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Liver Kinase B1 Controls T Follicular Helper Cell Differentiation and Germinal Center Formation through Regulation of Nuclear Factor kappa-light-chain-enhancer of Activated B cells

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Cellular and Molecular Pathology

by

Nicole Corinne Walsh

2015

ABSTRACT OF THE DISSERTATION

Liver Kinase B1 Controls T Follicular Helper Cell Differentiation and Germinal Center Formation through Regulation of Nuclear Factor kappa-light-chain-enhancer of Activated B cells

by

Nicole Corinne Walsh

Doctor of Philosophy in Cellular and Molecular Pathology

University of California, Los Angeles 2015

Professor Michael Teitell, Chair

Germinal centers (GCs) are a key feature during a T-dependent immune response. Mature B cells in secondary lymphoid organs, such as the spleen and lymph nodes, remain quiescent until they encounter foreign antigen. Antigen activated B cells that receive co-stimulation from cognate T cells form germinal centers. GC B cells undergo rapid proliferation and programmed DNA damage of the *immunoglobulin genes (IG)* that modifies the B cell receptor to increase its affinity to the activating antigen. B cells that successfully rearrange their B cell receptors exit the germinal center as long-lived, antibody secreting plasma cells or memory B cells. These terminally differentiated cells are the basis of long-term immunity.

We previously discovered a pathway involved at the end of the germinal center reaction important for plasma cell development involving Lkb1. To further investigate Lkb1's role in B cell biology, we knocked *Lkb1* out specifically in B cells. Unexpectedly, loss of *Lkb1* in B cells resulted in an increase of transitional B cells and a mixture of Lkb1+ and Lkb1- mature B cells. Lkb1- B cells aberrantly secreted IL-6 due to activation of NF-κB signaling. B cell derived IL-6 contributes to generation of an activated environment, inducing T cell activation and the

differentiation of T_{FH} cells. This activated environment spurred by loss of Lkb1 in a subset of B cells ultimately resulted in spontaneous GC formation. Our results suggest an interesting new role for Lkb1 in B cells as an essential negative regulator of activation.

The dissertation of Nicole Corinne Walsh is approved.

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University of California, Los Angeles

2015

DEDICATION

My thesis is dedicated to my parents, David and Maria. I would not be who I am or where I am in life if not for the values, motivation, and determination they have instilled in me.

I especially dedicate this to my father; he was the hardest working and most generous person I've ever known. He has been and will always be a driving force in my life and I can only hope that he will be as proud of me as I have been of him.

"You don't know strength until strength is the only choice you have".

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LIST OF ABBREVIATIONS

ACC:	acetyl-coA carboxylase
ACTH:	adrenocorticotropic hormone
AICDA (AID):	activation-induced cytadine deaminase
AMPK:	AMP-activated protein kinase
ASC:	antibody secreting cell
ATM:	ataxia-telangiectasia mutated
BCR:	B cell antigen receptor
BKO:	B cell specific knock out
BM:	bone marrow
BRSK:	brain specific kinase
cDNA:	complimentary DNA
CLP:	common lymphoid progenitor
CRTC2:	CREB-regulated transcription coactivator 2
CSR;	class switch recombination
DDR:	DNA damage response
Deptor:	DEP domain-containing mTOR-interacting protein
DLBCL:	diffuse large B cell lymphoma
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
DSB:	double strand break
Dvl:	Disheveled
FDC:	follicular dendritic cell
FO:	follicular
GALT:	gut-associated lymphoid tissue
GC:	germinal center

H&E:	hematoxylin and eosin
HCC:	hepatocellular carcinoma
HET:	heterozygous
HIGM:	Hyper-IgM syndrome
Hk2:	hexokinase 2
HSC:	hematopoietic stem cell
IFN:	interferon
Ig:	immunoglobulin
IHC:	immunohistochemistry
IL:	interleukin
LKB1:	liver kinase B1
MAP:	microtubule-associated proteins
MARK:	microtubule-affinity regulating kinase
MEF:	mouse embryonic fibroblast
MHC II:	major histocompatibility class II
mLST8:	mammalian lethal with Sec13 protein 8
MMP:	metalloproteinases
mTOR:	mammalian target of rapamycin
mTORC1:	mTOR complex 1
MZ:	marginal zone
NHEJ:	non-homologous end joining
NSCLC:	non-small cell lung cancer
NP-CGG:	4-hydroxy-3-nitrophenyl acetyl-chicken γ-globulin
NUAK:	novel (nua) kinase
PALS:	periarteriolar lymphoid sheath
PAMP:	pathogen-associated molecular patterns

PB:	plasmablast
PC:	plasma cell
PCR:	polymerase chain reaction
PID:	primary immunodeficiency
PJS:	Peutz Jeghers Syndrome
PNA:	peanut agglutinin
PRAS40:	Akt/PKB substrate 40 kDa
qRT-PCR:	quantitative real-time PCR
RAG:	recombinase activating gene
RFP:	red fluorescent protein
RNA:	ribonucleic acid
RT-PCR:	reverse-transcription PCR
S6K1:	S6 kinase 1
SHM:	somatic hypermutation
SIK:	salt inducible kinase
SLE:	systemic lupus erythematosus
SRBC:	sheep red blood cells
TCL-1:	T cell leukemia/lymphoma 1
T_{FH}:	T follicular helper
Tg:	transgenic
TLR:	toll-like receptor
TNFR:	tumor necrosis factor receptor
TR:	transitional
WCL:	whole cell lysate
WT:	wild type
YFP:	yellow fluorescent protein

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents. I have always felt encouraged and supported by them. I credit them for my ambition, motivation, and my work ethic-three things I've needed to get through this process.

I would like to thank my PhD mentor, Michael Teitell, for welcoming me in to the Teitell lab during my first year in graduate school and being instrumental in my intellectual and personal growth over the past few years. I am leaving graduate school a different person than when I started and a lot of that growth is based on the lessons I learned in this lab.

I would like to thank my former PI, John Manis, for hiring me as a technician out of college, for teaching me what academic science and life in a lab is really like, and for encouraging me to continue my education on this path.

I would like to acknowledge the Teitell Lab members-past and present-who I've worked with over the past 6 years. The people you see on a daily basis are what shape your experience and I've had the pleasure to work with some great scientists and great people. I especially want to thank the whole lab for putting up with me and holding me up over the last 3 years; I cannot express what your support has meant to me.

I would like to thank all of my friends and family back on the east coast. The last 3 years have been trying, but without all your love, support, and help I would not have been able to get through them in one piece.

I also want to thank all of my graduate school friends from ACCESS 12 and 13. The first few years of grad school were a lot of fun and you are all great people.

Chapter 2 is a version of a manuscript currently under revision for publication at EMBO Reports. Authors of this manuscript are Nicole C. Walsh, Lynnea R. Waters, Jessica A. Fowler, Mark Lin, Herbert C. Morse III, Jerold E. Rehg, and Michael A. Teitell. We thank Tammy Phung, Steven Bensinger, Laura Fahey, and David Brooks (UCLA) for assistance and feedback, and Jason Hong and Christopher Pang-Gonzalez for animal colony assistance. FACS was performed at the Broad Stem Cell Research Center FACS Facility and flow cytometry at the UCLA IMED Flow Cytometry Core. This study was supported by NIH grants T32CA009120 (N.C.W.), GM007185 (L.R.W.), CA90571, CA156674, GM073981, GM114188, and CA185189 (M.A.T.), ACS grant LIB-122716 (J.A.F.), and the NIH Intramural Research program, NIAID (H.C.M.).

Chapter 3 is a version of the published manuscript "Walsh NC and Teitell MA. (2011). B cell differentiation stimulated by physiologic DNA double stranded breaks. Cell Cycle 10:2, 1-2" reproduced here with permission freely granted from Landes Bioscience. The authors thank Dr. Barry Sleckman (Washington University, St. Louis) and Dr. Craig Bassing (University of Pennsylvania) for insightful discussions on this topic. Supported by grants from the National Institutes of Health CA009120 (N.C.W), CA90571, CA156674, GM073981, P01GM081621, and PNEY018228 (M.A.T.).

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CHAPTER 1:

Introduction

Adaptive Immunity and B Cell Development

The immune system is broken down into two types of immunity, innate and adaptive. Innate immunity, also called natural immunity, is characterized by the expression of general receptors called Pattern Recognition Receptors (PRRs) [1] on the surface of neutrophils and macrophages [2]. PRRs recognize Pathogen-Associated Molecular Patterns (PAMPs), general patterns of proteins on the surface of microbes [1]. This recognition results in a rapid response involving the phagocytosis of the microbe and an inflammatory response [1]. This system allows for a quick and generic response to any foreign microbe that expresses PAMPs on their cell surface.

Adaptive immunity allows for the recognition of diverse pathogens and the ability to mount an antigen-specific immune response. Lymphocytes are important mediators of adaptive immune responses. T cells are responsible for cell-mediated responses that involve the recognition of an intracellular pathogen and the subsequent destruction of pathogen-infected cells. B cells are vital for humoral immunity due to their ability to generate and secret high affinity, antigen specific antibodies [2]. Both T and B cells form memory cells that are able to mount a rapid, strong response upon reinfection with a previously encountered antigen, forming the basis for vaccination [3].

B cells express a diverse repertoire of B cell receptors (BCRs) on the cell surface that recognize and bind to foreign pathogens [4]. The BCR complex is made up of a membrane-bound immunoglobulin (Ig) and a heterodimer of Igα and Igβ transmembrane proteins [5]. The Igα/Igβ subunits constitute the signaling portion of the BCR. The Ig region constitutes the antigen-binding portion of the BCR. As with all antibodies, the Ig portion of the BCR is made up of 2 identical heavy chains (IgH) and 2 identical light chains (Igκ or Igλ) held together with disulfide bonds. Both the heavy and light chains are comprised of a N-terminus variable (V) region and a C-terminus constant (C) region [6].

To generate such a diverse repertoire, B cells undergo a process called V(D)J recombination [7], which changes the configuration of the variable region of antigen receptor genes. Double-stranded breaks (DSBs) are generated between variable (V), diverse (D), and joining (J) regions of the antigen receptor genes [4] by lymphocyte specific recombination activation gene (RAG) 1 and 2 [8]. After these regions undergo recombination, the DSBs are resolved by general repair machinery, specifically non-homologous end joining (NHEJ) [9, 10].

Before B cell lineage commitment, common lymphoid progenitors (CLPs) initiate the recombination of D_H and J_H gene segments of the IgH locus [11, 12]. CLPs that commit to the B lineage, now called pro-B cells, up regulate the transcription factors *EBF* and *E2A*. EBF is induced downstream of IL-7 receptor (IL-7R) signaling [13] and subsequently induces the expression of *Pax5* [14]. All these factors are essential for the resultant joining of the V_H gene segment to the already combined DJ_H [15-17]. Once V(D)J recombination of the heavy chain locus is completed, the new heavy chain allele associates with λ5 and Vpre B (the surrogate light chain (SLC)), and Igα/Igβ receptors to form the pre-BCR [18].

The assembling of the pre-BCR marks the pre B cell stage of B cell development. Initial signaling through the pre-BCR synergizes with IL-7R signaling to induce a clonal proliferative expansion that serves as a checkpoint for B cells that have successfully undergone $V(D)J_H$ recombination [19]. As IL-7R signaling becomes attenuated, signaling through the pre-BCR results in cell cycle exit due to the stage specific expression of *Aiolos* [19] and the expression of *Irf4*. *Aiolos* silences the expression of *Ccnd3* (Cyclin D3), which is necessary for cell cycle progression [20], and *Irf4* and *Irf8* inhibit the expression of the SLC [21], thereby down-regulating proliferative signaling through the pre-BCR [16, 22]. E2A expression is induced downstream of pre-BCR signaling and initiates the recombination of the immunoglobulin light chain, Igκ [19]. Once recombination of the VJ_κ has completed, the IgH and Igκ combine to form the BCR. This point in B cell development represents a second checkpoint where B cells with dysfunctional Igκ rearrangements undergo apoptosis through lack of survival signals [23]. B

cells with too high a self-affinity undergo receptor editing to lower the affinity of the BCR and discourage auto-reactivity. B cells with functional BCRs, now designated immature B cells, shut down further V(D)J recombination [24] and permitted to leave the bone marrow (BM).

Immature B cells immigrate to the spleen where they make up 15-20% of the B cell population. Immature B cells in the spleen are referred to as transitional B (TR) cells and are identified by the expression of AA41 [25], binding of mAb 493 [26], and high expression of heat soluble antigen (HSA) [27, 28]. TR B cells can be further differentiated into T1 (IgM^{high}CD23-), T2 (IgM^{high}CD23+) and T3 (IgM^{low}CD23+) populations through the expression of IgM and CD23 [25]. Immature B cells have a shorter half-life of 4 weeks compared to their mature counterparts, which have a half-life of 15-20 weeks [29]. Additionally, TR and mature B cells have different signaling outcomes downstream of the BCR, which is apparent in their responses to BCR crosslinking [27, 30]. Immature B cells undergo apoptosis whereas mature B cells proliferate and differentiate upon BCR crosslinking. However, it has been shown that some TR B cell populations are able to tolerate BCR crosslinking when accompanied by anti-CD40 or IL-4 stimulation. This suggests that TR B cells, specifically T2 and T3 cells, can respond to signals from T cells *in vivo*.

TR B cells differentiate into one of two different mature B2 cell types, follicular (FO) or marginal zone (MZ) B cells. Mature B cell fate is thought to be dependent on the strength of tonic signaling through the BCR [31]. TR B cells with weak BCR affinity up regulate Notch2 signaling and differentiate into MZ B cells, while TR B cells with intermediate BCR affinity differentiate into FO B cells [32]. These two different subsets that make up the mature B2 cell population in mice differ in their physical location within peripheral lymphoid organs and functionality [31]. FO and MZ B cells can be distinguished by their cell surface protein expression. Both subsets express B cell markers CD19 and B220, and lack the expression of the immature marker CD93. However, FO B cells express IgM, intermediate levels of CD21,

and CD23, while MZ B cells express higher levels of both IgM and CD21 and lack the expression of CD23 [32].

FO B cells reside in primary follicles located in the white pulp of the spleen and in lymph nodes (LNs). These B cells also recirculate through blood and lymphatics to the periphery and BM where they scavenge for foreign antigens. FO B cells express a diverse repertoire of mono-reactive BCRs that recognize unique T-dependent antigens [33]. As a result of antigenic stimulation, FO B cells differentiate into short-lived plasmablasts (PBs) or undergo a germinal center (GC) reaction to differentiate into antigen-specific memory cells or long-lived antigen-specific antibody secreting plasma cells (PCs) that home and reside in the BM (reviewed in [34, 35]).

MZ B cells reside in the spleen in an area around the marginal sinus between the red pulp and the white pulp. MZ B cells are not found in other peripheral lymphoid organs or in the BM. MZ B cells express a less diverse repertoire of BCRs than FO B cells, but these BCRs are multi-reactive and can bind related classes of T-independent antigens [33]. Along with expressing higher levels of CD21 and IgM than FO B cells, MZ B cells also express more toll-like receptors (TLRs). When activated by either BCR or TLR engagement, MZ B cells differentiate into short-lived, low affinity PCs. MZ B cells may also be able to shuttle antigen toward the follicles through binding of immune complexes with CD21 (reviewed in [32]).

A third class of mature B cells is B1 B cells. B1 B cells are thought to develop in the fetal liver and contribute to innate immunity. They reside in peritoneal and pleural cavities and are able to self-renew. They have a limited V gene usage in their BCRs that recognize bacterial antigens and have the potential to recognize self-antigens as well. They provide the body with natural IgM antibodies and can migrate to the gut-associated lymphoid tissue (GALT) to produce IgA antibodies and aid with mucosal immunity [36].

Immune Response and Terminal Differentiation

Mature B cells remain quiescent until antigen activation of the BCR. In secondary lymphoid organs such as spleen, lymph nodes and tonsils, naïve B cells encounter antigen through multiple mechanisms including passive diffusion, presentation from follicular dendritic cells (FDCs), transport from subcapsular macrophages, and antigenic regurgitation from dendritic cells [37]. MZ B cells are activated by blood-borne pathogens, specifically viruses and polysaccharide encapsulated bacteria [32, 38]. Engagement of these antigens, without assistance from other white blood cells (namely T cells), triggers a T-independent immune response. Upon activation, MZ B cells rapidly undergo differentiation in to low-affinity IgM or IgG secreting PBs [32, 38].

An antigen that is not recognized by MZ B cells is passed to the primary follicle area to be recognized by FO B cells and trigger a T-dependent immune response. Activated FO B cells then migrate to the T-B boundary where they interact with cognate T cells that reside in the periarteriolar lymphoid sheath (PALS) [39]. This interaction provides a co-stimulatory signal via CD40 ligand to survive and differentiate into low affinity, antibody secreting PCs or to proliferate and seed a GC [34].

GCs are histological structures that form in secondary lymphoid organs during a T-dependent immune response (reviewed in [35]). GCs can be divided into a dark zone and a light zone each with their own distinct cellular properties and functions [40]. Dark zones consist of small rapidly dividing B cell cells termed centroblasts [34, 41] that are amongst the most rapidly proliferating cells in the body. Centroblasts express BCL6, which represses key DNA damage-sensing and cell cycle proteins, namely p53, p21, and $p27^{cip}$ [42, 43]. Centroblasts undergo a process called somatic hypermutation (SHM) [44] during which activation-induced cytosine deaminase (AID) [45] introduces single base-pair mutations in the variable region of the *Ig* region. AID deaminates cytosine residues into uracil residues [45], which are then recognized by the mismatch repair mechanism and repaired using an error-prone repair system.

These mutations change the affinity of the *Ig* to the antigen, and will later contribute to the selection of high affinity B cells that proceed to terminal differentiation (reviewed in [46]).

Upon completion of SHM, centroblasts re-express their BCRs, exit the cell cycle and migrate to the light zone of GCs where they are termed centrocytes (reviewed in [40]). Along with centrocytes, light zones also consist of FDCs and T follicular helper (T_{FH}) cells [47]. FDCs bind antigens and present them to centrocytes as one mechanism of selection for B cells that have successfully increased their antigen affinity via SHM [48]. Centrocytes outnumber FDCs in the light zone, therefore creating competition for antigen binding. As a result, centrocytes with higher affinities are more likely to bind antigens and receive survival signaling through their BCRs [47]. Combined with co-stimulation through CD40-CD40L binding [49] and IL-21R signaling by T_{FH} cells [50], B cells are selected to continue through differentiation. Centrocytes also undergo a second *Ig* refinement in the light zone, class switch recombination (CSR) (reviewed in [40]). CSR is initiated, like SHM, by AID that generates DNA DSBs in the constant region of the *Ig* locus to swap the functional constant regions from Cμ or Cδ to Cγ, Cα, or Cε. DNA DSBs generated by AID are resolved by the alternative form of NHEJ machinery [51].

B cells that do not receive these signals can re-enter the dark zone and undergo additional cycles of SHM to increase their antigen affinity, or they may undergo death by neglect [47]. The exact mechanisms of these decisions are not well understood. Additionally, B cells that gain affinity to self-antigen during the antibody refinement processes undergo apoptosis through negative selection to limit the development of autoimmunity [40, 46].

B cells that have successfully undergone *Ig* refinement undergo terminal differentiation into either memory B cells [52] that are poised to rapidly respond upon antigen re-exposure or antibody secreting PCs. The mechanism through which B cells differentiate into memory B cells or PCs is not fully understood, but studies have indicated that B cells that attain the highest affinity for a given antigen terminally differentiate into long-lived PCs [46]. Memory B cells reside in the marginal zone of primary follicles in lymphoid organs and are able to quickly

respond to repeat antigenic exposure [52]. PCs home to the BM where they are relatively long-lived and continue to secret antigen specific antibodies. To differentiate into PCs, GC B cells must down regulate BCL6 as it acts as a repressor of *Prdm1*, the gene that encodes the BLIMP-1 protein. BLIMP-1 is the master regulator of PCs and is required for PC differentiation [53]. The down regulation of *Pax5* and *myc* and the up regulation of *Irf4* and *Xbp1* are also necessary for PC development [54].

Germinal Center Dysfunction

The GC reaction, from initial B cell activation and recruitment to selection and terminal differentiation, must be a tightly regulated process. GC B cells undergo rapid proliferation and physiological DNA damage to generate large numbers of B cells with high affinity antibodies, one of the characteristics of a robust immune response. Deregulation of the GC response can have catastrophic results, namely immunodeficiency [55], autoimmune disorders [56] and lymphomagenesis [57].

B cell lymphomas constitute ~4% of cancers and ~3% of cancer death in the USA [58]; 60-70% of which originate from GC B cells [57] based on the presence of somatically mutated *Ig* genes in tumor cells. The rapid proliferation and DNA damaging events paired with the loosened DNA damage check point in GCs can result in harmful mutations and DNA translocations resulting in lymphomagenesis [34, 40, 59]. DNA translocations found in B cell lymphomas can occur as a consequence of CSR. This is a result of translocations connecting the regulatory region of the *Ig* locus to the coding regions of proto-oncogenes [60]. Examples of common translocations found in mature B cell lymphomas arising from faulty CSR are *IgH/c-myc* in Burkitt's lymphoma [61], *IgH/Bcl6* in diffuse large B cell lymphoma (DLBCL) [62, 63], and *IgH/Pax-5* in lymphoplasmacytoid lymphoma [64]. These translocations result in the deregulated expression of the proto-oncogenes that generally result in either increased proliferation (*c-myc* and *Bcl-6*) or a block in differentiation (*Pax5*).

Aberrant SHM can also account for the deregulation of genes that encourage lymphomagenesis in GC B cells [59, 65]. SHM can generate mutations in the regulatory regions of proto-oncogenes that are not typically expressed in GC B cells, as well as disrupt the physiological modulation of gene expression during the GC reaction. Genes associated with aberrant SHM are *c-myc*, *Pim1*, *Pax5* [59], and *Prdm1* [66, 67]. The consequence of such genetic lesions, as with translocations, is enhanced proliferation or a block in differentiation.

Additionally, mutations obtained during B cell development unrelated to the GC reaction can predispose a B cell to undergo transformation once it enters the GC reaction. For example, the translocations of *IgH/Bcl2* that are found in follicular lymphoma [68], which has a GC cell of origin [57], are thought to originate during V(D)J recombination [60]. *Bcl2* is physiologically down regulated in GC B cells to create a pro-apoptotic state [69, 70]. When fused to *IgH*, *Bcl2* expression confers a survival advantage to GC B cells [40], increasing the likelihood that a GC B cell will undergo additional mutations that result in lymphomagenesis.

A second pathogenic consequence of increased survival of B cells, specifically GC B cells, is autoimmunity. One common feature of autoimmune diseases is the presence of antibodies against self-antigens such as dsDNA and other nuclear components [71]. Auto-reactive antibodies can arise from GCs based on evidence of affinity maturation. These self-reactive B cells are thought to originate from antigen-specific B cells that get recruited into GCs and then undergo SHM, which mutates the BCR to recognize self antigens [71]. GC B cells that gain affinity to self-antigens through SHM and are not effectively eliminated differentiate into antibody secreting PCs that cause autoimmunity [56]. Mutations that prevent the negative selection of autoreactive GC B cells include those that confer increased survival of these B cells, such as aberrant expression of *Bcl2*, $Bcl\text{-}X_L$, *or c-Flip*, or those that confer deficiency in pro-apoptotic proteins, such as *Bim* and *Fas* (reviewed in [56]). Additionally, a common feature of mouse models displaying autoimmune manifestations is the presence of spontaneous GCs [56].

A newly recognized mechanism contributing to autoimmunity development, along with failure to eliminate autoreactive B cells from GCs, is the aberrant positive selection of these B cells by T_{FH} cells. In physiological GCs induced by antigens, the number of T_{FH} cells recruited into these GCs is limited to drive competition between GC B cells for survival signals to generate high affinity antibody production [56]. A newly appreciated feature of both autoimmune mouse models and patients with autoimmune disorders is the increased presence of T_{FH} cells. As an example, *Sanroque* mice display spontaneous GC formation that originate from the presence of T_{FH} cells [72]; these cells can be transferred into WT mice and recapitulate the spontaneous GC formation and autoimmune manifestations seen in *Sanroque* mice [73]. Additionally, excessive IL-21 has also been associated with Systemic Lupus Erythematosus (SLE)-like disease in mice with the *Yaa* gene mutation and in the MRL/*lpr* mouse strain [56]. Patients with SLE, myasthenia gravis, and Sjorgren's syndrome have been found to have increased amounts of circulating CD4+ICOS+ or CD4+CSCR5+ICOS^{hi}PD-1^{hi} T cells, which correlates with disease severity and autoantibody titers (reviewed in [74]).

A third consequence of GC dysfunction is immunodeficiency. One prominent heritable immunodeficiency associated with the GC reaction is Hyper-IgM syndrome (HIGM). HIGM is characterized by the presence of serum IgM at either normal or elevated levels, as well as the absence of or decrease in other switched Ig isotypes. This is due to a defect in SHM and most prominently CSR during an adaptive immune response (reviewed in [75]). Additionally, mutations in genes required for GC formation and PC development disrupt the ability to undergo a proper immune response [55]. For instance, loss of BLIMP-1 blocks PC formation and lowers Ig titers after both primary and secondary immunizations [76], while loss of Grb2, a BCR signaling adaptor protein, results in impaired GC responses and IgG production [77, 78]. Recently, loss of function mutations in the IL-21 receptor gene has been reported in patients with primary immunodeficiency diseases (PID) whose B cells show decreased proliferation and decreased ability to undergo CSR. As described above, lymphoma, autoimmune disorders and

PIDs are all consequences of dysfunctional GC reactions. They can all occur separately or concurrently, and therefore highlight the necessity of the tight regulation of the GC response.

Regulation of TCL-1 by CRTC2

T cell leukemia/lymphoma 1 (TCL1) is an onco-protein that is expressed almost exclusively in lymphocytes, and functions in proliferation and survival. TCL1 was first identified as a protein that binds to the serine/threonine kinase AKT and augments its activity, thereby promoting cell survival and proliferation (28, 29). During B cell development, *TCL1* is highly expressed until antigen activation. Upon GC formation, *TCL1* expression is down regulated until it is silenced in terminally differentiated PCs. Some mature B cell lymphomas, display elevated TCL1 expression, suggesting that loss of *TCL1* down regulation during the GC reaction triggers lymphomagenesis (reviewed in [79]). A mouse model featuring enforced expression of *TCL1* in B and T cells resulted in the formation of mature B cell lymphomas in aged mice supporting this hypothesis [80].

Recent work from the Teitell lab has uncovered a novel signaling pathway in GC B cells originating from AID induced DSBs, resulting in the repression of *TCL1* [81]. AID generates DSBs in the *Ig* constant region that are sensed by ATM through auto-phosphorylation. Activated ATM phosphorylates liver kinase B1 (LKB1), which is hypothesized to phosphorylate and activate an unknown member of the AMPK family of protein kinases. This unknown AMPK family protein then phosphorylates CREB-regulated transcriptional co-activator 2 (CRTC2) [81], which results in its re-localization into the cytoplasm through its interaction with 14-3-3 [81]. CRTC2 is a co-activator of the transcription factor CREB and has been shown to regulate a subset of genes independent of CREB phosphorylation [82], including *TCL1* [83]. In a human *in vitro* B cell culture system, blockade of this signaling pathway at multiple points, including knockdown of ATM and LKB1 and mutation of CRTC2, results in a prolonged GC-like state of rapid proliferation and defective PC differentiation and antibody secretion [81]. Additionally,

ChIP-on-chip analysis revealed a transcriptional program regulated by this pathway integral to B cell terminal differentiation including *AICDA*, *BACH2*, *CDK6*, *MTA3*, *MYC*, *SMARCA2*, *SMARCA4*, *TCF3*, and *TCL1* [81]. These data suggest that this pathway is an important regulator of B cell terminal differentiation and therefore the adaptive immune response. Prior to this discovery, LKB1 was completely unstudied in the immune system as a whole and specifically in B cells.

Liver Kinase B1

LKB1 (also known as serine/threonine kinase 11, STK11) was discovered in 1998, linked to the human disease Peutz-Jeghers syndrome (PJS) [84, 85]. PJS is an autosomal dominant heritable condition (although there are cases that arise by *de novo* mutations) characterized by hamartatous polyps of the gastro-intestinal tract, hyperpigmentation of mucous membranes, and an increased risk for cancer development. Polyps of the GI tract are typically benign, but a frequent location for cancer development in these patients is the GI tract. Other common cancer sites include the intestines, the stomach and the pancreas, as well as the breast, lung, ovaries, uterus, and testis [86]. Over 144 mutations in LKB1 have been described, a majority of which are found in the kinase domain, affecting LKB1's catalytic activity [87]. Those that are not located in the kinase domain are found in the C-terminal region [88]. Loss of LKB1 activity is also found in sporadic cancers. While mutations are found infrequently in most epithelial cancers [89], up to 30% of non-small cell lung cancers (NSCLCs) [90] and ~20% of cervical cancers harbor mutations in LKB1 [91].

The *LKB1* gene is located in chromosome 19. It is a 23-kilobase gene made up of 10 exons, nine of which are coding. The human LKB1 protein is 433 amino acids, while the mouse is 436 amino acids. LKB1 contains a kinase domain, an N-terminal domain containing a nuclear localization signal (NLS), and a C-terminal domain thought to be regulatory [85]. Additionally, *LKB1* has an alternate splice variant [92, 93] ($LKB1_S$) that contains an alternative 9th exon with a

stop codon. LKB1 is not closely related to any other protein kinases but it is conserved in mice (*Lkb1*), *Xenopus laevis* (xenopus egg and embryonic kinase 1; *XEEK1*), *Drosophila melanogaster* (*lkb1*), and *Caenorbabditis elegans* (partitioning defective gene; *Par-4*) [94].

Expression of LKB1 in human cancer cell lines that do not endogenously express it, such as HeLa cells and G361 cells, results in G1 cell cycle arrest [95]. Cell cycle arrest in G361 cells upon expression of LKB1 was the result of induction of $p21^{WAF1/cip1}$ [96]. Additionally, expression of LKB1 in the A549 cell line, which also lacks LKB1 expression, resulted in a p53-dependent cell cycle arrest [97] as well as increased expression of PTEN, an inhibitor of PI3K which ultimately results in decreased AKT proliferation signaling [98]. These studies, combined with the causative role of *LKB1* mutation in PJS and sporadic tumors, reinforce the role of LKB1 as a tumor suppressor protein.

LKB1 has a NLS and localizes mainly to the nucleus, but is also found in cytoplasmic pools [96]. This cytoplasmic localization of LKB1 appears to be important for its ability to suppress growth, as a mutation (from a PJS patient) that retains catalytically-active LKB1 in the nucleus inhibits its ability to cause cell cycle arrest in G361 cells [99]. Additionally, mutations to the NLS that don't allow for nuclear localization of LKB1 are still able to suppress growth when expressed in LKB1 deficient cancer cell lines [96].

Important mediators of LKB1 cellular localization are the proteins STRAD (STRADα and STRADβ) and MO25 (MO25α and MO25β). LKB1 is found in a trimeric complex with these proteins in mammalian cells [100, 101]. This interaction localizes LKB1 to the cytosol [96, 99, 102] and increases the *in vitro* kinase activity of LKB1 by more than 10-fold [99]. STRAD is responsible for re-localization of nuclear LKB1 to the cytoplasm, as LKB1 has no nuclear export signal; STRAD facilitates the interaction of LKB1 with the nuclear export machinery CRM1 and exportin 7 [103]. Supporting the importance of this complex formation, mutations in LKB1 have been found in PJS patients that interrupt complex formation; when these mutants of LKB1 are

expressed in cells, LKB1 is retained in the nucleus and cannot induce a cell cycle arrest [99, 101, 102]. A second complex involving LKB1 includes HSP90 and Cdc37 [104, 105]. Although LKB1 is catalytically inactive in this complex, it is stable and protected from ubiquitin-mediated degradation [106].

LKB1 is a serine/threonine kinase with a preference for phosphorylating threonine residues. The phosphorylation motif for LKB1 is LxT [107]. AMP-activated protein kinase (AMPK) contains this sequence in its conserved activation loop (Thr172) and is the most well studied of LKB1's known substrates. In addition to activating AMPK, LKB1 also activates at least 12 other members of the AMPK family of protein kinases [108]. AMPK is a heterotrimeric protein complex of one AMPKα subunit, the catalytic subunit, and one of each of AMPKβ and AMPKγ, which are regulatory subunits. During periods of energetic stress, when AMP levels rise as ATP becomes depleted, AMP binds the AMPKγ subunit altering the conformation of AMPK complex and allowing it to be phosphorylated by LKB1 (reviewed in [109]). In this capacity, AMPK functions as an energetic regulator; when activated, AMPK promotes catabolic pathways to generate ATP such as glycolysis and oxidative phosphorylation and inhibits anabolic pathways such as lipid and protein synthesis to conserve ATP [110]. AMPK directly phosphorylates key enzymes in synthesis pathways to inhibit their functions, such as acetyl-CoA carboxylase 1 (ACC1) and HMG-CoA reductase [111]. Moreover, AMPK also regulates changes in key metabolic genes through the phosphorylation of transcription factors such as PGC1α and FOXOs, and transcriptional co-activators, such as CRTCs and p300 [111]. However, AMPK primarily conserves energy through its regulation of mammalian target of rapamycin complex 1 (mTORC1) [112].

mTORC1 regulates cell growth and proliferation through protein synthesis [113]. It consists of several subunits including mTOR, raptor, mammalian lethal with Sec13 protein 8 (mLST8), Akt/PKB substrate 40kDa (PRAS40), and DEP-domain-containing mTOR interacting

protein (deptor). Activated AMPK phosphorylates TSC2, which in complex with TSC1 leads to its activation as a Rheb GTPase [114]. GTP-bound Rheb activates mTORC1; therefore activation of TSC by AMPK leads to the deactivation of mTORC1. Additionally, AMPK can directly phosphorylate raptor [115] leading to inactivation of mTORC1 activity. mTORC1 activation leads to phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein (4EBP1) to promote cellular growth, proliferation, lipid synthesis and nucleotide synthesis (reviewed in [113]). Aberrant activation of mTORC1 is thought to contribute to cancer development, inflammatory diseases, type 2 diabetes, cardiology diseases and some neurological disorders [113]. Additionally, activation of mTORC1 has consistently been seen in tumors from PJS patients and in LKB1-deficient NSCLC tumors [111], as well as in some conditional knock-out animal models [116].

A second known role for LKB1 is in establishing cellular polarity. Disruption of *Par-4* (LKB1 homolog) in *C. elegans* results in failure to establish cell polarity during embryogenesis [117]. In *Drosophila*, *lkb1* mutations block anterior-posterior axis formation during embryogenesis, as well as polarity in epithelial cells [118]. In mammalian cells, LKB1 regulates polarity and tight junction formation across different cancer cell lines (colon and lung cancer), as well as in MDCK cells and pancreatic epithelium [119]. Additionally, induction of LKB1 expression in intestinal epithelial cell lines, lacking endogenous LKB1, leads to the reorganization of the actin cytoskeleton and the formation of a brush border [120]. Additional evidence supporting a role for LKB1 in cellular polarity as well as providing some mechanistic insight into how LKB1 affects polarity is that at least 6 members of the AMPK related proteins, microtubule affinity-regulating kinases 1-4 (MARK1-4) and brain-specific kinase 1 and 2 (BRSK1,2) have established roles in polarity [121] as well as AMPK itself [109].

MARK 1-4 belong to a conserved family that is found in *C. elegans* (Par-1), *Drosophila*, *Xenopus,* and mammals [122]. MARKs regulate tubulin dynamics through the phosphorylation of microtubule-associated proteins (MAPs) such as Tau. Activation of MAPs alters cell contacts

and the localization of E-cadherin [119]. In Drosophila and mammalian cells, Par-1 associates with an atypical PKC complex towards the basolateral membrane to establish polarity in cells [121]. Additionally, the four MARK proteins appear to have isoform-specific functions and may also participate in metabolic signaling similar to AMPK (reviewed in [121]). BRSK 1 and 2, which are also conserved in *C. elegans* (SAD-1) and *Drosophila* (CG6114), are mainly localized to the brain but are also expressed at low levels in the testis and pancreas [121]. BRSKs are known to participate in establishing neuronal polarity and may have a role in synapse development. MARK2 is also expressed in axons where it interacts with disheveled (Dvl) to activate β-catenin signaling during dendrite development (reviewed in [121]).

A third group of AMPK related proteins regulated by LKB1 are the salt inducible kinases (SIKs) 1, 2, and 3. Through their kinase activity, all 3 SIKs inactivate and re-localize CRTCs to the cytoplasm, resulting in the repression of their target genes [82, 123]. Adrenocorticotropic hormone (ACTH)-induced gene expression and gluconeogenesis induction are two transcriptional programs regulated by this signaling [124-126].

The final group of AMPK related a protein regulated by LKB1 consists of the novel (nua) kinase family (NUAK1 and 2). NUAK1 (also called ARK5), which is activated by both LKB1 and AKT [121, 127], is associated with tumorigenesis. NUAK1 promotes tumor progression by its putative roles in inducing cellular proliferation and survival through metabolic adaptation to low nutrients [127]. Additionally, it induces the expression of membrane type-1 metallopreoteinases (MMPs) to promote tumor invasion [121]. The direct mechanisms by which NUAK1 accomplishes these functions have not yet been elucidated. NUAK2 (also called SNARK) localizes to the nucleus and is activated by various cellular stresses, including nutrient deprivation and DNA damage. Activation of NUAK2 is cell type-dependent and may overlap with energetic activation of AMPK [121]. Additionally, NUAK2 may increase cell survival and inhibit apoptosis downstream of tumor necrosis factor receptor (TNFR)-signaling [127]. Similar to NUAK1, the mechanisms by which NUAK2 carries out these functions are unknown. While the

AMPK family proteins are not the only substrates reported for LKB1, their activation represents the most well characterized function for LKB1.

Mouse models of LKB1 loss

Lkb1 deletion is embryonic lethal by E11 and multiple developmental defects are seen starting at E8 (reviewed in [116]). Conditional knockout mouse models of *Lkb1* display a range of phenotypes demonstrating that Lkb1 (and its substrates) has cell type-specific roles. Unsurprisingly, multiple models show tumorigenesis upon deletion of *Lkb1*. *Lkb1*$^{+/-}$ mice develop PJS-like polyposis, hepatocellular carcinoma (HCC) and osteogenic legions, while specific loss of *Lkb1* in epidermal cells, uterine cells, and in prostate, mammary, and pancreatic epithelial cells all generate tumors (reviewed in [116]). Interestingly, many tissue-specific models display no tumorigenic phenotypes but display defects in polarity (pancreatic β-cells), metabolic phenotypes (hepatocytes, pancreatic β-cells, and skeletal myocytes), survival (hematopoietic stem cells and thymocytes), and differentiation (thymocytes and intestinal epithelial cells) [116]. While a number of these phenotypes can be attributed to Ampk signaling through mTorc1 or other Ampk protein family members, others cannot [116].

Recently, attention has focused on Lkb1's role in the immune system, resulting in the generation of *Lkb1* deficient hematopoietic stem cells (HSCs) [128-130], thymocytes and T cells [131-134]. Three separate groups used an inducible Cre system to delete *Lkb1* from HSCs [128-130]. Immediately upon excision, all three observed increased proliferation and expansion of HSCs, followed by increased apoptosis and pancytopenia by day 18 after excision. Additionally, multiple mitochondrial defects were observed including decreased mitochondrial DNA copy number [128, 130], decreased ATP levels and decreased mitochondrial membrane potential [128-130]. HSCs displayed increased mTorc1 signaling although treatment with rapamycin was unable to reverse the overall phenotype [128-130]. While some aspects of this

phenotype were also observed in A*mpk*1-/- HSCs, such as reduced mitochondrial DNA copy number, other aspects of the phenotype, such as mitochondrial membrane potential were not [128]. This indicates that Lkb1 signaling in HSCs has important survival functions by both Ampk-dependent and -independent mechanisms.

Lkb1 deletion in thymocytes resulted in an incomplete developmental block at the DN3/DN4 transition and a reduced number of mature T cells in the periphery [131, 133, 134]. Lkb1- thymocytes also displayed reduced survival *in vitro* [131, 133, 134], caused in part by the loss of Bcl-X_L [131, 134]. While one group found an increase in the percentage of Lkb1- thymocytes in S/G2/M phase of the cell cycle [131], other groups reported reduced proliferation in either Lkb1- thymocytes [133] or Lkb1- CD4+ T cells [134] when stimulated *in vitro*. Reflecting Lkb1's role in regulating cellular metabolism, Lkb1- T cells exhibit increased levels of *Glut1* and *Hexokinase 2* (*Hk2*), increased glucose uptake and glycolytic flux, and a defect in lipid oxidation [134]. Interestingly, loss of *Lkb1* resulted in hyperactivation of both CD4+ and CD8+ T cells [134]. This was reflected in the increased frequency of T cells expressing CD44 and a heightened response in activation marker induction and cytokine production when stimulated *in vitro* [134]. This enhanced cytokine response was specific to IFN-γ and IL-17 [134]. As in the HSCs, some aspects of these phenotypes were seen in Ampk- thymocytes and T cells, such as the metabolic and activated phenotypes [134], or were rescued by exogenous expression of a constitutively active Ampk construct [131]. Other features of the phenotype, however, appear to be Ampk-independent and occur through currently unknown mechanisms.

Based on the recent work from the Teitell lab highlighting an important role of LKB1 in the terminal differentiation of GC B cells into PCs and the specific phenotypes elicited in animal models of *Lkb1* deletion in related immune cell types, Lkb1 is poised to have an unique function in B cell biology. To determine the physiological role that Lkb1 plays in mature B cell homeostasis and immune response, we generated a B cell-specific Lkb1 knockout mouse and characterized the development and functional competence of the B cell populations.

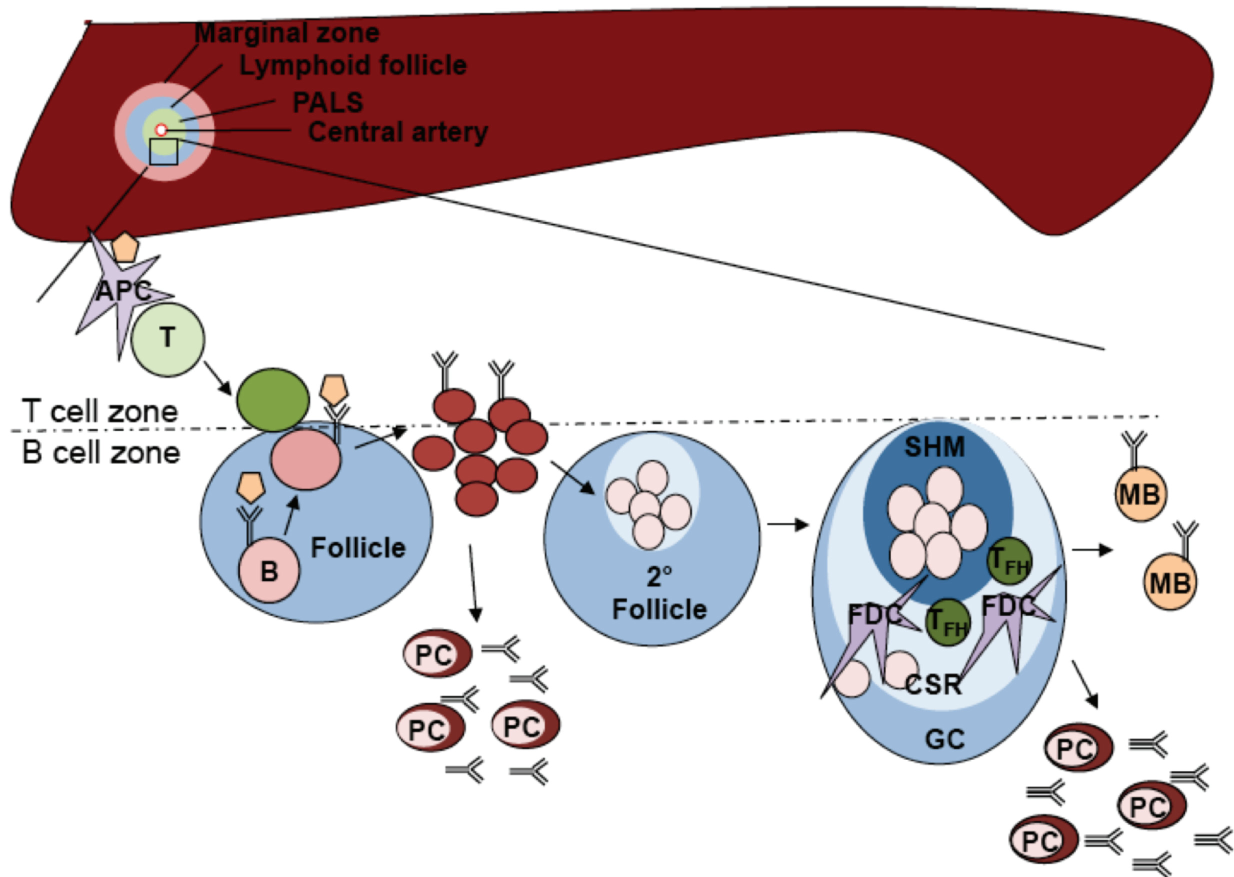
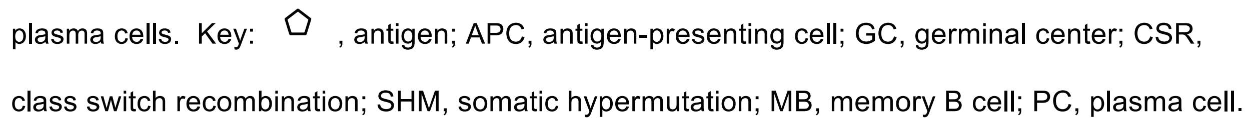


Figure 1-1: B Cell Activation and Differentiation in Peripheral Lymphoid Organs

B cells (red) are activated after interaction with antigens and cognate T cells (green). Subsequently, B cells either differentiate into short-lived, low affinity plasma cells or initiate germinal center formation to for antigen specific memory B cells or long-lived, high affinity plasma cells. Key:  , antigen; APC, antigen-presenting cell; GC, germinal center; CSR, class switch recombination; SHM, somatic hypermutation; MB, memory B cell; PC, plasma cell.

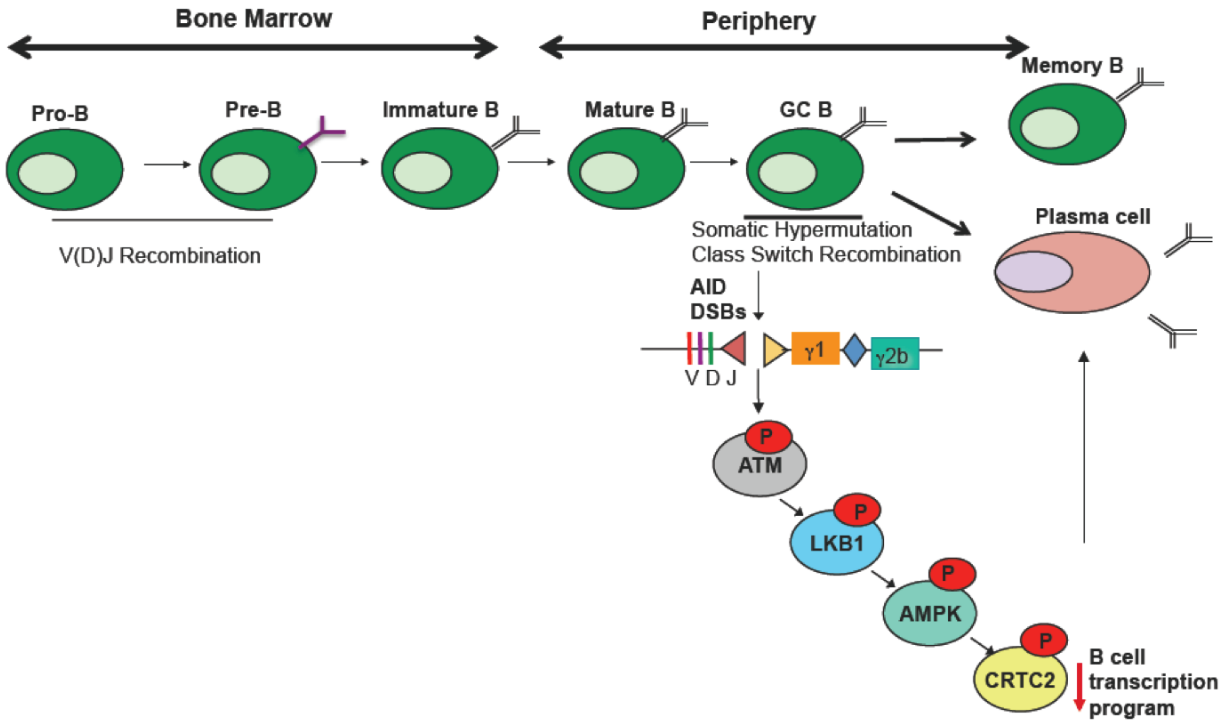


Figure 1-2. DNA DSBs activate signaling pathway to regulate genes important for terminal differentiation

During a T-dependent immune response in secondary lymphoid organs, GC B cells undergo DNA damage during SHM and CSR. AID-initiated DNA DSBs stimulate the activation of ATM, which then phosphorylates LKB1. LKB1 can activate AMPK and other members of the AMPK family of protein kinases, which in turn, phosphorylate CRTC2 to induce its cytoplasmic re-localization, resulting in the repression of CRTC2 target genes. A subset of these genes must be repressed for the terminal differentiation of GC B cells to PCs.

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CHAPTER 2:

T Follicular Helper Cell Differentiation and Germinal Center Formation is Prevented by Lkb1 Inhibition of NF-κB in B Cells

T cell dependent antigenic stimulation drives the differentiation of B cells into antibody secreting plasma cells, but it is unclear how B cells regulate this process. We show that Lkb1 expression in B cells maintains B cell quiescence and prevents the premature formation of germinal centers (GCs). *Lkb1* knockout in B cells (BKO) results in unprovoked B cell activation and secretion of multiple inflammatory cytokines that leads to splenomegaly from an unexpected exogenous T cell expansion. Within this cytokine response, increased IL-6 production occurs from heightened activation of NF-κB, which is suppressed by active Lkb1. Secreted IL-6 drives T cell activation and IL-21 production, promoting T follicular helper (T_{FH}) cell differentiation and expansion to support a ~100-fold increase in steady-state GC B cells. Blockade of secreted IL-6 from BKO B cells inhibits IL-21 expression, disfavoring T_{FH} cell differentiation and expansion. Together, these data reveal cell intrinsic and surprising cell extrinsic roles for Lkb1 in B cells that control T_{FH} cell differentiation and GC formation as a central regulator of T cell dependent humoral immunity.

Introduction

In adult mice, B lymphocytes develop in the bone marrow (BM) and emigrate to secondary lymphoid tissues, where they differentiate into memory B cells or plasma cells (PCs). Naïve B cells in primary lymphoid follicles and priming dendritic cells present processed protein antigen to cognate CD4+ T helper cells at the T–B border zones. This initial T–B encounter promotes the expansion and differentiation of B cells into low affinity, short-lived antibody secreting plasma cells (PCs) or germinal center (GC) founder B cells with the generation of T follicular helper (T_{FH}) cells [1, 2].

GCs are transient anatomic structures that develop in response to specific antigen exposure. The production and maintenance of both GC B cells and T_{FH} cells requires the expression of an essential transcriptional repressor, *Bcl6* [3, 4]. In GC B cells, BCL6 transiently suppresses DNA damage checkpoint genes, such as *p53* and *Atr* [5, 6], to allow rapid cell proliferation without triggering cell cycle arrest or apoptosis during somatic hypermutation (SHM) and class switch recombination (CSR) of immunoglobulin (*Ig*) genes. BCL6 also represses *Bcl2* expression [7, 8] and GC B cells upregulate *Fas* expression [9]. These changes generate a FASL-dependent apoptotic mechanism to eliminate GC B cells that do not receive an optimal antigen-dependent B cell receptor (BCR) rescue signal [10], thereby enforcing antigen specificity [11]. Antigen selected GC B cells then differentiate into memory B cells or long lived PCs [12] which remain quiescent until re-challenged or secrete high affinity antigen specific antibodies, respectively [13].

Recent studies uncovered a human GC B cell gene expression program that must be repressed for GC B cell differentiation into PCs [14]. Silencing of this program requires inactivation of the CREB transcriptional co-activator protein CRTC2 [15, 16]. Amongst 136 direct CRTC2 target genes in this silenced program are *AICDA, MYC, MTA3, CDK6, BACH2, TCF3, SMAD6, SMARCA2, SMARCA4,* and *TCL1*, all genes encoding proteins implicated in the

development and maintenance of GC B cells. Physiological CRTC2 inactivation begins with activation induced cytidine deaminase (AID) generated DNA double strand breaks during CSR and proceeds through a phospho-ser/thr signaling cascade from ATM to Lkb1 to an AMPK family member protein to CRTC2. Within this CRTC2 inactivation pathway, Lkb1 (liver kinase B1), a tumor suppressor ser/thr kinase responsible for Peutz-Jeghers syndrome [17], stands out as an understudied kinase in B cells with broad regulatory potential.

Lkb1 is a phosphorylation target of ATM [14, 18], protein kinase A [19], and ERK [20, 21], signaling kinases that can be activated in B cells. Lkb1 regulates AMPK activity, which in turn controls cellular metabolism and mTOR mediated protein synthesis [22, 23]. In mouse hematopoietic stem cells (HSCs), Lkb1 also regulates cell autonomous, partially AMPK-independent quiescence, proliferation, differentiation, energetics, and survival [24-26]. An initial proliferative burst with *Lkb1* knockout is followed by rapid depletion of HSCs and BM failure. Similar cell intrinsic and partial AMPK-independent Lkb1 control was also shown for mouse T cell development, physiology, and survival. T cell specific *Lkb1* knockout mice showed an incomplete block in thymocyte differentiation and decreased in vitro proliferation [27, 28] and survival [27-29], but increased activation of mature T cells that escaped to the periphery [27]. Despite these key findings in hematopoietic lineage cells, Lkb1 has not been assessed in B cells. The current study provides evidence that Lkb1 expressed in naïve B cells prevents premature, potentially spontaneous T_{FH} cell differentiation and GC formation in vivo.

Results

B cell specific *Lkb1* knockout (BKO) mice

Lkb1 was knocked out of post-pro/pre B cells in the BM by crossing *Lkb1*$^{fl/fl}$ mice [30] with *CD19-Cre*$^{+/-}$ knock-in mice [31] (Figure 2-1A). Although *mb1-Cre* expressing mouse B cells more efficiently delete floxed alleles, *CD19-Cre* expression is more specific, with *mb1-Cre* having activity also in T cells and germ cells [32, 33]. Therefore, to prevent complicating multi-lineage Lkb1 loss [34], *CD19-Cre* was used to inactivate *Lkb1* from B lineage cells.

Southern blot of CD43 depleted splenocytes showed robust allele excision in *Lkb1*$^{fl/wt}$*CD19-Cre*$^{+/-}$ (HET) mice, in contrast to significant steady-state retention of the floxed *Lkb1* alleles in *Lkb1*$^{fl/fl}$*CD19-Cre*$^{+/-}$ (BKO) mice (Figure 2-1B). qRT-PCR showed a similar ~2-fold reduction in *Lkb1* expression in both BKO and HET mice compared to *Lkb1*$^{wt/wt}$*CD19-Cre*$^{+/-}$ (WT) mice (Figure 2-1C). This prompted crosses with *Rosa26-YFP* mice [35] to generate BKO-YFP, HET-YFP, and WT-YFP mice in order to track Lkb1−YFP+ B cells (Figure 2-1A). In WT-YFP mice >85% of CD19+ splenocytes were Lkb1+YFP+ in contrast to <40% Lkb1−YFP+ splenocytes in BKO-YFP mice (Figure 2-2A). qRT-PCR and western blot confirmed loss of *Lkb1* mRNA and protein expression in YFP+ but not in YFP− splenic B cells in BKO-YFP mice (Figure 2-2B). Detailed analyses of WT-YFP and HET-YFP mice revealed phenotypic and functional equivalence so only data for WT-YFP control mice is shown. Overall, the YFP tracking data (Figure 2-2A) show that *CD19-Cre* mediated excision is incomplete and that Lkb1+YFP− B cells may have a competitive advantage over Lkb1−YFP+ B cells in BKO-YFP mice.

Pre-GC B cell development in BKO mice

In the BM, pro-B, pre-B, and immature B cells were similar in number between WT and BKO mice (Figure 2-1D). However, there was a ~3-fold decrease in recirculating B cells in the BM of BKO compared to WT mice. In the spleen, transitional (TR) B cells were increased ~2-fold in

BKO mice, whereas follicular (FO) and marginal zone (MZ) B cells were similar in number to WT mice (Figure 2-2C). In addition, no differences in the number of B-1a B cells in the spleen (Figure 2-1E), total cells and B cells in lymph nodes (Figures E2A and E2B), or total cells and B cells in the peritoneal cavity (Figures E2C and E2D) were detected between WT and BKO mice. Therefore, a decrease in recirculating BM B cells and an increase in splenic transitional B cells existed in BKO compared to WT mice. There was also a marked decrease in the frequency of Lkb1−YFP+ recirculating B cells in the BM (Figure 2-1D) and in the mature B cells in the spleen (Figure 2-2C and Figure 2-1E), lymph nodes (Figure 2-3B) and peritoneum (Figure 2-3D) of BKO-YFP compared to WT-YFP mice.

Splenomegaly from a T cell expansion in BKO mice

Despite modest differences in the total number of B cells (Figures 1C and E1D), spleens from BKO mice weighed ~3-fold more and contained almost 2-fold more cells compared to spleens from WT mice (Figure 2-2D). B cells were a lower percentage of the total spleen cells but equivalent in number between WT and BKO mice (Figure 2-2E). This resulted from an unanticipated increase in the number of mature T cells (Figure 2-2F) and, to a lesser extent, an increase in cells from multiple non-lymphoid white blood cell lineages in BKO versus WT spleens (Figure 2-2G). Histologic thin sections of BKO spleens showed alterations of the usual splenic architecture characterized by an expanded white pulp using hematoxylin and eosin (H&E) staining and enlarged splenic follicles by immunohistochemistry (IHC) for B and T lymphocytes (Figure 2-2H). Total thymocytes and development (Figures E2E and E2F), along with T cells in lymph nodes (Figure 2-3G) were similar between WT (WT-YFP) and BKO (BKO-YFP) mice, whereas peritoneal T cells were expanded ~4 to 5-fold in BKO mice (Figure 2-3H). The results show that Lkb1− splenic B cells induce a robust expansion of mature T cells in the spleen (and peritoneum) of BKO mice.

Lkb1 in B cells prevents spontaneous germinal center formation

Based on a marked white pulp expansion in BKO spleens (Figure 2-2H), IHC for GCs was performed. Spleens from WT mice showed occasional small clusters of peanut agglutinin (PNA) and BCL6 positive GC cells within small primary follicles in contrast to spleens from BKO mice, which showed large clusters of PNA+ and BCL6+ GCs within expanded follicles (Figure 2-4A). Analysis by flow cytometry revealed a robust increase in the number of GC B cells in BKO versus WT spleens and lymph nodes (Figure 2-4B and E3A). 75% of GC B cells in spleens and >90% of GC B cells in lymph nodes from BKO-YFP mice were Lkb1+YFP– (Figures 2B and E3A). Analysis by flow cytometry also detected a ~33-fold increased number of CD4+PD-1+CXCR5+ICOS+ T_{FH} cells in the spleens of BKO-YFP compared with WT-YFP mice, which contained few T_{FH} cells (Figure 2-4C). Additionally, expression of *Il21*, a canonical T_{FH} cytokine, was increased ~ 20 fold in CD4+ splenic T cells from BKO-YFP mice when compared with WT-YFP mice (Figure 2-4C). Thus, a subset of Lkb1 deficient B cells in BKO-YFP mice induced large GCs to form, with most GC B cells unexpectedly expressing Lkb1.

BKO GC B cells show increased apoptosis but still generate antigen-specific antibodies

Un-stimulated GCs in BKO mice prompted a functional assessment of CSR and antibody secretion. In the spleen and lymph nodes of BKO mice there was an increased percentage and number of IgG1+ B cells compared to WT mice (Figures 2D and E3B). Consistent with this increase, *Aicda* was expressed ~17-fold higher in BKO compared to WT B cells (Figure 2-4D). There was ~2-fold higher serum IgM in BKO compared to WT mice, but no difference in the amount of isotype-switched serum antibodies was detected (Figure 2-5C) despite a ~100-fold increase in GC B cells and a 4 to 5-fold increase in class switched B cells. Plasmablasts (PBs) in the spleen and PCs in the BM were statistically similar between BKO and WT mice (Figure 2-

5D). In BKO-YFP mice, <10% of PBs and PCs were YFP+, in contrast to ~70% YFP+ PBs and PCs in WT-YFP mice (Figure 2-5E), consistent with an advantage for mature Lkb1+YFP− compared to Lkb1−YFP+ B cells.

One hypothesis for the discrepant antibody secreting cells and isotype switched IGs in BKO mice is an increase in GC B cell apoptosis. In agreement, a ~2-fold increase in percentage of CD19+ B cells from BKO mice stained positive for activated, cleaved caspase 3 by flow cytometry (Figure 2-6F); however, apoptosis was not intrinsic for B cells that lost Lkb1 expression as the percentages of YFP+ and YFP− CD19+ B cells positive for cleaved caspase 3 was equivalent in BKO-YFP mice (Figure 2-5G). When partitioned into B cell subpopulations by flow cytometry, only GC B cells showed increased apoptosis (~10-fold) in BKO-YFP compared to control spleens (Figure 2-4E). Additionally, cleaved caspase 8, the caspase activated down-stream of FAS-ligand induced apoptosis [36, 37], was detected in whole cell lysate from CD43 depleted splenic B cells from BKO but not WT mice (Figure 2-4F). Increased GC B cell apoptosis coincides with a predominantly GC B cell expansion in BKO-YFP mice, which could prevent an increase in ASC production and isotype switched serum IGs in naïve BKO compared to WT mice. Even by controlling aberrant GC formation through apoptosis, anti-nuclear antibodies (ANA) were detected in 21% (4/19) of BKO mice aged over 6 months, whereas no anti-ANAs were detected from littermate controls (0/12) (Figure 2-5H). The data suggest that loss of Lkb1 in a subset of B cells drives GC formation, which over time may result in autoimmunity.

The presence of cleaved caspase 8 in B cells from BKO mice indicates lack of rescue signal provided by antigenic or costimulatory signaling upon antigen encounter [38, 39]. To determine whether BKO mice respond to antigenic stimuli, BKO were challenged with both T cell dependent and T cell independent antigens. BKO mice challenged with TNP-AECM-FICOLL, a T independent antigen, generate TNP-IgM and TNP-IgG3 at 7 days at levels similar

to WT mice (Figure 2-6A). Furthermore, BKO mice immunized with NP-CGG, a T dependent antigen, produced NP+ GC B cells at 10 and 28 days post-immunization (Figure 2-6B). At day 10 post-immunization, BKO-YFP spleens contained less NP+ GC B cells than WT-YFP spleens, however, serum NP-IgG1 at 14 and 28 days was similar between BKO-YFP and WT-YFP mice (Figure 2-6C). Unfortunately, the Lkb1−YFP+ or Lkb1+YFP− PC origins of secreted IGs cannot be determined using serum ELISAs.

B cells in BKO mice are hyperactive and proliferative

Since GC B cells were markedly increased (Figure 2-4B), B cell activation in BKO mice was examined. CD19+ B cells from spleen and lymph node showed increased expression of MHC II, CD86, and (for spleen) CD69 in BKO compared to WT mice (Figures 3A and E5A). Interestingly, only increased expression of MHC II was intrinsic to B cells that deleted Lkb1, as Lkb1−YFP+ B cells express higher levels of MHC II compared to Lkb1+YFP− CD19+ B cells in BKO-YFP mice (Figure 2-7B). Expression of CD86 and CD69 was similar between Lkb1+YFP− and Lkb1−YFP+ B cells in BKO-YFP mice (Figure 2-7B and Figure 2-8B), suggesting a possible pro-inflammatory cytokine environment with paracrine B cell activation. Pre-GC T2 and T3 transitional B cell subsets also show 2 to 3-fold higher MHC II surface expression in BKO compared to WT spleens (Figure 2-7C). Spontaneous in vivo activation was strongly suggested because freshly isolated B cells from BKO-YFP spleens incorporated ~8-fold more BrdU ex vivo in a 30 min pulse than WT-YFP splenic B cells (Figure 2-7D), which is consistent with the presence of GC B cells in BKO mice (Figure 2-4). Interestingly, the percent of Lkb1−YFP+ and Lkb1+YFP− splenic B cells that synthesized DNA was similar in BKO-YFP mice (Figure 2-7D), again suggesting a cell extrinsic influence of Lkb1−YFP+ B cells on Lkb1+YFP− B cells.

To examine the effect of Lkb1 on B cell proliferation, Celltracer dye dilution assays were performed on CD43 depleted B cells from WT-YFP and BKO-YFP spleens stimulated with anti-

CD40 mAb and IL-4. After 3 days, overall cell division was similar for WT-YFP and BKO-YFP B cells (Figure 2-8C). However, a greater percentage of Lkb1−YFP+ B cells divided by day 3 than did Lkb1+YFP− B cells from BKO-YFP spleens and underwent multiple rounds of division (Figure 2-8D). Additionally, BrdU incorporation studies showed similar levels of DNA synthesis between stimulated B cells from WT-YFP and BKO-YFP spleens (Figure 2-8E), whereas stimulated Lkb1−YFP+ B cells synthesize ~2-fold more DNA than Lkb1+YFP− B cells from BKO-YFP mice (Figure 2-8E). The data suggest that Lkb1 inhibits spontaneous B cell activation in BKO mice and negatively regulates B cell proliferation.

Lkb1 suppresses T cell activation

We next examined how Lkb1− B cells could stimulate a marked Lkb1+ GC B cell and massive T cell expansion. As MHC II and the T cell co-stimulatory protein CD86 were upregulated on BKO B cells, we investigated whether isolated BKO B cells could activate CD4+ T cells. In vitro co-incubation of CD4+CD62L+ naïve T cells from WT spleens with anti-CD3 and naïve BKO B cells induced T cell activation to a similar extent as anti-CD3 plus pre-activated WT B cells, as measured by the induction of the CD44 activation biomarker (Figure 2-7E). Naïve BKO B cells also induced T cell expression of activation biomarker CD25 (Figure 2-7F) as well as anti-CD3 and anti-CD28 co-stimulation.

In vivo, CD4+ T cells in BKO spleens showed a ~3-fold increase in the number of activated CD44+ T cells (Figure 2-7G), and a ~2 to 3-fold increase in the expression of CD69 or CD25 compared with CD4+ T cells from WT spleens (Figure 2-7H). Similarly, a ~2 to 3-fold increase in activated CD4+CD44+ T cells was detected in lymph nodes of BKO mice without an overall T cell expansion (Figures E5F). Combined, the data suggest that loss of Lkb1 in a subset of B cells is able to induce activation of CD4+ T cells in vivo.

Lkb1 blocks T_{FH} cell differentiation and IL-21 production through IL-6 inhibition

To determine how BKO B cells could influence the effector differentiation of activated CD4+ T cells, a cytokine and chemokine array was surveyed with conditioned media from un-stimulated cultures of CD43 depleted cells from WT-YFP and BKO-YFP mice. Strikingly, an inflammatory profile of 15 factors was elevated in BKO-YFP compared to WT-YFP enriched B cells, including IL-6, which was recently shown to induce the earliest stages of T_{FH} differentiation (Figure 2-9A) [40-44]. *Il6* transcripts from Lkb1−YFP+ B cells were elevated ~3-fold over Lkb1+YFP− B cells from BKO-YFP spleens and ~4 fold over WT B cells (Figure 2-10A). Additionally, un-stimulated Lkb1−YFP+ B cells produced and secreted ~ 3-4 fold more IL-6 protein over 24 hours, in vitro, than Lkb1+YFP− B or WT B cells (Figure 2-10B). These data strongly suggest that the loss of Lkb1 is responsible for increased IL-6 production. We focused on IL-6 because of its elevated expression in Lkb1−YFP+ B cells and its potential role in GC physiology as an early inducer of T_{FH} cell differentiation. IL-6 has been shown to induce not only T_{FH} cell, but also Th_{17} and T_{REG} cell differentiation from naïve CD4+ T cells [45-47]. Therefore, CD4+ T cells from BKO-YFP spleens were assessed for signature T cell subset transcripts. A ~20-fold increase in *Il-4* transcripts was detected in CD4+ T cells from BKO-YFP compared to WT-YFP spleens (Figure 2-9B). When paired with the ~20-fold increased expression of *Il21* (Figure 2-4C) the expression data is consistent with flow cytometry data indicating a robust T_{FH} expansion (Figure 2-4C). Increased expression of *Il10*, but not other Th subset canonical cytokine transcripts (Figure 2-9B), was detected in CD4+ T cells from BKO-YFP compared to WT-YFP spleens. This transcript profile suggests that along with an increase in T_{FH} cells, there was also an increase in T_{REG} cells in BKO mice. Consistent with this expression profile, increased expression of *Foxp3* was identified in CD4+T cells from BKO mice (Figure 2-9C). Further analysis by flow cytometry detected a marked increase in CD4+FoxP3+ T_{REG} cells and but not in CD4+PD-1+CXCR5+FoxP3+ T follicular regulatory (T_{FR}) [48] cells (Figure 2-9D). Importantly, as

previously shown [43] *Il21* induction required IL-6 secretion from Lkb1−YFP+ BKO B cells, as naïve WT T cell co-incubation with WT, or even pre-activated, B cells failed to induce *Il21* transcripts, whereas co-incubation with BKO B cells caused a significant induction of *Il-21* expression (Figure 2-10C). Blockade of secreted IL-6 with an anti-IL-6R antibody inhibited *Il-21* induction almost to WT or pre-activated B cell levels (Figure 2-10C).

Lkb1 expression inhibits NF-κB to block IL-6 production

To determine the impact of Lkb1 expression on IL-6 production without the influence of other cell types or cell extrinsic factors, WT and Lkb1− mouse embryonic fibroblasts (MEFs) were chosen as a study system. WT MEFs produced few *Il-6* transcripts, whereas Lkb1− MEFs showed a ~350-fold increase in *Il-6* transcript production (Figure 2-11A). Lkb1− MEFs also secreted ~10-fold more IL-6 over 24 hours than WT MEFs (Figure 2-11B). This increase in IL-6 production is specific to Lkb1 loss because reconstitution of Lkb1− MEFs with a mouse *Lkb1* expression vector (Figure 2-11C) results in both a repression of *Il-6* transcripts (Figure 2-11A) and IL-6 secretion (Figure 2-11B).

Multiple signaling pathways acting on a variety of transcription factors regulate the expression of *Il6* [49-52]. Lkb1− MEFs show an increase in nuclear p65, p52, and p50 NF-κB family member proteins compared to WT and Lkb1 reconstituted MEFs (Figure 2-11D) whereas other signaling pathways showed similar or reduced activity compared to WT MEFs (Figures 2-12A-E). To determine whether increased p65 NF-κB activity contributes to *Il-6* production, Lkb1− MEFs were incubated with an inhibitor of p65 nuclear import, JSH-23 [53]. Titration of JSH-23 below the established IC_{50} of 7.1 μM resulted in a dose-dependent response in *Il6* expression in Lkb1− MEFs (Figure 2-12F). Additionally, JSH-23 exposure decreased *Il-6* production by ~50% in Lkb1− MEFs compared to DMSO treated controls (Figure 2-11E). Similar to Lkb1− MEFS, Lkb1−YFP+ B cells from BKO spleens contain increased nuclear p65, p52, and

p50 proteins compared to both CD43 depleted WT B cells and Lkb1+YFP− B cells from the same BKO spleen (Figure 2-11F). These data indicate that Lkb1 inhibits spontaneous IL-6 production by repression of NF-κB activation, although this result does not exclude additional Lkb1-independent mechanisms of NF-κB regulation.

Lkb1 is phosphorylated downstream of B cell receptor signaling

NF-κB signaling is an important mediator for B cell responses such as proliferation and CSR [54] and is activated downstream of B cell receptor (BCR) signaling [55]. Interestingly, Lkb1 can be phosphorylated downstream of multiple signaling pathways [56] including MAPK/ERK pathway signaling [19], which is an important mediator of BCR signaling [57]. To determine whether Lkb1 is phosphorylated downstream of BCR engagement, CD43 depleted WT B cells were stimulated with anti-IgM F(ab^2) fragment. As expected, ERK was phosphorylated within 5 min upon stimulation (Figure 2-13). Additionally, Lkb1 was phosphorylated at Ser431 upon BCR signaling induction, with approximately the same kinetic profile as ERK phosphorylation (Figure 2-13). The regulation of Lkb1 by post-translational modification is complex, multi-faceted, and incompletely understood [56], although phosphorylation of S428 (S431 in mice) has been shown to be inhibitory in multiple cellular contexts [58-60] and could potentially link Lkb1 inactivation to the induction of NF-κB through BCR signaling.

Our BKO mice suggest that in B cells, Lkb1 expression likely prevents unprovoked cellular activation and IL-6 production by inhibiting NF-κB activation. Lkb1−YFP+ B cells show spontaneous activation and secrete inflammatory cytokines, including IL-6, which activates surrounding CD4+ T cells and induces *Il-21* expression, which promotes CD4+ T cell differentiation into T_{FH} cells to drive un-stimulated GC formation and expansion. These results suggest a model in which antigen-triggered Lkb1 inactivation in mature B cells is a physiologic

mechanism for stimulating the start of a GC reaction through B cell intrinsic and extrinsic immune system activation (Figure 2-14).

Discussion

Previously we identified a CSR-initiated signaling pathway in GC B cells that ends with inactivation of CRTC2 to silence GC B cell genes and enable the differentiation of PCs [14]. An essential intermediate in this signaling pathway is Lkb1, whose siRNA repression blocked human tonsil B cells from becoming IG-secreting PBs in an in vitro GC-like B cell differentiation system and whose loss of expression was detected in human lymphoma samples [14]. However, these studies did not examine Lkb1 in B cells in vivo where the microenvironment and non-B cell types influence GC formation, maintenance [42, 61-65], size regulation [66], and PC production. Therefore, *Lkb1* was deleted in developing and mature B cells in mice to assess its contextualized function. This resulted in reductions of mature Lkb1−YFP+ B cells, which resembled cell reductions in lineage specific *Lkb1* knockout T cell and HSC mice. Most interestingly, Lkb1− B cells also exerted unanticipated control over T cell activation, expansion, and differentiation that recruited naïve Lkb1+ and Lkb1− B cells into hyperplastic GC reactions (Figure 2-14).

Lkb1 knockout in mouse thymocytes or HSCs altered cell autonomous functions including quiescence, survival, differentiation, activation, and metabolism [24-29, 67]. Consistent with these studies, B cells with *Lkb1* deletion at the pro/pre B cell stage showed reductions in all mature B cell populations in the spleen, lymph nodes, and in the peritoneum of BKO mice. However, total B cell numbers in the spleen, lymph node, and peritoneum were equivalent between WT and BKO mice, indicating that Lkb1+ B cells in BKO mice compensated for these reductions, resulting in B cell homeostasis. An up to ~100-fold expansion of Lkb1+ and Lkb1− GC B cells in BKO-YFP mouse spleens and lymph nodes contrasts with reduced steady-state HSCs, thymocytes, T cells, and pre-GC B cells. This non-target Lkb1+ GC B cell expansion stimulated our focus on the cell extrinsic functions of Lkb1 loss in B cells as a potential explanation.

We showed that BKO B cells activate and expand T cells by increased expression of MHC class II proteins for antigen presentation, CD86 (B7-2) for co-stimulation, and augmented cytokine secretion. Secreted cytokines produced a mainly T_{FH} cell polarizing inflammatory response. Interestingly, *Lkb1* deletion in mature T cells resulted in autonomous cell activation and the production of interferon-γ and IL-17, potentially via mTORC1 hyperactivity [27]. A similar mTORC1 mechanism for cytokine secretion could operate in Lkb1– B cells, although mTORC1 is a general regulator of protein synthesis and an explanation for T_{FH} polarizing cytokine production is not revealed.

The loss of Lkb1 in both B cells and MEFs induced the expression of *Il-6*, which may occur by increased NF-κB activation [49]. Our data show an increase of nuclear NF-κB family member proteins in Lkb1– MEFs that, when inhibited, led to a decrease in *Il-6* levels. Together these results suggest that Lkb1 inhibits NF-κB. There is a low level of tonic NF-κB activity in mature B cells [68] and induced NF-κB activation is required for B cell proliferation, class switch recombination, and cytokine production [54]. However, excessive NF-κB signaling has been shown to be detrimental to B cells. Mice with constitutively active NF-κB display B cell hyperplasia [69-72], autoimmunity [70, 73, 74] and lymphomagenesis [71]. B cells from these mice respond by hyperactivation and hyperproliferation to stimulation [70, 71, 74], secrete IL-6 [70, 74] and form robust GCs [70, 72-74]. Regulation of NF-κB activity by Lkb1 could be a mechanism by which B cells survive with tonic BCR signaling, followed by heightened NF-κB activation from stimulation, such as by antigen, that provokes B cell activation and subsequent GC formation.

Recently, IL-6 induction in B cells during an antiviral immune response was reported to be dependent on Oct2 and OBF-1 transcription factors [44]. Karnowski et al., showed that in response to influenza, in vivo, and in response to TLR signaling, in vitro, IL-6 induction in B cells is dependent on the induction of Oct2 and particularly OBF-1 [44]. Similar to our model (Figure

2-14), IL-6 produced by B cells induces IL-21 production in T cells and these cytokines play overlapping roles in the anti-viral responses of T_{FH} cell differentiation and GC formation. In response to BCR signaling, however, *Oct2-/-* mice were able to induce *Il6* expression and while *Obf1-/-* mice were not, it is known that *Obf1-/-* B cells have defects in BCR signaling and that *Obf1-/-* mice cannot form GCs [75]. Given the emerging role of IL-6 as an early inducer of T_{FH} cell differentiation [40, 41, 43], multiple mechanisms could exist for initiation of IL-6 production in B cells depending on the triggering stimulus inducing an immune response. While inflammatory stimuli could provoke robust IL-6 secretion through *Oct2* and *OBF-1* induction [44], non-inflammatory activation of B cells through antigen-BCR signaling could induce IL-6 production through NF-κB activation to provide a mechanism for T_{FH} cell differentiation.

The identification of upstream regulators and downstream effectors of Lkb1 signaling in B cells is required to understand and potentially manipulate Lkb1 control of the GC reaction. To enable PC differentiation of GC B cells in human tonsil, Lkb1 relays signals from ATM during CSR to an unidentified AMPK family member protein to inactivate CRTC2. It is not known which of 13 potential AMPK family member proteins Lkb1 targets to repress genes that support GC B cells. These Lkb1 repressed CRTC2 target genes control key GC processes. This repressed gene program alone seems sufficient to terminate the GC reaction, but additional AMPK family targets of Lkb1 signaling are well positioned to regulate key GC and PC processes including energy metabolism (AMPK) [23], cell polarity (MARK proteins, others) [76] and anoikis (SIK1) [77]. Definitive evidence that Lkb1 inactivation of CRTC2 is a main control point for PC production requires generation of an in vivo model that replicates the results of the in vitro GC-like B cell differentiation system [14, 78, 79].

Surprisingly, Lkb1− B cells induced the activation and proliferation of both Lkb1+ and Lkb1− B cells. Lkb1− B cells produced inflammatory cytokines including IL-6 that induced T cell expansion and T_{FH} cell differentiation to support the generation of GCs that recruited both Lkb1+

and Lkb1− B cells. A key remaining question is whether Lkb1 controls the start of endogenous GC reactions. The earliest stage of GC formation at the outer T–B border zones requires signaling through BCR-cognate antigen recognition and CD40/CD40L B–T cell interactions. Lkb1 is a constitutively active ser/thr kinase [17] with potential for altered activity by post-translational modification, subcellular localization, or substrate availability [17, 27]. MAPK-ERK pathway signaling phosphorylates Ser325 and/or Ser428 in the C-terminal regulatory domain of Lkb1 [17, 18]. B-RAF V600E mutant melanoma cells also phosphorylate these sites through ERK signaling to negatively regulate Lkb1 and drive cancer cell proliferation [21]. Interestingly, BCR and CD40 signaling in B cells also activate MAPK-ERK pathway signaling. Further work is required to determine whether this signaling, changes in localization, or target protein availability attenuate Lkb1 kinase activity to generate T_{FH} polarizing cytokines that help initiate a GC reaction.

Lkb1 prevents naive B cells from expressing IL-6, a key in vivo cytokine that stimulates CD4+ T cells differentiate into T_{FH} cells [40, 41]. IL-16 secretion from activated B cells [80] results in the recruitment of T cells and dendritic cells at the B−T border. In turn, T_{FH} cells express CXCL13, to attract B cells into growing GCs [81, 82] and IL-21, to enhance the differentiation and expansion of T_{FH} cells to support larger GCs [41, 43]. Interestingly, the rigid requirement for antigen presentation by dendritic cells and B cells for T_{FH} differentiation and maintenance may be abrogated by *Lkb1* deletion from B cells [83, 84]. Future studies will focus on precisely how physiologic BCR, CD40, or other signaling relieves Lkb1 enforced B cell quiescence, how Lkb1 inhibition in B cells induces a T_{FH} cell polarizing inflammatory cytokine response, and whether manipulation of Lkb1 activity can enhance humoral immunity or block a deleterious autoantibody response.

Materials and Methods

Mice

Lkb1$^{fl/fl}$ mice were obtained from the National Cancer Institute repository. *Lkb1$^{fl/fl}$*, *CD19-Cre$^{+/-}$* and *Rosa26-YFP* mice (Jackson Laboratory) were housed in a pathogen-free animal facility at UCLA. All mouse studies were done between 6 to 12 weeks of age with approval (#1998-113-51) from the UCLA Animal Research Committee.

Reagents and antibodies

Anti-mouse antibodies included CD45R (RA3-6B2), CD5 (53-6.7), CD21/35 (7G6), CD43 (S7), CD44 (IM7), CD62L (MEL-4), CD86 (GL1), CD95, CD138 (281-2), IgM (R6-60.2), T-B cell activation antigen (GL-7) (all from BD); CD45R (RA3-6B2), CD4 (RM4-5), CD8α (5306.7), CD23 (B3B4), CD44 (IM7), CD69 (H1.2F3), CD93 (AA4.1), IgM (11/41), MHC II (114.15.2) (all from eBioscience); CD19 (6D5) (Biolegend), CD45R (RM) (Invitrogen), active caspase 3, BrdU-Red (BioVision) and β-tubulin (Sigma). Anti-rabbit antibodies included cleaved (D5B2) and total (D35G2) caspase 8, phospho-p42/44 (D13.14.4E), p42/44 (137F5), phosphor-p38 (D3F9), p38 (D13E1), phospho-JNK (81E11), JNK (56G8), p65 (D14E12), NF-κB p100/52 and Lkb1 (D60C5) (Cell Signaling), Actin (Sigma), NF-κB p50, HDAC1 (H-51), JunB, CREB, phosphor-Lkb1 (Ser431) (Santa Cruz) and phospho-CREB S133 (Millipore), and NP-PE (Biosearch Technologies).

Cell culture

WT and Lkb1− MEFs were obtained from Nabeel Bardeesy and were grown in DMEM supplemented with 10% FBS with or without JSH-23 (Santa Cruz Biotechnology) as indicated. Isolated mouse splenic B cells ($1X10^6$ cells/ml) were grown in RPMI 1640 supplemented with 10% FBS and β-mercaptoethanol and stimulated with 1 μg/ml anti-CD40 mAb (BD Pharmingen)

and 25 ng/ml IL-4 (R&D Systems) for up to 3 days or $F(ab^2)$ fragment of anti-IgM (Jackson Labs) for up to 1 hour. Mouse T cells were co-incubated with 1 µg/ml of plate bound anti-CD3 mAb (BioLegend) and either 1 µg/ml anti-CD28 mAb (BioLegend), WT B cells pre-activated for 24 hours, fresh WT B cells, or BKO B cells for 48 hours with or without of 10 µg/ml anti-IL-6R Ab (BioLegend).

qRT-PCR

RNA was isolated using Trizol (Life Technologies) and converted to cDNA with iScript (Bio-Rad). qRT-PCR was performed on a LightCycler480 (Roche) using SYBR green (Roche).

Immunoblotting

Cells were lysed with Triton-X lysis buffer or fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Extracts were separated by SDS-PAGE and transferred to either nitrocellulose or PVDF membranes. Membranes were incubated overnight with the indicated antibodies in TBS-Tween and 5% milk or 5% BSA.

Flow cytometry and cell sorting

Single cell suspensions were stained with conjugated antibodies; data was obtained on a BD LSRII (BD Biosciences) and analyzed with FlowJo software (Treestar). Cell sorting was performed on a BD FACSAria (BD Biosciences).

Histology and immunohistochemistry

Spleens were dehydrated and paraffin embedded. Thin 6µm sections were stained with hematoxylin and eosin or hematoxylin and antibodies using the avidin-biotin peroxidase complex method. Histologic images were viewed with an Olympus Ax70 microscope (40 /0.75

oil objective lens) and photographed with an Olympus DP71 camera; DP controller software (Version 3.3.1.292) was used for image acquisition.

B and T cell enrichment

RBC depleted splenocytes were enriched for B cells by CD43 depletion or naïve T cells by depletion of non-CD4+ T cells and positive selection of CD62L+ T cells (Miltenyi Biotec). All enrichments were ≥ 85% pure by flow cytometry.

BrdU labeling

BrdU was added to stimulation media for 30 minutes prior to harvest. Cells were prepared and stained using the BrdU for flow cytometry kit (BD Biosciences).

IL-6 ELISA

IL-6 in cultured media from overnight cultures was quantified by IL-6 ELISA (R&D Systems) per the manufacturer's instructions.

Retrovirus generation and infection

Mouse *Lkb1* was cloned into the *MSCV-IRES-tNGFR* retroviral expression vector [85] by standard methods. Viral supernatant was harvested from HEK293T cells 48 and 72 hours after transfection with FuGene (Roche). 2.0 X 10^6 cells were incubated with virus supplemented with 4μg polybrene for 6 hours. Prior to experimental assays, NGFR expressing cells were enriched by incubation with NGFR-PE antibody (Miltenyi Biotec) and anti-PE microbeads then passed through a magnetic column (Miltenyi Biotec).

Southern blot analysis

Cre-mediated excision of *Lkb1* exons 3 – 6 was determined by multiplex PCR or Southern blot analysis from tail genomic DNA as previously described [1] with an exon 5 – 8 digoxigenin-11-dUTP labeled probe (Roche). Blot hybridizations were visualized with an anti-digoxigenin-alkaline phosphatase conjugate (Roche) and chemilluminescent detection.

Immunizations

Mice were immunized with 25 µg TNP-AECM-FICOLL (Biosearch Technologies) in 1xPBS, pH 7.4, via intra-peritoneal injection and blood was collected and assayed 7 days post injection by enzyme-linked immunosorbent assay (ELISA). 100 µg of NP(20-29)-CGG (Biosearch Technologies) in alum was administered via intra-peritoneal injection and blood was collected and assayed 14 and 28 days post injection by ELISA.

ELISA

Serum IG concentrations were determine by ELISA using goat anti-mouse IG as a capture antibody and developed with an isotype specific goat anti-mouse antibody conjugated to horse-radish peroxidase (HRP) (Southern Biotech). Blood was collected via retro-orbital eye bleed. ELISA was performed with NP-BSA or TNP-BSA as a capture antibody (Biosearch Technologies) and developed with an isotype specific goat anti-mouse antibody conjugated to HRP (Southern Biotech).

ANA ELISA

Anti-nuclear antibodies in serum were detected by anti-ANA ELISA (Alpha Diagnostics) according to the manufacturer's instructions. The positivity index was calculated as follows:

mean OD of WT samples + 2SD. All samples were then compared to the positivity index, with values greater than 1.0 considered positive.

Proliferation assay

CD43 depleted B cells were incubated at 1X10^6 cell/ml with Celltracer Violet (Invitrogen), followed by culture with 1µg/ml anti-CD40 Ab (BD Bioscience) and 25 ng/ml IL-4 (R&D Technologies) for 3 days. Fluorescent intensity was measured daily by flow cytometry. Proliferation was analyzed using Flowjo software (TreeStar).

Cytokine proteome array

Single cell suspensions of CD43 depleted splenic B cells from WT (n=2) and BKO (n=2) mice were incubated in RPMI 1640 for 6 hr and culture media was harvested and analyzed using the Proteome Profiler Mouse Cytokine Array (R&D Systems). Briefly, cells were incubated with a detection antibody cocktail for 1 hr, added to the membranes, and incubated overnight. Membranes were developed with streptavidin-HRP and exposed to film. Pixel density was determined using ImageJ software.

FoxP3 staining

Red blood cell depleted single cell suspensions from spleens were stained with anti-bodies against CD4, CXCR5, PD-1, and SLAM, and then fixed and permeabilized using the Mouse FoxP3 Buffer Set (BD Biosciences), according to the manufacturer's instructions. Fixed and permeablized cells were stained with an antibody against FoxP3 (BD Horizon). Samples were run on an LSRII (BD Biosciences) and analyzed with Flowjo software (TreeStar).

Statistical analysis and experimental design

The two tailed, unpaired Student's t-test and the Mann-Whitney U test were employed to analyze parametric and non-parametric data, respectively. In the instances of more than 2 samples, the one way ANOVA test was employed, in all cases, $p < 0.05$ was also considered significant. Study sample size was determined using a Cohen's *d* between 1.5 and 2 with at least 80% power for all studies. Additionally, there was no blinding or randomization for animal studies.

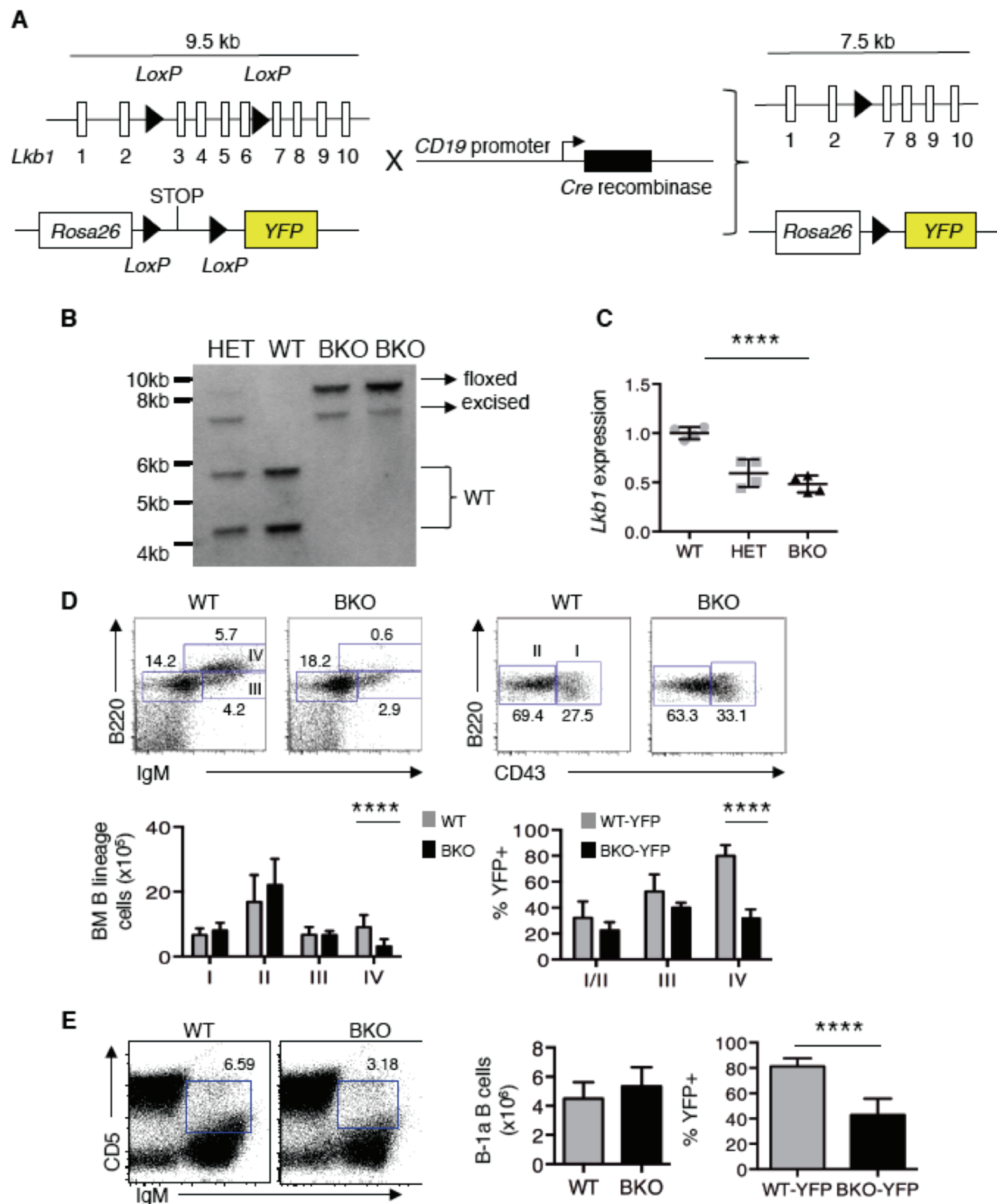

Figure 2-1. Decreased recirculating B cells in the BM of BKO mice

Figure 2-1. Decreased recirculating B cells in the BM of BKO mice

A. Strategy for generating *Lkb1* B cell lineage specific knockout (BKO, BKO-YFP) mice.

B. Southern blot of CD43 depleted splenocytes (>85% B cells) from HET, WT, and BKO mice evaluating excision of *Lkb1* exons 3 – 6.

C. *Lkb1* expression, relative to *36b4*, by qRT-PCR of CD43 depleted splenocytes from WT (n=4), HET (n=4), and BKO (n=4) mice. Mean ± SD, ****p=0.0001 by two tailed, unpaired Student's t test

D. Flow cytometry showing pro-B (I), pre-B (II), immature B (III), and recirculating B (IV) cells in the BM of WT (n=9) and BKO (n=10) mice and the frequency of YFP+ B cells in each subpopulation (n=5). Mean ± SD, ****p=0.0004 by Mann-Whitney U test and p=8.8E-006 by two-tailed, unpaired Student's t test, respectively

E. Flow cytometry showing splenic B-1a cells in WT (n=6) and BKO (n=9) mice and the frequency of YFP+ B-1a cells (n=9 and 8, respectively). Mean ± SD; ****p=1.8E-05 by two-tailed, unpaired Student's t test

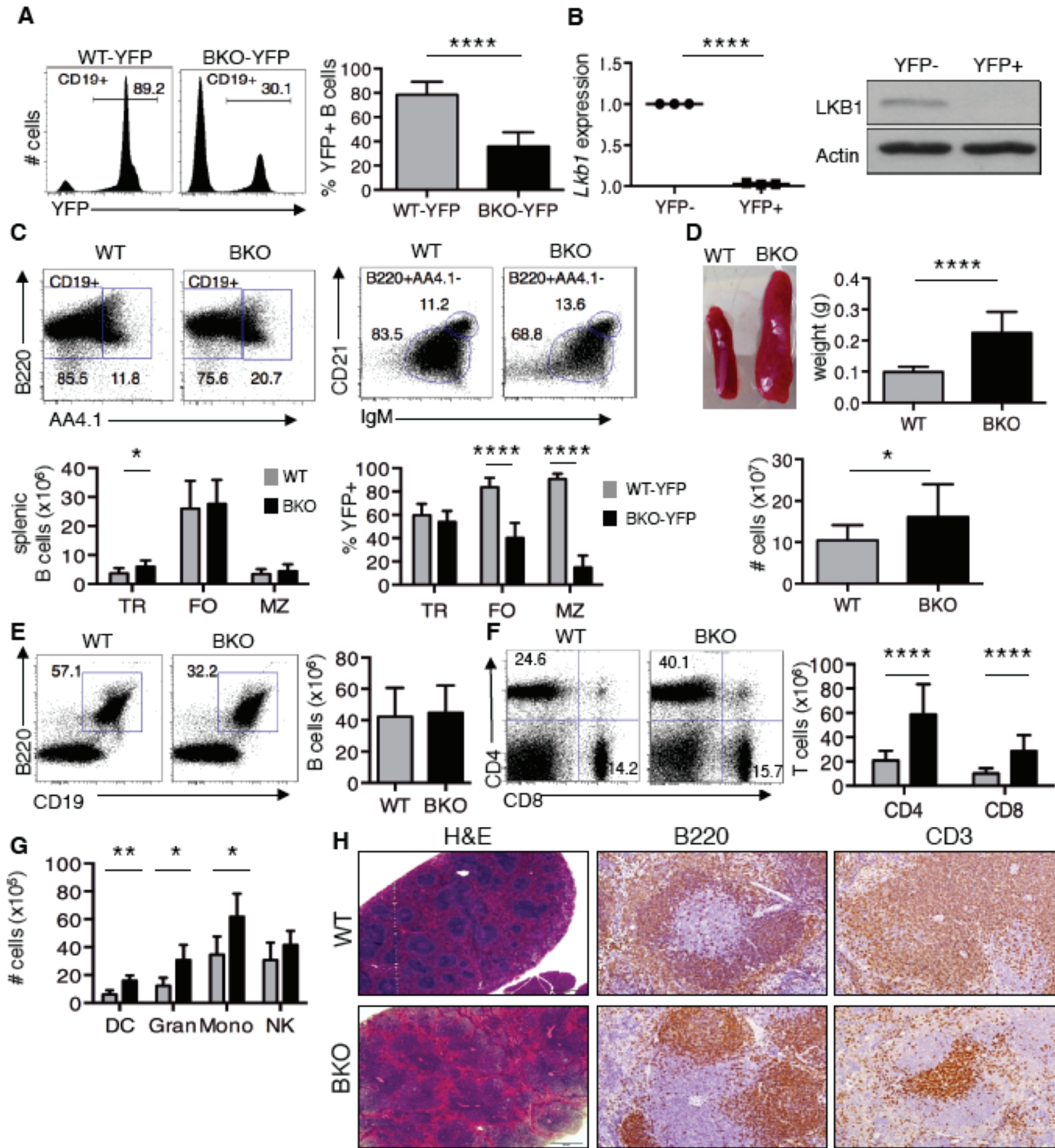

Figure 2-2. Reduced Lkb1− B cell subsets with splenomegaly from a T cell expansion in BKO-YFP mice

Figure 2-2. Reduced Lkb1− B cell subsets with splenomegaly from a T cell expansion in BKO-YFP mice

A. Flow cytometry for YFP expression in CD19+ splenocytes from WT-YFP (n=7) and BKO-YFP (n=8) mice. Mean ± SD, ****p=4.9E-06 by two=tailed, unpaired Student's t test

B. qRT-PCR (n=3) and a representative western blot for Lkb1 in YFP sorted splenocytes from BKO-YFP mice. *Lkb1* expression, relative to *36b4* expression, shown normalized to Lkb1+YPF− CD19+ BKO B cells. Mean ± SEM; ****p=3.8E-05 by two-tailed, unpaired Student's t test

C. Flow cytometry analysis for splenic transitional (TR: CD19+B220+AA41+), follicular (FO: CD19+B220+AA41-IgM+CD21+), and marginal zone (MZ: CD19+B220+AA41-IgM++CD21++) B cells in WT (n=8) and BKO (n=11) mice and the percentage of YFP+ cells for each subpopulation in WT-YFP (n=7) and BKO-YFP (n=8) mice. Mean ± SD; *p=0.03 by Mann-Whitney U test, ****p=3.5E-006 and 1.5E-010 by two-tailed, unpaired Student's t test, respectively

D. Representative images of spleens from WT and BKO mice. Weight of WT (n=34) and BKO (n=34) spleens and cell numbers recovered from WT (n=13) and BKO (n=18) spleens depicted as mean ± SD; p=0.011 and ****p=9.7E-13 by two-tailed, unpaired Student's t test

E. Flow cytometry and number of B220+CD19+ splenic B cells from WT (n=13) and BKO (n=18) mice. Mean ± SD

F. Flow cytometry and number of CD4+ and CD8+T cells from WT (n=11) and BKO (n=11) mice. Mean ± SD; ****p=1.1E-06 and 4.6E-06 by Mann-Whitney U test, respectively

G. Non-lymphocyte white blood cells in spleens calculated by flow cytometry from WT (n=5) and BKO (n=5) mice. Mean ± SD; **p=0.008, *p=0.03 and 0.032 by two-tailed, unpaired Student's t test, respectively

H. Histologic sections of WT and BKO spleens stained with hematoxylin and eosin (H&E), anti-B220, or anti-CD3 antibodies.

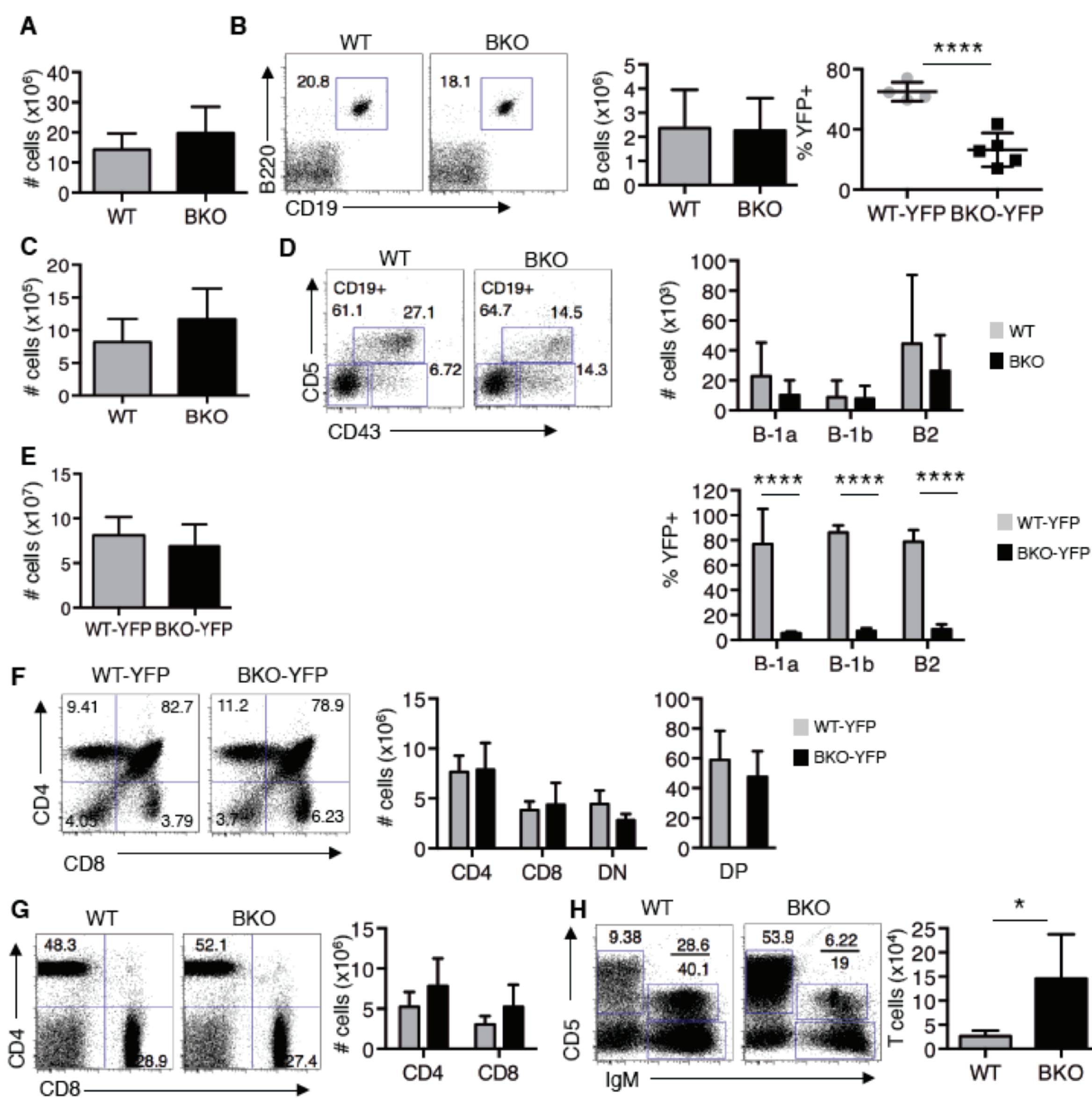

Figure 2-3. Reduced LKB1− B cells in lymphoid tissues, but increased T cells in the peritoneum of BKO mice

Figure 2-3. Reduced LKB1− B cells in lymphoid tissues, but increased T cells in the peritoneum of BKO mice

A. Lymph node cellularity in WT (n=7) and BKO (n=10) mice. Mean ± SD

B. (Left and center panels) Flow cytometry of B220+CD19+ B cells in lymph nodes of WT (n=7) and BKO (n= 10) mice. (Right panel) Flow cytometry for YFP+ expression in B220+CD19+ B cells in lymph nodes of WT-YFP (n=4) and BKO-YFP (n=5) mice. Mean ± SD; ****p=0.0005 by two-tailed, unpaired Student's t test

C. Peritoneal cavity cellularity in WT (n=5) and BKO (n=5) mice. Mean ± SD

D. (Upper panels) Flow cytometry of B cells in the peritoneal cavity of WT (n=5) and BKO (n=5) mice. (Lower panel) Frequency of YFP+ cells in total B-1a, B-1b, and B2 B cell subsets in the peritoneal cavity of WT-YFP (n=8) and BKO-YFP (n=8) mice. Mean ± SD; ****p=0.0002, 9.8E-012, and 6.7E-009 by two-tailed, unpaired Student's t test, respectively

E. Thymus cellularity in WT-YFP (n=5) and BKO-YFP (n=5) mice. Mean ± SD

F. Flow cytometry for CD4+ and CD8+ expression in thymocytes of WT-YFP (n=5) and BKO-YFP (n=5) mice. Mean ± SD

G. Flow cytometry for CD4+ and CD8+ T cells in lymph nodes from WT (n=7) and BKO (n=10) mice. Mean ± SD

H. Flow cytometry for CD5+ T cells in the peritoneum of WT (n=5) and BKO (n=5) mice. Mean ± SD; *p=0.016 by two-tailed, unpaired Student's t test

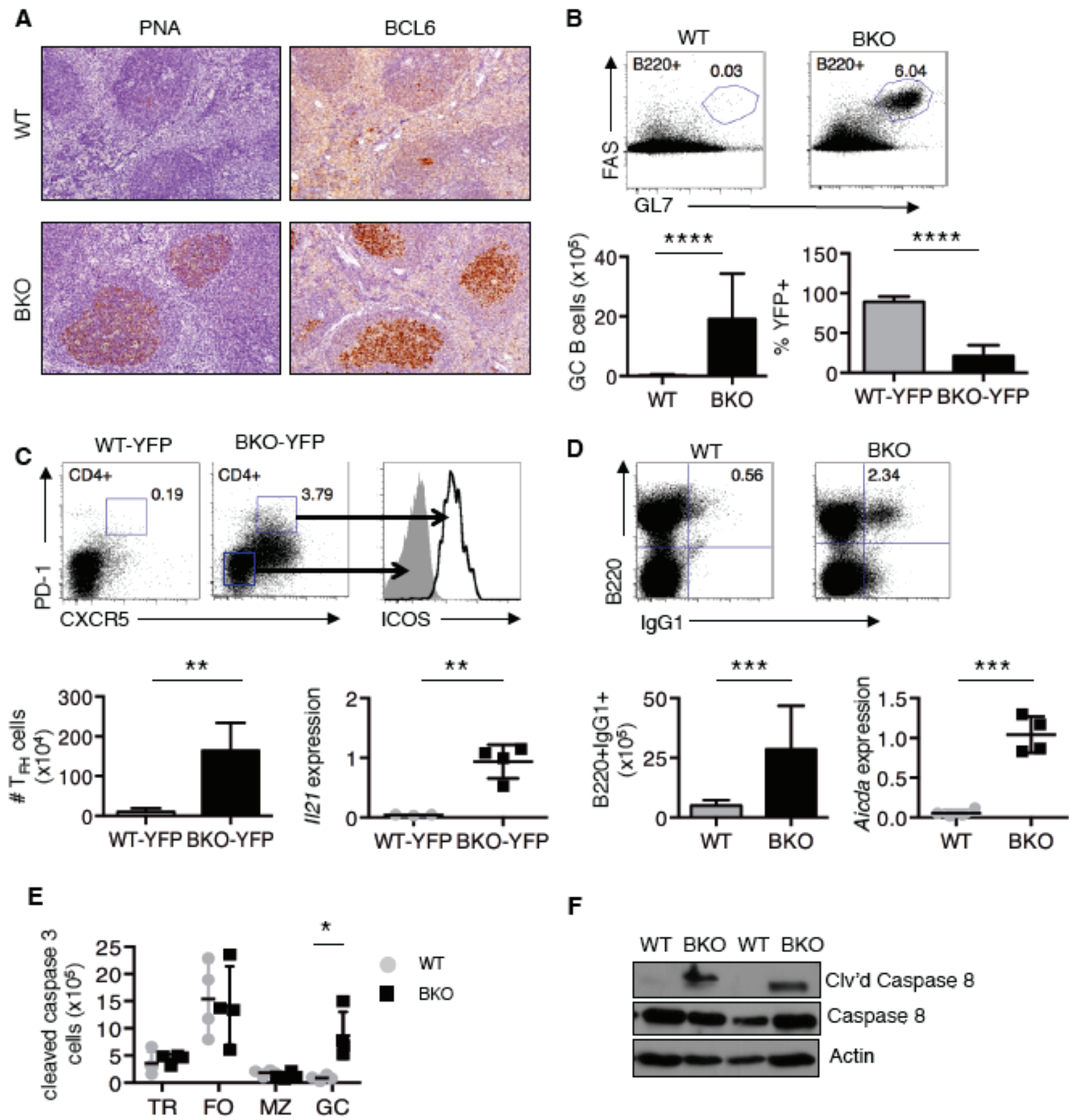

Figure 2-4. Large GCs with increased apoptosis in non-immunized BKO mice

Figure 2-4. Large GCs with increased apoptosis in non-immunized BKO mice

A. IHC sections of naïve WT and BKO spleens stained for PNA and BCL6 expression.

B. Flow cytometry and number of B220+GL7+FAS+ GC B cells from WT (n=8) and BKO (n=11) spleens, along with percentage of YFP+ GC B cells, by flow cytometry, from WT-YFP (n=8) and BKO-YFP (n=8) spleens. Mean ± SD; ****p=2.75E-05 and ****p=1.3E-07 by Mann-Whitney U test and two-tailedm unpaired Student's t test, respectively

C. Flow cytometry for CD4, PD-1, CXCR5, and ICOS expression in splenocytes from WT-YFP (n=5) and BKO-YFP (n=5) mice. Number of T_{FH} cells shown as mean ± SD; **p=0.008 by Mann-Whitney U test. qRT-PCR for *Il21* expression, relative to *36b4* expression*,* in CD4+ splenic T cells from WT-YFP (n=4) and BKO-YFP (n=4) mice. Mean ± SD; **p=0.0078 by two-tailed, unpaired Student's t test

D. Flow cytometry and number of B220+IgG1+ B cells from WT (n=6) and BKO (n=9) spleens, shown as mean ± SD; ***p=0.0048 by Mann-Whitney U test. qRT-PCR for *Aicda* expression, relative to *36b4* expression*,* from splenic B cells of WT (n=4) and BKO (n=4) mice. Mean ± SD; ***p=0.0027 two-tailed, unpaired Student's t test

E. Number of cleaved caspase 3+ TR, FO, MZ, and GC B cell subsets from WT (n=4) and BKO (n=4) mice. Mean ± SD; *p=0.029 by Mann-Whitney U test

F. Representative western blot analysis of CD43 depleted splenic B cells from WT and BKO mice for presence of cleaved caspase 8.

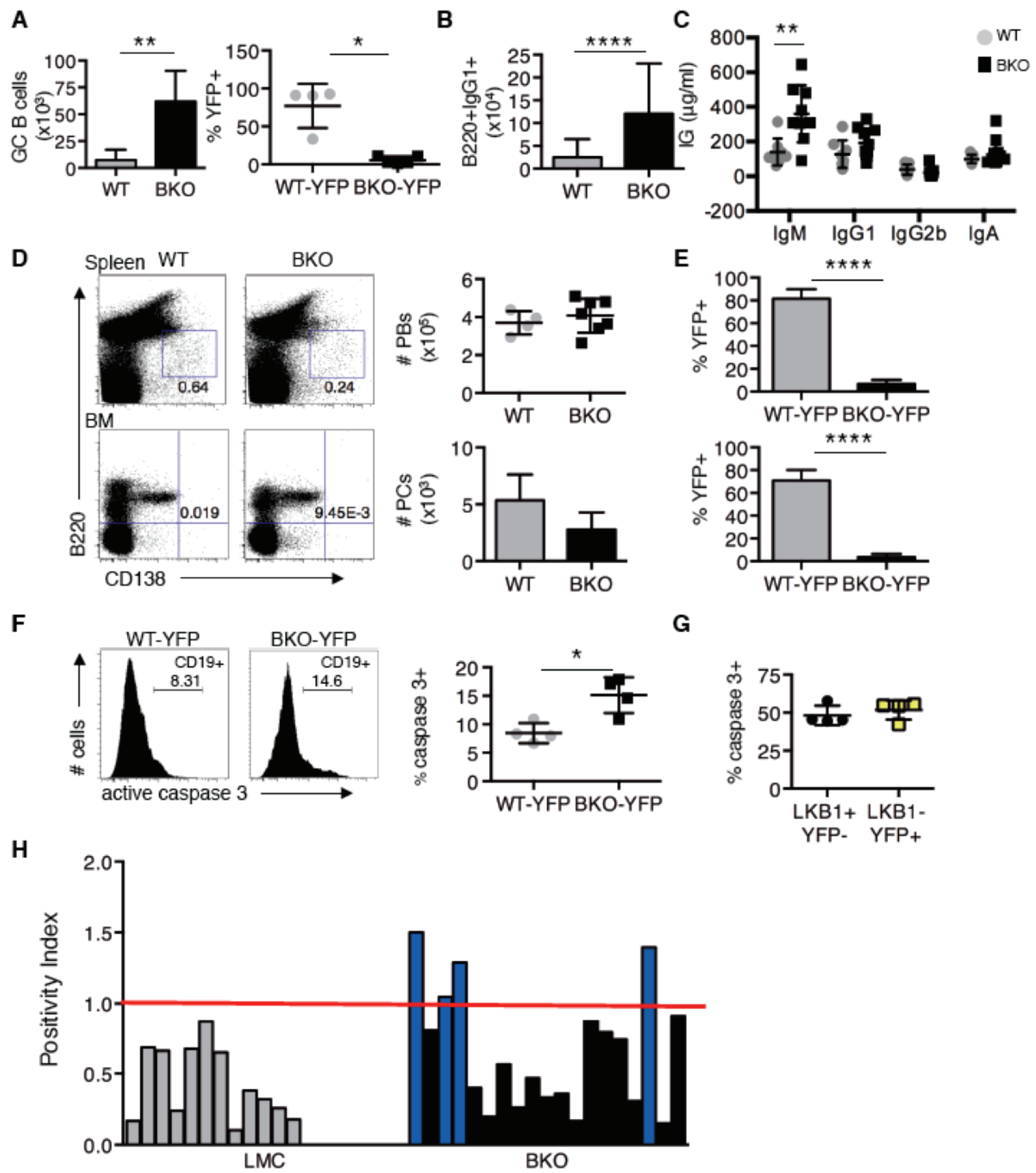

Figure 2-5. BKO mice contain abundant isotype switched and apoptotic GC B cells

Figure 2-5. BKO mice contain abundant isotype switched and apoptotic GC B cells

A. Number of B220+GL7+FAS+ GC B cells from WT (n=5) and BKO (n=8) lymph nodes and the frequency of YFP+ GC B cells from WT-YFP (n=4) and BKO-YFP (n=5) lymph nodes. Mean ± SD; **p=0.006 by Mann-Whitney U test and *p=0.015 by two-tailed, unpaired Student's t test

B. Number of B220+IgG1+ B cells from WT (n=5) and BKO (n=9) lymph nodes. Mean ± SD; ****p=0.0004 by Mann-Whitney U test

C. Serum IG isotypes from un-immunized WT (n=7) and BKO (n=10) mice. Mean ± SD; **p=0.01 two-tailed, unpaired Student's t test

D. Flow cytometry and number of B220^{low}CD138+ plasmablasts (n= 4 and 7, respectively) and B220−CD138+ plasma cells (n=5 and 5, respectively) from the spleen and BM of WT and BKO mice. Mean ± SD

E. Percentage of YFP+ to total plasmablasts (n=8) (top) and plasma cells (n=5) (bottom) from the spleen and BM of WT and BKO mice. Mean ± SD; ****p=2.4E-05 and 2.7E-05 two-tailed, unpaired Student's t test, respectively

F. Flow cytometry and percentage of CD19+ B cells with cleaved caspase 3 expression from spleens of WT-YFP (n=4) and BKO-YFP (n=4) mice. Mean ± SD; *p=0.016 two-tailed, unpaired Student's t test

G. Percentage of cleaved caspase 3+ CD19+YFP+ and CD19+YFP− B cells in spleens of BKO (n=4) mice. Mean ± SD

H. Anti-nuclear antibodies from the serum of WT or HET littermate controls (LMC) (n=12) and BKO (n=19) mice; blue bars represent animals scoring positive for ANAs.

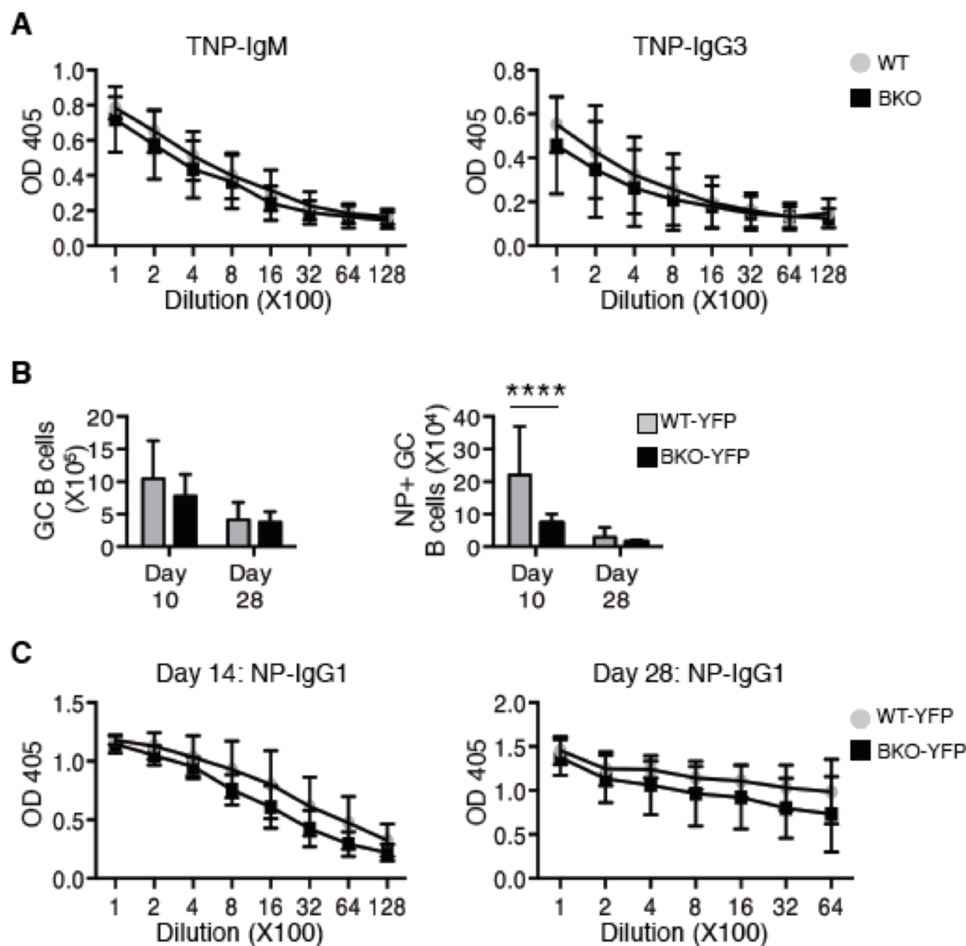

Figure 2-6. BKO mice respond to T cell independent and T cell dependent antigens

A. αTNP-IgM and IgG3 serum responses by ELISA to TNP-AECM-FICOLL 7 days after immunization of WT (n=8) and BKO (n=8) mice. Mean ± SD

B. Number of total GC and NP-specific GC B cells in response to NP-CGG 10 (n=8 and 8) and 28 days (n=7 and 7) after immunization of WT-YFP and BKO-YFP mice. Mean ± SD, ****p=0.0003 by Mann-Whitney U test

C. αNP-IgG1 serum response by ELISA to NP-CGG 14 (n=4 and 4) and 28 days (n=6 and 6) after immunization of WT-YFP and BKO-YFP mice. Mean ± SD

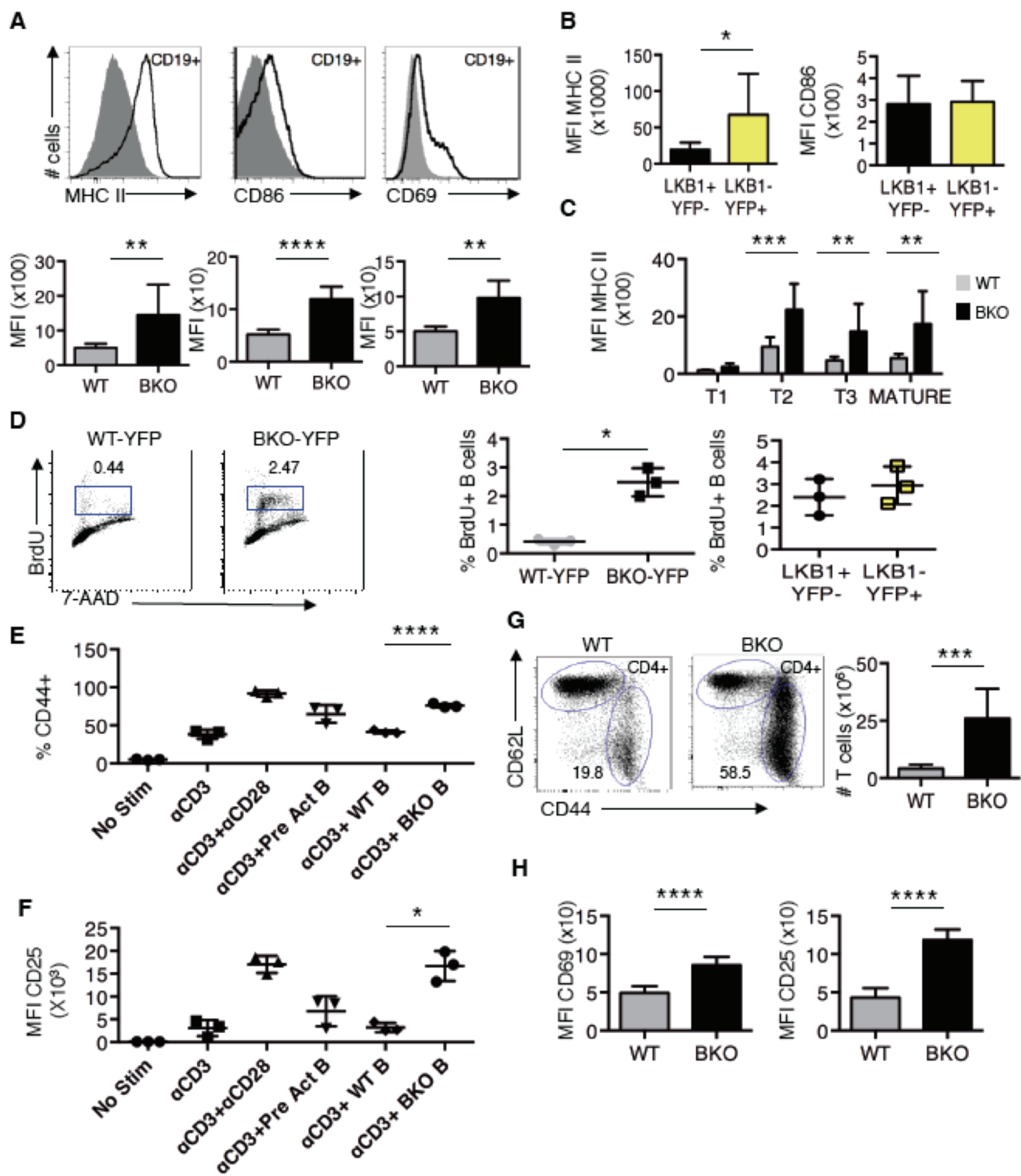

Figure 2-7. BKO mouse lymphocytes are hyperactivated

Figure 2-7. BKO mouse lymphocytes are hyperactivated

A. Flow cytometry for MHC II (n=8 and 11), CD86 (n=6 and 9) and CD69 (n=5 and 7) activation biomarkers and mean fluorescence intensity (MFI) for CD19+ splenocytes from WT and BKO mice. Mean ± SD; **p=0.005, ****p=9.1E-06, and **p=0.002 by two-tailed, unpaired Student's t test

B. MFI of MHC II (n=9 each) and CD86 (n=6 each) of Lkb1+YFP− and Lkb1−YFP+ CD19+ B cells from BKO mice. Mean ± SD; *p=0.02 by two-tailed, unpaired Student's t test

C. MFI of MHC II for transitional and mature B cell subsets from WT (n=8) and BKO (n=11) spleens. Mean ± SD; ***p=0.0007, **p=0.006 and 0.006 by two-tailed, unpaired Student's t test, respectively

D. Flow cytometry plot of BrdU incorporation into harvested splenic B cells during a 30 min pulse and BrdU incorporation in Lkb1+YFP− and Lkb1−YFP+ B cells from BKO-YFP mice. Mean ± SD for three independent experiments; *p=0.015 by two-tailed, unpaired Student's t test

E. and F. CD4 and CD62L enriched, naïve T cells were co-incubated with anti-CD3 Ab and either anti-CD28 Ab, WT B cells pre-activated for 24 hr, fresh CD43 depleted WT B cells or fresh CD43 depleted BKO B cells for 48 hr. Cultures were then harvested for analysis by flow cytometry. E. Percentage of CD4+CD62L+CD44+ activated T cells in listed culture conditions (n=3). Mean ± SD for three independent experiments; ****p=5.9E-05 by two-tailed, unpaired Student's t test. F. MFI of CD25 activation biomarker on CD4+ T cells in listed culture conditions (n=3). Mean ± SD for three independent experiments, *p=0.0135 by two-tailed, unpaired Student's t test

G. Flow cytometry for the expression of CD4, CD62L, and CD44 in splenocytes from WT (n=5) and BKO (n=7) mice. Mean ± SD; ***p=0.0025 by Mann-Whitney U test

H. MFI for CD69 and CD25 activation biomarkers in CD4+ splenic T cells from WT (n=5) and BKO (n=7) mice. Mean ± SD; ****p=0.0001 and 3.4E-06 by two-tailed, unpaired Student's t test, respectively

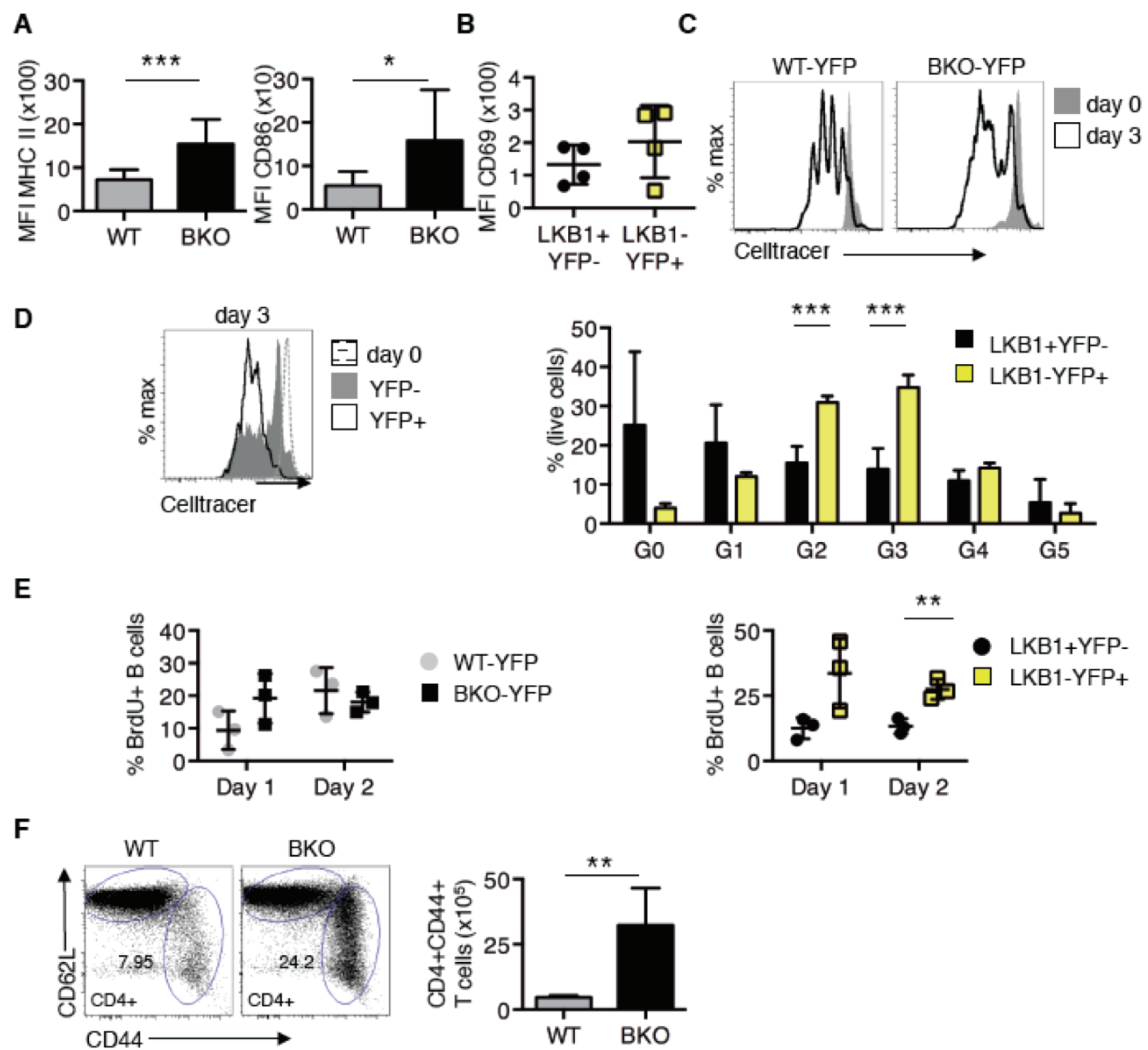

Figure 2-8. BKO mouse B cells are hyperactive and hyperproliferative

Figure 2-8. BKO mouse B cells are hyperactive and hyperproliferative

A. MFI of MHC II and CD86 activation biomarkers for CD19+ B cells in lymph nodes of WT (n=6) and BKO (n=9) mice. Mean ± SD; **p=0.0022 and *p=0.03 two-tailed, unpaired Student's t test, respectively

B. MFI of CD69 for CD19+ LKB1+YFP− and LKB1−YFP+ B cells from BKO-YFP mice (n=4). Mean ± SD

C. Flow cytometry of in vitro cell division over 3 days using Celltracer dye dilution for anti-CD40 Ab plus IL-4 stimulated splenic B cells from WT-YFP and BKO-YFP mice. Plots shown are representative of 3 independent experiments.

D. Flow cytometry of in vitro cell division over 3 days using Celltracer dye dilution for anti-CD40 Ab plus IL-4 stimulated splenic B cells BKO-YFP mice. (Right panel) The percentage of live LKB1−YFP+ and LKB1+YFP− B cells in each generation is graphed. Mean ± SD for three independent experiments, ***p=0.004 and 0.004 two-tailed, unpaired Student's t test, respectively

E. (Left panel) Percentage of harvested splenic B cells from WT-YFP and BKO-YFP mice that incorporate BrdU over 2 days and (Right panel) percentage of LKB1+YFP− and LKB1−YFP+ B cells from BKO-YFP mice that incorporate BrdU over 2 days. Mean ± SD from three independent experiments; **p=0.007 two-tailed, unpaired Student's t test

F. Flow cytometry for the expression of CD44 on CD4+ T cells in the lymph nodes of WT (n=5) and BKO (n=5) mice. Mean ± SD; **p=0.008 by Mann-Whitney U test

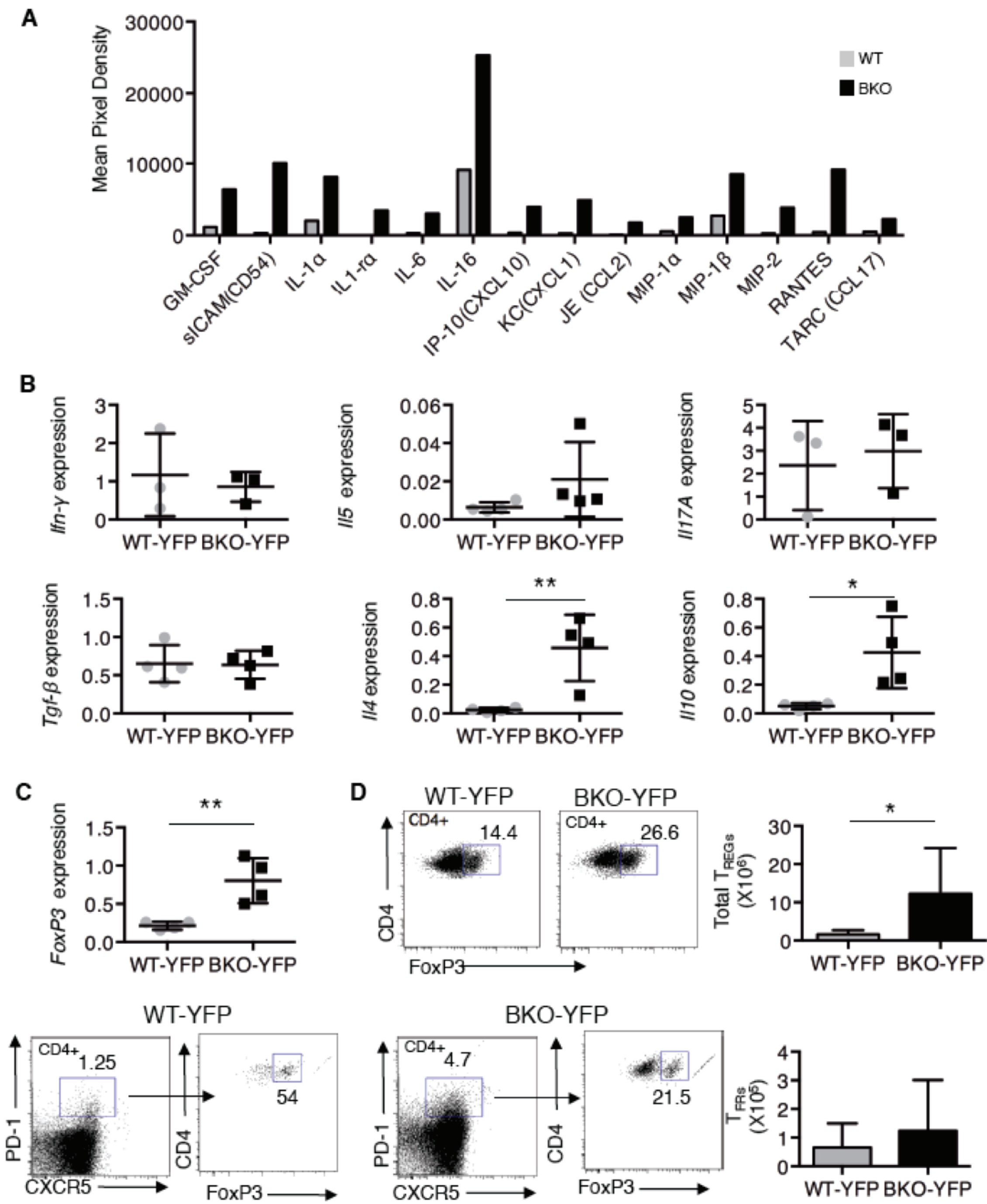

Figure 2-9. Activated T cells from BKO spleens are made up of T_{FH} and T_{regs}

Figure 2-9. Activated T cells from BKO spleens are made up of T_{FH} and T_{regs}

A. Cytokine and chemokine array surveyed with conditioned media from unstimulated CD43 depleted B cells from WT and BKO spleens. Mean values are plotted for proteins from two WT and two BKO mice that were increased 1.4-fold or more in BKO versus WT conditioned media.

B. qRT-PCR for *Ifn-γ, Il4, Il5, Il17A, Tgf-β, and Il10* expression, relative to *36b4* expression, in splenic CD4+ T cells from WT-YFP (n=4) and BKO-YFP (n=4) mice. Mean ± SD; **p=0.01 and *p=0.02 by two-tailed, unpaired Student's t test, respectively

C. qRT-PCR for *FoxP3* expression, relative to *36b4* expression, in splenic CD4+ T cells from WT-YFP (n=4) and BKO-YFP (n=4) mice shown as mean ± SD; **p=0.008 two-tailed, unpaired Student's t test.

D. Flow cytometry analysis for CD4+FoxP3+ T_{REG} and T_{FR} effector cell populations in WT-YFP (n=6) and BKO-YFP (n=6) mice. Mean ± SD; *p=0.04 by Mann-Whitney U test

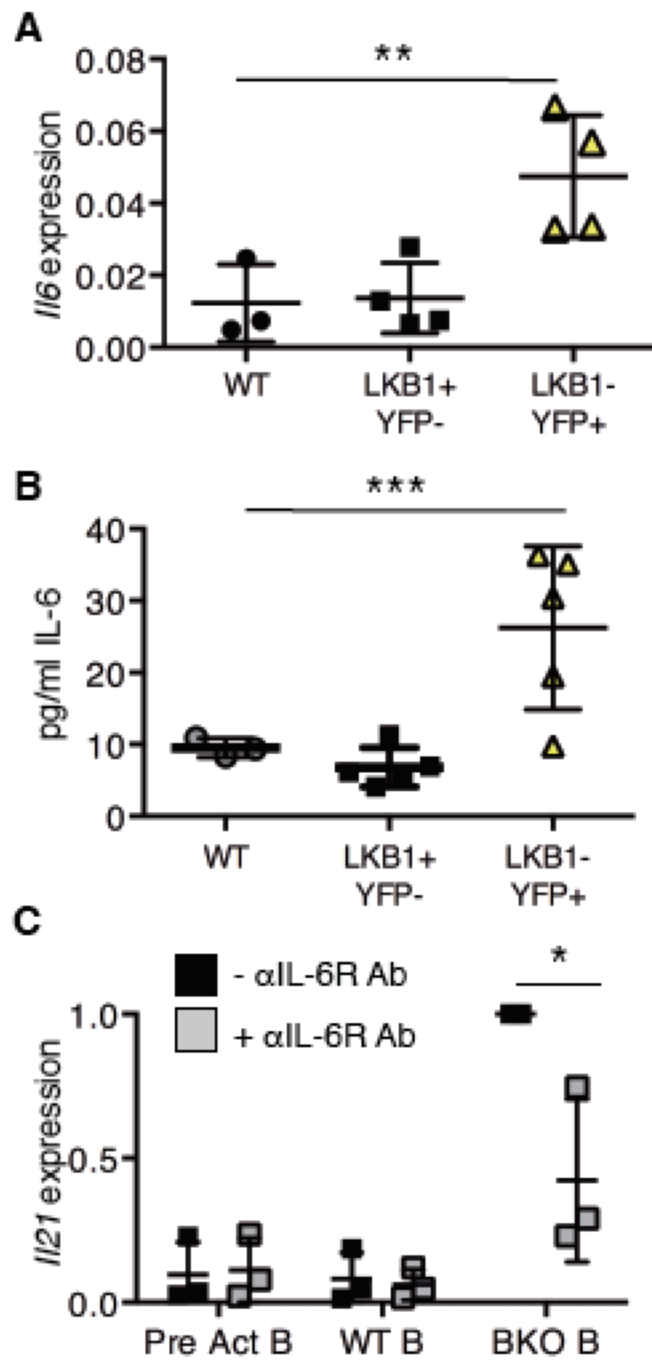

Figure 2-10: BKO B cells activate CD4+ T cells and induce T_{FH} cell differentiation by IL-6 secretion

Figure 2-10: BKO B cells activate CD4+ T cells and induce T_{FH} cell differentiation by IL-6 secretion

A. qRT-PCR for *Il-6* expression, relative to *36b4* expression, in CD43 depleted splenic B cells from WT-YFP (n=3), and Lkb1+YFP− (n=4) and Lkb1−YFP+ (n=4) sorted CD19+ splenic B cells from BKO-YFP mice. Mean ± SD; **p=0.0098 by one-way ANOVA

B. IL-6 secreted by WT-YFP (n=3), and Lkb1+YFP− (n=5), and Lkb1−YFP+ (n=5) CD19+ splenic B cells from BKO-YFP mice during 24 hours in culture. Mean ± SD; ***p=0.0047 by one-way ANOVA

C. CD4+CD62L+ naïve T cells were co-incubated with anti-CD3 Ab and WT B cells pre-activated for 24 hr, WT B cells, or B cells from BKO mice, with or without anti-IL-6R Ab for 48 hr. qRT-PCR for induced *Il21* expression, relative to *36b4* expression, and normalized to the induction of *Il21* expression in BKO B cell co-culture, is shown. Mean ± SD for three independent experiments; *p=0.024 by two-tailed, unpaired Student's t test

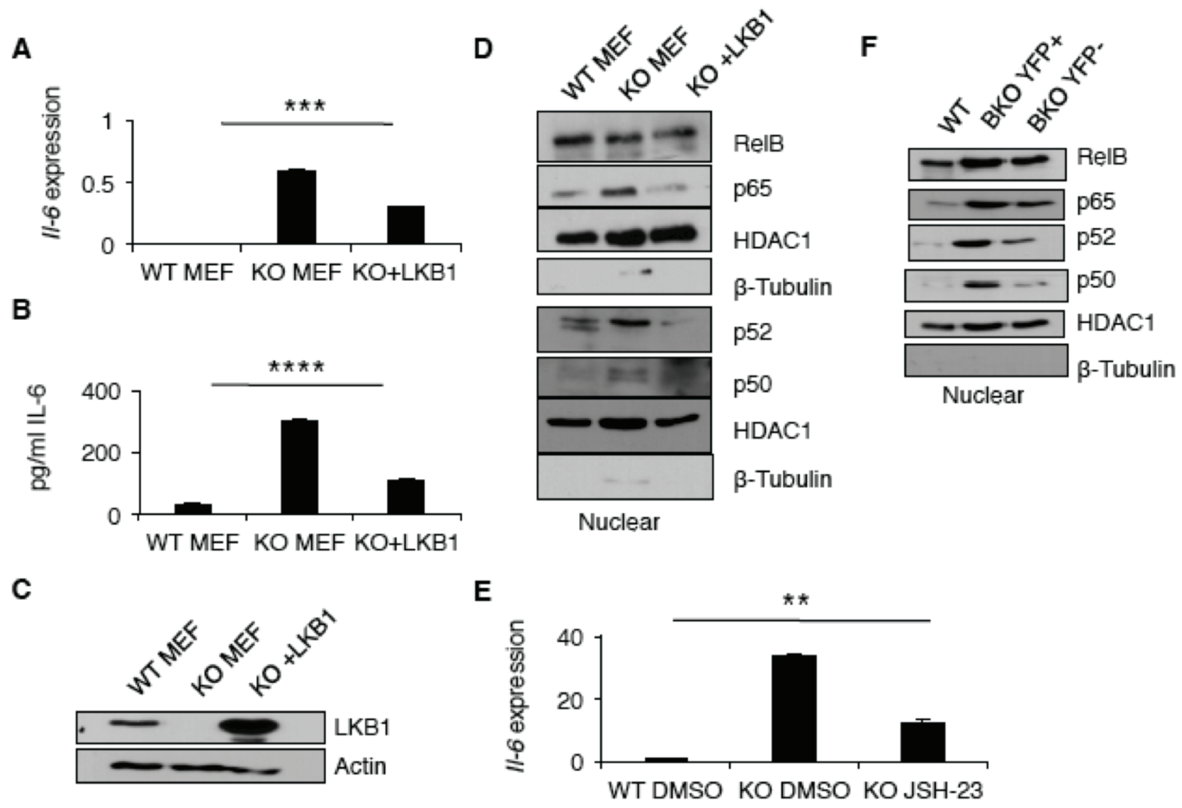

Figure 2-11: Loss of Lkb1 induces IL-6 production through NF-κB activation

Figure 2-11: Loss of Lkb1 induces IL-6 production through NF-κB activation

A. qRT-PCR for *Il-6* expression, relative to *36b4* expression in WT, Lkb1– (KO), and Lkb1– reconstituted MEFs (KO+Lkb1), in triplicate. Mean ± SEM; ***p=0.0017 by one-way ANOVA

B. IL-6 secreted from WT, KO, and KO+Lkb1 MEFs into culture media for 24 hours, in triplicate. Mean ± SEM; ****p=0.0001 by one-way ANOVA

C. Representative western blot of WT, Lkb1– (KO), and Lkb1– reconstituted MEFs (KO+Lkb1) for Lkb1 and Actin protein expression

D. Representative western blot analysis of NF-κB family member proteins in nuclear lysates from WT, KO, and KO+Lkb1 MEFs.

E. qRT-PCR for *Il-6* expression, relative to *36b4* expression, in WT MEFS incubated with DMSO, and Lkb1– MEFs incubated with either DMSO or 5 μM JSH-23. Mean ± SD for three independent experiments; **p=0.006 by one-way ANOVA

F. Representative western blot analysis of NF-κB family member proteins in nuclear lysates from CD43– B cells from WT, sorted YFP+, and sorted YFP– B cells from BKO mice (n=3)

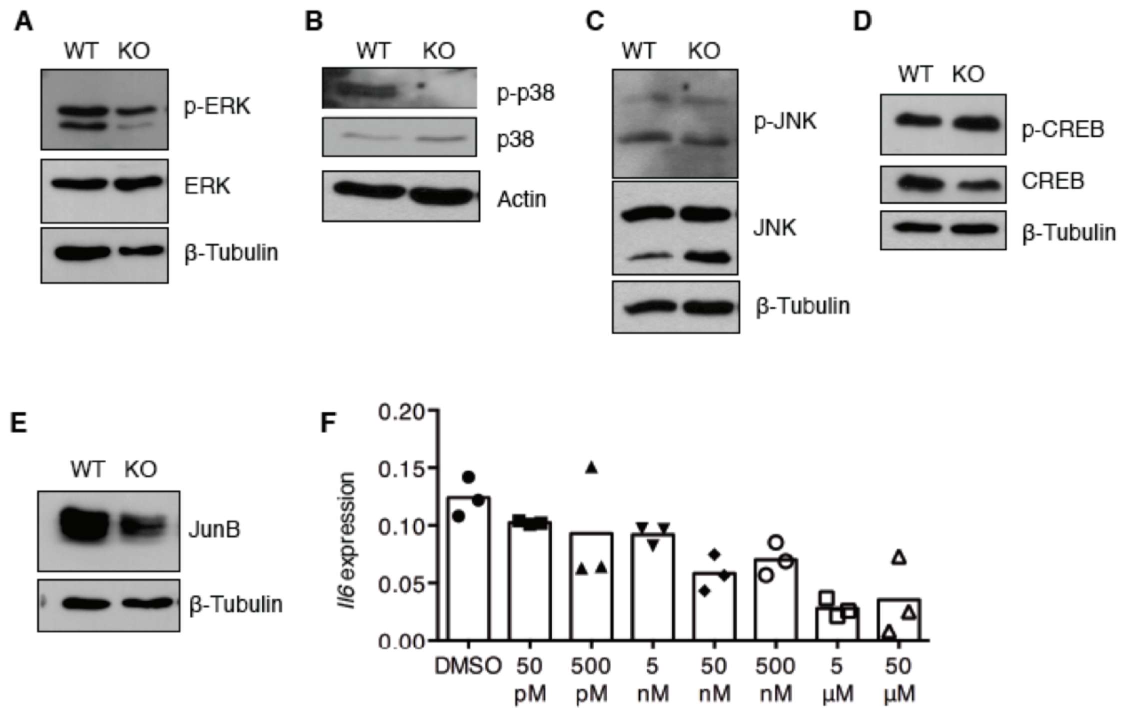

Figure 2-12. LKB1 regulates IL-6 production through NF-κB signaling

A. Western blot analysis of signaling through ERK1/2 in WT and LKB1− MEFs

B. Western blot analysis of signaling through p38 in WT and LKB1− MEFs

C. Western blot analysis of signaling through JNK in WT and LKB1− MEFs

D. Western blot analysis of signaling to CREB transcription factor in WT and LKB1− MEFs

E. Western blot analysis of JunB expression in WT and LKB1− MEFs

F. qRT-PCR analysis of *Il6* expression, relative to *36b4* expression, from LKB1− MEFs treated with DMSO or an escalating concentration of the NF-κB inhibitor JSH-23. Mean ± SEM

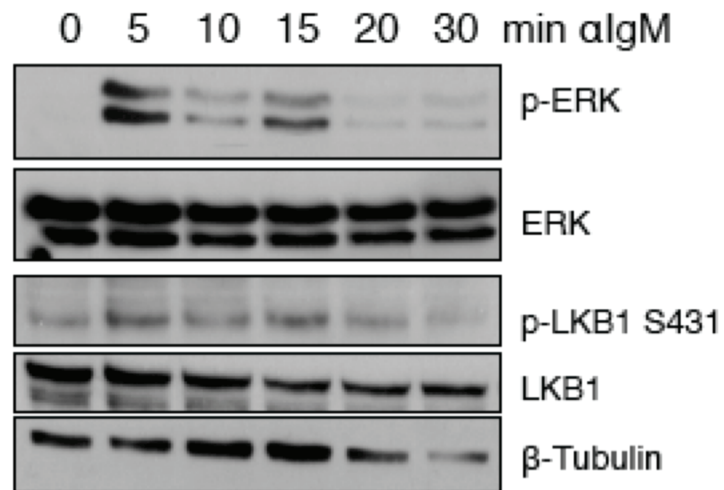

Figure 2-13: BCR signaling results in inhibitory Lkb1 S431 phosphorylation

Representative western blot analysis of phosphorylation of Lkb1 on serine-431 in CD43 depleted WT B cells stimulated with F(ab^2) fragments of anti-IgM for the indicated times.

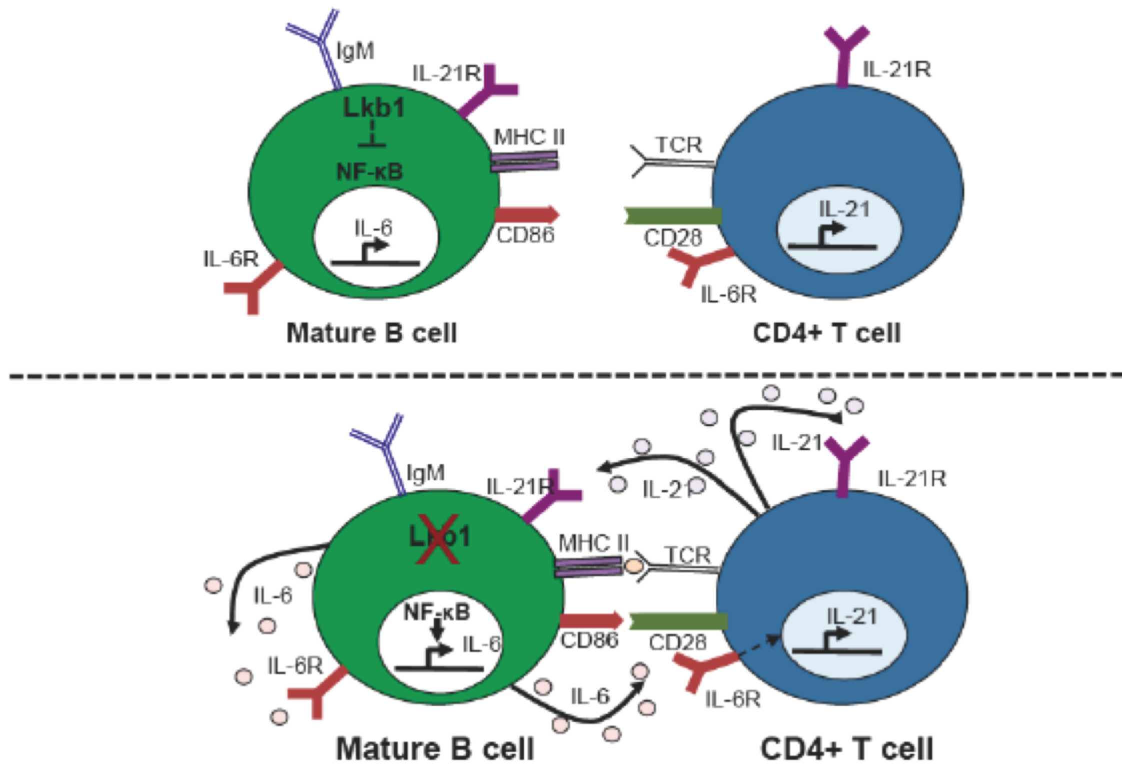

Figure 2-14: IL-6 from BKO B cells induces the differentiation of T_{FH} cells

Details for this model are given in the text.

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CHAPTER 3:

B Cell Differentiation Stimulated by Physiologic DNA Double Strand Breaks

DNA double strand breaks (DSBs) induced by stage-specific endonucleases are required for the assembly and diversification of B cell antigen receptor (BCR) genes in developing vertebrate B lymphocytes. BCR genes encode for antibodies that contain immunoglobulin (Ig) heavy (H) and light (L) chains and are assembled by the somatic recombination of variable (V), diversity (D), and joining (J) gene segments. In the bone marrow, the recombinase activating gene (RAG) endonuclease generates DNA DSBs in developing B cells at the border of two *Ig* gene segments and their flanking recombination signal sequences [1]. RAG DSBs activate a DNA damage response (DDR) program orchestrated by damage-sensing kinases, such as ATM, which phosphorylates hundreds of proteins that participate in DNA repair, tumor suppression, and cell cycle regulation [2, 3].

B cells with surface BCRs that survive bone marrow maturation immigrate into the circulation and home to lymphoid organs throughout the body. Here, naïve B cells in primary lymphoid follicles can be induced to form secondary follicles via T cell-dependent antigenic stimulation. Secondary follicles comprise a mantle zone of non-responding bystander B cells and a germinal center (GC) of antigen-responsive B cells. GC B cells undergo rapid expansion and a second round of *Ig* gene refinements mediated by another endonuclease, activation-induced cytidine deaminase (AID) [4]. GCs are sites of class switch recombination (CSR) and somatic hypermutation (SHM), which are AID-initiated DNA strand breaking and repair processes that result in the expression of high affinity antibodies of different isotypes. The generation of mature antibodies is required for the differentiation of responsive memory B cells and for plasma cells that secrete effective antibodies during a humoral immune response.

In general, three cellular outcomes are recognized for DDR signaling in response to DNA DSBs. These include transient cell cycle arrest coupled with DNA repair, apoptosis, or senescence [5]. However, two recent studies suggest an additional, fourth outcome for developing B cells with DNA DSBs, which is for DDR programs to promote B cell differentiation.

Bredemeyer and colleagues showed that RAG DSBs induced during *IgL* gene rearrangements in pre-B cells regulates the ATM-dependent and ATM-independent expression of ~300 genes [6]. ATM-dependent intracellular signaling activated NF-κB-dependent gene transcription, which was required for the migration and homing of pre-B cells. These results indicate that signaling from RAG DSBs through ATM and NF-κB links a DDR program with early B cell differentiation and function, which is beyond the known DDR that regulates DNA repair, the cell cycle, and apoptosis (Figure 3-1). More work is needed to determine whether RAG DSBs induced during *IgH* gene rearrangements in pro-B cells also regulate genes that control B cell differentiation and function.

Sherman and colleagues studied the role of AID DSBs in B cells stimulated to undergo a GC-like reaction. AID DSBs activated an ATM-dependent signaling pathway that ended by phosphorylating and inactivating the transcriptional co-activator CRTC2 [7]. CRTC2 was shown to activate 136 direct target genes, some of which are involved in processes that regulate GC B cell proliferation, self-renewal, and inhibit plasma cell differentiation. During CSR, AID DSBs activated ATM, which signaled successively through LKB1 and an unknown AMPK family member protein to inactivate CRTC2, resulting in the end of an ongoing GC reaction and differentiation of GC B cells into antibody-secreting plasma cells (Figure 3-1). GC B cells with ATM or LKB1 knocked down continued to proliferate, were impaired for antibody secretion, and failed to properly execute the plasma cell differentiation program. Molecular changes in the signaling pathway from ATM to CRTC2 inactivation were identified in human B cell lymphoma patient samples, including repressed ATM and LKB1 expression and sequence alterations in the CRTC2 kinase target domain, which suggested a potential role in malignant B cell transformation. More work is needed to determine whether defects in AID DSB-induced differentiation facilitate B lymphomagenesis.

Studies of pre-B and GC B cells also showed that chemical and physical agents causing DSBs outside of antigen receptor loci regulate gene expression and differentiation changes that are similar to the changes induced by RAG and AID DSBs, respectively [6, 7]. Findings from these non-endonuclease DSB studies also suggest that cell differentiation may be a general response to DNA DSBs beyond the lymphocyte lineage, which requires further investigations.

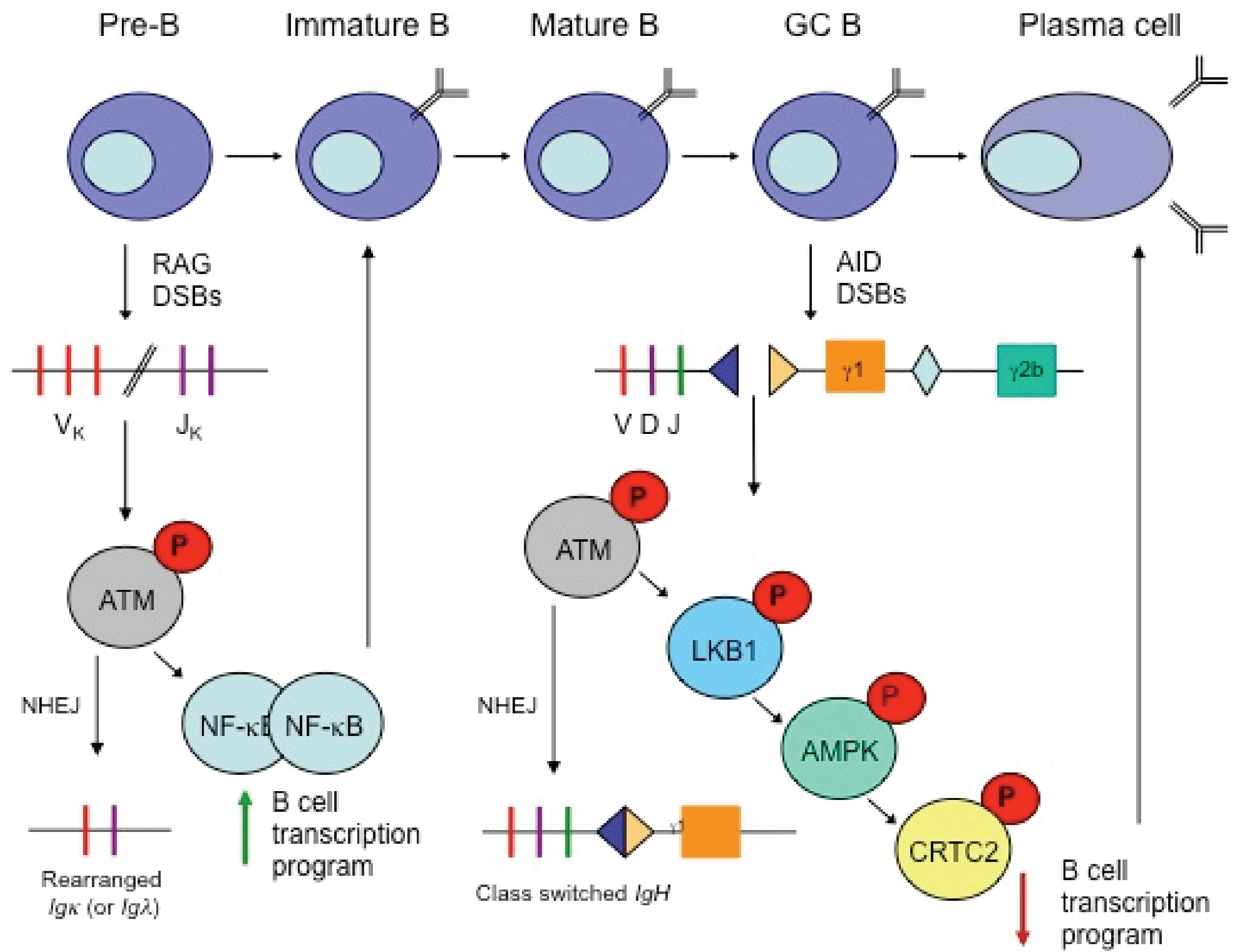

Figure 3-1. DNA DSBs promote B lymphocyte differentiation.

RAG-initiated DNA DSBs during VJ recombination of *Igκ* (or *Igλ*) light chain loci in pre-B cells activates an ATM-dependent DDR program that includes the induction of NF-κB target genes involved in regulating pre-B cell migration and homing. Later in B lineage development, AID-initiated DNA DSBs during class switch recombination of *Ig* heavy chain genes activates an ATM and LKB1-dependent DDR program that includes repression of CRTC2 target genes involved in regulating the physiology of germinal center B cells, resulting in the terminal differentiation of antibody-secreting plasma cells.

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CHAPTER 4:

Conclusions, Significance, and Future Directions

B cells are vital for adaptive immunity due to their ability to generate and secret high affinity, antigen specific antibodies. B cells originate from hematopoietic stem cells (HSCs) in the bone marrow. Pro and pre B cells undergo V(D) J recombination to generate a diverse repertoire of B cell receptors (BCRs) [1] able to recognize an array of antigens. Immature B cells emigrate to secondary lymphoid organs such as the spleen where they continue to develop into transitional and mature B cells. In secondary lymphoid organs mature naïve B cells encounter antigen and migrate to the T:B boundary where they interact with cognate T cells. This interaction provides co-stimulatory signals to survive and differentiate into low affinity, antibody secreting plasma cells or to proliferate and seed a GC (GC) [2]. GC B cells undergo rapid proliferation and the BCR refinement processes of somatic hypermutation (SHM) and class switch recombination (CSR). GC B cells that successfully undergo antibody refinement leave the GC as either a memory B cell or an antibody secreting plasma cell.

The GC reaction, from initial B cell activation and recruitment to selection and terminal differentiation, must be a tightly regulated process. The rapid proliferation and DNA damaging events paired with the loosened DNA damage check point can result in harmful mutations and DNA translocations resulting in lymphomagenesis [2-4]. A majority of human mature B cell lymphomas are believed to have GC origin [5]. GC B cells that gain affinity to self antigens through SHM that are not effectively eliminated can differentiate into antibody secreting plasma cells that cause autoimmunity [6]. There is also an emerging appreciation for activated, cytokine secreting B cells to regulate inflammatory and T cell mediated autoimmune diseases such as type 2 diabetes and multiple sclerosis [7].

We recently uncovered a signaling pathway important for terminal differentiation of B cells exiting GCs [8]. AID induced double stranded breaks at the *Ig* locus activate ATM, which signals through LKB1 to inactivate CRTC2 leading to the repression of genes important for the GC reaction [8]. Additionally, alterations in this signaling pathway are present in human B cell lymphoma samples [8]. To further dissect the importance of this pathway to B cell function as

part of a T-dependent immune response, we generated a *Lkb1* B cell specific mouse model (BKO mice). Interestingly, loss of *Lkb1* in immature B cell stages results in the aberrant activation of B cells and subsequent GC formation. An activated lymphoid environment is also generated (Figure 4-1) resulting in the expansion of all white blood cell types specifically mature T cell populations. Based on these data, we propose that Lkb1 has an integral role in B cell function, specifically in the initiation and termination of the GC response.

Conclusions and Significance

The data presented here show that Lkb1 has a cell intrinsic role in B cell biology. Loss of Lkb1 in mature B cells results in an increase in MHC II expression (Figure 2-7B), an increase in IL-6 production (Figure 2-10A), and an increase in proliferation *in vitro* (Figure 2-8D*)*. These results are not surprising given previous studies of Lkb1 in other systems. Lkb1 is a known tumor suppressor and has the ability to generate cell cycle arrest when expressed in tumor cell lines that lack endogenous *Lkb1* expression [9-11]. Additionally, loss of Lkb1 in T cells results in increased expression of the specific inflammatory cytokines, IFN-γ and IL-17A [12] although the mechanism of this increased production has not been deeply examined.

Loss of Lkb1 in B cells also affects the ability of mature B cells to survive and compete with Lkb1 sufficient B cells *in vivo*. This is evident by the sharp decrease in the percentage of Lkb1-YFP+ B cells during the differentiation from transitional B cells to mature B cell populations (Figure 2-2C). WT and BKO mice initially have comparable percentages of YFP+ and YFP- cells. Mature Lkb1-YFP+ B cells in BKO mice are significantly reduced compared to WT controls. One interesting hypothesis for this observation is that while loss of Lkb1 promotes increased proliferation, a cell lacking Lkb1 is not equipped to turn off anabolic processes when faced with a deficit in ATP. For example, Lkb1- MEFs have been shown to be more sensitive to energy stress than WT controls [13]. The most studied role for Lkb1 is as the upstream kinase to Ampk [13, 14]. As the AMP:ATP ratio increases in a cell, Ampk is activated to stop

processes, such as protein synthesis that consume energy, and to initiate processes that generate ATP such as oxidative phosphorylation (reviewed in [15]). Upon activation, B cells proliferate and undergo a metabolic switch to glycolysis [16] during the development of GCs. However, this proliferation is not sustainable and B cells need a mechanism to terminate the GC reaction to maintain or regain energy homeostasis. The loss of Lkb1 renders B cells unable to do this and this could be one potential explanation for the survival defect observed in Lkb1- mature B cells.

It is also possible that loss of Lkb1 results in a developmental defect in B cells. This scenario is a plausible explanation for the surprising lack of YFP+ marginal zone (MZ) B cells in BKO-YFP mice (Figure 2-2C). One consequence of loss of Lkb1 is the loss of Ampk activation [17-19]. Ampk is a negative regulator of mTor complex 1(mTorc1) though its activation of the tuberous sclerosis complex (Tsc) [20, 21]. Upon activation by phosphorylation by Ampk, Tsc inactivates Rheb. Active Rheb (GTP-bound) activates mTorc1, therefore inactivation of Rheb leads to the inactivation of mTorc1 and the subsequent inhibition of energy consuming processes such as protein synthesis and cell growth (reviewed in [22]). It has been reported that loss of Tsc1 in transitional B cells results in a block in MZ B cell development that can be alleviated by treatment with the mTorc1 inhibitor rapamycin [23]. These data suggest that tight regulation of mTorc1 signaling is important during the development of MZ B cells and if true, could be one potential reason why there could be a developmental block in Lkb1- B cells in MZ development. It would be of interest to further dissect the signaling pathways downstream of Lkb1-Ampk in sorted Lkb1- B cells to determine if mTorc1 (or any other signaling pathways regulated by Ampk) is increased.

It is interesting that loss of Lkb1 in transitional and mature B cells results in increased activation in the absence of provoking stimuli (Figure 2-7). Other groups have placed Ampk upstream to NF-κB signaling (reviewed in [24]) and my work here suggests that Lkb1 is an integral member of this pathway to regulate NF-κB. NF-κB signaling is present in mature B cells

[25, 26] and is up-regulated upon BCR engagement provoking B cell activation (reviewed in [27]). This suggests that tonic signaling in B cells, known to be vital to B cell survival has the ability to be mitogenic or activating if a negative regulator of the signaling is taken away (presumably here Lkb1). The Lkb1-Ampk signaling axis acts as a brake to mitogenic Akt signaling through inhibition of mTorc1 [20, 21] to turn down energy consumption. This opens the possibility that Lkb1-Ampk signaling isn't just a blunt brake to engage when a cell needs to slow down, but can be fine tuned to enforce quiescence or homeostasis for cells poised to undergo quick reactions.

Perhaps the most significant observation from the BKO mice is that loss of Lkb1 in just a subset of B cells was able to have a robust cell-extrinsic affect on the surrounding environment. Spleens from BKO mice were, on average, two fold larger with twice as many cells as WT controls (Figure 2-2D). These "extra" cells weren't B cells but populations of white blood cells such as dendritic cells, myeloid cells, granulocytes, and most significantly, T cells (Figures 2-2G). A cytokine profile on cultured supernatant from WT and BKO spleens suggests an increase in inflammatory cytokines being expressed by splenic cells in BKO mice (Figure 4-1) and T cells from these spleens display an activated CD44+ phenotype (Figure 2-7G). CD43- B cells from BKO mice express increased levels of IL-6 compared to WT controls and Lkb1-YFP+ B cells from BKO-YFP mice produce more IL-6 than their Lkb1+YFP- counterparts (Figures 2-10A and 2-10B). This suggests that loss of Lkb1 in B cells results in the increase of IL-6 *in vivo* although it does not discount the possibility that other cell types contribute to the splenic pool of IL-6. Additionally, secreted IL-6 can activate signaling in cells that express the IL-6R, which includes both B and T cells and this signaling can result in the further activation or differentiation of these cells. It is our belief that secretion of IL-6 by Lkb1-YFP+ B cells participates in the differentiation of activated CD4+ T cells into T_{FH} cells that secrete IL-21. The secretion of IL-6 and IL-21 combined with the interactions of B cells, T_{FH} cells, and other WBC populations result in the spontaneous GC formation seen in secondary lymphoid organs of BKO mice. This is

similar to the phenotype seen in *Sanroque* mice that carry a mutation that leads to the up regulation of Icos on T cells and the spontaneous differentiation into T_{FH} cells and formation of GCs [28].

Cell extrinsic effects have been reported in some *Lkb1* conditional knockout animals, with *Tgfβ* being decreased in multiple models [29, 30], Lkb1- MEFs [31], and in human umbilical vascular endothelial cells (HUVECs) treated with *Lkb1* siRNA [30]. Loss of *Lkb1*, for example, in smooth muscle cells, was found to reduce the production of *Tgfβ* and cause GI polyp formation in the epithelial tissues of the GI tract [29]. Additionally, in *Lkb1*-deficient embryos, *Vegf* has been found to be overexpressed contributing to the embryonic lethality of *Lkb1* excision [32]. Lkb1- T cells have been reported to be hyperactivated [12], similar to our described phenotype in Lkb1- B cells. Lkb1- T cells express increased levels of the inflammatory cytokines IL-17A and IFN-γ [12]. While the authors failed to note if this increased cytokine presence had an effect on the surrounding lymphoid environment or general health of the animals, it would be expected that there would be some physiological effect on the surrounding tissue microenvironments. Given the mounting evidence of the role of inflammation in human health [7, 33] and cancer initiation and progression [34] combined with the varied range of effects loss or mutation of LKB1 has on human health (namely its role as a tumor suppressor) (reviewed in [10, 35]), Lkb1's role in regulating inflammatory cytokine production seems to play an important part in the pathologies associated with its loss.

My thesis work combined with our recent publication detailing a novel signaling pathway integral to the termination of the GC reaction [8] place Lkb1 as an important regulator of the GC response in B cells. This is a conceptual breakthrough for the field because it places a protein that is not expressed in a B cell specific manner as a central regulator of an immune response. It also places an emphasis on metabolism as a regulator of B cell activation. My data suggest that upon BCR signaling, B cells downregulate expression or activity of Lkb1 to enable the rapid

proliferation necessary for an efficient immune response (Figure 4-2A). This would be the first reported scenario in which loss or suppression of Lkb1 would be physiologically motivated and imperative for proper cellular function.

Future Directions

To elucidate the potential of Lkb1 as a negative regulator of B cell activation and its role in maintaining a quiescent follicular environment more studies need to be performed to determine the regulation of Lkb1 during a B cell immune response, the requirement of Lkb1 down regulation for B cell activation, and the physiological consequence of dysregulated Lkb1 expression in B cells.

Determine Lkb1 post-translational modifications and re-localization during B activation

Hypothesis: Based on our data that shows loss of Lkb1 in CD19+ B cells results in aberrant activation, Lkb1 activity must be tightly regulated during B cell development and during an immune response. Preliminary data demonstrates that Lkb1 becomes phosphorylated downstream of BCR signaling, therefore we expect that post-translational modifications of Lkb1 will result in its sub-cellular re-localization or regulation of its kinase activity.

Rationale: Lkb1's role as a tumor suppressor stems from its ability to inhibit cell growth when added back to cell lines that lack its expression [9, 11] and that heritable loss of Lkb1 results in Peutz Jegher's Syndrome characterized by gastrointestinal polyposis and increased cancer susceptibility [36]. Additionally, somatic mutations in LKB1 have been found in cancers such as lung cancer [37, 38] and cervical cancer [39]. Mutations identified include deletions, frame-shifts, and point mutations [10]. Point mutations have been found in the kinase domain and in the C-terminal domain but not in the N-terminal domain [10] suggesting the C terminus is important for Lkb1 function and/or regulation [40].

Numerous phosphorylation sites in Lkb1 have been identified (Figure 4-3), 4 of which are auto-phosphorylation sites [10]. The other sites are phosphorylated by upstream kinases, some of which have not been identified yet. A majority of these sites are found at the end of the kinase domain or in the C-terminal domain supporting the idea that phosphorylation of Lkb1 could be an important mechanism for regulation. Upstream kinases that have been identified include ATM [41], RSK and PKA [42, 43], ERK [44], AKT [45], PKCζ [46, 47] Fyn [48], and Lck [49] . Interestingly, both ERK and AKT have prominent roles in B cell activation [50] while Lck and Lyn (a Src family kinase related to Fyn) have direct involvement in proximal BCR signaling. ERK signaling downstream from the B cell receptor is well established. RSK phosphorylates Lkb1 downstream of ERK at Ser431 (Ser428 in humans) [41, 43, 44, 51] and ERK, itself, phosphorylates Lkb1 directly on Ser325 [44]. We also have preliminary data showing phosphorylation of Lkb1 at Ser431 in primary B cells after stimulation with anti-mouse IgM $F(ab)^2$ fragment (Figure 4-2A). Phosphorylation at Ser325 and Ser431 downstream of ERK signaling in melanoma cells harboring $BRAF^{V600E}$ mutations is inhibitory and results in the inability of LKB1 to phosphorylate AMPK [44]. Additionally, latent membrane protein 1 (LMP1), the oncogenic product of Epstein-Bar Virus (EBV) which constitutively activates the MAPK/ERK signaling pathway, inhibits signaling through LKB1 to AMPK in nasopharyngeal carcinoma cell lines resulting in increased cell proliferation through phosphorylation of Ser428 [52].

AKT is also activated downstream of B cell receptor engagement activation of PI3K [50]. AKT phosphorylates LKB1 on Ser334, resulting in its association with 14-3-3 and its nuclear re-localization [45]. Additionally, Tyr261 and Tyr365 have been recently identified as phosphorylation sites of both Lck and Fyn kinases [48, 49]. In thymocytes, phosphorylation of Lkb1 by Lck was necessary for Lkb1 to act as an scaffold during TCR signaling, allowing for PLCγ1 to localize to the LAT signalosome to be phosphorylated and propagate TCR signaling [49]. Finally, in adipose tissues, loss or inhibition of Fyn had the indirect effect of increasing AMPK phosphorylation. This resulted from the loss of Tyr261 and Tyr365 phosphorylation on

Lkb1 by Fyn that serves as a negative regulator of Lkb1 activity. Phosphorylation by Fyn resulted in the nuclear localization and sequestration of Lkb1, which results in decreased Ampk phosphorylation and activity [48]. As there are many signaling pathways that converge on Lkb1 that are relevant to BCR signaling, it will be of use to determine which signaling events are important for Lkb1's function during an immune response. This study will determine the relevant signals to Lkb1 after stimulation with agents that result in B cell activation. Also, it will determine if sub-cellular re-localization is one mechanism in which Lkb1's function is regulated upon B cell activation.

Experimental Strategies: Our preliminary data show that Lkb1 becomes phosphorylated on serine 431 in CD43 depleted splenic B cells in response to treatment with anti-mouse IgM $F(ab)^2$ fragment (Figure 4-2A). To validate and expand our preliminary data, primary B cells will be enriched from C57B/6 spleens by CD43 depletion. Enriched B cells will be stimulated with $F(ab)^2$ fragment anti-mouse IgM, anti-CD40, anti-CD40+IL-4 and lipopolysaccharide (LPS) and collected at multiple time points. Whole cell lysate (WCL) will be collected followed by western blot analysis utilizing phosphorylation specific antibodies to Ser325, Ser334, and Ser431 to determine phosphorylation of Lkb1. To validate the detected Lkb1 phosphorylation is a result of the signaling cascade it has previously been attributed to, primary B cells will be pre-incubated with the Mek inhibitor U0126 and the Akt inhibitor Akti-1/2. B cells will be stimulated as above and phosphorylation will be assessed as previously outlined.

siRNA oligos for *Lkb1* have been designed and cloned into the pLKO.1 lentiviral vector system. Use of these vectors has resulted in 3 *Lkb1* knockdown (*Lkb1*-KD) lines in the mouse lymphoma cell line CH12 (Figures 4-4A and 4-4B). To determine if phosphorylation of Lkb1 is necessary for activation, we will utilize site-directed mutagenesis to generate Lkb1 mutant expression constructs, as before. cDNA for Lkb1 will be generated using specific primers and cloned into the EGFP cassette of the FUGW lentiviral vector [53] and will be used to generate

CH12-*Lkb1* mutant stable lines. Phosphorylation inhibiting mutants, Ser→Ala 325, 334, and 431 and Tyr→Phe 261, and 365, will be generated by site directed mutagenesis.

Stable lines will be stimulated as above and cellular proliferation assays such as MTS, BrdU incorporation and CFSE dilution assays will be utilized as readouts of activation as well as expression of *Il6* by qRT-PCR [54]. Additionally, CH12 cells can be stimulated with anti-CD40, IL-4 and TGF-β to undergo CSR from IgM to IgA *in vitro* [55]. To evaluate Lkb1's role in signaling that induces CSR, CH12-Lkb1 mutant cell lines will be used to perform *in vitro* class switch recombination assays, with switching from IgM to IgA monitored by flow cytometry (Figure 4-4C) [55].

To determine the consequence of phosphorylation of Lkb1 downstream of signaling that induces B cell activation, the sub-cellular localization of Lkb1 will be analyzed. First, both un-stimulated and stimulated CH12 cells will be fractionated into nuclear and cytoplasmic fractions and these fractions will be assessed for presence of Lkb1 by western blot. Additionally, these cells will also be used for immunofluorescence (IF) analysis of Lkb1 and will be counterstained with DAPI to identify the nuclear compartment. Additionally, CD43- B cells isolated from C57B/6 spleens will be stimulated and undergo the same western blot and IF analysis detailed above.

To identify other potential sites of phosphorylation on Lkb1 downstream of B cell activation signaling, we will perform nano-liquid chromatography and tandem mass spectrometry (nLC-MS/MS) as before [44]. Using the FUGW lentiviral system, we will infect CH12 B cells with Flag-tagged Lkb1. Lkb1 will be immunoprecipitated with an anti-flag antibody, separated by SDS-PAGE and stained with Sypro-Ruby. Trypsin digested gel slices will undergo nLC-MS/MS to determine the specific phosphorylated residues. Any additional phosphorylated residues will be analyzed as the previously published sites.

Anticipated Results and Interpretations: We expect that Lkb1 will be phosphorylated downstream of B cell activating stimuli based on our preliminary data and previously published

studies demonstrating phosphorylation of Lkb1 downstream of Erk and Akt kinases in different cell types [44, 45, 51, 52]. We expect that phosphorylation of Lkb1 downstream of B cell activation will have functional relevance based on the published data that show phosphorylation of Lkb1 regulates its function by altering its subcellular localization [45]. Demonstrating Lkb1 phosphorylation, kinase activity, and cellular localization as a result B cell activation will be the first study placing Lkb1 in the B cell activating signaling cascade.

Brief Perspectives, Significance, and Long-Range Objectives: Lkb1 signaling has not previously been described as part of the B cell activating signaling cascade. Our preliminary data is the first to place Lkb1 in a role as part of this cascade. While previous studies have linked Lkb1 to signaling that is known to be part of the B cell activating signaling cascades such as Erk [44, 51, 52] and Akt [45], the above study will be the first to establish Lkb1 in this role. This study will not only identify signaling to Lkb1, it will evaluate the consequence of phosphorylation of Lkb1 downstream of B cell activation and will be the first step in helping elucidate the molecular mechanism of Lkb1 regulation and function in antigen-activated B cells. This information will motivate future studies into not only Lkb1's but Ampk's role in B cell biology.

Examine the requirement of *Lkb1* downregulation for B cell activation

Hypothesis: Based on Lkb1's role as a tumor suppressor, our data showing that loss of *Lkb1* in transitional and mature B cells results in aberrant activation in BKO and BKO-YFP mice, and our preliminary data showing down regulation of *Lkb1* upon B cell activation *in vitro,* we expect *Lkb1* needs to be suppressed for effective activation of B cells during an immune response.

Rationale: Mature B cells in the periphery are quiescent until they encounter antigen and initiate an immune response [56, 57]. Once activated, B cells undergo rapid proliferation with a

reported cell division time of ~6-8 hours [58, 59] making activated B cells one of the most rapidly dividing cell type in the body. Quiescence of lymphocytes is a state of cell cycle exit that needs to be actively maintained [57]. Microarray data confirm the presence of quiescence transcriptional programs in B cells that regulate this state and upon activation there is an increase in expression of genes that control growth and differentiation but also repression of genes that make up the quiescence gene program [56]. This highlights the how tightly regulated proliferation is in both naïve and activated B cells.

One of Lkb1's roles as a tumor suppressor is its ability to inhibit cell growth when added back to cell lines that lack its expression such as HeLa cells [9]. Proliferation and cell cycle regulation by Lkb1 has been attributed to more than one mechanism. Lkb1 has been shown to regulate cell cycle arrest and cell death in a p53 dependent manner [11] through phosphorylation of both Ampk [60, 61] and Sik1 [62] during glucose starvation and anoikis respectively. Another mechanism in which Lkb1 regulates proliferation is as part of the AMPK metabolic checkpoint [63]. Lkb1 is the main upstream kinase to Ampk and 12 other members of the Ampk family of protein kinases [19, 64]. Ampk functions as an energy monitor; when a cell's AMP:ATP ratio increases, Ampk is activated, by Lkb1, and activates metabolic pathways focused on catabolism of ATP and inhibits biosynthetic pathways that consume ATP [15]. Loss of Lkb1 would result in a block in this signaling pathway would and enable cells to meet the high demands for biosynthesis necessary for rapid cell proliferation [65].

Preliminary data from our lab reveals a decrease in expression of *Lkb1* in CD43- B cells isolated from wild-type FVB X C57B/6 mice stimulated *in vitro*. This decrease is seen as early as 24 hours post-stimulation with anti-CD40 and IL-4 and is maintained out to 72 hours in stimulation (Figure 4-2B). Repression of *Lkb1*, based on our knowledge of Lkb1's functions as a tumor suppressor and upstream kinase to Ampk, would facilitate a B cell's ability to undergo the rapid proliferation that is observed upon activation and GC initiation. This study seeks to

determine if this downregulation of *Lkb1* we observe is necessary for efficient B cell activation, GC initiation, and T-dependent immune response.

Experimental Strategies: To validate our preliminary data indicating that *Lkb1* is down regulated in response to B cell stimulation *in vitro* (Figure 4-2B), CD43 depleted splenic B cells enriched from C57B/6 mice will be stimulated with anti-mouse IgM $F(ab)^2$ fragment, anti-CD40, anti-CD40+IL4, and LPS. Both WCL and cDNA will be generated from stimulated B cells at different time points over a 72 hour period, and expression of Lkb1 will be assessed by western blot and qRT-PCR.

Additionally, C57B/6 mice will be immunized with SRBCs to induce a robust GC reaction. CD19+IgM+CD21+ [66] follicular B cells will be sorted from un-immunized C57B/6 mice along with B220+GL-7+FAS+ GC B cells from immunized mice 10-12 days after immunization. Both WCL and cDNA will be generated from sorted populations and expression of Lkb1 will be assessed by western blot and qRT-PCR. Spleen sections from immunized and unimmunized mice will be formalin fixed and paraffin embedded and expression of Lkb1 will be analyzed by immunohistochemistry (IHC), as well as PNA, Bcl6, and OCA-B as performed before [67].

cDNA for *Lkb1* will be generated by RT-PCR and cloned into the pCMMP-IRES-mRFP retroviral expression vector. CD43 depleted B cells from C57B/6 spleens will be stimulated with anti-CD40 or LPS and then be retrovirally infected 24 hours later, as before [68]. Infected cells will be cultured in stimulation media (anti-CD40 and IL-4 or LPS) post-infection. Markers of activation such as MHC Class II, CD69, CD25, CD80 and CD86 will be assessed by flow cytometry daily for 3 days post-infection. A functional consequence of B cell activation*, in vitro*, is CSR to IgG1 (anti-CD40 and IL-4) and IgG3 (LPS). Switching to IgG1 and IgG3 will be analyzed by flow cytometry in RFP+ Lkb1 expressing B cells.

A transgenic cassette will be generated containing the coding region of mouse *Lkb1* that utilizes the B29 (Igβ) promoter to enforce expression of Lkb1 in the B lineage [69]. Transgenic (Tg) mice will be generated and founders will be identified by genomic PCR from tail isolated DNA. Three independent lines will be expanded and thoroughly characterized to ensure findings are a result of enforced *Lkb1* expression and not due to site of integration. The B cell compartment will be thoroughly characterized by flow cytometry to identify any alterations in the B cell populations due to expression of *Lkb1* transgene as performed in BKO mice. The ability for *Lkb1* Tg B cells to be activated will first be assessed *in vitro.* Briefly, CD43- enriched B cells will be stimulated with anti-CD40 and IL4. Expression of activation markers will be evaluated as well as ability to undergo CSR to IgG1.

The ability of *Lkb1* Tg mice to mount a T-dependent immune response *in vivo* to NP-CGG will be determined [70]. WT and Tg mice will be immunized via intra-peritoneal injection. On day 10 a cohort of mice will be euthanized and GC formation will be assessed by flow cytometry for presence of B220+GL-7+FAS+ B cells and spleen sections will be fixed and paraffin embedded for IHC as in section 2.1. On day 14 and day 28 eye bleeds will be performed and serum will be collected. NP-specific IgM and IgG1 anti-body titers will be determined by ELISA as performed before [70].

Anticipated Results and Interpretations: Based on the known functions of Lkb1 as a tumor suppressor, our data showing down regulation of *Lkb1* during B cell activation and the aberrant activation of transitional and mature B cells in BKO mice (Figure 2-9), we expect that the down regulation of *Lkb1* expression will be necessary for B cell activation and subsequent GC formation. We expect that our preliminary results will be validated *in vivo* and that *Lkb1* will be down regulated in GC B cells. If we do not see decreased expression of *Lkb1* in sorted GC B cells on day 10 it could be due to the kinetics of *Lkb1* regulation. It is possible that *Lkb1* is down regulated immediately upon B cell stimulation to allow activation, but then is re-expressed in

established GC B cells. We would proceed by sorting different subsets of GC B cells, such as centroblasts ($CXCR4^{high}CD86^{low}$) and centrocytes ($CXCR4^{low}CD86^{high}$) [71] to examine Lkb1 expression for each subtype. This result would not be surprising as we have shown a role for Lkb1 in terminal differentiation upon exit from the GC [8] and other proteins, such as c-myc [71, 72], are differentially regulated in specific GC subsets. In mice with enforced B cell expression of *Lkb1*, we expect to see an inability to form GCs or to mount an efficient antibody mediated immune response. If observed, these findings would establish Lkb1 has regulator of B cell quiescence, one that is both necessary and sufficient for blocking activation in both immature and mature B cells.

Brief Perspectives, Significance, and Long-Range Objectives: These studies will evaluate the physiological relevance of Lkb1 down regulation in a wild-type immune response. This is exciting because it would give more insight into the molecular mechanism of B cell activation. Reduction in Lkb1 expression, may prove to key to B cells' ability to undergo the rapid proliferation that is essential to mount a T-dependent immune response [73]. It would also provide insight into the phenotype we observe in BKO and BKO-YFP mice, where loss of Lkb1 in CD19+ B cells results in aberrant activation and *Il6* expression (Figures 2-7 and 2-10).

Evaluate the effects of long-term immune cell activation in BKO mice Hypothesis: Based on the presence of large spontaneous GCs in BKO mice and hyper activated lymphoid populations, we hypothesize that BKO mice will develop lymphoid pathology as they age, the most prominent and likely being autoimmune disease or B cell lymphoma.

Rationale: Germline loss of *LKB1* results in Peutz Jeghers Syndrome [36] (PJS) characterized by polyps of the gastro-intestinal tract and an increased risk of developing an array of cancers [36]. Somatic mutations in *LKB1* has been found in many cancers [35] including pancreatic [74],

melanoma [75, 76], breast [77, 78], and cervical [39]. Mutations in *LKB1* have never been detailed for lymphoma. Recently, we found in a panel of 17 human GC B cell lymphomas analyzed, *LKB1* expression was repressed more than 10-fold in 41% of the samples (7/17) [8].

Our lab has previously generated T cell leukemia-1 (TCL1) Tg mice that developed mature B cell malignancies by 12 months of age [69] despite expressing TCL1 in both B and T lineages and presenting with lymphocytosis of both lineages in young mice [69]. Additionally, when modeling BCL2 driven follicular lymphoma in the VavP-*Bcl2* mouse model [67] success was only accomplished when expressing Bcl2 in both lymphoid lineages. GC hyperplasia preceded lymphomagenesis in VavP-*Bcl2* mice [67] and GCs were found to be imperative to lymphomagenesis in TCL1 Tg mice [79]. These two models suggest that T cell help provided to B cells to form GCs is also needed to develop mature B cell tumors. Specifically it suggests that a prolonged GC reaction may promote lymphomagenesis by increasing the likelihood that a B cell will acquire additional genetic alterations that result in tumor formation [67]. Preliminary data from our lab shows loss of *Lkb1* in CD19+ B cells in BKO mice results in aberrant activation of both peripheral B and T cells (Figure 2-7) and spontaneous GC formation (Figure 2-4). This suggests that BKO-YFP mice may harbor an environment ideal for lymphomagenesis.

A hallmark in most (but not all) autoimmune disorders is the secretion of autoantibodies [6]. B cells undergo multiple genomic alterations during development and while undergoing immune responses, such as V(D)J recombination [1], SHM, and CSR [2], that left unchecked can result in the production of auto-reactive B cells. B cells also have a multiple mechanisms to curtail autoimmunity [80]. However, BKO mice have an increased number of activated lymphocytes (Figure 2-7) and spontaneous GC formation (Figure 2-4). While GC B cells appear to be undergoing apoptosis (Figure 2-4E and 2-4F) and there is no increase in plasma cells (Figure 2-5D) or switched Ig titers in BKO serum from young mice (Figure 2-5C), preliminary data show in mice aged to at least 6 months 21% (4/19) BKO serum were positive for anti-nuclear autoantibodies (ANAs) while 0% (0/5 and 0/7) of WT and HET mice were positive for

ANAs (Figure 2-5H). This suggests that as BKO-YFP mice age, autoimmunity could manifest itself.

Experimental Strategies: To validate our previous data of *LKB1* repression in a panel of 17 GC B cell lymphomas, we will analyze paired lymphoma and normal tissue samples for *LKB1* expression. Many samples from this bank have been characterized at the immunophenotypic and molecular levels and each is linked to treatment and patient outcome without use of patient identifiers. The tissue bank includes formalin-fixed and paraffin embedded samples from antibody staining and cryostat frozen sections for antibody staining, and DNA and RNA isolation. We will isolate RNA from samples and generate cDNA using standard techniques. cDNA will be analyzed by qRT-PCR for expression of *LKB1.* In samples where *LKB1* expression is similar to that of paired controls, we will sequence the coding region of LKB1 using standard methods. Sequencing will be focused on the C-terminus of *LKB1*, as that is where a majority of point mutations have been located [10].

To determine if the aberrant activation of both B and T cells in the BKO and BKO-YFP mice result in lymphomagenesis, sex-matched littermate WT, heterozygous (HET), and BKO mice will be followed and lymphoma formation will be assessed as we have done previously [69, 79]. Mice will be followed for a two-year period and monitored for disease development by usual methods. Additionally, mice will be periodically analyzed for development of pre-malignant changes. B cell populations in the bone marrow, spleen, lymph nodes and peritoneal cavity will be analyzed by flow cytometry [69, 79, 81], at 6, 9 and 12 months unless interrupted by tumor formation. Upon tumor formation, primary tumors and metastasis will be evaluated histologically for classification according to MMHCC guidelines for lymphoid malignancies [82]. Tumor clonality will be evaluated by PCR analysis of genomic DNA [69].

To validate and expand on the observation of presence of anti-ANAs in aged BKO mice, we will age a cohort of BKO-YFP mice. At 9, 12 and 18 months, serum will be collected via

retro-orbital eyebleeds and anti-ANA presence will be determined by ELISA, as well as secretion of IL-6 by ELISA. In young mice, BKO B cells express an increased amount of *Il6* (Figure 2-9E) but this is not detectable in serum (data not shown). Additionally, B and T cell populations in the bone marrow, lymph node, peritoneal cavity, and spleen will be analyzed by flow cytometry focusing on the plasmablast and plasma cell populations of B cells and memory populations (CD44+) T cells. Preliminary data also indicates by 6-8 weeks of age, BKO mice have lymphoid infiltrates in non-lymphoid organs such as liver and lungs (Figure 4-5). These data will be repeated with cohorts of mice at 3, 6, 9, and 12 months. Additionally, to expand upon these data, IHC will be performed to identify what specific lymphoid population (B or T or mixed cells) is responsible for these infiltrates. Additionally, kidneys will be collected and analyzed by IHC for presence of lymphocytes and immune complexes [83]. Briefly, kidneys will be OCT embedded, sectioned, and stained with FITC labeled anti-mouse Ig and IgG.

Anticipated Results and Interpretations: Based on previous data [8] we expect to see loss of LKB1 expression in tumor samples from GC B cell lymphomas compared to paired control tissue. If loss of LKB1 is not widely seen in tumor bank samples, we expect to discover mutations of LKB1 in these samples by analyses of sequencing of the coding region.

Based on preliminary data (Figures 2-5H and Figure 4-5) indicating early signs of autoimmune disease in BKO mice, we expect to see some manifestations of autoimmune disease as BKO-YFP mice are aged out. We expect activated lymphoid populations in BKO-YFP mice to continue to accumulate as the mice age, especially the activated T cell populations in which increased apoptosis has not been observed. There may not be much of an increase in the presence of autoantibodies detected in BKO-YFP mice as we have observed an increase in apoptosis of GC B cells in young (6-12 week) BKO-YFP mice.

Based on published data showing the necessity of T cell assistance and GC formation in the development of mature B cells lymphomas [67, 69, 79] and our observation of both of these

in BKO and BKO-YFP mice (Figures 2-4), it would not be surprising to see lymphomagenesis as BKO-YFP mice age. While loss of Lkb1 in a subset of B cells does lead to the systemic immune activation that we observe in BKO and BKO-YFP mice, it also appears to lead to apoptosis of GC B cells (Figure 2-4E and 2-4F). One possible consequence of this is that activated and proliferating B cells have a higher probability of gaining mutations that could block apoptosis and eventually lead to lymphoma formation. Another possibility is that activated B cells from BKO mice will continue to die before acquiring any survival enhancing mutations. This would result in no lymphoma formation in these mice and lead us to conclude that loss of Lkb1, in and of itself, does not lead to lymphogenesis.

Brief Perspectives, Significance, and Long-Range Objectives: The goal of this study is to provide pathological relevance to the studies of Lkb1 in B cells. Our group is, so far, alone in linking Lkb1 to lymphomagenesis and this study would further our understanding of Lkb1's role in mature B cell cancers. We originally proposed a role for Lkb1 in B cell lymphoma as signaling component in a DNA DSB induced signaling cascade in GC B cells undergoing terminal differentiation [8]. Our new data places Lkb1, not only at the termination of the GC response, but also as a regulator of aberrant activation in naïve transitional and mature B cells. Additionally, if loss of Lkb1 contributes autoimmune manifestations it would be the first implication for Lkb1 in disease pathology outside of PJS and cancer. Linking Lkb1 to lymphoma and/or autoimmunity would motivate studies on the Lkb1-Ampk signaling pathway as components for these diseases and the re-activation of this pathway through AMPK activators as potential therapeutics.

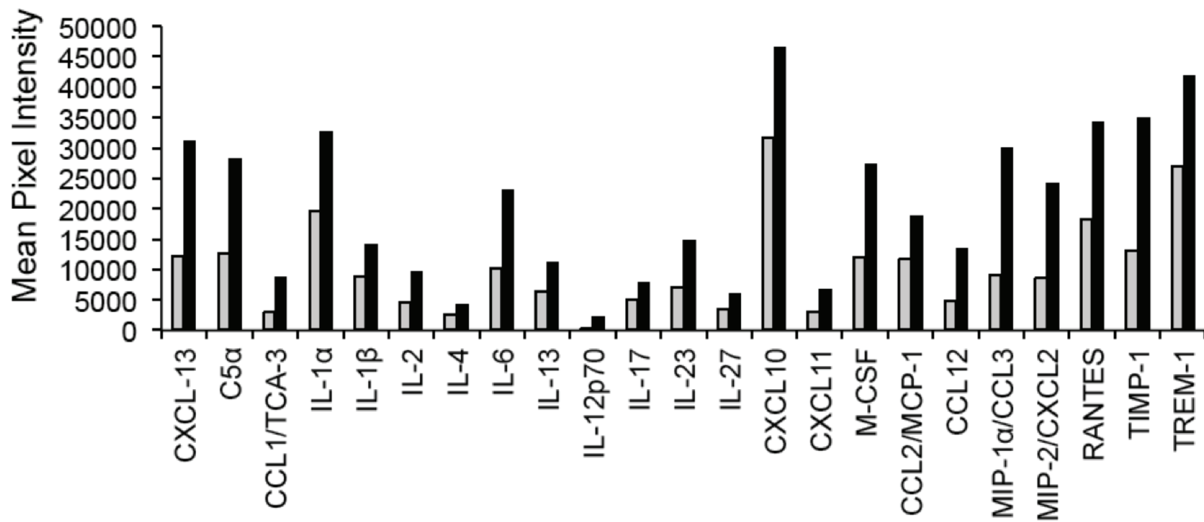
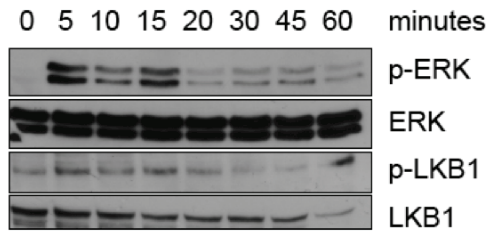


Figure 4-1. Inflammatory environment found in spleens from BKO mice

Cytokine array panel performed on cultured media from two WT (gray) and two BKO (black) spleens identified a profile of cytokines increased in BKO mice.

A



B

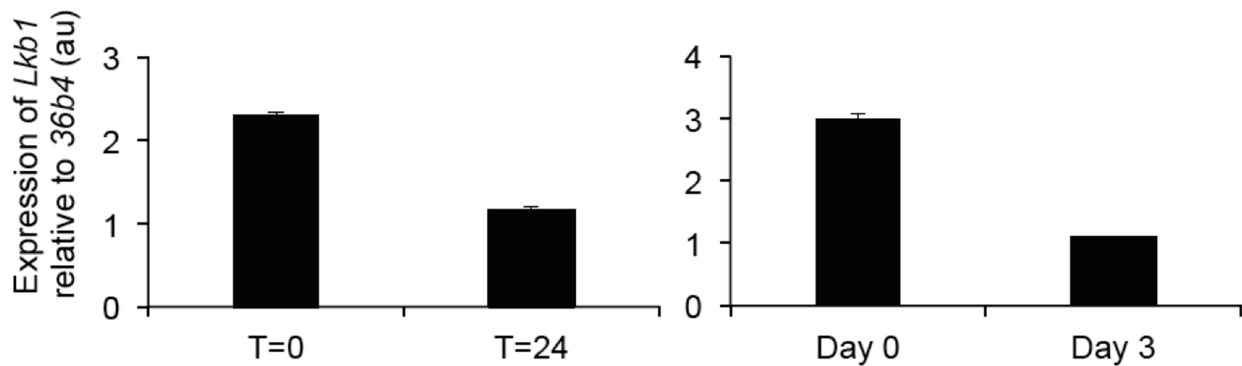


Figure 4-2. Lkb1 is modulated downstream of B cell activation

A. Lkb1 is phosphorylated on Serine 431 downstream of Erk signaling after stimulation with anti-IgM in CD43- splenic B cells by western blot analysis

B. *Lkb1* expression is reduced after activation of CD43- splenic B cells with CD40 ligand and interleukin 4 from 24 to 72 hours post activation by qRT-PCR analysis. Representative data represented as mean ± SEM

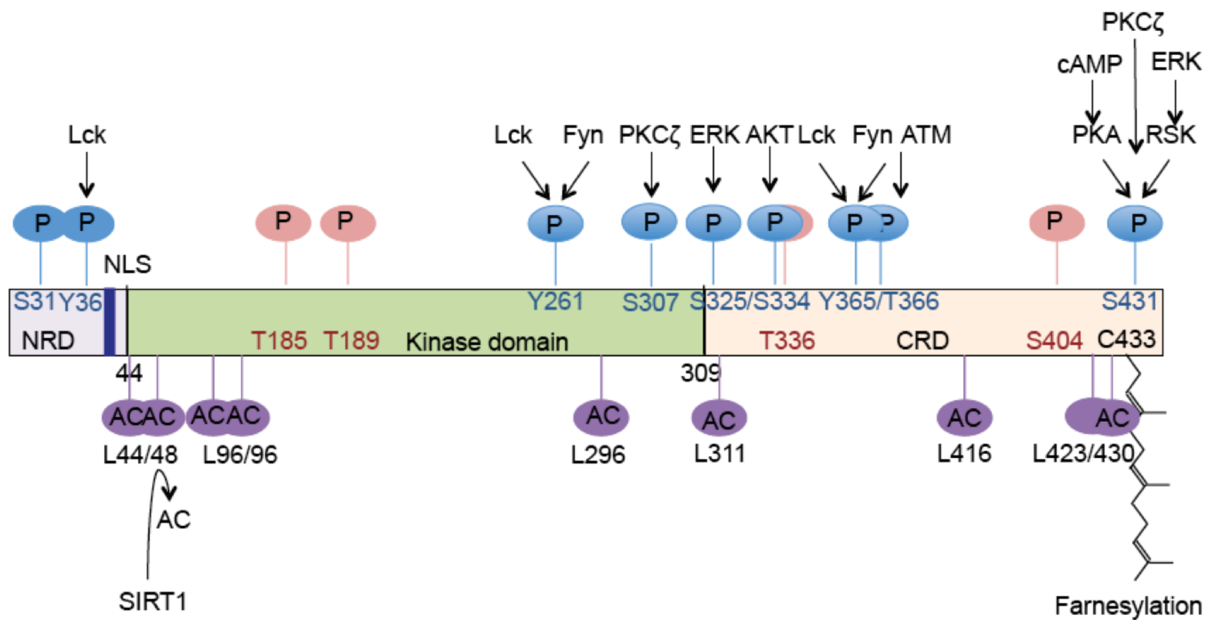


Figure 4-3. Post-translational modifications of Lkb1

(Adapted from [10]). Lkb1 is made up of a N-terminus regulatory domain (NRD) that contained a nuclear localization signal (NLS), a kinase domain, and a C-terminus regulatory domain (CRD) [10]. Mouse Lkb1 can be modified at multiple residues by upstream signaling. Lkb1 has four residues thought to be autophosphorylated, shown here in red, Thr185, Thr189, Thr336, and S404 [84]. Five serine (Ser31[84], Ser307[47], Ser325 [44, 84], Ser334 [45], Ser431 [42, 44]), three tyrosine (Tyr61, Tyr261, and Tyr365) [48, 49], and one threonine (Thr366 [41, 84]) residues can be phosphorylated by upstream kinases, shown here in blue. Human Lkb1 can be acetylated at multiple lysine residues (L44, L48, L96, L97, L296, L311, L416, L423 and L431 (mouse 430) [85]. L48 can be de-acetlyated by SIRT1 [85]. Finally, Lkb1 is prenylated on one cysteine residue, C433 [42].

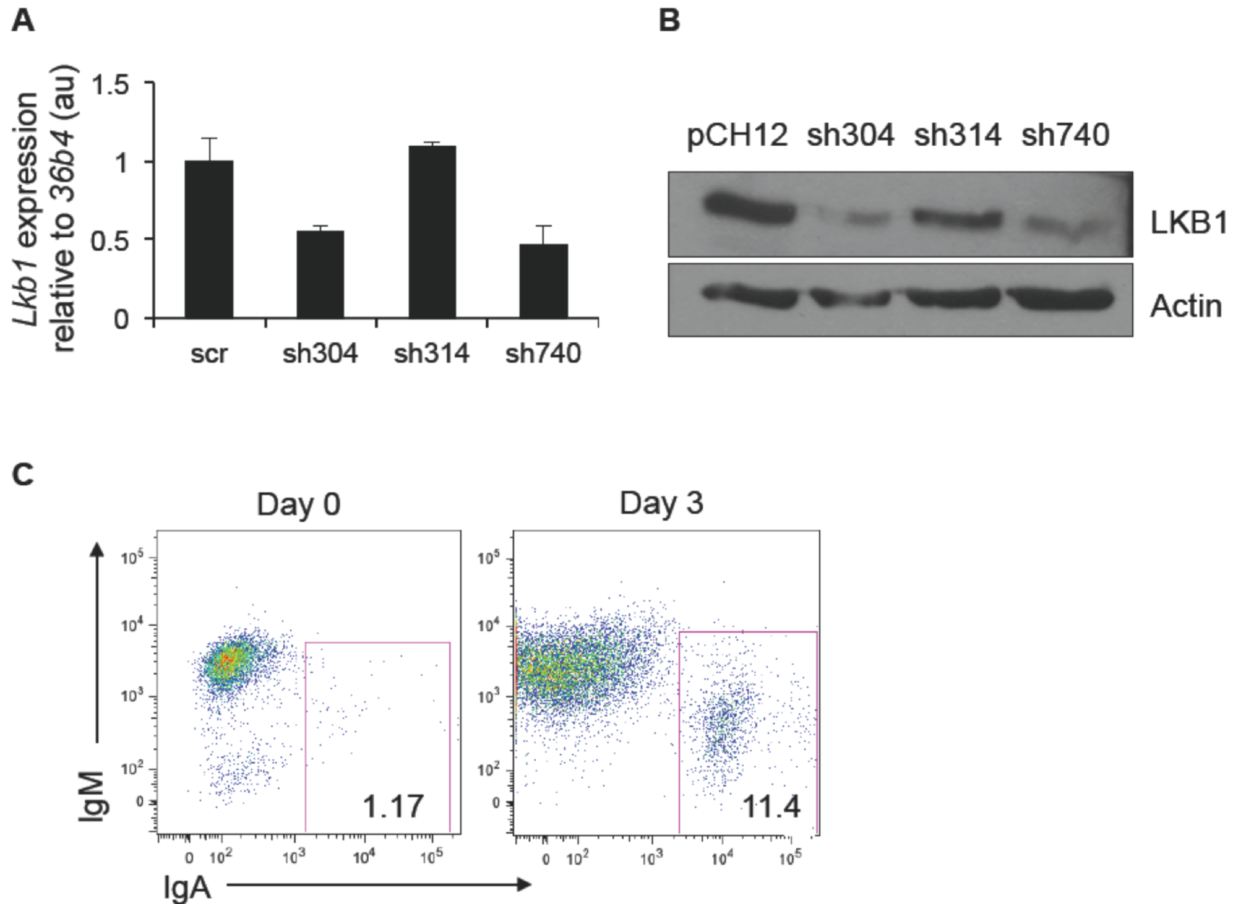


Figure 4-4. Generation of Lkb1-knockdown CH12 cells and CH12 class switch recombination assay

A. *Lkb1* expression in Lkb1-knockdown CH12 cells by qRT-PCR analysis. Data is represented as mean ± SEM

B. Lkb1 expression in Lkb1-knockdown CH12 cells by western blot analysis

C. Representative flow cytometry data showing class switching of IgM on CH12 cells to IgA after 3 day stimulation with CD40 ligand, IL-4, and TGF-β

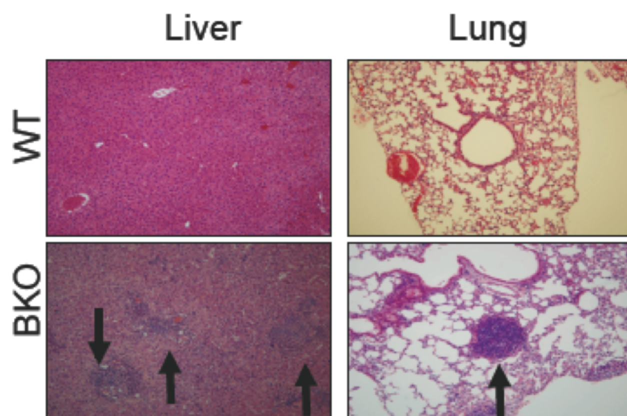


Figure 4-5. Autoimmune manifestations in BKO mice

Liver and lung sections from young, 6-8 week old, BKO mice were stained with H&E and imaged at 100X

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APPENDIX A:

Lkb1-Ampk Signaling Regulates *Il6* Transcription

Ampk is well known as the regulator of cellular energetic homeostasis, however it also plays a role in regulating inflammation. Signaling through Ampk, in multiple cell types, activates a number of proteins that negatively regulate inflammatory NF-κB signaling. Loss of Lkb1 in B cells and MEFs induces the production of IL-6 downstream of increased NF-κB activation. Additionally, inhibition of Ampk in WT MEFs resulted in an increase in *Il6* expression. Together these data suggest that Lkb1-Ampk signaling is one mechanism by which cells regulate inflammatory NF-κB activation.

Ampk is the regulator of cellular energy metabolism. Ampk consists 3 subunits, the catalytic Ampkα, Ampkβ, and Ampkγ. When there is an energy deficit, Ampk is activated via phosphorylation on threonine 172 in it activation loop. Activated Ampk phosphorylates proteins that activate processes to generate ATP, such as glycolysis and oxidative phosphorylation, and inhibit processes that consume energy, such as cell growth and protein synthesis (reviewed in [1] and [2]). Ampk can be phosphorylated by three upstream kinases; Lkb1 activates Ampk when the intracellular AMP:ATP ratio rises [3-5], CaMKKβ activates Ampk in response to Ca^{2+} signaling [3, 6], and TAK1 activates Ampk downstream of extracellular receptors, such as TRAIL mediated signaling [7].

There is an increasing literature supporting a role for Ampk signaling in the regulation of inflammation (Figure A-1). Activation of Ampk through pharmacological activators such as AICAR [8-12] and metformin [13, 14] have been shown to decrease the production of pro-inflammatory cytokines such as IL-6 and TNF-α in diverse cell types such as macrophages, T cells, neutrophils, mast cells, and endothelial cells. Additionally, Ampk activation has been shown to indirectly inhibit these inflammatory cytokines by inhibiting NF-κB activation [13, 15, 16]. As a consequence, treatment with Ampk activators such as metformin and AICAR decrease the severity of inflammatory symptoms in autoimmune disease models such as experimental autoimmune encephalopathy (EAE) [14] and chronic colitis [8].

The role for an upstream regulator for this signaling through Ampk has not been thoroughly established. Based on the increased production of IL-6 in both Lkb1- B cells and MEFs and the increased activation of NF-κB, we sought to investigate a possible role for Lkb1-Ampk signaling in the regulation of NF-κB and *Il6* expression. Lkb1- MEFs display decreased phosphorylated Ampk compared to WT MEFs and MEFs reconstituted with mouse *Lkb1*. Inhibition of Ampk in WT MEFs results in the increase of *Il6* expression. Taken together, these data support a role for Lkb1-Ampk signaling in the regulation of *Il6* expression, possibly through inhibition of NF-κB activation.

Results

Reintroduction of *Lkb1* in Lkb1- MEFs suppresses *Il6* expression

Loss of Lkb1 in mouse B cells resulted in the increased expression and secretion of IL-6 (Figure 2-9E). To gain insight into the signaling responsible for this increase, Lkb1- MEFs were analyzed and shown to produce increased IL-6 compared to WT MEFs (Figure 2-13A and 2-13B). To determine if absence of Lkb1 expression resulted in the increased induction of *Il6*, a retroviral expression construct was generated to reintroduce expression of Lkb1 into Lkb1- MEFs (Figure A-2A). This vector also contained an independently translated, truncated nerve growth factor receptor (tNGFR) to serve as a means of enrichment of successfully transduced cells (Figure A-2B). Lkb1- MEFs were infected with MSCV-mLkb1-IRES-tNFGR and enriched for tNFGR positive cells. Enriched Lkb1- MEFs infected with Lkb1-tNFGR (KO+Lkb1 MEFs) expressed Lkb1 to a higher extent that WT MEF controls (Figure 2-13C).

Subsequent analysis of known signaling pathways up stream of IL-6 induction revealed that transcription factors of the NF-κB transcription factor family were enriched in the nucleus of Lkb1- MEFS compared to WT MEF controls (Figure 2-13D). In KO+Lkb1 MEFs there was reduced nuclear p50, p52, and p65 (Figure 2-13D) similar to the nuclear levels of NF-κB family members found in WT MEF controls. These data support the hypothesis that in MEFs, loss of Lkb1 results in the activation of NFκB signaling.

Activation of Ampk contributes to the regulation of *Il6* expression

Expression of *Il6* was significantly repressed in KO+Lkb1 MEFs compared to Lkb1- MEFs, although *Il6* was still expressed more highly in KO+Lkb1 MEFs than in WT MEF controls (Figure 2-13A and 2-13B). Consistent with the role of Lkb1 as the main upstream kinase to Ampk [17-19], there was reduced phosphorylation of Ampk in Lkb1- MEFs compared to WT MEFs (Figure

A-2C). In KO+Lkb1 MEFs, phosphorylation of Ampk was restored to an extent seen in WT MEFs (Figure A-2C).

To determine if the decreased phosphorylation of Ampk in Lkb1- MEFs contributes to the up-regulation of *Il6* in Lkb1- MEFs, WT MEFs were treated with Compound C, an Ampk inhibitor, which reduced Ampk phosphorylation (Figure A-3A). Ampk inhibition in WT MEFs also increased the expression of *Il6* 13-fold compared to DMSO treated control (Figure A-3B). These data support the hypothesis that signaling through Ampk negatively regulates *Il6* expression in WT MEFs.

Discussion

Ampk signaling has been implicated in the negative regulation of inflammatory NF-κB activation (reviewed in [20]). Using WT and Lkb1- MEFs, we provide evidence that activation of Ampk by Lkb1 is an important event driving this signaling. Deletion of Lkb1 drives the activation of NF-κB in Lkb1- MEFs and the production of IL-6 (Figure 2-13). Exogenous expression of Lkb1 leads to the reduced activation of NF-κB and *Il6* expression (Figure 2-13). Additionally, inhibition of Ampk in WT MEFs results in increased *Il6* expression similar to that seen in Lkb1- MEFs.

As evidenced by the range of phenotypes exhibited in different tissue specific knockout models of Lkb1 [21], Lkb1 likely has different functions in different cell types. While Ampk has been shown to inhibit inflammation in different contexts, it has been shown to have this effect in multiple types of immune cells. In macrophage cell lines [22, 23], primary macrophages [22], and neutrophils [9] stimulation with LPS results in the induction of TNF-α and IL-6 and in B6J2 cells, LPS induces the suppression of the anti-inflammatory cytokine IL-10 [22]. Activation of Ampk with genistein [23], metformin [14], or ETC-1002 [24] resulted in the decreased induction of TNF-α and IL-6 as well as decreased nuclear NF-κB p65 [23]. Expression of a dominant-negative form of Ampk resulted in the increased induction of these inflammatory cytokines while expression of constitutive active Ampk mirrored chemical activation of Ampk with decreased inflammatory cytokine expression [22]. siRNA of Ampk in bone marrow derived macrophages stimulated with LPS resulted in an increased induction of TNF-α and IL-6 [22]. Additionally, T cells stimulated with anti-CD3 and anti-CD28 in the presence of AICAR failed to induce production of IFN-γ and IL-17 [8, 14] and mast cells stimulated with FcεRI in the presence of AICAR resulted in impaired activation including production of IL-6 [11]. Treatment of siRNA against *Ampkα2*, resulted in the increased activation of mast cells upon FcεRI stimulation and siRNA again *Lkb1* resulted in signaling similar to that of *Ampk* siRNA [11]. These data suggest

that Lkb1 to Ampk signaling in immune cells regulates induction of pro-inflammatory cytokines through regulation of NF-κB upon activation with a range of antigenic challenges.

Ampk activity is quickly inhibited by pro-inflammatory stimuli such as TNF-α [13] and LPS [9, 11, 14, 22, 24]. This could be accomplished in two ways; pro-inflammatory stimuli could increase the expression or activity of specific phosphatases that would de-phosphorylate Ampk or pro-inflammatory cytokines could affect the activity of Lkb1 (or other kinases upstream of Ampk) reducing the amount of phosphorylation events on Ampk and thereby diminishing its activity. These two hypotheses are not mutually exclusive and could work in concert to control Ampk activation in times when an inflammatory response in warranted. We also provide evidence to support a role of Lkb1-Ampk signaling in B cell activation. Deletion of Lkb1 in developing B cells results in the increase of IL-6 production (Figure 2-13F) and a cellular phenotype that resembles that of an activated B cell (Figure 2-9). *In vitro* stimulation of WT B cells resulted in the post-translational modification of Lkb1 on S431 (Figure 5-3A), a residue shown to inhibitory in other cell type [25].

Multiple signaling intermediates exist between Ampk and NF-κB that could be relevant in B cells. One intriguing potential intermediate is Foxo3. Foxo3 is activated directly by phosphorylation by Ampk [26] and Foxo3-/- mouse models display spontaneous splenomegaly after 8 months of age, increased inflammation and multi-organ lymphocytic infiltrations, as well as NF-κB hyperactivation and increased cytokine production (reviewed in [27]). Specifically in B cells, loss of Foxo3 results in the increased production of IgG2a, IgG3 and IgA serum immunoglobulins, a reduction in the pre-B cell populations and a reduction of re-circulating B cells found in the BM of Foxo3-/- animals [28]. Some aspects of these phenotypes are shared with BKO mice, specifically splenomegaly (Figure 2-4A), lymphocytic infiltrations (Figure 4-5B), NF-κB hyperactivation (Figure 2-13F), increased cytokine production (Figure 2-9E) and reduction of re-circulating B cells (Figure 2-2C). This suggests that loss of Foxo3 activity

downstream of Ampk contributes to the phenotype seen in BKO mice. Additional studies to determine the relevance of signaling through Lkb1 and Ampk to regulate NF-κB in B cells are necessary to sufficiently understand the importance of this pathway on B cell development, immune function, and potential pathological relevance.

Materials and Methods

Retro-viral construct generation

Mouse *Lkb1* was amplified by PCR and cloned into the pCR2.1-TOPO vector using the TOPO-TA cloning kit (Invitrogen). *Lkb1* was removed from TOPO by EcoRI digest and ligated into EcoRI sites in MSCV-IRES-tNGFR vector [29].

Viral Transduction

Retroviral particles were generated in 293T cells and harvested at 48 and 72 hours post transfection. MEFs were incubated with viral particles combined with polybrene overnight. Cells transduced with MSCV-mLkb1-IRES-tNGFR were enriched by incubation with CD271-PE (LNGFR) and anti-PE magnetic beads (Miltenyi). LNGFR positive cells were recovered and grown under standard conditions.

Cell Culture

WT and Lkb1– MEFs were obtained from Nabeel Bardeesy and were grown in DMEM supplemented with 10% FBS with or without 10 μM JSH-23 (Santa Cruz Biotechnology) or 20μM Compound C (Millipore).

qRT-PCR

RNA was isolated using Trizol (Life Technologies) and converted to cDNA with iScript (BioRad). qRT-PCR was performed on a LightCycler480 (Roche) using SYBR green (Roche). Target gene expression was normalized to expression of 36b4 [30].

Immunoblotting

Cells were lysed with Triton-X lysis buffer or fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Extacts were separated by SDS-PAGE and transferred to either nitrocellulose or PVDF membranes. Membranes were incubated overnight with the indicated antibodies in TBS-Tween and 5% milk or 5% BSA.

Statistical Analysis

The two tailed, unpaired student's t-test was employed to analyze parametric data, $p < 0.05$ was considered significant.

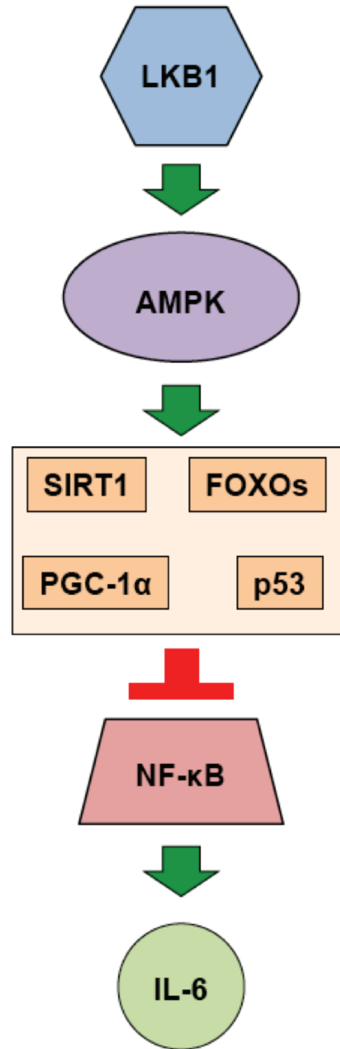

Figure A-1. Hypothesized signaling pathway regulating *Il6* expression through Lkb1 and Ampk

(Adapted from [20]). Lkb1 activation of Ampk results in the downstream activation of multiple protein kinases that can inhibit the activation of NF-κB, including FoxOs, Pgc-1α, Sirt1, and p53.

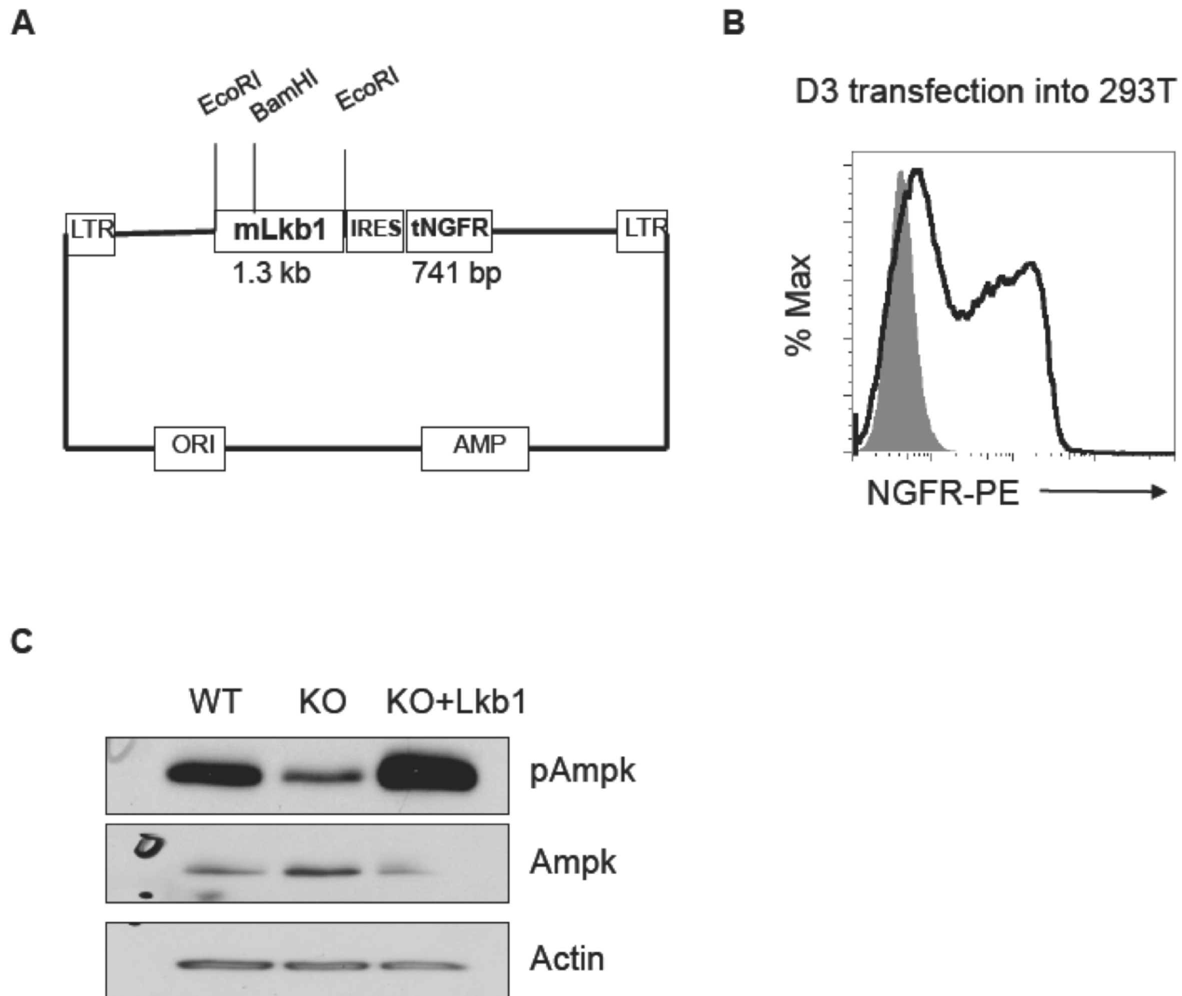

Figure A-2. Expression of mLkb1 in Lkb1- MEFs restores Ampk phosphorylation

A. Schematic of retro-viral construct generated to expression mouse Lkb1 and truncated NGFR in Lkb1- MEFs

B. Representative image of flow cytometry analysis of tNGFR expression after transfection of 293T cells with MSCV-Lkb1-IRES-tNGFR during generation of retro-viral particles

C. Western blot analysis of Ampk phosphorylation at threonine 172 in WT MEFs, Lkb1- MEFs (KO), and Lkb1- MEFs infected with MSCV-Lkb1-IRES-tNGFR (KO+Lkb1). Total Ampk expression and Actin are shown as loading controls

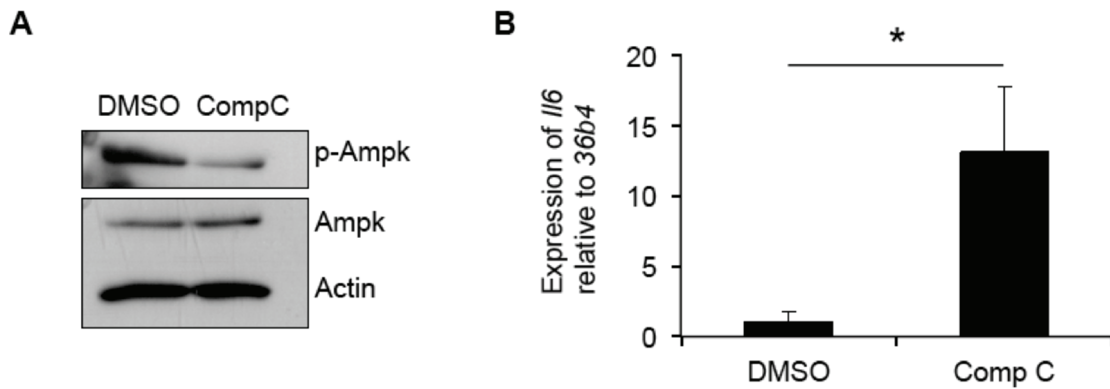

Figure A-3. Inhibition of Ampk in WT MEFs increases expression of *Il6*

A. Western blot analysis of WT MEFs treated with DMSO and Compound C for 2 hours to assess phosphorylation of Ampk at threonine 172. Expression of Ampk and Actin are shown as loading controls

B. qRT-PCR analysis of *Il6* expression in WT MEFs treated with DMSO or Compound C for 6 hours. Expression of *Il6* is shown relative to 36b4 and normalized to DMSO control. Data is shown as mean ± SD for three independent experiments, *p=0.011

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