ARTICLE

Sin1/mTORC2 regulate B cell growth and metabolism by activating mTORC1 and Myc

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Proper control of B cell growth and metabolism is crucial for B-cell-mediated immunity, but the underlying molecular mechanisms remain incompletely understood. In this study, Sin1, a key component of mTOR complex 2 (mTORC2), specifically regulates B cell growth and metabolism. Genetic ablation of Sin1 in B cells reduces the cell size at either the transitional stage or upon antigen stimulation and severely impairs metabolism. Sin1 deficiency also severely impairs B-cell proliferation, antibody responses, and anti-viral immunity. At the molecular level, Sin1 controls the expression and stability of the c-Myc protein and maintains the activity of mTORC1 through the Akt-dependent inactivation of GSK3 and TSC1/2, respectively. Therefore, our study reveals a novel and specific role for Sin1 in coordinating the activation of mTORC2 and mTORC1 to control B cell growth and metabolism.

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INTRODUCTION

The metabolic and biosynthetic demands placed on B cells vary greatly from homeostasis to acute infection and are directly influenced by environmental inputs sensed by growth factor receptors, cytokine receptors, and the antigen receptor.[1,2] Growth and metabolic control of B cells is critical in maintaining B cell survival and mitogenic responsiveness.[1,2] Mature resting B cells are quiescent but require growth and survival signals to maintain a baseline level of cellular metabolism that supports cell survival and provides the energy necessary for cell motility and immune surveillance.[1] The B cell receptor (BCR) provides one of the essential signals that is absolutely required for the growth and survival of mature and GC B cells.[2–6] The basal or tonic BCR signal activates the phosphoinisitol-3 kinase (PI3K) signaling cascade to sustain mature B cell survival and growth.[1,2] The PI3K-dependent inhibition of FoxO1 transcriptional activity is required for the pro-survival function of the tonic BCR signal.[2] In mature B cells, PKCbeta and Akt have also been reported to mediate BAFF signaling to maintain B cell metabolic fitness and survival.[1,7–9] When a resting B cell senses mitogenic antigen stimuli via either the BCR or Toll-like receptors (TLR), the B cell will dramatically increase its metabolic and biosynthetic capacity, resulting in a rapid increase in cell size (volume) that precedes cell division and proliferation.[10] PI3K-dependent signaling plays an essential role in regulating the metabolic reprogramming that occurs in B cells during this phase of mitogen-dependent blast cell growth.[11] This PI3K-mediated growth supports the rapid proliferation of the antigen-stimulated B cell clones that is essential for the development of the humoral immune response.[1]

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase that is one of the key downstream effectors of PI3K signaling in the mechanism regulating cellular growth and metabolism.[12–15] Inhibition of mTOR in B cells with rapamycin suppresses mitogen-dependent blast cell growth and inhibits cell proliferation by blocking cell cycle progression at G1 phase,[16,17] which highlights the importance of mTOR in B cell-mediated immune function. Recently, mTOR has been reported to regulate germinal center B cell growth, germinal center B cell selection, and affinity maturation.[18–22]

On the basis of the results from numerous studies conducted over the past decade, mTOR associates with two functionally distinct multi-protein complexes: mTOR complex 1 (mTORC1), containing mTOR, Raptor, mLST8 (GβL), PRAS40, and Deptor, and mTORC2, containing mTOR, Rictor, Sin1, mLST8, Deptor and PROTOR.[13,23–27] Nutrients, growth factors, hormones, and energy signals activate mTORC1, which in turn phosphorylates the translational regulators S6K and 4EBP1 to increase cellular protein synthesis and ribosome biogenesis.[13,14] On the other hand, mTORC2 regulates the actin cytoskeleton, PI3K-dependent Akt activity and specificity by phosphorylating the Akt hydrophobic motif (HM; Ser473), as well as the PI3K-independent stability of Akt and conventional PKC by phosphorylating the turn motif (Thr450 on Akt1; Thr641 on PKCβII).[24,28,29] Unlike mTORC1, mTORC2 is insensitive to acute rapamycin-induced inhibition, although a chronic rapamycin treatment may inhibit mTORC2 in many types of normal and cancerous cells.[25,28] A recent structural study of mTORC1 and mTORC2 using Cryo-EM revealed that the pocket of the FKBP12-Rapa complex was

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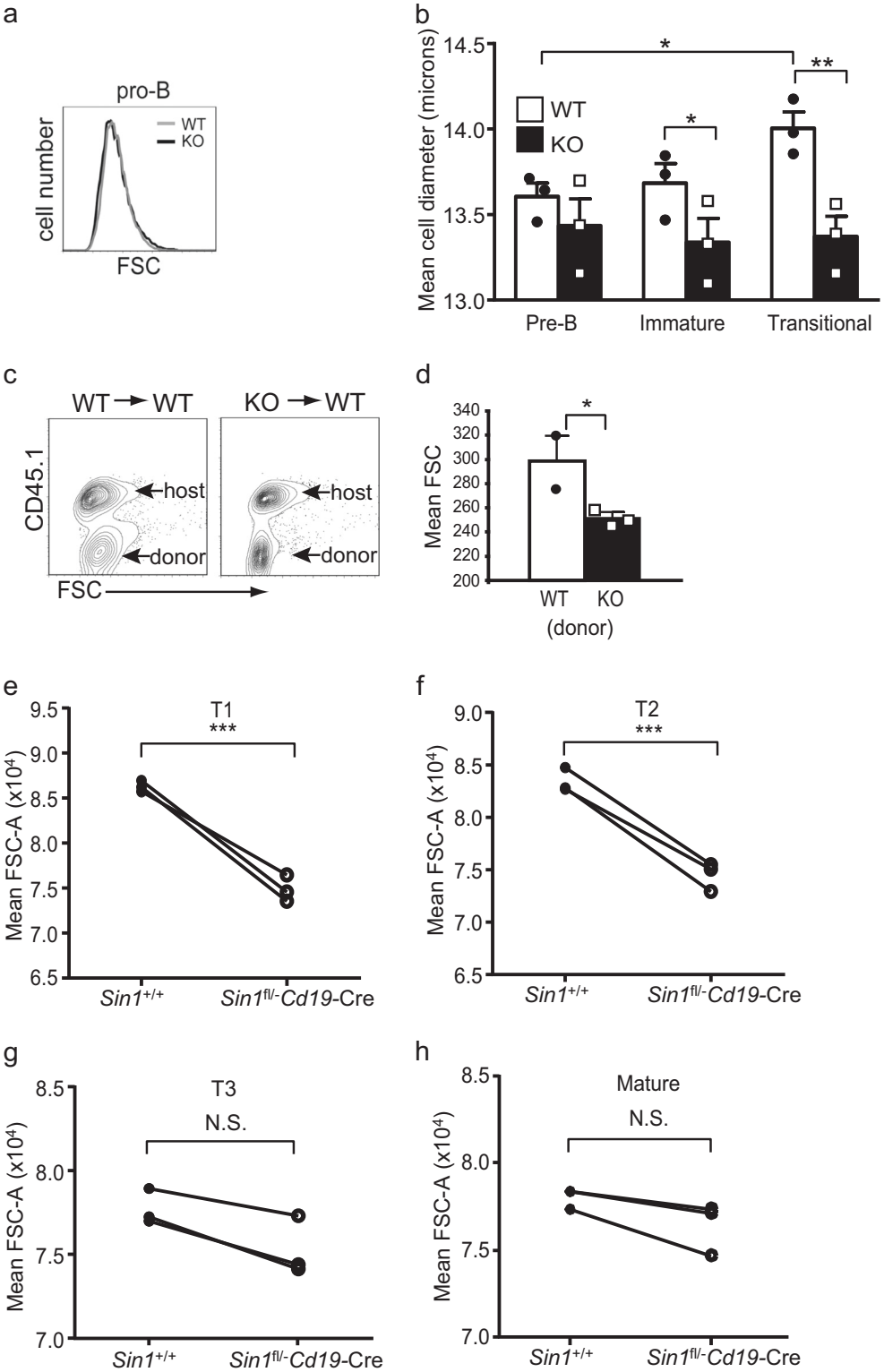

Fig. 1 Sin1 regulates B cell growth in a developmental stage-specific manner. (**a, b**) Sizes of *Sin1*$^{+/+}$ (WT) and *Sin1*$^{-/-}$ (KO) pro-B cells (**a**), and in vitro differentiated IgM^{-}IgD^{-} (pre B), IgM^{+}IgD^{-} (immature B) and IgM^{+}IgD^{+} (transitional B) cells (**b**) were measured by flow cytometry (FCM) using standard microbeads of known sizes. The data are presented as the averages of four independent experiments with mean standard deviations. The p-values were determined using a two-tailed unpaired *t* test. (**c**, **d**) The relative sizes of splenic B cells from *Sin1*$^{+/+}$ (WT→WT) or *Sin1*$^{-/-}$ (KO→WT) fetal liver HSC-chimeric mice were measured using forward light scattering (FSC). The fetal liver HSC-derived CD45.1^{-} WT or KO B cells (donor) and WT CD45.1^{+} (host) B cell populations within each mouse are indicated. The plots shown here were pre-gated on live, CD19^{+} lymphocytes and are representative of n=2 WT and n=3 KO chimeric mice (**c**). The bar graph shows the mean FSC of the splenic B cell populations within each mouse (**d**). (**e**–**h**) The relative cell sizes of indicated splenic B cell subsets (T1 B cells: B220^{+}AA4.1^{+}IgM^{hi}CD23^{lo}, T2 B cells: B220^{+}AA4.1^{+}IgM^{hi}CD23^{hi}, T3 B cells: B220^{+}AA4.1^{+}IgM^{lo}CD23^{hi} and mature B cells: B220^{+}AA4.1^{-}) were analyzed in *Sin1*$^{fl/-}$*Cd19*-Cre (cKO) and *Sin1*$^{+/+}$ (WT) mixed bone marrow chimeras ($n = 3$) using flow cytometry and presented as the mean forward light scatter (FSC) values, with control WT and cKO cells from individual mice linked. Bars represent means ± SEM. *$p \le 0.05$, **$p \le 0.01$, and ***$p \le 0.001$, unpaired two-tailed *t* test

blocked conformationally in mTORC1, but not mTORC2, which partially explains the insensitivity of mTORC2 to acute rapamycin treatment in many cell types.[30]

As shown in our previous study, Sin1 is specifically required for bone marrow B cell development via the Akt-mediated phosphorylation of FoxO1 to inhibit IL-7 receptor and recombinase-activating gene (RAG) expression.[31] Since the conditional Sin1-deficient mice are not available, researchers have been unable to clearly determine whether Sin1, either via an mTORC2-dependent or independent function, is critical for peripheral B cell maturation, differentiation, and immunity. Interestingly, Rictor, another essential component of mTORC2, has been reported to regulate B cell maturation using a hematopoietic cell-specific knockout system (Vav-Cre).[32] However, in another study using a B cell conditional knockout model (*Cd19*-Cre), B cell development was largely normal in the absence of Rictor.[33] Therefore, the impact of a Rictor deficiency on B cell development and maturation remains unclear.

In this study, we explored the functions of Sin1 and mTORC2 in peripheral B cell growth and metabolism. Unexpectedly, Sin1, via mTORC2, played a critical role in regulating B cell growth and development, specifically at the transitional and later maturation stages. Mice with a genetic deletion of Sin1 exhibited a smaller B-cell size. Conditional deletion of Sin1 in B cells led to a blockade of transitional B cell maturation, antibody production, and anti-viral immunity. Upon stimulation, Sin1-deficient B cells exhibited impaired blast cell growth, proliferation, and metabolism. Furthermore, Sin1/mTORC2 cross-regulated mTORC1 via the Akt-GSK3 axis to upregulate c-Myc expression in B cells. Based on our data, Sin1/mTORC2 was critically important for B cell growth and metabolism by integrating pro-growth signals to coordinate the anabolic activities of mTORC1 for antibody production and anti-viral responses.

RESULTS

Regulation of B-lineage cell growth in vitro by Sin1/mTORC2

Based on findings from emerging studies, mTOR signals regulate cell growth in response to nutrition and mitogen signals.[13,14] However, researchers have not precisely determined when the mTOR-mediated growth regulation becomes biologically significant in various types of cells.

To address this question, Sin1-deficient pro-B cells were induced to undergo differentiation in vitro, and their growth was examined at various stages according to their surface IgM and IgD expression, as described previously.[31] In vitro differentiation of the $Sin1^{+/+}$ or $Sin1^{-/-}$ pro-B cells was induced by withdrawing IL-7 from the OP9 culture medium, inducing pro-B cells to first differentiated into $IgM^{-}IgD^{-}$ pre-B cells, subsequently express cell surface immunoglobulin to become $IgM^{+}IgD^{-}$ immature B cells, and then further developed into $IgM^{+}IgD^{+}$ B cells.[34,35] These in vitro differentiated $IgM^{+}IgD^{+}$ B cells expressed high levels of IgM, similar to previously defined transitional T1 ($IgM^{hi}IgD^{low}$) and T2 ($IgM^{hi}IgD^{hi}$) B cells.[34] The relative cell size of B-lineage cells was quantified by flow cytometry using forward light scatter (FSC). As shown in Fig. 1a, b, no significant difference in cell size was observed between the $Sin1^{+/+}$ and $Sin1^{-/-}$ pro-B cells (Fig. 1a) and $IgM^{-}IgD^{-}$ pre-B cells (Fig. 1b). However, at the $IgM^{+}IgD^{-}$ and $IgM^{+}IgD^{+}$ B cell stages, the cell size of $Sin1^{-/-}$ B cells was significantly smaller than the corresponding WT $Sin1^{+/+}$ B cells, with the most obvious difference noted at the $IgM^{+}IgD^{+}$ stage (Fig. 1b). Thus, mTORC2 mediates growth in a developmental stage-specific manner and B cells likely require mTORC2-mediated growth signaling once IgM is expressed.

Regulation of B-lineage cell growth in vivo by Sin1/mTORC2

We generated chimeric mice with $Sin1^{-/-}$ fetal livers that lacked Sin1 in the hematopoietic system using a previously described method to investigate the role of Sin1 in mTOR-mediated B cell growth in vivo.[31] Host and donor hematopoietic cells were distinguished by the differential expression of the CD45.1 and CD45.2 congenic markers, which allowed us to evaluate the differentiation, maturation, and function of WT and $Sin1^{-/-}$ B cells in the exact same environment. As shown in Fig. 1c, d, the $Sin1^{-/-}$ fetal livers gave rise to a population of splenic $B220^{+}$ B cells, which is consistent with the findings in mice with B cell-specific deletion of Rictor.[32] Importantly, we observed a clear reduction in cell size in $B220^{+}CD45.1^{-}$ Sin1-deficient B-lineage cells compared to $B220^{+}CD45.1^{+}$ WT B-lineage cells in the same mice (Fig. 1c, d), indicating that Sin1 regulates B-lineage cell growth in vivo in a B cell-intrinsic manner.

We generated $Sin1^{fl/fl}$ mice (Supplemental Figure S1a) and crossed them with *Cd19*-Cre mice to further study the impact of the Sin1 deficiency on B-lineage cell growth in a B-cell-specific in vivo system.[36] We confirmed that Sin1 was efficiently deleted in splenic B cells (Supplemental Figure S1b), and the $Sin1^{fl}$ allele was efficiently deleted in B cells isolated from the spleens of $Sin1^{fl/fl}$*Cd19*-Cre and $Sin1^{fl/-}$*Cd19*-Cre mice (Supplemental Figure S1c). We generated mixed bone marrow chimeric mice reconstituted with $Sin1^{fl/-}$*Cd19*-Cre (*Cd45.2*$^{+/+}$) and WT control (*Cd45.1*$^{+}$*Cd45.2*$^{+}$) bone marrow to directly compare the cell size of Sin1-deficient and WT B-lineage cells in same mice. WT and $Sin1^{fl/-}$*Cd19*-Cre B-lineage cells exhibited a comparable cell size at the pro-B and pre-B stages (Supplemental Figures S1d-f). However, the Sin1-deficient $IgM^{+}IgD^{-}$ immature B cells were obviously smaller than the Sin1-sufficient immature B cells (Supplemental Figure S1g). Interestingly, a more remarkable reduction in cell size was observed in Sin1-deficient transitional B cell stages T1 and T2 compared to WT B cells, while the size disparity between WT and Sin1-defcient T3 or mature B cells was less than the difference observed in the T1 and T2 transitional B cells (Fig. 1e–h). Interestingly, we analyzed peripheral B cell development in $Sin1^{fl/+}$*Cd19*-Cre and $Sin1^{fl/fl}$*Cd19*-Cre mice, and observed a marked reduction in T2, T3 and FO B cell subpopulations with a concurrent increase in T1 B cells in Sin1 cKO mice (Supplemental Figs. S2a, 2b, 2d, 2e). Meanwhile, the numbers of peripheral B cell subsets in Sin1 cKO mice were dramatically reduced compared to control mice (Supplemental Figs. S2c and 2f). These data, together with our study of the in vitro differentiated B-lineage cells, indicate that the growth defect of Sin1-deficient B-lineage cells is stage-specific and appears after the functional B cell receptor is expressed.

Sin1-deficient B cells fail to grow and proliferate in response to BCR crosslinking

Since the growth defect only appeared in Sin1-deficient B-lineage cells after BCR was expressed, we reasoned that Sin1 regulates BCR-induced cell growth. As a test of this hypothesis, we stimulated splenic B cells from mixed bone marrow chimeric mice in vitro with an anti-IgM antibody for 24 h to observe BCR crosslinking-induced B cell growth in the absence of cell division. While anti-IgM stimulation induced the blast cell growth of both $Sin1^{+/+}$ and $Sin1^{fl/-}$*Cd19*-Cre B cells, the Sin1-deficient B cells were consistently smaller than $Sin1^{+/+}$ B cells (Fig. 2a). Using flow cytometry, we measured the size of the resting and stimulated $Sin1^{+/+}$ or $Sin1^{fl/-}$*Cd19*-Cre B cells and found that the mean cell size of activated $Sin1^{fl/-}$*Cd19*-Cre B cells was significantly smaller than the corresponding $Sin1^{+/+}$ B cells (Fig. 2b). Based on these data, Sin1 plays a critical role in regulating B-cell growth in response to BCR stimulation.

Proper blast cell growth is required for B-cell proliferation after BCR stimulation. Since the Sin1 deficiency impaired the blast cell growth of activated B cells, we asked if Sin1 was required for mitogen-dependent B cell proliferation. Splenic $Sin1^{+/+}$ or $Sin1^{fl/-}$*Cd19*-Cre B cells were labeled with CFSE and cultured for 72 h in vitro in the presence or absence of the anti-IgM antibody. Cell proliferation was measured using the CFSE dilution assay. The

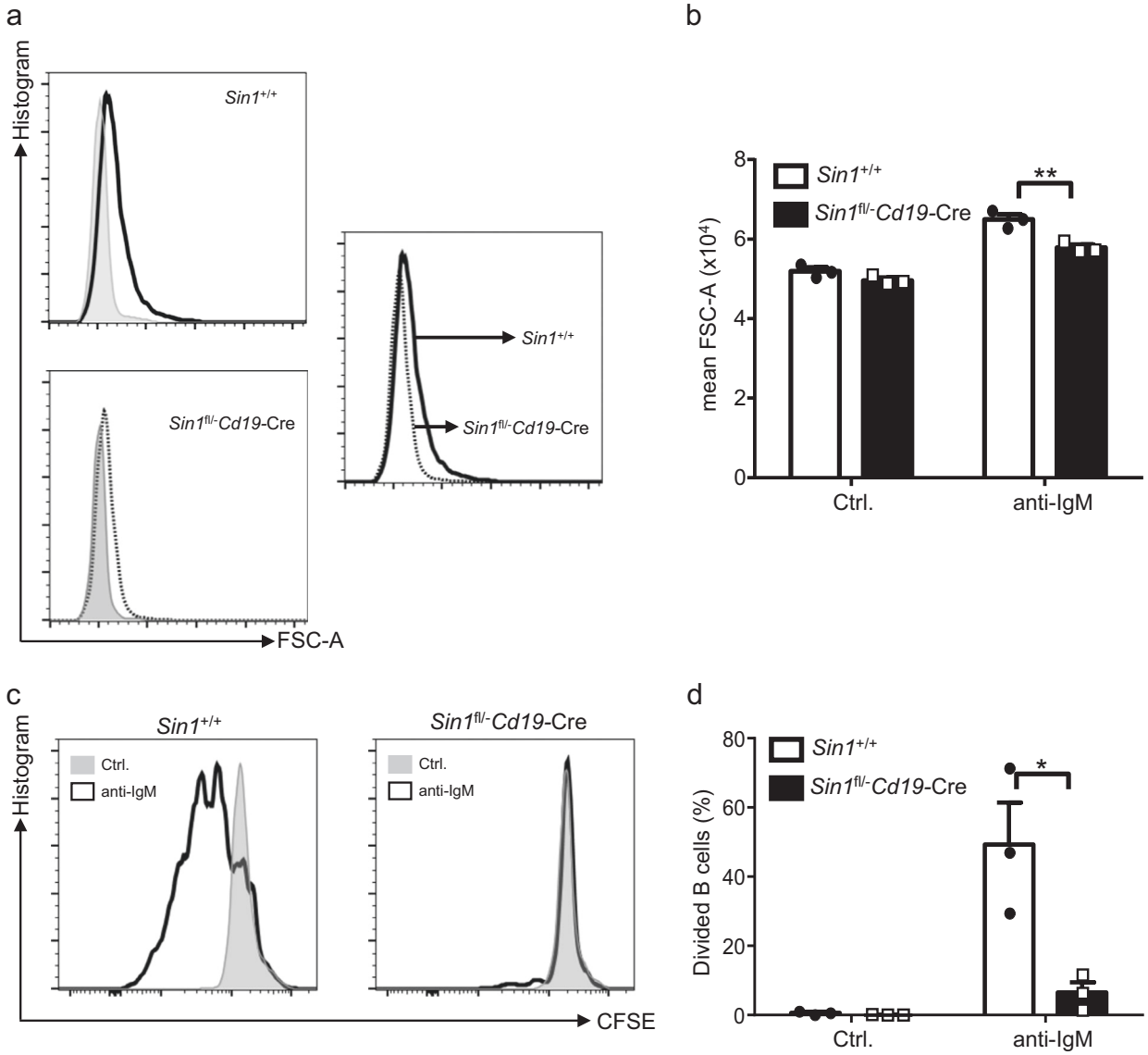

Fig. 2 Sin1 plays a critical role in regulating B cell growth in response to BCR stimulation. (**a**) Splenic B cells isolated from *Sin1*$^{fl/-}$*Cd19*-Cre and *Sin1*$^{+/+}$ mixed bone marrow chimeras were cultured in vitro with medium alone or 10 μg/ml anti-IgM F(ab')$_2$ for 24 h and the relative B-cell size was measured using forward light scattering (FSC). Unstimulated cells are indicated by the shaded lines, stimulated WT cells are indicated by the solid line and stimulated cKO cells are indicated by the dotted line. (**b**) The bar graph shows the mean sizes of *Sin1*$^{+/+}$ (WT) or *Sin1*$^{fl/-}$*Cd19*-Cre (cKO) splenic B cells cultured for 24 h in vitro as described in (**a**). (**c**) Splenic B cells isolated from *Cd19*^{Cre}*Sin1*$^{fl/-}$ and *Sin1*$^{+/+}$ mixed bone marrow chimeras ($n = 3$) were labeled with CFSE and cultured for 72 h in vitro after an anti-IgM (10 μg/ml) treatment. Cell proliferation was measured using FACS to determine the CFSE dilution, and presented as CFSE histogram. (**d**) The percentage of dividing B cells (CFSE^{lo}) in the experiments shown in (**c**) was determined using FACS to determine the CFSE dilution. The results shown here are representative of three independent experiments. Bars represent means ± SEM. *$p \le 0.05$ and **$p \le 0.01$, unpaired two-tailed *t* test

stimulation of *Sin1*$^{+/+}$ B cells with an anti-IgM antibody induced vigorous cell proliferation; however, *Sin1*$^{fl/-}$*Cd19*-Cre B cells were unable to proliferate in response to the anti-IgM treatment (Fig. 2c), and the percentage of dividing B cells in *Sin1*$^{fl/-}$*Cd19*-Cre mice was significantly decreased following anti-IgM stimulation (Fig. 2d). Meanwhile, we examined B cell viability 24 h after stimulation and found that the survival of resting or anti-IgM stimulated *Sin1*$^{fl/-}$*Cd19*-Cre B cells was comparable to *Sin1*$^{+/+}$ B cells (Supplemental Figure S3a). Thus, Sin1 is required for the blast cell growth, proliferation of resting or BCR-stimulated B cells.

Akt activity is required for B cell growth

Akt is an important downstream mediator of Sin1/mTORC2 signaling and a key regulator of cellular growth.[13,24,37] Thus, we postulated that Akt may mediate Sin1-dependent signaling to regulate B cell growth. We next generated Akt-deficient IgM$^-$IgD$^-$ pre-B and IgM$^+$IgD$^+$ B cells that were differentiated in vitro from *Akt1*$^{-/-}$*Akt2*$^{-/-}$ pro-B cells using a previously described method to test this possibility.[31] Indeed, the cell size of *Akt1*$^{-/-}$*Akt2*$^{-/-}$ IgM$^+$IgD$^+$ transitional B cells, but not IgM$^-$IgD$^-$ pre-B cells, was significantly smaller than the WT counterparts (Fig. 3a), suggesting that Sin1 and Akt may regulate B cell growth through a common mechanism.

Sin1-deficient B cells exhibit impaired Akt signaling

We examined both the activation and expression of the Akt protein in in vitro differentiated B cells to further determine if Sin1 and Akt regulate peripheral B cell growth through a common molecular mechanism. As shown in Fig. 3b, the level of the Akt protein was significantly reduced in *Sin1*$^{-/-}$ B cells compared to

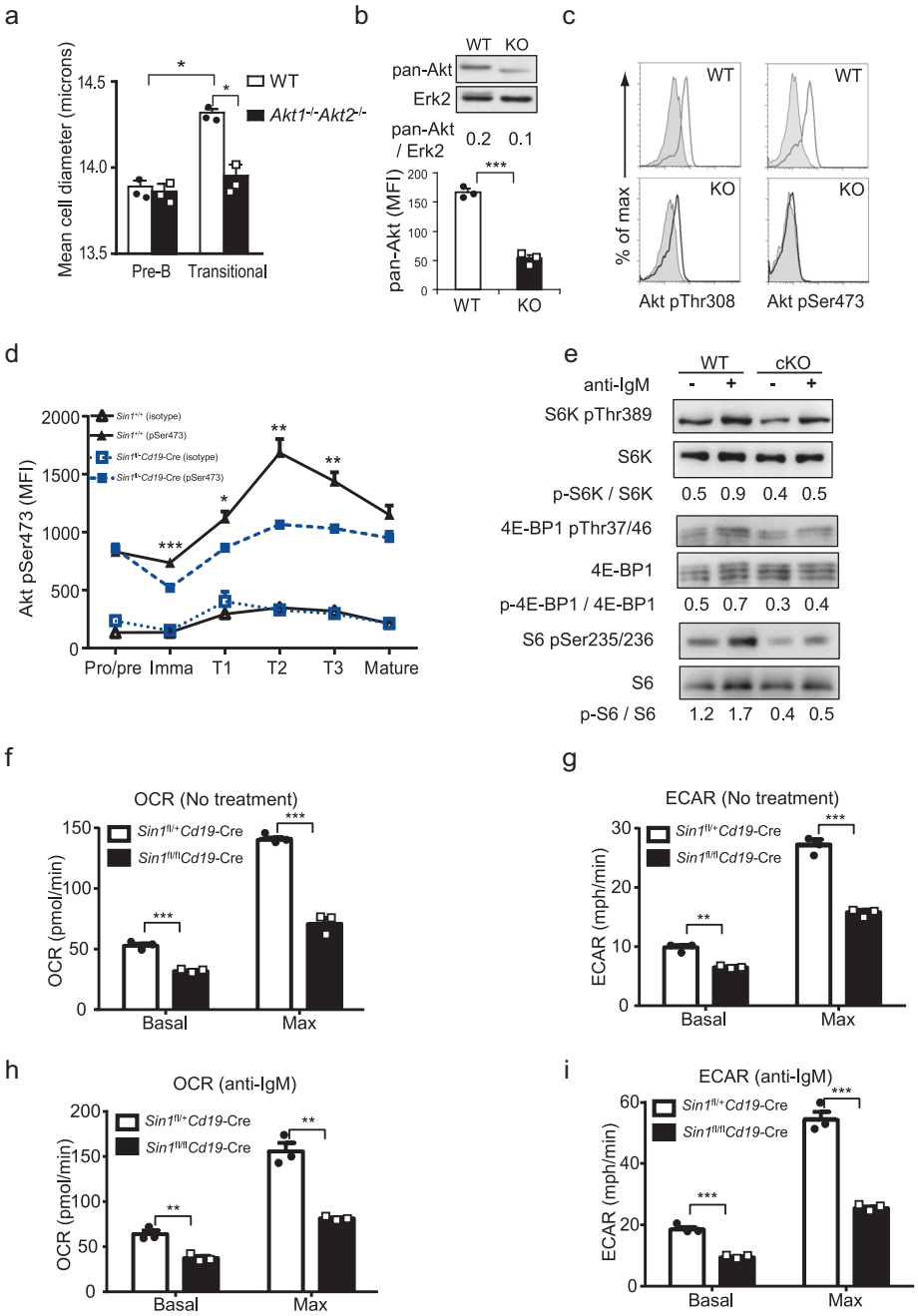

Fig. 3 Akt and mTORC1 signaling is impaired in Sin1-deficient B cells. (**a**) The relative sizes of WT and *Akt1*$^{-/-}$*Akt2*$^{-/-}$ pre-B and transitional B cells from in vitro differentiated pro-B cells were measured and presented as described in Fig. 1b. (**b**) In vitro differentiated *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) IgM^{+} transitional B cells were enriched and Akt expression was measured using immunoblotting (upper panel) and flow cytometry following intracellular staining with a pan-Akt antibody (lower panel). For the flow cytometry analysis, Akt expression was graphed as the average mean fluorescence intensity (MFI) of Akt with the mean standard error of triplicate samples. ***$p \le 0.001$, unpaired two-tailed *t* test. Representative data from one of the two independent experiments is shown. (**c**) In vitro differentiated *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) IgM^{+} transitional B cells were cultured with (line) or without (shaded) 10 μg/ml anti-IgM F(ab')$_2$ for 3 min, then fixed, permeabilized, and stained with the indicated phospho-Akt antibodies for the flow cytometry analysis. Representative data from three independent experiments are shown. (**d**) Akt p-Ser473 levels were analyzed using FACS intracellular staining in indicated bone marrow samples and splenic B cell subsets in *Cd19*^{Cre}*Sin1*$^{fl/-}$ and WT mixed bone marrow chimeras ($n = 3$) and presented as average MFIs with SEM. *$p \le 0.05$, **$p \le 0.01$, and ***$p \le 0.001$, unpaired two-tailed *t* test. (**e**) Splenic B cells were isolated from *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/-}$*Cd19*-Cre mice, cultured with or without 10 μg/ml anti-IgM F(ab')$_2$ for 15 min, then lysed and immunoblotted with the indicated antibodies against the phospho (p)-Thr389 S6K, total S6K, phospho (p)-Thr37/46 4E-BP1, total 4E-BP1, phospho (p)-Ser235/236 S6 and total S6 proteins, respectively. Representative data from two independent experiments are shown. (**f**–**i**) The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were analyzed in purified splenic B cells isolated from *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice ($n = 3$ for each group) treated with or without 10 μg/ml anti-IgM for 1 h, and the results are presented as the mean OCR or ECAR with SEM. Representative results from one of two independent experiments are shown. **$p \le 0.01$ and ***$p \le 0.001$, unpaired two-tailed *t* test. (**f**, **g**) Basal (Basal) or maximum (Max) OCR (**f**) and ECAR (**g**) were analyzed in purified splenic B cells isolated from *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice at the steady state (no treatment). (**h**, **i**) Basal or Max OCR (**h**) and ECAR (**i**) were analyzed in purified splenic B cells isolated from *Cd19*^{Cre}*Sin1*$^{fl/+}$ and *Cd19*^{Cre}*Sin1*$^{fl/fl}$ mice after an anti-IgM treatment for 1 h

Sin1$^{+/+}$ B cells, consistent with the role of Sin1/mTORC2 in increasing the stability of the Akt protein.[28] Akt phosphorylation at Thr308 and Ser473 was measured in response to anti-IgM antibody stimulation to explore the role of Sin1 in mediating BCR-dependent Akt activation. As shown in Fig. 3c, anti-IgM stimulation induced Akt Ser473 phosphorylation in *Sin1*$^{+/+}$ but not *Sin1*$^{-/-}$ B cells, indicating that BCR-dependent Akt Ser473 phosphorylation requires Sin1. However, Akt Thr308 was still phosphorylated in *Sin1*$^{-/-}$ B cells, which is consistent with previous studies showing that Sin1 and mTORC2 are not required for Akt Thr308 phosphorylation.[24,38] On the basis of these data, the impaired Akt activity in *Sin1*$^{-/-}$ B cells is likely due to both reduced Akt expression and insufficient Akt Ser473 phosphorylation. Furthermore, mature B cells from the spleens of *Sin1*$^{-/-}$ mice also failed to proliferate in response to anti-IgM stimulation, while WT B cells proliferated vigorously (Fig. 2c). Thus, Sin1-mediated Akt expression and its Ser473 phosphorylation are critical for the growth and proliferation of mature B cells.

Sin1 regulates Akt activation at different stages of B cell development

BCR-mediated signals are important for B cell development and growth.[2,4] We postulated that the developmental and growth defects of *Sin1*$^{-/-}$ B cells may be due to impaired BCR signaling to Akt in *Sin1*$^{-/-}$ B cells. Indeed, the levels Akt Ser473 phosphorylation varied substantially at different developmental stages, with a marked increase observed at the transitional stage, consistent with the change in BCR signaling (Fig. 3d). Interestingly, Akt Ser473 phosphorylation decreased in mature B cells, although stronger BCR signals were reported, suggesting a complex regulatory mechanism of Akt activation in mature B cells. Importantly, Akt Ser473 phosphorylation was markedly reduced in Sin1-deficient B cells at all developmental stages, from pro/pre-B cells to mature B cells, compared to control WT B cells (Fig. 3d). The reduced levels of Akt Ser473 phosphorylation correlated well with the decrease in cell size, indicating that Akt Ser473 phosphorylation may be a major determining factor of B cell growth.

Sin1 is required for mTORC1 activation and proper metabolism in B cells

According to previous studies by our group and other researchers, inactivation of mTORC2 via the deletion of either Sin1 or Rictor does not impair mTORC1, and in some cases, these changes increase mTORC1 activity.[24,29,31,32] However, to our surprise, the anti-IgM-stimulated mTORC1 activity, as measured by S6K Thr389, 4E-BP1 Thr37/46 and S6 Ser235/236 phosphorylation, was lower in *Sin1*$^{fl/-}$*Cd19*-Cre B cells than in *Sin1*$^{fl/+}$*Cd19*-Cre B cells (Fig. 3e). Cell surface IgM expression was equivalent between *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/-}$*Cd19*-Cre B cells (Supplemental Figure S3b), excluding the possibility of its contribution to the altered mTORC1 activity.

The impaired mTORC1 activation in *Sin1*$^{-/-}$ B cells prompted us to examine if B cell metabolism was also impaired by measuring the oxygen consumption rate (OCR) in splenic B cells isolated from *Sin1*$^{fl/fl}$*Cd19*-Cre and *Sin1*$^{fl/+}$*Cd19*-Cre control mice. Sin1-deficient B cells exhibited a reduced steady-state OCR (both basal and maximal) compared to control B cells (Fig. 3f and Supplemental Figure S3c). In addition, the basal and maximal levels of glycolysis, as measured by the extracellular acidification rate (ECAR), were also reduced in Sin1-deficient B cells compared to control B cells (Fig. 3g and Supplemental Figure S3e). Furthermore, anti-IgM antibody-stimulated glycolysis and mitochondria respiration were also impaired in Sin1-deficient B cells, indicating that Sin1 was required for the proper glycolytic and mitochondrial metabolic reprograming of B cells following antigen stimulation (Fig. 3h, i and Supplemental Figs. S3d and 3f). Therefore, Sin1 is crucial for maintaining proper B cell metabolic activity under both resting and anti-IgM-stimulated conditions.

Sin1 regulates B cell growth by controlling c-Myc expression

The impaired mTORC1 signaling, reduced metabolic activity, and smaller cell size of *Sin1*$^{-/-}$ B cells prompted us to investigate if c-Myc, a key metabolic regulator, was involved in the Sin1-mediated regulation of B cell growth.[39,40] Interestingly, a greater percentage of Sin1-deficient B cells were in G0/G1 phase and a lower percentage were in G2 phase, implying that Sin1 is involved in the B cell cycle (Supplemental Figure S4a), but is not involved in B cell apoptosis (Supplemental Figure S4b). Furthermore, the level of the c-Myc protein in *Sin1*$^{-/-}$ B cells was substantially decreased (Fig. 4a), although the expression levels of the *c-myc* transcript in *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ B cells were comparable (Fig. 4b), suggesting that the reduction in c-Myc expression occurred through a post-transcriptional mechanism. In addition, although BCR stimulation-induced *c-myc* mRNA expression was increased after Sin1 deletion (Fig. 4c), the increase in the level of the c-Myc protein was clearly impaired in in vitro differentiated *Sin1*$^{-/-}$ IgM^{+} B cells (Fig. 4d). Levels of the c-Myc protein in resting or anti-IgM stimulated splenic B cells from the mouse reconstituted with a mixed WT (*Sin1*$^{+/+}$) and *Sin1*$^{fl/-}$*Cd19*-Cre bone marrow cells were measured using flow cytometry to further confirm these results. As shown in Fig. 4e, the level of the c-Myc protein was much lower in Sin1-deficient B cells than in WT B cells in the same spleen under both resting and anti-IgM stimulated conditions. Consistent with these findings, when the human *myc* gene was ectopically expressed in *Sin1*$^{-/-}$ B cells, it significantly increased S6 expression/phosphorylation and the size of *Sin1*$^{-/-}$ B cells compared to a control empty vector (Fig. 4f). Based on these results, c-Myc is a major target of Sin1/mTORC2 in the mechanism regulating B cell growth.

Sin1 regulates the stability of the c-Myc protein by inhibiting GSK3

To understand the molecular mechanism through which Sin1 regulates c-Myc expression, WT B cells were cultured in vitro for 60 min with medium or with an anti-IgM antibody in the presence of vehicle, rapamycin, or the mTORC1/mTORC2 dual inhibitor pp242 to obtain an understanding of the molecular mechanism by which Sin1 regulates c-Myc expression. The expression of c-Myc was measured at 60 min. BCR stimulation increased the level of the c-Myc protein, and this change was inhibited by either rapamycin or pp242 (Fig. 5a). Interestingly, pp242 suppressed c-Myc expression to a greater extent than rapamycin, suggesting that both mTORC1 and mTORC2 are involved in regulating c-Myc protein expression. However, neither rapamycin nor pp242 inhibited the expression of the *c-myc* mRNA in BCR-stimulated B cells (Fig. 5b). Thus, mTOR regulates c-Myc expression by influencing c-Myc translation and/or c-Myc stability.

Because mTORC2 is a key activator of Akt, inhibition of mTOR with pp242 not only blocked Akt S473 phosphorylation but also Akt-mediated GSK3α phosphorylation (Fig. 5a). Consistent with these findings, GSK3 phosphorylation in resting or anti-IgM-stimulated Sin1$^{-/-}$ B cells was impaired compared to WT B cells (Fig. 5c). In addition, the Akt-mediated phosphorylation of TSC2, an upstream inhibitor of mTORC1,[41] was also impaired in Sin1$^{-/-}$ B cells (Fig. 5c), suggesting that the reduced mTORC1 activity observed in *Sin1*$^{-/-}$ B cells is due to the inability of Akt to inhibit TSC2.

The Akt-mediated phosphorylation of GSK3α at Ser21 or GSKβ at Ser9 is known to inhibit the kinase activity of GSK3,[42] which phosphorylates c-Myc to promote its degradation.[43] We stimulated WT B cells with anti-IgM for 1 h to increase c-Myc expression and then cultured those cells for an additional hour in the presence of vehicle, pp242 alone, or pp242 plus the GSK3 inhibitor SB216763 (GSKi) to test if the reduced level of the c-Myc protein observed in Sin1-deficient B cells was due to increased degradation of the c-Myc protein. The pp242 treatment decreased c-Myc

expression and increased GSK3-mediated c-Myc phosphorylation at Thr58/Ser62 compared to vehicle-treated cells (Fig. 5d). Consistent with these findings, the addition of the GSK3 inhibitor SB216763 to the pp242-treated B cells not only inhibited GSK3-mediated c-Myc phosphorylation but also rescued c-Myc protein levels (Fig. 5d). Therefore, Sin1/mTORC2 may inhibit GSK3-mediated c-Myc phosphorylation and degradation.

B cells were first stimulated with an anti-IgM antibody for 2 h to induce c-Myc protein expression and then treated for 1 additional hour with cyclohexamide (CHX) to prevent new protein synthesis, with CHX+pp242, or with CHX+pp242+SB216763 to further examine the role of the Sin1-mTOR-GSK3 axis in regulating c-Myc protein stability. Levels of the c-Myc protein were measured at 0, 10, 20, 40 and 60 min after treatment using flow cytometry. As shown in Fig. 5e, the CHX+pp242 treatment increased the rate of c-Myc degradation compared to CHX treatment alone at the 40- and 60-min time points. Importantly, the addition of SB216763 completely blocked this pp242-induced c-Myc degradation (Fig. 5e). Interestingly, the pp242 and SB216763 treatments alone or in combination had no effect on the rate of c-Myc protein degradation at the 10- and 20-minute time points, suggesting that c-Myc is stabilized in an mTOR- and GSK3-independent manner at the early time points. In addition, MG132-treated Sin1-deficient B cells showed an induction of c-Myc expression (Supplemental Figure S4f). Based on these data, Sin1 controls the stability of the c-Myc protein in B cells by negatively regulating the GSK3-mediated phosphorylation of c-Myc.

Sin1 is required for humoral immunity against viral infection

Given the severely impaired growth and proliferation of Sin1-deficient B cells in response to BCR activation, we speculated that the function of these B cells in immune responses was also impaired. *Sin1*$^{fl/fl}$*Cd19*-Cre mice and *Sin1*$^{fl/+}$*Cd19*-Cre control mice were immunized with the T-dependent model antigen NP-CGG to investigate the function of Sin1 in humoral immune responses in vivo. *Sin1*$^{fl/fl}$*Cd19*-Cre mice displayed significantly lower levels of high-affinity NP4-specific IgG antibodies than their littermate controls (Fig. 6a). Thus, Sin1 may exert a positive effect on B cell-mediated humoral immunity against viral infections in vivo.

Sin1$^{fl/fl}$*Cd19*-Cre mice and *Sin1*$^{fl/+}$*Cd19*-Cre control mice were immunized and boosted three weeks later with Influenza A viral hemagglutinin (HA) antigen to further test this hypothesis. The immunized mice were inoculated with 30 LD_{50} of the PR8 virus 9 days after the second immunization (Supplementary Figure S5a). Body weights of the experimental mice were monitored daily after PR8 infection, and animals with 25% or more loss of their initial bodyweight were euthanized, as previously described.[44] *Sin1*$^{fl/fl}$*Cd19*-Cre mice displayed a significantly greater body weight loss than *Sin1*$^{fl/+}$*Cd19*-Cre control mice (Fig. 6b). Strikingly, while all *Sin1*$^{fl/+}$*Cd19*-Cre control mice were protected (5/5), almost all *Sin1*$^{fl/fl}$*Cd19*-Cre mice reached the body weight loss endpoint (4/5) (Fig. 6c). In addition, hematoxylin and eosin (HE) staining of pulmonary tissue sections showed numerous infiltrating leukocytes in pulmonary alveolar and bronchial tissues isolated from *Sin1*$^{fl/fl}$*Cd19*-Cre mice with a more severe pathology than infected WT mice (Fig. 6d). However, we did not detect significant differences in inflammatory cytokine levels between *Sin1*$^{fl/fl}$*Cd19*-Cre and control mice (Supplementary Figures S5b-e).

When HA-specific antibody levels were analyzed in infected *Sin1*$^{fl/fl}$*Cd19*-Cre mice and *Sin1*$^{fl/+}$*Cd19*-Cre control mice, *Sin1*$^{fl/fl}$*Cd19*-Cre mice had moderately reduced HA-specific IgG antibody levels in the primary antibody response (Fig. 6e). Strikingly, the second immunization with the HA antigen failed to boost the IgG response in *Sin1*$^{fl/fl}$*Cd19*-Cre mice, whereas the control mice exhibited significantly more HA-specific IgG antibody (Fig. 6e), which explains the lack of protection of *Sin1*$^{fl/fl}$*Cd19*-Cre mice from PR8 infection. Thus, Sin1 is crucial for humoral immune protection from viral infections.

DISCUSSION

Mammalian TOR is known to regulate the B cell-mediated immune function,[1,45] but the precise molecular mechanisms remain incompletely understood. In this study, Sin1, one of the essential components of mTORC2, is essential for B cell growth and metabolism. Sin1-deficient B cells exhibit reduced Akt expression, impaired Akt Ser473 phosphorylation, and impaired Akt downstream signaling, leading to dramatic reductions of glycolysis and oxidative phosphorylation, as well as substantial reductions in the size and proliferation of B cells. Furthermore, Sin1 is required for antibody responses and humoral immunity against viral infections. At the molecular level, Sin1 transduces the BCR-mediated PI3K signals to activate Akt, which is required for the maintenance of mTORC1 activity and c-Myc expression that are both essential for optimal B cell metabolism and growth.

As shown in previous studies from our group and other researchers, Sin1 and mTORC2 are not required to maintain the cell size of embryonic fibroblasts, T cells, and leukemic pre-B cells.[46–48] Sin1 is also not required to maintain the size of proliferating IL-7 dependent pro-B cells nor to maintain the size of quiescent small pre-B cells.[31] However, in the present study, Sin1 was required for the increase in the size of B cells that had begun to express functional B cell receptors, particularly at the stages of immature and transitional B cells. Peripheral B cell development is largely blocked at the transitional developmental stage, with a marked reduction in Akt Ser473 phosphorylation. Based on these data, the transitional B cells require strong Sin1/mTORC2-mediated Akt signaling to sustain sufficient cell growth and metabolism, which would allow B cell selection and entry into the mature pool of B cells. Therefore, Sin1/mTORC2-mediated regulation of B cell growth is not only cell type-specific but also depends on the developmental stage.

The transitional B cell stage is a relatively short but critical period during B cell development. The development of transitional B cells is an essential step in generating mature B cells.[49] Our data reveals an essential role for Sin1/mTORC2 in the regulation of transitional B cell growth and intimately links mTORC2-mediated growth regulation to the development and maturation of B cells. Furthermore, upon maturation, B cells must undergo extensive anabolic reprograming when responding to antigens or other stimuli. The BCR signal required Sin1/mTORC2 to sustain mature B cell growth in the present study, highlighting the central importance of cell metabolism and growth.

Anti-BCR stimulation induces mature B cell growth and proliferation, leading to increased Glut1 expression, glucose uptake, and catabolism.[1,50] According to a previous study, mTORC2 is localized to the mitochondria-associated ER membrane (MAM), controlling MAM integrity and mitochondrial function through Akt, which regulates cell growth and metabolism.[51] Recently, GSK3, which is inactivated by Akt via the phosphorylation of Ser21 or Ser9 on GSK3α or GSK3β, respectively, was shown to be a metabolic sensor that restricts massive B cell accumulation.[52] Purified splenic B cells from GSK3-deficient mice show excessive glycolysis and respiration in response to anti-CD40 and IL-4.[52] Researchers have not clearly determined whether Sin1 or mTORC2 is involved in this regulatory mechanism. In our study, Sin1 deficiency led to constitutive GSK3 activity and reduced B cell glycolysis and respiration both at the basal level and in response to anti-IgM stimulation. This phenotype is the opposite to GSK3-deficient B cells, suggesting that Sin1/mTORC2 functions upstream of GSK in the pathway regulating B cell growth and metabolism.

BCR stimulation fails to induce Akt Ser473 phosphorylation in Sin1-deficient B cells, consistent with the known function of mTORC2 as the principle kinase mediating Ser473 phosphorylation.[24] However, researchers have not clearly determined if this reduced Akt activity also contributes to the reduction in B cell size, since mTORC2-deficient fibroblasts and T cells display impaired

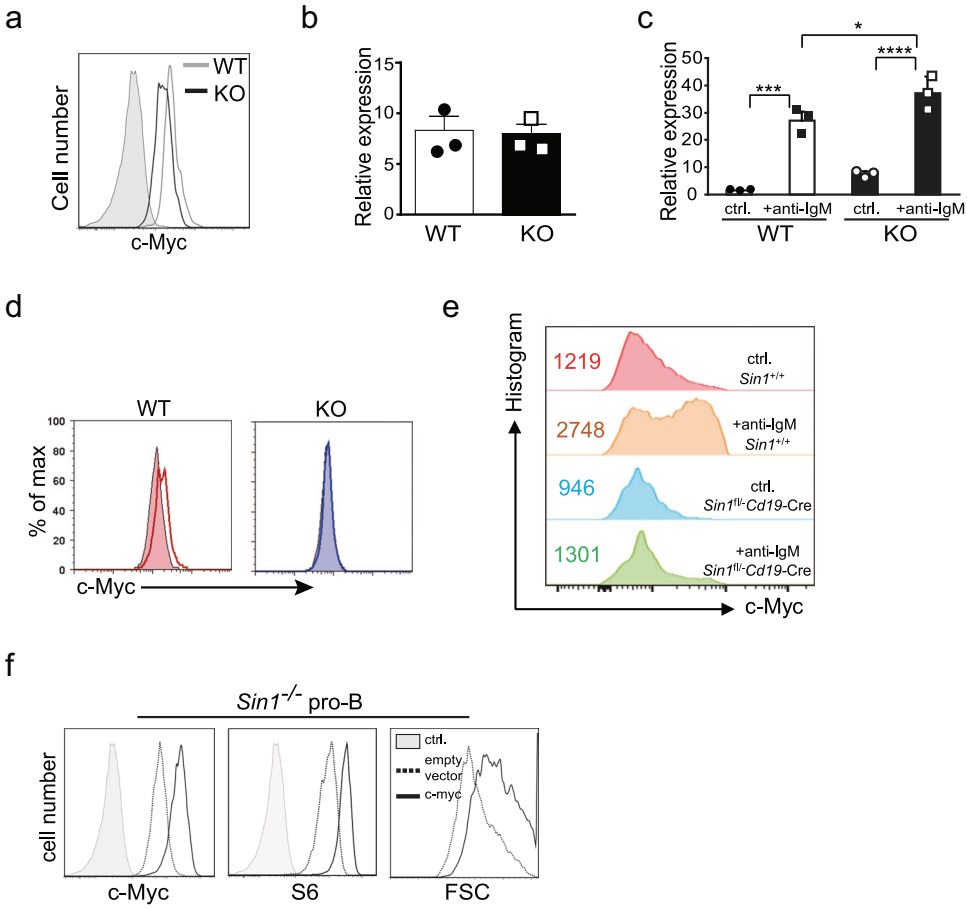

Fig. 4 Sin1 regulates c-Myc expression in B cells. (**a**) *Sin1*$^{+/+}$ (WT) (gray line) or *Sin1*$^{-/-}$ (KO) (black line) pro-B cells were differentiated in vitro and c-Myc expression was measured in IgM^{+} transitional B cells using FCM after intracellular staining. The shaded line represents the negative staining control. Representative data from three independent experiments are shown. (**b**) In vitro differentiated *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) transitional IgM^{+} B cells were enriched at 4 °C and immediately lysed using Trizol. The expression level of the *c-myc* mRNA was measured using quantitative (q) RT-PCR. Each sample was normalized to the expression of *GAPDH* and the graphs show the average expression recorded in triplicate experiments with the standard deviation. (**c**) *Sin1*$^{+/-}$ (WT) or *Sin1*$^{-/-}$ (KO) splenic B cells (CD45.1^{-}CD43^{-}B220^{+}) were enriched from *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ fetal liver chimeric mice and cultured for 1 h in vitro in the presence of absence (ctrl) of anti-IgM F(ab')$_2$ and then lysed with Trizol. The expression level of the *c-myc* mRNA was measured using qRT-PCR. Each sample was normalized to the expression of *GAPDH* and the graphs show the average expression recorded in triplicate measurements with the standard deviation. The p-value was determined using one-way ANOVA nonparametric comparison, * $p \le 0.05$, *** p≤0.001, and **** p≤0.0001. Representative results from two experiments are shown. (**d**) In vitro differentiated *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) IgM^{+} B cells were cultured with or without anti-IgM F(ab')$_2$ for 1 h, then fixed, permeabilized and stained with an anti-c-Myc antibody for the flow cytometry analysis. Representative data from two independent experiments are shown. (**e**) Intercellular staining for the c-Myc protein in IgM^{+} B cells of *Sin1*$^{fl/-}$*Cd19*-Cre and WT mixed bone marrow chimeras (n=3, WT vs. *Sin1*$^{fl/-}$*Cd19*-Cre) treated with (anti-IgM) or without (no treatment) anti-IgM for 1 h. Representative data from one of two independent experiments are shown. (**f**) *Sin1*$^{-/-}$ pro-B cells were infected with a control retrovirus or a retrovirus expressing human *MYC*. The expression of c-Myc or S6 in control infected (empty vector; dotted line) or *MYC* virus-infected (*MYC*; solid line) *Sin1*$^{-/-}$ pro-B cells was measured using FCM after intracellular staining. The relative cell size was measured using flow cytometry. Data from one of two independently derived *Sin1*$^{-/-}$ pro-B cell lines expressing human *MYC* are shown

Akt activation, but no effects on growth.[24,46,47] Interestingly, constitutively active Akt signaling contributes to the increased size of cardiomyocytes[53] and MCF-7 cells,[54] suggesting that Akt might be a key regulator of cell growth in certain cell types or during distinct developmental stages. Indeed, in the present study, *Akt1*$^{-/-}$*Akt2*$^{-/-}$ B cells also exhibit a reduced size, supporting our model that insufficient Akt signaling in *Sin1*$^{-/-}$ B cells is responsible for the reduction in B cell size.

The remarkable reduction in Akt signaling observed in *Sin1*$^{-/-}$ B cells may be due in part to the reduced expression of Akt in Sin1/mTORC2-deficient B cells. As shown in previous studies from our group and other researchers, the stability of the Akt protein is also regulated by Sin1/mTORC2.[28,29,55–58] Mammalian TORC2 associates with actively translating ribosomes and phosphorylates the turn motif of the nascent Akt polypeptide, thereby facilitating the folding and maturation of the newly synthesized Akt protein.[28,29] Indeed, total Akt expression is reduced approximately 3-fold in *Sin1*$^{-/-}$ B cells compared to *Sin1*$^{+/+}$ B cells, indicating that the Akt signaling in B cells is regulated at the both translational and post-translational levels.

The mechanism by which Akt signaling specifically regulates B cell growth and metabolism has not yet been identified. Based on the results from the present study, Akt activation by Sin1/mTORC2 phosphorylates and inhibits three main Akt targets: TSC1/2 and GSK3. Phosphorylation and inhibition of TSC1/2 increases mTORC1 activity, leading to enhanced S6K activation and S6 phosphorylation and subsequent increases in protein translation and cell growth. On the other hand, phosphorylation of GSK3 reduces c-Myc phosphorylation at Thr58/Ser62, resulting in the stabilization of the c-Myc protein. When Sin1 is deleted, a dramatic reduction in

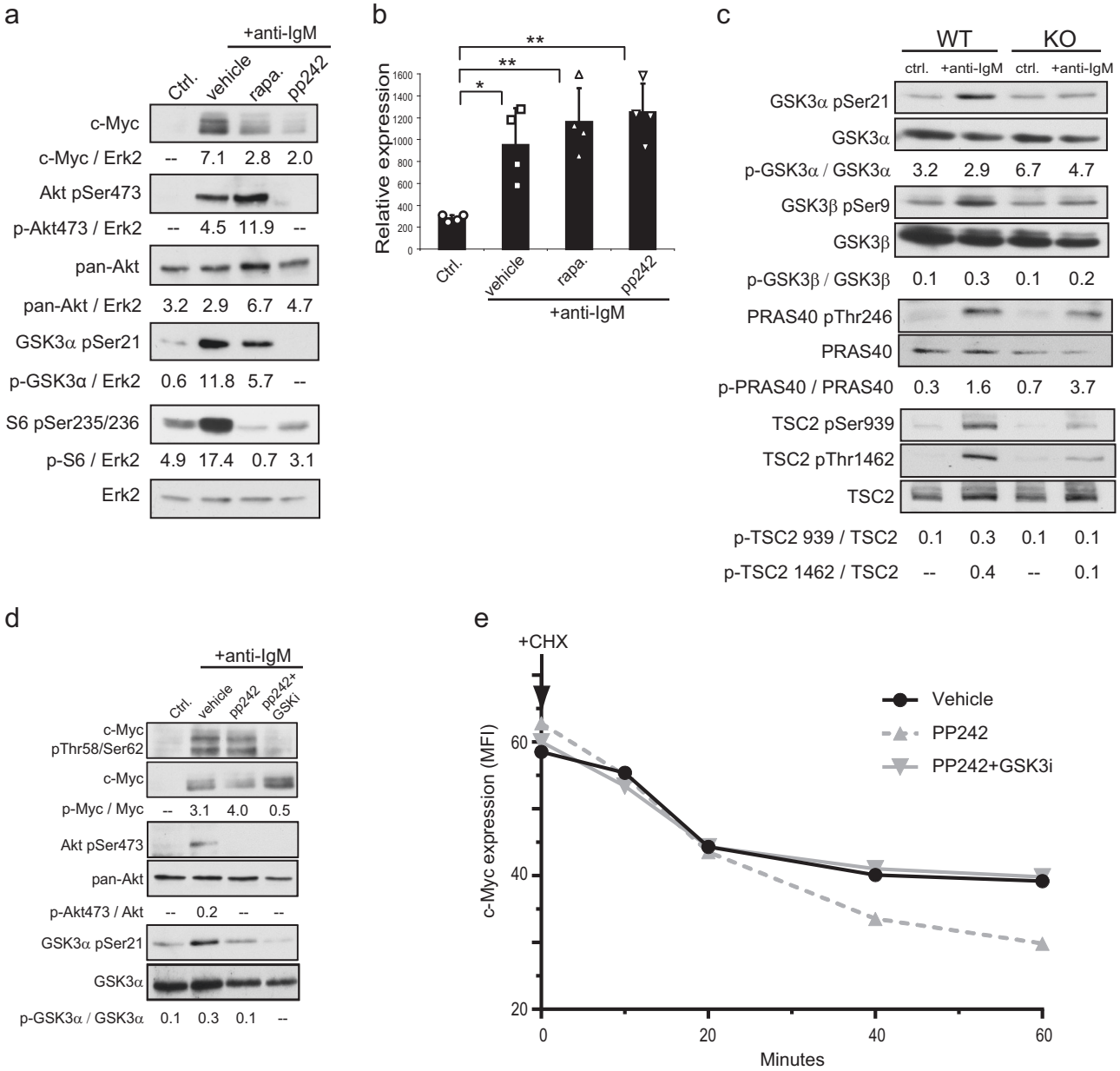

Fig. 5 mTORC2 regulates the stability of the c-Myc protein. (**a**) WT splenic B cells were enriched by negative selection and pretreated for 15 min with vehicle alone (vehicle), 20 nM rapamycin (rapa), or 50 nM pp242, and then cultured for an additional 60 min with medium alone (ctrl.) or 10 μg/ml anti-IgM F(ab')$_2$ antibody, as indicated. The expression level and phosphorylation of the indicated cellular proteins was measured using immunoblotting. Representative data from three independent experiments are shown. (**b**) WT splenic B cells were pretreated with the indicated mTOR inhibitors and then stimulated with an anti-IgM F(ab')$_2$ antibody as described in (**a**). Relative levels of the *c-myc* mRNA were measured using qRT-PCR and normalized to the expression level of *GAPDH*. The data are presented as the means ± standard deviations of four measurements and are representative of two independent experiments. The p-value was determined using one-way ANOVA nonparametric comparison, $*p \le 0.05$ and $**p \le 0.01$. (**c**) In vitro differentiated WT or *Sin1*$^{-/-}$ IgM$^+$ B cells were enriched and cultured with or without 10 μg/ml anti-IgM F(ab')$_2$ antibody for 15 min, as indicated, and then lysed and immunoblotted with the indicated antibodies against phosphorylated or total proteins. Representative data from two independent experiments are shown. (**d**) WT splenic B cells were cultured with (+anti-IgM) or without (ctrl.) anti-IgM for 1 h. The anti-IgM-stimulated B cells were then cultured for an additional hour with vehicle, pp242 or pp242 plus SB216763 (GSKi). Cells were then lysed and the indicated phospho-proteins and total proteins were analyzed using immunoblotting. Representative data from three independent experiments are shown. (**e**) Splenic B cells were cultured for 2 h with anti-IgM F(ab')$_2$ and treated with vehicle alone or the indicated inhibitors for an additional 0, 10, 20, 40 or 60 min The level of c-Myc was determined using FCM after intracellular immunostaining. The data are presented the MFIs of c-Myc staining and are representative of two independent experiments

mTORC1-mediated S6K activity and reduced S6 phosphorylation and protein translation are observed. At the same time, GSK3 activity in increased, subsequently increasing c-Myc phosphorylation and degradation. Because mTORC1 is a cardinal regulator of cell growth and metabolism, and c-Myc is a master transcriptional regulator of cell metabolism and growth, these data thus indicate that the underlying molecular mechanism by which Sin1/mTORC2 regulates B cell growth and metabolism is the Akt-mediated inhibition of TSC1/2 and GSK3, which leads to a sustained activation of mTORC1 and increased expression of c-Myc.

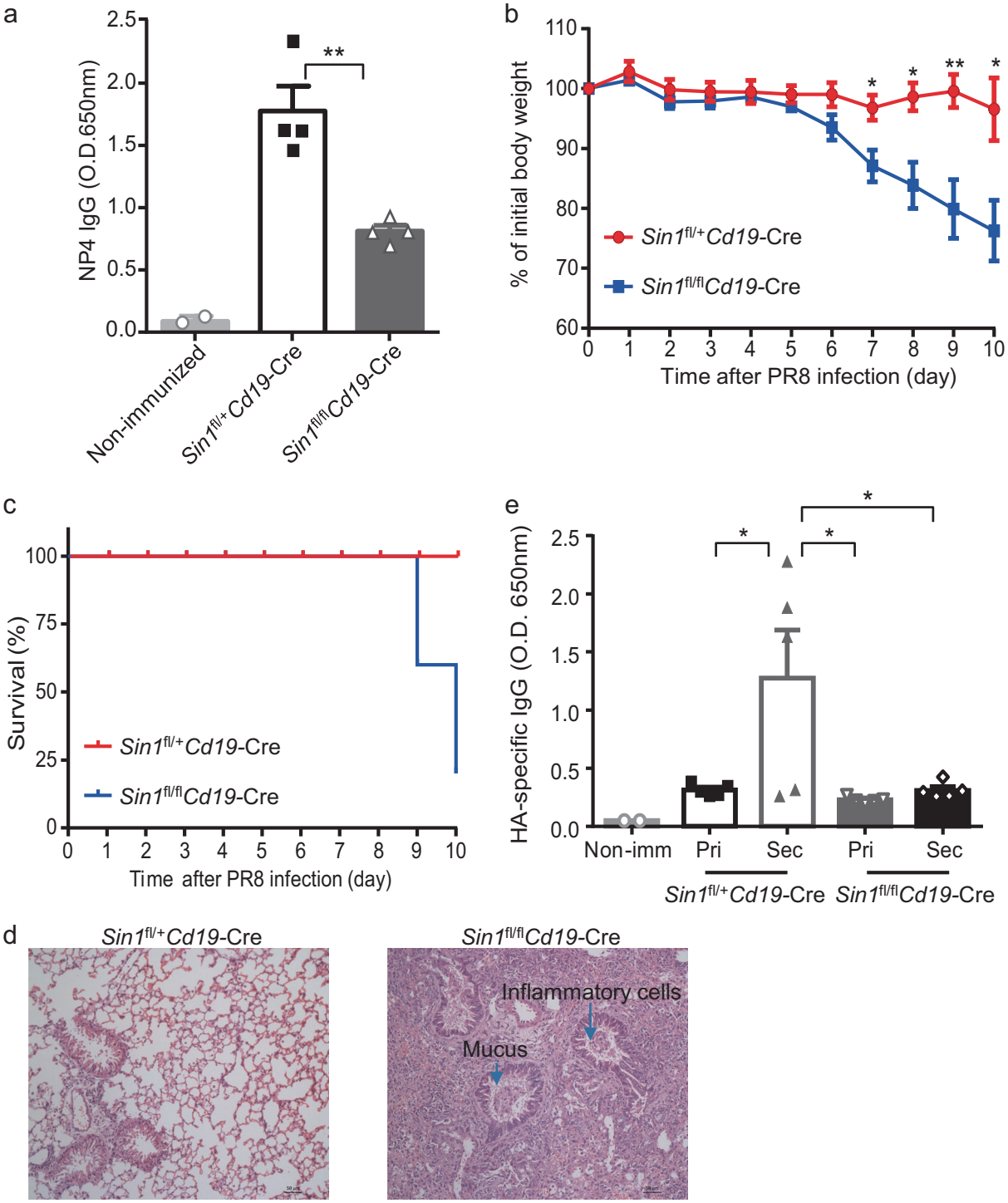

Fig. 6 Sin1 is essential for the T cell-dependent antibody response and humoral immunity against influenza virus infection. (**a**) High-affinity ($NP_{(4)}$) NP-specific serum IgG levels were analyzed in *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice (n=4 for each group) 14 days after intraperitoneal immunization with NP-CGG plus alum, and presented as the mean ELISA O.D. values with SEM. $^{**}p \le 0.01$, unpaired two-tailed *t* test. (**b**–**e**) *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice were immunized and then boosted with Influenza A viral hemagglutinin (HA), followed by H1N1 (PR8) viral infection 9 days later. The body weight loss (mean body weight loss with SEM. $^{*}p \le 0.05$ and $^{**}p \le 0.01$, unpaired two-tailed *t* test) (**b**), and survival (**c**) of these mice were examined; lung pathology (**d**) was examined using hematoxylin and eosin staining of lung sections. (**e**) HA-specific serum IgG levels were analyzed in primary and secondary responses (after the first immunization and boost, respectively) (n=5, WT vs. cKO) and presented as the mean ELISA O.D values with SEM. The data from one of two independent experiments are shown. The p-value was determined using one-way ANOVA nonparametric comparison, $^{*}p \le 0.05$

Supporting our findings, GSK3 was recently reported to serve as a metabolic sensor that restricts massive B cell accumulation,[52] whereas in Sin1-deficient B cells, GSK3 activity is not sufficiently inhibited by Akt, leading to diminished B cell glycolysis and respiration.

Given the critical roles of Sin1 in mediating B cell growth and proliferation, we were not surprised to find that Sin1 is also critically important for B cell-mediated immune responses in synthesizing immunoglobulins following model antigen immunization. Importantly, the Sin1 B cell cKO mice became highly susceptible to influenza virus infections, likely due to an inability to produce sufficient virus-specific antibodies as part of the antiviral response. Since the main defect in antibody production is a lack of IgG, which depends on the AID-mediated class switch reaction, future studies examining the role of Sin1 in the germinal center reaction may be fruitful.

Finally, one of the most specific targets of Sin1/mTORC2-dependent Akt Ser473 phosphorylation and activation, FoxO1, is a key regulator of B cell development and immune responses.[59–61] Sin1 is critically important for Akt-mediated inhibition of FoxO1 transcriptional activity in developing B cells.[31] Since the Sin1/mTORC2-Akt signaling axis integrates mitogen and growth factor signals to regulate the activities of FoxO1 and c-Myc, two key factors involved in cellular metabolism, future studies on the biochemistry and molecular mechanism by which mTORC2 regulates metabolic reprogramming will provide new insights and improve our understanding of B cell immunity in health and diseases.

MATERIALS AND METHODS

Mice

A Sin1 allele-targeted ES cell line (from EUCOMM) was injected into C57BL/6 blastocytes by the staff at the South China Mouse Facility Center (Shanghai, China) to produce the Sin1-FrtLacZNeoFrt-loxp chimeric mice. Seven founder lines were generated and used to breed to a CMV-Flp mouse line to obtain three germline-transmitted *Sin1*$^{flox/+}$ founder lines (to delete the FrtLacZNeoFrt cassette as well) that were subsequently crossed with *Cd19*-Cre[36] mice to generate inducible and B cell conditional Sin1-deleted mouse stains. At the same time, *Sin1*$^{+/-}$ mice[24] were also crossed with *Cd19*-Cre mice to generate *Sin1*$^{+/-}$*Cd19*-Cre mice.

Fetal liver transplantation

The host mice received a sub-lethal dose of radiation to ensure that a portion of host hematopoietic stem cells would survive to provide a population of wild type host B cells in recipient mice, which were directly compared with the transplanted cells. *Sin1*$^{-/-}$ KO mice were described previously.[24] *Cd45.1*$^{+/+}$ congenic (B6.SJL-*Ptprc*^{a}) mice were purchased from The Jackson Laboratory and used as recipients for the fetal liver hematopoietic cell transfers. Mice receiving fetal liver cell transplants were irradiated with 700–900 cGy. A single-cell suspension of fetal liver cells was prepared by gently passing the fetal liver through a 27-1/2 G needle 10 times and then suspended in 1× PBS for injection. Approximately 1×10^5–2×10^6 fetal liver cells were injected into each recipient mouse via the tail vein. Mice transplanted with fetal liver cells were housed in the animal facilities at Yale University and all animal procedures were approved by the Yale Institutional Animal Care and Use Committee.

Bone marrow chimeras

Two- to 3-month-old recipient mice (*Cd45.1*$^{+/+}$) were irradiated at a dose of 800 cGy and transplanted with *Cd45.1*$^{+}$ *Cd45.2*$^{+}$ B6 mouse bone marrow cells (2.5×10^6 cells) and *Sin1*$^{fl/-}$*Cd19*-Cre (*Cd45.2*$^{+/+}$) bone marrow cells (2.5×10^6 cells) to generate bone marrow chimeric mice. Sin1 cKO mice and BM chimeras were bred and housed in an SPF environment in a barrier facility in Shanghai Jiaotong University School of Medicine, and all animal procedures were reviewed and approved by the Animal Care Committee of Shanghai Jiaotong University School of Medicine.

Pro-B cell line cultures and splenic B cell culture

Pro-B cells were derived from paired E11.5-E12.5 *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ littermate embryos. The *Akt1*$^{-/-}$*Akt2*$^{-/-}$ pro-B cells were previously described.[31] All pro-B cells were cultured on OP9 stromal cells in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), penicillin/streptomycin (50 U/ml/50 μg/ml) (Life Technologies), gentamicin (25 μg/ml) (Life Technologies), 50 μM β-mercaptoethanol (Sigma-Aldrich), and recombinant mIL-7 (10 ng/ml, Peprotech or a 1:10 dilution of mIL-7 transfected 293T cell supernatant). The pro-B cell line was differentiated in vitro by plating 30×10^6 pro-B cells on a 10 cm plate containing confluent OP9 stromal cells in RPMI 1640 medium supplemented with 10% FBS, Pen/Strep, gentamycin and β-mercaptoethanol, without exogenous mIL-7. OP9 bone marrow stromal cells were cultured in DMEM high-glucose medium supplemented with 20% FBS, pen/strep and gentamicin. Spleen cells were isolated from *Sin1*$^{fl/-}$*Cd19*-Cre and *Sin1*$^{+/+}$ competitive chimeras and treated with 10 μg/ml anti-IgM F(ab')$_2$ (Jackson ImmunoResearch Laboratories, cat#115-006-020) in a medium containing 10% FBS, penicillin-streptomycin and β-mercaptoethanol. For the proliferation assay, splenic B cells were labeled with 5 μM CFSE (Life Technologies, C34554) and cultured with or without 10 μg/ml anti-IgM F(ab')$_2$ for 3 days, and B cell proliferation was subsequently measured using the CFSE dilution assay.

Inhibitors

Rapamycin (LC Laboratories) was prepared as a 10 μM stock solution in ethanol and used at a final concentration of 20 nM in all studies, unless indicated otherwise. A 10 μM stock solution of pp242 was prepared in DMSO, and the GSK3 inhibitor SB216763 was prepared at a stock concentration of 10 mM in DMSO. All inhibitor stocks were stored at -80 °C.

Flow cytometry

Single cell suspensions were first stained for surface antigens in cold FACS buffer (1× PBS pH 7.4 + 2% FBS) with the appropriate fluorophore or biotin-conjugated antibodies for 15 min on ice. For biotin-conjugated antibodies, cells were washed with FACS buffer and incubated with the appropriate streptavidin-conjugated fluorophores for 15 min on ice. Anti-CD23 (B3B4, eBioscience), anti-CD25 (3C7, BioLegend), anti-CD43 (S7, BD), anti-CD45.1 (A20, BioLegend), (A20, BD), CD45.2 (104, BioLegend), anti-CD93 (AA4.1, BioLegend), anti-IgM (RMM-1, BioLegend), anti-IgD (11-26c, eBioscience) and anti-B220 (RA3-6B2, BD) antibodies were used for surface marker staining. After surface staining, single cells were suspended in DAPI (D3571, Invitrogen) to exclude dead cells. PI (421301, BioLegend) and AnnexinV (550474, BD) were used to analyze the splenic B cell cycle and apoptosis. For the detection of intracellular antigens, cells were permeabilized with Fix Buffer 1 (BD) for 10 min at 37 °C and then washed and permeabilized with 1xPerm/Wash Buffer (BD) or Perm Buffer 3 (BD) for 5 mins at room temperature. Fixed and permeabilized cells were stained with the following antibodies diluted 1:100 in Perm/Wash buffer for 30 min at room temperature, anti-IgK-FITC and anti-IgM-PE (for intracellular IgH staining) (BioLegend), as well as one of the following unlabeled rabbit antibodies: anti-Akt p-Thr308 (rabbit polyclonal, Cat#9275), anti-Akt p-Ser473 (587F11), anti-Akt p-Ser473 (D9E), anti-pan-Akt (11E7), anti-S6 p-Ser235/236 (rabbit polyclonal, Cat#2211), anti-S6 (rabbit polyclonal, Cat#9202), anti-c-Myc (D84C12) (Cell Signaling Technology) or anti-Erk1/2 (rabbit polyclonal, Cat#9102). Cells were washed and stained with a 1:500 dilution of goat-anti-rabbit-IgG-Alexa Fluor647 or

FITC for 15 min in Perm/Wash Buffer. All cells were then washed and resuspended in FACS buffer for analysis with an LSRII or LSRFortessa instrument (BD).

Quantitative RT-PCR

Cells were lysed with TRIzol (Invitrogen), and total RNA was purified using isopropanol precipitation. Total RNA was treated with RNAse-free DNAse I (Sigma) and reverse transcribed with the iScript reverse transcriptase cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed with an iQ5 multicolor RT-PCR detection system (Bio-Rad) using the SsoFast EvaGreen Supermix PCR master mix kit (Bio-Rad).

B cell metabolic assay

Splenic B cells were purified from *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice using MACS (Miltenyi, Cat: 130-090-862). Purified splenic B cells from *Sin1*$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice were plated on Cell-Tak (BD Biosciences)-coated Seahorse cell culture plates (4×10^5 cells/well). The steady-state oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in B cells were measured using an XFe96 Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacturer's instructions. For the metabolic analysis of anti-IgM-induced cells, purified splenic B cells were pretreated with 10 μg/ml anti-IgM F(ab')$_2$ antibody for 1 h and then their ECAR and OCR were measured as described above.

Immunization and Ag-specific antibody detection

Sin1$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice were immunized with NP-CGG (0.1 mg/mouse) (Biosearch Technologies, N-5055E-5) plus Alum (Pierce, Cat#77161) via an intraperitoneal injection. After 14 days, serum samples were collected and levels of NP-specific antibodies were analyzed using an ELISA. NP_4 BSA (Biosearch Technologies, N-5050L-10) and NP_{30} BSA (Biosearch Technologies, N-5050H-10) were coated as antigens to detect high- and low-affinity NP-specific antibodies, respectively, as previously described.[62]

Vaccination and PR8 virus infection assay

Sin1$^{fl/+}$*Cd19*-Cre and *Sin1*$^{fl/fl}$*Cd19*-Cre mice were vaccinated with Influenza A virus hemagglutinin (HA) (1 μg/mouse) via an intramuscular (i.m.) injection. After 21 days, mice were boosted with another HA immunization (Sino Biological, 1 μg/mouse, i.m.). Nine days later, all mice were intranasally infected with 30 LD_{50} of influenza virus H1N1 (A/PR/8/34). Mouse body weights and survival rates were monitored daily after PR8 infection. Serum samples were collected after the primary vaccination, 7 days after the boost, and at the end of infection analyses (~day 40). Microtiter plates were coated with 2 μg/ml PR8 hemagglutinin (HA) protein overnight at room temperature. Serum HA-specific IgG titers were detected using an ELISA. For the histological analysis, lung tissues were fixed with formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin using a standard procedure.

Immunoblotting and antibodies

Cells were washed 2× with ice-cold 1x PBS and lysed in cold RIPA buffer with freshly added protease and phosphatase inhibitors. Total cell lysates were resolved on SDS-PAGE gels and blotted with the following antibodies: anti-Akt p-Thr308 (rabbit polyclonal, Cat#9275), anti-Akt p-Ser473 (587F11), anti-Akt p-Thr450 (rabbit polyclonal, Cat#9267), anti-panAkt (11E7), anti-PKCα/βII p-Thr638/641 (rabbit polyclonal, Cat#9375), anti-GSK3α/β p-Ser21/9 (rabbit polyclonal, Cat#9331), anti-GSK3α (D80E6), anti-GSK3β (27C10), anti-TSC2 p-Ser939 (rabbit polyclonal, Cat#3615), anti-TSC2 p-Thr1462 (5B12), anti-TSC2 (rabbit polyclonal, Cat#3612), anti-PRAS40 (rabbit polyclonal, Cat#21097-1-AP), anti-PRAS40 p-Thr246 (rabbit mAb, Cat#13175), anti-S6 p-Ser235/236 (rabbit polyclonal, Cat#2211), anti-S6 (mouse mAb, Cat#2317), anti-S6K p-Thr389 (rabbit mAb, Cat#9234), anti-S6K (rabbit polyclonal, Cat#9202), anti-4E-BP1 p-Thr37/46 (rabbit mAb, Cat#2855), anti-4E-BP1 (rabbit mAb, Cat#9644), anti-c-Myc p-Thr58/Ser62 (rabbit polyclonal, Cat#9401), anti-Erk1/2 (rabbit polyclonal, Cat#9102), anti-c-Myc (D84C12), anti-Erk1/2 p-Thr202/Tyr204 (mouse mAb, Cat#9106) anti-Foxo1/3a p-Thr24/Thr32 (rabbit polyclonal, Cat#9464) from Cell Signaling, anti-Sin1 (K87),[24] and anti-ERK2 (381A10) (Invitrogen). The densitometry analysis was performed with a Bio-Rad Molecular Imager Gel Doc XR system and Quantity One software (Bio-Rad).

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AUTHOR CONTRIBUTIONS

F.L., A.S.L. and B.S. conceived and designed the experiments and wrote the paper. M.L. and A.S.L. performed the experiments, analyzed the data and wrote the paper. X.O. generated the *Sin1*$^{fl/fl}$ mice. O.A., X.X., D.L., Q.W., L.Y., J.J. and Y.H. performed the experiments. A.S.L., O.A., X.X. and D.L. performed the fetal liver transplantation, in vitro pro-B cell culture and immunoblotting. B cell development analyses were performed by M.L. X.B.L, W.Q.Z and H.H.Z. Flow cytometry, model antigen immunization, the PR8 virus infection assay and B cell metabolic measurements were performed by M.L., X.O., G.Q., Y.F., L.L., S.J., and Q.W. C.H., C.R., and Z.Y. discussed the data.

ADDITIONAL INFORMATION

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REFERENCES

1. Jellusova, J. & Rickert, R. C. The PI3K pathway in B cell metabolism. *Crit. Rev. Biochem. Mol. Biol.* **51**, 359–378 (2016).
2. Srinivasan, L. et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell* **139**, 573–586 (2009).
3. Lam, K. P., Kuhn, R. & Rajewsky, K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* **90**, 1073–1083 (1997).
4. Kraus, M., Alimzhanov, M. B., Rajewsky, N. & Rajewsky, K. Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. *Cell* **117**, 787–800 (2004).
5. Avalos, A. M., Meyer-Wentrup, F. & Ploegh, H. L. B-cell receptor signaling in lymphoid malignancies and autoimmunity. *Adv. Immunol.* **123**, 1–49 (2014).
6. Khalil, A. M., Cambier, J. C. & Shlomchik, M. J. B cell receptor signal transduction in the GC is short-circuited by high phosphatase activity. *Science* **336**, 1178–1181 (2012).
7. Patke, A., Mecklenbrauker, I., Erdjument-Bromage, H., Tempst, P. & Tarakhovsky, A. BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. *J. Exp. Med.* **203**, 2551–2562 (2006).
8. Konigsberger, S. & Kiefer, F. The BAFFling function of Syk in B-cell homeostasis. *EMBO J.* **34**, 838–840 (2015).
9. Guo, B., Su, T. T. & Rawlings, D. J. Protein kinase C family functions in B-cell activation. *Curr. Opin. Immunol.* **16**, 367–373 (2004).
10. Rawlings, D. J., Schwartz, M. A., Jackson, S. W. & Meyer-Bahlburg, A. Integration of B cell responses through Toll-like receptors and antigen receptors. *Nat. Rev. Immunol.* **12**, 282–294 (2012).
11. Tsui, C. et al. Protein kinase C-beta dictates b cell fate by regulating mitochondrial remodeling, metabolic reprogramming, and heme biosynthesis. *Immunity* **48**, 1144–1159 e1145 (2018).

12. Su, B. & Jacinto, E. Mammalian TOR signaling to the AGC kinases. *Crit. Rev. Biochem. Mol. Biol.* **46**, 527–547 (2011).
13. Saxton, R. A. & Sabatini, D. M. mTOR signaling in growth, metabolism, and disease. *Cell* **168**, 960–976 (2017).
14. Wullschleger, S., Loewith, R. & Hall, M. N. TOR signaling in growth and metabolism. *Cell* **124**, 471–484 (2006).
15. Cornu, M., Albert, V. & Hall, M. N. mTOR in aging, metabolism, and cancer. *Curr. Opin. Genet. Dev.* **23**, 53–62 (2013).
16. Wicker, L. S. et al. Suppression of B cell activation by cyclosporin A, FK506 and rapamycin. *Eur. J. Immunol.* **20**, 2277–2283 (1990).
17. Aagaard-Tillery, K. M. & Jelinek, D. F. Inhibition of human B lymphocyte cell cycle progression and differentiation by rapamycin. *Cell. Immunol.* **156**, 493–507 (1994).
18. Ersching, J. et al. Germinal Center Selection and Affinity Maturation Require Dynamic Regulation of mTORC1 Kinase. *Immunity* **46**, 1045–1058 e1046 (2017).
19. Yang, J. *et al*. Critical roles of mTOR Complex 1 and 2 for T follicular helper cell differentiation and germinal center responses. *Elife* **5**, e17936 (2016).
20. Zeng, H. et al. mTORC1 and mTORC2 kinase signaling and glucose metabolism drive follicular helper T cell differentiation. *Immunity* **45**, 540–554 (2016).
21. Zhang, S. et al. B cell-specific deficiencies in mTOR limit humoral immune responses. *J. Immunol.* **191**, 1692–1703 (2013).
22. Luo, W., Weisel, F. & Shlomchik, M. J. B. Cell receptor and CD40 signaling are rewired for synergistic induction of the c-Myc transcription factor in germinal center B cells. *Immunity* **48**, 313–326 e315 (2018).
23. Sancak, Y. et al. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol. Cell* **25**, 903–915 (2007).
24. Jacinto, E. et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125–137 (2006).
25. Sarbassov, D. D. et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* **14**, 1296–1302 (2004).
26. Jacinto, E. et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell Biol.* **6**, 1122–1128 (2004).
27. Hara, K. et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110**, 177–189 (2002).
28. Facchinetti, V. et al. The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J.* **27**, 1932–1943 (2008).
29. Ikenoue, T., Inoki, K., Yang, Q., Zhou, X. & Guan, K. L. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J.* **27**, 1919–1931 (2008).
30. Chen, X. et al. Cryo-EM structure of human mTOR complex 2. *Cell Res.* **28**, 518–528 (2018).
31. Lazorchak, A. S. et al. Sin1-mTORC2 suppresses rag and il7r gene expression through Akt2 in B cells. *Mol. Cell* **39**, 433–443 (2010).
32. Lee, K. et al. Requirement for Rictor in homeostasis and function of mature B lymphoid cells. *Blood* **122**, 2369–2379 (2013).
33. Limon, J. J. et al. mTOR kinase inhibitors promote antibody class switching via mTORC2 inhibition. *Proc. Natl Acad. Sci. USA* **111**, E5076–E5085 (2014).
34. Holl, T. M., Haynes, B. F. & Kelsoe, G. Stromal cell independent B cell development in vitro: generation and recovery of autoreactive clones. *J. Immunol. Methods* **354**, 53–67 (2010).
35. Nakano, T., Kodama, H. & Honjo, T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* **265**, 1098–1101 (1994).
36. Rickert, R. C., Roes, J. & Rajewsky, K. B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res.* **25**, 1317–1318 (1997).
37. Engelman, J. A., Luo, J. & Cantley, L. C. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat. Rev. Genet.* **7**, 606–619 (2006).
38. Yang, G., Murashige, D. S., Humphrey, S. J. & James, D. E. A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation. *Cell Rep.* **12**, 937–943 (2015).
39. van Riggelen, J., Yetil, A. & Felsher, D. W. MYC as a regulator of ribosome biogenesis and protein synthesis. *Nat. Rev. Cancer* **10**, 301–309 (2010).
40. Iritani, B. M. & Eisenman, R. N. c-Myc enhances protein synthesis and cell size during B lymphocyte development. *Proc. Natl Acad. Sci. USA* **96**, 13180–13185 (1999).
41. Inoki, K., Zhu, T. & Guan, K. L. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**, 577–590 (2003).
42. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. & Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785–789 (1995).
43. Sears, R. C. The life cycle of C-myc: from synthesis to degradation. *Cell Cycle* **3**, 1133–1137 (2004).
44. He, B. et al. Intranasal application of polyethyleneimine suppresses influenza virus infection in mice. *Emerg. Microbes Infect.* **5**, e41 (2016).
45. Donahue, A. C. & Fruman, D. A. Distinct signaling mechanisms activate the target of rapamycin in response to different B-cell stimuli. *Eur. J. Immunol.* **37**, 2923–2936 (2007).
46. Chang, X., Lazorchak, A. S., Liu, D. & Su, B. Sin1 regulates Treg-cell development but is not required for T-cell growth and proliferation. *Eur. J. Immunol.* **42**, 1639–1647 (2012).
47. Lee, K. et al. Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity* **32**, 743–753 (2010).
48. Frias, M. A. et al. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr. Biol.* **16**, 1865–1870 (2006).
49. Chung, J. B., Silverman, M. & Monroe, J. G. Transitional B cells: step by step towards immune competence. *Trends Immunol.* **24**, 343–349 (2003).
50. Doughty, C. A. et al. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. *Blood* **107**, 4458–4465 (2006).
51. Betz, C. et al. Feature Article: mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology. *Proc. Natl Acad. Sci. USA* **110**, 12526–12534 (2013).
52. Jellusova, J. et al. Gsk3 is a metabolic checkpoint regulator in B cells. *Nat. Immunol.* **18**, 303–312 (2017).
53. Latronico, M. V., Costinean, S., Lavitrano, M. L., Peschle, C. & Condorelli, G. Regulation of cell size and contractile function by AKT in cardiomyocytes. *Ann. N. Y. Acad. Sci.* **1015**, 250–260 (2004).
54. Faridi, J., Fawcett, J., Wang, L. & Roth, R. A. Akt promotes increased mammalian cell size by stimulating protein synthesis and inhibiting protein degradation. *Am. J. Physiol. Endocrinol. Metab.* **285**, E964–E972 (2003).
55. Zhang, F., Lazorchak, A. S., Liu, D., Chen, F. & Su, B. Inhibition of the mTORC2 and chaperone pathways to treat leukemia. *Blood* **119**, 6080–6088 (2012).
56. Wu, Y. T. et al. mTOR complex 2 targets Akt for proteasomal degradation via phosphorylation at the hydrophobic motif. *J. Biol. Chem.* **286**, 14190–14198 (2011).
57. Liu, P. et al. Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis. *Nat. Cell Biol.* **15**, 1340–1350 (2013).
58. Liu, P. et al. PtdIns(3,4,5)P3-dependent activation of the mTORC2 kinase complex. *Cancer Discov.* **5**, 1194–1209 (2015).
59. Dengler, H. S. et al. Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. *Nat. Immunol.* **9**, 1388–1398 (2008).
60. Amin, R. H. & Schlissel, M. S. Foxo1 directly regulates the transcription of recombination-activating genes during B cell development. *Nat. Immunol.* **9**, 613–622 (2008).
61. Inoue, T. et al. The transcription factor Foxo1 controls germinal center B cell proliferation in response to T cell help. *J. Exp. Med.* **214**, 1181–1198 (2017).
62. Li, F., Smith, P. & Ravetch, J. V. Inhibitory Fcgamma receptor is required for the maintenance of tolerance through distinct mechanisms. *J. Immunol.* **192**, 3021–3028 (2014).