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

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Abstract

Signaling downstream of the B cell antigen receptor (BCR) is tightly regulated to allow for cells to gauge the strength and duration of antigen interactions and respond accordingly. In this study, we asked if metabolism of the second messenger diacylglycerol (DAG) by diacylglycerol kinase enzymes (DGKs) played a role in modulating the magnitude of signaling by this second messenger downstream of the BCR. In the absence of DGK ζ , 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 DGK ζ 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 DGK ζ 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.

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 (1). 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- γ 2 (PLC- γ 2) to generate the

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second messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG) (2-4). Whereas IP₃ is required for calcium mobilization and activation of the NFAT family of transcription factors, DAG signals through PKC β and the Ras guanine exchange factor, RasGRP, leading to activation of the NF- κ B and Ras-MEK-ERK mitogen-activated protein kinase (MAPK) pathways respectively (5-10). 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 (11). 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 (12, 13). 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 (5, 14). 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 (15), however its role in early formation of plasmablasts 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 (16), 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 (17). 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 (18-21).

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 DGK α and DGK ζ during B cell maturation in the spleen. Interestingly, while mature follicular B cells from mice deficient in DGK ζ exhibited enhanced ERK-MAPK signaling and had a reduced threshold for BCR-induced activation and proliferation, ablation of DGK α showed a lesser effect. In addition, in vivo experiments revealed that DGK ζ 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 DGK ζ -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 DGK ζ 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 (16). 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 (22). 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. 1A). In contrast, this inhibitor did not further enhance BCR-induced ERK activation in immature T1 or T2 B cells (Fig. 1A).

We next examined expression of *DGK α* and *DGK ζ* 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 showed nearly 2-fold higher expression of *DGK α* and *DGK ζ* mRNA than immature transitional T1 and T2 B cells sorted from spleens of WT mice (Fig. 1B). Notably, BCR stimulation of splenic B cells resulted in a substantial decrease in the abundance of *DGK α* mRNA within 4 hr of stimulation, as well as a smaller reduction in *DGK ζ* mRNA (Fig. 1C). The decrease in levels of *DGK α* mRNA following BCR stimulation was more pronounced in T2 and follicular B cells compared to T1 and T3 B cells sorted from the spleens of WT mice (Fig. 1C). Follicular B cells showed the largest decrease in *DGK ζ* mRNA following BCR stimulation, however there was less variability in mRNA reduction between the different B cell subsets for this isoform compared to *DGK α* (Fig. 1C). The decreased expression of DGK ζ and DGK α may be important to facilitate prolonged DAG signaling downstream of the BCR at later stages of activation of follicular B cells.

Next, we measured BCR-induced ERK phosphorylation in B cells from mice with targeted deletions of DGK α , DGK ζ , or both (19, 21). DGK α -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 DGK ζ -deficient follicular B cells (Fig. 1D and fig. S1A). The increased response of DGK ζ -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. 1D). Furthermore, this enhanced response was maintained at all time points tested (Fig. 1E). Similar to R59949 treatment, genetic deficiency in DGK ζ had only modest effects on BCR-induced ERK activation in T1 B cells (Fig. 1, D and E) indicating that DGK ζ -mediated regulation of DAG signaling was primarily a property of mature follicular B cells. Notably, DGK ζ -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. 1D). Follicular B cells from mice deficient in both DGK α and DGK ζ exhibited further enhancement of BCR-

induced ERK activation, indicating a secondary but significant role for DGK α in restraining BCR-mediated DAG signaling (fig. S1A). However, BCR-induced activation of mature B cells, as measured by surface CD69 induction, as well as antibody responses to the T cell-independent type 2 antigen NP-ficoll were unaffected by loss of DGK α , indicating a minor role for this isoform in restricting downstream responses to BCR stimulation (fig. S1, B and C). Given the dominant role of DGK ζ in limiting BCR-dependent 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 NF κ B activation (6), we examined degradation of the I κ B α inhibitory protein, which is required for nuclear translocation of NF κ B (23). I κ B α protein abundance was measured by intracellular staining and flow cytometry of permeabilized cells. BCR crosslinking of splenic B cells induced a rapid degradation of I κ B α in WT follicular B cells, which was clearly accelerated in DGK ζ -deficient follicular B cells, although by 30-60 min the amount of intracellular I κ B α was indistinguishable (Fig. 1F). Degradation of I κ B α in response to the TLR9 ligand CpG, which should be independent of DAG production (24), was not similarly affected (Fig. 1F), indicating specificity of DGK ζ for negative regulation of BCR-induced NF κ B activation. Similar to results obtained with intracellular staining and flow cytometry, measurement of BCR-induced ERK phosphorylation and I κ B α degradation by western blot of follicular B cells purified from lymph nodes of DGK ζ -deficient mice, also showed a substantial increase in ERK1/2 phosphorylation compared to WT follicular B cells, whereas the effect on I κ B α degradation was less pronounced (fig. S2).

Mice deficient in DGK ζ had normal numbers of the various B cell subpopulations in the bone marrow, spleen, and peritoneal cavity (fig. S3A). We did however observe a small but significant decrease in the amount of surface IgM present on all mature and immature splenic B cell subpopulations in the spleen of DGK ζ -deficient mice (fig. S3B). 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 DGK ζ -deficient follicular B cells (fig. S4, A and B), which may be a result of decreased surface BCR present on the cells.

DGK ζ deficiency reduces the threshold for BCR-induced activation and proliferation in vitro

As early BCR-induced ERK activation was strongly enhanced in DGK ζ -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 increase in the early activation marker CD69 was enhanced in DGK ζ -deficient B cells (Fig. 2A). Furthermore, in vitro proliferation of anti-IgM stimulated DGK ζ -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. 2B). In contrast, proliferation in response to stimulation with the TLR9 ligand, CpG oligonucleotide, or with anti-CD40 and IL-4 were minimally affected (Fig. 2B), 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. 2C), which is

downstream of Ras and ERK activation (25). The NF κ B-induced increase in *I κ B α* , which serves as a feedback inhibitory response for this pathway (26), as well as the survival factor *AI*, were not noticeably changed, however abundance of the survival factor *Bcl-XL*, which is also known to be a direct target of NF κ B (27) was moderately increased at later times in DGK ζ -deficient B cells (Fig. 2C). Taken together, these results demonstrate that DGK ζ 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.

DGK ζ -deficient mice have elevated serum IgM and enhanced responses to T cell-independent type 2 antigens

To determine if DGK ζ plays a role in limiting B cell activation in vivo, we first compared serum antibody titers between unimmunized WT and DGK ζ -deficient mice. DGK ζ -deficient mice had normal amounts of class-switched Ig isotypes in the blood, but a roughly 5-fold increase in IgM titers (Fig. 3A). Analysis of the spleen showed that DGK ζ -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. 3B). These results suggest that inhibition of DAG signaling by DGK ζ limits plasma cell generation or survival in the spleen.

We next looked at the response of DGK ζ -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 (28). DGK ζ -deficient mice generated elevated serum titers of NP-specific IgM and IgG3 on both day 4 and 7 after immunization (Fig. 3C), although the amount of NP-specific IgM was also substantially elevated in unimmunized DGK ζ -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. 3D), indicating an accelerated early plasma cell response. It must be noted however, that while amounts of NP-specific IgM and NP-IgM secreting plasma cells were elevated at all time points tested in DGK ζ -deficient mice, when measured as a fold change over the unimmunized controls, these differences were actually similar or somewhat reduced compared to WT mice. In contrast, the fold change in the amount of NP-specific IgG3 and numbers of NP-IgG3 secreting plasma cells was roughly 3-10 \times higher in DGK ζ -deficient mice compared to WT mice immunized with NP-ficoll. These results indicate that DGK ζ functions to limit the early response to antigens with strong BCR crosslinking properties.

DGK ζ limits early expansion as well as germinal center and plasma cell formation induced by T cell-dependent antigen immunization

We next wanted to determine whether inhibition of BCR-induced DAG signaling by DGK ζ 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 DGK ζ -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 (29, 30). In vitro stimulation of Ig^{HEL} transgenic B cells with intermediate affinity

HEL (HEL^{2×}) resulted in robust ERK phosphorylation in WT Ig^{HEL} follicular B cells, and this response was enhanced nearly 10-fold in DGK ζ -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^{3×}), which binds the Ig^{HEL} BCR with 50-fold less affinity than HEL^{2×} (30), was unable to induce significant ERK phosphorylation in WT B cells, however it induced a maximal phospho-ERK response in approximately 20% of DGK ζ -deficient B cells (Fig. 4A). These results indicate that in the absence of DGK ζ , the affinity threshold for antigen-induced BCR signaling to ERK is substantially reduced.

To compare the response of WT and DGK ζ -deficient Ig^{HEL} B cells to T cell-dependent antigen immunization, WT mice received a 1:1 mixture (1 \times 10⁵ total) of congenically marked WT (CD45.1⁺) Ig^{HEL} and DGK ζ -deficient (CD45.2⁺) Ig^{HEL} purified splenic B cells followed by immunization with intermediate affinity HEL^{2×}, or low affinity HEL^{3×}, conjugated to SRBCs (Fig. 4B). 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. 4C). Analysis of the allelic expression of these cells revealed a clear but modest enrichment of DGK ζ -deficient B cells within the HEL-binding population in response to both intermediate and low affinity HEL-SRBC immunization (Fig. 4, C and D). Furthermore CFSE labeling of transferred cells showed that these differences resulted from more robust proliferation rather than enhanced survival or engraftment of DGK ζ -deficient transferred cells (Fig. 4E). These results indicate that DGK ζ plays a role in limiting early B cell expansion in vivo in response to T cell-dependent antigens.

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 (31). 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^{2×} and HEL^{3×}-SRBC, indicated a further skewing in favor of DGK ζ -deficient cells, which comprised roughly 75% of the total HEL-binding GC B cell population (Fig. 5A and B). However, an even more pronounced advantage was seen within the donor-derived HEL-binding plasmablast compartment after immunization with either HEL^{2×}-SRBC or HEL^{3×}-SRBC (Fig. 5, B and C), as indicated by a roughly 8-12 fold enrichment for DGK ζ -deficient cells compared to WT cells with the same immunogen (Fig. 5B). The magnitude of the HEL-specific GC and plasmablast response was enhanced following immunization with intermediate affinity HEL^{2×} compared to low affinity HEL^{3×} for both WT and DGK ζ -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. 5, D and E) in agreement with previous reports (30, 32). Notably, deficiency in DGK ζ shifted the magnitude of the GC and plasmablast responses induced by immunization with low affinity HEL^{3×}-SRBC to a degree similar to that of WT cells responding to intermediate affinity antigen (fig 5, D and E) as

measured by the number of HEL-binding GC and plasmablast phenotype cell present in the spleen following immunization with the different affinity variants. Importantly, the differences observed were antigen specific, and were not due to an intrinsic ability of DGK ζ -deficient B cells to undergo spontaneous activation, as DGK ζ -deficient Ig^{HEL} B cells did not proliferate or differentiate into GC B cells or plasmablasts in the absence of immunization with HEL-SRBC's (fig. S5, A to C). Thus a critical function of DGK ζ is to promote affinity discrimination during the early phases of the T cell-dependent antibody response, and especially to limit early plasmablast formation or maintenance.

DGK ζ -deficient plasmablasts have enhanced proliferative capacity and higher amounts of the survival factor Bcl-XL

We next wanted to address whether there were differences in proliferation or survival of DGK ζ -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 DGK ζ -deficient cells undergoing active DNA synthesis on day 5 following immunization with HEL^{2 \times} -SRBC (Fig 6A). While these results indicate that DGK ζ -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 DGK ζ -deficient cells over WT within the plasmablast population. We therefore asked whether enhanced BCR signaling in the absence of DGK ζ resulted in differences in the abundance of the pro-survival factor Bcl-xL, as measured by intracellular staining and flow cytometry (Fig 6B). Whereas the amount of Bcl-xL was similar between WT and DGK ζ -deficient GC B cells (Fig. 6, B and C), there was a significant increase in Bcl-xL in donor-derived HEL-binding DGK ζ -deficient plasmablasts compared to their WT counterparts after HEL^{2 \times} -SRBC or HEL^{3 \times} -SRBC immunization (Fig. 6, B and C). Furthermore, consistent with the impaired survival capacity of plasmablasts generated in response to low affinity antigen immunization (32), Bcl-xL was reduced in WT plasmablasts generated from HEL^{3 \times} compared to HEL^{2 \times} -SRBC immunization (Fig. 6C). Interestingly, the amount of Bcl-xL present in DGK ζ -deficient plasmablasts remained high regardless of the initial antigen affinity (Fig. 6C). The increase in Bcl-xL seen in DGK ζ -deficient plasmablasts is in agreement with in vitro data showing that BCR stimulation induced higher mRNA induction of this survival factor in naïve DGK ζ -deficient B cells (Fig. 2C). Surface expression of the costimulatory molecule CD86, as well as MHC class II was similar between WT and DGK ζ -deficient GC B cells and plasmablasts generated from HEL^{2 \times} -SRBC immunization (fig. S6, A and B), suggesting that the effects of DGK ζ -deficiency on the early plasmablast response were unlikely due to superior antigen presentation to cognate T cells. Instead, these results point to an important role for DGK ζ , and the strength of BCR-signaling through DAG-dependent pathways, in mediating early plasmablast proliferation and survival based on the affinity of the antigen-BCR interaction.

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 PIP₂ by PLC- γ 2. 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 DGK ζ 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 DGK ζ -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- κ B. Whereas enhancement of DAG signaling by ablation of DGK ζ led to substantially enhanced ERK signaling in mature follicular B cells, the effect on NF- κ B activation was primarily kinetic in that I κ B α 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 (25), was enhanced in DGK ζ -deficient B cells, there was not greater induction of *I κ B α* or *AI*, which are both thought to be a direct targets of NF- κ B (26). Thus, the Ras-ERK pathway was highly sensitive to changes in the amount of DAG present following BCR engagement, whereas the NF- κ B 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 the amount of DAG present was unexpected, and future studies to define the mechanism of this differential sensitivity will be of considerable interest.

Deletion of DGK ζ enhanced ERK signaling in mature follicular B cells, and this signaling was further enhanced in B cells with combined deletion of DGK ζ and DGK α . Thus, although DGK α 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. DGK α deficiency enhanced in vitro responses to BCR stimulation to a minimal degree, and had little effect on in vivo responses to NP-Ficolin immunization, however it is possible that DGK α plays a more prominent role in regulation of DAG signaling in situations that were not analyzed in these experiments. Given that DGK α exhibited dramatic changes in mRNA expression during B cell development in the spleen, and following BCR stimulation, we speculate that its expression may be increased to attenuate ERK signaling in some circumstances. In this regard, we note that DGK α is important for antigen-induced anergy in T cells (19, 33, 34).

The use of DGK ζ -deficient B cells allowed us to probe the effects of different amounts of DAG signaling on B cell activation. These B cells were more sensitive to anti-IgM stimulation in vitro for CD69 induction and for proliferation. Taken together with previous evidence that CD69 is a Ras-regulated gene in T cells (35), and that MEK inhibitors block BCR-induced B cell proliferation and surface CD69 induction in vitro (14), these results are in agreement with the hypothesis that enhanced ERK signaling in DGK ζ -deficient B cells makes these cells more sensitive to BCR engagement for activation. We also observed

enhanced proliferation of HEL-specific DGK ζ -deficient B cells in vivo during the first three days following immunization with HEL² \times -SRBC, indicating that a similar regulation occurs during immune responses.

Among the most striking effects of DGK ζ -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 early 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 (36). 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 (37), and also was found to inhibit Pax5-dependent repression of Blimp1 transcription (38). 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 (12). Whether any of these mechanisms are responsible, in part, for the more rapid plasmablast response of DGK ζ -deficient mice to NP-Ficoll will require additional study.

We also saw a very large increase in early plasmablast numbers in DGK ζ -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 (29). In this system, increasing the affinity of antigen for the BCR dramatically increases the magnitude of the early antibody response, and this has been shown to be largely mediated by enhancing proliferation and survival of plasmablasts (30, 32). 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 DGK ζ -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 early 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 DGK ζ -deficient B cells, and we also did not observe any difference in the amount of surface MHC class II or the T cell costimulatory molecule CD86 on DGK ζ -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 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 DGK ζ -deficiency, versus the more common approach of completely ablating signaling reactions, for example by deletion of ERK1 and ERK2 (12, 15). 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 DGK ζ increases the elevation of DAG that occurs upon BCR engagement, but it may attenuate somewhat the amount 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 (39). 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 (40), 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 in lymphocytes 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* (41) 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 DGK ζ -deficiency on early B cell development. In addition, Guo *et al* (42) 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 be 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 (43-45). To what extent these alternate pathways for ERK activation downstream of the BCR are sensitive to changes in the amount of DAG present in the cell is not entirely clear. Mice lacking Bam32 have a substantial defect in T-independent type II antibody responses (43, 45), and have impaired germinal center maintenance in response to T cell dependent antigens (43, 45). 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 DGK ζ 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 DGK ζ is an important mechanism for limiting the numbers of short-lived plasmablasts generated that secrete 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 antibody responses by the presence of DGK ζ in B cells may protect against autoimmunity (46) or lymphoma development (47).

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. DGK $\alpha^{-/-}$ (*Dgka^{tm1Xpz}*) and DGK $\zeta^{-/-}$ (*Dgkz^{tm1Gak}*) mice were generated as previously described (19, 21) 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 DGK $\zeta^{-/-}$ 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.* (48) (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 (1×10^5 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 1×10^6 cells prior to immunization. SRBCs (Colorado Serum Company) were labeled with HEL^{2 \times} or HEL^{3 \times} (20 μ g/ml final concentration) on the day of immunization as described (30). 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 2×10^8 freshly conjugated HEL^{2 \times} - or HEL^{3 \times} -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 DGK ζ ^{-/-} 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 μ l PBS) on day 5 following HEL-SRBC immunization and sacrificed 60 min later. For *in vitro* proliferation analysis, purified WT and DGK ζ ^{-/-} splenic B cells were suspended in complete Iscove's medium with 10% FBS (1 \times 10⁶ cell/ml) and stimulated at 37°C with 0.25-10 μ g/ml anti-IgM F(ab')₂ (Jackson immunoresearch), 250 ng/ml CpG (Integrated DNA Technologies), or 2.5 μ g/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 μ M 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 I κ B α were performed as described (49). Briefly, 4 \times 10⁶ splenocytes (2 \times 10⁷/ml) were pre-warmed at 37°C 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 μ M 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-I κ B α rabbit Ab (both from Cell Signaling Technology), followed by staining with anti-rabbit IgG-APC (Jackson Immunoresearch) (for p-ERK and I κ B α) 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 (16).

ELISA and ELISPOT

To assess T cell-independent antibody production, mice were injected intraperitoneally with 10 μ g 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 (49). 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 NP₁₀-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 Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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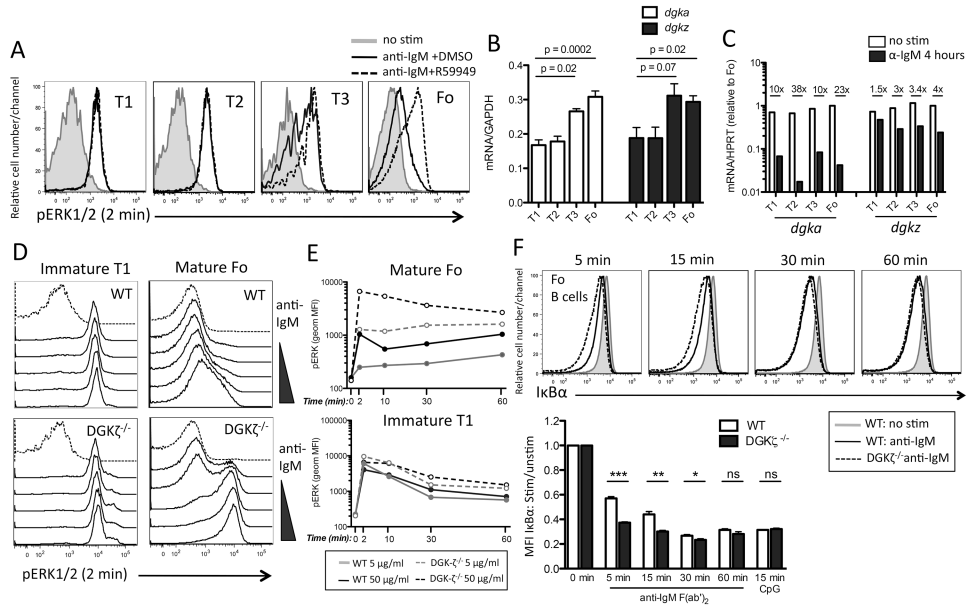


Figure 1. Activation of diacylglycerol-mediated signaling events is tightly regulated by DGK ζ in mature follicular B cells

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-int}), and follicular (CD93⁻, CD23⁺, IgM^{int-lo}) B cells following stimulation with 50 μ g/ml anti-IgM in the presence of 20 μ M R59949 or DMSO. (B) mRNA transcript expression of *DGK α* and *DGK ζ* 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 n=3 mice prior to cell sorting. Data are representative of 2 independent experiments. (C) Expression of *DGK α* and *DGK ζ* in response to BCR stimulation was measured in T1, T2, T3, and Fo B cells sorted from spleens of WT mice following *in vitro* stimulation for 4 hours with 10 μ g/ml anti-IgM F(ab')₂. Expression of each isoform was normalized to *HPRT* and is relative to expression in Fo B cells following BCR stimulation. B cells subsets were sorted from n=3 pooled WT spleens and data are representative of 2 independent experiments. (D) Comparison of BCR-induced ERK activation between WT and DGK ζ ^{-/-} 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 μ g/ml anti-IgM. (E) Time course (0-60 min) of BCR induced ERK activation in WT (solid lines) and DGK ζ ^{-/-} (dashed lines) T1 and follicular B cells stimulated with 5 μ g/ml (grey lines) and 50 μ g/ml (black lines) anti-IgM F(ab')₂. Relative amounts of phospho-ERK 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 intracellular I κ B α in Fo B cells following BCR stimulation of total splenic B cells from WT (solid black histogram) and DGK ζ ^{-/-} (dashed black histogram). Degradation of I κ B α was quantified (bottom) as the median fluorescence intensity of signal after stimulation with 50 μ g/ml anti-IgM F(ab')₂, or 500 ng/ml CpG ODN for the indicated times, and was normalized

to unstimulated samples. 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).

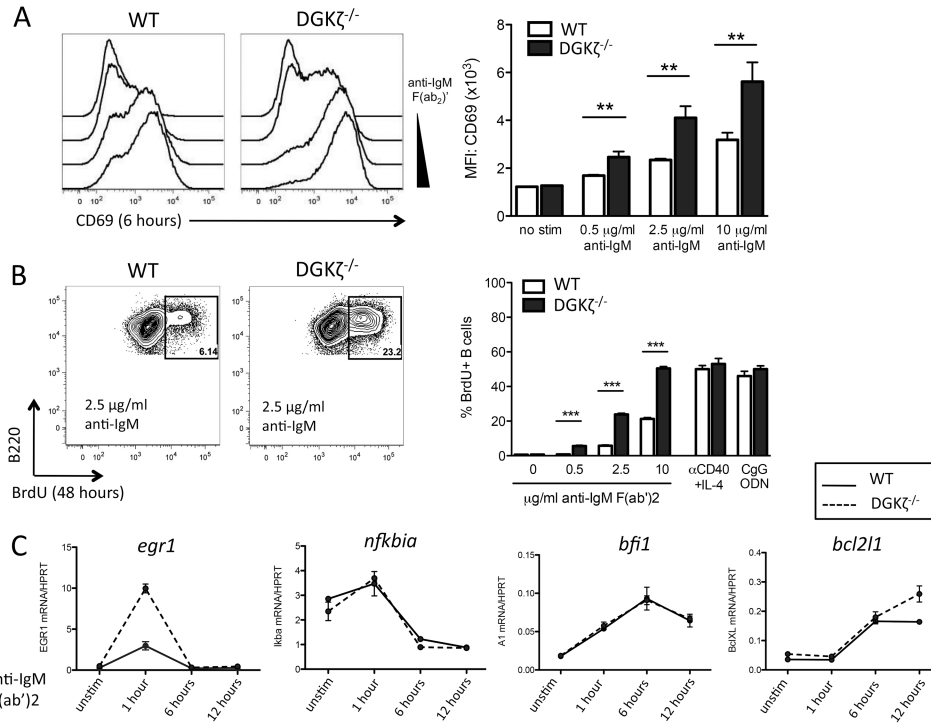


Figure 2. DGKζ deficiency reduces the threshold for BCR-induced activation and proliferation *in vitro*

(A) Early activation of WT and DGKζ^{-/-} purified splenic B cells measured by surface CD69 induction 6 hours after stimulation with increasing doses (0.5-10 μg/ml) of anti-IgM F(ab')₂ *in vitro*. Left panels show representative histograms for surface 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 36-48 hours 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*, *Al*, 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).

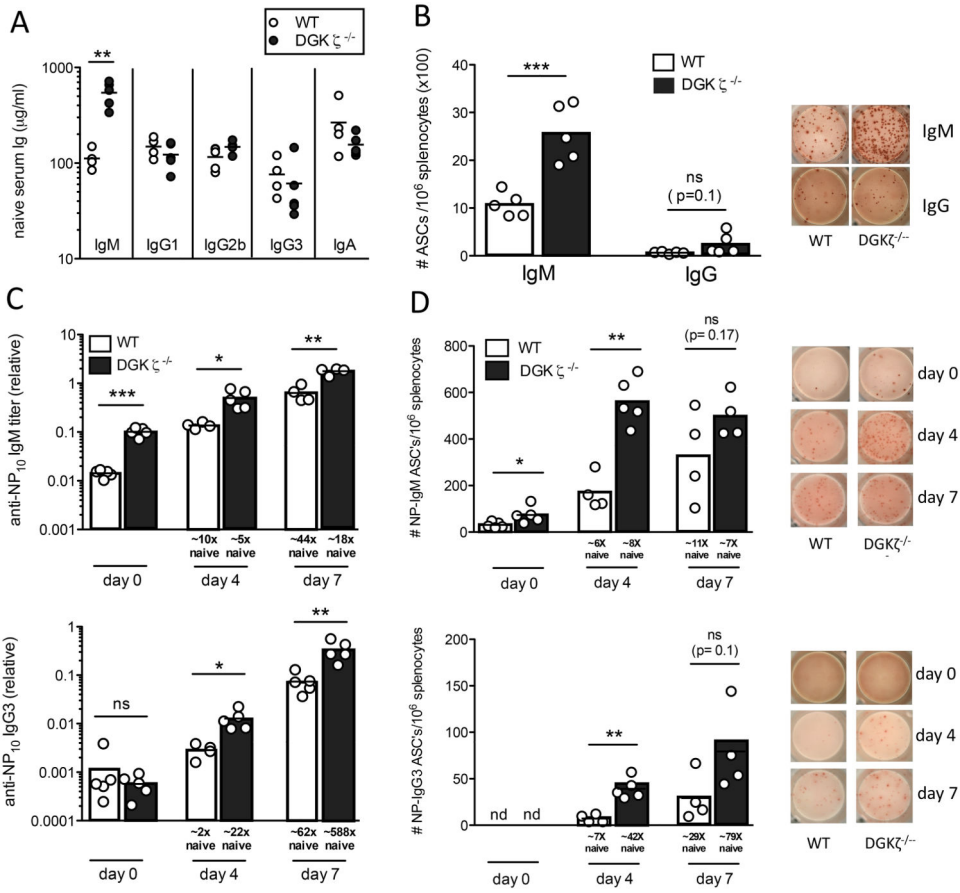


Figure 3. DGK ζ -deficient mice have elevated serum IgM and enhanced responses to T cell-independent type 2 antigens

Serum immunoglobulin titers were measured in unimmunized WT (open circles) and DGK $\zeta^{-/-}$ (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 DGK $\zeta^{-/-}$ (black bars) mice. Representative images of ELISPOT wells are shown in the right panels for total IgM and IgG ASC's. Data are representative of two separate experiments. (C-D) T-independent type 2 antibody response of DGK $\zeta^{-/-}$ 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 DGK $\zeta^{-/-}$ (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. The fold-increase in NP-specific Ig and ASC's was calculated by normalizing the average value at each time point to unimmunized levels. The fold increase in NP-IgG3 ASC's was determined by assigning a value of 1 for naïve WT and DGK $\zeta^{-/-}$ mice. Each circle represents an individual mouse for all data shown. *p<0.05, **P<0.01, ***p<0.001 (Student's *t*-test)

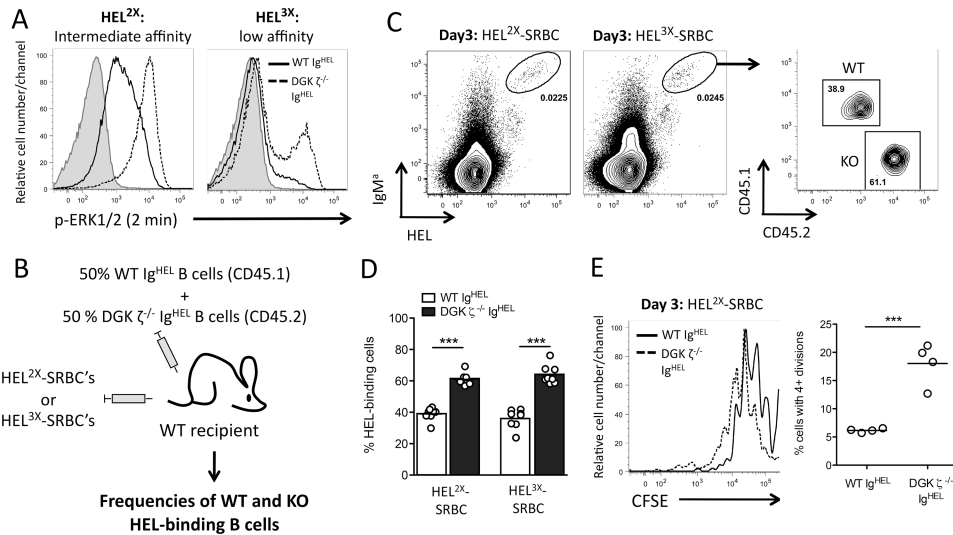


Figure 4. $DGK\zeta^{-/-}$ antigen specific B cells undergo enhanced early expansion in response to T cell-dependent antigen immunization

(A) BCR signaling induced by different affinity variants of HEL. ERK phosphorylation was measured by intracellular staining and flow cytometry as in Fig. 1D in WT/MD4 (solid black histograms) and $DGK\zeta^{-/-}$ /MD4 (dashed black histograms) splenic Fo B cells stimulated for 2 min with $1\mu\text{g}/\text{ml}$ of soluble $HEL^{2\times}$ (left) or $HEL^{3\times}$ (right). Data are representative of the response from pooled spleens of $n = 2-3$ mice, and similar results were found in a second experiment. (B) Experimental set up for measuring in vivo responses of WT/MD4 and $DGK\zeta^{-/-}$ /MD4 B cells. WT ($CD45.2^{+}$) mice received an equal mixture (1×10^5 total) of WT/MD4 ($CD45.1^{+}$) and $DGK\zeta^{-/-}$ /MD4 ($CD45.2^{+}$) purified splenic B cells followed by immunization with $HEL^{2\times}$ -SRBC or $HEL^{3\times}$ -SRBC. (C) Representative flow cytometry plots show staining of HEL-binding cells on day 3 following $HEL^{2\times}$ -SRBC or $HEL^{3\times}$ -SRBC immunization (left panels), and frequencies of WT and $DGK\zeta^{-/-}$ /MD4 B cells (right panel). Frequencies of WT/MD4 (white bars) and $DGK\zeta^{-/-}$ /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 $HEL^{2\times}$ -SRBC and $HEL^{3\times}$ -SRBC ($n = 5$ mice/group and representative of 3 experiments). (E) Proliferation of CFSE labeled WT/MD4 (solid black histogram) and $DGK\zeta^{-/-}$ /MD4 (dashed black histogram) HEL-binding B cells measured by CFSE dilution on day 3 following $HEL^{2\times}$ -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).

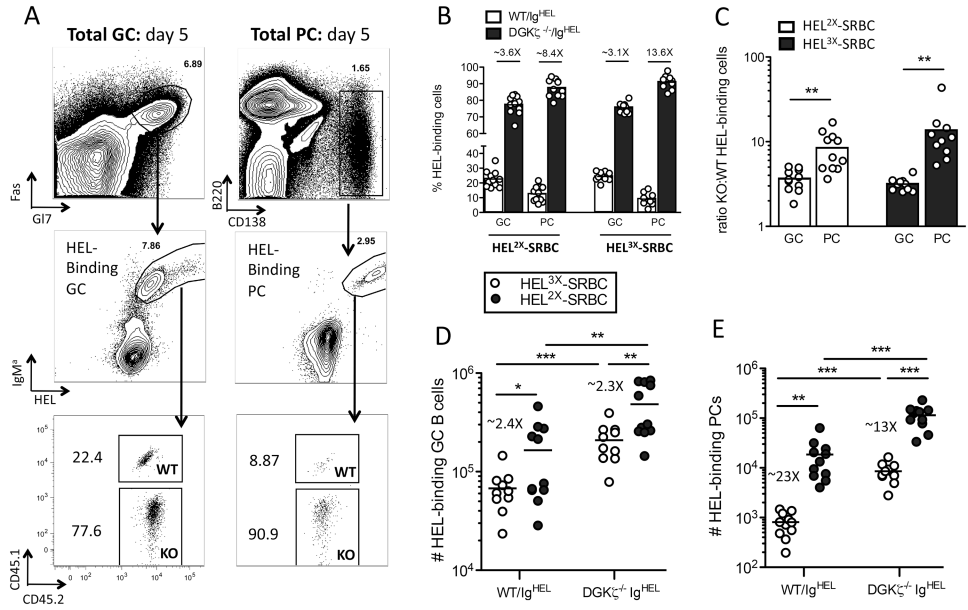


Figure 5. Deficiency in DGK ζ promotes robust early plasmablast and germinal center responses (A) Representative strategy for flow cytometric measurement of numbers of day 5 HEL-binding plasma cells (B220^{lo}, CD138^{hi}, IgM^{a-hi}, HEL^{hi}) and germinal center B cells (Fas^{hi}, GL7^{hi}, IgM^{a-int}, HEL^{int}) generated after HEL^{2x}-SRBC immunization using the same experimental design as illustrated in Fig. 4B, (B) Frequency of WT/MD4 (white bars) and DGK ζ ^{-/-}/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 ζ ^{-/-}/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 ζ ^{-/-}/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).

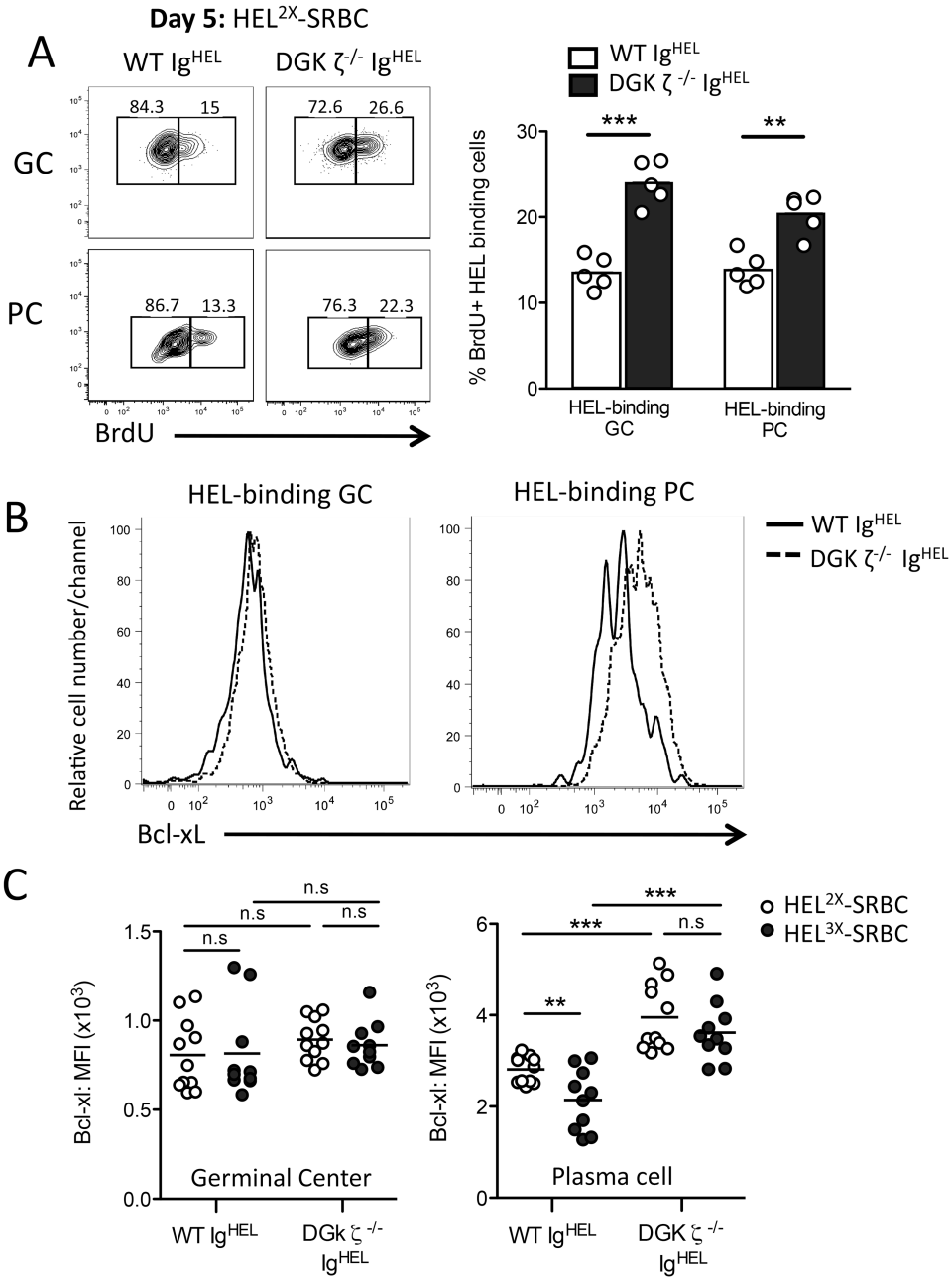


Figure 6. DGK ζ limits expansion of germinal center B cells and plasmablasts, and survival factor expression in plasmablasts

(A) Mice receiving equal mixtures of congenically marked WT and DGK $\zeta^{-/-}$ MD4 B cells were injected with BrdU on day 5 following immunization with HEL^{2X}-SRBC and BrdU incorporation was measured by flow cytometry within the HEL-binding germinal center and plasmablast populations. WT/MD4 and DGK $\zeta^{-/-}$ /MD4 cells were distinguished by CD45 alleles as in Fig. 5A, and the percentages of BrdU⁺ cells from each genotype were quantified within the HEL-binding germinal center and plasmablast population (right panel). Data are from n=5 mice and similar results were found in 2 independent experiment. (B) Representative histograms showing intracellular staining for Bcl-xL in day 5 WT/MD4 and

DGK $\zeta^{-/-}$ /MD4 HEL-binding germinal center B cells (left) and plasmablasts (right) generated in response to immunization with HEL^{2x}-SRBC. (C) Quantification of the amount of intracellular Bcl-xL (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).