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Fine-tuning of B cell antigen receptor signaling by reactive oxygen species and diacylglycerol metabolism

by

Matthew L. Wheeler

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

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By

Matthew Lewis Wheeler

Dedication

I would like to dedicate this thesis to my loving wife, Shan-shan, my parents, David and Wendy, and my crazy little dog Wallie.

Acknowledgements

None of the work presented in this thesis would have been possible without the support of a large group of people, and I am indebted to all of these individuals for their guidance over the years.

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be lucky enough to work with someone as knowledgeable and supportive as himself in the future.

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and the worst, and I love you with all of my heart. Thank you for being you, and for always brightening my day with your beautiful smile. Words cannot describe my gratitude to you.

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Prolonged production of reactive oxygen species in response to B cell antigen receptor stimulation promotes B cell activation and proliferation

Matthew L. Wheeler and Anthony L. DeFranco

The work presented in Chapter 3 is currently in revision for publication in Science Signaling

Critical role of diacylglycerol kinase-ζ in limiting B cell antigen receptor-induced ERK signaling and controlling the magnitude of the early antibody response

Matthew L. Wheeler, Matthew B. Dong, Robert Brink, Xiao-Ping Zhong, and Anthony L. DeFranco

Fine-tuning of B cell antigen receptor signaling by reactive oxygen species and diacylglycerol metabolism

By Matthew L. Wheeler

Abstract

Recognition of cognate antigen by the B cell antigen receptor (BCR) is one of the primary events that ultimately facilitate the production of pathogen-specific antibodies that are critical for host immunity. The process of B cell activation by antigen must be tightly regulated to ensure robust responses against pathogens while limiting responses directed at our own tissues. Much of this regulation is controlled at the level of signal transduction downstream of the BCR which requires a precise balance between signal amplification and feedback inhibition of pathways activated downstream of this receptor. The work presented in this thesis explores two distinct mechanisms by which signaling downstream of the BCR is regulated to govern the response of cells to cognate antigen stimulation.

Chapter 2 describes studies addressing the intracellular sources and physiological function of reactive oxygen species (ROS) produced in primary B cells in response to BCR stimulation. It was found that BCR stimulation of primary resting murine B cells induced the rapid production of ROS that occurred within minutes, and was maintained for at least 24 h following receptor stimulation. While the early production of ROS (0-2 h) was dependent on the Nox2 isoform of NADPH oxidase, at later stages of B cell activation (6-24 h) ROS were generated by a second pathway, which appeared to be dependent on mitochondrial respiration. B cells from mice deficient in the Nox2

NADPH oxidase complex lacked detectible early production of extracellular and intracellular ROS following BCR stimulation, but had normal proximal BCR signaling and BCR-induced activation and proliferation *in vitro,* and mounted normal or somewhat elevated antibody responses *in vivo.* In contrast, neutralizing both pathways of BCR-derived ROS with the scavenger N-acetylcysteine resulted in impaired *in vitro* BCR-induced activation and proliferation, and attenuated BCR signaling through the phosphatidylinositol 3-kinase pathway at later times. These results indicate that the production of ROS downstream of the BCR is derived from at least two distinct cellular sources and plays a critical role at the later stages of B cell activation by promoting sustained BCR signaling via the phosphatidylinositol 3-kinase signaling pathway, which is needed for effective B cell responses to antigen.

Chapter 3 describes studies addressing the role of metabolism of the second messenger diacylglycerol (DAG) by diacylglycerol kinase enzymes (DGKs), in modulating the magnitude of signaling by this second messenger downstream of the BCR. In the absence of DGKζ, the threshold for BCR signaling through the Ras-ERK MAP kinase pathway was markedly reduced in mature follicular B cells, resulting in exaggerated responses to antigen *in vitro* and *in vivo*. Inhibition of DAG signaling by DGKζ was especially important for limiting the number of antibody-secreting cells generated early in response to both T-independent type 2 antigens and T cell-dependent antigens. Furthermore, deficiency in DGKζ closely resembled the effects of increasing antigen affinity for the BCR during the T cell-dependent antibody response, strongly indicating that the magnitude of DAG signaling, likely through the degree of ERK activation, is

important for translating the affinity of the BCR for antigen into the amount of antibody produced during early stages of an immune response.

Together, these studies provide novel insight into the mechanisms involved in fine-tuning BCR signaling to promote high quality humoral immune responses while avoiding immunopathological consequences such as autoimmunity or lymphoma.

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Chapter 1

Introduction to B cell development, B cell receptor signaling, and peripheral B cell responses

Introduction to the immune system

Every day our bodies are bombarded with an array of pathogens including bacteria, fungi, and viruses, which if left uncontrolled can lead to serious tissue damage or death. To combat these microbial invaders we have evolved a highly sophisticated immune system which is tailored to target specific types of pathogens, and provide both short term and long lasting protection against re-infection.

The immune system can be divided into two main components; the innate immune system, and the adaptive immune system. The innate immune system provides our first line of defense through recognition of highly conserved molecular motifs that are common to most microbes, known as pathogen-associated molecular patterns (PAMPs). These PAMPs serve as "danger" signals to alert the immune system of a potential threat and allow for discrimination between "self" and "non-self". This type of response, although critical for initial host protection, is limited to a small number of pathogen associated molecules, and does not provide long lasting protection or immunological memory against re-infection. In addition, many bacteria and viruses have evolved ways to avoid recognition by the innate immune system, which can pose a serious threat to the host.

The adaptive immune system has evolved to overcome the restricted nature of the innate immune response, and is capable of responding to virtually any foreign antigen present on microorganisms. The key players in the adaptive immune system are the T and B lymphocytes which provide cell-mediated, and humoral immunity respectively. The

efficiency of the adaptive immune response lies in the fact that every lymphocyte expresses a single antigen receptor with a unique specificity, thus equipping the body with a highly diverse repertoire of cells with an unlimited range of specificities. However, it is important to note that the while innate and adaptive immunity are generally classified as two distinct types of responses, they are highly interconnected and co-dependent, and therefore both components of the immune system must function properly to efficiently protect us against infection.

B lymphocytes comprise the humoral component of the immune system which represents a critical arm of the adaptive immune response. Following antigen-mediated activation, B cells differentiate into plasma cells that secrete pathogen-specific antibody that provides systemic protection against extracellular microbes and many viruses. Antibodies can have a number of effector functions depending on the type of infection, their location, and isotype. They can have neutralizing effects by blocking the biological activity of toxins secreted by microbes, or in the case of viruses, by preventing their entry into target cells (1-3)**.** Antibodies also function by coating extracellular microbes and activating the complement system to target pathogens for uptake by phagocytic cells of the innate immune system through a process known as opsonization (4). The importance of the humoral immune response is evidenced in humans with mutations that render them unable to produce antibodies, such as patients with X-linked agammaglobulinaemia, who suffer from recurrent bacterial infections (5)**.** Furthermore, the success of most prophylactic vaccines depends on their ability to induce a high quality and long-lived antibody response (6). On the other hand a small percentage of the population generate

antibodies against self-components resulting in autoimmune diseases such as systemic lupus erythematosus (SLE) (7). It is therefore critical that the initial activation of B cells be tightly regulated to ensure efficient tolerance against self while also maintaining vigorous responses against pathogens.

Overview of B cell development, peripheral maturation and tolerance

Before discussing the mechanisms involved in B cell activation, it is first important to review the processes regulating early B cell development and maturation. Like most cells of the mammalian immune system, with the exception of T cells which develop in the thymus, B lymphocytes originate in the bone marrow from hematopoietic stem cells (HSCs) and undergo a series of defined maturation stages before they are functionally capable of mounting a response to foreign antigen. Commitment to the B lineage is imparted by a number of environmental cues as well as induction of a unique network of transcription factors that regulate expression of genes required for the B cell differentiation program**.** The stages of B cell development in the bone marrow can be defined both by expression of distinct cell surface markers (B220, CD43, CD24, CD25, c-kit) as well as the stage of immunoglobulin heavy (*IgH*) and light (*IgL*) chain expression and gene rearrangement, mediated by the enzymatic activity of the recombination activating genes (RAG1 and RAG 2) (8). This rearrangement of the immunoglobulin locus provides each B cell with unique antigen specificity thus contributing to the vast diversity within the B cell population.

Committed, or Pro-B cells, express and begin to rearrange their heavy chain locus. Once rearrangement generates a functional Ig heavy chain, it pairs with "surrogate" light chains during the pre-B stage of development (9). This pairing of the newly rearranged heavy chain with surrogate light chains, termed the pre-BCR, provides a way for the cell to test the functionality of the receptor and allow for subsequent developmental progression. This is mediated by antigen-independent, or "Tonic" signaling downstream of the pre-BCR which is required for expansion of pre-B cells, termination of further heavy chain rearrangement, and initiation of subsequent light chain rearrangement (10). The specifics of signaling downstream of the BCR, which is the primary focus of this thesis, will be discussed in greater detail in the next section. Once the cell has undergone successful Ig heavy and light chain rearrangement, and expresses a functional BCR in the form of IgM on the surface, the cell is now termed an immature or newly formed B cell, and can exit the bone marrow and migrate to the spleen where it will undergo further maturation stages at this peripheral site. Immature B cells that reach the spleen then mature through a series of "transitional" stages (T1, T2, and T3) before adopting a naïve mature B cell fate and each of these stages can be defined flow cytometrically by the combination of specific markers expressed on the surface of these cells (CD93, CD23, IgM, IgD, CD21/35) (11).

The reason behind this complex nature of B cell development is to impart a degree of quality control at each checkpoint to ensure that only cells with a functional BCR that does not recognize self will survive and contribute to the mature B cell repertoire. In the bone marrow, only B cells that have made successful *IgH* and *IgL* chain rearrangements

and express a functional BCR on their surface are able to receive tonic antigen-independent survival signals (8). In contrast, strong engagement of the BCR by cognate antigen at immature stages in bone marrow or spleen results in the induction of a number of tolerance programs which function to eliminate any potentially self-reactive B cells from the mature B cell pool (12). In the bone marrow, stimulation through the BCR induces a process known as receptor editing whereby the cell re-expresses Rag1/Rag2 and further rearranges the *IgL* chain locus with the goal of generating a new BCR that no longer retains autoreactive specificity (13, 14). During early transitional stages in the spleen (T1 and T2), strong BCR stimulation results in clonal deletion, once again to eliminate any potentially self-reactive B cells from the repertoire (15-18). Cells that are able to survive these developmental checkpoints can then adopt one of three distinct mature peripheral B cell fates: follicular, B1, or marginal zone (11), and these will be discussed in more detail below . In contrast to their immature B cell counterparts, mature naïve B cell respond to BCR stimulation by entry into the cell cycle, proliferation, and differentiation. Interestingly, while the original observation that immature B cells stimulated through their BCR undergo programmed cell death, whereas mature B cells become activated was made in 1976 (19), the mechanisms that mediate these functionally distinct responses at different stages of B cell maturation are still not entirely clear. Furthermore, the early B cell tolerance mechanisms described above are not sufficient to purge the body of all autoreactive B cells, and it has been estimated that even among healthy individuals, the BCR's expressed on as much as 20% of the mature B cell population possess some degree of autoreactivity or polyreactivity (20). These self-reactive cells that have survived early tolerance checkpoints cells are controlled by a

distinct form of tolerance in the periphery known as "anergy" whereby chronic BCR stimulation by self-antigen in the absence of any innate stimuli or T-helper cell derived signals induces a state of functional unresponsiveness, rendering these cells unable to mount a response to our own tissues (12). It is when one or more of these tolerance mechanisms fail that an individual is at risk for developing autoimmune diseases such as SLE.

The mature B cell repertoire is comprised of three main classes of naïve B cells which include the follicular B cells, marginal zone (MZ) B cells, and B1 B cells, each of which has distinct anatomical locations and physiological functions in our bodies (11). The follicular B cell is the most abundant subset of naïve B cells in the periphery and is found within the "follicular" region of secondary lymphoid organs. Unlike MZ B cells and B1 B cells, follicular B cells continually re-circulate through the spleen, lymph nodes, Peyers patches, and bone marrow in order to increase the likelihood of encountering their cognate antigen at any given time (21). Follicular B cells contribute to the classical T cell-dependent antibody response which gives rise to high affinity long lived plasma cells and memory B cells via the germinal center reaction, which will be discussed in more detail later. In contrast MZ B cells and B1 B cells are considered to be innate-like B cells which are capable of rapid differentiation into short live plasma cells that generate the initial wave of low affinity antibody to provide early protection against microbial infection (22). The rapid response of MZ and B1 B cells is mediated by a number of factors including elevated expression of toll like receptors and complement receptors, as well as their anatomical localization in the spleen and peritoneal cavity respectively (23,

24). Unlike follicular B cells, marginal zone B cells do not recirculate and instead remain localized in the spleen around the follicular perimeter in direct contact with blood flow, making them poised to respond efficiently to blood borne systemic pathogens (25). B1 cells localize to the peritoneal and pleural cavities, and similar to MZ B cells, are poised for rapid differentiation into short-lived plasma cells. B1 cells are also the primary source of natural IgM antibody, that is thought to be produced in an antigen-independent fashion, and provides preexisting immunity against a variety of pathogens (24). This division of labor among the different subsets of mature B cells ensures efficient responses to a wide array of infectious agents.

Regulation of B cell receptor signaling and B cell activation

The initial binding of cognate antigen to the B cell antigen receptor (BCR) is critical for transmitting signals intracellulary to dictate subsequent functional responses. The BCR consists of membrane immunoglobulin, which contains extracellular antigen binding sites within the variable regions of the heavy and light chain. The BCR itself does not possess any intrinsic signaling capabilities following antigen binding, and must rely on association with a heterodimer of the Igα and Igβ chains for initiation of downstream signaling (26, 27). This association of the BCR with Igα and Igβ occurs intracellularly in the ER and is required for trafficking of the BCR to the cell surface (28, 29). Signaling downstream of the BCR occurs upon stimulation with multivalent antigens, which are able to engage multiple receptors to bring necessary intracellular signaling molecules within close proximity to one another (30). The earliest signaling event following BCR stimulation involves the activation of the Src family kinases, which include Lyn, Fyn, and

Blk in B cells These kinases phosphorylate tyrosine residues within a conserved amino acid sequence in the cytoplasmic region of Igα and Igβ known as the Immunoreceptor tyrosine-based activation motif (ITAM) made up of the consensus sequence YXXL/IX7YXXL/I (31-33). ITAM phosphorylation of Igα and Igβ provides a binding site for the recruitment and activation of the protein tyrosine kinase (PTK) Syk which binds via its tandem SH2 domains (31, 33). The recruitment and activation of Syk at the membrane initiates a cascade of downstream phosphorylation events leading to the activation of a number of distinct, but interconnected signaling pathways required for various B cell functional responses. The pathways activated downstream of the BCR can be divided into four main classes which include the phosphatidylinositol 3-kinase (PI3K) pathway, the calcium/NFAT pathway, the NFκB pathway, and the Ras-ERK-MAP kinase pathway. Although B cell activation following cognate antigen stimulation requires coordination between all of the pathways mentioned above, each pathway is activated by distinct mechanisms downstream of the BCR, and has distinct functional outputs, and will therefore be discussed separately below.

PI3-kinase signaling

PI3-kinase (PI3K) is a heterodimeric enzyme consisting a regulatory subunit (p85α) and a catalytic subunit (p110δ), which catalyzes formation of the membrane phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) through addition of a phosphate group to the 3 position of the inositol ring of phosphatidylinositol (4,5)-bisphosphate (PIP_2) (34). The recruitment of PI3K to the membrane following BCR stimulation is mediated by phosphorylation, by Syk and Src family kinases, of adaptor molecules such as B-cell

PI3K adaptor protein (BCAP) and the BCR co-receptor CD19 which provide a docking sites for this enzyme (35). PIP_3 generated by the action of PI3Ks serves as a critical substrate for a number of signaling molecules that contain plekstrin homology (PH) domains, the most critical one being protein kinase B (PKB) or AKT. PI3K dependent generation of PIP_3 is also thought to be required for recruitment of the Tec kinase BTK to the membrane, which is critical for activating a number of important signaling molecules, most notably the enzyme PLCγ2 that generates the signaling second messengers IP_3 and diacylglycerol (DAG) (the importance of these messengers will be discussed in the following sections) (36). However, given that AKT is thought to be the most important mediator of PI3K signaling, signaling downstream of this kinase will be the focus of the remainder of this section.

The activation of AKT in response to BCR ligation by antigen is critical for a number of aspects of B cell function, including proliferation, differentiation, and most importantly B cell survival. Activation of AKT is mediated by phosphorylation at two key residues (threonine 308 and serine 473) by the kinase PDK1 and a protein complex known as mTORC2 (mammalian target of rapamycin complex 2) (37-41). Activated AKT can then phosphorylate targets leading to expression of genes involved in cell survival and proliferation. One of the main targets of AKT activity is the FOXO family of transcription factors which are important for inducing expression of pro-apoptotic molecules such as Bim and genes involved in maintaining cells in a "quiescent" or non-proliferative state, and for mediating repression of genes involved in cell survival and proliferation (36). Phosphorylation of FOXO family members by AKT induces their

nuclear export and subsequent proteosomal degradation, thus preventing expression of pro-apoptotic and anti-proliferative genes, and derepressing genes needed for cell survival and proliferation (42). Furthermore, activation of the mammalian target of rapamycin (mTOR) pathway by AKT is critical for mediating increased protein synthesis, glucose metabolism, and cell energy utilization, which are essential during clonal expansion, as would be the case following antigen-mediated activation (43). Chapter Two of this thesis focuses on a potential mechanism by which the signaling functions of the PI3K/AKT pathway can be sustained following BCR stimulation to ensure optimal cell activation and proliferation, through the production of reactive oxygen species.

The functions of AKT described above are not only critical for proliferation and survival in response to BCR stimulation by cognate antigen, but are also critical for B cell survival mediated by tonic signaling downstream of the BCR in resting mature B cells. The role of tonic BCR signaling in survival of B cells has been known for many years from studies showing that B cell development is severely compromised if membrane expression of the BCR is disrupted, or signaling downstream of the BCR is abolished (44-49). Subsequent studies in which the BCR was selectively deleted once cells reached the mature stage of development revealed that this tonic signaling function of the BCR was not only important for survival during B cell development, but also for survival of the resting mature B cell population (50, 51). Furthermore, more recent work showed that BCR-dependent survival of mature B cells is absolutely dependent on signaling through the PI3K pathway, as expression of a constitutively active version of PI3K was able to fully rescue mature B cell numbers in the absence of the surface BCR to provide tonic survival

signals (52). Consistent with the importance of this pathway both for tonic and antigen-dependent B cell survival, it is also well known that mutations that render PI3K/AKT signaling more sensitive, or constitutively active, such as loss of negative regulators of this pathway like PTEN or SHIP, can lead to defects in B cell tolerance (53-55) and contribute to the development of B cell lymphoma by providing survival signals to both autoreactive as well as malignant B cells (56).

Calcium Signaling

The release of calcium from intracellular stores, and subsequent influx of extracellular calcium into the cell is one of the earliest signaling events that occurs following ligation of the BCR. Calcium influx is critical in a number of cell types both for activation of signaling events as well as for altering ion gradients leading to cellular depolarization and excitation, as occurs in cells of the nervous system and heart.

In B lymphocytes, calcium signaling is initiated downstream of BCR ligation by action of the enzyme phospholipase C gamma 2 (PLCγ2), which is recruited to the membrane by association, via its SH2 domains, with phosphorylated tyrosine residues (mediated by Syk) on the scaffold, B cell-linker (BLNK). Phosphorylated BLNK also serves as a docking site for the Tec kinase BTK, which can initiate full activation of PLCγ2 by phosphorylating key tyrosine residues (Tyr753 and Tyr759) to maximize the lipid hydrolase activity of this enzyme (57). Activated PLCγ2 is required for generation of the signaling second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG)

through hydrolysis of PIP_2 stores in the membrane, and these second messengers are critical for propagating a number of downstream signaling events.

PLCγ2-dependent production of IP_3 is essential for initiating calcium signaling in B lymphocytes. IP_3 binds to its receptor ($InsP_3R$) present on the endoplasmic reticulum and causes the release of calcium stores from this organelle into the cytosol of the cell. This initial rise in free intracellular free calcium is then followed by a process known as store operated calcium entry (SOCE) whereby extracellular calcium flows into the cell to activate calcium-dependent signaling pathways (58). The depletion of ER calcium stores initiates SOCE by inducing oligimerization of the ER-associated calcium sensors STIM1 and STIM2. The oligimerization of STIM1 and STIM2 causes them to associate with the ORAI subunits of the calcium release-activated calcium channel (CRAC channel) present on the plasma membrane, leading to channel opening and influx of extracellular calcium into the cell. Once a sufficient amount of calcium is present in the cell to replenish ER stores, STIM proteins (which are monomeric in the presence of free calcium) will disassociate from each other and thus terminate further SOCE (59).

The function of calcium influx in modulating intracellular signaling events is through activation of signaling proteins that contain calcium-binding EF hands. Some examples of calcium regulated proteins are the Ras guanine exchange factors and protein kinase C isoforms (RasGRPs and PKCs) however their catalytic activity requires additional phosphorylation events, as well as binding to the second messenger DAG, and therefore calcium influx alone is not sufficient to activate signaling downstream of these enzymes.

The primary target of calcium signaling in lymphocytes is the NFAT family of transcription factors, of which NFAT1 and NFAT2 are the predominantly isoforms expressed in B and T cells (60). In the absence of free calcium, NFATs are normally highly phosphorylated and maintained in the cytoplasm, however the rise in intracellular free calcium causes activation of the phosphatase calcineurin, which rapidly dephosphorylates NFAT allowing for its nuclear translocation and subsequent alterations in gene expression to contribute to downstream responses to antigen (61). The transcriptional targets of NFAT vary depending on whether NFAT is present in a homodimeric complex, or in complex with the transcription factor AP-1, which is activated downstream of MAPK signaling. In the former situation, NFAT has been shown to mainly turn on genes associated with T and B cell anergy, whereas when complexed with AP1, the target genes are associated with lymphocyte activation, survival, and proliferation (62). These differential transcriptional effects of NFAT in the presence and absence of AP1 highlight the importance of coordination of the different signaling pathways activated downstream of antigen receptors.

The functional role of calcium signaling and the NFAT family members has mainly been studied in the context of T cells, where they have been shown to be critical for turning on various members of the cytokine family, and thus influencing downstream T cell polarization (62). However, the physiological role of calcium signaling through NFAT downstream of the BCR has not been quite as clear. B cell specific deletion of calcineurin, which presumably prevents NFAT activation in response to calcium influx, results in reduced B1 B cell development and abolished in vitro responses to BCR

stimulation (63). In contrast, these mice mounted enhanced T-cell independent type 1 responses, but had impaired T-cell dependent antibody responses. Another approach that was recently used to study calcium/NFAT signaling was to prevent SOCE by conditional deletion of calcium sensors STIM1 and STIM2 in the B cell lineage (64). However, the effects of loss of calcium influx in B cells were surprisingly mild, and in contrast to what was observed in the B cell-specific calcineurin deficient mice. Although B cells deficient in SOCE had large defects in survival and proliferation in response to BCR stimulation in vitro, there was little if any effect on B cell development or antibody responses in vivo. The main phenotype associated with lack of SOCE, and thus impaired NFAT activation, was in the inability to produce the immunosuppressive cytokine IL-10 from a population of B cells known as regulatory B cells, resulting in exacerbated autoimmune disease in a model of multiple sclerosis. Thus, one of the primary functions of calcium signaling in B cells may be to mediate transcriptional induction of cytokines via NFAT, as has been described in T cells. The reason for the discrepancies between these two studies in not clear, however it may lie in the fact that STIM1/2 deletion only abolishes SOCE, and not ER calcium release, which may be sufficient for some degree of NFAT activation. Calcium signaling is also thought to play an important role in B cell tolerance programs. For example, B cell anergy is associated with high basal levels of calcium influx, as well as uncoupling of BCR induced calcium and MAPK signaling (65). Furthermore, a recent study showed that SOCE was important for mediating negative selection of immature B cells in the bone marrow, through a pathway that involved calcium dependent activation of pro-apoptotic ERK signaling (66). Therefore, calcium/NFAT signaling may be important both for B cell immunogenic as well as tolerogenic responses, however there

are still a lot of unanswered questions regarding this signaling pathway in B cells that need to be addressed.

Nuclear factor κ B (NF-κB) signaling

The NFκB family of transcription factors consists of five members (NFκB1, NFκB2, RelA, RelB, and c-Rel) which function as homo- and hetero-dimers in the nucleus to activate and repress target gene transcription. Activation of the NF-κB downstream of the BCR is critical during B cell development, as well as for B cell survival and proliferation in response to antigen (67). In addition, a subset of diffuse large B cell lymphomas (DLBCLs) are characterized by constitutive activation of BCR-induced NFκB signaling, implicating this pathway as a prime target for treatment of B cell malignancies (68). NFκB activation in B cells also occurs in response to a variety of other stimuli including ligands for the toll-like receptors, CD40, and the BAFF receptor, however, given that the main topic of this thesis is BCR signaling, I will focus primarily on NFκB activation downstream of this receptor, which occurs via distinct upstream mechanisms compared to the stimuli listed above.

Similar to initiation of calcium signaling in response to BCR ligation described in the previous section, activation of NFκB downstream of the BCR also requires BTK-dependent activation of PLCγ2, and elaboration of the second messengers IP_3 and DAG. At the top of the signaling cascade required for NFκB activation is the β isoform of protein kinase C (69, 70). Catalytic activity of this kinase, similar to other classical PKC isoforms, requires binding to both calcium (via the C2 domain) and DAG (via the C1

domains) and is therefore absolutely dependent on the hydrolase activity of PLCγ2. Activated PKCβ has a number of cellular substrates for serine/threonine phosphorylation, however, critical for NFκB signaling is the scaffold molecule CARMA1 (or CARD11). Phosphorylation of CARMA1 by PKCβ results in a conformational change that allows for the formation of a signaling complex with the proteins Bcl-10 and MALT1, and subsequent activation of the IκB kinase (IKK) complex (69, 70). Deficiency in any of the factors listed above results in severely impaired NFκB activation downstream of the BCR (71-75). In resting B lymphocytes, the NFκB subunits are normally sequestered in the cytoplasm by the IκBα inhibitory protein, however, in response to BCR ligation this protein is phosphorylated by active IKK (downstream of the CARMA1/Bcl-10/MALT1 complex) and targeted for ubiquitin-mediated proteosomal degradation, allowing for NFκB to translocate to the nucleus where it can activate or repress transcription of target genes (76). Deficiency in PKCβ results in a complete loss in the signaling cascade described above following BCR ligation, however activation of NFκB in response to other stimuli such as TLR9 or CD40 ligation remained intact, indicating specificity of this signaling molecule for BCR-induced canonical NFκB activation (70). The third chapter of this thesis focuses on an enzyme that limits signaling through the second messenger DAG and is therefore highly relevant to activation of PKCβ and downstream NFκB signaling following BCR stimulation.

The importance of the NFκB pathway in B lymphocyte development and function has been demonstrated in a number of studies either investigating mice deficient in various members of the NFκB family, or in the signaling proteins required upstream for

activation of NFκB signaling. For example, combined deficiency in NFκB1 and NFκB2, or IKK1 and IKK2, results in a developmental block at the immature stage of B cell maturation which has been attributed to an important role for NFκB signaling in the induction of pro-survival genes such as Bcl-2 (77). Furthermore, NFκB has been implicated in receptor editing in immature B cells in the bone marrow and therefore may play an important role in preventing the development and/or survival of potentially autoreactive cells that arise during early B cell development (78). The NFκB pathway is also critical in mature B cells following BCR stimulation. This is evidenced by the fact that B cells deficient in PKCβ, or the different members of the CARMA1/Bcl-10/MALT1 complex fail to survive and proliferate in response to BCR stimulation, and have defective antibody responses (76). These effects are likely attributed to the importance of BCR-induced NFκB activation for induction of a number of genes involved in survival (e.g. Bcl-xL, Bcl-2, and A1) and proliferation (e.g. c-Myc). Interestingly, constitutive activation of this pathway is frequently observed in patients with the activated B cell like (ABC) form of DLBCL, which in a subset of patients results from mutations that give rise to chronic BCR signaling characteristics of the malignant cells (68). Therefore targeting proximal BCR signaling proteins such as Syk and BTK with small molecule inhibitors is actively being persued to treat this subset of lymphomas.

Ras-ERK-MAPK signaling

One of the most important signaling events that occurs downstream of antigen receptor stimulation is activation of the Ras GTPase, which is critical for growth and survival in multiple cell types, and is also frequently activated in a variety of cancers including

leukemia and lymphoma (79, 80). In lymphocytes, one of the primary downstream effects of Ras activation is activation of the ERK1 and ERK2 MAP kinase proteins, which play important roles in lymphocyte development, activation, survival, and cell fate decisions (81). The mechanisms by which BCR stimulation leads to activation of ERK-MAP kinase signaling, and the biological effects of activating this signaling pathway will be discussed below, and furthermore is the primary focus of studies related to Chapter Three of this thesis.

Activity of the Ras family members requires that these proteins be in the GTP-bound state, however due to the intinisic GTPase activity of Ras, these enzymes constantly shuttle between the GTP-bound (active) and GDP-bound (inactive) state. The full activation of Ras downstream of antigen receptors requires association with guanine nucleotide exchange factors (RasGEFs) which cause dissociation GDP from Ras. This allows for subsequent binding of GTP to Ras and adoption of the signaling active conformation (82). Lymphocytes express two main types of RasGEFs; the son-of-sevenless (SoS) and RasGRP families (82-85). Although both are capable of RasGTP production, the current model for Ras activation in lymphocytes is that Ras initially requires activation by RasGRP, and this activation is then amplified by SoS (85, 86). GTP-bound Ras activates members of the Raf family, which phosphorylate MEK1 and MEK2 that in turn phosphorylate ERK1 and ERK2 (81). Activated ERK can phosphorylate targets in the cytoplasm such as the ribosomal S6 kinase , and can also translocate to the nucleus where it can phosphorylate and activate a number of

transcription factors such as members of the Elk family leading to changes in gene expression required for B cell proliferation and survival (81, 87-89).

As mentioned above, the initial activation of Ras in B lymphocytes requires the guanine exchange factor RasGRP. In B cells, two isoforms of RasGRP are expressed, RasGRP1 and RasGRP3, which have somewhat overlapping contributions to downstream ERK activation, with RasGRP3 playing a predominant role in the mature B cell population (84, 90). RasGRP proteins contain calcium binding EF-hands as well as a DAG binding C1 domains and require binding to these substrates for catalytic activity (91, 92). Therefore, similar to activation of NFκB downstream of PKCβ, activation of ERK downstream of Ras is dependent on generation of the second messengers IP_3 and DAG, by the hydrolase activity of PLCγ2 downstream of BCR ligation. Furthermore, RasGRP activity is also promoted by PKC dependent phosphorylation, and therefore the activation of ERK and NFkB downstream of the BCR are largely coordinated (93, 94).

The importance of ERK signaling in B cells has been demonstrated utilizing various approaches, which include pharmacological inhibition of MEK (95), genetic deficiency in the signal intermediates RasGRP1 and RasGRP3 (90), or genetic deficiency in ERKs themselves (96, 97). B cell-specific deletion of ERK1 and ERK2 results in a complete block in early B cell development resulting from a loss of proliferative expansion of Pre-B cells mediated by signaling downstream of the pre-BCR (97). This study also revealed a number of important gene inductions that were mediated by ERK-dependent activation of the transcription factors Elk1 and CREB, and included a number of genes involves in

cell cycle regulation such as the Mef2 family, c-Myc, and Ilf2. Other studies have also implicated an important role for ERK in the induction of a number of early response genes including Egr1 and Nur77, as well as the transcription factor Fos which acts in a complex with Jun to influence expression a large set of genes involved in cell proliferation and differentiation in a variety of cell types (81, 89). In mature B cells, inhibition of MEK resulted in severely impaired proliferation and survival in response to BCR stimulation, and this was also true of B cells deficient in RasGRP1 and RasGRP3, which are unable to activate ERK in response to BCR crosslinking (90, 95). Furthermore, RasGRP deficient mice have defects in T cell-dependent and T cell-independent antibody responses, indicating an important role for Ras-ERK activation downstream of the BCR for B cell functional responses in vivo. Interestingly, RasGRP1/3 double knockout mice have mostly normal B cell development, so ERK signaling that is required for pre-B cell expansion must occur through a RasGRP independent mechanism (alternative pathways for ERK activation in B cells are discussed in Chapter Three). Lastly, conditional deletion of ERK1/2 within the germinal center prevented terminal differentiation of GC B cells into plasma cells through a mechanism that involved loss of Elk1-dependent induction of the master regulator of plasma cell differentiation, BLIMP1 (96). Therefore activation of this pathway is absolutely critical for B cell development and optimal responses to antigen.

In Chapter Three of this thesis I describe a mechanism by which BCR-induced ERK activation is regulated at the level of the second messenger DAG, and furthermore provide evidence that implicates ERK signaling as a key regulator of the extrafollicular

plasmablast response that provides the initial wave of protective antibody during infection.

Feedback inhibition of BCR signaling

All of the signaling events described above function as positive regulators of mature B cell responses through their ability to promote cell growth, survival, proliferation, and differentiation in response to cognate antigen stimulation. However, B cell activation through these pathways must be tightly regulated to ensure that cells maintain vigorous responses to invading pathogens while also remaining unresponsive to our own tissues. Furthermore, unchecked signaling through many of these pathways is known to be a cardinal feature of numerous B cell lymphoma subtypes (98). To avoid these unwanted consequences of deregulated signaling, B cells are equipped with a number feedback inhibitory pathways that ensure proper regulation of BCR signaling in response to cognate antigen, and also allow for the cell to interpret the quantity and quality of the signaling input based on the strength of intracellular signaling events induced by ligand binding. This is both important to create a threshold for B cell activation, which ensures that only cells with higher affinities for antigen contribute to the antibody response, and also to limit the response of self-reactive cells that can potentially contribute to autoimmune disease.

One of the best characterized feedback inhibitory pathways downstream of the BCR involves the Src kinase Lyn, which in addition to phosphorylating ITAMs on Igα and Igβ chains to initiate BCR signaling, also phosphorylates inhibitory tyrosine residues within

immunoreceptor-based tyrosine inhibitory motifs (ITIMs) present in the cytoplasmic tail of a number of cell surface receptors including CD22 and the inhibitory Fc receptor, FcγRIIB (99, 100). This phosphorylation event facilitates the recruitment of phosphatases such as SHP1 to the membrane that can broadly inhibit BCR signaling by dephosphorylating a number of proximal signaling mediators such as Syk and BLNK (101, 102). Therefore, B cells require strong enough stimulation in the form of high affinity or high avidity antigens to overcome the attenuation provided by this inhibitory pathway and allow for subsequent cellular activation. In addition, this inhibitory pathway is critical for limiting activation of autoreactive B cell in the periphery as evidenced by the fact that mice deficient in different components of this pathway such as Lyn, CD22, or SHP1 have severe defects in B cell tolerance and develop antinuclear antibodies as well as a number of features of SLE (103-105). Furthermore, a subset of mutations in patients with diffuse large B cell lymphoma were shown to indirectly attenuate Lyn kinase activity which promoted constitutive BCR signaling and survival of these malignant cells (106).

Because the Lyn-CD22-SHP1 feedback inhibitory pathway targets proximal BCR signaling, it results in broad inhibition of all signaling events downstream of the BCR. However there are also a number of examples of pathway-specific feedback inhibition that occur in response to BCR stimulation. Signaling through the PI3K pathway is limited by the actions of the lipid phosphatases PTEN and SHIP, both of which function to dephosphorylate membrane PIP_3 to prevent membrane recruitment and activation of AKT (107, 108). B cell specific deficiency in PTEN and SHIP causes a lethal

lymphoproliferative disease that resembles features of B cell lymphoma (56). Moreover, B cells deficient in either PTEN or SHIP are resistant to anergy induction in transgenic mouse models, pointing to a critical role for limiting this signaling pathway in maintinance of B cell tolerance (53, 55). Interestingly, in Chapter Two of this thesis I describe a mechanism whereby signaling through the PI3K pathway can be sustained by the production of reactive oxygen species, which have the potential to inhibit PTEN phosphatase activity (109-111). Sustained AKT signaling is likely required for cell cycle entry and survival of antigen stimulated B cells.

Similar pathway-specific feedback inhibition for NFκB signaling has been described. As mentioned previously, NFκB is normally sequestered in the cytoplasm by the inhibitory protein IκBα, which is degraded in response to BCR stimulation allowing for NFκB to translocate to the nucleus and activate target genes (76). As a mechanism to terminate this signaling pathway, activated NFκB induces de novo synthesis of IκBα, which can then bind NFκB and retain it in the cytoplasm (112). Interestingly a subset of patients with Hodgkin's lymphoma were shown to have mutations in IκBα that presumably enhanced NFκB signaling (113). It will be interesting to determine if deficiency in IκBα also leads to defects in B cell tolerance mechanisms. In addition, the ubiquitin editing enzyme A20 (*tnfaip3*) is also an NFκB induced gene which is involved in ubiquitin mediated regulation of a number of positive regulators of NFkB signaling such as MALT1 (114, 115). Mice with B cell specific deletion of A20 develop a lupus like autoimmune disease that is mediated by hyperresponsive NFκB signaling (116). A20 has also been identified as a susceptibility locus for SLE and B cell lymphomas in humans

(114). These observations strongly imply that tight regulation of NFκB signaling by feedback inhibition is critical for maintenance of B cell tolerance and likely protective against development of B cell lymphoma.

In the case of the ERK signaling pathway, a number of potential feedback inhibitory mechanisms exist. One potential mechanism by which this pathway can be selectively targeted is through the action of dual specificity phosphatases (DUSPs), which are known to directly dephorphorylate ERK1 and ERK2 in other cell types (117). Currently it is not known if ERK signaling is limited by this mechanism in B lymphocytes, however B cells do express several isoforms of these phosphatases (http://www.immgen.org) (118), and it will be interesting to determine if they are important for controlling the magnitude of MAP kinase signaling in these cells. These phosphatases have been implicated as negative regulators of basal and TCR-induced ERK activation in T cells, so it is conceivable that BCR signaling is regulated in a similar fashion (119).

In Chapter Three of this thesis I have identified a novel mechanism through which the magnitude of Ras-ERK signaling can be controlled at the level of the second messenger DAG by the enzyme diacylglycerol kinase ζ (DGKζ). This enzyme phosphorylates DAG, converting it to phosphatidic acid (PA), thus limiting signaling through DAG-dependent pathways downstream of the BCR. In the absence of DGKζ, B cells have a reduced threshold for BCR-induced ERK activation and mount more robust responses to antigen in vitro and in vivo. Furthermore, I found that this enzyme is upregulated as B cells mature to the follicular stage in the spleen, and this appears to be important for

promoting efficient affinity discrimination in response to antigen immunization. I hypothesize that this inhibitory function of DGKζ may be critical to ensure that only cells with high affinity for antigen contribute to the antibody response. It will also be interesting to determine if upregulation of DGKζ in mature B cells plays a role in maintaining peripheral B cell tolerance or protects against lymphoma development.

The antibody response

Given that the goal of B cell activation through the pathways described above is ultimately the production of pathogen-specific antibodies, this section will briefly go through the different stages of the antibody response, from initial encounter with cognate antigen to terminal differentiation into antibody-secreting plasma cells.

Antibody responses are divided into two classes based on whether or not the response is dependent on help from cognate T cells, and furthermore differ in the magnitude, duration, isotype, as well as the quality, or affinity, of the antibody produced. T cell-independent antibody responses are classified as type 1 (TI-1) or type 2 (TI-2), based on the physical properties of the immunogen used to elicit the response (see below), and have unique characteristics that bypass the need for T cell help to initiate plasma cell differentiation and antibody secretion. Antigens that are coupled to mitogenic factors such as TLR ligands, like bacterial cell wall derived lipopolysacharride (LPS), induce TI-1 antibody responses, whereas antigens that have to ability to strongly crosslink the BCRs on cognate B cells, such as polysaccharide-based antigens, induce TI-2 responses (120, 121). The primary difference between these two types of responses is that the latter

depends on the ability of the antigen to induce robust signaling downstream of the BCR, whereas the former does not. The dependence of TI-2 responses on robust BCR signaling is evidenced by the observation that this response is completely abolished when BCR signaling is compromised, as in the case with deficiency in the Tec kinase BTK (49). Both TI-1 and TI-2 responses are generally thought to give rise to short-lived plasma cells that secrete unmutated, low-affinity antibodies of the IgM and IgG3 isotypes predominantly, and furthermore are not thought to give rise to memory B cell populations, although the latter characteristic is somewhat debated (122, 123). However, these types of responses, particularly with regard to the TI-2 subtype, are highly relevant given that polysaccharide-based vaccines have been used for years to protect against a number of bacteria infections such as *Streptococcus pneumoniae*. More recently many of these polysaccharide-based vaccines have been conjugated to protein carriers in order to promote T-cell dependent antibody responses, which are discussed below, and provide higher quality and longer lasting humoral immunity.

Unlike the T cell-independent antibody responses, the efficiency of T cell-dependent antibody responses requires coordination between a number of cell types following infection or immunization. For this type of response to be elicited, antigens must be protein-based so that they can be processed and presented as peptides in the context of MHC class II to cognate T cells by antigen presenting cells of the immune system. Cognate T cell activation initially occurs through interactions with antigen presenting dendritic cells, whereas the initial activation of cognate B cells occurs following binding antigen in its native form via the BCR. This initial binding of antigen not only promotes

early activation of B cells through the signaling processes described in the previous sections, but also allows for antigen uptake and processing, and as well as re-localization of the activated B cells to the border between the B cell follicle and T cell zone, where they are able to present antigen to previously activated cognate $CD4^+$ helper T cells, and receive "help" in the form of co-stimulation via CD40-CD40 ligand interactions and cytokines such as IL-4 and IL-21 produced by these cells (124). In situations where these cognate T-B cell interactions are disrupted, such as deficiency in CD40, or deficiency in cell-cell adhesion signaling molecules like SAP, T cell-dependent humoral immunity is severely compromised (125).

The initial T-B cell interaction takes place between days 1 and 3 following immunization, during which both B and T cell clonal expansion occurs. Following this initial activation and expansion phase, cognate B cells will either move to extrafollicular regions of the spleen or lymph node and differentiate directly into short-lived plasma cells (the "extrafollicular response"), or move to the center of the follicle and form a highly specialized structure known as the germinal center (GC). The series of cell movements that occurs during the early phases of the T-dependent response is mediated by a number of directional cues provided by chemokines, and will not be discussed further here, however this has been elegantly described in recent reviews if the reader is interested (21, 124, 126). Although the extrafollicular response and germinal center response are distinct, they are not mutually exclusive, and each provides a critical arm of T-dependent humoral immunity. The extrafollicular plasma cell response occurs rapidly, within the first 5 days of infection and/or immunization, and is the primary source of the early wave

of antibody needed to provide initial protection against pathogens. However, similar to T-independent responses, this response is short-lived, and the antibodies produced are typically of moderate to low affinity and show somewhat limited class switching (127). In contrast, the germinal center response is a much slower process, but gives rise to memory B cells and long-lived plasma cells that secrete high affinity antibody to provide, in some cases, life-long protection against re-infection (128). For this reason, current vaccines are designed to maximize the latter type of response. Although the mechanisms that lead to the generation of memory B cells from the germinal center reaction are not entirely clear, much more is known about the processes leading to selection of high-affinity clones from this response. Within the germinal center, cognate B cells undergo multiple rounds of proliferation, and are constantly competing for a limited supply of antigen displayed on the surface of follicular dendritic cells (FDC) (129). In this way, only the cells with the highest affinity BCR's will be selected, and this is mediated largely by the ability of higher affinity GC B cells to acquire and present antigen more efficiently to T follicular helper cells (T_{FH}), which are also limited in numbers within the germinal center (130). The other unique feature of the germinal center reaction that leads to the production of high-affinity antibody is the process of somatic hypermutation, in which the cells turn on an enzyme (Activation-induced cytidine deaminase or AID) that actively mutates DNA encoding the variable regions of the IgH and IgL chains to give rise to variants, some of which have increased affinity for the antigen (128, 131). Of course this process will also give rise to variants with reduced-affinity or altered specificity, however these cells will be rapidly outcompeted by higher affinity GC B cells, thus representing a unique form of Darwinian selection at the cellular level. The

cells that arise from this reaction differentiate into plasma cells that home to specialized niches in the bone marrow where they can potentially reside and secrete protective antibody for the lifetime of an individual (131). Furthermore, as mentioned above, the germinal center is also the primary source of memory B cells, which initiate a more rapid and robust response upon secondary infection (132). However, due to the slow nature of this response, antibodies derived from the germinal center reaction may be less protective during the early phases of a primary infection, and are therefore primarily effective in providing long-term protection against re-infection. In this way, the division of labor between the rapid extrafollicular plasma cell response, which provides early protection, and the germinal center response, which provides memory and long-lasting protection, is absolutely critical for productive humoral immunity.

In Chapter Three of this thesis I have investigated how the magnitude of signaling by the second messenger DAG influences these two components if the T cell-dependent antibody response, as well as it’s role in T cell independent type-2 antibody responses.

Chapter 2

Prolonged production of reactive oxygen species in response to BCR stimulation promotes B cell activation and proliferation.

Introduction

Engagement of the B cell antigen receptor initiates a series of tightly controlled signaling events that in self-reactive B cells promote tolerance, and in B cells responding to foreign antigen promotes activation (57). Signaling downstream of the BCR is initiated by the Src family protein tyrosine kinases Lyn, Fyn, and Blk, which phosphorylate targets to induce activation of a number of signaling pathways required for subsequent B cell responses (31, 32). Among the targets of these kinases are the BCR Ig-α and Ig-β subunits, and their phosphorylation recruits Syk, representing an amplification loop that is countered by several feedback inhibitory pathways (31, 102). An especially well characterized feedback inhibitory pathway involves activation and recruitment, in proximity to the BCR, of the protein tyrosine phosphatase (PTPase) SHP-1, which broadly inhibits signaling by dephosphorylating proximal BCR signaling intermediates such as Syk (100, 101). Feedback inhibition of BCR signaling can also target specific pathways downstream of the BCR, as is the case with the lipid phosphatases PTEN and SHIP, which negatively regulate signaling through the phosphatidylinositol 3-kinase (PI3K) pathway (133). The balance between the activities of BCR-stimulated protein kinases and the opposing phosphatases is likely critical for controlling the ultimate outcome of antigen encounter by the B cell.

Reactive oxygen species (ROS) have long been known to be potent inhibitors of PTPases and the lipid phosphatase PTEN, through their ability to oxidize catalytic-site cysteines in these enzymes to render them transiently inactive (109, 111, 134, 135). While NADPH oxidase-derived ROS production by phagocytic cells is well appreciated for its role in

anti-microbial defense, non-phagocytic cells, including lymphocytes, are also known to express functional machinery of the NADPH oxidase complex, and can generate ROS in response to activating signals such as antigen receptor engagement (136-138). Activated cells also generate ROS as a by-product of normal mitochondrial respiration (139). Thus, it has been hypothesized that low-level production of ROS serves as a mechanism to inhibit phosphatase activity and thereby promote BCR signal amplification (140). Interesting in this regard, growth factor receptor signaling, which has many parallels to antigen receptor signaling, has been shown to be amplified by low-level ROS generation (141, 142).

Several studies have investigated whether low-level production of ROS downstream of the BCR or TCR can influence the magnitude of signaling or cell activation in response to cognate antigen stimulation (136, 143-146), however the results have been inconclusive, and were focused mainly on the signaling function of ROS generated early after antigen receptor engagement. In this study, we sought to examine the role of ROS production in regulating BCR signaling both at early times upon initial stimulation, and later during the activation of mature B cells. We found that ROS production in response to BCR stimulation arises from two distinct sources; one that is dependent on the Nox2 isoform of NADPH oxidase and occurs within minutes of BCR crosslinking, and a second that occurs at later times following BCR stimulation and is likely derived from mitochondrial sources. Importantly, we found that B cells deficient in early Nox2-dependent ROS production had no major defects in proximal BCR signaling or B cell activation, and mounted normal or enhanced antibody responses to a T cell-dependent

antigen. In contrast, neutralizing ROS produced at later times attenuated BCR-dependent sustained PI3K signaling, and ultimately resulted in defective activation and proliferation in response to BCR stimulation. These results show that while early NADPH oxidase-derived ROS is dispensable for BCR signal amplification in naïve resting B cells, the continuous production of ROS at later times during the activation process is critical for maintaining some components of BCR signal transduction, and for optimal antigen-induced B cell activation and proliferation.

Results

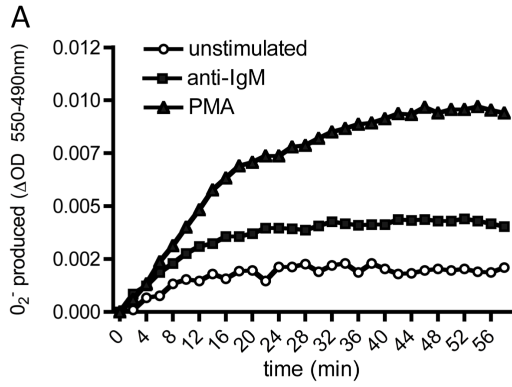
BCR stimulation of primary mouse B cells induces the rapid production of reactive oxygen species.

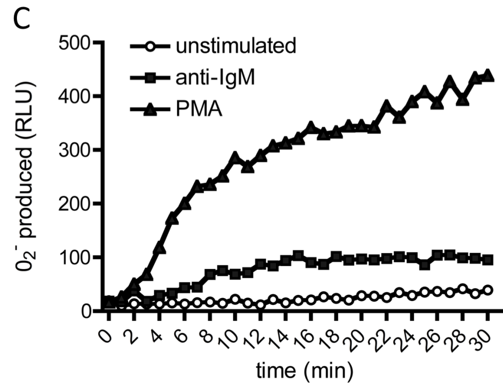
Non-phagocytic cells are known generate low levels of ROS in response to various stimuli such as growth factors or antigen receptor engagement (147). Using several assays that detect superoxide (O_2^-) and/or hydrogen peroxide (H_2O_2), we observed that BCR stimulation of primary splenic B cells induced a measurable oxidative burst in response to BCR stimulation. For example anti-IgM F(ab')$_2$ stimulation induced the extracellular production of O_2^- which could be observed as early as 6 min and was maintained for at least 1 h following BCR stimulation, as measured by reduction of cytochrome C added to the outside of cells (Fig. 1A). In addition, stimulation of B cells with the diacylglycerol (DAG) analogue PMA resulted in robust superoxide production, suggesting that BCR-induced ROS production by B cells is dependent on a DAG-stimulated signaling pathway downstream of the BCR (Fig. 1A). Similarly, stimulation of MD4 Ig-transgenic B cells, which express a transgenic BCR specific for hen egg lysozyme (HEL), with soluble HEL induced robust production of O_2^-, while non-transgenic B cells stimulated with HEL showed no detectible response (Fig. 1B). Highly reproducible, albeit low level, production of O_2^- in response to anti-IgM was also detected using a chemiluminescent oxidation sensitive probe, in agreement with a previous report (145). The PMA induced O_2^- burst detected by this assay was similar to what was observed by cytochrome C reduction (Fig. 1C). Finally, as a measure of BCR-localized ROS production, we chemically conjugated anti-IgM F(ab')$_2$ antibodies to the H_2O_2 detection probe, H_2-DCFDA, and used this probe to stimulate splenic B cells as was

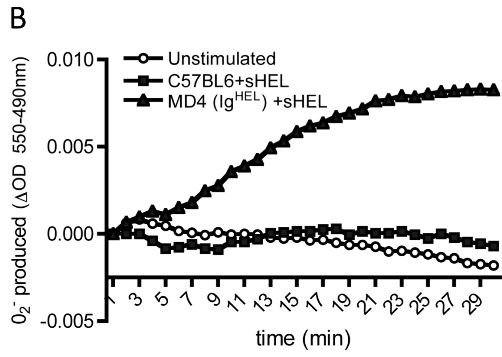
recently reported (143). This method allows for flow cytometric measurement of local ROS production that is adjacent to the BCR. Superoxide generated extracellularly can be rapidly converted to H_2O_2, which is capable of diffusing across the plasma membrane into the cytoplasm (16) where it could act on cytoplasmic targets. In addition, this method can be used to demonstrate that ROS production is from B cells and not from contaminating phagocytic cells by co-staining cells for surface markers to exclude non-B cell populations from the analysis. Using this reagent we observed robust production of ROS specifically from mature splenic B cells as early as 5 min after BCR stimulation, whereas non-B cells did not show detectible fluorescence of this probe (Fig. 1D). Thus, ROS were produced in proximity to the BCR in a rapid and sustained fashion in response to BCR crosslinking.

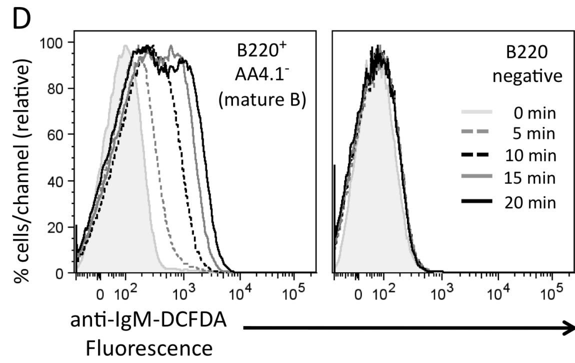
Figure 1. BCR stimulation of primary mouse B cells induces the rapid production of reactive oxygen species.

Purified mouse primary B cells (A-C) or total spleen cells (D), isolated from wild-type (A and C) or MD4/Ig^{HEL} (B) spleens, were stimulated for the indicated times with 0.5 μg/ml PMA, 25 μg/ml anti-IgM F(ab')$_2$, 1 μg/ml soluble HEL, or 20 μg/ml anti-IgM-DCFDA. **A)** O_2^- production was measured by cytochrome C reduction from WT B cells which were unstimulated (open circles), or stimulated with PMA (filled triangles) or with anti-IgM (filled squares). Relative O_2^- levels are shown as ΔOD 550nm-490nm at 2 min intervals over 1 h. **B)** O_2^- production by WT non-transgenic (filled squares) and WT/MD4 Ig^{HEL} B cells (filled triangles) stimulated with soluble HEL (solid symbols) or left unstimulated (open circles) was measured by cytochrome C reduction as in A. **C)** O_2^- production by WT B cells was detected using the chemiluminescent reagent Diogenes, and is shown as relative light units (RLU) following stimulation with PMA (triangles) or anti-IgM (squares) **D)** H_2O_2 production in the vicinity of the BCR was measured at the indicated time points by flow cytometry after activation with anti-IgM conjugated to DCFDA. H_2O_2 production was measured as fluorescence in the FITC channel at each time point, and comparing levels in control $B220^-$ (non-B cells) and $B220^+CD93^-$ mature B cells. All data are representative of at least 2-3 independent experiments.


A
unstimulated
anti-IgM
PMA
0.012
0.010
0.007
0.005
0.002
0.000
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56
time (min)


C
unstimulated
anti-IgM
PMA
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
time (min)


B
Unstimulated
C57BL6+sHEL
0.010
0.005
0.000
-0.005
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
time (min)


D
% cells/channel (relative)
B220 negative
0 min
5 min
10 min
15 min
20 min
anti-IgM-DCFDA
Fluorescence

Early BCR-induced ROS production is dependent on B cell expression of the Nox2-containing NADPH oxidase.

While phagocytic cells such as neutrophils are known to generate ROS utilizing the classical Nox2 isoform of NADPH oxidase, a number of additional isoforms of this enzyme have been identified and have been shown to have unique functions in a wide variety of tissues (137). Both the Nox2-containing NADPH oxidase as well as a calcium-regulated isoform, Duox1, have been suggested to be important for lymphocyte ROS production (136, 138, 144, 145). To investigate the source of BCR-induced ROS generation in primary murine B cells, we first assessed the expression of all known Nox/Duox catalytic subunit isoforms by semi-quantitative RT-PCR. Expression of each isoform was compared to a positive control tissue to verify specificity of the PCR products. Messenger RNA encoding the Nox2 "phagocyte oxidase" catalytic subunit was readily detected in B cells, whereas expression of other NADPH oxidase isoforms was very low or undetectable (Fig. 2A). The low level amplification of Nox3 was also observed in control RNA samples that were not reverse transcribed, likely resulting from genomic DNA contamination (data not shown). These data are in agreement with results of transcript microarray data from the Immunological Genome Project (http://www.immgen.org).

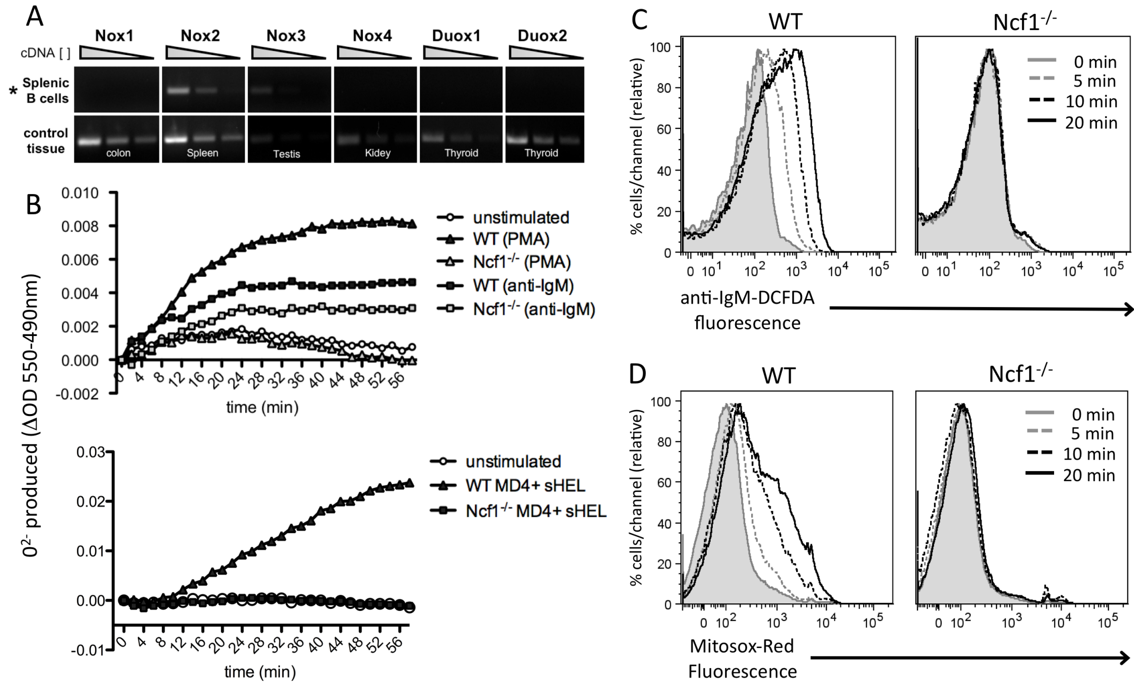
We next asked whether the Nox2 phagocyte oxidase complex is responsible for BCR-mediated ROS production by using mice that harbor a loss-of-function mutation in *Ncf1,* which encodes the essential $p47^{phox}$ subunit of the Nox2-containing NADPH oxidase complex (148). Whereas $p47^{phox}$ is required for Nox2 activity, it is dispensable for other

Nox/Duox isoforms (137). B cells from Ncf1-deficient mice showed no detectible O_2^- production in response to PMA stimulation, and a substantially reduced response to anti-IgM, as measured by cytochrome C reduction (Fig. 2B). In addition, B cells from Ncf1-deficient mice crossed to the MD4 Ig^{HEL} transgene failed to generate O_2^- upon stimulation with soluble HEL, further indicating that activation of Nox2 downstream of BCR stimulation was mainly responsible for acute ROS production by B cells (Fig 2B). These results are in agreement with a previous study (145) and our own independent observations (data not shown), showing that BCR-mediated ROS, detected by a chemiluminescent probe, were absent in mice genetically deficient in Nox2 ($gp91^{phox}$). In addition, Ncf1-deficient B cells completely lacked early (5-20 min) production of BCR-localized ROS, as measured with anti-IgM-DCFDA, as well as intracellular production and/or accumulation of ROS following BCR stimulation as measured with the intracellular ROS detection probe Mitosox-Red (Fig. 2C and 2D). While the latter reagent is generally used to detect mitochondrial ROS, in our hands this probe readily detected the early wave of ROS produced after BCR stimulation, which was highly dependent on the Nox2 NADPH oxidase (Fig. 2D).

Taken together, these results show that BCR stimulation induces rapid local production and intracellular accumulation of ROS that is highly dependent on B cell expression and activation of the Nox2 isoform of NADPH oxidase. Therefore, Ncf1-deficient B cells serve as an appropriate model for investigating the possible role for early B cell ROS production in regulating proximal BCR dependent signaling events.

Figure 2. Early BCR-induced ROS production is dependent on B cell expression of the Nox2-containing NADPH oxidase.

A.) Expression of NADPH oxidase catalytic subunit isoforms in purified splenic B cells was measured by semi-quantitative RT-PCR using primers specific for Nox1-4 and Duox1-2. Shown are relative amounts of each transcript compared to a positive control tissue for each isoform. Wedges indicate 5-fold serial dilutions of cDNA (starting with 50 ng cDNA). **B.)** (Top panel) O_2^- was measured as in Fig 1A by cytochrome C reduction from B cells isolated from spleens of WT (filled symbols) and Ncf1$^{-/-}$ (open symbols) mice stimulated with PMA (triangles) or anti-IgM (squares) for the indicated times. (Bottom panel) O_2^- was measured by cytochrome C reduction from B cells purified from WT/MD4 (filled triangles) or Ncf1$^{-/-}$/MD4 (filled squares) spleens and stimulated with soluble HEL for the indicated times. Basal ROS levels (open circles; top and bottom panel) were measured from unstimulated WT non-transgenic (top), or WT/MD4 (bottom) B cells. **C)** H_2O_2 production within the vicinity of the BCR was measured by flow cytometry as in Fig 1C from WT and Ncf1$^{-/-}$ B cells stimulated with 20 μg/ml anti-IgM-DCFDA for the indicated times. Shown are relative H_2O_2 levels from mature B cells (B220$^+$CD93$^-$), represented as a histogram for anti-IgM-DCFDA fluorescence. **D)** Intracellular O_2^- production by WT and Ncf1$^{-/-}$ B cells was measured by flow cytometry from cells loaded with the O_2^- indicator dye Mitosox-Red and stimulated for the indicated times with 20 μg/ml anti-IgM. O_2^- production by mature splenic B cells (B220$^+$CD93$^-$) was measured as fluorescence in the PE channel for the indicated time points.

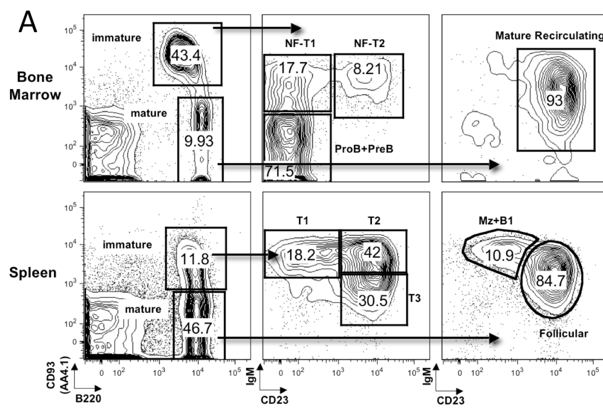

A
Nox1
Nox2
Nox3
Nox4
Duox1
Duox2
cDNA [ ]
* Splenic B cells
control tissue
colon
Spleen
Testis
Kidey
Thyroid
Thyroid
B
unstimulated
WT (PMA)
Ncf1-/- (PMA)
WT (anti-IgM)
Ncf1-/- (anti-IgM)
time (min)
02- produced (ΔOD 550-490nm)
WT MD4+ sHEL
Ncf1-/- MD4+ sHEL
C
WT
Ncf1-/-
0 min
5 min
10 min
20 min
% cells/channel (relative)
anti-IgM-DCFDA fluorescence
D
Mitosox-Red Fluorescence

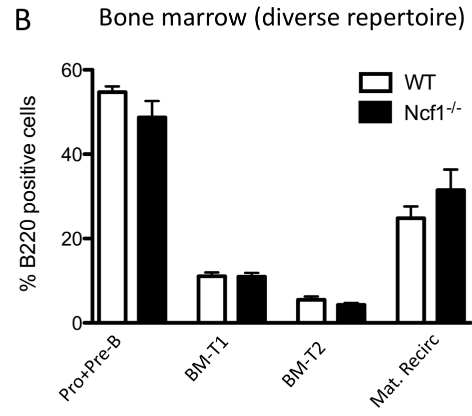
Ncf1-deficient mice have relatively normal B cell development and maturation. Because mutations that affect BCR signaling may influence B cell development and maturation, we looked at frequencies of mature and immature B cell sub-populations from Ncf1-deficient mice. Ncf1-deficient mice showed nearly normal ratios of pro- and pre-B cells, newly formed B cells, and mature re-circulating B cells in the bone marrow (Fig. 3B). Ncf1-deficiency did however result in a small but significant increase in the frequency of mature B cells, and concomitant decrease in immature B cell populations in the spleen (Fig. 3C). We also looked at the frequencies of splenic B cell sub-populations in MD4-transgenic Ncf1-deficient mice, as subtle alterations in B cell development can sometimes be masked by changes in the repertoire of expressed BCR's. Fixing the BCR led to small changes in peripheral B cell frequencies, namely a small but significant increase in the percentage of immature T1 cells, as well as a decreased combined frequency of splenic marginal zone and B1 B cell populations (Fig. 3D). Although there were small changes in B cell subpopulation frequencies, deficiency in Nox2 NADPH oxidase activity had only modest effects on B cell development and maturation.

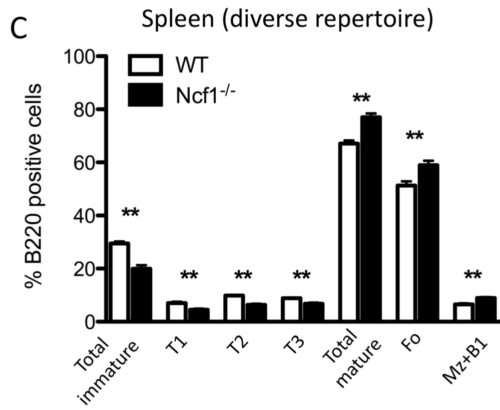
Figure 3. Ncf1-deficient mice have relatively normal B cell development and maturation.

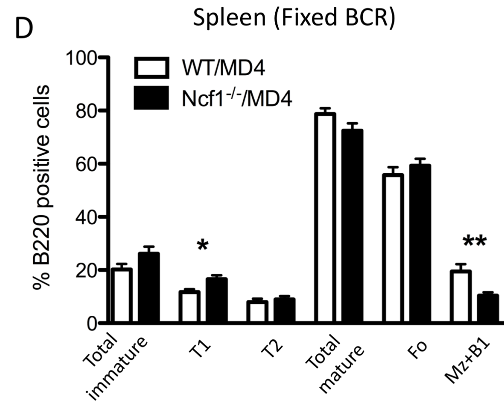
A) Representative flow cytometry plots showing gating strategy for bone marrow and spleen B cell sub-populations. Mature and immature B cells in the spleen and BM are distinguished by surface CD93, while follicular, T1, T2, T3, and Mz+B1 (spleen), or mature re-circulating, pro-pre-B, newly formed-T1, and newly formed-T2 (bone marrow) were distinguished by surface CD23 and IgM levels as indicated in representative plots.

B-D) Frequencies of bone marrow (B) and spleen (C and D) B cell sub-populations are shown as percentages of $B220^+$ cells from WT and $Ncf1^{-/-}$ mice with a diverse (B and C) or fixed (MD4) BCR repertoire (D). Data represent n = 6-12 mice per group and similar results were found in 2-3 independent experiments. (* $p < 0.05$, ** $p < 0.01$ Student's T test)


A
Bone Marrow
immature
43.4
mature
9.93
NF-T1
NF-T2
17.7
8.21
71.5
ProB+PreB
Mature Recirculating
93
Spleen
immature
11.8
mature
46.7
T1
T2
18.2
42
30.5
T3
Mz+B1
10.9
84.7
Follicular
CD93 (AA4.1)
B220
IgM
CD23


B
Bone marrow (diverse repertoire)
% B220 positive cells
WT
Ncf1-/-
Pro+Pre-B
BM-T1
BM-T2
Mat. Recirc


C
Spleen (diverse repertoire)
% B220 positive cells
WT
Ncf1-/-
Total immature
T1
T2
T3
Total mature
Fo
Mz+B1


D
Spleen (Fixed BCR)
% B220 positive cells
WT/MD4
Ncf1-/-/MD4
Total immature
T1
T2
Total mature
Fo
Mz+B1

Deficiency in Nox2 NADPH oxidase-dependent early ROS production does not alter proximal BCR signaling or downstream B cell activation and proliferation.

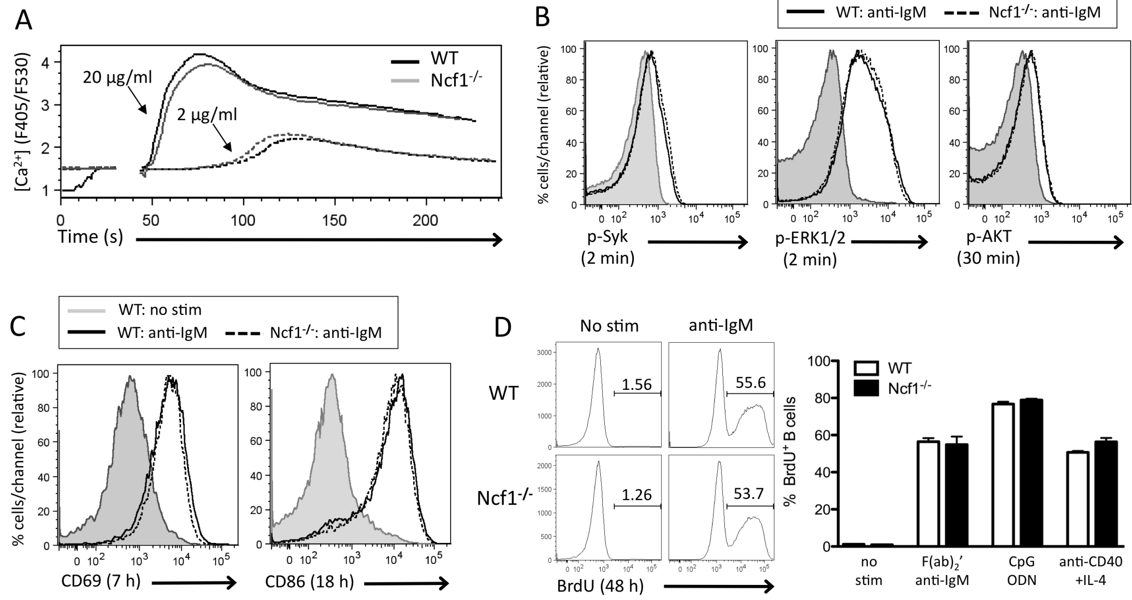
ROS molecules such as H_2O_2 have the ability to transiently inhibit phosphatase activity in the cytoplasm through oxidation of catalytic-site cysteine residues within these enzymes (135). Therefore it has been proposed that production of ROS downstream of the BCR may function as a positive feedback signal, by preventing phosphatases such as SHP-1 from inhibiting downstream BCR signaling (140). Since early BCR-induced ROS production was dependent on the Nox2 NADPH oxidase, we compared the magnitude of a variety of signaling reactions in B cells from WT and Ncf1-deficient mice. Ncf1-deficient B cells were similar to WT cells in their ability to flux calcium in response to BCR stimulation with anti-IgM (Fig. 4A). Furthermore, at several time points tested after anti-IgM stimulation (2-30 min), WT and Ncf1-deficient B cells had indistinguishable activation of proximal and downstream BCR signaling pathways, as measured by phosphorylation of the proximal tyrosine kinase Syk, as well as downstream signaling intermediates ERK and AKT (Fig. 4B and data not shown). Syk is thought to be a primary substrate for SHP-1 mediated dephosphorylation (101), so normal activation of this kinase in Ncf1-deficient B cells argues against a role for NADPH oxidase-derived ROS in inhibiting SHP-1 activity downstream of the BCR. Ncf1-deficient B cells were also indistinguishable from WT cells in activation-induced upregulation of the early and late activation markers CD69 and CD86 (B7-2) (Fig. 4C), and they also proliferated normally *in vitro* in response to BCR stimulation with anti-IgM, or BCR-independent stimulation with CpG, or CD40 and IL-4 (Fig. 4D). These results show that while the

Nox2 NADPH oxidase complex is required for early BCR-induced ROS production, it is dispensable for BCR signal amplification and downstream activation and proliferation.

Figure 4. Deficiency in Nox2 NADPH oxidase-dependent early ROS production does not alter proximal BCR signaling or downstream B cell activation and proliferation.

A) Cytoplasmic free calcium was measured by indo-1 fluorescence emission ratio at 405 and 530 nm in WT (black lines) and Ncf1$^{-/-}$ (grey lines) follicular B cells (B220^{+}, CD93^{-}, CD23^{+}, IgM^{int-lo}) stimulated with 2 μg/ml (dashed) or 20 μg/ml (solid) anti-IgM. Data are representative of n= 3 mice each from 3 independent experiments. **B)** BCR-induced phosphorylation of proximal (Syk) and downstream (ERK and AKT) targets was measured by flow cytometry in WT (solid black) and Ncf1$^{-/-}$ (dashed black) follicular B cells (B220^{+}, CD24^{-}, CD23^{+}, IgM^{int-lo}) stimulated with 25 μg/ml anti-IgM, and stained intracellulary with phospho-specific antibodies against activating phosphorylation sites of Syk, ERK1/2, and AKT. Basal phosphorylation status is represented by intracellularly stained unstimulated WT B cells (filled grey). Data are representative of n = 2-3 mice each from 3 independent experiments **C)** Upregulation of activation markers CD69 and CD86 was measured by flow cytometry from WT (solid black) and Ncf1$^{-/-}$ (black dashed) purified splenic B cells stimulated for 6 h (CD69) or 18 h (CD86) with 10 μg/ml anti-IgM. Data are representative of n = 2-3 mice in 3 independent experiments. **D)** Proliferation of WT and Ncf1$^{-/-}$ B cells was measured by BrdU incorperation *in vitro* 48 h after stimulation with anti-IgM (10 μg/ml), CpG ODN (500 ng/ml) or anti-CD40 + IL-4 (10 μg/ml and 10 ng/ml). Proliferation was measured by intracellular staining with anti-BrdU and flow cytometry, following an 18 h pulse with BrdU. Left panel shows representative histograms of BrdU^{+} WT (top) and Ncf1$^{-/-}$ (bottom) unstimulated, and anti-IgM stimulated B cells. Data are quantified in right panel as the percentage of WT (white

bars) and Ncf1$^{-/-}$ (black bars) that have incorperated BrdU. Data are representative of n = 3 mice/group from 2 independent experiments.


A
B
C
D
20 µg/ml
2 µg/ml
WT
Ncf1-/-
[Ca2+] (F405/F530)
Time (s)
WT: no stim
WT: anti-IgM
Ncf1-/-: anti-IgM
% cells/channel (relative)
p-Syk (2 min)
p-ERK1/2 (2 min)
p-AKT (30 min)
CD69 (7 h)
CD86 (18 h)
No stim
anti-IgM
1.56
55.6
1.26
53.7
BrdU (48 h)
% BrdU+ B cells
no stim
F(ab)2' anti-IgM
CpG ODN
anti-CD40 +IL-4

Deficiency in Nox2 NADPH oxidase-dependent ROS production does not alter T cell-dependent germinal center formation or B cell antigen presentation, but enhances T cell-dependent antibody production.

While we did not observe any major differences in BCR signaling or activation of naïve resting B cells *in vitro*, it is possible that subtle, or activation-dependent differences in signaling may become more apparent in an *in vivo* setting such as during an antibody response. Surprisingly, Ncf1-deficient mice immunized with the T cell-dependent antigen NP-CGG, produced somewhat elevated antigen-specific serum IgM and IgG1 titers (Fig. 5A). This difference did not appear to be due to differences in germinal center formation, as Ncf1 deficient mice had comparable numbers of IgD^{lo}, Fas^{+}, $GL7^{+}$ GC phenotype B cells on days 7 and 14 after immunization (Fig. 5B). We also did not observe any differences in the numbers of NP-specific plasma cells measured by ELISPOT to detect anti-NP-IgG producing antibody secreting cells (ASCs) from spleens of day 14 immunized mice (Fig. 5C).

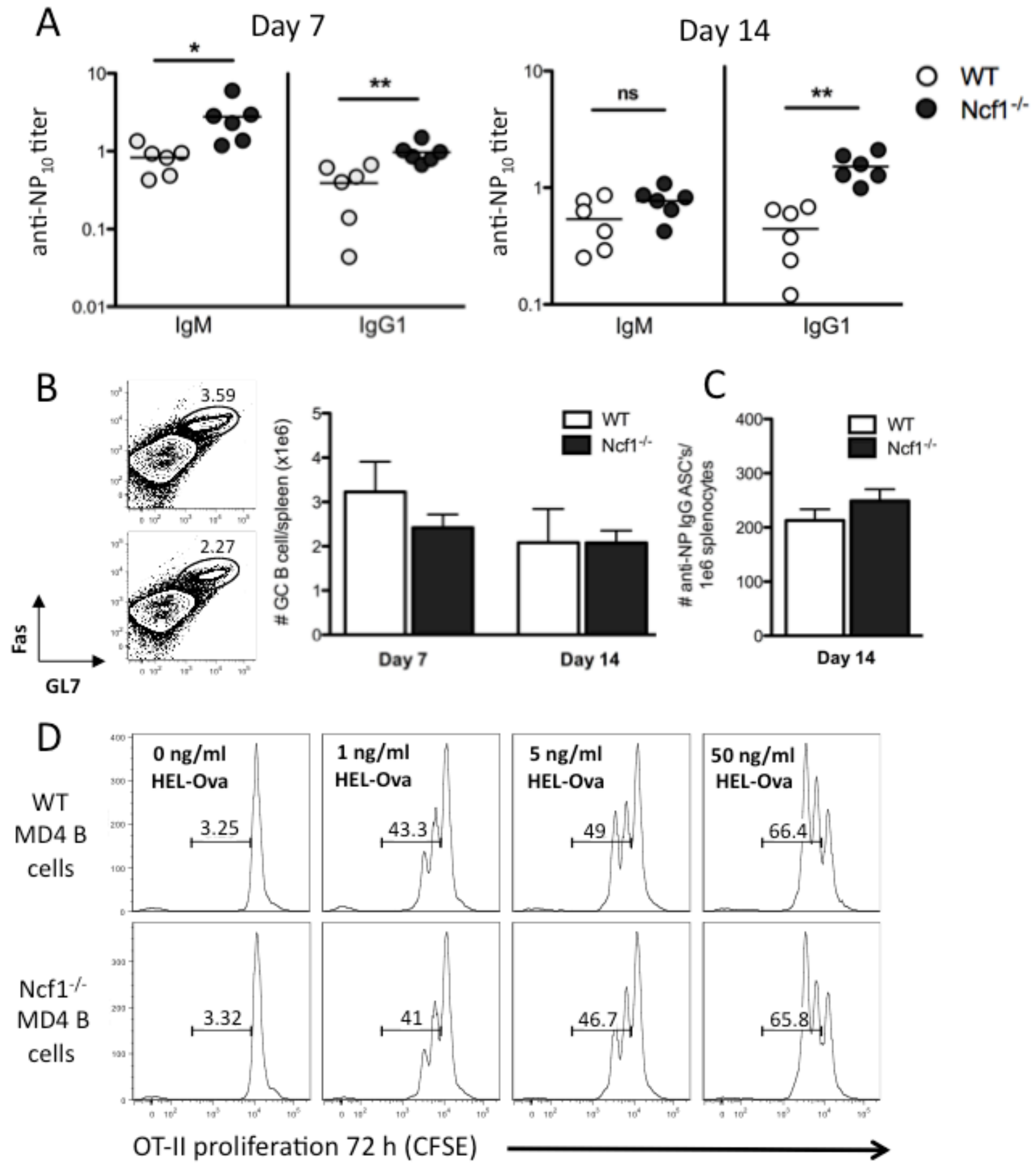
Previous studies have shown that Nox2 NADPH oxidase-dependent ROS generation is accompanied by ion and proton fluxes that promote antigen processing and presentation in dendritic cells (149-151). We were therefore curious whether Ncf1-deficient B cells had an altered ability to present antigen to cognate T cells. To test this possibility, Ncf1-deficient MD4 Ig-transgenic B cells were pulsed *in vitro* with various concentrations of a HEL-Ova conjugate and co-cultured with ovalbumin-specific OT-II TCR transgenic T cells to test their antigen presentation capability. Proliferation of OT-II T cells was measured by CFSE dilution as a readout of the cognate T cell response to antigen. Based

on this assay, the antigen presentation ability of MD4 transgenic B cells was indistinguishable regardless of whether they had functional Nox2 activity or not (Fig. 5D). Thus, the reason that Ncf1-deficient mice generated somewhat higher titers of antigen-specific antibody was not evident.

While we cannot rule out a B cell intrinsic effect on antibody production resulting from deficiency in Ncf1, these differences could be due to an absence of myeloid-derived ROS, which have been suggested to be immunosuppressive in some contexts (152, 153). In any case, these results indicate that early BCR-mediated production of Nox2-derived ROS does not enhance *in vivo* B cell responses to a T cell-dependent antigen.

Figure 5. Deficiency in Nox2 NADPH oxidase-dependent ROS production does not alter T cell-dependent germinal center formation or B cell antigen presentation, but enhances T cell-dependent antibody production.

A) NP-specific IgM and IgG1 serum antibody titers were measured by ELISA from day 7 and day 14 NP-CGG immunized WT (open circles) and Ncf1$^{-/-}$ (filled circles) mice. Each circle represents an individual mouse (n = 6) (* $p < 0.05$, ** $p < 0.01$). Similar results were found a second independent experiment. **B)** Germinal center formation in response to immunization with T cell-dependent antigen (50 μg NP-CGG in alum IP) in WT and Ncf1$^{-/-}$ mice. Total numbers of GC B cells (IgD^{-low}, GL7^{+}, FAS^{+}) per spleen are shown from day 7 and day 14 NP-CGG immunized WT (white bars) and Ncf1$^{-/-}$ (black bars) mice. **C)** Numbers of anti-NP IgG producing plasma cells per spleen from day 14 NP-CGG immunized WT (white bars) and Ncf1$^{-/-}$ (black bars) mice. **D)** Antigen presentation to cognate T cells by WT and Ncf1$^{-/-}$ B cells was measured by co-culturing CFSE-labeled OT-II T cells with WT/MD4 (top panel) or Ncf1$^{-/-}$/MD4 (bottom panel) B cells pulsed with increasing amounts of HEL-OVA. Presentation of Ova to cognate T cells was determined by measuring OT-II proliferation by CFSE dilution following 72 h of co-culture. Data are representative of n= 3 mice/group from 2 independent experiments.



A
Day 7
Day 14
anti-NP10 titer
IgM
IgG1
ns
WT
Ncf1-/-
B
3.59
2.27
Fas
GL7
GC B cell/spleen (x1e6)
Day 7
Day 14
C
anti-NP IgG ASC's/ 1e6 splenocytes
D
0 ng/ml HEL-Ova
1 ng/ml HEL-Ova
5 ng/ml HEL-Ova
50 ng/ml HEL-Ova
WT MD4 B cells
Ncf1-/- MD4 B cells
3.25
43.3
49
66.4
3.32
41
46.7
65.8
OT-II proliferation 72 h (CFSE)

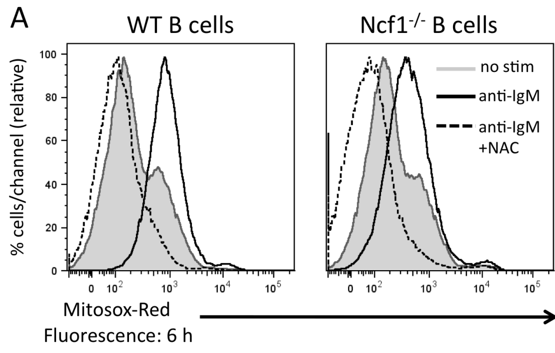
BCR stimulation for 6 h or more leads to sustained production of ROS independently of Nox2 NADPH oxidase.

Although our results, along with results of an independent study (145), indicate that early BCR-induced ROS production derived from the Nox2 NADPH oxidase does not substantially promote BCR signaling or B cell activation, it should be noted that there are other sources of ROS in cells, including the mitochondria which generate ROS as an intermediate during normal cellular respiration (139). While acute ROS production by BCR-stimulated resting B cells was dependent on the Nox2 NADPH oxidase complex, as described above, we found that after 6 h of anti-IgM stimulation, significant levels of intracellular ROS were present in Ncf1-deficient B cells (Fig. 6A). By 24 h of stimulation, the levels of intracellular ROS seen in Ncf1-deficient B cells were nearly identical to those observed in BCR-stimulated WT B cells (Fig. 6B). This late production of ROS was measured in purified splenic B cells using the intracellular superoxide detection reagent Mitosox-Red, which has been reported to preferentially detect mitochondria-derived ROS (154). Pretreatment of cells with the ROS scavenger N-acetylcysteine (NAC) blunted ROS production in wild type B cells during the first 2 h and blocked further ROS production for at least 12 h. ROS production by Ncf1-deficient B cells was decreased compared to wild type B cells, particularly at early times, and was completely blocked by 5 mM NAC (fig 6A and B). These data indicate that intracellular ROS detected by Mitosox-red fluorescence was generated by Nox2 as well as by other mechanisms. The Nox2-independent production of ROS at later times was unlikely to be derived from other NADPH oxidase family members as BCR stimulation for 6 or 12 h did not induce expression of any additional Nox/Duox isoforms (Fig 6C). In contrast, we

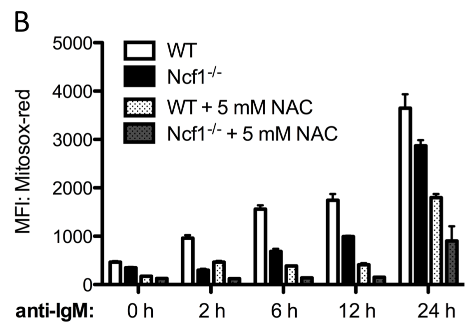
found the production of ROS in BCR-stimulated B cells could be substantially enhanced by transient treatment with antimycin-A, which blocks complex-III of the electron transport chain and leads to accumulation of mitochondria-derived O_2^- (Fig. 6D) (155). This result indicates B cells stimulated via their BCR for 12 h have highly active mitochondrial respiration, which is known be a significant source of intracellular ROS production in a variety of cell types (139). Taken together, these results demonstrate that B cell activation is characterized by a delayed production of ROS that is produced independently of the Nox2 NADPH oxidase, and is maintained during prolonged BCR stimulation.

Figure 6. BCR stimulation for 6 h or more leads to sustained production of ROS independently of Nox2 NADPH oxidase.

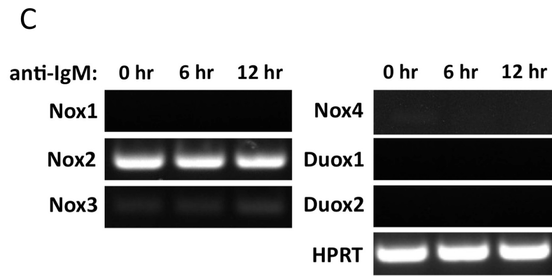
A-B) Purified splenic B cells from WT and Ncf1$^{-/-}$ mice were stimulated with 10 μg/ml anti-IgM for the indicated times, and superoxide levels were measured with the ROS indicator dye Mitosox-Red loaded into cells 30 min prior to harvesting cells at each time point. Specificity of the dye for ROS levels was determined by comparing the median fluorescence intensity (MFI) of Mitosox-Red in WT and Ncf1$^{-/-}$ B cells stimulated in the presence or absence of 5 mM N-acetylcysteine. Histograms (A) represent mitochondrial ROS levels at 0 h (grey filled) and 6 h after anti-IgM stimulation of WT (left) or Ncf1$^{-/-}$ (right) B cells, in the presence (black dashed) or absence (black solid) of 5mM NAC. **B)** Quantification of Mitosox-Red fluorescence (represented as MFI) from WT (white and light grey bars) and Ncf1$^{-/-}$ (black and dark grey bars) B cells measured at the indicated times over 24 h of anti-IgM stimulation in the presence (light grey and dark grey bars) or absence (white and black bars) of NAC. Data are representative of n =3 mice/group from 3 independent experiments. **C)** mRNA Expression of NADPH oxidase isoforms was measured, as described in Fig 2A, from purified resting splenic B cells (0 h), or in purified B cells stimulated for 6 h or 12 h with 10 μg/ml anti-IgM. Data are representative of two independent experiments. **D)** Intracellular superoxide levels were measured as described above with Mitosox-Red from unstimulated (filled grey histogram) and anti-IgM-stimulated WT B cells (12 hrs). Cells were treated for the last 2 h with 20 μg/ml antimycin-A (dashed black) or vehicle control (solid black) to enhance mitochondrial ROS production. Data are representative of n = 2-3 mice/group and similar results were obtained in 2 independent experiments.



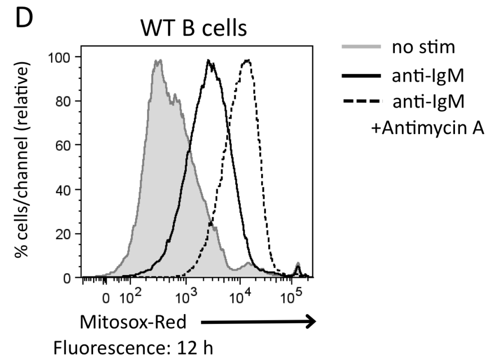
A
WT B cells
Ncf1-/- B cells
no stim
anti-IgM
anti-IgM
+NAC
% cells/channel (relative)
Mitosox-Red
Fluorescence: 6 h



B
WT
Ncf1-/-
WT + 5 mM NAC
Ncf1-/- + 5 mM NAC
MFI: Mitosox-red
5000
4000
3000
2000
1000
0
anti-IgM:
0 h
2 h
6 h
12 h
24 h



C
anti-IgM:
0 hr
6 hr
12 hr
Nox1
Nox2
Nox3
0 hr
6 hr
12 hr
Nox4
Duox1
Duox2
HPRT



D
WT B cells
no stim
anti-IgM
anti-IgM
+Antimycin A
% cells/channel (relative)
100
80
60
40
20
0
Mitosox-Red
Fluorescence: 12 h

Prolonged ROS production is required for optimal activation and proliferation, and to sustain PI3K signaling in response to BCR stimulation.

As prolonged BCR signaling resulted in ROS production from a second source, we next asked if neutralizing the ROS generated by both mechanisms at later times would affect downstream B cell activation. Pretreatment of WT and Ncf1-deficient B cells with 5 mM NAC was sufficient to substantially attenuate intracellular ROS accumulation in wild type B cells, and completely block it in Ncf1-deficient B cells (Fig. 6A and B). This dose of NAC resulted in significantly reduced BCR-induced activation as measured by upregulation of the early activation marker CD69 (Fig. 7A). Furthermore, this treatment resulted in a nearly complete block in proliferation of WT and Ncf1-deficient B cells stimulated with anti-IgM (Fig. 7B). Interestingly, NAC pretreatment did not impact proliferation in response to BCR-independent stimulation with anti-CD40 plus IL-4, or with CpG ODN (Fig. 7B), indicating that the effect of 5 mM NAC on anti-IgM-induced proliferation was likely not due to toxicity, but rather that prolonged ROS production is a specific requirement for optimal responses to antigen receptor stimulation.

Treatment of WT and Ncf1-deficient primary B cells with 5 mM NAC had only minimal effects on proximal BCR signaling at early times, as determined by normal phosphorylations of Syk and ERK kinases in response to BCR crosslinking (Fig. 7D). A small reduction in BCR-induced calcium in both WT and Ncf1-deficient B cells was observed (Fig 7C), but it was unclear whether this was due to scavenging ROS or some other effect, and furthermore was unlikely to explain the near complete block in BCR-induced proliferation observed with 5 mM NAC. Higher concentrations of NAC (25 mM)

further attenuated calcium signaling in agreement with a previous report (21) (data not shown), however this likely reflects additional effects on B cells beyond simply scavenging ROS. As treatment of Ncf1-deficient B cells with 5 mM NAC clearly depleted all residual ROS produced over the first 6-12h of anti-IgM stimulation (Fig. 6B), but did not substantially impact early BCR signaling, these results further support the conclusion that early production of ROS plays little if any role in amplifying proximal BCR signaling upon initial stimulation of resting B cells.

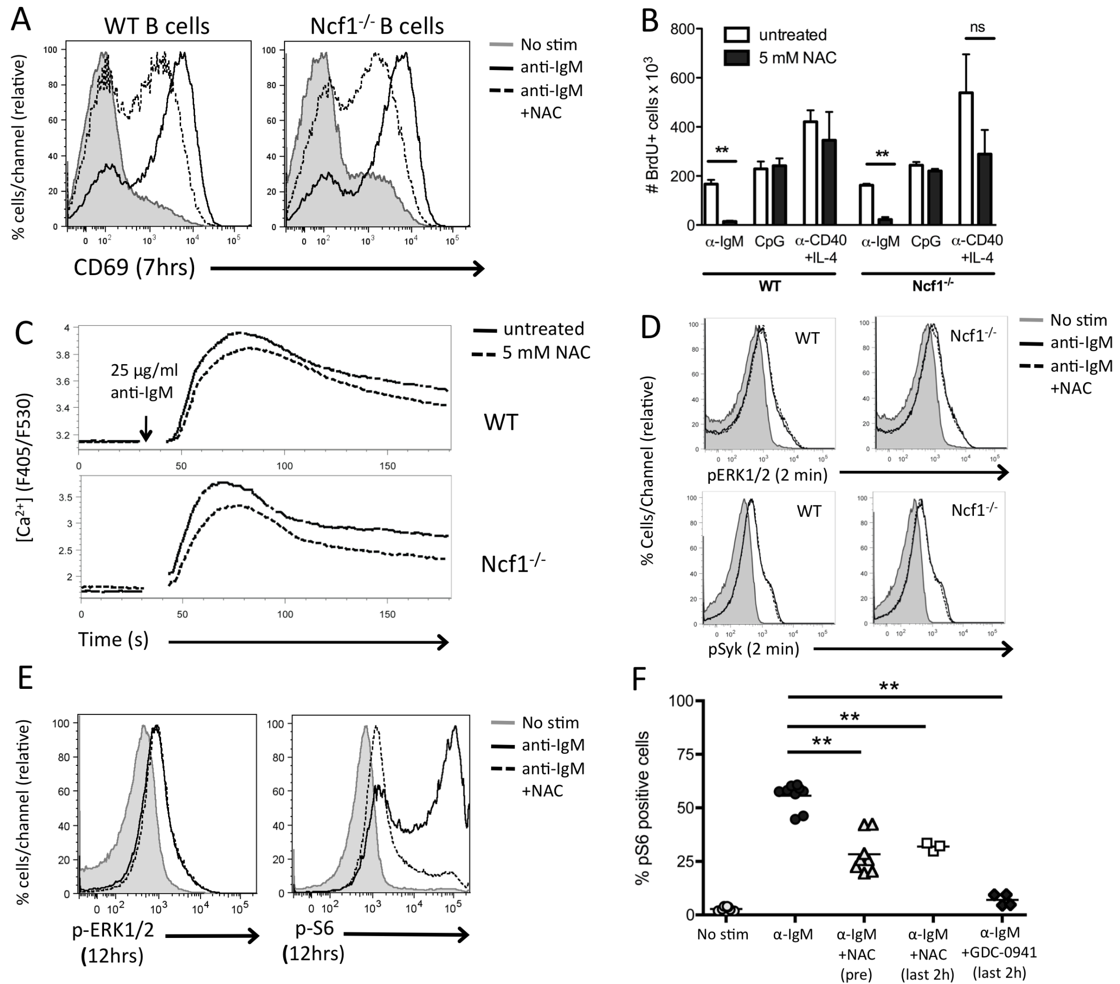
To address the mechanism by which ROS promoted B cell activation and proliferation, we asked if this sustained ROS generation could be functioning to maintain signaling downstream of the BCR. After 12 h of anti-IgM stimulation, both WT and Ncf1-deficient B cells, pretreated with NAC to neutralize both mitochondrial and Nox2 NADPH oxidase-derived ROS, showed unaffected levels of ERK1/2 phosphorylation indicating intact MAPK signaling (Fig. 7E). However signaling through the PI3K pathway was severely attenuated by neutralizing intracellular ROS, as phosphorylation of the ribosomal protein S6, a downstream readout of PI3K activity, was largely reduced by pre-treatment with NAC (Fig. 7E). Phosphorylation of S6 at these late time points remained dependent on continuous PI3K signaling downstream of the BCR, as this signal was completely blocked by addition of the highly specific class I PI3K inhibitor, GDC-0941 (156), for the last 2 h of stimulation (Fig. 7F). Importantly, phospho-S6 levels could also be similarly reduced by transient treatment with NAC for the final 2 h of stimulation (Fig. 7F). These results show that continuous signaling through the PI3K pathway requires the sustained production of ROS, and that ROS-dependent

amplification of this signaling pathway likely promotes efficient B cell activation and proliferation in response to BCR stimulation.

Figure 7. Prolonged ROS production is required for optimal B cell activation and proliferation and for sustained PI3K signaling in response to BCR stimulation.

A) Upregulation of the early activation marker CD69 was measured by flow cytometry 7 h after stimulation of WT (left) or Ncf1$^{-/-}$ (right) B cells with 10 μg/ml anti-IgM in the presence (black dashed) or absence (solid black) of 5 mM NAC. Data are representative of n = 2-3 mice/ group. **B)** Proliferation of WT and Ncf1$^{-/-}$ B cells in the presence or absence of NAC was measured by BrdU incorperation as in Fig 3D. Data are represented as the total number of BrdU positive B cells 48 h after stimulation with anti-IgM (10 μg/ml), CpG ODN (500 ng/ml), or anti-CD40 (10 μg/ml) + IL-4 (10 ng/ml) in the presence or absence of 5 mM NAC (** p<0.01). Data from A and B are representative of B cells from n = 2-3 mice/ group, and similar results were found in 3 independent experiments. **C)** BCR-induced calcium mobilization was measured as in Fig 4A in WT (top) and Ncf1-/- (bottom) follicular B cells stimulated with 25 μg/ml anti-IgM in the presence (dashed black lines) or absence (solid black lines) of 5 mM NAC. **D)** BCR-induced phosphorylation of Syk and ERK were measured as in Fig 4B in WT (left panels) and Ncf1-/- (right panels) follicular B cells. B cells stimulated with 25 μg/ml anti-IgM in the presence (dashed black) or absence (solid black) of 5 mM NAC. Data are representative of 4 (C) and 3 (D) independent experiments **E)** Phospho-ERK and phospho-S6 levels were measured by intracellular staining and flow cytometry, as described in figure 4b, of purified WT B cells stimulated for 12 h with anti-IgM (10 μg/ml) in the presence (dashed black) or absence (solid black) of 5mM NAC. **F)** Quantification of phospho-S6 (represented as the percentage of p-S6^{+} cells) from purified WT B cells stimulated with anti-IgM, as in E, in the presence or absence of NAC for the

entire stimulation (12 h), or incubated for the last 2 h with NAC or 1 μM of the class I PI3K inhibitor GDC-0941. Data represent n=2-3 mice/group, and are pooled from 2-4 independent experiments. (** $p<0.01$ Student's T test)



A
WT B cells
Ncf1-/- B cells
No stim
anti-IgM
anti-IgM +NAC
% cells/channel (relative)
CD69 (7hrs)
B
untreated
5 mM NAC
BrdU+ cells x 10³
α-IgM
CpG
α-CD40 +IL-4
WT
Ncf1-/-
ns
C
untreated
5 mM NAC
25 µg/ml anti-IgM
[Ca²⁺] (F405/F530)
WT
Ncf1-/-
Time (s)
D
No stim
anti-IgM
anti-IgM +NAC
% Cells/Channel (relative)
pERK1/2 (2 min)
pSyk (2 min)
E
No stim
anti-IgM
anti-IgM +NAC
% cells/channel (relative)
p-ERK1/2 (12hrs)
p-S6 (12hrs)
F
% pS6 positive cells
No stim
α-IgM
α-IgM +NAC (pre)
α-IgM +NAC (last 2h)
α-IgM +GDC-0941 (last 2h)

Discussion

In this study, we investigated the intracellular sources and biological function of ROS produced by primary B cells at early and late times following BCR stimulation. We found that the immediate production of ROS in resting B cells was dependent on the Nox2-containing NADPH oxidase complex, as B cells deficient in an essential component of this complex, $p47^{phox}$ (*Ncf1*), were unable to generate detectible amounts ROS over the first several hours of BCR stimulation. However, examination of proximal BCR signaling, B cell activation, and B cell proliferation of BCR-stimulated Ncf1-deficient B cells indicated the lack of a unique role for early Nox2 NADPH oxidase-derived ROS production in these processes. Moreover, Ncf1-deficient mice mounted normal, or even elevated, antibody responses *in vivo*, further demonstrating that early BCR-induced ROS production does not function to enhance the initial response of B cells to antigen stimulation. In contrast, after several hours of BCR stimulation, a second major source of ROS emerged, likely generated as a product of mitochondrial respiration. Blocking accumulation of ROS at these later times with the ROS scavenger N-acetylcysteine resulted in severely attenuated PI3K signaling, and led to marked defects in downstream B cell activation and proliferation following BCR stimulation. Thus, ROS did not substantially enhance proximal BCR signaling early after stimulation of resting B cells, but prolonged production of ROS played a critical role in enhancing BCR-mediated activation, at least in part by promoting sustained BCR-induced PI3K signaling.

Our results demonstrating that BCR-induced early ROS production did not contribute measurably to the magnitude of BCR signaling, are in agreement with a recent report

(145) which showed that B cells deficient in the catalytic subunit of Nox2, gp91^{phox}, also failed to generate ROS in response to BCR stimulation, and had no significant defects in proximal BCR signaling. Furthermore, while both Ncf1-deficient mice (this study) and gp91^{phox} deficient mice (145) exhibit some alterations in antibody responses, these differences were modest in magnitude, and furthermore were elevated compared to wild-type mice with respect to levels of antigen-specific antibody titers. The reason for the enhanced T cell-dependent antibody response observed in Ncf1-deficient mice is not evident from these studies, however it is possible that lack of Nox2 NADPH oxidase-derived ROS production in other immune cells is responsible for these differences. In particular, ROS produced by myeloid cells such as macrophages is known to have immunosuppressive effects on T cells during inflammation (152), and furthermore, regulatory T cells deficient in Nox2 have been shown to have reduced suppressive capacity (157, 158) which may be relevant to FoxP3+ T follicular regulatory cells that have recently been demonstrated to regulate germinal center responses (159, 160).

In contrast to our results with resting primary B cells, a previous study by Singh *et al* (146) found that BCR stimulation of a B lymphoma cell line led to ROS production via the calcium-regulated NADPH oxidase isoform Duox1, resulting in transient inactivation of the PTPase SHP-1 and subsequent BCR signal amplification. A recent study also found that TCR stimulation of human T cell blasts induced rapid Duox1-dependent ROS production that was critical for early TCR signal amplification (144). We were unable to detect expression of Duox1 mRNA in primary resting B cells, and moreover, we did not detect any ROS production at early times following BCR stimulation of Ncf1-deficient

resting B cells, indicating that Duox1 is not a significant contributor to BCR-induced ROS during the early activation of resting mature B cells. It is possible that expression of Duox1 is a feature of activated, germinal center, or memory B cells, or was acquired by the B lymphoma cell line used (A20) as one of the changes that contributed to immortalized growth and survival. Although we did not observe upregulated expression of Duox1 in BCR-activated B cells *in vitro* it is possible that this may differ in an *in vivo* setting, or in the context of B cell activation by other stimuli such as TLR ligands or helper T cell-derived signals.

Another recent study examining B cells deficient in the proton channel HVCN1 suggested a positive signaling role for BCR-induced ROS production (143). HVCN1-mediated proton fluxes have previously been shown to be required for maintaining NADPH oxidase activity in neutrophils undergoing oxidative burst (161, 162). B cells deficient in HVCN1 showed a significant, albeit partial, reduction in ROS produced downstream of BCR stimulation, presumably due to the function of HVCN1 in transporting protons out of the cytosol to balance ionic movements created in the process of ROS production by NADPH oxidase. Interestingly, deficiency in HVCN1 also resulted in largely attenuated proximal BCR signaling and B cell proliferation *in vitro*, and impaired antibody responses *in vivo*. These defects were attributed to a role for HVCN1 in B cells to maintain early NADPH oxidase-dependent ROS production that was required for transient inhibition of SHP-1 to promote BCR signal amplification. Our data showing that Ncf1-deficient B cells lack nearly all early BCR-induced ROS production, but have no defect in BCR signaling or activation *in vitro* or *in vivo* indicate

that the defects observed in HVCN1-deficient B cells probably reflect other mechanisms by which HVCN1 function contributes to proximal BCR signaling. As lack of HVCN1 in B cells resulted in cytosolic acidification and mitochondrial dysfunction, the BCR signaling and antibody response defects seen in HVCN1-deficient B cells could result from effects on a number of biochemical processes. Therefore, while an important role for proton translocation is identified by analysis of HVCN1-deficient B cells, the exact mechanism by which this contributes to BCR signaling remains to be defined.

While Richards *et al* (145) and we found that the early Nox2 NADPH oxidase-dependent ROS production does not play a significant role in amplifying proximal BCR signaling, it is known that B cells require continual BCR stimulation for at least 24 h in order to enter the cell cycle and commit to cell division (163, 164). We found that BCR stimulation of naïve resting B cells resulted in prolonged ROS generation, and by 6 h after anti-IgM stimulation a second source of ROS became significant that was independent of the Nox2 NADPH oxidase. Importantly, blocking accumulation of ROS from both sources during this time period with the anti-oxidant N-acetylcysteine severely impaired anti-IgM-induced activation and proliferation of both WT and Ncf1-deficient B cells. In contrast, this anti-oxidant did not inhibit proliferation induced by the TLR9 ligand CpG ODN, or by anti-CD40 plus IL-4, demonstrating that the importance of ROS was restricted to activation through the BCR.

A potential source of Nox2-independent ROS in BCR-stimulated B cells are the mitochondria, which give rise to ROS in the form O_2^- as an intermediate during the

electron transport chain (139). Oxygen radicals are normally neutralized with high efficiency by enzymes in the mitochondria, however a small percentage of this ROS is known to leak out of the mitochondria in the form of H_2O_2, which can diffuse through the mitochondrial membrane to potentially oxidize protein cysteine residues and thereby regulate signaling or other physiological processes in the cytosol. It is likely the ROS observed at these later times was derived from the mitochondria, as B cells were found to express negligible amounts of other NADPH oxidase isoforms both in the resting state, or after BCR-induced activation *in vitro*. Moreover, ROS levels could be further enhanced by treating BCR-stimulated cells with a mitochondrial complex III inhibitor, a treatment that results in a build up of O_2^- generated from the electron transport chain (165), indicating that activated B cells at these times had highly active mitochondrial respiration. Mitochondrial ROS have recently emerged as critical mediators of signaling in a number of contexts including macrophage cytokine production, inflammasome activation, and growth and proliferation of cancer cells (154, 166, 167). Our data indicate that the functions of mitochondrial ROS can likely be extended to signaling pathways important for B cell activation and proliferation.

The mechanism by which prolonged production of ROS enhanced B cell activation and proliferation is not totally evident from our studies, however, one likely contribution is enhanced activation of the PI3K signaling pathway, which is an important regulator of cell energy uptake and utilization (168, 169). This pathway is negatively regulated by the lipid phosphatase PTEN which is known to be inactivated by ROS through oxidation of an active-site cystine (109). We found that after 12 h of BCR stimulation, neutralizing

ROS with the anti-oxidant N-acetylcysteine had little effect on sustained signaling through the ERK pathway, but this resulted in a marked reduction in phosphorylation of the ribosomal protein S6, which is consistent with an attenuation of upstream PI3K signaling. Furthermore, neutralization of ROS for just the last 2 h of stimulation in B cells treated for 12 h with anti-IgM also reduced S6 phosphorylation, indicating that the continued presence of ROS was required for maintaining this signaling pathway at these later times. We focused on S6 phosphorylation as a readout of PI3K activity in B cells due to the increased sensitivity of this signaling event compared to phosphorylation of AKT at these later times following BCR stimulation, however it will be important to determine if other events downstream of PI3K activation are also similarly reduced by ROS neutralization. In particular, activated AKT is known to phosphorylate and inactivate the transcription factor FOXO1 which normally drives the transcription of genes that maintain cells in a quiescent state (170, 171), and this is consistent with our observation that neutralization of ROS with N-acetylcysteine prevented BCR-induced entry into the cell cycle.

Signaling through the PI3K pathway is critical for a number of cellular processes in lymphocytes including cell proliferation, survival, protein synthesis, and energy utilization, and therefore maintaining signaling through this pathway is likely essential for B cells to respond efficiently to cognate antigen (43, 133, 168, 169). Tonic low-level activation of this pathway is known to be required for the maturation and survival of naïve B cells (52, 172), however, it has also been demonstrated that long-term maintenance of PI3K signaling is critical for B cell proliferation and survival following

BCR stimulation (173). The production of ROS at these later stages of B cell activation may therefore represent a mechanism to promote signal amplification when BCR signaling is suboptimal due to moderate antigen affinity, reduced antigen concentration, and/or a reduced number of BCRs present on the cell surface following receptor internalization in response to initial antigen encounter. These results suggest that the amplification of late BCR signaling by ROS is likely critical for efficient B cell responses to T cell-independent antigens, and/or for maintaining the activation status of antigen-stimulated B cells during the early stages of a T cell-dependent antibody response prior to receiving cognate-T cell help.

Materials and Methods.

Mice

Mice were between the ages of 7-12 weeks for most experiments. B6 (000664; C57BL/6J) and Ncf1 mutant mice (004742; C57BL/6J-*Ncf1m1J*/J) (148) were purchased from Jackson laboratory. MD4 transgenic (Ig^{HEL}) mice were obtained from J. Cyster (University of California, San Francisco) and crossed to the *Ncf1m1J*/J background for some experiments. All animals were housed in a specific pathogen-free facility at the University of California San Francisco, according to University and National Institutes of Health guidelines. Animal use was approved by the University of California Institutional Animal Care and Use Committee.

Antibodies, flow cytometry analysis, and B cell purification

Fluorophore-conjugated Abs directed against the following molecules were used: B220 (RA3-6B2), CD23 (B3B4), CD86 (Gl1), CD69 (H1.2F3), CD95(Fas), GL7 (Ly77), IgD (11-26c.2a) and CD19 (ID3) all from BD Pharmingen; CD24 (M1/69) from Biolegend; CD93 (AA4.1) from eBioscience; IgM (goat polyclonal F(ab) monomer, μ chain specific) from Jackson immunoresearch. Cells were analyzed on an LSR-II (BD Pharmingen). B cell populations in the spleen and bone marrow were stained for 30 min with anti-B220-PE-Cy7, CD93-APC, CD23-PE, and IgM-FITC F(ab) monomer, and mature and immature B cell subpopulations were distinguished using the Allman protocol (174). Dead cells were excluded by propidium iodide (BioChemika) uptake. Purified B cells were isolated from spleens of 7-12 week old mice by negative selection using CD43 microbeads (Miltenyi Biotech) according to the manufacturer's instructions, and passage

through MACS LS separation columns (Miltenyi Biotech). All FACS data were analyzed with FlowJo version 9.3.3 (Tree Star software).

Detection of ROS

Cytochrome-C reduction to measure extracellular ROS production was performed as described (175). Briefly, purified splenic B cells were resuspended in phenol red free HBSS with Ca/Mg salts + 1% BSA at $5x10^6$ cells/ml, equilibrated at 37^0C for 10 min. Cells (100 μl) were aliquoted into wells of Immulon-4 96 well plates (Thermo Scientific) containing cytochrome C (sigma) (0.1 mM final concentration). Immediately following addition of stimulus (50 μg/ml anti-IgM $F(ab')_2$, 500 μM PMA, or 500 ng/ml HEL for MD4 B cells) OD recordings at 550nm and 490nm were read at 2 min intervals in a 96 well kinetic plate reader (Molecular Devices; Spectra MAX plus). Relative superoxide production was derived from change in OD at 550-490nm.

Chemiluminescent detection of superoxide was performed as described (145) using Diogenes (National Diagnostics). Briefly, purified B cells were stimulated as described above except in the presence of Diogenes, and luminescence was recorded at 5 min intervals on a luminometer (Molecular Devices; Spectra MAX M5).

BCR-localized ROS production was measured as described (143), by flow cytometry, by stimulating cells with 20 μg/ml anti-IgM $F(ab')_2$ conjugated to OxyBURST Green H_2DCFDA, SE (invitrogen) in phenol red free HBSS w/ Ca/Mg salts + 10mM HEPES + 1% FBS. ROS production was recorded at 488nm at 5 min intervals by flow cytometry

using an LSR-II and gating B220$^+$CD93$^-$ splenic B cells or B220$^-$ non-B cells as a control.

Intracellular superoxide was recorded on total spleen cells loaded intracellularly with 5 μM Mitosox-Red (Invitrogen) for 20 min at 37^{0}C, and stained for surface B220 and CD93 as described above. For short term recordings, B cells were stimulated at 37^{0}C with 20 μg/ml anti-IgM F(ab')$_{2,}$ and Mitosox-Red fluorescence was recorded at 561 nm at 5 min intervals over 30 min by flow cytometry using an LSRII, and gating on mature B cells and non-B cells as described above for anti-IgM-DCFDA. For longer time points, purified B cells were pre-stained with anti-B220 and CD93, stimulated with 10 μg/ml anti-IgM F(ab')$_2$ in complete Iscove's medium containing 10% FBS for 2, 6, 12, and 24 h, and loaded with 5 μM Mitosox-Red for 30 min at 37^{0}C prior to harvesting the cells at each time point. Cells were then washed and analyzed by flow cytometry as described to measure Mitosox-Red fluorescence. In some cases, cells were pre-incubated with 5 mM N-acetylcysteine (Sigma) for 30 min prior to stimulation, or treated for the last 2 h of stimulation with 20 μg/ml Antimycin-A (Sigma).

Analysis of calcium mobilization

Measurement of intracellular-free calcium levels was performed as described (176). Briefly, splenocytes were loaded with Indo-1-AM (Molecular Probes, Invitrogen) and stained for B220, CD93 (AA4.1), CD23, and surface-IgM (using F(ab) monomer). Cells were resuspended in RPMI-1640 medium with 1% BSA and 10 mM HEPES, warmed for 3 min at 37^{0}C, and baseline indo-1 fluorescence was recorded on an LSR-II for 30 s.

Cells were then stimulated with 2 or 20 μg/ml goat anti-mouse anti-IgM F(ab')$_2$ (Jackson Immuno Research) and median intracellular calcium levels measured for an additional 3 min, indicated by the ratio of fluorescence 405 nm emission to 530 nm emission over time. For some experiments cells were pre-incubated for 15 min with 5 mM N-acetylcysteine prior to stimulation.

Intracellular Flow cytometry

Intracellular measurement of phospho-ERK, phospho-AKT, and phospho-Syk were performed as described (176). Briefly, $4x10^6$ splenocytes were resuspended in Iscove's medium containing 1% BSA, 10 mM HEPES and 2-ME, and pre-warmed at 37^0C for 30 min. In some cases, cells were stained for surface IgM (with anti-IgM F(ab) monomer) for the final 10 min of warming. Cells were stimulated with 25 μg/ml anti-IgM F(ab')$_2$ for 2, 10, and 30 min and immediately fixed with 2% Paraformaldehyde and permeabilized with 100% ice-cold methanol (both from Electron Microscopy Services). Cells were labeled with anti-phospho-Syk-Alexa-647 (BD Pharmingen), anti-phospho-ERK1/2 (thr202/Tyr204) rabbit mAB, or anti-phospho-AKT (Ser473) rabbit mAB (both from Cell Signaling Technology), followed by staining for anti-rabbit IgG-APC (Jackson Immunoresearch) (for p-ERK and p-AKT) and B220, CD24 (in place of CD93), and CD23. Cells were analyzed by flow cytometry, and the phosphorylation status of each signaling molecule was analyzed within the mature follicular B cell population ($B220^+$, $CD24^-$. $CD23^+$, IgM^{int-lo}). For analysis of late time points purified splenic B cells were stimulated for 12 h with 10 μg/ml anti-IgM F(ab')$_2$ in complete Iscove's medium containing 10% FBS, followed by intracellular staining and flow cytometry as described

above using anti-phospho-ERK1/2 or anti-phospho-S6 (Cell Signaling Technology) rabbit mAB's. In some cases stimulation was carried out in the presence of 5 mM NAC (added prior to stimulation or for the last 2 h of stimulation) or 1 μM of the class I PI3K inhibitor GDC-0941 (Chemdea) added for the last 2 h of stimulation.

B cell activation and proliferation

For *in vitro* B cell activation analysis, purified splenic B cells were resuspended at 1 x 10^6 cells/ml in complete Iscove's medium containing 10% FBS, in the presence or absence of 5 mM NAC, and equilibrated at 37^0C for 30 min prior to stimulation. Following stimulation with anti-IgM $F(ab')_2$, cells were harvested at indicated times, and stained with fluorescent antibodies against surface CD69 (6-7 h) or CD86 (18 h). For *in vitro* proliferation, purified B cells, in the presence or absence of 5 mM NAC, were stimulated as described above with 10 μg/ml anti-IgM $F(ab')_2$, 500 ng/ml CpG (Integrated DNA Technologies), or 10 μg/ml anti-CD40 (BD Pharmingen) + 10 ng/ml IL-4 (Roche). BrdU (BD Pharmingen) was added to cells (final concentration 10 μM) 36 h after stimulation, and cells were harvested for analysis 48 h after stimulation. Proliferation was assessed by incorperation of BrdU within $B220^+$ cells by intracellular staining using a BrdU flow kit (BD Pharmingen) following manufacturers instructions. Cells were analyzed by flow cytometry using an LSR-II, and proliferation was derived from the percentage or absolute number of BrdU positive B cells.

Immunizations, Germinal center cell analysis, ELISA, and ELISPOT

To assess T cell-dependent antibody production, mice were injected intraperitoneally with 50 μg NP-CGG precipitated in Alum. Sera were collected on days 7 and 14 after immunization, and NP-specific IgM and IgG_1 titers were measured by ELISA. For ELISA, 96 well plates (BD Falcon) were coated overnight with NP_{10}-BSA and blocked with 2% FBS in 1xPBS (1-2 h), followed by incubation with serial dilutions of serum for 2 h at room temperature. NP-specific IgM and IgG1 were detected with Horseradish peroxidase (HRP)-conjugated anti-mouse IgM and IgG1 Abs (Southern Biotech) and developed with TMB substrate (Vector laboratories). OD (450-570 nm) measurements were recorded with a 96 well microplate plate reader (Molecular Devices; VERSA max). Serum antibody titers were derived from the slope of the titration curve and normalized to a standard serum sample from a hyper-immunized mouse. Germinal center cell development was measured on days 7 and 14 from NP-CGG immunized mice by staining total spleen cells with antibodies against B220, CD4, IgD, CD95 (Fas), and GL7, and defining GC B cells as $B220^+$, IgD^{-lo}, Fas^+, and $GL7^+$. Numbers of NP-specific plasma cells were determined by ELISPOT. Briefly, splenocytes from NP-CGG immunized mice were resuspended in complete Iscove's medium containing 10% FBS, and incubated overnight in wells of multiscreen HTS 96 well filter plates (Millipore) that were previously coated with NP_{10}-BSA. The following day, plates were washed of cells and spots were detected with HRP-conjugated anti-mouse IgG (Southern Biotech) and developed with a 3-Amino-9-ethylcarbazole (AEC) chromogen kit (Sigma), according to manufacturer's instructions.

B cell antigen presentation

Splenic cells (2 x 10^5) from WT and Ncf1$^{-/-}$/ MD4 Ig-transgenic were pulsed with indicated concentrations of HEL-Ova (gift from J. Cyster UCSF) for 2 h, and washed to remove excess antigen. Ag-pulsed B cells were then incubated with 1x 10^5 CFSE-labeled ovalbumin-specific OT-II TCR transgenic CD4+ T cells. After 72 h of co-culture, cells were harvested and stained for CD19 and CD4, and OT-II proliferation was determined by monitoring CFSE dilution by flow cytometry, as described. Dead cells were excluded by propidium iodide uptake.

Chapter 3

Critical role of diacylglycerol kinase-ζ in limiting B cell antigen receptor-induced ERK signaling and controlling the magnitude of the early antibody response

Introduction

Engagement of the B cell antigen receptor (BCR) by specific antigen induces a complex cascade of intracellular signaling events that play critical roles in B cell development, activation, survival, and proliferation (57). Early signaling by the BCR involves the activation of Src and Syk family protein tyrosine kinases, which stimulate a number of downstream signaling events, including activation of phospholipase C-γ2 (PLC-γ2) to generate the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (31, 177, 178). Whereas IP_3 is required for calcium mobilization and activation of the NFAT family of transcription factors, DAG signals through PKCβ and the Ras guanine exchange factor, RasGRP, leading to activation of the NF-κB and Ras-MEK-ERK mitogen-activated protein kinase (MAPK) pathways respectively (69, 90, 91, 179-181). Activation of these signaling pathways downstream of the BCR results in rapid transmission of signals to the nucleus and alterations in gene expression necessary for subsequent B cell functional responses.

The ERK MAPK signaling cascade is critical for a number of aspects of B cell function and fate decisions (81). During early B cell development, ERK signaling is required for proliferative expansion induced by signaling through the pre-BCR, as well as for differentiation of immature transitional B cells to the mature follicular stage in the spleen (81, 182). In mature B cells, pharmacological inhibition of MEK, or genetic deficiency in the key signaling intermediates for Ras activation, RasGRP1 and RasGRP3, severely impairs survival and proliferation in response to BCR stimulation (90, 95). Antigen stimulation of mature B cells *in vivo* induces antibody production through the rapid

formation of extrafollicular plasma cells, as well as a slower germinal center response, which gives rise to plasma cells that secrete higher affinity antibodies. ERK signaling in germinal center B cells is required for terminal differentiation to antibody-secreting plasma cells through induction of the key transcription factor Blimp1 (96), however its role in the early extrafollicular plasma cell response has not been examined.

Previous work has shown that B cell maturation from the immature transitional stage to the mature follicular stage in the spleen is accompanied by an attenuation in BCR-induced ERK activation (176), suggesting the possibility that ERK is differentially regulated in a pathway-specific manner during B cell maturation. One possible mechanism of such regulation is by the action of diacylglycerol kinase (DGK) family members, which phosphorylate DAG and convert it to phosphatidic acid, therefore limiting signaling by this second messenger (183). Interesting in this regard, previous studies in T cells found that the degree of ERK activation is controlled at the level of DAG metabolism through the actions of the α and ζ isoforms of DGK (183-186).

Here we report evidence for an important role for DGK-dependent regulation of DAG signaling in mature B cells. We observed that inhibition of DGK enzymatic activity enhanced BCR-mediated activation of ERK selectively in mature follicular B cells, and this correlated with increased mRNA expression of DGKα and DGKζ during B cell maturation in the spleen. Interestingly, while mature follicular B cells from mice deficient in DGKζ exhibited enhanced ERK-MAPK signaling and had a reduced threshold for BCR-induced activation and proliferation, ablation of DGKα showed a lesser effect. In

addition, *in vivo* experiments revealed that DGKζ plays a role in limiting B cell activation during immune responses, and is especially important for limiting the number of antibody-secreting plasma cells generated early in response to both T cell-independent type 2 and T cell-dependent antigen immunization. Strikingly, the effect of enhanced ERK signaling in DGKζ-deficient B cells closely mimics the effect of increasing the affinity of antigen for the BCR, strongly suggesting that the magnitude of ERK signaling in B cells is an important determinant of affinity discrimination during the antibody response.

Results

Activation of diacylglycerol-mediated signaling events is tightly regulated by DGKζ in mature follicular B cells

Previously, it was observed that low amounts of BCR crosslinking are sufficient to induce full activation of ERK in immature transitional B cells, whereas even at high doses of BCR crosslinking, activation of ERK is markedly reduced in the mature follicular B cell population (176). To determine if these differences might be due to differences in negative regulation by DGK enzymes, we examined the effect on BCR-induced activation of ERK1 and ERK2 of the DGK inhibitor, R59949, which is known to block activities of several DGK isoforms (187). Pretreating splenic B cells with this inhibitor resulted in substantially enhanced BCR-induced ERK phosphorylation within the immature T3 and mature follicular B cell populations, as measured by flow cytometry using a phospho-specific antibody against the activation sites of ERK1 and ERK2 (Fig. 8A). In contrast, this inhibitor did not further enhance BCR-induced ERK activation in immature T1 or T2 B cells (Fig. 8A). We next examined expression of DGKα and DGKζ mRNAs in B cells at different stages of peripheral maturation in the spleen. Consistent with attenuated BCR-induced ERK signaling in follicular B cells, this population expressed nearly 2-fold greater mRNA levels of DGKα and DGKζ than immature transitional T1 and T2 B cells sorted from spleens of WT mice (Fig 8B). Notably, BCR stimulation of purified splenic B cells resulted in rapid downregulation of DGKα mRNA within 2 hr of stimulation, as well as a smaller but substantial reduction in DGKζ mRNA within 6 hours of BCR stimulation (Fig 8C), which may be important to facilitate prolonged DAG signaling downstream of the BCR at later stages of B cell activation.

Next, we measured BCR-induced ERK phosphorylation in B cells from mice with targeted deletions of DGKα, DGKζ, or both (184, 186). DGKα-deficient follicular B cells had slightly elevated BCR-induced ERK phosphorylation compared to WT cells (Fig. S1A), however this response was enhanced nearly 10 fold in DGKζ-deficient follicular B cells (Fig 8D and S1A). The increased response of DGKζ-deficient follicular B cells was evident over a wide range of anti-IgM doses used to cluster membrane IgM isoforms of the BCR to trigger downstream signaling (Fig. 8D). Furthermore, this enhanced response was maintained at all time points tested (Fig. 8E). Similar to R59949 treatment, genetic deficiency in DGKζ had only modest effects on BCR-induced ERK activation in T1 B cells (Fig 8D, 8E) indicating that DGKζ-mediated regulation of DAG signaling was primarily a property of mature follicular B cells. Notably, DGKζ-deficiency appeared to shift the phospho-ERK response of follicular B cells from a graded, analogue response seen in WT cells, to a digital or bimodal response, especially at intermediate doses of anti-IgM (Fig. 8D). Follicular B cells from mice deficient in both DGKα and DGKζ exhibited further enhancement of BCR-induced ERK activation, indicating a secondary but significant role for DGKα in restraining BCR-mediated DAG signaling (Fig. S1A). Given the dominant role of DGKζ for regulating DAG signaling in follicular B cells, we focused on this isoform in subsequent experiments.

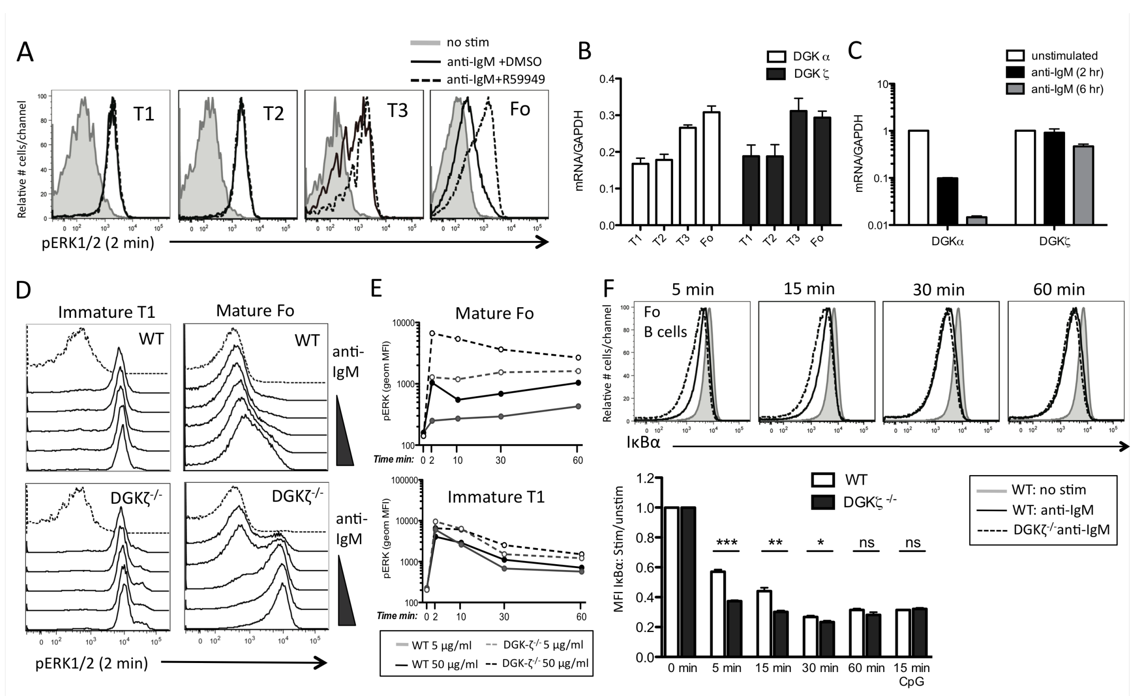
As DAG is also an important second messenger for BCR-induced canonical NFκB activation (69), we examined degradation of the IκBα inhibitory protein, which is required for nuclear translocation of NFκB (72). IκBα levels were measured by

intracellular staining and flow cytometry of permeabilized cells. BCR crosslinking of splenic B cells induced a rapid degradation of IκBα in WT follicular B cells, which was clearly accelerated in DGKζ-deficient follicular B cells, although by 30-60 min the levels of IκBα were indistinguishable (Fig. 8F). Degradation of IκBα in response to the TLR9 ligand CpG, which should be independent of DAG production (188), was not similarly affected (Fig. 8F), indicating specificity of DGKζ for negative regulation of BCR-induced NFκB activation.

Mice deficient in DGKζ had normal numbers of the various B cell subpopulations in the bone marrow, spleen, and peritoneal cavity (Fig. S2A). We did however observe a small but significant decrease in the levels of surface IgM on all mature and immature splenic B cell subpopulations in the spleen of DGKζ-deficient mice (Fig. S2B). As expected, DAG-independent components of BCR signaling including calcium elevation and phosphorylation of the proximal tyrosine kinase Syk were not enhanced, and were even somewhat reduced in DGKζ-deficient follicular B cells (Fig. S3A and S3B), which may result from decreased surface BCR levels.

Figure 8. Activation of diacylglycerol-mediated signaling events is tightly regulated by DGKζ in mature follicular B cells. BCR-induced ERK phosphorylation was measured by flow cytometry in splenic $B220^+$ T1 ($CD93^+$, $CD23^{neg}$, IgM^{hi}), T2 ($CD93^+$, $CD23^+$, IgM^{hi}), T3 ($CD93^+$, $CD23^+$, $IgM^{lo\text{-}int}$), and follicular ($CD93^-$, $CD23^+$, $IgM^{int\text{-}lo}$) B cells following stimulation with 50 μg/ml anti-IgM in the presence of 20 μM R59949 or DMSO. (B) mRNA transcript expression of DGKα and DGKζ during peripheral B cell maturation was measured by quantitative RT-PCR from sorted WT splenic B cells (T1, T2, T3, and Fo) and normalized to GAPDH. Splenocytes were pooled from 3 mice prior to cell sorting. Data are representative of 2 independent experiments. (C) Expression of DGKα and DGKζ in response to BCR stimulation was measured in purified WT splenic B cells following *in vitro* stimulation for indicated times with 10 μg/ml anti-IgM $F(ab')_2$. Expression of each isoform was normalized to GAPDH and is representative of the fold change in expression compared to unstimulated B cells. B cells were purified from n =2-3 spleens and data are representative of 3 independent experiments. (D) Comparison of BCR-induced ERK activation between WT and $DGK\zeta^{-/-}$ T1 (*left panels*) and follicular (*right panels*) B cells following stimulation with anti-IgM $F(ab')_2$ for 2 min. Wedge indicates a 50-fold dose response range, starting with 1 μg/ml anti-IgM. (E) Time course (0-60 min) of BCR induced ERK activation in WT (solid lines) and $DGK\zeta^{-/-}$ (dashed lines) T1 and follicular B cells stimulated with 5μg/ml (grey lines) and 50 μg/ml (black lines) anti-IgM $F(ab')_2$. Relative phospho-ERK levels are derived from the geometric median fluorescence intensity. Data for D-E are representative of the response from stimulated Fo and T1 B cells from n = 3 pooled spleens. Similar results were found in at least 3 independent experiments. (F) Representative histograms showing IκBα levels in

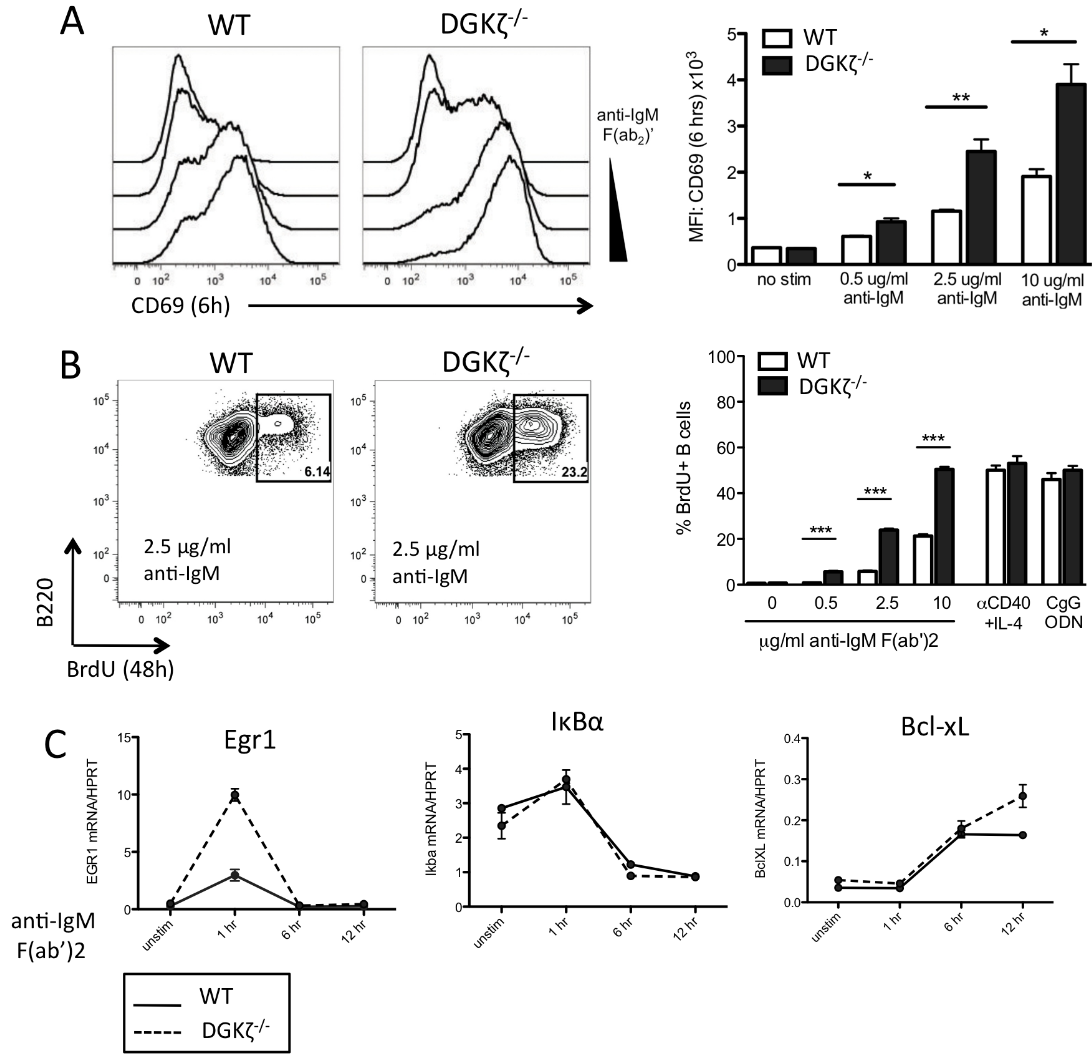
Fo B cells following BCR stimulation of total splenic B cells from WT (solid black histogram) and DGKζ$^{-/-}$ (dashed black histogram). Degradation of IκBα was quantified (bottom) as the median fluorescence intensity of signal after stimulation with 50 µg/ml anti-IgM F(ab')$_2$, or 500 ng/ml CpG ODN for the indicated times, and was normalized to unstimulated levels. Data are from n = 3 mice and are representative of 3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Student's *t*-test).


A
no stim
anti-IgM +DMSO
anti-IgM+R59949
T1
T2
T3
Fo
Relative # cells/channel
pERK1/2 (2 min)
B
DGK α
DGK ζ
mRNA/GAPDH
T1
T2
T3
Fo
C
unstimulated
anti-IgM (2 hr)
anti-IgM (6 hr)
DGKα
DGKζ
D
Immature T1
Mature Fo
WT
anti-IgM
DGKζ-/-
pERK1/2 (2 min)
E
Mature Fo
Immature T1
pERK (geom MFI)
Time min:
WT 5 μg/ml
WT 50 μg/ml
DGK-ζ-/- 5 μg/ml
DGK-ζ-/- 50 μg/ml
F
5 min
15 min
30 min
60 min
Fo
B cells
IκBα
WT
DGKζ -/-
WT: no stim
WT: anti-IgM
DGKζ-/- anti-IgM
MFI IκBα: Stim/unstim
0 min
5 min
15 min
30 min
60 min
15 min CpG
***
**
*
ns
ns

DGKζ deficiency reduces the threshold for BCR induced activation and proliferation *in vitro*

As early BCR-induced ERK activation was strongly enhanced in DGKζ-deficient follicular B cells, we then asked if there was a reduced threshold for BCR induced activation and proliferation of these cells. Indeed, *in vitro* BCR-induced upregulation of the early activation marker CD69 was enhanced in DGKζ-deficient B cells (Fig. 9A) whereas this response was largely normal in B cells lacking DGKα (Fig. S1B). Furthermore, *in vitro* proliferation of anti-IgM stimulated DGKζ-deficient B cells was markedly increased compared to that of WT B cells, representing a roughly five-fold shift in the dose response curve (Fig. 9B). In contrast, proliferation in response to stimulation with the TLR9 ligand, CpG oligonucleotide, or with anti-CD40 and IL-4 were minimally affected (Fig 9B), as expected given the different signaling mechanisms of these receptors. Correspondingly, enhancement of some early gene inductions in response to BCR stimulation was observed, most prominently of the mRNA encoding the transcription factor *Egr-1* (Fig. 9C), which is downstream of Ras and ERK activation (189). The NFκB-induced increase in IκBα, which serves as a feedback inhibitory response for this pathway (112), was not noticeably changed**,** however upregulation of survival factor Bcl-xL, which is also known to be a direct target of NFκB (190) was moderately increased at later times in DGKζ-deficient B cells (Fig 9C). Taken together, these results demonstrate that DGKζ is a critical negative regulator of DAG signaling downstream of the BCR in mature follicular B cells, which primarily targets the ERK-MAPK signaling pathway and reduces the sensitivity for BCR-induced B cell activation and proliferation.

Figure 9. DGKζ deficiency reduces the threshold for BCR-induced activation and proliferation *in vitro*. (A) Early activation of WT and DGKζ$^{-/-}$ purified splenic B cells measured by CD69 upregulation 6h after stimulation with increasing doses (0.5-10 μg/ml) of anti-IgM F(ab')$_2$ *in vitro*. Left panels show representative histograms for CD69 induction comparing the response of WT (left) and DGKζ$^{-/-}$ (right) B cells to increasing amounts of BCR stimulation. Data are quantified on the right as the median fluorescence intensity of the CD69 signal from n = 3 mice/group, and are representative of 3 independent experiments. (B) *In vitro* proliferation of WT and DGKζ$^{-/-}$ B cells measured by BrdU incorporation 36-48h after stimulation of purified splenic B cells with anti-IgM F(ab')$_2$ (0.5-10 μg/ml), anti-CD40 plus IL-4 (2.5 μg/ml and 1 ng/ml) or CpG ODN (250 ng/ml). Representative flow cytometry plots are shown on the left, and data from n =3 mice/group are quantified on the right as the percentage of BrdU$^+$ cells for each stimulation condition, and are representative of 2 independent experiments. (C) Induction of ERK and NFκB dependent genes (Egr1, IκBα, and Bcl-xL) was measured by quantitative RT-PCR in purified splenic B cells from WT (solid black) and DGKζ$^{-/-}$ (dashed black) mice (n = 3) stimulated for the indicated times with 2.5 μg/ml anti-IgM F(ab')$_2$. Data are representative of 2 independent experiments. *$p<0.05$, **$p<0.01$, ***$p< 0.001$ (Student's *t*-test).

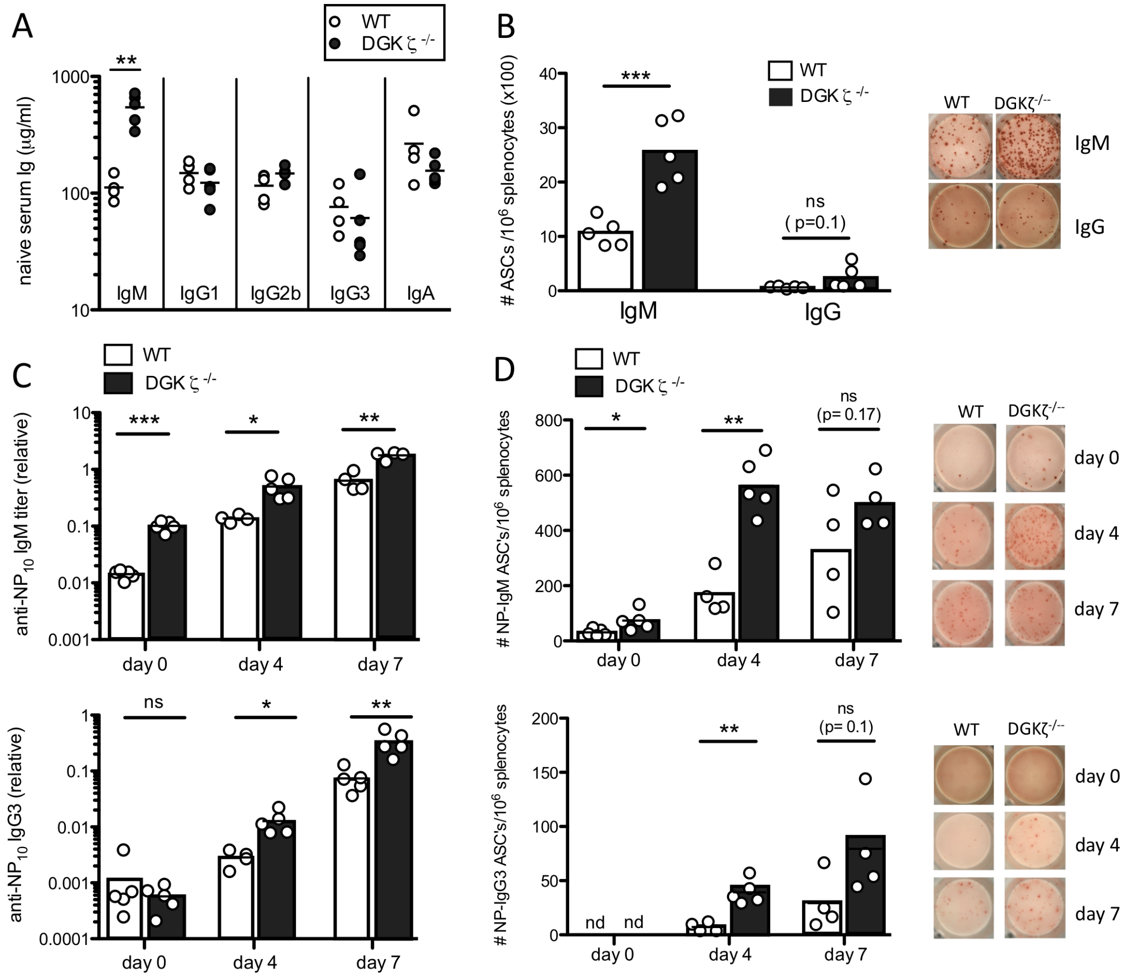

A
WT
DGKζ-/-
anti-IgM F(ab2)'
CD69 (6h)
MFI: CD69 (6 hrs) x10^3
WT
DGKζ-/-
no stim
0.5 ug/ml anti-IgM
2.5 ug/ml anti-IgM
10 ug/ml anti-IgM
B
WT
DGKζ-/-
6.14
23.2
2.5 μg/ml anti-IgM
2.5 μg/ml anti-IgM
B220
BrdU (48h)
% BrdU+ B cells
0
0.5
2.5
10
μg/ml anti-IgM F(ab')2
αCD40 +IL-4
CgG ODN
C
Egr1
IκBα
Bcl-xL
EGR1 mRNA/HPRT
Ikba mRNA/HPRT
BclXL mRNA/HPRT
unstim
1 hr
6 hr
12 hr
anti-IgM F(ab')2
WT
DGKζ-/-

Elevated levels of serum IgM and enhanced response to T cell-independent type 2 antigens in DGKζ-deficient mice.

To determine if DGKζ plays a role in limiting B cell activation *in vivo,* we first compared serum antibody titers between unimmunized WT and DGKζ-deficient mice. DGKζ-deficient mice had normal levels of class-switched Ig isotypes in the blood, but a roughly 5-fold increase in IgM levels (Fig 10A). Analysis of the spleen showed that DGKζ-deficient mice have a large increase in the number of IgM-secreting plasma cells, as well as a trend towards increased numbers of IgG-secreting plasma cells as measured by ELISPOT (Fig. 10B). These results suggest that inhibition of DAG signaling by DGKζ limits extrafollicular plasma cell generation and/or survival in the spleen.

We next looked at the response of DGKζ-deficient mice to the T cell-independent type 2 antigen NP-Ficoll, which triggers vigorous BCR crosslinking on antigen-specific B cells, bypassing their need for T cell help (121). DGKζ-deficient mice generated elevated serum titers of NP-specific IgM and IgG3 on both day 4 and 7 after immunization (Fig. 10C), although the levels of NP-specific IgM were also substantially elevated in unimmunized DGKζ-deficient mice. The increased response was also reflected in the number of plasma cells secreting NP-specific IgM and IgG3 as detected by ELISPOT, especially at day 4 (Fig. 10D), indicating an accelerated extrafollicular plasma cell response. In contrast, mice deficient in DGKα mounted a normal antibody response to NP-Ficoll immunization (Fig. S1C). These results show that DGKζ functions to limit the early response to antigens with strong BCR crosslinking properties.

Figure 10. Elevated levels of serum IgM and enhanced response to T cell-independent antigens in DGKζ-deficient mice. Serum immunoglobulin titers were measured in unimmunized WT (open circles) and DGKζ$^{-/-}$ (filled circles) mice by ELISA. Data are representative of titers from three separate cohorts of mice containing n= 4-5 mice/group. (B) Total spleen IgM and IgG secreting plasma cells were measured by ELISPOT from unimmunized WT (white bars) and DGKζ$^{-/-}$ (black bars) mice. Representative images of ELISPOT wells are shown in the right panels for total IgM and IgG Antibody secreting cells (ASC's). Data are representative of two separate experiments. (C-D) T-independent type 2 antibody response of DGKζ$^{-/-}$ mice. NP-specific serum IgM (top) and IgG3 (bottom) titers were measured by ELISA (C), and numbers of NP-specific IgM (top) and IgG3 (bottom) secreting plasma cells in the spleen were measured by ELISPOT (D), for indicated times before and after NP-Ficoll immunization of WT (white bars) and DGKζ$^{-/-}$ (black bars) mice. Representative images of ELISPOT wells are shown on the right. Data are representative of n =4-5 mice/group, and similar results were found in 2 independent experiments. Each circle represents an individual mouse for all data shown. *$p<0.05$, **$P<0.01$, ***$p<0.001$ (Student's *t*-test)


A
WT
DGK ζ -/-
**
naive serum Ig (μg/ml)
1000
100
10
IgM
IgG1
IgG2b
IgG3
IgA
B
ASCs /10^6 splenocytes (x100)
***
ns
(p=0.1)
IgM
IgG
WT
DGKζ-/-
IgM
IgG
C
anti-NP10 IgM titer (relative)
***
*
**
day 0
day 4
day 7
anti-NP10 IgG3 (relative)
ns
D
NP-IgM ASC's/10^6 splenocytes
ns
(p= 0.17)
NP-IgG3 ASC's/10^6 splenocytes
(p= 0.1)
nd

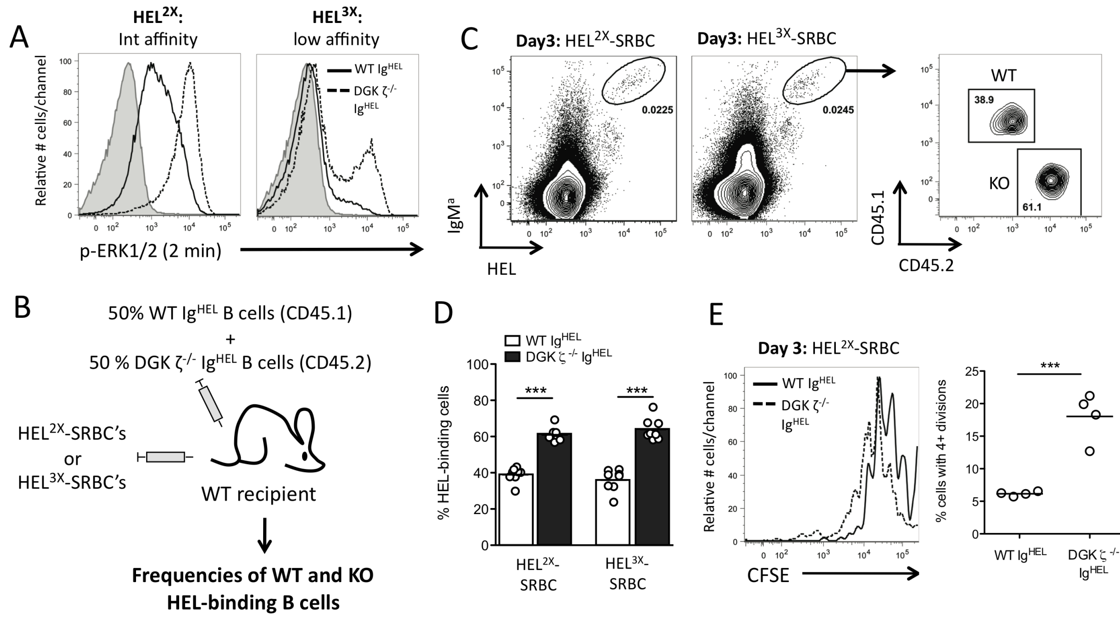
DGKζ limits antigen induced ERK activation and early B cell expansion induced by T cell-dependent antigen immunization

We next wanted to determine whether inhibition of BCR-induced DAG signaling by DGKζ is important for regulating the T cell-dependent antibody response. For this purpose we used an adoptive transfer approach in which small numbers of WT or DGKζ-deficient MD4/Ig^{HEL} transgenic B cells, specific for the hen egg lysozyme (HEL) antigen, were transferred into WT recipient mice, followed by immunization with sheep red blood cells (SRBC) coupled to mutant forms of HEL with different affinities for the transgene-encoded Ig^{HEL} BCR (191, 192). *In vitro* stimulation of Ig^{HEL} transgenic B cells with intermediate affinity HEL (HEL^{2X}) resulted in robust ERK phosphorylation in WT Ig^{HEL} follicular B cells, and this response was enhanced nearly 10-fold in DGKζ-deficient Ig^{HEL} B cells (Fig 4A) similar to what was observed with BCR crosslinking by anti-IgM. Notably, stimulation with the same dose of low affinity HEL (HEL^{3X}), which binds the Ig^{HEL} BCR with 50-fold less affinity than HEL^{2X} (192)**,** was unable to induce significant ERK phosphorylation in WT B cells, however it induced a maximal phospho-ERK response in approximately 20% of DGKζ-deficient B cells (Fig 11A). These results indicate that in the absence of DGKζ, the affinity threshold for antigen-induced BCR signaling to ERK is substantially reduced.

To compare the response of WT and DGKζ-deficient Ig^{HEL} B cells to T cell-dependent antigen immunization, WT mice received a 1:1 mixture ($1x10^5$ total) of congenically marked WT ($CD45.1^+$) Ig^{HEL} and DGKζ-deficient ($CD45.2^+$) Ig^{HEL} purified splenic B cells followed by immunization with intermediate affinity HEL^{2X}, or low affinity HEL^{3X},

conjugated to SRBCs (Fig. 11B). As early as day 3 following immunization, small numbers of HEL-binding B cells could be detected by flow cytometry by co-staining with fluorescently labeled HEL and IgM^a to distinguish donor derived B cells from host B cells responding to the HEL-SRBC conjugates (Fig. 11C). Analysis of the allelic expression of these cells revealed a clear but modest enrichment of DGKζ-deficient B cells within the HEL-binding population in response to both intermediate and low affinity HEL-SRBC immunization (Fig. 11C and 11D). Furthermore CFSE labeling of transferred cells showed that these differences resulted from more robust proliferation rather than enhanced survival or engraftment of DGKζ-deficient transferred cells (Fig. 11E). These results indicate that DGKζ plays a role in limiting early B cell expansion *in vivo* in response to T cell-dependent antigens.

Figure 11. Enhanced early expansion of DGKζ$^{-/-}$ antigen specific B cells in response to T cell-dependent antigen immunization. (A) BCR signaling induced by different affinity variants of HEL. ERK phosphorylation was measured by intracellular staining and flow cytometry as in Fig. 8D in WT/MD4 (solid black histograms) and DGKζ$^{-/-}$/MD4 (dashed black histograms) splenic Fo B cells stimulated for 2 min with 1μg/ml of soluble HEL^{2X} (left) or HEL^{3X} (right). Data are representative of the response from pooled spleens of 2-3 mice, and similar results were found in a second experiment. (B) Experimental set up for measuring *in vivo* responses of WT/MD4 and DGKζ$^{-/-}$/MD4 B cells. WT (CD45.2^{+}) mice received an equal mixture (1x10^{5} total) of WT/MD4 (CD45.1^{+}) and DGKζ$^{-/-}$/MD4 (CD45.2^{+}) purified splenic B cells followed by immunization with HEL^{2X}-SRBC or HEL^{3X}-SRBC. (C) Representative flow cytometry plots show staining of HEL-binding cells on day 3 following HEL^{2X}-SRBC or HEL^{3X}-SRBC immunization (left panels), and frequencies of WT and DGKζ$^{-/-}$/MD4 B cells (right panel). Frequencies of WT/MD4 (white bars) and DGKζ$^{-/-}$/MD4 B cells (black bars) on day 3 are quantified in (D) as the percentage of each genotype within the HEL-binding population in response to HEL^{2X}-SRBC and HEL^{3X}-SRBC (n =5 mice/group and representative of 3 experiments). (E) Proliferation of CFSE labeled WT/MD4 (solid black histogram) and DGKζ$^{-/-}$/MD4 (dashed black histogram) HEL-binding B cells measured by CFSE dilution on day 3 following HEL^{2X}-SRBC immunization. Proliferation is quantified as the percentage of cells that have undergone 4 or more divisions. n=4 mice/group and representative of 2 independent experiments). ***p<0.001 (Student's *t*-test).

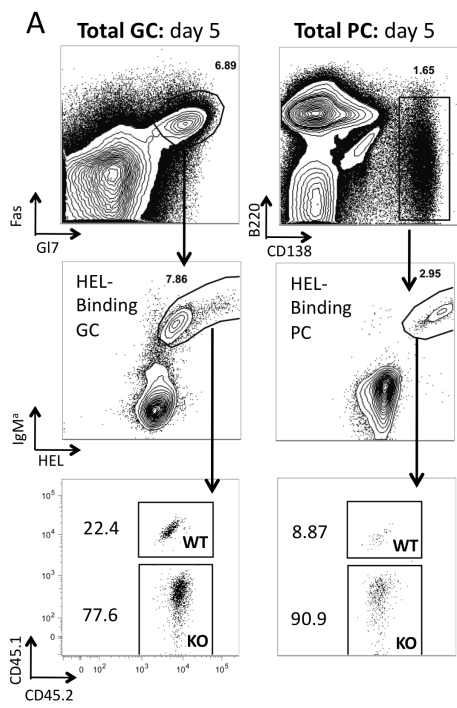

A
HEL2X: Int affinity
HEL3X: low affinity
Relative # cells/channel
WT IgHEL
DGK ζ-/- IgHEL
p-ERK1/2 (2 min)
B
50% WT IgHEL B cells (CD45.1)
+
50 % DGK ζ-/- IgHEL B cells (CD45.2)
HEL2X-SRBC's
or
HEL3X-SRBC's
WT recipient
Frequencies of WT and KO HEL-binding B cells
C
Day3: HEL2X-SRBC
Day3: HEL3X-SRBC
0.0225
0.0245
IgMa
HEL
WT
38.9
KO
61.1
CD45.1
CD45.2
D
WT IgHEL
DGK ζ -/- IgHEL
% HEL-binding cells
***
***
HEL2X-SRBC
HEL3X-SRBC
E
Day 3: HEL2X-SRBC
WT IgHEL
DGK ζ-/- IgHEL
Relative # cells/channel
CFSE
% cells with 4+ divisions
***
WT IgHEL
DGK ζ -/- IgHEL

DGKζ limits early germinal center and extrafollicular plasmablast formation induced by T cell-dependent antigen immunization

Between days 3 and 5 during the T cell-dependent antibody response, activated cognate B cells partition between a rapid short-lived extrafollicular plasmablast response, analogous to that seen in response to the T-independent antigens described above, and a germinal center (GC) response that involves repeated rounds of somatic mutation of the genes encoding the immunoglobulin heavy and light chains and selection for higher affinity variants (124). Eventually this GC response gives rise to long-lived high affinity antibody-secreting plasma cells, as well as memory B cells. Analysis of the early GC response on day 5 following immunization with HEL^{2X} and HEL^{3X}-SRBC, indicated a further skewing in favor of DGKζ-deficient cells, which comprised roughly 75% of the total HEL-binding GC B cell population (Fig. 12A and 12B). However, an even more pronounced advantage was seen within the donor-derived extrafollicular HEL-binding plasmablast compartment after immunization with either HEL^{2X}-SRBC or HEL^{3X}-SRBC (Fig. 12B and 12C), as indicated by a roughly 8-12 fold enrichment for DGKζ-deficient cells compared to WT cells with the same immunogen (Fig. 12B). The magnitude of the HEL-specific GC and extrafollicular plasmablast response was enhanced following immunization with intermediate affinity HEL^{2X} compared to low affinity HEL^{3X} for both WT and DGKζ-deficient Ig^{HEL} transferred cells, although this affinity-dependent difference was much more apparent in the plasmablast population (13-23 fold increase in plasmablasts vs 2.4-2.5 fold increase in GC B cells) (Fig. 12D and 12E) in agreement with previous reports (53, 192). Notably, deficiency in DGKζ shifted the magnitude of the GC and plasmablast responses induced by immunization with low affinity HEL^{3X}-

SRBC to levels similar to that of WT cells responding to intermediate affinity antigen (Fig. 12D and 12E) as measured by the numbers of HEL-binding GC and plasmablast phenotype cells present in the spleen following immunization with the different affinity variants. Thus a critical function of DGKζ is to promote affinity discrimination during the early phases of the T cell-dependent antibody response, and especially to limit extrafollicular plasmablast formation and/or maintenance.

Figure 12. Deficiency in DGKζ promotes robust early plasmablast and germinal center responses. (A) Representative strategy for flow cytometric measurement of numbers of day 5 HEL-binding plasma cells ($B220^{lo}$, $CD138^{hi}$, $IgM^{a\text{-}hi}$, HEL^{hi}) and germinal center B cells (Fas^{hi}, $GL7^{hi}$, $IgM^{a\text{-}int,}$ HEL^{int}) generated after HEL^{2x}-SRBC immunization using the same experimental design as illustrated in Fig. 11B, (B) Frequency of WT/MD4 (white bars) and $DGK\zeta^{-/-}$/MD4 (black bars) HEL-binding cells in the plasma cell and germinal center B cell compartments in response to HEL^{2X}-SRBC (left) and HEL^{3X}-SRBC (right). Data are pooled from 2 individual experiments with n=5-6 mice/group; similar results were obtained in a third independent experiment. (C) Ratio of $DGK\zeta^{-/-}$/MD4: WT/MD4 HEL-binding cells within the germinal center and plasma cell compartments on day 5 in response to HEL^{2X}-SRBC and HEL^{3X}-SRBC. (D-E) Total numbers of WT/MD4 and $DGK\zeta^{-/-}$/MD4 HEL-binding germinal center B cells (D) and plasma cells (E) generated on day 5 in response to HEL^{2X}-SRBC (open circles) and HEL^{3X}-SRBC (filled circles). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Student's *t*-test).


A
Total GC: day 5
Total PC: day 5
6.89
1.65
Fas
Gl7
B220
CD138
HEL-
Binding
GC
7.86
HEL-
Binding
PC
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IgMa
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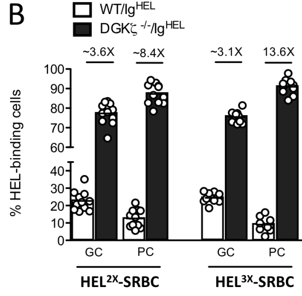
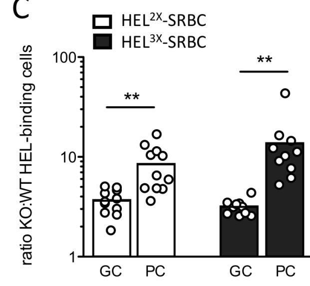
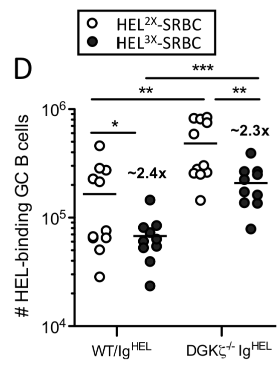
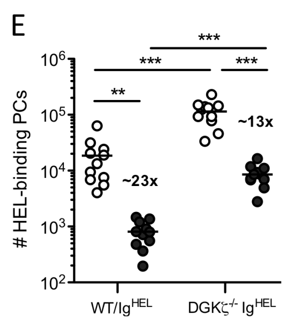
8.87
WT
90.9
KO
CD45.1
CD45.2


B
WT/IgHEL
DGKζ -/-/IgHEL
~3.6X
~8.4X
~3.1X
13.6X
% HEL-binding cells
GC
PC
GC
PC
HEL2X-SRBC
HEL3X-SRBC


C
HEL2X-SRBC
HEL3X-SRBC
ratio KO:WT HEL-binding cells
**
**
GC
PC
GC
PC


HEL2X-SRBC
HEL3X-SRBC
D
HEL-binding GC B cells
~2.4x
~2.3x
WT/IgHEL
DGKζ-/- IgHEL

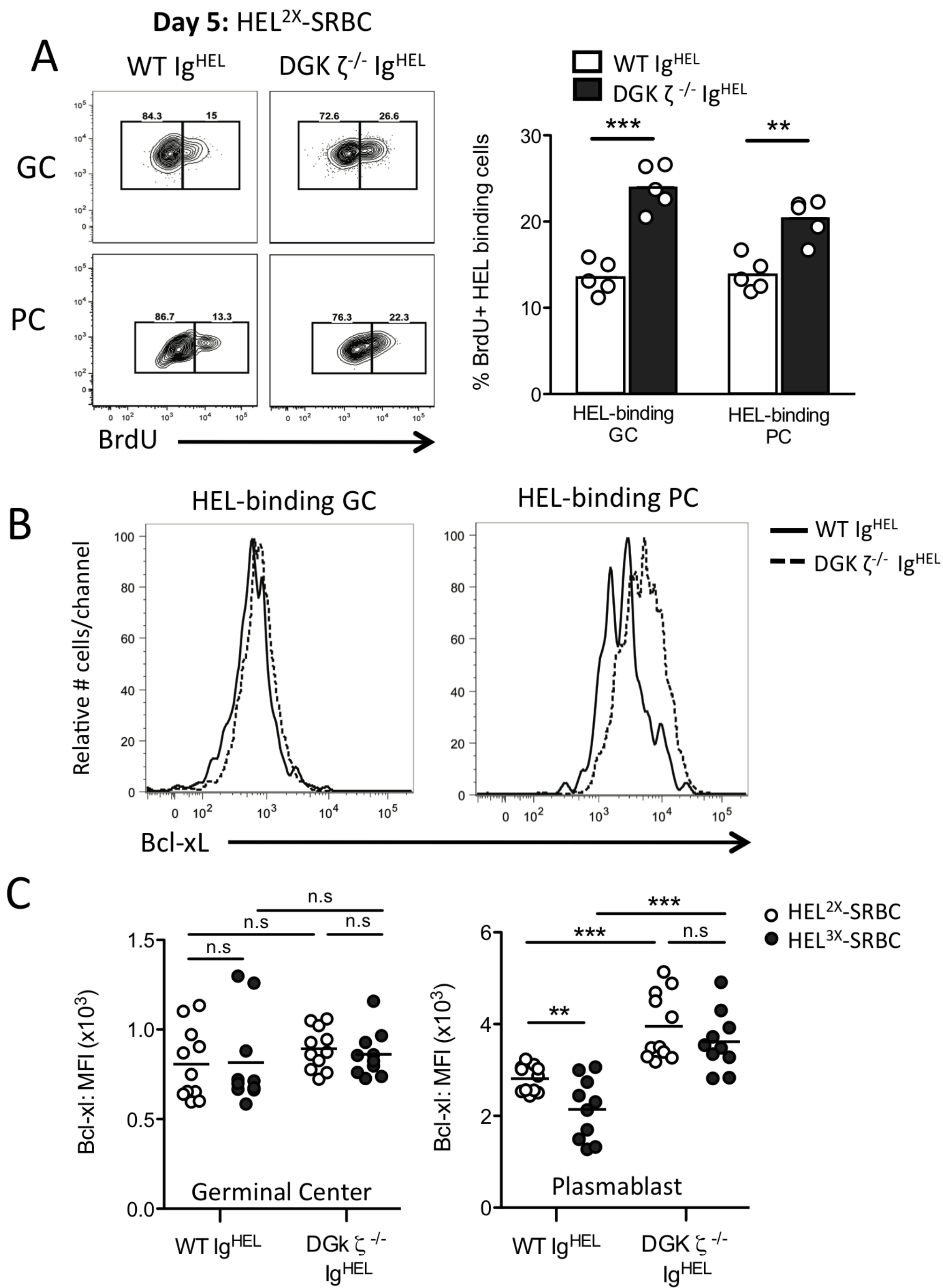

E
HEL-binding PCs
~23x
~13x
WT/IgHEL
DGKζ-/- IgHEL

Proliferation and survival factor expression of DGKζ-deficient GC B cells and extrafollicular plasmablasts

We next wanted to address whether there were differences in proliferation and/or survival of DGKζ-deficient Ig^{HEL} B cells that might account for their enrichment in the GC and plasmablast populations. BrdU labeling of HEL-binding GC B cell and plasmablast populations revealed a moderate increase in the percentage of DGKζ-deficient cells undergoing active DNA synthesis on day 5 following immunization with HEL^{2X}-SRBC (Fig 13A). While these results indicate that DGKζ-deficient cells maintain an enhanced proliferative capacity upon differentiation to the GC B cell and plasmablast stages, it was not clear whether this was sufficient to fully explain the roughly 10-fold increase in DGKζ-deficient cells over WT within the extrafollicular plasmablast population. We therefore asked whether enhanced BCR signaling in the absence of DGKζ resulted in differences in expression of the pro-survival factor Bcl-xL, as measured by intracellular staining and flow cytometry (Fig 13B). Whereas levels of Bcl-xL were similar between WT and DGKζ-deficient GC B cells (Fig. 13B and 13C), there was a significant increase in Bcl-xL levels in donor-derived HEL-binding DGKζ-deficient plasmablasts compared to their WT counterparts after HEL^{2X}-SRBC or HEL^{3X}-SRBC immunization (Fig. 13B and 13C). Furthermore, consistent with the impaired survival capacity of plasmablasts generated in response to low affinity antigen immunization (53), levels of Bcl-xL were reduced in WT plasmablasts generated from HEL^{3X} compared to HEL^{2X}-SRBC immunization (Fig 13C). Interestingly, the levels of Bcl-xL in DGKζ-deficient plasmablasts remained high regardless of the initial antigen affinity (Fig. 13C). The increase in Bcl-xL levels seen in DGKζ-deficient plasmablasts is in agreement with *in*

vitro data showing that BCR stimulation induced higher mRNA induction of this survival factor in naïve DGKζ-deficient B cells (Fig. 9C). Levels of the costimulatory molecule CD86, as well as MHC class II were similar between WT and DGKζ-deficient GC B cells and plasmablasts generated from HEL^{2X}-SRBC immunization (Fig. S4A and B), suggesting that the effects of DGKζ-deficiency on the extrafollicular response were unlikely due to superior antigen presentation to cognate T cells. Instead, these results point to an important role for DGKζ, and the strength of BCR-signaling through DAG-dependent pathways, in mediating extrafollicular plasmablast proliferation and survival based on the affinity of the antigen-BCR interaction.

Figure 13. DGKζ limits expansion of germinal center B cells and plasmablasts, and survival factor expression in plasmablasts. (A) Mice receiving equal mixtures of congenically marked WT and DGKζ$^{-/-}$ MD4 B cells were injected with BrdU on day 5 following immunization with HEL^{2X}-SRBC and BrdU incorperation was measured by flow cytometry within the HEL-binding germinal center and plasmablast populations. WT/MD4 and DGKζ$^{-/-}$/MD4 cells were distinguished by CD45 alleles as in Fig. 12A, and the percentage of BrdU^{+} cells from each genotype was quantified within the HEL-binding germinal center and plasmablast population (right panel). Data are from n=5 mice and similar results were found in one independent experiment. (B) Representative histograms showing intracellular staining for Bcl-xL in day 5 WT/MD4 and DGKζ$^{-/-}$/MD4 HEL-binding germinal center B cells (left) and plasmablasts (right) generated in response to immunization with HEL^{2X}-SRBC. (C) Quantification of Bcl-xL levels (median fluorescence intensity) in HEL-binding germinal center B cells (left panel) and plasma cells (right panel) generated upon immunization with HEL^{2X}-SRBC (open circles) vs. HEL^{3X}-SRBC (filled circles). Data are pooled from 2 independent experiments with n = 5-6 mice each. **$p<0.01$, ***$p<0.001$ (Student's *t*-tests).


Day 5: HEL2X-SRBC
A
WT IgHEL
DGK ζ-/- IgHEL
GC
PC
84.3
15
72.6
26.6
86.7
13.3
76.3
22.3
BrdU
WT IgHEL
DGK ζ -/- IgHEL
% BrdU+ HEL binding cells
***
**
HEL-binding GC
HEL-binding PC
B
HEL-binding GC
HEL-binding PC
WT IgHEL
DGK ζ-/- IgHEL
Relative # cells/channel
Bcl-xL
C
HEL2X-SRBC
HEL3X-SRBC
n.s
***
**
Bcl-xl: MFI (x103)
Germinal Center
Plasmablast
WT IgHEL
DGk ζ -/- IgHEL
DGK ζ -/- IgHEL

Discussion

Antigen stimulation of the B cell antigen receptor (BCR) induces a variety of intracellular signaling events, including those promoted by the second messenger diacylglycerol (DAG), which is generated through hydrolysis of PIP_2 by PLC-γ2. In this work, we have examined the regulation of DAG-dependent signaling events by diacylglycerol kinase (DGK) enzymes, which convert DAG to phosphatidic acid to terminate signaling by this second messenger. Ablation of DGKζ substantially enhanced BCR signaling to ERK in mature follicular B cells and reduced the threshold for BCR-dependent activation and proliferation *in vitro* and *in vivo*. The most striking effects of DGKζ-deficiency in B cells related to greatly enhanced plasmablast responses that closely mimicked the effect of increasing antigen affinity for the BCR. These results indicate that DAG signaling is likely an important determinant of affinity-based regulation of the early plasmablast response.

In antigen-stimulated B and T cells, DAG binds to RasGRP and protein kinase C isoforms, leading to activation of Ras and the ERK MAP kinase as well as the transcription factor NF-κB. Whereas enhancement of DAG signaling by ablation of DGKζ led to substantially enhanced ERK signaling in mature follicular B cells, the effect on NF-κB activation was primarily kinetic in that IκBα was degraded more rapidly but not to an obviously greater extent. In agreement with the signaling results, whereas anti-IgM-induction of the early response gene *Egr-1*, which is mediated by the Ras-ERK pathway (189), was enhanced in DGKζ-deficient B cells, there was not greater induction of IκBα, which is thought to be a direct target of NF-κB (112). Thus, the Ras-ERK

pathway was highly sensitive to changes in the amount of DAG present following BCR engagement, whereas the NF-κB pathway was relatively insensitive to these changes, and instead exhibited more of an all-or-none response. This differential responsiveness of the two major pathways downstream of DAG in B cells to alterations in DAG levels was unexpected, and future studies to define the mechanism of this differential sensitivity will be of considerable interest.

Deletion of DGKζ enhanced ERK signaling in mature follicular B cells, and this signaling was further enhanced in B cells with combined deletion of DGKζ and DGKα. Thus, although DGKα was largely dispensable for proper regulation of DAG signaling in resting follicular B cells, it is clearly capable of acting on DAG and attenuating signaling. DGKα deficiency enhanced *in vitro* responses to BCR stimulation to a minimal degree, and had little effect on *in vivo* responses to NP-Ficoll immunization, however it is possible that DGKα plays a more prominent role in regulation of DAG signaling in situations that were not analyzed in these experiments. Given that DGKα exhibited dramatic changes in mRNA expression during B cell development in the spleen, and following BCR stimulation, we speculate that it may be upregulated to attenuate ERK signaling in some circumstances. In this regard, we note that DGKα is important for antigen-induced anergy in T cells (184, 193, 194).

The use of DGKζ-deficient B cells allowed us to probe the effects of different levels of DAG signaling on B cell activation. These B cells were more sensitive to anti-IgM stimulation *in vitro* for CD69 upregulation and for proliferation. Taken together with

previous evidence that CD69 is a Ras-regulated gene in T cells (195), and that MEK inhibitors block BCR-induced B cell proliferation and CD69 upregulation *in vitro* (95), these results are in agreement with the hypothesis that enhanced ERK signaling in DGKζ-deficient B cells makes these cells more sensitive to BCR engagement for activation. We also observed enhanced proliferation of HEL-specific DGKζ-deficient B cells *in vivo* during the first three days following immunization with HEL^{2X}-SRBC, indicating that a similar regulation occurs during immune responses.

Among the most striking effects of DGKζ-deficiency in B cells for *in vivo* antibody responses was the enhancement of plasmablast numbers seen in response to immunization with both T-independent type 2 antigens and T-cell dependent antigens. In the case of immunization with NP-Ficoll, there appeared to be a more rapid extrafollicular plasmablast response, which correlated with increased serum antibody titers. Interestingly, previous studies have implicated the amount of BCR signaling as well as the ERK pathway in regulation of plasma cell differentiation. For example, in an *in vitro* system, the degree of BCR signaling translated into the amount IRF4 induction, which is required to promote expression of the transcriptional regulator of plasma cell differentiation, Blimp1 (196). Other studies have directly linked ERK signaling to plasma cell differentiation. For example, ERK activation downstream of the BCR has been shown to promote proteosomal degradation of the Blimp1 transcriptional repressor Bcl6 (197), and also was found to inhibit Pax5-dependent repression of Blimp1 transcription (198**).** Moreover, deletion of ERK1 and ERK2 in B cells after they reach the germinal center stage prevented plasma cell differentiation from germinal center B

cells, apparently by preventing ERK dependent phosphorylation of ELK1 and indirect induction of Blimp1 (81). Whether any of these mechanisms are responsible, in part, for the more rapid plasmablast response of DGKζ-deficient mice to NP-Ficoll will require additional study.

We also saw a very large increase in extrafollicular plasmablast numbers in DGKζ-deficient HEL-specific B cells on day 5 after immunization with mutant forms of HEL that have different affinities for the anti-HEL MD4 BCR (191). In this system, increasing the affinity of antigen for the BCR dramatically increases the magnitude of the extrafollicular response, and this has been shown to be largely mediated by enhancing proliferation and survival of plasmablasts (53, 192). It was not clear from the previous studies, however, whether this effect of affinity was mediated by the magnitude of BCR signaling in plasmablasts, or whether it was related to the amount or quality of T cell help that had been received by B cells of different affinities. In the experiments described here, the effects of DGKζ-deficiency were strikingly similar to the effects of higher affinity. These results point to the magnitude of BCR signaling, and especially DAG signaling, as a primary determinant of the magnitude of the extrafollicular plasmablast response. Although we cannot fully rule out higher quality T cell help as a contributor to the effect of antigen affinity on plasmablast numbers, we observed that *in vitro* responses to CD40 were normal in DGKζ-deficient B cells, and we also did not observe any difference in levels of MHC class II or the T cell costimulatory molecule CD86 on DGKζ-deficient germinal center B cells or plasmablasts from HEL-SRBC immunized mice. These observations strongly suggest that the mechanism by which affinity is

translated into extrafollicular plasmablast proliferation and survival is largely due to the magnitude of DAG signaling, likely through the ERK pathway.

The findings of this study illustrate the virtue of modulating signaling reactions, as was done here with DGKζ-deficiency, versus the more common approach of completely ablating signaling reactions, for example by deletion of ERK1 and ERK2 (81, 96). Whereas ablation of a signaling pathway demonstrates when that pathway is required, and this is highly informative, modulation of the magnitude of a signaling pathway makes it possible to assess whether regulation of biological responses takes advantage of changes in the amount of signaling, as was the case for affinity regulation of B cells during the early plasmablast response. Of course, each approach to perturbing signaling reactions has its advantages and disadvantages. Ablation of DGKζ increases the elevation of DAG that occurs upon BCR engagement, but it may attenuate somewhat the levels of phosphatidic acid (PA), and this molecule has been reported to modulate the activity of some signaling pathways. For example, PA has been shown to promote mTOR signaling in HEK293 cells (199). However, we think this signaling function of PA is unlikely to be a major factor in our studies given that DGKs have been shown to limit mTOR signaling in T cells through a mechanism involving Ras and MEK1 (200), which is opposite of what would be expected if DGK-dependent PA production was positively regulating this signaling pathway downstream of antigen receptor stimulation.

Whereas the canonical pathway of ERK activation involves DAG-mediated activation of RasGRP, which activates Ras, leading through Raf1 and MEK1/2 to ERK1/2, recent

studies have identified several alternative pathways of ERK activation in B cells stimulated by antigen. For example, Limnander *et al* (66) showed that in immature B cells in the bone marrow, BCR signaling to ERK is mediated primarily through a mechanism involving store-operated calcium entry rather than DAG, which may explain why there was not an obvious effect of DGKζ-deficiency on early B cell development. In addition, Guo *et al* (201) have demonstrated an alternative pathway by which the BCR activates ERK in IL-4-pretreated B cells, and this pathway seems to operate in addition to the canonical pathway. Furthermore, the adaptor protein Bam32 has been shown to important for a significant proportion of ERK activation downstream of the BCR in naïve resting B cells, apparently by a pathway involving MEKK1 rather than Raf1 as the kinase upstream of MEK1/2 (202-204). To what extent these alternate pathways for ERK activation downstream of the BCR are sensitive to changes in DAG levels is not entirely clear. Mice lacking Bam32 have a substantial defect in T-independent type II antibody responses (202, 204)**,** and have impaired germinal center maintenance in response to T cell dependent antigens (202, 204). These observations are in good agreement with the results of enhancing ERK signaling in the experiments presented here, however it is possible that additional effects of Bam32 deficiency beyond reduced ERK signaling contribute to impaired antibody responses in these mice.

In conclusion, we have demonstrated a critical role for DGKζ in B cells for regulating BCR signaling through the second messenger DAG to the ERK MAP kinase pathway. We have shown that attenuation of DAG signaling by DGKζ is an important mechanism for limiting the numbers of extrafollicular plasmablasts secreting low affinity antibody

against the immunizing antigen. We propose that this inhibitory mechanism is important to maintain a higher affinity initial antibody response, which may be more protective against pathogens during the early stages of the infection before high affinity class switched antibody is produced by the germinal center response. Alternatively or additionally, more stringent regulation of B cell activation and/or antibody responses by the presence of DGKζ in B cells may protect against autoimmunity (100) or lymphoma development (98).

Materials and Methods.

Mice

Mice were used between the ages of 7-12 weeks for most experiments. B6 (000664; C57BL/6J) and CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) were purchased from Jackson laboratory. DGKα$^{-/-}$ (*Dgka*^{tm1Xpz}) and DGKζ$^{-/-}$ (*Dgkz*^{tm1Gak}) mice were generated as previously described (184, 186) and were backcrossed to the C57BL/6J background for 8 generations. MD4 transgenic (Ig^{HEL}) mice were obtained from J. Cyster (University of California, San Francisco) and bred to the DGKζ$^{-/-}$ background for some experiments. All animals were housed in a specific pathogen-free facility at the University of California San Francisco, according to University and National Institutes of Health guidelines. Animal use was approved by the UCSF Institutional Animal Care and Use Committee.

Antibodies, flow cytometry analysis, B cell purification, and cell sorting

Fluorophore-conjugated Abs directed against the following molecules were used: B220, CD23, CD69, CD95 (Fas), CD4, GL7, IgD, IgM^{a}, IgM^{b}, CD45.1, CD45.2, CD138, (syndecan), CD86, I-A^{b} (MHCII) and CD19 (all from BD Pharmingen); CD24 from Biolegend; CD93 (AA4.1) from eBioscience; IgM (goat polyclonal F(ab) monomer, μ chain specific) from Jackson Immunoresearch. For most experiments RBC lysed splenocytes were surface stained on ice following FcR blocking with anti-CD16/32 (UCSF Hybridoma core) and analyzed on an LSR-II (BD Pharmingen). Dead cells were excluded by propidium iodide (BioChemika) uptake. For cell sorting, splenic T1, T2, T3, and follicular B cell subpopulations were identified using the method described by

Allman et al. (174) (B220, CD93, CD23, IgM), and sorting was performed with a MoFlo sorter (DakoCytomation). Purified B cells were isolated from spleens of 7-12 week old mice by negative selection using CD43 microbeads (Miltenyi Biotech) according to the manufacturer's instructions, and passage through MACS LS separation columns (Miltenyi Biotech). All FACS data were analyzed with FlowJo version 9.3.3 (Tree Star software).

Adoptive transfers, HEL-SRBC conjugation, and immunizations.

Splenic B cells were purified from WT/MD4 (Ig^{HEL}) ($CD45.1^+$ or $CD45.1^+.2^+$) and DGK$\zeta^{-/-}$ /MD4 (Ig^{HEL}) ($CD45.2^+$ or $CD45.1^+.2^+$) mice by negative selection as described above. Purified WT/MD4 and DGK$\zeta^{-/-}$/MD4 B cells were mixed at a 50:50 ratio and injected intravenously ($1x10^5$ total) into WT C57BL/6J $CD45.2^+$ hosts. To track cell proliferation *in vivo*, cells were pre-labeled with CFSE and prepared as described above except mice received $1x10^6$ cells prior to immunization. SRBCs (Colorado Serum Company) were labeled with HEL^{2X} or HEL^{3X} (20 µg/ml final concentration) on the day of immunization as described (192). HEL labeling efficiency was measured by flow cytometry by staining the conjugated SRBC with an anti-Hy9HEL-PE-Cy5.5 antibody (Gift from Dr. Jason Cyster UCSF). The following day after adoptive transfer, mice were immunized by intraperitoneal injection with $2x10^8$ freshly conjugated HEL^{2X}- or HEL^{3X}-SRBC. Spleens were harvested and analyzed on day 3 and day 5 following immunization, and donor derived cells were distinguished from host cells by costaining for IgM^a and with hen egg lysozyme (Sigma) conjugated to Alexa-647 (Invitrogen)

fluorescent dye. WT and DGKζ$^{-/-}$ HEL-binding cells were distinguished by CD45.1 and CD45.2 surface staining combinations.

Analysis of HEL-binding germinal center B cells and plasma cells generated from HEL-SRBC immunization

HEL-binding germinal center B cells and plasma cells were analyzed in the spleens of day 5 HEL-SRBC immunized mice. For improved plasma cell recovery spleens were injected with type 4 collagenase (Worthington) and incubated at 37°C for 45 min prior to isolating cells for staining. HEL-binding germinal center B cells were defined as CD4^{-}, IgM^{a+} HEL-A647^{+}, B220^{+}, Fas^{+}, GL7^{+}. HEL-binding plasma cells were identified from total CD4^{-}, B220^{lo}, CD138^{hi} cells, and further distinguished from germinal center B cells by intracellular staining for IgM^{a} and HEL using cytofix and cytoperm (BD Biosciences).

***In vivo* and *in vitro* BrdU labeling**

For *in vivo* BrdU labeling experiments, mice were injected IP with 2.5 mg BrdU (in 250 μl PBS) on day 5 following HEL-SRBC immunization and sacrificed 60 min later. For *in vitro* proliferation analysis, purified WT and DGKζ$^{-/-}$ splenic B cells were suspended in complete Iscove's medium with 10% FBS (1x10^{6} cell/ml) and stimulated at 37°C with 0.25-10 μg/ml anti-IgM F(ab')$_2$ (Jackson immunoresearch), 250 ng/ml CpG (Integrated DNA Technologies), or 2.5 μg/ml anti-CD40 (BD Pharmingen) + 2.5 ng/ml IL-4 (Roche) for 48 h. BrdU (BD Pharmingen) was added to cells at a final concentration of 10 μM at 36 h after stimulation, and cells were harvested for analysis 48 h after stimulation. For *in vitro* and *in vivo* analysis, cells were stained for appropriate surface markers, and BrdU

incorporation was measured by intracellular staining and flow cytometry using a BrdU Flow kit (BD Pharmingen) according to manufacturer's instructions.

Intracellular flow cytometry

Intracellular measurement of phospho-ERK, phospho-Syk, and total IκBα were performed as described (205). Briefly, $4x10^6$ splenocytes ($2x10^7$/ml) were pre-warmed at 37^0C for 30 min and stimulated with anti-IgM $F(ab')_2$ followed by immediate fixation and permeabilization with 2% PFA and ice cold methanol respectively (both from Electron Microscopy Services). For some experiments, cells were pretreated for 20 min with 20 μM of the DGK inhibitor II R59949 (Alexis biochemicals) or vehicle (% DMSO). Cells were labeled with anti-phospho-Syk-Alexa-647 (BD Pharmingen), anti-phospho-ERK1/2 (Thr202/Tyr204) rabbit mAB, or anti-IκBα rabbit Ab (both from Cell Signaling Technology), followed by staining with anti-rabbit IgG-APC (Jackson Immunoresearch) (for p-ERK and IκBα) and surface staining to distinguish T1, T2, T3, and Fo B cells. Cells were analyzed by flow cytometry, and for most experiments the analysis was performed on the T1 ($B220^+$, $CD24^{hi}$, $CD23^{neg}$, IgM^{hi}) and follicular ($B220^+$, $CD24^{lo}$, $CD23^+$, IgM^{lo-int}) populations. Measurement of intracellular free calcium levels was performed as described (176).

ELISA and ELISPOT

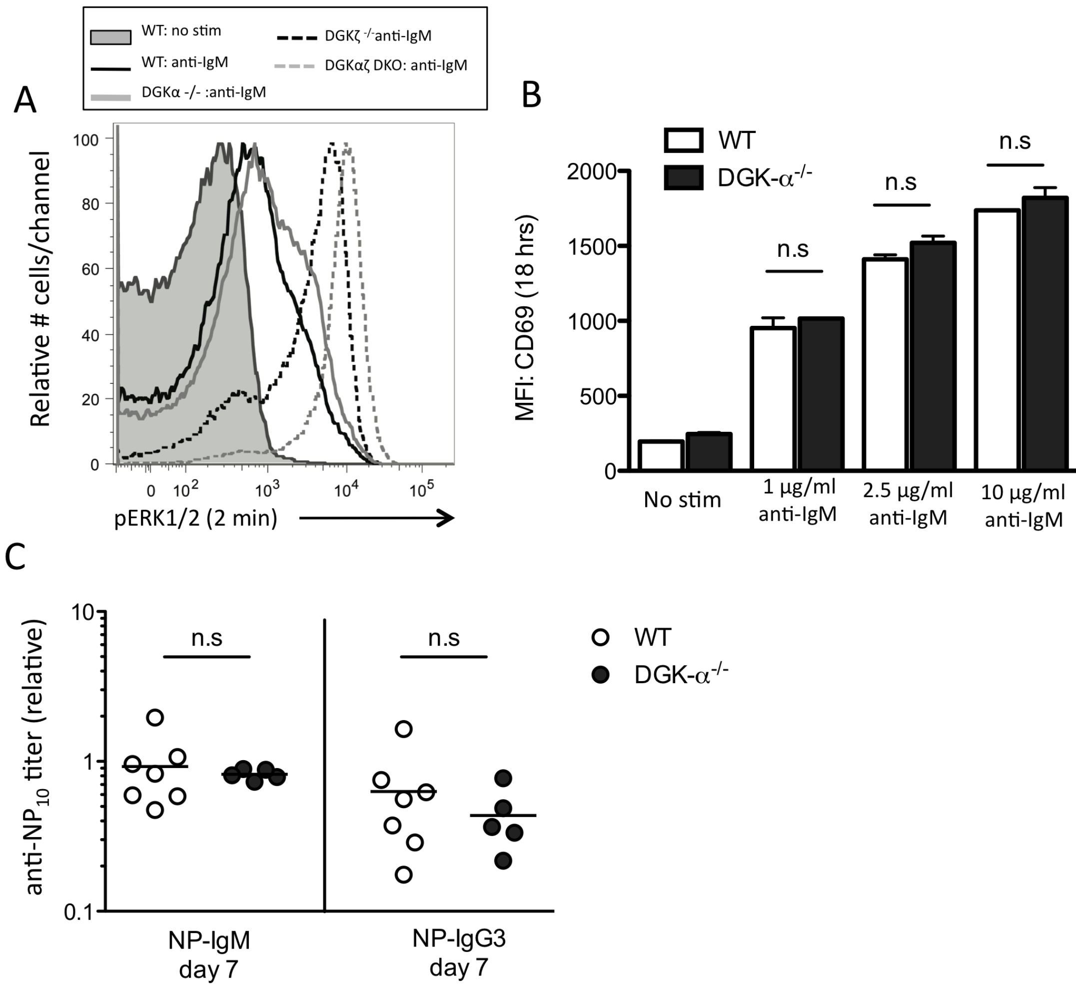
To assess T cell-independent antibody production, mice were injected intraperitoneally with 10 μg NP-AECM-Ficoll (Biosearch Technologies Inc.). Pre-immune and immune sera (day 4 and 7) were collected, and NP-specific IgM and IgG3 titers were measured by

ELISA as described (205). Total naïve serum immunoglobulin was captured with goat anti-mouse antibodies against IgM, IgA, or total IgG (Southern Biotech), and detected with isotype-specific HRP-conjugated anti-mouse antibodies. Serum concentration was extrapolated from a standard curve generated for each isotype.

Numbers of NP-specific and total IgM and IgG secreting plasma cells were determined by ELISPOT. Briefly, splenocytes from naïve or NP-Ficoll immunized mice were resuspended in complete Iscove's medium containing 10% FBS, and incubated overnight in wells of multiscreen HTS 96 well filter plates (Millipore) that were previously coated with NP_{10}-BSA or with anti-mouse capture antibodies against IgM and IgG. The following day, plates were washed of cells and spots were detected with HRP-conjugated anti-mouse IgM, total IgG, or IgG3 (Southern Biotech) and developed with a 3-amino-9-ethylcarbazole (AEC) chromogen kit (Sigma), according to manufacturer's instructions. Spots were counted with an ELISPOT reader.

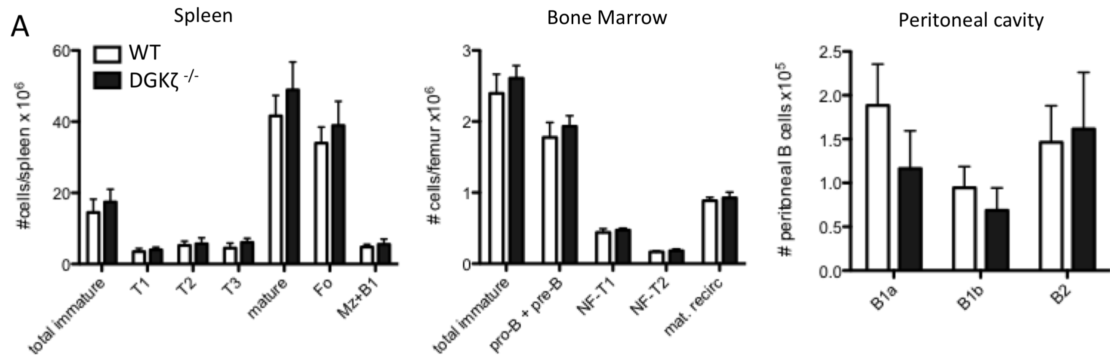
Supplementary figures related to Chapter 3

Figure S1. DGKα plays a minor role in negative regulation of BCR-induced ERK activation, but does not limit B cell activation or T independent type II antibody responses. (A) ERK activation in follicular B cells from WT, DGKα$^{-/-}$, DGKζ$^{-/-}$, and DGKα/ζ$^{-/-}$ mice stimulated for 2 minutes with 50 μg/ml anti-IgM F(ab')$_2$. Data are representative of 2 independent experiments using pooled cells from n = 3 mice. (B) BCR-induced CD69 upregulation on WT and DGKα$^{-/-}$ purified splenic B cells stimulated with indicated doses of anti-IgM for 18h *in vitro*. Data are representative of two experiments with n = 3 mice/group (C) Serum NP-specific IgM and IgG3 titers measured by ELISA from day 7 NP-ficoll immunized WT and DGKα$^{-/-}$ mice. Data are representative of two independent experiments. Each dot represents a single mouse. n.s = not significant.



A
WT: no stim
WT: anti-IgM
DGKα -/- :anti-IgM
DGKζ -/- anti-IgM
DGKαζ DKO: anti-IgM
Relative # cells/channel
pERK1/2 (2 min)
B
WT
DGK-α-/-
MFI: CD69 (18 hrs)
n.s
No stim
1 μg/ml anti-IgM
2.5 μg/ml anti-IgM
10 μg/ml anti-IgM
C
anti-NP10 titer (relative)
n.s
NP-IgM day 7
NP-IgG3 day 7
WT
DGK-α-/-

Figure S2. B cells from DGKζ$^{-/-}$ mice develop normally but have reduced surface BCR levels. (A) B cell development and maturation in the bone marrow, spleen and peritoneal cavity of WT (white bars) and DGKζ$^{-/-}$ (black bars) mice. Data are representative of 3 independent experiments with n = 5 mice/group. In the spleen and bone marrow, immature and mature B cells subsets were distinguished by surface B220, IgM, CD93 (AA4.1), and CD23 using the Allman protocol as described (205). In the peritoneal cavity, B cells were defined as IgM^{+} and IgD^{+} and were further were separated into B1-a (B220^{int-lo}, CD5^{hi}), B1-b (B220^{lo}, CD5^{lo}), and B2 (B220^{hi}, CD5^{lo}) subpopulations. Total numbers were back calculated from the percentage of total white blood cells obtained from a single femur (bone marrow), total spleen, or peritoneal lavage. (B) Surface IgM levels on splenic T1, T2, T3 and follicular B cells were measured from the median fluorescence intensity (MFI) of signal by flow cytometry. Data are representative of two independent experiments with n=4 mice/group. ***$p<0.001$ (student's T-test).


A
Spleen
WT
DGKζ -/-
#cells/spleen x 10^6
total immature
T1
T2
T3
mature
Fo
Mz+B1
Bone Marrow
cells/femur x10^6
total immature
pro-B + pre-B
NF-T1
NF-T2
mat. recirc
Peritoneal cavity
peritoneal B cells x10^5
B1a
B1b
B2

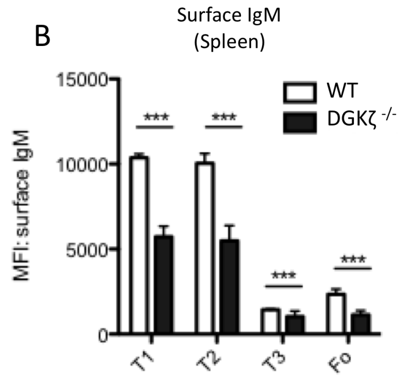
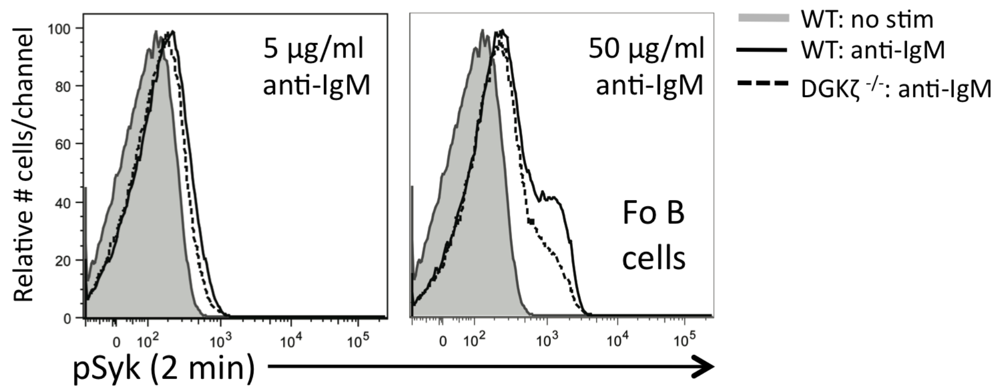

B
Surface IgM
(Spleen)
WT
DGKζ -/-
MFI: surface IgM
***
T1
T2
T3
Fo

Figure S3. DAG-independent proximal BCR signaling is mostly normal in DGKζ$^{-/-}$ follicular B cells. DAG-independent signaling in DGKζ$^{-/-}$ follicular B cells. Flow cytometric measurement of Syk phosphorylation (A) and intracellular calcium mobilization (B) in response to stimulation with 5 and 50 μg/ml anti-IgM F(ab')$_2$, and comparing the response of WT and DGKζ$^{-/-}$ follicular B cells. Data are representative of 2 independent experiments using pooled cells from n = 3 mice.

A



B

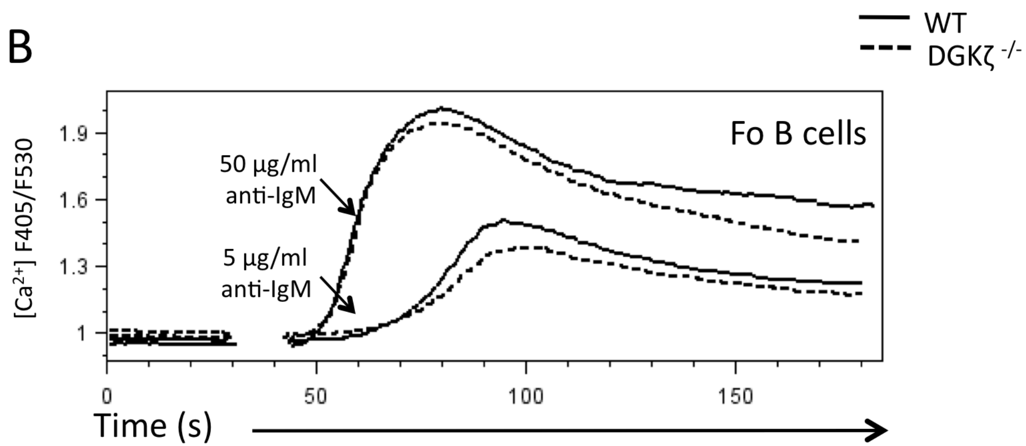
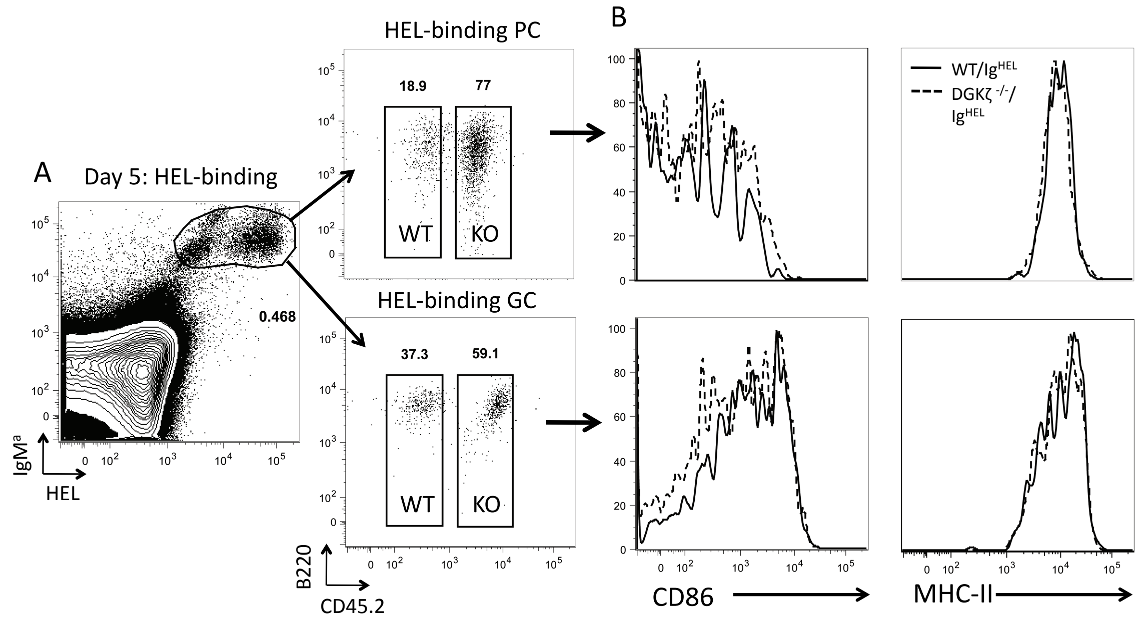


Figure S4. DGKζ deficiency does not alter CD86 or MHC II levels on HEL-binding plasmablasts or GC B cells. Adoptive co-transfers of WT and DGKζ$^{-/-}$ congenically marked MD4/Ig^{HEL} B cells were performed as in Fig. 11B followed by immunization with HEL^{2X}-SRBC. (A) Gating strategy to define total HEL-binding cells (left), and WT/Ig^{HEL} (CD45.1) and DGKζ$^{-/-}$/Ig^{HEL} (CD45.2) cells within the HEL-binding plasmablast (top) and germinal center (bottom) populations on day 5 following HEL^{2X}-SRBC immunization. Plasma cells were defined as CD4^{neg}, B220^{lo}, cytoplasmic IgMa^{hi}, cytoplasmic HEL^{hi}. HEL-binding GC B cells were defined as CD4^{-}, B220^{hi}, cytoplasmic IgMa^{int}, cytoplasmic HEL^{int}. (B) Representative histograms comparing the levels of CD86 (left panels) and MHCII (I-A^{b}) (right panels) between WT and DGKζ$^{-/-}$ HEL-binding plasmablast (top) and germinal center (bottom) B cell populations. Data are representative of 2 independent experiments with n = 5-6 mice.


A
Day 5: HEL-binding
0.468
IgMa
HEL
HEL-binding PC
18.9
77
WT
KO
HEL-binding GC
37.3
59.1
WT
KO
B220
CD45.2
B
WT/IgHEL
DGKζ -/-/ IgHEL
CD86
MHC-II

Chapter 4

Conclusions and future direction related to B cell ROS production and DGK-dependent inhibition of DAG signaling

Summary

In Chapters 2 and 3 of this thesis I have described unique mechanisms by which BCR signaling is fine tuned to ultimately influence the response of B cells to cognate antigen stimulation. In Chapter 2, I described a potential mechanism by which BCR signaling through the PI3K pathway can be amplified, or sustained, through B cell intrinsic production of reactive oxygen species, whereas in Chapter 3, I Identified a unique inhibitory mechanism involving metabolism of the second messenger DAG, resulting in decreased BCR signaling through the ERK-MAPK pathway. Taken together, these studies highlight the importance of interplay between signaling amplification and inhibition downstream of the BCR to maintain a balanced response to antigen stimulation. Ultimately this balance is also critical for providing a safeguard against the activation of self-reactive B cells that have the potential to contribute to autoimmune disease, as well as protecting against development of B cell lymphoma. In this chapter, I discuss future directions to extend both of these studies that will provide a better understanding of the mechanisms that control these two modes of BCR signal regulation, as well as address the physiological relevance of these pathways in the context of productive humoral immunity and disease.

Identifying the mechanism by which ROS promote PI3K signaling.

In Chapter 2, I was interested in determining if the feedback inhibitory pathways that are engaged in response to BCR stimulation could be countered by B cell-intrinsic production of ROS as a mechanism to promote BCR signal amplification in response to cognate antigen stimulation. The mechanism by which ROS have previously been shown to promote receptor signaling is through their ability to oxidize catalytic-site cysteine residues within tyrosine phosphatases such as SHP-1 and the lipid phosphatase PTEN, to render them transiently inactive (111). Although my findings do not support a role for BCR-induced ROS production in amplification of proximal BCR signaling, the finding that blocking accumulation of ROS at later times specifically attenuated signaling through the PI3K/AKT pathway suggests that PTEN may be a potential target of the sustained production of ROS generated in response to BCR stimulation. Future directions for this project will be to utilize various biochemical and genetic strategies to determine if PTEN is in fact a target for oxidation by ROS, and if this has any biological relevance for B cell responses to antigen.

Measurement of a protein's oxidation status is not trivial, and furthermore the methods for this type of analysis are not well established. Whereas specific phosphorylations of proteins can be assessed by the use of antibodies that recognize these modifications, antibodies that can specifically recognize oxidized cysteine residues do not currently exist. One method that has been reported makes use of a chemical compound known as dimedone, which reacts with cysteine sulfenic acid (SOH) groups that are formed upon oxidation of cysteine thiol groups (SH) by ROS (206). Biotinylated derivatives of this

compound have been generated which can theoretically be used to assess the cysteine oxidation status of any protein, assuming that the protein can be successfully immunoprecipitated (207, 208). Given that blocking ROS production/accumulation in my experiments only appeared to affect signaling downstream of the PI3K pathway, and that this effect was really only relevant to long term maintenance of signaling through this pathway, it will be important to determine the oxidation status of PTEN at these later stages of B cell activation (between 6-24 h). A previous study utilizing similar methods found that PTEN is oxidized downstream of epidermal growth factor receptor (EGFR) signaling, and that this oxidation transiently inhibits it's lipid phosphatase activity (109). Therefore, a reagent such as biotinylated dimedone could be used to label oxidized cysteines residues in cell lysates prepared from BCR-stimulated B cells stimulated for 6-24 h in the presence or absence of NAC to scavenge ROS. Immunoprecipitation of PTEN from these lysates could then be performed, followed by detection with strepavidin-conjugated fluorophores or horseradish peroxidase by standard western blotting methods. Furthermore, oxidation of PTEN has been shown to result in the formation of an intramolecular disulfide bond between Cys-124 and Cys-71 (110), which causes this protein to migrate faster than it's reduced counterpart on an SDS-PAGE gel under non-reducing conditions. The later method can be used with standard mobility-shift western blotting techniques to determine if the oxidized version of PTEN appears at these later stages of B cell activation, when the effects of ROS scavenging on PI3K signaling are the greatest.

Genetic approaches to understanding how ROS regulate PI3K signaling downstream of the BCR

The main issue with the techniques described above is that they must be performed very carefully under anaerobic conditions, such as in a nitrogen chamber, to prevent further oxidation within the protein lysate. Thus, proper controls must be used to ensure specificity of this probe for oxidation by ROS produced in response to cellular stimulation. This includes being able to show that the oxidized version of PTEN only appears in BCR-stimulated B cell lysates, or B cells treated with H_2O_2, and also that pre-treating BCR-stimulated cells with NAC to block intracellular ROS accumulation prevents formation of oxidized PTEN. However, even with these methods it will still be difficult to determine if any oxidation that occurs is truly inhibiting the phosphatase activity of PTEN. Use of phosphatase assays to compare the activity of immunoprecipitated oxidized vs. reduced PTEN has been described (109, 110), however these assays are purely *in vitro,* and therefore may not accurately reflect the intracellular effects of PTEN oxidation. One method to indirectly test if PTEN phosphatase activity is required to limit PI3K signaling and prevent proliferation in NAC pre-treated B cells, would be to assess the effect of PTEN deficiency on the response to BCR stimulation in the presence of ROS scavengers. If the hypothesis is correct, that the sustained production of ROS is needed to keep PTEN activity low and PI3K signaling high, then there should be little or no effect of NAC-pretreatment on the response of PTEN-deficient B cells to BCR stimulation. This could be accomplished by deleting PTEN specifically in the B cell lineage using MB1-Cre knockin mice crossed to the PTEN-

flox/flox background, as these mice are viable and do not have any major abnormalities in B cell development (209, 210) (and unpublished observations).

Identification of the intracellular source(s) of the ROS produced at later stages of B cell activation downstream of the BCR is also an important and interesting area for further investigation. The data presented in Chapter 2 of this thesis indicate that the most likely source is the mitochondria, however additional experiments will be required to test this hypothesis. A recent study described mice that can be conditionally deleted for a component of complex III of the mitochondrial respiratory chain, *Uqcrfs*, (and thus lack mitochondrial ROS production) (211). Surprisingly this study found that T cell-specific deletion of this protein did not have any major effects on T cell development, nor did it affect *in vitro* and *in vivo* proliferation in response to cytokine stimulation. In contrast, T cells from these mice were unable to proliferate in response to TCR stimulation, and furthermore did not expand *in vivo* in response to immunization with the LCMV peptide GP61, or following infection with *Listeria monocytogenes*, ultimately making them more susceptible to infection. It is not clear however if the defects observed in *Uqcrfs* deficient T cells were specifically due to the loss of mitochondrial ROS production, or to some other effect of disrupting mitochondrial respiration. Despite this uncertainty, these results fit well with my findings that the prolonged production of ROS, presumably from the mitochondria, is specifically required for B cell proliferation in response to BCR stimulation, but not in response to innate stimuli such as TLR stimulation, or T helper cell derived signals (CD40 and IL-4). It will be interesting to determine if B cells deficient in this protein behave similarly to NAC treated B cells, and furthermore if B

cell-specific deletion of *Uqcrfs* leads to impaired antibody responses *in vivo*, which would provide more definitive evidence to support my *in vitro* observations.

Final thoughts related to BCR-induced ROS production

The experiments described above will be important to the field of BCR signaling, as there has been some degree of controversy, as well as conflicting results, surrounding the true function of ROS production downstream of the BCR. Furthermore, having a better understanding of the mechanism by which sustained ROS production impacts BCR signaling could be highly relevant to diseases such as SLE or B cell lymphoma. Previous studies have shown that malignant cells from T cell acute lymphoblastic leukemia (T-ALL) patients have elevated levels of intracellular mitochondrial-derived ROS, and this was found to be required for maintaining constitutive PI3K/AKT signaling through a mechanism involving PTEN inhibition (212). Whether B cell lymphomas show similar characteristics, or dependence on elevated ROS production, remains to be determined. It will also be interesting to determine if circumstances that give rise to elevated ROS levels, such as deficiency in intracellular antioxidant proteins like the peroxiredoxins (213) results in B cell hyperresponsiveness that could exacerbate autoimmune disease in mouse models of SLE, or contribute to the progression and/or survival of lymphomas. Furthermore, given that autoimmune diseases are generally characterized by high levels of inflammation, the production of ROS by bystander cells such as phagocytes could have the potential to enhance activation of autoreactive cells, given that H_2O_2 is able to freely diffuse between membranes. Thus, a large amount of investigation will be required to determine the true biological function of ROS production

in B cells, as well as the more general role for these molecules in mediating intracellular signaling events in any given cell type under normal and diseased conditions.

Conclusions and future directions related to DGK-ζ inhibition of DAG signaling.

The results presented in Chapter 3 of this thesis showed a critical role for the enzyme Diacylglycerol kinase-ζ in limiting BCR signaling through the ERK-MAP kinase pathway, and for regulating the magnitude of the early extrafollicular plasmablast response. This is the first study to investigate the role of DGK enzymes in B lymphocyte responses, and therefore a number of important questions need to be addressed regarding the physiological relevance of these enzymes for proper B cell function. In particular, while my findings clearly show a strong inhibitory role for DGK-ζ in the T cell-independent and T cell-dependent antibody responses, there is still a question as to why this inhibitory mechanism is in place. If loss of this enzyme leads to more robust antibody production, then presumably this would be more protective during an infection. This section will discuss the potential beneficial functions of DGK-ζ in limiting DAG signaling downstream of the BCR as a means to improve the quality of the antibody produced. In addition the potential role of DGK enzymes in protecting against the development of autoimmune diseases such as SLE, as well as B cell lymphoma will also be discussed.

Role of DGK-ζ in improving the quality of the T cell-dependent antibody response

The initial observations that provided the motivation for this project came from earlier studies of a former postdoctoral fellow in the lab, Andrew Gross, which showed that as B

cells develop from immature "transitional" stages, to the mature follicular stage in the spleen, their capacity to signal downstream of BCR engagement was severely diminished (176). This was true both for intracellular calcium mobilization, as well as for BCR induced activation of the ERK signaling pathway as described in Chapter 3, and furthermore was mediated in part by upregulated expression and activity of components of the Lyn-CD22-SHP-1 inhibitory pathway. The results presented in Chapter 3 extend these observations by showing B cell maturation is also accompanied by upregulated mRNA expression of DGK-α and DGK-ζ, and that the attenuated phospho-ERK response of mature follicular B cells is largely mediated by the actions of these enzymes, in particular DGK-ζ.

The differential expression and/or activity of inhibitory pathways during B cell maturation likely plays an important role both in promoting B cell tolerance mechanisms, as well as fine tuning the humoral immune response. For example, the heightened sensitivity of immature B cells to BCR crosslinking may permit more efficient induction of tolerance programs such as receptor editing in the bone marrow or clonal deletion in the spleen, to ensure that potentially autoreactive cells will not contribute to the mature B cell pool. On the other hand, the reduced sensitivity of mature B cells for BCR signaling is likely important to establish an activation threshold in the periphery to limit responses of B cells with autoreactive specificity or with low initial antigen affinity, while enriching for higher affinity B cells to contribute to the response. Inhibition of BCR-induced DAG signaling by DGK-ζ may also be relevant to the germinal center response by promoting more efficient affinity discrimination to select for cells containing mutations that confer

high affinity specificity for foreign antigen. Thus a critical function of DGK-ζ may be to improve the overall quality of antibodies produced throughout the course of the T cell-dependent response by increasing the stringency for selection early during the extrafollicular response as well as later in the germinal center.

There are a number of approaches one can take to study the quality of an antibody response. In most cases "quality" refers an antibody's affinity for antigen, and whether or not it possesses neutralizing properties, which is a desirable property of antibodies produced during most viral infections (1). To determine if the overall quality of antibody decreases in the absence of DGK-ζ in B cells, mixed bone marrow chimeras could be generated by reconstituting lethally irradiated mice with a mixture of bone marrow from B cell-deficient (μMT) mice with WT or DGK-ζ deficient mice at a ratio of 80:20 respectively. This would give rise to mice in which all of the B cells will be derived from either WT or DGK-ζ-deficient bone marrow, while the majority (80%) of the other hematopoietic cells will come from the μMT bone marrow, and thus are DGK-ζ sufficient. This system would allow for separation of the effect of DGK-ζ deficiency in the B cell lineage from other cell types such as T cells or dendritic cells during the T cell-dependent antibody response. An alternative approach would be to generate mixed bone marrow chimeras as above, except to use a 50:50 mixture of donor cells from mice in which the heavy chain of the BCR is marked by the "A" allotype (IgH^a), with cells from either WT or DGK-ζ deficient mice that express the "B" allotype heavy chain (IgH^b). In this situation WT (IgH^a) and DGK-ζ-deficient (IgH^b) B cells will be responding in the same host, and thus will receive equivalent T cell help. Furthermore, the antibodies

derived from each of the donor cell population can be effectively distinguished with allotype-specific detection antibodies by standard ELISA methods. While both approaches have their limitations, both can be used to address the B cell-intrinsic role for DGK-ζ in determining the overall quality of the antibody response to T cell-dependent antigen immunization.

The chimeras described above could then be immunized with the T-dependent model antigen, NP-CGG, after which levels of NP-specific IgM and IgG antibodies in the serum would be tracked by ELISA, and the overall affinity of the antibody for NP could be measured using one of a number of different methods. The most common approach for measuring affinity in this system is to look at binding efficiency of serum antibody to NP conjugated with BSA at low (1:1 or NP_1) or high (20:1 or NP_{20}) densities by ELISA. Only the high affinity fraction of antibody will stay bound to NP_1-BSA (and therefore will be detected), whereas both low and high affinity antibody will bind NP_{20}-BSA. The ratio of NP_1 to NP_{20} binding by serum antibody will therefore increase as the antibody affinity increases. This ratiometric measure could be used to determine the relative affinity of antibody produced from extrafollicular plasma cells during the early stages of the response (i.e. day 5), as well as that derived from the germinal center reaction (day 7 and on). If the hypothesis that inhibition of BCR signaling by DGK-ζ is important to enrich for higher affinity cells for the early protective wave of antibody produced from the extrafollicular response is correct then the NP_1/NP_{20} ratio should be reduced in situations where the B cell compartment lacks DGK-ζ. Furthermore, if affinity maturation in the germinal center is compromised by loss of DGK-ζ, this ratio would also

be reduced at later times (i.e. day 14-21), and also would not increase with time as should occur for the WT response.

An alternative, and potentially more physiological, approach would be to use the μMT mixed bone marrow chimeras described in the previous section in a viral infection model to look at the production of neutralizing antibodies in situations where the B cell compartment lacks DGK-ζ. The "neutralizing" property of an antibody is defined as the ability to block viral entry into host cells, and this property increases with antibody affinity and/or avidity (1, 214). In the case of cytopathic viral infection models such as vesicular stomatitis virus (VSV), neutralizing IgM antibody is produced within the first 5 days of infection, and is absolutely required for host protection, as mice lacking B cells die within the first week of VSV infection (215, 216). Given the timing of this response, the neutralizing antibody generated against VSV must be derived from the rapid extrafollicular plasma cell response, which is described in detail in chapters 1 and 3 of this thesis. Because the extrafollicular response does not involve affinity maturation in the germinal center, efficient protection against this virus therefore will likely depend on selection of the highest affinity B cells early on to give rise to antibody secreting cells. Although my results show that the extrafollicular response is more robust in the absence of DGK-ζ, I believe that the reduced threshold for activation of DGK-ζ-deficient cells will result in less stringent early selection, and therefore a poorer quality of antibody produced. The VSV infection model would be ideal to test this hypothesis, and I would expect that mice in which the B cell compartment lacks DGK-ζ would have overall higher titers of anti-VSV antibody, but reduced titers of neutralizing antibodies against

this virus, and potentially compromised survival or viral clearance. The titer of neutralizing antibodies against VSV can be determined by incubating the virus with serial dilutions of serum from infected mice, and then looking at the ability of the virus to infect a host cell line using a plaque forming assay. This would provide direct evidence that increasing the threshold for BCR signaling is important for increasing the stringency for selection of high affinity B cells for the early extrafollicular response. On the other hand, to determine if DGK-ζ is important for mediating efficient selection during the germinal center reaction, I would use the non-cytopathic lymphocytic choriomeningitis virus (LCMV) infection model, as neutralizing antibodies in this model are typically not seen until around day 60 post infection (217, 218), which is consistent with a requirement for affinity maturation. Therefore, if the absence of DGK-ζ results in less stringent selection in the germinal center, I would expect that mice in which the B cell compartment lacks DGK-ζ would have reduced titers of IgG anti-LCMV neutralizing antibodies at these later times and as well as compromised viral clearance.

In addition to the production of high affinity neutralizing antibody, a high quality humoral immune response is also characterized by the formation of memory B cell as well as long lived plasma cells, which together have the potential to provide protection against re-infection for the lifetime of the host (124). The formation of both memory B cells and long lived plasma cells is typically thought to be a property of the germinal center reaction, although populations of memory B cells are known to be generated independently of the GC during T cell-independent and T cell-dependent responses (122, 219-222). The formation of memory B cells in the mice described above could be

indirectly assessed by measuring overall titers, as well as the affinity or neutralizing ability of antibodies produced upon secondary immunization or infection (50-60 days after the primary response). It is unclear what one would expect to see for this type of response when DGK-ζ is absent in the B cell compartment, as the role of BCR signaling in memory B cell formation is not entirely clear. However, it is possible that enhanced and/or premature differentiation of GC B cells into short-lived plasma cells will occur at the expense of memory B cell and long-lived plasma cell formation. Interestingly, a previous study looking at the response of $CD8^+$ T cells to LCMV infection found that deficiency in DGK-α or DGK-ζ resulted in enhanced primary CD8 responses, but poor memory responses following secondary infection (185). Whether or not this will also be true for B cells deficient in DGK-ζ remains to be tested, however if this is the case then this would provide an important clue about the physiological role of DGK-ζ in promoting efficient humoral immunity.

In summary, the experiments described above will provide a great deal of insight into the role of DGK-ζ, as well as the general role of inhibitory pathways downstream of the BCR, in shaping the antibody response. Furthermore, these studies may demonstrate the importance of maintaining a fine balance between the quantity and quality of antibody produced to provide efficient protection against pathogens.

Role of DGK enzymes in protection against autoimmune disease and B cell lymphoma

-Autoimmunity in DGK-deficient mice

In addition to the potential role of DGK-ζ in shaping the antibody response discussed above, the most obvious reasons why this inhibitory pathway would be present in follicular B cells may be to limit the activation of self-reactive B cells and/or protect against development of B cell lymphoma. As mentioned previously, deficiency in inhibitory molecules such as components of the Lyn-CD22-SHP1 inhibitory pathway lead to loss of peripheral B cell tolerance resulting in the appearance of anti-nuclear antibodies as well as many immunopathological features of SLE (103-105). Furthermore, loss of other inhibitory molecules such as combined deficiency of PTEN and SHIP in the B cell lineage has been shown to cause B cell lymphoma in mice as well as defects in B cell tolerance (53, 55, 56). In addition, survival of malignant cells in some subsets of diffuse large B cell lymphoma is known to be mediated by constitutive signaling downstream of the BCR (106). Whether or not inhibition of DAG signaling by DGK's is important for preventing some of these unwanted consequences of deregulated BCR signaling is unknown, and will be the focus of this section.

Preliminary data looking for the presence of autoreactive anti-dsDNA antibodies in the serum of aged mice (by ELISA) so far has not revealed any significant effects of deficiency in DGK-α or DGK-ζ alone. However, mice deficient in both DGK-α and ζ do develop moderate levels of double-stranded DNA-specific IgM antibodies around 8-10 months (data not shown). Thus, while the phenotype is somewhat mild, this does suggest

a potential for defects in B cell tolerance in the absence of these enzymes. Furthermore, these mice have been maintained on a relatively autoimmune resistant background (C57BL/6) which could mask, to some degree, the effects of DGK deficiency. Lastly, the presence of autoantibodies against other nuclear antigens such as chromatin or RNA in these mice still needs to be addressed before any conclusions regarding B cell tolerance defects can be made. Therefore, a more detailed analysis of B cell tolerance should be performed using transgenic mouse models of self-antigen exposure, as well as other models of SLE to determine if lack of DGK's either individually, or in combination, results in loss of B cell tolerance and/or exacerbation of lupus-like autoimmune disease.

In order to determine if there is a B cell intrinsic role for DGK-ζ in maintaining peripheral B cell tolerance, a mixed bone marrow chimera approach, similar to what was discussed above, would be utilized to minimize any effects of DGK deficiency in other hematopoietic cell lineages. For simplicity, all of the experiments discussed below will make use of mixed bone marrow chimeras generated from an 80:20 mixture of μMT and WT or DGK-ζ deficient bone marrow to give rise to mice that lack DGK-ζ primarily in the B cell compartment. As a first approach I propose to use BCR transgenic models such as the MD4/ML5 system (223) in which HEL serves a soluble self-antigen for HEL-specific MD4 B cells, or the 3H9/Vκ8 transgenic system (224) in which B cells express a BCR that recognizes single stranded DNA (ssDNA) with low affinity. In both systems, WT transgenic B cells that develop and mature in the presence of self-antigen are rendered anergic, and therefore cannot give rise to autoantibodies against HEL or DNA. However, if B cell tolerance is compromised, as I would expect in the absence of DGK-ζ,

then anti-HEL or anti-ssDNA antibodies will be readily detected in the serum of these mice. For both transgenic systems, an 80:20 mixture of μMT and WT or DGK-ζ deficient Ig transgenic bone marrow cells would be used to reconstitute lethally irradiated WT or ML5 (sHEL transgenic) recipients. Effects of DGK-ζ-deficiency on the anergic phenotype of Ig transgenic B cells could be measured *ex vivo* by comparing signaling events such as calcium flux and ERK phosphorylation from B cells that developed in the presence or absence of self-antigen (for the MD4/ML5 system). Given that T cells deficient in either DGK-α or DGK-ζ have been reported to be more resistant to TCR induced anergy induction *in vitro* (184, 225), it is highly possible that anergy will also be compromised in B cells lacking one or both of these enzymes.

The BCR transgenic models described above would be highly useful to dissect the role of DGK-mediated inhibition of DAG signaling on maintenance of peripheral B cell tolerance, however these models may not recapitulate the full range of B cell tolerance mechanisms that occur on a daily basis with a diverse repertoire of BCR specificities. The most efficient way to determine if B cell tolerance is compromised in this situation is by assaying for the presence of serum anti-nuclear antibodies (ANA's). This could be done by ageing the μMT mixed bone marrow chimeras described in the previous section (except using non-transgenic BM from WT, DGK-ζ KO, or DGK-α/ζ DKO mice as donors) and screening for the presence of IgM and IgG ANA's by immunofluorescent staining of nuclei of HEp-2 cells. If ANA's are present when the B cell compartment lacks one or both DGK isoforms, then the specific self-antigens that are recognized by DGK-deficient B cells could be identified with protein microarrays that contain a number

of the self-antigens commonly observed in mice and humans with SLE (226). Furthermore, other features of SLE such as glomerulonephritis could be measured by assessing immune complex and complement deposition in the kidneys. As an additional approach, the bone marrow chimeras could be immunized with pristane, which is a saturated hydrocarbon that causes lupus-like autoimmune disease in mice (227), to determine whether DGK-deficiency in B cells leads to exacerbated disease as measured by readouts described above. This approach would be useful if spontaneous disease is not observed, or is mild in the absence of one or both DGK isoforms.

-B cell lymphoma and myeloma in DGK-deficient mice

In addition to the potential role of DGK-ζ in maintaining peripheral B cell tolerance, another possible protective function of DAG metabolism by DGK's in B cells would be to protect against the development of B cell lymphoma. Indeed, combined heterozygosity for DGKα and ζ has previously been shown to promote T cell derived thymic lymphomas in a TCR transgenic mouse model (183). Mice deficient in either DGK-α, DGK-ζ, or both do not appear to spontaneously develop B cell lymphomas within the first year of life (unpublished observations), however, this is not entirely surprising given that these types of malignancies typically require a number of oncogenic hits for full disease development. Thus, loss of DGK enzymes alone may not be sufficient to drive disease. However, given that both ERK and NFκB activation are driven by the second messenger DAG, and both of these signaling pathways are implicated in the survival of malignant cells in diffuse large B cell lymphoma (68, 228, 229), it is reasonable to predict that loss of DGK-dependent inhibition of these pathways

in combination with other mutations could drive or exacerbate disease. This could be tested in various mouse models of B cell lymphoma such as the Eμ-Myc transgene model in which the oncogene *c-Myc* is expressed under control of the immunoglobulin heavy chain μ enhancer (230). This transgene gives rise to a mixture of different subtypes of B cell lymphoma with characteristics that resemble B cells at various stages of B cell development and activation (231), and could therefore be used in combination with DGK-deficiency to determine if this leads to more rapid onset of disease.

One particular B cell-derived malignancy that would be interesting to investigate in the context of DGK-deficiency is multiple myeloma (MM), which is a cancer that arises from antibody-secreting plasma cells. One of the most predominant features of DGK-ζ-deficient mice was the large increase both in basal and immunization-induced plasma cell numbers, which appeared to be a result of increased plasmablast proliferation and survival. Therefore, the loss of DGK-dependent inhibitory function could potentially drive this disease in combination with other mutations. Another piece of evidence supporting this hypothesis is the observation that DGK-ζ deficient plasmablasts express higher levels of Bcl-XL protein, which is commonly upregulated in MM cell lines as well as primary patient samples, where it has been shown to confer a survival advantage for these cells (232-235). Furthermore, in a different transgenic a mouse model in which c-Myc is driven by the C-α immunoglobulin heavy chain enhancer, plasma cell tumors that resembled human MM were only observed if a Bcl-XL transgene was coexpressed, indicating an important role for this survival factor in promoting this subset of malignancies (236). It would therefore be interesting to determine if plasma cell tumors

could also be initiated when this c-Myc transgene is expressed on a DGK-ζ-deficient background instead of in combination with the Bcl-XL transgene.

Final thoughts on the role of DGK's in disease

If the potential for development of SLE or malignancy were observed upon loss of DGK-mediated inhibition, it would be interesting to screen patients with autoimmune disease and/or lymphoma for inactivating mutations in one or more of these enzymes. On the other hand, constitutive activation of DGK's could result in B or T cell immunodeficiencies that could potentially be reversed by small molecule inhibitors of DGK enzymatic activity. Collectively, the experiments described in this section, in combination with the results presented in Chapter 3 of this thesis, will be important for understanding how tight regulation of DAG signaling downstream of the BCR promotes productive humoral immunity while minimizing immunological disease.

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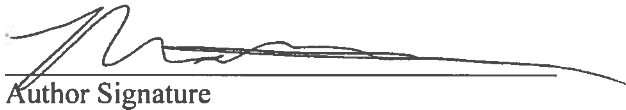

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