Interaction of TAPP adapters with the phosphoinositide PI(3,4)P2 regulates B cell activation and differentiation

by

Ivan Landego

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree

MASTER OF SCIENCE

Department of Immunology
University of Manitoba
Winnipeg, Manitoba

THESIS ABSTRACT

Phosphoinositide 3-kinase is a family of lipid kinases that function by phosphorylating the D3 position of phosphoinositide (PI) lipids generating PI(3)P, PI(3,4)P2 and PI(3,4,5)P3. These D3 phosphoinositides regulate various cellular processes through the recruitment of effector proteins containing lipid specific pleckstrin homology (PH) domains. PI phosphatases such as PTEN and SHIP function to restrain PI3K signaling by limiting the amount of D3 PI available for binding. Deletion of either PTEN or SHIP significantly alters B cell function and humoral immune responses. TAPP1 and TAPP2 are dual PH domain containing adaptors which selectively bind the phosphoinositide PI(3,4)P2 via their C-terminal PH domains. PI(3,4)P2 is a lipid messenger generated by PI3K and through the inositol phosphatase activity of SHIP. The function of PI(3,4)P2 remains incompletely understood. To identify the functional role of TAPP-PI(3,4)P2 interactions, we utilized a knock-in (KI) mouse bearing mutations within the PI-binding pocket of both TAPPs. Our study assessed the effect of PI3K dependent KI mutation on B lymphocyte development, activation and antibody production. Flow cytometry analyses of lymphoid tissues found that TAPP KI mice develop relatively normal frequencies of mature B cell populations with the exception of peritoneal B1 cells, which are increased by approximately 50%. Strikingly, TAPP KI mice developed substantially elevated serum antibody levels. TAPP KI mice were able to generate high affinity antigen-binding antibodies upon immunization with NP-OVA in alum adjuvant; however, total immunoglobulin production was markedly increased under this immunization condition.

We further assessed the germinal centre (GC) response, which are known to require PI3K signaling and a hallmark of T cell dependent (TD) antibody responses. TAPP KI mice generated larger germinal centers (GC) upon immunization, which was associated with increased GC B cell survival. We further assessed whether uncoupling of TAPPs from PI(3,4)P2 alters B cell signaling and functional responses in vitro. B cells purified from TAPP KI mice were found to have altered functional responses in vitro, with significantly increased survival and cell division following antigen receptor cross-linking. Consistent with increased cell survival, TAPP KI B cells show increased Akt phosphorylation on Ser473 and Thr308 after antigen receptor cross-linking. However, reconstitution of B cell deficient mice with either WT or TAPP KI B cells was found to generate similar GC responses, suggesting that activation of other cells may contribute to the enhanced in vivo responses. Consistently, when we examined the CD4+ T follicular helper cells, a subset providing critical cues to GC responses, we found increased expression of ICOS activation marker. Our results indicate the interactions of TAPP adapters with PI(3,4)P2 serve to restrain lymphocyte activation and limit antibody production, providing the first in vivo evidence that this interaction is important for immune function.

ACKNOWLEDGEMENTS

I would like to extent my sincere gratitude to my supervisor and mentor, Dr. Aaron Marshall, for extending the opportunity to become a part of his research team. Throughout the years, your continued support and guidance has contributed substantially to my personal and professional successes. Thank you for challenging me and seeing my potential as a scientist.

I would also like to extend many thanks to my committee members, Drs. Jude Uzonna and Hao Ding, for their support, constructive criticisms and encouragement throughout my graduate program.

I am indebted to the contributions of Tingting Zhang and Sen Hou who provided me with my initial training and who have helped me 'TAPP' into the project. I would also like to thank all the past and present members from the Dr. Marshall lab for their insightful discussions, technical assistance and friendship.

Lastly, I would like to thank all the members from Department of Immunology for making the lab an enjoyable environment to learn. I would also like to extend a special thanks to the administrative staff, Karen Morrow and Susan Ness, for all their hard work in the department.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS:

4-Phosphatases	Inositol polyphosphate 4-phosphatase
Ag	Antigen
AID	Activation induced cytidine deaminase
Ab	Antibody
Alum	Alumumin potassium sulfate
BAFF	B cell activating Factor
Bam32	B lymphocyte adapter molecule of 32 kDa
BCR	B cell antigen receptor
BCAP	B cell activator adapter for PI3K
BLNK	B cell linker
BM	Bone marrow
BPK	B cell progenitor kinase
Btk	Bruton tyrosine Kinase
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CSR	Class switch recombination
DAPI	4, 6 diamidino-2-phenylindole
DAPP1	Dual adaptor for phosphotyrosine and 3-phosphoinositides-1
FDC	Follicular Dendritic Cell
FO	Follicular
Foxo	Forkhead box class O
FYVE	Fab1, YOTB, Vac1, EEA1

GC	Germinal centre
H_2O_2	Hydrogen peroxide
IC	Immune complex
ICOS	Inducible co-stimulator molecule
Ig	Immunoglobulin
IL	Interleukin
INPP4	Inositol polyphosphate 4-phosphatase
ITIM	Immunoreceptor tyrosine based inhibitory motif
KI	Knock-in
KO	Knock-out
LFA-1	Lymphocyte function associated antigen
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mTORC	Mammalian target of rapamycin complex
MZ	Marginal zone
NP-OVA	(4-hydroxy-3-nitrophenyl) Acetyl - ovalbumin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PALS	Periarteriolar lymphoid sheath
PDK1	3-phosphoinositide-depenedent protein kinase-1
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-kinase
PI	Phosphoinositide

PIP5K	Phosphatidylinositol-4-phosphate 5-kinases
PKB	Protein kinase B – also known as Akt
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTPL1	Protein tyrosine phosphatase like protein-1
PX	Phox homology
RAG-1/2	Recombination activation gene-1/2
SH2/3	Src homology domain 2/3
SHM	Somatic hypermutation
SHIP	SH2 domain containing inositol 5’ phosphatase
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
TAPP	Tandem PH domain containing protein
Tfh	T follicular helper cells
T1, T2, T3	Transitional type 1, type 2, type 3
Tg	Transgenic
TI-I	Type I T-independent Antigen
TI-II	Type II T-independent Antigen
TD	T cell Dependent Antigen
Tec	Tyrosine kinase expressed in hepatocellular carcinoma
TNP	Trinitrophenyl
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

INTRODUCTION

1.1.0 The immune system

The immune system is a collection of cells, tissues and organs that protects the body from invading foreign pathogens and cancer. The mammalian immune system is comprised of an innate component, capable of rapid non-specific response, and an adaptive component, which provides a slower, more highly specific longer lasting response. The adaptive immune response has evolved in vertebrates due to the shortcoming of the innate immune response and rapidly evolving nature of pathogens. The adaptive immune response is mediated by two main cell types, the B and T lymphocytes. Lymphocytes contain antigen specific receptors that promote their differentiation into effector cells following engagement by antigen (Ag).

B lymphocytes originate in the bone marrow, where they undergo extensive selective pressures and maturation. B cells emigrating from the bone marrow display an antigen specific antibody known as a B cell antigen receptor (BCR). B cells then home to secondary lymphoid tissues where they continue the maturation process. Mature B cells can elicit responses to either T cell dependent (TD) or T cell independent (TI) Ags. B cells that encounter T cell dependent (TD) antigens require T cell specific help in order to mount an effective Ab response. While T cell independent Ags can activate B cells and generate an Ab response in the absence of T cell help. Antibodies also have different forms or isotypes depending on the type of infection. Antibodies function to help clear the infection and aid in the activation of other immune cells.

The process of B cell development, activation and differentiation depends on intracellular signaling events that occur following engagement of the BCR. The BCR functions by activating or inhibiting particular signaling events depending on the Ag it encounters. Intracellular signaling events regulate all aspects of B cell function including proliferation, survival, motility, growth and metabolism, and differentiation (1). Signal transduction propagates extracellular cues into complex, intricately regulated signaling cascades involving protein and lipid kinases, phosphatases, lipases and adapter proteins. In B cells one of the most important signaling pathways activated downstream of the BCR is the phosphoinositide 3-kinase pathway (PI3K). Regulation of the PI3K pathway has been shown to be critical for B cell development, activation and differentiation (2). However, many avenues of PI3K regulated signaling are still not well understood. We are particularly interested into two adaptor proteins, TAPP1 and TAPP2, which have been found to selectively bind to a unique PI3K derived lipid.

1.2.0 B cell development

B cell development is an intricately regulated process, occurring within both the primary and secondary lymphoid organs. Throughout B cell development, B cells pass through checkpoints to delete autoreactive B cells or to positively select functional B cell subsets (3). Thus, even though the murine bone marrow produces approximately 1 to 2*10^7 IgM+ B cells per day, only about 1-3% of these survive in the periphery and reach maturity (4, 5). Although mature B cells are stringently selected, these checkpoints can

breakdown allowing the escape of autoreactive B cells leading to the development of autoimmune disease (6).

B cell maturation proceeds through discrete stages regulated by transcription factors (7), cytokines (8) and cell surface receptors (9). B cell development can be divided into two phases, an antigen independent phase occurring in the bone marrow and an antigen dependent phase occurring in the periphery (10). B cell development is a stepwise process beginning with pro-B cells which then transition through pre-B and immature stages until finally maturing into functional B lymphocytes (**Figure 1**).

Pro-B cells transiently express recombination activation gene 1 and 2 (RAG1/2), which are enzymes involved in VDJ heavy chain rearrangement (11). B cells that successfully recombine their heavy chains genes bind a surrogate light chain and display a pre-BCR complex on the surface (pre-B cell stage). Pre-BCR signaling requires the Igα/β complex and appears to share some common signaling mechanisms with mature BCR (12, 13). Pre-B cells with productive in frame heavy chain rearrangements are positively selected and undergo clonal expansion (14). In contrast, mutation or deletion of the μ heavy chain, (9) Igβ or Igα (15, 16) significantly decreases B cell development beyond the pro-B cell stage. Signaling through the pre-BCR reactivates RAG genes and induces light chain rearrangement (17). Following successful rearrangement, B cells replace the surrogate light chain with either Igκ or Igλ light chains and express a mature BCR (10).

Immature B cells that are capable of binding to self-antigen either undergo negative selection (deletion) (18), receptor editing (19, 20), or become anergic (inactivated) (21-23). One mechanism for inducing tolerance is through the elimination of self-reactive B cells. Immature B cells are highly susceptible to apoptosis with extensive crosslinking of the BCR by autoantigens leading to death (18, 24). In contrast, B cells can become anergic, a state in which they no longer respond to antigen (B cells are hyporesponsive) (21, 22). Anergy is induced through engagement of the BCR by intermediate levels of antigen (25). Lastly, autoreactive B cells can undergo a process termed receptor editing, where the heavy or light chain can undergo secondary DNA rearrangements to change the Ag binding specificity (19, 20, 26). Immature B cells initially only express surface IgM, then emigrate from the bone marrow to the spleen to either die or complete the maturation process and upregulate IgD (3).

Once B cells acquire IgM, they then immigrate to the spleen and are known as transitional B cells (27). Transitional B cells are an intermediate population of B cells capable of differentiating into mature B cell subsets. Transitional B cells can be subdivided into T1, T2 or T3. These B cell subsets differ in their cell surface expression and functional responses. Transitional type 1 (T1) B cells are highly susceptible to negative selection, eliminating B cells that react with self antigens in the peripheral tissues (28). Crosslinking of the BCR on T1 B cells triggers apoptosis, while conversely in T2 B cells it promotes B cell proliferation and maturation (29). Consistently, T1 B cells were also found to express lower levels of anti-apoptotic proteins compared to T2 B cells (27, 29). Another factor important for B cell maturation is B cell activating factor

(BAFF). BAFF is a cytokine of the tumor necrosis family that functions by activating the noncanonical NF-κB pathway and influencing B cell survival by upregulating anti-apoptotic factors (28). BAFF deficient mice were found to halt B cell development at the T1 stage (30), paralleling NF-KB subunit knockout mice (28, 31). BAFF influences B cell development by selectively enhancing the survival of T2 cells (32). Interplay between BAFF and BCR signaling dictates the fate of transitional B cell, either eliminating autoreactive B cells or promoting further maturation.

Mature B cells require both the BCR (33) and BAFF (34, 35) for maintenance. Ablation of the BCR using a interferon inducible cre, leads to rapid death of peripheral B cells (33). Further studies elucidated that constitutive or 'tonic' signaling via the Igα/β heterodimer is required for peripheral B cell survival (36). BCR deficient B cells can be rescued by constitutive PI3K activation, potentially inactivating FOXO pro-apoptotic signals (37). BCR ligation has been found to increase BAFF-R (BR3) expression in the late transitional and follicular B cell subsets (38). BAFF binds BAFF-R (BR3) on peripheral B cells and induces Akt phosphorylation and Bcl-X_L (anti-apoptotic) expression, promoting B cell survival (35, 39, 40). Interestingly, BAFF is often found upregulated in patients with lupus-like autoimmune diseases (41). Consistently, BAFF transgenic (Tg) mice were found to have expanded mature B cell populations and develop severe autoimmune manifestations (42). In contrast though, BAFF deficient mice or Tg mice expressing a soluble BAFF receptor (TACI) show dramatic reductions in follicular and marginal zone B cells, while peritoneal B1 cells remain intact (30, 43).

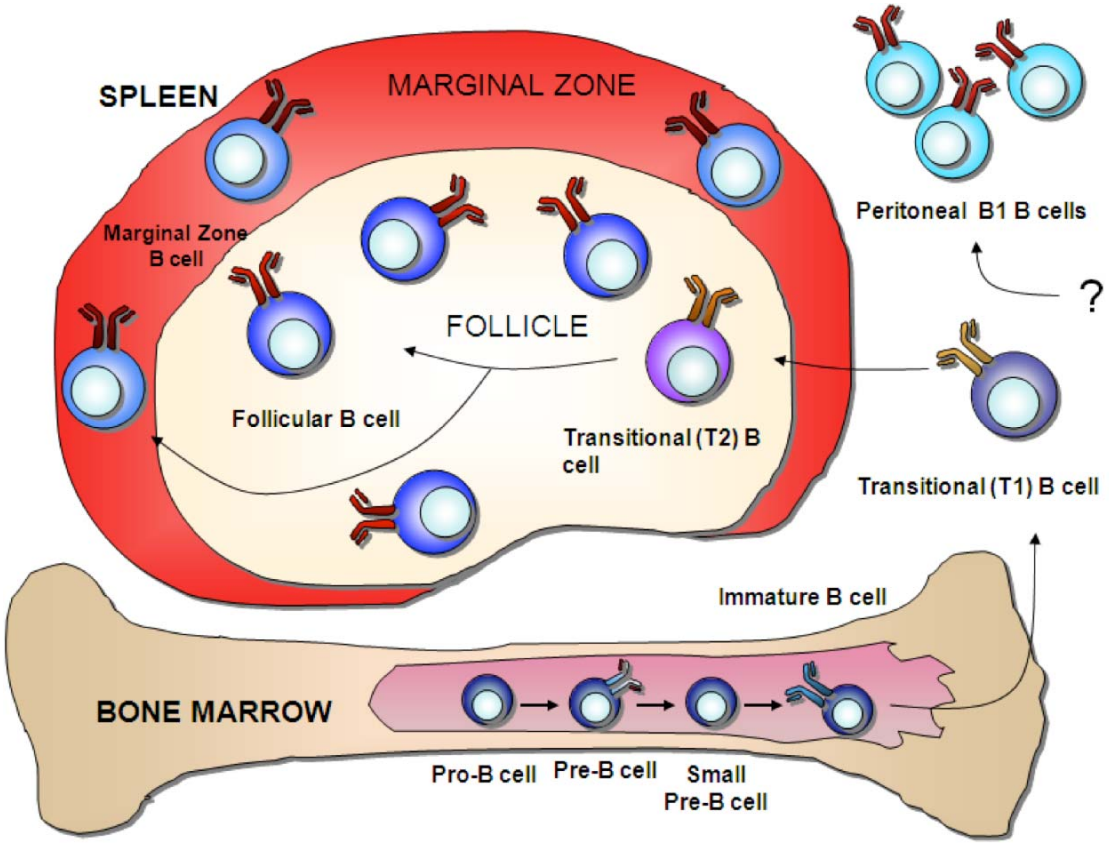

Figure 1: B cell development

B cell development is a stepwise process, initially occurring in the bone marrow where B cells progress from pro-B cells to immature B cells. Immature B cells immigrate to the spleen where the maturation process continues as B cells transition through an intermediate before fully maturating into follicular or marginal zone B cells. The origin of peritoneal B1 B cells is still relatively unknown.

1.2.1 Mature B cell subsets

Mature B cell subsets can be divided into either B2 or B1 B cells. B2 or conventional B cells can be subdivided into major 2 subsets: marginal zone (MZ) and follicular (FO) B cells. These B cell subsets populate distinct locations and differ in their ability to respond to certain Ags. These subsets differ not only in their functional responses, but also in their relative lifespans, with the half-life of FO B cells being approximately 4.5 months, while the number of MZ and B1 B cells remain relatively unchanged over a year (44).

Follicular B cells

Follicular B cells are the largest subset of mature B cells. They primarily populate B cell follicles in secondary lymphoid organs, however they also recirculate between tissues and bone marrow (45). Follicular B cells colonize primary follicles that become seeded by antigen activated germinal centre B cells, forming secondary follicles (46). Follicular B cells are responsible for generating long-lived antibody producing cells in response to immunization with T cell dependent antigen (45, 47).

Marginal zone and B1 B cells

Specialized B cell populations known as MZ and B1 have been shown to be responsible for basal immunoglobulins and generating antibodies responses to T cell independent (TI) antigens (48, 49). Development of B1 and MZ B cell subsets is highly dependent on the PI3K pathway (2, 50). Marginal zone and peritoneal B1 B cells are ‘innate like’ B cells and express a limited repetitore of invariant BCRs (51). MZ and B1 B cells exist in a pre-activated state and are weakly autoreactive (48, 52).

Marginal zone (MZ) B cells are a group of non circulating, rapidly responding B cells and are defined by their anatomical location within the spleen (53). In contrast to follicular B cells, MZ B cells are characterized by the increased surface expression of CD21 (complement receptor) and IgM (54, 55), as well as adhesion molecules β2 integrin, LFA1 and α4β1 integrin (56). Increasing evidence suggests that marginal zone B cells are positively selected based on the specificity of their BCR (57). Marginal zone B cells differentiate into plasmablasts upon stimulation with LPS and secrete more IgM and IgG3 then follicular B cells. (54, 58).

B1 cells are a long-lived, self-renewing population located predominantly in the pleural and peritoneal cavities, with a small percentage found in the spleen (48). The origin of B1 cells still remains unclear, originating from either a distinct B cell lineage precursor, while others argue that they are derived from follicular B2 cells (48). Consistent with MZ B cells, B1 cells are also suggested to be positively selected, expressing BCRs of pre-defined specificity, reactive against phosphatidyl choline and bacterial carbohydrates (10). B1 cells are responsible for the production of basal antibody and are capable of responding to TI-II antigens (48). B1 cells can be identified by the surface expression of CD5 and IgM (48). Increases in B1 B cells have also been found to contribute to the development of autoimmune disease in both human and mouse (48).

Signal Strength Model

One model of B cell development, the 'signal strength' model proposes that the strength of signal through the BCR determines the fate a particular B cell adopts. During B cell development, signaling through the BCR, either through interactions with self-antigens or ligand independent tonic signaling, functions to positively select B cells and direct lineage commitment (59). In general, the signal strength model proposes that relatively weak BCR signals are required for MZ B cell development, while intermediate and high level of BCR signals are required for FO and B1 B cell development, respectively (59, 60). However, inconsistencies have been identified, and alternatively suggest that FO B cells require lower level of tonic signaling as compared to MZ and B1 B cells (57).

1.3.0 Humoral immunity

Antigens that provoke humoral immune responses can be grouped together as either T cell dependent (TD) or independent (TI) antigens. While TD responses require MHC class II presentation of the antigen to T cells to fully activate B cells, TI Ags are able to generate antibody responses in the absence of T cell Ag presentation (61). To study Ag-specific Ab responses, antigens are conjugated to a hapten such as NP or TNP, which by themselves are unable to elicit a response; however, when coupled to a carrier compound (ie. OVA) are able to generate hapten specific antibody responses. However, these conjugates are unable to generate large responses without the presence of an adjuvant. Adjuvants function by increasing the immunogencity of the antigen by stimulating the innate immune system and/or prolonging the duration of antigen release (62).

TD or TI antigens differ not only structurally but also in the type of response they elicit. In contrast to TD antigens, TI antigens generate relatively rapid responses, characterized by little to no affinity maturation or isotype switching (61). Affinity maturation is characterized by the progressive increase in affinity of the Ab for antigen over time (due to somatic hypermutation (SHM) in the variable region of the Ab) (47). Isotype switching is the rearrangement of the Ab heavy chain locus from IgM to an effector subtype (ie. IgG). TI antigens induce rapid production of low affinity IgM and IgG3 specific responses (63). TI antigens activate the innate arm of the humoral immune system mediated by B1 and marginal zone B cells and often lack memory (63), whereas TD antigens activate follicular B cells and generate robust antibody responses and generate memory (46).

1.3.1 T cell independent responses

Antigens that can activate B cells independent of MHC class II presentation to T cells are known as T cell independent antigens. TI antigens often induce rapid responses by directly activating B cells without the need of cognate or Ag specific T cell help. T cell independent antigens can be subdivided into class I and class II TI antigens and differ in the mechanism by which they activate B cells.

Type I TI antigens are polyclonal B cell activators capable of stimulating proliferation and antibody secretion irregardless of the BCR antigenic specificity (64). A model type I TI antigen, lipopolysaccharide (LPS), a common component of bacterial cell walls, has been shown to stimulate antibody secretion in athymic nude mice (deficient in T cells)

(65). At low doses of LPS, B cells become activated in an antigen specific manner, while high doses of LPS induce polyclonal B cell activation (66). C3H/HeJ mice, which contain a spontaneous mutation in a receptor (TLR4) that recognizes LPS, fail to proliferate or generate Ab responses to LPS challenge (67). Secondary responses to TNP-LPS immunization showed a similar degree of response suggesting that memory B cell are not generated (65).

Type II T independent antigens are composed of highly repetitive (multimeric) Ag units such as the polysaccharide ficoll. In contrast to type I, type II TI antigens are unable to elicit a response in an immature or hosts deficient in Btk (downstream BCR kinase) (68). Type II TI antigens function by cross-linking multiple BCRs (>10) (69), leading to Btk activation and sustained Ca^{2+} flux (70). Although, type II TI antigens do not require classical antigen presentation, resting B cells still require additional factors or cytokines (IL-2 and IL-5) to induce antibody production and class switching (71). In contrast to type I TI antigens, type II TI responses can occur in the absence of functional TLR signaling (72), suggesting that B cells might be capable of "missing-self" recognition. Missing self-recognition suggests that B cells may contain inhibitory receptors to self-antigens blunting response to self-antigens and preventing the development of autoimmune responses (73). However, engagement of the BCR alone, without ligation of inhibitory receptors leads to a Type 2 TI response (74).

1.3.2 T cell dependent responses

Most protein antigens require CD4+ T cell help in order to mount an effective response and are therefore called T cell dependent Ags. In experimental immunization studies, adjuvants are required to generate the germinal centre response which typically reaches maturity and peaks around day 10 to 14 post-immunization (75, 76). However, other antigens such as sheep red blood cells (SRBC) induce robust B and T cell activation in the absence of adjuvant (77). Primary B cell follicles neighbor T cell rich regions or PALS (periarteriolar lymphoid sheath) and are responsible for responding to TD antigens. Dendritic cells (DC) process protein antigen and present it to antigen specific CD4+ T cells, resulting in their activation. Mature B cells that encounter the protein antigen can process and present antigen to pre-activated T cells. Together with BCR signaling, costimulation provided by B-T cognate interactions activates B cells allowing them to either differentiate into a plasmablast or migrate to primary B cell follicles to establish germinal centres (called secondary B cell follicles once the germinal centre is formed) (78). Plasmablasts are short-lived plasma cells responsible for secreting Ab prior to the generation of long-lived plasma and memory cells. In contrast to GC B cells, plasmablasts also lack somatic hypermutations in the variable regions of the Ab (79, 80).

1.3.3.1 Germinal centres

As the short lived plasma cells diminish, structures known as germinal centres form and generate long-lived antibody secreting cells (75). As illustrated in figure 2, germinal centres are microenvironments in secondary lymphoid tissues (ie. spleen, lymph nodes and peyers patches) where activated B cells undergo extensive proliferation, somatic

hypermutation and selection based on affinity of the BCR for antigen (81). Germinal centres typically develop in response to infection or immunization with TD antigens (81), although have also be found to form spontaneously in autoimmune patients or mouse strains (82). GCs are also normally present in tissues with persistent microbial exposure such as mesenteric lymph nodes, peyers patches and tonsils. Germinal centre B cells are characterized by expression of activation markers such as Fas, GL7, PNA, and loss of IgD (83). As the germinal centre reaction proceeds, competition between GC B cells and serum Ab increases, perpetuating the continual increase in Ab affinity (47). Although affinity maturation occurs in germinal centres, considerable increases in antibody affinity can still be observed in the absence of these structures (84). B cells with higher affinity for Ag receive T cell help, while lower affinity BCRs undergo apoptosis. Surviving GC B cells can then differentiate into either long-lived plasma or memory B cells.

Characteristic GCs form histologically distinct dark and light regions (**figure 2**). The dark region contains densely packed B cells termed centroblasts, while the light zone contains a dense network of follicular dendritic cells (FDC). Chemokines CXCL12 (SDF-1) and CXCL13 are often upregulated within the dark and light zone of the germinal centre, respectively (85, 86). CXCR4 (CXCL12 receptor) is upregulated on germinal centre B cells concentrated within the dark zone (85). Centroblasts downregulate their surface Ig and undergo extensive proliferation (46). Although cell division occurs predominately in the dark zone, recent studies have also found that B cells within the light zone also undergo mitosis (87, 88). Activation induced cytidine deaminase (AID) is a DNA modifying enzyme upregulated in centroblasts and is

essential for SHM and CSR (89). SHM introduces single nucleotide mutations into the variable regions of the Ab, generating B cells of varying affinity for Ag (79). Centroblasts then downregulate CXCR4 (85) and migrate to the light zone, however they may return to the dark zone to undergo further division and selection (88).

Conversely, the light zone of the GC is where B cells undergo affinity selection, class-switch recombination and differentiation into either plasma or memory B cell subsets (90). Light zone germinal centre B cells or centrocytes re-express their newly generated surface Ig (46, 83) and compete for survival signals. FDC capture antigen immune complexes through Fc and complement receptors (91). Classical modeling suggested that centrocytes were selected based on their ability to capture and process antigen from FDCs (46, 83). Interestingly though, mice devoid of serum Ig and detectable immune complex, still develop robust germinal center responses (92). Centrocytes with higher affinity BCRs exhibit a competitive advantage compared to their lower affinity counterparts (83, 93). However, SHM typically introduces deleterious mutations producing lower affinity or non-functional BCRs, resulting in a high rate of death within the GC (46). Surviving centrocytes can either return to the dark zone to undergo further proliferation and selection, or process and present MHC-Ag to CD4+ T cells (94). A specialized subset of antigen experienced CD4+ T cells called T follicular helper cells (Tfh) provide cytokines and survival cues such as CD40L to selected B cells allowing them to differentiation into long-lived plasma or memory B cells (95). Furthermore, blockage of B-T interactions by administration of CD40L blocking antibody prevented

further antibody maturation (96). Recent studies using two photon microscopy have suggested that GC T cells are the limiting factor in the GC reaction (88, 97)

Following processing through the germinal centre, GC B cells either differentiate into plasma or memory B cells, capable of secreting copious amounts of immunoglobulin or generating a long-lived B cell population, respectively. However, the decision to commit to an effector or memory B cell subset still remains unclear (90). Plasma cells home to the bone marrow where they can live up to a year (98), while memory cells can recirculate in the periphery where they secrete antibody upon secondary infection or immunization (99).

T follicular helper cells

T follicular helper cells (Tfh) are a subset of specialized CD4+ T cells that participate in TD responses by providing critical signals such as cytokines and cell contacts to germinal centre B cells (95). Tfh cells are an antigen experienced lineage of activated T cells, located within B cell follicles. They are characterized by the expression of chemokine receptor CXCR5, the costimulatory molecule inducible costimulatory molecule (ICOS) and the programmed death-1 (PD-1) (100). Tfh produce cytokines such as IL-4 (101), IL-10 (102) and IL-21 (103). IL-21 can function in both an autocrine and a paracrine manner, influencing both Tfh development and GC formation and differentiation, respectively (104-106). The cytokine milieu produced by Tfh cells supports GC B cell proliferation and differentiation into Ab secreting cells (100).

Cell contacts between GC B cells and Tfh cells are required for maintenance and progression of the GC response (95). Tfh provide critical cell contacts such as CD40L and ICOS to GC B cells (95). Deficiency in CD40L or ICOS has been found to significantly impair germinal centre formation and antibody responses to TD antigens (107, 108). Naïve T cells typically express low levels of ICOS; however is upregulated following T cell activation (109). Previous studies have shown that co-stimulation through ICOS on CD4+ T cells increases CD40L expression (110). In the absence of ICOS, anti-CD40 treatment restores antibody responses (110), suggesting that ICOS may regulate Ab production through CD40L. ICOS functions by binding ICOSL on B cells (111). Elevated ICOS expression through loss of function of the ubiquitin ligase, roquin, results in a severe autoimmune disease paralleling systemic lupus erythematosus (SLE) (112). Sanroque mice (roquin loss of function) have increased Tfh cells and have significantly enhanced germinal centre formation and antibody production (112).

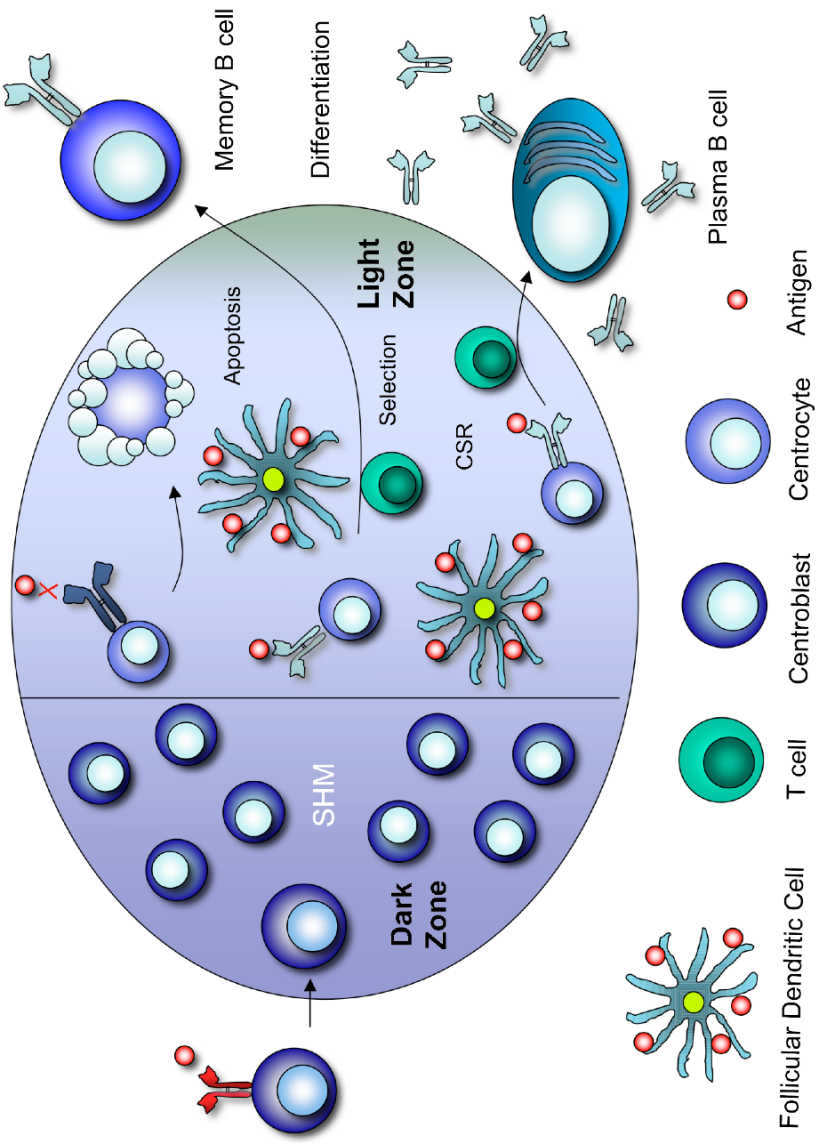

Figure 2: Germinal centre

Antigen activated B cells enter the dark zone of the germinal centre and undergo extensive proliferation and somatic hypermutation (SHM) in the variable regions of the

Ab. Centroblasts then differentiate into centrocytes which compete for Ag on FDCs and survival signals from CD4+ Tfh cells. Tfh cells also provide soluble factors that act on B cells to induce class switch recombination (CSR). B cells that express higher affinity BCRs differentiate into either plasma or memory B cells, while those B cells with lower affinity BCRs fail to receive survival cues and undergo apoptosis.

1.4.0 Introduction to phosphoinositide 3-kinase

Phosphoinositide 3-kinases (PI3K) are a family of enzymes that selectively phosphorylate the D3 hydoxyl group of the inositol head group of phosphoinositide (PI) lipids (**figure 3**). PI3Ks can be broken down into three families based on their structure and PI specificity. Class I PI3K enzymes are heterodimeric complexes composed of a single regulatory (p85α, p85β, p55γ, p55α, and p50α) and catalytic subunit (p110α, p110β, p110δ or P110γ). While the p110α and β catalytic subunits are ubiquitiously expressed, the p110δ (B and T cells) and γ (T cells) subunits are largely restricted to leukocytes (113, 114). Class I PI3K enzymes can either be activated by receptors triggering tyrosine kinase activity (class IA) or through G protein coupled receptors (GPCRs) (115). Class I PI3Ks enzymes are involved in the regulation of PI(3,4,5)P3 and PI(3,4)P2 levels and have been shown to play an essential role in immune cell activation (115, 116). In contrast, class II PI3K enzymes selectively phosphorylate PI and PI(4)P to produce PI(3)P and PI(3,4)P2, whereas class III only produce PI(3)P (115). However, little is known about the role of class II and III PI3Ks in humoral immune responses. The class I PI3K signaling pathway is linked to a number of key cellular processes including cell survival, proliferation, differentiation and motility (2).

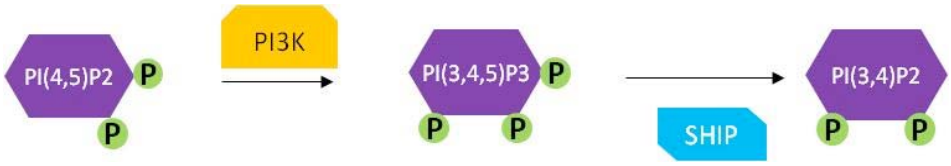

Figure 3: PI3K lipids

PI3K functions by phosphorylating the D3 position of the inositol head group of PIs. Class I PI3K enzymes function to preferentially convert either PI(4,5)P2 or PI(4)P into PI(3,4,5)P3 or PI(3,4)P2, respectively.

1.4.1 PI3K activation in B lymphocytes

B lymphocytes express a large array of receptors that have been shown to signal through PI3K including antigen receptors, costimulatory molecules, adhesion molecules, toll like receptors and cytokine receptors (2, 117). However, our understanding of the mechanism by which many these receptors activate PI3K is still poorly understood. One of the most extensively studied receptors in activation of PI3K is the B cell antigen receptor (BCR) (**figure 4**). The BCR signaling complex is composed of a membrane bound immunoglobulin and a heterdimeric Igα/β (ITAM containing complex) signal transducing complex. Crosslinking of the BCR either by antigen or receptor agonist results in phosphorylation of the ITAM motifs of the Igα/β via Src family tyrosine kinases (Lyn, Fyn or Blk) (118). These phospho-tyrosine (p-tyr) residues function as docking sites for protein tyrosine kinases such as Syk (115). Deletion of Syk impairs BCR induced PI(3,4,5)P3 accumulation (119). Activated Syk phosphorylates tyrosine residues on CD19, cbl and BCAP, which function as docking sites for p85 regulatory subunit of PI3K (120-122). Defective BCR signaling can result in impaired B cell development, activation and differentiation (123).

The recruitment and membrane localization of class I PI3K results in the rapid accumulation of PI(3,4,5)P3 and PI(3,4)P2 (124) and activation of complex signaling cascades. CD19 is a transmembrane co-receptor that associates with the BCR and primarily functions in the recruitment and activation of PI3K (120). CD19 functions in a complex with CD21 and CD81 complement receptors to lower the threshold of BCR activation by approximately 100 fold (125). Deficiencies in CD19 significantly abrogate

PI3K activation in B cells (126) and activation of PI3K regulated protein kinases, such as Akt and Btk (127, 128). The p85 regulatory subunit of PI3K binds to p-Tyr residues on CD19 via it SH2 domains. Of the eight tyrosines of CD19, residues Y482 and Y513 have been shown to be critical in the recruitment and activation of PI3K (120, 129). The binding of the p85 regulatory subunit to pTyr motifs on CD19 allows Ras-GTP induced activation of the p110 catalytic subunit (130). Although CD19 is involved in PI3K activation, CD19 deficiency does not completely abrogate PI3K signaling in B cells (127), suggesting that there are alternate mechanisms of PI3K activation. B cell activator adapter for PI3K (BCAP) was identified as a novel adapter protein containing four YxxM, phospho-tyrosine binding motifs. These motifs are phosphorylated by Syk and can be bound by the SH2 domain of the p85 subunit of PI3K (121). Furthermore, BCAP deficient DT40 cells show a 50% reduction in PI(3,4,5)P3 production (121). Consistently, when CD19 and BCAP are simultaneously deleted, there is a dramatic reduction in Akt phosphorylation, which is indicative of reduced PI3K activity (131).

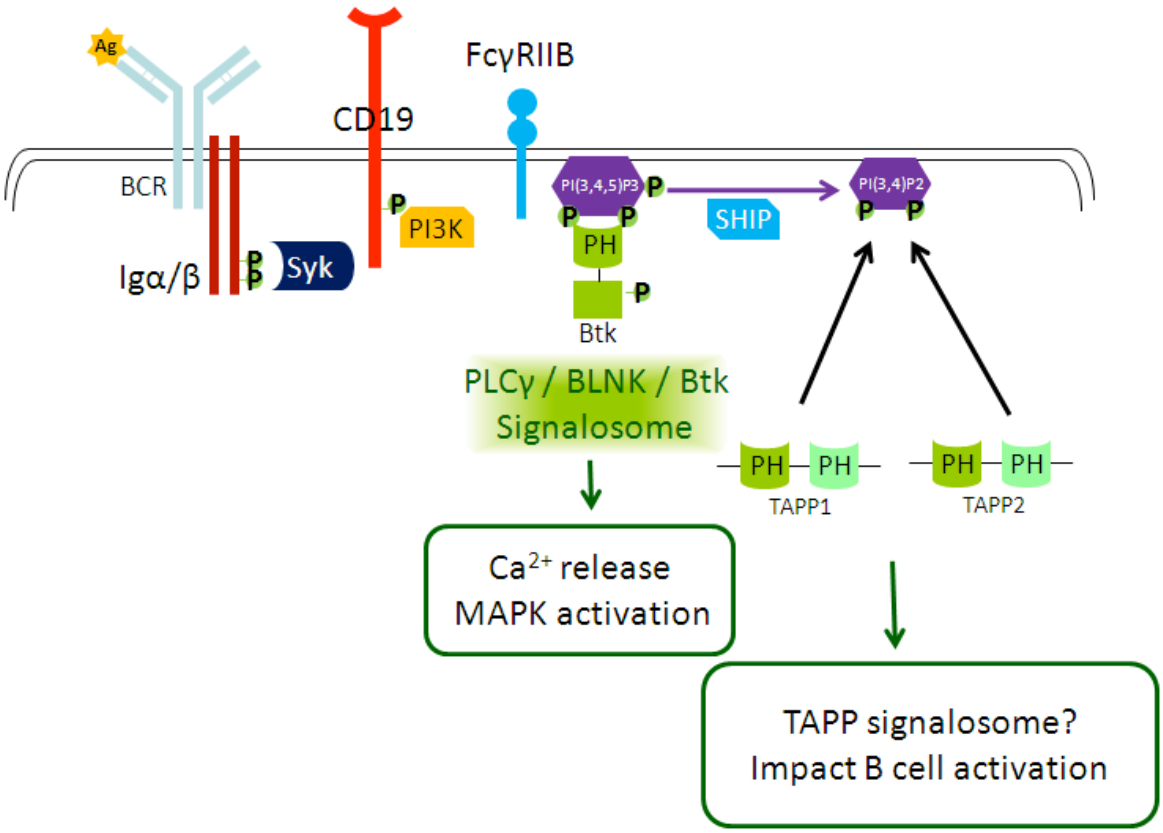

Figure 4: PI3K activation in B cells

Engagement of the BCR by antigen initiates phosphorylation of the Igα/β complex recruiting Syk kinase. Syk kinase phosphorylates the YxxM motif of CD19 and other adapter proteins recruiting PI3K. PI3K functions to selectively phosphorylate the D3 position of PI(4,5)P2 and PI(4)P, generating PI(3,4,5)P3 and PI(3,4)P2, respectively. These lipid second messengers function as docking sites for PH domain proteins. Recruitment of Btk to the plasma membrane initiates the formation of a signalosome complex resulting in Ca^{2+} mobilization and MAPK activation. On the other hand, TAPP adapter bind specifically to PI(3,4)P2, however their role in B cell activation and humoral immune responses remains to be defined.

1.4.2 Phosphoinositide dynamics

Phosphoinositides are a class of membrane phospholipids containing an inositol head group. PI3K functions by phosphorylating the D3-position of membrane phosphoinositides, generating PI(3)P, PI(3,4)P2 and PI(3,4,5)P3 (132). Interestingly, while only 10% of the total eukaryotic lipids are phosphoinositides, less than 0.25% of total inositol lipids are phosphorylated at the D3 position (133). Of the D3 phosphoinositides, the PI(3)P is the most abundant and remains relatively stable before and after antigen stimulation (116, 123, 124, 133). In contrast, PI(3,4)P2 and PI(3,4,5)P3 levels are transiently increased following BCR crosslinking (116, 124). These D3-phosphoinositides regulate various cellular processes through the recruitment of effector proteins containing specialized domains. Interestingly, while PI(3,4,5)P3 is clearly implicated in activation of key mitogenic signaling pathways, the unique signaling events downstream of PI(3,4)P2 are still not well understood (116).

1.4.3 Phosphoinositde phosphatases

Regulation of D3 phosphoinositides is crucial for the prevention of leukocyte-derived cancers as well development of autoimmune diseases (134, 135). PI phosphatases function by restraining PI3K signaling by limiting the amount of PI(3,4,5)P3 and PI(3,4)P2 available (**figure 5**). PTEN (phosphatase and tensin homolog deleted on chromosome 10) and SHIP (SH2- domain containing inositol polyphosphate phosphatase) function by metabolizing PI(3,4,5)P3. PTEN is a tumor suppressor and functions to directly antagonize PI3K signaling by dephosphorylating the D3 phosphate

of PI(3,4,5)P3 (136). Homozygous PTEN deletion in mice results in embryonic lethality (137), while heterozygous mice survive but develop malignances (137) and autoimmune disease (138). In contrast, SHIP selectively dephosphorylates the D5 phosphate of PI(3,4,5)P3 generating PI(3,4)P2 (139). SHIP is classically activated following recruitment to the immunoreceptor tyrosine based inhibitory motif (ITIM) of FcγRIIB (140). SHIP deficient mice are viable but show significant mortality by 14 weeks of age due to expansion and infiltration of myeloid cells into the lungs (141). Hematopoetic cells from the bone marrow of SHIP deficiency mice were found to have enhanced responsiveness to various cytokines, suggesting that SHIP is also an important negative regulator of cytokine signaling (141).

Inositol 4-phosphatases (4-Pases) are another family of phosphatases that have emerged as critical negative regulators of the PI3K pathway. 4-Pases function to preferentially dephosphorylate the D4 phosphate of PI(3,4)P2 to generate PI(3)P (142). Recent studies have found that type 2 4-Pases function as tumor suppressors (143) and have been found lost in breast (144) and prostate cancers (145). However, the function of 4-Pases in the immune system is still poorly understood.

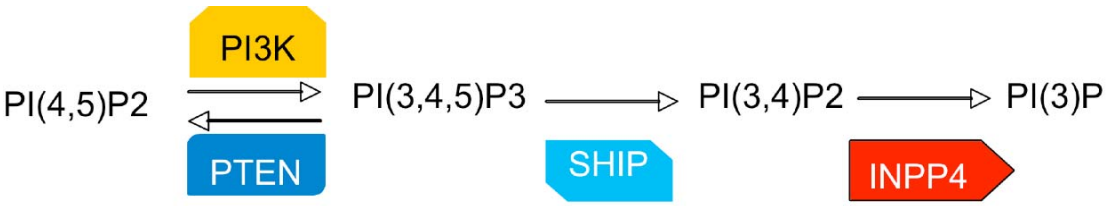

Figure 5: Phosphoinositide phosphatases

Phosphoinositide phosphatases function to selectively remove phosphate groups from the inositol ring of PIs. PTEN directly antagonizes PI3K signaling by removing the D3 phosphate. SHIP selectively dephosphorylates the D5 position of PI(3,4,5)P3, generating PI(3,4)P2. INPP4 or inositol polyphosphate 4-phosphatases selectively remove the D4 phosphate of PI(3,4)P2, generating PI(3)P.

1.4.4 Phosphoinositide-binding proteins domains

Phosphoinositides promote responses to extracellular stimuli by binding specialized lipid binding domains of kinases, lipases or adaptor proteins. The pleckstrin homology (PH), phox-homology (PX), or Fab1, YOTB, Vac1, and EEA1 (FYVE) domains demonstrate binding to phosphoinositides and initiate activation of acute signaling cascades or participate in membrane and protein trafficking (146).

The pleckstrin homology (PH) domain is a specialized region composed of approximately 100 to 120 amino acids that are found in more than 200 proteins in the human proteome (146). PH domains have a characteristic seven strand beta sandwich structure composed of basic amino acids that allow for electrostatic stabilization and hydrogen bonding to negatively charged molecules, including lipids (147). PH domains, unlike other PI binding domains, can specifically bind with high affinity and specificity to PI with adjacent phosphates (148). Although certain PH domains are known for their high affinity and specificity for phosphoinositides, it is estimated that only 10% may actually participate in such highly specific interactions (148). The role of the remaining 90% remains relatively unknown, however some may participate in low affinity interactions with various phosphoinositides or may facilitate protein-protein interactions (147, 149, 150). However, the functional consequences of these low affinity interactions is still under debate. Among the PH domains that have high affinity and selectivity for PI3K products, a unique recognition motif in the β1 and β2 loop of the PH domain is present and is critical for binding to phosphoinositides (151). Interestingly, mutations that

abolish or promote PH domain binding are associated with the development and progression of certain diseases (146).

1.5.0 Function of PI3Ks and PI-binding proteins in B cell biology

1.5.1 The role of PI3K signaling in B cell development

As discussed above, the pre-BCR and BCR expressed on the B cell surfaces regulate developmental checkpoints and select for functional lymphocytes. BCR-induced PI3K signaling events play critical roles in early B cell selection and differentiation into mature B cell subsets, including follicular, marginal zone and B1 subsets. Deletion, genetic inactivation or pharmacological inhibition of the subunits of PI3K have been used to elucidate the role of the class I isoforms in B cell development, activation and differentiation.

All class I catalytic isoforms are expressed within leukocytes, however, deletion of the p110α or p110β results in early embryonic lethality (152, 153). Mutations targeting the predominant p85α regulatory or p110δ catalytic subunits have been shown to retard B cell maturation at the pro B cell stage within the bone marrow (154-156). Subsequently, there is a profound reduction in mature B cells within the spleen and lymph nodes, and decreased B1a B cells within the pleural and peritoneal cavities (154-158). In contrast, deletion of p110γ did not affect B cell development, however it did have a profound impact on thymocyte development and mature T cell activation (159).

B cell development is regulated by both positive and negative signaling events. Interestingly, although CD19 has an important role in B cell responsiveness, CD19-/- mice develop normal B cell populations within the bone marrow (160). However, examination of peripheral lymphoid tissues shows dramatically reduced peritoneal B1a and marginal zone B cells (160). Conversely, enhancing PI signaling by conditional deletion of PTEN in B cells results in the expansion of B1 and marginal zone B cell subsets (161). Interestingly, deletion of PTEN can restore the marginal zone and B1 subsets within CD19 deficient mice, suggesting that PI3K pathway activation is a key function of CD19 in driving MZ and B1 B cell development (161). However, deletion of PTEN only partially restores the marginal zone subset in p110δ deficient mice (162). CD19/BCAP double knockout mice show a significant reduction in the number of peripheral B cells and show severe impairment in the generation of immature and mature B cell subsets within the spleen and bone marrow (131).

Another phosphatase known to negatively regulate PI3K signaling is the inhibitory phosphatase SHIP. SHIP functions by limiting the amount of PI(3,4,5)P3 by selectively removing the D5 phosphate, producing PI(3,4)P2 (139). Deletion of SHIP results in a significant reduction in B cell development within the bone marrow (163). However, despite the decrease in B cell precursors, SHIP deficient mice have increased frequency and number of mature B cells within the spleen (164). Furthermore, analysis of the kinetics of B cell reconstitution after irradiation found that SHIP deficiency accelerates mature B cell development (164).

1.5.2 Role of PI3K on humoral immunity

PI3K activation has been shown to be critical in the induction of humoral immune responses. Inactivation of either the p85 regulatory or p110 catalytic subunits has been found to significantly perturb B cell functional responses, including reduced proliferation, increased susceptibility to apoptosis and dramatically reduced both basal and antigen induced antibody production (2, 155). Consistent with the defects in B cell development and activation, p110δ mutant mice were found to have significantly diminish GC formation and antibody production in response to TD antigens (157, 158). In parallel, deletion of CD19 was also found to significantly impair GC differentiation and Ab responses to TD antigens (165). However, inactivation of PTEN in CD19 deficient mice was found to restore GC formation upon immunization with TD antigens (161). Interestingly, increasing PI3K signaling through B cell specific deletion of PTEN has been found to impair CSR resulting in increased IgM production while inhibiting switching to IgG isotypes (166). Deletion of SHIP, however, resulted in increased basal Ig and enhanced Ab responses to TD antigens (163). Consistent with the impairments in TD humoral immune responses, p110δ mutant and p85α deficient mice were also found to have severely diminished antibody responses to TI-2 antigens (155, 158).

Table 1: Summary of PI3K mutations on humoral immunity

	Targeted Subunit	Viability	Immunological Defects
Catalytic	p110α	Embryonic Lethal	N/A (152)
	p110β	Embryonic Lethal	N/A (153)
	p110δ	Viable	Severe reduction in B cell development, with absence of marginal zone and B1 B cells. Corresponding reductions in basal and TI II antibody responses. Reduced B cell proliferation to mitogenic stimuli. Reduced germinal centre formation and TD antibody responses (156-158).
	p110γ	Viable	Regulates thymocyte development, T cell activation, and neutrophil migration (159)
Regulatory	p85	Viable	Reduced mature B cells. Reduced basal Ig and TI II responses. Reduced B cell functional responses, including decreased proliferation to various mitogenic stimuli. Intact TD responses (154, 155).

1.5.3 Tec kinases

Pleckstrin homology domain containing effector proteins are recruited to D3 PIs on the plasma membrane and initiate intracellular signaling cascades. The Tec (tyrosine kinase expressed in hepatocellular carcinoma) kinases are a family of non-receptor tyrosine kinases activated downstream of various cell surface receptors that phosphorylate target proteins involved in various cellular processes including adhesion, migration, actin reorganization, apoptosis and cellular transformation (167). The Tec kinase family is largely restricted to cells of hematopoetic origin and is made up of Tec, Btk, Itk, Rlk and Bmx. These kinases are highly expressed in leukocytes and are characterized by a proline rich region and a PH domain highly specific for PI(3,4,5)P3 (168).

Activation of Tec kinases is most well understood in the context of antigen receptor signaling. Activation of Tec kinases occurs in part by plasma membrane localization (169) followed by phosphorylation by Src family of kinases (170). Btk is one of the most well studied Tec kinases and is expressed predominately in the B cell lineage (171). Primary phosphorylation of the Y551 residue leads to the subsequent autophosphorylation on Y223 residue, resulting in Btk activation (170). Once activated Btk can bind BLNK and can phosphorylate and activate PLC-γ, a phospholipase involved in the cleavage of PI(4,5)P2 into IP3 and DAG (172). IP3 and DAG are involved in the release of endoplasmic reticulum calcium stores and activation of PKC-MAPK pathway (123). Btk deficiency results in decreased PLC-γ phosphorylation and reduced calcium flux (70). In addition to diminishing PI(4,5)P2, Btk can also replenishes PI(4,5)P2 levels through the membrane localization of phosphatidylinositol-4-phosphate-5-kinase (PIP5K)

(173). Btk can be inhibited by activation of SHIP through the FcγRIIB which reduces the availability of PI(3,4,5)P3 for binding (174, 175).

Btk has been shown to be a critical regulator of B cell development and maturation (176). Initially identified in a screen of cytoplasmic tyrosine kinases involved in B cell development, BPK or Btk was cloned and found to be expressed in B cell lines at various stages of development (171). Interestingly, patients with X-linked agammagloblemia (XLA), an inherited humoral immunodeficiency characterized by a blockage in early B cell development (177), showed reduced or absent Btk mRNA and protein (171). Consistent with this, CBA/N mice were found to carry an X-linked immunodeficiency (Xid) mutation mapped to a conserved region of the Btk PH domain (178, 179). In contrast to XLA patients, Xid mutant and Btk deficient mice develop a milder phenotype, although still have impairments in B cell activation and cannot respond to type II T cell independent antigens (180-182). To determine redundant features of the Tec family of kinase, knockout of both the Tec and Btk have been shown to greatly diminish B cell development as compared to either KO alone (183).

1.5.4 Akt

Akt (also referred to as PKB) is a proto-oncogene serine/threonine protein kinase, often dysregulated in many human cancers (184) and metabolic disorders (185, 186). Three isoforms of Akt have been identified and are all ubiquitously expressed (187). Akt contains an N-terminal PH domain capable of interacting with both PI(3,4,5)P3 and PI(3,4)P2 (188) and is targeted to the plasma membrane in a PI3K dependent manner (189-191). Mutations within the PH domain of Akt have been found to abrogate cell

activation (189, 192). Interestingly though, N-terminal myristoylation of Akt caused constitutive activation in the absence of a functional PH domain, suggesting that the main role of the PH domain is to mediate membrane localization (192). Binding of Akt to the plasma membrane induces a conformational change exposing sites for phosphorylation (193).

In order for Akt to become fully activated it must be dually phosphorylated in the activation loop (Thr308 – PDK) and regulatory hydrophobic motif (Ser473 – mTORC2) (190). Interestingly, substitution of these regulatory sites with negatively charged aspartic acid residues, mimicking phosphorylation, resulted in constitutive Akt activation (190). Phosphorylation of the threonine 308 and serine 473 residues is carried out by PDK1 and mTOR kinases, respectively. PDK1 (3-phosphoinositide-depenedent protein kinase-1) contains a PH domain and is capable of interacting with PI(3,4,5)P3 and PI(3,4)P2 (194). PDK1 functions by phosphorylating the activating threonine 308 residue of Akt (195). Mutation of the PH domain of PDK1, preventing PDK1-phosphoinositide interactions, diminished Akt phosphorylation on the threonine 308 residue (196). Interestingly, the serine 473 residue of Akt is insensitive to acute rapamycin treatment, although knockdown of either mTOR (mammalian target of rapamycin) or its binding partner rictor, decreased the total phosphorylation of Akt (197). Furthermore, the phosphorylation status of the serine 473 residue of Akt appears to be critical in allowing threonine 308 phosphorylation, therefore components affecting 473 phosphorylation were also found to affect 308 phosphorylation (197, 198).

Akt is linked to a number of key cellular processes including cell survival, proliferation and metabolism (199). Akt functions by phosphorylating a number of downstream effector kinases and transcription factors (200). Engagement of the BCR activates Akt in a PI3K dependent manner (201, 202). B cells deficient in p110δ catalytic subunit shows decreased Akt activation and are highly susceptible to apoptosis (158). Conversely, overexpression of Akt in B cell lymphoma (DT40 – immature B cell line) cell line inhibited BCR induced apoptosis, suggesting Akt plays an important role in B cell survival (203). One of the important downstream targets of Akt is the forkhead box, class O (FoxO) family of transcription factors, known to regulate both pro-apoptotic and anti-proliferative proteins (204). Three FoxO transcription factors are expressed in B cells including FoxO1, FoxO3a, and FoxO4. FoxO transcription factors are inactivated downstream of the PI3K-Akt signaling axis, resulting in nuclear export to the cytosol and degradation (204). Overexpression of Akt independent Foxo mutants in activated primary murine B lymphocytes leads to increased cell cycle arrest and apoptosis (205).

Negative regulation of the PI3K-Akt signaling axis is important for the prevention of various types of cancers (134). Co-ligation of the BCR and the inhibitory receptor, FcγRIIB1, reduces Akt membrane localization and activity (downstream phosphorylation) in a SHIP-dependent manner (202, 206). Expression of mutant FcγRIIB1, lacking the ITIM motif, or in B cells lacking SHIP fail to negatively regulate Akt activation (207). SHIP deficient B cells show enhanced and prolonged Akt phosphorylation (163). Consistently, PTEN deficient cell lines show enhanced Akt phosphorylation through upregulation of PI(3,4,5)P3 lipid (161), whereas expression of

PTEN in tumor cell lines led to decreased Akt phosphorylation and enhanced cell death (208, 209). In vitro stimulation of PTEN deficient splenic B cells with $F(ab)_2$ anti-IgM showed increased PI(3,4,5)P3 lipid levels and a corresponding increase in Akt activation (161). Lastly, deficiency or knockdown of either type I or type II 4-pases, which generate PI(3)P, were found to have a significant increase in Akt phosphorylation following growth factor stimulation(143, 210).

1.5.5 PI binding adapter proteins

Adapter proteins play essential roles in orchestrating intracellular signaling events by facilitating protein-protein or protein-lipid interactions (211). Adapter proteins can function within the cytosol or on the membrane. Although adapter proteins lack intrinsic enzymatic activity, they possess multifunctional binding domains such as src homology 2/3 (SH2/3), pleckstrin homology (PH), or PDZ binding domains allowing them to function as molecular scaffolds (212). The phosphorylation status of adapter proteins also allows interaction with other proteins within the cell (211, 212). Adapter proteins can function in the recruitment and binding of essential kinases and phosphatases facilitating their localization in the cell (211, 212). Adapter proteins have been found to function both positively and negatively in immune cell development and activation (211, 212).

PI3K dependent adapter proteins are a set of highly specific adapter proteins found to be recruited to the plasma membrane upon 3-PI generation. Interaction with 3-PI occurs through interaction with specialized domains, such as the PH, PX and FYVE. While

these domains are wide spread throughout the genome, only a limited number of adapters families can be classified as 'PI3K regulated adapters' (213). The Gab, SKAP and Bam32/DAPP1/TAPP families of adapters have all been found bind D3 PI and be recruited to the plasma membrane in a PI3K dependent manner (213). However, the function of these adapter proteins in humoral immunity is still not well understood and they may have differential roles depending on their cellular context.

Bam32 is an adapter protein originally isolated from human GC B cells which has been found to be recruited to the plasma membrane in a PI3K dependent fashion (214, 215). Bam32 deficient mice were found to develop normal B cell populations within the spleen and bone marrow, however they were found to have a significant reduction in peritoneal B1 B cells (216). Consistent with the reduction in peritoneal B1 B cells, Bam32 deficient mice were found to have deficiencies in TI-II responses (216). As for TD responses, Bam32 deficient mice show premature dissolution of the germinal centre, showing increased GC B cell apoptosis and decreased Ab affinity maturation (217). Further analysis of spleen sections from TD immunized Bam32 deficient mice found less CD4+ T cell recruitment to the GC (217). Administration of anti-CD40 agonist was found to restore the GC response and bypassed the requirement of Bam32 (217). These results demonstrate the importance of adapter proteins in regulating humoral immune responses.

1.5.5.1 Structure and expression of TAPPs

TAPP1 and TAPP2 are dual PH domain containing proteins initially identified by a database search for putative 3-PI binding domains proteins (218). In parallel, the

Marshall lab identified TAPP1 and TAPP2 based on sequence homology to a related adapter protein, Bam32 (215). TAPP adapters are ubiquitously expressed proteins, with TAPP2 being more highly expressed in hematopoetic cells (215). TAPP adapters are highly homologous structures containing a highly conversed N-terminal region, termed the 'TAPP conserved region' (215). Additionally, TAPP1 and TAPP2 contain two consecutive PH domains and a PDZ (postsynaptic density protein) binding motif at the C-terminus (218, 219). While only the C-terminal PH domain has shown strong PI binding, the function of the N-terminal PH domain remains to be defined. TAPP1 also contains a proline rich motif in the C-terminal region resembling an SH3 binding domain (215).

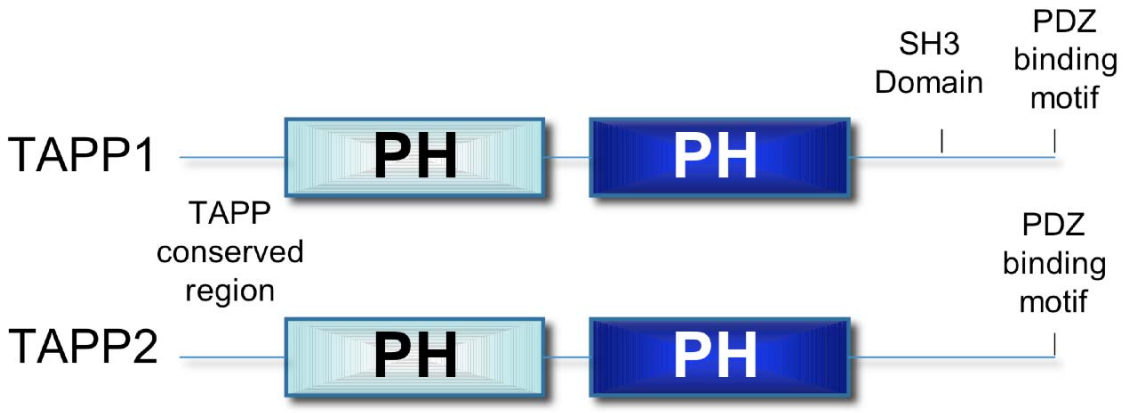

Figure 6: TAPP adapter proteins

Schematic representation of the major domains of TAPP adapters. The C-terminal PH domain of TAPP1 and TAPP2 have shown to preferentially bind PI(3,4)P2, while the function of the N-terminal PH domain has yet to determined. TAPP1 also contains a proline rich motif resembling an SH3 domain. TAPP1 and TAPP2 also contain a PDZ binding motif.

1.5.5.2 TAPP-PI(3,4)P2 interactions

PH domains can mediate both protein-protein or protein-lipid interactions. However, only a subset of PH domains have been found to bind D3 PIs strongly (146). BCR stimulation of BJAB cells, a B cell lymphoma cell line, expressing EGFP labeled TAPP1 and TAPP2 showed recruitment of TAPPs to the plasma membrane, which was abolished following treatment with PI3K inhibitors (215). Interestingly, only the C-terminal PH domain of TAPPs was necessary for membrane recruitment, while the N-terminal PH domain showed no detectable membrane interactions (215). Similarly, in T cells stimulation through the CD28 costimulatory molecules led to recruitment of TAPP -PH domain to the plasma membrane (220). In vitro PI interaction assays demonstrated highly selective binding of TAPP C-PH domain to PI(3,4)P2 (218). Mixed vesicles of various 3-PI, demonstrate that TAPP adapters specifically bind PI(3,4)P2 in vitro (221). Consistently, TAPP2 recruitment in BCR stimulated BJAB cells demonstrated membrane recruitment paralleling PI(3,4)P2 production, showing delayed but sustained recruitment kinetics (215).

The crystal structure of the C-terminal PH domain of TAPP1 has provided insight into the specificity and affinity for PI(3,4)P2 (222). Although TAPPs were discovered based on sequence homology to Bam32 their PH domains show unique PI binding specificity (218). While Bam32 shows relatively equal affinity for PI(3,4,5)P3 and PI(3,4)P2 (218), TAPPs are highly specific for PI(3,4)P2 (218, 221, 222). Crystal structures of Bam32-PH domain and TAPP1 C-PH domain revealed an alanine residue in TAPP1 C-PH domain sterically hindering its ability to bind PI(3,4,5)P3 (222). Substitution of the

alanine with a glycine residue converts Bam32 into a PI(3,4)P2 TAPP like PH containing domain protein (222). Point mutations R212L and R218L in the C-terminal PH domains of TAPP1 and TAPP2, respectively, completely abolished binding to PI(3,4)P2 (215, 223).

1.5.5.3 Regulation of TAPP membrane recruitment

Phosphoinositide phosphatases function by selectively removing phosphates from the inositol ring in turn activating or inhibiting particular cellular processes. SHIP functions by selectively removing the D5 phosphate of PI(3,4,5)P2 generating PI(3,4)P2 (140), the binding lipid of TAPP1 and TAPP2. SHIP dephosphorylation alters membrane phospholipids dynamics, shutting down certain PI3K dependent pathways while activating others (113). Coligation of BCR and FcγRII was found to inhibit Btk recruitment, but did not affect TAPP2 membrane localization (224). Therefore, SHIP activation can terminate Btk recruitment, while increasing levels of PI(3,4)P2 and enhancing recruitment of TAPPs (224). In contrast, 4-phosphatases have been shown to preferentially dephosphorylate the D4 position of PI(3,4)P2, generating PI(3)P (142, 143). Membrane recruitment of GFP-PH-TAPP1 was significantly reduced when co-transfected with wild-type 4-phosphatases as compared to empty vector control (225), terminating PI(3,4)P2 signaling.

Hydrogen Peroxide or H_2O_2 has been found to regulate PI3K dependent proteins through the generation of PI(3,4)P2 (226). Peroxide stimulation induces preferential increases of PI(3,4)P2 over PI(3,4,5)P3 (226). Transient transfection of BJAB lymphoma cell line

with EGFP labeled PI(3,4)P2 binding proteins, including TAPP2, showed plasma membrane recruitment following dose dependent stimulation with peroxide (227). Interestingly, costimulation through the antigen receptor and treatment with hydrogen peroxide leads to synergistic increases in TAPP2 membrane recruitment (227).

1.5.5.4 TAPP interacting partners

Identification of the TAPP-phosphoinositide interaction has provided the first insights into the functional roles of these adapters; however, the downstream signaling mechanism remains to be determined. Biochemical studies of TAPP protein interaction partners have tried to characterize the signaling complexes associated with TAPP adapters. A yeast two hybrid screen of the C-terminal PDZ binding motif of TAPP1 identified MUPP1, a multi-PDZ containing protein with no known catalytic activity (219). TAPP mutants lacking C-terminus PDZ binding motif abolished MUPP1 binding (219). TAPP1 has also been shown to interact with PTPL1 or protein tyrosine phosphatase like protein-1 (228), a phosphatase possessing both positive and negative regulatory roles (229). Although TAPP-PTPL1 interaction did not appear to influence phosphatase activity, TAPP1 was found to colocalize with PTPL1 in the cytosol of unstimulated cells and at the plasma membrane following stimulation H_2O_2 (228). Depletion or knockout of PTPL1 also showed enhanced Akt and STAT4/6 phosphorylation suggesting that this phosphatase play an important negative regulatory role (230, 231). Consistently, TAPP1 knockdown in IGF1 stimulated HEK-293 cells showed enhanced Akt phosphorylation (228), suggesting it may have a regulatory role by

targeting the localization of PTPL1. The functional significance of these interactions in the immune system has yet to be investigated.

To determine the role of TAPP adapters in lymphocytes a mass spectrometry analysis of TAPP2 interacting proteins identified 40 potential partners involved in signal transduction, cytoskeletal rearrangement and endocytic trafficking (232). TAPP2 was found to interact with the cytoskeletal protein utrophin and utrophin associated protein, β2-syntrophin (232). Utrophin, a homologue of dystrophin, is often found to be upregulated in patients with muscular dystrophy (233), while syntrophin function as scaffold protein binding to utrophin and dystrophin. The utrophin/synthrophin complex has been shown regulate adhesion through interactions with the dystrophin-glycoprotein complex (234). Interestingly, BCR stimulated BJAB cells overexpressing wildtype or membrane targeted TAPP2 showed enhanced adhesion to both fibronectin and laminin (232). In contrast, knockdown of TAPP1/2 or inhibition of PI3K showed dramatic reduction in adhesion to ECM proteins (232). The C-terminal PDZ binding motif of TAPP1 was also found to interact with the PDZ domains of γ1-, α1- and β2-synthrophin isoforms in circular membrane ruffles (235), suggesting that complex may play a role in regulating actin dynamics. Additionally, siRNA knockdown of PTPL1 in MCF breast cancer cells decreased adhesion to ECM proteins, including fibronectin and showed increased invasiveness on matrigels (236). Although TAPPs have been found to regulate adhesion, the mechanism is still unclear.

1.6.0 Thesis Overview

1.6.1 Study rationale

Phosphoinositide 3-kinase (PI3K) signaling pathway is linked to a number of key cellular processes including cell survival, proliferation, differentiation and motility (2). Regulation of this pathway in immune cells is crucial for prevention of leukocyte-derived cancers as well as development of autoimmune diseases (138, 237-239). The BCR activates PI3K (240) and this receptor plays critical roles in B lymphocyte development and induction of antibody responses (154, 158). PI3K functions by phosphorylating the D3-position of membrane phosphoinositides generating PI(3)P, PI(3,4)P2 and PI(3,4,5)P3. These secondary messengers regulate various cellular activities by functioning as membrane docking sites for PH domain containing proteins. While PI(3,4,5)P3 is clearly implicated in activation of key mitogenic signaling pathways, the unique signaling events downstream of PI(3,4)P2 are not well understood. TAPP1 and TAPP2 are ubiquitously expressed PH domain containing adaptor proteins, which have been found to selectively bind to PI(3,4)P2 in a delayed but sustained fashion following BCR stimulation (215). Interestingly, TAPP1 was previously reported to associate with PTPL1 (228), a phosphatase known to play a critical role in STAT (230) and Akt (231) activation, indicating that TAPPs may function as a negative regulator of immune cell activation.

1.6.2 Study hypothesis

TAPP adaptor proteins function as negative regulators of B cell activation. Genetic disruption of TAPP protein recruitment will result in abnormally enhanced B cell functional responses.

1.6.3 Experimental approach

To identify the functional role of TAPP-phosphoinositide interactions, a knock-in (KI) mouse model was generated bearing mutations within C-terminal PH domains of both TAPP1 and TAPP2 preventing their binding to PI(3,4)P2 (223). Here we report the impact of these mutations on humoral immune responses and B lymphocyte activation.

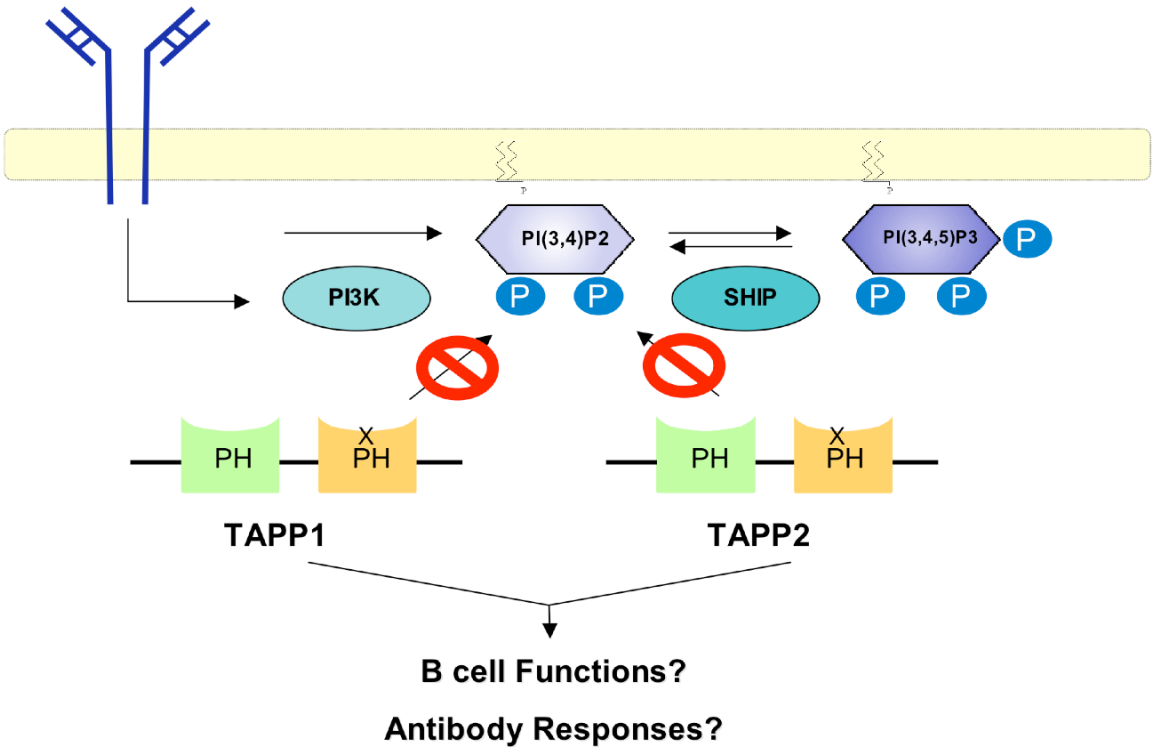

Figure 7: TAPP KI model

TAPP1/2 KI mice were created by introduction of mutations into the C-terminal PH domains of TAPP1 (R211L) and TAPP2 (R218L), and then crossed to TAPP1 and TAPP2 double homozygous mutants. The mutations introduced substituted critical arginine residues with leucines and were found to abolish TAPP-PI(3,4)P2 interactions. $TAPP1^{R211L/R211L}$ and $TAPP2^{R218L/R218L}$ mutate proteins were expressed at normal levels in various tissues.

1.6.4 Significance

Dysregulation of the PI3K pathway, leading to excessive activation, has been linked to a number of diseases including allergy and asthma (241, 242), lymphomas and leukemias (243, 244), and autoimmune disorders (138). However, many avenues of PI3K signaling are still not well understood and are required for increasing our understanding about the origin of PI3K related diseases, as well as for the development of more safe and effective drug treatments. These studies will help define a specific downstream signaling pathway and cellular functions regulated by TAPP-PI(3,4)P2 interactions in lymphocytes. In addition, they could provide insights into the functional roles of TAPP1 and TAPP2 in the immune system and identify new regulatory mechanisms governing germinal centre and antibody responses.

MATERIALS AND METHODS

Mice

TAPP1$^{R211L/R211L}$ TAPP2$^{R218L/R218L}$ mutant mice were generated as described (223). Mice were generated and maintained on a C57BL6 background and a TAPP wild-type line generated from littermates was used as controls in all experiments. μMT mice (Igh-6^{tm1cgn}) were purchased from The Jackson Laboratory. All animals were housed at the Central Animal Care Facility (University of Manitoba, Winnipeg, MB.) in compliance with the guidelines established by the Canadian Council on Animal Care.

Immunizations

To study T cell-dependent antibody responses, TAPP KI or littermate control mice were injected intraperitoneally (i.p.) with either 10ug of NP-OVA precipitated in 2.0mg of alum or 500ul of 10% sheep red blood cells (Innovative Research) diluted in PBS. In the indicated experiments, mice were boosted on day 29 post-immunization with 10ug of NP-OVA without alum. To study T cell-independent antibody responses, mice were immunized by intraperitoneal injection with either 12.5ug of NP(41)-AECM-Ficoll (Biosearch Technologies, Inc.) or NP(0.7)-LPS (Biosearch Technologies, Inc.) in HBSS. Blood samples were collected at the indicated days from either the tail vein or by cardiac puncture.

Serum antibody measurements

Serum was harvested from blood samples by incubation at 37°C for 30 min or 4°C overnight to clot red blood cells and then centrifugation at 3000 rpm for 5 min. For ELISA assays, ninety-six well assay plates were coated overnight at 4°C with capturing antibody or antigen diluted in carbonate coating buffer (0.015M Na_2CO_3, 0.035 M $NaHCO_3$, 0.05% NaN_3, pH 9.6). Antibodies binding to nitrophenyl (NP) were assayed by coating wells with NP_3-BSA or NP_{20}-BSA (Biosearch Technologies, Inc.) to detect antigen-specific antibody responses. Total antibodies levels were determined by coating with either anti-mouse IgM (Jackson ImmunoResearch Laboratories), IgG (Jackson ImmunoResearch Laboratories) or IgE antibodies (BD Pharmigen). Coated plates were then incubated with blocking buffer (2% BSA in washing buffer) for 2 hours at 37°C and then washed (PBS, 0.05% Tween 20, 0.02% NaN_3, pH 7.4). Serum samples and standards were serially diluted using ELISA dilution buffer (1:10 dilution of blocking buffer with wash buffer) and incubated at 37°C for 2 hours or overnight at 4°C. Plates were washed and bound antibody levels were detected using biotinylated anti-mouse IgM, IgG1, IgG2c or IgG3 antibodies (all Southern Biotech). After incubating overnight at 4°C, plates were washed and incubated with streptavidin alkaline phosphatase for 50 minutes at room temperature (RT). Following washing, p-nitrophenyl phosphate tablets (Sigma Aldrich) were dissolved in ELISA substrate solution and then added to each well. Absorbance at 405nm and 690nm was read using a Molecular Devices plate reader.

Table 2: ELISA

Total Ig	Coat	Sample Dilution	Standard	Detection
IgM	5ug/ml	Basal 1:5K Primary 1:10K Secondary 1:100K	20ng/ml	0.13ug/ml
IgG1	5ug/ml	Basal 1:5K Primary 1:10K Secondary 1:100K	20ng/ml	0.1ug/ml
IgG2c	5ug/ml	Basal 1:5K Primary 1:10K Secondary 1:100K	20ng/ml	0.66ug/ml
IgE	0.5ug/ml	Basal 1:10 Primary 1:50	100ng/ml	0.1ug/ml

Flow cytometry analyses

Single cell suspensions were generated from the spleen, lymph nodes, bone marrow or peritoneal cavity. Erythrocytes were depleted using ACK lysis buffer (0.15M NH_4Cl, 1 mM $KHCO_3$, 0.1mM Na_2EDTA-2H20, pH 7.4) for 2 minutes on ice, followed by neutralization with PBS. Suspensions were filtered using cell strainers (BD Falcon 40μm nylon) and counted using hemocytometer. Cells were incubated with 2.4G2 antibody for 10 minutes on ice to block Fc receptors and then washed with FACS buffer (PBS+2% FBS). Approximately $2x10_6$ cells were incubated with biotinylated Abs for 25 minutes on ice. Following washing with FACS buffer, directly conjugated Abs and streptavidin conjugates were incubated for 15 minutes on ice. Cells were then washed and resuspended

in approximately 350ul of FACS buffer and acquired immediately on a FACS Canto II flow cytometer (BD Biosciences). All data were analyzed using FlowJo software (TreeStar).

The following antibodies used for flow cytometry were purchased from BD Pharmingen, unless otherwise stated: fluorescein isothiocyanate (FITC) Rat-Anti-Mouse CD4, Phycoerythrin (PE) anti-mouse B220, FITC anti-mouse CD21, PE anti-mouse CD19, APC Streptavidin, PE antimouse CD5, PE anti-mouse CD23, APC Rat anti-mouse CD45R/B220, PerCP Rat anti-mouse CD45R/B220, PE conjugated Hamster Anti-Mouse CD95 (Fas), PE anti-mouse CD5FITC anti-mouse T-and B-Cell activation antigen GL7, Biotin Rat Anti-Mouse CXCR5 Rat anti-mouse CD19-APC (Southern Biotechnology Associates, Inc.), rat Anti-mouse IgD-Biotin (Southern Biotechnology Associates, Inc.), Pacific Blue conjugated anti-mouse/human CD45R/B220 (eBioscience)), PE conjugated anti-mouse ICOS (Cat. No. 12-9942-81; eBioscience), and AlexaFluor647 anti-mouse CD4 (eBioscience).

Ex vivo apoptosis was determined using Annexin V-FITC (Biovision) and DAPI (Invitrogen) staining. Cells were collected and stained according to the manufacturers protocol. Briefly, approximately $1x10^6$ cells were resuspended in Annexin V Binding Buffer (BD Pharmingen), and incubated with 1ul of Annexin V-FITC and 1ul DAPI (100ng/ul working solution) at RT for 10 minutes. Cells were then analyzed immediately on the FACS Canto II.

BrdU labeling and detection

To measure in vivo DNA synthesis of germinal center B cells, TAPP KI and littermate controls were immunized with 10ug NP-OVA precipitated in 2mg of alum. Five hours before sacrifice on day 14, mice were injected with 5mg of 5-bromo-2'-deoxyuridine (BrdU) (Sigma Aldrich) in PBS. After cell surface staining of splenocytes as described above, cells were fixed and permeabilized on ice for 30 min using BD Cytofix/Cytoperm solution, washed with PBS containing 0.1% saponin, and then incubated in PBS+1% saponin for 10 minutes. Fixed and permeabilized cells were treated with deoxyribonuclease I (0.15M NaCl, 4.2 mM $MgCl_2$, 10uM HCl, 50 Kunitz units/ml DNase I) (Sigma Aldrich) for 30 minutes at 37°C to expose the incorporated BrdU. Incorporated BrdU was detected using a FITC labeled anti-BrdU antibody (eBioscience).

Tissue sectioning, confocal microscopy and image analysis

Spleens were harvested 14 days after NP-OVA immunization, embedded in O.C.T. compound (Tissue Tek) and snap frozen in liquid nitrogen. Sections were cut at 8μm using a cryostat and placed onto glass slides (Fisher Superfrost/Plus). Frozen sections were fixed for 15-20 minutes using ice cold acetone, blocked with 5% goat serum for 30 minutes at room temperature and then incubated with biotinylated anti-IgD for 2 hours at RT or overnight at 4_oC. Sections were then washed with PBS+0.1% Tween20 and stained with streptavidin-Alexa 647, anti-GL7-FITC, and anti-CD4-PE in PBS+2% BSA for

approximately one hour at RT. After washing, anti-fade mounting solution (Prolong Gold, Molecular Probes) and cover slips were applied. Sections were imaged using a confocal microscope (Ultraview LCI, Perkin-Elmer). The size of germinal centers was determined using Ultraview image analysis software to calculate the relative areas of GL7+IgD- regions and the number of GCs per section was determined by visual inspection.

B cell isolation, cultures and adoptive transfer experiments

Spleen or pooled lymph nodes (mesenteric, inguinal, popliteal) were harvested from naïve TAPP KI or littermate control mice. Erythrocytes were depleted using ACK buffer for 2 minutes on ice. Splenic B cells were purified by MACS depletion of CD43+ cells using magnetic beads, according to manufacturers protocol (Miltenyi Biotech). Briefly, cell suspensions were incubated with anti-CD43 microbeads on ice for 15 minutes, washed and passed through a magnetic column with 0.5% BSA/PBS solution.

For cell division assays, purified splenic or lymph node B cells were resuspended in 2% FBSPBS solution at 6 million cells/ml and labeled using 2.5μM of carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) for 5 minutes. Cells were then washed with 5% FBS-PBS and resuspended at 2 million/ml in complete medium (RPMI 1640 containing penicillin-streptomycin, 2-mercaptoethanol and 10% FBS). Purified B cells ($4x10^5$ cells/200ul) were then stimulated and cultured for 72 hours in 96 well round bottom tissue culture plates. For microscopic assessment of homotypic adhesion cells

were plated in flat-bottomed plates and cultured for 3hr or 20hr. Stimulants included: Purified anti-mouse CD40 (HM40-3) (BD Pharmingen), $F(ab')_2$ goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, Inc.), lipopolysaccharides from Escherichia coli 026:B6 (Sigma Aldrich) and IL-4 (PeproTech). For assessment of cell viability, splenic or lymph node B cells were resuspended in complete medium at 2 million/ml and plated in 96 well round bottom plates and stimulated with the indicated stimuli for approximately 72 hours. During the last 2 hours of incubation, 20ul of 5mg/ml working solution of thiazoylyl blue tetrazolium bromide (MTT) (Alfa Aesar) was added to each well and mixed thoroughly. At the end of culture, formazen crystals generated in the cell pellets were dissolved in 200ul of DMSO and transferred to an ELISA plate. Absorbance were determined at both 560nm and 670nm using ELISA plate reader.

For adoptive transfer experiments, purified splenic B cells from either TAPP KI or WT mice were injected intravenously (22.5-30 $*10^6$/recipient) into the tail vein of B cell deficient hosts (μMT). Five day post-transfer, reconstituted mice were immunized intraperitoneally with 10ug of NP-OVA precipitated in 2mg of alum. Fourteen days post-immunization, recipient mice were analyzed for germinal centre B cell formation and T cell activation.

Western blotting

Purified splenic or lymph node B cells were stimulated with $F(ab)_2$ anti-IgM for the indicated times and then immediately lysed using NP40. Protein samples were mixed with

laemmli buffer (Sigma), boiled for 5 minutes and then run on 4-15% polyacrylamide gradient gels (Biorad) and then transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at RT with TBS containing 0.1% tween-20 and 3-5% skim milk (Santa Cruz). Membranes were then washed 3 times with TBST and then incubated with primary antibodies (anti-phosphoAkt Ser 473 or Thr 308 or total Akt; Cell Signaling) in TBST containing 5% BSA and kept gently shaking overnight at 4 degrees. Blots were then washed 3 times with 1X TBS with 0.1% tween-20 and primary Abs were detected using anti-rabbit-HRP (Cell Signaling). Blots were then developed using enhanced chemiluminescence substrate (GE Healthcare) and signals were detected with the Fluorchem 8800 chemiluminescence imager (Alpha Innotech).

Intracellular phosphoprotein staining

Fresh splenocytes were equilibrated for 1 to 2 hr at 37°C in complete medium, stimulated for 5 min with 10 ug/ml $F(ab)_2$ goat anti-mouse anti-IgM, then immediately fixed using pre-warmed BD Cytofix buffer (BD Biosciences) incubated at 37°C for 15 min. Cells were then washed with FACS staining buffer (PBS, 0.2% BSA) and permeabilized with BD Phosflow Perm Buffer III (BD Biosciences) on ice for at least 30 min or -20°C overnight. Cells were then washed twice and equilibrated at RT in FACS staining buffer for 15 min prior to staining for 30 min using a 1:10 dilution of labeled anti-phosphoprotein Abs (all BD Bioscience): PE-mouse anti-Akt (pT308), Alexa488 mouse anti-Akt (pSer473), PE mouse anti-Syk (pY348), Alexa647 mouse anti-PLCγ2 (pY759),

PE mouse anti-BLNK (pY84), pacific blue mouse anti-ERK1/2 (pT202/pY204) Ab. Cells were washed once, suspended in 250 ml FACS buffer and analyzed on a FACSCanto-II instrument (BD Biosciences).

Statistical analysis

T-tests were performed using GraphPad Prism 4. *< 0.05; **< 0.01; ***<0.001

RESULTS

B cell populations in TAPP1$^{R211L/R211L}$ TAPP2$^{R218L/R218L}$ mutant mice

We investigated B lymphocyte development and function in TAPP1$^{R211L/R211L}$ TAPP2$^{R218L/R218L}$ (TAPP KI) mice, in which TAPP proteins are expressed at normal levels but cannot bind PI(3,4)P2 due to mutations in the PI-binding pocket of their C-terminal PH domains (223). PI3K signaling is known to affect development of mature B cell subsets (2), but the contribution of PI(3,4)P2 binding proteins TAPP1 and TAPP2 to this process has not been determined. Initial examination of the lymphoid tissues of TAPP KI mice did not reveal substantial alterations in the proportions of B and T lymphocytes within lymphoid tissues or blood, however a small reduction in the proportion of B cells within the spleen was observed (**Figure 8**). Absolute cell numbers from the spleens of TAPP KI mice were similar to those of the WT mice. The composition of B cell subsets within the spleen was further assessed by flow cytometry, and it was found that TAPP KI mice have intact populations of both mature follicular and marginal zone B cells (**Figure 9**). Interestingly, significant decreases in newly-generated B cell subsets (transitional 1 and 2) were seen in TAPP KI spleens (**Figure 9**). Small reductions in the frequencies of pro-B, pre-B and immature B cells in the bone marrow were also observed (**Figure 10**). We also examined B1 cells, a B cell subset residing in the peritoneal cavity whose development is known to be highly dependant on PI3K signaling (2, 161). It was found that TAPP KI mice have a significant increase in the percentage of

B220+CD5+IgM+ B1 cells in the peritoneal cavity (**Figure 9**). These data indicate that TAPP-PI(3,4)P2 interactions are not essential for development of mature B cell populations, but may influence the dynamics of early B cell maturation or selection within the spleen, bone marrow and peritoneal cavity.

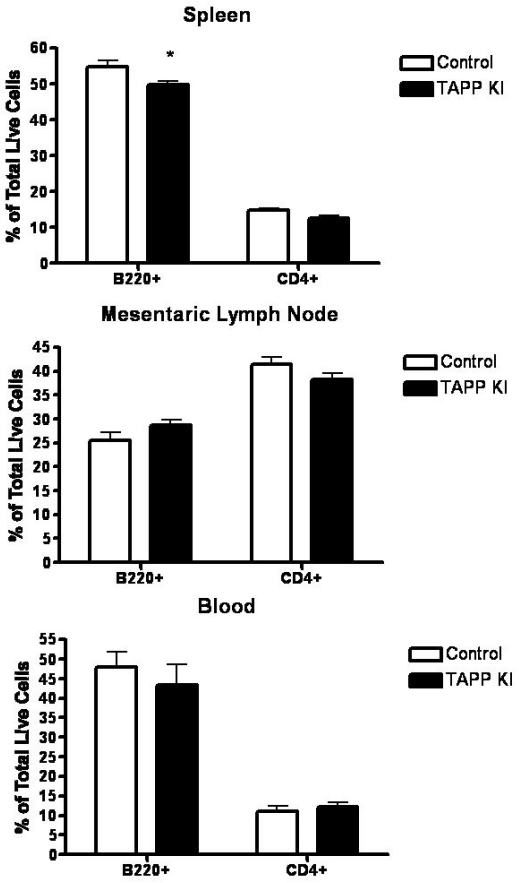

Figure 8: Frequency of B220+ and CD4+ cells in the spleen, mesenteric lymph node and blood

Frequency of B220+ B cell and CD4+ T cell populations were assessed in the blood, spleen and mesenteric lymph node using flow cytometry. The indicated cell populations were gated as indicated and graphed as proportion of total live cells. Graphs represent the mean and standard deviation with a minimum of 4 mice per group.

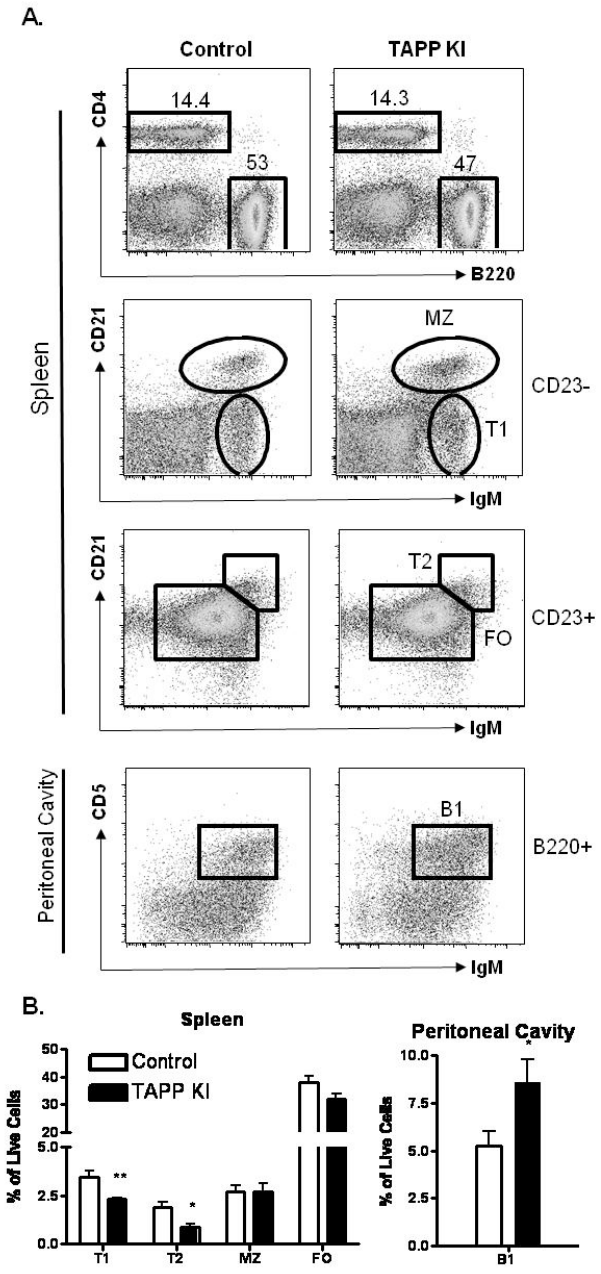

Figure 9: B cell population in TAPP KI mice

A. Flow cytometry analyses were performed on splenocytes and peritoneal wash cells of 8-10 week old TAPP KI and control mice. The indicated cell populations were gated on singlet live cells. The top panel shows staining for B220+ B cells and CD4+ T cells. In

the middle two panel, total live cells were gated on either CD23- or CD23+ populations and further characterized by IgM and CD21 staining to differentiate transitional – type 1 (CD21-IgM+), transitional – type 2 (CD21+IgM+), marginal zone (CD21+IgM+) and follicular B cells ($CD21^{lo}IgM^{lo}$). In the bottom panel, peritoneal wash cells were stained for B1 cells (B220+CD5+IgM+). FACS plots are representative of 4-8 mice examined per group. **B.** Mean percentages of B lymphocyte populations within the spleen and peritoneal cavities. The graphs indicate the frequency of B cell (B220+CD4-) subpopulations as a percentage of total live cells (singlets, DAPI-). Data represent the mean and standard deviation of 4-8 mice per group.

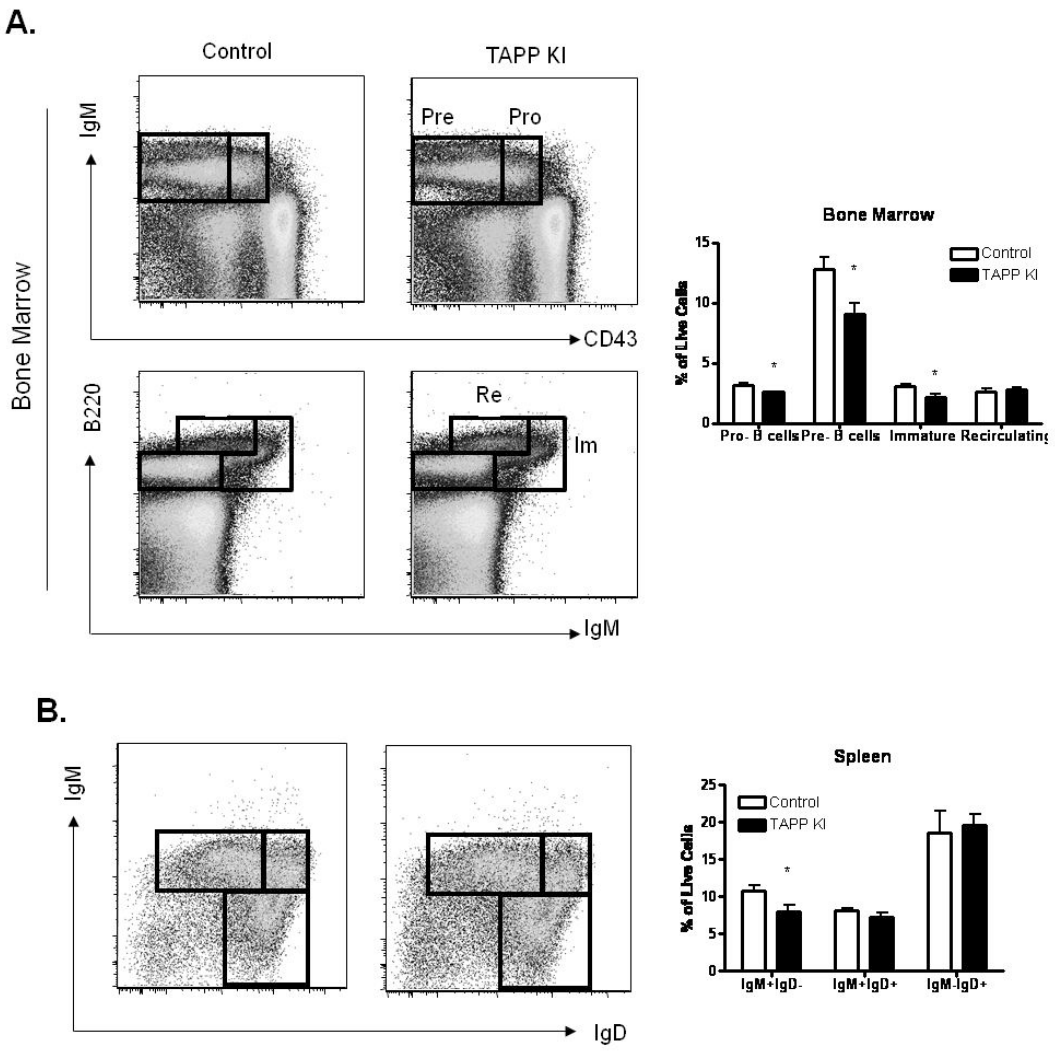

Figure 10: B cell development in the bone marrow and spleen of TAPP KI mice

A. Flow cytometry analyses were performed on the bone marrow of naïve 8-10 week old TAPP KI and control mice. The top panel shows plots of IgM negative cells, gating B220+CD43+ pro- B and B220+CD43- pre B cells. The bottom panel shows gating of immature ($B220^{lo}IgM^{hi}$) and recirculating mature ($B220^{hi}IgM^{lo}$) B cells. The indicated cell populations were gated on singlet live cells. FACS plots are representative of 4 mice per group and the mean percentages of cell populations are shown the right panel. **B.** IgM and IgD staining of splenocytes from naïve 8-10 week old TAPP KI and control mice was

used to distinguish immature (IgD^{lo}, IgM^{hi}), mature (IgD^{hi}, IgM^{lo}) and transitional ($IgD^{hi}IgM^{hi}$) B cells. Plots of 8 mice per group are shown, with right panel showing the mean and standard deviation. The indicated cell populations were gated on B220+DAPI- cells and graphed as a percentage of total live cells (DAPI-).

Antibody responses in TAPP1$^{R211L/R211L}$ TAPP2$^{R218L/R218L}$ mutant mice

As an initial assessment of mature B cell function we examined the impact of TAPP PH domain mutations on serum antibody levels. Serum from TAPP KI and control mice was collected and analyzed for the levels of immunoglobulin (Ig) isotypes. Interestingly, TAPP KI mice showed a significant increase in basal levels of all Ig isotypes examined (**Figure 11**). We went on to determine whether TAPP KI mutations also affected antibody production after immunization with antigen (NP-OVA). Serum was collected at various time points post-immunization and levels of total IgM, IgG1 and IgG2c antibody were measured. Compared to control mice, TAPP1/2 KI mice produced significantly higher quantities of serum IgM, IgG1 and IgG2c, over the 35 day period post-immunization (**Figure 12A**). Similar increases in total serum Ig levels were observed after immunization with 10% sheep red blood cells (**Figure 12B**). Consistent with the increases in total Ab titres, TAPP KI mice were also found to have increased frequency of plasma cells within the spleen at day 14 post-immunization (**Figure 13**). Together these results demonstrate that uncoupling TAPP adaptors from PI(3,4)P2 leads to substantial increases in antibody production.

Immunization with T-dependant Ags such as NP-OVA leads to selective production of antibodies binding the Ag with high affinity, and the average Ab affinity increases over time as rare B cells producing high affinity antibody gain a selective advantage during the response. To examine the levels of antigen-specific antibodies generated in TAPP KI

mice, we used NP_3-BSA and NP_{20}-BSA coated ELISA plates to measure Abs binding the NP molecule with high or low affinity, respectively. The results indicated that, despite elevated levels of total antibody, levels of NP_3- (**Figure 14A**) and NP_{20}-binding Abs (**Figure 14B**) were not markedly altered in the TAPP KI mice.

We further examined Ag specific Ab production after immunization with either type 1 or type 2 T-independent Ags. We also found that levels of NP-binding Abs produced after immunization with T-independent Ags were relatively normal (**Figure 15A,B**). Together these results indicate that TAPP KI mice are capable of mounting antigen-specific antibody responses but exhibit a dysregulation in controlling overall Ab production.

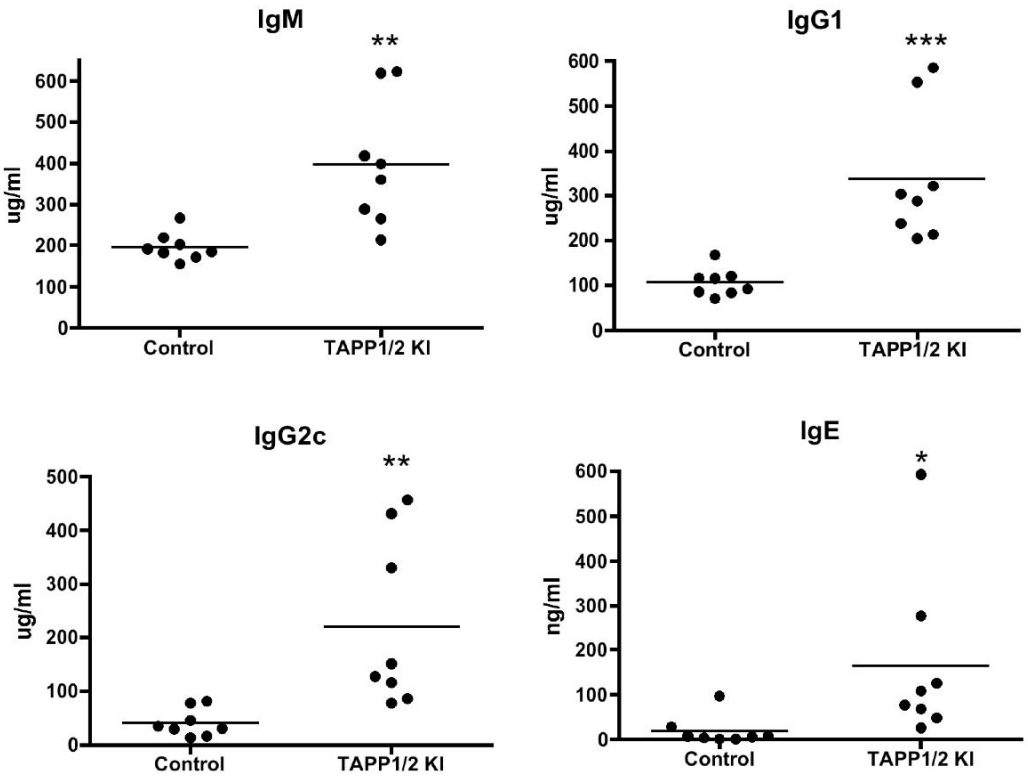

Figure 11: Enhanced basal antibody levels in TAPP KI mice

Sera from naïve TAPP KI or control mice were collected and analyzed for total IgM, IgG1, IgG2c and IgE immunoglobulin production using specific ELISA assays. Individual mice are plotted as single dots, with black lines representing the means.

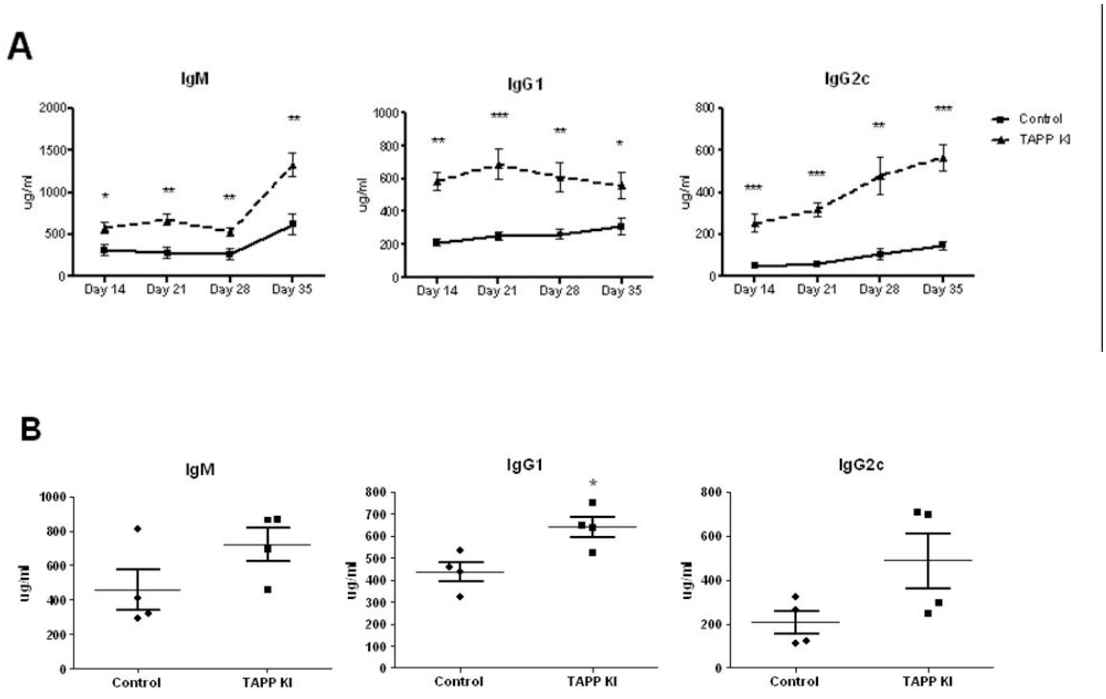

Figure 12: TAPP KI mice show increased total antibody production

A. Control or TAPP KI mice were immunized i.p. with NP-OVA-alum and boosted on day 29. Blood was collected and sera were collected over a 35 day period and analyzed for total antibodies using specific ELISA assays. **B.** Similarly, mice were also immunized i.p with 10% sheep red blood cells. At day 14 post-immunization, sera was collected and levels of the indicated immunoglobulin isotypes measured using specific ELISA assays

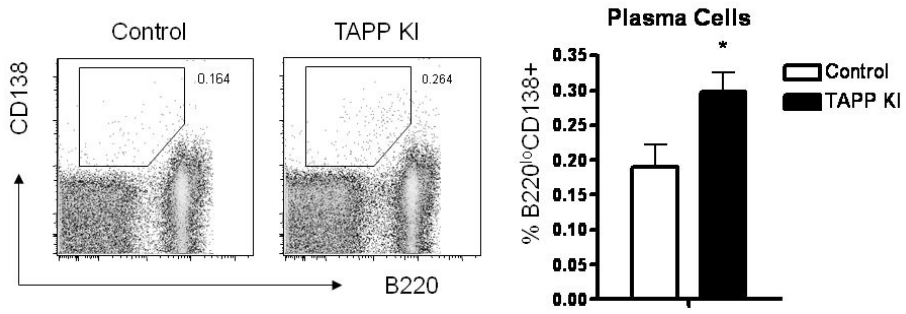

Figure 13: Increased plasma cell differentiation in TAPP KI mice

Flow cytrometry analysis of splenocytes from day 14 NP-OVA immunized mice were found to have increased B220+CD138^{lo} plasma cell population. FACS plots are representative of 4 mice per group. The indicated cell populations were gated on CD4- singlet cells and graphed as a percentage of live lymphocytes (DAPI-).

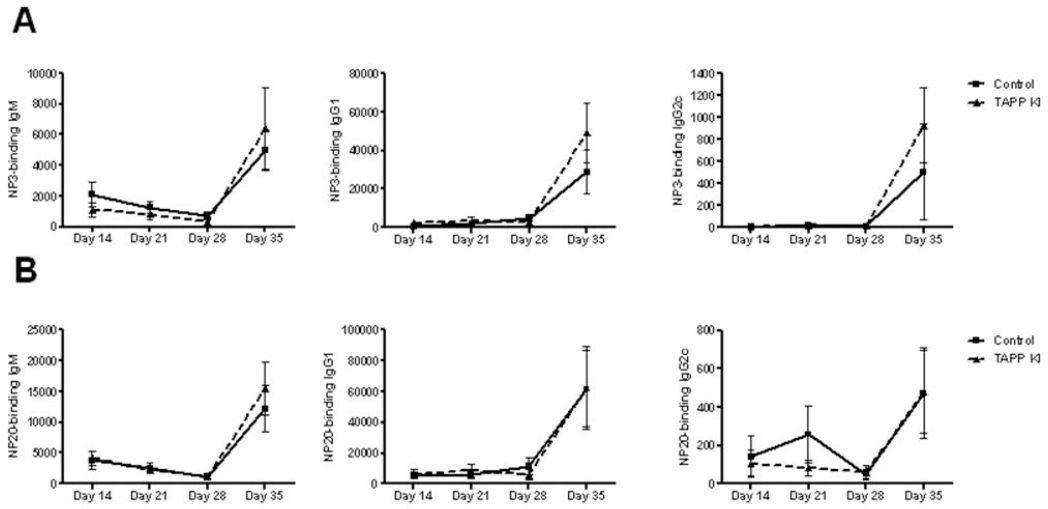

Figure 14: NP specific antibody responses in TAPP KI mice

Mice were immunized with NP-OVA-alum and boosted on day 29. Blood was collected and sera analyzed for NP-binding (**A,B**). To measure generation of antibodies binding NP with high affinity or low affinity, sera were analyzed using NP_3-BSA (**A**) and NP_{20}-BSA (**B**) coated ELISA plates respectively. Note that specific antibody responses remain relatively unaltered in the TAPP KI mice. Graphs represent the mean and standard deviation of at least 4-6 mice per time point.

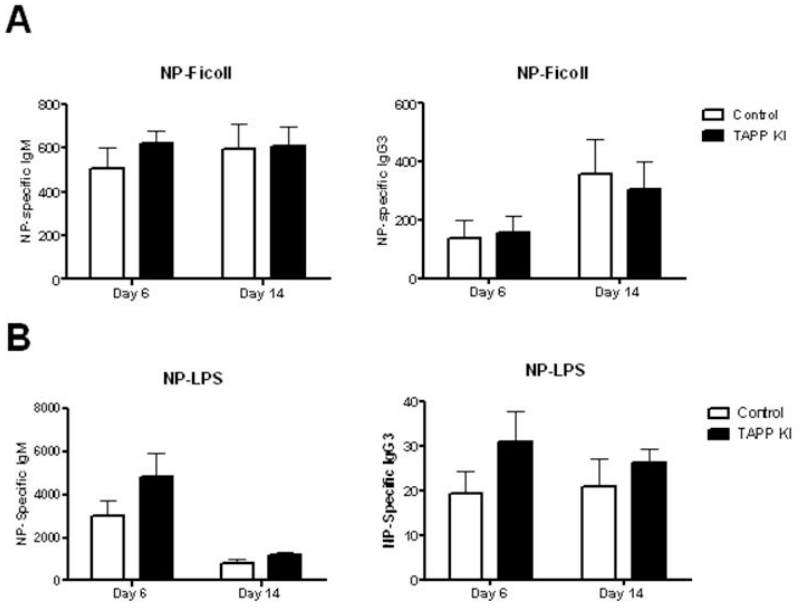

Figure 15: T-independent antibody responses in TAPP KI mice

Mice were immunized i.p. with either (A) NP-Ficoll or (B) NP-LPS. Blood was collected at day 6 and 14 by tail vein or cardiac puncture, respectively. Serum was analyzed for NP specific IgM or IgG3 by using NP_{20}-BSA coated ELISA plates. **A.** TAPP KI mice displayed no significant difference in antigen specific IgM and IgG3 in response to NP-Ficoll. **B.** TAPP KI mice show a trend towards in increase in response to NP-LPS. Graphs are representative of 8-12 mice per group.

TAPP interactions with PI(3,4)P2 modulates B cell activation and survival

To determine if TAPP KI B cells have altered functional responses to antigen receptor stimulation, B cell activation, proliferation and survival were assessed in vitro. Analyses of cell division using CFSE dilution assay showed that TAPP KI B cells underwent more divisions than control B cells during a 3 day stimulation period (**Figure 16A**). Spleen or lymph node B cells show similarly increased cell division induced by anti-IgM (**Figure 16A**); however, no consistent differences in division were found using other mitogens such as LPS, anti-CD40 or IL4. Additional experiments using MTT assay to measure cell proliferation and viability supported the conclusion that TAPP KI B cells mount more robust proliferative responses when stimulated with $F(ab)_2$ anti-IgM (**Figure 16B**).

Co-ligation of the BCR with the inhibitory receptor FcγRIIB was previously found to increase membrane recruitment of TAPP2 associated with increased generation of PI(3,4)P2 by SHIP (215). We thus asked whether TAPP-PI(3,4)P2 interaction may contribute to the inhibitory action of FcγRIIB on B cell proliferation. B cells were stimulated with various doses of intact anti-IgM, co-ligating both the BCR and FcγRIIB. As expected, wild-type B cells show reduced viability and fewer divisions when stimulated with intact anti-IgM, compared to $F(ab)_2$ anti-IgM (**Figure 17A**). TAPP KI B cells appear to be partially resistant to the inhibitory effect of FcγRIIB co-ligation, and show substantially improved cell viability and more divisions than control cells (**Figure 17B,C**). These data suggest that TAPP interactions with the SHIP product PI(3,4)P2

may contribute to B cell regulation via FcγRIIB.

B cell activation is well known to induce the expression of Fas (245), a cell surface receptor that triggers apoptosis following ligation. To determine whether TAPP KI B cells are defective in their ability to undergo Fas induced apoptosis, we activated TAPP KI and WT B cells with anti-CD40 and IL-4 for 2 days and then treated these B cells with an anti-Fas Ab for the last 18 hours. We then measured their ability to undergo Fas-induced cell death using annexin V and DAPI to detect early and late apoptosis, respectively. However, in contrast to Ag induced B cell survival, TAPP KI B cells exhibited similar levels of Fas induced B cell survival as compared to WT B cells, when treated with apoptotic stimuli (**Figure 18**). Taken together, these results suggest that although TAPP KI B cells survive better after Ag induced activation, however they are still susceptible to extrinsic apoptotic signals.

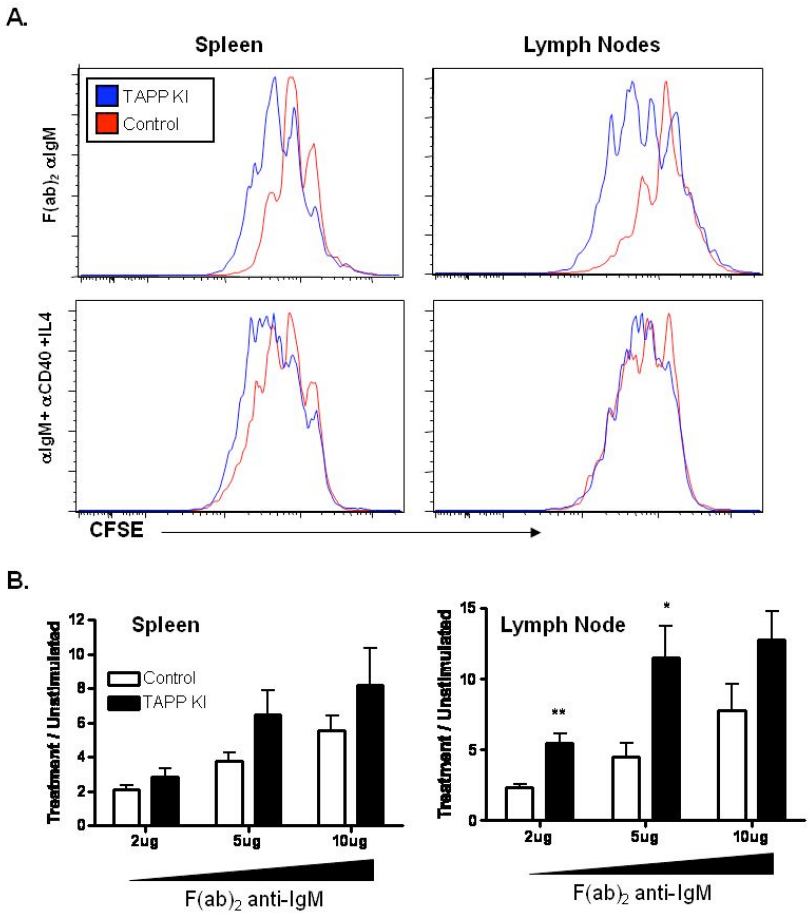

Figure 16: TAPP KI B cells show increased proliferative responses in vitro

Purified splenic or lymph node B cells from TAPP KI or control mice were labeled with CFSE and stimulated in vitro with the indicated mitogens for 72 hours. **A.** TAPP KI B cells show increased division (CFSE dilution) in response to BCR cross-linking using F(ab)2 anti-IgM, however, did not show consistent differences when stimulated with anti-IgM plus anti-CD40+IL-4. FACS histograms are representative of a minimum of 4 independent experiments. **B.** Cell proliferation was assessed 3 days after stimulation using the MTT colorimetric assay. Proliferation index was calculated by normalizing to unstimulated control B cells. Results represent mean and standard deviation of 4 independent experiments.

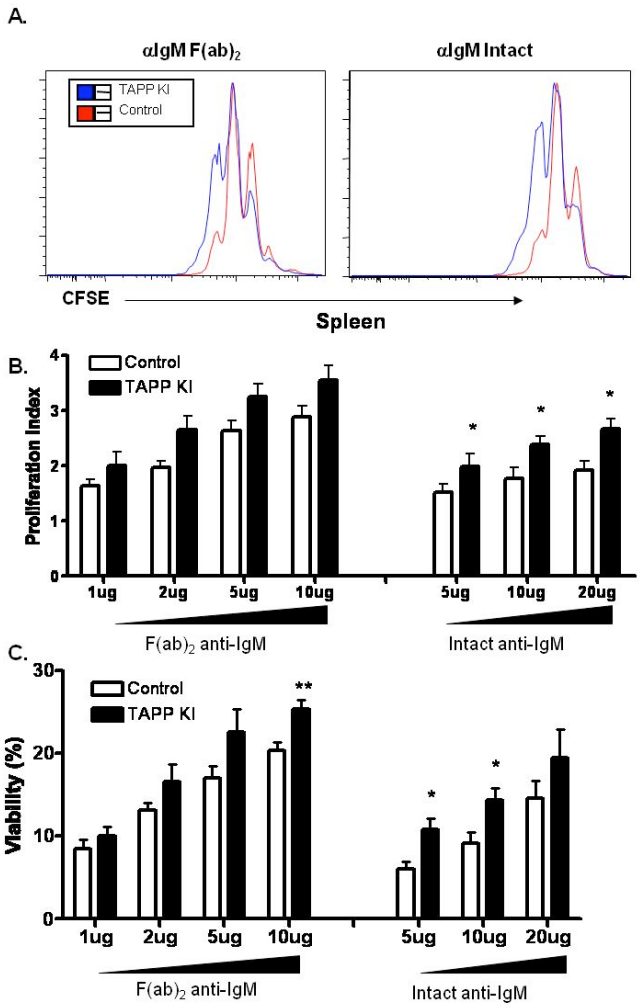

Figure 17: TAPP KI B cells are partially resistant to the inhibitory actions of the FcγRIIB

To compare the response of B cells following engagement of the BCR alone or in combination with FcγRIIB, purified splenic B cells were stimulated with 10ug of intact or F(ab)2 anti-IgM for 72 hours and CFSE dilution was measured by flow cytometry. **A.** FACS plots indicating that TAPP KI B cells have enhanced cell division in the presence

of intact anti-IgM. Results are representative of 5 independent experiments. **B.** Relative CFSE dilution values (calculated as CFSE MFI unstimulated / CFSE MFI stimulated), indicating that TAPP KI B cells are partially resistant to reduced cell division following intact Ab stimulation. **C.** Viability of purified splenic and lymph node B cell cultures after 3 days of stimulation as assessed by flow cytometry.

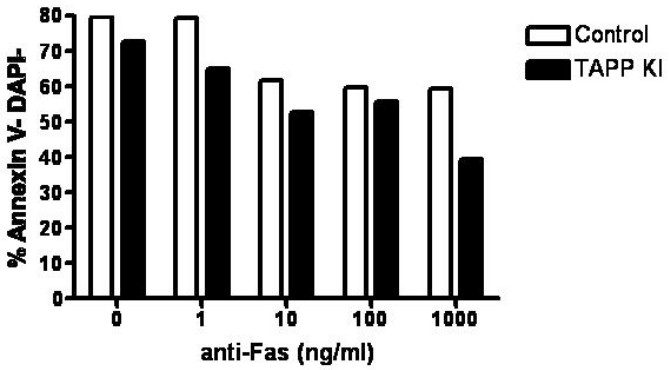

Figure 18: Fas mediated apoptosis of TAPP KI B cells

Purified splenic B cells from TAPP1/2 KI or control mice were stimulated for 48 hours with 1ug/ml of anti-CD40 and 10ng/ml of IL4. B cell cultures were treated with logarthimic doses of anti-Fas for the last 18 hours of culture. Cell survival was determined using Annexin V and DAPI staining. Cell viability was plotted as a percentage of Annexin V and DAPI negative. Graphs are representative of one independent experiment.

TAPP interactions with PI(3,4)P2 modulate Akt phosphorylation

To determine whether disruption of TAPP-PI(3,4)P2 interactions affects PI3K-dependent signaling pathways in B cells, we examined the ability of TAPP KI B cells to activate the Btk/PLCγ2 and Akt signaling axes after antigen receptor (BCR) cross-linking. Initially we determined whether there are any differences in signaling events immediately downstream of the BCR by stimulating splenic B cells with $F(ab)_2$ anti-IgM and measuring phosphorylation of Syk and BLNK by flow cytometry. Consistent with the relatively late membrane recruitment of TAPPs (215), no significant differences were observed in these early tyrosine phosphorylation events (**Figure 19**). We also found that PLCγ2 phosphorylation and calcium flux responses appeared normal in TAPP KI B cells (**Figure 19A,B**). We then examined serine-threonine phosphorylation of Akt, which is known to contribute to B cell survival (203). We observed significantly increased phosphorylation of the serine 473 and threonine 308 residues of Akt upon BCR cross-linking using $F(ab)_2$ anti-IgM, as assessed by Western blot (**Figure 20**) and flow cytometry (**Figure 19A**). These results suggest that TAPP interactions with PI(3,4)P2 are not essential for initiation of BCR signaling but can impact downstream signaling events involving Akt.

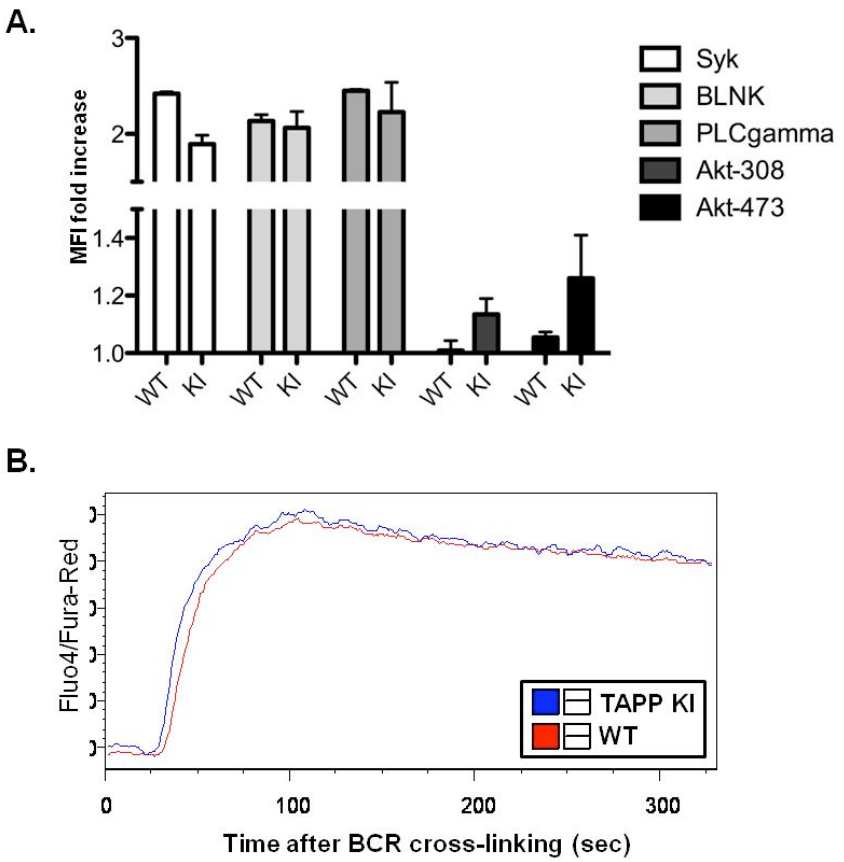

Figure 19: Early BCR signaling events in TAPP KI splenocytes

A. Splenocytes were stimulated with 10ug/ml of F(ab)2 anti-IgM for 5 minutes or left unstimulated, and then fixed, permeabilized and stained with the indicated phospho-specific antibodies. The graph presents fold increases in mean fluorescence intensity (MFI) of staining after stimulation, pooled from two independent experiments. Phosflow performed by Kennedy Makondo **B.** Calicum flux was measured by loading purified splenic B cells with calcium sensitive dyes Fluo-4 and Fura-Red, and stimulated with 10ug of $F(ab)_2$ anti-IgM. The Fluo-4/Fura-Red ratio was monitored by flow cytometry. Results are representative of two independent experiments.

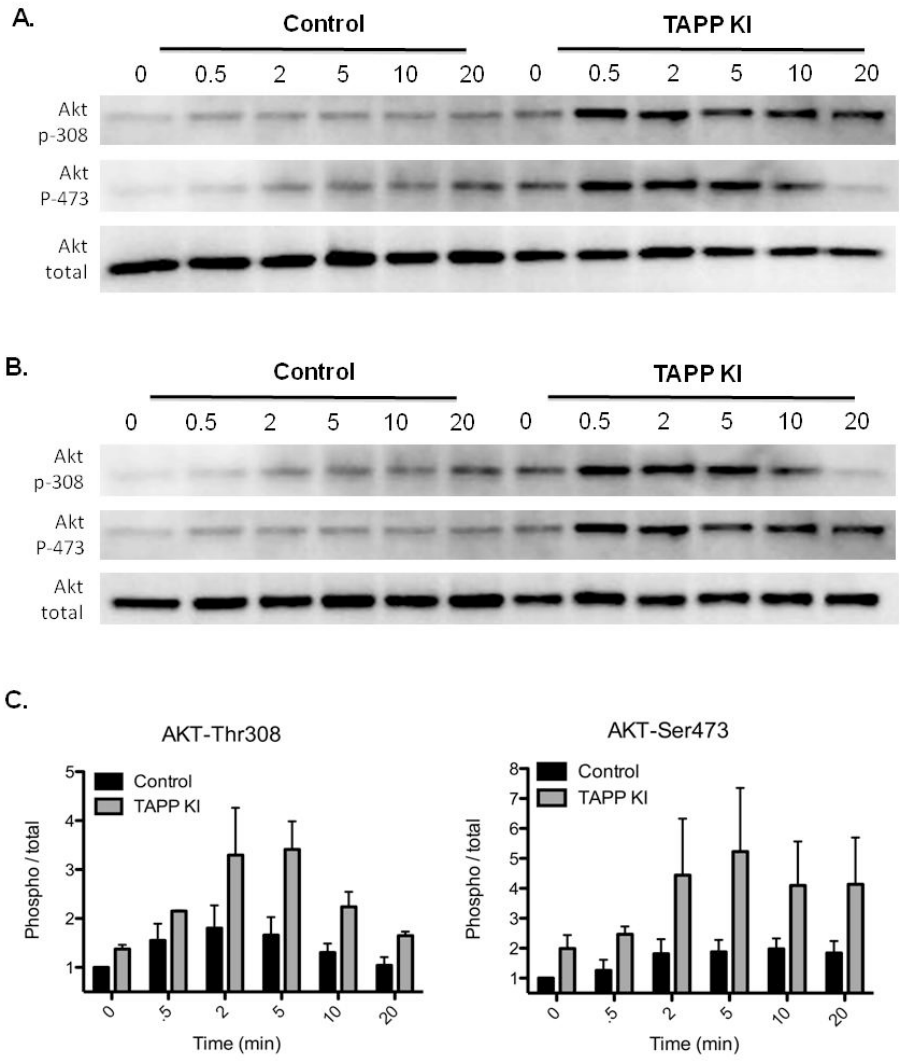

Figure 20: Increased Akt phosphorylation in TAPP KI B cells following BCR cross-linking

Purified splenic (**A**) or lymph node (**B**) B cells were stimulated with $F(ab)_2$ anti-IgM for the indicated time (min) and Akt phosphorylation on ser473 and thr308 was detected by Western blotting. **C.** Graphs showing pooled quantitative data from 3 independent experiments (phospho/total Akt signals normalized to control unstimulated cells). Western blots performed by Sen Hou.

Germinal center responses in TAPP1$^{R211L/R211L}$ TAPP2$^{R218L/R218L}$ mutant mice

B cell responses to T cell-dependent antigens involve extensive B cell proliferation and selection within germinal centers (GC), which is dependant on signaling via PI3K (158, 161). We therefore examined the GC response generated in TAPP KI and control mice after immunization. Flow cytometric analysis of the spleen revealed a significant increase in the frequency of germinal center B cells on days 7 and 14 post-immunization, however, the response declined to normal levels by day 21 (**Figure 21A**). Immunofluorescence microscopy of spleen sections revealed visibly larger clusters of activated B cells expressing the GC marker GL7 at day 14 post-immunization (**Figure 21B**). Quantification of GL7+ cell clusters revealed that TAPP KI mice have a 1.6-fold increase in average GC size, whereas the number of GC observed per section was similar to controls (**Figure 21C**). We assessed whether the increased GC size is associated with enhanced GC B cell division or decreased GC B cell apoptosis. To assess cell division, we labeled cells undergoing DNA replication by administering the thymidine analog BrdU at day 14 after immunization. Five hours later, spleens were harvested and BrdU-labeled cells were detected by flow cytometry. Splenic GC B cells (B220+Fas+) showed similar frequencies of BrdU incorporation in TAPP1/2 KI and control mice (**Figure 22A**), indicating similar rates of DNA synthesis. We then examined GC B cell apoptosis by staining splenocytes at day 14 post-immunization with annexin V and DAPI, to detect loss of plasma membrane and nuclear integrity respectively. As expected, GC B cells (B220+Fas+) show a high frequency of cells expressing apoptosis markers compared to non-GC B cells (**Figure 22B**). However, the GC B cell population from TAPP KI mice

contained significantly more viable cells (**Figure 22B**). These results indicate that the increased GC response resulting from disrupted TAPP-phosphoinositide interaction is associated with increased GC B cell survival.

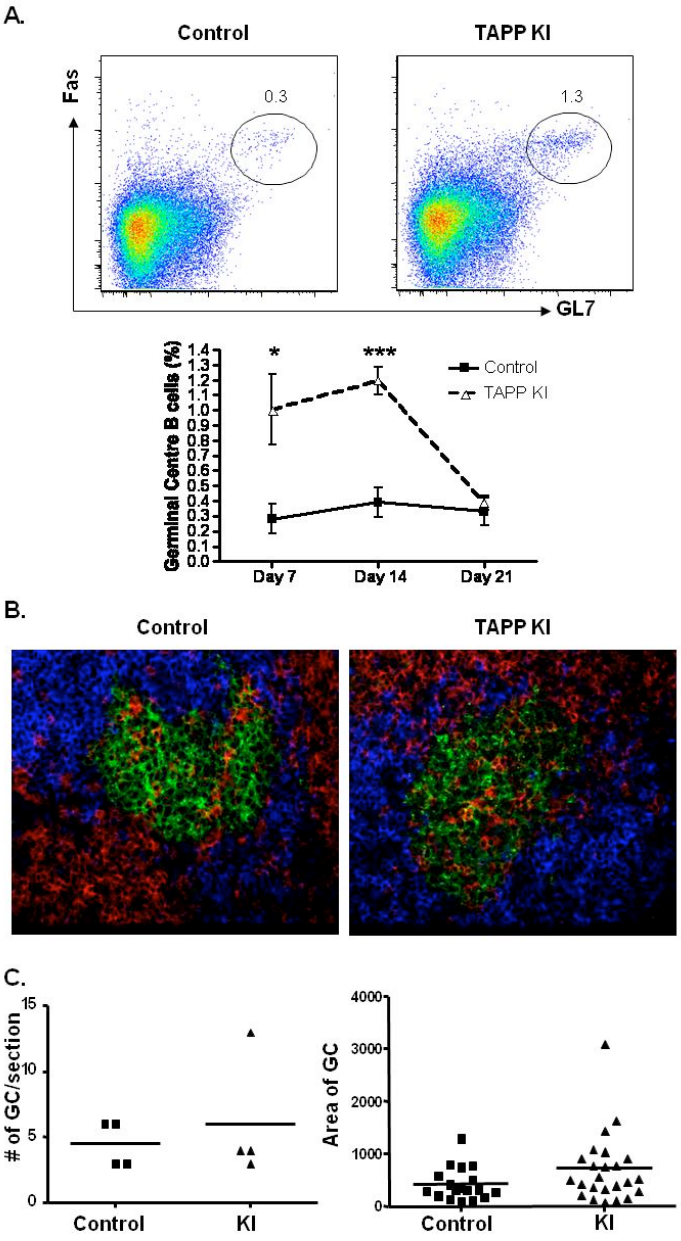

Figure 21: Increased germinal center responses in TAPP KI mice

A. Flow cytometry analysis of splenocytes at day 7, 14 and 21 after NP-OVA immunization, indicating increased GC B cell frequencies. FACS plots are representative of day 14 NP-OVA immunized mice and illustrate the increased frequency of GL7+Fas+

among B220+DAPI- lymphocytes. The graph below indicates that TAPP KI mice have a significant increase in the frequency of germinal centre B cells on day 7 and 14, but no difference by day 21 post-immunization. Data represent the average and standard deviation of a minimum of 3 mice per time point. **B.** Germinal centers were visualized using immunofluorescence microscopy. Spleen sections from day 14 NP-OVA immunized mice were stained with anti-GL7 (green), anti-CD4 (red) and anti-IgD (blue). GL7+ clusters were quantified as number per section and relative GL7+/IgD- staining area of GCs from 4 different mice per group.

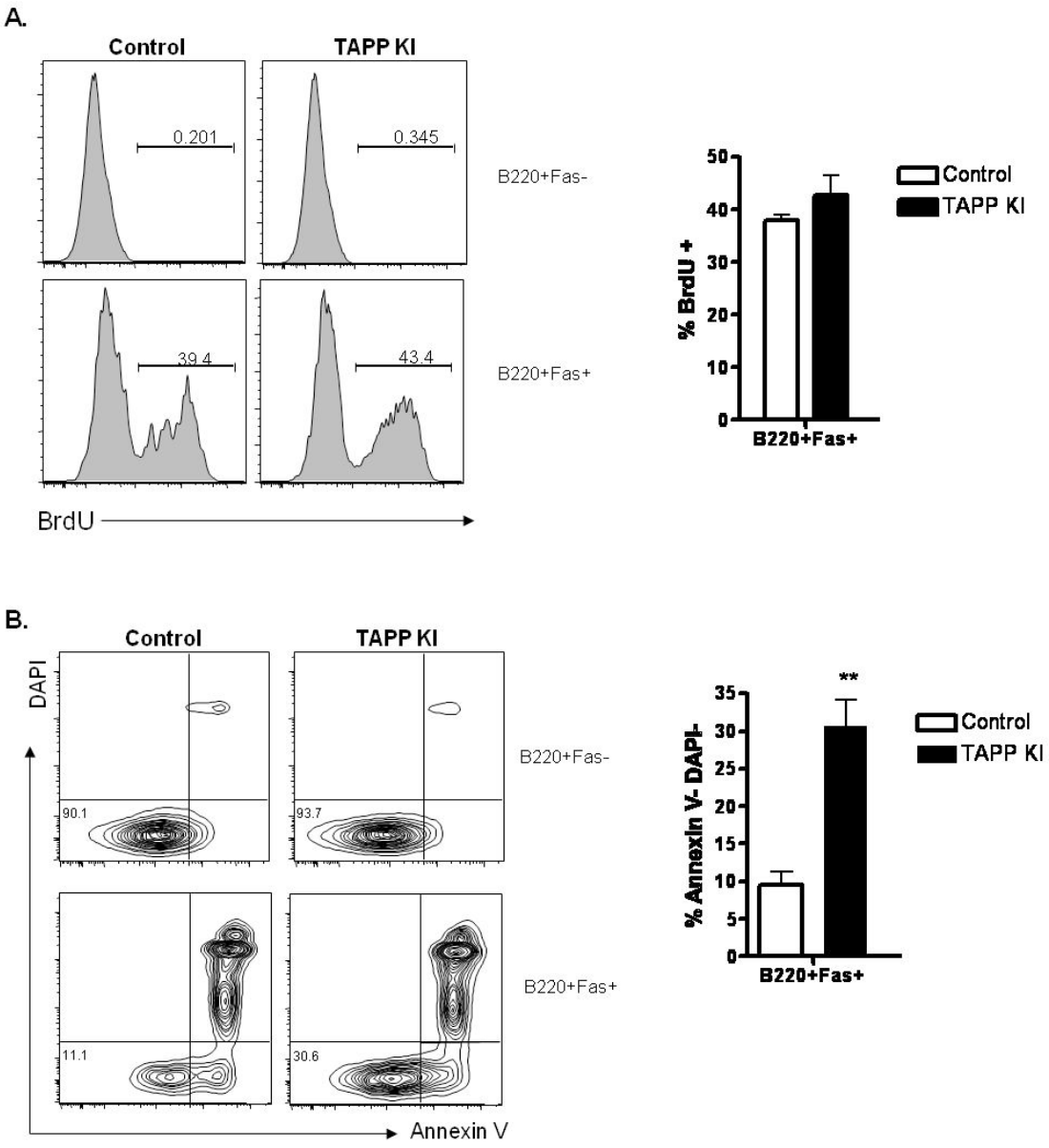

Figure 22: Increased germinal centre B cell survival in TAPP KI mice

A. TAPP KI GC B cells show normal levels of DNA synthesis. At day 14 post-immunization, TAPP KI and control mice were injected with BrdU and sacrificed 5 hours later. Isolated splenocytes were cell surface stained, then fixed, permeabilized, and stained using anti-BrdU antibodies. *Left:* DNA synthesis among GC (B220+Fas+) or non-GC (B220+Fas-) lymphocyte populations was determined as percentage of BrdU

incorporation. *Right:* Similar average BrDu incorporation among GC B cells from TAPP KI or control mice. **B.** Splenocytes from day 14 NP-OVA/alum immunized mice were stained using Annexin V and DAPI to detect cell survival, early apoptosis and loss of nuclear integrity. Representative FACS plots illustrate the cell survival profile among B220+Fas+ and B220+Fas- lymphocytes. *Right:* Graph showing percent viability (Annexin V-DAPI-) among GC B cells from a group of 4 mice. A similar trend was found in 2 independent experiments with 4 mice/group.

Analysis of humoral immune responses in B cell deficient mice reconstituted with TAPP1$^{R211L/R211L}$ TAPP2$^{R218L/R218L}$ B cells

To determine if the enhanced germinal centre and antibody responses in TAPP KI mice is due to a B cell-intrinsic signaling defect, we performed adoptive transfer experiments by reconstituting B cell deficient hosts (μMT) with purified TAPP KI or WT B cells. TAPP KI and WT recipient mice were found to have no significant differences in B cell reconstitution in the spleen (**Figure 23**). Immunization of recipient mice with NP-OVA/alum, revealed no significant difference in the frequency of germinal centre B cells at day 14 post-immunization (**Figure 24A**). Consistently, TAPP KI and WT recipient mice were found to have similar levels of GC B cell (B220+Fas+) apoptosis as assessed by annexin V and DAPI staining (**Figure 24B)**. However, analysis of the immunoglobulin production revealed enhanced switched IgG1 in TAPP KI recipient mice sera (**figure 25**). Taken together, these results suggest that abnormal activation of other immune cell types may contribute to enhanced GC responses within TAPP KI mice. On the other hand, disruption of TAPP-PI(3,4)P2 interactions only in B cells has a measurable impact on IgG1 secretion in an adoptive transfer model.

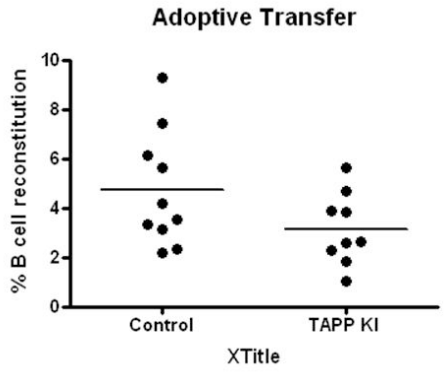

Figure 23: Reconstitution of TAPP KI and WT B cells in B cell deficient hosts.

Purified B cells from either TAPP KI or WT mice were injected into B cell deficient μMT hosts. Five days after reconstitution mice were immunized to with NP-OVA/alum. At day 14 post-immunization, B cell reconstitution in the spleen was then measured using flow cytometry. Graph showing the percentage of CD19+ live lymphocytes. Individual mice are plotted as a single dot, with black lines representing the means.

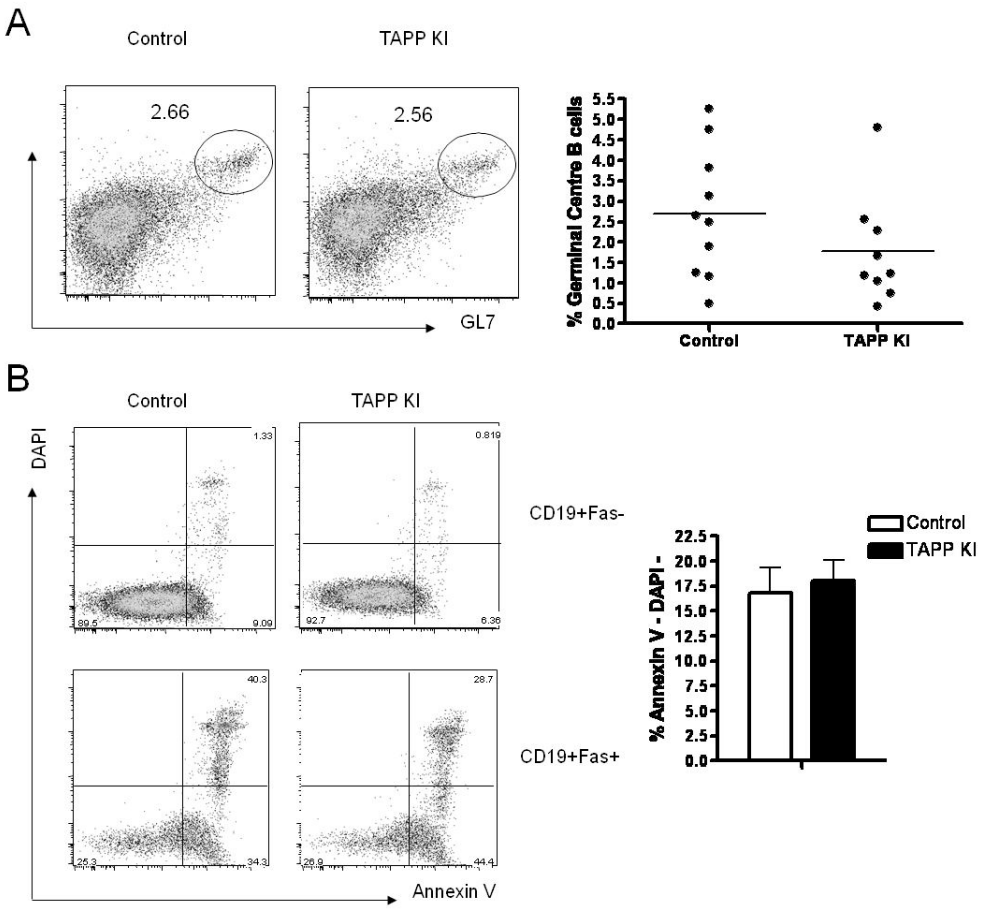

Figure 24: Germinal centre response in B cell deficient mice reconstituted with TAPP KI B cells

Fourteen days after NP-OVA/alum immunization, frequency of germinal centre B cells and survival was determined using flow cytometry. **A.** Germinal centre B cells were gated on CD19+DAPI- singlet lymphocytes. Representative plots and percentages of GC B cells are shown. **B.** Splenocytes from day 14 NP-OVA/alum immunized recipient mice were also stained using Annexin V and DAPI to detect cell survival, early apoptosis and loss of nuclear integrity. Representative FACS plots illustrate the cell survival profile among CD19+Fas+ and CD19+Fas- lymphocytes. *Right:* Graph showing percent viability (Annexin V-DAPI-) among GC B cells from a group of 8-9 mice.

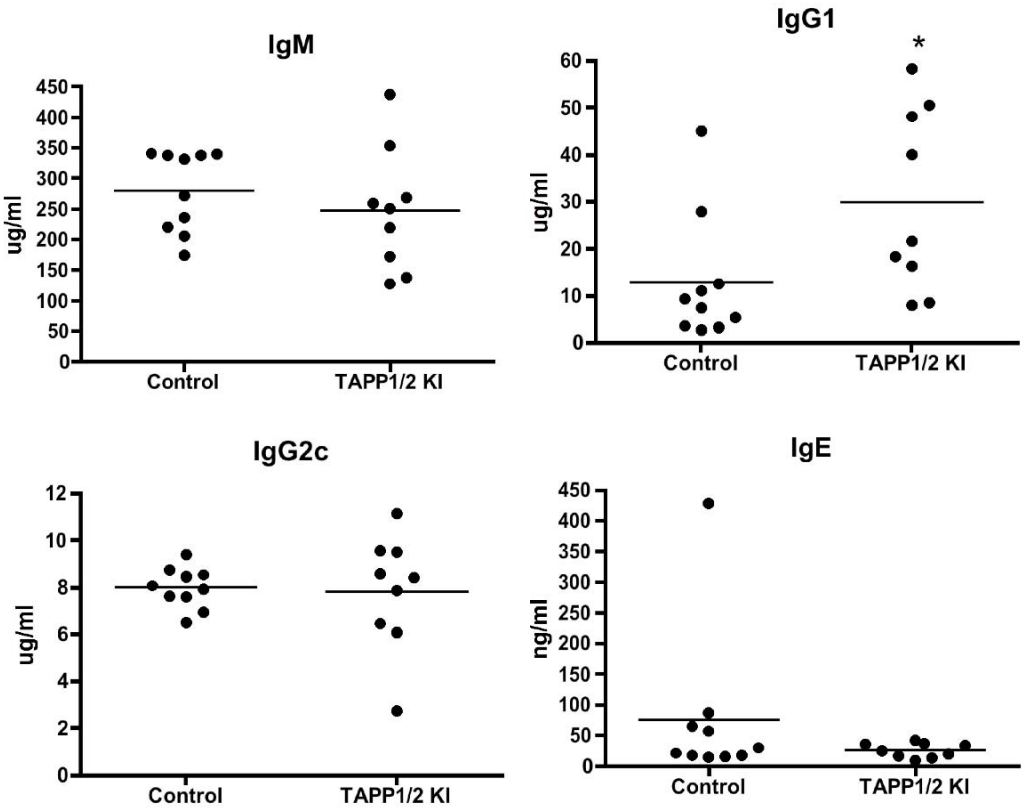

Figure 25: Antibody response in adoptive transfer mice

Purified TAPP KI or control B cells were injected into B cell deficient μMT hosts. Fourteen days after NP-OVA/ alum immunization blood was collected and sera analyzed for total immunoglobulin production using ELISA. Individual mice are plotted as a single dot, with black lines representing the means.

TAPP-phosphoinositide interactions regulate ICOS expression on CD4+ T cells

Another critical component of germinal centre response is CD4+ T cells or T follicular helper cells (Tfh), which are a specialized subset of T lymphocytes that participate in the germinal centre response by providing critical signals, such as cytokines and cell contact-dependants to germinal center B cells (95). We thus examined markers associated with Tfh, including CXCR5 and ICOS. Flow cytometry analysis of the CD4+ T cell population within the spleens of TAPP KI mice revealed a significant increase in ICOS expression on day 14 post-immunization (**Figure 26**). Nonetheless, transfer of TAPP KI B cells into B cell deficient hosts was unable to augment the increased ICOS expression in host CD4+ T cells (**Figure 27**), suggesting that the enhanced T cell activation found in the TAPP KI mice is not due secondary to B cell defects.

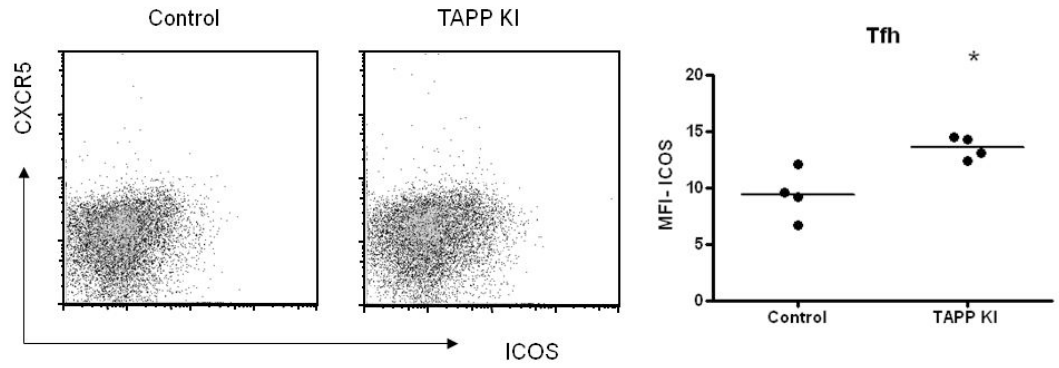

Figure 26: Analysis of T follicular helper cell population in TAPP KI mice.

Flow cytomety analysis of the spleens of day 14 NP-OVA immunized mice were stained with the indicated markers. Cells were gated on CD4+B220- singlet live lymphocytes. Representative FACS plots are shown. Mean fluorescence intensity (MFI) of PE-anti-ICOS was used to determine the relative shift. Individual mice are plotted as a single dot, with black lines representing the means. Similar trends have been found in 2 independent experiments with 4 mice/group.

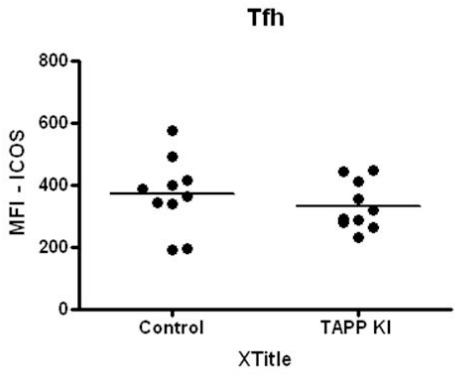

Figure 27: T follicular helper cells in μMT recipient mice

Flow cytometry analysis of CD4+ T cell population in the spleens of μMT recipient mice. Cell population was gated on CD4+DAPI- lymphocytes. Mean fluorescence intensity (MFI) of PE-anti-ICOS was used to determine the relative shift. Individual mice are plotted as a single dot, with black lines representing the means.

DISCUSSION

Previous studies have indicated that PI3K-dependent generation of PI(3,4)P2 is a feature of B cell antigen receptor signaling (124, 215, 246); however the function of this phosphoinositide in B cell activation is unknown. In the absence of functional data, PI(3,4)P2 is often considered to represent an intermediate by-product generated when PI3K signaling is terminated by inhibitory phosphatases SHIP1 and SHIP2 which hydrolyze PI(3,4,5)P3 (139). However, several studies have indicated that PI(3,4)P2 can be selectively generated under certain conditions including oxidant stimulation (219, 247) and integrin outside-in signaling (248). The specific binding of PI(3,4)P2 by TAPP adaptors suggests that these molecules mediate cellular functions that are sensitive to levels of this lipid messenger. The present results provide the first direct evidence that the interaction of TAPPs with PI(3,4)P2 indirectly impacts B cell activation, differentiation and survival. Moreover, disruption of this interaction results in significant systemic perturbations in antibody production.

B cell development and maintenance is clearly dependent on "tonic" BCR signals that require PI3K activity (37, 249). In particular, the marginal zone and B1 B cell subsets appear to require a higher threshold of PI3K signal, as these cells are selectively absent in mice deficient in p110δ catalytic subunit of PI3K (158), or treated with PI3K inhibitors (50). Our data indicate that TAPP interactions with PI(3,4)P2 are not essential for development of mature B cell subsets within the spleen. Development of B1 B cells also

did not require TAPP interaction with PI(3,4)P2; however our data suggest a role in regulating the accumulation or expansion of this population within the peritoneal cavity. The observed subtle reductions in B cell precursors within the bone marrow and spleen might be related to altered pre-BCR or BCR signaling leading to accelerated differentiation into mature B cell subsets and increased clonal deletion at the immature/transitional B cell stages.

Despite the relatively small effect of the TAPP PH domain mutations on mature B cell populations, serum Ab responses were significantly elevated, and this affected all Ag isotypes tested. Levels of serum Abs were also strikingly elevated in TAPP KI mice after immunization. These results clearly suggest that absence of TAPP-PI(3,4)P2 interactions does not impair B cell activation, but rather de-regulates Ab production at some level. Interestingly, the levels of Ag binding Abs produced after immunization are in the normal range, suggesting that excess serum Ig production originates from low affinity or polyclonal B cell activation. Consistent with this idea, we find that TAPP KI mice have increased plasma cell differentiation in the spleen. Furthermore, in adoptive transfer studies, B cell deficient hosts receiving TAPP KI B cells were also found to have increased IgG1 as compared to hosts receiving WT cells.

B cell proliferation in germinal centres of TAPP KI mice seems to follow normal kinetics, however GC B cells accumulated to significantly higher levels at the peak of the response. One possibility to explain this finding is that GC B cells of low affinity for the

immunizing Ag survive and accumulate to a greater extent in TAPP KI mice. Consistent with this idea, we find that a lower proportion of GC B cells expressing markers of apoptosis in the TAPP KI mice. In adoptive transfer experiments where we reconstituted B cell deficient hosts with either TAPP KI or WT B cells, we found that the TAPP KI recipients elicited similar germinal centre responses as compared to those receiving WT cells, suggesting that the enhanced germinal centre response found in the TAPP KI mice is not solely due to the absence of TAPP-PI(3,4)P2 interactions in B cells. However, it should be noted that the B cell adoptive transfer system may not be sensitive enough to detect subtle differences in GC B cell survival and proliferation. Alternatively, competitive adoptive transfer experiments with mixtures of WT and TAPP KI B cells might be more sensitive to detect any advantage of TAPP KI.

While our findings suggests that the enhanced germinal centre response in the TAPP KI mice may in part be due to increased activation of other immune cells, we examined the CD4+ cell population in the TAPP KI mice. Consistent with the hyper-responsiveness of B cells, we found that CD4+ T cells had increased expression of the inducible co-stimulator molecule (ICOS) following immunization with TD antigen. Previous studies have shown that co-stimulation through ICOS contributes to CD40L expression (110), potentially contributing to the increased GC survival in the TAPP KI mice. While it is possible that this increased expression of ICOS on CD4+ T cells may directly contribute to the survival of the TAPP KI GC B cells, it is still possible that activation of by other immune cells may contribute to the enhanced ICOS expression and GC response found in

the TAPP KI mice. However, it should be noted that transfer of TAPP KI B cells into B cell deficient hosts was insufficient to induce upregulation of ICOS on WT T cells.

Examination of BCR signaling in TAPP KI B cells indicated that early signaling events are intact, whereas phosphorylation of Akt was increased. This result is consistent with our previous data implicating TAPPs in Akt activation following IGF-1 stimulation of HEK293 cells (228) or insulin stimulation of MEFs (223). We observed significant increases in B cell viability both in vivo and in vitro consistent with enhanced Akt activity. However, when we treated TAPP KI B cells in vitro with CD40+IL4, we found that the TAPP KI B cells underwent Fas induced death comparable to that of the WT B cells, suggesting that TAPP KI B cells are still susceptible to extrinsic apoptotic stimuli. Increased numbers of cell divisions were also observed after BCR stimulation, which may be partly due to enhanced probability of cell survival at each division. Conversely, we did not see difference in proliferation with other mitogenic stimuli such as anti-CD40 or LPS; however the mechanism by which these stimuli activate the PI3K pathway is still not well understood. We also did not see any difference in proliferation in the presence of IL-4, suggesting that alternate survival pathways are activated, compensating the marginal effect seen with BCR cross-linking. Since BCR signaling is known to simultaneously activate pro- and anti-apoptotic signaling pathways (28), our interpretation of the present data is that uncoupling TAPPs from PI(3,4)P2 leads to increased anti-apoptotic signaling, potentially via modulating Akt, tipping the balance towards cell survival. Our working model is that TAPPs exert their regulatory function by recruiting specific inhibitory

signaling molecules to the plasma membrane. For example it was previously found that TAPPs interact with the inhibitory phosphatase PTPL1/FAP1/PTP-BL (228), and disrupting TAPP-PI(3,4)P2 interactions may inactivate such a regulatory circuit. Since Akt interacts with both PI(3,4,5)P3 and PI(3,4)P2 (250), and its activation appears sensitive to PI(3,4)P2 levels (246), an additional possibility is that TAPPs operate as competitive inhibitors by limiting the pool of PI(3,4)P2 available for binding.

Engagement of SHIP via FcγRII is known to inhibit B cell proliferation, Akt activation and survival (164, 251, 252). Since TAPP adaptors bind the product of SHIP, PI(3,4)P2, and their recruitment is enhanced by SHIP (224), it is tempting to speculate that TAPP recruitment may, in part, mediate inhibitory signals downstream of SHIP/FcγRIIB. Consistent with this idea, MEFs derived from TAPP KI mice and treated with insulin were found to have 1.5 to 2 fold increases in PI(3,4,5)P3 levels MEFs (223), suggesting that TAPPs may help mediate the inhibitory action of SHIP. Similarly, we found that TAPP KI B cells were also able to proliferate and survive more effectively than wildtype B cells when FcγRII is co-ligated with the BCR. In addition there are striking similarities between TAPP KI and SHIP deficient mice, which both show reduction in B cells within the bone marrow and spleen, yet have increased number and frequency of mature B cells in the periphery (164). SHIP deficiency was also found to impact antibody production, leading to a significant increase in basal immunoglobulin production (163), consistent with that found in the TAPP KI mice. Furthermore, SHIP-/- B cells were also found to be resistant to $F(ab)_2$ anti-IgM induced cell death and have increased Akt phosphorylation

upon BCR cross-linking (163, 164). Together these results suggest that TAPP-PI(3,4)P2 interaction may mediate an important component of SHIP-mediated feedback inhibition.

In summary, we demonstrate here the first evidence for function of TAPP adaptors in regulating B cell mediated immune responses. Our results support the functional significance of TAPPs in B cell activation and underline the need for further investigations into the mechanism by which TAPPs mediate their inhibitory signaling function.

Future Directions

Determine the inhibitory signaling mechanism associated with TAPPs

Previous studies have demonstrated that TAPP1 interacts via PDZ binding domain with the inhibitory phosphatase PTPL1 (228). PTPL1 is a tyrosine phosphatase previously shown to negatively regulate Akt (231) and STAT signaling (230). Initially we would need to determine whether PTPL1 is expressed in B lymphocytes and validate TAPP-PTPL1 interaction. Then examine whether TAPP-PTPL1 interaction mediates a functionally significant role in lymphocytes.

Alternatively, since TAPPs bind to the breakdown product of SHIP and their recruitment is enhanced by SHIP activity, it is interesting to speculate that TAPPs may influence SHIP activity through an inhibitory feedback loop. To determine whether TAPP KI mutation influences SHIP activation, we will examine the phosphorylation status of SHIP upon BCR cross-linking alone and when co-stimulated through the FcγRIIB. One

possibility is that in the absence of TAPP recruitment to the plasma membrane levels of PI(3,4,5)P3 could be increased due to reduced SHIP activation.

Determine the immunopathological effect of Ab deregulation in TAPP KI mice

To determine the long term effect of deregulated Ab production in TAPP KI mice, we will measure anti-nuclear, anti-DNA and total antibodies from serum samples collected over a one-year period. Anti-nuclear and anti-DNA Abs are produced in both human and mouse autoimmune diseases involving B cell dysregulation and tend to accumulate with age. Specifically, we will determine whether aged TAPP KI mice show signs of antibody deposition in kidneys leading to dysfunction and excretion of creatinine and protein, which are known signs of lupus-like autoimmune syndromes.

Determine the role of TAPPs in T cell activation and germinal centre responses

T cells stimulated through CD28 costimulatory molecule were previously found to recruit TAPP1-PH domain to the plasma membrane (220); however, the functional significance of TAPPs in T cells has yet to be determined. To identify potential T cell-intrinsic defects, purified TAPP KI T cells will be cultured with anti-CD3 and anti-CD28 which are known activators of the PI3K pathway (253, 254). Proliferation and apoptosis will be determined using cell division and cell death assays, respectively.

ICOS is a homolog of CD28 that is rapidly up regulated on activated T lymphocytes following TCR and CD28 engagement (109). We have repeatedly observed increased ICOS expression in immunized TAPP KI mice. To determine if the increased ICOS

expression is a T cell intrinsic defect, we will use purified CD4+ and stimulate TCR/CD28 and measure the kinetics of ICOS induction. Furthermore, we would like to determine whether uncoupling of TAPPs from PI(3,4)P2 would alter the expression of other activation or co-stimulatory molecules following TCR/CD28 stimulation. Previous studies have suggested that co-stimulation of ICOS on T cells leads to upregulation of CD40-L (110). Furthermore, we would like to determine if the increased ICOS expression and enhanced germinal centre responses are due to T cell intrinsic defects, we will perform adoptive transfer experiments by reconstituting lymphocyte deficient mice with TAPP KI T cells and wild-type B cells. We predict that TAPPs may have distinct yet overlapping roles in regulating T lymphocyte activation versus B lymphocyte activation, which may both contribute to deregulated germinal centre responses. These studies will help elucidate the in vivo role of TAPP1 and TAPP2 in regulating lymphocyte activation.

Investigate the PI3K independent functions of TAPP1 and TAPP2

To investigate PI3K-independent functions of TAPP1 and TAPP2, generate TAPP knockout (KO) mice. The TAPP KI mice were designed to both mutate the PH domains and introduce LoxP sites flanking central exons of the TAPP genes (223). To generate TAPP KO mice, Cre transgenic mice specific for B cells (CD19-Cre) will be crossed with floxed TAPP KI mice to generate complete B cell specific KO. Examination of TAPP KO B cells are expected to show a more severe phenotype than the PH mutants we have been working with, as both PI3K dependent and independent functions will be lost.

TAPP KO B cells will be examined for their ability to proliferate, survive and generate antibody responses.

REFERENCES

1. Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* 17: 555-592.
2. Okkenhaug, K. and B. Vanhaesebroeck. 2003. PI3K in lymphocyte development, differentiation and activation. *Nat. Rev. Immunol.* 3: 317-330.
3. Hardy, R. R. and K. Hayakawa. 2001. B cell development pathways. *Annu. Rev. Immunol.* 19: 595-621.
4. Allman, D. M., S. E. Ferguson, V. M. Lentz, and M. P. Cancro. 1993. Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol.* 151: 4431-4444.
5. Osmond, D. G. 1986. Population dynamics of bone marrow B lymphocytes. *Immunol. Rev.* 93: 103-124.
6. von Boehmer, H. and F. Melchers. 2010. Checkpoints in lymphocyte development and autoimmune disease. *Nat. Immunol.* 11: 14-20.
7. Nutt, S. L., P. Urbanek, A. Rolink, and M. Busslinger. 1997. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev.* 11: 476-491.
8. Corcoran, A. E., A. Riddell, D. Krooshoop, and A. R. Venkitaraman. 1998. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. *Nature* 391: 904-907.
9. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 350: 423-426.
10. Meffre, E., R. Casellas, and M. C. Nussenzweig. 2000. Antibody regulation of B cell development. *Nat. Immunol.* 1: 379-385.
11. Li, Y. S., K. Hayakawa, and R. R. Hardy. 1993. The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. *J. Exp. Med.* 178: 951-960.
12. Guo, B., R. M. Kato, M. Garcia-Lloret, M. I. Wahl, and D. J. Rawlings. 2000. Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex. *Immunity* 13: 243-253.
13. Herzog, S., M. Reth, and H. Jumaa. 2009. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat. Rev. Immunol.* 9: 195-205.
14. Jumaa, H., R. W. Hendriks, and M. Reth. 2005. B cell signaling and tumorigenesis. *Annu. Rev. Immunol.* 23: 415-445.
15. Gong, S. and M. C. Nussenzweig. 1996. Regulation of an early developmental checkpoint in the B cell pathway by Ig beta. *Science* 272: 411-414.
16. Minegishi, Y., E. Coustan-Smith, L. Rapalus, F. Ersoy, D. Campana, and M. E. Conley. 1999. Mutations in Igalpha (CD79a) result in a complete block in B-cell development. *J. Clin. Invest.* 104: 1115-1121.

17. Reth, M., E. Petrac, P. Wiese, L. Lobel, and F. W. Alt. 1987. Activation of V kappa gene rearrangement in pre-B cells follows the expression of membrane-bound immunoglobulin heavy chains. *EMBO J.* 6: 3299-3305.
18. Hartley, S. B., J. Crosbie, R. Brink, A. B. Kantor, A. Basten, and C. C. Goodnow. 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature* 353: 765-769.
19. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* 177: 999-1008.
20. Tiegs, S. L., D. M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* 177: 1009-1020.
21. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, and K. Raphael. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* 334: 676-682.
22. Goodnow, C. C., J. Crosbie, H. Jorgensen, R. A. Brink, and A. Basten. 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature* 342: 385-391.
23. Sandel, P. C. and J. G. Monroe. 1999. Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter. *Immunity* 10: 289-299.
24. Nemazee, D. A. and K. Burki. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* 337: 562-566.
25. Pike, B. L., A. W. Boyd, and G. J. Nossal. 1982. Clonal anergy: the universally anergic B lymphocyte. *Proc. Natl. Acad. Sci. U. S. A.* 79: 2013-2017.
26. Hertz, M. and D. Nemazee. 1997. BCR ligation induces receptor editing in IgM+IgD- bone marrow B cells in vitro. *Immunity* 6: 429-436.
27. Carsetti, R., G. Kohler, and M. C. Lamers. 1995. Transitional B cells are the target of negative selection in the B cell compartment. *J. Exp. Med.* 181: 2129-2140.
28. Niiro, H. and E. A. Clark. 2002. Regulation of B-cell fate by antigen-receptor signals. *Nat. Rev. Immunol.* 2: 945-956.
29. Su, T. T. and D. J. Rawlings. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *J. Immunol.* 168: 2101-2110.
30. Schiemann, B., J. L. Gommerman, K. Vora, T. G. Cachero, S. Shulga-Morskaya, M. Dobles, E. Frew, and M. L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293: 2111-2114.
31. Franzoso, G., L. Carlson, L. Xing, L. Poljak, E. W. Shores, K. D. Brown, A. Leonardi, T. Tran, B. F. Boyce, and U. Siebenlist. 1997. Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev.* 11: 3482-3496.
32. Batten, M., J. Groom, T. G. Cachero, F. Qian, P. Schneider, J. Tschopp, J. L. Browning, and F. Mackay. 2000. BAFF mediates survival of peripheral immature B lymphocytes. *J. Exp. Med.* 192: 1453-1466.
33. Lam, K. P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 90: 1073-1083.
34. Thompson, J. S., P. Schneider, S. L. Kalled, L. Wang, E. A. Lefevre, T. G. Cachero, F. MacKay, S. A. Bixler, M. Zafari, Z. Y. Liu, S. A. Woodcock, F. Qian, M. Batten, C. Madry, Y. Richard, C. D. Benjamin, J. L. Browning, A. Tsapis, J. Tschopp, and C.

Ambrose. 2000. BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. *J. Exp. Med.* 192: 129-135.
35. Hsu, B. L., S. M. Harless, R. C. Lindsley, D. M. Hilbert, and M. P. Cancro. 2002. Cutting edge: BLyS enables survival of transitional and mature B cells through distinct mediators. *J. Immunol.* 168: 5993-5996.
36. Kraus, M., M. B. Alimzhanov, N. Rajewsky, and K. Rajewsky. 2004. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. *Cell* 117: 787-800.
37. Srinivasan, L., Y. Sasaki, D. P. Calado, B. Zhang, J. H. Paik, R. A. DePinho, J. L. Kutok, J. F. Kearney, K. L. Otipoby, and K. Rajewsky. 2009. PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 139: 573-586.
38. Smith, S. H. and M. P. Cancro. 2003. Cutting edge: B cell receptor signals regulate BLyS receptor levels in mature B cells and their immediate progenitors. *J. Immunol.* 170: 5820-5823.
39. Patke, A., I. Mecklenbrauker, H. Erdjument-Bromage, P. Tempst, and A. Tarakhovsky. 2006. BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. *J. Exp. Med.* 203: 2551-2562.
40. Thompson, J. S., S. A. Bixler, F. Qian, K. Vora, M. L. Scott, T. G. Cachero, C. Hession, P. Schneider, I. D. Sizing, C. Mullen, K. Strauch, M. Zafari, C. D. Benjamin, J. Tschopp, J. L. Browning, and C. Ambrose. 2001. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science* 293: 2108-2111.
41. Cancro, M. P., D. P. D'Cruz, and M. A. Khamashta. 2009. The role of B lymphocyte stimulator (BLyS) in systemic lupus erythematosus. *J. Clin. Invest.* 119: 1066-1073.
42. Mackay, F., S. A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, and J. L. Browning. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* 190: 1697-1710.
43. Schneider, P., H. Takatsuka, A. Wilson, F. Mackay, A. Tardivel, S. Lens, T. G. Cachero, D. Finke, F. Beermann, and J. Tschopp. 2001. Maturation of marginal zone and follicular B cells requires B cell activating factor of the tumor necrosis factor family and is independent of B cell maturation antigen. *J. Exp. Med.* 194: 1691-1697.
44. Hao, Z. and K. Rajewsky. 2001. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *J. Exp. Med.* 194: 1151-1164.
45. Pillai, S. and A. Cariappa. 2009. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat. Rev. Immunol.* 9: 767-777.
46. MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12: 117-139.
47. Tarlinton, D. M. and K. G. Smith. 2000. Dissecting affinity maturation: a model explaining selection of antibody-forming cells and memory B cells in the germinal centre. *Immunol. Today* 21: 436-441.
48. Berland, R. and H. H. Wortis. 2002. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol.* 20: 253-300.
49. Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* 14: 617-629.
50. Durand, C. A., K. Hartvigsen, L. Fogelstrand, S. Kim, S. Iritani, B. Vanhaesebroeck, J. L. Witztum, K. D. Puri, and M. R. Gold. 2009. Phosphoinositide 3-kinase p110 delta

regulates natural antibody production, marginal zone and B-1 B cell function, and autoantibody responses. *J. Immunol.* 183: 5673-5684.
51. Kearney, J. F. 2005. Innate-like B cells. *Springer Semin. Immunopathol.* 26: 377-383.
52. Oliver, A. M., F. Martin, and J. F. Kearney. 1999. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J. Immunol.* 162: 7198-7207.
53. Pillai, S., A. Cariappa, and S. T. Moran. 2005. Marginal zone B cells. *Annu. Rev. Immunol.* 23: 161-196.
54. Oliver, A. M., F. Martin, G. L. Gartland, R. H. Carter, and J. F. Kearney. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur. J. Immunol.* 27: 2366-2374.
55. Gray, D., I. C. MacLennan, H. Bazin, and M. Khan. 1982. Migrant mu+ delta+ and static mu+ delta- B lymphocyte subsets. *Eur. J. Immunol.* 12: 564-569.
56. Lu, T. T. and J. G. Cyster. 2002. Integrin-mediated long-term B cell retention in the splenic marginal zone. *Science* 297: 409-412.
57. Wang, H. and S. H. Clarke. 2004. Regulation of B-cell development by antibody specificity. *Curr. Opin. Immunol.* 16: 246-250.
58. Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* 14: 617-629.
59. Pillai, S., A. Cariappa, and S. T. Moran. 2004. Positive selection and lineage commitment during peripheral B-lymphocyte development. *Immunol. Rev.* 197: 206-218.
60. Cariappa, A. and S. Pillai. 2002. Antigen-dependent B-cell development. *Curr. Opin. Immunol.* 14: 241-249.
61. Janeway, C. 2001. Immunobiology 5: the immune system in health and disease. 5thed.Garland Pub., New York. 732.
62. Marrack, P., A. S. McKee, and M. W. Munks. 2009. Towards an understanding of the adjuvant action of aluminium. *Nat. Rev. Immunol.* 9: 287-293.
63. Mond, J. J., A. Lees, and C. M. Snapper. 1995. T cell-independent antigens type 2. *Annu. Rev. Immunol.* 13: 655-692.
64. Coutinho, A. and G. Moller. 1973. B cell mitogenic properties of thymus-independent antigens. *Nat. New Biol.* 245: 12-14.
65. Fidler, J. M. 1975. In vivo immune response to TNP hapten coupled to thymus-independent carrier lipopolysaccharide. *Cell. Immunol.* 16: 223-236.
66. Coutinho, A., E. Gronowicz, W. W. Bullock, and G. Moller. 1974. Mechanism of thymus-independent immunocyte triggering. Mitogenic activation of B cells results in specific immune responses. *J. Exp. Med.* 139: 74-92.
67. Coutinho, A. and E. Gronowicz. 1975. Genetical control of B-cell responses. III. Requirement for functional mitogenicity of the antigen in thymus-independent specific responses. *J. Exp. Med.* 141: 753-760.
68. Mosier, D. E., J. J. Mond, and E. A. Goldings. 1977. The ontogeny of thymic independent antibody responses in vitro in normal mice and mice with an X-linked B cell defect. *J. Immunol.* 119: 1874-1878.
69. Dintzis, H. M., R. Z. Dintzis, and B. Vogelstein. 1976. Molecular determinants of immunogenicity: the immunon model of immune response. *Proc. Natl. Acad. Sci. U. S. A.* 73: 3671-3675.

70. Fluckiger, A. C., Z. Li, R. M. Kato, M. I. Wahl, H. D. Ochs, R. Longnecker, J. P. Kinet, O. N. Witte, A. M. Scharenberg, and D. J. Rawlings. 1998. Btk/Tec kinases regulate sustained increases in intracellular Ca2+ following B-cell receptor activation. *EMBO J.* 17: 1973-1985.
71. Pecanha, L. M., C. M. Snapper, F. D. Finkelman, and J. J. Mond. 1991. Dextran-conjugated anti-Ig antibodies as a model for T cell-independent type 2 antigen-mediated stimulation of Ig secretion in vitro. I. Lymphokine dependence. *J. Immunol.* 146: 833-839.
72. Gavin, A. L., K. Hoebe, B. Duong, T. Ota, C. Martin, B. Beutler, and D. Nemazee. 2006. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* 314: 1936-1938.
73. Nemazee, D. and A. Gavin. 2003. Do B cells take advantage of 'missing self' recognition? *Curr. Dir. Autoimmun.* 6: 245-264.
74. Duong, B. H., H. Tian, T. Ota, G. Completo, S. Han, J. L. Vela, M. Ota, M. Kubitz, N. Bovin, J. C. Paulson, and D. Nemazee. 2010. Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo. *J. Exp. Med.* 207: 173-187.
75. Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173: 1165-1175.
76. Shapiro-Shelef, M. and K. Calame. 2005. Regulation of plasma-cell development. *Nat. Rev. Immunol.* 5: 230-242.
77. Stenzel, K. H., A. L. Rubin, and A. Novogrodsky. 1981. Mitogenic and co-mitogenic properties of hemin. *J. Immunol.* 127: 2469-2473.
78. Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173: 1165-1175.
79. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centres. *Nature* 354: 389-392.
80. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell* 67: 1121-1129.
81. MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12: 117-139.
82. Vinuesa, C. G., I. Sanz, and M. C. Cook. 2009. Dysregulation of germinal centres in autoimmune disease. *Nat. Rev. Immunol.* 9: 845-857.
83. Manser, T. 2004. Textbook germinal centers? *J. Immunol.* 172: 3369-3375.
84. Matsumoto, M., S. F. Lo, C. J. Carruthers, J. Min, S. Mariathasan, G. Huang, D. R. Plas, S. M. Martin, R. S. Geha, M. H. Nahm, and D. D. Chaplin. 1996. Affinity maturation without germinal centres in lymphotoxin-alpha-deficient mice. *Nature* 382: 462-466.
85. Allen, C. D., K. M. Ansel, C. Low, R. Lesley, H. Tamamura, N. Fujii, and J. G. Cyster. 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat. Immunol.* 5: 943-952.
86. Cyster, J. G., K. M. Ansel, K. Reif, E. H. Ekland, P. L. Hyman, H. L. Tang, S. A. Luther, and V. N. Ngo. 2000. Follicular stromal cells and lymphocyte homing to follicles. *Immunol. Rev.* 176: 181-193.

87. Wang, Y. and R. H. Carter. 2005. CD19 regulates B cell maturation, proliferation, and positive selection in the FDC zone of murine splenic germinal centers. *Immunity* 22: 749-761.
88. Allen, C. D., T. Okada, H. L. Tang, and J. G. Cyster. 2007. Imaging of germinal center selection events during affinity maturation. *Science* 315: 528-531.
89. Cattoretti, G., M. Buttner, R. Shaknovich, E. Kremmer, B. Alobeid, and G. Niedobitek. 2006. Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. *Blood* 107: 3967-3975.
90. Klein, U. and R. Dalla-Favera. 2008. Germinal centres: role in B-cell physiology and malignancy. *Nat. Rev. Immunol.* 8: 22-33.
91. Kosco-Vilbois, M. H. 2003. Are follicular dendritic cells really good for nothing? *Nat. Rev. Immunol.* 3: 764-769.
92. Hannum, L. G., A. M. Haberman, S. M. Anderson, and M. J. Shlomchik. 2000. Germinal center initiation, variable gene region hypermutation, and mutant B cell selection without detectable immune complexes on follicular dendritic cells. *J. Exp. Med.* 192: 931-942.
93. Shih, T. A., E. Meffre, M. Roederer, and M. C. Nussenzweig. 2002. Role of BCR affinity in T cell dependent antibody responses in vivo. *Nat. Immunol.* 3: 570-575.
94. Allen, C. D., T. Okada, and J. G. Cyster. 2007. Germinal-center organization and cellular dynamics. *Immunity* 27: 190-202.
95. Vinuesa, C. G., S. G. Tangye, B. Moser, and C. R. Mackay. 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat. Rev. Immunol.* 5: 853-865.
96. Takahashi, Y., P. R. Dutta, D. M. Cerasoli, and G. Kelsoe. 1998. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J. Exp. Med.* 187: 885-895.
97. Victora, G. D., T. A. Schwickert, D. R. Fooksman, A. O. Kamphorst, M. Meyer-Hermann, M. L. Dustin, and M. C. Nussenzweig. 2010. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell* 143: 592-605.
98. Slifka, M. K., M. Matloubian, and R. Ahmed. 1995. Bone marrow is a major site of long-term antibody production after acute viral infection. *J. Virol.* 69: 1895-1902.
99. Tarlinton, D. 2006. B-cell memory: are subsets necessary? *Nat. Rev. Immunol.* 6: 785-790.
100. King, C. 2009. New insights into the differentiation and function of T follicular helper cells. *Nat. Rev. Immunol.* 9: 757-766.
101. Reinhardt, R. L., H. E. Liang, and R. M. Locksley. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat. Immunol.* 10: 385-393.
102. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397: 263-266.
103. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29: 138-149.
104. Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian, and C. Dong. 2008. Generation of T follicular

helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29: 138-149.
105. Vogelzang, A., H. M. McGuire, D. Yu, J. Sprent, C. R. Mackay, and C. King. 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29: 127-137.
106. Zotos, D., J. M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L. M. Corcoran, D. I. Godfrey, K. M. Toellner, M. J. Smyth, S. L. Nutt, and D. M. Tarlinton. 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J. Exp. Med.* 207: 365-378.
107. Renshaw, B. R., W. C. Fanslow 3rd, R. J. Armitage, K. A. Campbell, D. Liggitt, B. Wright, B. L. Davison, and C. R. Maliszewski. 1994. Humoral immune responses in CD40 ligand-deficient mice. *J. Exp. Med.* 180: 1889-1900.
108. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. Chan, G. Duncan, B. Odermatt, A. Ho, A. Itie, T. Horan, J. S. Whoriskey, T. Pawson, J. M. Penninger, P. S. Ohashi, and T. W. Mak. 2001. ICOS is essential for effective T-helper-cell responses. *Nature* 409: 105-109.
109. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397: 263-266.
110. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature* 409: 102-105.
111. Yoshinaga, S. K., J. S. Whoriskey, S. D. Khare, U. Sarmiento, J. Guo, T. Horan, G. Shih, M. Zhang, M. A. Coccia, T. Kohno, A. Tafuri-Bladt, D. Brankow, P. Campbell, D. Chang, L. Chiu, T. Dai, G. Duncan, G. S. Elliott, A. Hui, S. M. McCabe, S. Scully, A. Shahinian, C. L. Shaklee, G. Van, T. W. Mak, and G. Senaldi. 1999. T-cell co-stimulation through B7RP-1 and ICOS. *Nature* 402: 827-832.
112. Vinuesa, C. G., M. C. Cook, C. Angelucci, V. Athanasopoulos, L. Rui, K. M. Hill, D. Yu, H. Domaschenz, B. Whittle, T. Lambe, I. S. Roberts, R. R. Copley, J. I. Bell, R. J. Cornall, and C. C. Goodnow. 2005. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* 435: 452-458.
113. Vanhaesebroeck, B., M. J. Welham, K. Kotani, R. Stein, P. H. Warne, M. J. Zvelebil, K. Higashi, S. Volinia, J. Downward, and M. D. Waterfield. 1997. P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* 94: 4330-4335.
114. Chantry, D., A. Vojtek, A. Kashishian, D. A. Holtzman, C. Wood, P. W. Gray, J. A. Cooper, and M. F. Hoekstra. 1997. P110delta, a Novel Phosphatidylinositol 3-Kinase Catalytic Subunit that Associates with P85 and is Expressed Predominantly in Leukocytes. *J. Biol. Chem.* 272: 19236-19241.
115. Deane, J. A. and D. A. Fruman. 2004. Phosphoinositide 3-kinase: diverse roles in immune cell activation. *Annu. Rev. Immunol.* 22: 563-598.
116. Fruman, D. A. and G. Bismuth. 2009. Fine tuning the immune response with PI3K. *Immunol. Rev.* 228: 253-272.
117. Koyasu, S. 2003. The role of PI3K in immune cells. *Nat. Immunol.* 4: 313-319.
118. Kurosaki, T. 1999. Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* 17: 555-592.

119. Beitz, L. O., D. A. Fruman, T. Kurosaki, L. C. Cantley, and A. M. Scharenberg. 1999. SYK is upstream of phosphoinositide 3-kinase in B cell receptor signaling. *J. Biol. Chem.* 274: 32662-32666.
120. Tuveson, D. A., R. H. Carter, S. P. Soltoff, and D. T. Fearon. 1993. CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase. *Science* 260: 986-989.
121. Okada, T., A. Maeda, A. Iwamatsu, K. Gotoh, and T. Kurosaki. 2000. BCAP: the tyrosine kinase substrate that connects B cell receptor to phosphoinositide 3-kinase activation. *Immunity* 13: 817-827.
122. Kim, T. J., Y. T. Kim, and S. Pillai. 1995. Association of activated phosphatidylinositol 3-kinase with p120cbl in antigen receptor-ligated B cells. *J. Biol. Chem.* 270: 27504-27509.
123. Marshall, A. J., H. Niiro, T. J. Yun, and E. A. Clark. 2000. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cgamma pathway. *Immunol. Rev.* 176: 30-46.
124. Gold, M. R. and R. Aebersold. 1994. Both phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase products are increased by antigen receptor signaling in B cells. *J. Immunol.* 152: 42-50.
125. Carter, R. H. and D. T. Fearon. 1992. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* 256: 105-107.
126. Buhl, A. M., C. M. Pleiman, R. C. Rickert, and J. C. Cambier. 1997. Qualitative regulation of B cell antigen receptor signaling by CD19: selective requirement for PI3-kinase activation, inositol-1,4,5-trisphosphate production and Ca2+ mobilization. *J. Exp. Med.* 186: 1897-1910.
127. Otero, D. C., S. A. Omori, and R. C. Rickert. 2001. Cd19-dependent activation of Akt kinase in B-lymphocytes. *J. Biol. Chem.* 276: 1474-1478.
128. Buhl, A. M. and J. C. Cambier. 1999. Phosphorylation of CD19 Y484 and Y515, and linked activation of phosphatidylinositol 3-kinase, are required for B cell antigen receptor-mediated activation of Bruton's tyrosine kinase. *J. Immunol.* 162: 4438-4446.
129. Wang, Y., S. R. Brooks, X. Li, A. N. Anzelon, R. C. Rickert, and R. H. Carter. 2002. The physiologic role of CD19 cytoplasmic tyrosines. *Immunity* 17: 501-514.
130. Jimenez, C., C. Hernandez, B. Pimentel, and A. C. Carrera. 2002. The p85 regulatory subunit controls sequential activation of phosphoinositide 3-kinase by Tyr kinases and Ras. *J. Biol. Chem.* 277: 41556-41562.
131. Aiba, Y., M. Kameyama, T. Yamazaki, T. F. Tedder, and T. Kurosaki. 2008. Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase. *Blood* 111: 1497-1503.
132. Vanhaesebroeck, B., S. J. Leevers, K. Ahmadi, J. Timms, R. Katso, P. C. Driscoll, R. Woscholski, P. J. Parker, and M. D. Waterfield. 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* 70: 535-602.
133. Rameh, L. E. and L. C. Cantley. 1999. The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* 274: 8347-8350.
134. Chalhoub, N. and S. J. Baker. 2009. PTEN and the PI3-kinase pathway in cancer. *Annu. Rev. Pathol.* 4: 127-150.
135. Oak, J. S. and D. A. Fruman. 2007. Role of phosphoinositide 3-kinase signaling in autoimmunity. *Autoimmunity* 40: 433-441.

136. Maehama, T. and J. E. Dixon. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 273: 13375-13378.
137. Di Cristofano, A., B. Pesce, C. Cordon-Cardo, and P. P. Pandolfi. 1998. Pten is essential for embryonic development and tumour suppression. *Nat. Genet.* 19: 348-355.
138. Di Cristofano, A., P. Kotsi, Y. F. Peng, C. Cordon-Cardo, K. B. Elkon, and P. P. Pandolfi. 1999. Impaired Fas response and autoimmunity in Pten+/- mice. *Science* 285: 2122-2125.
139. Damen, J. E., L. Liu, P. Rosten, R. K. Humphries, A. B. Jefferson, P. W. Majerus, and G. Krystal. 1996. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 93: 1689-1693.
140. Ono, M., S. Bolland, P. Tempst, and J. V. Ravetch. 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc(gamma)RIIB. *Nature* 383: 263-266.
141. Helgason, C. D., J. E. Damen, P. Rosten, R. Grewal, P. Sorensen, S. M. Chappel, A. Borowski, F. Jirik, G. Krystal, and R. K. Humphries. 1998. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev.* 12: 1610-1620.
142. Norris, F. A. and P. W. Majerus. 1994. Hydrolysis of phosphatidylinositol 3,4-bisphosphate by inositol polyphosphate 4-phosphatase isolated by affinity elution chromatography. *J. Biol. Chem.* 269: 8716-8720.
143. Gewinner, C., Z. C. Wang, A. Richardson, J. Teruya-Feldstein, D. Etemadmoghadam, D. Bowtell, J. Barretina, W. M. Lin, L. Rameh, L. Salmena, P. P. Pandolfi, and L. C. Cantley. 2009. Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling. *Cancer. Cell.* 16: 115-125.
144. Fedele, C. G., L. M. Ooms, M. Ho, J. Vieusseux, S. A. O'Toole, E. K. Millar, E. Lopez-Knowles, A. Sriratana, R. Gurung, L. Baglietto, G. G. Giles, C. G. Bailey, J. E. Rasko, B. J. Shields, J. T. Price, P. W. Majerus, R. L. Sutherland, T. Tiganis, C. A. McLean, and C. A. Mitchell. 2010. Inositol polyphosphate 4-phosphatase II regulates PI3K/Akt signaling and is lost in human basal-like breast cancers. *Proc. Natl. Acad. Sci. U. S. A.* 107: 22231-22236.
145. Hodgson, M. C., L. J. Shao, A. Frolov, R. Li, L. E. Peterson, G. Ayala, M. M. Ittmann, N. L. Weigel, and I. U. Agoulnik. 2011. Decreased expression and androgen regulation of the tumor suppressor gene INPP4B in prostate cancer. *Cancer Res.* 71: 572-582.
146. Lemmon, M. A. 2008. Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* 9: 99-111.
147. Lemmon, M. A., K. M. Ferguson, and J. Schlessinger. 1996. PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* 85: 621-624.
148. Lemmon, M. A. 2007. Pleckstrin homology (PH) domains and phosphoinositides. *Biochem. Soc. Symp.* (74): 81-93.
149. Kavran, J. M., D. E. Klein, A. Lee, M. Falasca, S. J. Isakoff, E. Y. Skolnik, and M. A. Lemmon. 1998. Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. *J. Biol. Chem.* 273: 30497-30508.

150. Yu, J. W., J. M. Mendrola, A. Audhya, S. Singh, D. Keleti, D. B. DeWald, D. Murray, S. D. Emr, and M. A. Lemmon. 2004. Genome-wide analysis of membrane targeting by S. cerevisiae pleckstrin homology domains. *Mol. Cell* 13: 677-688.
151. Isakoff, S. J., T. Cardozo, J. Andreev, Z. Li, K. M. Ferguson, R. Abagyan, M. A. Lemmon, A. Aronheim, and E. Y. Skolnik. 1998. Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast. *EMBO J.* 17: 5374-5387.
152. Bi, L., I. Okabe, D. J. Bernard, A. Wynshaw-Boris, and R. L. Nussbaum. 1999. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. *J. Biol. Chem.* 274: 10963-10968.
153. Bi, L., I. Okabe, D. J. Bernard, and R. L. Nussbaum. 2002. Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. *Mamm. Genome* 13: 169-172.
154. Fruman, D. A., S. B. Snapper, C. M. Yballe, L. Davidson, J. Y. Yu, F. W. Alt, and L. C. Cantley. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science* 283: 393-397.
155. Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu. 1999. Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. *Science* 283: 390-392.
156. Jou, S. T., N. Carpino, Y. Takahashi, R. Piekorz, J. R. Chao, N. Carpino, D. Wang, and J. N. Ihle. 2002. Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. *Mol. Cell. Biol.* 22: 8580-8591.
157. Clayton, E., G. Bardi, S. E. Bell, D. Chantry, C. P. Downes, A. Gray, L. A. Humphries, D. Rawlings, H. Reynolds, E. Vigorito, and M. Turner. 2002. A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation. *J. Exp. Med.* 196: 753-763.
158. Okkenhaug, K., A. Bilancio, G. Farjot, H. Priddle, S. Sancho, E. Peskett, W. Pearce, S. E. Meek, A. Salpekar, M. D. Waterfield, A. J. Smith, and B. Vanhaesebroeck. 2002. Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* 297: 1031-1034.
159. Sasaki, T., J. Irie-Sasaki, R. G. Jones, A. J. Oliveira-dos-Santos, W. L. Stanford, B. Bolon, A. Wakeham, A. Itie, D. Bouchard, I. Kozieradzki, N. Joza, T. W. Mak, P. S. Ohashi, A. Suzuki, and J. M. Penninger. 2000. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 287: 1040-1046.
160. Engel, P., L. J. Zhou, D. C. Ord, S. Sato, B. Koller, and T. F. Tedder. 1995. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity* 3: 39-50.
161. Anzelon, A. N., H. Wu, and R. C. Rickert. 2003. Pten inactivation alters peripheral B lymphocyte fate and reconstitutes CD19 function. *Nat. Immunol.* 4: 287-294.
162. Janas, M. L., D. Hodson, Z. Stamataki, S. Hill, K. Welch, L. Gambardella, L. C. Trotman, P. P. Pandolfi, E. Vigorito, and M. Turner. 2008. The effect of deleting p110delta on the phenotype and function of PTEN-deficient B cells. *J. Immunol.* 180: 739-746.
163. Helgason, C. D., C. P. Kalberer, J. E. Damen, S. M. Chappel, N. Pineault, G. Krystal, and R. K. Humphries. 2000. A dual role for Src homology 2 domain-containing

inositol-5-phosphatase (SHIP) in immunity: aberrant development and enhanced function of b lymphocytes in ship -/- mice. *J. Exp. Med.* 191: 781-794.
164. Brauweiler, A., I. Tamir, J. Dal Porto, R. J. Benschop, C. D. Helgason, R. K. Humphries, J. H. Freed, and J. C. Cambier. 2000. Differential regulation of B cell development, activation, and death by the src homology 2 domain-containing 5' inositol phosphatase (SHIP). *J. Exp. Med.* 191: 1545-1554.
165. Rickert, R. C., K. Rajewsky, and J. Roes. 1995. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* 376: 352-355.
166. Suzuki, A., T. Kaisho, M. Ohishi, M. Tsukio-Yamaguchi, T. Tsubata, P. A. Koni, T. Sasaki, T. W. Mak, and T. Nakano. 2003. Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination. *J. Exp. Med.* 197: 657-667.
167. Takesono, A., L. D. Finkelstein, and P. L. Schwartzberg. 2002. Beyond calcium: new signaling pathways for Tec family kinases. *J. Cell. Sci.* 115: 3039-3048.
168. Kojima, T., M. Fukuda, Y. Watanabe, F. Hamazato, and K. Mikoshiba. 1997. Characterization of the pleckstrin homology domain of Btk as an inositol polyphosphate and phosphoinositide binding domain. *Biochem. Biophys. Res. Commun.* 236: 333-339.
169. Lewis, C. M., C. Broussard, M. J. Czar, and P. L. Schwartzberg. 2001. Tec kinases: modulators of lymphocyte signaling and development. *Curr. Opin. Immunol.* 13: 317-325.
170. Rawlings, D. J., A. M. Scharenberg, H. Park, M. I. Wahl, S. Lin, R. M. Kato, A. C. Fluckiger, O. N. Witte, and J. P. Kinet. 1996. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* 271: 822-825.
171. Tsukada, S., D. C. Saffran, D. J. Rawlings, O. Parolini, R. C. Allen, I. Klisak, R. S. Sparkes, H. Kubagawa, T. Mohandas, and S. Quan. 1993. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 72: 279-290.
172. Rhee, S. G. 2001. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* 70: 281-312.
173. Saito, K., K. F. Tolias, A. Saci, H. B. Koon, L. A. Humphries, A. Scharenberg, D. J. Rawlings, J. P. Kinet, and C. L. Carpenter. 2003. BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity* 19: 669-678.
174. Bolland, S., R. N. Pearse, T. Kurosaki, and J. V. Ravetch. 1998. SHIP modulates immune receptor responses by regulating membrane association of Btk. *Immunity* 8: 509-516.
175. Krahn, A. K., K. Ma, S. Hou, V. Duronio, and A. J. Marshall. 2004. Two distinct waves of membrane-proximal B cell antigen receptor signaling differentially regulated by Src homology 2-containing inositol polyphosphate 5-phosphatase. *J. Immunol.* 172: 331-339.
176. Satterthwaite, A. B. and O. N. Witte. 2000. The role of Bruton's tyrosine kinase in B-cell development and function: a genetic perspective. *Immunol. Rev.* 175: 120-127.
177. Conley, M. E. 1985. B cells in patients with X-linked agammaglobulinemia. *J. Immunol.* 134: 3070-3074.
178. Rawlings, D. J., D. C. Saffran, S. Tsukada, D. A. Largaespada, J. C. Grimaldi, L. Cohen, R. N. Mohr, J. F. Bazan, M. Howard, and N. G. Copeland. 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* 261: 358-361.

179. Thomas, J. D., P. Sideras, C. I. Smith, I. Vorechovsky, V. Chapman, and W. E. Paul. 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science* 261: 355-358.
180. Sieckmann, D. G., R. Asofsky, D. E. Mosier, I. M. Zitron, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response. *J. Exp. Med.* 147: 814-829.
181. Scher, I., A. D. Steinberg, A. K. Berning, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in CBA/N mice. II. Studies of the mechanisms underlying the immune defect. *J. Exp. Med.* 142: 637-650.
182. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, and L. A. Herzenberg. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity* 3: 283-299.
183. Ellmeier, W., S. Jung, M. J. Sunshine, F. Hatam, Y. Xu, D. Baltimore, H. Mano, and D. R. Littman. 2000. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. *J. Exp. Med.* 192: 1611-1624.
184. Carpten, J. D., A. L. Faber, C. Horn, G. P. Donoho, S. L. Briggs, C. M. Robbins, G. Hostetter, S. Boguslawski, T. Y. Moses, S. Savage, M. Uhlik, A. Lin, J. Du, Y. W. Qian, D. J. Zeckner, G. Tucker-Kellogg, J. Touchman, K. Patel, S. Mousses, M. Bittner, R. Schevitz, M. H. Lai, K. L. Blanchard, and J. E. Thomas. 2007. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* 448: 439-444.
185. Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw 3rd, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292: 1728-1731.
186. Pearce, L. R., D. Komander, and D. R. Alessi. 2010. The nuts and bolts of AGC protein kinases. *Nat. Rev. Mol. Cell Biol.* 11: 9-22.
187. Chan, T. O., S. E. Rittenhouse, and P. N. Tsichlis. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* 68: 965-1014.
188. Frech, M., M. Andjelkovic, E. Ingley, K. K. Reddy, J. R. Falck, and B. A. Hemmings. 1997. High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J. Biol. Chem.* 272: 8474-8481.
189. Franke, T. F., S. I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81: 727-736.
190. Alessi, D. R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B. A. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* 15: 6541-6551.
191. Burgering, B. M. and P. J. Coffer. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376: 599-602.
192. Andjelkovic, M., D. R. Alessi, R. Meier, A. Fernandez, N. J. Lamb, M. Frech, P. Cron, P. Cohen, J. M. Lucocq, and B. A. Hemmings. 1997. Role of translocation in the activation and function of protein kinase B. *J. Biol. Chem.* 272: 31515-31524.

193. Milburn, C. C., M. Deak, S. M. Kelly, N. C. Price, D. R. Alessi, and D. M. Van Aalten. 2003. Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. *Biochem. J.* 375: 531-538.
194. Alessi, D. R., S. R. James, C. P. Downes, A. B. Holmes, P. R. Gaffney, C. B. Reese, and P. Cohen. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* 7: 261-269.
195. Williams, M. R., J. S. Arthur, A. Balendran, J. van der Kaay, V. Poli, P. Cohen, and D. R. Alessi. 2000. The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.* 10: 439-448.
196. Bayascas, J. R., S. Wullschleger, K. Sakamoto, J. M. Garcia-Martinez, C. Clacher, D. Komander, D. M. van Aalten, K. M. Boini, F. Lang, C. Lipina, L. Logie, C. Sutherland, J. A. Chudek, J. A. van Diepen, P. J. Voshol, J. M. Lucocq, and D. R. Alessi. 2008. Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. *Mol. Cell. Biol.* 28: 3258-3272.
197. Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098-1101.
198. Scheid, M. P., P. A. Marignani, and J. R. Woodgett. 2002. Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Mol. Cell. Biol.* 22: 6247-6260.
199. Brazil, D. P. and B. A. Hemmings. 2001. Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem. Sci.* 26: 657-664.
200. Chan, T. O., S. E. Rittenhouse, and P. N. Tsichlis. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* 68: 965-1014.
201. Gold, M. R., M. P. Scheid, L. Santos, M. Dang-Lawson, R. A. Roth, L. Matsuuchi, V. Duronio, and D. L. Krebs. 1999. The B cell antigen receptor activates the Akt (protein kinase B)/glycogen synthase kinase-3 signaling pathway via phosphatidylinositol 3-kinase. *J. Immunol.* 163: 1894-1905.
202. Aman, M. J., T. D. Lamkin, H. Okada, T. Kurosaki, and K. S. Ravichandran. 1998. The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J. Biol. Chem.* 273: 33922-33928.
203. Pogue, S. L., T. Kurosaki, J. Bolen, and R. Herbst. 2000. B cell antigen receptor-induced activation of Akt promotes B cell survival and is dependent on Syk kinase. *J. Immunol.* 165: 1300-1306.
204. Calnan, D. R. and A. Brunet. 2008. The FoxO code. *Oncogene* 27: 2276-2288.
205. Yusuf, I., X. Zhu, M. G. Kharas, J. Chen, and D. A. Fruman. 2004. Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. *Blood* 104: 784-787.
206. Astoul, E., S. Watton, and D. Cantrell. 1999. The dynamics of protein kinase B regulation during B cell antigen receptor engagement. *J. Cell Biol.* 145: 1511-1520.
207. Aman, M. J., T. D. Lamkin, H. Okada, T. Kurosaki, and K. S. Ravichandran. 1998. The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J. Biol. Chem.* 273: 33922-33928.

208. Myers, M. P., I. Pass, I. H. Batty, J. Van der Kaay, J. P. Stolarov, B. A. Hemmings, M. H. Wigler, C. P. Downes, and N. K. Tonks. 1998. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc. Natl. Acad. Sci. U. S. A.* 95: 13513-13518.
209. Stambolic, V., A. Suzuki, J. L. de la Pompa, G. M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J. M. Penninger, D. P. Siderovski, and T. W. Mak. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 95: 29-39.
210. Ivetac, I., R. Gurung, S. Hakim, K. A. Horan, D. A. Sheffield, L. C. Binge, P. W. Majerus, T. Tiganis, and C. A. Mitchell. 2009. Regulation of PI(3)K/Akt signalling and cellular transformation by inositol polyphosphate 4-phosphatase-1. *EMBO Rep.* 10: 487-493.
211. Jordan, M. S., A. L. Singer, and G. A. Koretzky. 2003. Adaptors as central mediators of signal transduction in immune cells. *Nat. Immunol.* 4: 110-116.
212. Tomlinson, M. G., J. Lin, and A. Weiss. 2000. Lymphocytes with a complex: adapter proteins in antigen receptor signaling. *Immunol. Today* 21: 584-591.
213. Zhang, T. T., H. Li, S. M. Cheung, J. L. Costantini, S. Hou, M. Al-Alwan, and A. J. Marshall. 2009. Phosphoinositide 3-kinase-regulated adapters in lymphocyte activation. *Immunol. Rev.* 232: 255-272.
214. Marshall, A. J., H. Niiro, C. G. Lerner, T. J. Yun, S. Thomas, C. M. Disteche, and E. A. Clark. 2000. A novel B lymphocyte-associated adaptor protein, Bam32, regulates antigen receptor signaling downstream of phosphatidylinositol 3-kinase. *J. Exp. Med.* 191: 1319-1332.
215. Marshall, A. J., A. K. Krahn, K. Ma, V. Duronio, and S. Hou. 2002. TAPP1 and TAPP2 are targets of phosphatidylinositol 3-kinase signaling in B cells: sustained plasma membrane recruitment triggered by the B-cell antigen receptor. *Mol. Cell. Biol.* 22: 5479-5491.
216. Han, A., K. Saijo, I. Mecklenbrauker, A. Tarakhovsky, and M. C. Nussenzweig. 2003. Bam32 links the B cell receptor to ERK and JNK and mediates B cell proliferation but not survival. *Immunity* 19: 621-632.
217. Zhang, T. T., M. Al-Alwan, and A. J. Marshall. 2010. The pleckstrin homology domain adaptor protein Bam32/DAPP1 is required for germinal center progression. *J. Immunol.* 184: 164-172.
218. Dowler, S., R. A. Currie, D. G. Campbell, M. Deak, G. Kular, C. P. Downes, and D. R. Alessi. 2000. Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. *Biochem. J.* 351: 19-31.
219. Kimber, W. A., L. Trinkle-Mulcahy, P. C. Cheung, M. Deak, L. J. Marsden, A. Kieloch, S. Watt, R. T. Javier, A. Gray, C. P. Downes, J. M. Lucocq, and D. R. Alessi. 2002. Evidence that the tandem-pleckstrin-homology-domain-containing protein TAPP1 interacts with Ptd(3,4)P2 and the multi-PDZ-domain-containing protein MUPP1 in vivo. *Biochem. J.* 361: 525-536.
220. Freeburn, R. W., K. L. Wright, S. J. Burgess, E. Astoul, D. A. Cantrell, and S. G. Ward. 2002. Evidence that SHIP-1 contributes to phosphatidylinositol 3,4,5-trisphosphate metabolism in T lymphocytes and can regulate novel phosphoinositide 3-kinase effectors. *J. Immunol.* 169: 5441-5450.
221. Manna, D., A. Albanese, W. S. Park, and W. Cho. 2007. Mechanistic basis of differential cellular responses of phosphatidylinositol 3,4-bisphosphate- and

phosphatidylinositol 3,4,5-trisphosphate-binding pleckstrin homology domains. *J. Biol. Chem.* 282: 32093-32105.
222. Thomas, C. C., S. Dowler, M. Deak, D. R. Alessi, and D. M. van Aalten. 2001. Crystal structure of the phosphatidylinositol 3,4-bisphosphate-binding pleckstrin homology (PH) domain of tandem PH-domain-containing protein 1 (TAPP1): molecular basis of lipid specificity. *Biochem. J.* 358: 287-294.
223. Wullschleger, S., D. H. Wasserman, A. Gray, K. Sakamoto, and D. R. Alessi. 2011. Role of TAPP1 and TAPP2 adaptor binding to PtdIns(3,4)P2 in regulating insulin sensitivity defined by knock-in analysis. *Biochem. J.* 434: 265-274.
224. Krahn, A. K., K. Ma, S. Hou, V. Duronio, and A. J. Marshall. 2004. Two distinct waves of membrane-proximal B cell antigen receptor signaling differentially regulated by Src homology 2-containing inositol polyphosphate 5-phosphatase. *J. Immunol.* 172: 331-339.
225. Ivetac, I., A. D. Munday, M. V. Kisseleva, X. M. Zhang, S. Luff, T. Tiganis, J. C. Whisstock, T. Rowe, P. W. Majerus, and C. A. Mitchell. 2005. The type Ialpha inositol polyphosphate 4-phosphatase generates and terminates phosphoinositide 3-kinase signals on endosomes and the plasma membrane. *Mol. Biol. Cell* 16: 2218-2233.
226. Van der Kaay, J., M. Beck, A. Gray, and C. P. Downes. 1999. Distinct phosphatidylinositol 3-kinase lipid products accumulate upon oxidative and osmotic stress and lead to different cellular responses. *J. Biol. Chem.* 274: 35963-35968.
227. Cheung, S. M., J. C. Kornelson, M. Al-Alwan, and A. J. Marshall. 2007. Regulation of phosphoinositide 3-kinase signaling by oxidants: hydrogen peroxide selectively enhances immunoreceptor-induced recruitment of phosphatidylinositol (3,4) bisphosphate-binding PH domain proteins. *Cell. Signal.* 19: 902-912.
228. Kimber, W. A., M. Deak, A. R. Prescott, and D. R. Alessi. 2003. Interaction of the protein tyrosine phosphatase PTPL1 with the PtdIns(3,4)P2-binding adaptor protein TAPP1. *Biochem. J.* 376: 525-535.
229. Abaan, O. D. and J. A. Toretsky. 2008. PTPL1: a large phosphatase with a split personality. *Cancer Metastasis Rev.* 27: 205-214.
230. Nakahira, M., T. Tanaka, B. E. Robson, J. P. Mizgerd, and M. J. Grusby. 2007. Regulation of signal transducer and activator of transcription signaling by the tyrosine phosphatase PTP-BL. *Immunity* 26: 163-176.
231. Bompard, G., C. Puech, C. Prebois, F. Vignon, and G. Freiss. 2002. Protein-tyrosine phosphatase PTPL1/FAP-1 triggers apoptosis in human breast cancer cells. *J. Biol. Chem.* 277: 47861-47869.
232. Costantini, J. L., S. M. Cheung, S. Hou, H. Li, S. K. Kung, J. B. Johnston, J. A. Wilkins, S. B. Gibson, and A. J. Marshall. 2009. TAPP2 links phosphoinositide 3-kinase signaling to B-cell adhesion through interaction with the cytoskeletal protein utrophin: expression of a novel cell adhesion-promoting complex in B-cell leukemia. *Blood* 114: 4703-4712.
233. Hoffman, E. P., R. H. Brown Jr, and L. M. Kunkel. 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 51: 919-928.
234. Haenggi, T. and J. M. Fritschy. 2006. Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue. *Cell Mol. Life Sci.* 63: 1614-1631.

235. Hogan, A., Y. Yakubchyk, J. Chabot, C. Obagi, E. Daher, K. Maekawa, and S. H. Gee. 2004. The phosphoinositol 3,4-bisphosphate-binding protein TAPP1 interacts with syntrophins and regulates actin cytoskeletal organization. *J. Biol. Chem.* 279: 53717-53724.
236. Glondu-Lassis, M., M. Dromard, M. Lacroix-Triki, P. Nirde, C. Puech, D. Knani, D. Chalbos, and G. Freiss. 2010. PTPL1/PTPN13 regulates breast cancer cell aggressiveness through direct inactivation of Src kinase. *Cancer Res.* 70: 5116-5126.
237. Borlado, L. R., C. Redondo, B. Alvarez, C. Jimenez, L. M. Criado, J. Flores, M. A. Marcos, C. Martinez-A, D. Balomenos, and A. C. Carrera. 2000. Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. *FASEB J.* 14: 895-903.
238. Longo, P. G., L. Laurenti, S. Gobessi, S. Sica, G. Leone, and D. G. Efremov. 2008. The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells. *Blood* 111: 846-855.
239. Niedermeier, M., B. T. Hennessy, Z. A. Knight, M. Henneberg, J. Hu, A. V. Kurtova, W. G. Wierda, M. J. Keating, K. M. Shokat, and J. A. Burger. 2009. Isoform-selective phosphoinositide 3'-kinase inhibitors inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukemia: a novel therapeutic approach. *Blood* 113: 5549-5557.
240. Gold, M. R., V. W. Chan, C. W. Turck, and A. L. DeFranco. 1992. Membrane Ig cross-linking regulates phosphatidylinositol 3-kinase in B lymphocytes. *J. Immunol.* 148: 2012-2022.
241. Ali, K., A. Bilancio, M. Thomas, W. Pearce, A. M. Gilfillan, C. Tkaczyk, N. Kuehn, A. Gray, J. Giddings, E. Peskett, R. Fox, I. Bruce, C. Walker, C. Sawyer, K. Okkenhaug, P. Finan, and B. Vanhaesebroeck. 2004. Essential role for the p110delta phosphoinositide 3-kinase in the allergic response. *Nature* 431: 1007-1011.
242. Nashed, B. F., T. Zhang, M. Al-Alwan, G. Srinivasan, A. J. Halayko, K. Okkenhaug, B. Vanhaesebroeck, K. T. Hayglass, and A. J. Marshall. 2007. Role of the phosphoinositide 3-kinase p110delta in generation of type 2 cytokine responses and allergic airway inflammation. *Eur. J. Immunol.* 37: 416-424.
243. Ringshausen, I., F. Schneller, C. Bogner, S. Hipp, J. Duyster, C. Peschel, and T. Decker. 2002. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. *Blood* 100: 3741-3748.
244. Longo, P. G., L. Laurenti, S. Gobessi, S. Sica, G. Leone, and D. G. Efremov. 2008. The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells. *Blood* 111: 846-855.
245. Mizuno, T., X. Zhong, and T. L. Rothstein. 2003. Fas-induced apoptosis in B cells. *Apoptosis* 8: 451-460.
246. Ma, K., S. M. Cheung, A. J. Marshall, and V. Duronio. 2008. PI(3,4,5)P3 and PI(3,4)P2 levels correlate with PKB/akt phosphorylation at Thr308 and Ser473, respectively; PI(3,4)P2 levels determine PKB activity. *Cell. Signal.* 20: 684-694.

247. Van der Kaay, J., M. Beck, A. Gray, and C. P. Downes. 1999. Distinct phosphatidylinositol 3-kinase lipid products accumulate upon oxidative and osmotic stress and lead to different cellular responses. *J. Biol. Chem.* 274: 35963-35968.
248. Sultan, C., M. Breton, G. Mauco, P. Grondin, M. Plantavid, and H. Chap. 1990. The novel inositol lipid phosphatidylinositol 3,4-bisphosphate is produced by human blood platelets upon thrombin stimulation. *Biochem. J.* 269: 831-834.
249. Ramadani, F., D. J. Bolland, F. Garcon, J. L. Emery, B. Vanhaesebroeck, A. E. Corcoran, and K. Okkenhaug. 2010. The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development. *Sci. Signal.* 3: ra60.
250. Frech, M., M. Andjelkovic, E. Ingley, K. K. Reddy, J. R. Falck, and B. A. Hemmings. 1997. High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J. Biol. Chem.* 272: 8474-8481.
251. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J. V. Ravetch. 1996. Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. *Nature* 379: 346-349.
252. Liu, Q., A. J. Oliveira-Dos-Santos, S. Mariathasan, D. Bouchard, J. Jones, R. Sarao, I. Kozieradzki, P. S. Ohashi, J. M. Penninger, and D. J. Dumont. 1998. The inositol polyphosphate 5-phosphatase ship is a crucial negative regulator of B cell antigen receptor signaling. *J. Exp. Med.* 188: 1333-1342.
253. Prasad, K. V., Y. C. Cai, M. Raab, B. Duckworth, L. Cantley, S. E. Shoelson, and C. E. Rudd. 1994. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc. Natl. Acad. Sci. U. S. A.* 91: 2834-2838.
254. Coyle, A. J., S. Lehar, C. Lloyd, J. Tian, T. Delaney, S. Manning, T. Nguyen, T. Burwell, H. Schneider, J. A. Gonzalo, M. Gosselin, L. R. Owen, C. E. Rudd, and J. C. Gutierrez-Ramos. 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity* 13: 95-105.