Regulation of B cell metabolic activation, germinal center responses and autoimmunity by Tandem PH domain-containing proteins

By

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Abstract

Regulation of PI3K signaling is critical for B cells to generate an effective adaptive immune response and prevent the development of autoimmunity and malignancy. PI3K pathway activated upon B cell antigen receptor (BCR) crosslinking generates membrane phosphoinositide such as PI(3,4,5)P3 and PI(3,4)P2. These are lipid second messenger molecules that recruit various effector proteins to the membrane to mediate downstream signaling. Down regulation of PI3K signaling via inositol 5-phosphatases such as SHIP is associated with increased generation of PI(3,4)P2. However, PI(3,4)P2 dependent mechanisms are not well understood although the role of PI(3,4,5)P3 is extensively studied in PI3K signaling. Tandem PH domain containing proteins (TAPPs) are adapter proteins which exist as TAPP1 or TAPP2 isoforms. TAPPs strictly bind to PI(3,4)P2 via its C-terminal PH domain to get recruited to the membrane upon PI3K activation.

To investigate the role of TAPPs interacting with PI(3,4)P2 in B cell signaling, studies were carried out in mice bearing targeted mutations within their C-terminal PH domains that disrupt their ability to interact with the membrane (TAPP KI mice). Previous findings from the lab showed that TAPP KI B cells exhibited enhanced PI3K signaling upon BCR crosslinking, and showed increased B cell proliferation. Additionally, young adult TAPP KI mice exhibited hypergammaglobulinemia in the serum. Therefore our central hypothesis is that, **uncoupling of TAPPs from the membrane leads to pathological dysregulation of B cell function in TAPP KI mice.**

Specific aims of this project

1. To investigate whether TAPP KI mice develop autoimmunity and to identify its link to chronic germinal centers in these animals.

2. To investigate TAPP KI B cell intrinsic defect contributing to these abnormalities.
3. To determine the PI3K dependent metabolic abnormality driving B cell hyper activation in TAPP KI mice.

Upon investigating aging TAPP KI mice, I found that these animals developed splenomegaly, antinuclear antibodies and an increase in titre of anti-dsDNA antibodies with age. Aged TAPP KI mice (above 40 weeks) showed IgG deposition within the glomeruli of the kidney together resembling the characteristics exhibited in other lupus prone mice and SLE patients.

I subsequently examined the role germinal centres (GC) play in development of autoimmune disease in TAPP KI mice. TAPP KI mice exhibited elevated frequencies of GC B cells and exhibited a spontaneous increase in B cells displaying markers of activation or memory such as CD80 and CD86. Expansion of age associated B cells (ABCs) and peritoneal B1a cells in middle aged (28-30 weeks old) TAPP KI mice resembled that of SLE. Most of these B cell hyper-activation phenotypes showed gene dose dependence based on number of TAPP KI alleles. Disrupting the GC, by blocking the GCB (germinal centre B cell) – TFH (T follicular helper cell) interaction was achieved by knocking out ICOS from TAPP KI mice. Both acute and chronic GC development was impaired in *Icos*-/- TAPP KI mice. Importantly, all measured autoimmune characteristics were also reversed in these *Icos*-/- TAPP KI mice. I further assessed the B cell intrinsic role of TAPPs contributing to the development of chronic GC and autoimmunity. Using mixed bone chimeras, I found evidence that mice having TAPP KI mutations only in B cells develop chronic GCs and autoantibodies, indicating that abnormal signaling within TAPP KI B cells is sufficient to drive these phenotypes.

TAPP KI B cell abnormalities were characterized in detail using in vitro analyses. TAPP KI B cells were hyper-responsive to multiple stimuli in a TAPP KI gene dose dependent manner. Metabolic abnormalities were detected in TAPP KI B cells as they exhibited an increase in rate of glycolysis and mitochondrial respiration. These cells also exhibited enhanced expression of glucose transporter GLUT1 and an increase in glucose uptake in vitro. Inhibition of PI3K signaling using the novel therapeutic agent Idelalisib reversed TAPP KI B cell hyper responsiveness as well as defects in glucose metabolism in vitro. TAPP KI GC B cells exhibited a significant increase in glucose uptake and GLUT1 expression in vivo. Treatment of TAPP KI mice with the inhibitor of glycolysis 2-deoxyglucose reversed chronic GC and autoimmunity.

Together these data suggest that interaction of TAPPs with PI(3,4)P2 regulates PI3K signaling to attenuate B cell metabolic activation. Disruption of this regulatory circuit results in chronic B cell activation and germinal center responses, leading to autoantibody production.

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List of Publications

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List of Abbreviations

2-DG	2- Deoxy– D- glucose
2- DG-6- P	2- Deoxy-glucose-6-phosphate
2 – NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-deoxyglucose
ABC	Age associated B cells
ADCC	Antibody- dependent cell- mediated cytotoxicity
AID	Activation induced cytidine deaminase
AKT	A serine threonine kinase downstream of PI3K
ALPS	Autoimmune lymphoproliferative syndrome
ANA	Anti-nuclear antibody
APDS	Activated PI3K delta syndrome
ATP	Adenosine triphosphate
BAFF	B cell activating factor
B-ALL	B cell acute lymphoblastic leukemia
Bam32	B-cell adapter molecule of 32 kDa
BCAP	B cell adapter protein
B cell	B lymphocyte

B-CLL	B – cell derived chronic lymphocytic leukemia
BCL6	B -cell lymphoma 6
BCR	B-cell antigen receptor
BM	Bone marrow
BSA	Bovine serum albumin
Btk	Bruton’s tyrosine kinase
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
cGy	Centigray
CSR	Class switch recombination
CXCR	C-X-C motif chemokine receptor
DAG	Diacylglycerol
DC	Dendritic cell
DCA	Dichloroacetic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA

EBV	Epstein–Barr virus
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
ELISA	Enzyme-linked immunsorbent assay
ERK	Extracellular- signal- regulated kinase
ETC	Electron transport chain
FACS	Fluorescence - activated cell sorting
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FcγR	Fc gamma receptor
FDA	Food and drug administration
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FO B	Follicular B cells
FYVE	Fab1, YOTB,Vac1 and EEA1 homology domain
GC	Germinal center
GCB	GC B-cells
GTP	Guanosine triphosphate

G6P	Glucose-6-phosphate
HEK293	Human embryonic kidney 293 cell
HEp-2 cells	Human epithelial type 2 cells
HIF	Hypoxia –inducible factor
HRP	Horseradish peroxidase
H_20_2	Hydrogen peroxide
ICOS	Inducible T-cell costimulator
ICOSL	Inducible T-cell costimulatory ligand
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INPP4	Inositol phosphate-4-phosphatase
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KI	Knock –in

LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MBC	Memory B-cells
MFGE8	Milk fat globule- EGF factor 8
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyl tetrazolium bromide
mTORC	Mammalian target of rapamycin complex
MUPP1	Multi-PDZ domain protein 1
MZ B cell	Marginal zone B cell
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor- kappa B
NSAID	Nonsteroidal anti-inflammatory drugs
NZB	New Zealand black
NZW	New Zealand white

OCR	Oxygen consumption rate
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with tween 20
PC	Plasma cell
PDH	Pyruvate dehydrogenase
PDHK	Pyruvate dehydrogenase kinase
PDK1	3- phosphoinositide-dependent protein kinase 1
PD1	Programmed cell death-1
PDZ domain	PSD-95/discs large/ZO-1 domain
PE	Phycoerythrin
PGI	Phosphoglucose isomerase
PH domain	Pleckstrin homology domain
PI	Phosphatidylinositol
PIP3	PI(3,4,5)P3
PI3K	Phosphoinositide 3- kinase
PI3Kδ	Phosphoinositide 3- kinase delta

PKC	Protein kinase C
PKM2	Pyruvate kinase M2
PLEKHA1	Pleckstrin homology domain containing A1
PLEKHA2	Pleckstrin homology domain containing A2
PLC	Phospholipase C
PTEN	Phosphatase and tensin homologue
PTPL1	Protein tyrosine phosphatase L1
PX	Phox homology
P(3,4)P2	Phosphatidylinositol 3,4- bisphosphate
PI(3,4,5)P3	Phosphatidylinositol 3,4,5 -trisphosphate
PI(4,5)P2	Phosphatidylinositol 4,5- bisphosphate
P110 d	P110 delta (P110 δ)
RA	Rheumatoid arthritis
RAG	Recombination- activating genes
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SHM	Somatic hyper mutation

SHIP	SH2 domain- containing inositol 5’ – phosphatase
SH2	Src homology 2
SLE	Systemic lupus erythematosus
SPR	Surface plasmon resonance
SRBC	Sheep red blood cells
Syk	Spleen tyrosine kinase
T cells	T lymphocyte
TAPP	Tandem PH domain containing protein
TCR	T cell antigen receptor
TD	T cell- dependent antigen
TFH	T follicular helper cells
TH cell	T helper cell
TI	T cell- independent antigen
TLR	Toll-like receptors
TNFR	Tumor necrotic factor receptor
T1, T2	Transitional type 1 and 2
WT	Wild type

XHM	X- linked hyper IgM
XLA	X- linked agammaglobulinemia
µM	Micro molar
µg	Microgram
µl	Microliter

Chapter 1

Introduction

1.1 B cell development

B cells are lymphocytes that mediate protection from invading pathogens primarily by producing antibodies and therefore play a significant role in the adaptive immune response. The discovery of B cells and their characterization started around the mid-1960's using experimental models. B cells were named after their discovery in the Bursa of Fabricius which is a lymphoid organ present only in birds. Transplant models in mice have shown the origin of these antibody producing cells. Mammalian B cell development occurs in primary lymphoid organs which is fetal liver during embryonic development but bone marrow (BM) becomes the site in adults. B cell development occurs in a similar fashion within mice and humans as it takes place throughout their life time, whereas in species like rabbits and chicken, B -cell development is restricted to early life [1-3].

Medullary cavity of the bone comprises a variety of cells that provide a supportive environment for B cell development. A combination of both intrinsic and extrinsic signaling mechanisms tightly regulates B cells development in the BM. The presence of stromal cells in this micro environment, providing support in the form of extrinsic signals, is key for these primitive B cells [4]. Precursors of B cells are derived from common lymphoid progenitors which were developed during hematopoiesis [5]. Based on the differential expression of surface markers by these precursor B cells, they are divided into various stages of early B cell development within the BM, before they are released into the periphery [6, 7].

As B cells undergo development in the BM, they differentially express Immunoglobulin (Ig) heavy and light chains and rely upon Ig gene rearrangement mechanisms. This error prone mechanism is termed as V(D)J recombination which generates B cell antigen receptors (BCRs) with a diverse range of affinities for antigen. The role of recombination activating genes (RAG1 and 2) is crucial for this Ig gene rearrangement mechanism [8]. Apart from B cells, T cells also rely upon RAG, for the generation of a diverse repertoire of TCR (T cell antigen receptor). Generation of BCR and TCR is so important for B and T cell development, that *Rag*1-/- mice and *Rag2*-/- mice lack mature B and T lymphocytes in the periphery [9, 10].

The earliest identifiable precursor of B cells is termed as pro B cells which is a stage independent of Ig rearrangement. Stromal cell derived IL-7 contributes to the survival and activation of these early B cells and also in the initiation of V(D)J recombination. As this subset progresses further in development, they start exhibiting pre–BCR which is a combination of heavy chain with surrogate light and therefore called pre-B cells [6, 11]. Mice deficient in RAG abort B cell development at the pro-B cell stage, as they are unable to perform Ig heavy chain rearrangement [9, 10]. The signals derived from pre-BCR, help in the expansion of the pre-B cell pool in the BM. Towards the later stages of pre-B cells, rearrangement of the light chain loci begins, thus resulting in the generation of immature B cells that exhibit membrane bound IgM but not IgD [6, 12].

Within the BM, there is a balance between B cell proliferation, apoptosis and differentiation to maintain a steady state. Those B cells which show defects in Ig gene recombination or antigen recognition are removed by apoptosis [12]. During the stages of development in the BM, these cells need to pass through certain checkpoints to ensure selection of healthy B cells. One of these check points is at the pre-B cell stage where only the B cells exhibiting functional pre-BCR will

be selected for further development [6, 13]. Those B cells with pre-BCR will receive the survival advantage and will be positively selected while others undergo apoptosis. Another checkpoint in B cell development within the BM occurs during the differentiation of immature B cells. During the immature B cell stage, B cells exhibit a mature, fully functional BCR in the form of membrane bound IgM. However, more than 50% of the BCRs generated in this process are autoreactive. Those B cells with strong affinity towards self-antigens are negatively selected, with the rest being allowed to exit the BM into the periphery [14].

Immature B cells which are not selected are those exhibiting auto-reactivity and undergo cell death by a process termed as clonal deletion. However, before they undergo cell death these cells are given another chance by allowing them to edit their BCR. These IgM^+ immature B cells re-express RAGs, internalize these self-reactive BCRs and are allowed to perform multiple rounds of Ig light chain gene rearrangement. Those BCRs edited by these mechanisms that display lower affinity towards self-antigen, will be allowed to proceed further. However, B cells that fail to perform receptor editing undergo clonal deletion. This process of selection that occurs during B cell development within the BM is termed as central tolerance [12, 13]. The receptor editing mechanism is not restricted to the BM but can also occur within peripheral B cells in response to exposure with self-antigen [15].

1.2 B cell maturation in the periphery

Once naïve immature B cells are released from the bone marrow they enter into secondary lymphoid organs such as lymph nodes and spleen where they differentiate into transitional and mature B cells. B cells within the periphery require tonic or basal BCR signaling for their survival. This is an antigen independent BCR signaling essential for their survival. Tonic BCR

signaling is essential for stoppage of RAG expression and V(D)J recombination while exiting the BM. A certain threshold of these tonic signals is required for their differentiation into transitional B cells. There is a striking similarity between mice and human B cell selection within the BM and their maturation in the periphery [16-18].

1.2.1. Transitional B cells

The transitional B cell subsets represent a link between the immature B cells from the bone marrow compartment and the mature B cells in the periphery. Although a larger proportion of these subsets are seen in the periphery, a small fraction of those begin to differentiate within the bone marrow itself [19-21]. Defects in the transitional B cell frequencies have been detected in immunodeficiency and autoimmunity. Therefore it is important to understand the biology of this population as they have relevance in B cell related diseases [21].

Transitional B cells which are extensively studied belong to two major categories, namely T1 and T2 which are identified by differential surface expression of IgM and CD23 markers. T1 subsets are identified by IgM^{hi} $CD23^{-}$ population, while IgM^{hi} $CD23^{+}$ represents T2 subset of B cells. Human transitional B cells, like those of mice, showed a reduced response to BCR signaling when compared to mature B cells. Upon BCR crosslinking the transitional B cells instead of getting activated, are programmed to undergo apoptosis. To prevent BCR crosslinking by self-antigen the late transitional B cells down regulate surface IgM and start expressing IgD as they fully mature [20, 22].

1.2.2. B cell anergy and tolerance to peripheral antigens

A fraction of B cells released from the BM which are in the transitional state exhibits BCRs that are self-reactive. The generation of strong BCR signaling upon self-antigen recognition, drives a portion of these cells to go through selection by anergy while some undergo cell death by apoptosis. Whether B cells undergo tolerance or anergy is determined by the nature of self-antigen. Soluble monovalent antigen drives anergy while multivalent antigens lead to clonal deletion of these self-reactive B cells [23, 24].

Anergy is an important tolerance mechanism within the periphery. It requires chronic activation of B cells by sustained antigen receptor binding and signaling resulting in an induced state of unresponsiveness [25]. The main features discriminating anergic B cells from normal B cells include a reduced lifespan and diminished antibody response, thus limiting their function [26]. Anergic cells can be recruited into GCs if given T cell help, but fail to become antibody secreting cells unless abnormalities within the GC cause them to lose self-reactivity [27].

B cells, when chronically exposed to antigen in mice, become anergic and the cells are arrested at the transitional stage preventing them from undergoing further maturation. Although they exhibit normal B cell development, the absolute number of mature follicular cells in the periphery is significantly reduced. In addition to that, they exhibit a reduced ability to mount an antibody response upon immunization [28, 29].

An important mediator of B cell survival in the periphery is a cytokine called BAFF (B- cell activating factor) which is secreted by a number of cells such as macrophages, monocytes and dendritic cells. Although elevated BAFF levels do not alter B cell development in the BM, it can significantly affect B cell numbers in the periphery. The absence of BAFF can lead to a complete

loss of mature B cells in the periphery [30]. A mouse model deficient in BAFF-R shows a similar phenotype with that of *Baff*-/- mice as in both cases mature B cells are drastically reduced [31]. Immature B cells exiting the BM express BAFF-R as this is important for their differentiation into transitional B cells [32]. In contrast, self-reactive B cells in the periphery that have undergone anergy can be reversed by an abnormal elevation of BAFF. Abnormally upregulated BAFF is associated with B cell hyperplasia and development of autoantibodies [33].

1.3 B cell subsets

Immature B cells circulate through the blood to enter into lymphoid organs such as spleen, lymph node and Peyer's patch. To generate an appropriate B cell mediated immune response, localization into a specific microenvironment within these secondary lymphoid organs is necessary. These transitional B cells differentiate into marginal zone (MZ) or Follicular (FO) B cells depending on various aspects that include BCR signal strength and antigen specificity. B1 cells are another subset of B cells generated through a unique developmental process during early life [34].

1.3.1 Marginal Zone B cells

MZ B cells constitute nearly 5-10% of the total splenic B cells which are identified by high levels of IgM expression but not IgD at the cell membrane. Although MZ B cells rely on BCR signaling for their survival, additional mechanisms are known to contribute to their cell fate decision [35, 36]. According to a recent study, those transitional B cells that upregulate Notch2 signaling is key in driving them to differentiate into MZ B but not FO B cells. The conditional deletion of Notch2 in B cells resulted in significant loss of the MZ B population without effecting FO B cells [37].

On differentiation into MZ B cells, they become non-circulating which prevents them from cycling between blood and spleen. These B cells reside in the region surrounding the white pulp and are associated with the marginal sinuses of the spleen. These are the regions which are responsible for filtering blood and thus exposing MZ B cells to blood borne infections as they respond strongly to both innate and BCR signals. Depletion of MZ B cells in mice makes them susceptible to blood borne infections [38, 39].

When antigen recognition occurs, MZ B cells undergo proliferation and differentiate into short lived plasma cells that secrete low affinity antibodies in a T cell independent (TI) manner. This response is very rapid when compared to FO B cells that require time to generate the antigen specific response. The TI antibody response mediated by MZ B cells acts as a first line of defense against a wide range of pathogens and thus demonstrating their innate like behavior [36]. However, at times MZ B cells shuttle between the marginal sinuses and the follicular region. As they exhibit high levels of complementary receptor CD21, MZ B cells are able to capture antigens which are trapped by immune complexes which are then transported to the follicular B cells. This behavior of MZ B cells indirectly helps in contributing to the antibody response that occurs within the follicles [40].

1.3.2 B-1 cells

B-1 cells are another innate like B cell that mediate protection by secreting IgM similar to marginal zone B cells and therefore mediating an early response to infection. These cells are mainly localized within the peritoneal and pleural cavities with a small proportion of these subsets within the spleen. B-1 B cells are classified into B-1a and B-1b cells which are derived from separate precursors and differ in CD5 expression which is absent in the B-1b population.

Adoptive transfer approaches have demonstrated the origin of these subsets. B-1 cells develop during early embryonic development. Fetal liver cells but not adult bone marrow is capable of reconstituting B1 cells. However, in adult mice, these cells are continuously replenished by undergoing self-renewal within the peritoneum [41, 42].

B-1 cells spontaneously secrete natural antibodies even without antigenic exposure. More than 80% of the natural IgM circulating in the blood is produced by these cells under steady state. These antibodies are germ line derived and exhibit broad reactivity. As they do not undergo somatic mutation these antibodies show limited diversity or specificity. B-1 cell derived antibodies can recognize a wide variety of bacterial antigens including some self- antigens. Apart from producing natural antibodies, B-1 cells can be activated by TI stimulations such as TLR agonists or other microbial products [42, 43]. Another protective role of natural IgMs circulating in the blood is to facilitate clearance of apoptotic cells. B cells getting exposed to intracellular antigens especially DNA, RNA or nuclear proteins due to defective apoptotic cell clearance, leads to the development of autoantibodies [44].

1.3.3 Follicular B cells

A portion of transitional B cells entering into the secondary lymphoid organs can differentiate into follicular B cells. Identified by $CD23^{+}$ $CD21^{int}$ expression, this population is localized within the follicular region of the spleen and hence got its name. This mature B cell subset exhibits intermediate to low levels of membrane IgM and high levels of IgD. FO B cells constitute the majority of the B cell population in the periphery and constitute nearly 80 to 90 percent of the total splenic B cells. Unlike MZB cells of the spleen, this population can circulate

between the blood and spleen. It coordinates with T cells in generating specific antibody responses via the germinal response and thus contributing to the adaptive immunity [39].

1.4 Germinal centre responses.

Effective and long lasting antigen specific antibody responses require development of germinal centers (GC). These are transiently developed structures that develop in response to T cell dependent antigen within peripheral lymphoid organs. GC is required for the development of high affinity antibodies and generation of plasma and memory cells. This is essential for final clearance of pathogens and thus mediating long term protection [45].

1.4.1 Initiation of the GC response.

The lymphoid organs are comprised of specialized regions called follicles constituted of naïve B cells which are separated from their surroundings by the interfollicular region. These follicles are bordered by a T cell rich area called the T cell zone. The initial steps in the process of GC formation involve activation of naïve B cells by exogenous antigen. The B cells migrate towards the border of the follicles to interact with the T cells at the interfollicular region which were initially primed by dendritic cells. This interaction leads to further activation of these subsets but only a portion of that population will be allowed to enter into the GC. Some of these activated B cells differentiate into short lived plasma blasts secreting low affinity antibodies against the pathogen [46, 47] .

1.4.2 Germinal centre dynamics.

The development of intravital microscopy has given us more insights on the cellular dynamics within and around the GC. Those B cells that exhibit the highest relative affinity for the antigen

in that activated pool will obtain access to the center of the follicles where they differentiate to become the GC B cells. This subset of B cells is identified based on expression of markers such as GL7 and FAS. Similarly, there are T cells from the interfollicular region that are committed to enter follicles by differentiating into T Follicular helper cells (TFH). This subset of $CD4^+$ T cells that acquired TFH phenotype exhibit markers such as CXC-chemokine receptor 5 (CXCR5), programmed cell death protein 1 (PD1) and Inducible co–stimulatory molecule (ICOS). Expression of transcription factor, B cell lymphoma 6 (BCL6) is key in driving the differentiation of both GCB and TFH cells [48-50] .

It will take nearly 7 days after antigenic exposure for the GC to fully develop and generate an appropriate antibody response. At this stage, GC is polarized into light zone and dark zone compartments which is essential for an optimum performance. The light zone is populated by a mixture of sparsely populated cells that include TFH cells, Follicular dendritic cells (FDC) and others residing along with GCB cells. The dark zone got its name from its histological appearance where actively proliferating cells are densely packed in this region. There are some cell intrinsic programs that can drive the transitioning of B cells between light and dark zones. B cells within the dark zone are identified by increased expression of chemokine receptor CXCR4. They are programmed to downregulate the expression of CXCR4 gene within the light zone compartment. Studies have shown that loss of CXCR4 can restrict B cells within the light zone and exhibit defects in generating an effective antibody response against an antigen [51-53].

The microenvironment within the GC is highly dynamic as there is a continuous circulation of GCB cells between light and dark zone that helps in achieving multiple rounds of mutation and selection. This is essential for affinity maturation of antigen receptor, a process ultimately leading to the generation of high affinity antibodies against wide range of epitopes exhibited by

the invading pathogen. B cells that undergo somatic hyper mutation (SHM) of genes within the dark zone diversify immunoglobulin variable region to develop antigen receptors with heterogeneous affinities [45, 54, 55].

1.4.3. Affinity maturation and role of follicular helper T cells

Once GC B cells enter into the light zone, there is a competition for antigen recognition and T cell help before they re-enter the dark zone for further expansion. Only those B cells with highest affinity for antigen are positively selected within the light zone [56, 57]. Within the light zone compartment, FDCs displays antigens for recognition by B cells using their BCR. TFH helps in facilitating B cell selection within the light zone which requires direct contact with GCB cells. The antigen sequestered by B cells is processed and presented to these TFH cells using MHC. Those B cells displaying BCR with the highest antigen affinity have the ability to capture antigen more efficiently than those with low affinity antigen receptors. Therefore those B cells displaying a high density of antigenic peptides through MHC, exhibits a stronger and extensive GCB – TFH interaction thus driving the selection of these B cells [58].

Signals derived from GCB and TFH contact is crucial for B cell selection within the light zone and their division after entry into the dark zone. These subsets are mutually supportive for their survival and activation. The light zone B cells exhibit increased levels of CD40 which are ligated by CD40L displayed by TFH cells [59]. In addition to this co-stimulation , the production of IL-4 and IL-21 cytokines by TFH cells together contributes to light zone GCB cells activation [60]. Similarly, TFH activation is facilitated by crosslinking of ICOS which is dependent on ICOSL exhibited by GCB [58, 59]. Absence of ICOS signaling in humans as well as in mice, leads to

loss of germinal centers [61]. In contrast, enhanced ICOS expression in mice has resulted in an aberrant GC response, contributing to the development of autoimmunity [62].

Upon re-entry into the dark zone after selection, those B cells that obtained the greatest magnitude of help from TFH are programmed to divide more robustly than others. SHM within Ig variable region is closely associated with the proliferative ability of B cells. Every division can introduce a random mutation at a rate of one nucleotide per thousand bases. The cells with higher proliferative potential can undergo more affinity enhancing mutations than others. As high affinity BCR exhibiting cells are repeatedly instructed to re-enter dark zone for further rounds of division and SHM, they eventually become more dominant than the low affinity B cells [56, 63]. Apart from the role of cellular interactions in regulating the GC, there are molecular mechanisms involving epigenetic and transcriptional mediators along with post translational events that program the behavior of these subsets [58, 59].

1.4.4. Class switch recombination and the role of AID

Another mechanism that contributes to the diversification of the antibody response against a pathogen is called class switch recombination (CSR). Antibody isotypes exhibited by naive mature B cells in the periphery are mainly IgM and IgD. Upon antigenic exposure, these B cells which were initially secreting predominantly IgM undergo CSR within the GC. The constant region of immunoglobulin heavy chain gene is switched without modifying the variable region. Isotype switching generates different classes of Igs such as IgG, IgA and IgE that exhibit diverse effector functions without altering their antibody affinity [64, 65]. Defects in Ig class switching due to a rare mutation in the gene encoding CD40 or CD40 L impairs the GC function [66, 67].

This can lead to hyper IgM syndrome which is characterized by an increase in levels of IgM but a lack of other isotypes of Igs required in mediating protection against pathogens [68].

Similar to SHM, the signals that initiate CSR within GC requires T cell help that triggers CD40 signaling in B cells. In addition to that, cytokines derived from TFH cells can also mediate the process of isotype switching in B cells. Unlike SHM where point mutation is introduced into the immunoglobulin variable region, CSR involves cutting and joining heavy chain loci of immunoglobulin genes. A key enzyme involved in both CSR and SHM mechanism is AID (Activation induced cytidine deaminase) which contributes to the diversification of immunoglobulin gene repertoire. AID activity is mainly restricted to immunoglobulin genes and they are tightly regulated [64]. This minimizes undesirable mutations targeting non immunoglobulin loci resulting in B cell malignancies. Absence of AID activity in mice and humans leads to defects in class switching and therefore increases susceptibility to infections [69, 70].

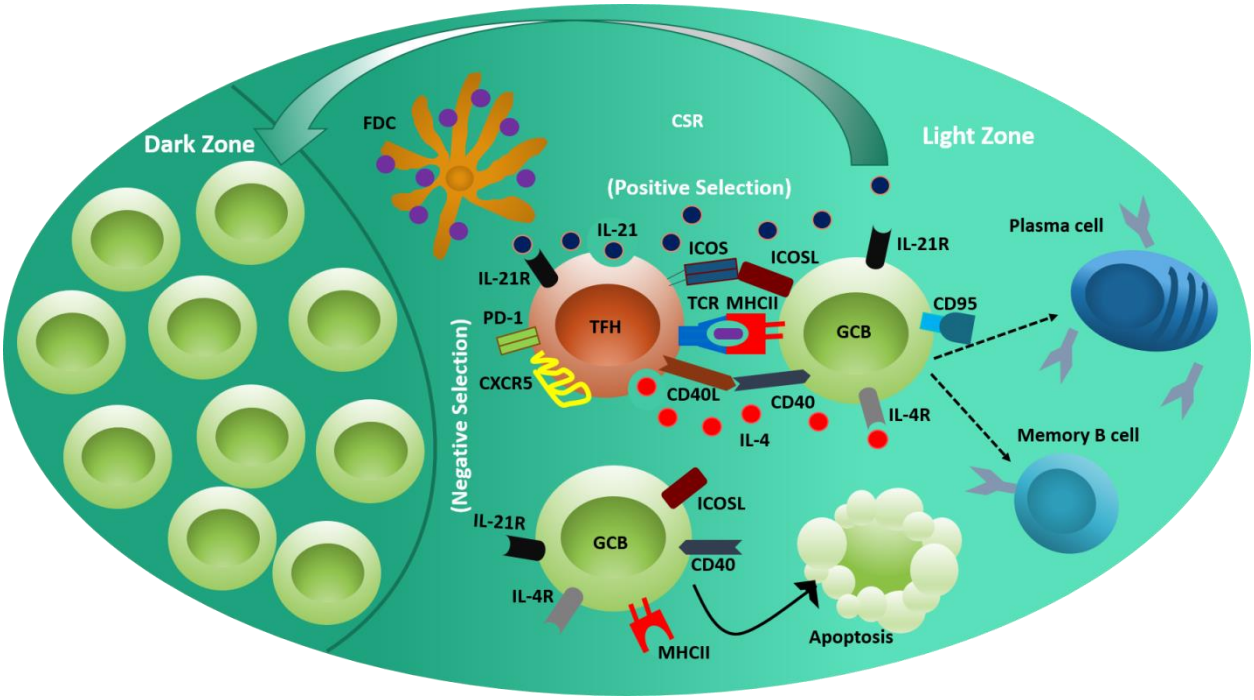

Figure 1.1 - Germinal B cell dynamics

GCB cells that obtain antigens from follicular dendritic cells (FDC) within the light zone compartment are selected to enter into the dark zone where it divides and undergo somatic hyper mutation (SHM) before re-entering into the light zone. Those GCB cells that exhibit high antigenic affinity BCR gets positively selected by obtaining survival help from TFH cells and differentiates into plasma and memory cells. On the other hand, those GCB cells exhibiting low affinity BCRs don't receive TFH help and therefore undergo apoptosis.

1.5. Effector and Memory B cells

GC B cells undergo terminal differentiation to generate memory B cells (MBCs) and long lived plasma cells (PCs) that mediate long term protection from infections. MBCs are antigen experienced B cells that persist even after clearing the infection. They have an intrinsic ability to respond faster than naïve B cells. Upon re-exposure to the antigen, they exhibit a proliferative burst and differentiate into plasma cells to mediate a robust antibody response. As these MBCs have already undergone affinity maturation and selection within the GC, upon secondary exposures they can generate high affinity class switched antibodies [71] . The memory response can last for years and these cells have the capability to self-renew as they differentiate into PCs [72, 73].

Long lived PCs are quite distinct from those short lived plasma cells which are derived during an early exposure to antigen prior to the formation of GC. The short lived PCs that generate low affinity antibodies and exist in small percentages in the spleen survive only for few days. However, the long lived PCs cells migrate into the BM and survive for several years. Within this microenvironment, they obtain support from stromal cells which are essential for their survival. Residing in the protective niches of the BM, these PCs are capable of secreting pathogenic autoantibody during autoimmune diseases and are difficult to be eliminated. Upon re-exposure to an infection, these plasma cells can emigrate from BM into peripheral blood and secrete high affinity antibodies without a delay. Memory B cells are able to differentiate and replenish these PCs if required [72, 74].

1.6 Age associated B cells

Age associated B cells (ABCs) are a phenotypically distinct B cell subset that accumulate progressively with age and is detected more often during viral, bacterial and parasitic infections. This subset is associated with the generation of anti-pathogen or autoantibody responses. An increase in ABCs is detected prematurely with autoimmunity and it correlates with the onset of the disease. ABCs are mostly in resting state and respond poorly to BCR crosslinking alone but in combination with TLR agonist or in the presence of IFNγ they undergo proliferation and secrete cytokines [75, 76].

While investigating the source of ABC, it was found that an increase in frequency of ABCs corresponds to decrease in FO B cells. In addition to that, adoptive transfer of FO B cells but not BM cells generated ABC in the recipient mice [75]. Recent findings show that these cells are antigen experienced and share similarities with long lived memory B cells. However, further investigation is required to confirm whether this memory like B cell is capable of developing a recall response. The generation of ABCs is dependent on TD response that occurs within the GC and thus exhibiting a diverse antibody repertoire. As the mutation frequency exhibited by ABC is lower than GC B cells, it appears that they exit the GC much earlier before the selection process is completed [77].

1.7 Immunoglobulin structure and function

Immunoglobulins are large glycoproteins with a size of 150kDa that are secreted by plasma cells. B cell mediated protection from a wide range of infections depends on antibody production and therefore constitutes an important component of humoral immunity. Apart from the secretory form, they also exist in membrane bound form which is BCR. The transmembrane region of this

molecule has the potential to mediate downstream signaling when triggered for activation [78]. While in the immature stage within the BM, B cells only exhibit IgM on the surface, however, naive mature B cells co- express IgD and IgM [79].

Antibodies are Y shaped molecules that mediates protection from invading microbes by a variety of ways. They constitute 4 polypeptide chains with 2 large heavy chains and 2 small light chains which are joined together by disulphide bonds. These heavy or light chain domains are encoded by distinct gene loci present on different chromosomes. These heavy and light chains are further divided into variable and constant regions which have unique functions. The structure is also composed of a hinge region that adds flexibility to the molecule. The two types of light chains that exist are κ or λ where only one type can exist in an antibody at a time. Unlike light chains, the heavy chains exists as α, δ, ε ,μ and γ which determines the 5 different classes or isotypes of antibody which includes IgA, IgD, IgE , IgM or IgG respectively. These isotypes differ in their localization and effector function. Isotypes such as IgD , IgE and and IgG are monomeric while IgA is a dimer and IgM is pentameric in structure [78].

IgM production remains the first B cell mediated antibody response on antigenic exposure. Although these IgMs have low affinity towards these molecules, being pentameric they also have high avidity that will help in controlling infection to certain degree during this early stage. IgM also exist in a membrane bound form alone in immature B cells or along with IgD in mature B cells to mediate downstream signaling. IgG on the other hand exist as various subclasses and constitute one of the major isotypes that mediates protection from an invading pathogen. This is the only isotype that can move across placenta to mediate passive immunity to the growing fetus.

IgA is an isotype which is mostly associated with the mucosal regions and are also present in body secretions such as saliva or breast milk. Another class of antibody called IgE is known for its role in generating an allergic response and also in mediating protection from parasitic worms [80].

The portion of the antibody molecule that can recognize the antigen is called Fab (fragment antigen binding). This fragment is composed of one variable and one constant region for each heavy and light chain. The variable portion of Fab is called complementarity determining region or hyper variable region which is located at the tip of the molecule. This region is responsible for specificity towards the antigen. The ability to vary amino acid sequences in this region can lead to generation of antibodies with diverse affinities and help them to recognize a wide range of epitopes. The direct binding of antibody with the epitope of the microbe may neutralize it by preventing the survival or invasion of the organism within the body. Apart from that they can also precipitate soluble antigens in the form of clumps which can become a target for phagocytosis [78].

The base of the Y shaped molecule is called Fc (Fragment, crystalizable) portion with two heavy chains comprised of only the constant region. Fc portion determines the isotype of the antibody that mediates protection by modulating the immune system. The Fc portion of antibody can bind to the Fc receptor (FcR) expressed on various immune cells. Depending on the type of FcR that gets activated, it can have a diverse biological outcome [81].

The Fc portion of IgE can bind to FcRε on the mast cell resulting in its degranulation and secretion of histamines as a part of an allergic response to an antigen. The binding of the Fc portion of the antibody can also trigger FcR of effector cells and mediate antibody dependent cell

cytotoxicity (ADCC). IgE can drive ADCC to clear parasitic worms by coating them with antibody followed by activating other immune cells that mediate the cytotoxic function by triggering their FcR [78, 81].

Another mechanism driven by the Fc portion of the antibody is complement activation. When a microbe is coated by antibody and complement proteins they are marked for phagocytosis. Immune cells such as macrophages or dendritic cells are chemotactically attracted to the site to mediate phagocytosis. Another mechanism mediated by complement pathway is the formation of the membrane attack complex that mediates lysis of the bacterial cell [81].

1.8 Antibody-independent B cell functions.

One of the antibody independent functions of B cells includes their role as antigen presenting cells (APC) where they are able to prime T cells with antigenic peptide even under low antigen concentration. Unlike other APCs such as dendritic cells or macrophages which rely on pinocytosis for antigen uptake, B cells internalize antigen following its binding with BCR. The role of B cells as antigen presenting cells is substantially increased under autoimmune condition as demonstrated in RA [82, 83].

B cells modulate immune response by secreting cytokines to either promote or suppress inflammation. They secrete pro inflammatory cytokines such as IL-4, IL6 and IFNγ which can activate other immune cells around the site of infection. In addition to that some of these cytokines also have an autocrine effect on B cells that facilitates its further activation [84, 85]. Unlike those B cells that mediate inflammation, the activation of regulatory B cells (B regs) contributes to its suppression of T cells by secreting IL-10 and thus reducing inflammation. Absence of B regs in autoimmune mice has shown to increase the severity of the inflammation.

Adoptive transfer of WT B cells but not IL-10 deficient B cells were able to suppress inflammation [86, 87]. The regulatory function of this subset is functionally impaired in lupus patients [88].

1.9 Diseases related to B cell dysfunction

Apart from the protective role of B cells, dysregulation of B cell function leading to their hyper activation results in the development of malignancies or autoimmune disease. On the other hand, loss of B cell function is known to cause B cell deficiency diseases.

1.9.1 Immune Deficiencies

X linked agammaglobulinemia (XLA) otherwise known as Brutons agammaglobulinemia is a condition resulting from attenuated BCR signaling. Mutation in the *BTK* gene leads to deficiency in Bruton's tyrosine kinase resulting in this impairment. Targeted disruption of the *BTK* gene in mice showed similar disease characteristics as that of human [89]. This defect effects B cell maturation in the BM by preventing them from differentiating into pre-B cells. In both cases there is a complete loss of circulating B cells in the periphery [90, 91].

Another immunodeficiency disease caused by B cell dysfunction is hyper IgM syndrome which is again an X-linked immunodeficiency disease. Mutation in CD40 ligand (CD40L) leads to the loss of CD40-CD40L interaction which is crucial in mediating antibody class switching. Mouse models lacking either CD40 or CD40L resemble that of human XHM (X linked hyper IgM) and showed defects in the development of GC. Under this condition, because of their inability to undergo class switching it leads to increased levels of IgM. This defect that prevents the development of an appropriate antibody response makes these patients susceptible to various infectious diseases [92-95].

Mouse models of B cell deficiency are widely used in research to understand the B cell biology and are also used as a model of immunodeficiency disease in human. One of the popular models is the muMT mice, which was developed by the Rajewsky lab in early 90s. These mice were developed by a targeted mutation within the immunoglobulin heavy chain gene. Homozygous loss of mu heavy chain in these mice blocked B cell development at the pre B cell stage as they lack functional pre BCR required for its differentiation. Although this mutation drastically impairs B cells, these animals exhibited a normal T cell frequency in the periphery [96].

1.9.2 B cell malignancies

There are different types of malignancies which are driven by aberrant B cell activation. Constitutive activation of BCR signaling is detected in many of these B cell malignancies. B – CLL (B-cell chronic lymphocytic leukemia) is one of the most common types of leukemia seen in elderly individuals. These malignant B cells originate within the BM and enter into the peripheral blood and lymphoid organs where it gets progressively accumulated. Approaches to target abnormal BCR signaling using small molecule inhibitors are being tested in C-LL [97-99].

B–ALL (B cell - acute lymphoblastic leukemia) is another type of B cell cancer that originates from within the bone marrow. It is characterised by an abnormal expansion of malignant precursor B cells leading to their accumulation within the BM. Chromosomal translocations leading to the production of defective fusion proteins contribute to the malignant transformation of precursor B cells [100, 101].

Non-Hodgkin lymphoma is a lymphoproliferative disease with more than 80% of the cases driven by B cell defects [102]. These types of cancers are developed within the lymphoid organs,

originating from germinal centres. These B cells exhibit altered BCR expression due to abnormal re-arrangement of immunoglobulin genes [103, 104].

1.9.3 Autoimmune diseases

Autoimmune diseases are broadly classified into T cell mediated and autoantibody mediated. Productions of defective antibodies by B cells that recognize self-antigens are associated with loss of B cell tolerance and hyper responsiveness. Multiple sclerosis, Rheumatoid arthritis, and Systemic lupus erythematosus are some of the most common diseases where autoreactive B cells play a crucial role [105]. Below I will focus on SLE as it is most relevant to this thesis.

Systemic lupus erythematosus is a chronic autoimmune disease driven predominantly by hyper responsive B cells. Some of their characteristics include production of high titers of antibodies (hyper gamma globulinemia) including autoantibodies contributing to systemic inflammation leading to organ damage. In addition to the effects caused by autoantibodies, B cells contribute to lupus pathogenesis by antibody independent mechanisms. These functions include secretion of proinflammatory cytokines, presentation of self-antigens and co-stimulation of T cells [106].

The etiology of SLE is not fully understood as the disease pathogenesis is contributed by interaction between varieties of factors. A combination of genetic, hormonal, age and environmental factors contribute to the disease onset. Therefore these individuals exhibit a wide range of clinical heterogeneity. In addition to that, infections caused by Epstein- Barr viruses or parvo viruses are known to trigger the development of lupus. Apart from that cigarette smoking or exposure to silica dust are some of the environmental stimuli [107].

1.9.3.1 B cells and autoantibodies in SLE

Apart from the external clinical symptoms, a wide variety of autoantibodies that are generated in lupus are used in the diagnosis of the disease. Specificity of autoantibody is an important aspect for diagnosis where some of these autoantibodies can be detected before the onset of the clinical symptoms [108]. Among the classification criteria outlined by American College of Rheumatology, only a few which are specific for the disease has been considered for the diagnosis in the laboratory. Antibodies that are very specific to lupus include anti-dsDNA antibodies and anti-smith antibody. In addition to that presence of ANA (antinuclear antibodies) is also considered as a diagnostic marker. Antibodies against RNA binding proteins such as Ro and La are prevalent but not specific as they are also seen in Sjogren's disease [109, 110].

Pathogenic role of autoantibodies in lupus include those which deposit within the tissues activating complement cascade leading to tissue damage. Anti-dsDNA antibody which is one of the most commonly detected antibodies in lupus contributes to the development of glomerulonephritis eventually causing kidney damage. Other examples include anti-Ro or anti-cardiolipin antibodies that cause cardiac dysfunction or thrombosis respectively. Autoantibodies also help in the internalization of self-antigens upon Fc receptor binding [111]. Passive transfer of anti-dsDNA antibodies to healthy animals demonstrated the role of these antibodies in the development of lupus nephritis [112].

B cells also contribute to the lupus pathogenesis in an antibody independent manner. They have been shown to exhibit enhanced antigen presentation to break T cell tolerance. In addition to that increased levels of pro-inflammatory cytokines or reduced IL-10 secretion contribute to the disease severity [88, 113, 114]. Therefore, clinical symptoms in certain individuals don't

necessarily correlate with auto-antibody levels. Approaches to deplete B cells have reduced the disease severity in mouse models and in SLE patients. Understanding the B cell driven mechanisms contributing to lupus is crucial in the development of B cell therapies.

1.9.3.2 Genetic susceptibility to SLE

Understanding the genetic basis of SLE helps in predicting the pathophysiology and also in delivering appropriate treatment methods. There are certain genetic risk factors altering the immunological pathways that contribute to their dysregulation and drives hyper responsiveness of these cells. Mutations leading to over expression of signal enhancing molecules or deficiency of inhibitors of the signaling mediate this defect. Genome wide association studies screening variations in the human genome has identified several lupus susceptible gene loci [115].

Around 50 different lupus susceptibility loci have been identified and several of those are associated with B cell signaling [116]. Genetic mutations favoring survival of autoimmune B cells helping them to escape selection have been associated with lupus in mice and humans. Polymorphism associated with *LYN*, is linked to the development of lupus in European – American women. In addition to that, B cells derived from lupus patients showed reduced levels of LYN [117, 118]. Mutations within several Lyn regulating proteins are also known for its role in this disease. BANK1 is a scaffold protein that associates with lyn to mediate calcium mobilization upon BCR crosslinking. *PTPN22*, a gene that encodes tyrosine phosphatase LYP or its interacting protein CSK are both highly susceptible to mutations driving B cell hyper responsiveness and thus leading to the development of lupus. Another gene mutated within B cell signaling is a kinase predominantly expressed in B cells called *BLK*. Polymorphism in *BLK* is associated with lupus in certain Asian populations [119].

Several different variants are available for *FcγRIIB*, which are known for their contributions in the development of lupus. FcγRIIB mediate the inhibitory signaling mechanism of BCR signaling. They also promote clearing of immune complexes preventing them from depositing within the tissues. *FcγRIIB* polymorphism inhibiting these protective mechanisms contributes to B cell hyper activation and autoimmunity [122, 123]. Mice deficient in *FcγRIIB* spontaneously develop autoimmune disease resembling human lupus [124].

1.9.3.3 Female sex bias in SLE

One of the characteristics of lupus is that it is highly prevalent in females when compared to males with a female to male ratio of 9:1. Sex hormones remain one of those factors contributing to these differences. Sex hormones that orchestrate the immune response mediate this by regulating antibody production or release of various pro inflammatory cytokines. There are reports of female SLE patients experiencing flares during pregnancy where sex hormones play a predominant role contributing to this [125]. Sex hormones like estrogen have shown to influence the survival, maturation and repertoire selection of self-reactive B cells [126, 127]. SLE patients respond strongly to estrogen when compared to healthy controls. Estrogen treatment of PBMC has demonstrated elevated levels of anti-dsDNA antibodies produced by SLE B cells when compared to healthy controls. However, testosterone treatment has an antagonistic effect as they block the production of autoantibody [128]. Performing ovariectomy in mouse models resembling human lupus before attaining their puberty reduced the symptoms of the disease. However, orchiectomy of male mice accelerated the disease onset in these mice [129].

Toll like receptors (TLRs) 7, 8 and 9 are X chromosome linked genes that contribute to the development of SLE by driving the production of anti-dsDNA or anti RNA antibodies. These are

endosomal receptors which act as intracellular nucleic acid sensors. Deficiency in one copy of the X- linked TLR8 reduced the SLE symptoms in female mice making them comparable to level observed in their male counterparts [130].

Another aspect that contributes to the differences in males and females is the difference in microbiota and the inflammatory environment within the gut. The disease symptoms are reduced in female mice maintained under a germ free condition. In addition to that pro inflammatory response that is generated in the gut mucosa contributes to the progression of lupus. Upon comparison with males, female SLE mice showed higher number of plasma cells in the gut along with increased levels of pro inflammatory cytokines. This difference was detected long before the start of puberty suggesting an estrogen independent effect contributing to the sex bias [131-133].

1.9.3.4 Mouse models of SLE

Mouse models were developed to understand the genetic and cellular contribution to the development of SLE. One of the most common spontaneous lupus models includes the F1 hybrid of New Zealand black (NZB) and New Zealand white (NZW) strains which is represented as NZB/W F1. Unlike their parents, the F1 hybrid develops severe lupus like disease resembling lupus patients where they displayed a strong female sex bias. The disease characteristics exhibited by them include splenomegaly, autoantibodies against dsDNA and immune complexes within kidney [134].

Mapping of the genome in these mice has identified several loci within NZB/W F1 that predispose to the disease. Morel et al has identified susceptibility loci Sle1-3 within NZM2410 mice, a strain that was originally derived from NZB/W F1. They have performed a detailed

investigation on the outcome of individual loci on the immune response. Using congenic mice carrying a combination of all three loci is essential for the mice to fully develop lupus characteristics. However, a further validation is required to determine the relevance of these loci in human lupus [135, 136].

Another popular model of lupus is the MRL/lpr strain of mice which unlike NZB/W F1, both male and female are effected significantly and showed a higher mortality rate. The accelerated phenotype exhibited by these mice is due to an autosomal recessive mutation called lymphoproliferation (lpr). This mutation alters transcription of the FAS receptor which is known for its role in apoptosis. Absence of functional FAS receptor on B and T cells drives enhanced proliferation of these cells leading to a lupus like phenotype [137, 138]. Defects in FAS mediated apoptosis in humans leads to autoimmune lymphoproliferative syndrome (ALPS) which shows similarities with lupus but lacks development of glomerulonephritis [139] .

Apart from those spontaneous models of lupus which are caused by genetic factors, this disease can also be induced in mice on exposure to certain environmental agents. Pristane a chemical derived from mineral oil, when administered into normal mice by intraperitoneal injection can induce the production of autoantibodies. In spontaneous lupus model, pristane administration has demonstrated an increase in severity of the disease. Pristane exposure that accelerates apoptosis exposes various autoantigens which are essential to break the tolerance eventually leading to the development of lupus [140, 141].

A number of different gene-targeted mice with introduced mutations in B cell signaling and regulatory genes develop lupus-like disease. Some of these are discussed below.

Lyn -/- mice

LYN is a molecule that belongs to the SRC family of protein tyrosine kinases. They are found to be associated with BCR complex and rapidly activated to play a crucial role in signaling [142-144]. Although LYN is associated with both activatory and inhibitory pathways [145], B cell deficiency in LYN results in a net loss of inhibitory signals leading to hyper responsiveness [146]. Phosphorylating the tyrosine residues of inhibitory receptors such as FcγRIIB by LYN, it recruits inhibitory phosphatases [147]. *Lyn*-/- mice develop progressive autoimmunity resembling lupus. B cells from these mice exhibits an activated phenotype with expression of CD80 and CD86. Cellular abnormalities such as B cell lymphopenia and complete absence of MZ B cells were reported in these animals. Autoreactive antibodies are detected even in 8 weeks old young *Lyn*-/- . As they get older the severity increases and they develop splenomegaly and glomerulonephritis. The loss of multiple inhibitory receptor signaling due to LYN deficiency make the phenotype stronger than those models with a loss of a single inhibitory receptor function [120, 148].

Fcγr2b-/- mice

Fc receptor exhibited by B cells namely, FcγRIIB is known for inhibiting BCR mediated signaling. Upon colligation of these receptors, FcγRIIB promotes the membrane recruitment of phosphatase that mediates down regulation of BCR signaling and thus attenuating B cell activation [149]. Absence of this inhibitory receptor signaling in *Fcγr2b* -/- mice leads to the development of immune complex mediated autoimmune disease resembling lupus. B cells from these mice exhibit loss of peripheral tolerance and generates autoantibodies against chromatin and dsDNA. Other lupus characteristics include immune complex mediated glomerulonephritis

leading to renal failure with nearly 50% of the mice dying before 9 months of age. Although FcγRIIB is exhibited by myeloid cells, the BM marrow chimera approach showed the B cell intrinsic role of *Fcγr2b* -/- in the development of autoimmunity in these mice [124, 150].

Ship -/- mice

SHIP (SH2–containing inositol phosphatase) is an inhibitory membrane phosphatase. The activity of SHIP is predominantly through FcγRIIB. SHIP is expressed in myeloid and lymphoid cells including B cells. The inhibitory role of this phosphatase is mediated by dephosphorylating a membrane lipid PIP3, which is generated by PI3K activation [151-153]. In the absence of this inhibitory signaling from SHIP, B cells exhibited hyper responsiveness to BCR mediated stimulation. These B cells exhibited increased calcium flux and enhanced rate of proliferation in vitro. They also exhibited elevated levels of antibodies in their serum. These mice died prematurely, within 10-12 weeks, which is too young to develop autoantibodies [154]. However, B cell specific deletion of SHIP in mice resulted in lupus like autoimmunity [155, 156].

Pten +/- mice

PTEN (Phosphatase and Tensin Homolog) is a 3' phosphatase that negatively regulates PI3K signaling by dephosphorylating membrane phospholipid PIP3. PTEN is also known as a tumor suppressor and is mutated in different types of cancers. Partial loss of PTEN in mice leads to enhanced PI3K signaling and is associated with the development of autoimmunity. Female *Pten*+/- mice developed more severe disease characteristics much earlier than male mice and died mostly due to glomerulonephritis. These mice developed abnormal GC and produced high levels of autoantibodies against nuclear antigens [157].

1.9.3.5 Cellular abnormalities in SLE

SLE is characterized by production of autoantibodies and development of immune complexes that deposit within the kidneys. The generation of self-reactive B cells arise much prior to the development of clinical symptoms in lupus. The loss of B cell tolerance can occur during early B cell development in the BM. A large portion of the autoreactive B cells are removed at the check point within the BM at the immature B cell stage. Defects within these mechanisms result in high numbers of mature naïve self-reactive B cells circulating in the periphery which could potentially increase the chances of developing autoimmunity [14, 158].

In addition to abnormalities occurring during early B cell stages leading to loss of self-tolerance, autoreactive B cells may also develop within the periphery. Development of spontaneous GC along with high titers of autoantibodies driving systemic inflammation has been detected in lupus patients as well as in mouse models [159, 160]. GCB cells in the process of undergoing somatic mutation as well as antibody class switching; have a high chance of losing tolerance to autoantigens [161, 162]. With advances in the studies using murine models of lupus, investigating these abnormalities has increased our understanding of the disease.

GCB cell activation is tightly regulated within the GC by a balance between pro-survival and pro-apoptotic signals. FAS is a death receptor highly expressed by GCB cells and deficiency of this molecule is another cause of GC dysregulation [163].Generation of autoreactive GC B cells is prevented by selection mechanisms that occur within the GC. Those B cells exhibiting high affinity to the foreign antigen will receive survival signals and are positively selected. These cells eventually differentiate into memory B cells and long lived plasma cells. However, those B cells exhibiting low antigen affinity or developing auto reactivity do not receive the survival

signals and they undergo apoptosis by negative selection. This mechanism is compromised in many lupus prone mouse models as well as in patients. Studies in mice showed that engagement of BCR in the absence of T cell help results in deletion of those B cells within the GC. Mutation that drives excessive activation of GCB cells and escaping the selection is associated with abnormal GC response and autoimmunity in mouse models [164, 165].

Although GC B cells and TFH physically interact within the GC and provide survival support to each other, the cell intrinsic role of B cells driving the TFH cell expansion and generation of autoimmunity has been extensively studied [164, 166]. Elimination of total B cells or GCB cells from autoimmune mice substantially reduces the TFH population. These findings indicate that maintenance of TFH cells require persistent GC B cell interactions. This benefits the TFH cells by activating ICOS and CD28 signaling which is required for their survival [167, 168].

Another characteristic of lupus is that they develop ectopic germinal centers within inflamed tissues. These structures are similar to the GCs but are detected outside lymphoid organs. Hyper responsive B and T cells entering into the sites of chronic inflammation eventually drives the differentiation of FDC and contributes to the development of these ectopic GCs. The plasma cells residing within these structures secrete antibodies and contribute to the disease pathology [169, 170].

Apart from GC abnormalities, another B cell compartment altered during lupus includes MZ B cells. Pathogenicity of MZ B cells has been implicated in certain models of lupus as these B cells can respond faster than follicular B cells to autoantigens. Several lupus models have shown expansion of this subset and secretion of anti-dsDNA [171, 172]. However, expansion of MZ B

cells does not necessarily correlate with the disease as in certain cases this population is drastically reduced [173].

Another innate like B cell subset that exhibits a defect in lupus is the peritoneal B1 cell. Natural antibodies secreted by B1 cells show certain degree of auto reactivity and therefore its abnormal activation contributes to lupus in certain mouse models. Although they do not necessarily play a role in disease induction, they mediate lupus pathogenesis through the production of low affinity antibodies [174]. Depletion of this subset attenuated the disease severity in autoimmune mice [175, 176]. Expansion of B1a population showed a correlation with lupus characteristics in some mouse models as well as in SLE patients [177-179].

ABC (age associated B cells) is a distinct subset of B cell that is prematurely accumulated within young autoimmune mice and showed a female sex bias. It is also detected in autoimmune patients and is associated with autoantibody production. These cells secrete high levels of autoantibodies under in vitro stimulation. ABCs being potent antigen presenting cells may contribute to autoimmunity by activating T cells by presenting self-peptides [180-182].

Abnormalities in non-B cells such as dendritic cells, macrophages and TFH cells are also associated with SLE as they have a direct or indirect role contributing to the disease. Follicular Dendritic cells (FDCs) that reside within the germinal centres, trap antigens for exposing it to the B cells [183, 184]. Apoptotic materials containing self-antigens were found to be displayed by FDCs in a certain group of lupus patients [185]. Impairment of FDC homeostasis leads to autoimmunity. FDCs secrete MFGE8 (milk fat globule EGF factor 8 protein) that bind to the apoptotic GC B cells which enable their phagocytosis and clearance by macrophages. [186, 187]. Defective apoptotic clearance by phagocytes can lead to a severe inflammatory response. This

defect is observed in lupus prone mice as well as in SLE patients [185, 186]. The cytokines derived from DCs and macrophages can drive the activation of low affinity autoreactive B cells. The elevated levels of type 1 interferons which are present in SLE patients and lupus prone mice are mainly derived from those myeloid cells. These interferons play a direct or indirect role in regulating B or T cell peripheral tolerance. Abnormal levels of IFNα in lupus drives the secretion of BAFF in excess by myeloid cells contributing to B cell hyper activation. These interferons can also enhance CD8+ T cell mediated cytotoxicity to generate more apoptotic materials, thus exposing autoantigens [188, 189]. Through enhanced presentation of autoantigens such as DNA, histones and ribonucleoproteins by DCs and macrophages it leads to excessive activation of autoreactive T cells [190].

Another important non-B cell that contributes to the development of lupus is TFH (T follicular helper cells). TFH - GC B cells interactions are crucial in the development of germinal centres and generation of a high affinity antibody response during an infection. Some of the TFH mediated help includes secretion of IL21 and crosslinking of CD40 expressed by GC B cells, together contributing to the development of GC [191]. The pathogenic role of TFH in lupus prone mice has been well established. ICOS signaling being crucial in promoting TFH differentiation, upon dysregulation in mice, results in expansion of the TFH population. This TFH defect contributes to aberrant GC response leading to lupus like syndrome [62]. In humans, circulating TFH is detected in lupus and correlates with disease activity [192]. Targeting TFH cell differentiation or blocking their interactions with GCB can reverse the development of lupus. Some of the approaches that have been found to be effective in mice or humans include blocking ICOS–ICOSL [193] or CD40L-CD40 [194] interactions and using neutralizing antibody against IL21 [195].

1.9.3.6 Development of drugs to treat SLE

Conventional drugs for treatment of lupus like autoimmune disease include NSAID (Non-steroidal anti-inflammatory drug), hydroxychloroquine, corticosteroids and immunosuppressive drugs like cyclophosphamide. Increased toxicity associated with many of these drugs on prolonged usage can lead to greater complications. Therefore better treatment methods are required to minimize the toxicity and to improve efficacy over those standard treatment methods [196].

With improved understanding of the disease, the treatment methods have evolved in the past few years. Discovery of novel and targeted approaches has improved the survival rate of the patients living with the disease. A more specific approach to treat autoimmune disease like lupus is by depleting B cells using monoclonal antibody directed against CD20 on its cell surface. Rituximab is one of those known antibodies used for this treatment. This can mediate killing of B cells by ADCC, complement activation or by antibody driven apoptosis. One of the limitations of rituximab is that, these antibodies can only deplete mature B cells from the periphery without targeting long lived plasma cells or precursor B cells that do not express CD20. In addition to that, peritoneal B cells also exhibit certain degree of resistance to this treatment although they exhibit CD20 [197, 198].

After rituximab treatment and once the drug clears the system, relapse is observed in 50% of patients within a few months as B cell repopulation occurs within the periphery. Loss of B cells in these patients making them increasingly susceptible to infections remains one of the major side effects of this treatment. Heterogeneity is observed between the patients responding to

rituximab as factors such as age, gender and immunological profile vary between individuals [199].

BAFF is one of those factors that contributes to the survival of autoreactive B cells in the periphery. Overexpression of BAFF in transgenic mice exhibited expansion of peripheral B cells and increase in titer of autoantibodies leading to lupus like disease in mice [33] . Neutralization of BAFF reduced the severity of autoimmunity in mice [200]. Blocking BAFF from lupus prone mice treated the disease in these animals [201].

Since there is elevated BAFF levels detected in the serum of patients with SLE, targeting this protein remains an approach to treat individuals with lupus [202]. Human monoclonal antibody directed against BAFF is also known as Belimumab. This is the first drug approved by FDA in more than 50 years for the treatment of lupus [203]. Autoreactive B cells have a greater dependency of BAFF over healthy B cells and therefore becoming more susceptible than the other [204]. However, rituximab target CD20 expressing cells without being able to differentiate between autoreactive and normal B cells. As lupus is a complex disease that exhibit heterogeneity between patients, blockade of BAFF shows variability in efficacy which is a challenge in addition to the high cost of this drug [205].

Apart from those drugs that are currently available for treatment there are additional approaches which are being tested under clinical trial. One of the key pathogenic cytokines in SLE is type 1 interferon (IFN). This includes cytokines such as IFN α that is known to drive activation of autoreactive B cells and promote the secretion of autoantibodies [206]. Genome wide association studies have recognized lupus susceptibility loci in the IFN signaling pathway [207]. Elevated levels of type 1 interferons are detected in the serum of SLE patients [208, 209]. A monoclonal

antibody targeting IFNα or its receptor is under clinical trial and has demonstrated improvement in disease symptoms in lupus patients [210].

To prevent hyper activation of immune cells, small molecule inhibitors that target antigen receptor signaling can be an approach to treat SLE. SYK (Spleen tyrosine Kinase) is a kinase that is recruited to the immune receptors upon activation. They are associated with amplification of the antigen receptor signaling to regulate a diverse array of cellular response. Signaling pathways involving SYK was enhanced within lymphocytes derived from SLE patients [211]. Expression levels of SYK were found to be higher in these cells and are linked to their abnormal cellular activation [212]. Inhibition of SYK showed amelioration of disease symptoms in mice [213] . T cells derived from SLE patients showed reduced intracellular calcium signaling upon inhibiting SYK in vitro [212].

Another approach to target hyper responsive B cells in lupus is by blocking BTK, as they play a crucial role in mediating B cell activation upon BCR crosslinking. Mutation in the BTK gene is associated with complete loss of peripheral B cells and immunoglobulin [89-91]. Autoimmune mice treated with BTK inhibitor reduced levels of anti-ds DNA antibodies and exhibited reduction in glomerulonephritis [214]. As both Syk and Btk inhibition showed beneficial outcome in lupus prone mice, they will be considered as possible candidates for lupus clinical trials in the future. In addition to that, targeting PI3K signaling or metabolic pathways to reverse lupus in mice (discussed later in detail) appears to be a promising approach to treat lupus.

1.10 Signal transduction pathways controlling B cell activation

1.10.1 Major B cell receptors and signaling pathways

B cells can be activated by a variety of receptors at its membrane. These include activation of B cell antigen receptor (BCR), toll like receptors, cytokine receptor, chemokine receptors and costimulatory molecules [215].

CD40 is a costimulatory molecule expressed by antigen presenting cells including B cells. It is a 40-45kDa transmembrane receptor that belongs to the TNFR (tumor necrotic factor receptor) superfamily. Upon crosslinking, the signaling is mediated by recruitment of TRAF (TNF receptor associated factor) activating pathways like PI3K, NFkB and JAK-STAT downstream [216]. CD40-CD40L interactions between B and T cells are essential for mediating the T cell dependent antibody response. Mouse models lacking functional CD40 or CD40L exhibit impairment in GC formation [92, 93].

IL-4R (Interleukin 4 receptor) is one of the cytokine receptors exhibited by B cells. It is a common gamma chain family of receptors which signal through a type 1 receptor complex in immune cells and type 2 in non-immune cells. IL-4R expressed by immune cells is comprised of IL-4Rα subunit and common gamma chain [217, 218]. IL-4 promotes B cell proliferation and survival within the GC. IL-4R can activate PI3K and JAK-STAT signaling upon activation and regulate the B cell response [219]. Both CD40 and IL-4 receptor signaling is essential in mediating class switching of B cells within the GC [220].

BCR is a transmembrane protein complex, which has an antigen binding immunoglobulin structure at the membrane. The heavy chain of this molecule is associated with heterodimeric subunits such as Igα and Igβ (Immunoglobulin α and β) which are linked by disulfide bond. The cytoplasmic tail of these subunits has a portion called ITAM (Immunoreceptor tyrosine based activation motif) that is capable of signaling on BCR crosslinking. Receptor associated Src

family of tyrosine kinases such as LYN, FYN and BLK, phosphorylate the tyrosine residues of ITAM. Phosphorylation of ITAM mediates binding of SH2 domain containing thryrosine kinase SYK to mediate downstream signals for B cell activation [221-223]. Tonic BCR signaling is required for B cell survival and therefore ablation of BCR in mice resulted in loss of peripheral B cells [224, 225].

A wide variety of signaling mechanisms are activated by BCR crosslinking. PI3K pathway is one of the key signaling pathways down stream of BCR. Phosphorylation of AKT downstream of PI3K activation is crucial in mediating B cell function [226]. BCR activation also regulates migration through remodeling of the cytoskeleton by GTPases such as Rac or Rho. PI3K pathway plays a crucial role in controlling the activation of these GTPases [227].

Another pathway activated upon engagement of BCR is calcium signaling. The release of calcium from intracellular stores raises the level of cytosolic calcium that triggers signaling mediated by NFAT, NFkB and MAPK/ERK pathways. Activation of BCR signaling regulates B cell survival, proliferation, migration and metabolism [228]. BCR signaling regulating B cell metabolism will be covered in detail in a later section.

1.10.2 PI 3-kinase structure and mechanisms for activation in B cells

The PI3K pathway remains one of the major signaling pathways required for survival, differentiation and effector function of B cells. PI3K belongs to a family of lipid modifying enzymes which are evolutionarily conserved among species. They are categorized into classes 1, 2 and 3 based on their structure, specificity and regulation. Among those classes of PI3K enzymes, class 1 is shown to be most relevant to immune cells. The class 1 PI3K is heterodimeric proteins constituting regulatory and enzymatic subunits. Unlike the regulatory

subunits which exist in different forms or sizes, the enzymatic portion of class 1 PI3K are always 110kDa sized subunits. The class 1 PI3K can be further categorized into Class 1 A and B. Antigen receptor which has a tyrosine kinase activity is required to activate class 1A PI3K. While Class1B PI3K requires activation through G protein coupled receptors. While p110α, p110β or p110δ subtypes of catalytic subunit comes under class 1A PI3K, P110γ is associated with Class1B PI3K [229, 230].

In B cells, PI3K activation is driven by molecules such as CD19 or BCAP (B-cell PI3K adapter protein) in a SYK dependent manner. The interaction with CD19 or BCAP to PI3K requires its p85 regulatory subunit [231]. Loss of p85 alone or double knock out of CD19 and BCAP in mice significantly abolish PI3K activation, thus mediating impairment in B cell development and function [232, 233]. CD19 being an important component of BCR signaling when hyper expressed results in B cell hyper activation in mice, thus contributing to a substantial increase in antibody titer within the serum [234].

1.10.3 Downstream effectors of PI3K

Membrane lipids generated as a product of PI3K activation are binding sites of several cytoplasmic proteins. This interaction facilitates a transient recruitment of these molecules to the membrane. There are 3 major classes of membrane phosphoinositide binding domains which include PH (Pleckstrin homology) domain, PX domains or FYVE domains. The PX and FYVE domains are associated with class 2 and 3 PI3Ks and participate in endosomes and vesicular trafficking [235, 236]. PH domain proteins are linked to class 1 PI3K activation and thus more relevant to immune cell function. Therefore PH domain proteins are very crucial in regulating

the B cell response on PI3K activation and thus mediate a wide variety of immunological functions [237, 238].

One of the downstream effectors of PI3K signaling is AKT which is otherwise known as protein kinase B (PKB). It is a serine/ threonine kinase targeted to the membrane on PI3K activation via its PH domain. Complete activation of AKT requires phosphorylation of ser473 residues by mTORC2 in the cytosol and Thr308 residues by PDK1 at the membrane [239]. Activated AKT targets a wide range of molecules downstream that controls B cell metabolism, survival, and proliferation and effector functions [230, 240].

B cell activation that is driven by PI3K signaling also mediates initiation of calcium signaling. Membrane recruitment of BTK (bruton's tyrosine kinase) promotes phosphorylation of phospholipase C (PLC). Activation of PLC mediates enzymatic hydrolysis of PI(4,5)P2 to generate second messengers such as soluble IP3 and membrane bound DAG (diacyl glycerol). Release of calcium from intracellular calcium stores is mediated by IP3.This mechanism raise the levels of free $Ca2^{+}$ within the cytosol that mediate activation of transcription factors that regulate B cell proliferation and secretion of cytokines [241, 242].

1.10.4 Impact of PI3K deficiency on B cell development and function

During early B cell development within the BM, PI3K signals derived from pre-BCR are essential for their survival and maturation. Absence of pre-BCR signals shows developmental defects of B cell progenitors within the BM. Genetic approaches to target PI3K by knocking out regulatory subunit P85 [232, 243] or doubly knocking out catalytic subunits such as P110γ and P110δ [244] showed a severe defect in B cell differentiation in mice. This resulted in

accumulation of precursor B cells within the BM and impairment in B cell population in the periphery [243, 245].

PI3K is also essential for peripheral B cell survival, maturation and homing. The tonic survival signal from BCR in the absence of antigen crosslinking is primarily derived from PI3K. P110δ isoform being predominantly expressed in lymphocytes, inactivating its catalytic activity by introducing a point mutation in mice has demonstrated severe impairment in B cell homeostasis and function. In the periphery, B1 cells showed a significant reduction and MZ B cell subsets were almost undetectable. These animals also exhibited lower basal immunoglobulin levels compared to normal mice. PI3K signaling is essential for the development of GC response. Upon immunization of these P110δ mutant mice with a T cell dependent antigen, they were unable to develop GC when compared to WT mice. The B cell from these mice showed reduced AKT phosphorylation upon BCR crosslinking and showed impairment in B cell proliferation in vitro [245].

On the other hand, a gain of function mutation in the gene that encodes the p110δ subunit results in constitutive PI3K signaling leading to a condition called Activated PI3Kδ Syndrome (APDS) in certain individuals. This is an immunodeficiency characterized by B cell lymphopenia and hyper IgM levels due to reduced class switching [246]. Inability to develop an appropriate antibody response makes these individuals highly susceptible to a variety of infectious diseases [246].

Targeting PI3K using small molecule inhibitors for P110δ can be an approach to treat diseases that are driven by PI3K dysregulation in B cells. Compared to Pictilisib (GDC-0941), which is a pan PI3K inhibitor that targets different isoforms of PI3K, Idelalisib (CAL-101) is a selective

inhibitor of P110δ. Treating B cell malignancies using idelalisib is currently under clinical trial. Lupus prone mice exhibiting B cell defect [247] showed improvement in disease symptoms when treated with a P110δ inhibitor [248] .

1.10.5 Regulation of PI3K pathway activity and consequences of its dysregulation in B cells

Down regulation of PI3K signaling involves activation of an inhibitory Fc receptor called FcγRIIB in B cells. The activation of this receptor is essential to maintain B cell homeostasis and prevents the development of autoimmune diseases. Loss of this feedback mechanism can lead to sustained PI3K activation leading to B cell hyper responsiveness. Deficiency in FcγRIIB signaling in mice leads to lupus like disease with abnormal germinal centers and autoantibodies [124]. B cells derived from lupus patients show polymorphisms in this *FcR* gene resulting in its reduced expression on the membrane [123]. Inhibitory function of FcγRIIB is contributed by recruitment of phosphatase SHIP which hydrolyzes one of the PI products of PI3K (further discussed below).

Dysregulation of PI3K feedback mechanism is associated with loss of B cell tolerance in the periphery. Anergy is an important tolerance mechanism disrupted by such a signaling defect. Chronic antigen exposure to BCR desensitizes the receptor activity and attenuates PI3K signaling. In these anergic B cells, upon BCR crosslinking, phosphorylation of signaling chains Igα or Igβ subunits and the proximal kinases are reduced. Such B cells exhibit reduced calcium signaling, and shows impairment in B cell proliferation and expression of activation markers [29, 249, 250].

PI3K activation generates membrane phosphoinositide which act as lipid messengers of this pathway. PI(3,4,5)P3 which is generated by phosphorylation of PI(4,5)P2 by PI3K is required to

mediate downstream signaling [251]. Down regulation of PI3K signaling requires hydrolysis of PI(3,4,5)P3 which is carried out by phosphatases such as PTEN or SHIP [252, 253]. SHIP (Src homology 2 domain containing inositol 5- phosphatase) generates PI(3,4)P2 by dephosphorylating PI(3,4,5)P3 and thus promote dampening of PI3K to attenuate B cell activation [253].

Once BCR is co-ligated with FcγRIIB, it recruits SHIP to the membrane. Membrane localization of SHIP is crucial to mediate its phosphatase activity. Cytoplasmic domain of FcγRIIB namely, ITIM (Immuno receptor tyrosine based inhibitory motif) get phosphorylated at its tyrosine residues upon receptor colligation. SH2 domain of SHIP interacts with the phosphorylated residues of ITIM to mediate SHIP recruitment towards the membrane [253].

This feedback mechanism mediated by SHIP activation prevents inappropriate action of B cells and therefore preventing B cell hyper activation and autoimmunity. Both FcγRIIB and SHIP are linked to the regulation of autoreactive B cells. Loss of SHIP phosphatases in mice leads to enhanced PI3K signaling in B cells and hyper responsiveness [154]. A B cell specific knock out of SHIP has shown to develop autoimmunity in mouse model [156].

Another phosphatase that mediates down regulation of PI3K signaling is PTEN (Phosphatase and tensin homologue deleted on chromosome 10). PTEN is mutated in different types of cancers and therefore they are identified as a tumor suppressor. It is a second messenger activated downstream of PI3K signaling. Being a 3' phosphatase, PTEN hydrolyzes membrane phospholipid PI(3,4,5)P3 by removing the 3rd phosphate at its inositol ring to generate PI(4,5)P2. Loss of PI(3,4,5)P3 prevents AKT phosphorylation at the membrane and thus down regulates B cell activation. In the absence of PTEN activity, PIP3 levels are increased leading to enhanced

AKT phosphorylation. *Pten*+/- mice developed B cell hyperplasia and autoimmunity [157]. However, anergic B cell express high levels of PTEN. B cell specific knock out of PTEN from these B cells reverse the anergy in mice [254].

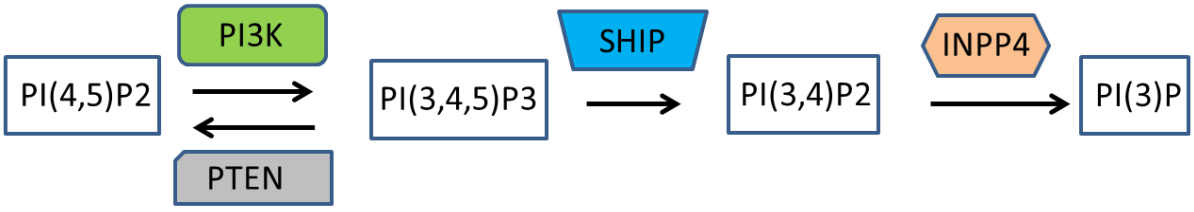

Figure 1.2 - Regulation of membrane phospholipids

Lipid phosphatases controlling membrane phosphoinositides - PI(3,4,5)P3, a product of PI3K activation is dephosphorylated by SHIP which removes the phosphate group from the 5th position of this phospholipid's inositol ring to generate PI(3,4)P2 . PTEN reduces the levels of PI(3,4,5)P3 by hydrolyzing its phosphate at 3rd position to generate PI(4,5)P2 at the membrane. INPP4 (inositol phosphate 4 phosphatase), is a 4' phosphatase that generates PI(3)P by degrading PI(3,4)P2.

1.11 Regulation of cellular metabolism by PI3K

1.11.1 Activation of anabolic metabolism and cell growth by Akt and mTOR

One of the mechanisms by which the PI3K pathway contributes to cellular function is by controlling anabolic metabolism. Synthesis of biomolecules such as lipids, proteins and nucleic acids is regulated by a protein kinase complex called mTORC1 (mammalian target of rapamycin complex1). Approaches targeting AKT can inhibit PI3K mediated activation of mTORC1 signaling [255].

mTORC1 differs from mTORC2 by its composition of protein subunits, enzymatic function and upstream regulation. One of the major differences between the two with regards to PI3K/AKT signaling is that, mTORC2 phosphorylates AKT contributing to its activation [256]. On the other hand, mTORC1 can be activated by an AKT dependent mechanism [255]. Abnormal activation of mTORC1 is associated with B cell cancers [257-259]. Loss of mTORC1 in B cells impairs B cell development and effector functions [260].

mTORC1 signaling mainly relies on the abundance of cellular nutrients such as amino acids, glucose and ATP for its activation. The outcome of this anabolic metabolism facilitated by mTORC1 is an increase in cell growth which is largely contributed by an increase in accumulation of cellular macromolecules. Down regulation of mTORC1 signaling occurs under nutrient limiting conditions, as the cell cannot afford to spend cellular ATP on synthesizing macromolecules [261].

1.11.2 Regulation of catabolic metabolism: glycolysis and mitochondrial respiration

Cellular biosynthesis of macromolecules is a complex process demanding an enormous amount of cellular energy. This requirement of energy is derived from ATP that is generated from catabolic metabolism. ATP is generated from a variety of metabolic pathways which are tightly controlled within the cells. Glycolysis takes place within the cytosol and does not require oxygen to carry out the process. This is one of the quickest but a less efficient pathway to derive energy as the net gain is only 2 ATPs per molecules of glucose. Pyruvate that is generated from glucose can either get converted to lactate or transfer into mitochondria. Under low oxygen tension or hypoxia, pyruvate is not taken for oxidative phosphorylation into the mitochondria. Switching predominantly to glycolysis under such a situation is required to maintain energy homeostasis. As pyruvate generated in this pathway is converted to lactic acid, measuring the extracellular acidification rate remains a reliable technique to measure glycolysis [262]. In lymphocytes as well as in cancer cells, glucose is preferentially converted to lactic acid even in the presence of oxygen and therefore known as aerobic glycolysis or the Warburg effect [263, 264].

The PI3K pathway promotes trafficking of the glucose transporter to the membrane and uptake of glucose into the cell [265]. Activation of hexokinase, a key enzyme in this pathway, is driven by AKT activation [266]. PI3K signaling regulates glucose metabolism by promoting the expression of HIF1α through mTORC1 activation. HIF1α (Hypoxic inducible factor 1) is a transcription factor that regulates adaptive immunity through transcription of genes that encodes mediators of glycolysis. Low oxygen tension that favors the stabilization of HIF1α promotes anaerobic glycolysis to meet the cellular energy demand [267, 268].

One of the mechanisms by which HIF1α positively regulates glycolysis is by promoting transcription of the lactate dehydrogenase (LDH) gene. LDH is an enzyme that mediates the conversion of pyruvate to lactate [269, 270]. In addition to that, pyruvate entry into mitochondria is negatively regulated by HIF1α by promoting the transcription of pyruvate dehydrogenase kinase (PDHK). The presence of increased PDHK activity blocks the enzymatic conversion of pyruvate into Acetyl CoA, thus downregulating mitochondrial respiration [271].

Upon triggering PI3K signaling in lymphocytes, it can also induce expression of another transcription factor MYC. Regulation of metabolic genes by MYC contribute to lymphocyte activation and proliferation. They regulate glycolysis by upregulating GLUT1 expression. In addition to that, pyruvate kinase (PKM2) which is involved in the generation of pyruvate, is transcriptionally regulated by MYC [272, 273].

Unlike glycolysis, aerobic respiration takes place within the mitochondria and requires oxygen. Therefore, oxygen consumption rate is used as a measure for mitochondrial respiration. Within the mitochondria, they oxidize the products of glycolysis such as pyruvate in the process of generating ATPs. Pyruvate that enters into the mitochondrial matrix is enzymatically converted to acetyl-CoA and feeds into the Krebs cycle. The second phase of mitochondrial respiration takes place within the inner mitochondrial membrane. The electron transport chain (ETC) leading to generation of membrane potential is essential for ATP synthesis. This oxygen dependent mechanism is together called oxidative phosphorylation. Mitochondrial respiration makes an efficient way of energy production with 34 ATPs synthesized per molecule of glucose [262].

One of the mechanisms by which PI3K activation regulates mitochondrial respiration is through MYC. Mitochondrial biogenesis and oxidative phosphorylation are positively regulated by MYC [274] . They promote transcription of glutaminase, an enzyme that contributes to the production of a Krebs cycle intermediate [275]. Deficiency of MYC in B or T lymphocytes causes impairment in glycolysis and mitochondrial respiration [272, 276].

These pathways are not only essential for ATP production but also provide various precursors molecules essential for different biosynthetic pathways. Intermediates of glycolysis such as Glucose-6- phosphate can enter into pentose phosphate pathway contributing to the synthesis of nucleic acids [277, 278]. NADPH derived from glycolysis can act as a cofactor in lipid biosynthesis. Citrate produced in Krebs cycle within the mitochondria can also participate in the production of fatty acids and cholesterol [278, 279]. Likewise, intermediates from other pathways apart from pyruvate can also feed into the Krebs cycle such as glutamate or fatty acids. Therefore pathways of biosynthesis and cellular energy production are interdependent [278].

1.11.3 Pharmaceutical targeting of catabolic metabolism

Those cells exhibiting a unique metabolic profile can be targeted by blocking specific pathways of metabolism. Cells that are highly reliant upon glycolysis are more sensitive to its inhibition and therefore are selectively killed. Small molecule inhibitors to target glycolysis can lead to impairment in ATP generation within the cell. Glucose being an important carbon source for anabolic metabolism it will also affect cell growth and proliferation [280, 281].

One of the commonly used inhibitors of glycolysis is 2-DG. This molecule has a glucose structure with the 2nd hydroxyl group replaced by hydrogen. The altered structure of this molecule does not affect its uptake ability across the cell by glucose transporters. As glucose

transport is bi-directional, a rate limiting glycolytic enzyme namely hexokinase converts glucose into glucose-6 phosphate (G6P) thus trapping the molecule within the cell. However, in the presence of 2-DG, they get converted to 2- deoxy-glucose-6-phosphate (2-DG-6-P) instead. This 2-DG-6-P that cannot be further metabolized and is trapped within the cell, inhibits hexokinase in a non-competitive manner. In addition to that, phosphoglucose isomerase (PGI) which is a downstream glycolytic enzyme that utilizes G6P as its substrate is inhibited in a competitive manner by 2-DG-6-P [282-284].

Another crucial mechanism that regulates glycolysis is the enzymatic conversion of pyruvate that is generated during glycolysis, to a Krebs cycle intermediate by pyruvate dehydrogenase (PDH). This prevents the conversion of pyruvate into lactate and thus down regulating glycolysis. PDHK (pyruvate dehydrogenase kinase) is an inhibitor of mitochondrial PDH that downregulates its activity. DCA (Dichloroacetic acid) is a small molecule inhibitor of glycolysis which works by inhibiting PDHK and therefore enabling PDH activity. Apart from the inhibitory effect on glycolysis, by switching to mitochondrial respiration these cells become more susceptible to cell death due to the production of more pro-apoptotic mediators including ROS through mitochondrial activity [285, 286]. Both 2-DG and DCA are currently tested in clinical trials to develop a cure for various types of cancers [287].

Mitochondrial respiration on the other hand can be targeted by blocking components of the electron transport chain. A clinically relevant drug which is being tested as a mitochondrial inhibitor is Metformin. It is an ETC complex 1 protein inhibitor which is currently used in the treatment of certain diseases and in the clinical trial for cancer. Lupus prone mice treated with a combination of 2-DG and metformin to target glycolysis and mitochondrial respiration

respectively reverse lupus in mice [288]. Therefore the approaches to target metabolic pathways can potentially be taken forward for clinical trials in developing treatment for lupus.

1.12 Metabolic regulation of Immune cells

Metabolic programming is essential in regulating immune activation and homeostasis of these cells. Defects in these regulatory mechanisms can lead to diseases of the immune system such as autoimmunity or immunodeficiency. Immune cells which are under a state of rest or anergy have low metabolic requirements. However, on activation they reprogram their metabolic pathways to satisfy the energy and biosynthetic demands to generate an appropriate immune response. The metabolic requirements may vary depending upon the type of immune cell and their status of activation. As metabolic regulation of immune cells is required for their effector function, targeting metabolic pathways can potentially be useful in the treatment of various immune related diseases [289].

1.12.1 Metabolic reprogramming in T cells

$CD4^+$ T lymphocytes when activated can switch to glycolysis to mediate their effector function which otherwise favors mitochondrial respiration at the naïve state for their survival. $CD4^+$ T cells when stimulated in vitro exhibited an increased rate of GLUT1 expression and glycolysis with a reduced rate of beta oxidation of fatty acids [290-292]. Deprivation of glucose or use of a glycolysis inhibitor suppresses the activation of these cells and inhibits the production of cytokines such as IFN-γ which is a key in mediating a T cell response [293].

Enhanced glycolysis can give rise to hyper responsiveness as seen in GLUT1 transgenic $CD4^+$ T cells. These mice exhibited autoimmune characteristics with age [291, 294]. This metabolic

behavior of activated $CD4^+$ T cells to rely on aerobic glycolysis resembles of that of a cancer cell, where faster production of ATP is preferred to fulfil the energy demands of the rapidly responding cell. Glucose-6-phosphate, a glycolysis intermediate that feeds into the pentose phosphate pathway for the generation of nucleic acid will be rapidly elevated by switching to glycolysis and thus becomes beneficial for an actively proliferating cell [292, 295].

Immune cells exhibit metabolic heterogeneity to meet their specific requirements. Activated $CD4^+$ T cells that differentiate into various subsets depending on the cytokine milieu have distinct metabolic requirements. Although inflammatory effectors such as TH1 or TH17 subsets cells rely on glycolysis, the immunosuppressive T regs require mitochondrial respiration for their function. The long lived memory T cells rely upon oxidative phosphorylation and suppress lipid oxidation under resting condition. Once triggered, these cells respond faster by switching to fatty acid oxidation and thus responding faster than naïve T cells [294, 296, 297].

1.12.2 Metabolic reprogramming in B cells

Metabolic regulation of B cells is crucial for its homeostatic regulation and generation of effector responses such as proliferation and antibody production. Recognizing the metabolic targets of B cells and exposing their vulnerabilities can potentially help in the development of drugs against B cell mediated autoimmune diseases [298]. However, the mechanisms regulating B cell metabolism is not well understood. Metabolic phenotyping of activated B cells has demonstrated that B cells exhibit a balanced increase in both glycolysis and mitochondrial respiration on BCR crosslinking which is quite distinct from those observed in $CD4^+$ T cells. Although both the pathways seem to be elevated in B cells, glycolysis plays an important role in the production of antibodies. Targeting glycolysis with drugs such as 2-DG or DCA significantly suppressed B cell

proliferation and antibody production. B cell specific deletion of GLUT1 resulted in impaired B cell numbers or antibody production [276].

PI3K signaling can determine the activation status of B cells by regulating metabolic pathways such as glycolysis and mitochondrial respiration [298]. GLUT1 expression is a PI3K dependent mechanism in B cells. Inhibition of PI3K signaling by BCR and FcγRIIB colligation suppresses glycolysis in these cells [265]. Sustained AKT activation is sufficient to increase glucose utilization in B cells. Anergic B cells with reduced activation of PI3K are metabolically quiescent and their ability to produce antibody is impaired. However, chronically active B cells exhibit an enhanced rate of metabolism which corresponds to sustained PI3K activity in these cells [276, 299, 300].

Loss of B cell tolerance mechanisms can lead to SLE like autoimmune disease. Dysregulation of PI3K signaling in B cells may be one of the major contributors [299, 300]. A vast majority of lupus patients exhibit increased levels of a cytokine called BAFF [202]. As BAFF can trigger PI3K signaling in these cells, a sustained exposure to this cytokine remains one of the reasons for B cells being able to override tolerance mechanisms in these individuals. Mouse models of lupus have shown that enhanced PI3K signaling is associated with an increase in glucose uptake within these autoreactive B cells [33, 299-301].

Metabolic heterogeneity among different B cell subsets is not entirely understood. However, unlike non GC B cells, the light zone compartment of GC is a hypoxic and relies on glycolysis to meet the energy requirements. This loss of oxygen tension is required to stabilize HIF1α which suppresses B cell proliferation and antibody class switching within the light zone compartment. The HIF1α mediated inhibition of AID gene expression is achieved by suppressing mTORC1

signaling. These restrictions created by hypoxia within light zone give more priority to the selection over proliferation. Those B cells selected from this light zone are then allowed to proceed to the dark zone for further expansion [302]. Expression of MYC transcription factor is induced by T cell dependent activation in light zone B cells. As the light zone B cells are highly glycolysis dependent, it is tempting to speculate a possible MYC dependent mechanism in controlling GC B cell metabolism, by increasing GLUT1 expression [302, 303]. When compared to MYC deficient GCB, MYC sufficient GCB undergo a greater degree of affinity enhancing mutations, consistent with this idea [304, 305].

1.13 PH domain proteins in the regulation of PI3K signaling

Pleckstrin-homology (PH) is a small protein domain containing nearly 120 amino acid residues. This domain was first identified in the early 90s where it was found to be associated with a protein called pleckstrin. Although it is present in a wide variety of signaling molecules, the PH domain primary structure exhibits low sequence similarity they exhibited a highly conserved 3D structure among them [306].

PH domains are diverse in terms of affinity and specificity towards the membrane phosphoinositide. This interaction helps them in transiently recruiting the PH containing proteins towards the membrane. An electrostatic interaction between negatively charged inositol phosphates on the membrane with positively charged amino acid residues within the PH domain is crucial for this interaction. The membrane binding pocket of the PH domain mainly constitutes positively charged amino acids such as lysine, arginine and histidine [307].

Membrane localization of a variety of PH domain proteins is triggered upon PI3K activation. Some of these proteins that bind to the lipid products of PI3K includes kinases such as AKT,

PDK1 and BTK, adapter proteins such as Bam32 and TAPPs and guanine nucleotide exchange factor like Vav. Unlike BTK which are selective PI(3,4,5)P3 binders, the binding affinity of PDK1, AKT and Bam32 remains similar towards PI(3,4,5)P3 and PI(3,4)P2 and others such as TAPPs are strictly PI(3,4)P2 binders [308-312] . Identifying the role of PH proteins will provide us with more insights on how the PI3K pathway is regulated and thus contributing to the development of novel approaches to deal with B cell related diseases. Below I will focus my discussion on adaptor proteins and particularly TAPPs which are relevant topic of my thesis.

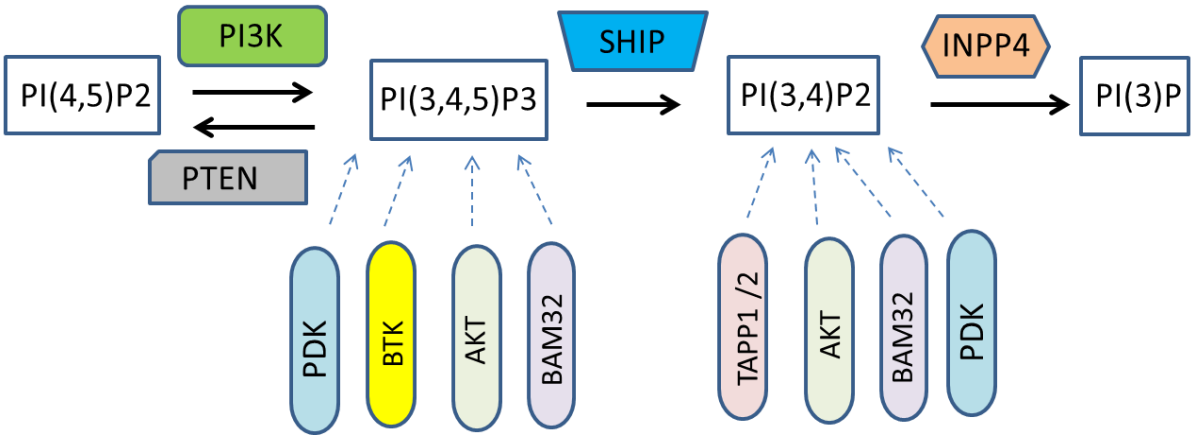

Figure 1.3 – Phosphoinositide binding proteins

PI(3,4,5)P3 and PI(3,4)P2 generated during PI3K signaling recruits various PH domain proteins to the membrane. PDK, BAM32 and AKT have similar affinity for both the lipids while BTK selectively bind to PI(3,4,5)P3. TAPPs on the other hand are strictly PI(3,4)P2 binding proteins and require their presence for its membrane translocation.

1.13.1 Role of Bam32 in GCB cell response

Bam32 (B lymphocyte adapter molecule of 32 kDa) is another membrane phosphoinositide binding adapter protein which is also known as DAPP1 [313]. Its expression is the highest in B cells when compared to other immune cells. They play a crucial role in B cell function downstream of PI3K signaling. Some of the major functions of Bam32 are involved in the regulation of signaling molecules associated with B cell survival, adhesion and cytoskeletal rearrangement [314, 315].

Bam32-/- B cells show impairment in antigen presentation to T cells due to their inability to form conjugates between the cells. The loss of cell adhesion along with defects in cytoskeletal rearrangement contribute to this defect. This abnormality in antigen presentation can be reversed in the presence of T cell dependent stimulation such as anti-CD40 and IL-4 in the culture. Bam32 is a protein highly expressed in GCB cells. Although, the absence of Bam32 showed no alterations in the initiation of germinal center in mice on immunization, there was a rapid decrease in size of the GC over time when compared to normal GC. This defect in maintaining the GC is due to their inability to form GCB-TFH conjugates which trigger important signals that are essential for GCB cell survival [314, 316].

1.13.2 Understanding the role of TAPPs in B cell signaling

Generation of PI(3,4)P2, which is a product of SHIP activity, is associated with a feedback role that dampens PI3K signaling. However, this PI(3,4)P2 dependent mechanism remained unclear for a long time [152]. TAPPs are Tandem PH domain containing proteins that interact with PI(3,4)P2 which is essential for its recruitment from the cytosol to the plasma membrane [317-

319]. The localization of TAPPs to the membrane happens in a PI3K dependent manner and can be disrupted by using pharmacological inhibitors of PI3K [320].

1.13.2.1 TAPP gene and protein structure

TAPP genes are found to be expressed in a variety of vertebrates that includes mice, rats and humans but are not detected in invertebrates [320]. TAPP1 and TAPP2 are derived from genes, PLEKHA1 and PLEKHA2 which are located in separate chromosomes (10 and 8 respectively) [319]. TAPP1 and TAPP2 isoforms are found to be expressed in a wide variety of tissues. However, TAPP2 is found to be more abundantly expressed in lymphoid tissues than TAPP1 [319, 320] .

TAPPs are adapter proteins that share a lot of characteristics similar to Bam32. Both are small sized adapter proteins where TAPPs are 47kDa and Bam32 is 32kDa molecules. These proteins lack enzymatic activity, but play important roles in PI3K signaling. Although TAPPs are exclusively PI(3,4)P2 binders, Bam32 on that other hand can interact with PI(3,4)P2 and PI(3,4,5)P3 with similar affinity. Unlike TAPP adapter proteins which possess two PH domains, one at the C-terminus and the second at the N-terminus, Bam32 has only one PH domain at the C-terminus. Although the N-terminal domain is highly conserved between TAPP1 and TAPP2 and also among the species, the C-terminal domain of TAPPs has less homology between the isoforms. However, the C-terminal PH domain of TAPPs has sequence homology with the PH domain of Bam32 [310, 314, 317-320].

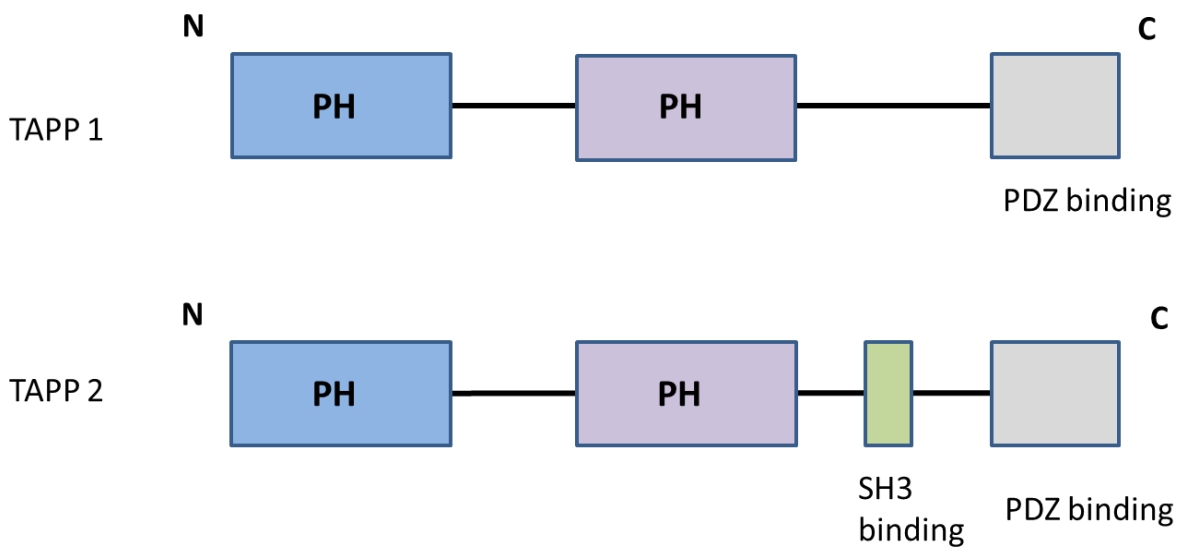

Figure 1.4 - Structure of TAPP adapter proteins

The C-terminal PH domains of TAPP1 and TAPP2 selectively bind to PI(3,4)P2 generated at the membrane. However, role of the N-terminal PH domain is not clear. Both TAPP1 and TAPP2 have a PDZ binding domain at the C-terminus that interacts with other proteins. Unlike the C-terminal domain, the N-terminus is highly conserved between TAPP1 and TAPP2.

1.13.2.2 TAPP recruitment to the plasma membrane

Protein lipid overlay and SPR (Surface Plasmon Resonance) analysis has shown that the C-terminal PH domain of TAPP can interact with PI(3,4)P2 while the N-terminal PH domain cannot. The TAPP membrane binding is facilitated by the interaction between the basic residues within the C-terminal PH domain with that of the 3rd and the 4th phosphate group of PI(3,4)P2. The presence of an alanine residue within the C-terminal PH domain that is close to the 5th phosphate position in PI(3,4,5)P3 causes a stearic hindrance resulting in its low binding affinity. However, replacing this alanine residue with glycine can allow its binding with both PI(3,4)P2 and PI(3,4,5)P3 [310, 319, 321]. Mutating the PH domain by replacing the conserved arginine residues of TAPP1 or TAPP2 with leucine residues has inhibited TAPP membrane localization in BJAB cells [320].

TAPP membrane recruitment kinetics was investigated in a PH domain transfected B cell line called BJAB which is stimulated by BCR crosslinking to trigger PI3K signaling. This study demonstrated a slow and sustained membrane recruitment kinetics of TAPP C-terminal PH domain, that corresponds to the synthesis of PI(3,4)P2 and not PI(3,4,5)P3. In contrast, BTK exhibited a rapid localization to the membrane followed by a faster return to the cytosol, closely corresponds to the PI(3,4,5)P3 synthesis and degradation at the membrane. This study showed distinct binding preferences among PH domain containing proteins and therefore indicating their diversity in cellular function [320].

H_2O_2 (hydrogen peroxide) stimulation that can selectively upregulate PI(3,4)P2 shows a corresponding increase in TAPPs at the plasma membrane in BJAB cells [322]. The endogenous ROS (reactive oxygen species) which are generated in B cells during an inflammatory response

are essential for amplification of BCR signaling [323]. A defect in peroxide catabolizing enzymes was shown to exhibit enhanced AKT phosphorylation and immune hyper responsiveness [324, 325] . This preferentially induced PH proteins that are PI(3,4)P2 binders and not those like BTK which are exclusively PI(3,4,5)P3 binding protein. This peroxide induced recruitment of TAPPs is entirely dependent on Class1A PI3K. Blocking the enzymes involved in the production of superoxide prevented the BCR induced localization of TAPPs [322, 326].

The membrane association of TAPPs is regulated by lipid phosphatases such as SHIP, PTEN and INPP4 (Inositol polyphosphate 4-phosphatase) which are known to alter the PI(3,4)P2 dynamics at the membrane [152, 252, 327]. Co-ligation of BCR with FcγRIIB in B cells inhibited recruitment of BTK, due to the depletion of PI(3,4,5)P3. However, TAPP recruitment was not inhibited but instead was elevated at the membrane due to the increase in levels of PI(3,4)P2. Similarly, over expression of SHIP prevents BTK recruitment without inhibiting the translocation of TAPPs to the membrane [328] .

In contrast, an increase in PTEN activity that hydrolyze PI(3,4,5)P3 to generate PI(4,5)P2 can restrict TAPP localization to the membrane due to reduced PI(3,4)P2 levels. As PTEN is susceptible to peroxide, its inhibitory effect on TAPP membrane localization can thus be reversed by targeting them. Therefore enhanced TAPP membrane translocation in the presence of peroxide is contributed in part by PTEN inactivation where the SHIP activity remains unaffected [322]. Loss of SHIP in these peroxide treated cells, elevate the levels of PIP3 as either of the phosphatase activity remains functional [329]. A third phosphatase that is known to regulate TAPP membrane translocation is INPP4A. PI(3,4)P2 being a substrate for this enzyme is broken down to PI(3)P. INPP4A overexpression leaves TAPPs with significantly reduced levels of PI(3,4)P2 available for binding even on PI3K activation [330].

1.13.2.3 TAPP interactions with other proteins

TAPPs possess a PDZ binding domain at its C-terminus which is known for its role in protein binding. This suggests that TAPPs might have other biological functions apart from their role at the membrane. One of the TAPP1 binding proteins is MUPP1 (Multi PDZ domain protein1) which has a role in tight junctions of some non-immune cells but its role in B cells has not been understood [318, 331].

While investigating the role of TAPPs in PI3K signaling, it was demonstrated that knock down of TAPPs in HEK 293 cells upregulates AKT phosphorylation, suggesting their role in inhibition of PI3K signaling. It was identified that a PDZ domain containing phosphatase, PTPL1 interacts with both TAPP1 and TAPP2 and was found to inhibit phosphorylation of AKT [317]. PTPL1 expression was shown in T lymphocytes but its absence from these cells resulted in enhanced activation and differentiation [332]. Therefore it is tempting to speculate that TAPPs mediate inhibition of B cell signaling by associating with PTPL1. However the role of PTPL1 in B cells is yet to be investigated.

Previous studies in our lab aimed at identifying the binding partners of TAPPs, used immunoprecipitation from lysates of B cells transfected with TAPP2 and activated by BCR crosslinking. Nearly 40 different proteins were identified by mass spectrometry that could potentially be interacting with TAPP2. This included proteins involved in cytoskeletal rearrangement, protein trafficking and signal transduction. Among those molecules, cytoskeletal proteins such as utrophin and syntrophin were confirmed to interact with TAPP2 [333]. Another study demonstrated that TAPP1 co-localized with syntrophin using their PDZ domain [334]. These findings remain consistent with the observation in BJAB cells, were TAPPs are found to

be accumulated in the F actin rich areas within the membrane ruffles, indicating its association with cytoskeletal proteins [320].

1.13.2.4. TAPP2 functions in B cell adhesion and migration

Upon BCR crosslinking, B cells showed an increase in adhesion to extracellular matrix (ECM) that was blocked in the presence of PI3K inhibitors. B cells were unable to attach to the ECM when TAPP2 was knocked down. However, overexpressing TAPP2 in B cells enhanced the adhesion but not when its C- terminal PH domain was mutated. While TAPP1 knockdown alone showed a smaller reduction in adhesion, a greater effect was observed in the absence of both TAPPs. When utrophin, a TAPP binding protein is knocked down, adhesion was impaired suggesting that TAPP mediated adhesion to matrix could potentially require its binding to this protein [333].

Interaction of cells to ECM is known to promote cell motility and cellular chemotaxis. PI3K activation is known to regulate migration of cells by establishing cell polarity and by sensing chemotactic gradient. TAPP2 being the isoform predominantly expressed in B cell leukemia cells which is known for its high migratory potential, its role in cell migration was also explored. In the absence of TAPP2, impairment in cell polarity and cytoskeletal rearrangement was observed. The speed and directionality of B cells were reduced in the absence of TAPP2. One of characteristics of the leukemic B cells is that they infiltrate into the BM where they interact with the stromal cells to obtain survival signals. In the absence of TAPP2, migration of these cells into BM stromal layers was impaired [335].

1.14 TAPP KI mouse model

To further understand the role of TAPP in B cell function we collaborated with Alessi's group who provided us with the mice with a germline mutation in its C-terminal PH domain. TAPP1/2 knock-in mice was generated by mutating the C-terminal PH domain which is critical for its interaction with PI(3,4)P2. Arginine residues within this binding pocket of PH domain are essential for this binding. Arg^{211} in TAPP1 and Arg^{218} in TAPP2 are mutated to leucine to abolish this interaction. The mutation with TAPPs only uncoupled it from the membrane without altering the expression of both TAPP1 and TAPP2. Embryonic stem cells separately transfected with a construct of mutant TAPP1 or TAPP2 were screened to confirm vector insertion before injecting into the blastocyst. $TAPP1^{R211L/\ R211L}$ and TAPP2 $^{R218L/\ R218L}$ mice that were separately developed by this method were maintained on a C57BL/6 background. The two genotypes were then crossed with each other to develop $TAPP1^{R211L/\ R211L}$ x TAPP2 $^{R218L/\ R218L}$ mice which were born in a Mendelian ratio [336].

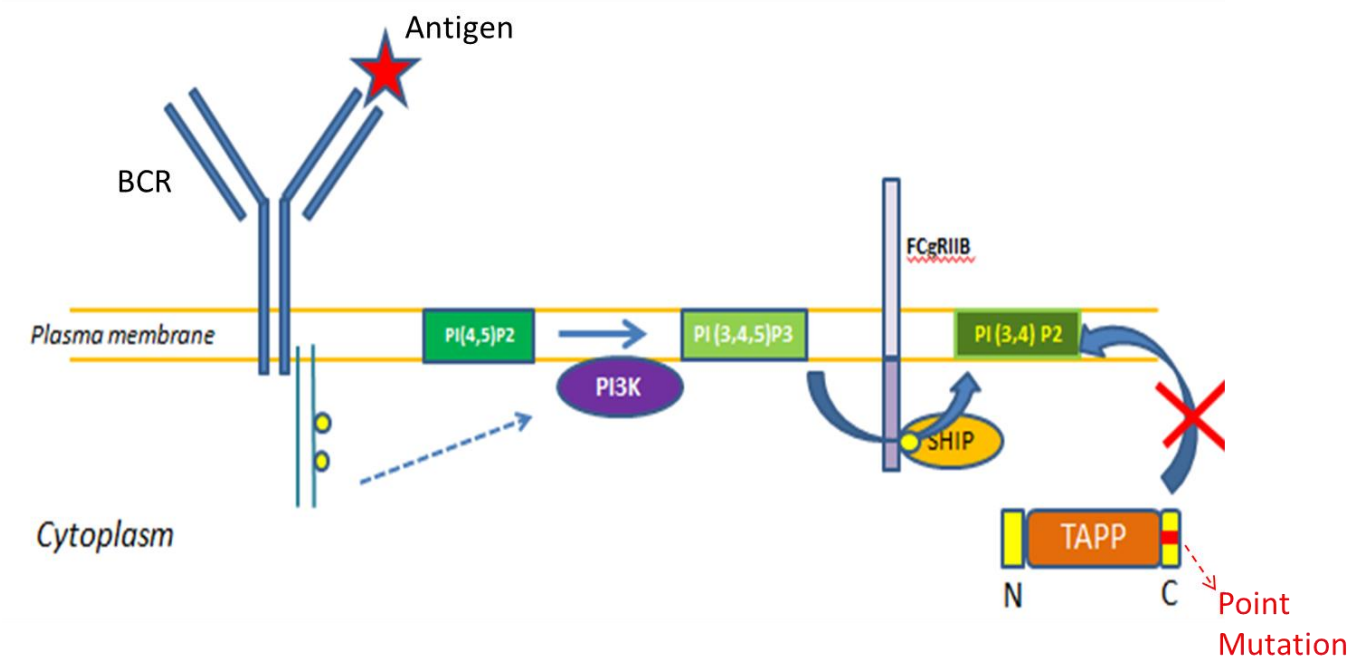

Figure 1.5 – TAPP 1/2 KI mouse model

A point mutation was introduced within the C-terminal PH domain of TAPP1 and TAPP2. This prevents TAPPs from translocating to the membrane as they can no longer bind to PI(3,4)P2. However, this mutation did not affect the expression of TAPPs within the cell.

1.15 Rationale of the thesis, central hypotheses and specific questions

An initial characterization of TAPP KI mice were carried out prior to starting my thesis project. The characteristics observed in TAPP KI mice were strikingly similar to that of *Ship-/-* mice. For example, in both cases PI3K signaling was dysregulated and they exhibited B cell hyper responsiveness and hypergammaglobulinemia in young mice [337, 338]. A B cell specific knockout of SHIP resulted in the development of lupus like characteristics in these animals [156]. FcγRIIB which is an inhibitory receptor in B cells, functions in part through SHIP activation. Polymorphism in the *fcgr2b* gene is associated with the development of autoimmunity in humans and mouse models [124, 339]. **We thus hypothesized that TAPP KI mice would develop lupus-like disease with age.**

One of the characteristics of lupus is the development of spontaneous germinal centres [160]. The PI3K pathway is critical for the development and functioning of GCs [124]. TAPPs are PI(3,4)P2 binding proteins downstream of SHIP activation contributing to the feedback regulation of PI3K signaling. When TAPPs are dissociated from the membrane, enhanced PI3K activity and B cell hyper activation was observed in vitro [340]. **We thus hypothesized that TAPP KI mice would have dysregulated B cell activation and abnormal GC in in vivo, contributing to development of autoimmunity.**

In vitro stimulation of TAPP KI B cells by BCR crosslinking exhibited enhanced PI3K signaling and increased proliferation [340]. However, PI3K dependent mechanisms driving this B cell hyper activation are not well understood. There is evidence indicating that PI3K signaling defect is associated with B cell metabolic abnormalities contributing to the hyper-responsiveness [265]. Blocking PI3K signaling or metabolic pathways has reversed B cell hyper activation [276, 340].

We thus hypothesized that TAPP KI B cells have defective control of metabolic pathways underlying their hyper-responsiveness to stimulation.

Therefore our central hypothesis is that, **uncoupling of TAPPs from the membrane leads to pathological dysregulation of B cell function in TAPP KI mice.**

Specific questions

4. Do TAPP KI mice develop autoimmunity and are chronic germinal centers linked to this?
5. Do TAPP KI mice exhibit a B cell intrinsic defect contributing to these abnormalities?
6. Is there a PI3K dependent metabolic abnormality driving hyper activation of TAPP KI B cells?

Chapter 2

Materials and methods

2.1 Mice and immunizations

TAPP1$^{R211L/\ R211L}$ and TAPP2 $^{R218L/\ R218L}$ mice were generated as described by Wullschleger et al. [336]. TAPP KI mice were bred and maintained on C57BL/6 background along with their littermate controls. A cohort of mice was aged up to a period of 65 weeks to study the development of autoimmunity in these animals. P110δ mutant mice (*P110δ* $^{D910A/\ D910A}$) that has a mutation in the P110δ domain to inactivate its PI3K activity was used as a negative control in some of the experiments (will be indicated as P110d in the figure panels). TAPP KI mice were crossed with *Icos*-/- mice resulting in the *Icos*+/- TAPP KI or *Icos*-/- TAPP KI were used to determine the role of TAPP KI GC in autoimmunity. μMT is a B cell deficient model which was used for the BM chimera experiment to study the B cell intrinsic role of TAPPs. *Icos*-/- mice and μMT were purchased from Jackson Laboratory. To assess the acute GC development, young adult mice were intraperitoneally injected with 200μl of sheep red blood cells (Cedarlane#CL2581-100A) with approximately $2x10^9$ cells per mouse. Most experiments used young mice between 8-12 weeks except for those involving aging mice where the ages were specified. All these animals were housed in a pathogen free facility according to the guidelines of the Canadian Council on Animal Care.

2.2 Ig Isotype ELISA

Blood was collected by tail vein puncture from WT and TAPP KI mice every five weeks while they are aging and by cardiac puncture at the end point. Serum was collected from blood, after

letting it to coagulate at room temperature for 30 mins, and centrifuged at a 4000 rpm for 10 minutes. The supernatant was carefully collected and stored at -20^{0}C. To measure total Igs in the serum, ELISA plates were coated with capture antibody (anti-mouse IgM or IgG from Jackson ImmunoResearch Laboratories) diluted in carbonate buffer and incubated overnight at 4^{0}C. These plates are then incubated in blocking buffer (2% BSA in washing buffer) for 2 hrs at room temperature (RT). Standards (starting at 20ng/ml) as well as serum samples were serially diluted and added to these wells which were incubated for 2 hrs at RT or 4^{0}C overnight. The detection antibody which is a biotinylated anti-mouse IgM or IgG isotype was added to this plate for 1 hr at room temperature (RT). This is followed by addition of streptavidin alkaline phosphatase to these wells for 1 hr at RT. P-nitrophenyl phosphate tablets (Sigma Aldrich) are dissolved in substrate buffer and added to these wells which turn yellow in a few minutes. Each of these steps mentioned above is followed by washing the plate with wash buffer (PBS, 0.05% Tween 20, 0.02% azide. Using a Molecular devices plate reader, the absorption is measured between 405 and 690nm.

2.3 Anti-dsDNA antibody ELISA

Anti-dsDNA antibodies (IgG or IgM isotypes) in the mouse serum were tested using a kit from Alpha Diagnostics International (Catalogue #5120). Serum was diluted at 1:100 in sample diluent and then added as duplicates into the dsDNA coated wells along with the calibration controls provided with the kit and incubated for 1 hr. The wells were washed and HRP conjugated IgG was added for detection and incubated for 30 mins. A blue color developed after addition of TMB substrate as a result of enzymatic reaction was stopped by adding stop solution. The absorbance was measured between wavelengths of 450-630nm using Molecular Devices plate reader.

2.4 Flow cytometry

Blood, spleen, lymph node and peritoneal cells were obtained from aged TAPP KI mice. Spleen and lymph nodes were homogenized into single cell suspensions. Blood was collected in heparin coated tubes (purchased from Sarstedt) to prevent coagulation. Ack lysis buffer was used to deplete RBC in splenocytes or blood for 2 mins on ice. Cells were then filtered, re-suspended and counted using a hemocytometer. $2x10^6$ cells were then distributed into each FACS tube. Prior to staining, the Fc receptors were blocked for 20 mins by incubating with 2.4G2 monoclonal antibody. The cells were washed with FACS buffer (2% FCS in PBS) and surface stained with fluorescently labelled rat anti-mouse antibodies such as anti-B220-PercepCy5.5, anti-CD4–V500, anti-CD19–Pacific blue, anti-CD21–FITC, anti-CD23–PE, anti-GL7-FITC, anti-FAS- PECy7, anti-CD5–APC, anti-ICOS–APC, anti-PDI-PECy7 all from BD biosciences. The cells were stained for 15 mins, washed and re- suspended in FACS buffer for acquisition. GLUT1 expression in different B cell subsets ex vivo required surface staining as before followed by fixing and permeabilization of the cell using Fix perm buffer (eBioscience). The cells were blocked with normal rat serum for 15 mins and were stained with anti-GLUT1-Alexa 647 (1:1000 dilution in FACS buffer) for 30 mins at room temperature. After washing and re-suspending the cells, they were assessed using a BD FACS Canto II instrument. For multicolor staining appropriate compensation controls were used to adjust the voltage for each fluorochrome before acquiring the samples. The data obtained from this machine was analyzed using FlowJo software.

2.5 Anti-nuclear antibody staining

Serum isolated from the peripheral blood as described above is tested for antinuclear antibody. HEp-2 coated slides (from Biorad) were blocked for 1 hr in normal horse serum prior to sample

addition. The serum samples diluted at 1:40 in PBS were incubated over these cells for 2 hrs at RT or 4^0C overnight. The slides were washed in PBST (0.01% tween in PBS) and then incubated with secondary antibody which is rabbit anti-mouse IgG-Alexa 488 (1:2000 dilution) for 30 mins. After washing, the slide is mounted using anti-fade reagent (Molecular probes) and the coverslip is properly sealed and stored at -20^0C. Imaging was done using an AxioObserver spinning disk confocal microscope at a magnification of 100X.

2.6 Immunofluorescence

To detect immune complex deposition within the glomerulus of the kidney, the freshly harvested kidneys are embedded in OCT and snap frozen in liquid nitrogen. The frozen block is cut into 8uM thick cryosections and attached to frost free slides which were stored at -80^0 C. The section is fixed in cold acetone, air dried and blocked before it was stained with anti-mouse IgG-Alexa 488 at a dilution of 1:2000. For both experiments secondary antibodies were diluted in PBS containing 0.1% BSA and 0.01% Tween 20. Imaging was done using AxioObserver spinning disk confocal microscope at a magnification of 20X or 100X.

To detect GC staining, the spleen was freshly harvested from mice on day 14 after NP-OVA immunization. It was embedded in OCT compound and snap frozen in liquid nitrogen. These frozen blocks are cut into 8uM thick cryosections and attached to frost free slides. These sections were fixed and blocked as described above. The staining was done with biotinylated anti-IgD for 2 hrs at room temperature followed by a cocktail of antibodies such as streptavidin-Alexa 647, anti-GL7-FITC and anti-CD4–PE (from BD bioscience) for 1 hr at room temperature. Imaging was done at 100X magnification using Ultra view confocal microscope from Perkin–Elmer. GC size within the $GL7^+$ IgD^- regions was determined with the help of image analysis software.

2.7 Development of bone marrow chimera

The BM recipient µMT mice (6-8 weeks old) were lethally irradiated with split doses of radiation 450 cGy, 2 hrs apart using RS2000 irradiator (Rad source technologies). BM was harvested from 4-6 weeks old WT or TAPP KI mice from the femur bone. RBCs were lysed as before and counted using a hemocytometer before mixing the BM. WT or TAPP KI BM was mixed with uMT mice BM at 1:4 ratios in PBS. The BM mix from the donors was then injected through the tail vein into the irradiated recipient uMT mice. Each mouse received a total of $20x10^6$ cells in a 200µl volume. The recipient mice were provided with water containing antibiotics and rested to allow recovery from the radiation. The mice were monitored and weighed every 12 hrs for 2 weeks to assess their recovery. Engraftment of the transferred cells and repopulation of the immune cells in the peripheral blood were confirmed using FACS after 6 weeks. These animals were aged and blood was collected for ELISAs every 5 weeks until they were sacrificed by around 25 weeks.

2.8 B cell isolation and culture

B cells were isolated from splenocytes by negative selection using an easysep mouse B cell isolation kit (Stemcell technologies, catalog#19854) according to the procedure recommended by the manufacturer. Briefly, splenocytes were re-suspended at a cell concentration of 100 $x10^6$ cells/ml in FACS buffer. These cells were blocked with normal rat serum followed by incubation with an antibody cocktail containing biotinylated antibodies directed against non-B cells. Tubes containing cell suspension were placed between strong magnets after adding streptavidin coated magnetic beads. B cells were poured out by inverting the tube, while the non-B cells remain attached to the wall of the tube. B cells obtained by this procedure showed over 95% purity.

Purified B cells were re-suspended in RPMI 1640 medium (containing 2-Mercaptoethanol, 10% FCS, 1% pen-strep) and cultured in 96 well flat bottom plates at a density of $2x10^5$ cells / well. B cells were stimulated with anti-mouse antibodies such as intact anti-IgM, anti-IgM F(ab)'2 both at 10µg/ml concentration (Jackson ImmunoResearch Laboratory), 1µg/ml anti-CD40 (BD Bioscience) and 5ng/ml IL-4 (Peprotech). PI3K inhibitor CAL-101 (Idelalisib- a P110δ inhibitor) was purchased from Selleck chemicals. The glucose analogue, 2-DG was purchased from Cayman chemicals.

2.9 Phospho AKT (ser 473) ELISA

ELISA to detect Phospho-AKT (Ser 473 residues) was performed in B cell lysates using a kit (eBioscience, catalog #85-86042-11) and following the manufacturer's protocol. Briefly, B cells were isolated and suspended in RPMI media for serum starvation up to 2 hrs. These B cells ($1x10^6$ cells) were stimulated with 10µg/ml of IgM F(ab')2 for 5 mins at 37^0 C and immediately transferred to ice, then pelleted and lysed. The cell lysate was mixed with antibody cocktail constituting a mixture of capture and detection antibodies which was then loaded into micro well plates and incubated for 1 hr in a shaker. The plates were washed and then incubated with substrate until the color developed. The reaction was stopped using stop solution and the plate was read at 450-650nm in a plate reader.

2.10 $CD4^+$ T cell isolation and culture

$CD4^+$ T cells were isolated freshly from splenocytes by negative selection using a kit from Stemcell technologies (Catalog # 19852A). Isolation was according to the kit protocol. Briefly, splenocytes were re-suspended in FACS buffer at 100 $x10^6$ cells/ml. The cells were blocked with normal rat serum and then incubated with an antibody cocktail which was a combination of

biotinylated antibodies against non-$CD4^+$ T cells. After addition of streptavidin bound magnetic beads, the mixture was placed between strong magnets. As non-$CD4^+$ T cells are attached to the walls of the tube, highly purified (> 90% purity) $CD4^+$ T cells were easily poured out by inverting the tube.

The isolated $CD4^+$ T cell were counted using a haemocytometer and cultured at a density of $2x10^5$ cells/well in culture medium (RPMI 1640 containing 2-mercaptoethanol, 10% FCS, 1% pen-strep) for 72 hrs. For stimulation, the 96 well flat bottom plate used for culturing $CD4^+$ T cells was coated with anti-CD3 (1µg/ml) in PBS and stored at 4^0 C for overnight prior to the day of culture. In addition, anti-CD28 was dissolved in the culture medium in a dose range up to 50ng/ml.

2.11 MTT and survival assay

The MTT assay was performed after culturing cells for 24 or 72 hrs. 5µg/ml of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) purchased from Invitrogen was added to the culture and incubated for 30 mins. The insoluble purple formazan crystals developed during incubation were detected under a microscope. These crystals were pelleted and dissolved in DMSO where the color intensity is measured using ELISA plate reader at 560-670 nm absorption.

To perform the survival assay for the cultured cells, they were harvested from the plates after 24 hrs of incubation and washed with annexin binding buffer. These cells were stained with annexin V- FITC and 7AAD (BD biosciences) at 1:500 dilution (30 mins incubation at 4^0C). The cells were washed and re-suspended in annexin binding buffer for acquisition.

2.12 In vitro glucose uptake assay

Purified splenic B cells were cultured as before for 24 hrs with anti-CD40 (1µg/ml) + IL-4 (5ng/ml) + anti-IgM $F(ab')_2$ (10µg/ml). The cells were harvested from 96 well culture plates, washed and re-suspended in glucose free medium. This was followed by incubation at room temperature with 2-NBDG (Cayman chemical, Catalog #600470) at a concentration of 150µg/ml for 0, 15 and 30 mins and then immediate transferred to ice. The cells were washed in cold FACS buffer, pelleted and re-suspended in FACS buffer. Glucose uptake was measured by flow cytometry.

2.13 Seahorse assay

Glycolysis and mitochondrial respiration was measured in real time using the seahorse metabolic analyzer. B cells were freshly harvested from mice to study the effects ex vivo or cultured as mentioned above. B cells harvested from the culture were washed in assay medium and attached over XF 24 well plate (Seahorse bioscience) that was coated with CellTak (BD bioscience) at a density of $1.5x10^6$ cells/ml. Mitochondrial respiration was measured in the presence of drugs (1uM each) such as oligomycin - (ATP synthase inhibitor), FCCP (alter the membrane potential) to obtain basal and maximal response and followed by antimycin + rotenone (that target electron transport chain complexes) to shutdown oxidative phosphorylation. Glycolysis was measured starting with glucose free medium into which glucose (10mM) is added followed by oligomycin to obtain maximum glycolytic capacity and 2-DG (100mM, glycolysis analogue) to block the pathway and thus validating the process.

2.14 2-DG treatment

We aged TAPP KI female mice up to 24 weeks, which is the time required for them to develop characteristics of lupus. These animals were divided into two groups where one group was provided with 2-DG in drinking water at a concentration of 5mg/ml which was freshly weighed and dissolved in water that was replaced 3 times a week. The control group on the other hand was provided with plain drinking water. Blood was collected prior to the start of treatment to measure antibody titer. The treatment continued until these animals were euthanized on its 4^{th} week of treatment to harvest spleen, kidney and blood for the study.

2.15 Glucose uptake in vivo

To assess glucose uptake in vivo, mice were injected intraperitoneally (i.p) with $2x10^9$ sheep red blood cells as explained before. On day seven post immunization, the mice were injected with 2-NBDG and rested for an hour before they are sacrificed to collect the spleen. Cells were processed and surface stained as indicated above. Uptake of glucose was measured by detecting the 2-NBDG fluorescence by gating different B cell subsets. Control immunized mice which were not injected with 2-NBDG was used for background correction. 2-NBDG, purchased from Cayman chemicals, was reconstituted in ethanol and stored. For injection, the stock was diluted to a concentration of 2mM in PBS which was injected at a volume of 100µl per mice though the tail vein.

2.16 Statistical analysis

Statistical analysis was done to obtain p-values using two-tailed unpaired Student's t-test unless specified. Graph pad prism software was used for these tests and p-value is represented as *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ for significant and NS for not significant data. The bar graphs

and line graphs represents the mean and standard error for the indicated number of mice or experiments.

Chapter 3

Uncoupling of TAPP adaptors from PI(3,4)P2 leads to chronic B cell activation and autoimmunity

This chapter is based on

(1) Part of a published paper along with additional data

Landego I, Jayachandran N, Wullschleger S, Zhang TT, Gibson IW, Miller A, Alessi DR, Marshall AJ. Interaction of TAPP adapter proteins with phosphatidylinositol (3,4)-bisphosphate regulates B-cell activation and autoantibody production. Eur J Immunol. 2012. PMID: 22777911

(2) Part of a published paper along with additional data

Jayachandran N, Landego I, Hou S, Alessi DR, Marshall AJ. B-cell-intrinsic function of TAPP adaptors in controlling germinal center responses and autoantibody production in mice. Eur J Immunol. 2017. PMID: 27859053

3.1 Introduction

Activation of the PI3K pathway generates lipid second messengers such as PI(3,4,5)P3 and PI(3,4)P2. The generation of these phospholipids is tightly regulated by inositol phosphatases. This is an essential mechanism to regulate membrane translocation of phosphoinositide binding proteins that mediate the downstream response [341]. Tonic BCR signaling, predominantly PI3K activity is required for survival of mature B cells [224, 225]. However, aberrant chronic BCR signaling is associated with B cell malignancies. Therefore targeting this pathway is one of the promising therapeutic approaches [342].

Feedback regulation of PI3K signaling is mediated by activation of FcγRIIB and recruitment of SHIP. However, the mechanisms downregulating PI3K signaling are not fully understood [253]. TAPP is a PH domain protein that binds to PI(3,4)P2 which is a product of SHIP activity. However, the role of TAPP in PI3K signaling in B cells was not clear. [320]. Studies on TAPP KI mice done just prior to starting my thesis project showed that uncoupling of TAPPs from the membrane leads to B cell hyper responsiveness. Upon in vitro stimulation by BCR crosslinking, TAPP KI B cells showed a significantly increased rate of proliferation as measured by CFSE dilution assay. Enhanced activation of TAPP KI B cells is associated with dysregulated PI3K signaling which was detected by enhanced phosphorylation of AKT. The B cell hyper responsiveness demonstrated in the presence of the TAPP mutation was reversed in vitro in the presence of AKT inhibitor. These findings also showed that TAPPs are not involved in the initiation of BCR signaling but regulate AKT mediated downstream events [340]. Young adult TAPP KI mice (8-12 weeks) exhibited significantly elevated levels of IgM and IgGs compared to normal mice [340].

TAPP KI mice showed some striking similarities with *Ship*-/- mice as in both cases there was an increase in AKT phosphorylation on BCR ligation contributing to B cell hyper responsiveness, along with enhanced CD86 expression in vitro. These animals showed a basal elevation of Igs at a young age similar to TAPP KI mice [337, 338, 340]. Hyper-activation of PI3K signaling has been associated with chronic B cells activation and autoimmunity in a variety of mouse models [156, 157]. B cell specific deletion of SHIP showed B cell hyper responsiveness and development of lupus like autoimmunity in mice [156]. In mouse models, the characteristics resembling clinical signs of lupus include presence of anti-dsDNA antibodies, glomerulonephritis and splenomegaly [124, 156, 157]. In this chapter, I have explored the autoimmune characteristics in aged TAPP KI mice along with abnormalities associated within their B cell compartments.

3.2 Results

3.2.1 TAPP KI mice develop lupus-like autoimmune characteristics

To investigate whether TAPP KI mice develop autoreactive antibodies, serum was collected every 5 weeks up to the end point at 65 weeks. Female TAPP KI mice exhibited strikingly elevated levels of anti-dsDNA antibody from week 20 and beyond. The autoantibody titer progressively increased in these animals and was almost 5 times higher than WTs at 65 weeks (Fig 3.1A left). Similar to lupus in humans and other mouse models, we found a female sex bias: TAPP KI male mice did not develop anti-dsDNA antibody (Fig 3.1A right) with age and their levels were comparable with that of the WT controls. Consistent with the increased presence of anti-dsDNA antibodies, female mice showed the presence of antinuclear antibodies (ANA) at week 65 (Fig 3.1B). Apart from those autoantibodies in the serum, another characteristic of SLE is enlargement of the spleen. The size (Fig 3.1C above) and weight (Fig 3.1C below) of the spleen in aged TAPP KI mice was higher than that of the wild type controls. In addition, immunofluorescence staining showed IgG deposition within the glomeruli of TAPP KI kidney (Fig 3.1D). In addition to the presence of immune complexes within TAPP KI kidney, they also exhibited infiltration of cells within the glomerulus and tissue scarring resembling lupus nephritis in humans. Peripheral blood of TAPP KI mice exhibited a trend of reduction in percentage of both B and T cells along with an increase in trend among the myeloid population with age (Fig 3.2A and 3.2 B) which resembled the abnormalities reported previously in lupus [343, 344]. Together these data suggested that uncoupling TAPPs from the membrane lead to autoimmunity in TAPP KI mice.

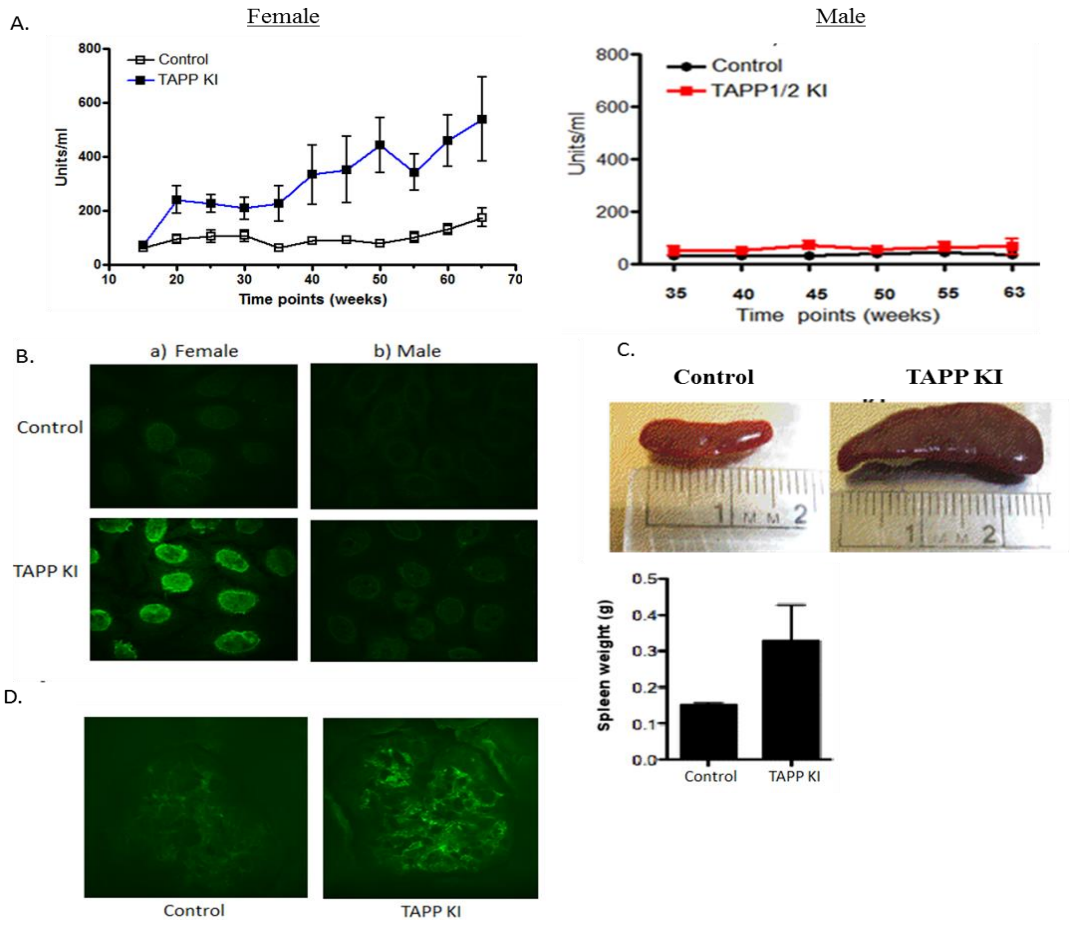

Figure 3.1 TAPP KI mice develop lupus-like pathology.

(A) Serum collected from aging TAPP KI or WT control mice every 5 weeks were tested for anti-dsDNA antibody (IgG isotype) titer by ELISA and also performed a comparison between both female (left) and male (right) mice. (B) Immunofluorescence staining of HEp2 cells incubated with aged mice serum (week 65) to detect the presence of anti-nuclear antibody and to compare between female (left) and male mice (right). (C) A comparison of spleen size between TAPP KI and WT control (above) and their net weight (below) at 65 weeks of age. (D) Immune complex deposition in the glomerulus detected by immunofluorescence staining of female kidney cryosections with fluorescently labelled IgG secondary antibody (week - 65). Although anti-dsDNA antibody titre in TAPP KI females (A-left) and spleen weight (C- below) was not

statistically significant in this cohort, a trend of increase was observed. In male TAPP KI mice, anti-dsDNA antibody titre (A-right) clearly showed no difference when compared to WTs for all the time points indicated. All experiments combined two cohorts with a total of n=7 mice under each group.

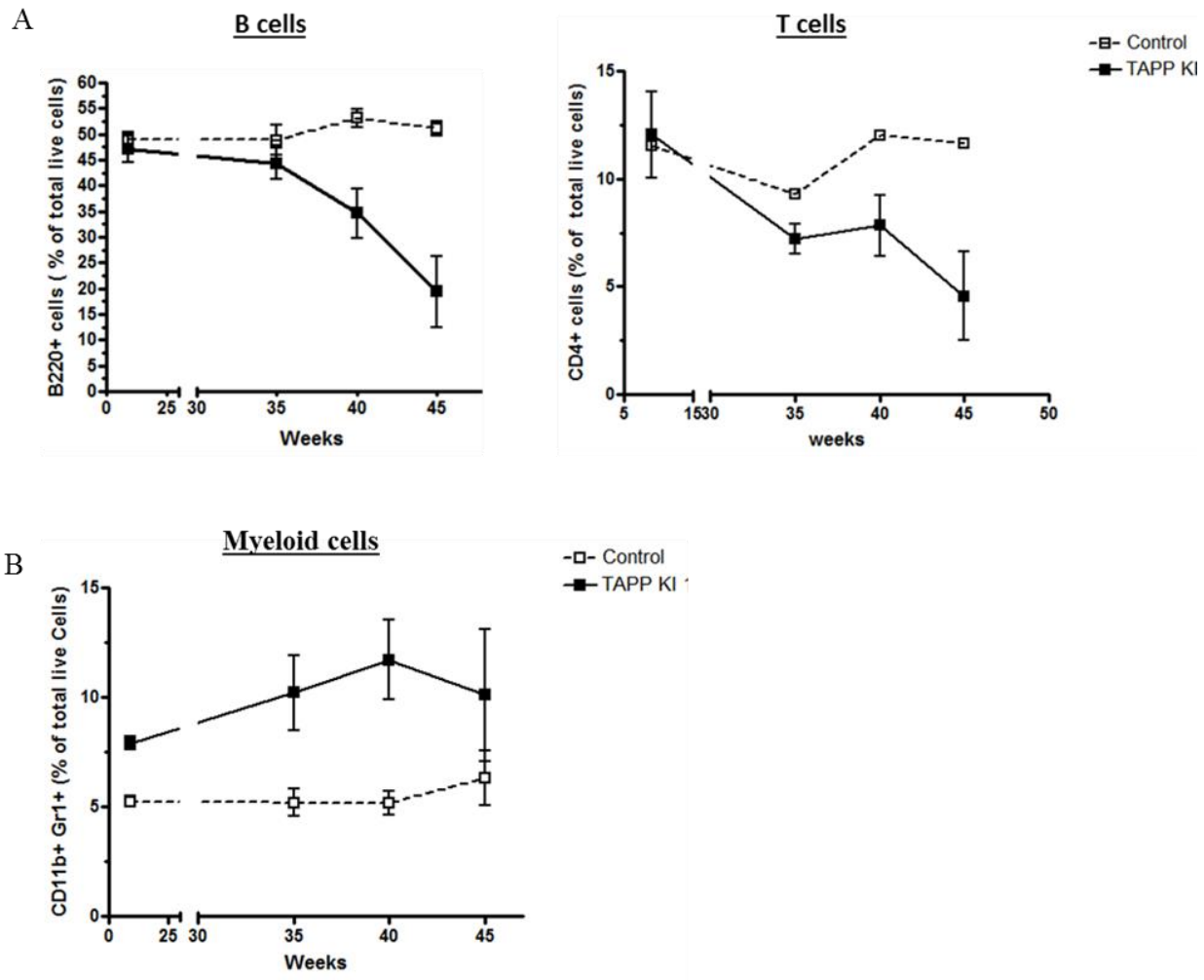

Figure 3.2 TAPP KI mice exhibit abnormalities in lymphoid and myeloid populations within the peripheral blood

Peripheral blood was collected at different time points (as indicated) from an aging cohort of WT control or TAPP KI mice. The RBC lysed population was stained for (A) B cells ($B220^+$) on the left and T cells ($CD4^+$) cells on the right (B) Myeloid ($CD11b^+$ $Gr1^+$) cells. Although not statistically significant, all these data showed a trend of decrease in lymphocytes and increase in myeloid cells within TAPP KI mice. Data is derived from a single aging cohort with 4 mice per group.

3.2.2 TAPP KI mice exhibits spontaneous B cell activation and chronic GC

TAPP KI mice exhibit autoimmune characteristics which can be detected after 20 weeks of age. To understand the cellular mechanism underlying autoimmune pathology, abnormalities in the B cell population in middle aged mice (28-30 weeks) were assessed. TAPP KI mice exhibited an increase in frequency of the germinal center B cell (GCB) population in the spleen identified as $GL7^{+}Fas^{+}$ B cells (Fig 3.3A). This germinal center abnormality exhibited by middle aged TAPP KI mice is accompanied by a proportional increase in follicular helper T cell (TFH) population in the spleen, identified as $CD4^{+}ICOS^{+}PD1^{+}$ T cells (Fig 3.3B). Age and sex matched P110δ mutant mice are known to lack both these populations [245, 345] and were used as negative controls. These middle aged TAPP KI mice also exhibited B cells with significantly elevated levels of the co-stimulatory molecule CD80 and a trend towards increased CD86 (Fig 3.4A and B). Mesenteric lymph nodes (MLN) from TAPP KI mice showed a similar increase in GCB cells, CD80 and CD86 populations (Fig 3.5).

Consistent with other models of chronic inflammatory diseases, TAPP KI mice showed a substantial increase in the frequency of the age associated B cell (ABC) population [76, 182] which are identified as $CD23^{-}CD21^{-}$ $B220^{+}$ cells. However, the percentage of follicular (FO) B cells ($B220^{+}$ $CD23^{+}$ $CD21^{-}$) and marginal zone (MZ) B cells ($B220^{+}$ $CD23^{-}$ $CD21^{+}$) population was not significantly altered in these middle aged mice (Fig 3.6). Expansion of the B1a ($CD19^{+}$ $B220^{+}$ $CD5^{+}$) subset in the peritoneum was detected in TAPP KI mice, resembling the peritoneal B cell abnormalities observed in other mouse models of lupus [178, 179] (Fig 3.7). Together these findings tell us that middle aged TAPP KI mice exhibits spontaneous B cell activation and develop chronic germinal centers.

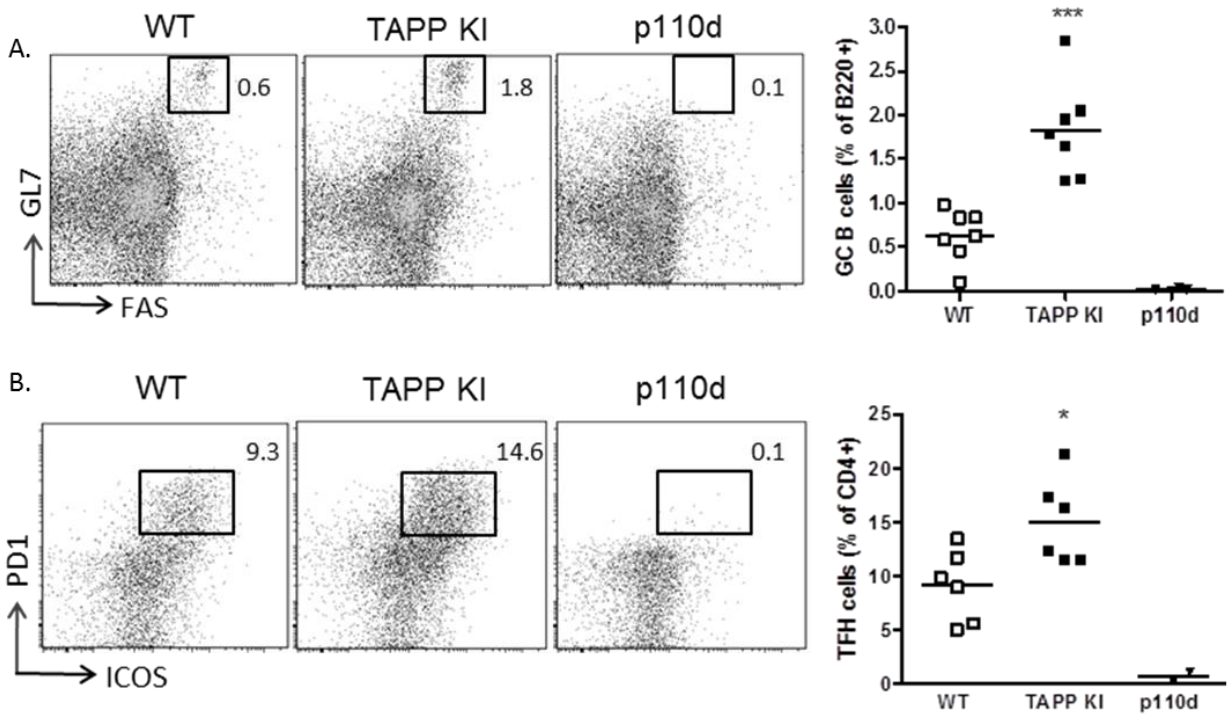

Figure 3.3 TAPP KI mice develop chronic germinal centers in the spleen.

Spleen harvested from middle aged female WT or TAPP KI mice or P110δ mutant mice (28-30 weeks) were analyzed by flow cytometry. (A) Splenocytes were gated for $GL7^+$ FAS^+ $B220^+$ GCB cells, represented by dot plots (left) and summary graph (right). (B) Splenic TFH cells were gated within total $CD4^+$ T cell population which are identified by $PD1^+$ $ICOS^+$ gating, represented by dot plots (left) and summary graph (right). The data combines two independent experiments with a total of 6-7 mice per group (except P110δ mutant mice which is 2-3 mice). WT to TAPP KI statistical comparison was performed using a two-tailed unpaired Student's t-test and significance is as indicated as *P< 0.05, ***P< 0.0001.

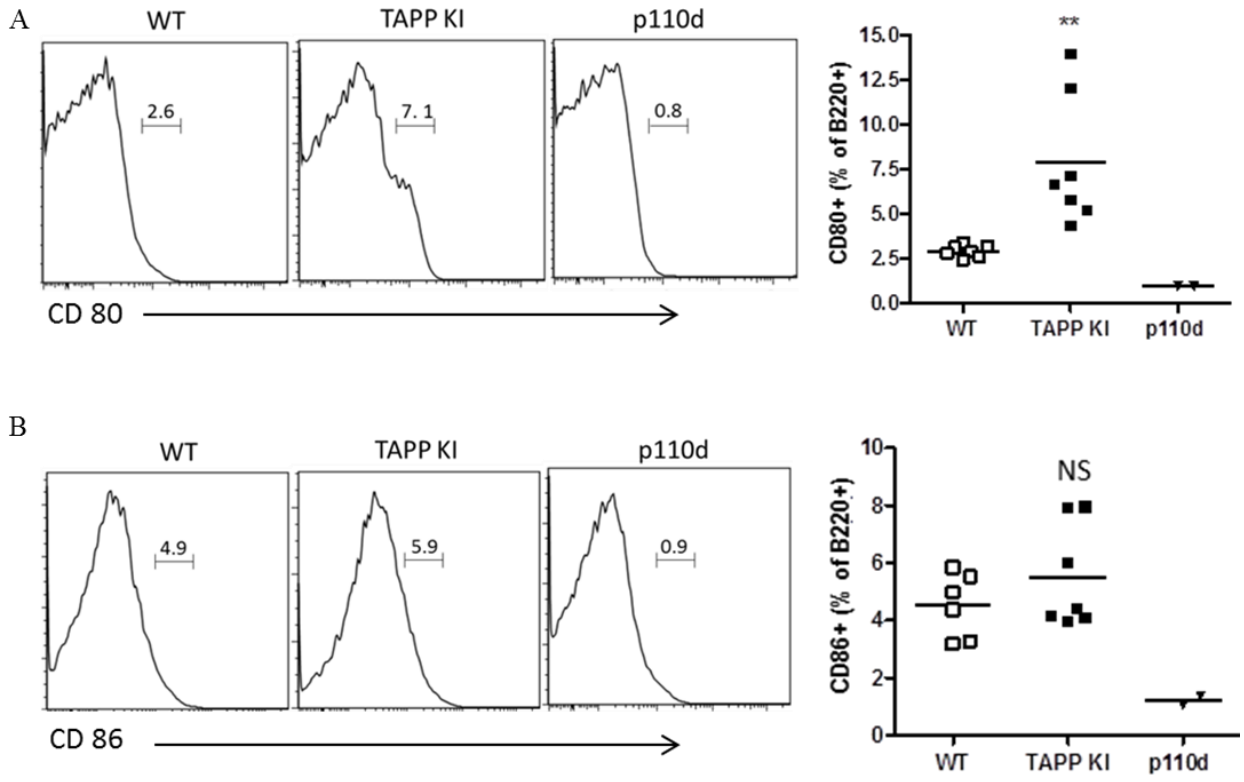

Figure 3.4 Spontaneous B cell activation in TAPP KI spleen

Spleen harvested from middle aged female WT or TAPP KI mice or P110δ mutant mice (28-30 weeks) were analyzed by flow cytometry. (A and B) Expression of B cell activation markers such as CD80 and CD86 within splenic $B220^+$ cells. Representative histograms on the left (line segments indicating percentage of $CD80^+$ or $CD86^+$ B cells) and summary graph on the right. The data combines two independent experiments with a total of 6-7 mice per group (except P110δ mice which is 2-3 mice). WT to TAPP KI statistical comparison was performed using a two-tailed unpaired Student's t-test and significance is as indicated as NS, $P>0.05$, $^{**}P< 0.001$.

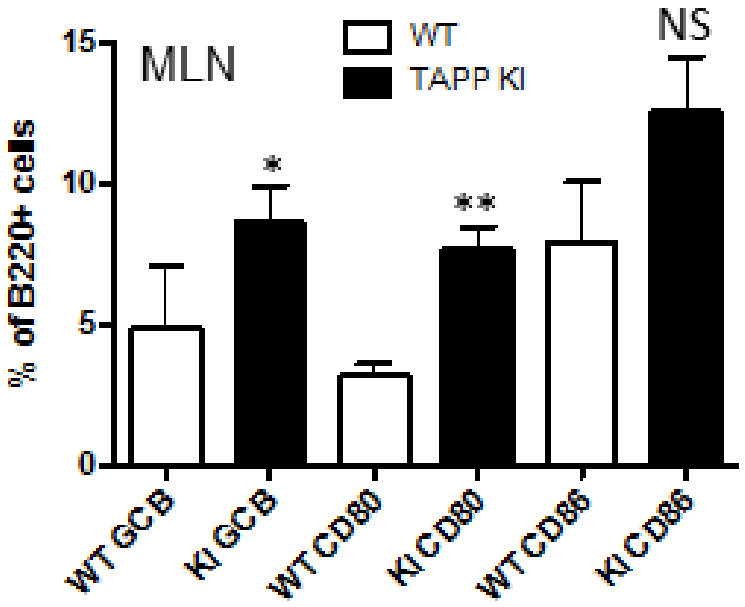

Figure 3.5 Spontaneous B cell activation and chronic GC in the mesenteric lymph node of TAPP KI mice

Mesenteric lymph node (MLN) harvested from middle aged female WT or TAPP KI mice (28-30 weeks) were analyzed by flow cytometry. Bar graph showing percentage of GCB cells, CD80 and CD86 within MLN were gated in the same way as it was done in spleen. The data combines two independent experiments with a total of 6-7 mice per group. WT to TAPP KI statistical comparison was performed using a two-tailed unpaired Student's t-test and significance is as indicated as NS, $P>0.05$, *$P< 0.05$, **$P< 0.001$.

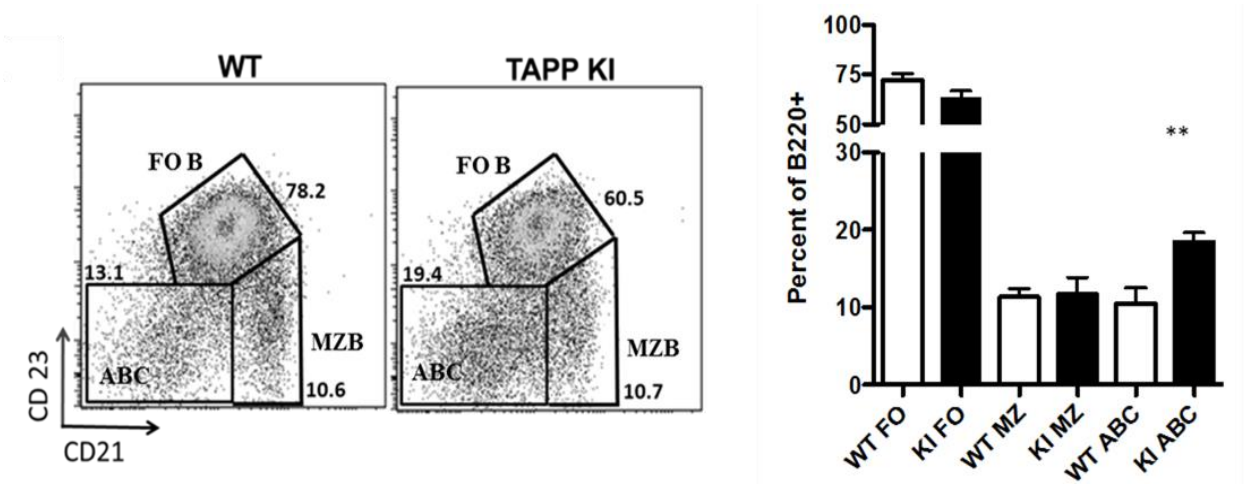

Figure 3.6 Abnormalities in B cell compartments within the TAPP KI spleen.

Spleen harvested from middle aged female WT or TAPP KI mice (28-30 weeks of age) were analyzed by flow cytometry. Gating for age associated B cells (ABC) which are $CD23^{-}$ $CD21^{-}$ $B220^{+}$ cells, Marginal Zone (MZ) B cells ($B220^{+}$ $CD21^{+}$ $CD23^{-}$ population) and follicular (FO) B cells ($B220^{+}$ $CD21^{-}$ $CD23^{+}$) represented by dot plots (left) and summary graph (right). The data combines two independent experiments with a total of 6-7 mice per group. WT to TAPP KI statistical comparison was performed using a two-tailed unpaired Student's t-test and significance is NS (FO, MZ B cells) , **P< 0.001(ABC population).

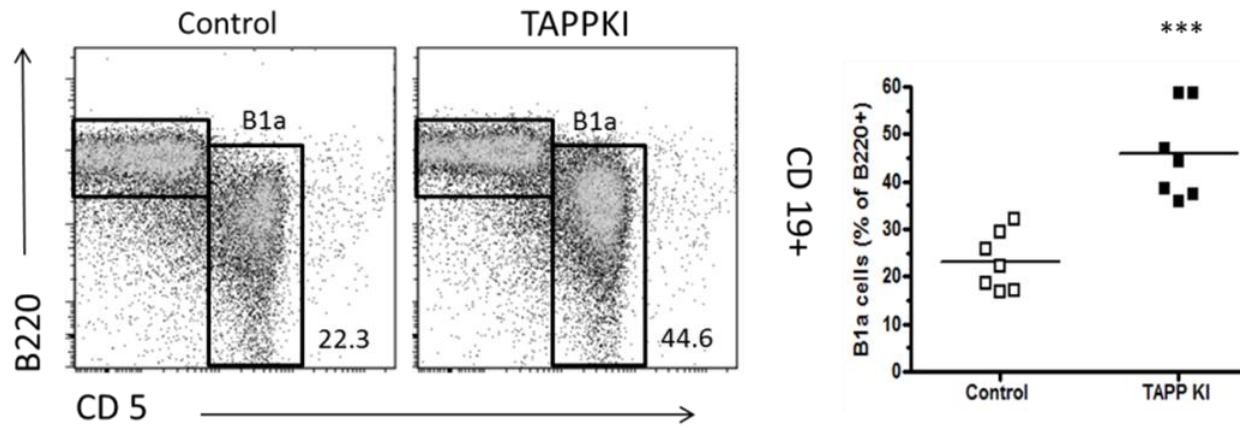

Figure 3.7 Expansion of peritoneal B cell subset in TAPP KI mice

The peritoneal cavity of WT or TAPP KI mice (28-30 weeks of age) was flushed with PBS to harvest the cells. The cells were stained for B1a population which was analyzed by flow cytometry. The total $CD19^+$ B population were gated to determine the percentage of B1a ($CD19^+$ $B220^+$ $CD5^+$) subset, represented by dot plot (left) and summary graph (right). The data combines two independent experiments with a total of 7 mice per group. WT to TAPP KI statistical comparison was performed using two-tailed unpaired Student's t-test and significance is as indicated as ***P< 0.0001.

3.3 Discussion

PI3K signaling triggered by BCR crosslinking is crucial for B cell activation and to mediate its effector function. Downregulation of PI3K signaling requires enzymatic hydrolysis of PIP3. PI(3,4)P2 that is produced as a product of PIP3 hydrolysis is the binding site for TAPPs but their function was unknown [253, 310]. Our lab has previously shown that, interaction of TAPPs with PI(3,4)P2 promotes its membrane translocation in B cells [320].

Recruitment of SHIP that is involved in PIP3 hydrolysis is activated via inhibitory FcγRIIB. Loss of FcγRIIB in mice as well as its gene polymorphism in humans is associated with the development of severe autoimmunity [124, 339]. Based on the similarities between TAPP KI mice and B cell specific SHIP deficient mice, [156, 340] it is tempting to speculate that the inhibitory effect of SHIP could be partially mediated by TAPP – PI(3, 4)P2 interaction.

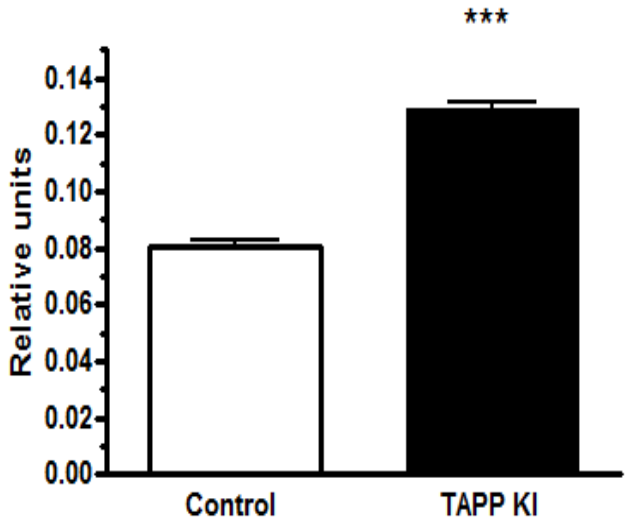

Figure 3.8 - Enhanced AKT phosphorylation by TAPP KI B cells

B cells freshly purified ($1x10^6$ cells) from WT control or TAPP KI were stimulated with 10µg/ml of IgM F(ab')2 for 5 mins. The cell lysate was tested for AKT phosphorylation (Ser 473 residues) by ELISA. The data is representative of 3 or more independent experiments. WT control to TAPP KI statistical comparison was performed using a two-tailed unpaired Student's t-test and significance is as indicated as ***P< 0.0001.

B cell hyper responsiveness driving the development of autoimmunity has been associated with a number of autoimmune models [137, 148, 156]. ANA that was detected in aged TAPP KI mice indicates the possibility of broad spectrum of autoantibodies that includes anti-dsDNA, anti-nucleosomes, anti-ribonucleoproteins, anti-chromatin antibodies and others. Anti-dsDNA antibodies being one of the specific markers of lupus, exhibited a progressive increase in titer clearly suggesting a breach in tolerance mechanism. It is quite possible that autoreactive B cells which are expected to undergo death by neglect in the absence of T cell help within the GC are receiving excessive activation signals through PI3K activation, which is defective in TAPP KI mice. Although we're favoring the idea of a defect in a peripheral tolerance mechanism to remove autoreactive cells arising in GC, it is possible that these mice may also have defective elimination of autoreactive cells during B cell development in bone marrow due to abnormalities in central tolerance.

The findings in aged TAPP KI mice that showed females with autoimmune characteristics that was not detected in male mice resembles many autoimmune models as well as human SLE patients [346]. One of the mechanisms contributing to the sex bias is the female sex hormones. Approaches to target these sex hormones in female autoimmune mice have reduced the disease severity in other animal models [347].

To investigate the mechanisms underlying autoimmunity in TAPP KI mice, we tested the cellular abnormalities in middle aged TAPP KI mice. Lupus like autoimmune disease is characterized by development of chronic GC. Defective GC is driven by abnormalities in the GCB and TFH populations as they are mutually supportive and therefore both can contribute to the development of autoimmunity [159, 160, 348]. GC responses greatly rely upon PI3K signaling and they are crucial in antibody class switching and generation of antigen specific antibodies [349]. Therefore

P110δ mutant mice defective in the major hematopoietic cell PI3K isoform are unable to develop germinal center response [245, 350]. TAPP KI mice on the other hand exhibit enhanced PI3K signaling (Fig 3.8) thus contributing to chronic GC with increased frequencies of GCB and TFH cells. Expression of costimulatory molecules such as CD80/CD86 is dependent on PI3K signaling [351] which is enhanced in TAPP KI B cells and thus contributing to their increased expression in these middle aged mice.

The current approaches to treat SLE include depleting B cells or targeting BAFF using monoclonal antibodies. As these methods show certain limitations, new treatment methods are to be considered for developing a more effective cure. The findings in TAPP KI mice indicate the dysregulation of PI3K signaling in B cells. As defective regulation of B cells contribute to the development of abnormal GC and autoimmunity, targeting PI3K/Akt pathway can potentially be used as an approach to treat SLE.

Chapter 4

B-cell-intrinsic function of TAPP adaptors in controlling germinal center responses and autoantibody production in mice.

This chapter is based on

(1) Part of a published paper along with additional data

Jayachandran N, Landego I, Hou S, Alessi DR, Marshall AJ. B-cell-intrinsic function of TAPP adaptors in controlling germinal center responses and autoantibody production in mice. Eur J Immunol. 2017. PMID: 27859053

4.1 Introduction

Development of GC is essential to mediate protection against invading pathogens by generating a high affinity antibody response [352]. However, generation of chronic germinal centers and autoantibodies is associated with lupus in mice and humans[162]. GC B cells interacting with TFH cells in the light zone compartment are crucial in generating such a response and the PI3K pathway plays an important role in this [348, 349]. Blocking PI3K signaling by mutating the P110δ subunit results in the disruption of the GC response [245].

One of the co-stimulatory molecules required for GCB-TFH crosstalk is ICOS, which is exhibited by TFH cells. ICOSL displayed by GCB cells can mediate crosslinking of ICOS during the cellular interaction within the GC. PI3K signaling derived from ICOS is crucial for survival of TFH cells [353]. Loss of ICOS in mice results in impairment in the development of GC even after immunization [61]. However, abnormal expression of ICOS results in aberrant chronic GC and autoimmunity in mice [62]. Disrupting the GCB-TFH interaction has been achieved using a monoclonal antibody to block ICOS crosslinking has reversed abnormal GC and autoimmunity in mice [193].

Dysregulated PI3K signaling is associated with expansion of GCB cells and TFH populations contributing to autoimmunity [124]. B cell intrinsic role in the generation of GC has been reported in lupus prone mice [156]. TAPPs when uncoupled from the membrane showed aberrant PI3K signaling and B cell intrinsic defects upon in vitro stimulation. Aged TAPP KI mice exhibited chronic B cell activation, spontaneous GC and anti-dsDNA antibodies, with their titer increasing with age. In this chapter, I have explored the role of TAPP KI GC in the

development of autoimmunity, investigating the significance of GCB-TFH interactions and determining the B cell intrinsic role of TAPPs contributing to the disease in these mice.

4.2 Results

4.2.1 Abnormal GC response in TAPP KI mice on immunization

To determine the effect of uncoupling TAPPs from the membrane on the acute GC response, young 8-12 weeks TAPP KI mice were immunized with sheep red blood cells (SRBC), which is a T cell dependent antigen known to generate an acute GC response in 7 days post immunization. A near 2 fold increase in the frequency of the GC B cell population was detected in TAPP KI mice compared to controls as assessed by flow cytometry (Fig 4.1A). In addition to that, immunofluorescence staining of spleen sections showed TAPP KI mice have a relatively larger GC area (Fig 4.1C). A corresponding increase in the percentage of the T-follicular helper cell subset in these immunized TAPP KI mice was observed (Fig 4.1B). B cells expressing costimulatory molecules such as CD80 and CD86 were also significantly increased in these young immunized TAPP KI mice relative to control immunized mice (Fig 4.2A and B). Together these data suggest that TAPP KI mice develop increased acute B cell activation and generate abnormally large GC responses upon immunization.

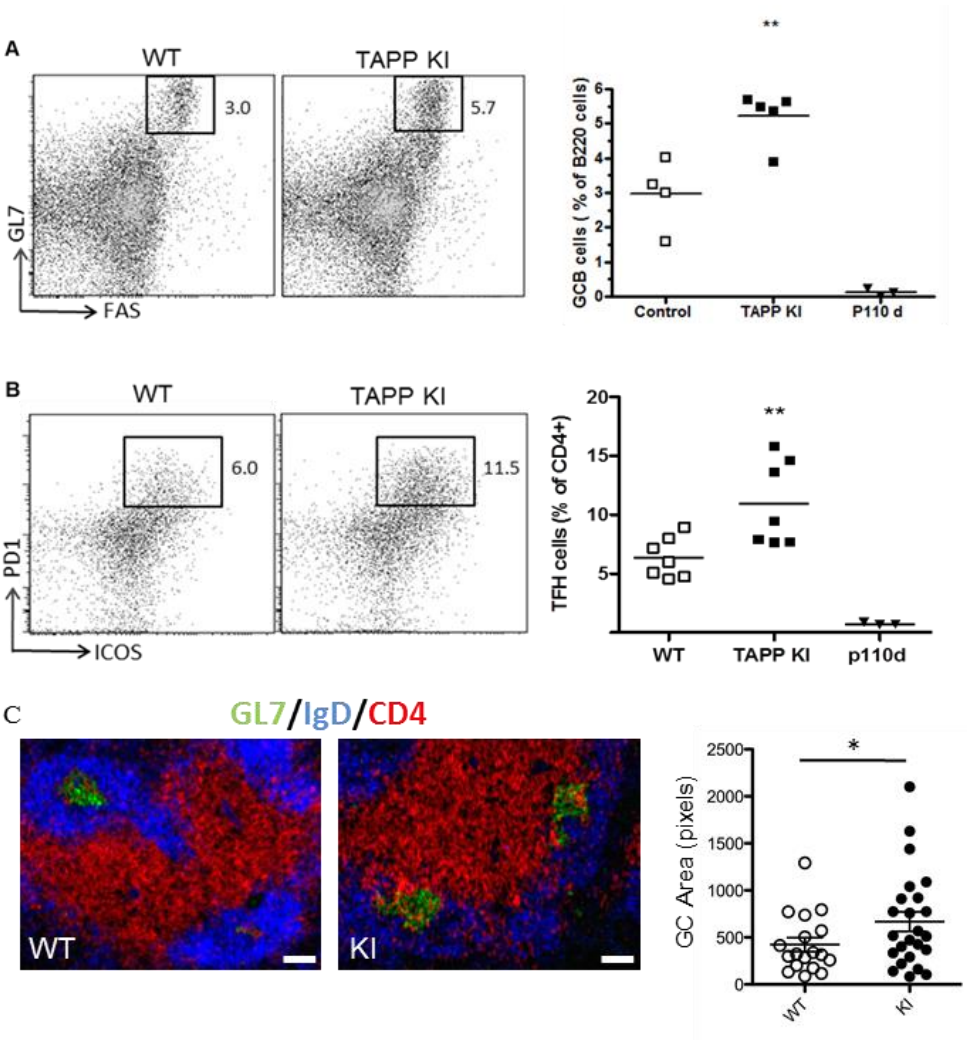

Figure 4.1 TAPP KI mice develop acute germinal centers

Young adult (8-12 weeks old) WT or TAPP KI mice were immunized with sheep red blood cells (SRBC) and sacrificed after 7 days. P110δ mutant mice were used as a negative control for this experiment (A) Splenocytes from these mice were stained for $GL7^+$ FAS^+ $B220^+$ for GCB cells and their frequency was determined by flow cytometry. The results are represented as a dot plot on the left and a summary graph showing GCB frequency on the right. (B) TFH population was assessed based on the presence of $PD1^+$ $ICOS^+$ markers within total $CD4^+$ T cells with

representative dot plot on the left and summary graph on the right. (C) Left - Cryosections of the spleen from immunized mice stained for $GL7^{+}$ IgD^{-} GCs by confocal microscopy (scale bar - 200μM). Right – Graph compares the area of GCs combined from 3 mice per group. The dots represent pixel count within a GC. All other data is pooled from 2 experiments with 4-7 mice per group. WT to TAPP KI statistical comparison was performed using a two-tailed unpaired Student's t-test and significance is as indicated as **P< 0.001.

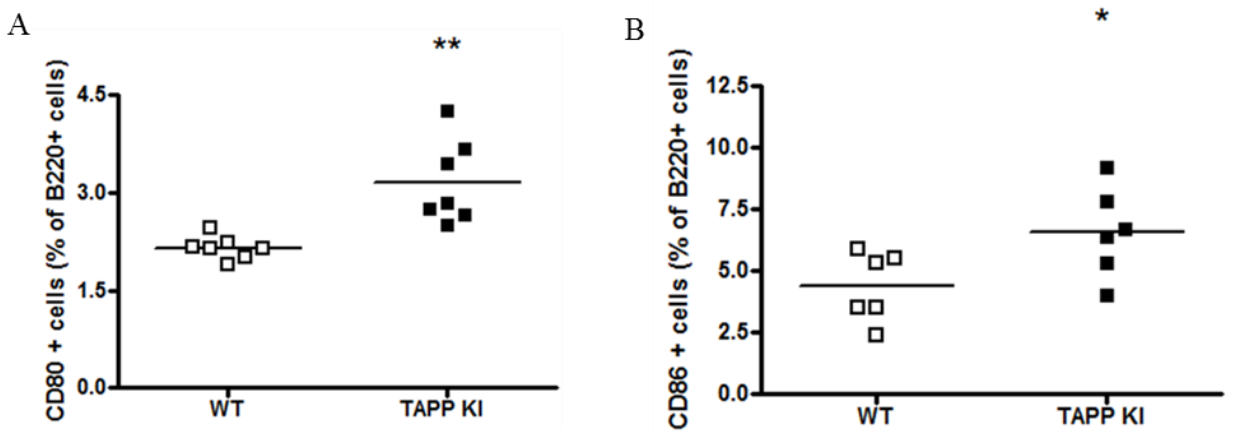

Figure 4.2 Acute activation of B cells in TAPP KI mice

Young adult (8-12 weeks old) WT or TAPP KI mice were immunized with sheep red blood cells (SRBC) and sacrificed after 7 days. (A and B) The costimulatory molecules, CD80 (left) and CD86 (right) expressed by B220 cells were gated as shown in chapter 3. All data is combined from 2 experiments with 4-7 mice per group. WT to TAPP KI statistical comparisons were performed using a two-tailed unpaired Student's t-test and significance is as indicated as *P< 0.05 and **P< 0.001.

4.2.2 Chronic GC of TAPP KI is age and gene dose dependent

Spontaneous chronic germinal centers are associated with autoimmunity in humans as well as mouse models. Therefore further investigation was done on TAPP KI GC to understand their kinetics and gene dose dependence. An increase in GCB (left panel) and TFH (right panel) populations was detected in young (8-12 weeks) TAPP KI mice and this increase progressed with age (Fig 4.3A and B). GC B and TFH populations showed high sensitivity to the dosage of TAPP KI genes. The heterozygous mice even with a single normal allele encoding TAPP 1 or TAPP 2 displayed a drastic reduction in both populations making them comparable with that of the WT controls (Fig 4.3C and D). $CD80^{+}$ splenic B cells also exhibited an increase in frequency over time (Fig 4.4A) and here again they showed gene dose dependence with WT or TAPP KI alleles (Fig 4.4B) suggesting that chronic activation of these B cells could be associated with TAPP KI GC.

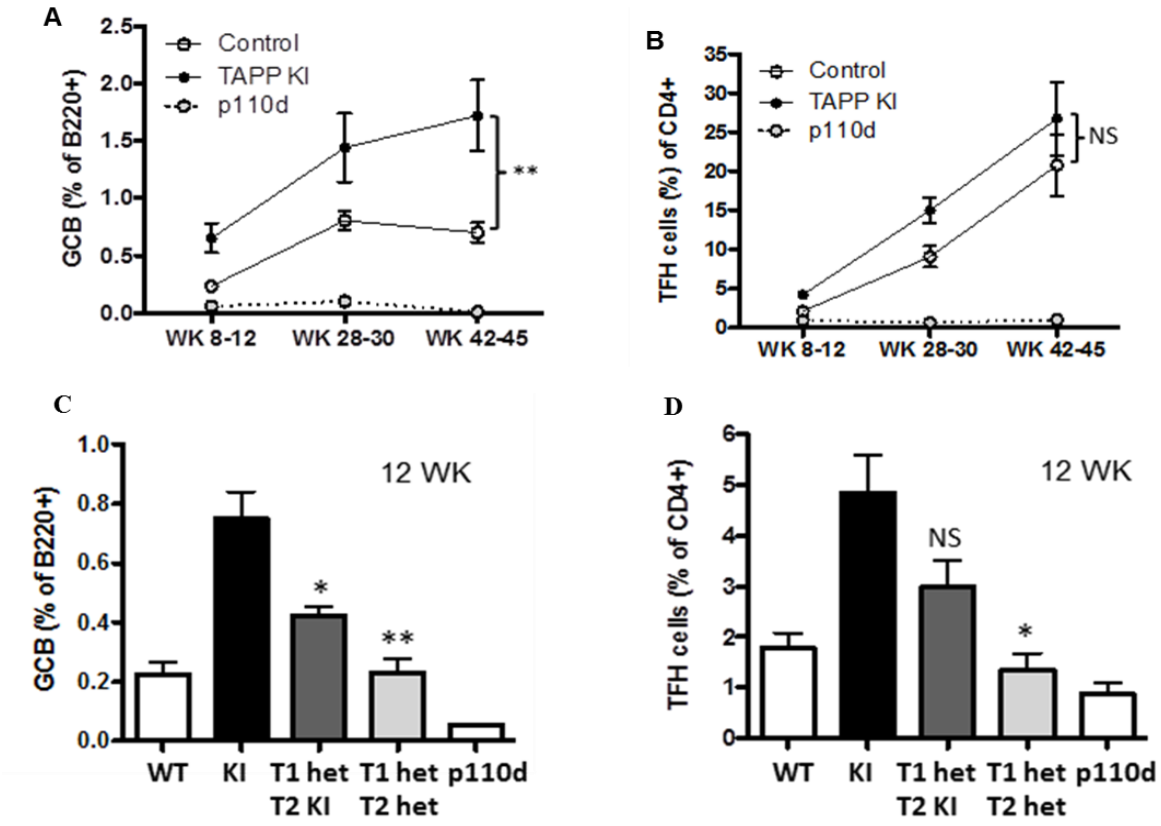

Figure 4.3 Age and gene dose dependent development of chronic GC in TAPP KI mice

Spleens were harvested from naïve young adult (8-12 weeks), middle aged (28-30 weeks) and old (42-45) mice. The splenocytes were stained for different GC subsets and analyzed by flow cytometry. (A) GC B cell frequency over time for WT control, TAPP KI and p110δ mutant mice for the age groups indicated. (B) Percentage of TFH cells at different ages for TAPP KI and p110δ mutant mice. (C) Gene dose effect of TAPP1 and TAPP2 mutation on the percentage of GC B cells in young adult mice (D) Gene dose effect of TAPP1 and TAPP2 mutation on the percentage of TFH cells in young adult mice. Panels A and B represent data where a statistical comparison between WT and KI were performed using two-way ANOVA. Panels C and D represents data where a comparison between homozygous TAPP KI with the indicated

heterozygous genotypes were performed using a two-tailed unpaired Student's t-test. All the data represent the mean and standard error from 2 or more independent experiments with 3-7 mice per group. Statistical significance is represented as NS, $P>0.05$,*$P< 0.05$, **$P< 0.001$.

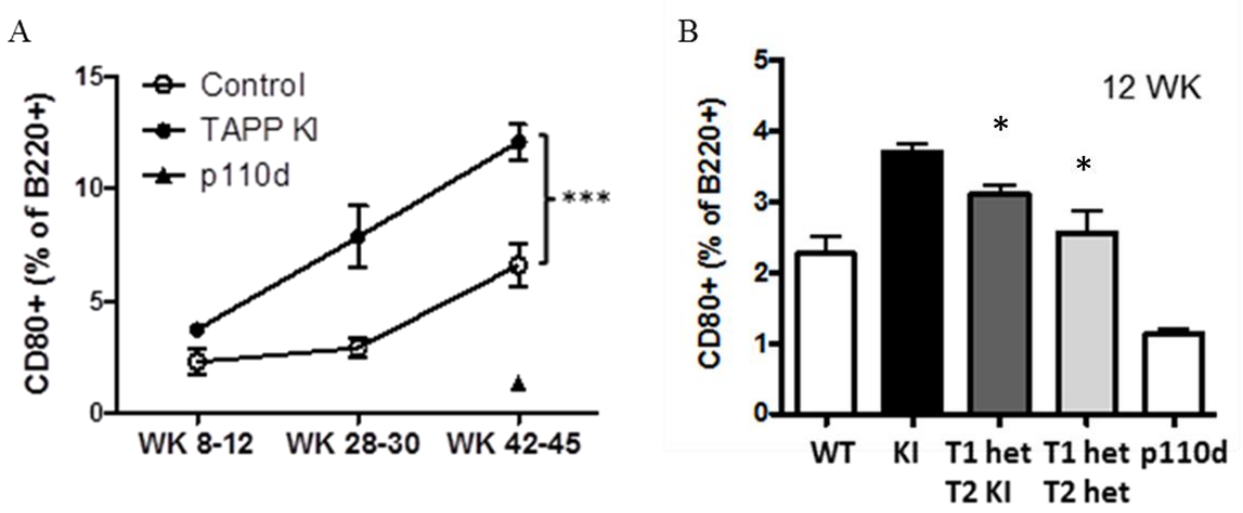

Figure 4.4 Age and gene dose dependent spontaneous B cell activation in TAPP KI mice

Spleens harvested from naïve young adult (8-12 weeks), middle aged (28-30 weeks) and old (42-45) mice. The splenocytes were stained for $CD80^+$ B cell populations and analyzed by flow cytometry. (A) Percentage of CD80 expressing B cells with age. (B) Gene dose response of TAPP1 and TAPP2 mutation on $CD80^+$ B cells in unimmunized young adult mice. Panel A represents data where a statistical comparison between WT and KI were performed using two-way ANOVA. Panels B represent data where a comparison between homozygous TAPP KI mice with indicated heterozygous genotypes were performed using a two-tailed unpaired Student's t-test. All the data represent the mean and standard error from 2 or more independent experiments with 3-7 mice per group. Statistical significance is represented as *P< 0.05, ***P< 0.0001.

4.2.3 Absence of GC prevents development of autoimmunity in TAPP KI mice

To determine the role of TAPP KI chronic GC in the development of autoimmunity, *Icos* -/- mice were crossed with TAPP KI mice to generate *Icos*+/- TAPP KI and *Icos*-/- TAPP KI mice. Upon immunization of young adult mice, absence of a single *Icos* allele in TAPP KI mice significantly reduced the size of the acute GC in these animals, while complete deletion of *Icos* ablated the GC response in TAPP KI mice (Fig 4.5A). A similar pattern of *Icos* gene dose dependence was observed in the development of chronic GC in TAPP KI mice at middle age (Fig 4.5B). Abnormal accumulation of CD80 or CD86 B cell population relies upon *Icos* gene dose within middle aged TAPP KI mice (Fig 4.5C and D). Loss of *Icos* in TAPP KI mice led to reduction of anti-dsDNA antibodies (both IgG and IgM isotypes) with age (Fig 4.6A and B). Anti-nuclear antibodies (ANA) and IgG deposition in the kidney glomerulus which was detected in middle aged TAPP KI mice was absent in *Icos*-/- TAPP KI mice (Fig 4.6C and D). Together these data suggests that targeting ICOS signaling by knocking out this gene in TAPP KI mice impairs the development of chronic GC and autoimmunity.

The results from *Icos*-/- TAPP KI mice could indicate a requirement for GCB, TFH or likely both contributing to aberrant GC and autoimmunity. We investigated whether isolated B cells or T cells are intrinsically hyper-responsive. B cells are clearly hyper-responsive as previously published and it is investigated in depth in the next chapter; however T cells also exhibited hyper-responsiveness to anti-CD3 + anti-CD28 stimulation (Fig 4.7). The effect of TAPP mutation on other cell types remains to be investigated. Thus, it is important to determine the B cell intrinsic contribution to autoimmunity in TAPP KI mice.

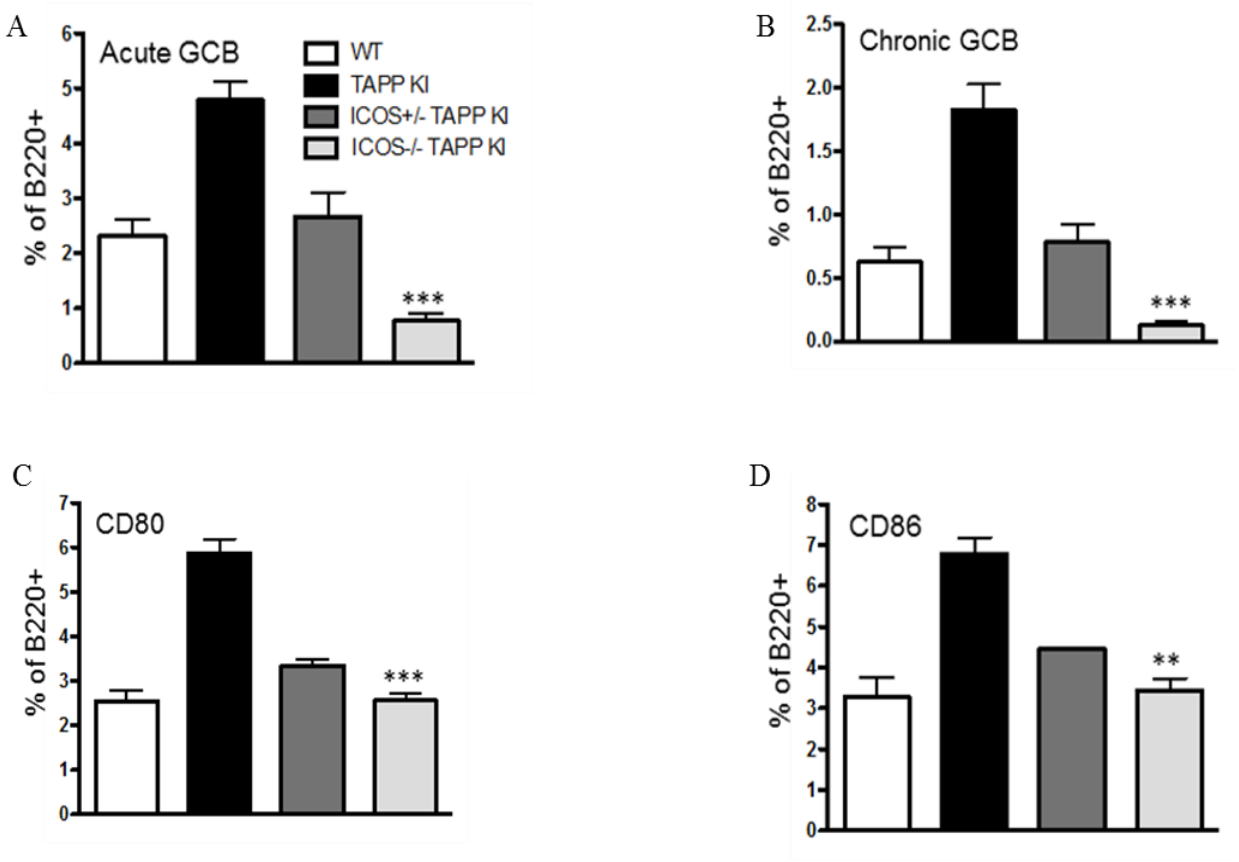

Figure 4.5 Impaired germinal center in TAPP KI mice in the absence of ICOS

(A) Young adult WT, TAPP KI, ICOS+/- TAPP KI and *Icos*-/- TAPP KI mice were immunized with sheep red blood cells and sacrificed after day 7. The splenocytes from these mice were stained for GCB cells ($GL7^+FAS^+$ $B220^+$ cells) and was analyzed by flow cytometry. (B) Middle aged (25 weeks) WT, TAPP KI, *Icos*+/- TAPP KI and *Icos*-/- TAPP KI were tested for chronic GCB and (C,D) percentage expression of CD80 and CD86 within total B cells. The bar graph represents the mean and standard error pooled from at least 2 separate experiments with 3-5 mice per group. Panels A to D, statistical comparisons between TAPP KI with *Icos*+/- TAPPKI or *Icos* -/- TAPPKI were performed using a two-tailed unpaired Student's t-test. Significance is represented as **P< 0.001 and ***P< 0.0001.

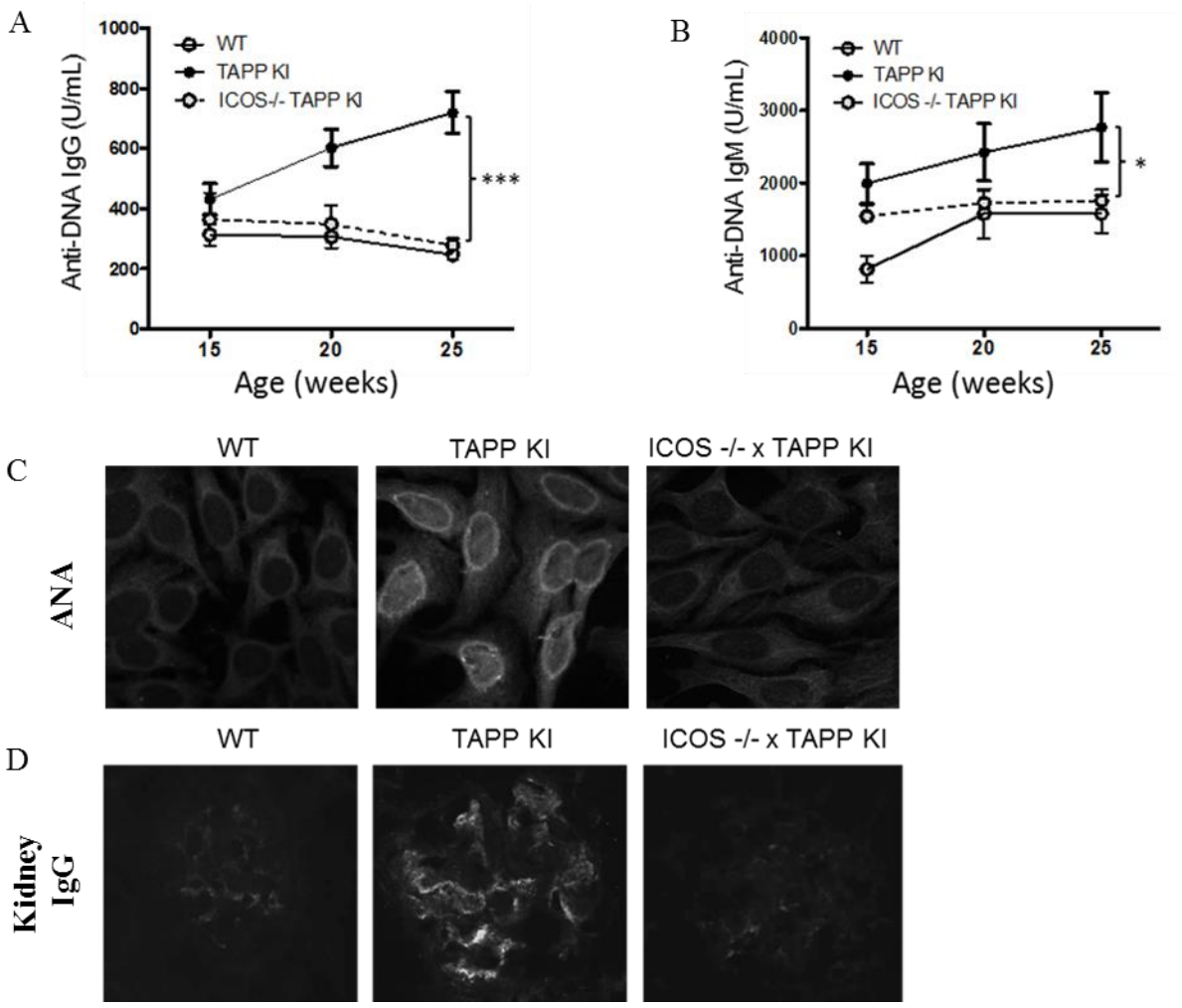

Figure 4.6 Chronic GC is essential for aging TAPP KI mice to develop autoantibodies

(A and B) Aging WT, TAPP KI and *Icos*-/- TAPP KI mice from which serum collected at 15th, 20th and 25th week were tested for anti-dsDNA (IgG and IgM) by ELISA. (C) Immunofluorescence staining of HEp2 cells to detect anti-nuclear antibodies (ANA) within the serum of middle aged mice (25 weeks). (D) Immunofluorescence staining representing IgG deposition within the kidney of middle aged mice. A and B graphs represent the mean and standard error where a comparison between TAPP KI and *Icos*-/- TAPPKI is made using 2-way ANOVA. Significance is represented as *P< 0.05 and ***P< 0.0001.

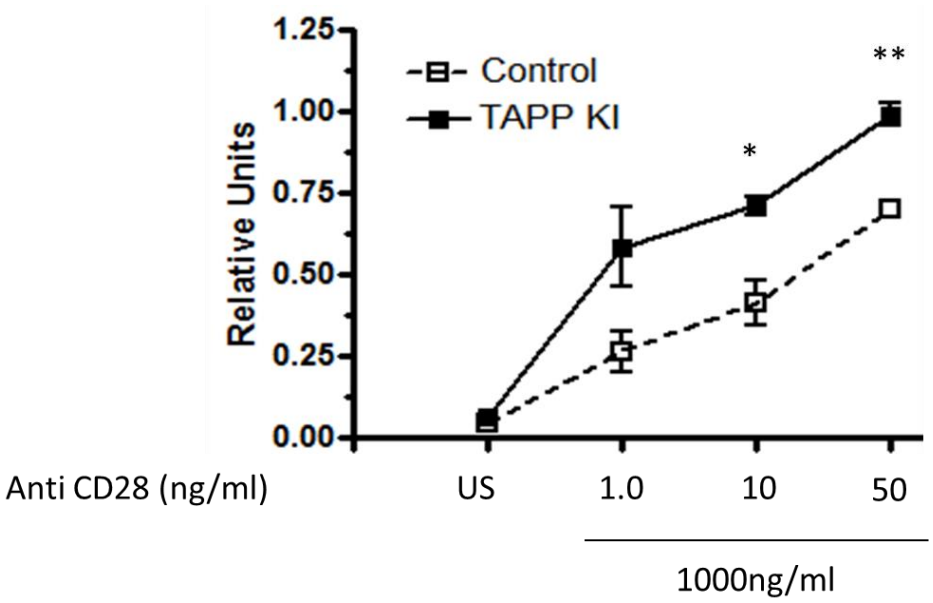

Figure 4.7 TAPP KI CD4$^+$ T cell intrinsic defect

CD4$^+$ T cells purified from WT control or TAPP KI splenocytes were stimulated with plate bound anti-CD3 (1000ng/ml) along with soluble anti-CD28 in doses indicated above. MTT assay was performed after 72 hours of stimulation. The data is representative of 3 independent experiments. The statistical comparisons between WT and TAPPKI CD4$^+$ T cells were performed using a two-tailed unpaired Student's t-test. Significance is represented as *P< 0.05 and **P< 0.001.

4.2.4 B cell intrinsic role of TAPP-membrane interaction mediating protection from autoimmunity

We asked if a B cell intrinsic defect contributed to the development of spontaneous chronic GC and autoimmunity in TAPP KI mice. Mixed bone marrow (BM) chimera experiments were performed by reconstituting irradiated B cell deficient mice (μMT mice) with WT or TAPP KI bone marrow to determine whether B cells derived from TAPP KI donors alone can drive chronic GC and autoimmunity within the recipient mice. WT and KI B cells in the peripheral blood showed uniform reconstitution in the recipient μMT mice (Fig 4.8A). TAPP KI BM recipient μMT mice (chimeric mice) also showed a percentage increase in CD80 and CD86 expressing B cells in the blood 6 weeks after reconstitution (Fig 4.8B). Middle aged TAPP KI BM recipient μMT mice exhibited splenic GCB and TFH percentages substantially increased on comparison with WT BM recipients (Fig 4.8C). TAPP KI BM chimeric mice developed anti-dsDNA antibody in the serum with age (Fig 4.9A), anti-nuclear antibodies (Fig 4.9B) and deposition of IgG within the kidney (Fig 4.9C) which was absent in WT BM recipients. Together these findings indicate the B cell intrinsic role of the TAPP mutation in mediating B cell hyper responsiveness and development of autoimmunity.

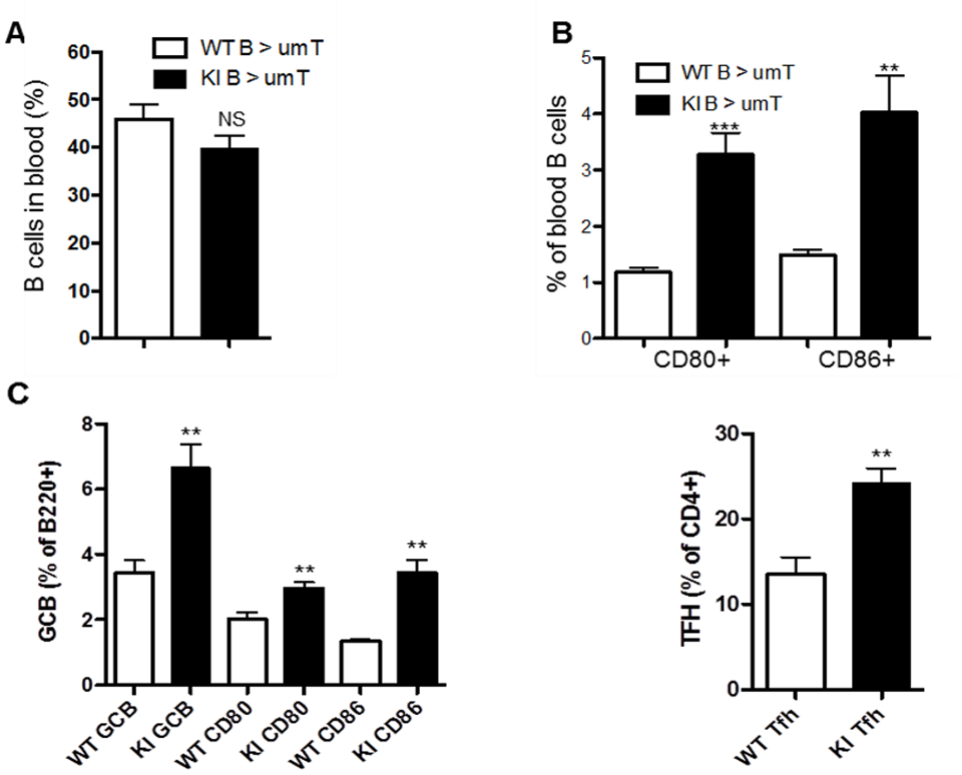

Figure 4.8 B cell intrinsic role of TAPP KI in spontaneous B cell activation and chronic GC

Mixed bone marrow chimera was developed by mixing BM from WT or TAPP KI mice with BM from B cell deficient mice (μmT mice) at a 1:4 ratio followed by transfer into irradiated μmT recipient mice. Peripheral blood collected 6 weeks after reconstitution was tested by flow cytometry (A) to confirm B cell reconstitution and to determine (B) expression of CD80 and CD86 costimulatory molecules by B cells. (C) Splenocytes derived from middle aged mice (at 25 weeks) were stained for GC B cells, B cells expressing CD80 and CD86 (left) and TFH population (right). All these graphs represent the mean and standard error pooled from 2 experiments with 6-7 mice per group. Statistical comparison between WT and TAPP KI BM recipients were performed using two-tailed unpaired Student's t-test. Significance is represented as NS, **P< 0.001 and ***P< 0.0001.

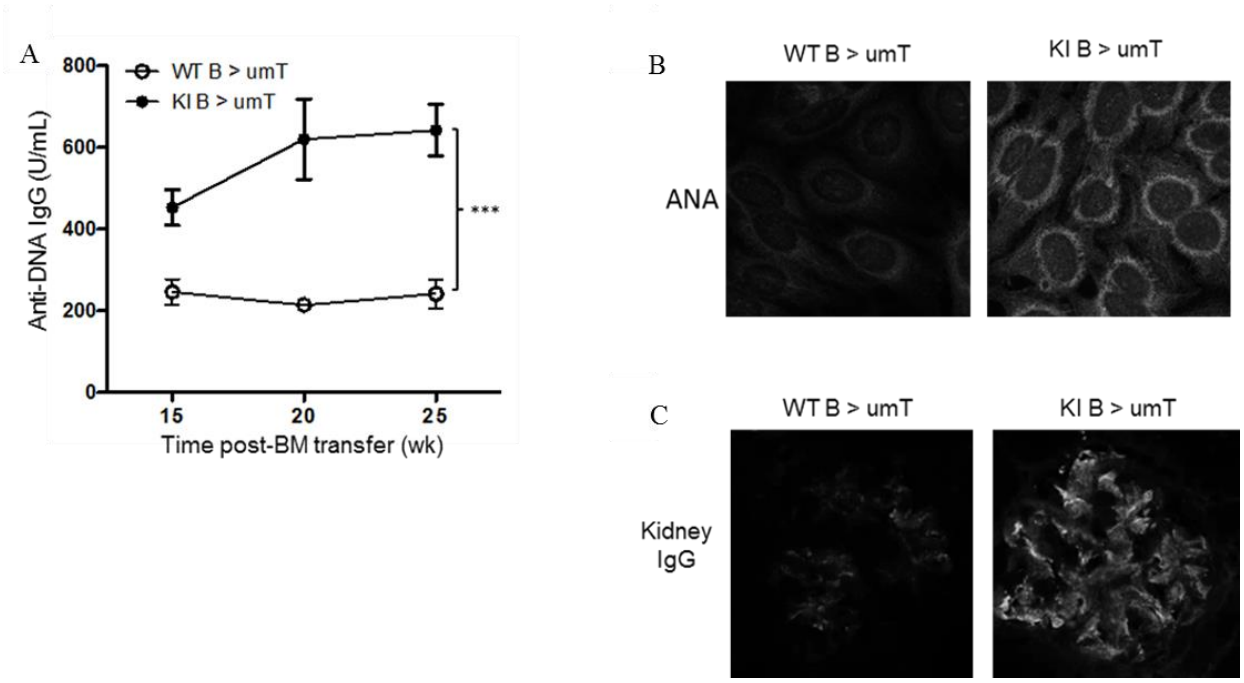

Figure 4.9 B cell intrinsic role of TAPP KI in the development of autoimmunity

Mixed bone marrow chimera was developed by mixing BM from WT or TAPP KI mice with BM from B cell deficient mice (μmT mice) at a 1:4 ratio followed by transfer into irradiated μmT recipient mice. (A) Serum collected from recipient mice at 15, 20 and 25 weeks were tested for anti-dsDNA antibody (IgG isotype). (B) Serum derived from mice at week 25 was tested for ANA by staining Hep2 cells. (C) Kidney harvested at week 25 were snap frozen and sectioned to detect IgG deposition in the glomerulus by immunofluorescence staining. Data was combined from 2 experiments with 6-7 mice per group. Statistical comparison between WT and TAPP KI BM recipients was done for (A) with 2 - way ANOVA. Significance is represented as ***P< 0.0001.

4.3 Discussion

The development of germinal centers (GC) is essential to generate class switched and antigen specific antibody responses during infections. The presence of abnormal chronic germinal centers is a characteristic feature of lupus like autoimmune disease in mice as well as humans [159, 162]. PI3K/AKT pathway activation is known to be associated with the generation of the GC response. Therefore P110δ mutant mice with impaired PI3K signaling in lymphocytes lack GC [245]. However, TAPP KI mice that exhibited enhanced PI3K pathway developed chronic GC and autoimmunity.

The GC response results from interactions between different subsets that include GC B cells and TFH cells [348]. Although blocking this cellular interaction can ablate the GC response, an abnormal increase in these subsets is associated with aberrant GC response and autoantibody production. The percentage of GCB and TFH populations was found to be significantly higher in TAPP KI mice at middle age. GCB cells in particular exhibited a progressive increase in TAPP KI mice that was significantly higher than that of normal aging mice. These findings in the GC could be correlated with an increased anti-dsDNA antibody in their serum as shown in chapter-3. Another feature of this GC is that they are TAPP KI gene dose dependent. Presence of a single WT allele of TAPP1 or 2 is sufficient to reverse the abnormal GC in these mice.

ICOS is an important molecule expressed by TFH cells that is essential for the GCB-TFH interaction [61]. To further understand the role of TAPP KI GC in the generation of autoantibodies, we ablated the GC by crossing *Icos*-/- mice with TAPP KI mice. The *Icos*-/- TAPP KI mice showed severe impairment in the development of a chronic GC response and autoimmunity. Therefore these findings from *Icos*-/- TAPP KI mice indicate that chronic GCs

are critical in driving autoimmunity in TAPP KI mice. While TAPP KI B cells are clearly hyper-responsive, my data indicated there is also a T cell intrinsic role of TAPPs (Fig 4.7).

To investigate whether lupus like characteristics in TAPP KI mice is B or T cell mediated, mixed bone marrow chimera experiment was performed. Findings from this approach tell us that B cells are sufficient to drive the development of chronic GC, including expansion of TFH cells. Presence of autoimmune characteristics within the middle aged muMT (B cell deficient) recipients that obtained BM cells from TAPP KI mice suggests a B cell intrinsic role of TAPPs in the generation of autoantibodies. However, my data does not rule out an additional role for TAPP KI T cell abnormalities in contributing to the disease. Future studies can include investigating the involvement of other immune subsets in the development of autoimmunity in TAPP KI mice.

Abnormal germinal center response being crucial in the development of lupus, disrupting the GC by blocking GCB –TFH interactions or targeting the mechanisms regulating GC B cells can be a promising approach to design treatment methods for SLE. Further studies could use monoclonal antibodies blocking the co-stimulatory molecules such as ICOS or CD40 which are essential for GCB–TFH interactions.

Chapter 5

TAPP KI B cells exhibit metabolic defects contributing to B cell hyper activation and autoimmunity

This chapter is based on

(1) Part of the manuscript that is under preparation along with some additional data

Nipun Jayachandran, Edgard M. Mejia, Kimia Sheikholeslami, Sen Hou, Grant M. Hatch, Dario R. Alessi and Aaron J. Marshall. Control of B cell glycolysis via the PI3K/Akt pathway: modulation by TAPP adaptors.

5.1 Introduction

Metabolic regulation of B cells is essential for their survival, activation, proliferation, antibody production and for mediating anergy. Unlike $CD4^+$ T cells, B cell upon activation exhibits a balanced increase in both glycolysis and mitochondrial respiration. Although both of these aspects of metabolism are upregulated in activated B cells, blocking glycolysis showed a more severe impairment in proliferation and antibody production. Targeting glycolysis by knocking out GLUT1 or using small molecule inhibitors negatively regulates B cell response [276].

One of the mechanisms by which B cell metabolism is regulated is though PI3K activation. PI3Ks play a crucial role in glycolysis by regulating glucose transporter expression, glucose uptake and activation of enzymes in the pathway [265, 276, 354]. Downregulation of glycolysis can be achieved by targeting PI3K signaling with small molecule inhibitors or co-ligating BCR along with the inhibitory FcγRIIB receptor [265]. Chronically active B cells which are metabolically active, and anergic B cells that are metabolically quiescent, show a correlation with their PI3K activity [276, 299, 355]. Loss of B cell tolerance due to PI3K dysregulation is associated with lupus like autoimmune disease. Autoreactive B cells isolated from these mouse models exhibit enhanced glucose uptake activity [276].

TAPP KI mice showed chronic activation of B cells and developed autoimmunity with age. Spontaneous GC is crucial in the development of autoimmunity in TAPP KI mice. The bone marrow chimera experiment showed a B cell intrinsic role of TAPPs in the development of chronic GC and autoimmunity. TAPP KI B cells exhibited enhanced PI3K signaling upon BCR crosslinking. Metabolic defects driving B cell hyper activation is demonstrated in lupus prone mice [288]. In this chapter, I have explored the role of glycolysis contributing to TAPP KI B cell hyper activation, development of chronic GC and production of autoantibodies.

5.2 Results

5.2.1 TAPP KI B cells exhibit hyper-responsiveness to multiple stimuli, in a gene dose-dependent manner

Purified TAPP KI B cells were stimulated with BCR crosslinking and T cell dependent stimulation in vitro. When compared to normal B cells, TAPP KI B cells exhibited a significantly enhanced proliferative response as measured by MTT assay (Fig 5.1A to C). Unlike anti-IgM F(ab)'2 that crosslink only the BCR, intact IgM that co-ligate the BCR along with the inhibitory receptor FcγRIIB, significantly reduce the overall cellular response. TAPP KI B cells were still able to display a greater response under this condition, clearly exhibiting a much higher cell intrinsic activity than WT (Fig 5.1B). The hyper-responsiveness exhibited by TAPP KI B cells is gene dose dependent. B cells derived from heterozygous mice carrying a single normal allele for TAPP1 and TAPP2 completely reverses this defect under both anti-IgM F(ab)'2 and anti-CD40+IL-4 stimulation conditions (Fig 5.2A and B). It should be noted that MTT assay measures total live cells at the endpoint via activity of a metabolic enzyme, thus this readout is influenced by total number of viable cells at the endpoint, as well as their level of metabolic activity. Although, the cell survival remains comparable with that of the WT (Fig 5.3B) TAPP KI B cells exhibited a significant increase in cell size upon stimulation (Fig 5.3A). Increased cell size is also considered an indicator of increased metabolic activity in lymphocytes [299].

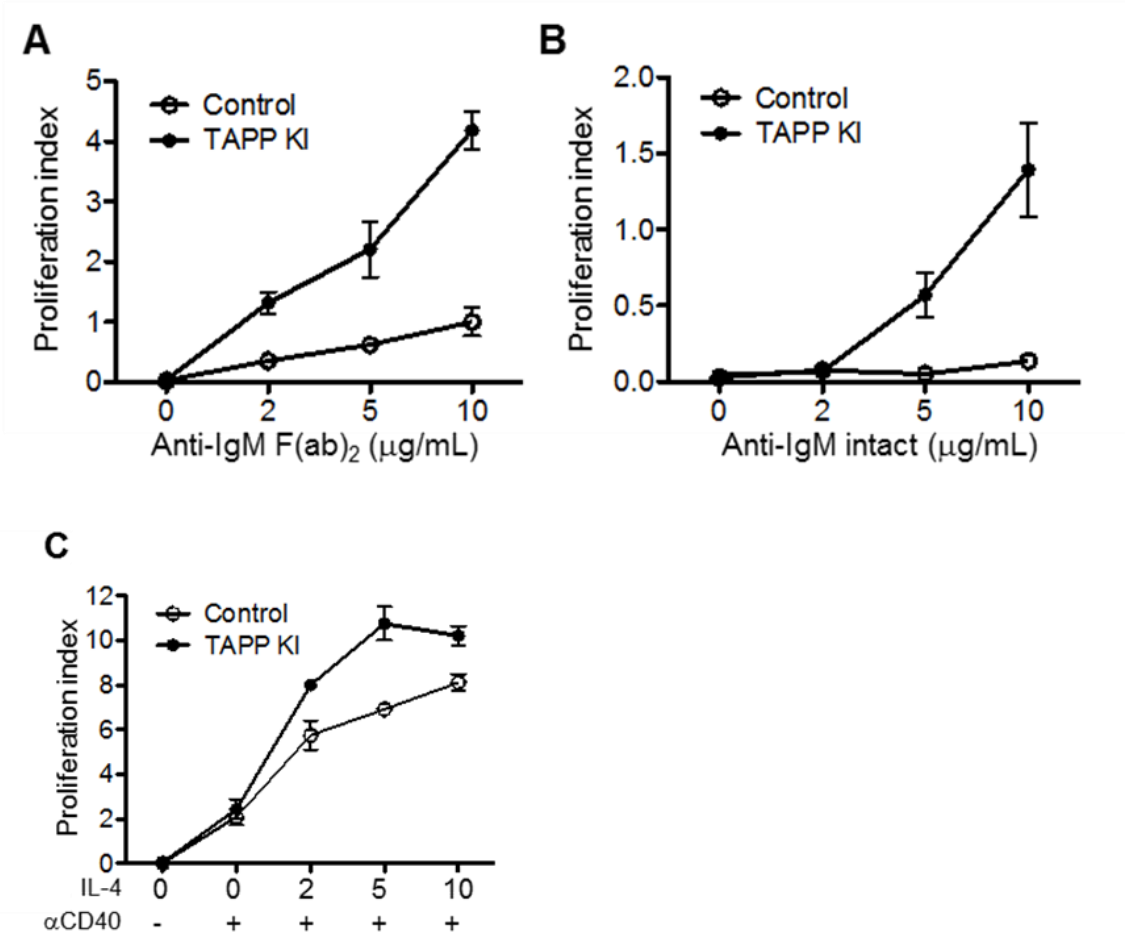

Figure 5.1 TAPP KI B cells exhibiting hyper responsiveness upon PI3K activation

Splenic B cells purified from young adult TAPP KI mice were stimulated for 72 hrs to measure proliferation using MTT assay. B cells stimulated with (A) Anti-IgM F(ab')$_2$, (B) intact anti-IgM or (C) a combination of anti-CD40 (1μg/ml) and IL-4 with doses indicated. All these data are representative figures from 2 or more experiments.

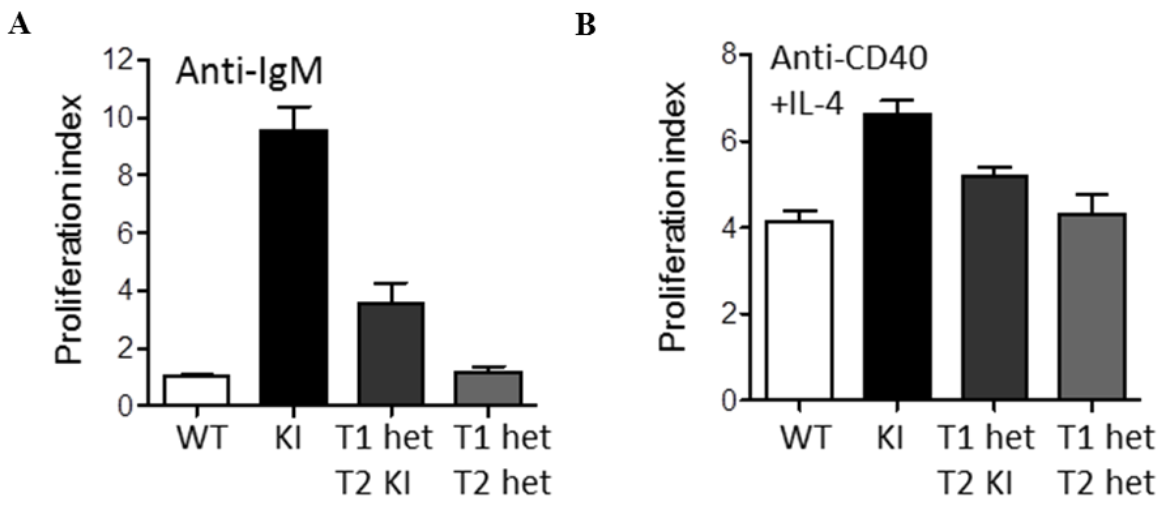

Figure 5.2 TAPP KI B cell hyper -activation is gene dose dependent

Splenic B cells purified from young adult TAPP KI mice are stimulated for 72 hrs to measure proliferation using the MTT assay. (A) To determine the effect of gene dose on hyper responsiveness of B cells stimulated with Anti-IgM $F(ab')_2$ - comparing WT (white), TAPP1 KI/KI x TAPP2 KI/KI (black), single heterozygous TAPP1 WT/KI x TAPP2 KI/KI (dark grey) or double heterozygous TAPP1 WT/KI x TAPP2 WT/KI (light grey). (B) B cell stimulated with 1µg/ml anti-CD40 and 5ng/mL IL-4 for the same genotypes indicated in A. All these data are representative figures from 2 or more experiments.

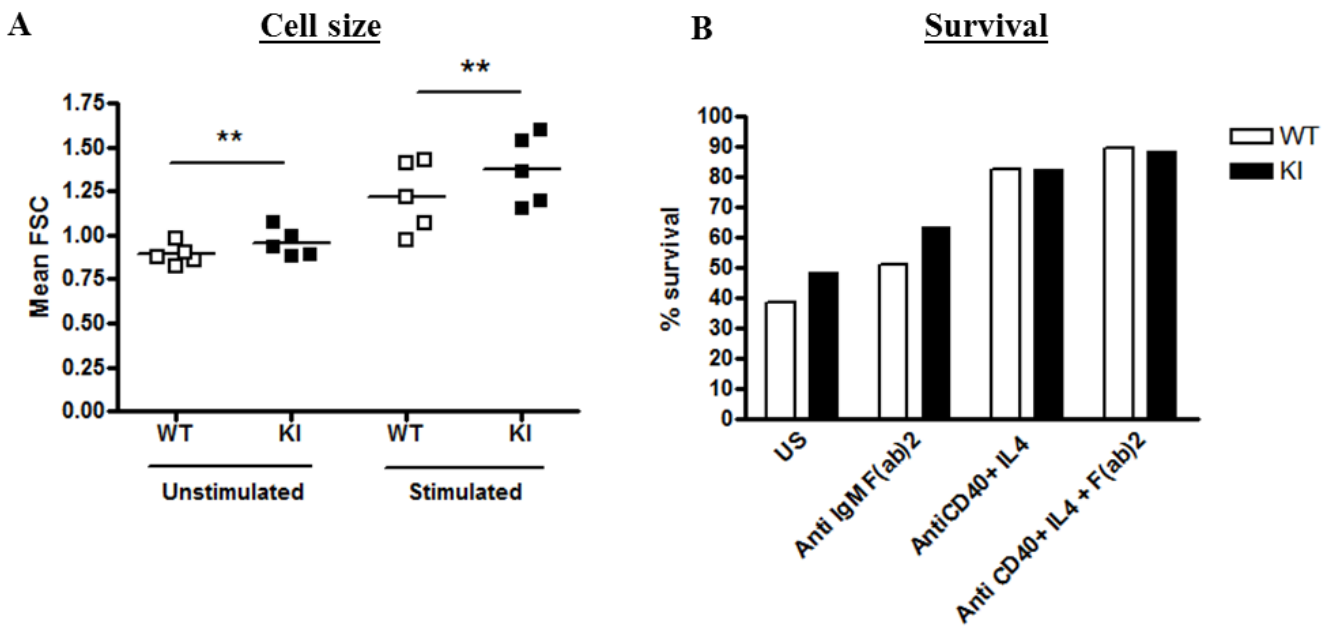

Figure 5.3 TAPP KI B cells exhibit an increase in cell size

B cells isolated from spleen is stimulated with (anti-CD40 (1µg/ml) +IL-4 (5ng/ml) + anti-IgM $F(ab')_2$ (10µg/ml)) for 24 hrs. (A) Cell size measurement was taken by gating total live cells to obtain the mean forward scatter. (B) The percentage survival of B cells was assessed by flow cytometry by gating the cells that are negative for apoptotic markers such as Annexin V and 7AAD. The data for (A) was combined from 5 experiments and (B) is a representative figure from 2 or more experiments. Statistical comparison was done between WT and TAPP KI B cells with a two-tailed unpaired Student's t-test. Significance is represented as **P< 0.001 for panel A, while panel B did not show any significant difference for conditions indicated above.

5.2.2 Defective regulation of cellular metabolism in TAPP KI B cells

To determine whether the hyperresponsiveness exhibited by TAPP KI B cells is associated with defective regulation of metabolic activity, we measured mitochondrial respiration and glycolysis 24 hours after B cell stimulation using seahorse metabolic flux analyzer. Mitochondrial respiration, which is assessed by measuring oxygen consumption rate (OCR), showed an increase in both basal and maximal respiration rate under anti-IgM or anti-CD40+IL-4 stimulations (Fig 5.4A). Measurement of extracellular acidification rate (ECAR) as a proxy for lactate production (a major product of glycolysis) revealed that TAPP KI B cells have substantially increased glycolytic activity under different stimulation conditions (Fig 5.4B).

PI3K/AKT signaling is previously found to regulate glucose transporter (GLUT) expression [354]. To investigate the mechanism underlying increased glycolysis in TAPP KI B cells, the expression of GLUT1 in these cells was tested. In vitro stimulation of TAPP KI B cells revealed a greater increase in expression of GLUT1 when compared to wildtype controls as indicated by flow cytometry (Fig 5.5A).Upon gating freshly isolated splenocytes for B cell subsets ex vivo, only TAPP KI germinal center B cells showed significant upregulation of GLUT1 expression (Fig 5.5B). We then determined whether increased GLUT1 expression is associated with increased rate of glucose uptake, using an assay based on the fluorescent labelled glucose analogue, 2-NBDG. TAPP KI B cells on in vitro stimulation exhibited a substantial increase in 2-NBDG uptake (Fig 5.5C) which is consistent with increased glucose transporter activity. We further investigated glucose in the B cell subsets in vivo by injecting mice with 2-NBDG and sacrificing 1 hr later for flow cytometry analysis. TAPP KI B cells showed significantly increased 2-NBDG uptake, with GC B cell subset showing the greatest elevations (Fig 5.5D).

These findings together suggest that TAPP uncoupling from PI(3,4)P2 results in dysregulation of B cell metabolic programing including increased glucose uptake within GC B cells.

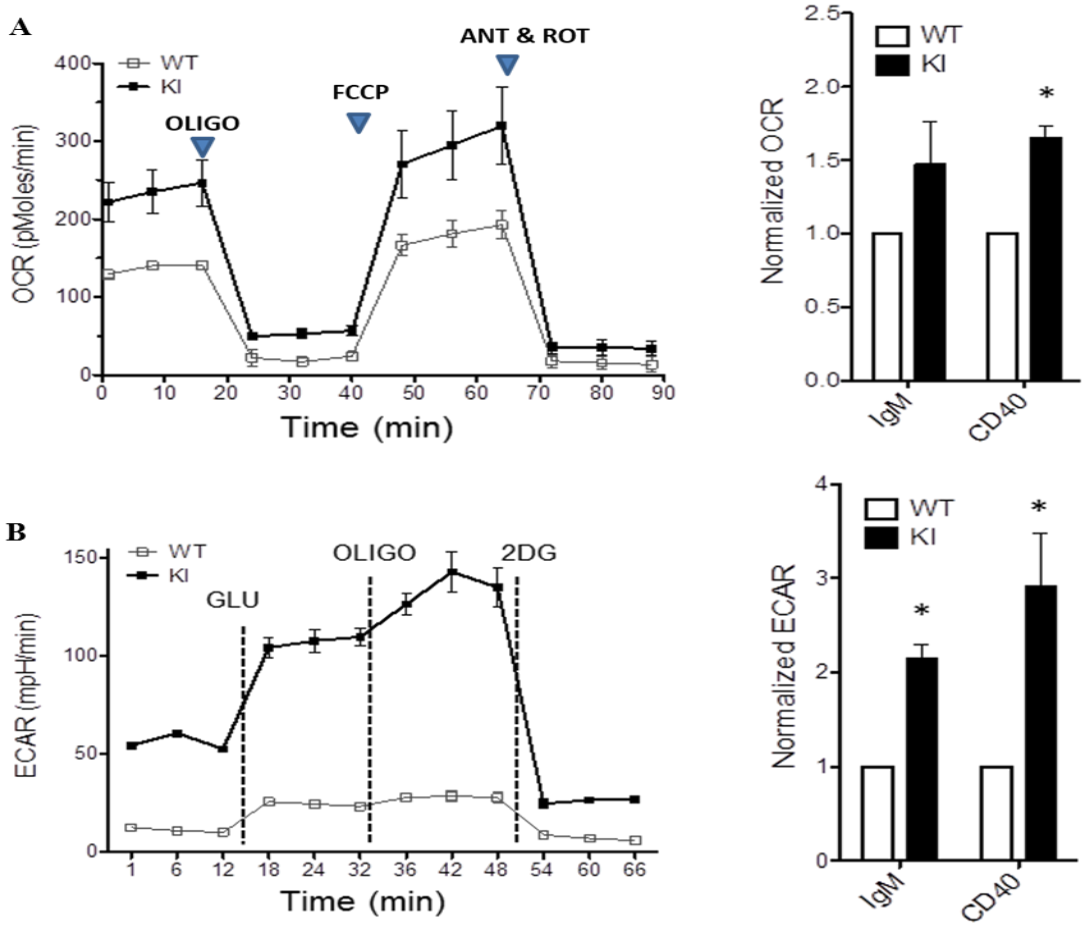

Figure 5.4 Defective regulation of cellular metabolism by TAPP KI B cells

Purified B cells from spleen were stimulated with anti IgM or 1 µg/ml anti-CD40 + 5 ng/ml IL-4 for 24 hrs, and then freshly harvested cells from culture were attached over seahorse assay plate to measure metabolic response. (A) Mitochondrial respiration was measured based on oxygen consumption rate (OCR) tested in the presence of drugs – oligomycin (OLIGO), FCCP and Antimycin & Rotenone (ANT & ROT) to determine basal and maximal respiration as described under materials and methods (B) Glycolytic activity of the cell was measured based on extracellular acidification rate (ECAR), tested in presence of glucose (GLU) followed by drugs

such as oligomycin (OLIGO) and 2-Deoxy-D-glucose (2-DG) to measure basal and maximal glycolytic activity. For the experiments, real time measurements on the left and the normalized data is shown on the right. Data is representative of 3-4 independent experiments. Statistical comparison between WT and TAPP KI was done with a two-tailed unpaired Student's t-test. Significance is represented as *P< 0.05.

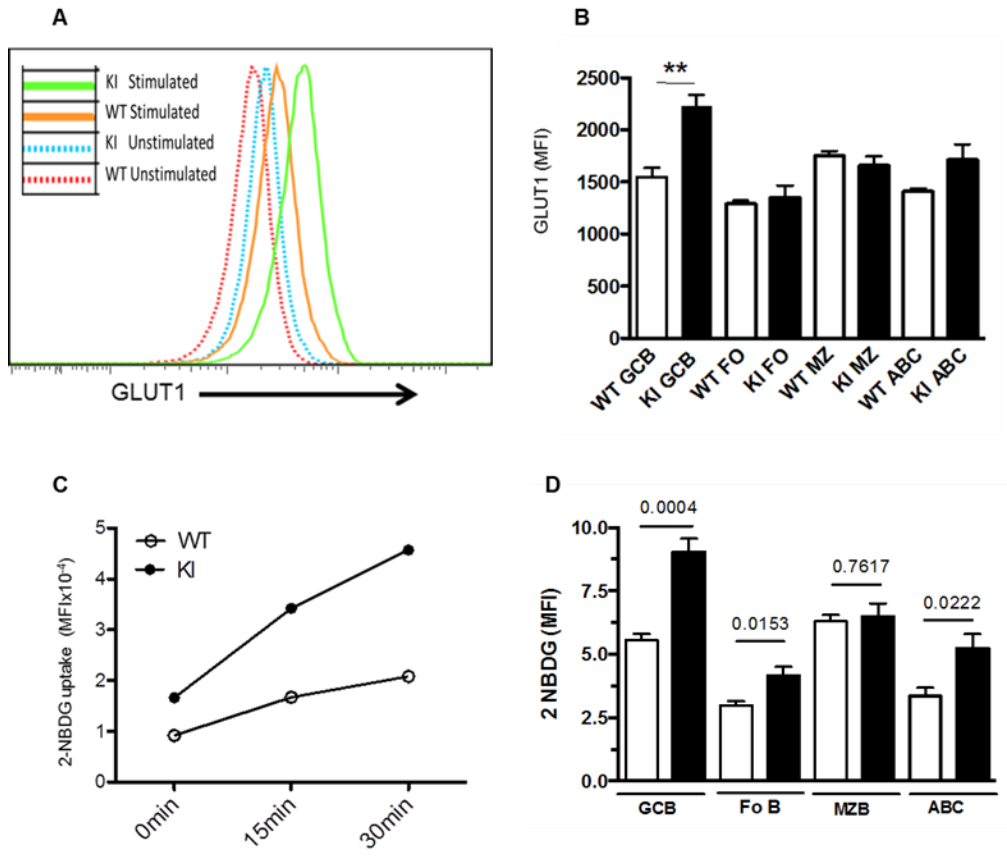

Figure 5.5 Defects in GLUT1 expression and glucose uptake by TAPP KI B cells

(A) B cell stimulated for 24 hrs in culture with anti-CD40 (1µg/ml) +IL-4 (5ng/ml) + anti-IgM F (ab')$_2$ (10µg/ml) were fixed permeabilized and stained for GLUT1 was tested by flow cytometry. (B) Splenocytes freshly isolated from mice were surface stained to identify different B cell subset; these cells were then stained with anti-GLUT1 antibody and were assessed by FACS. (C) B cell stimulated for 24 hrs in culture with anti-CD40 (1µg/ml) + IL-4 (5ng/ml) + anti-IgM F(ab')$_2$ (10µg/ml) were washed in glucose free medium and incubated with 2-NBDG (150µg/ml) for 0-30 mins to measure the uptake of this molecule by FACS. (D) Mice were immunized with SRBC for 7 days and was injected with 2mM 2-NBDG for 1 hr. Freshly harvested splenocytes

were surface stained as before to measure uptake of 2-NBDG under each subset of B cells. The data (A) and (C) is a representative figure from 3 or more independent experiments, (B) and (D) is data combined from 2 experiments with at least 5 mice per group. Statistical comparison between WT and TAPP KI was done using a two-tailed unpaired Student's t-test. Significance is represented as **P< 0.001.

5.2.3 TAPP KI B cell defect reversed by blocking PI3K or glycolysis

PI3K mediated control of cellular metabolism has been extensively studied [298, 354]. We asked whether dysregulated PI3K/AKT in TAPP KI B cells contributed to the observed metabolic defects. Addition of PI3K inhibitor Idelalisib reversed the B cell hyper-responsiveness in MTT assay in a dose dependent manner (Fig 5.6A). Consistent with earlier findings that demonstrated the role of PI3K/AKT signaling in glucose transport mechanisms, presence of Idelalisib in TAPP KI B cell culture down regulated the expression of GLUT1 and 2-NBDG uptake (Fig 5.6 B and C). Together these findings indicate that dysregulated PI3K signaling in TAPP KI B cells contributes to enhanced glucose uptake and hyper-activation. We further investigated whether the hyper-proliferative response of TAPP KI B cells could be normalized by directly targeting glycolysis using 2-DG. Addition of 2-DG reversed B cell hyper responsiveness in a dose dependent manner (Fig 5.6D), suggesting that TAPP KI B cell hyper response may be contributed largely due to glycolytic defect.

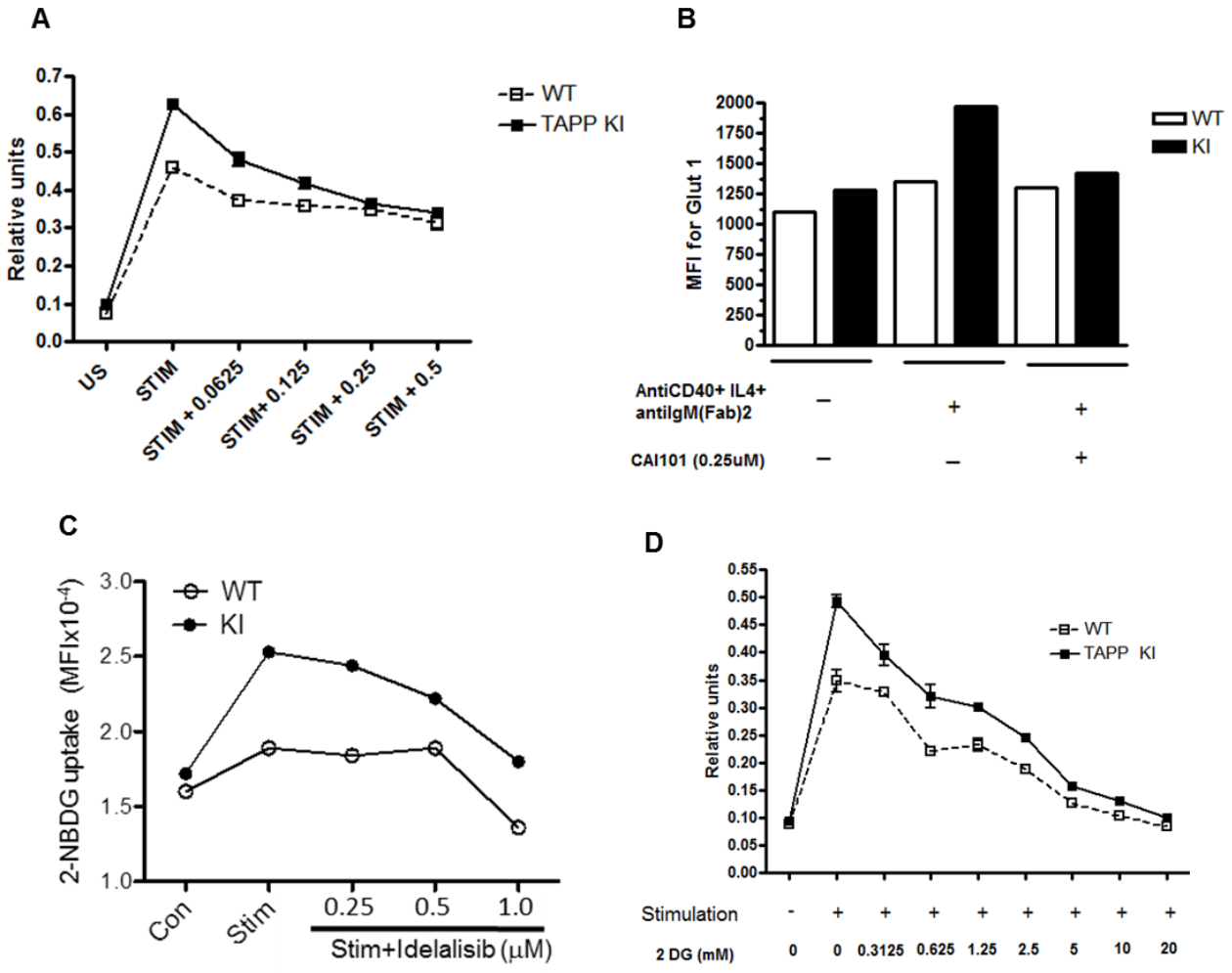

Figure 5.6 TAPP KI B cell hyper-activation inhibited by blocking PI3K or glycolysis

Purified splenic B cells were stimulated for 24 hrs in culture with anti-CD40 (1μg/ml) + IL-4 (5ng/ml) + anti-IgM F(ab')$_2$ (10μg/ml). (A) Different doses of PI3Kδ inhibitor, CAL-101 (Idelalisib) were added and proliferation was measured by MTT assay. (B) GLUT1 expression by B cells in the presence of Idelalisib, measured by flow cytometry. (C) Glucose uptake by B cells in the presence of varying doses of idelalisib, and measured by FACS. (D) TAPP KI B cell hyper responsiveness measured in the presence of indicated doses of 2-DG and measured by MTT assay. All these data are representative figures from 2 or more independent experiments.

5.2.4 Chronic GC and autoimmunity in TAPP KI mice can be treated by blocking glycolysis

The role of glycolysis in the development and maintenance of germinal centres has been demonstrated in a mouse model of systemic autoimmunity [288]. As TAPP KI mice develop spontaneous GCs with age, the role of glycolysis in TAPP KI GCB cells was investigated. Middle aged female TAPP KI mice were either treated with 2-DG in drinking water for 4 weeks or given normal water. The 2-DG treated group showed significant reduction in the frequency of GCB cells compared to the untreated group (Fig 5.7A), while total B cell percentages were unaltered. B cells exhibiting markers of chronic activation or memory such as CD86 were significantly reduced in frequency. Percentage of $CD80^+$ B cells also showed a decrease in trend upon treatment (Fig 5.7B). Importantly, the serum from these mice showed a significant reduction in titre of anti-dsDNA antibody detectable within two weeks of treatment (Fig 5.8A). The 4 fold reduction of the anti-dsDNA antibody levels at the end point showed a close correlation with the reduction of chronic GC B cell frequency (Fig 5.7A). Our published data on aging TAPP KI mice demonstrated additional lupus-like characteristics such as anti-nuclear antibody as well as IgG deposition within the glomerulus of kidney. An improvement in these disease characteristics were also observed on treating these animals with 2-DG (Fig 5.8B and C). Together these findings indicate that the autoimmune disease in TAPP KI mice can be effectively treated by attenuating glycolysis. These finding are consistent with the hypothesis that dysregulated glycolytic pathways within B cells may drive the development of chronic GCB cells and autoantibody production in TAPP KI mice.

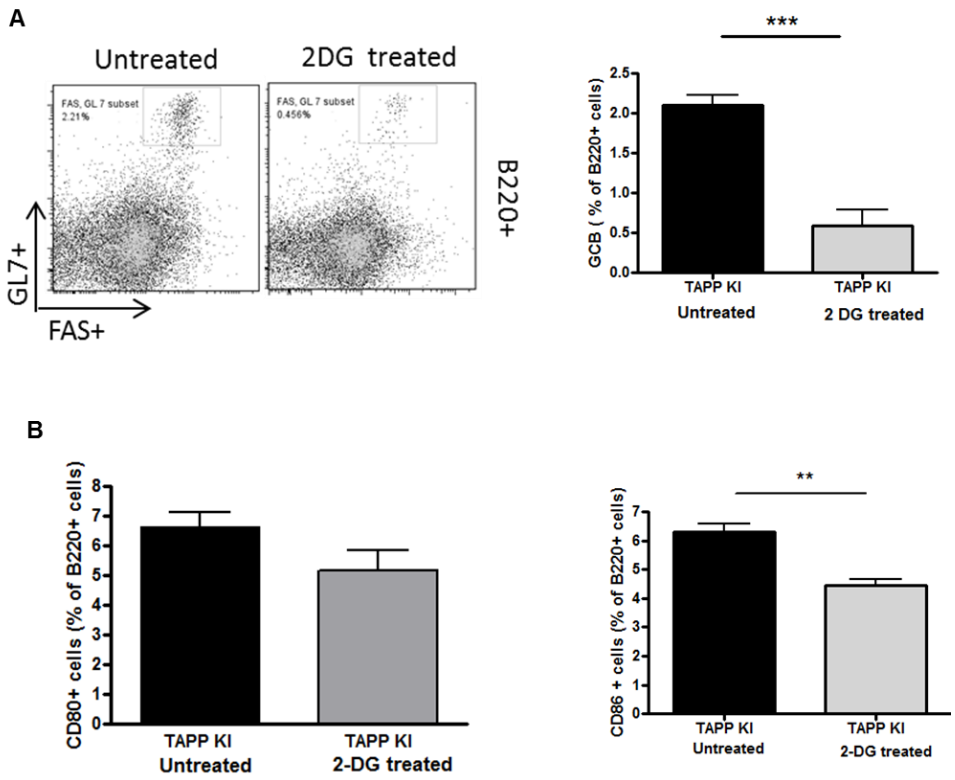

Figure 5.7 Chronic GC in TAPP KI mice reversed by 2-DG treatment

Middle-aged female TAPP KI mice treated with 2-DG in drinking water for 4 weeks were compared with untreated age/sex matched TAPP KI mice. Splenocytes were stained for B cell subsets and was assessed by flow cytometry (A) GC B cell gating ($B220^+$ $GL7^+$ FAS^+) is shown on the left and summary bar graph on the right for the mean percentage of GC B cells. (B) Percentage expression of CD80 (left) and CD86 (right) within total splenic B cell population. The bar graphs represent the mean and standard error of 4 mice per group. Statistical comparison between untreated and treated TAPP KI mice was done with a two-tailed unpaired Student's t-test. Significance is represented as ***P< 0.0001 and **P< 0.001 (A- right and B - right) while panel (B -left) although not statistically significant, it showed a decrease in trend, after treatment.

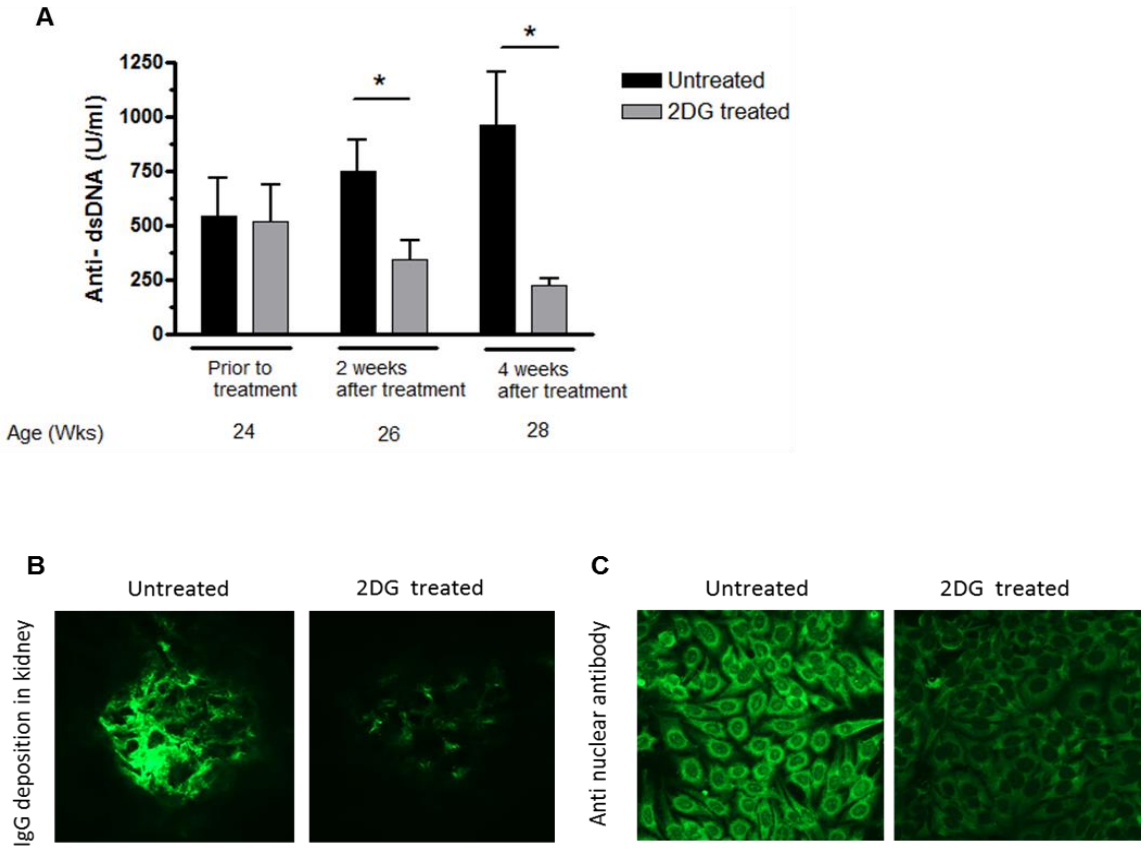

Figure 5.8 Autoimmunity in TAPP KI mice reversed by 2-DG treatment

Middle-aged female TAPP KI mice treated with 2-DG in drinking water for 4 weeks were compared with untreated age/sex matched TAPP KI mice. (A) Serum collected from these mice before and during the treatment was tested for anti-dsDNA antibody (IgG isotype). (B) Kidneys obtained during the endpoint were cryopreserved, sectioned and stained with fluorescent labelled IgG to measure immune complex deposition within the glomerulus. (C) HEp2 coated slides incubated with the endpoint serum and stained with fluorescently labeled secondary antibody to measure ANA. Data is representative of 4 mice per group. Statistical comparison between untreated and treated TAPP KI mice was done with a two-tailed unpaired Student's t-test. Significance is represented as *P< 0.05.

5.3 Discussion

Metabolic regulation of immune cells is strictly controlled by signals from activation and inhibitory receptors [265] . The proper regulation of these receptors is thought to be critical for mediating tolerance and B cell effector functions. Defective regulation of PI3K/AKT pathway has recently been implicated in driving altered metabolic activity in B cells [298]. Chronically stimulated B cells from BAFF transgenic mice were found to be highly metabolically active, whereas metabolism is suppressed in anergic B cells [276]. Metabolically active cells exhibit an increase in cell size which requires AKT mediated activation of mTORC1 signaling downstream [299]. Stimulation of TAPP KI B cells although did not display defects in survival, the hyper responsiveness which was measured by MTT along with increase in cell size, was an indication of metabolic defect. In addition to their increased metabolic activation after stimulation, TAPP KI B cells may be chronically more metabolically active prior to stimulation. I have observed markers of chronic activation expressed by TAPP KI B cells from young adult mice ex vivo (Fig 4.4B). Freshly purified B cells from these animals also showed increase in cell size and mitochondrial respiration (Fig 5.9), thus showing some similarity with BAFF transgenic B cells [276] as they also develop chronic B cell activation and autoimmunity [33].

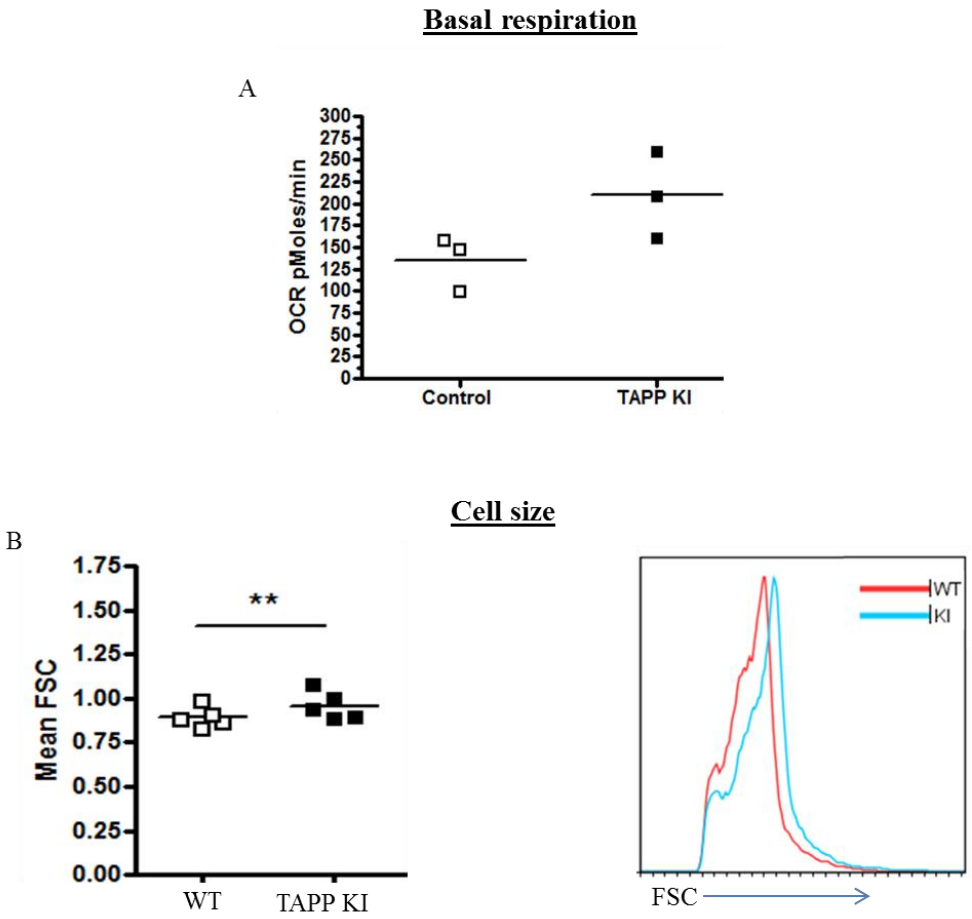

Figure 5.9 Enhanced metabolic activity exhibited by TAPP KI B cells ex vivo

B cells were freshly isolated from WT control or TAPP KI splenocytes. (A) Sea horse assay to measure mitochondrial respiration ex vivo - basal respiration. (B) Cell size was measured by FACS, by gating total live cells to obtain the mean forward scatter summary graph (left) and histogram overlay on the (right). The data is representative of 3-5 independent experiments. WT to TAPP KI statistical comparison was performed using 2-tailed unpaired Student's t-test. Significance is indicated as **P< 0.001 (B-left) while (A) although was not statistically significant, it showed an increase in trend.

Available data from others indicate that B cell metabolic reprogramming upon activation is distinct from that of T cells. Activation of PI3K signaling in $CD4^+$ T cell upon in vitro stimulation, exhibited a metabolic reprograming that promotes switching to glycolysis from mitochondrial respiration [272]. Contrastingly, B cells upon stimulation, showed both the pathways of metabolism activated in a balanced manner. However, blocking glycolysis had a much stronger effect on antibody production when compared to inhibition of mitochondrial respiration [276]. Mechanisms regulating B cell metabolism is relatively poorly understood and requires further investigation.

One of the mechanisms by which glycolysis is regulated by PI3K pathway is by expression of glucose transporter [354]. Among different families of GLUTs, loss of GLUT1 showed the greatest impairment in B cell effector function [356]. Both B and T cells show a PI3K dependent upregulation of GLUT1 which is essential for their activation [265, 290]. Targeting glycolysis by 2-DG treatment or conditional knock out of GLUT1 in B cells impaired antibody response in mice [276, 288]. However, the impact of increased GLUT1 expression levels on antibody response remains unclear. My findings demonstrate that GLUT1 is differentially expressed among different B cell subsets and showed a correlation with in vivo glucose uptake. GCB cells showing the highest level of GLUT1 expression and glucose uptake, suggests the importance of glucose metabolism within this subset. As GC is maintained in a hypoxic environment, to support B cell selection mechanism, it may partially explain why glycolysis is favored by GCB cells [302] . As TAPP KI GCB cells exhibit a significantly higher upregulation of glucose uptake and GLUT1 levels compared to normal GCB, it is also possible that dysregulation of glycolysis occurs within TAPP KI GC. Based on these findings it is tempting to speculate that, this

metabolic defect in TAPP KI mice contributes to chronic and abnormally enlarged GC as well as autoantibody production.

Chronic activation of B cells is linked to autoimmunity in BAFF transgenic mice [33]. B cells from these mice exhibited a high metabolic profile. On the other hand, anergic B cells which are metabolically quiescent were unable to restore normal metabolic activity even after stimulation [276]. However, anergy is not the only tolerance mechanisms essential for peripheral B cell tolerance. In TAPP KI mice, that develop abnormal GCs, the defect in the selection mechanism that removes low affinity or autoreactive B cells may be disrupted. Excess activation of PI3K signaling contributing to metabolic upregulation within the TAPP KI GC could possibly be helping these B cells to escape cell death during selection and thus driving autoantibody production.

TAPP KI hyper-responsiveness could be reversed using inhibitors that target PI3Kδ and this was associated with normalized GLUT1 expression and glucose uptake. This finding is consistent with the hypothesis that increased PI3K signaling is driving the hyper activation and metabolic dysregulation of TAPP KI B cells. Defective regulation of PI3K signaling is associated with chronic GC and autoimmunity [124]. Lupus like disease in MRL/lpr mice was reversed using P110δ inhibitor [248]. TAPP KI GC was shown to exhibit enhanced AKT phosphorylation previously by our lab, indicating elevated PI3K activity. My recent findings demonstrate high glycolytic activity within TAPP KI GC which was indicated by increased glucose uptake and GLUT1 expression. Cells which are highly dependent on glycolytic pathway such as cancer cells become increasingly susceptible to inhibition of glycolysis. I found that 2-DG treatment in aging TAPP KI mice reversed chronic GC, suggesting that GCB cells are highly sensitive to blockade of glycolysis. It is interesting to note that, the treatment has only impaired the GCB population

while the frequency of other B cell subsets were unaltered for the given dose and thus indicating the specificity of the treatment. In addition to the disrupted GC response, these animals also showed a corresponding reduction in anti-dsDNA antibody titer.Although GC response is clearly reduced in treated TAPP KI mice, we are not excluding the possibility of long lived plasma cells that could have been targeted upon 2-DG treatment. Plasma cells within the hypoxic environment of BM being highly glycolysis dependent, their status within TAPP KI mice are to be explored further.

As 2-DG treatment reversed autoantibody-mediated disease in these animals, it confirms the role of dysregulated glycolysis contributing to the defects in TAPP KI mice. Therefore, targeting PI3K/AKT dependent metabolic regulation of B cells, using drugs that block PI3Ks or downstream metabolic enzymes, can be a promising approach to treat SLE.

Chapter 6

General discussion

6.1 Major findings

- Uncoupling of TAPPs from the membrane leads to lupus like characteristics in aging TAPP KI mice.
- Chronic germinal centers are essential for TAPP KI mice to develop autoimmunity.
- TAPP KI mice exhibit a B cell intrinsic defect contributing to chronic GC and autoimmunity.
- TAPP KI B cells exhibit an abnormality in glycolysis that is reversed by blocking PI3K pathway.
- Chronic GC and autoimmunity in TAPP KI mice is treated by blocking glycolysis.

6.2 Significance

B cell mediated diseases such as lupus are prevalent in North America. According to lupus Canada nearly 1 in 10,000 Canadians are living with lupus and women are more susceptible to this disease than men. Lupus is a systemic autoimmune disease characterized by presence of autoantibodies, where chronic activation of B cells plays a crucial role contributing to the development of the disease. The conventional treatment methods using anti-inflammatory drugs, lack specificity and show a range of side effects. Although the modern treatment methods are more specific as they are designed to target B cells, they still exhibited limitations of their own as discussed before. In addition to that, heterogeneity among lupus patients remains one of the

major challenges as these individuals don't respond uniformly to treatment. Therefore, it is important to consider new approaches to develop a more improved treatment for the disease.

More than 50 lupus susceptibility loci are known today and many of those are associated with B cell antigen receptor (BCR) signaling. Others have shown that dysregulation of PI3K signaling by targeting regulatory phosphatases drives the development of lupus-like disease in mice. Although at this point, polymorphisms in TAPP associated with the development of lupus in humans is not clear, my findings identifying the role of TAPPs in B cells signaling contribute to our understanding of the biology of B cell regulation relevant to autoantibody generation. Identifying novel regulatory mechanisms in B cells signaling will expose new targets for developing treatment for lupus.

The role of chronic GC associated with lupus is widely reported in patients and mouse models. Targeting GC is a possible approach to reverse autoimmunity as we have demonstrated in TAPP KI mice. Identifying the importance of blocking chronic GC as an approach to treat development of autoantibody mediated disease like lupus is an approach can be considered. Monoclonal antibody blocking the key molecules essential for GCB – TFH interaction will be one possible way to disrupt the GC in these individuals. In addition to that, GCB cells being metabolically distinct, allows their specific targeting.

We are the first to show that B cells subsets have distinct glycolytic requirements in vivo. This will distinguish these subsets based on their glycolytic needs and thus exposing their vulnerabilities. Chronic GC and autoimmunity in aging TAPP KI mice was reversed by blocking glycolysis which is consistent with the studies previously done by Morell's group by blocking

pathways of metabolism in another lupus model. These findings in lupus prone mice are quite encouraging and will be potentially useful in developing treatment for lupus in humans.

Dysregulation of PI3K signaling and B cell hyperresponsiveness is also found to be linked to other diseases such as rheumatoid arthritis and B cell malignancies. Therefore some of those approaches to treat lupus may be applied against other B cell mediated diseases.

6.3 Integrated model of TAPP KI

My findings focused on understanding the role of TAPPs in B cells have indicated a negative feedback mechanism associated with PI3K signaling. Mutation that prevents TAPPs from translocating to the membrane on BCR crosslinking showed enhanced AKT phosphorylation and B cell hyper responsiveness. These mice develop high titres of antibodies at a young age, and as they get older, female TAPP KI mice progressively develop autoantibodies. While investigating the cellular mechanisms, I have identified the role of chronic germinal centres which are crucial in the production of these autoantibodies. Although TAPP mutation can affect other cell types, its B cell intrinsic role driving chronic GC and autoimmunity has been understood in this model.

Purified B cells from TAPP KI mice exhibit a metabolic defect with an increase in both mitochondrial respiration and glycolysis upon BCR crosslinking. Glycolytic abnormality in TAPP KI B cells can be reversed by blocking PI3K. This is consistent with the earlier findings that showed PI3K mediated control of metabolism. B cell subsets in mice show distinct glycolytic requirements with TAPP KI GCB cells exhibiting substantial increase in GLUT1 expression and glucose uptake. Inhibiting glycolysis by 2-DG, reversed TAPP KI B cell hyper responsiveness in vitro. In addition to that, treating aged TAPP KI mice with 2-DG blocked chronic GC and autoimmunity in these animals. Together these findings suggest that TAPP-membrane interaction is essential to prevent B cell hyper-activation and to mediate protection from the development of autoimmunity.

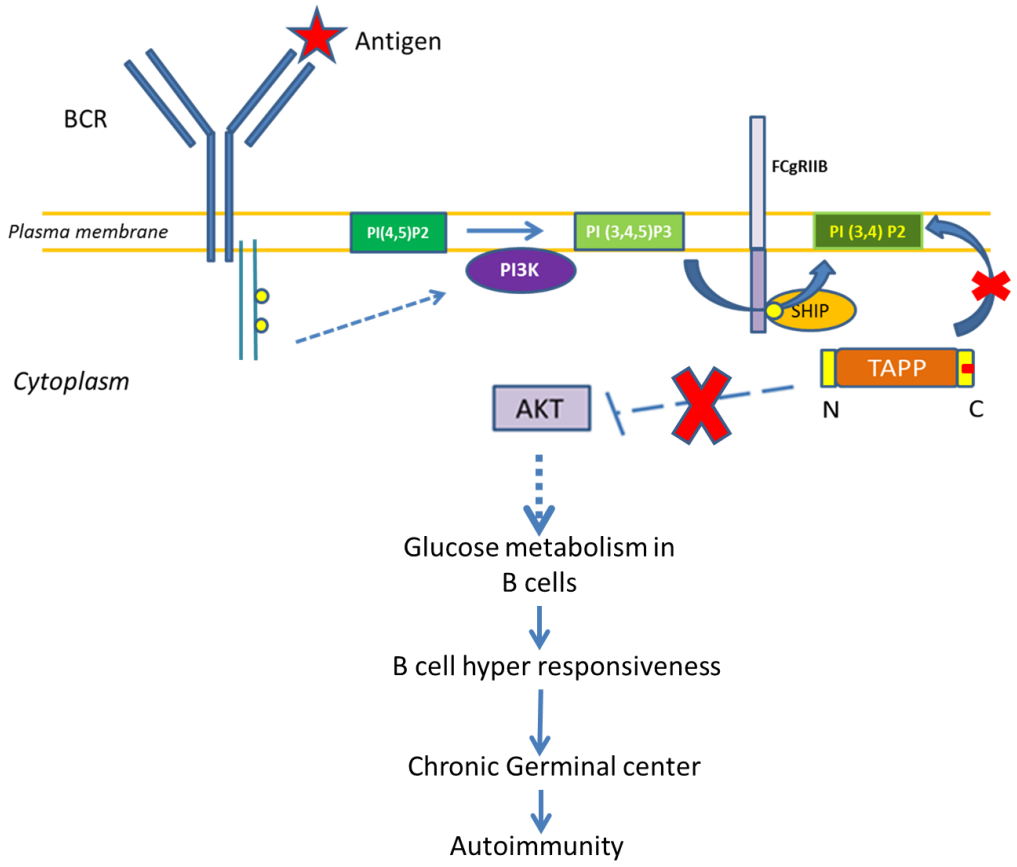

Figure 6.1 An overview of B cell regulation by TAPPs

Mutating the C-terminal PH domain of TAPPs prevents it from translocating to the membrane and thus dysregulating the feedback mechanism associated with PI3K signaling. Enhanced activation of PI3K/AKT signaling contributes to metabolic abnormalities in B cells. The B cell hyper responsiveness driven by these defects in TAPP KI mice is linked to the development of spontaneous GC and autoimmunity.

6.4 Limitations and future direction

My findings in TAPP KI mice have identified the crucial role of TAPPs in B cell signaling by mediating protection from B cell hyper responsiveness and development of lupus like autoimmune disease. However, one of the limitations is that the TAPP associated inhibitory mechanism on PI3K pathway is not fully understood. Based on the study conducted by Sen Hou from our lab (unpublished), we understand that the intact PH domains of TAPPs compete with AKT to interact with PI(3,4)P2 . When the PH domain of TAPP is mutated, PI(3,4)P2 is more available for AKT to bind effectively and thus enhancing the AKT phosphorylation in these cells.

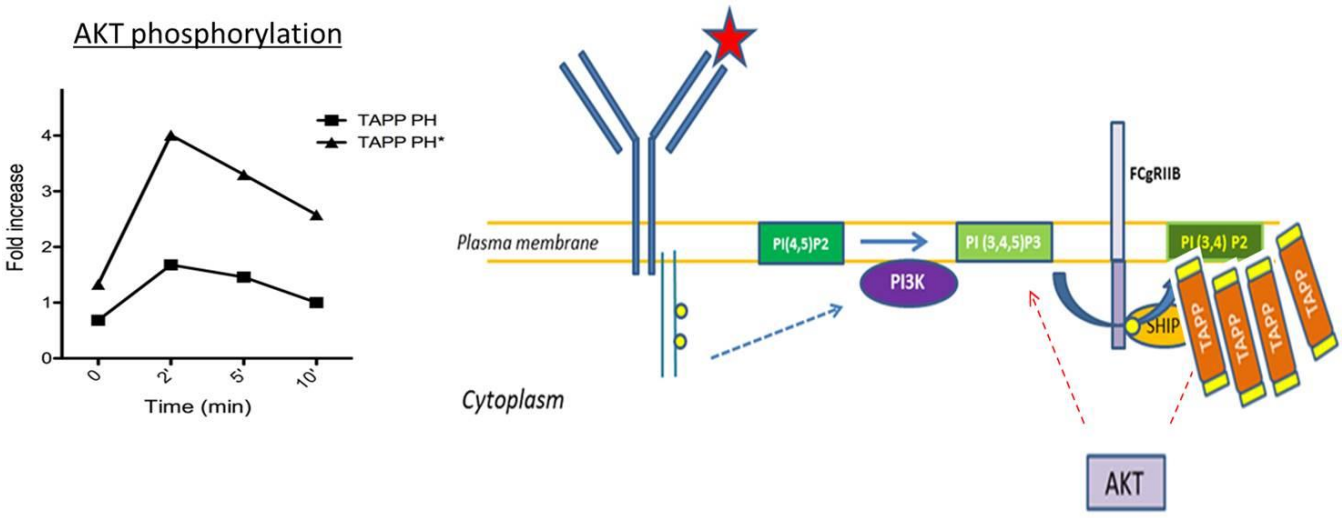

Figure 6.2 Competition for PI(3,4)P2 binding by TAPPs partially interferes with AKT membrane recruitment

On the left, a B cell line transfected with intact PH domain of TAPP that can bind to PI(3,4)P2 (square), cells transfected with mutated PH domain of TAPPs that doesn't interact with PI(3,4)P2 (triangle). These cells upon BCR cross-linking showed a time dependent effect on AKT phosphorylation. Those cells overexpressing mutated TAPP PH domain showing enhanced AKT phosphorylation compared to those with unmutated TAPP PH domain. On the right is a model illustrating the competition for binding to PI(3,4)P2 in the presence of TAPP interaction which reduce AKT recruitment to the membrane.

Another possibility is that TAPPs are introducing some phosphatases to the membrane. TAPP has a PDZ binding domain that can interact with other proteins. A TAPP binding protein phosphatase called PTPL1 is brought to the membrane upon generation of PI(3,4)P2 [317] . PTPL1 is known to down regulate AKT phosphorylation and therefore inhibits PI3K pathway [357]. Therefore this possibility of TAPP associating phosphatases cannot be excluded. As a first step to detect the presence of any phosphatase associating with TAPPs in B cell is to perform a co-immunoprecipitation for TAPP followed by a phosphatase assay. This will tell whether or not there is any phosphatase associating with TAPPs in B cells.

Dysregulated PI3K signaling in TAPP KI B cells is required for increasing glycolytic metabolism in these cells. However, the signaling mechanisms linking TAPPs, and the PI3K signaling pathway in general, to GCB metabolism require further investigation. Isolating large numbers of viable purified GCB is extremely challenging as they exist in very small percentage in naïve mice (<1%), and they are very susceptible to apoptosis in vitro. So as an alternative method, to closely resemble a GC environment, B cells can be grown in a hypoxic chamber providing T cell dependent stimulation condition. Another approach shown by others is to generate GC B like cells in a co-culture system constituting CD40L and BAFF expressing cells along with cytokines [358]. A PCR array can be done to identify the genes within the metabolic pathway that are differentially regulated in these cells. Further studies will be on those specific proteins that are coded by these genes to measure their expression and activation within TAPP KI B cells. This approach will determine the role of TAPP associated signaling downstream of PI3K is regulating metabolism.

The mouse model that has a mutation in TAPPs is not tissue specific and has an effect on all the immune subsets. Although we observed a B cell intrinsic role of TAPP mutation in the

development of autoimmunity, the contribution of other immune cells in the development of the disease need to be addressed. As I found preliminary evidence for TAPP KI T cell hyper-reactivity and T cells are one of the major subset that is interacting with B cells in the GC, T cell intrinsic contribution need to be investigated further. To determine T cell detect in TAPP KI mice, the purified T cells has to be stimulated for PI3K activation to determine the status of AKT phosphorylation and to measure proliferation, survival and metabolic activity in vitro. In addition to that, adoptive transfer of T cells to T cells deficient mice to study the T cell intrinsic role in the development of spontaneous GC and autoimmunity.

B cells exhibit enhanced PI3K signaling when TAPP is uncoupled from the membrane. Although TAPP KI B cells exhibiting hyper responsiveness and enhanced glucose metabolism can be reversed upon blocking PI3Kδ, all these studies were conducted in vitro. To further validate the findings in vivo, aging TAPP KI mice will be treated with Idelalisib. Blood will be collected before and after the start of the treatment to monitor autoantibody levels. At the endpoint, spleen will tested to determine the status of chronic GC and kidneys for immune complex deposition. These experiments will tell us whether or not the inhibition of PI3K signaling will have an effect on development of spontaneous GC and autoimmunity.

As TAPPs have the ability to interact with other proteins, it might have additional functions in B cells apart from membrane translocation. As we already have TAPP genes floxed in the TAPP KI mice, it will be possible to develop the B cell specific knock out of the protein in mice as a long term goal. This will help us to explore membrane independent function of TAPPs and to determine whether complete loss of TAPPs in B cells has an effect on germinal center development. Another long term goal of this project will be to knock down TAPPs in human B cells to study their effect on cell proliferation, survival and metabolism. Together these studies

will help us understand TAPP dependent mechanisms that control B cell signaling. These studies will contribute to our understanding in regulation of B cells and will benefit in the development of new approaches to discover treatment for B cell mediated diseases in the future.

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