Thus, Btk regulates actin dynamics and antigen transport by activating WASP via Vav and phosphatidylinositides. This represents a novel mechanism by which BCR-mediated signaling regulates BCR-mediated antigen processing and presentation.

2.2 Introduction

The BCR can serve as both signal transducer and antigen transporter. By increasing the kinetics and specificity of antigen capture, uptake and transport, the BCR increases the efficiency of antigen processing and presentation by B cells [167, 242], which enables B cells to present even sparsely occurring antigens. Key signaling intermediates, such as the tyrosine kinase Syk and the adaptor protein BLNK [178, 243], are involved in the timely transport of BCR-antigen complexes from the cell surface to antigen processing compartments. BCR signaling blockade by the tyrosine kinase inhibitor genistein or PP2 [181, 244], or loss-of-function mutants for Lyn or Syk [177, 178], has been shown to impede antigen uptake. Moreover, tyrosine phosphorylation of clathrin in lipid rafts upon BCR cross-linking is required for BCR internalization [168], revealing the entwined nature of signaling and antigen transport pathways of the BCR.

The binding of the BCR to antigens not only induces the reorganization of the actin cytoskeleton, but also triggers its association with the BCR and signaling molecules, including Lyn, Syk, and GTP binding proteins [190, 191, 195]. Tyrosine kinase inhibitors block BCR-induced actin polymerization [197], suggesting that actin remodeling is downstream of BCR proximal signaling. Accordingly, disrupting the actin cytoskeleton does not inhibit BCR-induced tyrosine phosphorylation or the translocation of the BCR to lipid rafts [131]. However, it blocks BCR internalization by inhibiting the pinching off of clathrin-coated vesicles from the plasma membrane (PM) [171]. An actin-dependent, but clathrin-independent, internalization pathway for the BCR has recently been observed [245], underscoring the importance of actin in conventional and non-conventional BCR

internalization. These studies have led to the hypothesis that a dynamic actin cytoskeleton is a determining factor for the correct intracellular routing of BCR-bound antigens and that there is a regulatory relationship between the actin cytoskeleton and BCR signaling and antigen transport pathways.

The mechanistic links between the interrelated pathways of BCR signaling, antigen transport and the actin cytoskeleton have not been well studied. Wiskott-Aldrich syndrome protein (WASP) is potentially such a link. WASP is a hematopoietic-cell specific actin regulator that links upstream signals to actin polymerization and branching by stabilizing Arp2/3 complexes [246]. WASP contains multiple interacting domains, including WASP homology-1 (WH1), basic (B), GTPase-binding (GBD), and proline-rich domains (PRD), and C-terminal verprolin-homology, cofilin homology and acidic domains (VCA) [207]. The interaction of GTP-Cdc42 with the GBD, phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) with the B domain, and phosphorylation at tyrosines 256 and 291 and serines 242 and 483/484 of WASP regulate its activity [212, 215, 216, 219]. Antigen binding to the BCR has been shown to induce the phosphorylation of Rho-family GTPase guanidine nucleotide exchange factor (GEF) Vav [247], the activation of Rho-family GTPases [248], and modulation of phosphatidylinositide metabolism [249]. While all of these signaling activities potentially regulate WASP, the exact mechanism that links BCR signaling to WASP activation remains to be defined.

Bruton's tyrosine kinase (Btk) belongs to the Tec tyrosine kinase family. The significance of Btk in B cell function was revealed by the discovery that Btk mutations cause inherited

immunodeficiencies in both humans (XLA, for X-linked agammaglobulinemia) and mice (*Xid*, for X-linked immunodeficiency) [138]. Xid mice have a point mutation at arginine 28 to cysteine (R28C), which lies in the pleckstrin homology (PH) domain of Btk. The xid mutation prevents Btk from binding to PtdIns-3,4,5-P_3 and recruiting to the PM, consequently inhibiting its activation [110, 147]. This particular mutation leads to B cell developmental defects, with 50% reduction in mature B cells, a virtual absence of the B1-subset of cells, and a pronounced decrease in serum levels of IgM and IgG_3 [137]. In addition to its kinase domain, Btk has PH, Tec homology (TH), Src homology 3 (SH3), and SH2 domains [138]. Both the kinase and PH domains are indispensable for Btk activity in mature B-cells. Upon BCR activation, Btk is recruited to the PM by its PH domain binding PtdIns-3,4,5-P_3 [109]. At the PM, Btk binds the scaffolding adaptor protein BLNK, and is phosphorylated by Src kinases at Tyr^{551} in the kinase activation loop which then leads to the autophosphorylation of tyrosine 223 in its SH3 domain [96, 97]. Phosphorylated Btk activates PLCγ2in concert with Syk, by phosphorylating it on key residues, thus modulating the Ca^{2+} influx [153]. Additionally, it facilitates phosphatidylinositide metabolism at the membrane by its interaction with phosphatidylinositol 4-phosphate 5-kinase (PIP5K) [149], thereby generating substrates for PI3-Kinase and PLCγ2. Thus, Btk's role in B-cell signaling is well delineated though its role in modulating the actin cytoskeleton is not as distinct. Nonetheless, there are some clues to its possible involvement in regulating cellular actin dynamics. For instance, PIP5K mediated actin changes are known to be articulated via its modulation of the Rho GTPases [250]. Thus, by controlling the cellular location of PIP5-K, Btk modulates actin reorganization at least indirectly. There is *in vitro* evidence for the association of Btk with WASP and its role in phosphorylating Tyr^{291} on WASP [143, 251,

252]. Moreover, recent studies in T cells from mice deficient in Tec kinases Itk and/or Rlk show impaired actin polarization and defects in the recruitment of GTP-Cdc42 and its GEF Vav1 to the TCR at the immune synapse [161, 164]. As both Cdc42 and Vav are implicated in modulating the actin cytoskeleton, these studies indicate a role for Tec kinases in linking upstream signaling to actin dynamics.

In this study, we explore the role of Btk, a Tec kinase expressed in B cells, in linking BCR signaling to the actin cytoskeleton and BCR-mediated antigen processing pathways. We report that the BCR-triggered signaling regulates the dynamics of the actin cytoskeleton through WASP in a Btk-dependent manner. Btk function is required for BCR-induced WASP and Vav activation, increased cellular PtdIns-4,5-P_2 levels, actin rearrangement, and ultimately for BCR-mediated antigen processing and presentation.

2.3 Materials and Methods

2.3.1 Mice and Cells

Splenic B cells were isolated from wt (CBA/CaJ) and xid (CBA/CaHN-*Btk*^{xid}/J) mice (Jackson Laboratories). Mononuclear cells were obtained by Ficoll density-gradient centrifugation (Sigma-Aldrich). T cells were deleted by anti-Thy1.2 mAb (BD Biosciences) and guinea pig complement (Rockland Immunochemicals). All procedures involving mice were approved by the Animal Care and Use Committee of University of Maryland. B cell lymphoma A20 IIA1.6 cells (H-2^{d}, IgG_{2a}^{+}, FcγIIBR^{-}) were cultured in DMEM supplemented with 10% FBS.

2.3.2 DNA constructs and transfection.

B cell lymphoma A20 IIA1.6 cells (H-2^d, IgG_{2a}^+, FcγRIIB$^-$) were cultured at 37^{o}C in DMEM supplemented with 10% FBS. The DNA construct encoding the eGFP fusion protein of actin (eGFP-actin) was obtained from Clonetech and was introduced into A20 B cells by electroporation using the Nucleofector and Nucleofection kit V from Amaxa (Gaithersburg, MD).

2.3.3 Flow Cytometric Analysis

B cells were stimulated with 20 μg/ml $F(ab')_2$-goat-anti-mouse IgG+M [$F(ab')_2$-anti-Ig] (Jackson ImmunoResearch) for indicated times. To determine the effect of the Btk inhibitor, B cells were preincubated with LFM A-13 (100 μM, Calbiochem) at 37^{o}C for 1 h, and the inhibitor was included in the cell medium during B cell stimulation and chase. A non-active derivative of LFM A-13, LFM A-11 (100 μM) was used as a control (data not shown). Cells were fixed with 4% paraformaldehyde, washed, permeabilized with 0.05% saponin, and stained with Alexa Fluor (AF) 488-phalloidin (Invitrogen) or AF488-anti-phosphorylated Vav Y174 (pVav) (Santa Cruz Biotechnology). The cells were analyzed using a FACSCalibur (BD Biosciences) flow cytometer. The data are represented as mean fluorescence intensity (MFI). To compare the levels of phosphorylated WASP (pWASP) in different B cell subpopulations, splenic B cells from wt and xid mice were incubated with PE-Cy5-anti-mouse B220, AF488-anti-mouse IgM, and PE-anti-mouse IgD at 4^{o}C with or without warmed up to 37^{o}C for 5 min. The cells were then fixed, permeabilized, and

incubated with anti-mouse pWASP (S483/S484). The cells were analyzed using CyAN^{TM} (Dako, Carpinteria, CA) flow cytometer. Three subsets of splenic B cells were gated, including follicular (FO) ($B220^{+}$ $IgM^{lo}IgD^{hi}$), T2 ($B220^{+}$ $IgM^{hi}IgD^{hi}$), and T1 ($B220^{+}$ $IgM^{hi}IgD^{lo}$) B cells.

2.3.4 Immunofluorescence Microscopy Analysis

In order to analyze differences in the cellular distributions of BCR and F-actin in the presence of various inhibitors, untreated A20 B cells or A20 cells pre-treated with either PP2 (25μm, Src kinase inhibitor) or U0126 (25μM, MEK/Erk Inhibitor) were incubated with Cy3 F(ab) anti-mouse IgG (10μg/mL Jackson Immunoresearch) for 10 min at 4^{o}C to label the surface BCR, followed by rabbit anti-mouse IgG (20 μg/ml, Jackson Immunoresearch) for indicated times at 37^{o}C to crosslink the BCR. Cells were fixed with 4% paraformaldehyde, permeabilized and stained with Alexa Fluor (AF) 488-conjugated Phalloidin (Invitrogen/Molecular Probes). Cells were mounted and analyzed using a confocal fluorescence microscope.

To analyze BCR internalization, B cells were incubated with 5 μg/ml Cy3-Fab-rabbit anti-mouse IgM (Cy3-Fab-anti-μ, Jackson ImmunoResearch) at 4^{o}C for 30 min in the presence of 10 μg/ml F(ab')$_2$-anti-Ig to label and cross-link the surface BCR. Cells were chased at 37^{o}C for varying lengths of time and incubated with AF488-cholera toxin subunit B (CTX-B) (Invitrogen) at 4^{o}C to label the plasma membrane.

To analyze the movement of the BCR from early to late endosomes, B cells were incubated with Cy3-Fab-anti-μ in the presence or absence of F(ab')$_2$-anti-Ig at 18°C for 30 min to allow for BCR internalization. The cells were warmed up to 37°C for varying lengths of time. To

mark early endosomes, AF488-holo-transferrin (Tf) (10 μg/ml, Invitrogen) was included in the incubation medium at both 18°C and 37°C. After fixation and permeabilization, the cells were incubated with anti-CD32/CD16 mAb (BD Biosciences) to block FcγR, anti-LAMP-1 mAb (1D4B) (ATCC) to mark late endosomes, and AF488-phalloidin to label F-actin.

To analyze the cellular distribution of active and phosphorylated WASP (oWASP/pWASP), pVav, and PtdIns-4,5-P_2, splenic B cells were pulsed with Cy3-Fab-anti-μ at 37°C for 10 min and chased in the presence or absence of F(ab')$_2$-anti-Ig for varying lengths of time at 37°C. The cells were fixed, permeabilized, pre-incubated with anti-CD32/CD16 mAb, and incubated with antibody specific for oWASP (Upstate Biotechnology), pWASP S483/S484 (Bethyl Laboratories), pVav, or PtdIns-4,5-P_2 (Invitrogen), followed by their corresponding secondary antibodies. Cells were analyzed under a laser-scanning confocal microscope (Zeiss LSM 510) or a Deltavision deconvolution microscope. **Pearson's correlation coefficients (R)** of differently stained proteins were determined using the LSM 510 software. Pearson's correlation coefficient is one of the standard measures in pattern recognition and is defined by:

$$R_r = \frac{\sum_i (S1_i - S1_{aver}) \cdot (S2_i - S2_{aver})}{\sqrt{\sum_i (S1_i - S1_{aver})^2 \cdot \sum_i (S2_i - S2_{aver})^2}}$$

where S1 represents signal intensity of pixels in the channel 1 and S2 represents signal intensity of pixels in the channel 2; $S1_{aver}$ and $S2_{aver}$ reflect the average intensities of these respective channels. It is used for describing the correlation of the intensity distributions

between channels. It takes into consideration only similarity between shapes, while ignoring the intensities of signals. Its values range between −1.0 and 1.0, where 0 indicates no significant correlation or random distribution and −1.0 indicates complete negative correlation. The correlation coefficient here indicates the strength and direction of a linear relationship between the cellular locations of two proteins.

2.3.5 Live cell imaging.

The construct encoding eGFP-actin was introduced into A20 B cells by electroporation using Nucleofection kit (Amaxa). 24 hours after transfection, cells were placed into chambered polylysine (10 μM)-coated cover glasses (Nalgene Nunc Int. Rochester, NY) and incubated at 37°C, 5% CO_2 for 30 min. Cells were then incubated with Cy3-Fab-goat anti mouse IgG (5 μg/ml; Jackson Immunoresearch) at room temperature for 10 min, washed with 1%FBS in PBS. Cells were activated by crosslinking the BCR with rabbit-anti-mouse IgG (20 μg/ml; Jackson Immunoresearch) and images were acquired every 3 seconds. eGFP actin expressing cells were also photo-bleached for 30 sec in selected regions of interest (ROI) to see recovery of fluorescence in the photo-bleached area, the LSM 510 confocal and LSM510 Image Examiner were used to analyze this data. Images of eGFP actin expressing cells were acquired using the Zeiss LSM510 confocal microscope.

2.3.6 Analysis of Actin Nucleation Sites

Actin nucleation sites were labeled as previously described [253]. Briefly, B cells were serum starved for 1 h and then incubated with Cy3-Fab-anti-μ and $F(ab')_2$-anti-Ig at 37^{o}C for indicated times. In the last minute of incubation, cells were treated with 0.45 μM AF488-G-

actin (Cytoskeleton Inc.) in the presence of 0.025% saponin and then immediately fixed. The cells were analyzed using a confocal fluorescence microscope and quantified using the LSM510 software.

2.3.7 Immunoblotting

Untreated A20 B-cells or B-cells treated with Latrunculin B (10 μM) were activated with $F(ab')_2$-anti-Ig for indicated times and lysed with a lysis buffer containing 0.5% Triton X100 RIPA-base, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4, and protease inhibitors (Roche, Basel Switzerland). Cell lysates were analyzed with SDS-PAGE and Western blot, probed for total phosphorylated tyrosines using anti-phospho-tyrosine mAb (4G10, Upstate Biotech, Lake Placid, NY) and phosphorylated Erk using rabbit anti-phosphorylated ERK (pERK) (Cell signaling) respectively. The blots were stripped and reprobed with anti-mouse β-tubulin (Sigma) for establishing loading controls. B cells untreated or treated with LFM A-13 (100 μM) were activated with $F(ab')_2$-anti-Ig for indicated times and lysed. Cell lysates were analyzed with SDS-PAGE and Western blot, probed for pWASP S483/484 and pVav Y174 respectively. The blots were stripped and reprobed with anti-mouse β-tubulin (Sigma) for establishing loading controls. The blots were quantified by densitometry. The levels of pWASP and pVav were normalized against tubulin and expressed as fold increases over unstimulated levels.

2.3.8 Analysis of BCR Internalization by Flow Cytometry

B cells were incubated with 10 μg/ml of biotin-$F(ab')_2$-anti-mouse IgM at 4^oC and chased for 0, 5, 10 and 30 min at 37^oC. Biotin-$F(ab')_2$-anti-IgM left on the cell surface after the chase

was stained with PE-streptavidin and quantified using a flow cytometer. The data were expressed as percentages of the cell surface-associated biotin-F(ab')$_2$-anti-IgM antibody at time 0. To identify different subpopulations of splenic B cells, cells were co-labeled with FITC-anti-IgD and PE-Cy5-anti-B220 antibodies (BD Bioscience). BCR internalization in different subpopulations of splenic B cells was compared by gating on $B220^{+}IgM^{lo}IgD^{hi}$ (FO), $B220^{+}IgM^{hi}IgD^{hi}$ (T2), and $B220^{+}IgM^{hi}IgD^{lo}$ (T1) subsets, respectively.

2.3.9 Antigen Presentation Assay

Splenic B cells were incubated with hen egg lysozyme (HEL) alone or with the following antibodies in sequence at 4^{o}C to target HEL to the BCR: anti-CD32/CD16 mAb to block FcγRs, rabbit anti-mouse IgM (5 μg/ml) to bind the BCR, goat anti-rabbit IgG (5 μg/ml) to link rabbit anti-HEL and rabbit anti-mouse IgM antibodies, rabbit anti-HEL antibody (5 μg/ml), and finally HEL (0.5 or 1 μg/ml) as the antigen. Cells were warmed to 37^{o}C with the antigen-antibody complex for 15 min, washed, and incubated at 37^{o}C for 14 h. HEL–I-A^{k} complexes on the cell surface were detected by AF488-C4H3 mAb and quantified by flow cytometry. To test the ability of B cells to present antigen to T cells, splenic B cells were pulsed with HEL (1 μg/ml) with or without the antibody complex for varying lengths of time. After washing, the B cells (1 X 10^{6}) were co-cultured overnight with KZH T-cells (1 X 10^{6}) that are specific for HEL_{46-61}:I- A^{k} and express a lacZ reporter gene under the control of the IL-2 promoter [254] (a kind gift from Dr. Nilabh Shastri and Kenneth Frauwirth). The lacZ activity was assayed using chlorophenol red β-galactopyranoside [255]. The reaction product was quantified by its absorbance at 595 nm, with 655 nm as the reference wavelength.

2.4 Results

2.4.1 Actin dynamics are regulated by signaling cues upstream and downstream of BCR proximal signaling.

The integrity of the actin cytoskeleton is required for internalization of the BCR and trafficking of the antigen-BCR complex to the right compartments [171]. Here, we set out to establish a system to examine actin dynamics in live cells and real-time. GFP-actin was introduced into A20 B cells by electroporation, and A20 cells stably expressing GFP-actin were selected and cloned. In order to test the functionality of A20 B cells expressing GFP-actin, we labeled the surface BCR and analyzed the distribution of the actin cytoskeleton relative to the BCR in response to BCR cross-linking. Unstimulated cells moved randomly, and their actin cytoskeleton distributed evenly under the plasma membrane without any significant polarized association with the BCR (Fig. *2.1A, a-f*). Upon BCR cross-linking (Fig *2.1A, g-l*), the BCRs clustered at one pole of the cell surface, forming caps from where the BCR internalized. The actin cytoskeleton in these cells was rapidly polarized to areas at the cell membrane that were rich in the BCR. Some of the GFP-actin remained colocalized with the BCR even after it had been internalized. Initial studies with fluorescence recovery after photobleaching (FRAP) show that BCR associated actin is thread milling. Photobleaching GFP-actin in areas of BCR-actin colocalization showed dramatic recovery of GFP-levels shortly after the bleach was performed (data not shown). This preliminary study shows that there is no significant effect of GFP-actin expression on the intracellular trafficking of the BCR and

demonstrated the value of GFP-actin expressing cells in delineating relationships between the BCR and the actin cytoskeleton.

The structure and dynamics of the actin cytoskeleton are regulated by extracellular signals. In chapter 1 the impact of signaling kinases on the regulation of the actin cytoskeleton is discussed. Thus, in order to further understand the molecular mechanism for BCR-mediated regulation of actin dynamics, we looked to see if disrupting varied signaling components in the BCR signaling pathways has any effect on the F-actin levels of B cells. F-actin levels were measured using phalloidin-AF488 by immunofluorescence. In the absence of an inhibitor, A20 B cells transiently increased the levels of F-actin at early times (<2 min) in response to BCR cross-linking and decreased their F-actin level at later time (>5 min) after the stimulation (Fig. *2.1B, a-d*). At 10 min, the BCR was internalized and colocalized with intracellular patch of F-actin (Fig. *2.1Ba*). The MEK/Erk inhibitor U0126, which is downstream of Btk in the BCR signaling pathway, inhibited both the initial increase and the later decrease in F-actin levels induced by BCR cross-linking (Fig. *2.1B, e-h*). At 10 min, F-actin formed clustered patches under the plasma membrane. Moreover, BCR internalization appeared to be blocked, as most of the stained BCR remained at the cell surface. Meanwhile, BCR-induced increases in F-actin appeared to be delayed (5 min), and high levels of F-actin remained at 10 min after BCR cross-linking, on treatment with the Src kinase inhibitor PP2, which disrupts signaling upstream of Btk (Fig. *2.1B*). BCR internalization in PP2-treated cells was also blocked, consistent with previous findings (Fig. *2.1C*) [177]. Interestingly, BCR internalization was also blocked with the MEK/ERK inhibitor U0126 (Fig *2.1C*). These results reinforce

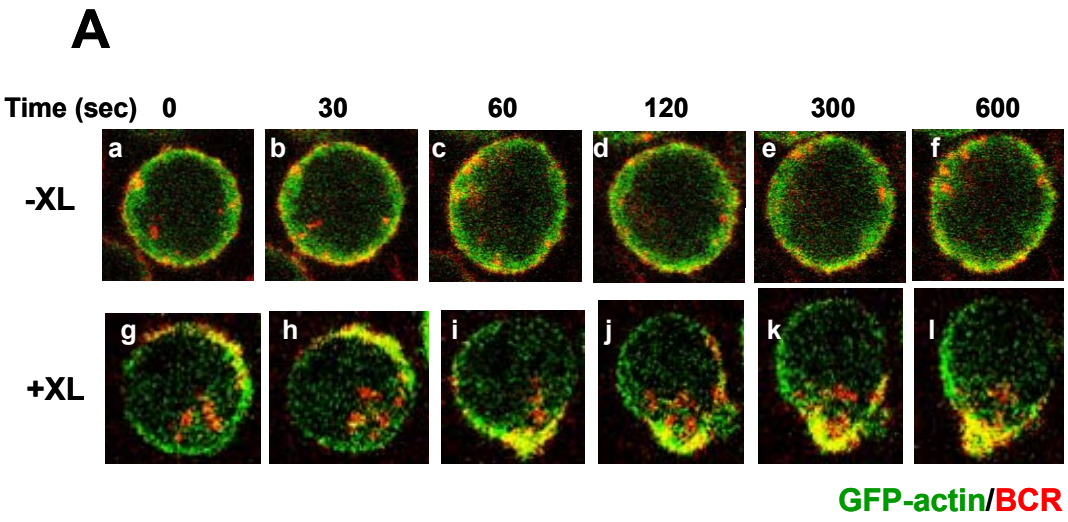
the idea that actin regulation in B-cells is mediated by multiple components of BCR signaling pathways, perhaps in both direct and indirect ways. Additionally, these results show a clear functional correlation of BCR internalization and BCR-triggered actin reorganization. To test the presence of feedback loops from the actin cytoskeleton to BCR signaling, I determined the effect of actin disruption on the key MAP kinase ERK. The phosphorylation levels of ERK (pERK) in response to BCR cross-linking in A20 B cells treated with or without Latrunculin B (sequesters G-actin monomers and induces actin depolymerization) were determined using Western blot (Fig. *2.1D*). Surprisingly, disrupting the actin cytoskeleton by using Latrunculin B increased the levels of pERK at early times after B-cell activation when compared to untreated cells (Fig. *2.1D*). This suggests that bilateral regulatory relationship between BCR signaling pathways and the actin cytoskeleton are extant.

2.4.2 BCR Crosslinking Induces Btk-Dependent Actin Rearrangement

To investigate the relationship between BCR signaling pathways and the actin cytoskeleton, we determined changes in the overall levels of cellular F-actin and actin polymerization in response to BCR cross-linking. Upon antigen binding, the levels of total F-actin, quantified by phalloidin staining and flow cytometry, increased reproducibly by 2 min, followed by a decrease at later time points in both splenic and A20 B cells (Fig. *2.2A*). This result suggests biphasic actin reorganization, with polymerization and depolymerization dominated phases. To follow actin polymerization, AF488-G-actin was introduced to cells in the presence of detergent in the last minute of

FIGURE 2.1. Actin dynamics are regulated by proximal and downstream BCR signals.

(A) Image frames from live cell microscopy of A20 B cells expressing GFP-actin. A20 B-cells stably expressing eGFP actin were plated on poly-L-lysine coated chambered coverglasses at 37^{o}C for 15 min. Cells were incubated with Cy3-Fab-rabbit-anti-mouse IgG (10 μg/mL) for 10 min at 37^{o}C in order to visualize the BCR and goat-anti-mouse IgG (10 μg/mL) to activate the B-cells. Images were acquired every 5 sec after addition of antigen. Shown are representative images of three independent experiments. (B-C) A20 B-cells were either left untreated or were pretreated with the Src kinase inhibitor PP2 (25 μM) or MEK/ERK inhibitor U0126 (25 μM) for 1 h at 37^{o}C and 5% CO_2. Cells were then incubated with Cy3-Fab-anti-mouse IgG (10 μg/mL) for 10 min at 37^{o}C to visualize the BCR and anti-mouse IgG (10 μg/mL) at 37^{o}C for varying lengths of time in the presence of the inhibitors. Cells were fixed, permeabilized, and incubated with Alexa Fluor (AF)-488 conjugated phalloidin for F-actin. Shown are representative images from three independent experiments. (C) Images are representatives from 10 min time points for the conditions noted from three independent experiments. (D) Effect of latrunculin B (LatB) on ERK phosphorylation in response to BCR activation. A20 B-cells, which were either pretreated with 10 μM LatB for 1 h at 37^{o}C or left untreated, were incubated with anti-mouse IgG (10 μg/mL) to cross-link the BCR and lysed at denoted time points. Levels of phosphorylated ERK (pERK) were determined by western blotting using pERK-specific antibody. Shown are representative blots from two independent experiments and densitometry analysis.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60
120
300
600
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+XL
a
b
c
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e
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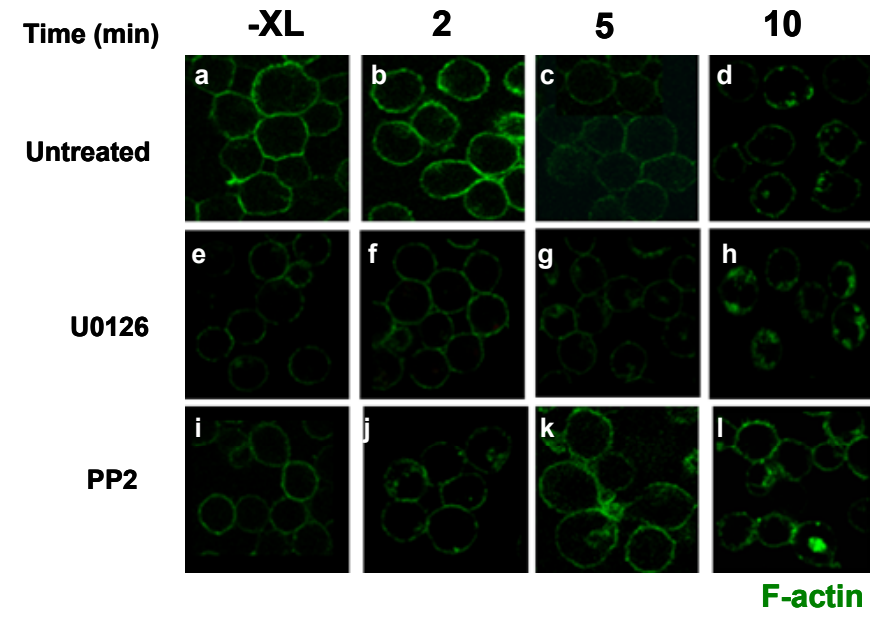
g
h
i
j
k
l
GFP-actin/BCR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Time (min)
-XL
2
5
10
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U0126
PP2
a
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k
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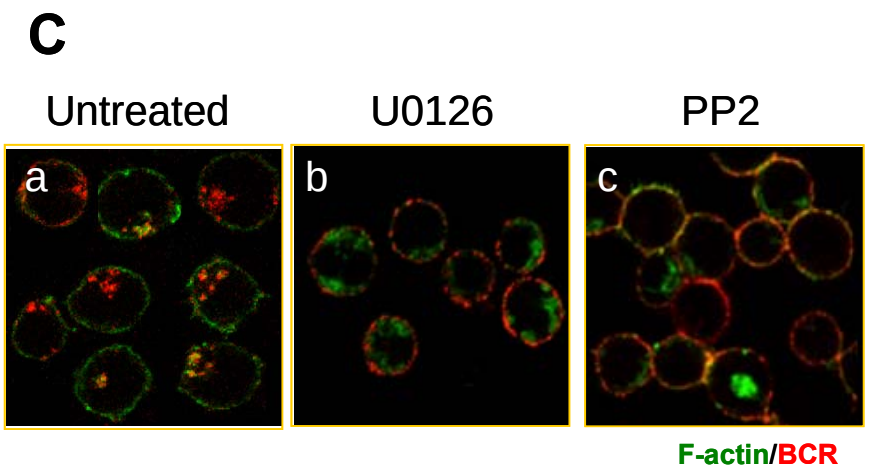
F-actin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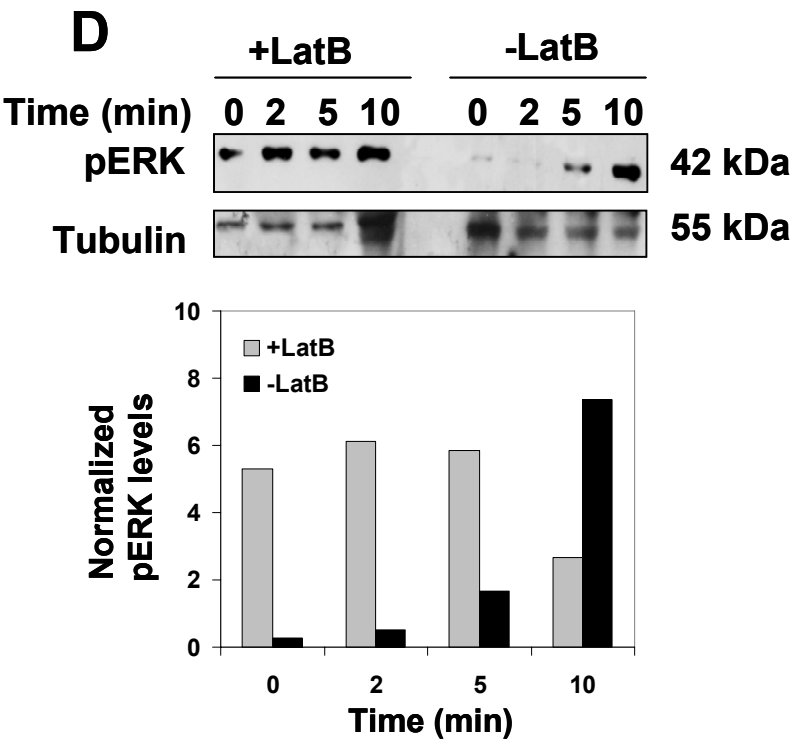
PP2
a
b
c
F-actin/BCR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-LatB
Time (min)
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0 2 5 10
pERK
42 kDa
Tubulin
55 kDa
Normalized pERK levels
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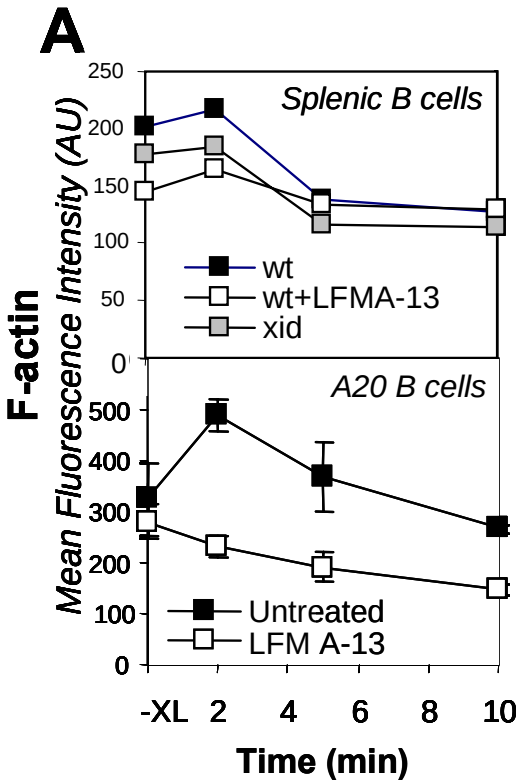
-LatB
10
8
6
4
2
0
0
2
5
10
Time (min)

stimulation. The incorporation of AF488-G-actin into polymerizing ends of F-actins marks *de novo* actin nucleation sites. Similar to the cellular levels of F-actin, G-actin incorporation increased significantly 1-2 min after stimulation, after which the levels of G-actin incorporation plateaued (Fig. *2.2 Ba-d* and *C*). Statistical analysis showed that the initial increase from 0 min to 1, 2 or 5 min in wt cells was significant (*$p \leq 0.05$), however subsequent increases *between* timepoints 1, 2 and 5 were not significant, implying beyond the initial rapid incorporation of G-actin, its levels do not change (data not shown). In order to study the subcellular location of this incorporation relative to the BCR, the correlation indices of individual BCR and G-actin pixels were calculated. Cross-linking the BCR significantly increased the correlation between staining for actin nucleation sites and the BCR and then the correlation stayed fairly constant in wt B-cells for the duration tested (Fig. *2.2D*). Thus, BCR activation increases actin polymerization in the vicinity of the antigen-bound BCR.

To test whether Btk plays a role in linking BCR signal transduction to the actin cytoskeleton, we used xid mice and a Btk inhibitor, leflunomide metabolite analog 13 (LFM A-13) [256]. LFM A-13, a membrane permeable inhibitor, binds exclusively to the SH1 domain of Btk, inhibiting the kinase activity of Btk. The inhibitor allows for inhibition of Btk activity independent of developmental defects seen in xid B cells [137]. LFM A-13 and Btk xid mutation showed similar inhibitory effects on both BCR-triggered gross and Btk tyrosine phosphorylation (data not shown). The effect of the Btk mutation and inhibitor on BCR-induced actin rearrangement was determined. LFM A-13 inhibited BCR-induced increases in F-actin levels in both splenic and A20 B cells, while the xid

FIGURE 2.2. BCR stimulation induces the reorganization of the actin cytoskeleton and this actin remodeling is dependent on Btk.

A, Wt splenic and A20 B cells that were treated or untreated with Btk inhibitor LFM-A13 (100 μg/ml) and untreated xid splenic B cells were stimulated with $F(ab')_2$-anti-mouse IgG+M ($F(ab')_2$-anti-Ig, 10 μg/ml) for 0, 2, 5 and 10 min. The cells were fixed, and F-actin was stained with Alex Fluor (AF) 488-phalloidin. The cells were analyzed using flow cytometry. Shown are the average fluorescence intensities (±S.D.) of AF488-phalloidin staining at the indicated times from three independent experiments. *B-D*, Splenic B cells from wt and xid mice were incubated with Cy3-Fab-anti-mouse μ chain (Fab-anti-μ) and stimulated with $F(ab')_2$-anti-Ig for 1, 2 and 5 min at 37°C. In the last minute of the stimulation, cells were incubated with AF488-G-actin in the presence of detergent to label newly polymerizing F-actin. The cells were immediately fixed and analyzed using a confocal fluorescence microscope. Shown are representative images from three independent experiments (*B*). Bar, 5 μm. Images were quantitatively analyzed to determine the fluorescence intensity of cell-associated AF488-G-actin (*C*) and the correlation coefficients between the labeled BCR and AF488-G-actin (*D*). Shown are mean values (±S.D.) from three independent experiments where over 300 cells were individually analyzed using Zeiss LMS 510 software (*, $p \leq 0.01$).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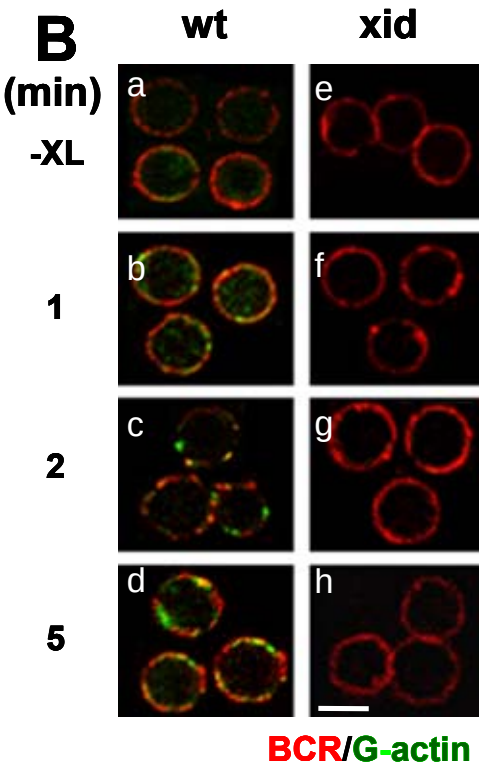
Splenic B cells
A20 B cells
F-actin
Mean Fluorescence Intensity (AU)
wt
wt+LFMA-13
xid
Untreated
LFM A-13
-XL
Time (min)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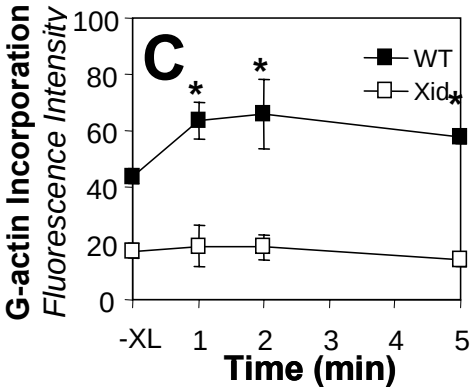
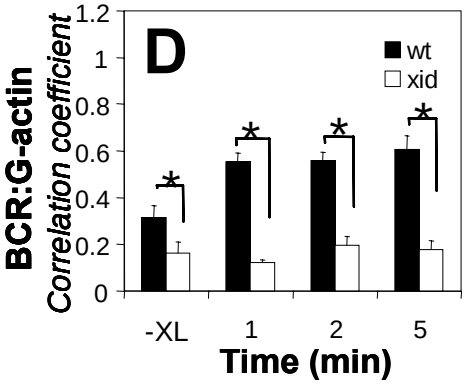
BCR/G-actin


C
G-actin Incorporation
Fluorescence Intensity
WT
Xid
Time (min)


D
BCR:G-actin
Correlation coefficient
wt
xid
Time (min)

mutation only slightly reduced F-actin levels (Fig. *2.2A*). The xid mutation not only blocked BCR-induced increases in actin polymerization, but also drastically reduced the basal level of actin polymerization (Fig. *2.2, Be-h* and *C*) and the colocalization of the BCR with actin nucleation sites (Fig. *2.2D*). Thus, Btk is required for both constitutive and BCR-induced actin reorganization in B cells.

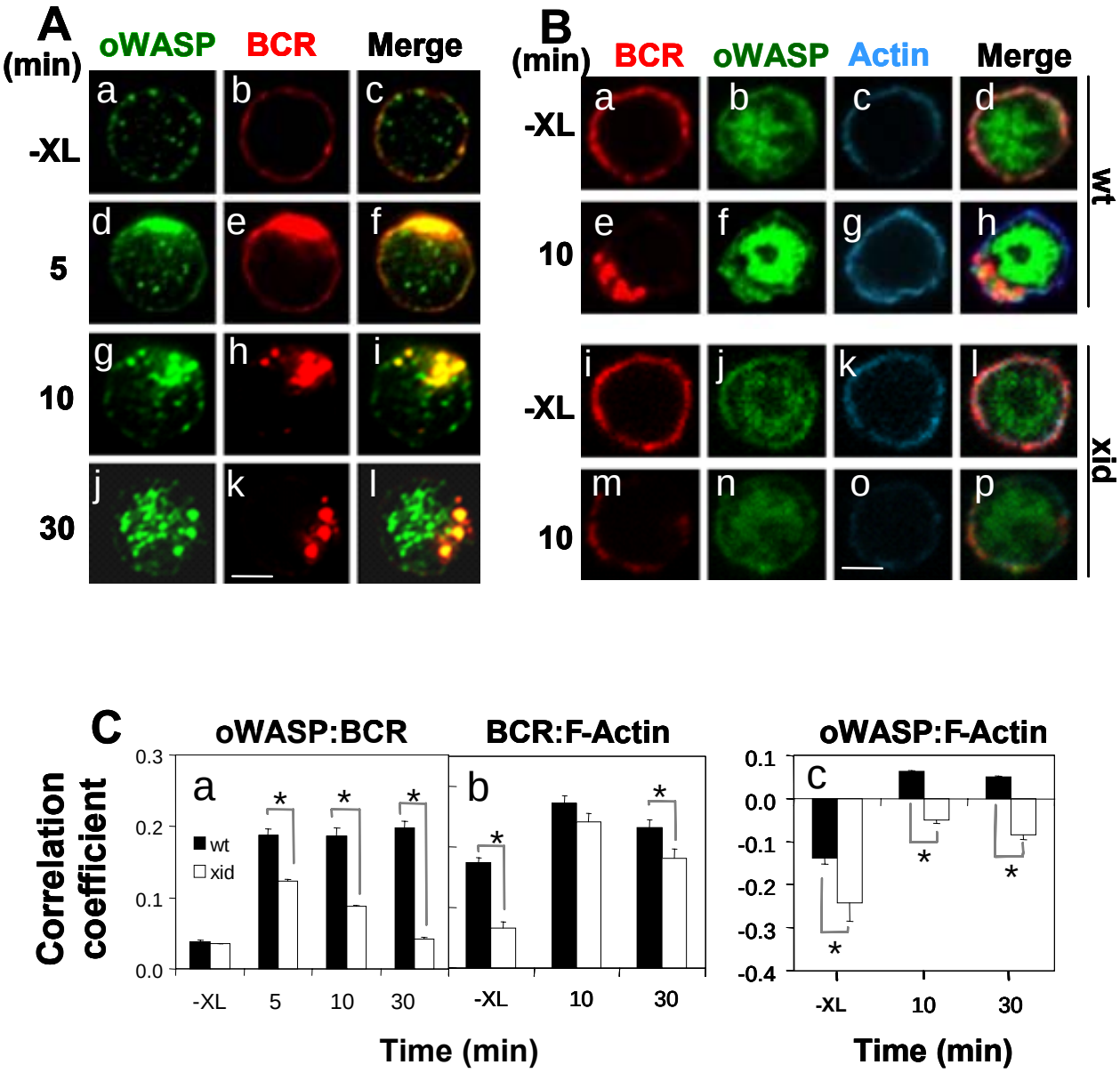
2.4.3 Antigen Engagement of the BCR Induces Btk-Dependent Activation of WASP

In order to understand the mechanism for BCR-induced actin reorganization, we examined the cellular behavior of WASP, an actin nucleation promoting factor. The activation of WASP was

followed by changes in its conformation and phosphorylation using antibodies specific for WASP in its open, active conformation (oWASP) or WASP phosphorylated at S483/S484 (pWASP), respectively. Immunofluorescence microscopic studies found that upon antigenic stimulation, oWASP was relocated from the cytoplasm to cell surface under BCR caps (Fig. *2.3, Aa-f*). At 10 min after the stimulation, oWASP was recruited to BCR^+-vesicles at the perinuclear location (Fig. *2.3 Ag-i*). By 30 min, while some of oWASP remained with BCR^+-vesicles, the rest appeared to move into the nuclei (Fig. *2.3 Aj-l*). Consistent with these results, BCR activation significantly increased the correlation coefficient between oWASP and the BCR by 5 min, and it remained high until at least 30 min (Fig. *2.3Ba*). While the BCR colocalized with F-actin in both stimulated and unstimulated cells, BCR cross-linking further increased this co- localization (Fig. *2.3Bb* and *Ca-h*). Furthermore, BCR cross-linking

FIGURE 2.3. BCR activation induces Btk-dependent WASP activation.

A, The surface BCR on wt splenic B cells was labeled with Cy3-Fab-anti-μ and either left unstimulated (-XL) or stimulated with $F(ab')_2$-anti-Ig at 37°C for indicated times. The cells were fixed, permeabilized, and stained with an antibody specific for WASP with an open, active conformation (oWASP). Cells were analyzed using the Deltavision deconvolution microscope. Shown are representative images from three independent experiments. Bar, 3 μm. *B,* The surface BCR of splenic B cells from wt (*a-h*) and xid (*i-p*) mice were labeled and stimulated as described in (*A*). After fixation and permeabilization, cells were labeled with AF488-phalloidin and an antibody specific for oWASP, and analyzed using a confocal fluorescence microscope. Bar, 3 μm. *C*, The colocalization coefficients between oWASP and BCR (*a*), BCR and F-Actin (*b*), and oWASP and F-Actin (*c*) for wt and xid B cells were quantified using the LSM 510 software. Shown are the average values (±S.D.) from three independent experiments of ≥300 cells (*, $p \leq 0.01$).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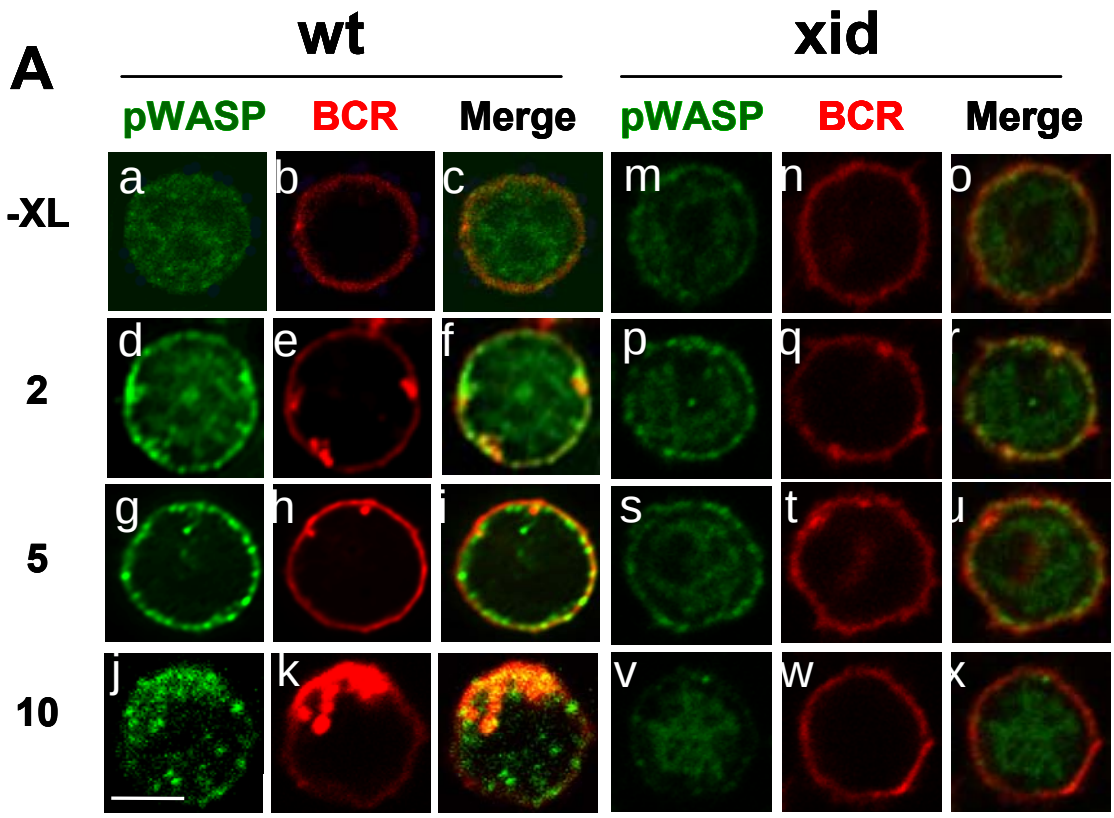
(min)
oWASP
BCR
Merge
-XL
5
10
30
B
(min)
BCR
oWASP
Actin
Merge
-XL
10
-XL
10
wt
xid
C
Correlation coefficient
oWASP:BCR
BCR:F-Actin
oWASP:F-Actin
wt
xid
Time (min)
Time (min)

increased the correlation between the staining of oWASP and F-actin from negative to positive values (Fig. *2.3Bc*). The phosphorylation of WASP was analyzed by both immunofluorescence microscopy and Western blot. Immunofluorescence microscopic studies showed a significant increase in pWASP staining over time in the splenic B cells upon BCR cross-linking (Fig. *2.4, Aa, Ad, Ag, Aj* and *B*). The average level of pWASP staining peaked at 2 min (Fig. *2.4B*). Meanwhile, pWASP was relocated from the cytoplasm to the PM where it colocalized with the surface BCR (Fig. *2.4*, *Aa-i* and *C-D*). At 5 min, ~50% of cells showed predominant cell surface staining of pWASP, in contrast to ~5% of unstimulated cells (Fig. *2.4C*). By 10 min, the number of cells showing significant surface staining of pWASP were reduced to ~25% (Fig. *2.4C*). Correlation analyses showed continued increase in the colocalization between pWASP and the BCR with time (Fig. *2.4D*). pWASP colocalized with BCRs at the cell surface at early time points (Fig. *2.4Ad-i* and *C- D*) and with internalized BCRs at later time points (Fig. *2.4*, *Aj-l* and *D*). Quantitative analysis of pWASP by Western blot showed that cross-linking the BCR significantly increased the level of pWASP in both A20 (Fig. *2.4E* and *G*) and splenic B cells (Fig. *2.4, F* and *H*), which peaked at 2 min after stimulation. Thus, BCR activation induces phosphorylation and conformational changes in WASP and the recruitment of WASP to the cell surface and BCR^+-vesicles. The induction of WASP activation by BCR cross-linking suggests that BCR-derived signals regulate the actin cytoskeleton through WASP. The lack of BCR-triggered actin reorganization in the Btk-deficient models implicates Btk in regulating WASP functions. We thus measured the effect of the xid mutation and Btk inhibitor on the cellular distribution and levels of oWASP and pWASP. In comparison with wt splenic B cells, the staining level of oWASP in xid splenic B cells was much lower (Fig. *2.3C*), suggesting a

FIGURE 2.4. BCR activation increases the phosphorylation and colocalization of phosphorylated WASP with the BCR in a Btk-dependent manner.

A, Splenic B cells from wt and xid mice were stained with Cy3-Fab-anti-μ for the BCR and stimulated with $F(ab')_2$-anti-Ig at 37°C for indicated times. The cells were fixed, permeabilized, and stained with an antibody specific for WASP phosphorylated at S483/S484 (pWASP). The cells were analyzed using a confocal fluorescence microscope. Shown are representative images of three independent experiments**.** Bar, 3 μm. *B*, Shown are the means (±S.D.) of pWASP fluorescence intensity of >300 cells from three independent experiments (*, $p \leq 0.005$). *C-D*, Cells showing membrane redistribution of pWASP were visually determined and quantified. The data were plotted as percentages of total cells in images (*C*). The correlation coefficients between the BCR and pWASP in wt and xid B cells were determined using the LSM 510 software (*D*). Shown are the average results of three independent experiments where >300 cells were analyzed (*, $p \leq 0.005$). *E-H*, A20 B cells that were treated with or without LFM A-13 (*E* and *G*) and splenic B cells from wt and xid mice (*F* and *H*) were stimulated with $F(ab')_2$-anti-Ig for indicated times. The cells were lysed, and the cell lysates were analyzed using SDS-PAGE and Western blot, probed for pWASP S483/S484. The blots were stripped and reprobed for tubulin as loading controls. The blots were analyzed by densitometry. pWASP levels were normalized against tubulin levels, and the data were plotted as fold increases over unstimulated levels (*G-H*). Shown are representative blots and plots of three independent experiments. *, $p \leq 0.05$.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wt
xid
pWASP
BCR
Merge
pWASP
BCR
Merge
-XL
2
5
10
a
b
c
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e
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s
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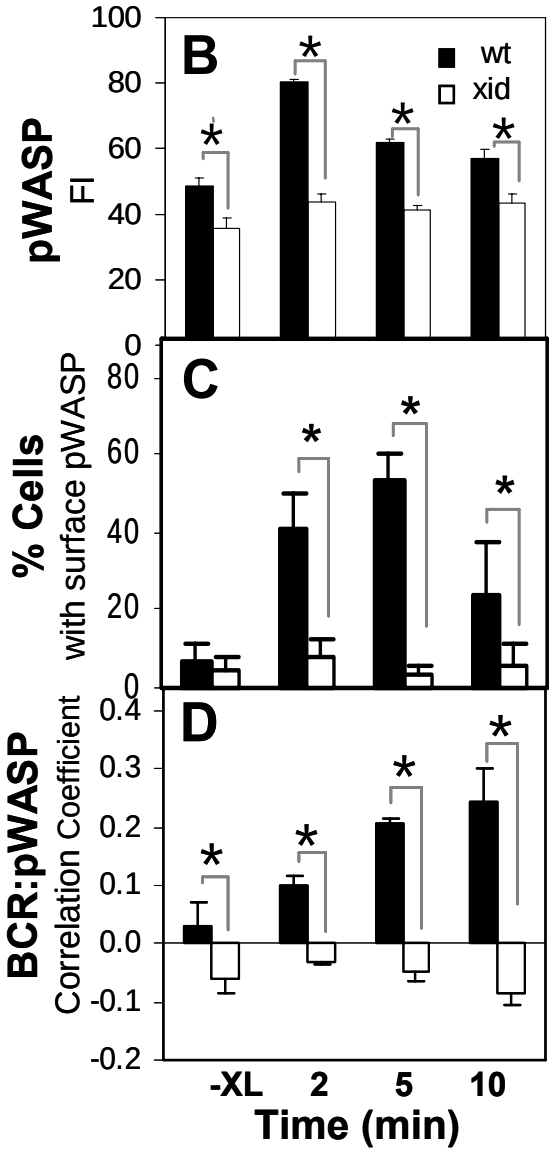
u
j
k
v
w
x


B
wt
xid
pWASP
FI
100
80
60
40
20
0
C
% Cells
with surface pWASP
80
60
40
20
0
D
BCR:pWASP
Correlation Coefficient
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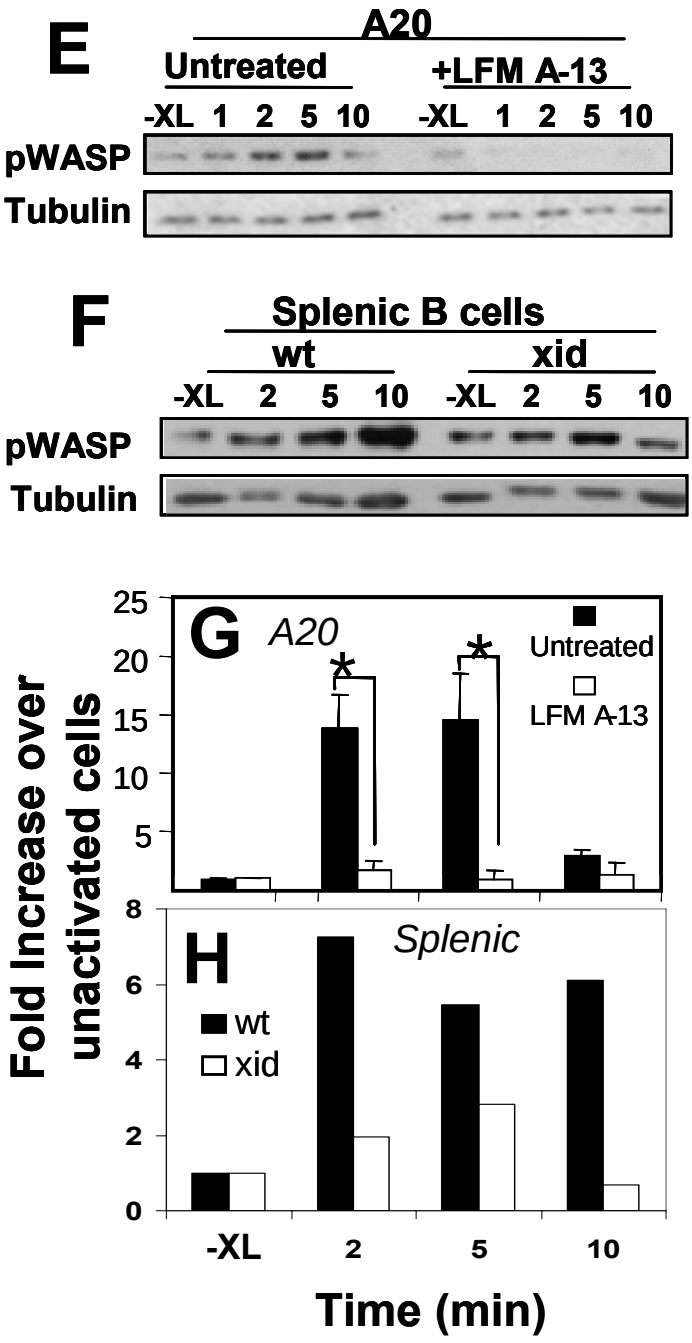
0.3
0.2
0.1
0.0
-0.1
-0.2
-XL
2
5
10
Time (min)


E
A20
Untreated
+LFM A-13
-XL 1 2 5 10 -XL 1 2 5 10
pWASP
Tubulin
F
Splenic B cells
wt
xid
-XL 2 5 10 -XL 2 5 10
pWASP
Tubulin
G
A20
Untreated
LFM A-13
H
Splenic
wt
xid
Fold Increase over unactivated cells
Time (min)
-XL
2
5
10

defect in WASP activation in xid B cells. While BCR activation did cause an initial increase in the colocalization between the BCR and oWASP in xid B cells, the correlation was significantly lower than in wt B cells (Fig. *2.3Ca*). In contrast to wt splenic B cells, where the colocalization of the BCR and oWASP was sustained, this colocalization declined swiftly in xid B cells over time (Fig. *2.3Ca*). While the xid mutation had less of an inhibitory effect on the correlation of the BCR with F-actin (Fig. *2.3Cb*), it significantly decreased the correlation of oWASP with F-actin (Fig. *2.3Cc*). BCR activation failed to significantly increase the staining level of pWASP (Fig. *2.4, Am-x* and *B*) or induce comparable redistribution of the pWASP to the cell surface in xid B cells (Fig. *2.4, Am-x* and *C*), similar to the results with oWASP. In xid B cells, the BCR and pWASP showed a negative correlation (Fig. *2.4D*), indicating that the BCR and pWASP do not colocalize, rather they exclude from each other. Western blot analysis further confirmed that LFM A-13 (Fig. *2.4, E and G*) and the xid mutation (Fig. *2.4, F and H*) both block BCR-induced phosphorylation of WASP. Thus inhibition of Btk blocks BCR-induced activation and recruitment of WASP to the BCR, indicating that a role for Btk in regulating WASP activity.

To test whether the inhibition of WASP activation in xid mice is an indirect effect of B cell developmental detects caused by the Btk mutation, we compared the levels of pWASP between different subpopulations of splenic B cells by flow cytometry (Fig. *2.5*). We found that BCR cross-linking increased pWASP levels in all the splenic B cell subpopulations from wt mice, including $IgD^{hi}IgM^{lo}$ follicular B cells, $IgD^{hi}IgM^{hi}$ T2 B cells, and $IgD^{lo}IgM^{hi}$ T1 B cells, but failed to increase the pWASP levels of all the splenic B cell subpopulations from xid mice (Fig. *2.5*). This indicates that it is Btk's xid

mutation, but not delayed B cell development, which inhibits BCR-induced WASP activation.

2.4.4 BCR-Induced Biogenesis of PtdIns-4,5-P_2 Depends on Btk

To examine the mechanism for Btk-mediated activation of WASP, we determined the effect of Btk deficiency on the cellular level and distribution of PtdIns-4,5-P_2, a co-activator of WASP, using immunofluorescence microscopy and flow cytometry. Significant increases in PtdIns-4,5-P_2 staining levels were observed in both activated splenic (Fig. *2.6*, *Aa-i*) and A20 B cells (Fig. *2.6B*), comparing to unstimulated cells (-XL). Additionally, PtdIns-4,5-P_2 appeared to be recruited to the cell periphery and BCR^+-vesicles after activation for 10 min (Fig. *2.6*, *Ag-i*). This BCR-induced increase in the levels of PtdIns-4,5-P_2 and its redistribution were severely dampened in xid splenic B cells (Fig. *2.6, Aj-r*). LFM A-13 treatment not only blocked BCR-triggered increases in PtdIns-4,5-P_2, but also dramatically reduced constitutive levels of PtdIns-4,5-P_2 in unstimulated A20 B cells (Fig. *2.6B*). Thus, cellular biogenesis of PtdIns-4,5-P_2 in response to BCR stimulation is dependent on the unimpaired activity of Btk.

2.4.5 BCR-Triggered Vav Activation Requires Btk

In addition to PtdIns-4,5-P_2 binding to the B domain, the coordinated binding of GTP-Cdc42 to the GBD domain of WASP is required for WASP activation [212]. Vav serves as a GEF for Cdc42 [257]. To test whether Btk-dependent WASP activation is mediated through Vav,

FIGURE 2.5. The xid mutation inhibits BCR-induced WASP phosphorylation in all subsets of splenic B cells.

Splenic B cells from wt and xid mice were incubated with PE-Cy5-anti-mouse B220, AF488-anti-mouse IgM, and PE-anti-mouse IgD at 4^{o}C with or without warmed up to 37^{o}C for 5 min. The cells were then fixed, permeabilized, and incubated with anti-mouse pWASP (S483/S484). The cells were analyzed using flow cytometry. Three subsets of splenic B cells were gated, including follicular (FO) B cells ($B220^{+}$ $IgM^{lo}IgD^{hi}$), T2 ($B220^{+}$ $IgM^{hi}IgD^{hi}$), and T1 ($B220^{+}$ $IgM^{hi}IgD^{lo}$) B cells (*A*). Shown are representative histograms of pWASP levels in each B cell subset from wt and xid spleens, with (+XL) and without (-XL) BCR cross-linking, from three independent experiments (*B*).

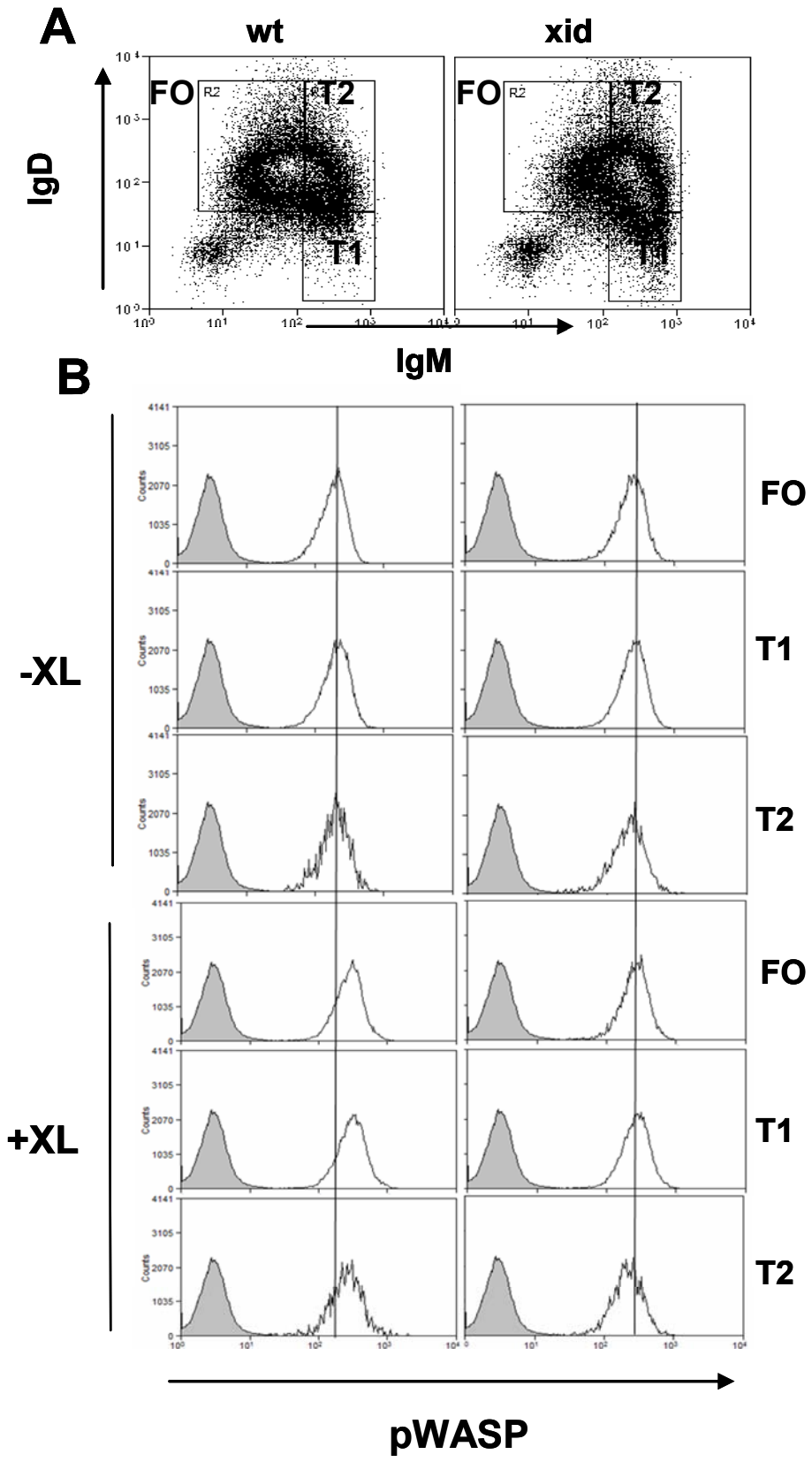
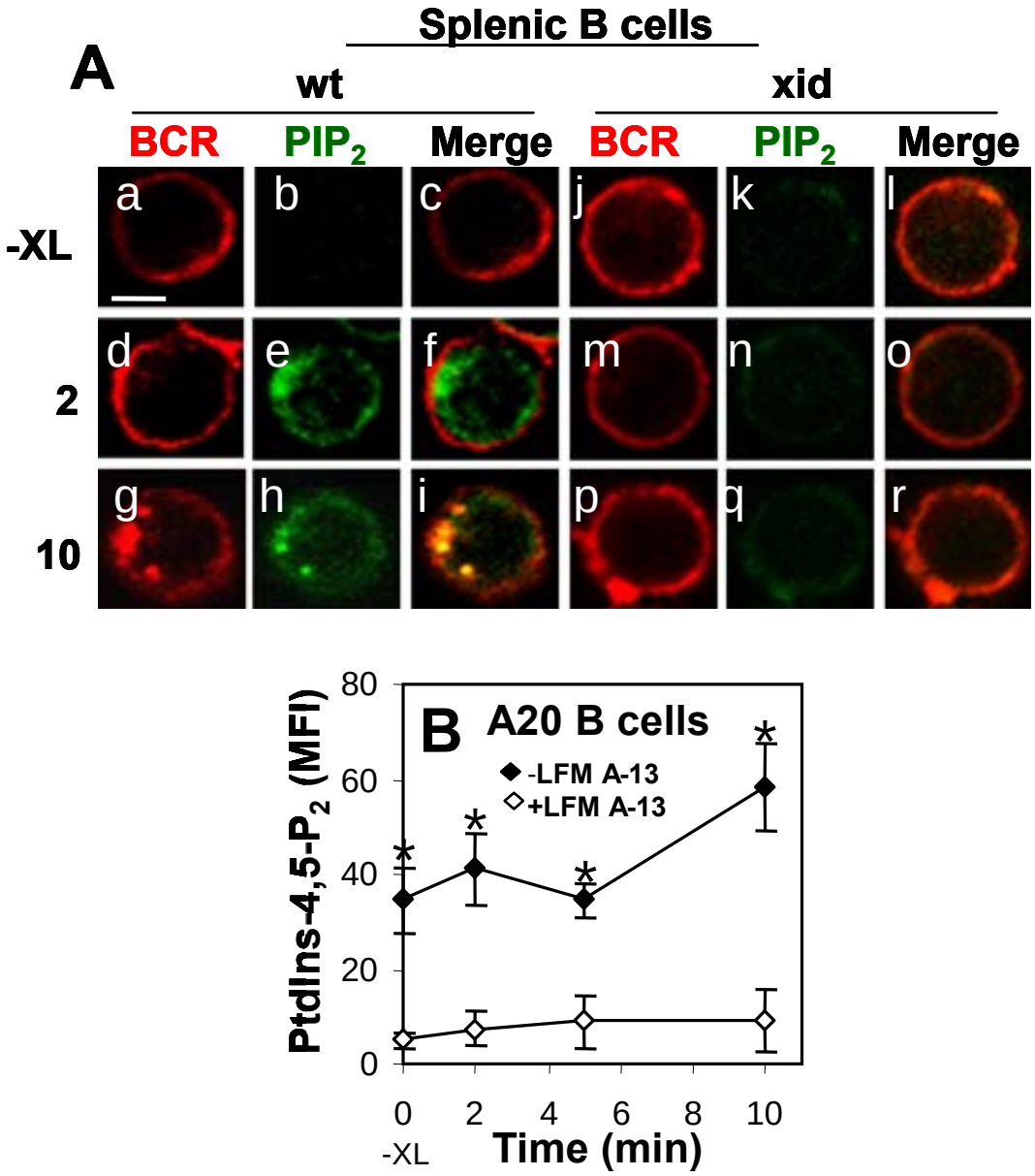

A
wt
xid
FO
T2
T1
R2
IgD
IgM
B
-XL
+XL
FO
T1
T2
Counts
pWASP

FIGURE 2.6. BCR activation induces Btk-dependent production of PdtIns-4,5-P_2. Splenic B cells from wt (*Aa-i*) and xid (*Aj-r*) mice were incubated with Cy3-Fab-anti-Ig to label the BCR and activated with F(ab')$_2$-anti-Ig for indicated times at 37^{o}C. The cells were fixed, permeabilized, and stained with an anti-PtdIns-4,5-P_2 mAb followed by a Cy2-conjugated secondary antibody. The cells were analyzed by a confocal fluorescence microscope. Shown are representative images from three independent experiments (*A*). Bar, 3 μm. PtdIns-4,5-P_2 levels in untreated and LFM A-13 treated A20 cells were analyzed by flow cytometry. Shown are the mean fluorescence intensities (±S.D.) of PtdIns-4,5-P_2 that were plotted against time (*B*) (*, $p \leq 0.01$).


A
Splenic B cells
wt
xid
BCR
PIP2
Merge
BCR
PIP2
Merge
-XL
2
10
a
b
c
j
k
l
d
e
f
m
n
o
g
h
i
p
q
r
B
A20 B cells
-LFM A-13
+LFM A-13
PtdIns-4,5-P2 (MFI)
80
60
40
20
0
0
2
4
6
8
10
-XL
Time (min)

we determined the effect of the xid mutation and LFM A-13 on Vav activation. Vav activation was followed by its recruitment to the cell surface and its phosphorylation in response to BCR stimulation [258] using an antibody specific for Vav phosphorylated at Y174 (pVav) by immunofluorescence microscopy, flow cytometry, and Western blot. In wt splenic B cells, BCR cross-linking noticeably increased the staining levels of pVav, compared to unstimulated B cells (-XL) (Fig. *2.7*, *Aa-i*). This BCR-triggered increase in pVav staining was drastically reduced in the xid B cells (Fig. *2.7*, *Aj-r*). pVav accumulated primarily at the cell surface where it heavily colocalized with the BCR at early times after activation (~2 min) (Fig. *2.7*, *Ad-f*). At later times (>10 min), pVav colocalized not only with the surface BCR, but also the internalized BCR at the perinuclear location (Fig. *2.7*, *Ag-i*). Correlation analysis showed an increase in the colocalization of pVav and the BCR upon BCR cross-linking (data not shown). Both flow cytometry (Fig. *2.7B*) and Western blot (Fig. *2.7*, *C-F*) analyses confirmed that BCR cross-linking increased pVav levels in wt splenic and A20 B cells, which was reduced by the xid mutation and LFM A-13 treatment. Thus, BCR-triggered Vav phosphorylation and colocalization of pVav with the BCR are dependent on the activity of Btk.

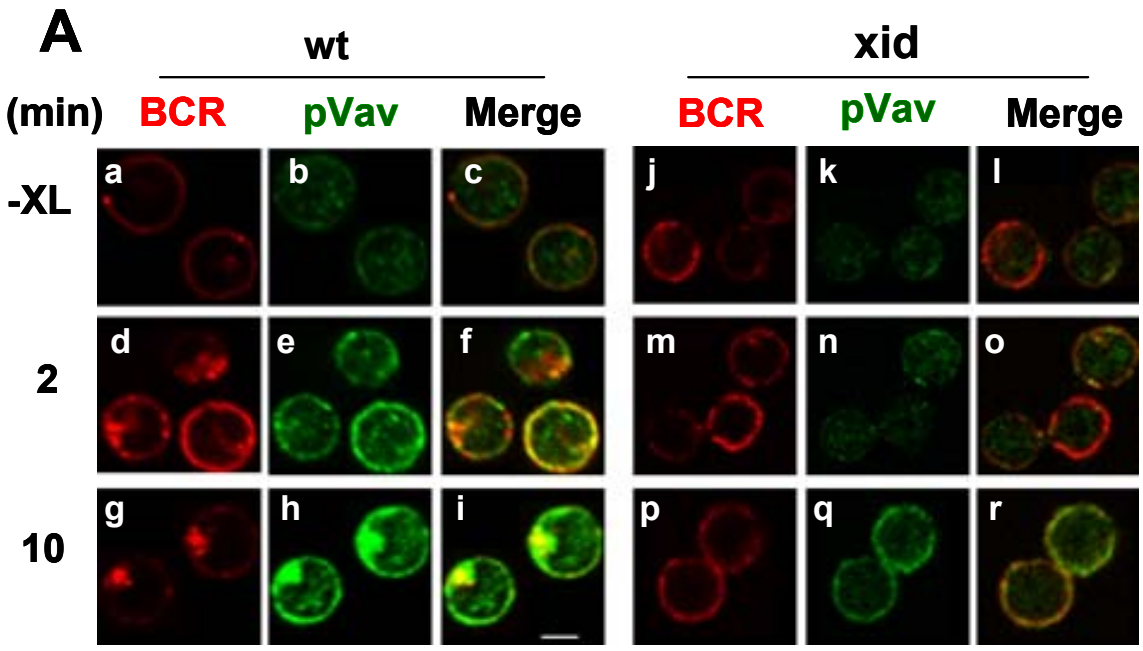
2.4.6 Btk Inhibitor and Deficiency Inhibit BCR-Mediated Antigen Internalization and Transport to Antigen-Processing Compartments

BCR-mediated antigen uptake and transport is dependent upon the integrity of signaling pathways and the actin cytoskeleton. Btk's roles in modulating both signaling and the actin cytoskeleton implicate its role in BCR-mediated antigen processing. To test this hypothesis, we determined the effects of Btk deficiency on the internalization and movement of the BCR

from the cell surface to antigen processing compartments (Fig. *2.8*). The surface-labeled BCR was chased for 30 min at 37°C. The cell surface was identified with cholera toxin subunit B (CTX-B) which binds G_{M1}, early endosomes with holotransferrin (Tf), and late endosomes/lysosomes by LAMP-1. After 30 min of antibody cross-linking, the BCR moved from the PM to a perinuclear location (Fig. *2.8Aa* and *Ac*), where most of BCRs colocalized with LAMP-1 (Fig. *2.8Bg-i*) while a small portion was found with Tf (Fig. *2.8Ba-c*). In contrast, after the same treatment, the surface labeled BCR in splenic xid B cells remained colocalization with CTXB at the PM (Fig. *2.8Ad*), and showed little to no internalization and colocalization with Tf (Fig. *2.8Bd-f*) or LAMP-1 (Fig. *2.8Bj-l*). The correlation between the BCR and LAMP-1 staining increased with time in wt splenic B cells, but this increase was abrogated in xid splenic B cells (Fig. 2.8*C*). We next determined the effect of Btk deficiency on the kinetics of BCR internalization, which was followed by decreases in the levels of surface-labeled BCR after the chase using flow cytometry. We found that the rate of BCR internalization was dramatically reduced in the xid B cells, in comparison with the wt B cells (Fig. *2.8D*). Similarly, LFM A-13 significantly reduced BCR internalization rates in both splenic and A20 B cells (Fig. *2.8D*). Furthermore, by gating on $IgM^{lo}IgD^{hi}$ FO, $IgM^{hi}IgD^{hi}$ T2, and $IgM^{hi}IgD^{lo}$ T1 B cells, we found that both mature and immature B cells from the spleen of xid mice showed reduced rates of BCR internalization compared to respective wt B cell subsets (Fig. *2.9*), indicating that the decrease in the rate of BCR internalization is not the result of B cell developmental defects in xid mice. These demonstrate a requirement for Btk-mediated signals in efficient BCR internalization and transport of antigen to antigen processing compartments.

FIGURE 2.7. BCR activation induces Btk-dependent phosphorylation of Vav and recruitment of phosphorylated Vav to the BCR.

A, The surface BCR of splenic B cells from wt (*a*-*i*) and xid mice (*j*-*r*) were labeled with Cy3-Fab-anti-μ and activated with F(ab')$_2$-anti-Ig for varying lengths of time. The cells were fixed, permeabilized, and stained with an antibody specific for phosphorylated Vav at Y174 (pVav). Images were acquired using a confocal fluorescence microscope. Shown are representative images from three independent experiments. Bar, 3 μm. *B***,** Wt splenic B cells that were treated and untreated with LFM A-13 and xid splenic B cells were activated with F(ab')$_2$-anti-Ig for varying lengths of time. The cells were fixed, permeabilized, and stained with an antibody specific for pVav Y174. The mean fluorescence intensity (MFI) of pVav was quantified using flow cytometry. Shown is a representative plot of pVav MFI versus the time from three independent experiments. *C*-*F*, A20 B cells that were treated with LFM A-13 or left untreated (*C*-*D*) as well as splenic wt and xid B cells (*E*-*F*) were stimulated with F(ab')$_2$-anti-Ig for indicated times and lysed. The lysates were analyzed by SDS-PAGE and Western blot, and probed for pVav Y174. The blots were stripped and reprobed for tubulin as loading controls. The blots were analyzed using densitometry. The pVav levels were normalized against tubulin levels, presented as fold increases over unstimulated B cells, and plotted as a function of time. Shown are representative blots and average pVav levels from three independent experiments.


A
wt
xid
(min)
BCR
pVav
Merge
BCR
pVav
Merge
-XL
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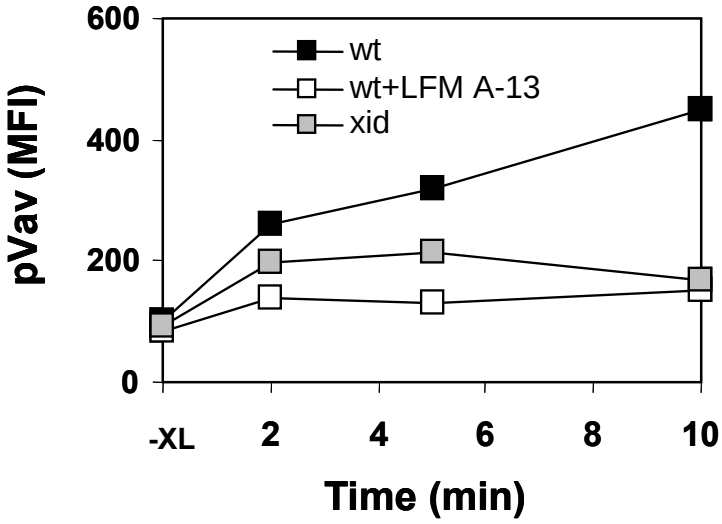
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400
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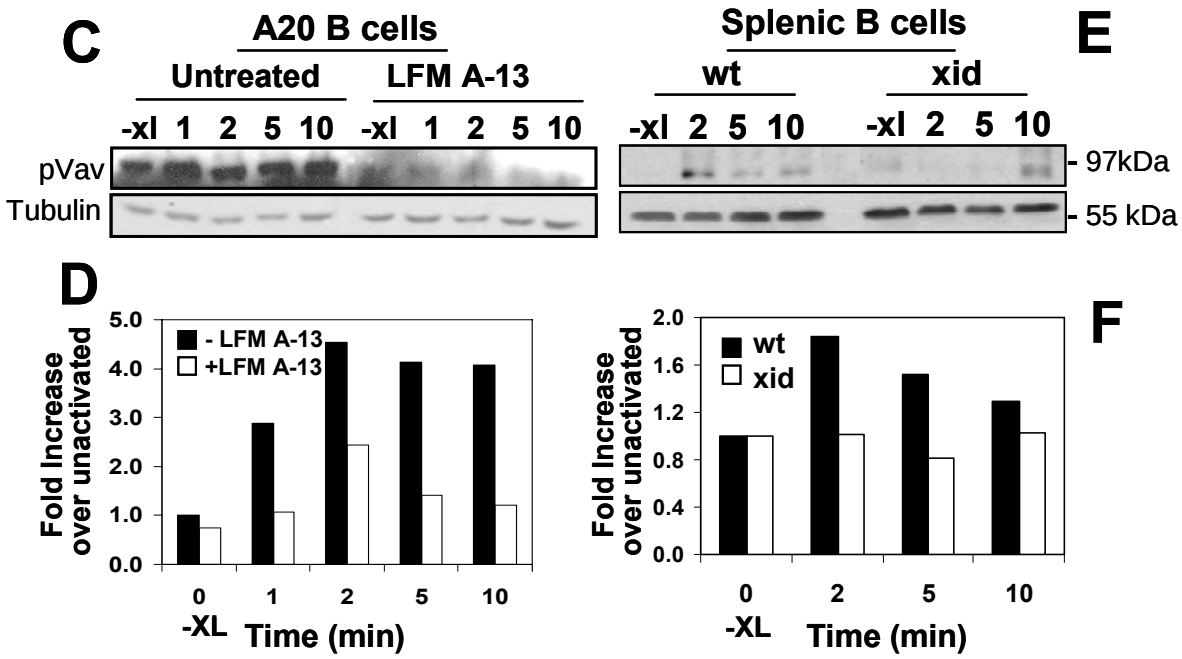
wt
wt+LFM A-13
xid
-XL
2
4
6
8
10
Time (min)

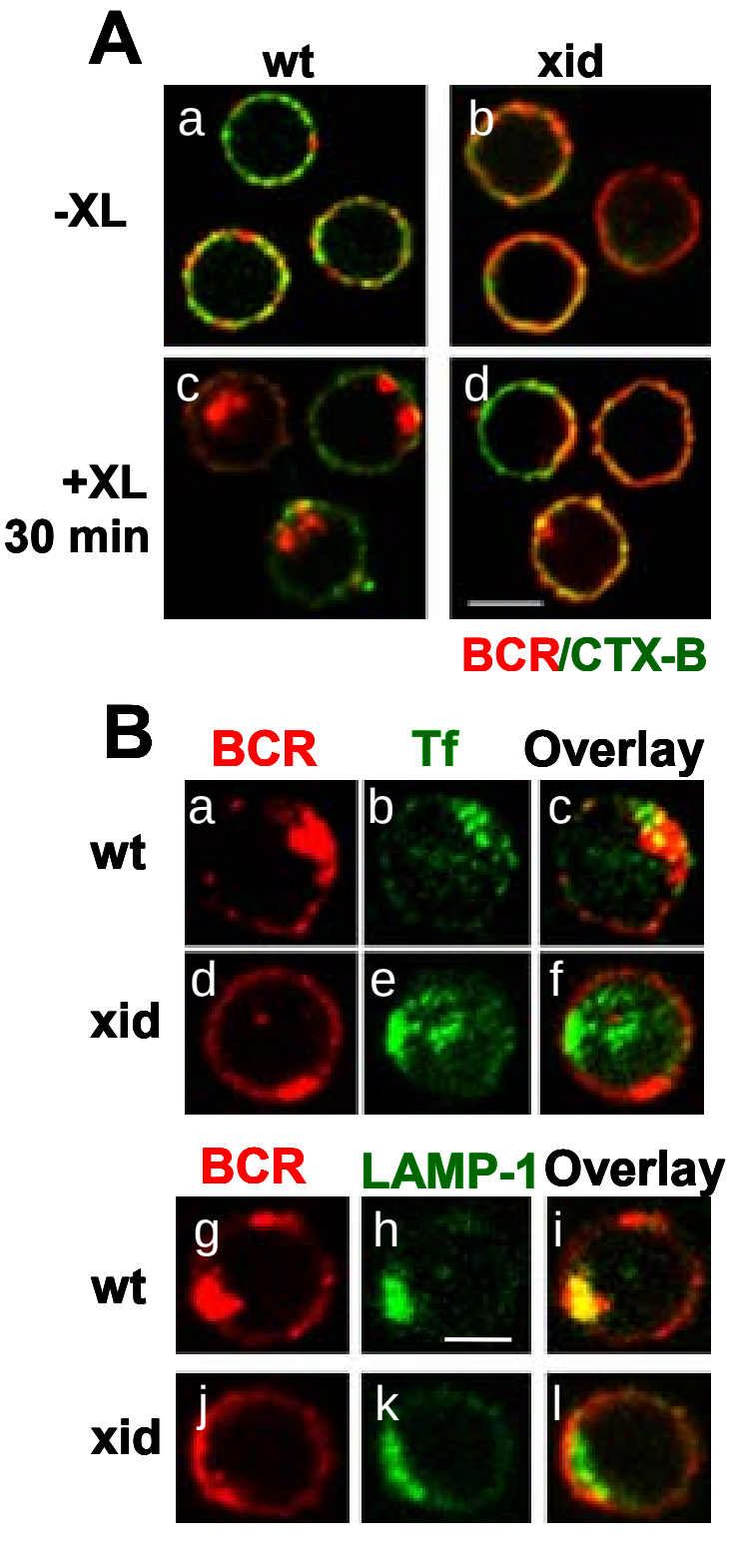

C
A20 B cells
Untreated
LFM A-13
-xl 1 2 5 10 -xl 1 2 5 10
pVav
Tubulin
Splenic B cells
E
wt
xid
-xl 2 5 10 -xl 2 5 10
- 97kDa
- 55 kDa
D
Fold Increase over unactivated
- LFM A-13
+LFM A-13
5.0
4.0
3.0
2.0
1.0
0.0
0 1 2 5 10
-XL
Time (min)
F
Fold Increase over unactivated
wt
xid
2.0
1.6
1.2
0.8
0.4
0.0
0 2 5 10
-XL
Time (min)

2.4.7 Btk-Deficient B Cells are Defective in BCR-Mediated Antigen Presentation

The inhibitory effect on BCR internalization and transport to late endosomes suggests that Btk deficiency interferes with BCR-mediated antigen processing. To test this, we compared the efficiencies of BCR-mediated antigen presentation by wt and xid B cells. A model antigen, hen egg lysozyme (HEL), was targeted to the BCR for BCR-mediated antigen processing and presentation using an antibody complex. The surface levels of HEL_{46-61}-loaded MHC class II I-A^k (HEL_{46-61}:I-A^k) was determined by mAb C4H3 [259] and flow cytometry. To test the sensitivity and efficiency of antigen processing and presentation, splenic B cells were pulsed with HEL (0.5 or 1 μg/ml) either alone (for pinocytosis-mediated antigen processing) or in presence of the antibody complex (for BCR-mediated antigen processing) for 15 min and incubated at 37^{o}C for 14 h. Wt B cells pulsed with HEL plus the antibody complex displayed much higher levels of surface HEL_{46-61}:I-A^k than those pulsed with HEL alone (Fig. *2.10A*). This indicates that BCR-mediated antigen processing and presentation has a higher efficiency than non-specific pinocytosis. Wt and xid B cells pulsed with HEL alone showed similar surface levels of HEL_{46-61}:I-A^k (Fig. *2.10A*, dotted line), suggesting that Btk deficiency does not significantly affect pinocytic rates of antigen processing and presentation. While both wt and xid B cells show similar levels of MHC class II I-A^k on their surfaces (data not shown), the surface levels of HEL_{46-61}:I-A^k on xid B cells pulsed with HEL-antibody complex were much lower than those on wt B cells, even though they were slightly higher than those of xid B cells pulsed with HEL alone (Fig. *2.10*, *A-B*). While both wt and xid B cells increased surface HEL_{46-61}: I- A^k levels as the antigen concentration increased, the increase shown by xid B cells was much smaller than that by wt

FIGURE 2.8. Btk inhibitor and xid mutation inhibit BCR internalization and intracellular movement to late endosomes.

A-C, Splenic B cells from wt and xid mice were incubated with Cy3-Fab-anti-μ at 4^{o}C to label the surface BCR and treated with or without F(ab')$_2$-anti-Ig for 30 min at 37^{o}C. Then cells were incubated with AF488-cholera toxin B subunit (CTX-B) at 4^{o}C to demarcate the cell surface (*A*). The cells were fixed, permeabilized, and stained for LAMP-1 using a mAb (ID4B) for marking late endosomes (*Bg-l*). To mark early endosomes, splenic B cells from wt and xid mice were labeled with Cy3-Fab-anti-μ in the presence of F(ab')$_2$-anti-Ig at 18^{o}C for 30 min and chased at 37^{o}C for 30 min in the presence of AF488-holo-Transferrin (Tf) (*Ba-f*). The cells were analyzed using a confocal fluorescence microscope. Representative images from three independent experiments are shown. Bar, 3 μm. *C,* The correlation coefficients between BCR and LAMP-1 staining were determined from images of ≥300 cells from three independent experiments using the Zeiss LSM 510 software (*, $p \leq 0.05$). *D*, Splenic B cells from wt and xid mice and LFM-A13-treated wt and A20 B cells were incubated with biotinylated F(ab')$_2$-anti-Ig at 4^{o}C to label the surface BCR. After washing, cells were incubated at 37^{o}C for indicated times. Biotin-F(ab')$_2$-anti-Ig remaining on the cell surface after the chase was detected with PE-streptavidin and quantified using flow cytometry. Shown are the average percentages (±S.D.) of biotin-F(ab')$_2$-anti-Ig remaining on the cell surface from three independent experiments (*, $p \leq 0.05$).


A
wt
xid
a
b
-XL
c
d
+XL
30 min
BCR/CTX-B
B
BCR
Tf
Overlay
wt
a
b
c
xid
d
e
f
BCR
LAMP-1
Overlay
wt
g
h
i
xid
j
k
l

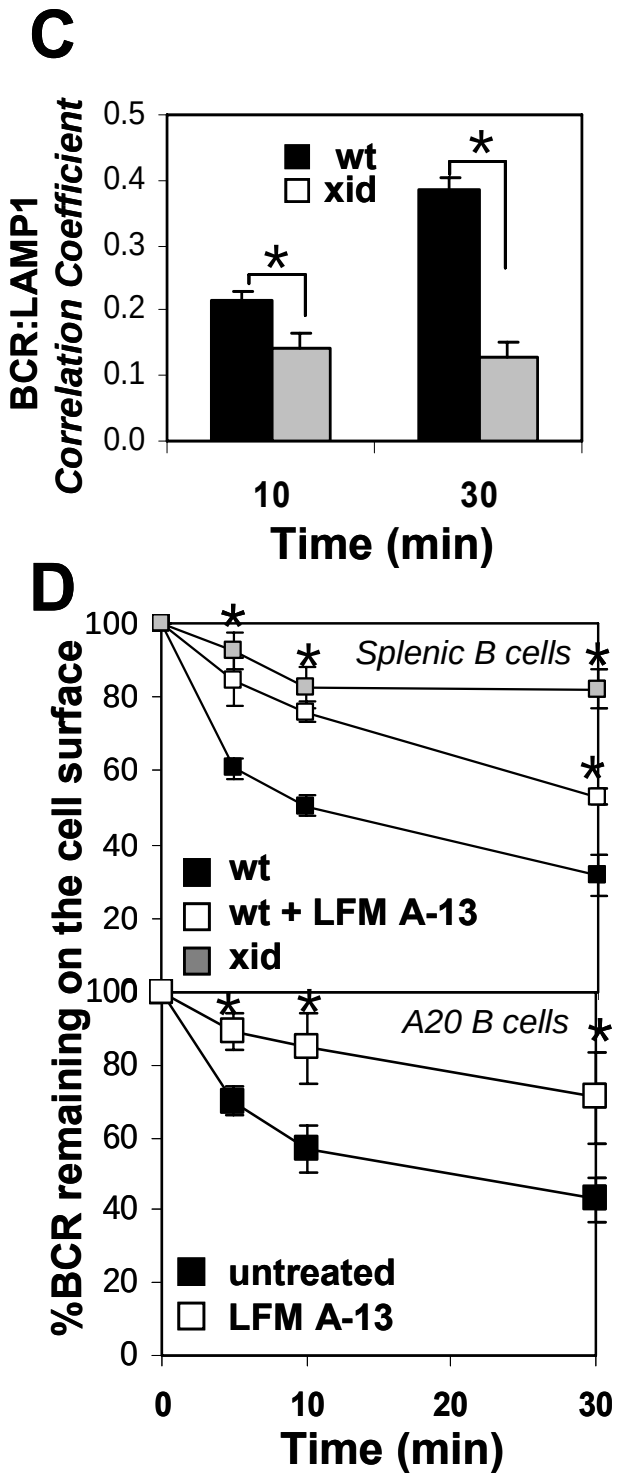
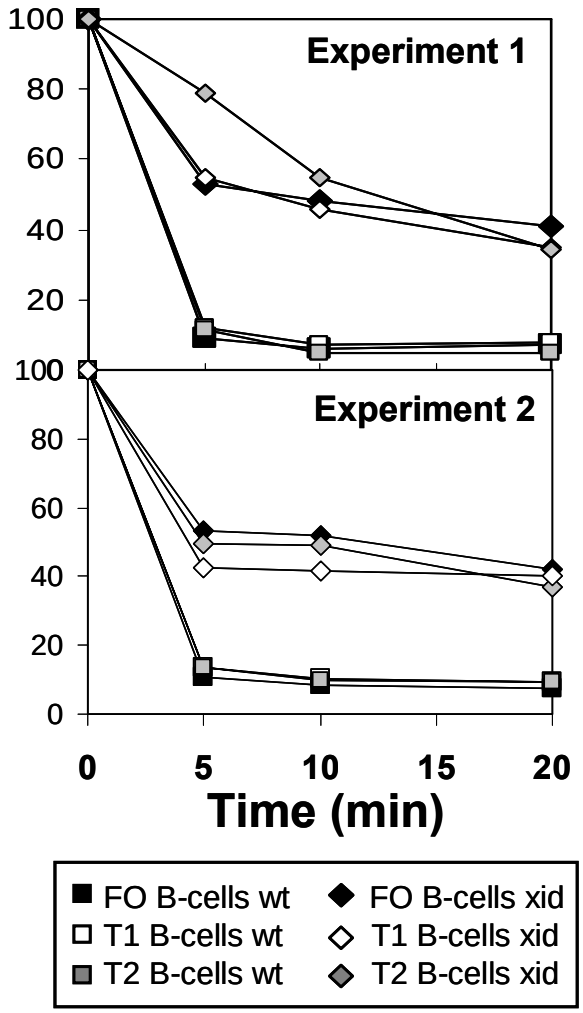

C
BCR:LAMP1
Correlation Coefficient
0.5
0.4
0.3
0.2
0.1
0.0
wt
xid
*
*
10
30
Time (min)
D
%BCR remaining on the cell surface
100
80
60
40
20
0
Splenic B cells
wt
wt + LFM A-13
xid
A20 B cells
untreated
LFM A-13
0
10
20
30
Time (min)

FIGURE 2.9. The Btk xid mutation decreases the rate of BCR internalization in all the subsets of splenic B cells.

Splenic B cells from wt and xid mice were labeled with PE-Cy5 anti-mouse B220, biotin-F(ab')$_2$-anti-mouse IgM, and AF488-anti-mouse IgD antibodies, and BCR internalization was analyzed as described in Fig. *2.7*. BCR internalization in different B cell subsets were measured by individually gating FO ($B220^+IgM^{lo}IgD^{hi}$), T2 ($B220^+$ $IgM^{hi}IgD^{hi}$), and T1 ($B220^+$ $IgM^{hi}IgD^{lo}$) B cell subsets. Shown are experimental data from two independent experiments.



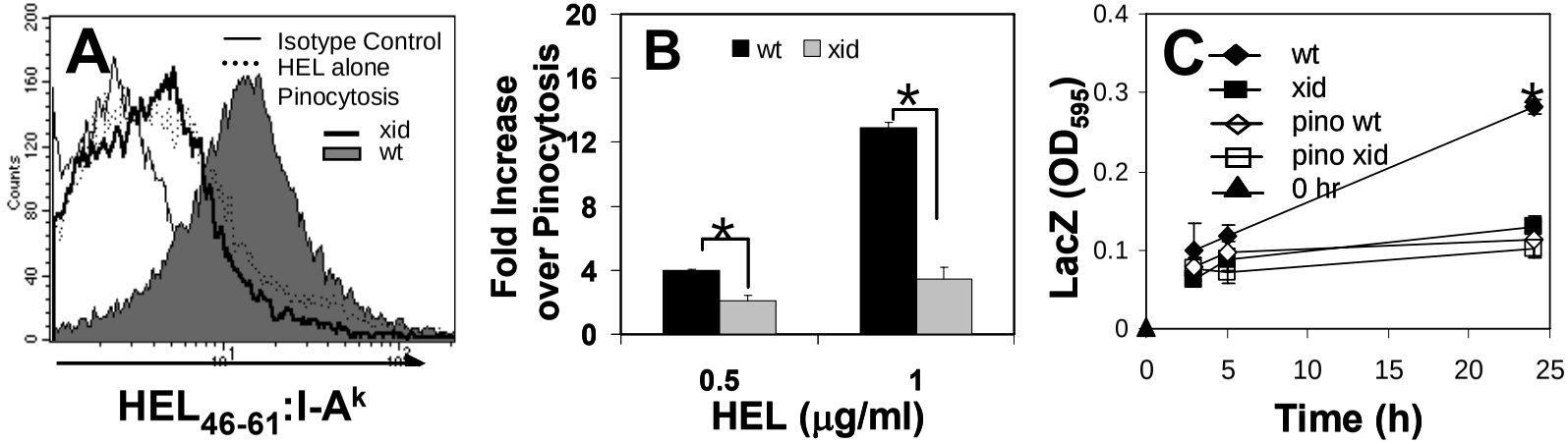
B cells (Fig. *2.10B*). In order to compare the abilities of wt and xid B cells to present antigen and activate T cells, splenic B cells pulsed with HEL or HEL-antibody complex for varying lengths of times were incubated with KZH T cells. The KZH T cells, specific for the same peptide–MHC class II complex recognized by the C4H3 mAb, express a lacZ reporter gene under the control of the IL-2 promoter [254]. T cell activation was monitored by the expression of the reporter lacZ. Similar to the surface HEL_{46-61}: I- A^k levels, wt and xid B cells pulsed with HEL alone activated the KZH T cells to similar extents (Fig. *2.10C*). However, when HEL was targeted to the BCR by the antibody complex, wt B cells stimulated the KZH T cells to a much higher level than xid B cells, especially at a later time point (Fig. *2.10C*). These data demonstrate a role for Btk in regulating BCR-mediated antigen processing and presentation.

2.5 Discussion

The binding of antigens to the BCR induces a series of cellular evens that are essential for B cell activation, including signaling cascades, actin reorganization, antigen internalization for processing and presentation. While there is a regulatory relationship between these cellular events, no distinct link between these cellular pathways has been defined. Here, we identify Btk as a linker that transduces signals from the BCR into actin reorganization by controlling the activity of WASP, Vav and PtdIns-4,5-P_2 biogenesis. Significantly, Btk-dependent actin cytoskeleton remodeling is required for the high efficiency of BCR-mediated antigen uptake, processing and presentation.

FIGURE 2.10. The antigen presentation efficiency is reduced in B cells from xid mice.

A, Splenic B cells from xid and wt mice were pulsed with hen egg lysozyme (HEL) (1 μg/ml) alone or with the antibody complex that targets HEL to the BCR at 37°C for 15 min. After washing, cells were incubated at 37°C for 14 h. MHC class II I-A^k loaded with HEL peptides (HEL_{46-61}: I-A^k) on the cell surface was detected using a mAb (C4H3) and quantified using flow cytometry. Shown are representative histograms of three independent experiments. *B*, Shown are the ratios of MFI of HEL_{46-61}:I-A^k on the surface of B cells that were pulsed with the HEL-antibody complex, which targets HEL to the BCR, versus those B cells that were pulsed with HEL alone, where HEL was internalized through pinocytosis. Shown are average values (±S.D.) of three independent experiments (*, $p \leq 0.01$). *C*, Splenic B cells (1×10^6) from wt and xid mice were either incubated with HEL (1μg/ml) alone or with the antibody complex for varying lengths of time. After washing, the B cells ($1X10^6$) were co-cultured overnight with KZH T-cells ($1X10^6$). The activity of LacZ that is under the control of IL-2 promoter in the T cells was measured using a colorimetric LacZ substrate. Shown is the OD of the LacZ enzymatic product over time and representative data of three independent experiments with triplicates (*, $p \leq 0.005$).


A
Isotype Control
HEL alone
Pinocytosis
xid
wt
Counts
HEL46-61:I-Ak
B
wt
xid
Fold Increase over Pinocytosis
HEL (μg/ml)
C
wt
xid
pino wt
pino xid
0 hr
LacZ (OD595)
Time (h)

Using GFP-actin expressing B-cell lines and live cell imaging, I show the dynamic reorganization of the actin cytoskeleton in B cells responding to antigenic stimuli. This then leads to the hypothesis that signaling cues emanating from the BCR determine the plasticity of the actin cytoskeleton. Previous studies presented in Chapter 1 demonstrate that early signals from the BCR serve to modulate the actin cytoskeleton. This may then primes the actin cytoskeleton for the later events of BCR endocytosis, and trafficking. Preliminary studies presented here show the involvement of signaling proteins like Src kinases and MAP kinase ERK participate in cytoskeletal reorganization, suggesting a step-wise regulatory relationship between BCR signaling and the actin cytoskeleton. Similar to a previous report, depolymerization of F-actin by Latrunculin B in my system also lead to sustained ERK phosphorylation [192]. In that particular study, Hao and August further showed that actin disruption interfered with the downstream activation of transcription factors [192]. These findings suggests that in addition to the forward-facing link from BCR signaling to the actin cytoskeleton, there are regulatory feedback loops from the actin cytoskeleton to BCR signaling pathways. Together these provide an clue to the integration of signaling, actin reorganization and receptor endocytosis for optimal B-cell activation.

BCR activation is known to trigger changes in the actin cytoskeleton [190, 192]. We further characterize it as a biphasic process with a rapid but transient increase in cellular F-actin in the first few minutes post BCR cross-linking, followed by a decline during the next few minutes. Moreover, cross-linking of the BCR triggers site-directed actin polymerization near the BCR. Localized actin polymerization shown here provides an explanation for the dependency of BCR internalization on the actin cytoskeleton we reported previously [171].

BCR activation induces actin assembly at BCR internalization sites and BCR^+-vesicles, where this polymerization may provide the driving force for fission of clathrin-coated vesicles and inward movement of BCR^+-vesicles. BCR-triggered depolymerization of F-actin, on the other hand, may loosen the "fence" formed by the cortical actin cytoskeleton, allowing for inward movement of BCR-containing vesicles. Thus, these biphasic actin dynamics may be essential for restructuring the actin cytoskeleton in response to BCR activation.

The abrogation of BCR-triggered actin cytoskeleton rearrangement in the presence of tyrosine kinase inhibitors and Syk deficiency [177, 178, 244] indicates a regulatory role for BCR-mediated signaling in actin dynamics. Using two model systems, xid mice and Btk inhibitor LFM A-13, we demonstrate that BCR-dependent actin polymerization and even the constitutive level of actin polymerization is dependent on the functionality of Btk. This study reveals for the first time that Btk is the major signaling component that links BCR signals to the actin cytoskeleton in B cells.

While the Btk xid mutation and LFM A-13 exhibit similar inhibitory effects on Btk activity and actin dynamics in B cells, each of them could influence actin dynamics through a mechanism different from Btk, as the xid mutation causes B cell developmental delays [144] and LFM A-13 can inhibit Jak2 [260]. Our finding that different subpopulations of splenic B cells increase the level of phosphorylated WASP to similar degrees and exhibit similar BCR internalization rates demonstrates that the observed inhibitory effect of the xid mutation is not caused by B cell developmental defects. Because BCR activation does not induce Jak2

phosphorylation and BCR-induced STAT activation is independent of Jaks [261], the effect of LFM A-13 on actin polymerization is unlikely due to its inhibition of Jak2.

The results presented here demonstrate that BCR-triggered, Btk-dependent actin cytoskeleton rearrangement in B cells is mediated through WASP. Binding of antigen to the BCR increases the levels of open, active WASP, triggers its phosphorylation at S483/484, and recruits activated WASP to the PM and the BCR. The concordance of antigen-bound BCR, actin nucleation sites, and active WASP strongly suggests that WASP mediates actin polymerization and branching at BCR internalization sites. A fraction of the active WASP was found to maintain its colocalization with the BCR, even after it had been internalized, suggesting a role for WASP beyond internalization, possibly in driving the inward movement and/or membrane fusion of BCR^{+}-vesicles to late endosomes. The relationship of defects in BCR-triggered WASP activation and actin reorganization in xid and Btk inhibitor-treated B cells reinforces that Btk can funnel BCR signaling cues to the cytoskeleton through WASP.

The activation mechanism for WASP has been extensively studied and a general model for its activation has emerged. In the absence of stimuli, WASP is present in an autoinhibited state mediated by the interaction of its GBD with VCA region. This autoinhibition is released when its GBD, B, and PRD domains coordinately bind to GTP-Cdc42, PtdIns-4,5-P_2, and a SH3 domain-containing protein, respectively, freeing the VCA region for binding Arp2/3 [207]. The phosphorylation of WASP at Y291 and S483/484 [215] increases the actin polymerization activity of WASP, by stabilizing its open, active conformation. In B cells, BCR activation has been shown to induce transient tyrosine phosphorylation of WASP [143]

and, an interaction of WASP with Btk *in vitro* has been reported [143, 251, 252]. Our results show that BCR activation induces the cell surface recruitment and phosphorylation of Cdc42 GEF Vav and increases the cellular biogenesis of PtdIns-4,5 P_2 and the surface recruitment and serine phosphorylation of WASP, all of which occur in a Btk-dependent manner. These results suggest that Btk activates WASP by several related mechanisms, by regulating the activity of Vav, the generation of PtdIns-4,5-P_2, and the phosphorylation of WASP. The direct interaction of WASP with Btk reported previously [143, 251, 252] provides another mechanism for Btk to regulate the phosphorylation and sub-cellular location of WASP, whereby Btk recruits WASP to the plasma membrane where WASP interacts with PtdIns-4,5-P_2 and is phosphorylated by kinases.

Reports on the involvement of Tec kinases in regulating Vav activity in T cells [161, 162] and our study support a general function of Tec kinases in regulating WASP activity by controlling the activity of Vav. How Btk activates Vav in B cells remains to be elucidated. Possible mechanisms include direct phosphorylation of Vav by Btk or Btk-mediated recruitment of Vav to BCR signaling microdomains, where it is phosphorylated by Src or Syk. The adaptor function of Btk brings to the cell surface PIP5K, the primary PtdIns-4,5-P_2-generating lipid kinase [149]. Here, we show that Btk is able to regulate the local metabolism of PtdIns-4,5-P_2 that in turn is a substrate or co-activator for downstream effectors of Btk, such as PLCγ2 and WASP [153, 212]. Btk-dependent PtdIns-4,5-P_2 generation activates WASP in cooperation with GTP-Cdc42, the product of Btk-dependent Vav activation.

Btk's role in BCR signaling has been well characterized [105, 111, 114, 148]. The results of this study show for the first time that Btk is part of a regulatory mechanism for efficient antigen uptake and transport that leads to antigen processing and presentation. The high efficiency of B cells to process and present antigen relies on the abilities of the BCR to capture antigens with high specificity and affinity, to rapidly internalize them, and specifically target them to the antigen processing compartment. This allows B cells to present antigens even when exposed to low levels of antigens for short periods of time. The disruption of Btk function, either by the R28C mutation or the inhibitor, reduces the rates of BCR internalization and its movement to the antigen processing compartment. This consequently decreases the amount of antigen available to the processing and presentation machinery. It is important to note that the processing and presentation of antigens endocytosed by pinocytosis is not significantly affected by the Btk xid mutation. This is in line with a previous finding that B cells from WASP knockout mice could process and present soluble antigens that were not specifically targeted to the BCR at normal efficiencies in the presence of LPS and CD40 ligation [227], while the ability of $WASP^{-/-}$ B cells to process BCR specific antigens has not been tested. This implies that Btk's role in this process is instigated by the BCR binding to cognate antigens. The defects of BCR-mediated antigen processing and BCR-triggered actin cytoskeleton rearrangement in the xid B cells suggest that Btk connects BCR signaling activity to its antigen transport and processing functions by mobilizing the actin cytoskeleton.

A working model for the interactions among BCR signaling, the actin cytoskeleton, and BCR antigen processing pathway, thus, emerges. The binding of the BCR by multivalent antigens

triggers the formation of surface signaling microdomains. The production of PtdIns-3,4,5-P_3 by PI3-kinase recruits Btk to the signaling microdomains where Btk is activated by phosphorylation. Subsequently, Btk recruits WASP to the signaling microdomains and activates it by inducing its phosphorylation, activating Vav and consequently Cdc42, and increasing local PtdIn-4,5-P_2 levels. Activated WASP promotes actin assembly and branching in the vicinity of the BCR. This provides the driving force for the formation of BCR^+ clathrin-coated vesicles and the inward movement and fusion of these antigen-containing vesicles with the antigen processing compartment. This cross-talk mechanism between signaling, actin cytoskeleton, and membrane transport machineries might be important for all lymphocytes to transduce antigenic signals into cellular responses. The functional and physical interaction between Btk and WASP may not be the only link between BCR signaling and the actin cytoskeleton. B cells from WASP knockout mice only show mild defects in general [222], suggesting the presence of additional links. Other members of the WASP family proteins, N-WASP and WAVE [262], potentially compensate for WASP downstream BCR signaling. BCR signaling could also regulate the actin cytoskeleton through other actin regulators that do not belong to WASP family, such as the regulation of HS1, an actin-binding protein and a homolog of cortactin expressed in lymphocytes, by Syk kinases [263] and actin-binding protein 1 by BCR signaling [264]. Moreover, redundant functions provided by the other Tec-family kinase, Tec, cannot be eliminated, as the recently created Btk/Tec double knockout mice [145] display defects that are more severe than the Btk knockout alone. Future studies will examine additional molecular links and interaction mechanisms between the actin cytoskeleton and BCR-mediated signaling and antigen processing pathways.

Chapter 3: N-WASP and WASP, two to tango

3.1 Abstract

The WASP family proteins are actin-nucleation promoting factors which regulate actin dynamics. WASP deficiency is associated with a severe X-linked immunodeficiency in both humans and mice and underscores the importance of actin regulatory elements in initiating and propagating the immune response. B-cell dependent humoral responses are not much altered in WASP deficient mice, leading to the hypothesis that WASP activity may be complemented by a closely related family member N-WASP, which is present in B-cells. In this study, I have discovered a bilateral regulatory relationship between BCR signaling and actin cytoskeleton. Using WASP knockout mice, I have shown that WASP plays a role in the development of marginal zone B-cells and the transition between immature subsets of B cells, while not having a significant role in BCR-mediated signaling and internalization in mature B-cells. Increased expression levels of other two members of WASP family, N-WASP and WAVE-2, were detected in WASP knockout mice. Similar to the activation of WASP, BCR activation induced the phosphorylation of N-WASP. Over expression of GFP tagged full length N-WASP and hemagglutinin (HA)-tagged constructs of N-WASP containing the PRD, inhibited BCR internalization and movement to late endosomes. These results provide evidence for a role for N-WASP in B cells.

3.2 Introduction

A number of actin regulatory proteins involved in a variety of cellular processes, have been discovered in recent years. These proteins are highly conserved and expressed either ubiquitously or in a cell-specific manner, linking cytoskeletal cellular responses to environmental prompts. It has since become clear that cytoskeleton-mediated spatial and temporal regulation is essential for cell integrity, motility, membrane trafficking and cell division especially during cancer and microbial pathogen invasion [187, 200, 265-267].

B-cell-mediated humoral responses to antigens are initiated when the BCR encounters antigen. The binding of antigens to the BCR is known to induce dramatic changes in the state of the actin cytoskeleton. Recent studies reveal that upon antigen encounter, BCR-proximal signaling regulates actin cytoskeleton dynamics and reorganization in B cells, mainly through tyrosine kinases Syk and Btk ([178] and Chapter2). In turn, BCR-triggered rearrangement of the actin cytoskeleton regulates the signaling and antigen processing function of the BCR. As shown in Chapter 2, one of the targets of BCR signaling pathways for actin regulation is Wiskott-Aldrich syndrome protein (WASP). The WASP family of proteins is well known to be capable of translating signals into dynamic structural changes in the actin cytoskeleton [211, 246, 262]. It contains multiple members, including WASP, neural WASP (N-WASP), and the WAVE/Scar proteins. While WASP is expressed only within the hematopoietic lineage, N-WASP is more ubiquitously distributed. WAVE1 and 3 are found preferentially in the neural tissue, and WAVE-2 is preferentially expressed in lymphocytes.

A seminal observation with regards to the biological significance of these proteins has been the association of a deficiency or mutation of WASP with a rare X-linked recessive disease in humans and corresponding severity seen in N-WASP and WAVE-2 null mice, both of which are embryonically lethal [267-269]. Patients afflicted by Wiskott-Aldrich Syndrome (WAS), for which the protein is named, exhibit variable immunodeficiency [207].WASP-deficiency primarily impacts T-cell and macrophage function, presenting with abnormal morphology, defects in chemotaxis, migration, phagocytosis and adhesion (macrophages), defective CD3-mediated T cell proliferation, faulty thymocyte development, impaired capping of actin and T-cell receptors, and the inability of T cells to form a functional immune synapse with antigen presenting cells, thereby compromising the T-dependent responses to antigens [210]. There is a preponderance of evidence for the role of WASP in T-cells from WASP null mice, where a deficiency of this protein leads to dramatic cellular defects. Equivalent studies on the role of WASP in B-cell biology have not been as thorough. B-cells in WAS patients have an abnormal morphology and developmental defects [207, 224]. Moreover the germinal center reaction is delayed and accompanied by lower frequencies of germinal center precursors [224, 228]. In WAS patients and WASP knockout mice, antibody production, isotype switching and particularly responses to T cell-independent polysaccharide antigens are impaired, presenting with low IgM levels [207]. There appears to be a slight defect in antigen-induced B-cell proliferation, which is overcome with the addition of LPS and/or IL-4 [223]. This suggests that the impairment seen in B-cells is BCR specific and may correspond to signaling defects. However, B-cell responses to T-dependent antigens are not significantly modified [207, 223, 227, 228].

While the actin cytoskeleton is essential for B cell activation, the deficiency of WASP, a key actin regulator, has much milder effect on B cells than on T cells. This implicates the potentially redundant function of WASP family proteins in compensating deficiencies. This hypothetical redundancy is supported by the relatively high level of conserved homology between WASP family proteins: of consequence is the high degree of homology (68%) in the domains responsible for actin nucleation [229, 230]. Significantly, the finding that the role of N-WASP in vesicular movement can be fully replaced by WASP [231] and the similarity in cellular roles played by these proteins [207] reinforces functional redundancy of these two proteins, especially in case of deficiency. However the high level of homology and shared binding partners could be a physiological constraint which limits distribution and activity of the proteins in the cell. As noted in Chapter 1, WASP and N-WASP share binding partners which are able to affect their activation variably. Moreover, the differences in domain structure could also account for differential recruitment, activity and distribution of these two proteins. These differences may be helpful in pinpointing unique roles played by these two proteins in B-cells while also helping us understand how they may compensate for each other.

In order to test the redundancy hypothesis in B-cells we examined the role of N-WASP in B-cells in the presence and absence of WASP and also studied the normal roles of WASP and N-WASP in B-cell activation. WASP knockout mice and dominant negative transfections of different N-WASP domains were used as model systems. Here, I demonstrate that BCR activation induces Btk dependent phosphorylation of both WASP

and N-WASP, and that N-WASP is required for BCR-mediated antigen uptake and transport to the antigen processing compartment. Importantly, WASP deficiency increased the total expression level of N-WASP and WAVE2. Moreover, BCR-induced phosphorylation of N-WASP is higher in WASP-deficient B cells. Additionally, WASP-deficiency revealed a role for WASP in B-cell development. These results provide evidence for both redundant and non-redundant functions of N-WASP in B-cells.

3.3 Materials and Methods

3.3.1 Mice, cells and cell culture.

B cell lymphoma A20 IIA1.6 cells (H-2^d, IgG_{2a}^+, FcγIIBR$^-$) were cultured in DMEM supplemented with 10% FBS. Wt (CBA/CaJ) mice that were 6-8 weeks old were purchased from Jackson Laboratories. WASP knockout mice ($WASP^{-/-}$) on a CBA/CaJ background were established and kindly provided by Dr. Scott Snapper (Harvard Medical School, MA). To isolate splenic B cells, single-cell suspensions of splenocytes were subjected to density-gradient centrifugation (2300 x g) in Ficoll (Sigma-Aldrich, St Louis, MO) to obtain mononuclear cells, treated with anti-Thy1.2 mAb (BD biosciences, San Jose, CA) and guinea pig complement (Rockland Immunobiochemicals, Gilbertsville PA) to remove T cells, and panned for 2 h to remove monocytes. All procedures involving mice were approved by the Animal Care and Use Committee of University of Maryland.

3.3.2 PCR probes, DNA constructs and transfection.

PCR probes to identify the insertion of the neomycin cassette in the $WASP^{-/-}$ locus were obtained from the Jackson Laboratory. Specifically 5'-TTTGAATCCACTCGGTGCTC-3' (oIMR3153), 5'-CCCATCAGGTGGTCCACTA-3' (oIMR3152) and 5'-GCTATCAGGACATAGCGTTGG-3' (oIMR1100) were used to amplify the 650bp insert. B cell lymphoma A20 IIA1.6 cells (H-2^d, IgG_{2a}^+, FcγRIIB$^-$) were cultured at 37^{o}C in DMEM supplemented with 10% FBS. The DNA constructs for eGFP fusion protein of full length N-WASP (eGFP-N-WASP) and HA-fusion proteins of N-WASP mutants with their PRD and WA domains deleted (HA-ΔPWA) and those which expressed only PRD and WH2 domains (HA-PW) were kindly provided by Drs. Michael Kessels and Britta Qualman, (Leibniz Institute for Neurobiology, Germany). DNA constructs for eGFP fusion proteins of N-WASP mutants such as WA domain deleted (eGFP- ΔWA), those expressing only the C-terminal WH2 and acidic domains i.e. WA or VCA domains (eGFP-VCA) were kindly provided by Drs. Michael Way and Susan Gunst. DNA constructs were introduced into A20 B cells by electroporation using the Nucleofector and Nucleofection kit V from Amaxa (Gaithersburg, MD).

3.3.3 Flow Cytometric Analysis.

To identify various subsets of B-cells i.e. mature and immature B cells, B1 cells and Marginal Zone B-cells (MZB), splenic B-cells from WASP knockout and wt mice were co-labeled with PE anti-IgM, FITC anti-IgD and PE-Cy5 anti-B220 for analysis by FACSCalibur flow cytometer for three-color analysis. And then for more comprehensive studies cells were co-labeled with PE anti-CD21, FITC-anti-IgM, Pacific Blue anti-

CD24, Allophycocyanin (APC) anti-CD23 and PE-Cy5-anti-B220 antibodies (BD Bioscience). These cells were analyzed using the Dako-Cytomation (BD Biosciences) flow cytometer for t multi-color analysis. The data are represented as mean fluorescence intensity (MFI). To determine total levels of N-WASP and WAVE-2 in splenic B-cells from $WASP^{-/-}$ and wt B-cells as well as A20 B-cells, these cells were first fixed and permeabilized and the endogenous levels of N-WASP and WAVE-2 were measured using rabbit anti-NWASP and rabbit anti-WAVE-2 antibodies and PE-conjugated secondary antibodies and analyzed on a FACS-Calibur. To determine the kinetics of N-WASP phosphorylation, phosphorylated levels of N-WASP (pN-WASP) were measured as follows: A20 B-cells were cross-linked with anti-mouse IgG (20 μg/ml; Jackson Immunoresearch) for various times at 37°C and fixed with 4% paraformaldehyde. The cells were then permeabilized and levels of pN-WASP were measured using rabbit anti-pN-WASP (Tyr^{256}) and a PE-conjugated secondary on a FACS-Calibur (BD Biosciences).

3.3.4 Immunofluorescence Microscopy Analysis.

In order to analyze differences in the cellular distributions of BCR and eGFP or HA-tagged domains of N-WASP in transfected or untransfected A20 B-cells, cells were incubated with Cy3-F(ab') (10μg/mL Jackson Immunoresearch) for 10 min at 4°C to label the surface BCR, followed by rabbit anti-mouse IgG (20 μg/ml, Jackson Immunoresearch) for indicated times at 37°C to crosslink the BCR. Cells were fixed with 4% paraformaldehyde, permeabilized and stained with Alexa Fluor (AF) -633 conjugated Phalloidin and HA-tags were visualized

with an AF488 conjugated-anti-HA mAb. Cells were mounted and analyzed using a confocal fluorescence microscope.

Endogenous levels of phosphorylated N-WASP in B-cells from $WASP^{-/-}$ and wt splenic B-cells were measured by first adhering splenocytes to poly-L-lysine coated slides for 20 min at 37°C. The BCRs on these cells were then labeled with Cy3 conjugated anti-mouse IgM F(ab') fragments (10μg/mL) at 37°C for 10 min. The cells were then cross-linked with anti-mouse IgM for varying times and then fixed and permeabilized. Intracellular, endogenous levels of pN-WASP were visualized with rabbit anti-pNWASP (Tyr^{256}) and an AF488 conjugated secondary antibody.

In order to analyze of the movement of the BCR from the cell surface to late endosomes, A20 B-cells untransfected or transfected with either eGFP-N-WASP or the various eGFP and HA-tagged domain mutants of N-WASP were incubated with Cy3-conjugated $F(ab')_2$-goat anti-mouse IgG (Jackson Immunoresearch) for 20 min at 4°C to label the surface BCR. Cells were washed and then adhered to poly-lysine-coated slides (Sigma-Aldrich) for 20 min at 4°C and then chased at 37°C for varying lengths of time to allow for the internalization of the BCR. At the end of each time point, cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin in DMEM, 10%FBS, 10mM HEPES and 10mM glycine, and incubated with a mAb specific for lysosome-associated membrane protein 1 (LAMP-1) (ID4B, ATCC, Manassas, VA) and an AF633-conjugated secondary antibody. Cells were mounted with gel mount (Biomeda, Foster City, CA) and analyzed using a laser-scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany). For quantitative analysis of images, the cellular distribution of the BCR was divided into two different categories: the BCR mainly distributed on the cell surface without colocalization with LAMP-1 or

extensively colocalized with LAMP-1 in the perinuclear region of cells. Furthermore, the correlation of $LAMP1^+$ compartments and $HA^+/eGFP^+$ vesicles was calculated by using correlation analysis LSM 510 software. Cells were categorized by visual inspection to determine the localization pattern of the BCR. Over 100 cells from three independent experiments were analyzed for each time point.

3.3.5 Analysis of BCR internalization.

A20 B-cells untransfected or transfected with either eGFP-N-WASP or the various eGFP and HA-tagged domain mutants of N-WASP or splenic B-cells from $WASP^{-/-}$ and wt mice were incubated with biotinylated goat anti-mouse IgG/IgM (20 μg/ml; Jackson Immunoresearch) for 30 min at 4°C to label the surface BCR. After washing off unbound antibodies, cells were chased at 37°C for varying lengths of time. The chase was terminated by adding ice-cold DMEM containing 6 mg/ml BSA (DMEM-BSA). The biotinylated antibodies remaining on the cell surface after the chase were stained with PE conjugated-streptavidin (5 μg/ml; Qiagen, Valencia, CA) at 4°C. The cells were then fixed with 2% paraformaldehyde and in the case of HA-tagged constructs, permeabilized and the HA-tag was visualized using an Alexa Fluor (AF)-488 conjugated anti-HA antibody. Cells were then analyzed using a flow cytometer (FACSCalibur, BD Bioscience, and San Jose, CA). eGFP or HA-expressing cells were gated for analysis. The data was plotted as a percentage of the mean fluorescence intensity of cell-surface PE-streptavidin at time 0.

3.3.6 Live cell imaging.

Constructs encoding eGFP-N-WASP, or eGFP- ΔWA were introduced into A20 B cells by electroporation using Nucleofection kit (Amaxa). 24 hours after transfection, cells were placed into chambered polylysine (10 μM)-coated cover glasses (Nalgene Nunc Int. Rochester, NY) and incubated at 37°C, 5% CO_2 for 30 min. Cells were then incubated with Cy3-Fab-goat anti mouse IgG (5 μg/ml; Jackson Immunoresearch) at room temperature for 10 min, washed with 1%FBS in PBS. Cells were activated by crosslinking the BCR with rabbit-anti-mouse IgG (20 μg/ml; Jackson Immunoresearch) and images were acquired every 3 seconds. Images of eGFP N-WASP expressing cells were acquired using the Zeiss Live-DUO confocal microscope while images for the other construct (eGFP- ΔWA) were obtained using the Zeiss LSM510.

3.3.7 Immunoblotting.

Endogenous levels of total phosphorylated tyrosines (4G10mAb), pN-WASP (Tyr^{256}), phosphorylated Vav (Tyr^{174}), pWASP (Ser483/484), pERK and pBLNK (Tyr^{96}) were measured in splenic B cells from wild type CBA/CaJ and $WASP^{-/-}$ mice, as well as B-splenocytes from wt mice that were either treated with the Btk inhibitor: LFM A-13 or left untreated and A20 B cells. Cells were activated by crosslinking the BCR with goat anti-mouse IgG+M (20 μg/ml, Jackson Immunoresearch) for splenic B-cells, or goat anti-mouse IgG (20 μg/ml, Jackson Immunoresearch) for A20 B-cells, for indicated times and lysed with above lysis buffer additionally containing Na-Pervanadate (made by mixing equimolar volumes of 20μM $Na_3VO_4$and 20μM H_2O_2 and adding it to the lysis buffer at a final concentration of 0.05μM,) and Na-pyrophosphate (100μM). Cell lysates were analyzed using

SDS-PAGE and Western blotting, probing with anti-phospho-tyrosine mAb (4G10, Upstate Biotech, Lake Placid, NY), rabbit anti-phosphorylated N-WASP (pN-WASP), rabbit anti-phosphorylated WASP (Bethyl Laboratories), rabbit anti-phosphorylated BLNK (Cell Signaling), rabbit anti-phosphorylated Vav (Santa Cruz Biotechnology) and mouse anti-tubulin (IgG1 specific) or rabbit-anti-actin (Sigma) and HRP-conjugated secondary antibodies (Jackson ImmunoResearch). The blots were quantified using densitometry and normalized to tubulin or actin levels.

3.4 Results

3.4.1 The role of actin regulatory protein WASP in B-cells

As shown in Chapter 2, BCR signaling regulates actin dynamics in B cells through Btk and WASP. Btk-deficiency abolished both BCR-triggered actin rearrangement and WASP activation, suggesting a role for WASP in actin regulation in B cells. However, the lack of general physiological or significant actin-related defects in WASP-deficient B-cells argues against this hypothesis [207, 222]. To study the role of WASP in B cells, we examined B cells from WASP knockout mice [222]. The lack of WASP gene and protein expression in splenic B-cells from $WASP^{-/-}$ mice was verified by PCR and western blotting (Fig *3.1A* and *B*).

We next determined the effect of WASP-deficiency on BCR internalization in response to antigenic crosslinking. Splenic B-cells from wt and $WASP^{-/-}$ mice were labeled with biotinylated anti-IgM antibody at $4^{o}C$ to label the surface BCR and chased at $37^{o}C$ for the

indicated times. Endpoint levels of remaining surface BCR was measured with PE-conjugated streptavidin. The rate of BCR internalization was only slightly reduced in splenic B-cells from $WASP^{-/-}$ mice, compared to that in wt mice (Fig. *3.2*). This is in agreement with previously reported findings that there were no significant defects in antigen presentation by B-cells from $WASP^{-/-}$ mice. Moreover, WASP-deficiency did not significantly alter BCR-triggered tyrosine phosphorylation (data not show).

Although $WASP^{-/-}$ B-cells are able to internalize and signal adequately upon antigenic stimulus, when stained with the pan B-cell surface marker B220, the $B220^{+}$ B-cell population from $WASP^{-/-}$ spleens unlike the wt population is significantly segregated into $B220^{hi}$ and $B220^{lo}$ subsets (data not shown). B220 staining was originally used to identify B-cells and study BCR internalization within that subset. $B220^{lo}$ staining profiles are traditionally indicative of an immature phenotype while $B220^{hi}$ indicate mature recirculating B-cells. The percentage of $B220^{lo}$ population was much higher in the $WASP^{-/-}$ spleens indicating a progressively immature population. Although, the original study of this knockout did not report any discrepancies in B220 staining or B-cell development, our observations were incongruous and we decided to thoroughly study the effect of WASP gene knockout on B cell development. We initially sought to examine the peripheral B-cell subsets as they seemed the affected population in our B220 profile and thus we analyzed splenic B cells from $WASP^{-/-}$ mice using flow cytometry (Fig. *3.3A-C*). We labeled the surface of splenic B-cells with anti-B220, IgM, IgD, CD21, and CD23 antibodies. Follicular and mature (FO/B2, phenotypes), immature transitional (T1 – $B220^{+}$ IgM^{hi} IgD^{lo} $CD21^{lo}$ $CD23^{-}$, and T2- $B220^{+}$ IgM^{hi} IgD^{hi} $CD21^{lo}$ $CD23^{lo}$)

FIGURE 3.1. Confirming WASP gene disruption and absence of WASP expression in WASP$^{-/-}$ B-cells.

WASP gene disruption in the WASP$^{-/-}$ mice was confirmed by PCR analysis of tail DNA isolated from female homozygous WASP$^{-/-}$ and wt mice (A) and Western blot analysis of cell lysates from splenic B-cells from wt and WASP$^{-/-}$ mice using WASP-specific antibody (B). WASP gene disruption in the WASP$^{-/-}$ mice was accomplished by the insertion of a neomycin resistance cassette in the reverse orientation with respect to transcription. Reverse probes flanking the neomycin insert were used to amplify the presence of the insert which produces a 650bp product. Adding a forward neo primer along with the reverse neo primer and primers that flank the insert amplifies a 175bp product in the case of wt allele, which can produce a read through product.

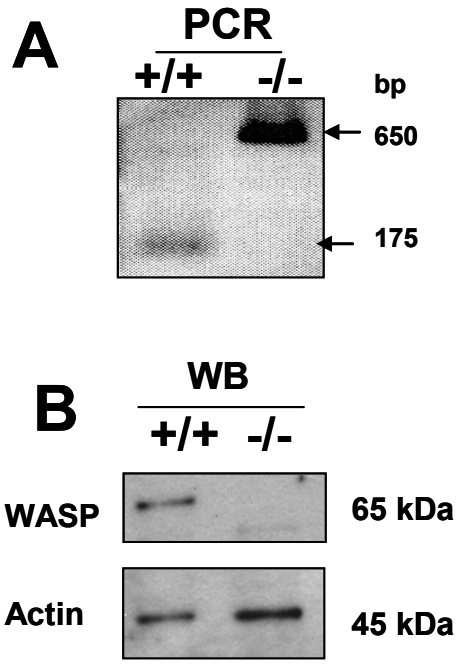
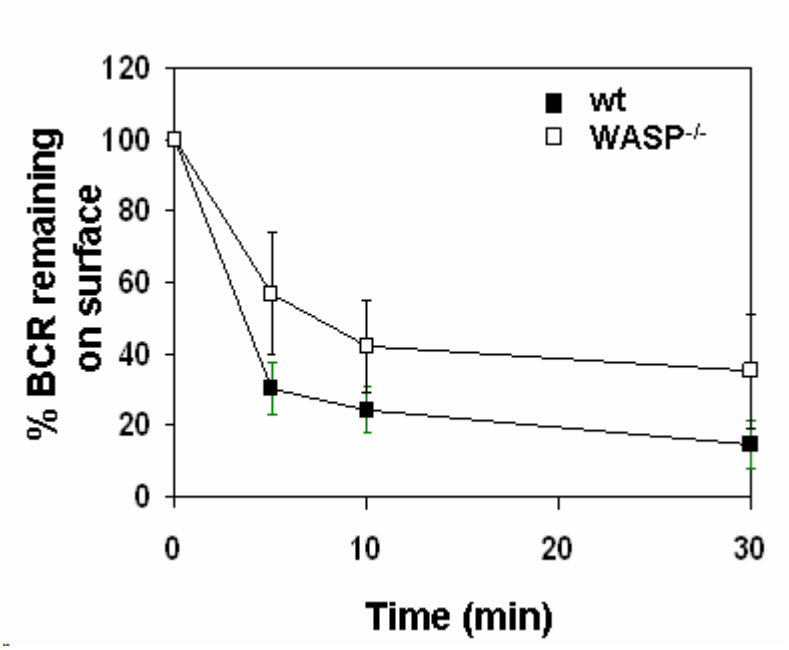


FIGURE 3.2. WASP-deficiency has no significant effect on BCR internalization. The surface BCR was labeled and cross-linked with biotin-F(ab')$_2$-goat anti-mouse IgM at 4^{o}C for 30 min. The cells were then chased at 37^{o}C for indicated times. Biotin-anti-mouse IgM left on the surface after the chase was detected by PE-streptavidin and quantified using a FACS Calibur. The data was plotted as the percentage of the surface-labeled BCR remaining at the cell surface. Shown are the averages (±S.D.) of three independent experiments.



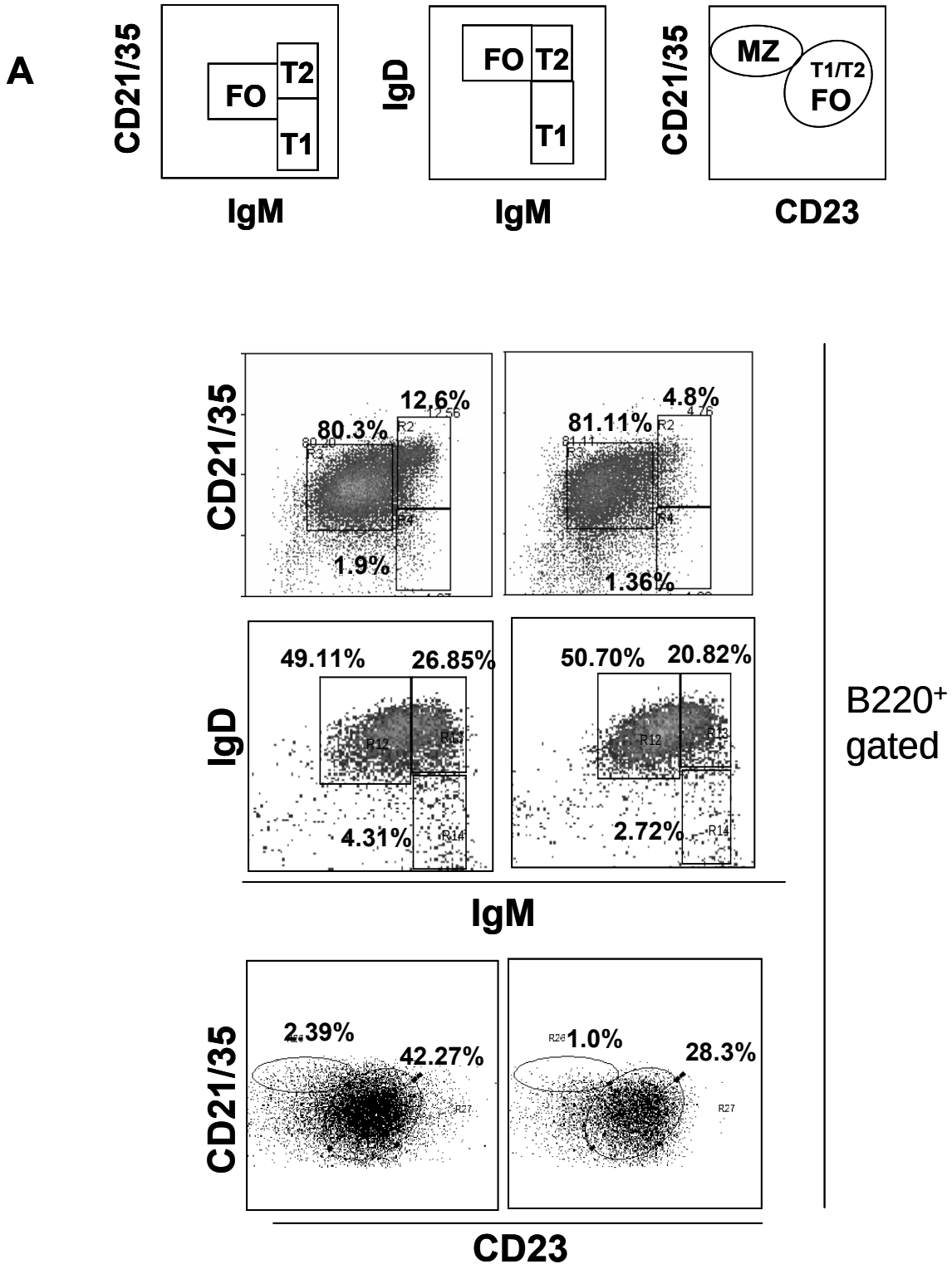
and marginal zone B-cells (MZB - $B220^{+}IgM^{hi}CD21^{hi}CD23^{lo}$) were identified and quantified based on distinct surface marker profiles. Initial analysis showed a decrease in the numbers of both T1 and T2 immature B-cells but normal follicular/mature B cells numbers (Fig. *3.3A, B*). Further analyses revealed a severe decrease in the numbers of MZB cells in $WASP^{-/-}$ mice (Fig. *3.3A,B*). While such detailed analysis has not been reported previously, these results are in general agreement with other studies [228]. Taken together, the subtle defects in BCR signaling and antigen uptake functions in $WASP^{-/-}$ mice imply the presence of additional factors compensating for WASP function in WASP-deficient B-cells. However the defects in B cell development, though not restrictively severe, may highlight the non-redundant role of WASP in lymphocyte development.

3.4.2 The presence and modulation of N-WASP in B-cells

The possibility of functional redundancy in B-cells by participating members of the WASP family of proteins lead us to testing for the presence of a likely candidate. We picked N-WASP as it is known to be ubiquitously distributed and shares significant domain and functional homology with WASP. We first tested for the presence of N-WASP in splenic B-cells and A20 B-cell lymphoma line using specific antibodies and Western blotting. The results show a detectable level of N-WASP from lysates of both splenic and A20 B cells (Fig. *3.4 A- D*). To test whether BCR activation is able to regulate the activity of N-WASP like that of WASP, we determined the tyrosine phosphorylation level of N-WASP and cellular distribution of phosphorylated N-WASP

FIGURE 3.3. B cell developmental block in WASP$^{-/-}$ mice.

(A-B) Purified splenic B cells from wt and WASP$^{-/-}$ mice were stained with anti-IgM, IgD, CD23, CD24 and B220 and analyzed by flow cytometry. Surface levels of other markers on B220^{+} cells were used to characterize the phenotypic distribution of B-cell subsets. Cells for each double plot were B220 gated and characterized as shown in representative plots. Percentages of gated samples are shown for representative experiments (n = 3). Data are displayed as dot plots on a logarithmic scale.


A
CD21/35
FO
T2
T1
IgM
IgD
FO
T2
T1
IgM
CD21/35
MZ
T1/T2
FO
CD23
CD21/35
12.6%
80.3%
1.9%
4.8%
81.11%
1.36%
IgD
49.11%
26.85%
4.31%
50.70%
20.82%
2.72%
IgM
B220+
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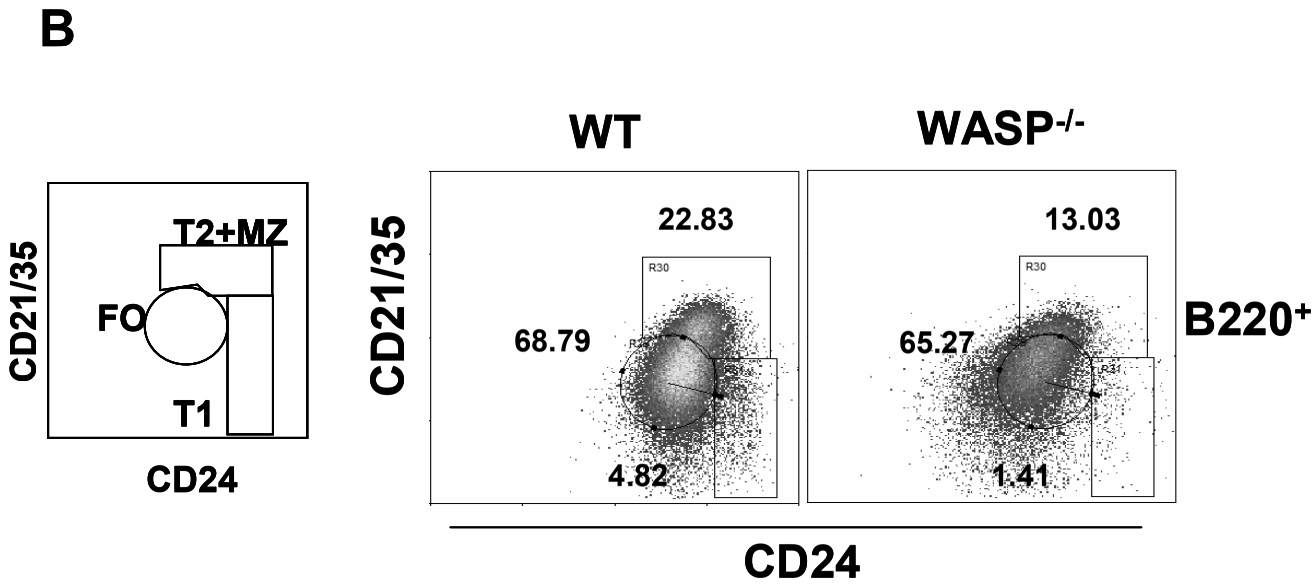
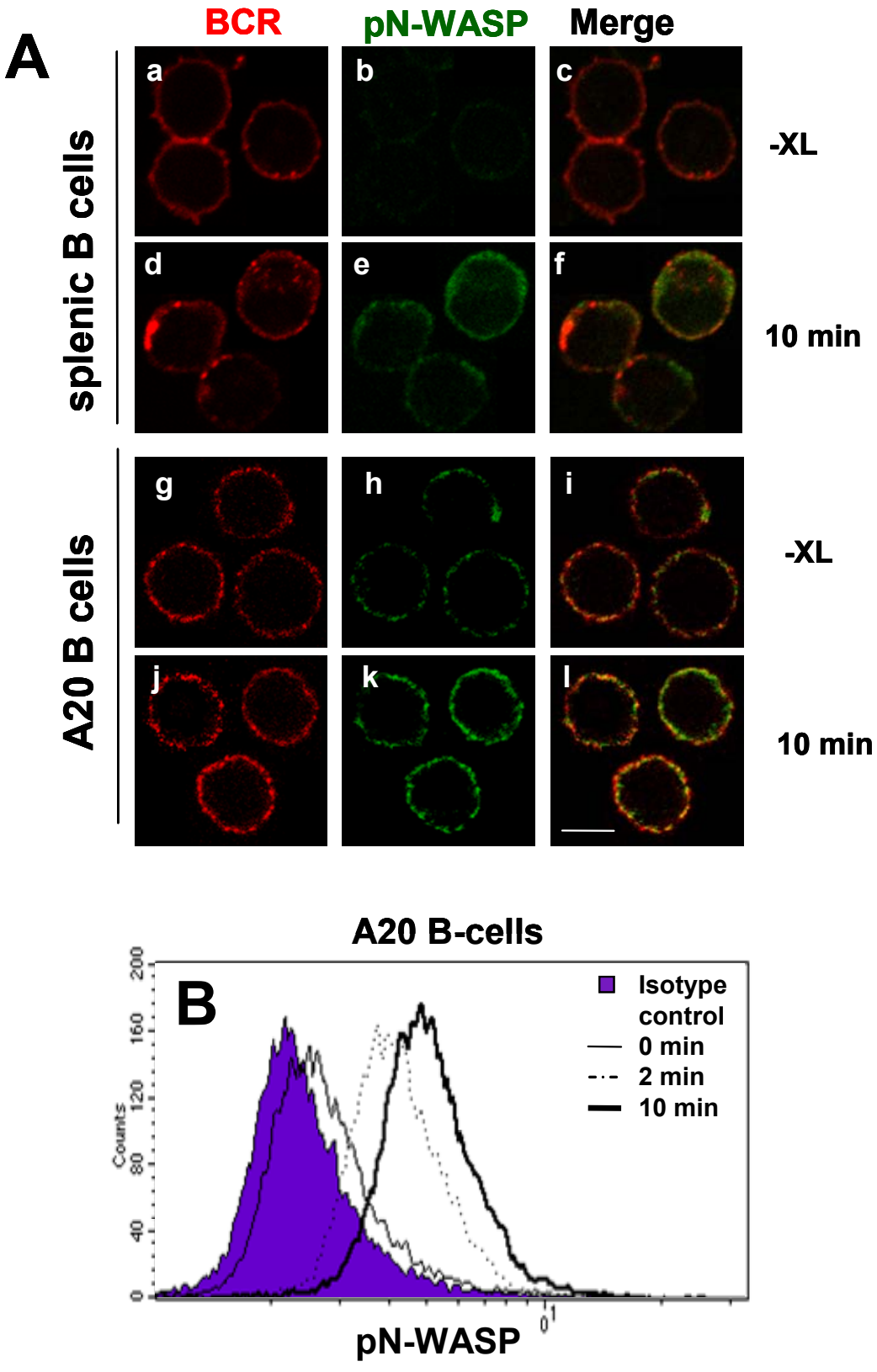
CD21/35
2.39%
42.27%
1.0%
28.3%
CD23

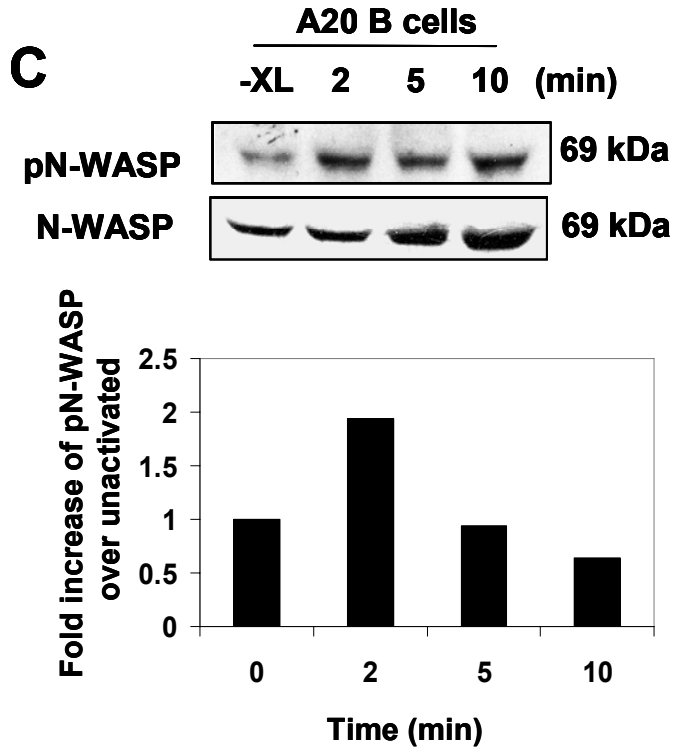

B
T2+MZ
FO
T1
CD21/35
CD24
WT
WASP-/-
22.83
13.03
R30
68.79
65.27
4.82
1.41
B220+
CD21/35
CD24

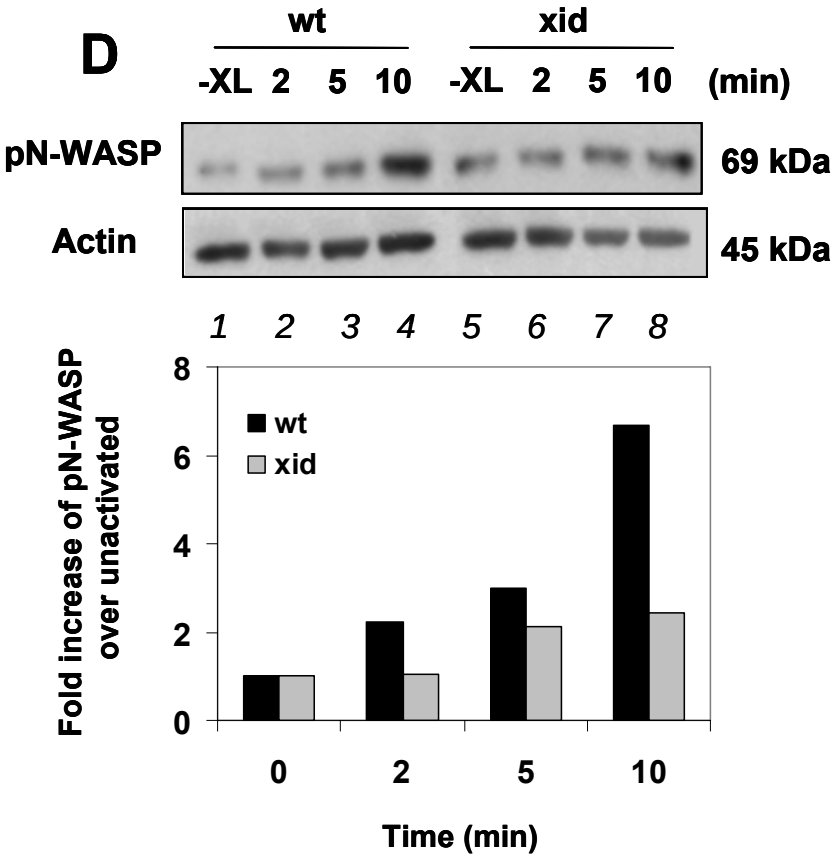
(pN-WASP) using an antibody specific for N-WASP phosphorylated on Tyr^{256}. We explored the distribution of pN-WASP in response to BCR activation using confocal immunofluorescence microscopy. pN-WASP staining levels appeared to be increased at 10 min post BCR cross-linking in both splenic and A20 B-cells (Fig. *3.4A*). In splenic B-cells, pN-WASP was nearly undetectable in unstimulated cells and appeared at the plasma membrane in the vicinity of the BCR after BCR cross-linking (Fig. *3.4A a-f*). In A20 cells pN-WASP was localized at the plasma membrane before and after BCR cross-linking (Fig. *3.4A g-l*). The levels of pN-WASP were further quantified by flow cytometry,(Fig. *3.4B*). Similar to immunofluorescence, BCR activation increased pN-WASP levels over time in A20 B-cells, with an increase most distinctive at 10 min after

FIGURE 3.4. N-WASP is expressed in both splenic and A20 B-cells and is phosphorylated in response to BCR activation.

(A) Splenic B-cells from wt mice and A20 B-cells were incubated with Cy3-conjugated F(ab) of goat anti-mouse to label the BCR and anti-mouse IgM+IgG (20 μg/mL) to cross-linking the BCR for indicated times. The cells were then fixed and permeabilized, and phosphorylated N-WASP (pN-WASP) was labeled with rabbit anti-mouse pN-WASP (Tyr^{256}) and an AF-488 conjugated secondary antibodies. Cells were analyzed using a confocal microscope. Shown are representative images from two independent experiments (Bar, 10 μm). (B) Levels of pN-WASP were quantified by flow cytometry. A20 B-cells were activated with goat anti-mouse IgG for indicated times. Cells were fixed, permeabilized, labeled for pN-WASP, and analyzed using FACS Calibur. Data shown is representative of two independent experiments. (C & D) N-WASP phosphorylation was verified by western blotting. Lysates from A20 B-cells (C) and splenic B-cells (D) were resolved by SDS-PAGE and analyzed by Western blotting. Phosphorylated N-WASP was detected using an antibody specific for N-WASP phosphorylated Tyr^{256}. The blots were stripped and reprobed for N-WASP or actin to serve as loading controls. Levels of pN-WASP were quantified using densitometry and normalized to total N-WASP or actin levels for A20 and splenic lysates respectively and reported as fold increases over unstimulated levels. Shown are representative blots and their densitometry quantification from two independent experiments.


A
BCR
pN-WASP
Merge
a
b
c
d
e
f
g
h
i
j
k
l
splenic B cells
A20 B cells
-XL
10 min
-XL
10 min
B
A20 B-cells
Isotype control
0 min
2 min
10 min
Counts
0
40
80
120
160
200
pN-WASP


C
A20 B cells
-XL 2 5 10 (min)
pN-WASP
69 kDa
N-WASP
69 kDa
Fold increase of pN-WASP over unactivated
2.5
2
1.5
1
0.5
0
0 2 5 10
Time (min)


D
wt
xid
-XL 2 5 10 -XL 2 5 10 (min)
pN-WASP
69 kDa
Actin
45 kDa
1 2 3 4 5 6 7 8
Fold increase of pN-WASP over unactivated
8
6
4
2
0
wt
xid
0 2 5 10
Time (min)

crosslinking. Increase in N-WASP phosphorylation is perceptible as early as 2 min after BCR crosslinking when measured by flow cytometry (Fig. *3.4B*). pN-WASP was detected in both unstimulated splenic and A20 B-cells (Fig. *3.4C*) by western blotting, implying a role for N-WASP in housekeeping actin dynamics. Upon crosslinking the BCR, pN-WASP levels increased with time, however, the kinetics differ between A20 B-cells and splenic B-cells (Fig *3.4C & D*). Baseline levels of pN-WASP in A20 cells are higher when compared to wt naïve splenic B-cells, and levels of pN-WASP increase much quicker becoming prominent as early as 2 min after BCR stimulation (Fig *3.4C*) consistent with data from flow cytometric analysis (Fig *3.4B*). However, a sustained increase until 10 min was not perceptible in A20s by western blotting. Splenic B-cells, on the other hand, seem to increase the levels of pN-WASP moderately up to 10 min (Fig. *3.4D lanes 1-4*), when the gross increase over baseline is the most prominent. As Btk has been shown to regulate WASP phosphorylation, we sought to test the effect of Btk deficiency on N-WASP phosphorylation using the xid mice. The levels of phosphorylated N-WASP were found to be significantly reduced in splenic B-cells from xid mice at 10 min after activation, when the increase in pN-WASP in wt splenic B-cells is most dramatic (Fig. *3.4Cb lanes 5-8*). This implies a role for Btk in regulating N-WASP in addition to WASP.

3.4.3 Increased expression levels of N-WASP and WAVE-2 in WASP-deficient B cells

As the effect of WASP deficiency did not seem to affect the ability of B-cells to initiate signaling or internalize the antigen, we speculated that functionally redundant family members of WASP were likely compensating this deficiency. If other members of

WASP-family proteins functionally compensate for WASP, we then hypothesized that these proteins will increase their expression in WASP-deficient B cells. As we have previously already established that N-WASP is indeed present in B-cells we next compared the expression level of N-WASP and another WASP-family protein - WAVE-2, in wt and $WASP^{-/-}$ splenic B cells using Western blotting and flow cytometry. Western blot analysis showed an increase in total N-WASP protein levels in $WASP^{-/-}$ splenic B cells, in comparison with wt splenic B cells (Fig. *3.5A*). Additionally levels of tyrosine phosphorylated N-WASP were also increased this may imply increased activity of N-WASP in $WASP^{-/-}$ B-cells. Furthermore, the intracellular staining level of WAVE-2 protein in $WASP^{-/-}$splenic B cells was higher than that of wt splenic B cells, when measured by flow cytometry. This indicates that the expression levels of both N-WASP and WAVE-2 increase when WASP gene is knocked out. In support of this notion, immunofluorescence studies showed that staining levels of pN-WASP were increased in both unstimulated and stimulated $WASP^{-/-}$ B-cells (Fig. *3.5Cg-m*) when compared to wt splenic B-cells (Fig. *3.5Ca-f*). Similar to wt splenic B cells, pN-WASP was recruited to the plasma membrane where it colocalized with the BCR, upon BCR activation (Fig. *3.5Cg-m*).

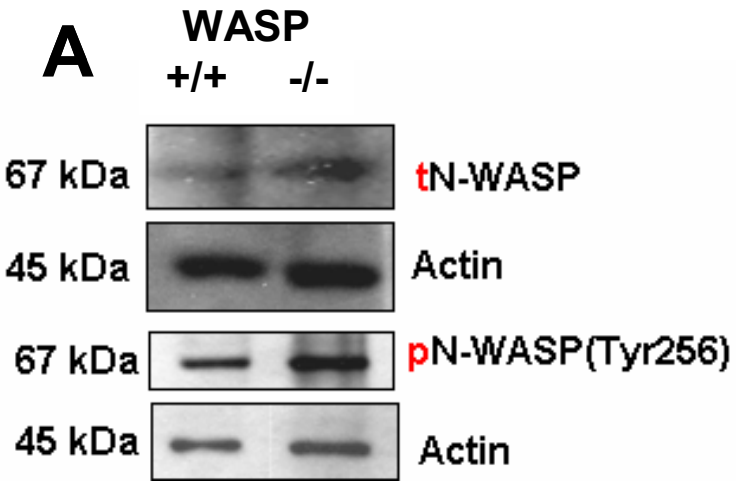
3.4.4 The role of N-WASP in B-cells

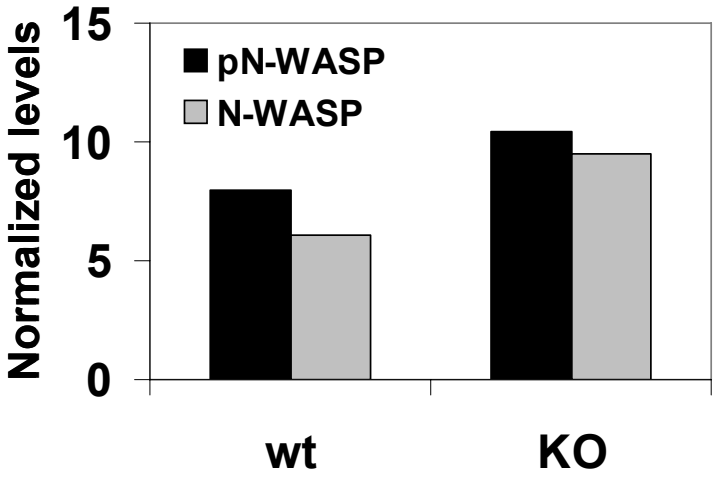
Previous studies on the nature of N-WASP involvement in endocytic events and intracellular trafficking of the vesicles implicate the proline rich domain and the C-terminal WA domain as being important for the full activity of N-WASP in endocytic events and vesicular movement [231, 270]. The constructs used in these previous

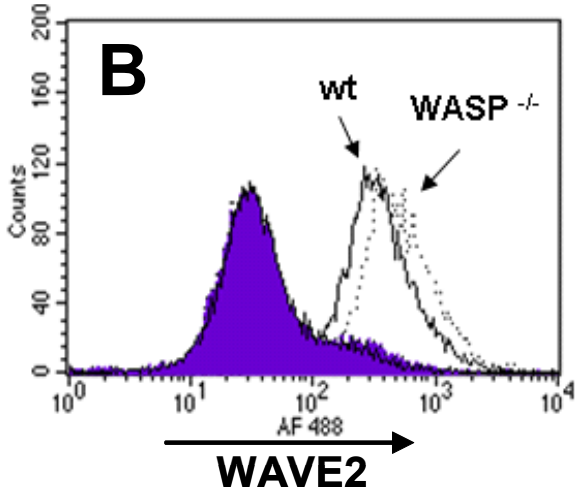
investigations were used to study the role of N-WASP in BCR internalization and movement from cell surface to late endosomes. Thus, transient transfection was next used to elaborate the role of N-WASP in B-cells and I introduced GFP fusion- or HA tagged-full-length and truncated domain constructs of N-WASP into A20 B cells by nucleofection. Over-expression of eGFP tagged-full-length N- WASP leads to a reduction in the rate of BCR internalization (Fig. *3.6*) when compared to the internalization kinetics of the internal control (i.e. cells that were subjected to the transfection procedure but have not taken up the construct and thus, do not express eGFP). A similar phenotype has been reported by our collaborators M. Kessels and B. Qualmann, in fibroblast cells transfected with GFP fusion protein of full length N-WASP [270]. They report that the rate of endocytosis is reduced in cells transfected with the full length protein. Furthermore, on comparing the effects of full-length and domain constructs of N-WASP, they conclude that full length N-WASP functions in a dominant negative capacity as a result of the concomitant overexpression of its PRD. They show that this inhibition of endocytosis can be relieved by the addition of the SH3 domain of a key interacting partner, syndapin. In our studies, over expression of N-WASP mutants that contain the PRD also showed reduced rates of BCR internalization (Fig. *3.6*), whereas over-expressing N-WASP constructs that lack the PRD and the actin binding WA domain (ΔPWA) (Fig. *3.6*) did not affect the rate of BCR internalization significantly, when compared to control cells not expressing the pertinent construct. Immunofluorescence analysis of A20 cells transfected with the same N-WASP constructs as above yielded similar results. The BCR remained on the cell surface (Fig. *3.7Aa, b, and e*) and failed to traffick to LAMP-1^{+}-compartments (Fig. *3.7B*) when N-

FIGURE 3.5. The protein expression levels of N-WASP and WAVE-2 are increased in WASP$^{-/-}$ B-cells.

(A) The protein expression and tyrosine phosphorylation levels of N-WASP in wt and WASP$^{-/-}$ splenic B cells were compared using western blotting. Splenic B cells were incubated with goat-anti-mouse IgM for 10 min to cross-link the BCR. The cells were lysed, and the cell lysates were resolved by SDS-PAGE and analyzed by Western blotting, probing with rabbit anti-mouse N-WASP, rabbit anti-mouse pN-WASP, or rabbit anti-actin antibodies. Shown is a representative blot from two independent experiments. The blots were quantified by densitometry. The protein expression and phosphorylation levels of N-WASP were normalized to actin levels. (B) The protein expression levels of WAVE-2 were measured by flow cytometry. The splenic B-cells from wt and WASP$^{-/-}$ were stained with PE-Cy5 anti-mouse B220 at 4^{o}C to gate for B cells. Cells were fixed and permeabilized, and WAVE-2 were stained using rabbit anti-human WAVE-2 antibodies and an AF488 conjugated secondary antibodies. Shown is a representative histogram from three independent experiments (C) Cellular distribution of pN-WASP in wt and WASP$^{-/-}$ splenic B-cells was analyzed using immunofluorescence microscopy. Cells were incubated with Cy3-Fab-goat anti-mouse IgM for 15 min at 37^{o}C to label the BCR and biotinylated- F(ab$^{'}$)$_2$-anti-mouse IgM for 10 min at 37^{o}C to activate the BCR. Cells were fixed, permeabilized, and stained for pN-WASP Y256 using a specific antibody and an AF-488 conjugated secondary. Cells were analyzed using a confocal fluorescent microscope. Bar, 5μm.


A
WASP
+/+
-/-
67 kDa
tN-WASP
45 kDa
Actin
67 kDa
pN-WASP(Tyr256)
45 kDa
Actin


Normalized levels
15
10
5
0
pN-WASP
N-WASP
wt
KO


B
wt
WASP -/-
Counts
200
160
120
80
40
0
10^0
10^1
10^2
10^3
10^4
AF 488
WAVE2

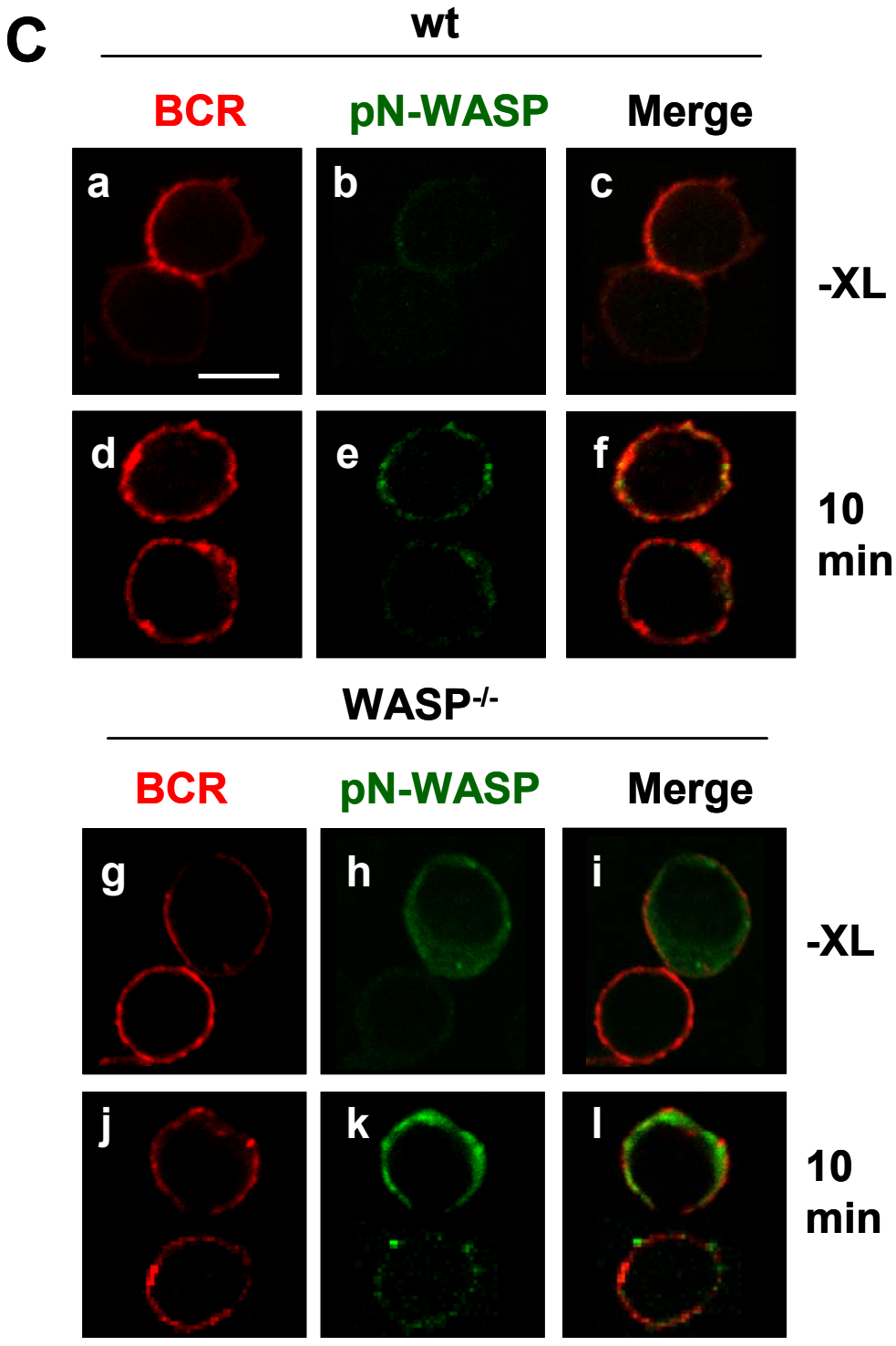
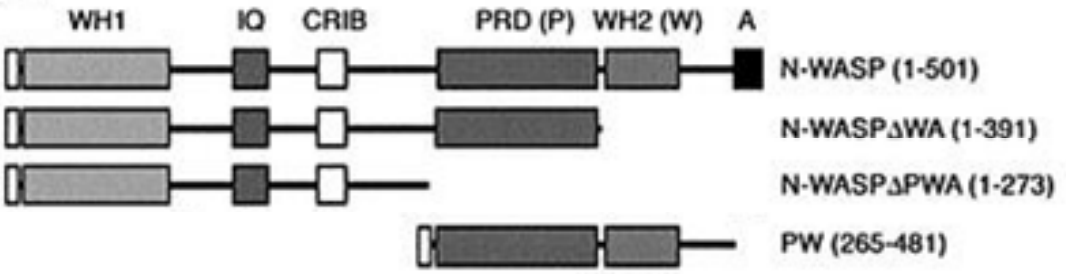

C
wt
BCR
pN-WASP
Merge
a
b
c
-XL
d
e
f
10 min
WASP-/-
BCR
pN-WASP
Merge
g
h
i
-XL
j
k
l
10 min

FIGURE 3.6. Over-expression of GFP-N-WASP and PRD of N-WASP inhibits BCR internalization.

On the top are shown illustrations of the domains used for the following experiment. This part of the figure has been adapted from [270]. A20 cells were transiently transfected with GFP-fusion protein of full length N-WASP (GFP-N-WASP) or N-WASP mutants with its WH2 and A domains deleted (GFP-ΔWA), or HA-tagged N-WASP mutants with PRD, WH2, and A domains deleted (HA-ΔPWA) and only containing PRD and WH2 domains (HA-PW). Twenty-four hours later the surface BCR was labeled with biotin-$F(ab')_2$-goat anti-mouse IgG at 4^oC, washed, and chased at 37^oC for indicated times. Biotin-anti-mouse IgG left on the surface was detected by PE-streptavidin. To visualize the HA-tagged domains, after PE-streptavidin staining, cells were fixed with 2% PFA and permeabilized to label the HA-tags with anti-HA AF488 and quantified using a FACS Calibur. The data was plotted as the percentage of the surface labeled BCR remaining at the cell surface. Cells expressing GFP or showing HA-staining were gated on as positive transfectants. The mean fluorescence intensity (MFI) of the gated expressors was - GFP N-WASP – 30.4 arbitrary units (A.U.); GFP-ΔWA – 30.2 A.U.; HA-PW –;144.5 A.U, HA-ΔPWA – 116.3 A.U. The rate of BCR uptake of the transfectants was compared to the internal control (i.e. cells subjected to the transfection protocol but not showing GFP or HA staining; MFI of eGFP/HA staining for internal control = 1.2 A.U.). Shown are the averages of three independent experiments for each construct. Mean transfection efficiencies for the various constructs are as follows: GFP N-WASP –40%; GFP-ΔWA – 25.98%; HA-PW – 35.15%; HA-ΔPWA – 23.25%


WH1
IQ
CRIB
PRD (P)
WH2 (W)
A
N-WASP (1-501)
N-WASPΔWA (1-391)
N-WASPΔPWA (1-273)
PW (265-481)

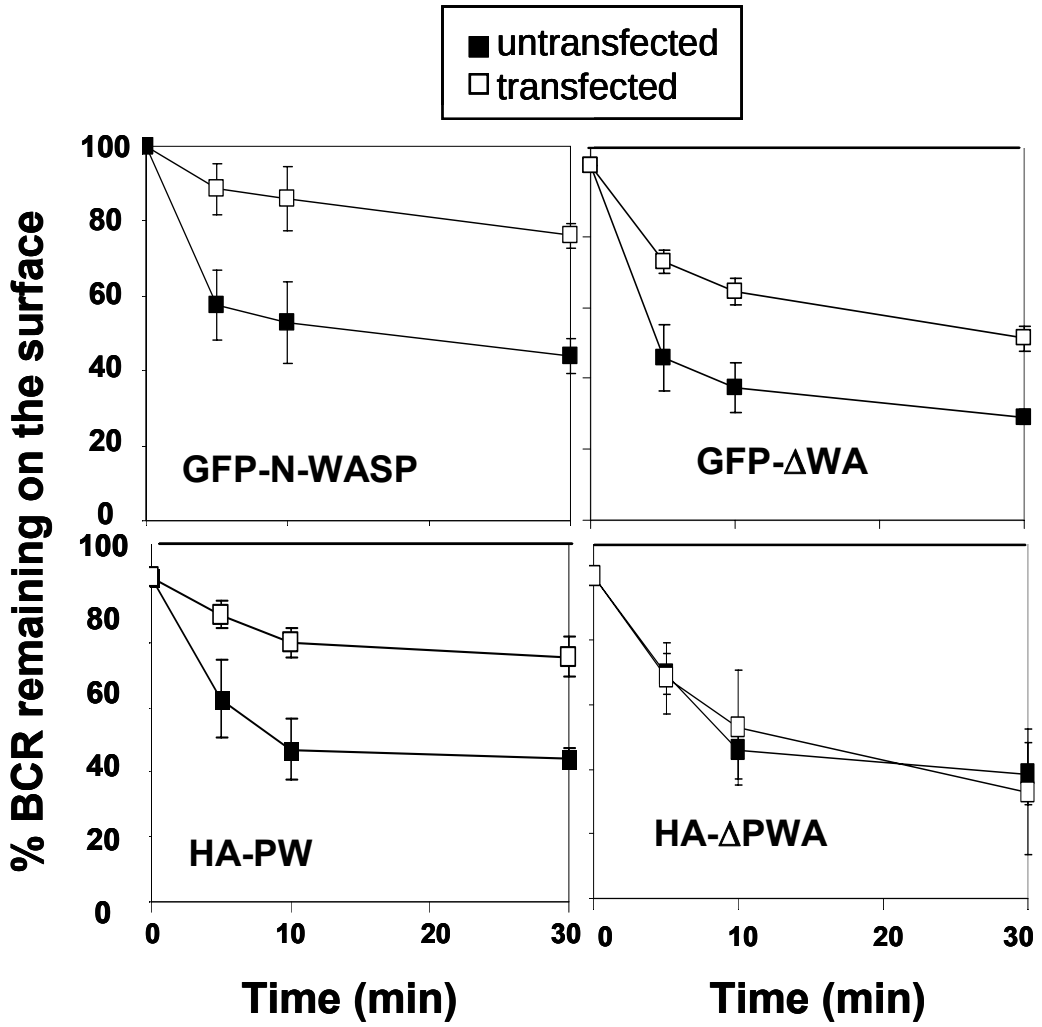
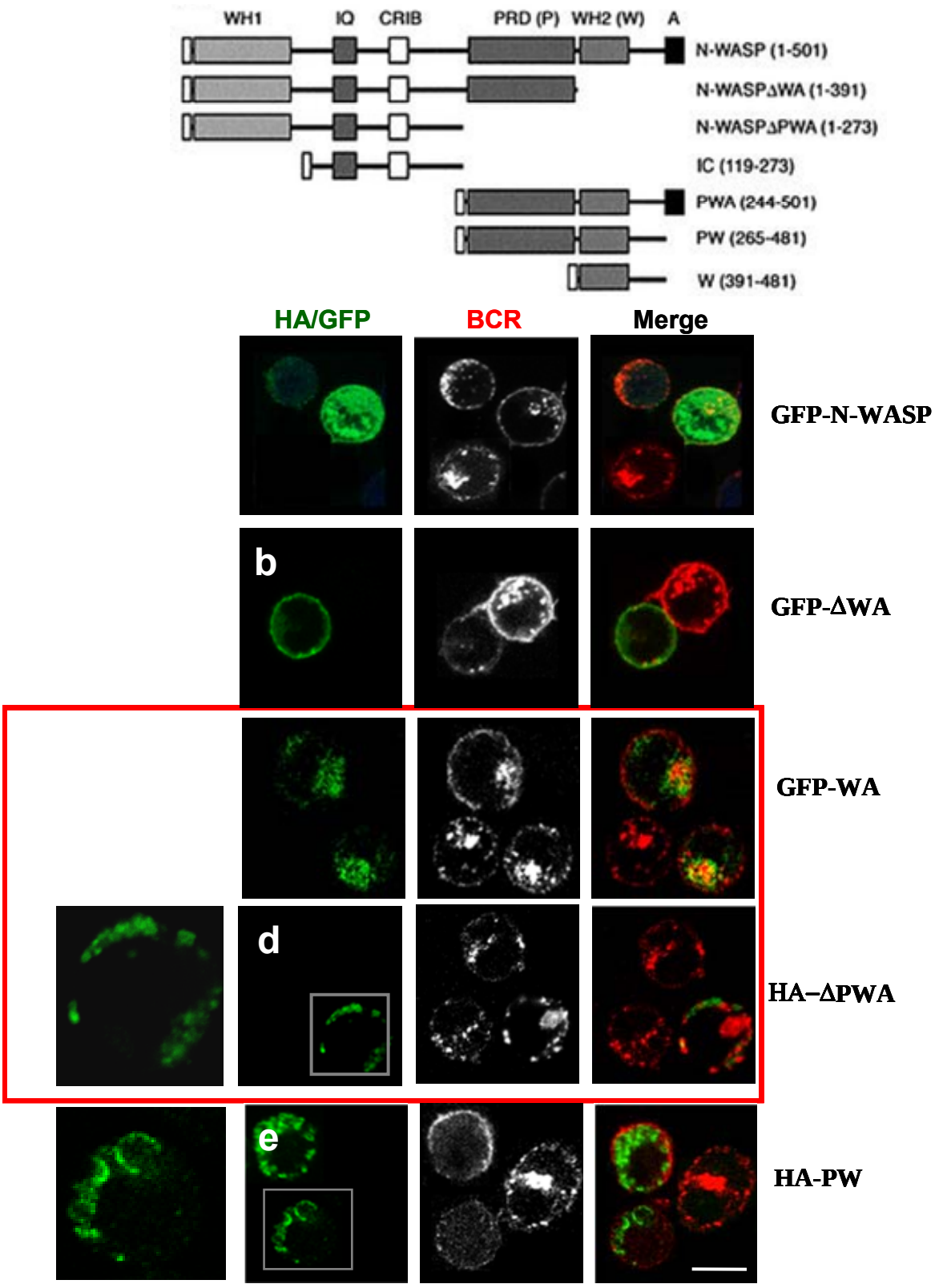
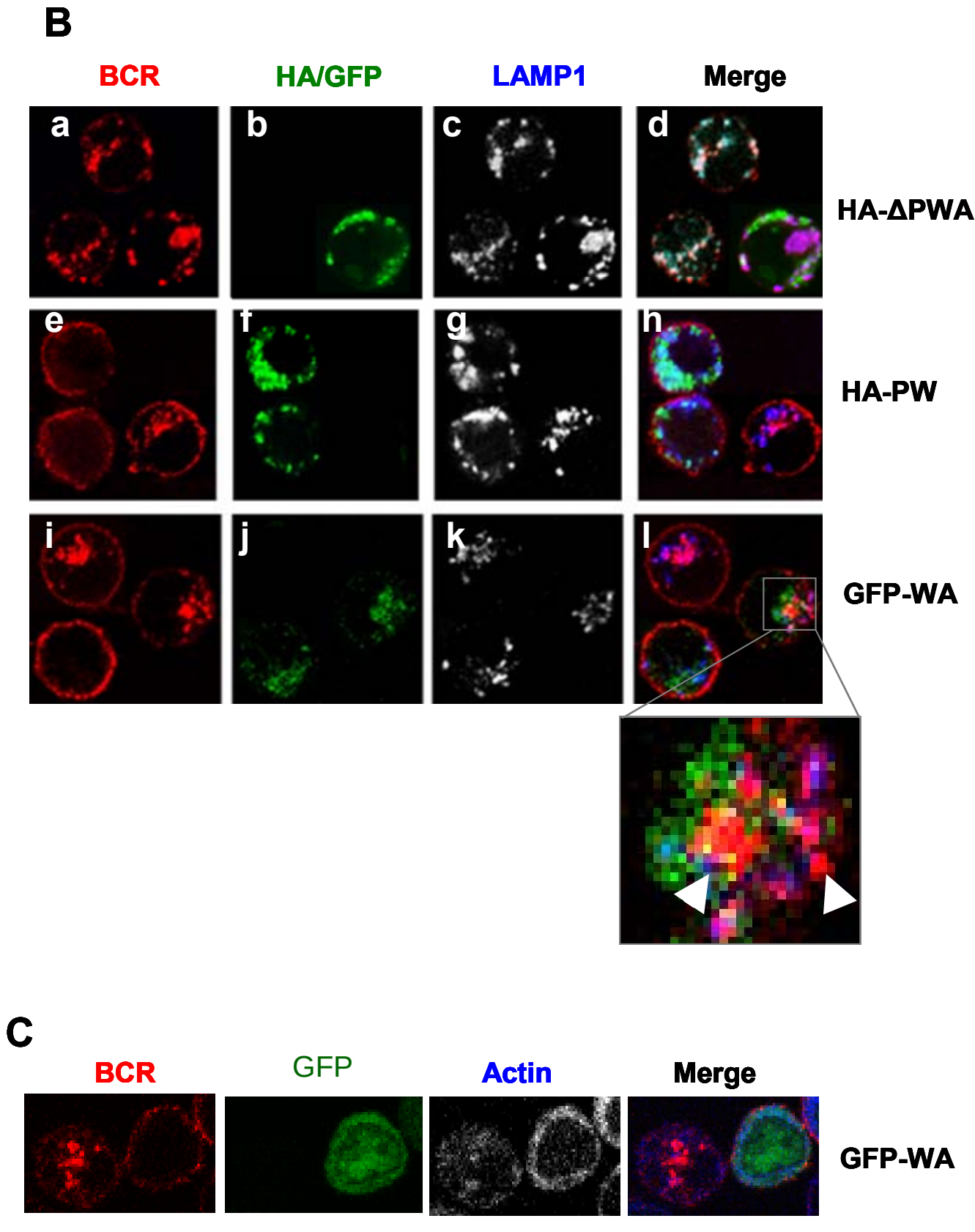
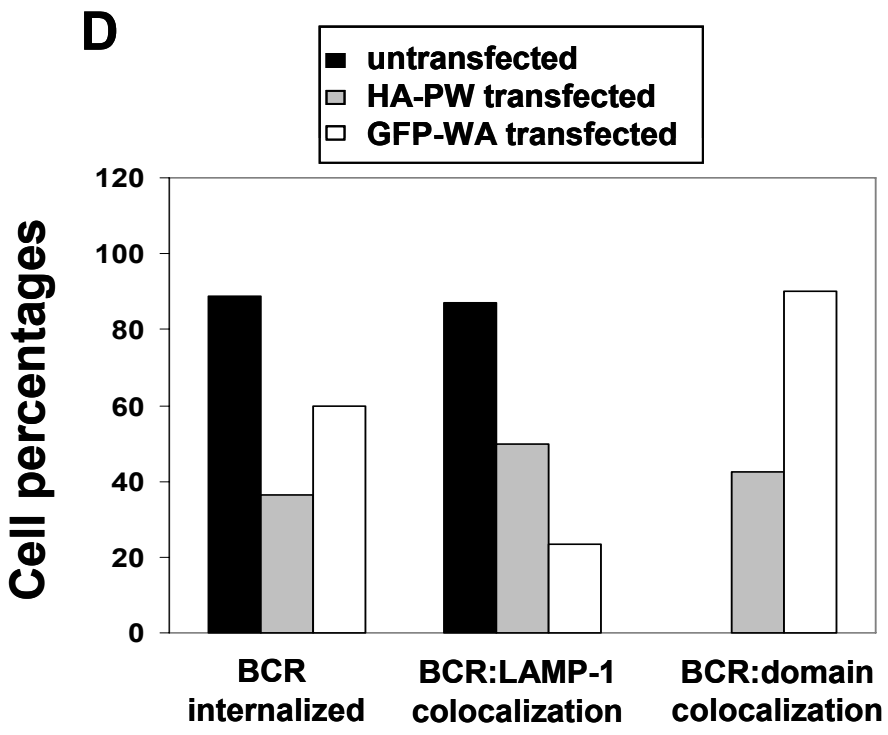

untransfected
transfected
% BCR remaining on the surface
GFP-N-WASP
GFP-ΔWA
HA-PW
HA-ΔPWA
Time (min)
Time (min)

FIGURE 3.7. Over expression of the proline rich domain of N-WASP inhibits BCR trafficking from cell surface to LAMP1$^+$ compartments.

(A) A20 B cells were transiently transfected with GFP or HA tagged full-length protein of N-WASP (GFP-N-WASP), N-WASP lacking its WH2 and Acidic domains or WA domain (GFP-ΔWA), N-WASP missing the PRD and WA domains (HA-ΔPWA), the WA domain (GFP-WA) and the PRD and WH2 domains (HA-PW) of N-WASP. Twenty-four hours after transfection, the surface BCR were labeled with Cy3-goat anti-mouse IgG for 20 min at 4^{o}C and chased for 30 min at 37^{o}C. Cells were fixed, permeabilized, and labeled with anti-HA antibody for HA-tagged N-WASP constructs where necessary. Late endosomes were labeled with anti-LAMP-1 mAb (B), while actin was labeled with AF633-phalloidin (C) HA staining and GFP levels indicate the expression of transfected proteins. Shown are representative images from two independent experiments each. Arrows show areas where BCR and LAMP1 staining do not colocalize. Shown are cells from the same experiment but from different fields of view for ease of comparison between untransfected and transfected B-cells. Bar, 10 μm (D) Shown are quantitative bar graphs of the effects of domain overexpression from two independent immunofluorescence experiments each. Graphs depict percentages of total cell population on the y-axis that display the phenotypes noted on the x-axis. Mean transfection efficiencies are as follows: GFP N-WASP – 40.1%; GFP-ΔWA – 25.98%; GFP-WA –69.7%; HA-PW – 35.13%; HA-ΔPWA – 23.25%. On the top are shown illustrations of the domains used for the above experiment. This part of the figure has been adapted from [270].


WH1
IQ
CRIB
PRD (P)
WH2 (W)
A
N-WASP (1-501)
N-WASPΔWA (1-391)
N-WASPΔPWA (1-273)
IC (119-273)
PWA (244-501)
PW (265-481)
W (391-481)
HA/GFP
BCR
Merge
GFP-N-WASP
b
GFP-ΔWA
GFP-WA
d
HA–ΔPWA
e
HA-PW


B
BCR
HA/GFP
LAMP1
Merge
a
b
c
d
HA-ΔPWA
e
f
g
h
HA-PW
i
j
k
l
GFP-WA
C
BCR
GFP
Actin
Merge
GFP-WA


D
untransfected
HA-PW transfected
GFP-WA transfected
Cell percentages
120
100
80
60
40
20
0
BCR internalized
BCR:LAMP-1 colocalization
BCR:domain colocalization

WASP mutants containing the PRD were over-expressed. Over expression of N-WASP mutants that contain the C-terminal actin/Arp2/3 binding domain (Fig. *3.7Ac*) or the N-terminal segment of the protein (Fig. *3.7Ad*), both of which lack the PRD, did not perturb BCR internalization very much. When the HA-tagged PW domain is over-expressed in B- cells (Fig. *3.7Ae*), uncharacteristically large vesicular bodies coated with the HA-tag are observed. Only 42% of these HA-PW^{+} vesicles actually correlate with the staining patterns of the BCR (Fig. *3.7D*). This could mean that only 42% of these vesicles are derived from the BCR containing vesicles that are budding at the membrane, these are likely derived from the plasma membrane (PM) as only the BCR at the PM was labeled. These characteristic vesicular structures were not observed with the expression of the other GFP-fusion constructs containing the N-WASP PRD. The GFP-tagged ΔWA protein, which lacks the actin and Arp2/3 binding domains but possesses the PRD, shows primarily cytoplasmic staining but is able to redistribute to the cell periphery upon BCR stimulation. Interestingly, GFP-ΔWA and HA-ΔPWA expressing transfectants show adherence defects and were unable to adhere to poly-L-lysine coated slides. Unfortunately, this did not leave enough transfected cells for effective quantification. Preliminary studies by over-expression of the domain responsible for actin-polymerization (WA) (the C-terminal WH2, actin and Arp2/3 binding domains) hint that this domain may in part also be responsible for the efficient internalization of the BCR (Fig. *3.7Ac*). Statistical analysis of the immunofluorescence images suggests that almost 40% of the cells are unable to internalize their BCRs upon antigen encounter (Fig. *3.7D*). Even though this is a significant fraction, these studies need to be corroborated by flow cytometric analysis of the rates of BCR internalization in these transfectants. There is

some indication that this domain may actually play a bigger role in specifically targeting the BCR^+ vesicles to the $MIIC^+LAMP1^+$ compartments (Fig. *3.7B i-l* and inset & *3.7D*). The distribution of the GFP-tagged WA construct, is striking, as it seems to colocalize with the internalized BCR in over 80% of the transfected cell population able to internalize the antigen. BCR internalization in 60% of the GFP-WA transfected cells proceeds normally, but upon $LAMP1^+$ visualization, it is apparent that the internalized BCR does not co-localize with $LAMP1^+$ endosomes at later time points when compared to untransfected internal controls. Although BCR and LAMP-1 staining are perinuclear in location at 30 min, staining patterns indicate exclusion of the BCR from the $LAMP1^+$ compartment and this phenotype is aberrant, indicating a possible trafficking/vesicular fusion defect (Fig. *3.7D*). In fact, only 23% of cells that internalize their BCR show any colocalization of the LAMP1 and BCR. WA over expression does increase the levels of F-actin seen in $eGFP\text{-}WA^+$ cells (Fig. *3.7C*). This aberrant increase in F-actin content may play a role in disrupting normal BCR trafficking to the late endosomal compartments.

3.5 Discussion

The cell cytoskeleton is dynamic, and constantly reorganizing to adapt to environmental changes. Cytoskeletal dynamics are particularly important for lymphocytes because of their induced mobility and ability to form polarized immunological synapses with interacting cells. In this chapter, I focus on the regulation of the actin cytoskeleton in the immediate context of antigenic encounter by the B-cell. WASP-family proteins are key regulators of actin in all cells. In Chapter 2, it was demonstrated that Btk regulates the

actin cytoskeleton through WASP via activating Vav, the GEF for Cdc42 and Rac1, and altering phosphatidylinositide metabolism. The Btk xid mutation, which blocks Btk-mediated signaling, inhibits BCR triggered WASP activation and actin reorganization, consequently reducing BCR-mediated antigen internalization, processing and presentation to T-cells. In this chapter, WASP knockout mice were used to further study the role of WASP in B-cell. In contrast to B cells from Btk-deficient mice, B cells from WASP knockout mice did not show any significant alteration in BCR internalization and BCR-mediated signaling. Contrary to the B cell phenotype, WASP-knockout mice exhibit defective TCR-mediated signaling, endocytosis and activation of T-cells [222, 223]. Despite the lack of significant cellular defects in peripheral mature naïve B-cells, WASP deficiency significantly compromises B-cell development. It presents with exaggerated splenomegaly (data not shown), decreases in the number of immature T1/T2 B-cells, and a striking reduction in MZ B-cells. It remains to be clarified what results in splenomegaly and its exact effect on the mature B-cell population. Besides, fractional analysis of B-cell subsets indicates a problem, total numbers of mature and immature B-cells in these mice may be otherwise abnormal due to the splenomegaly. Further analysis is necessary of absolute numbers of cells obtained from these mice. Furthermore, B-cells from WASP knockout mice display a reduced ability to migrate, aggregate, adhere, spread, form polarized BCR caps, in response to LPS, IL-4 or anti-CD40 stimuli and express normal microvilli or protrusions in response to LPS alone but not LPS+IL-4, indicating an intrinsic defect in actin cytoskeletal reorganization [102].

If WASP was the sole actin regulator targeted by Btk, a deficiency in WASP would result in B cell defects similar to those in Btk-deficiency. The absence of significant cellular defects and partial morphologic and developmental defects in $WASP^{-/-}$ B-cells indicate the presence of additional actin regulators downstream of Btk. These may help rescue the complete ablation of B-cell responses. Because WASP and the related family member N-WASP share 50% homology at the protein level [229], it is tempting to speculate that N-WASP can partially substitute WASP function. To support the idea of redundancy, we found that N-WASP is naturally expressed in both mouse B cell lines and splenic B cells. Moreover, BCR activation increases the levels of phosphorylated N-WASP (pN-WASP) which are found in the vicinity of the BCR. In partial confirmation of the idea of functional substitution, the total levels of N-WASP were found to be higher in $WASP^{-/-}$ splenic B-cells. Concurrently, I also observed higher levels of pN-WASP in $WASP^{-/-}$ B-cells, this may be tied to the upregulation of total N-WASP expression or may reflect the heightened phosphorylation and activation of N-WASP under scenarios of WASP deficiency. Further confirmation of this increase will be needed by RT-PCR studies, which will look at the levels of N-WASP mRNA. The inconsistencies in the kinetics of pN-WASP increase between western-blotting and immunofluorescence or flow cytometry may reflect the limits and sensitivity of the techniques used. Thus, it may be important to follow up these experiments controlling rigidly for cell numbers and protein concentration.

In a bid to understand the endogenous role for N-WASP in B-cells, dominant negative transfection studies were used where various domains of N-WASP were overexpressed.

Over expression of the PRD of N-WASP reduced BCR internalization and trafficking of the BCR-antigen complex to the antigen processing compartments, suggesting a role for N-WASP in these processes. While over expression of the PRD of N-WASP may competitively inhibit both endogenous WASP and N-WASP as well as other SH3 domain containing proteins, these data provide the first indication for a potential role for N-WASP in BCR function and an ability for N-WASP to substitute for WASP in $WASP^{-/-}$ B cells. The increase in expression level of N-WASP observed here further supports this hypothesis. Interestingly, over-expression of the full-length GFP-tagged N-WASP had a dominant negative effect. In effect this could be because over-expression of the full-length protein mimics the over-expression of the N-WASP PRD and thus sequesters the same components which result in its dominant negative phenotype. Alternately, the dominant negative phenotype caused by the full-length protein could also be due to different protein-protein/protein-lipid/protein-substrate interactions sequestering away some important component(s). However, it also feasible that the normal function of N-WASP *is* in *deregulating* BCR internalization and trafficking perhaps by hyper-polymerizing actin and forming stable cortical actin structures. We also cannot exclude the possibility that the GFP-tag could interfere by blocking the function of certain domains of N-WASP, thereby leaving the unobstructed domains to participate in dominant negative interactions. However cumulative data from this study and another suggests that it may indeed be the PRD which is responsible for this abnormal endocytic phenotype seen with full-length N-WASP [270].

The exact role of WASP and N-WASP in BCR functions needs to be further examined. In addition to overlapping functions, these two proteins may function independently and yet collaboratively. For instance, N-WASP has been found to be localized on endocytic vesicles with actin comets in *Xenopus* extracts [271, 272], while WASP has been implicated in podosome and filopodia formation [102]. A clue to the involvement of N-WASP in vesicular movement can be seen with the characteristic staining pattern of some of the dominant negative N-WASP constructs. As can be seen in the overexpression of the HA-tagged PRD-WH1 fusion construct 'HA-PW', staining of the HA-tag corresponds with irregular vesicular bodies within the transfected cells. The nature of these abnormally large vesicular structures and role of N-WASP in their creation/function is thus far unclear. There is also no definitive proof that these vesicles are originating from the plasma membrane as at least 58% of these vesicles do not correlate with BCR staining in the cell. Furthermore, it seems that this particular domain shows a more severe internalization defect than the others, which may reflect the effect of over-expressing the PRD while simultaneously lacking the Acidic, actin-Arp2/3 binding domain. One could speculate that vesicular trafficking and intracellular fission/fusion events, which may determine the size and distribution of the vesicles formed, are regulated by N-WASP activity. In this model, the PRD may be important for recruiting and retaining the protein to the right sub-compartments. The subsequent coordination of the functions of the PRD and the actin-binding domain would ensure vesicular propulsion and precise fusion. Indeed, active cytoskeletal rearrangements have been shown to accompany many vesicle transport, fission and fusion events in a variety of cell systems [273-275]. Moreover, the PRD of N-WASP has been shown to interact with, an SH3 domain containing-accessory

protein syndapin [270]. Syndapin forms heterocomplexes with dynamin, and the interaction of N-WASP, syndapin and dynamin was shown to determine the formation of endocytic vesicles containing the transferrin receptor and their subsequent trafficking within the cell [270, 276]. Thus, N-WASP's PRD and WA domains may play a role in this process of membrane fission and vesicle transport. Some hints as to how N-WASP is recruited to sites of action is obtained by studying the redistribution of the PRD containing construct GFP-ΔWA. GFP-ΔWA, although lacking the actin and Arp2/3-binding domains, seems to be recruited to the cell periphery upon antigenic crosslinking of the BCR. This is may be significant as it implies that the interaction between N-WASP's PRD and its binding partners is a major determinant of its cellular location and function. This corroborates another study which found that interactions between N-WASP's PRD and SH3 domain containing proteins is significant in recruiting N-WASP to vesicles [231]. Also, the consistent association of the overexpressed GFP-WA domain (which expresses only the N-terminal WA domain of N-WASP) with the BCR is an important observation as this implies that the actin binding WA domain of N-WASP may interact with the BCR and may be responsible for its consistent association with the BCR. Thus, a role for N-WASP in endocytosis and intracellular transport is probable. Notably, this role seems particularly dependent on its PRD and to a lesser extent its actin and Arp2/3-binding domains.

Surprisingly, studies with $WASP^{-/-}$ mice suggest that WASP is not obligatory for endocytosis of the BCR or in BCR signaling. However, WASP seems to play an important role in B-cell development. Although the cellular deficiencies in T1/T2

immature B cells are not as severe as noted for some other immunodeficiencies [277, 278], the absolute reduction in cellularities and the severe decrease in MZB is an indication of the importance of WASP in B-cell development and lineage commitment. As MZB development is known to proceed via the activation of different signaling molecules than those in follicular B2 cells [52, 279], it may be that WASP plays a predominant role in the development of this lineage, this, however, remains to be tested. Previous findings from WASP deficient mice show that B-cells display reduced adhesion, migration and homing, abnormal splenic architecture and diminished germinal centers responses [228]. It is possible that the decreased motility of B-cells contributes to the diminished humoral responses and developmental delays in WASP-deficient mice. In this aspect WASP may play a role in cellular motility and migration, thereby contributing to the development of immunodeficiency by inhibiting cellular migration and adhesion, although this hypothesis has not been tested. Thus, under conditions of full-sufficiency N-WASP probably coordinates receptor-mediated endocytosis, intracellular vesicular movement and subsequent fusion events while WASP may be involved in mobilizing and organizing actin-rich structures at the cell-surface for cellular motility and formation of adhesion foci. Under conditions of WASP deficiency however, it is likely that N-WASP can complement WASP function albeit not perfectly, and this may be a consequence of its level of expression and constraints posed by its normal cellular functions. Further delineation of normal WASP and N-WASP function in B-cells would provide the necessary clues to understanding in what other pathways these molecules function, how the cell regulates two such closely related molecules and if this regulation is distinct or similar.

The presence and modulation of another WASP family protein i.e. N-WASP, in murine B-cells from $WASP^{-/-}$ mice further points to the idea that functional redundancy between members of the same family of proteins can determine the extent of observed phenotypic deficiency. Homologs of WASP, SCAR/WAVE proteins have been shown to be involved in actin-mediated cellular changes in several cell types [214, 280-282]. Whereas WAVE1 and WAVE3 are expressed primarily in neuronal cells, WAVE2 shows a more ubiquitous distribution with relatively high expression levels in hematopoietic cells [283]. More recently, WAVE-2 was shown to be required for formation of the immunological synapse in T-cells by recruiting actin binding proteins such as vinculin and talin [284]. WAVE family proteins lack the CRIB domain which is a hallmark of WASP and N-WASP and important in their auto-regulation [285]. These proteins also have a modular design and possess a WAVE homology domain (WHD) at their N terminus, followed by a basic region, a proline-rich region (PRR), and a C-terminal verprolin-cofilin-acidic (VCA/WA) region that binds to actin and the Arp2/3 complex. The basic region of WAVE-2 binds PtdIns-3,4,5-P_3, which is important for the cellular localization of WAVE-2 [283, 286, 287] and its activation is also dependent on the small GTPase Rac1[214, 285]. Since WAVE family proteins form a less stringently regulated family of actin modulating proteins and are known to be involved in the immune system, it was important to determine whether WAVE-2 is naturally found in B-cells from $WASP^{-/-}$ mice. Indeed up-regulation of WAVE-2 is observable in $WASP^{-/-}$ B-cells. However, its current role in B-lymphocyte activation is unclear. Previous data hints at the presence of a WASP/N-WASP independent mechanism of actin regulation, implicating another WASP-family

member [267]. The up-regulation of WAVE-2 in conditions of WASP deficiency thus, implies a role for WAVE-2 in B-cell function.

In this study, the role of actin regulators WASP and N-WASP in B-cell function was explored. I found that WASP deficient mice display a developmental delay in the B-cell subsets, with absolute numbers of immature (T1 and T2) B-cells reduced, while the marginal zone B-cell subset was severely diminished. WASP deficiency however, did not translate into a either reduction or significant cellular defect in mature B-cells. WASP deficient B-cells were able to internalize their BCR and carry out signaling normally. However, it was found that N-WASP plays an important role in BCR internalization and transport in B-cells Moreover, N-WASP's proline-rich domain is important in its role for BCR endocytosis and subsequent trafficking. Significantly, total levels of N-WASP and WAVE-2 are upregulated in unstimulated $WASP^{-/-}$ B-cells. Phosphorylated levels of N-WASP are concomitantly higher in $WASP^{-/-}$ B-cells and are constitutively associated with the BCR. Lastly I also show that actin modulation is regulated by BCR proximal signals and the phosphorylation of both WASP and N-WASP is modulated by Btk. With these studies I provide compelling evidence for the unique and redundant roles of WASP and N-WASP in B-cell activation and underscore the importance of regulated actin-remodeling for B-cell function. Further delineation of specific components involved in the activation and regulation of N-WASP will be important in establishing its role in B-cells and the degree to which it is able to complement WASP function. Moreover, a double knockout model is needed to verify whether or not WASP/N-WASP perform redundant functions in B-cells. Some headway has been made on that front with the

development of an siRNA model which effectively knocks down WASP and N-WASP to 20-30% of their original levels (Appendix A). Studies on the cell biology of WASP/N-WASP double negative B-cells are underway. It will also be important to clarify the exact role of WASP in B-cell development and further study the effect of WASP deficiency on the B1-subset of B-cells, known to be responsible for responses to T-independent antigens. This will be a particularly interesting study, as humoral responses to T-independent antigens are severely diminished in $WASP^{-/-}$ mice and WAS patients [207, 210, 228]. With the clarification of the roles of WASP and N-WASP in B-cells it is possible that a general understanding of their hierarchy and functions in cells emerges, which may be of immense therapeutic value in cancer, immunodeficiency and microbial pathogenesis.

Chapter 4: General Discussion and Future Directions

The actin cytoskeleton is an essential cellular component that drives cell migration, cell-cell adhesion, cell morphological changes, and cell surface spatial organization. Therefore, the actin cytoskeleton is vital for lymphocyte-mediated immune responses [241]. Lymphocytes rearrange their actin cytoskeleton in response to various extracellular effectors & most prominently during antigenic stimulation. This necessitates a mechanism which can translate the extracellular interaction of the antigen and antigen receptor to specific intracellular remodeling of the actin cytoskeleton. In B-cells several candidate molecules involved in early signaling are implicated in actin regulation. For example, CD19, Lyn, Syk, PLCγ2, Vav, Abp1, Bam32, Rap, Rac and Cdc42 are all thought to be vital in transducing signals from the BCR to the actin cytoskeleton [101, 102, 178, 194-196, 248, 288-291]. Antigen receptor-triggered actin rearrangement is required for the formation of immune synapses [292, 293] and the internalization and intracellular trafficking of antigens by the BCR for antigen processing and presentation [171, 191, 202, 245]. I have provided evidence that Btk can modulate changes in the actin cytoskeleton by controlling the activity of actin regulatory proteins WASP and N-WASP. Btk is able to accomplish this by controlling the phosphorylated levels of WASP and N-WASP. In addition it increases the levels of activated, phosphorylated Vav and initiates accelerated PtdIns-4,5-P_2 biogenesis at the membrane. PtdIns-4,5-P_2 affects the activation of WASP and possibly N-WASP by binding directly to the Basic domains of these proteins, while Vav generates GTP-bound Cdc42 which is also a crucial effector for their full activity. By controlling the activity of these components, Btk is able to direct the role

of actin in regulating antigen-uptake and transport by the BCR. Thus, Btk's modulation of actin dynamics ultimately determines the efficiency of antigen presentation.

Immunodeficiencies caused by the gene knockout or mutation of components involved in BCR signaling and actin regulation are incredibly similar in character. For instance, Btk (*xid*), Tec/Btk, CD19, PI3-K p85α, BLNK, WASP and Vav deficiencies in mice all block lymphocyte development in the same B-cell subsets and some of these deficiencies display abnormal activation of mature B-cells ([93, 94, 137, 145, 223, 228, 294-298] and Chapters 2 and 3). The similarity in their phenotypes suggests the importance of relaying BCR-mediated signals to the cell cytoskeleton in B cell activation and development. Priming of the cytoskeleton by early signals may be responsible for an appropriate cellular response to environmental cues such as antigen recognition and chemokine gradients. It is particularly important to note that the effect of Btk (*xid*), Tec/Btk, CD19, CD45, PI3-K p85α, BLNK, and especially WASP, Vav ([93, 94, 137, 145, 223, 228, 294-298] & Chapters 2 and 3), and Abp1 deficiency (personal communications, unpublished observations) on B-cell subsets is chiefly seen in marginal zone B-cells (MZBs) and to a lesser extent on B1 cells. Both subsets have specialized functions associated with the particular niches they occupy i.e. the peritoneal cavity (B1) and the marginal zone of splenic follicles (MZB). As pointed out before, these two subsets of B-cells play a central role in T-cell independent phases of the humoral response. More recently, intra-vital multi-photon microscopic studies demonstrated a high level of motility of MZBs in the mantle and marginal zones of the spleen and a distinct ability to capture and transport antigens after they are introduced into the system [52, 299]. It is likely that antigen

binding to the BCRs on MZBs directs this actin-driven cell migration. The reason why these cells are particularly affected is still not known. Perhaps the ability to migrate is a unique developmental constraint for MZB and B1-cells. This would explain the dependence of their developmental programs on proteins able to regulate cytoskeletal changes resulting in cellular motility. It will be interesting to see what actin-regulatory proteins are expressed in these cells.

The role of actin in the mature immune response is complex and multidimensional. It is further complicated by the presence of *several* closely related proteins of the WASP-family, which regulate the function of the actin cytoskeleton in lymphocytes. Thus, non-redundant functions of the WASP-family in lymphocytes have been traditionally hard to define. Any segregation of their roles would likely be based on their selective enrichment in certain cell populations, the differences they display in binding partners/affinities and distinct cellular functions. Of this family, two proteins, WASP and N-WASP gain precedence because they are represented together in several cells of the haematopoietic lineage, including B-cells [102]. Some indications as to the differential and perhaps non-overlapping functions of WASP and N-WASP have emerged from the studies in Chapter 3 and what is currently known about these proteins. For instance, models of N-WASP deficiency have revealed a pivotal role for it in embryogenesis (N-WASP deficient embryos abort spontaneously by day 12) [267], formation of focal adhesions[217], neuronal propagation [300, 301], clathrin-coated pit assembly during receptor mediated endocytosis [302, 303] and in actin-based motility of vesicles [272, 304] and certain intracellular pathogens such as *Shigella* and vaccinia virus [265, 267]. Indeed, the actin-

based spreading proteins of *Shigella* seem particularly adapted to use N-WASP and are unable to substitute WASP for this function [305]. Alternately, non-obligatory functions of N-WASP were discovered in fibroblasts, where an N-WASP deficiency did not affect the ability of the cell to spread via lamellipodia or protrude filopodia [267]. Initial studies in B-cells (Chapter 3) and fibroblasts [270] indicate that N-WASP seems to play a pivotal role in receptor-mediated endocytosis and intracellular movement of vesicles. This is not surprising, as it has been found to be responsible for the endocytosis of a variety of other cell surface receptors [270, 306], assist in clathrin coated pit formation [302], initiate actin comet tails on vesicles, propel vesicles [231], and aid the fusion of ligand rich vesicles with endosomes/lysosomes rich in proteolytic components [272]. Thus, N-WASP activity in B-cells is at least evident in endocytic events and vesicular trafficking. Perhaps more surprising is the fact that WASP expression is *not* mandatory for the internalization of ligand-bound BCRs in B-cells (Chapter 3). However, $WASP^{-/-}$ mice do show blocks in B-cell development (Chapter 3). By way of this and other studies [102, 228], it is credible to suppose that WASP may be primarily involved in cortical actin rearrangement. This would allow WASP to play main roles in cell motility and in the projection of cell surface structures such as filopodia and lamellipodia for antigen detection and accumulation. A combination of these roles by WASP would instruct B-cell development by directing competent migratory responses. This premise relies on the fact that normal haematopoietic cell development is highly dependent on their ability to migrate.

Thus far, relatively autonomous roles of WASP and N-WASP in the current model systems can be perceived in B-cell motility and development and during receptor-mediated endocytosis, respectively. However, a stipulation for the role of WASP in B-cells needs to be made here, especially since it seems to be in apparent conflict with the results from Chapter 2. In chapter 2, I showed that defective BCR internalization in Btk deficient systems correlates with the deregulation of WASP activity. This implicates WASP in receptor-mediated internalization. However, this discrepancy can be incorporated into the model if we consider the results from Chapter 3 which show that Btk is able to regulate N-WASP phosphorylation as well. Thus, a Btk deficiency would also affect the activation of N-WASP which may in fact result in the endocytic defects seen in that system. Direct proof for the preferential involvement of one protein over another would require the development of several more models of deficiency. In fact, the partisan roles for N-WASP and WASP in B-cell processes may be determined by a master regulator such as Btk. If true, this would underscore the ultimate importance of *BCR-specific* signals in actin-remodeling and receptor internalization.

There are certain additional caveats to the model of 'independent-functions' for WASP and N-WASP. For instance, the ability of WASP and N-WASP to compensate for each other cannot be ignored. WASP's role in the initial events of endocytosis and also in vesicle transport cannot be discounted on the basis that only N-WASP is able to conduct endocytosis under normal circumstances. Indeed, there is sufficient evidence that WASP plays a main role in receptor mediated endocytosis [223, 307, 308] and vesicular transport [271]. Furthermore, the unitary model for the role of WASP in B-cell

development is challenged by the recent development of a conditional knockout of N-WASP in T-cells. Studies with this knockout indicate that N-WASP, in addition to WASP, is a crucial part of the T-cell developmental programme [309]. Because B and T-cells are closely related lineages, this may also hold true for B-cell development. Instead it is plausible that WASP and N-WASP share roles in B-cells and their expression and distribution in the cell lead to the preferential activation of one over the other in certain scenarios. Moreover, certain signals may act redundantly to regulate the activity of these proteins in B-cells. Further delineation of the roles of WASP and N-WASP in B-cells will be achieved with the development of additional single and double-negative systems.

The presence and upregulation of an additional WASP family protein in B-cells – WAVE-2, adds another layer of complexity to BCR mediated changes to the actin cytoskeleton. Previous studies on the WAVE proteins show that they are particularly adapted to modulate changes at the leading edge of the cell membrane during cellular motility and are thus, involved in the protrusion of lamellipodia (WAVE-2) and dorsal ruffle formation (WAVE-1) [310, 311]. Based on these observations, it can be speculated that the relatively dilute nature of the block in B-cell development in $WASP^{-/-}$ mice is due to the continuing function of WAVE-2 in many compartments of developing B-cells. This assumes that WASP is most important in modulating cellular changes for cell migration and consequently steers normal B-cell development. Thus, it is this particular activity of WASP that the WAVE proteins are able to substitute. Yet, the *presence* of a developmental block indicates that some activity of WASP in B-cell development is non-redundant and cannot be replaced. This is an especially important distinction and may be

related to the fact that although members of the WAVE-family require several of the same binding partners as other WASP family proteins, they lack the *functional regulation* imposed on WASP and N-WASP. Thus, these developmental blocks may in fact reflect the necessity of a highly regulated system to manipulate actin and cell migration, one which is particularly responsive to BCR/pre-BCR mediated signals.

Specific antigen recognition by the BCR is the first step in the initiation of B cell signaling and activation. The subsequent step in a humoral immune response involves the acquisition of antigens via the surface immunoglobulin. B cells can respond to antigens presented to them in a variety of forms. Within the lymph nodes (LNs), they can bind and recognize antigens in an acellular, soluble context, [312-315] and it is now generally accepted that they also recognize immobilized *native* antigen presented to them on the surface of a number of different cell types, including follicular dendritic cells (FDCs), dendritic cells (DCs) and macrophages [316-318].The advent of high-resolution imaging techniques like total internal reflection microscopy (TIRFM), has allowed the study of detailed events at the plasma membrane very early after receptor crosslinking. One such study discovered that BCRs are able to form microclusters containing 10-100 BCR molecules upon encountering membrane-bound antigen [288]. These microclusters were further found to be sites of active signaling and recruited signaling active molecules Syk, PLCγ2, Vav and CD19 while simultaneously excluding phosphatases such as CD45 [194, 288].Thus, it seems that microclusters act as sites of assembly for numerous ‘microsignalosomes’ and may be responsible for signal initiation.

In order to explain the formation of microclusters and subsequent accumulation of antigen, a model of B-cell plasma membrane spreading and contraction has been proposed [193]. The rapid spreading response, mediated by the extension of lamellipodial protrusions, allows B cells to engage more antigens. The ensuing contraction phase allows for the collection of the antigen into a central aggregate, which is subsequently internalized. Interestingly, the early peak in actin polymerization seen in our initial studies with F-actin (Chapter 2) coincidences very well with spreading kinetics (1-3 minutes after antigen encounter) [193]. Moreover the kinetics of subsequent contraction of the cell surface (4-15 minutes) also correlates excellently with the gross depolymerization seen in the F-actin assays of Chapter 2. Expectedly, the disruption of early signaling with the Src kinase inhibitor PP2 or actin destabilization with agents like latrunculin A or cytochalasin B leads to a failure of the cells to spread and accumulate antigen [193]. This reinforces the roles for signaling and actin reorganization in the formation and sustenance of BCR microclusters and thus in B-cell activation. Indeed, actin is very important in the formation of TCR microclusters and to maintain signaling during T-cell activation [319]. Analogously, the same seems to be true of actin in B-cells. What remains to be determined is the exact regulation of actin-cytoskeletal dynamics during BCR microcluster formation. Studies in this dissertation help formulate a mechanism to control actin dynamics, which may regulate microclustering. For instance, the activation of Btk in the vicinity of such BCR microclusters and subsequent mobilization of phosphoinositides like PtdIns-4,5-P_2 and adaptor protein Vav (found to be associated with co-receptor CD19 [249, 320, 321]) could initiate the process of actin reorganization via WASP. Thus, since a constant association of rapidly-polymerizing

actin and activated WASP with the BCR is dependent on Btk (Chapter 2), Btk activation may be important in clustering the BCR. Furthermore, CD19 is also implicated in BCR-induced changes in cell shape, indicating its involvement in the molecular signalosome that forms at sites of BCR microclusters [288]. Here, it may direct changes in cellular shape by regulating membrane spreading and contraction to ensure antigen accumulation. Recent studies [194, 288] provide further proof of this by implicating not only CD19 in the spreading and contraction model but by also showing that PLCγ2 and Vav play an exhaustive role in this process. This is interesting as this doctoral study provides proof that Btk is important in the activation of Vav while Btk's role in PLCγ2 activation is already well-established [100, 153, 322]. This activation of Vav may be accomplished when CD19 co-clusters with the BCR, providing Btk with the opportunity to phosphorylate and fully activate Vav. Importantly, we now know that Vav activation is important for the activity of WASP, and the activity of both correlates very well with actin reorganization (Chapter 2 and [164, 323]). Thus, co-ligation of CD19 with the BCR may serve to lower the signaling threshold *and* retain all required signals for sustained WASP activation during antigen accumulation, internalization and trafficking from the microcluster.

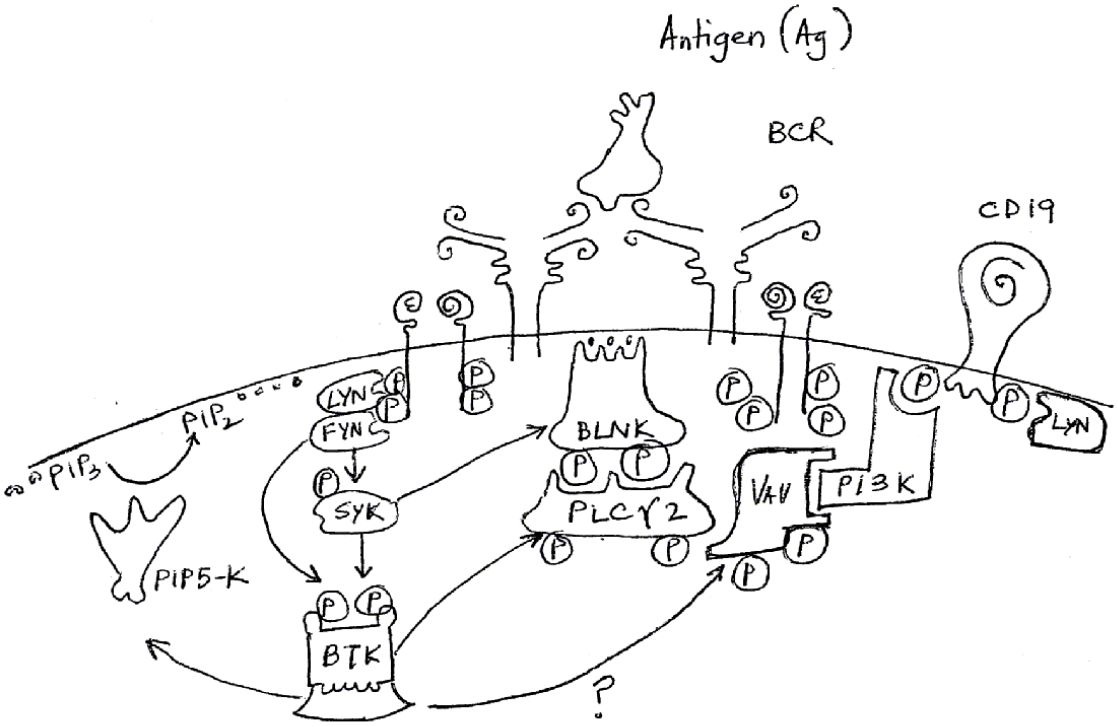
By integrating observations from my studies into the current understanding of the initial molecular events in B-cell activation, it is possible to extend these findings and formulate a model of B-cell activation (Fig. *4.1*). In this model, key molecules like Btk are able to regulate the ability of the B-cell to cluster the BCR on their cell surface by modulating the reorganization of the actin cytoskeleton in response to signals at the BCR. They

afford this manipulation by controlling the activity of several important factors like PLCγ2, Vav and ultimately WASP. Syk would be an important upstream component in mobilizing and fully activating Btk and maintaining a scaffold for signaling by phosphorylating adaptor proteins like BLNK. The subsequent activity of Btk would be directed in controlling the activities of specific proteins assembled around the scaffold. Btk is known to phosphorylate and activate PLCγ2 directly [100, 153, 322] and thus initiate the Ca^{2+} flux and subsequent PKC-β activation. This study provides evidence that Btk is involved in the phosphorylation and activation of both Vav and WASP. Furthermore, by increasing the levels of PtdIns-4,5-P_2 in the vicinity of the BCR, Btk provides additional mechanisms for the activation of WASP-dependent and independent [250, 324] mechanisms of actin reorganization. The activation of WASP would productively rearrange the actin cytoskeleton to constrain BCR microclusters for the formation of immunological synapses, to drive the pinching off of the clathrin-coated vesicles containing antigen-BCR complexes, and to direct the transport of the antigen to the MHC II containing vesicles. Subsequent surface presentation of antigenic peptide-MHC II would recruit T-cell help and launch the adaptive immune response.

The migration of the B cell into the correct splenic/LN capsular areas, interaction with cells that are able to immobilize antigens on the cell surface, spreading and contracting of the B cell, and microclustering of BCR to initiate signaling and antigen accumulation are excellent examples of the degree of flexibility expected of the B cell architecture so early in the immune response. In light of new evidence and the studies presented here, it appears that the events of signal initiation, actin reorganization and the internalization of

FIGURE 4.1. A model for early B-cell activation.

Incorporating results from Chapter 2 and 3, a hand drawn model summarizes the sequence of events in the B-cell after encountering an antigen. Antigenic crosslinking of the BCR causes conformational changes in the BCR and Igα/Igβ heterodimer and leads to the activation of Src kinases and subsequently the activation of kinases Syk and Btk. This leads to the recruitment and phosphorylation of adaptor protein BLNK in a BCR dependent manner. Simultaneously the phosphorylation of CD19 by Src kinases leads to the recruitment of PI3-K and Vav via the CD19 dependent pathway. Btk's recruitment to the plasma membrane (PM) via the interaction of its PH domain with PtdIns-3, 4, 5-P_3 or its interaction with BLNK, brings PIP5-K to the PM. The activity of PIP5-K at the membrane generates PtdIns-4, 5-P_2, which is a substrate for both PLCγ2 and PI3-K, as well as an activating factor for WASP. Btk is subsequently able to initiate the phosphorylation of both WASP and Vav at the PM. This has a three-fold effect on the activity of WASP. Firstly, Vav is able to generate a crucial WASP activating factor – GTP-bound Cdc42 which binds to WASP and initiates the opening up of its autoinhibitory loop. Secondly, Btk dependent PtdIns-4,5-P_2 increases promote the binding of PtdIns-4,5-P_2 to WASP and also help activate it. Thirdly, Btk-mediated phosphorylation of WASP further augments its ability to nucleate and polymerize actin. Eventually actin polymerization in the vicinity of the BCR leads to membrane deformation and probably aids in the formation and pinch-off of vesicles containing antigen bound BCR. Subsequent directed polymerization of actin may help direct correct intracellular vesicular trafficking.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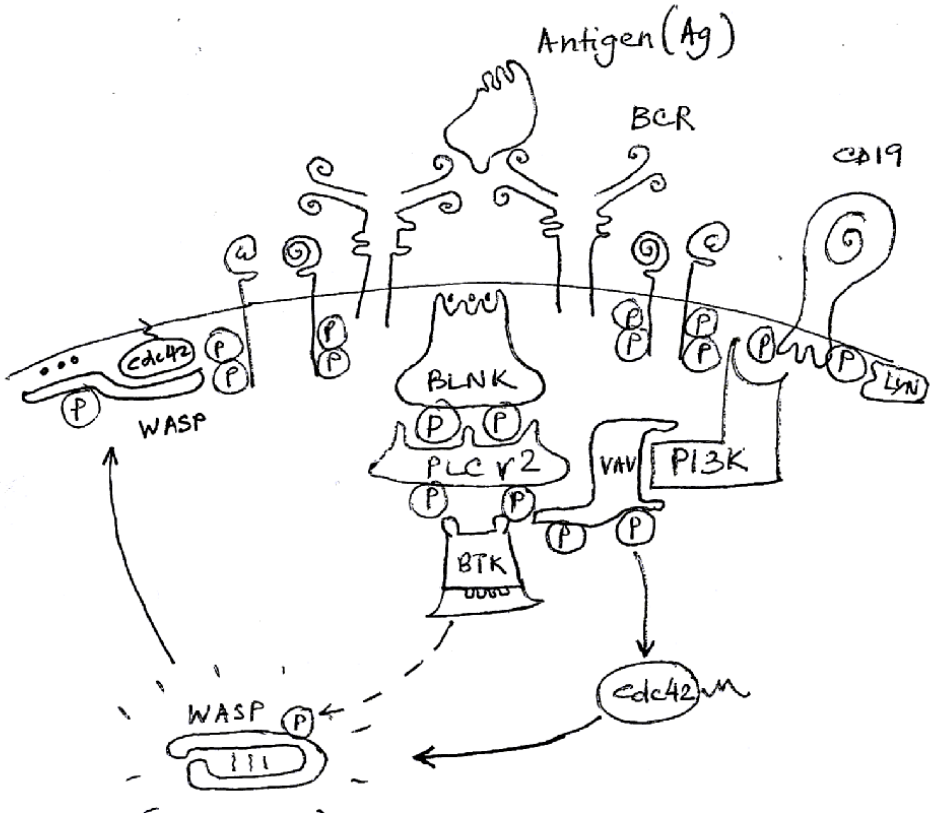
BCR
CD19
LYN
FYN
SYK
BLNK
PLCγ2
VAV
PI3K
LYN
PIP2
PIP3
PIP5-K
BTK
?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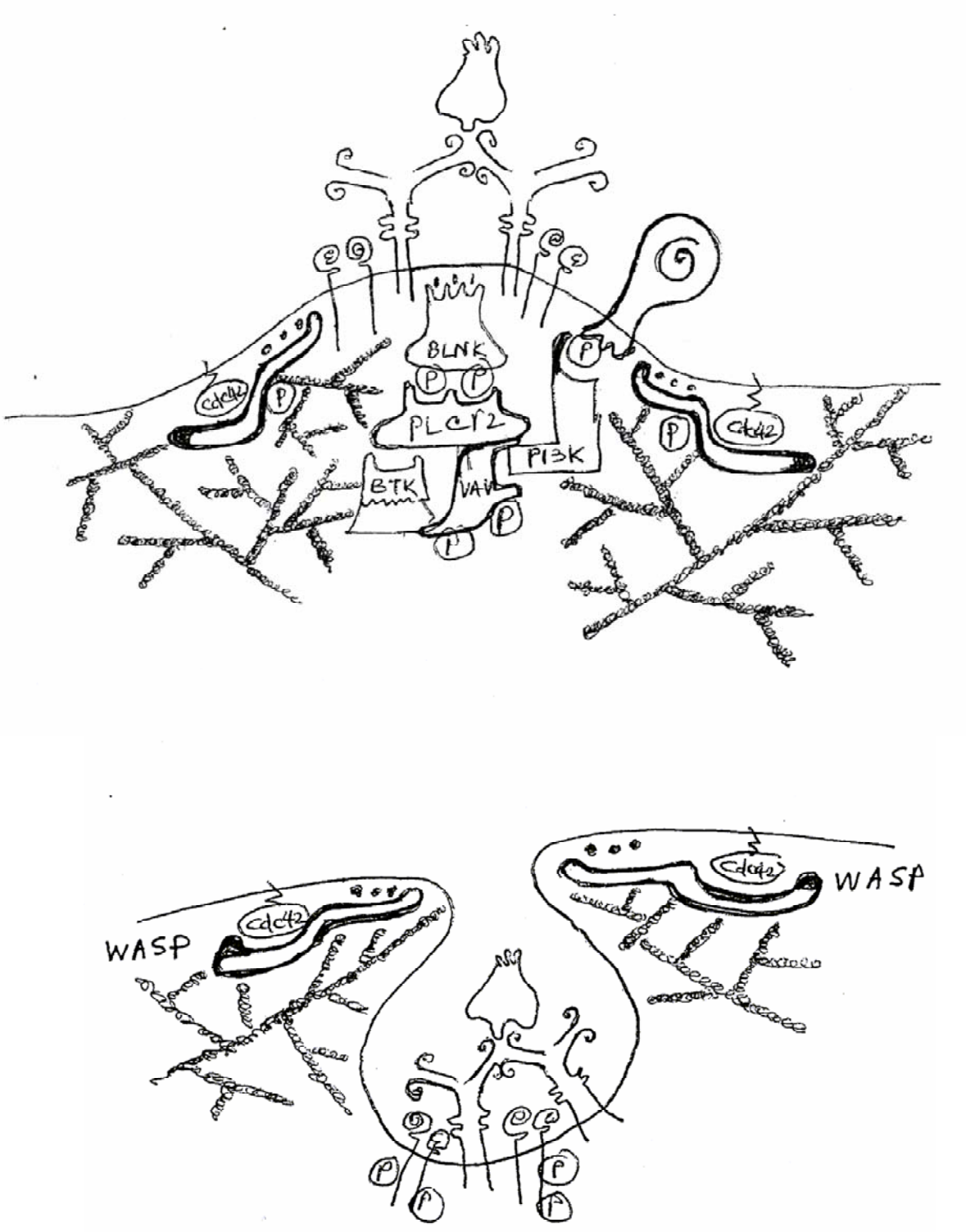
BLNK
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PLCγ2
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VAV
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Cdc42
WASP
Cdc42
WASP
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the BCR- antigen complex are intimately connected in establishing a seamless pathway for the antigen to be accumulated by the B cell. The integrity of this pathway is often hijacked during infectious diseases, lost in immunodeficiency, or subverted in autoimmunity and cancer. A contemporary working model of the complex biology of lymphocytes and the determinants for their activation plays an important role not only in developing therapeutic solutions to these problems but in gaining fuller awareness of the elegant laws of nature behind them.

4.1 Future Directions

As noted above, a WASP deficiency seems to entail progressive developmental defects associated with B-lymphocytes. The subset most affected is the marginal zone B-cell subset, widely regarded as more motile and fast-reacting than the conventional B-2 cells. The B-2 or follicular B-cell subsets are also affected somewhat in terms of absolute cellularity in the absence of WASP, although any significant impairment in signaling and other functions remains to be verified. This implies that B-cells from a model of WASP-deficiency are unable to mature/differentiate normally. Some questions that arise as a consequence of this observation are – what role does WASP play in developing B-cells? Is the role of WASP largely restricted to migration/chemotaxis? Are there intrinsic defects in the stromal milieu of the bone-marrows of WASP deficient mice, associated with/directly dependent on WASP? Interestingly, responses to T-independent polysaccharide antigen in WAS patients is abnormal, such differences are also seen in $WASP^{-/-}$ mice which show a much diminished response to T-independent antigens [224, 228]. Thus, it would also be important to study the effect of WASP deficiency on the

development and activation of B1 cells, which are primary mediators of responses to T-independent antigens.

Several methods of inquiry could be used to answer the above queries. Initially it would be important to test whether or not WASP$^{-/-}$ mice have a B1 subset of cells. This can be accomplished by extracting the gut associated lymphoid tissue (GALT) from wt and WASP$^{-/-}$ mice and harvesting the B-cells. Subsequent labeling of cell surface markers specific for B1 cells as well as analysis by multi-parameter flow will help determine if this subset of B-cells is indeed lacking. In order to follow the maturation and migration of MZB and B1 cells in WASP$^{-/-}$ mice, bone-marrow chimeras can be created where donor WASP$^{-/-}$ or wt bone marrows enriched for previously described B1 and MZB precursors [325, 326] are transplanted into lethally irradiated host mice of wt origin. 8 weeks after bone marrow transplants, the spleens, bone marrows and the gut associated lymphoid tissue (GALT) can be harvested from the host mice. The ability of these cells to home to and populate the right compartments (i.e. spleen and GALT) can be measured by staining for cell-specific markers. Multi-parameter flow-cytometry can be used to designate each subset and to measure cell numbers within each subset. If the specific precursors from WASP$^{-/-}$ bone marrows are not able populate the right compartments in comparable numbers to wt bone marrow transplants, it would indicate an intrinsic deficiency of these cell subsets in WASP$^{-/-}$ mice to home to their designated niches. Moreover, they would be retained in the bone marrow and will be measurable there as a surviving precursor populations. Alternately, bone marrows from WASP$^{-/-}$ and wt mice can be transplanted in to irradiated μMT mice. Since the μMT mice lack all B-cell subsets, the repopulation of

the different B-cell compartments in these μMT host mice would be a direct consequence of the donor bone marrows. Thus, comparing the cellularities of repopulated B-cell subsets from wt and WASP$^{-/-}$ donors, by the means mentioned above, will afford some delineation of the defects associated with the WASP$^{-/-}$ phenotype. If MZB and B1-precursors can be effectively labeled, we can follow the actual migration and development of these subsets via intra-vital multi-photon microscopy. Intra-vital multi-photon microscopy has been instrumental in understanding cell location, movement and cell-cell interaction in live animals. In this instance, intra-vital staining and multiphoton microscopy can be used to follow the kinetics of maturation for precursors of B1 and MZB-cell subsets using CFSE/cell specific labeling and bone marrow transplants.

With regards to the involvement of other WASP-family proteins in lymphocyte development and migration, it would be extremely interesting to see whether or not a conditionally doubly deficient model of WASP and N-WASP is able to develop B-cells properly. This may be achieved by extending our collaboration with Dr. Scott Snapper from Harvard University, to obtain their conditional doubly deficient WASP/N-WASP model in B-cells in mice. Just as important as these studies, would be the necessity to detect the presence of endogenous N-WASP in MZB and B-1 cells. Since the lack of WASP likely affects these cells severely it may be important to detect not only N-WASP but any other potentially redundant proteins such as WAVE-family proteins (specifically WAVE-2) in these cells. It is speculated that these particular subsets of B-cells, lack the redundant WASP-family proteins. It is hugely important to understand the role of actin nucleating proteins like WASP, N-WASP and WAVE in B-cell development and

activation as sources of therapeutic relevance for not only Wiskott Aldrich Syndrome and related immunodeficiencies, but for microbial infections may emerge in these studies.

The role of WASP in B-cell signaling has been confused by the presence of contradictory data; it would be very exciting to challenge the present model of WASP activation and observe what feedback roles it plays in BCR signaling. Its role in FcR signaling has not been very well explored. Thus, the balance between positive and negative signaling could rest on proteins like WASP which are responsible for transducing cellular changes and which may feedback to the signaling pathways once the changes have been made. Moreover, the kinetics of recruitment of a wide variety of signaling molecules to the BCR microcluster is not well established. With the advent of Total Internal Reflection Fluorescence Microscopy (TIRFM) combined with the power of Fluorescence Resonance Energy Transfer (FRET), detailed studies on the involvement of key interacting proteins at the BCR on the cell-surface have begun. By way of using GFP- and GFP-variant (like Red Fluorescent protein, RFP; Yellow Fluorescent protein, YFP; Cyan Fluorescent protein, CFP) fusion proteins of Btk, N-WASP, WASP, actin, dynamin, Vav, PKC, etc. and studying their sub-cellular location before and after BCR crosslinking, a kinetic model for B-cell activation can be established. Additionally, the roles of WASP and N-WASP at the cell surface can be clarified by using GFP -fusion protein versions of both proteins and studying their distribution during receptor endocytosis and cell spreading. Intriguingly, a seminal study by Merrifield et al. [303] simultaneously imaged actin and clathrin at the ventral plasma membrane of live cells using time-lapse evanescent field or total internal reflection (TIRF) illumination to reveal transient bursts of actin

accumulation, which frequently coincide with the disappearance of clathrin-coated structures (CCS) containing the transferrin receptor (TfR). Interestingly, both N-WASP and the Arp2/3 complex also accumulate at sites of clathrin pit endocytosis [302]. These studies combined with the observations presented in this dissertation provide further impetus for similar studies in B-cells.

The observation that WASP and N-WASP can localize in the nuclei of B-cells and recent reports of the role of N-WASP nuclear localization in the expression of heat shock protein 90 (hsp90) and RNA-polymerase mediated nuclear transcription in fibroblasts by way of nuclear actin polymerization [327-329], may hint at an hitherto unexplored role of WASP and N-WASP in nuclear transcription in B-cells. It would be highly advantageous to study this process in B-cells and identify nuclear factor binding partners and perhaps extend the role of these proteins in the cell cytoplasm into the nucleus as well, establishing a unique role for them in the progression of the B-cell cycle.

The development of disease and infection is a complex mechanism thwarting the optimal immune response and its entire arsenal. Every component of the immune system is integrally involved in responding with force and alacrity to danger signals. Universal components of regulation are often the reducible common factors between the cells – of which the cell cytoskeleton forms a significant shared feature. The dysregulation of the cell-cytoskeleton has far-reaching consequences as it affects a plethora of cellular activities. As it cannot be easily accomplished because of the degree of redundancy in proteins that regulate it, any deficiency that presents itself within set criteria is

significant. When such deficiencies present themselves they indicate a severe deregulation of their components. An appropriate understanding of all components in the regulation of the cytoskeleton is thus required to study critical processes like the progression of cancer, immunodeficiency and infection, as the cytoskeleton is implicated in all of them.

Appendix A: A system for siRNA-mediated knockdown of WASP and N-WASP in B cells.

In order to further delineate the role of WASP and N-WASP in B-cells, I have optimized the siRNA method to knock down the gene expression of WASP and N-WASP individually or together. Information on this system/model of double deficiency is presented in this section.

A.1 Methods

A.1.1 siRNA transfection and analysis

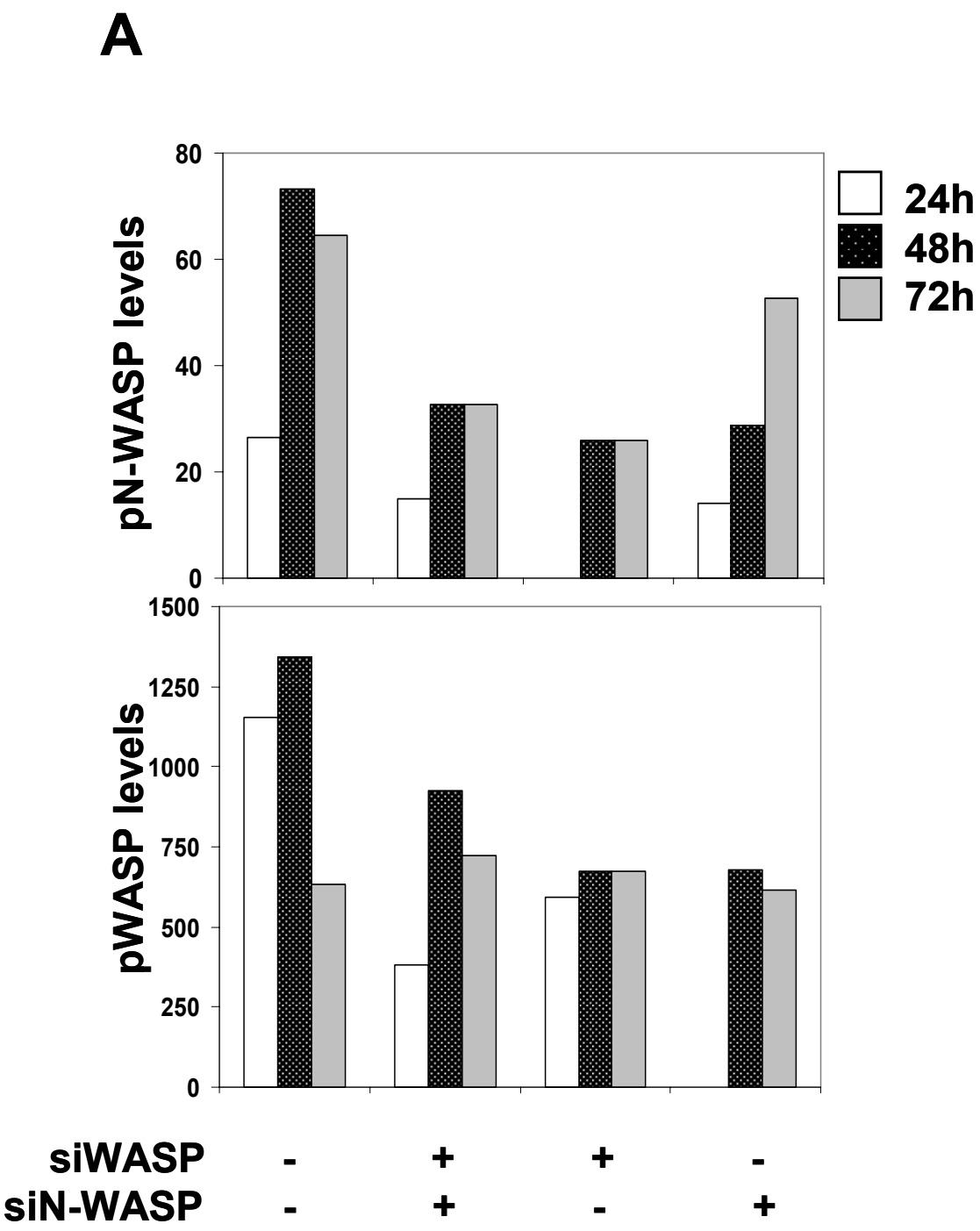
Sequences with the potential to target mouse WASP and N-WASP mRNA were pre-designed by Santa Cruz Biotechnology and provided as a mixture of three putative sequences. *WASP siRNA (m)* Duplex 1 Sense Strand: CCACAGUUGUUCAGCUCUAtt; Duplex 2 Sense Strand: CGAGUGGAUUCAAACAUGUtt; Duplex 3 Sense strand: GAACCUCAUACCCAAUCUAtt. mRNA accession: NM_009515. *N-WASP siRNA (m)* Duplex 1 Sense Strand: GACAGGGCAUUCAAUUGAATT; Duplex 2 Sense Strand: CCUGUCUAUUGAUCUGUAATT; Duplex 3 Sense Strand: CCUACCUACUUUCAAUACATT. mRNA accession:NM_028459. siRNA concoction obtained from Santa Cruz were re-suspended in RNA-ase free water and nucleofected into A20 cells using Amaxa, at varying concentrations (0, 1, 2, 4, 8 μg/mL). The empty vector, pMax eGFP (2μg/mL), was co-transfected with the siRNA to aid in identifying $siRNA^+$ cells, as efficiency of vector uptake is increased by prior uptake of siRNA and vice versa. Transfected cells were washed and fixed at varying time points

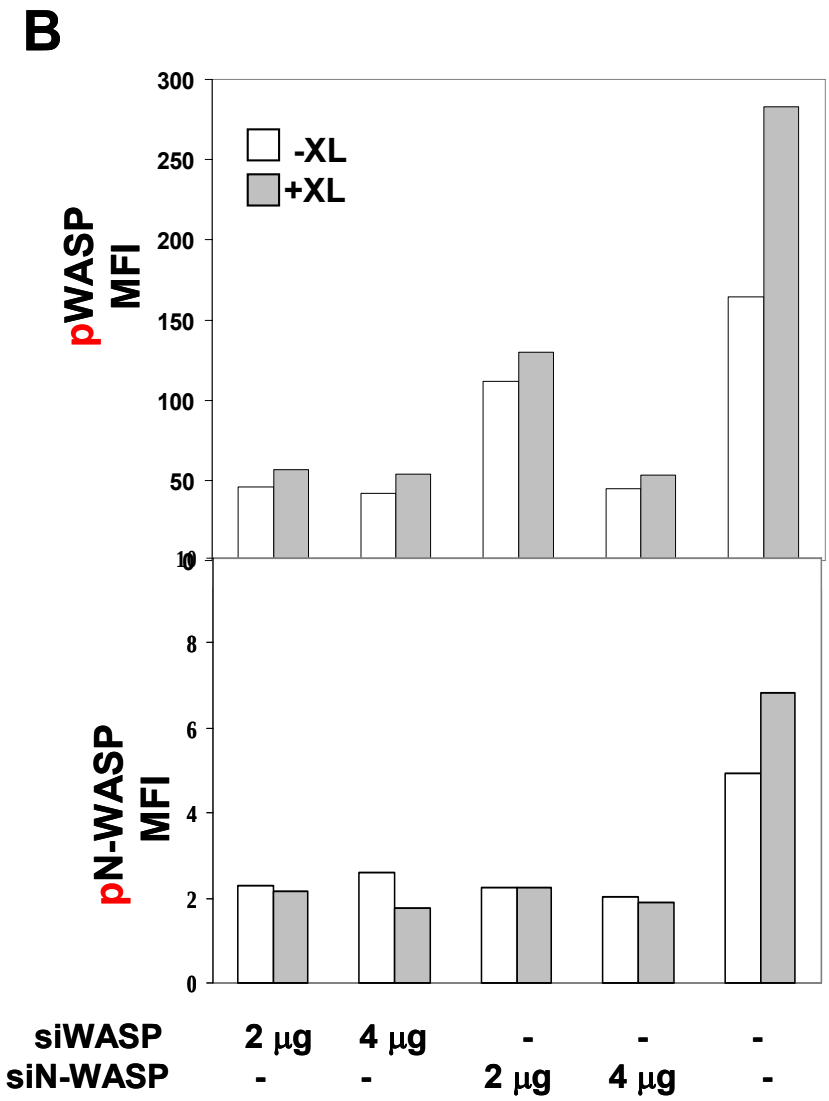
after transfection – 3, 5, 12, 24, 48 and 72 h. They were permeabilized and levels of endogenous phosphorylated WASP and phosphorylated N-WASP were measured in unactivated B-cells using anti-phosphorylated WASP (Ser483/484) and anti-phosphorylated NWASP (Tyr^{256}) and PE-labeled secondary antibodies. Cells were then analyzed using FACS-Calibur and cells were gated for both eGFP expression as well as eGFP non-expression, as the transfection efficiency of the smaller siRNA molecules is far greater than the transfection efficiency of the eGFP-vector. The levels of siRNA mediated protein shutdown within each gate were measured with specific protein staining levels. Percent shutdown for each protein was calculated by measuring the mean fluorescence intensity (MFI) of phosphorylated levels of WASP and N-WASP and comparing these levels between untransfected and $eGFP^+/eGFP^-$ $siRNA^+$ cells. For immunofluorescence assays, siRNA transfected cells were washed and adhered to poly-lysine-coated slides (Sigma-Aldrich) for 20 min at 37°C, the BCR was labeled using Cy3-Fab anti-IgG for 20 min at 37°C. The cells were then fixed with 4% paraformaldehyde, permeabilized (0.05% saponin, 10mM HEPES, 10mM Glycine and 10% Fetal bovine serum in DMEM) and stained for pWASP or pNWASP using rabbit anti-pWASP [330] or rabbit anti-pNWASP [Tyr^{256}] and AF 488 conjugated anti-rabbit pAb. Cells were mounted with gel mount (Biomeda, Foster City, CA) and analyzed using a laser-scanning confocal microscope (LSM 510; Zeiss, Oberkochen, Germany). WASP/N-WASP knockdown was confirmed by looking at comparative staining intensities of transfected and untransfected cells.

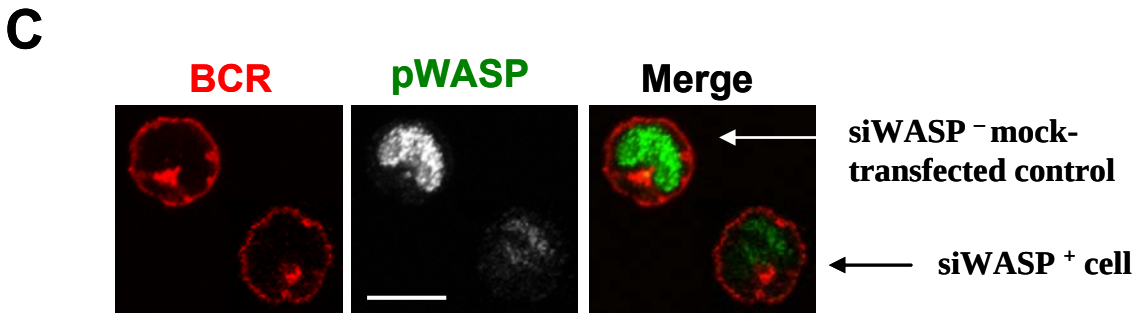
FIGURE A.1. siRNA-mediated silencing of WASP and N-WASP.

(A) Putative siRNAs against WASP (siWASP) and N-WASP (siN-WASP) were introduced into A20 B-cells by electroporation either together or individually. Cells were harvested at 24, 48 and 72h and basal levels of pN-WASP and pWASP in cells were measured using rabbit anti-mouse pN-WASP (Y256) or pWASP (S483/S484) and PE-conjugated secondary antibodies by flow cytometry. siRNA-transfected cell populations were compared to mock-transfected cells which served as a control. Cell populations were divided with population markers on the basis of their eGFP expression and fluorescent staining levels of pWASP or pN-WASP. The basal and activated levels of pWASP and pN-WASP in siRNA-transfected populations were normalized to basal and activated levels of pWASP and pN-WASP in the mock-transfected cells. Populations showing differences in pWASP and pN-WASP levels when compared to mock-transfected controls were gated and their mean fluorescence intensity measured for comparison. (A) Time-dependence of siRNA-mediated silencing of WASP and N-WASP was measured and standardized. Shown are mean fluorescent intensities (MFI) of pWASP and pN-WASP staining graphed against the conditions of the experiment at three separate times - 24h, 48h or 72h after siRNA electroporation. Levels of these proteins were measured in cells which were transfected with 2μg/ml of each, siRNA for WASP (siWASP) (+/-), siRNA for N-WASP (siN-WASP) (-/+), siRNAs for both (+/+) or neither (-/-). (B) Efficacy of siRNA-mediated silencing was tested using two different concentrations of siWASP and siN-WASP and under conditions of BCR crosslinking after transfection. Cells were electroporated with siRNA, harvested 24 h (for siWASP) or 48 h (for siN-WASP) later, and incubated with goat anti-mouse IgG (20 μg/mL) at 37^{o}C

for 10 min to cross-linking the BCR. Cells were fixed, permeabilized, labeled with pWASP and pN-WASP specific antibodies, and analyzed using flow cytometry. Shown are representative MFI from two independent experiments. (C) A20 B cells which were either mock-transfected or transfected with WASP siRNA (siWASP) were fixed, permeabilized, and labeled with rabbit anti-pWASP and an AF 488-conjugated secondary antibody individually and also mixed together and stained together. The Zeiss 510 software was used to measure intensity of pWASP staining in mock and siWASP transfected samples, compensating for background. Cells displaying reduced staining levels of pWASP in siRNA transfected samples when compared to mock-transfected cells were considered to be $siRNA^{+}$. Shown are cells from the same experiment but different transfection conditions for ease of comparison (Bar, 10 μm).


A
80
60
40
20
0
pN-WASP levels
24h
48h
72h
1500
1250
1000
750
500
250
0
pWASP levels
siWASP - + + -
siN-WASP - + - +


B
300
250
200
150
100
50
-XL
+XL
pWASP MFI
8
6
4
2
0
pN-WASP MFI
siWASP 2 µg 4 µg - - -
siN-WASP - - 2 µg 4 µg -


C
BCR
pWASP
Merge
siWASP⁻ mock-transfected control
siWASP⁺ cell

A.2 Results

As mentioned above, the siRNAs for WASP and N-WASP were introduced into A20 cells by electroporation wither individually or together. The protein expression levels of WASP and N-WASP were monitored by flow cytometry. The most effective knockdown was observed at 24 h for WASP and 48 h for N-WASP after the electroporation (Fig. *A1A*) and at 2 μg/ml siRNA for WASP but 4 μg/ml siRNA for N-WASP (Fig. *A1B*). Under optimal conditions, WASP expression was knocked down to 20%, but N-WASP expression only downed down to 30% of their original levels (Fig. *A1B*). Unfortunately, siRNAs designed to be specific for either WASP or N-WASP inhibited the expression of both WASP and N-WASP. For instance, the ability of both siRNAs against WASP (siWASP) and N-WASP (siN-WASP) to knockdown pWASP levels are almost identical at all concentrations tested (Fig. *A1B*). Additionally, among the $siRNA^+$ cells, both concentrations of siWASP (2μg/mL and 4μg/mL) and 4μg/mL of siN-WASP were able to knockdown pWASP levels most efficiently by 85-92%. The knockdown of pWASP was verifiable by immunofluorescence analysis; $siRNA^+$ cell showed markedly reduced levels of pWASP, compared to $siRNA^-$ cells (Fig. *A1C*). Interestingly, similar to $WASP^{-/-}$ B-cells these cells were also able to internalize their BCRs normally upon crosslinking. Taken together, this data shows that a system is available for the study of double deficiency of WASP and N-WASP in the same cellular system. Further verification of knockdown would be assessed by Quantitative RT-PCR. Moreover, with the advent of highly finessed electroporation techniques, siRNA mediated knockdown of N-WASP in $WASP^{-/-}$ splenic B-cells is also foreseeable.

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