Gsk3 is a metabolic checkpoint regulator in B cells

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B cells predominate in a quiescent state until an antigen is encountered, which results in rapid growth, proliferation and differentiation of the B cells. These distinct cell states are probably accompanied by differing metabolic needs, yet little is known about the metabolic control of B cell fate. Here we show that glycogen synthase kinase 3 (Gsk3) is a metabolic sensor that promotes the survival of naive recirculating B cells by restricting cell mass accumulation. In antigen-driven responses, Gsk3 was selectively required for regulation of B cell size, mitochondrial biogenesis, glycolysis and production of reactive oxygen species (ROS), in a manner mediated by the co-stimulatory receptor CD40. Gsk3 was required to prevent metabolic collapse and ROS-induced apoptosis after glucose became limiting, functioning in part by repressing growth dependent on the myelocytomatosis oncoprotein c-Myc. Notably, we found that Gsk3 was required for the generation and maintenance of germinal center B cells, which require high glycolytic activity to support growth and proliferation in a hypoxic microenvironment.

B cell responses are initiated by antigen uptake and presentation to CD4+ T cells, which in turn co-stimulate B cells via CD40 engagement and leads to the secretion of interleukins. Some of these antigen-primed B cells undergo further differentiation in the germinal center (GC), which is a unique microenvironment that coordinates the antigen-driven clonal selection of B cells. B cells proliferate and undergo somatic hypermutation in the histologically distinct ‘dark zone’ of the GC and subsequently migrate to the ‘light zone’ to bind antigen that is retained by resident follicular dendritic cells and to receive prosurvival and differentiative cues from follicular helper T cells. Although B cells in the dark zone express genes associated with cell division, B cells in the light zone show genetic signatures that are associated with B cell antigen receptor (BCR) and CD40 stimulation, as well as with c-Myc activity. The signaling events that mediate B cell selection in the GC are poorly understood and, as illustrated by c-Myc expression, probably apply to a small and temporally restricted fraction of B cells.

Although resting lymphocytes have low metabolic requirements, activated cells face increased energetic and biosynthetic demands to support cell growth, proliferation and effector function. In B cells, enhanced glycolytic activity has been observed after BCR, CD40 or interleukin 4 (IL-4) stimulation. The phosphatidylinositol-3-OH kinase (PI(3)K) signaling pathway has been implicated in regulating glucose catabolism after BCR stimulation, but it appears to be dispensable for IL-4-mediated glucose utilization. However, an understanding of many fundamental aspects of metabolic regulation in B cells is lacking. Specifically, it is unclear how B cell metabolism is maintained in the quiescent state, how cytokine- and BCR-induced signaling affect metabolic reprogramming and how B cell survival is affected in metabolically challenging situations.

Here we identified Gsk3 as a metabolic sensor that integrates cytokine-induced cell growth and proliferation with nutrient availability. Gsk3 is a ubiquitously expressed kinase with more than 50 known targets that can strongly affect cell differentiation, proliferation, survival and transformation. It is expressed in the α and β isoforms, which are highly homologous and show similar substrate specificities. Notably, Gsk3 is constitutively active in resting and nutrient-deprived cells, but it is disabled by phosphorylation-dependent degradation after growth factor stimulation. This phosphorylation event on Ser9 or Ser21 can be mediated by many kinases such as PKA, Akt, p70S6K and PKC. There is also evidence showing that Gsk3 activity promotes distinct outcomes on proliferation and cell fate, depending on the cell type and the effects of other signaling events.

Relatively little is known about the role of Gsk3 in lymphocytes, perhaps owing to the redundant functions of the α and β isoforms. In a previous study, we showed that Gsk3β is inactivated in a PKA-dependent manner in GC B cells, allowing for the accumulation of cyclin D3 and promoting proliferative expansion. Here we show that Gsk3 activity restrains cell mass accumulation in resting B cells, as well as B cell growth, metabolic activity and proliferation. This effect is most prominent after co-stimulation with CD40 and IL-4, suggesting that Gsk3 limits responses to T cell help. However, Gsk3 activity also attenuates ROS production to maintain the redox state and prevent apoptosis. These opposing roles of Gsk3 are critical for the regulation of the GC reaction.

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RESULTS

Germline center B cells face increased metabolic demands

Because GC B cells are under strong proliferative stress, we posited that they would have increased energy and nutrient demands to fuel biosynthesis. Indeed, we found that mouse GC B cells are larger (Fig. 1a) and have increased protein content (Fig. 1b), enhanced glucose uptake (Fig. 1c) and increased mitochondrial content (Fig. 1d) relative to those in follicular B cells. Because the GC microenvironment arises as a poorly vascularized site of intense cell proliferation, we also reasoned that it may be oxygen limited. In fact, injection of mice with pimonidazole, which forms thiol-containing protein adducts in hypoxic cells, correspondingly, GC B cells selectively expressed the transcription factor hypoxia-inducible factor 1α (HIF-1α), which drives the expression of several genes involved in glycolysis (Fig. 1e). Consistently with the idea of increased glucose uptake by GC B cells in comparison to resting B cells, inhibition of glycolysis with the hexokinase inhibitor 2-deoxy-D-glucose (2-DG) resulted in a significant decrease in the percentage of GC B cells (Fig. 1g), whereas the overall percentage of B cells, the ratio of CD4+ T cells to CD8+ T cells, and the percentage of follicular helper T (CD4+ PD1+ ) cells were not substantially altered (Supplementary Fig. 1a–c). Thus, glycolysis is an important feature of GC B cells, supporting anabolism in a hypoxic microenvironment.

Profiling BCR and metabolic signaling in the germline center

Signaling events that determine the individual fates of B cells within the GC are poorly understood. To interrogate GC B cell signaling, we performed mass cytometric analysis of intracellular effectors associated with BCR activation and metabolism. Gsk3 was of central interest because of its presumptive role in GC B cell growth, proliferation and survival through the regulation of c-Myc, cyclin D3 and Mcl-1 stability, respectively15. In addition to assessing Gsk3 inactivation, as determined by the level of phosphorylation on Ser9 and Ser21 of Gsk3 (phospho-Gsk3(Ser9,Ser21)), we also examined the abundance of c-Myc and Bcl-6, which regulate the transcription of numerous genes whose products are involved in metabolism (Fig. 1h). Enhanced protein synthesis driven by the mechanistic target of rapamycin complex 1 (mTORC1) pathway was measured by phosphorylation of ribosomal protein S6 on Ser235 and Ser236 (phospho-S6(Ser235,Ser236)). To assess BCR signaling, phosphorylation of phospholipase Cγ2 (PLC-γ2) on Tyr759, B cell linker (BLNK) on Tyr84 and extracellular-signal-regulated kinase (Erk) on Thr202 and Tyr204 was measured. For reference, anti-IgM-induced signaling was examined in marginal zone B cells (Supplementary Fig. 1d). The viSNE algorithm was used to identify cells in the GC that showed similar signaling profiles18. As expected, PLC-γ2 and BLNK identified a similar fraction of GC B cells (Fig. 1h). Although Bcl-6 was expressed by most GC B cells, c-Myc was mainly expressed by cells signaling via the PLC-γ2–BLNK pathway (Fig. 1h). Gsk3 inactivation and consequent β-catenin accumulation was found in a large fraction of GC B cells that encompassed BCR-activated cells (Fig. 1h and Supplementary Fig. 1e). Although Gsk3 has been reported to regulate both c-Myc accumulation and mTOR signaling, and thus S6 phosphorylation, S6 phosphorylation was confined to a small subpopulation of GC B cells that was also positive for...
Ig1 (Fig. 1h). Moreover, large amounts of c-Myc and phosphorylated S6 were found in distinct subsets of GC B cells (Fig. 1h), suggesting that mTOR- and c-Myc-dependent signaling pathways may function in parallel to regulate cell metabolism in the GC.

**B cell quiescence and homeostasis are promoted by Gsk3**

To directly address Gsk3 function in the regulation of B cell homeostasis and function, we intercrossed mice harboring loxP-flanked (floxed) Gsk3a (Gsk3afl/L) or Gsk3b (Gsk3bfl/L) alleles with Cd19Cre mice, which inducibly activate Cd19-Cre in mature B cells, splenic B cell lysates were subjected to immunoblot analysis for Gsk3. A small amount of Gsk3 was present in control B cell lysates, but normal follicular architecture in the spleen (Fig. 2a and Supplementary Fig. 2a) and lymph nodes (Fig. 2b and Supplementary Fig. 2b), but normal follicular architecture in the spleen (Supplementary Fig. 2c). Decreased B cell numbers can result from defective B cell generation, maturation or survival. Because Cd19Cre-mediated deletion is incomplete in early B cell development, we did not anticipate defects in B cell development. Consistently, pro-B (B220+CD43+BP1), large pre-B (B220+CD43+BP1+), small pre-B (B220+CD43+IgM-) and immature B (B220+CD43+IgM+) cells were present in 

The frequency of mature follicular (B220+CD21+IgM+) B cells was significantly increased, and the percentage of transitional T1 (B220+CD21+IgMhi) cells was slightly increased (Fig. 2c) relative to those in control mice. To determine the efficiency of Gsk3a and Gsk3b deletion in Cd19CreGsk3afl/LGsk3bfl/L B cells, splenic B cell lysates were subjected to immunoblot analysis for Gsk3. A small amount of Gsk3 was detectable in B cell lysates from Cd19CreGsk3afl/LGsk3bfl/L mice, which inducibly activate Cd19-Cre in mature B cells, Cd19CreGsk3afl/LGsk3bfl/L mice, respectively (Fig. 2d). However, residual expression of both Gsk3 isoforms was detected in cell lysates from Cd19CreGsk3afl/LGsk3bfl/L mice (Fig. 2d), suggesting a selective advantage for B cells that maintain Gsk3 expression. Moreover, Gsk3-deficient B cells also downregulated IgD (Supplementary Fig. 2e) and showed significant increases in cell size (Fig. 2e) and protein content (Fig. 2f) and a marked increase in glucose consumption (Fig. 2g), suggesting that Gsk3 may be required for B cells to persist in a quiescent state.

To better assess the acute role of Gsk3 in the survival of mature B cells, we crossed hCd20-TamCre mice, which inducibly activate Cre in mature B cells following tamoxifen treatment, to Gsk3afl/LGsk3bfl/L mice, resulting in efficient loss of Gsk3 protein after tamoxifen treatment in hCd20-TamCreGsk3afl/LGsk3bfl/L mice (Fig. 2h). The hCd20-TamCre mice and derived crosses also carry the Rosa26-flox-STOP-YFPfl-transgene, whose expression identifies cells that express Cre as positive for yellow fluorescent protein expression (YFP+). By comparing the decline in the percentage of YFP+ cells in hCd20-TamCre (Ctrl) and hCd20-TamCreGsk3afl/LGsk3bfl/L (double-knockout, or dKO) mice after tamoxifen treatment, we were able to assess the turnover of mature Gsk3-deficient B cells. In control mice, the percentage of YFP+ follicular B cells in the spleen on days 26 and 47 after tamoxifen treatment was ~77% and 60%, respectively (Fig. 2i),
which is consistent with the expected half-life of mature B cells. By contrast, in hCd20-TamCre;Gsk3aL/L;Gsk3bL/L mice, the percentage of YFP+ follicular B cells was ~35% and ~13% on days 26 and 47, respectively, relative to the frequency of YFP+ B cells in blood on day 7 after tamoxifen treatment (Fig. 2i). Although the frequency of YFP+ marginal zone B cells, which are known to be long-lived, was not significantly decreased in wild-type (WT) mice over the course of the experiment, the population of YFP+ marginal zone B cells in hCd20-TamCre;Gsk3aL/L;Gsk3bL/L mice was significantly (P = 0.0121) decreased on day 47 relative to those in control mice (Supplementary Fig. 2f). These findings indicated that Gsk3 was needed for the survival of both follicular and marginal zone B cells.

The IgM–IgD profile of Gsk3-deficient B cells was normal early after Gsk3 ablation, but IgD was substantially downregulated at later time points (data not shown). Thus, low IgD expression on Gsk3-deficient B cells is not due to impaired B cell maturation, but it may reflect a perturbed quiescent state. To evaluate the effect of acute versus developmental loss of Gsk3 expression, we measured spontaneous cell mass accumulation after inducing Gsk3 ablation in mature B cells. We found that the acute loss of Gsk3 caused a slow accumulation of cell mass (Fig. 2j). B cells from hCd20-TamCre;Gsk3aL/L;Gsk3bL/L mice were comparable in size to B cells from control mice (hereafter referred to as control B cells) 1 d after tamoxifen-induced Gsk3 deletion, but they slowly increased in size and were significantly larger than control B cells by day 14 after tamoxifen treatment (Fig. 2j). Thus, Gsk3 controlled the metabolic state and long-term survival of mature resting B cells.

Gsk3 is required for T cell–dependent B cell responses

We have shown that Gsk3 is phosphorylated on the inhibitory residue Ser9 in GC B cells. Therefore, to determine the role of Gsk3 in the GC response, we immunized hCd20-TamCre;Gsk3aL/L;Gsk3bL/L mice with the T cell–dependent antigen, sheep red blood cells (SRBCs). hCd20-TamCre;Gsk3aL/L;Gsk3bL/L mice showed an impaired immune response, as indicated by significantly reduced SRBC-specific IgG1 and IgM titers (Fig. 3a), slightly decreased percentage of plasma cells (Fig. 3b) and a significant reduction in the GC B cell compartment (Fig. 3c,d). Because the GC is the site of antibody affinity maturation, we performed additional immunizations with 4-hydroxy-3-nitrophenyl acetyl (NP) that was conjugated to keyhole limpet hemocyanin (KLH) at a conjugation ratio of 25:1 (NP32) and measured titers of high-affinity (NP4) and low-affinity (NP23) antigen-specific serum IgG and IgM. In contrast with control mice, hCd20-TamCre;Gsk3aL/L;Gsk3bL/L mice showed poor production of both high- and low-affinity antigen-specific antibodies (Fig. 3e). These data demonstrated that Gsk3 was required in B cells for humoral responses to T cell–dependent antigens.

CD40-induced B cell proliferation is inhibited by Gsk3

To determine whether naive Gsk3-deficient B cells were capable of differentiating into ‘induced’ GC B cells, we used a fibroblast feeder layer expressing membrane-bound CD40 ligand and secreted B cell–activation factor (BAFF) and added exogenous IL-4. We found that Gsk3-deficient B cells efficiently acquired a GC phenotype, as indicated by the upregulation of the cell surface receptor Fas and the GC marker GL7 (Fig. 4a). To analyze the effect of individual and combined stimuli on cell proliferation, we cultured B cells with anti-CD40 alone, anti-CD40 plus IL-4, anti-IgM or lipopolysaccharide (LPS). Gsk3-deficient B cells that were stimulated with anti-CD40 alone or with anti-CD40 plus IL-4 showed more proliferation than similarly treated control B cells (Fig. 4b,c and Supplementary Fig. 3a); however, anti-IgM- or LPS-induced proliferation was comparable to the proliferation of control B cells (Fig. 4b,c). Because B cells can be exposed to multiple stimuli in the GC, we mimicked these events by first stimulating Gsk3-deficient and control B cells with anti-IgM for 16 h and then adding anti-CD40, IL-4 and BAFF for an additional 3 d. Gsk3-deficient B cells cultured under these conditions showed more proliferation than that observed in WT cells (Supplementary Fig. 3b), suggesting that the presence of Gsk3 limits CD40-mediated proliferation in the context of other stimuli.

To confirm that the increase in CD40-mediated proliferation observed in vitro reflects the properties of Gsk3-deficient B cells in vivo, we administered an agonistic CD40-specific antibody by
Gsk3 inhibits CD40-induced B cell proliferation. (a) Expression of GL7 and Fas on induced GC B cells (iGBs) generated from the B cells of hCd20-TamCre or Gsk3a−/−Gsk3b−/− (control (Ctrl)) mice and Gsk3a−/−Gsk3b−/−hCd20-TamCre (dKO) mice (n = 3 per genotype) with the feeder cell line CD40LB and exogenous IL-4. (b,c) Proliferation (b) and average divisions (c) of B cells from control and dKO mice (as in a) after 3 d of culture with the various stimuli (above b) or below (c) plots. (d) B cell proliferation in Cd19Cre or Gsk3a−/−Gsk3b−/− (control (Ctrl)) mice and Gsk3a−/−Gsk3b−/−Cd19Cre (dKO) mice 3 d after injection of anti-CD40, as measured by BrdU incorporation (left); right, summary of results at left. (e) FSC-A values of B cells obtained from control and dKO mice (as in a) and left unstimulated (US) or treated overnight (treatment conditions, horizontal axis). Each symbol (c–e) represents an individual mouse; small horizontal lines indicate the mean. **P = 0.0093 (c), **P = 0.0043 (d) and **P = 0.0079 (e) (Mann–Whitney test). Data are representative of three (a), two (d,e) or three (LPS stimulation) four (anti-CD40 stimulation) or six (CD40+IL4 and anti-IgM stimulation) independent experiments.

CD40-induced metabolic activity is limited by Gsk3
To assess the role of Gsk3 in the regulation of B cell growth and metabolism, B cells were isolated from hCd20-TamCreGsk3a−/−Gsk3b−/− mice 7 d after tamoxifen treatment. At this time point, Gsk3-deficient B cells were comparable in cell size to unstimulated control B cells (Fig. 4e). After anti-IgM stimulation, the sizes of both Gsk3-deficient and control B cells increased similarly (Fig. 4e), indicating that Gsk3 does not regulate cell growth after anti-IgM stimulation. By contrast, Gsk3-deficient B cells were significantly larger than control B cells after stimulation with anti-CD40 plus IL-4 (Fig. 4e). Similarly, Gsk3-deficient B cells that were stimulated with anti-IgM, anti-CD40, IL-4 and BAFF were slightly larger than similarly treated WT B cells (Supplementary Fig. 3c). To determine whether glucose was differentially catabolized in control versus Gsk3-deficient B cells, we measured glucose consumption and lactate production in vitro. Unstimulated B cells showed little metabolic activity, but glucose consumption by both control and Gsk3-deficient B cells increased after anti-IgM stimulation (Fig. 5a). However, anti-IgM-stimulated Gsk3-deficient B cells produced lower amounts of lactate than control B cells (Fig. 5a). By contrast, Gsk3-deficient B cells stimulated with anti-CD40 plus IL-4 showed more lactate production and glucose consumption than control B cells (Fig. 5a). Notably, although the average number of cell divisions of B cells stimulated with anti-CD40 plus IL-4 was comparable to that of B cells that were stimulated with anti-IgM (1.1 versus 1.4, respectively; Fig. 4c), Gsk3-deficient B cells stimulated with anti-CD40 plus IL-4 produced more lactate than Gsk3-deficient B cells stimulated with IgM (Fig. 5a). This finding suggested that increased lactate production in Gsk3-deficient B cells that were stimulated with anti-CD40 and IL-4 was not simply a byproduct of increased proliferation. However, to exclude the contribution of accelerated proliferation to increased metabolic activity in Gsk3-deficient B cells, we used Seahorse XF technology to analyze oxidative phosphorylation and glycolysis in real time. The oxygen-consumption rate (OCR) was used as a measurement of oxidative phosphorylation, and the extracellular acidification rate (ECAR) was used as a measurement of glycolysis. After measuring the basal rate of respiration, we treated cells first with oligomycin to block mitochondrial ATP production and then with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) to induce maximal amounts of oxygen consumption and rotenone plus antimycin to inhibit the electron transport chain. We found that, after stimulation of the cells with anti-CD40 and IL-4, both basal and maximal rates of respiration were greater in Gsk3-deficient B cells than in WT B cells (Fig. 5b,c). Inhibition of ATP generation from respiration causes an increase in the rate of glycolysis to supply cells with energy. Anti-CD40- and IL-4-stimulated Gsk3-deficient and control B cells showed an increase in the ECAR after oligomycin inhibition, but the ECAR was slightly greater in Gsk3-deficient B cells under all of the conditions tested (Fig. 5b,c). Similar experiments were performed with anti-IgM-stimulated cells; however, no significant differences were observed between Gsk3-deficient and control B cells (Supplementary Fig. 4). These findings suggested that B cell activation was limited by Gsk3 through the inhibition of anti-CD40- and IL-4-induced metabolic adaptations.

Rapamycin sensitivity and c-Myc degradation are promoted by Gsk3
Our data demonstrated that Gsk3 limited cellular growth, metabolic activity and proliferation of anti-CD40- and IL-4-stimulated B cells. To determine the biochemical basis of these findings, we first focused on the role of mTORC1 as a central regulator of cell metabolism. Treatment with the mTORC1 inhibitor rapamycin efficiently inhibited anti-CD40- and IL-4-mediated proliferation and growth of control B cells, but it did not affect growth, and only marginally inhibited...
proliferation, of Gsk3-deficient B cells (Fig. 6a–c). In contrast to that observed after anti-CD40 and IL-4 stimulation, anti-IgM-stimulated Gsk3-deficient B cells showed little proliferation and a comparable cell size as that in control B cells after mTORC1 inhibition (Fig. 6d–f). To determine whether mTORC1 activity was enhanced in Gsk3-deficient B cells, we measured the abundance of mTORC1-phosphorylated p70S6K at Thr389, as well as p70S6K-phosphorylated ribosomal protein S6 at Ser235, Ser236, Ser240 and Ser244 in anti-CD40- and IL-4-stimulated B cells (Fig. 6g). Both p70S6K and S6 were phosphorylated in control and Gsk3-deficient B cells, and their phosphorylation was abolished after rapamycin treatment (Fig. 6g). By contrast, the Gsk3 target β-catenin was protected from degradation in Gsk3-deficient B cells, and β-catenin expression was unaffected by rapamycin treatment (Fig. 6g). These findings suggest that Gsk3-deficient B cells are able to proliferate and grow independently of S6 phosphorylation, and thus are less sensitive to inhibition of mTORC1.

Gsk3 is known to phosphorylate the transcription factor c-Myc, which facilitates c-Myc degradation26. Consistent with this finding, anti-CD40- and IL-4-stimulated Gsk3-deficient B cells had more c-Myc than that in control and freshly isolated B cells (Fig. 6a and Supplementary Fig. 5a,b). c-Myc accumulated to a similar degree in anti-IgM-stimulated Gsk3-deficient B cells and control B cells (Fig. 6h and Supplementary Fig. 5c), indicating that c-Myc stability was distinctly regulated downstream of BCR signaling versus that downstream of anti-CD40 and IL-4 stimulation. To address whether enhanced c-Myc expression could account for the altered growth of anti-CD40- and IL-4-stimulated Gsk3-deficient B cells, we compared the effects of c-Myc overexpression and Gsk3 loss in B cells. Mice overexpressing Myc specifically in B cells (Myc-tg mice) were generated by crossing Rosa26-StopMyc (ref. 27) and Cd19cre mice. In contrast to control B cells, naive B cells from Myc-tg mice (hereafter referred to as Myc-tg B cells) expressed substantial amounts of c-Myc protein (Fig. 7a). After B cell activation, c-Myc accumulated in both control and Myc-tg B cells (Fig. 7a); however, c-Myc abundance remained higher in stimulated Myc-tg B cells than in stimulated control B cells (Fig. 7a). Myc-tg mice showed normal B cell maturation (Supplementary Fig. 6); however, the cells were significantly larger than control B cells, indicating that Myc overexpression was sufficient to induce accumulation of cell mass in unstimulated mature B cells (Fig. 7b). Increased c-Myc expression was also associated with enhanced proliferation and cell size in response to anti-CD40 and IL-4 stimulation or, to a lesser extent, to anti-IgM stimulation (Fig. 7c,d). Thus, higher c-Myc expression functionally correlates with the selective responsiveness of Gsk3-deficient B cells to anti-CD40 and IL-4 stimulation.

c-Myc is known to drive a transcriptional program that promotes glycolysis28. Indeed, Myc-tg B cells showed more lactate production after stimulation with anti-CD40 and IL-4 or with anti-IgM than control B cells (Fig. 7e). Because most of the c-Myc-positive cells in the GC did not show phosphorylation of S6 (Fig. 1h), we sought to determine whether c-Myc could induce proliferation independently of mTORC1 signaling. To this end, we treated anti-CD40- and IL-4-stimulated or anti-IgM-stimulated B cells with rapamycin, which inhibited cell division in both anti-IgM-stimulated and anti-CD40- and IL-4-stimulated WT and Myc-tg B cells (Fig. 7f). Thus, unlike Gsk3-deficient B cells (Fig. 6), Myc-tg B cells remained sensitive to mTORC1 inhibition.

B cell survival under glucose restriction is promoted by Gsk3

Although Gsk3-deficient B cells showed increased metabolism, growth and proliferation in vitro, they failed to mount an efficient T cell–dependent immune response in vivo. To help resolve this enigma, we analyzed the survival of Gsk3-deficient B cells that were stimulated with IL-4, anti-CD40, anti-CD40 plus IL-4, anti-IgM, LPS or BAFF. Gsk3-deficient B cells showed survival comparable to that of control B cells in response to each treatment, except IL-4 (Fig. 8a); however, this defect could be rescued by the addition of anti-CD40. To assess the role of Gsk3 in GC B cell survival independently of its function in the initiation of the GC response, we immunized control and hCd20-TamCreGsk3aL/L-Gsk3bL/L mice with SRBCs and administered tamoxifen on days 5–7 after immunization. Fewer GC B cells were recovered from hCd20-TamCreGsk3aL/L-Gsk3bL/L mice than from control mice (Fig. 8b), and annexin V staining revealed an increased frequency of apoptosis in Gsk3-deficient GC B cells (Fig. 8c). However, deletion of Gsk3 during an ongoing immune response promoted GC B cell proliferation (Fig. 8d). Taken together, these data suggested that Gsk3 deletion led to a proliferative burst of GC B cells, but this was offset by increased amounts of apoptosis.

To explain why Gsk3-deficient B cells showed differential susceptibility to apoptosis under in vitro versus in vivo growth conditions, we hypothesized that the B cells were cultured in nutrient-rich medium, whereas the GC microenvironment was likely to be nutrient-limited. To analyze the survival of Gsk3-deficient B cells under nutrient-stressed conditions, anti-CD40- and IL-4-stimulated cells were cultured in glucose-free medium. Whereas Gsk3-deficient B cells...
Figure 8 Gsk3 promotes rapamycin sensitivity and c-Myc degradation. (a–c) Proliferation (a), average cell divisions (b) and size (FSC-A) (c) of B cells obtained from hCd20-Tam22 or Gsk3aL−L Gsk3bL−L (control (Ctrl)) and Gsk3aL−L Gsk3bL−L hCd20-Tam22 (dKO) mice treated with tamoxifen on 3 consecutive days, then cultured in vitro for 3 d with anti-CD40 and IL-4 in the presence or absence of 25 nM rapamycin (Rap). (d–f) Proliferation (d), average cell divisions (e) and size (FSC-A) (f) of B cells obtained from control and dKO mice (as in a–c) and cultured for 3 d with anti-igM in the presence or absence of 25 nM rapamycin. (g,h) Immunoblot analysis of lysates of B cells obtained from control and dKO mice (as in a–c) and stimulated overnight with anti-CD40 plus IL-4 (g) or anti-IgM (h) in the presence or absence of 25 nM rapamycin. Each symbol (b,c,e,f) represents an individual mouse; small horizontal lines indicate the mean. n.s., not significant; **P = 0.0023 (b), *P = 0.02 (b, left), *P = 0.014 (b, right); **P = 0.007 (c, top) **P = 0.0012 (c, left), ***P = 0.0003 (c, right); ***P = 0.0006 (e), **P = 0.0043 (e), *P = 0.035 (e); and **P = 0.0022 (f) (Mann-Whitney test). Data are representative of six (a–f), two (g) or three (h) independent experiments.

had comparable viability to that of control B cells in the presence of glucose, viability was significantly reduced after culturing in glucose-free medium (Fig. 8e). Cells respond to metabolic stress by limiting mTORC1 signaling, and inhibition of mTORC1 can improve cell survival under glucose starvation. Correspondingly, rapamycin treatment significantly increased the survival of both control and Gsk3-deficient B cells in glucose-free medium (Fig. 8e), but the degree of rescue was modest, suggesting that mTORC1-independent pathways also contributed to Gsk3-dependent B cell survival (Fig. 8e).

In addition to serving as a source of energy, glucose is also needed in the pentose phosphate pathway that provides cells with NADPH, thus supporting intracellular redox balance. Because Gsk3-deficient B cells showed enhanced respiration, we hypothesized that Gsk3-deficient B cells could accumulate mitochondrial mass, leading to increased production of ROS. Indeed, Gsk3-deficient B cells had increased mitochondrial content relative to that in control B cells after overnight stimulation with anti-CD40 and IL-4 but not after stimulation with anti-IgM (Fig. 8f,g). Consistent with the known role of c-Myc in supporting mitochondrial biogenesis28, we found that B cells from Myc-tg mice showed increased mitochondrial mass after anti-CD40 and IL-4 stimulation, as well as after anti-IgM stimulation (Fig. 8g,h). Gsk3-deficient B cells that were stimulated with anti-CD40 and IL-4 did not show a significant increase in ROS production, as compared to control B cells, when cultured in complete medium; however, in glucose-free medium they produced more ROS than control B cells (Fig. 8i). Excessive ROS production can lead to oxidation of macromolecules and ultimately to cell death29. Indeed, when the ROS scavenger N-acetyl cysteine (NAC) was added to the cultures, survival of Gsk3-deficient B cells in glucose-free medium was significantly enhanced (Fig. 8i). Correspondingly, freshly isolated Cd19CreGsk3aL−L Gsk3bL−L B cells accumulated more ROS than control B cells, consistent with their increased cell size and decreased survival in vivo (Fig. 8i). Collectively, these data highlight a critical role for Gsk3 in curtailing anabolic growth and promoting cell viability under glucose deprivation.
DISCUSSION

Here we showed that rapid growth and proliferation in the GC occurred in a hypoxic environment and thus was limited in blood-derived oxygen and nutrients, findings that are consistent with two reports that were published during the review of our manuscript\textsuperscript{30,31}. We further demonstrated that GC B cells underwent metabolic adaptation by increasing mitochondrial biogenesis, glucose uptake and induction of the HIF-1α-dependent glycolytic program\textsuperscript{16}. Indeed, the GC response collapsed after glycolysis was halted by the administration of 2-DG. These findings are consistent with a recent study\textsuperscript{32}, although HIF has been shown to counter Myc-dependent mitochondrial biogenesis and respiration\textsuperscript{33}. By analogy, the synergy of the c-Myc and HIF pathways is also likely to be critical in the GC. We found that Gsk3 restrained c-Myc-dependent glycolysis and respiration in the regulation of catabolism and energy production, and it attenuated the cytotoxic effects of ROS production. Gsk3 was inhibited in GC B cells engaged in BCR signaling, but it was also inhibited in B cells that were not actively signaling via the BCR, including in a small subpopulation of B cells with abundant pS6. These cells may be responding to distinct growth stimuli, such as the CD40L and IL-4 provided by cognate follicular helper T cells. In support of this view, this same population was enriched for IgG1-expressing B cells, which are a product of CD40L plus IL-4 signaling.

The enhanced presence of c-Myc may partially account for the increased growth, metabolic activity and proliferation of Gsk3-deficient B cells. On one hand, we showed that levels of c-Myc were increased in Gsk3-deficient B cells that were stimulated with anti-CD40 and IL-4 and that c-Myc overexpression in Gsk3-sufficient B cells led to increased B cell proliferation and cell growth. On the other hand, anti-IgM-stimulated Gsk3-deficient B cells showed comparable amounts of c-Myc as their WT counterparts and had normal rates of proliferation. Indeed, by using an inducible system, we demonstrated a B cell–specific deletion of the glucose transporter Glut1 by increasing mitochondrial biogenesis, glucose uptake and respiration, findings that are consistent with two reports that were published during the review of our manuscript\textsuperscript{30,31}. We further demonstrated that GC B cells underwent metabolic adaptation by increasing mitochondrial biogenesis, glucose uptake and induction of the HIF-1α-dependent glycolytic program\textsuperscript{16}. Indeed, the GC response collapsed after glycolysis was halted by the administration of 2-DG. These findings are consistent with a recent study\textsuperscript{32}, although HIF has been shown to counter Myc-dependent mitochondrial biogenesis and respiration\textsuperscript{33}. By analogy, the synergy of the c-Myc and HIF pathways is also likely to be critical in the GC. We found that Gsk3 restrained c-Myc-dependent glycolysis and respiration in the regulation of catabolism and energy production, and it attenuated the cytotoxic effects of ROS production. Gsk3 was inhibited in GC B cells engaged in BCR signaling, but it was also inhibited in B cells that were not actively signaling via the BCR, including in a small subpopulation of B cells with abundant pS6. These cells may be responding to distinct growth stimuli, such as the CD40L and IL-4 provided by cognate follicular helper T cells. In support of this view, this same population was enriched for IgG1-expressing B cells, which are a product of CD40L plus IL-4 signaling.

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In addition to c-Myc, mTORC1 is a central regulator of cell metabolism and its activity is dependent on nutrient availability. Gsk3 has been previously shown to phosphorylate tuberous sclerosis complex 2 (TSC2), an inhibitor of mTORC1 (ref. 35). This phosphorylation event leads to TSC2 activation and thus inhibition of mTORC1. Accordingly, chemical inhibition of Gsk3, or Gsk3 knockdown has been shown to enhance mTORC1 signaling\textsuperscript{36}. Conversely, Gsk3...
has also been shown to promote mTORC1 signaling by facilitating assembly with raptor.36,37 We did not find any evidence of augmented mTORC1 signaling in the absence of Gsk3, as phosphorylation of S6K and its substrate S6 were unaffected. However, proliferation and growth of Gsk3-deficient B cells was largely resistant to rapamycin inhibition, suggesting that Gsk3 repressed protein synthesis by an S6-independent pathway.

In summary, our data demonstrated a critical role for Gsk3 in the maintenance of the quiescent state of naive recirculating B cells and in the regulation of GC B cell growth and proliferation in response to nutrient stress and oxygen debt. This regulation is delimited to CD40L–IL-4 signaling and thus is a direct outcome of follicular helper T cell interactions in the GC.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

I.J. designed and performed the majority of the experiments, analyzed the data and, together with R.C.R., wrote the manuscript; M.H.C. performed and analyzed the NP–KLH immunization experiment and contributed to the initial phenotypic analysis of Gsk3-deficient mice; J.R.A. performed and analyzed the CyTOF experiments and annexin V stainings; P.R.-R. performed and analyzed the in vivo CD40 stimulations and contributed to the experiments analyzing B cell proliferation in vivo and in vitro; C.R.L. and C.C. provided technical assistance with the experiments; A.D.R. helped perform and interpret the analysis of B cell metabolism; E.M.C. and R.J.B. provided advice, resources and assistance with the CyTOF experiments; J.R.W. provided mice and conceptual input to the manuscript; and R.C.R. conceived of and coordinated the study, interpreted the data and wrote the manuscript.
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

**Mice.** Gsk3α-/- and Gsk3β-/- mice were intercrossed with Cd19Cre mice. In addition, Gsk3α-/- and Gsk3β-/- mice were intercrossed with hCD20-TamCre mice and Rosa26-flx-STOP-YFP mice. For Cre induction, mice were injected i.p. with 1 mg tamoxifen (Sigma-Aldrich) + 10% ethanol in olive oil on three consecutive days. Gsk3α-/-Gsk3β-/- and hCD20-TamCre mice were used as controls. Controls were injected with tamoxifen the same way as the experimental animals.

R26StopFL-Myc mice were intercrossed with Cd19Cre mice. The mice used were heterozygous for the R26StopFL-Myc locus. All animals were maintained in the animal facility of the Sanford-Burnham Prebys Medical Discovery Institute. Experiments were carried out in accordance with institutional guidelines and regulations.

**Cell sorting.** Naive B cells were obtained by negative sorting using CD43-specific beads (Miltenyi Biotec) according to the manufacturer’s instructions. GC B cells and non-GC B cells were obtained by negative sorting using biotinylated antibodies (anti-CD3 (S7), anti-CD11c (N418) and anti-IgD (11-26) to obtain GC B cells, and anti-CD3 (S7), anti-CD11c (N418) and GL-7 to obtain non-GC B cells; all antibodies from eBioscience), alkaliphycocyanin (APC)-conjugated streptavidin (eBioscience) and anti-APC beads (Miltenyi Biotec).

**Flow cytometry and antibodies.** To measure the cell surface expression of selected markers, single-cell suspensions were prepared and stained according to standard procedures. The following antibody clones were obtained from eBioscience: anti-B220 (RA3-6B2), anti-CD23 (B384), anti-CD21 (4E3), anti-IgM (II/41), anti-IgD (11-26), anti-BP-1, anti-CD3 (S7) and anti-GL7 (GL-7). The following antibodies were obtained from BD Pharmingen: anti-CD138 (281-2) and anti-CD95 (anti-Fas) (Jo2). PBS + 1% FBS + 0.01% sodium azide was used as flow cytometry buffer. To measure mitochondrial content, MitoTracker Red CMXRs (Life Technologies) was used. Cells were stained for 30 min at 37 °C with 60 nM MitoTracker Red CMXRos in complete RPMI medium (Corning Cellgro) and washed twice before measurement. Cells were analyzed by flow cytometry and microscopy. To measure protein abundance, cells were stained with eFluor670 (eBiosciences) for 10 min in PBS at 19–25 °C, washed with complete RPMI medium, resuspended in complete RPMI medium, incubated for 5 min, washed again and resuspended in flow cytometry buffer. To detect ROS, cells were stained with the oxidative stress indicator carboxy-H2DCFDA (Invitrogen Molecular Probes; 10 µM in 1 ml PBS) for 20 min at 37 °C and washed twice before measurement. All data were collected using a FACSCanto Flow Cytometer (BD Biosciences).

**Mass cytometry (CyTOF).** Mice were immunized with SRBCs to induce GCs. Spleen cells were collected in a small volume of ice-cold DPBS (Corning Cellgro) containing 50 µM cisplatin (Enzo) to stain dead cells. After 1 min, a 20-fold volume of lyse/fix buffer (BD Biosciences) was added for 10 min at 37 °C. The cells were stained for surface markers followed by permeabilization in 90% methanol for 30 min at ~80 °C. Antibodies to intracellular markers and secondary antibodies were added for 45 min. DNA was stained using 191/193Ir (DVS) for 15 min followed by one wash in DPBS and one wash in water. The following antibodies (conjugate, supplier) were used to analyze cell signaling in GC B cells: anti-B220 (167Yb, DVS Sciences), anti-β-catenin (147Sm, DVS), anti-CD19 (149Sm, DVS), anti-IgD (150Nd, DVS), anti-IgM (151Eu, DVS), anti-IgG1 (154Sm, BD; conjugation by BD Biosciences), anti-CD43 (S7) and anti-GL7 (GL-7). The following antibodies were obtained from BD Pharmingen: anti-CD138 (281-2) and anti-CD95 (anti-Fas) (Jo2). PBS + 1% FBS + 0.01% sodium azide was used as flow cytometry buffer. To measure mitochondrial content, MitoTracker Red CMXRs (Life Technologies) was used. Cells were stained for 30 min at 37 °C with 60 nM MitoTracker Red CMXRos in complete RPMI medium (Corning Cellgro) and washed twice before measurement. Cells were analyzed by flow cytometry and microscopy. To measure protein abundance, cells were stained with eFluor670 (eBiosciences) for 10 min in PBS at 19–25 °C, washed with complete RPMI medium, resuspended in complete RPMI medium, incubated for 5 min, washed again and resuspended in flow cytometry buffer. To detect ROS, cells were stained with the oxidative stress indicator carboxy-H2DCFDA (Invitrogen Molecular Probes; 10 µM in 1 ml PBS) for 20 min at 37 °C and washed twice before measurement. All data were collected using a FACSCanto Flow Cytometer (BD Biosciences).

**Glucose uptake.** To measure glucose uptake in GC B cells, mice were immunized with H-2Dk SRBCs and intravenously (i.v.) injected with 100 µl 2 mM 2-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2NBDG, Cayman Chemical Company) on day 7. Mice were sacrificed 1 h after the 2NBDG injection. 2NBDG uptake in different B cell populations was measured by flow cytometry.

**Glucose consumption.** Mice were immunized with SRBCs (day 0), i.p. injected with 300 µl 2% (w/v) glucose in PBS on days 6 and 9, and sacrificed on day 10. Spleens were collected and stained with antibodies (conjugate, supplier) for flow cytometry, using CellTracker dye (Life Technologies) for cell viability. Glucose consumption was measured by flow cytometry.

**Immunizations and detection of antigen-specific antibodies.** Mice were injected intraperitoneally (i.p.) with 100 µl 5% BSA in PBS on days 1 and 4, and sacrificed on day 7. Spleens were collected and stained with antibodies (conjugate, supplier) for flow cytometry. Glucose consumption was measured by flow cytometry.

**Immunizations and detection of antigen-specific antibodies.** Mice were injected i.p. with 100 µl 2% (w/v) glucose in PBS on days 1 and 4, and sacrificed on day 7. Spleens were collected and stained with antibodies (conjugate, supplier) for flow cytometry. Glucose consumption was measured by flow cytometry.

**In vivo CD40 stimulation.** Mice were injected with 250 µl functional-grade purified anti-CD40 (1C10) or corresponding rat IgG2a isotype control (eBioscience) on day 0. To analyze B cell proliferation, mice were i.p. injected with 2 µg BrDU (in PBS) on day 3 and sacrificed 2 h later. Cells that incorporated BrDU were detected using the BrdU Flow Kit according to the manufacturer’s instructions (BD Biosciences).

**Metabolic assays.** Glucose consumption and lactate production were measured in cell supernatants obtained at day 3 of culture using the YSI 2900 metabolic analyzer (YSI Life Sciences). The Seahorse XF24 metabolite analyzer was used to measure oxygen consumption and proton production. CD43- B cells were cultured overnight with the indicated stimulations and counted; 1 × 10⁶ cells (anti-CD40- and IL-4-stimulated cells) or 2 × 10⁶ cells (anti-IgM-stimulated cells) were plated on Cell-Tak (BD Biosciences) coated Seahorse cell culture plates. Cells were incubated for 1.5 h in Seahorse assay medium supplemented with 10 mM glucose and 1 mM sodium pyruvate before measurement. The Seahorse Bioscience XF Cell Mito Stress Test kit was used to examine different parameters of respiration, basal respiration, coupling efficiency and spare respiratory capacity. Experiments were performed at the Sanford Burnham Prebys Cancer Metabolism Core.

**Glucose uptake.** Glucose uptake in vivo was measured in naive and GC B cells. To measure glucose uptake in GC B cells, mice were immunized with SRBCs and intravenously (i.v.) injected with 100 µl 2 mM 2-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2NBDG, Cayman Chemical Company) on day 7. Mice were sacrificed 1 h after the 2NBDG injection. 2NBDG uptake in different B cell populations was measured by flow cytometry.

**2-deoxy-D-glucose injection.** Mice were immunized with SRBCs (day 0), i.p. injected with 300 µg/kg 2-DG (Sigma) on days 4.5 and 6, and sacrificed on day 7. GC B cells were analyzed by flow cytometry.
(BD Biosciences). To study affinity maturation, mice were injected with tamoxifen on three consecutive days, rested for 2 d and immunized with 100 µg alum-precipitated NP25–KLH (Biosearch Technologies). Serum was collected on days 0, 7 and 14. Antibody titers were determined by ELISA. NP2-BSA and NP25-BSA-coated plates were used to detect high-affinity antibodies and total titers, respectively.

**Histology.** Spleens were embedded in Tissue Tek O.C.T. (Sakura Finetek) and frozen at −80 °C. Sections were fixed with acetone, blocked with PBS + 5% FBS for 1 h at 19–25 °C and stained with a combination of various antibodies: peanut agglutinin (Vector Labs), anti-B220 (RA3-6B2, eBioscience), metallophilic macrophages 1 (Moma-1, Abcam), anti-IgM (II/41, eBioscience), anti-CD5 (53-7.3, eBioscience), anti-CD35 (8C12, BD Pharmingen) and anti-CD3e (145-2C11) for 2 h at 19–25 °C. Streptavidin–Cy3 (Jackson) was used in a second step to detect biotinylated antibodies. Sections were washed three times in-between every step with PBS + 0.5% Tween 20. Images were acquired on a Zeiss Axio ImagerM1 Microscope (Zeiss) using SlideBook software (Intelligent Imaging Innovations) at 19–25 °C with EC Plan–NEOFLUAR objectives (Zeiss).

**Detection of hypoxic regions in the spleen.** Mice were immunized with SRBCs and i.p. injected with 60 µg per g body weight (µg/g) Hypoxyprobe-1 (Hypoxyprobe) and sacrificed by cervical dislocation 1 h later. Frozen spleen sections were prepared. An APC-labeled mouse monoclonal antibody detecting pimonidazole adducts (Hypoxyprobe Red APC kit, Hypoxyprobe) was used to visualize hypoxic cells.

**Immunoblotting.** Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, 1 mM EDTA), and immunoblotting was performed following standard procedures. Antibodies specific for the following proteins were obtained from Cell Signaling Technologies: cyclin D3 (DCS22), MEK1/2 (DIA5), Gsk3 (D7SD3), β-catenin (polyclonal, Lot 3), c-Myc (D84C12), p27kip1 (polyclonal, Lot 5), phospho-p70S6K at Thr389 (108D2), phospho-S6 at Ser235 and Ser236 (D57.2.2E). A horseradish-peroxidase-coupled goat anti-rabbit IgG (Jackson) was used as a secondary antibody. 1% ovalbumin in PBS + 0.1% Tween 20 was used as blocking buffer.

**Generation of induced germinal center B cells.** Induced germinal center B cells were generated by culturing B cells on the feeder cell line CD40LB (ref. 24) in the presence of IL-4 for 5 d.

**Software and statistical analysis.** Gimp (GNU Image Manipulation Program) and GraphPad Prism (GraphPad Software) were used for image editing and statistical analysis, respectively. The presented western blots were cropped for clarity. The viSNE algorithm was used to visualize data obtained from mass cytometry. Statistical significance of observed differences was evaluated as indicated in the figure legends. Significant differences between the control and the experimental groups are marked with an asterisk. Obtained P values are indicated in the figure legends. Unless stated otherwise, statistical data are shown as mean values. In graphs showing data as dot plots, each symbol represents a biological replicate (an individual mouse or a cell culture sample originating from an individual mouse). The statistical tests used are specified in the respective figure legends.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon request.