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Award Number:
W81XWH-10-1-0259

TITLE:

The Role of mTOR Signaling in the Regulation of RAG Expression and Genomic Stability During B Lymphocyte Development

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REPORT DATE:
July 2014

TYPE OF REPORT:
Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

XX Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2014	Final report	15 Apr 2010 - 14 Apr 2014

4. TITLE AND SUBTITLE
The Role of mTOR Signaling in the Regulation of RAG Expression and Genomic Stability During B Lymphocyte Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0259

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University

New Haven CT 065210-8089

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The developing lymphocytes, which are a type of white blood cells, routinely create chromosome breaks while generating unique receptors to recognize foreign pathogens. The enzyme which cuts lymphocyte DNA to facilitate the construction of an immune cell receptor is encoded by two genes collectively known as the recombinase activating genes (*rag*)s. Expression of the *rag* genes is tightly controlled by cellular signals that ensure *rag* is only active in lymphocytes when immune receptor formation is occurring, after which RAG expression is shut down. Our research has revealed that mTOR controls *rag* expression in B cells by participating in a multi-protein complex called mTOR complex 2 (mTORC2). mTORC2 actively inhibits expression of the *rag* genes in B cells thereby preventing inappropriate *rag* expression and protecting the B cell DNA from excessive damage caused by *rag* activity. We found that mTORC2 suppresses *rag* expression by controlling the activity of a signaling mediator called Akt. Abnormal Akt activity is commonly associated with a wide range of cancers and our research has revealed that mTORC2 plays a key role in controlling Akt activity in B cells raising the possibility that mTORC2 inhibition may be good target for the treatment of certain B cell tumors. We show that inhibition of mTORC2 plus the chaperon protein HSP90 *in vitro* and *in vivo* elicits a potent anti-leukemic effect which is greater than inhibiting mTORC2 or HSP90 alone, suggesting that combination of mTOR inhibitors and chaperon inhibition may enhance anti-leukemic activity in blood cancer patients. Over the funding period, we have explored the role of Sin1 in B cell growth and proliferation, either under normal physiological or when become malignant. We have also revealed a combination retreatment for leukemia cells with both mTORC2 inhibitor and molecular chaperon inhibitor. At the molecular mechanistic level, we discover that Sin1 could affect both mTORC1 and mTORC2 in regulating cell growth via a previously unknown function of mTORC2 through a GSK3-involved c-Myc expression in B cells.

15. SUBJECT TERMS
Sin1, mTOR, Rag1/2, p53, B cells, leukemia and lymphoma, blood cancer.

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	85	USAMRMC

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INTRODUCTION:

Mechanistic target of rapamycin (mTOR) is an important mediator of phosphoinositol-3-kinase (PI3K) signaling [1]. PI3K signaling regulates B cell development, homeostasis and immune responses [2]. However, the molecular mechanism of mTOR signaling in B cell growth and differentiation is not fully understood. Sin1 is a highly conserved adaptor molecule required for mTOR activation. Previous studies have established Sin1 as an indispensable component and regulator of mTOR complex 2 (mTORC2) for its integrity and function [3, 4]. We previously demonstrate that Sin1 is essential for mTORC2 dependent regulation of Akt and FoxO activity using mouse genetics and biochemical approaches [3]. Most importantly, our studies show that Sin1 is crucial for the integrity of mTORC2 but not mTORC1. We hypothesize that Sin1 is likely to be important for B cell tumor growth through its regulation of mTOR function. Based on our recent studies on B cell development, we also propose that Sin1 promotes genomic stability and suppresses tumor formation in developing B cell by negatively regulating recombinase activating gene (RAG) expression and Immunoglobulin (Ig) gene somatic recombination [V(D)J recombination]. As we pursue the studies outlined in this proposal we provided the first evidence linking mTOR function to the regulation of *Rag* gene expression and V(D)J recombination and developed unique and innovative new Sin1/mTORC2 deficient progenitor B cell lines and B cell leukemia cell models. Furthermore, we have explored the role of Sin1 in B cell growth and proliferation and found that Sin1/mTORC2 plays an essential role in regulating B cell growth and proliferation. These studies identified the pro-growth transcriptional regulator c-Myc as a target of mTORC2 mediated signaling in B cells thereby revealing a novel role for Sin1/mTORC2 in B cell growth and metabolic regulation. Finally, we utilized our unique *Sin1*$^{-/-}$ BCR-Abl leukemia cell lines to study the role of Sin1 maintaining leukemia cell viability under conditions of cellular stress such as nutrient depravation. These studies reveal that Sin1 and possibly mTORC2 play an important role in regulating the adaptive leukemic cellular response to amino acid depravation and raise the possibility that mTORC2 inhibition may sensitize leukemia cells to therapeutics that target cell metabolism.

BODY:

With this generous DOD support, we have successfully completed the key goals outlined in the SOW of this proposal and, in the process of completing these goals, we have revealed new and unexpected functions of Sin1 in B cell growth, development and immune function. Since we have listed most of the key research accomplishments in each of the annual progress reports submitted in the previous years, in this final progress report, we will not present in the main body of the report all the figures but instead to include the published papers and also the submitted manuscript as attachments at the end of the report. We will try to summarize only the main findings from this proposed study. In **Task 1**, we hypothesized that Sin1 regulates RAG expression and Ig gene V(D)J recombination through a mechanism involving mTORC2. Our results show that Sin1 is required for B cell development and Ig gene recombination and showed that Sin1, as part of mTORC2, suppresses Ig gene V(D)J recombination by regulating the expression of the RAG recombinase genes *rag1* and *rag2*. Mammalian TORC2 mediates pre-B cell receptor or B cell receptor signaling and phosphorylates Akt2 at S473. S473 phosphorylated Akt2 phosphorylation directs Akt2 substrate specificity towards the transcriptional regulator FoxO1 resulting in the inactivation of FoxO1 and reduction of FoxO1 dependent RAG gene expression. The increased RAG expression in Sin1 deficient B cells correlated with increased V(D)J recombinase activity as measured by an artificial virally expressed V(D)J recombinase substrate and by increased Ig light chain receptor editing in Sin1 deficient immature B cells.

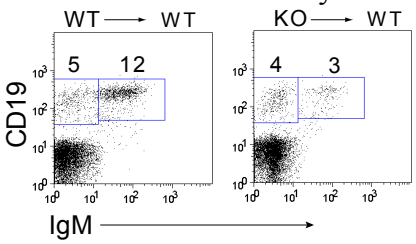

Figure 1: Bone marrow from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ chimeric mice was analyzed by flow cytometry and the percentage of CD19^{+} IgM^{-} and CD19^{+} IgM^{+} B cells is indicated. The data are representative of *Sin1*$^{+/+}$ (n=3) and *Sin1*$^{-/-}$ (n=4) fetal liver chimeric mice.

In research outlined in **Task 2**, we have elucidated the function of Sin1 in the regulation of B cell growth and development, and successfully developed an *in vivo* model to study Sin1 function in the immune system of mice by adoptively transferring *Sin1*$^{-/-}$ fetal liver hematopoietic stem cells (FL-HSC) into lethally irradiated wild type or *rag1*$^{-/-}$ recipient mice. We also successfully developed an *in vitro* pro-B cell culture system to study Sin1 function in pro-B cell and early developing B cell differentiation. Furthermore, we used chimeric mice generated by adoptively transferring FL-HSCs into lethally irradiated wild type CD45.1^{+} congenic mice so that lymphocytes arising from the FL-HSCs are easily distinguished from the host lymphocytes due to differential expression of the cell surface markers CD45.1 (exclusively expressed on host cells) and CD45.2 (expressed on donor cells). We found that *Sin1*$^{-/-}$ FL-HSCs successfully engrafted and contributed to the long term reconstitution of the hematopoietic system of both strains of recipient mice. Analysis of the bone marrow and spleen of *Sin1*$^{-/-}$ FL-HSC chimeric mice revealed that loss of Sin1 is required for B cell development. Sin1 deficiency results in a reduction of IgM^{+} immature B cells in the bone marrow (**Fig. 1**).

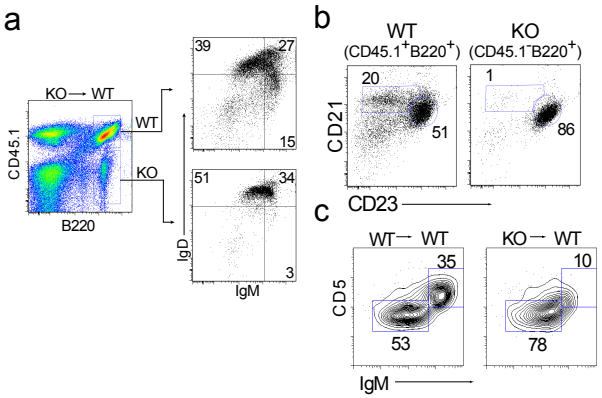

Figure 2: **a)** Total splenocytes from a *Sin1*$^{-/-}$ fetal liver HSC chimeric mouse were stained with the indicated antibodies and analyzed by flow cytometry. Host wild type (WT) B cells (CD45.1^{+}B220^{+}) and donor *Sin1*$^{-/-}$ (KO) B cells (CD45.1^{-}B220^{+}) were gated and analyzed for IgM and IgD expression. The proportion of host and donor IgM^{hi}IgD^{low}, IgM^{hi}IgD^{hi} and IgM^{low}IgD^{hi} splenic B cells is indicated next to each respective quadrant. The data shown are representative of three individual *Sin1*$^{-/-}$ fetal liver HSC chimeric mice. **b)** The proportion of WT and KO marginal zone B cells (CD21^{high}CD23^{low}; rectangular gate) and follicular B cells (CD21^{int}CD23^{hi}; circular gate) in the spleen of the *Sin1*$^{-/-}$ chimeric mouse described in **a** are indicated next to the corresponding FACS gates. The plots shown are pre-gated on B220^{+}CD45.1^{+} host wild type (WT) or B220^{+}CD45.1^{-} donor *Sin1*$^{-/-}$ (KO) B cells and are representative of three *Sin1*$^{-/-}$ fetal liver HSC chimeric mice. **c)** FACS plots showing donor derived B cells obtained from the peritoneal cavity of a *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ fetal liver HSC chimeric mice were stained for CD5 and IgM. The plots shown are pre-gated on CD45.1^{-} CD19^{+} donor B cells and are representative of n=2 *Sin1*$^{+/+}$ and n=2 *Sin1*$^{-/-}$ chimeric mice. The percentage of B1a B cells (CD5^{high}IgM^{high}) were reduced in Sin1 deficient chimeric mice when compared to WT chimeric mice (10% vs. 35% respectively).

Interestingly, we found that Sin1 deletion in B cells impaired development of mature peripheral B cells with a specific loss of the splenic marginal zone B cells and the loss of peritoneal B1a B cells (**Fig. 2**). These data clearly show that Sin1 plays a critical role for B cell development.

Previously we show that Sin1 plays an essential role in mTOR signaling. Analysis of peripheral B cells from *Sin1*$^{-/-}$ FL-HSC chimeric mice revealed that *Sin1*$^{-/-}$ mature splenic and lymph node B cells indeed had a smaller size than that of wild type B cells (**Fig. 3**). These data indicate that Sin1 may play an important role in regulating B cell metabolism and/or macromolecular synthesis. Cell growth is essential for the development of a functional B cell immune response. Therefore, we asked if Sin1 is also required for the blast cell growth and proliferation of mitogen activated B cells. We stimulated splenic B cells isolated from *Sin1*$^{-/-}$ or *Sin1*$^{+/+}$ FL-HSC chimeric mice with lipopolysaccharide (LPS), a potent polyclonal B cell mitogen, and measured B cell growth and proliferation. We found that Sin1 deficient B cells showed severely impaired blast cell growth and proliferation in response to LPS stimulation (**Fig. 4**). We observed similar results in *Sin1*$^{-/-}$ B cells stimulated through the B cell receptor which strongly suggests that Sin1 plays an important role in mediating mitogen dependent signals that promote B cell growth and immune function. Surprisingly, we found that Sin1 is not required for the growth and proliferation of T cells indicating that Sin1 and, most likely, mTORC2 play a specific and key role in regulating B cell growth and

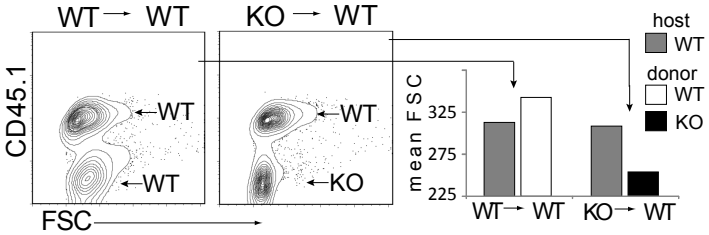

Figure 3: The relative size of splenic B cells from *Sin1*$^{+/+}$ (WT-->WT) or *Sin1*$^{-/-}$ (KO-->WT) fetal liver HSC-chimeric mice was measured by forward light scatter (FSC). The fetal liver HSC derived CD45.1^{-} WT or KO B cells (donor) and WT CD45.1^{+} (host) B cell populations within each mouse are indicated. The plots shown are pre-gated on live, CD19^{+} lymphocytes and are representative of n=2 WT and n=3 KO chimeric mice.

immunity (**Reprint 3**).

Using *in vitro* *Sin1*$^{-/-}$ pro-B cell culture system, we prepared primary pro-B cell lines from the fetal liver cells of *Sin1*$^{-/-}$ or *Sin1*$^{+/+}$ embryos in the presence of recombinant mouse IL-7. These progenitor *Sin1*$^{-/-}$ or *Sin1*$^{+/+}$ B cells can be expanded and cultured for an extended period of time. Without exogenous IL-7, B cell differentiated within 5-7 days, gave rise to populations of developing B cells that resemble bone marrow IgM^{-} pre-B, IgM^{+} immature B and IgM^{+}IgD^{+} transitional B cells. We measured the cell size of pro-B, pre-B, immature B and transitional B cells from these cultures and found that Sin1 deficiency results in a decrease in B cell size at the later immature and transitional B cell developmental stages but not at the earlier pro-B and pre-B developmental stages (**Fig. 5**). These data reveal that Sin1 regulates B cell growth in a developmental stage specific manner.

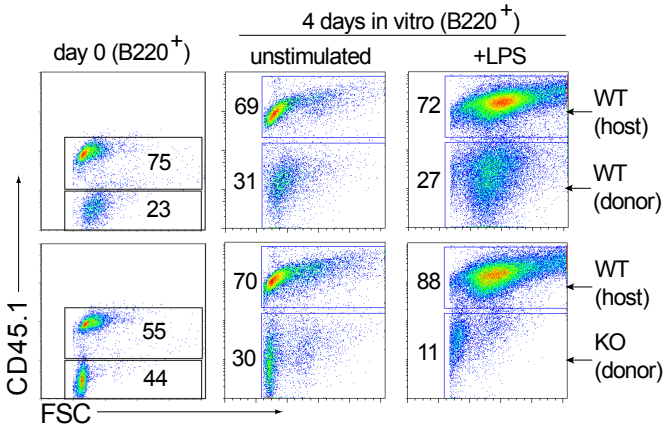

Figure 4: Total splenocytes from *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) fetal liver HSC chimeric mice were cultured in vitro with medium alone (unstimulated) or with 10 µg/ml LPS (+LPS) for 4 days. The relative size of the WT CD45.1^{+} (host) B cells and CD45.1^{-} (donor) WT or KO B cells was measured by FSC. The plots show freshly isolated splenic B cells (day 0) and splenic B cells cultured in vitro for 4 days with or without LPS. All of the plots shown are pre-gated on live, B220^{+} B cells and the numbers adjacent to each gate indicate the percent of B cells within the gate.

Our data showing that Sin1 regulates the size of developing and mature B cells strongly suggested that Sin1, and possibly via mTORC2, may act as an upstream regulator of one or more key growth or metabolic regulators in B cells. The transcription factor c-Myc is a potent B cell growth and metabolism activator whose expression is normally up-regulated in proliferating B cells. Abnormal up-regulation of c-Myc expression is a common feature of many B cell cancers. Therefore we examined c-Myc expression in *Sin1*$^{-/-}$ B cells and found that c-Myc expression was significantly lower in *Sin1*$^{-/-}$ B cells when compared to *Sin1*$^{+/+}$ B cells. We found that Sin1 was not required for c-Myc gene expression indicating that Sin1 regulates the c-Myc protein translation or degradation (**Fig. 6a & 6b**). Importantly, the mTOR kinase inhibitor pp242, which inhibits both mTORC1 and mTORC2, completely blocked the up-regulation of c-Myc expression in activated B cells whereas rapamycin, which only inhibits mTORC1, partially blocked the up-regulation of c-Myc expression in B cells (**Fig. 6c & 6d**). These data suggest that Sin1/mTORC2 regulates c-Myc expression through a mechanism which is independent of mTORC1. Indeed, we found that mTORC2 signaling promotes the stability of c-Myc protein through a pathway which is dependent on Akt and GSK3. As described in more detail the Attachment 4, these studies reveal a novel role for Sin1/mTORC2 in the regulation of c-Myc dependent B cell growth.

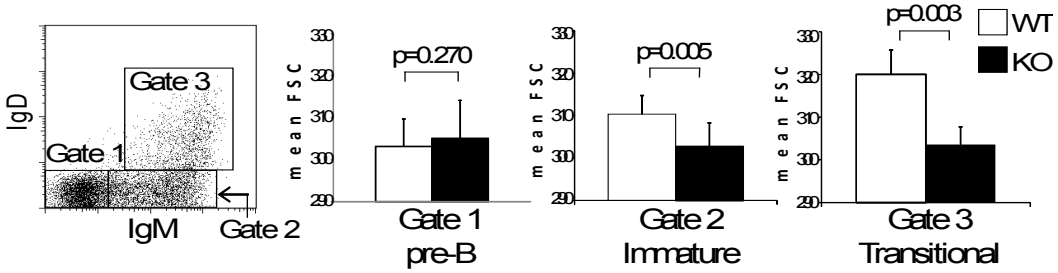

Figure 5: *Sin1*$^{+/+}$ (WT) and *Sin1*$^{-/-}$ (KO) pro-B cells were differentiated in vitro for 7 days on OP9 stromal cells and the mean FSC of pre-B (Gate 1), immature B (Gate 2) or transitional B (Gate 3) cells was measured by flow cytometry. The bar graphs for each of the gated B cell populations are the average FSC measurements from 4 independent experiments with standard deviation indicated by the error bars.

In completing the experiments outlined in **Task 3**, we found that crossing our outbreed B6/129 Sin1 knockout mice with either the B6 *RAG*^{GFP} and B6 *p53*$^{-/-}$ stains has resulted in a significant reduction in the number of viable E12.5 *Sin1*$^{-/-}$ embryos. We believe that the reduced survival of the Sin1 knockout embryos is due to a greater contribution of alleles from the B6 background since all B6 backcrossed *Sin1*$^{-/-}$ mice die at E10.5 (unpublished data).

Therefore, we used a pharmacologic approach to mimic the effects of Sin1/mTORC2 disruption in RAG^{GFP} or $p53^{-/-}$ mice. We treated mice with the mTOR kinase inhibitor pp242 to directly block mTORC2. Using the cell and mouse models we developed to exploring Sin1/mTORC2 function in specific blood cancer models, we found, surprisingly, that inhibition of mTOR with the TOR kinase inhibitor pp242 suppressed leukemia cell proliferation but failed to induce significant tumor cell death. We observed this phenomenon in both wild type and $Sin1^{-/-}$ p210 BCR-Abl transformed mouse B leukemia cells. We also found that disruption of Sin1/mTORC2 is absolutely required for the phosphorylation of a highly conserved phosphorylation motif on Akt and classical (c)PKC proteins called the turn motif (TM) in leukemia cells. We have previously shown in MEF cells that mTORC2 dependent Akt TM phosphorylation stabilizes the Akt protein. Furthermore we demonstrated that the stability of Akt proteins that lack TM is dependent on the chaperon protein HSP90 while HSP90 was not required for the stability of Akt proteins that were TM phosphorylated. Therefore we speculated that inhibition of HSP90 would specifically inhibit Akt expression and induce cell death in Sin1/mTORC2 deficient leukemia cells but not wild type leukemia cells. Indeed, we found that the HSP90 inhibitor 17-AAG preferentially inhibited the expression of Akt and cPKC in $Sin1^{-/-}$ when compared to wild type cells. We then demonstrated that 17-AAG preferentially induced cell death of $Sin1^{-/-}$ cells over wild type pre-B leukemia cells *in vitro* and *in vivo*. Finally we found that combining the mTOR inhibitors rapamycin or pp242 with 17-AAG resulted in a greater anti-leukemic effect than either drug alone in wild type leukemia cell lines. These studies reveal a novel and innovative new strategy to treat leukemia and provide a strong rational for further testing of dual mTOR/chaperon pathway inhibition for the treatment human blood cancers. These studies were described in details in **Reprint 2** (Zhang F *et al*. 2012. *Blood*).

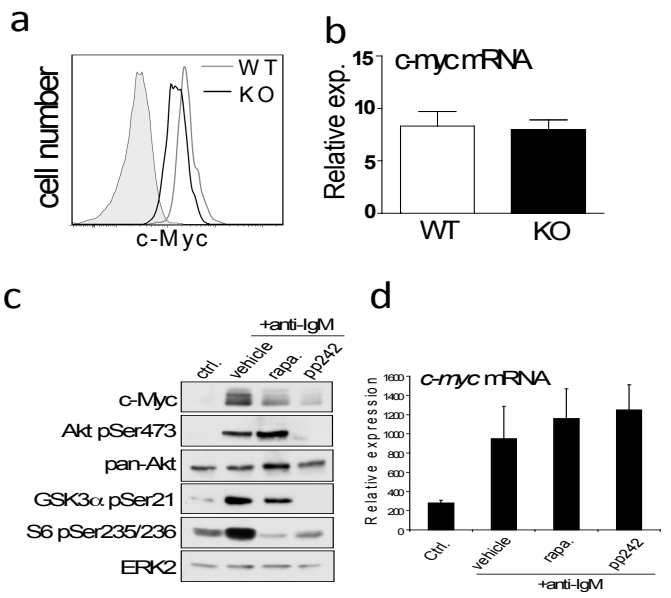

Figure 6: **a)** $Sin1^{+/+}$ (WT) or $Sin1^{-/-}$ (KO) immature B cells were stained and c-Myc expression was measured by intracellular staining and flow cytometry. The shaded line is the negative staining control. **b)** The expression level of *c-myc* mRNA was then measured by quantitative (q)RT-PCR in WT or KO IgM^+ B cells. Each sample was normalized to the expression of GAPDH. **c)** Wild type splenic B cells were enriched by negative selection and pretreated for 15 minutes with vehicle only (vehicle), 20 nM rapamycin or 50 nM pp242, and then cultured for an additional 60 minutes with medium only (ctrl.) or 10 μg/ml anti-IgM $F(ab')_2$. The expression level and phosphorylation status of the indicated cellular proteins was measured by immunoblotting. **d)** Wild type splenic B cells were pre-treated with indicated mTOR inhibitors and then stimulated with anti-IgM $F(ab')_2$ as described in panel **a**. Relative expression levels of *c-myc* mRNA was measured by qRT-PCR and normalized to the expression level of GAPDH.

Finally, we explored the role of Sin1 in regulating the response of leukemic B cells to nutrient and energy depravation. Mechanistic TOR is a master regulator of cellular metabolism which may activate an adaptive starvation response when cells are deprived of nutrients (i.e. amino acids) or energy (i.e. glucose). To explore the role of mTORC2 in regulating the cellular response to nutrient or energy depravation, we derived pre-B leukemia cells from $Sin1^{+/+}$ or $Sin1^{-/-}$ pro-B cells through transformation with the human oncogene p210BCR-Abl. Interestingly, Sin1/mTORC2 is not required for BCR-Abl leukemia cell growth, proliferation or survival when nutrients and energy sources are abundant. However, we found that $Sin1^{-/-}$ BCR-Abl leukemia cells showed significantly reduced survival compared to $Sin1^{+/+}$ leukemia cells when subjected to conditions of amino acid starvation. We found that $Sin1^{+/+}$ p210BCR-Abl leukemia cells grown in low cysteine or low glutamine medium were capable of maintain their cell size under amino acid starvation conditions however, $Sin1^{-/-}$ p210BCR-Abl pre-B leukemia cells underwent a significant reduction in cell size when subjected to starvation conditions (**Fig. 7**). Consequently, $Sin1^{+/+}$ leukemia cells were able to continue to proliferate, albeit at

a slower rate, under nutrient starvation conditions while *Sin1*$^{-/-}$ leukemia cells failed to proliferate and ultimately died under these same conditions. These studies reveal a novel role for Sin1 in the regulation of the leukemic cell response to nutrient stress. We are currently pursuing the underlying mechanism through which Sin1 mediates this cellular adaptation to nutrient depravation and will determine if Sin1 acts through mTORC2 to mediate this cellular stress response.

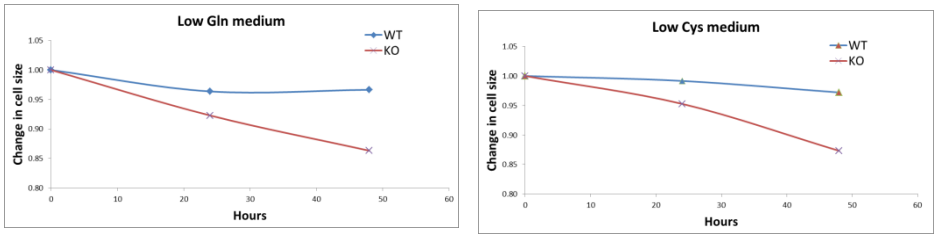

Figure 7: Sin1$^{+/+}$ and Sin1$^{-/-}$ p210BCR-Abl were co-cultured together in cell culture medium which was specifically deficient in glutamine or cystine. The change in cell size relative to 0 hrs was determinded by FSC using flow cytometry and Sin1$^{+/+}$ and Sin1$^{-/-}$ cells were distinguished by the differential expression of GPF.

In summary, in finishing the experiments outlined in this proposal, we have studied the physiological role of Sin1, one of the most critical components of mTORC2 in normal B cell development, differentiation, and growth. We have also revealed a combination retreatment for leukemia cells with both mTORC2 inhibitor and molecular chaperon inhibitor. At the molecular mechanistic level, we discover that Sin1 could affect both mTORC1 and mTORC2 in regulating cell growth via a previously unknown function of mTORC2 through a GSK3-involved c-Myc expression in B cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Sin1 maintains mTORC2 integrity and regulates B cell development
- Sin1-mTORC2 suppresses *il7r* and *rag* gene expression in developing B cells
- Sin1-mTORC2 utilizes Akt2 but not Akt1 to suppress *il7r* and *rag* gene expression
- Akt hydrophobic motif site phosphorylation is required to suppress FoxO1 target gene expression
- Disruption of mTORC2 function promotes V(D)J recombinase activity in developing B cells and B cell leukemia cells
- Sin1 regulates Ig gene receptor editing in developing B cells
- P53 deficiency suppresses the *Sin1*$^{-/-}$ B cell developmental defect
- Sin1-mTORC2 mediates Akt and cPKC turn motif phosphorylation in leukemic B cells
- 17-AAG preferentially inhibits the expression of Akt and PKCβII in Sin1 deficient leukemia cells
- 17-AAG preferentially inhibits the growth and survival of *Sin1*$^{-/-}$ leukemia cells *in vitro* and *in vivo*
- mTOR inhibitors plus 17-AAG elicit a stronger anti-leukemic effect on wild type tumor cells than either drug alone
- Sin1 is required for the development of peripheral mature B cells
- Sin1 regulates B cell growth in a cell type specific and cell developmental stage specific manner
- Mammalian TORC2 regulates B cell growth through c-Myc and mTORC1

- Sin1 promotes BCR-Abl B cell leukemia growth under conditions where nutrient availability is limiting

REPORTABLE OUTCOMES:

Manuscripts:

Lazorchak AS, Liu D, Facchinetti V, Di Lorenzo A, Sessa WC, Schatz DG, Su B. 2010. Sin1-mTORC2 suppresses rag and il7r gene expression through Akt2 in B cells. *Mol Cell.* 39(3):433-43.

Zhang F, Lazorchak AS, Liu D, Chen F, Su B. 2012. Inhibition of the mTORC2 and chaperon pathways to treat leukemia. Blood. 2012 Jun 21;119(25):6080-8.

Xing Chang, Adam S. Lazorchak, Dou Liu and **Su B.** Sin1 regulates Treg-cell development but is not required for T-cell growth and proliferation. Eur J Immunol. 2012 Jun;42(6):1639-47.

CONCLUSION:

DNA damage which results in the breaking of a chromosome is inherently dangerous to a cell and may result in mutations which are a common cause of blood cancers such as leukemia and lymphoma. The developing lymphocytes, which are a type of white blood cells, routinely create chromosome breaks while generating unique receptors to recognize foreign pathogens. The enzyme which cuts lymphocyte DNA to facilitate the construction of an immune cell receptor is encoded by two genes collectively known as the recombinase activating genes (*rag*)s. Expression of the *rag* genes is tightly controlled by cellular signals that ensure *rag* is only active in lymphocytes when immune receptor formation is occurring, after which RAG expression is shut down. Over the past year we have investigated how a specific mediator of cellular signals called mammalian target of rapamycin (mTOR) regulates *rag* expression in B lymphocytes. Our research has revealed that mTOR controls *rag* expression in B cells by participating in a multi-protein complex called mTOR complex 2 (mTORC2). mTORC2 actively inhibits expression of the *rag* genes in B cells thereby preventing inappropriate *rag* expression and protecting the B cell DNA from excessive damage caused by *rag* activity. Our studies have revealed that mTORC2 suppresses *rag* expression by controlling the activity of a signaling mediator called Akt. Abnormal Akt activity is commonly associated with a wide range of cancers and our research has revealed that mTORC2 plays a key role in controlling Akt activity in B cells raising the possibility that mTORC2 inhibition may be good target for the treatment of certain B cell tumors. Over the past year we have explored how Sin1 and mTORC2 regulates the growth of B cells. We have revealed a novel role for mTORC2 in the regulation of B cell growth and shown that the proto-oncogene c-Myc is a target of Sin1/mTORC2 regulation. We have also explored the role of Sin1 the adaptive response of leukemia cells to nutrient depravation stress. We have revealed a combination retreatment for leukemia cells with both mTORC2 inhibitor and molecular chaperon inhibitor. At the molecular mechanistic level, we discover that Sin1 could affect both mTORC1 and mTORC2 in regulating cell growth via a previously unknown function of mTORC2 through a GSK3-involved c-Myc expression in B cells. We will continue exploring the molecular basis of this Sin1 dependent nutrient stress response which we strongly believe will provide new insights to guide the development of novel therapeutics to treat blood cancers.

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APENDECESE:

Reprint 1
Reprint 2
Reprint 3
Manuscript 1

Molecular Cell
Article

Sin1-mTORC2 Suppresses *rag* and *il7r* Gene Expression through Akt2 in B Cells

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DOI 10.1016/j.molcel.2010.07.031

SUMMARY

Mammalian target of rapamycin (mTOR) is an important mediator of phosphoinositol-3-kinase (PI3K) signaling. PI3K signaling regulates B cell development, homeostasis, and immune responses. However, the function and molecular mechanism of mTOR-mediated PI3K signaling in B cells has not been fully elucidated. Here we show that Sin1, an essential component of mTOR complex 2 (mTORC2), regulates B cell development. Sin1 deficiency results in increased IL-7 receptor (*il7r*) and RAG recombinase (*rag1* and *rag2*) gene expression, leading to enhanced pro-B cell survival and augmented V(D)J recombinase activity. We further show that Akt2 specifically mediates the Sin1-mTORC2 dependent suppression of *il7r* and *rag* gene expression in B cells by regulating FoxO1 phosphorylation. Finally, we demonstrate that the mTOR inhibitor rapamycin induces *rag* expression and promotes V(D)J recombination in B cells. Our study reveals that the Sin1/mTORC2-Akt2 signaling axis is a key regulator of FoxO1 transcriptional activity in B cells.

INTRODUCTION

Mammalian TOR is a constitutively expressed, evolutionarily conserved protein kinase that plays a central role in the regulation of cell growth, proliferation, apoptosis, and metabolism (Wullschleger et al., 2006). Mammalian TOR resides in two distinct protein complexes termed mammalian TOR complex 1 (mTORC1) and mammalian TORC2 (mTORC2) (Guertin and Sabatini, 2009; Wullschleger et al., 2006). mTORC1 contains mTOR, raptor, mLST8 (GβL), and PRAS40, and its function is acutely inhibited by rapamycin, a potent immunosuppressant with antitumor effect (Guertin and Sabatini, 2009; Wullschleger et al., 2006). Upon stimulation by nutrients, growth factors, hormones, and energy signals, mTORC1 phosphorylates the translational regulators S6K and 4EBP1, which leads to increased cellular protein synthesis and ribosome biogenesis (Gingras et al., 2004; Harris and Lawrence, 2003; Wullschleger et al., 2006). mTORC2 contains Rictor, Sin1, and mLST8 in addition to mTOR, and regulates actin polymerization and cytoskeleton function (Guertin and Sabatini, 2009; Wullschleger et al., 2006). Mammalian TORC2 is resistant to acute rapamycin inhibition. However, chronic rapamycin exposure also inhibits mTORC2 in vitro and in vivo (Facchinetti et al., 2008; Sarbassov et al., 2006; Zeng et al., 2007). Recent studies show that mTORC2 regulates Akt/PKB in both a PI3K-dependent and PI3K-independent manner (Facchinetti et al., 2008; Jacinto et al., 2006; Sarbassov et al., 2005).

Akt/PKB is one of the most-studied members of the AGC kinase family, which also includes S6K, RSK, SGK, and PKC (Peterson and Schreiber, 1999; Woodgett, 2005). Like most members in this family, Akt is phosphorylated at two key residues that are located in the catalytic center (activation loop or T loop) and the C-terminal hydrophobic motif (HM). Phosphorylation of Akt/PKB at the T loop site (Thr308) is mediated by PDK1 and is essential for Akt catalytic activity (Alessi et al., 1997; Stephens et al., 1998). Phosphorylation of Akt at the HM site (Ser473) is independently mediated by mTORC2 (Jacinto et al., 2006; Sarbassov et al., 2005). Although Akt Ser473 phosphorylation is widely used as an indicator of Akt activation, the precise physiological function of this phosphorylation is still not fully understood. Phosphorylation at the Akt HM site may facilitate the PDK1-mediated phosphorylation of the T loop site, thereby enhancing Akt activity upon growth factor stimulation and PI3K activation (Alessi et al., 1996; Biondi, 2004; Scheid et al., 2002). Surprisingly however, genetic studies reveled that mTORC2 disruption, which abolishes Akt HM site phosphorylation, does not inhibit T loop phosphorylation (Jacinto et al., 2006). Rather, Akt HM site phosphorylation regulates the substrate specificity of Akt (Jacinto et al., 2006). More recently, mTORC2 was shown to phosphorylate Akt at the turn motif (TM) residue Thr450, which controls Akt protein stability (Facchinetti et al., 2008).

B lymphocyte development is divided into distinct stages where immunoglobulin (Ig) variable (V), diversity (D), and joining (J) genes of the Ig heavy (IgH) chain and V and J genes of the Ig

light (IgL) chain undergo somatic recombination, generally referred to as V(D)J recombination, to generate the B cell antigen receptor (BCR) (Schatz et al., 1989; Schlissel, 2003; Spicuglia et al., 2006). V(D)J recombination is mediated by the recombination activation genes *rag1* and *rag2*, which associate and form the V(D)J recombinase (Leu and Schatz, 1995; Schatz et al., 1989). IgH gene recombination occurs first in progenitor B (pro-B) cells and, if successful, leads to the expression of the pre-BCR. Pre-BCR signals provide critical feedback about the functionality of the IgH chain, allowing only those developing B cells with a functional pre-BCR to further differentiate into precursor B (pre-B) cells and begin IgL gene rearrangement (Herzog et al., 2009; Martensson et al., 2007). Pre-BCR signals promote cell survival and proliferation and suppress *rag* expression to prevent further IgH recombination (Geier and Schlissel, 2006; Schlissel, 2003). The pre-BCR-dependent suppression of RAG expression contributes to allelic exclusion of IgH genes, terminates additional V(D)J recombination that could disrupt a productively rearranged IgH gene, and prevents aberrant V(D)J recombination that may result in genomic instability in proliferating pre-B cells. Subsequent IgL recombination leads to the expression of the BCR on immature B cells.

PI3K and Akt negatively regulate RAG expression and V(D)J recombination (Amin and Schlissel, 2008; Llorian et al., 2007; Verkoczy et al., 2007). The Forkhead family transcription factor FoxO1 is a direct regulator of *rag* genes downstream of PI3K and Akt (Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008, 2009). Genetic or pharmacological inhibition of the PI3K pathway in B cells increases the expression of FoxO1 target genes and results in abnormal B cell function (Donahue and Fruman, 2004; Llorian et al., 2007; Suzuki et al., 1999; Verkoczy et al., 2007). These studies suggest that Akt may mediate PI3K signaling to control FoxO1 activity in B cells; however, it is unclear how PI3K signals are integrated through Akt to regulate FoxO1 and it is not known if Akt is the sole mediator of PI3K-dependent signals that regulate FoxO1. Additionally, the Akt isoform that regulates FoxO1 phosphorylation and function in B cells has not been identified and the molecular mechanism through which pre-BCR/BCR signals activate Akt and suppress FoxO1 activity is unknown.

In this study, we reveal the function of mTORC2 in B cells and elucidate mechanisms of mTORC2 regulation of B cell development. We show that genetic ablation of Sin1 in mice disrupts mTORC2 and abolishes Akt phosphorylation at Ser473 and Thr450 but not at Thr308 in developing B cells. Developing *Sin1*$^{-/-}$ B cells show increased IL-7 receptor expression, enhanced response to IL-7, augmented RAG expression, and elevated V(D)J recombinase activity. We demonstrate that the mTORC2-dependent Akt HM phosphorylation is specifically required for the suppression of *rag* gene expression and FoxO1 phosphorylation is dependent on both Sin1 and Akt2 in B cells. Finally, we show that the mTOR inhibitor rapamycin increases *rag1* expression and promotes V(D)J recombination in B cells. These data reveal that the Sin1/mTORC2-Akt2 signaling axis regulates IL-7 responsiveness, RAG expression, and V(D)J recombination in developing B cells.

RESULTS

Sin1 Regulates B Cell Development

To investigate the function of Sin1 in vivo, we generated Sin1 KO mice (Jacinto et al., 2006). The *Sin1*$^{-/-}$ embryos die during gestation between embryonic day (E) 10.5 and 15.5 due to severe defects in cardiovascular development (this will be described in another study). We successfully reconstituted lethally irradiated CD45.1 congenic mice with wild-type and *Sin1*$^{-/-}$ fetal liver hematopoietic cells from E11.5–E12.5 embryos, demonstrating that *Sin1*$^{-/-}$ hematopoietic stem cells are capable of reconstituting the hematopoietic system of adult mice (data not shown).

We examined bone marrow of chimeric mice and found that the proportion of immature IgM$^+$ bone marrow B cells was reduced in *Sin1*$^{-/-}$ chimeric mice when compared to *Sin1*$^{+/+}$ chimeras (Figure 1A). Specifically, in a representative pair of *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ chimeric mice, 70% (12% CD19$^+$IgM$^+$ / 17% CD19$^+$) of the bone marrow B cells were IgM$^+$, while only 43% (3% CD19$^+$IgM$^+$ / 7% CD19$^+$) of *Sin1*$^{-/-}$ bone marrow B cells were IgM$^+$. We also analyzed a chimeric mouse that contained a 1:1 ratio of host (wild-type) to *Sin1*$^{-/-}$ donor cells, allowing us to directly compare developing *Sin1*$^{-/-}$ B cells and wild-type B cells within the same animal. The proportion and number of *Sin1*$^{-/-}$ IgM$^+$ B cells was reduced by approximately 3-fold when compared to the wild-type IgM$^+$ B cells (Figures 1B and 1C). We also observed a 4-fold increase in the total number of *Sin1*$^{-/-}$ IgM$^-$ bone marrow B cells when compared to the wild-type IgM$^-$ B cells. The CD19$^+$IgM$^-$ population contains pro-B cells, which are the most immature bone marrow B cell population that also express the pro-B cell surface marker CD45R (B220$^+$) and high levels of CD43 (CD43^{hi}). Analysis of IgM$^-$ bone marrow B cells from the *Sin1*$^{-/-}$ chimeric mice revealed a 2-fold increase in the proportion of *Sin1*$^{-/-}$ B220$^+$CD43^{hi}IgM$^-$ pro-B cells relative to wild-type cells (Figure 1D). Together, these data show that the developmental defect is intrinsic to the *Sin1*$^{-/-}$ B cells and Sin1 deficiency results in expansion of pro-B cells.

Sin1 Deficiency Perturbs B Cell Development In Vitro

We established primary pro-B cell lines from the fetal livers of paired E12.5 *Sin1*$^{-/-}$ and *Sin1*$^{+/+}$ littermate embryos using OP9 stromal cells supplemented with IL-7 (Vieira and Cumano, 2004). We generated four independent pairs of wild-type and *Sin1*$^{-/-}$ pro-B cell lines and observed no defect in the ability in *Sin1*$^{-/-}$ fetal liver hematopoietic cells to give rise to pro-B cells when compared to the wild-type fetal liver hematopoietic cells (Figures S1A–S1C).

To determine the differentiation potential of *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells, we differentiated those cells in vitro on OP9 cells in the absence of IL-7 for 7 days. We found that *Sin1*$^{-/-}$ pro-B cells differentiated in vitro gave rise to a smaller proportion of IgM$^+$ cells than the wild-type cells (Figure 1E). We also observed 3-fold more Igμ^- pro-B cells in the *Sin1*$^{-/-}$ culture than in the *Sin1*$^{+/+}$ B cell culture (Figure 1F). Together, these results show that Sin1 is required for the proper development of IgM$^+$ B cells and suggest that *Sin1* deficiency may enhance the survival and/or proliferation of pro-B cells when IL-7 is limiting.

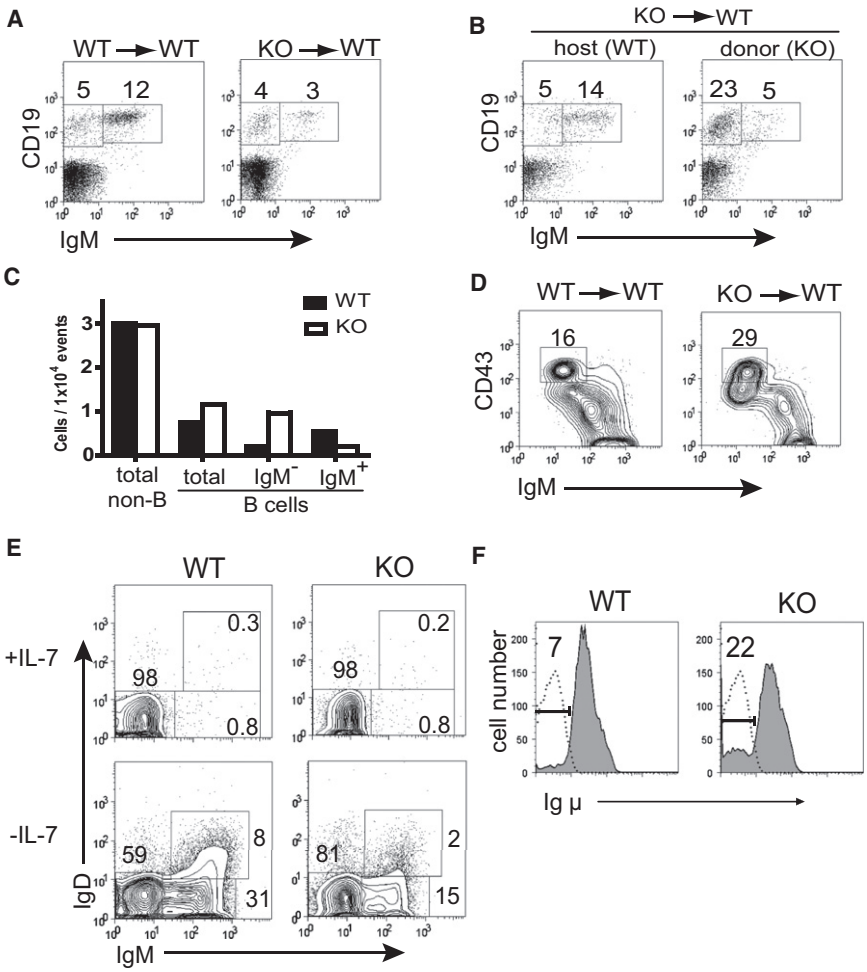

Figure 1. Sin1 Regulates B Cell Development

(A) Bone marrow from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ chimeric mice was analyzed by flow cytometry and the percentage of CD19^{+} IgM^{-} and CD19^{+} IgM^{+} B cells is indicated. The data are representative of *Sin1*$^{+/+}$ (n = 3) and *Sin1*$^{-/-}$ (n = 4) fetal liver chimeric mice.

(B) Bone marrow from a chimeric mouse containing a 1:1 ratio of wild-type host and *Sin1*$^{-/-}$ donor cells was analyzed by flow cytometry. The plots shown are pregated on CD45.1^{+} host cells or CD45.1^{-} *Sin1*$^{-/-}$ donor cells, and the percentage of CD19^{+}IgM^{-} and CD19^{+}IgM^{+} B cells is indicated.

(C) Bar graph illustrating the total number of *Sin1*$^{+/+}$ host or *Sin1*$^{-/-}$ donor bone marrow cells from the chimeric mouse shown in (B).

(D) The proportion of B220^{+}CD43^{hi}IgM^{-} bone marrow pro-B cells from wild-type or *Sin1*$^{-/-}$ fetal liver chimeric mice. The plots shown are pregated on CD45.2^{+}B220^{+} donor cells and are representative of *Sin1*$^{+/+}$ (n = 3) and *Sin1*$^{-/-}$ (n = 4) fetal liver chimeric mice.

(E) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured in vitro on OP9 cells with or without IL-7 for 7 days and surface IgM and IgD expression was analyzed. The plots are representative of four independent experiments.

(F) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured on OP9 cells without IL-7 for 7 days, fixed, permeablized, and stained for Igμ chain expression (shaded area). The proportion of Igμ^{-} pro-B cells is indicated. *Rag1*$^{-/-}$ pro-B cells are used as a negative control for Igμ staining (dotted line). Representative plots are shown from three independent experiments.

Sin1$^{-/-}$ Pro-B Cells Exhibit Increased IL-7Rα Expression and Enhanced IL-7-Dependent Survival

IL-7 provides the primary pro-B cell survival signal. Since we observed an increased proportion of *Sin1*$^{-/-}$ pro-B cells in the bone marrow and in the OP9 coculture differentiation assay, we speculated that Sin1 deficiency might enhance pro-B cell responsiveness to IL-7. We examined IL-7 receptor (IL7R) expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells and found that *il7r* mRNA levels were increased approximately 2-fold in *Sin1*$^{-/-}$ pro-B cells relative to *Sin1*$^{+/+}$ pro-B cells (Figure 2A). The expression of membrane bound IL-7R was also increased on *Sin1*$^{-/-}$ pro-B cells when compared to the *Sin1*$^{+/+}$ pro-B cells (Figure 2B). IL-7R was not expressed on *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ IgM^{+} immature B cells indicating that Sin1 only regulates *il7r* expression in pro-B cells (Figure S2).

These data suggested that the increased IL-7R expression on *Sin1*$^{-/-}$pro-B cells may render these cells more sensitive to IL-7 than *Sin1*$^{+/+}$ pro-B cells. Indeed, we found that the number of *Sin1*$^{-/-}$B cells recovered at each time point after IL-7 withdraw was substantially greater than that of *Sin1*$^{+/+}$ cells (Figure 2C). Most notably, the number of viable *Sin1*$^{+/+}$ B cells decreased by approximately 90% 72 hr after IL-7 withdraw while the number of viable *Sin1*$^{-/-}$ B cells at 72 hr was similar to the number of cells initially plated at time 0. These data suggest that *Sin1*$^{-/-}$ pro-B cells exhibit enhanced survival to the IL-7 produced by the OP9 cells.

To further confirm that *Sin1*$^{-/-}$ pro-B cells exhibit enhanced IL-7 responsiveness, we measured IL-7-dependent survival of *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells in the absence of OP9 cells. *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were washed and cultured in medium containing a low concentration of IL-7 (0.1 ng/ml). The number of live cells was measured at 10 hr or 24 hr after plating. We observed significantly more live *Sin1*$^{-/-}$ pro-B cells than wild-type pro-B cells after 10 hr in culture (Figure 2D). There was little difference between *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ cell viability by 24 hr, indicating that IL-7 is absolutely required for the survival of both wild-type and *Sin1*$^{-/-}$ pro-B cells. We also cultured pro-B cells in medium supplemented with a high concentration of IL-7 (5 ng/ml) and found that *Sin1*$^{-/-}$ pro-B cells showed enhanced survival over *Sin1*$^{+/+}$ pro-B cells under these conditions (Figure 2E). Together, these data show that *Sin1* deficient pro-B cells have increased IL-7 receptor expression and exhibit enhanced IL-7-dependent survival.

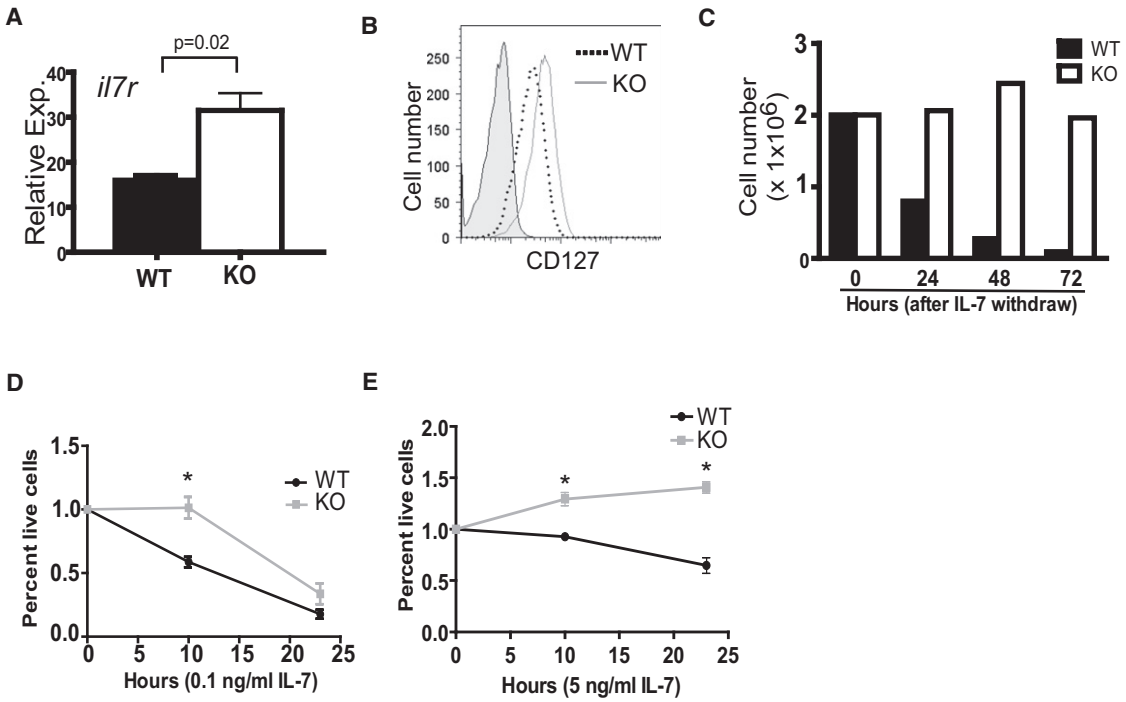

Figure 2. *Sin1*$^{-/-}$ Pro-B Cells Exhibit Increased *il7r* Expression and Enhanced IL-7-Dependent Survival

(A) IL-7 receptor (*il7r*) mRNA levels in *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) pro-B cells were measured by qPCR and normalized to *GAPDH* expression. Samples were run in triplicate and data are representative of two independent experiments.

(B) Expression of IL-7R (CD127) on *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells was measured by flow cytometry. Shaded area is the isotype control staining. The plots are representative of three independent experiments.

(C) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured on OP9 cells without exogenous IL-7. The total number of live cells recovered at each time point was measured. Dead cells were excluded from the analysis by trypan blue staining. Data are representative of two independent experiments.

(D) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured without OP9 cells in medium supplemented with 0.1 ng/ml IL-7. The number of live cells at 10 and 24 hr after plating was determined by trypan blue dye exclusion. Each data point represents triplicate wells from one of two independent experiments (* = $p < 0.01$).

(E) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured without OP9 cells in medium supplemented with 5 ng/ml IL-7. The number of live cells at various time points after plating was determined by trypan blue dye exclusion. Each data point represents triplicate wells from one of two independent experiments (* = $p < 0.01$). Error bars indicate standard deviation.

Sin1$^{-/-}$ B Cells Lack Functional mTORC2 and Exhibit Defective Akt Phosphorylation

To investigate the mechanism of Sin1 function in B cells we established Abelson murine leukemia virus (Ab-MuLV) transformed pre-B cells from *Sin1*$^{+/+}$, *Sin1*$^{+/-}$, and *Sin1*$^{-/-}$ pro-B cells. *Sin1*$^{-/-}$Ab-MuLV pre-B cells expressed B lineage surface markers and showed no defect in growth, proliferation, or survival when compared to *Sin1*$^{+/+}$ or *Sin1*$^{+/-}$ pre-B cells (data not shown). To determine if Sin1 is essential for mTORC2 integrity in B cells, the endogenous mTORC2 was immunoprecipitated from *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells using an anti-Rictor antibody. As expected, mTOR and Sin1 coimmunoprecipitated with Rictor in the *Sin1*$^{+/-}$ but not *Sin1*$^{-/-}$ pre-B cells (Figure 3A). In addition, Rictor and mTOR co-immunoprecipitated with Sin1 in the *Sin1*$^{+/-}$ cells (data not shown). These data show that Sin1 is required for the mTORC2 integrity in B cells. Consistent with these results, phosphorylation of Akt at the mTORC2 target sites Ser473 and Thr450 was abolished in the *Sin1*$^{-/-}$ pro-B cells and Ab-MuLV pre-B cells but not in the Sin1-sufficient B cells (Figure 3B). The Akt T loop (Thr308) phosphorylation was approximately 1.5 fold more in *Sin1*$^{-/-}$ pro-B cells and about 10-fold more in *Sin1*$^{-/-}$ Ab-MuLV pre-B cells than that in *Sin1*$^{+/+}$ pro-B and pre-B cells, respectively (Figure 3B). Furthermore, Akt expression in both primary and transformed *Sin1*$^{-/-}$ B cells was decreased, as well, consistent with our previous studies showing that the TM phosphorylation regulates Akt protein stability (Facchinetti et al., 2008).

Sin1 Is Required for FoxO1 Phosphorylation in B Cells

The FoxO transcription factors are evolutionarily conserved targets of Akt and disruption of mTORC2 results in the selective impairment of FoxO1/3a phosphorylation in embryonic fibroblasts (Jacinto et al., 2006). Therefore, we examined FoxO1 phosphorylation in control and *Sin1*$^{-/-}$ Ab-MuLV pre-B cells and found that FoxO1 phosphorylation at Thr24 was impaired (Figure 3C). The Akt-dependent phosphorylation of FoxO proteins is known to promote the ubiquitination and subsequent proteasome-dependent degradation of FoxO proteins (Plas and Thompson, 2003). Consistently, more FoxO1 protein was detected in *Sin1*$^{-/-}$ Ab-MuLV pre-B cells than the control cells (Figure 3C). FoxO1 Thr24 phosphorylation was also impaired and FoxO1 protein levels were increased in *Sin1*$^{-/-}$pro-B cells

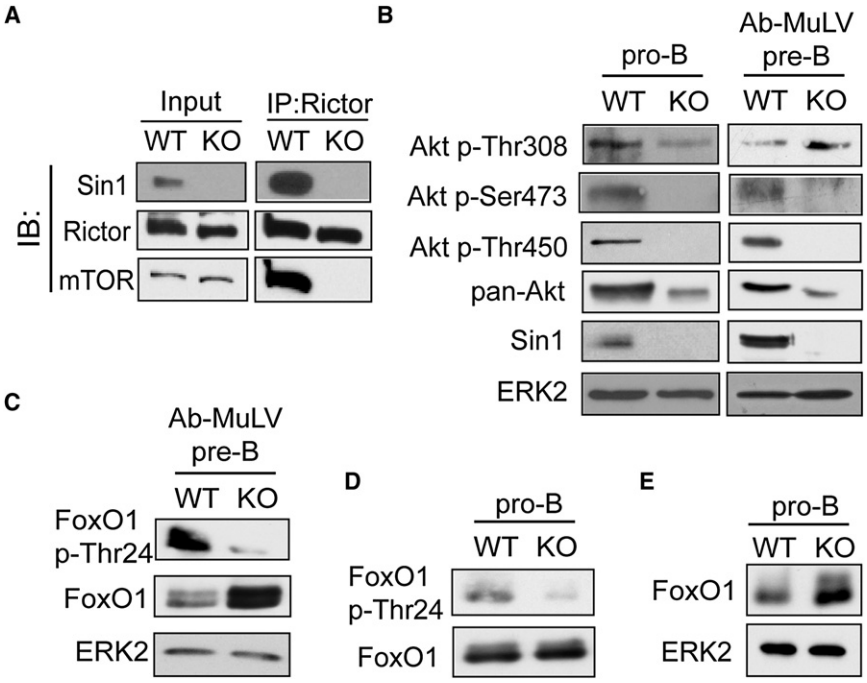

Figure 3. Sin1 Is Required for mTORC2-Dependent Akt Hydrophobic Motif and Akt Turn Motif Phosphorylation in B Cells

(A) The mTORC2 complex integrity in *Sin1*$^{+/-}$ (WT) or *Sin1*$^{-/-}$ (KO) Ab-MuLV pre-B cells was determined by immunoprecipitation (IP) of Rictor followed by immunoblotting (IB) of Sin1, Rictor, and mTOR.

(B) Akt phosphorylation and expression was measured in primary *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells cultured on OP9 cells with IL-7 and Ab-MuLV transformed *Sin1*$^{+/-}$ (WT) or *Sin1*$^{-/-}$ (KO) pre-B cells. Samples were normalized to total cellular protein and blotted as indicated.

(C) FoxO1 phosphorylation and expression was measured in *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells. Samples were normalized to total cellular protein and blotted for FoxO1 or FoxO1 p-Thr24.

(D) FoxO1 phosphorylation was measured in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells cultured with serum and IL-7. The samples were normalized to total FoxO1 protein then blotted for FoxO1 p-Thr24.

(E) FoxO1 expression was measured in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells. The samples were normalized to total cellular protein.

when compared to *Sin1*$^{+/+}$ pro-B cells (Figures 3D and 3E). Together, these data show that Sin1 deletion impairs FoxO1 phosphorylation in B developing cells.

Sin1 Suppresses RAG Expression in Developing B Cells

Our observation that FoxO1 phosphorylation is impaired and FoxO1 expression is increased in *Sin1*$^{-/-}$ B cells provides a possible mechanism to explain the elevated expression of *il7r,* which was recently identified as a FoxO1 target gene (Kerdiles et al., 2009). The genes *rag1* and *rag2* are also regulated by FoxO1 in B cells (Amin and Schlissel, 2008; Herzog et al., 2008; Llorian et al., 2007; Verkoczy et al., 2007). We measured *rag1* transcript levels in *Sin1*$^{+/-}$ and *Sin1*$^{-/-}$ Ab-MuLV pre-B cells and found that *rag1* expression was significantly elevated in *Sin1*$^{-/-}$ pre-B cells compared to the *Sin1*$^{+/-}$cells (Figure 4A). *Rag* expression is suppressed by the v-Abl kinase and can be rapidly induced by inhibiting v-Abl activity with Imatinib (Muljo and Schlissel, 2003). We found that Imatinib treatment substantially increased *rag1* expression in both control and *Sin1*$^{-/-}$ pre-B cells. However, *rag1* expression was increased an additional 3-fold in Imatinib treated *Sin1*$^{-/-}$ pre-B cells when compared to the *Sin1*$^{+/-}$ pre-B cells (Figure 4A). In addition, RAG1 protein was readily detectable in *Sin1*$^{-/-}$ but not *Sin1*$^{+/-}$ Ab-MuLV pre-B cells cultured in the absence of Imatinib (Figure 4B).

Next, we examined *rag1* expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells cultured on OP9 cells with IL-7 and found that *rag1* expression was approximately 2-fold more in *Sin1*$^{-/-}$ pro-B cells than in *Sin1*$^{+/+}$ pro-B cells (Figure 4C, at the 0 hr time point). IL-7 attenuates *rag1* expression in pro-B cells (Melamed et al., 1997). Therefore we measured *rag1* expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells 20 hr after IL-7 withdrawal and found that *rag1* expression increased 2-fold in *Sin1*$^{+/+}$ pro-B cells while *rag1* expression showed little increase in *Sin1*$^{-/-}$ pro-B cells (Figure 4C). At 48 hr after IL-7 withdrawal, *Sin1*$^{+/+}$ pro-B cells showed a 5-fold increase in *rag1* expression while *Sin1*$^{-/-}$ pro-B cells showed a 3-fold increase in *rag1* expression relative to the 0 hr time point. These data suggest that IL-7 signals suppress *rag* expression through a mechanism that is not dependent on *Sin1*.

We also examined *rag1* expression in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pre-B cells and immature B cells differentiated in vitro on OP9 cells. We found that *rag1* expression was 3-fold higher in *Sin1*$^{-/-}$ pre-B cells than *Sin1*$^{+/+}$ pre-B cells and 2-fold higher in *Sin1*$^{-/-}$ immature B cells than *Sin1*$^{+/+}$ immature B cells (Figure 4D). Expression of *rag2* was also higher in both *Sin1*$^{-/-}$ pre-B and immature B cells than that in *Sin1*$^{+/+}$ B cells (Figure 4D). To determine if Sin1 deficiency influences IgL recombination we examined the Igκ and Igλ chain expression on immature IgM^{+} B cells. Analysis of IgL chain expression on immature IgM^{+} *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ B cells revealed a 2-fold increase in the proportion of Igλ light chain expressing *Sin1*$^{-/-}$ B cells when compared to *Sin1*$^{+/+}$ B cells (Figure S3). Together, these results show that Sin1 regulates *rag* expression in B cells and suggest that Sin1 influences IgL recombination in developing B cells.

Finally, we reconstituted the *Sin1*$^{-/-}$ Ab-MuLV pre-B cells with human *Sin1* cDNA and examined RAG1 protein levels in these cells. Restoration of Sin1 expression in *Sin1*$^{-/-}$ Ab-MuLV pre-B cells decreased RAG1 protein expression while a control virus lacking human Sin1 expression did not decrease RAG1 expression (Figure 4E). These data confirm that Sin1 is a negative regulator of *rag* expression in developing B cells.

Sin1 Suppresses V(D)J Recombinase Activity in Developing B Cells

To determine if the elevated *rag* gene expression and RAG1 protein observed in the *Sin1*$^{-/-}$ pre-B cells correlates with

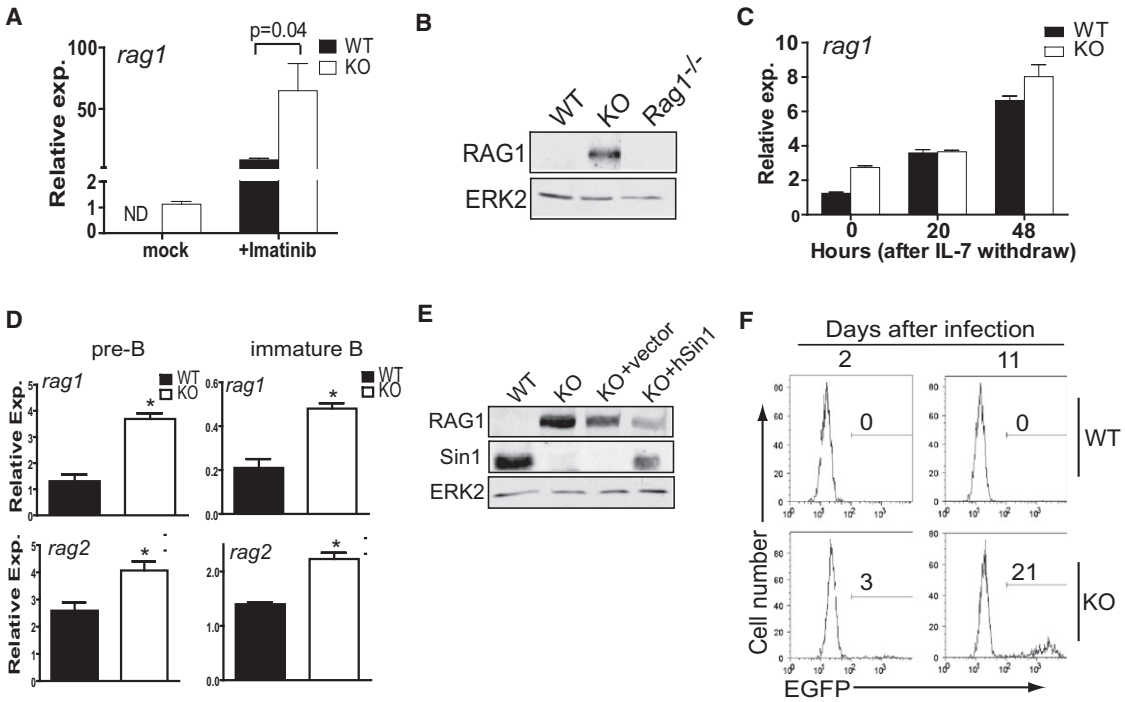

Figure 4. Increased RAG Expression and V(D)J Recombinase Activity in Developing *Sin1*$^{-/-}$ B Cells

(A) *Rag1* mRNA expression was measured by qPCR in *Sin1*$^{+/-}$ (WT) or *Sin1*$^{-/-}$ (KO) Ab-MuLV pre-B cells cultured for 5 hr in the presence or absence of Imatinib. *Rag1* mRNA was normalized to *GAPDH* mRNA. ND: not detected.

(B) RAG1 protein in *Sin1*$^{+/-}$ (WT), *Sin1*$^{-/-}$ (KO) or *rag1*$^{-/-}$ Ab-MuLV pre-B cells was measured by immunoblotting. The ERK2 protein is used as a loading control.

(C) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells were cultured on OP9 cells without exogenous IL-7 for 0, 20, or 48 hr. *Rag1* expression was measured by qPCR. *Rag1* expression was normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments.

(D) *Sin1*$^{+/+}$ (KO) or *Sin1*$^{-/-}$ (WT) pro-B cells were differentiated in vitro on OP9 cells for 7 days, and *rag1* or *rag2* expression in pre-B and immature B cells obtained from these cultures was measured by qPCR as described in (A). Samples were run in triplicate and are representative of two independent experiments (*$p < 0.01$).

(E) *Sin1*$^{-/-}$ Ab-MuLV pre-B cells were infected with a retrovirus expressing human Sin1 or a control empty retrovirus, and the RAG1 expression was measured by immunoblotting. Data are representative of two independent experiments.

(F) *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells were infected with a retrovirus containing an EGFP RAG recombinase reporter. The infected Ab-MuLV pre-B cells were then grown under normal culture conditions, and the EGFP^{+} cells were determined by flow cytometry 2 days and 11 days later. The plots shown are gated on infected cells (hCD4^{+} cells). The data are representative of three independent experiments. Error bars indicate standard deviation.

V(D)J recombinase activity, we infected wild-type or *Sin1*$^{-/-}$ Ab-MuLV pre-B cells with a retrovirus containing an EGFP-based V(D)J recombinase reporter. The reporter contains an anti-sense orientated EGFP cDNA flanked by 12- and 23-recombination signal sequences. RAG-mediated recombination flips the EGFP cDNA sequence to the sense orientation permanently marking the cell with EGFP expression. *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ pre-B cells infected with the reporter virus were assayed 2 days and 11 days postinfection by flow cytometry. The infected cells were first identified by human CD4 expression from an IRES-hCD4 cassette in the retroviral vector and then analyzed for EGFP expression. At day 2, no *Sin1*$^{+/-}$ pre-B cells expressed EGFP while 3% of the *Sin1*$^{-/-}$ hCD4^{+} pre-B cells expressed EGFP. Eleven days after infection, 21% of the hCD4^{+} *Sin1*$^{-/-}$ pre-B cells expressed EGFP while none of the *Sin1*$^{+/-}$ hCD4^{+} pre-B cells expressed EGFP (Figure 4F). These data show that Sin1 deficiency results in increased V(D)J recombinase activity in developing B cells.

Akt2 Regulates FoxO1 Phosphorylation and Suppresses *il7r* and *rag* Expression in B Cells

Our data show that Sin1/mTORC2 regulates the expression of the FoxO1 target genes *il7r*, *rag1*, and *rag2*. Studies from our group and other laboratories suggest that Sin1/mTORC2 mediates PI3K signals to activate Akt, which in turn suppresses FoxO1 activity. However, genetic evidence supporting this model is currently lacking. If this model is correct, we predict that the Sin1 and Akt-deficient B cells will share a common phenotype showing defective FoxO1 regulation and augmented FoxO1 target gene expression. To test this model, we established primary pro-B cell lines from *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ mice. We first examined *rag* expression in these cells by quantitative RT-PCR, and found that Akt1 deficiency had no effect on *rag1* expression in pro-B cells (Figure 5A). In contrast, the *rag1* expression was elevated approximately 3-fold in *Akt2*$^{-/-}$ pro-B cells relative to the wild-type cells (Figure 5A). Consistent with the increased *rag1* mRNA expression, RAG1 protein levels were also significantly increased in

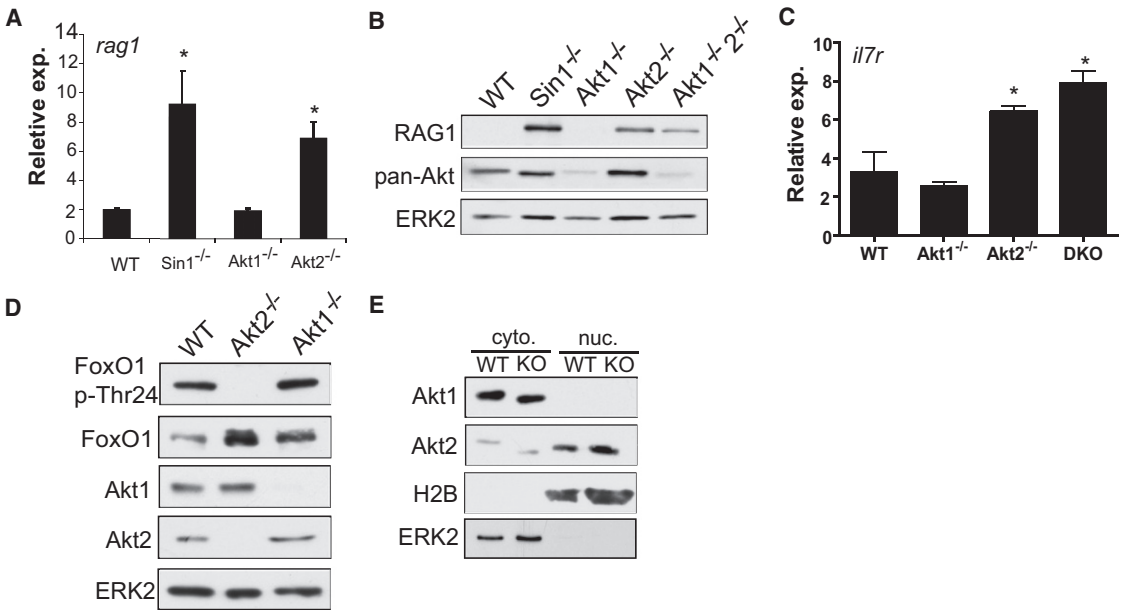

Figure 5. Akt2 Regulates Rag and IL-7R Expression in Pro-B Cells

(A) Wild-type (WT), *Sin1*$^{-/-}$, and Akt-deficient pro-B cells were cultured on OP9 cells with IL-7. *Rag1* mRNA levels were measured by qPCR and normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments (* = $p < 0.01$).

(B) RAG1 protein was measured in primary pro-B cells cultured on OP9 cells with IL-7. Total Akt expression was measured with a pan-Akt antibody. Samples were normalized to total cellular protein.

(C) *Il7r* mRNA expression in WT, *Akt1*$^{-/-}$, *Akt2*$^{-/-}$, or *Akt1*$^{-/-}$ *Akt2*$^{-/-}$ (DKO) pro-B cells cultured on OP9 cells with IL-7 was measured by quantitative RT-PCR and normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments (* = $p < 0.01$).

(D) FoxO1 phosphorylation was measured in wild-type, *Akt2*$^{-/-}$, or *Akt1*$^{-/-}$ pro-B cells. Samples were normalized to total cellular protein.

(E) Cytoplasmic and nuclear Akt1 and Akt2 was determined in *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) pro-B cells cultured on OP9 cells with IL-7. ERK2 and H2B proteins were used to verify the quality of the cytoplasmic and nuclear fractions. Three times more nuclear proteins (based on the total cell numbers) than cytosolic proteins were loaded in this assay to enhance the nuclear signals. Standard deviation is indicated by all error bars.

Sin1$^{-/-}$ *Akt2*$^{-/-}$, and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ pro-B cells but not wild-type or *Akt1*$^{-/-}$ pro-B cells (Figure 5B). We also examined IL-7R expression in Akt deficient pro-B cells and found that *il7r* expression was increased in *Akt2*$^{-/-}$ and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$but not wild-type or *Akt1*$^{-/-}$ pro-B cells (Figure 5C). These results indicate that Akt2 is the principle mediator of Sin1/mTORC2 signaling, which suppresses *il7r* and *rag* expression in developing B cells.

To explore the mechanism though which Akt2 regulates *rag1* and *il7r* in B cells, we analyzed FoxO1 phosphorylation in *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ pro-B cells and found that the deletion of Akt2 but not Akt1 blocked FoxO1 Thr24 phosphorylation (Figure 5D). These results prompted us to examine how Akt2 might specifically regulate FoxO1 phosphorylation in B cells. We speculated that the differential subcellular localization of Akt1 and Akt2 proteins within pro-B cells may contribute to the specific regulation of FoxO1 by Akt2. Therefore, we fractionated cytosol and nuclear proteins from *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells and determined the distribution of Akt1 and Akt2 proteins in these subcellular fractions. We found that Akt1 was localized exclusively in the cytosol while Akt2 was localized in both cytosol and nucleus of pro-B cells (Figure 5E). These studies also revealed that the differential subcellular localization of Akt1 and Akt2 proteins in pro-B cells is not dependent on Sin1. Together, these data show that Akt2 specifically regulates FoxO1 phosphorylation in pro-B cells and suggests that the selective localization of Akt2 to the B cell nucleus contributes to the specific regulation of FoxO1 by Akt2.

Rapamycin Induces *rag* Expression in B Cells by Blocking mTORC2-Dependent Akt HM Phosphorylation

Prolonged rapamycin treatment is known to disrupt mTORC2 and inhibit Akt Ser473 phosphorylation in some cell types (Facchinetti et al., 2008; Sarbassov et al., 2006). Therefore we asked if rapamycin treatment may disrupt mTORC2, inhibit Akt HM phosphorylation, and induce *rag* gene expression in B cells. We treated wild-type Ab-MuLV pre-B cells with rapamycin for 5 hr or 25 hr and measured *rag* expression by quantitative RT-PCR. The acute rapamycin treatment (5 hr) did not alter *rag* expression, but prolonged rapamycin treatment (25 hr) substantially increased *rag1* and *rag2* mRNA levels (Figure 6A). In addition, rapamycin treatment also markedly increased the RAG1 protein level in Ab-MuLV pre-B cells (Figure 6B). We also observed that FoxO1 protein levels were increased after rapamycin treatment (Figure 6B). Furthermore, we observed that phosphorylation of Akt Ser473 was inhibited after 25 hr but not 5 hr of rapamycin treatment (Figure 6C). The overall Akt protein level was also reduced after 25 hr of rapamycin treatment. As expected, phosphorylation of the mTORC1 target 4E-BP1 was inhibited by rapamycin at 5 and 25 hr points

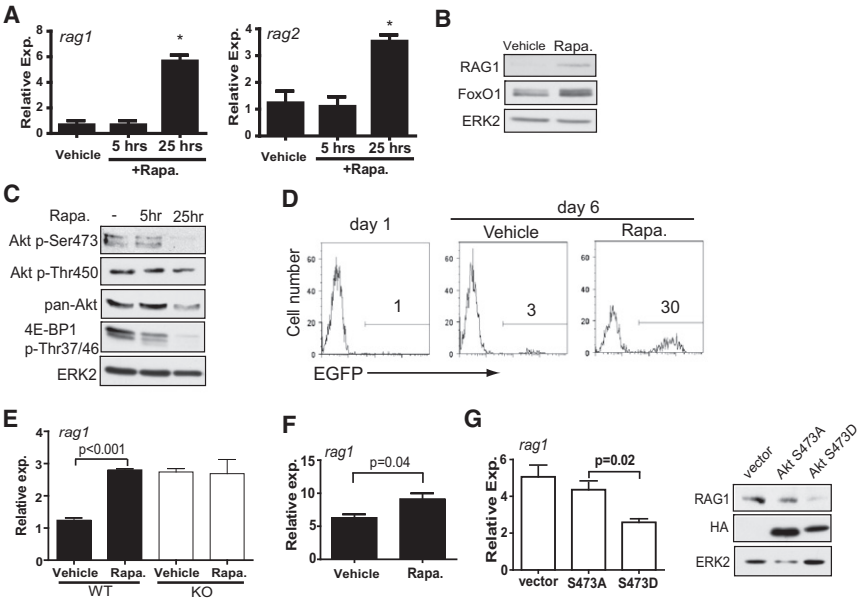

Figure 6. Rapamycin Induces RAG Gene Expression and Inhibits Akt Ser473 Phosphorylation in B Cells

(A) *Rag* mRNA levels were measured by qPCR in *Sin1*$^{+/-}$ Ab-MuLV pre-B cells treated with rapamycin for the indicated periods of time. *Rag1* and *rag2* expression was normalized to *GAPDH* mRNA. Samples were run in triplicate and are representative of two independent experiments (* = $p < 0.01$).

(B) *Sin1*$^{+/-}$ Ab-MuLV pre-B cells were cultured in the presence or absence of rapamycin for 25 hr and RAG1 or FoxO1 protein was measured. ERK2 is used as a loading control.

(C) *Sin1*$^{+/-}$ Ab-MuLV pre-B cells were vehicle or rapamycin treated for 5 or 25 hr. Cell lysates were normalized to total protein and immunoblotted as indicated.

(D) *Sin1*$^{+/-}$ Ab-MuLV pre-B cells were infected with a retrovirus containing an EGFP RAG recombinase reporter. The infected cells were continuously cultured in the presence (Rapa.) or absence (vehicle) of rapamycin for 6 days. The EGFP^{+} cells were determined by flow cytometry at 1 day and 6 days after infection. The plots are gated on infected cells (hCD4^{+} cells) and are representative of three independent experiments.

(E) *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) pro-B cells cultured on OP9 cells with IL-7 were treated with rapamycin or vehicle for 25 hr and *rag1* expression was measured by qPCR. Samples were normalized to *GAPDH* expression. Samples were run in triplicate and the data are representative of two independent experiments.

(F) Resting *Sin1*$^{+/-}$ splenic B cells were enriched by negative selection and cultured in vitro in the presence (Rapa) or absence (Vehicle) of rapamycin for 25 hr. *Rag1* expression was measured by qPCR and normalized to *GAPDH* expression. Samples were run in triplicate, and the data are representative of two independent experiments.

(G) *Sin1*$^{-/-}$ Ab-MuLV pre-B cells were infected with retrovirus expressing human Akt with a null HM mutation (S473A) or a phosphomimetic HM mutation (S473D). *Rag1* mRNA expression was measured by qPCR and normalized to *GAPDH*. RAG1 protein levels were measured by immunoblotting and the HA blotting verifies expression of the virally expressed Akt. Quantitative RT-PCR samples were run in triplicate and the data are representative of two independent experiments. Standard deviation is indicated by all error bars.

(Figure 6C). These data show that rapamycin induces *rag* gene expression in a manner that correlates with the loss of Akt Ser473 phosphorylation in B cells.

The increased *rag* expression in *Sin1*$^{+/-}$ pre-B cells following rapamycin treatment suggested that rapamycin may also increase V(D)J recombinase activity in B cells. We infected *Sin1*$^{+/-}$ Ab-MuLV pre-B cells with the EGFP RAG recombinase reporter and cultured these cells in the presence or absence of rapamycin. We observed that about 30% of the rapamycin treated, infected pre-B cells expressed EGFP while only 3% of the vehicle treated, infected cells expressed EGFP after 6 days (Figure 6D). These data show that rapamycin mimics the effect of Sin1 deficiency (Figure 4F) and promotes V(D)J recombinase activity in B cells.

Next, we asked if rapamycin induces *rag* expression in nontransformed B cells. We cultured *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells on OP9 cells with IL-7 in the presence or absence of rapamycin for 25 hr then measured *rag1* expression by quantitative RT-PCR. Rapamycin induced *rag1* expression in the *Sin1*$^{+/+}$ pro-B cells by approximately 3-fold, while rapamycin had no effect on *rag1* expression in *Sin1*$^{-/-}$pro-B cells (Figure 6E). Additionally, we also observed that rapamycin induced *il7r* mRNA levels by 2-fold in *Sin1*$^{+/+}$ pro-B cells (Figure S4). To further explore the effect of rapamycin on *rag* expression in B cells, we purified total splenic B cells from a *Sin1*$^{+/-}$ mouse and cultured these cells in vitro for 24 hr with or without rapamycin. We observed that *rag1* expression was increased in the rapamycin treated *Sin1*$^{+/-}$ splenic B cells when compared to vehicle treated B cells (Figure 6F). These data show that rapamycin treatment induces *il7r* expression in pro-B cells and *rag* expression in pro-B and splenic B cells.

Since Akt HM phosphorylation is dependent on mTORC2, we asked if Akt HM phosphorylation specifically mediates the Sin1/mTORC2-dependent inhibition of *rag* expression in B cells. We infected *Sin1*$^{-/-}$ Ab-MuLV pre-B cells with retrovirus expressing a human *Akt* cDNA with either a Ser473 to Ala null mutation or a phosphomimetic Ser473 to Asp mutation. Infected cells were sorted based on virally expressed GFP and *rag1* expression was measured by quantitative RT-PCR. Ectopic expression of the Ser473Ala Akt mutant failed to inhibit *rag1* expression in *Sin1*$^{-/-}$ pre-B cells. In contrast, expression of the Ser473Asp Akt mutant markedly reduced *rag1* expression in *Sin1*$^{-/-}$ pre-B cells (Figure 6G). Consistently, expression of the Ser473Asp Akt mutant but not the Ser473Ala Akt mutant suppressed RAG1 protein levels in Sin1$^{-/-}$ pre-B cells (Figure 6G). In addition, Ser473Asp Akt mutant also induced more FoxO1 phosphorylation than the Ser473Ala Akt mutant (data not shown). These data demonstrate that Akt HM site function is necessary and sufficient to complement Sin1 deficiency and suppress *rag* expression in developing B cells.

DISCUSSION

In this study we show that Sin1, an essential component of mTORC2, plays a critical role in B cell development. As illustrated in the model in Figure 7, Sin1/mTORC2 mediates PI3K-dependent signals (i.e., pre-BCR or BCR) to phosphorylate the hydrophobic motif (Ser473) of Akt2. PI3K-dependent PDK1 phosphorylates the T loop (Thr308) of Akt2 resulting in full Akt2 activation. Ser473 phosphorylation directs Akt2 activity toward its substrate FoxO1 Thr24 resulting in phosphorylation of FoxO1 thus suppressing the expression of FoxO1 target genes *il7r*, *rag1*, and *rag2* in developing B cells. Our study demonstrates the specific role of Sin1/mTORC2 and Akt2 as key regulators of *il7r* and *rag* gene expression in B cells.

Interestingly, Sin1 is not required for pro-B cell proliferation and survival (Figures S1A and S1B). Rather, *Sin1*$^{-/-}$ pro-B cells exhibit enhanced survival when cultured in the presence of IL-7. Our data indicates that this enhanced response to IL-7 is due to increased IL-7R expression on *Sin1*$^{-/-}$ pro-B cells. Our data also indicates that *Sin1* regulates pro-B cell to pre-B cell differentiation since we observe an accumulation of pro-B and a reduction of IgM^{+} immature B cells in the bone marrow of *Sin1*$^{-/-}$ chimeric mice and in *Sin1*$^{-/-}$ B cells differentiated in vitro on OP9 cells. Therefore, we propose that Sin1 mediates the PI3K-dependent pre-BCR signaling to suppress *rag* and *il7r* expression, which inhibits further IgH recombination.

It is of note that, mice lacking key structural components of pre-BCR such as Igμ, surrogate light chain genes λ5 and VpreB, and mice deficient in key pre-BCR signaling mediators such as Igα or Igβ all exhibit a B cell developmental block at the pro-B to pre-B transition (Herzog et al., 2009). The Sin1/mTORC2-Akt2-FoxO1 axis may also operate in a similar manner in transducing BCR signals in immature B cells following IgL recombination since the downstream mediators of pre-BCR and BCR signaling are conserved (Herzog et al., 2009). Therefore we predict that Sin1 deficiency may also perturb BCR signaling in immature and mature B cells. PI3K-dependent BCR signaling suppresses *rag* expression and inhibits IgL receptor editing in immature B cells (Verkoczy et al., 2007). We observe that *rag* gene expression is elevated in *Sin1*$^{-/-}$ immature B cells and that Igλ chain usage is increased in *Sin1*$^{-/-}$ B cells (Figures 4D and S3), suggesting that Sin1 mediates BCR-dependent PI3K signaling as well.

Sin1 deletion does not appear to impair Ig recombination. Rather, we observe increased *rag* expression and V(D)J recombinase activity in *Sin1*$^{-/-}$ B cells. Furthermore, the induction of Igκ germline transcription, which is an indicator of locus activation, is not impaired in *Sin1*$^{-/-}$ pre-B cells indicating that Sin1 is not required for the IgL locus accessibility (data not shown). In fact, the augmented V(D)J recombinase activity in developing *Sin1*$^{-/-}$ B cells may even promote IgL recombination since we observe an increase in the percentage of immature *Sin1*$^{-/-}$ B cells that express Igλ (Figures S3 and 4D).

Sin1 is an evolutionally conserved adaptor molecule and is essential for the integrity of mTORC2 in a diverse array of organisms and cell types (Jacinto et al., 2006; Yang et al., 2006). Sin1 deficiency blocks the phosphorylation of Akt at Ser473 and Thr450 in both primary pro-B and transformed pre-B cells without impairing Akt Thr308 phosphorylation. These results are consistent with our previous studies in nonimmune cells (Facchinetti et al., 2008; Jacinto et al., 2006). Our results also demonstrate that the mTORC2 is the sole kinase (PDK2) for Akt HM site phosphorylation in B cells.

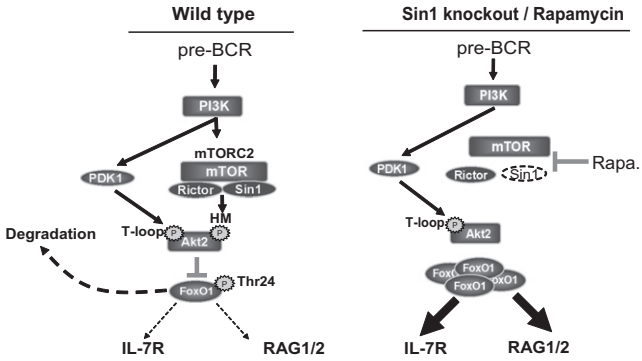

Figure 7. A Model Illustrating the Regulation of *il7r* and *rag* Expression by the Sin1/mTORC2-Akt2 Signaling Axis in Developing B Cells

PDK 1 and Sin1/mTORC2 mediate the PI3K signals to phosphorylate Akt2 at Thr308 and Ser473, respectively. Activated Akt2 then phosphorylates FoxO1, resulting in FoxO1 degradation and downregulation of *il7r* and *rag* gene expression. In the absence of Sin1 or during chronic rapamycin treatment, mTORC2 is disrupted and Akt2 is not fully activated, resulting in FoxO1 hypophosphorylation and accumulation; this leads to the augmented *il7r* and *rag* gene expression.

The expression of *rag1*, *rag2* and *il7r* is stringently regulated in developing B cells through a mechanism, which involves multiple signaling pathways. Our study reveals Sin1 as a key negative regulator of these genes in B cells whose activity is most likely mediated by FoxO1 since *rag1*, *rag2*, and *il7r* are direct targets of FoxO1 regulation (Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008). We present evidence showing that disruption of Sin1 or Akt2 impairs FoxO1 phosphorylation, which correlates with increased *rag1/2* and *il7r* expression. Furthermore, we demonstrate the functional importance of Akt HM phosphorylation in regulating FoxO1 activity by showing that ectopic expression of the phosphomimetic Ser473Asp Akt mutant but not the phosphorylation null Ser473Ala Akt mutant inhibits *rag* expression in *Sin1*$^{-/-}$ pre-B cells. Interestingly, we observed that the relative amount of nuclear localized FoxO1 protein is similar in *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells (data not shown). These data suggest that the relative abundance of nuclear FoxO1 protein is not tightly correlated with FoxO1 transcriptional activity and that regulation of FoxO1 localization in B cells is Sin1/mTORC2 independent.

Although Sin1/mTORC2 regulates both Akt1 and Akt2 HM and TM site phosphorylation, Akt1 and Akt2 appear to be differentially utilized by developing B cells with respect to the regulation of *rag1*, *rag2*, and *il7r*. We were particularly surprised to find that Akt1 does not regulate FoxO1 phosphorylation or *rag1*, *rag2*, and *il7r* expression since Akt1 is the most abundantly expressed Akt isoform in developing B cells (Figure 5B). We show that Akt2, but not Akt1, is selectively localized to the nucleus of B cells. Based on these data we propose that nuclear localized Akt2 is responsible for phosphorylating FoxO1. Exactly how this selective,

isoform specific, cellular distribution of Akt proteins is achieved and regulated in B cells remains to be elucidated. However, we observe that Akt2 is nuclear localized in *Sin1*$^{-/-}$ pro-B cells suggesting that Akt HM and TM phosphorylation are not required for Akt2 import into the nucleus. These results indicate that Akt2 HM phosphorylation may be required to specifically facilitate interaction of Akt2 with FoxO1 or promote the Akt2-dependent phosphorylation of FoxO1 at Thr24.

V(D)J recombination is normally suppressed in proliferating B cells by coupling RAG2 protein degradation with mitosis (Li et al., 1996). Cell-cycle-dependent regulation of V(D)J recombination helps to ensure that aberrant recombination products are not generated during the cell cycle, which may result in mutations that promote tumor formation. Our data reveals that Sin1/mTORC2 signaling provides an additional level of protection against abnormal V(D)J recombinase activity by suppressing expression of *rag1* and *rag2* in proliferating B cells. Surprisingly, we observed substantial V(D)J recombinase activity in *Sin1*$^{-/-}$ Ab-MuLV pre-B leukemia cells that were actively proliferating (Figure 4F). These findings raise the possibility that mTOR inhibitors, which disrupt mTORC2 function and induce *rag* expression, may promote genome instability in B cells by promoting aberrant V(D)J recombinase activity. This is a very important point in light of our finding that rapamycin increases *rag* expression in mature B cells. Our data argues that the *rag* locus is not irreversibly silenced upon B cell maturation and suggests that mTORC2-dependent signaling actively suppresses *rag* expression in mature B cells. This has significant implications with regard to the mechanisms that lead to genome instability and B cell tumors. Perturbations of mTORC2 signaling that induce *rag* expression in immature or mature B cells may increase the likelihood of coexpression of *rag* with activation-induced cytidine deaminase (AID), a circumstance that has been strongly implicated in the generation of chromosomal translocations (Tsai et al., 2008; Wang et al., 2008, 2009). Future studies will elucidate the role of mTORC2 in promoting B cell genome stability and determine if pharmacologic mTOR inhibition increases the likelihood of generating B cell tumors.

EXPERIMENTAL PROCEDURES

A detailed explanation of the experimental methods can be found in the Supplemental Experimental Procedures.

Mice

Sin1 knockout mice were described previously (Jacinto et al., 2006). Akt1 and Akt2 knockout mice were described previously (Di Lorenzo et al., 2009). 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. All mice were housed in the animal facilities at Yale University, and all animal procedures were approved by the Yale Institutional Animal Care and Use Committee.

B Cell Cultures

Pro-B cells were derived from paired *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ littermate E12.5 embryos. *Akt2*$^{-/-}$ and *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ pro-B cells were derived from E13.5 embryos, and *Akt1*$^{-/-}$ pro-B cells were derived from the bone marrow of a 6 week old Akt1$^{-/-}$ mouse. All pro-B cells were cultured on OP9 stromal cells in medium supplemented with recombinant mIL-7 (PeproTech). Abelson murine leukemia virus transformed pre-B cells were generated by infecting cultured pro-B cells with viral supernatant (kindly provided by Dr. Yuan Zhuang, Duke University).

Inhibitors

Imatinib (10 mM, LC Laboratories) stocks were prepared in sterile water and used at a final concentration of 10 μM. Rapamycin (LC Laboratories) was prepared as a 10 μM stock in ethanol and used at a final concentration of 10 nM in all studies unless otherwise indicated.

Flow Cytometry

Single cell suspensions were stained in cold FACS buffer (1× PBS pH7.4 + 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-IgM μ chain specific F(ab′)$_2$ fragment (Jackson ImmunoResearch) was used for intracellular IgH staining. All cells were washed and resuspended in FACS buffer for analysis with a FACSCalibur (BD).

Quantitative RT-PCR

Cells were lysed in TRIzol (Invitrogen); total RNA was purified by isopropanol precipitation. Total RNA was treated with RNase free DNase I (Sigma) and reverse transcribed with Super-Script II reverse transcriptase (Invitrogen) using random primers. Quantitative RT-PCR was performed with an iQ5 multicolor RT-PCR detection system (Bio-Rad) using the Power SYBR green PCR master mix kit (Applied Biosystems).

V(D)J Recombinase Activity Reporter

Ab-MuLV pre-B cells were infected with the retroviral vector pMX-RSS-GFP/IRES-hCD4, which contains a RAG recombinase activity reporter cassette and an IRES-hCD4 expressing cassette (Liang et al., 2002). The infected cells were gated for human CD4 expression and analyzed for the EGFP-positive cells at various times following infection.

Immunoblotting and Antibodies

Cells were washed 2× with ice cold 1× PBS and lysed in cold RIPA buffer with freshly added protease and phosphatase inhibitors. Total cell lysates were resolved by SDS-PAGE and blotted with the following antibodies: anti-Akt p-Thr308, anti-Akt p-Ser473 (587F11), anti-panAkt (11E7), anti-Akt2 (D6G4), anti-PKCα/βII p-Thr641/641, anti-Foxo1/3a p-Thr23/32, anti-Foxo1 (C29H4), anti-4E-BP1 p-Thr37/36 from Cell Signaling, anti-Akt1 (E45W), anti-H2B (EP819Y) (Epitomics), anti-Sin1 (K87) (Jacinto et al., 2006), anti-Rictor (Bethyl Inc.), anti-mTOR (N5D11) (IBL), anti-RAG1 (35.2) (Leu and Schatz, 1995), ERK2 (381A10) (Invitrogen). Densitometry analysis was performed with a BioRad Molecular Imager Gel Doc XR system and Quantity One software (BioRad). Immunoprecipitation of mTORC2 was performed as previously described (Facchinetti et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2010.07.031.

ACKNOWLEDGMENTS

We thank Drs. Mark Shlomchik and Adriano Flora for critically reading the manuscript and providing helpful suggestions and Dr. Yuan Zhuang for the gift of Abelson murine leukemia virus. This work is supported in part by grant AI 063348 (NIH) and W81XWH-10-1-10259 (DoD) (to B.S.) and an America Heart Association grant 0765060Y (AHA) (to V.F.). A.S.L. is a recipient of Brown-Cox Fellowship from Yale University and a Leukemia & Lymphoma Society fellow. D.G.S. is an investigator of the Howard Hughes Medical Institute.

A.S.L. designed and performed experiments, analyzed data, and wrote the paper; D.L and V.F. performed experiments; A.D.L., W.C.S., and D.G.S. provided key experimental reagents; D.G.S. assisted in experiment design; and B.S. designed experiments, analyzed data, and wrote the paper.

Received: December 14, 2009
Revised: April 22, 2010
Accepted: July 16, 2010
Published: August 12, 2010

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blood

2012 119: 6080-6088
doi:10.1182/blood-2011-12-399519 originally published online May 7, 2012

Inhibition of the mTORC2 and chaperone pathways to treat leukemia

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Inhibition of the mTORC2 and chaperone pathways to treat leukemia

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Constitutive activation of the kinases Akt or protein kinase C (PKC) in blood cancers promotes tumor-cell proliferation and survival and is associated with poor patient survival. The mammalian target of rapamycin (mTOR) complex 2 (mTORC2) regulates the stability of Akt and conventional PKC (cPKC; PKCα and PKCβ) proteins by phosphorylating the highly conserved turn motif of these proteins. In cells that lack mTORC2 function, the turn motif phosphorylation of Akt and cPKC is abolished and therefore Akt and cPKC protein stability is impaired. However, the chaperone protein HSP90 can stabilize Akt and cPKC, partially rescuing the expression of these proteins. In the present study, we investigated the antitumor effects of inhibiting mTORC2 plus HSP90 in mouse and human leukemia cell models and show that the HSP90 inhibitor 17-allylaminogeldanamycin (17-AAG) preferentially inhibits Akt and cPKC expression and promotes cell death in mTORC2 deficient pre-B leukemia cells. Furthermore, we show that 17-AAG selectively inhibits mTORC2 deficient leukemia cell growth in vivo. Finally, we show that the mTOR inhibitors rapamycin and pp242 work together with 17-AAG to inhibit leukemia cell growth to a greater extent than either drug alone. These studies provide a mechanistic and clinical rationale to combine mTOR inhibitors with chaperone protein inhibitors to treat human blood cancers. (*Blood*. 2012;119(25):6080-6088)

Introduction

Constitutive activation of the AGC kinase protein kinase B/Akt is observed frequently in blood cancers and is associated with poor patient survival.[1-3] Constitutive phosphorylation of Akt at residues S473 and T308, which are required for full Akt activation, is observed in most patients with acute myelogenous leukemia (70%-80%).[1,2] Constitutive Akt activity promotes tumor-cell proliferation by phosphorylating and suppressing the cell-cycle inhibitor $p27^{Kip1}$ and the F-box–containing transcription factor FoxO1.[4-6] Akt activation also promotes tumor-cell survival by directly phosphorylating and inhibiting the proapoptotic protein BAD and by suppressing the degradation of the antiapoptotic protein Mcl-1 by inhibiting GSK3.[3,7,8] Akt activity is regulated by mammalian target of rapamycin complex 2 (mTORC2), which phosphorylates Akt at S473.[9-11] Pharmacologic or genetic inhibition of mTORC2 abolishes growth-factor–dependent Akt S473 phosphorylation and impairs Akt signaling.[9-12]

mTORC2 is composed of mTOR, Rictor, MAPK-associated protein 1 (Mapkap1/Sin1), mLST8, Protein observed with Rictor (Protor/PRR5,) and DEP-domain–containing mTOR-interacting protein (DEPTOR).[13] mTORC2 function may be inhibited by rapamycin in cells that are chronically exposed to the drug; however, the precise mechanism through which this inhibition occurs is unclear.[9,14] Genetic deletion of Rictor, Sin1, or mLST8 in mammalian cells results in the disruption of the mTORC2 complex and the loss of mTORC2 function.[9,10,15] mTORC2 regulates the activity and stability of the AGC family kinases Akt and conventional protein kinase C (cPKC) in both a growth-factor–dependent and growth-factor–independent manner.[9-11,15-17] Growth factor signals activate mTORC2, which in turn phosphorylates a conserved serine residue in the hydrophobic motif (HM, S473 in Akt1) of Akt and directs Akt kinase activity toward a selected subset of substrates such as FoxO1 and FoxO3a.[9,13]

The mTOR inhibitor rapamycin and the rapamycin-related rapalogs temsirolimus and everolimus suppress leukemic cell growth.[18] However, these compounds generally do not promote tumor cell death due, at least in part, to the inability of these drugs to effectively block mTORC2 and Akt signaling.[18] Small-molecule mTOR kinase inhibitors are capable of inhibiting the rapamycin-sensitive function of both mTOR and mTORC2 directly. Consequently, the mTOR kinase inhibitors impair Akt signaling by blocking mTORC2-dependent Akt S473 phosphorylation.[12,19] mTOR kinase inhibitors exhibit an improved ability to suppress leukemic cell growth and to promote leukemic cell death in models of BCR-Abl^+ B-cell acute lymphoblastic leukemia (B-ALL), T-ALL, and acute myelogenous leukemia.[20-23] However, mTORC2 is not the sole kinase capable of phosphorylating Akt at the hydrophobic motif, and other kinases such as DNA-PK and TBK1 may function independently of mTOR to regulate Akt signaling.[24,25] Therefore, the clinical utility of mTOR kinase inhibitors may be reduced against tumors that use alternative pathways to activate Akt signaling.

mTORC2 also regulates the stability of Akt and cPKC proteins in a growth-factor–independent manner.[17] mTORC2 is required for the phosphorylation of the conserved turn motif (TM) of Akt (T450 in Akt1) and cPKC (T638 on PKCα and T641 in PKCβII).[16,17] mTORC2 associates with actively translating ribosomes and phosphorylates the TM sites of nascent Akt and cPKC polypeptides during translation.[26] TM site phosphorylation promotes the proper folding of newly synthesized Akt or cPKC polypeptides. In $Sin1^{-/-}$ or $Rictor^{-/-}$ cells, in which mTORC2 is disrupted, TM phosphorylation of Akt and cPKC is abolished, leading to reduced stability of Akt and cPKC.[16,17] However, the stability of Akt proteins lacking TM phosphorylation is partially rescued through association with

Submitted December 19, 2011; accepted April 19, 2012. Prepublished online as *Blood* First Edition paper, May 7, 2012; DOI 10.1182/blood-2011-12-399519.

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the chaperone protein HSP90.[17] Inhibition of HSP90 in *Sin1*$^{-/-}$ mouse embryonic fibroblasts results in the rapid reduction of Akt protein expression.[17]

Because Akt is abnormally activated in many human cancers, in the present study, we explored the idea that inhibition of both mTORC2 and HSP90 in leukemia cells would synergistically decrease Akt expression and inhibit tumor cell proliferation and survival. We tested this novel therapeutic strategy by investigating the effect of 17-allylaminogeldanamycin (17-AAG) on Akt expression, cell proliferation, and survival in *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ mouse v-Abl and p210 BCR-Abl–transformed pre-B leukemia cell lines. We show that 17-AAG inhibits Akt expression preferentially and promotes the cell death of *Sin1*$^{-/-}$–deficient leukemia cells, but not wild-type leukemia cells. Furthermore, we demonstrate that 17-AAG specifically inhibits the in vivo tumor growth of *Sin1*$^{-/-}$, but not *Sin1*$^{+/+}$, p210 BCR-Abl–transformed leukemia cells in mice. Finally, we show that chronic rapamycin treatment inhibits mTORC2 and sensitizes wild-type mouse pre-B and human T-cell leukemia to 17-AAG–dependent inhibition of Akt expression and cell death. The results of the present study suggest that combining the chaperone protein inhibitor 17-AAG with mTOR inhibitors may be a promising anticancer strategy.

Methods

Mice

B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mice were purchased from The Jackson Laboratory and used as recipients for the p210 BCR-Abl leukemia cell transfers. Mice receiving leukemia cells were irradiated with 300 cGy 24 hours before leukemia cell transfer, and 1×10^6 leukemia cells were injected via the tail vein. Mice were treated with vehicle only (sterile corn oil) or 17-AAG (80 mg/kg/d in sterile corn oil) delivered by 1 IP injection per day. All mice were housed in the animal facilities at Yale University and all animal procedures were approved by the Yale institutional animal care and use committee.

Abs

Anti–phospho-PKC α/βIIT638/641, anti–phospho-Akt T450, anti–phospho-Akt S473, anti–phospho-Akt T308, anti–pan-Akt, anti–phospho-S6 S235/236, and anti-S6 were purchased from Cell Signaling Technology. Anti–PKCβ2 and anti-ERK2 were from Santa Cruz Biotechnology. Anti-Sin1 Ab was described previously.[9] Reagents used for flow cytometry were as follows: annexin V-PE (BD Pharmingen), propidium iodide (PI) and CD45.2-PE (eBiosciences), and NGFR-Alexa Fluor 647 (a kind gift of Warren Schlomchik, Yale University). All reagents were used at a 1:100 dilution of the stock from the company.

Inhibitors

17-AAG (10mM; LC Laboratories) stocks were prepared in DMSO and used at a final concentration of 5μM, which was used for the in vitro experiments. For in vivo experiments, 17-AAG was first prepared at a stock concentration of 150 mg/mL in DMSO, further diluted with corn oil to a final concentration of 8 mg/mL, and used at a dose of 80 mg/kg/d delivered by IP injection. Imatinib (10mM; LC Laboratories) stocks were prepared in sterile water and used at a final concentration of 10μM. Rapamycin (LC Laboratories) was prepared as a 10mM stock in ethanol and used at a final concentration of 100nM. Wortmannin and LY294002 (10mM; Sigma-Aldrich) stocks were prepared in DMSO and used at a final concentration of 20μM. pp242[12] (20mM) stock was prepared in DMSO and used at a final concentration of 400nM.

Retroviral vectors and generation of virus stocks

Abelson murine leukemia virus supernatant was kindly provided by Dr Yuan Zhuang (Duke University, Durham, NC). The p210 BCR-Abl retroviral supernatant was prepared by transfecting 293T cells with the retroviral plasmid plus ecotropic packaging plasmids. All viral supernatants were sterile filtered and stored at 4°C.

Generation of transformed cell lines

Sin1$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells were described previously.[27] Wild-type or Sin1-knockout pro-B cells were infected with Abelson murine leukemia virus or p210 BCR Abl retrovirus supernatants in the presence of 4 μg/mL of polybrene and centrifuged for 90 minutes at 800*g* at 37°C with RPMI 1640 + 10% FBS culture medium supplemented with recombinant mouse IL-7 (PeproTech). Infected cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 50μM β-mercaptoethanol, and antibiotics (without IL-7) at 37°C in a 5% CO_2 incubator.

Cell lines and culture

Jurkat cells, Abelson murine leukemia virus (Ab-MuLV) cells, or p210 BCR-Abl–transformed mouse pre-B leukemia cells were maintained in RPMI 1640 medium with 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 5 μg/mL of gentamicin, and 50μM β-mercaptoethanol at 37°C in a 5% CO_2 incubator. Cells were monitored daily and fresh medium were exchanged when needed.

Analysis of cell death

Control or treated cells were incubated with propidium iodide (PI) and annexin V-PE in annexin V binding buffer (10mM HEPES, pH 7.3, 150mM NaCl, and 1.8mM $CaCl_2$) at room temperature for 15 minutes, and then analyzed by flow cytometry.

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer containing 50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1mM EDTA, 1mM EGTA, 1mM PMSF, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 25mM NaF, 1mM Na_3VO_4, 25mM β-glycerophosphate, and 2.5mM p-nitrophenyl phosphate. Total cell lysates were resolved on 8% SDS-PAGE gels and transferred to an Immobilon P membrane (Millipore). Membrane was blocked in 5% nonfat dry milk in TBS-T (0.1% Tween 20/TBS) and blotted with appropriate Abs, as per the manufacturer's instructions.

Flow cytometry

Single-cell suspensions were stained in cold FACS buffer (1× PBS, pH 7.4, with 2% FBS) with the appropriate fluorophore-conjugated Abs for 30 minutes on ice. All cells were washed and resuspended in FACS buffer for acquisition on an LSRII or FACSCalibur flow cytometer (BD Biosciences) using FACSDiva Version 6.0 or CellQuest Pro Version 6.0 software (BD Biosciences). Postacquisition analysis was performed with FlowJo Version 7.6.3 software (TreeStar).

In vivo transplantation experiments with mouse p210 BCR-ABL leukemia cells

BCR-Abl–transformed pre-B cells were harvested in culture and injected via tail vein into sublethally irradiated (300 cGy) syngeneic Peb3b (CD45.1^{+}) mice. 17-AAG was administered by 1 IP injection immediately after cell transfer and was repeated daily for 5 days. Mice were killed at day 7 (mice were not treated on day 6) to harvest BM and spleens. Leukemic engraftment was determined by flow cytometry.

Statistical analysis

The Student *t* test was used to determine the statistical significance of the differences between groups of samples. $P < .05$ was considered statistically significant. The number of sample replicates and the number of experimental replicates are indicated in the figure legends.

Results

mTOR inhibitors show limited cytotoxicity against T-ALL and pre-B-ALL

The mTOR signaling pathway is commonly activated in blood cancers to promote uncontrolled cellular growth and proliferation. Because mTOR is a master regulator of cell growth and metabolism, there is great interest in the clinical potential of mTOR inhibitors in the treatment of blood cancers.[18,28-30] Rapamycin and the related rapalogs are approved for clinical use, and newly developed mTOR kinase inhibitors are currently in the early stages of clinical development. To determine the effect of mTOR inhibitors on leukemia-cell proliferation and survival, in the present study, we treated human Jurkat T-ALL cells with rapamycin or the dual PI3K/mTOR kinase inhibitor LY294002. We observed that rapamycin only slightly reduced Jurkat cell growth and had no effect on cell viability relative to mock-treated cells, whereas LY294002 dramatically reduced cell growth and promoted the death of Jurkat cells (Figure 1A-B). We also tested the effect of rapamycin and LY294002 on p210 BCR-Abl–transformed mouse pre-B-ALL cells. Similar to our observations in Jurkat cells, rapamycin reduced cell proliferation but had only a modest cytotoxic effect (Figure 1C-D). In contrast, LY294002 or the Abl kinase inhibitor imatinib were significantly more effective than rapamycin in inhibiting cell proliferation and promoting the death of p210 BCR-Abl leukemia cells (Figure 1C-D). These data show that rapamycin alone can suppress cell proliferation, but only induces minimal cytotoxicity in T-ALL and pre-B-ALL cells.

Rapamycin is an incomplete mTOR inhibitor that may not inhibit mTORC1 fully and may only inhibit mTORC2 in some cell types after chronic treatment.[14] Therefore, we derived mTORC2-deficient, p210 BCR-Abl–transformed pre-B leukemia cell lines from a *Sin1*$^{-/-}$ mouse and analyzed the phosphorylation of Akt at the mTORC2 target site S473 in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ p210 BCR-Abl leukemia cells. We found that Sin1/mTORC2 deficiency did not abolish Akt S473 phosphorylation, suggesting that a kinase other than mTORC2 is responsible for phosphorylating the hydrophobic motif of Akt in p210 BCR-Abl pre-B leukemia cells (Figure 1E). However, we observed that mTORC2-dependent phosphorylation of the Akt and PKCβII TM (T450 and T641, respectively) was abolished in *Sin1*$^{-/-}$ p210 BCR-Abl pre-B leukemia cells (Figure 1E). To better understand the effect of complete mTOR inhibition on leukemia-cell proliferation and survival, we treated Jurkat and p210 BCR-Abl leukemia cells with the mTOR kinase inhibitor pp242. We found that a concentration of 400nM pp242 was sufficient to inhibit mTORC1-dependent S6 S235/236 phosphorylation in both Jurkat and wild-type p210 BCR-Abl leukemia cells (Figure 1F). This dose of pp242 inhibits Akt S473 phosphorylation in Jurkat cells, but only partially inhibits Akt S473 phosphorylation in p210 BCR-Abl leukemia cells (Figure 1F).

We also investigated whether mTOR inhibition with pp242 is sufficient to inhibit leukemia-cell proliferation and promote cell death. First, we cultured Jurkat cells in vitro with or without 400nM pp242 and counted live cells at 24, 48 and 72 hours. We observed that pp242 treatment did not inhibit Jurkat cell proliferation significantly compared with untreated cells (Figure 1G). Furthermore, pp242 did not reduce the viability of Jurkat cells significantly (Figure 1H). We also cultured *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ p210 BCR-Abl pre-B leukemia cells with or without 400nM pp242 and counted live cells at 24, 48, and 72 hours. We observed that pp242 inhibited the proliferation of both *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ p210 BCR-Abl pre-B cells significantly relative to untreated cells (Figure 1I). We also found that pp242 treatment resulted in a modest reduction in p210 BCR-Abl cell viability; however, the inner-cell viability between *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ cells was similar (Figure 1J; 83% live *Sin1*$^{+/+}$ untreated cells vs 71% *Sin1*$^{+/+}$ pp242 treated and 85% live *Sin1*$^{-/-}$ untreated cells vs 69% *Sin1*$^{-/-}$ pp242 treated). These results suggest that mTORC2 is not required for p210 BCR-Abl leukemia cell growth and that inhibition of mTOR alone is insufficient to induce substantial cytotoxicity in pre-B-ALL and T-ALL compared with the dual PI3K/mTOR inhibitor LY294002 or the Abl kinase inhibitor imatinib. These data also suggest that leukemia cells may be able to survive despite the loss of mTOR activity.

17-AAG destabilizes Akt and preferentially induces cell death in *Sin1*$^{-/-}$ leukemia cells

Although Akt is critical for the survival of tumor cells, our data suggested that Akt HM and TM phosphorylation are not essential for leukemia cell survival. Rather, our results suggested that the Akt T-loop (T308) phosphorylation may be necessary and sufficient to promote tumor-cell survival. We have shown previously that Akt and PKCα/βII TM phosphorylation is abolished in *Sin1*$^{-/-}$ p210 BCR-Abl leukemia cells and *Sin1*$^{-/-}$ MEF cells (Figure 1E).[17] Consistent with those results, we observed in the present study that Akt and PKCβII TM phosphorylation was absent in *Sin1*$^{-/-}$ Ab-MuLV–transformed pre-B-ALL cells (Figure 2A). In *Sin1*$^{-/-}$ MEF cells, the stability of Akt and PKCα/βII is partially maintained by HSP90, and inhibition of HSP90 with 17-AAG reduces Akt and PKC protein expression significantly.[17] Therefore we investigated whether 17-AAG could inhibit Akt and PKCβII expression in *Sin1*$^{-/-}$ Ab-MuLV leukemia cells. *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells were treated with 17-AAG for 4 or 8 hours and total Akt or PKCβII protein levels were measured. We found that 17-AAG treatment reduced Akt and PKCβII expression in *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells to a greater extent than in *Sin1*$^{+/-}$ Ab-MuLV cells at the 4- and 8-hour time points (Figure 2B). We also observed that Akt and PKCβII expression was reduced *Sin1*$^{-/-}$ Ab-MuLV pre-B cells relative to *Sin1*$^{+/-}$ pre-B cells before the addition of 17-AAG (Figure 2B), which is consistent with our previous results showing that Akt and PKC protein stability is partially impaired in *Sin1*$^{-/-}$ MEFs.[9]

We also examined the effect of 17-AAG on *Sin1*$^{+/-}$ and *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL survival. *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells were cultured for 24 hours with 17-AAG or vehicle only and cell viability was assessed by flow cytometry using PI and annexin V staining. We observed that 17-AAG treatment decreased *Sin1*$^{+/-}$ cell viability by 10% (81% annexin V$^-$PI$^-$ vehicle–treated cells vs 73% annexin V$^-$PI$^-$ 17-AAG–treated cells) (Figure 2C). In contrast, 17-AAG treatment decreased *Sin1*$^{-/-}$ cell viability by 77% (64% annexin V$^-$PI$^-$ vehicle–treated cells vs 15% annexin V$^-$PI$^-$ 17-AAG–treated cells; Figure 2C). We also observed a reduced proportion of live, vehicle-treated, *Sin1*$^{-/-}$ Ab-MuLV pre-B cells compared with vehicle-treated *Sin1*$^{+/-}$ cells (81% live *Sin1*$^{+/-}$ cells vs 64% live *Sin1*$^{-/-}$ cells). However, 17-AAG treatment of *Sin1*$^{-/-}$ cells resulted in a significantly greater reduction in the total number of viable leukemia cells in culture compared with *Sin1*$^{+/-}$ cells (Figure 2D). These data show that loss of Sin1 and disruption of mTORC2-dependent Akt and PKCβII TM phosphorylation enhances the 17-AAG–mediated inhibition of Akt and PKCβII expression in *Sin1*$^{-/-}$ Ab-MuLV

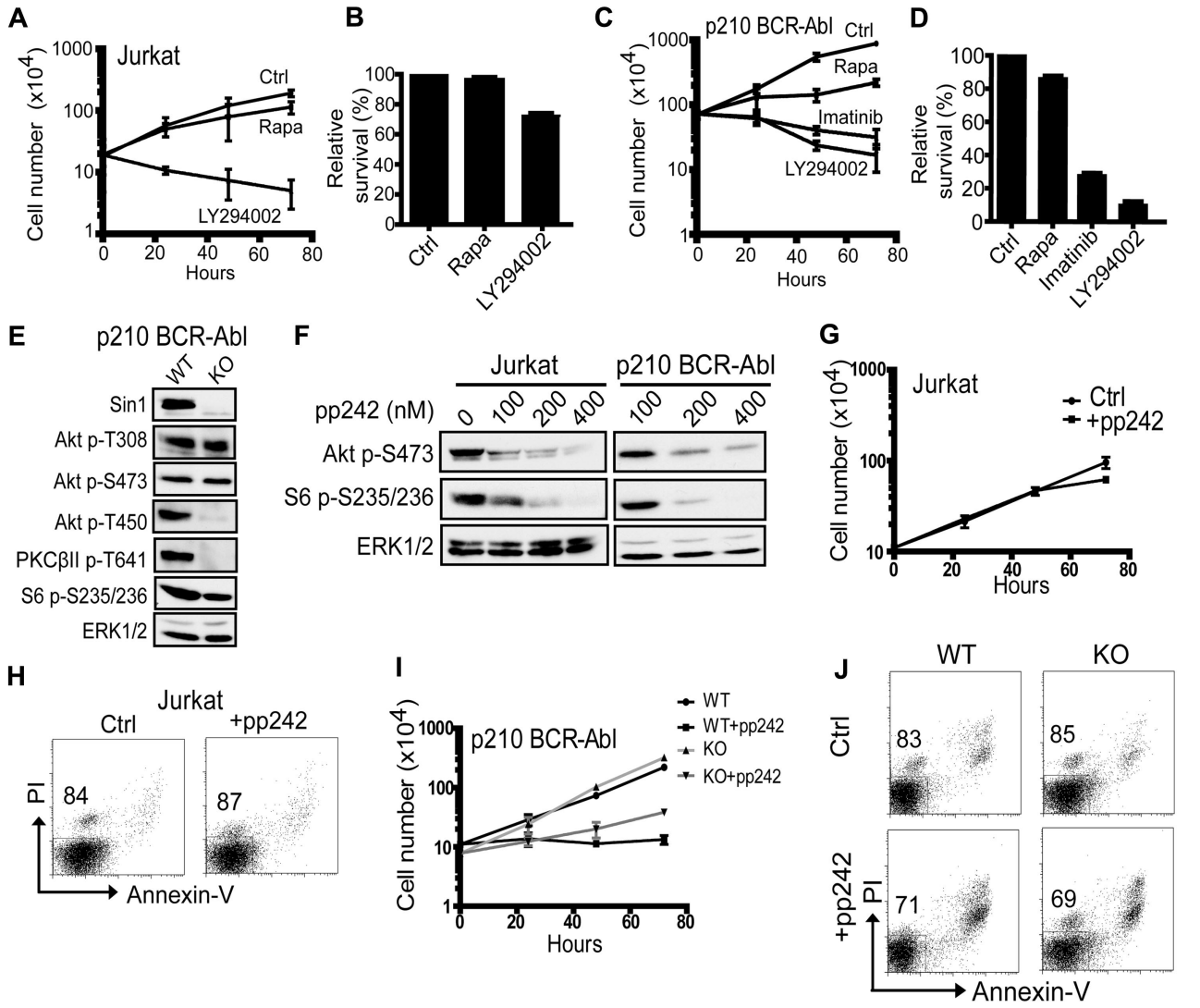

Figure 1. The role of mTOR in leukemia-cell proliferation and survival. (A) Jurkat cells were cultured with vehicle (Ctrl) only or with 100nM rapamycin (Rapa) or 25μM LY294002 as indicated. The total number of live cells at 0, 24, 48, and 72 hours was determined by a trypan blue exclusion cell-viability assay. Each data point shown is the average of triplicate samples from 1 of 3 independent experiments. (B) Jurkat cells from panel A were stained with PI and analyzed by flow cytometry at 24 hours. The number of viable cells in each drug-treated group relative to the untreated control (Ctrl) group (set as 100%) is shown. The data presented are the average of triplicate samples with SD and are representative of 3 independent experiments. (C) Wild-type p210 BCR-Abl–transformed mouse pre-B leukemia cells were cultured with vehicle (Ctrl) or with 100nM rapamycin (Rapa), 10μM imatinib, or 25μM LY294002 for the indicated times. The total number of live cells at 0, 24, 48, and 72 hours was determined as described in panel A. Each data point shown is the average of triplicate samples from 1 of 3 independent experiments. (D) Cells in panel C were stained with PI and analyzed by flow cytometry at 24 hours. The number of live cells in the drug-treated groups relative to the control (Ctrl) group (100%) is shown. The data shown are the average of duplicate samples and are representative of 3 independent experiments. (E) Total cellular proteins from *Sin1*$^{+/+}$ (wild-type; WT) or *Sin1*$^{-/-}$ (knockout; KO) p210 BCR-Abl leukemia cells were analyzed by immunoblotting for the indicated proteins and phosphoproteins. (F) Jurkat or *Sin1*$^{+/+}$ p210 BCR-Abl pre-B cells were cultured with the indicated doses of pp242 for 18 hours and then analyzed by immunoblotting for the indicated proteins. (G) Jurkat cells were cultured with (+pp242) or without (Ctrl) 400nM pp242 for the indicated periods of time and live cells were counted by a trypan blue exclusion assay. Fresh cell-culture medium with or without pp242 was added to the cells every 24 hours. The data shown are the average of triplicate samples from 1 of 2 independent experiments. (H) Cells from panel G were stained with PI and annexin V and analyzed by flow cytometry at the 24-hour time point to determine cell viability. The percentage of live cells (annexin V$^-$PI$^-$) is indicated. (I) Sin1 WT and KO p210 BCR-Abl cells were cultured with (+pp242) or without 400nM pp242 for the indicated periods of time and live cells were counted by a trypan blue exclusion cell-viability assay. Fresh cell-culture medium with or without pp242 was added to the cells every 24 hours. The data shown are the average of triplicate samples from 1 of 3 independent experiments. (J) Sin1 WT and KO cells from panel I were stained with PI and annexin V and analyzed by flow cytometry at the 24-hour time point to determine cell viability. The percentage of live cells (annexin V$^-$PI$^-$) is indicated.

pre-B-ALL cells. Furthermore, disruption of Sin1/mTORC2 function sensitizes Ab-MuLV pre-B-ALL leukemia cells to 17-AAG–dependent cell death.

Restoration of mTORC2 function protects *Sin1*$^{-/-}$ pre-B-ALL cells from 17-AAG–mediated cell death.

To confirm that the 17-AAG–dependent reduction in Akt expression observed in *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells was due to loss of Sin1/mTORC2 function, we reconstituted Sin1-deficient cells with human Sin1α (hSin1α). Retroviral reconstitution of hSin1α restored mTORC2-dependent TM phosphorylation of Akt and PKCβII in Ab-MuLV pre-B-ALL cells (Figure 3A) and prevented the 17-AAG–dependent decrease in Akt expression (Figure 3B). We next investigated whether hSin1α expression protects *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells from 17-AAG–dependent cell death. *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells were infected with an empty retrovirus or an hSin1α–expressing virus and then cultured for 24 hours with 17-AAG or vehicle only. Cell

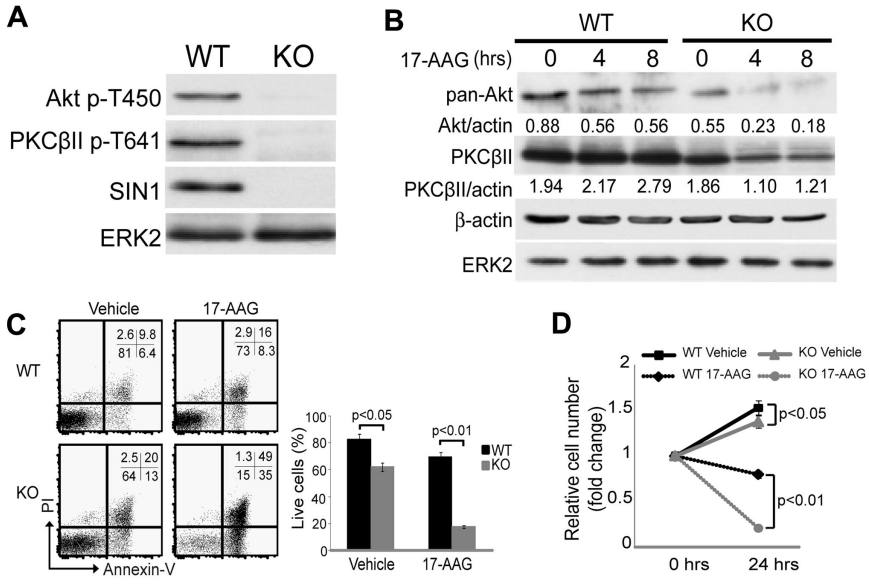

Figure 2. 17-AAG destabilizes Akt and PKCβII and induces cell death of *Sin1*$^{-/-}$ Ab-MuLV pre-B leukemia cells preferentially. (A) Total cellular proteins from *Sin1*$^{+/-}$ (wild-type; WT) or *Sin1*$^{-/-}$ (knockout; KO) Ab-MuLV pre-B cells were assayed by immunoblotting for TM phosphorylation of Akt (p-T450) and PKCβII (p-T641). Sin1 expression is shown and ERK2 expression was used as a loading control. (B) Sin1 WT or KO Ab-MuLV pre-B cells were cultured in the presence or absence of 5μM 17-AAG for the indicated periods of time. Total Akt or PKCβII expression at each time point was measured by immunoblotting. ERK2 and β-actin expression served as loading controls. The Akt/actin or PKCβII/actin ratios were calculated by dividing the total pixel volume of Akt or PKCβII by the total pixel volume of β-actin. The results shown are representative of 2 independent experiments. (C) Sin1 WT or KO Ab-MuLV pre-B cells were cultured with or without 5μM 17-AAG for 24 hours. Cell viability was measured by flow cytometry with PI and annexin V staining. A representative FACS plot is shown on the left. The numbers in the plot show percentages of the gated populations in each quadrant. The data shown on the right graph are the average of triplicate samples from 1 of 3 independent experiments. (D) Sin1 WT or KO Ab-MuLV pre-B cells were cultured for 24 hours with or without 5μM 17-AAG and the relative change in viable cell number was determined. The data shown are the average of triplicate samples from 1 of 3 independent experiments. The *P* values shown were calculated by a 2-tailed *t* test.

viability was assessed by PI/annexin V staining and flow cytometry. We observed that the reconstitution of *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells with hSin1α resulted in a 50% increase in the proportion of viable cells after 17-AAG treatment compared with the cells infected with a control retrovirus (23% annexin V$^-$PI$^-$ viable control cells vs 46% annexin V$^-$PI$^-$ viable hSin1α–expressing cells; Figure 3C). Furthermore, we observed that there were significantly more live hSin1α reconstituted cells in culture

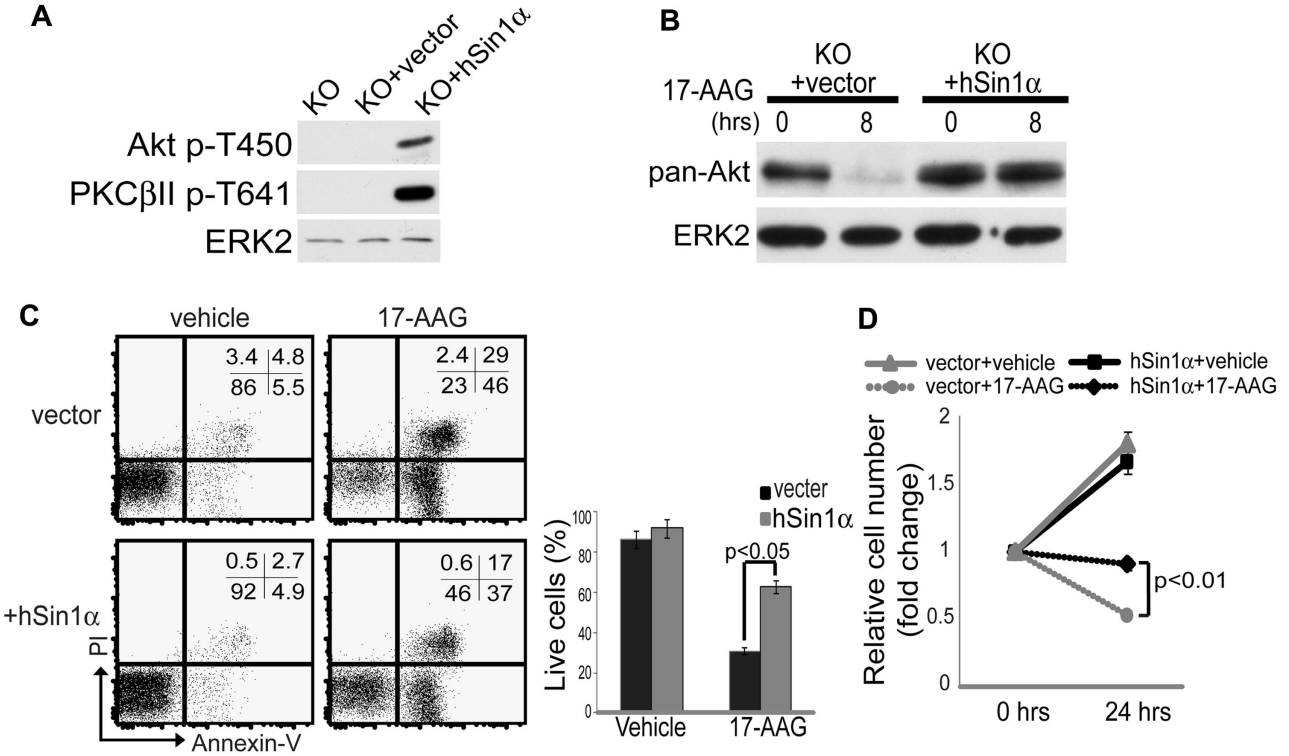

Figure 3. Rescue of mTORC2-dependent TM phosphorylation protects *Sin1*$^{-/-}$ Ab-MuLV pre-B cells from 17-AAG–mediated Akt degradation and cell death. (A) Total proteins from *Sin1*$^{-/-}$ (knockout; KO), empty vector (KO + vector), or human Sin1 (KO + hSin1α)–reconstituted *Sin1*$^{-/-}$ Ab-MuLV pre-B leukemia cells were assayed by immunoblotting for Akt and PKCβII TM phosphorylation as described in Figure 2A. ERK2 expression was used as a loading control. (B) Total cellular proteins were extracted from empty vector (+vector) or human Sin1 (+hSin1α)–reconstituted *Sin1*$^{-/-}$ Ab-MuLV–transformed leukemia cells that were treated with 5μM 17-AAG for 0 or 8 hours. Total Akt protein levels were then measured by immunoblotting, with ERK2 serving as a loading control. The data shown are representative of 2 independent experiments. (C) Empty vector (vector) or hSin1α–reconstituted *Sin1*$^{-/-}$ Ab-MuLV leukemia cells were treated with vehicle or 5μM 17-AAG for 24 hours. Cell viability was measured by PI and annexin V staining and flow cytometric analysis. A representative FACS plot is shown on the left. The numbers in the plot show the percentages of the gated populations in each quadrant. The graph on the right shows the relative proportion of live cells (PI$^-$annexin V$^-$) in each culture condition after 24 hours. The data shown are the average of triplicate samples with SD from 1 of 3 independent experiments. (D) The relative change in total cell number of vector or hSin1α–transduced *Sin1*$^{-/-}$ Ab-MuLV pre-B cells cultured with or without 5μM 17-AAG for the indicated amount of time is shown. The data are the average of triplicate samples with SD from 1 of 3 independent experiments. The *P* values shown were calculated by a 2-tailed *t* test.

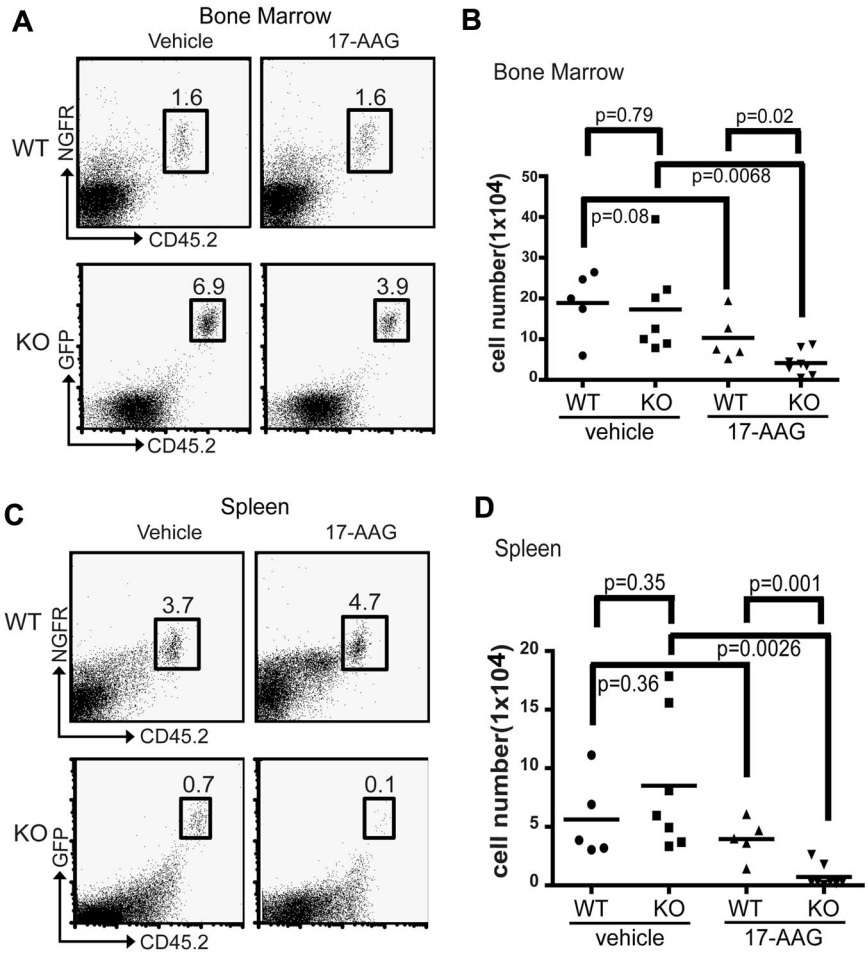

Figure 4. 17-AAG inhibits *Sin1*$^{-/-}$ leukemia growth preferentially in vivo. Sublethally irradiated wild-type CD45.1 congenic mice were transplanted with 1×10^6 *Sin1*$^{+/+}$ (CD45.2$^+$NGFR$^+$; wild-type; WT) or *Sin1*$^{-/-}$ (CD45.2$^+$GFP$^+$; knockout; KO) p210 BCR-Abl–transformed mouse leukemia cells by tail vein injection. The transplanted mice were treated with 17-AAG (80 mg/kg/d in corn oil) or vehicle (corn oil) daily for 5 consecutive days beginning 24 hours after tumor-cell transplantation. Mice were killed 24 hours after the last drug treatment and the percentage and total number of *Sin1*$^{+/+}$ (wild-type; WT) or *Sin1*$^{-/-}$ (knockout; KO) pre-B leukemia cells in the BM (A-B) and spleen (C-D) were analyzed by flow cytometry. The total number of mice in each treatment group are as follows: WT vehicle-treated, n = 5; WT 17-AAG–treated, n = 5; KO vehicle-treated, n = 7; and KO 17-AAG–treated, n = 7. (A) Representative FACS plots showing the percentages of transplanted WT and KO leukemia cells (numbers in the boxes) in BM. (B) Summary of total number of transplanted leukemia cells in the whole BM extracted from one long leg bone (tibia) of WT or KO mice treated with vehicle or 17-AAG. Each symbol represents the cells from one tibia of the indicated recipient mice. (C) Representative FACS plots showing the percentages of transplanted WT and KO leukemia cells (numbers in the boxes) in the spleen. (D) Summary of total number of transplanted leukemia cells in the spleen of WT or KO mice treated with vehicle or 17-AAG. Each symbol represents the cells from the spleens of the indicated recipient mice.

24 hours after 17-AAG treatment compared with empty vector–infected *Sin1*$^{-/-}$ Ab-MuLV pre-B-ALL cells (Figure 3D). These results show that Sin1 protects pre-B leukemia cells from 17-AAG–mediated cell death and indicate that mTORC2 mediates this protection by phosphorylating the Akt and PKCβII TM site.

17-AAG preferentially inhibits the growth of Sin1$^{-/-}$ p210 BCR-Abl B-ALL cells in vivo

Our in vitro data strongly suggest that 17-AAG treatment will be highly effective at inhibiting the growth of tumors that lack mTORC2 function in vivo. To test this hypothesis, we transplanted 1×10^6 *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ p210 BCR-Abl–transformed mouse pre-B-ALL cells into wild-type CD45.1-congenic mice. Starting 24 hours after the leukemia cell transfer, the mice were treated with either vehicle or 17-AAG for 5 consecutive days. All of the mice were killed 1 day after the final drug treatment and analyzed for the presence of pre-B-ALL cells in the BM and spleen by flow cytometry. We observed that pre-B-ALL cells were present in the BM and spleen of all mice regardless of whether they received *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ cells. However, we found that 17-AAG treatment resulted in a significant decrease in the proportion and total number of *Sin1*$^{-/-}$ pre-B-ALL cells in the BM, whereas 17-AAG treatment caused no significant decrease in the number of *Sin1*$^{+/+}$ leukemia cells (Figure 4A-B). Furthermore, we observed that there was no difference in the number of *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pre-B-ALL cells obtained from the BM in the vehicle-treated cohort (Figure 4B). Similar to our observations in BM, we found that 17-AAG treatment resulted in a significant reduction in the proportion and total number of *Sin1*$^{-/-}$, but not *Sin1*$^{+/+}$, pre-B-ALL cells obtained from the spleen (Figure 4C-D) and, again, we observed no significant difference in the total number of *Sin1*$^{-/-}$ or *Sin1*$^{+/+}$ pre-B-ALL cells obtained from the spleens of the vehicle-treated cohort (Figure 4D). These data demonstrate that inhibition of the chaperone pathway by 17-AAG works together with mTORC2 deficiency to suppress leukemia cell growth and survival in vivo.

Coadministration of mTOR inhibitors and 17-AAG promotes leukemia cell death

Our in vitro and in vivo data provide strong evidence that inhibition of both mTORC2 and HSP90 will produce a synergistic antitumor effect that is greater than the inhibition of the mTOR or chaperone pathway alone. Therefore, we chose to investigate whether 2 pharmacologic methods of mTORC2 inhibition, chronic exposure of cells to rapamycin[17,27] or to pp242, could synergize with 17-AAG to suppress wild-type leukemia-cell proliferation in vitro. We first compared the growth rates of Jurkat cells treated with rapamycin alone, 17-AAG alone, or rapamycin plus 17-AAG with untreated cells. We observed that the combination of rapamycin plus 17-AAG inhibited Jurkat cell proliferation to a significantly greater extent than either drug alone (Figure 5A). We then treated Jurkat cells with the combination of pp242 plus 17-AAG, which also inhibited Jurkat cell proliferation; however, pp242 alone had little effect on cell proliferation (Figure 5B).

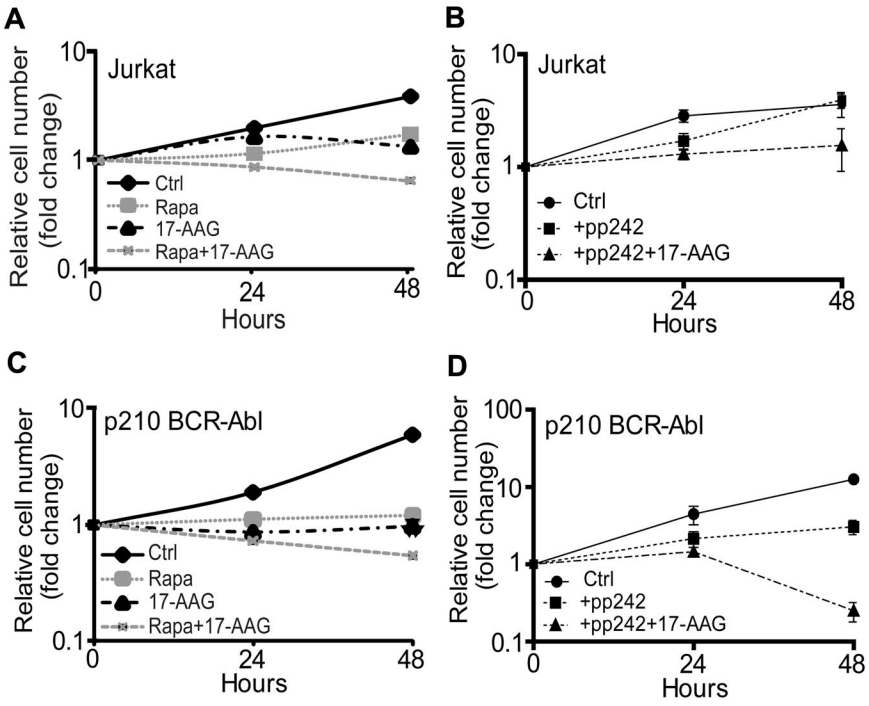

Figure 5. mTOR inhibitors synergize with 17-AAG to promote leukemia cell death. (A) Jurkat cells were treated with vehicle (Ctrl), 100nM rapamycin (Rapa), 5μM 17-AAG, or rapamycin (Rapa) plus 17-AAG for 0, 24, or 48 hours. The relative change in the number of viable cells at each time point was determined by a trypan blue exclusion assay. The total number of viable cells at 0 hours was set at 1. The data shown are the average of triplicate samples from 1 of 3 independent experiments. (B) Jurkat cells were treated with vehicle, 400nM pp242 (+pp242), or pp242 plus 5μM 17-AAG for 0, 24, or 48 hours. The relative change in the number of viable cells at each time point was determined by a trypan blue exclusion assay. The total number of viable cells at 0 hours was set at 1. The data shown are the average of duplicate samples from 1 of 2 independent experiments. (C) Wild-type p210 BCR-Abl mouse pre-B leukemia cells were treated with vehicle, 100nM rapamycin (Rapa), 5μM 17-AAG, or rapamycin (Rapa) plus 17-AAG for 0, 24, or 48 hours. The relative change in the number of viable cells at each time point was determined by a trypan blue exclusion assay. The total number of viable cells at 0 hours was set at 1. The data shown are the average of triplicate samples from 1 of 3 independent experiments. (D) Wild-type p210 BCR-Abl pre-B leukemia cells were treated with vehicle, 400nM pp242 (pp242), or pp242 plus 5μM 17-AAG for 0, 24, or 48 hours. The relative change in the number of viable cells at each time point was determined by a trypan blue exclusion assay. The total number of viable cells at 0 hours was set at 1. The data shown are the average of duplicate samples from 1 of 3 independent experiments.

We also treated wild-type p210 BCR-Abl pre-B-ALL cells with rapamycin or 17-AAG alone or together. Although treatment with rapamycin or 17-AAG alone inhibited leukemia-cell proliferation significantly, cotreatment with rapamycin plus 17-AAG elicited an even greater suppression of leukemia cell growth than either drug alone (Figure 5C). Consistently, cotreatment of p210 BCR-Abl cells with pp242 plus 17-AAG also resulted in a significantly greater inhibition of leukemia cell growth compared with pp242 alone (Figure 5D). These data show that mTOR and chaperone inhibitors act in synergy to inhibit leukemia-cell proliferation and survival.

Discussion

Constitutively activated Akt signaling is found frequently in blood cancers, resulting in an increased resistance of tumor cells to cytotoxic chemotherapies. Therefore, the inhibition of Akt activity is a logical strategy to include in the treatment of these cancers. mTORC2 regulates the growth-factor–dependent activation of Akt by phosphorylating Akt HM at S473. However, we and others have shown that the inhibition of Akt HM phosphorylation does not fully inhibit Akt signaling.[9,10,15,27] Furthermore, mTORC2 is not essential for the growth or proliferation of multiple cell types, including but not limited to embryonic fibroblasts, T cells, developing B cells, and v-Abl or p210 BCR-Abl oncogene-transformed pre-B leukemia cells.[9,10,27,31,32] Furthermore, our analysis of *Sin1*$^{-/-}$ p210 BCR-Abl pre-B-ALL cells revealed that Akt S473 phosphorylation is not fully dependent on mTOR. This conclusion is supported by our observation the high dose of pp242 used herein failed to abolish Akt S473 phosphorylation completely in wild-type p210 BCR-Abl leukemia cells. Several recent studies have also shown that kinases in addition to mTORC2 may phosphorylate Akt in different type of cells.[24,25] Therefore, the inhibition of mTOR alone may not be sufficient to induce the growth arrest and cell death of cancer cells.

mTORC2 also phosphorylates the TM of Akt and cPKC proteins.[16,17] In the absence of mTORC2, Akt and cPKC TM phosphorylation is abolished and the stability of these proteins is reduced.[16,17] HSP90 associates with Akt and cPKC proteins, which lack TM phosphorylation, and rescues the stability of the newly synthesized Akt and cPKC proteins.[17] This is consistent with our observation that Akt and PKCβII expression is reduced by approximately 2-fold in *Sin1*$^{-/-}$ pre-B leukemia cells compared with *Sin1*$^{+/+}$ leukemia cells (Figure 2B). In the present study, we have shown that HSP90 maintains Akt expression in *Sin1*$^{-/-}$ pre-B leukemia cells, but not in *Sin1*$^{+/+}$ leukemia cells. We have also shown that treatment of *Sin1*$^{-/-}$ pre-B leukemia cells with the HSP90 inhibitor 17-AAG results in a dramatic reduction of Akt and PKCβII proteins. These data strongly support a model in which the sensitivity of Akt to 17-AAG is dependent on the loss of mTORC2-dependent Akt TM phosphorylation. Our present results also show that reexpression of Sin1 in *Sin1*$^{-/-}$ leukemia cells restores Akt TM phosphorylation and prevents the reduction of Akt expression on 17-AAG treatment in pre-B leukemia cells (Figure 3). Furthermore, chronic rapamycin treatment or pp242 inhibited mTORC2 in wild-type cells to sensitize p210-BCR-Abl or Jurkat leukemia cells to 17-AAG. These data demonstrate that the combined inhibition of mTORC2 and HSP90 destabilizes Akt and cPKC proteins and synergizes the ability of mTOR inhibitors and 17-AAG to elicit a more effective antileukemic effect.

Because Akt plays a critical role in regulating cell survival, we predicted that the 17-AAG–dependent reduction of Akt expression would promote the cell death of *Sin1*$^{-/-}$ pre-B leukemia cells preferentially over *Sin1*$^{+/+}$ leukemia cells. Indeed, 17-AAG treatment induced substantially more cell death in *Sin1*$^{-/-}$ pre-B leukemia cells than wild-type pre-B leukemia cells. Furthermore, reexpression of human Sin1 in *Sin1*$^{-/-}$ pre-B leukemia cells led to increased resistance to 17-AAG–mediated cytotoxicity. These in vitro studies were supported by in vivo experiments in which we transplanted *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ p210 BCR-Abl–transformed mouse leukemia cells into wild-type mice and treated the recipients with 17-AAG or vehicle for 5 days. Sin1 gene status does not alter tumor growth in vivo, becasuse we were able to recover equivalent

numbers of $Sin1^{+/+}$ and $Sin1^{-/-}$ leukemia cells from the BM and spleens of vehicle-treated mice (Figure 4). However, consistent with our in vitro studies, 17-AAG reduced $Sin1^{-/-}$ pre-B cell tumor burden significantly both in the BM and spleen, whereas the $Sin1^{+/+}$ tumor cell numbers were not affected by 17-AAG. These data provide the first in vivo evidence that the inhibition of mTORC2 sensitizes leukemia cells to 17-AAG and strongly suggest that the dual inhibition of mTOR plus HSP90 may serve as an effective anticancer therapy.

Recently, De Raedt et al reported that the HSP90 inhibitor IPI-504 induced tumor regression when combined with rapamycin in a mouse model of Ras-driven lung cancer.[33] The investigators presented evidence showing that IPI-504 induces reactive oxygen species in lung cancer tumor cells and that mTOR protects tumor cells from ROS by supporting tumor-cell production of the antioxidant glutathione. That study also showed that rapamycin/IPI-504 treatment promotes ER stress and mitochondrial damage, which ultimately results in tumor cell death. The mechanism of rapamycin-induced sensitivity to IPI-504 could be mediated through mTORC1, because knock-down of the essential mTORC1 component raptor mimicked the effect of rapamycin.[33] However, the prolonged rapamycin treatment may have inhibited mTORC2 at later time points (> 1 day), which could also explain in part the synergistic antitumor effects of rapamycin and IPI-504 observed by De Raedt et al.[33] It is unlikely that the enhanced cytotoxic effects of 17-AAG observed in $Sin1^{-/-}$ pre-B leukemia cells are due to impaired mTORC1 function, because Sin1 deficiency does not inhibit mTORC1-dependent phosphorylation of S6K and 4E-BP1 in pre-B leukemia cells[27] (data not shown). Our data and those presented by De Raedt et al indicate that mTOR inhibitors could synergize with HSP90 inhibitors through 2 independent mechanisms, the first of which involves mTORC2-dependent regulation of Akt/cPKC protein stability and the second mTORC1-dependent regulation of the cellular antioxidant response.

Rapamycin and the rapalogs do not inhibit mTORC2 directly. However, accumulating evidence indicates that chronic rapamycin treatment may block mTORC2 complex assembly in many cell types.[9,14,17,27,34] Therefore, it is reasonable to propose that HSP90 inhibitors such as 17-AAG will synergize with rapamycin to induce a cytotoxic response causing tumor regression or remission in human blood cancer patients. Substituting rapamycin with mTOR kinase inhibitors that block both mTORC1 and mTORC2 directly will likely increase the antitumor effectiveness of HSP90 inhibitors. Our present data also provide a rational for developing mTORC2-specific inhibitors that can be combined with 17-AAG to treat blood cancers. A therapeutic strategy that does not inhibit mTORC1 will prevent many of the immunosuppressive and metabolic side effects of rapamycin and preserve the mTORC1-mediated negative feedback regulation of PI3K. Based on the results of the present study, we predict that mTORC2 will be an important new target for the development of specific inhibitors that can be used in combination with chaperone inhibitors to treat a wide range of cancers.

Acknowledgments

The authors thank Kevan M. Shokat (University of California, San Francisco) for generously providing the pp242; Warren Schlomchik (Yale University) for the generous gift of p210 BCR-Abl retrovirus and human NGFR–Alexa Fluor 647 Ab; and Dr Yuan Zhuang (Duke University) for generously providing the Abelson Murine leukemia virus supernatant.

This work was supported in part by the Department of Defense (grant PR093728 to B.S.). A.S.L. is the recipient of a Brown-Cox Fellowship from Yale University and is currently a Leukemia & Lymphoma Society fellow. F.Z. is partially supported by the Chinese Scholarship Council, Ministry of Education, China.

Authorship

Contribution: F.Z., A.S.L., and D.L. performed the experiments; F.Z., A.S.L., and B.S. analyzed the data, wrote the manuscript, and designed the experiments; and F.C. analyzed the data and assisted in manuscript preparation.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Sin1 regulates Treg development but is not required for T cell growth and proliferation

Journal:	*European Journal of Immunology - 2*
Manuscript ID:	eji.201142066
Wiley - Manuscript type:	Regular Article
Date Submitted by the Author:	25-Aug-2011
Complete List of Authors:	Chang, Xing; Yale University, Immunobiology Lazorchak, Adam; Yale University, Immunobiology Liu, Dou; Yale University, Immunobiology Su, Bing; Yale University, Immunobiology
Keywords:	T cells, Signal transduction, CD4 T cells

Sin1 regulates Treg development but is not required for T cell growth and proliferation

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Running title: Sin1-mTORC2 function in T cells

Summary

Mammalian Sin1 plays key roles in the regulation of mitogen activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) signaling. Sin1 is an essential component of mTOR complex (mTORC) 2. The function of Sin1 and mTORC2 is largely unknown in T cells. Here we investigate Sin1 function in T cells using mice which lack *Sin1* in the hematopoietic system. *Sin1* deficiency blocks the mTORC2 dependent Akt phosphorylation in T cells during development and the immune response. Sin1 deficient T cells exhibit normal thymic cellularity and percentages of double negative, double positive and single positive CD4 and CD8 thymocytes. *Sin1* deficiency does not impair T cell receptor (TCR) induced growth and proliferation, and normal $CD4^+$ helper cell differentiation. However *Sin1* deficiency results in an increased proportion of $Foxp3^+$ natural T regulatory (nTreg) cells in the thymus. We show that the TGF-β dependent differentiation of $CD4^+$ T cells *in vitro* is enhanced by the inhibition of mTOR but not loss of Sin1 function. Our results reveal that Sin1 and mTORC2 are dispensable for the development and activation of T cells but play a role in natural Treg cell differentiation.

Introduction

Mammalian target of rapamycin (mTOR) is a conserved serine/threonine protein kinase that regulates cell growth and metabolism [1]. Mammalian TOR is inhibited by rapamycin which is potent suppressor of T cell-mediated immune responses [2]. Rapamycin inhibits IL-2 dependent T cell proliferation, promotes the expansion of regulatory T (Treg) cells and has recently been shown to promote the development of memory $CD8^+$ T cells [3-5]. Mammalian TOR function is mediated by at least two distinct multi-protein complexes called mTOR complex 1 (mTORC1), containing mTOR, raptor, mLST8 (GβL) and PRAS40, and mTORC2, containing Rictor, Sin1, and mLST8 in addition to mTOR. Nutrients, growth factors, hormones, and energy signals, activate mTORC1 to phosphorylate the translational regulators S6K and 4EBP1 leading to increased cellular protein synthesis and ribosome biogenesis [1]. Mammalian TORC2 regulates actin polymerization and cytoskeleton function [1], controls Akt activation and specificity in a PI3K-dependent manner by phosphorylating the Akt hydrophobic motif (S473 on Akt1), and regulates the stability of Akt and conventional PKC in a PI3K-independent manner by phosphorylating the turn motif (T450 on Akt1) [6-8]. Mammalian TORC2 is resistant to rapamycin inhibition therefore previous studies utilizing rapamycin to study mTOR were unable to properly evaluate the contribution of mTORC2 to T cell immunity. In addition, mTOR also posses rapamycin independent mTORC1 function [9]. Therefore, it is unclear how mTORC1 and mTORC2 each specifically contribute to T cell function.

Recent genetic studies have begun to elucidate the mechanism of mTOR function and regulation in T cells. Delgoffe *et al* recently reported that CD4-Cre mediated T cell specific mTOR deletion impairs T cell proliferation and inhibits T_H1, T_H2, and T_H17 differentiation without blocking early T cell activation [10]. Mammalian TOR deficiency also greatly enhanced Treg differentiation *in vitro*. T cells lacking Rheb, a small GTPase which positively regulates mTORC1 function, fail to spontaneously differentiate into Treg cells upon activation suggesting that mTORC2 may play a prominent role in regulating Treg dependent [10]. Two recent studies from independent labs explored have explored the function of mTORC2 in T cells using mice that specifically lack Rictor expression in T cells [11, 12]. In the first study, Lee *et al* show that *rictor*$^{-/-}$ T cells lack functional mTORC2 and exhibit defects in Akt and PKCθ phosphorylation as well as decreased NF-κB activity, reduced proliferation, impaired T helper cell differentiation and increased $CD4^+FoxP3^+$ Treg differentiation [12]. While in the second study, Delgoffe *et al*

show that *rictor*$^{-/-}$ T cells exhibit defects in proliferation and T_H2 differentiation, they do not observe deficiencies in T_H1, T_H17 or Treg differentiation [11].

In this study, we reconstituted lethally irradiated wild type mice with *Sin1*$^{-/-}$ fetal liver hematopoietic stem cells (HSC) and examined the T cell development, growth, proliferation, and $CD4^+$ effector cell differentiation in cells obtained from these mice. We show that the loss of Sin1 in T cells disrupts mTORC2 function and blocks Akt phosphorylation at the HM and TM sites. Although mTORC2 function is abolished in *Sin1*$^{-/-}$ T cells, we find that Sin1 is not required for thymic T cell development. These data reveal that Akt HM and TM phosphorylation are not required for thymic T cell development even though Akt plays an essential role in maintaining the metabolism and viability of thymocytes undergoing TCR β selection. Furthermore, mature T cell growth, proliferation or $CD4^+$ helper T cell differentiation is unaffected by Sin1 deficiency. However, we observe that *Sin1*$^{-/-}$ thymic T cells give rise to a greater proportion of natural Treg cells than wild type thymocytes. These data support a role for mTORC2 in the regulation of Treg differentiation. We also provide evidence that Akt1 and Akt2 are not required for mTORC2 mediated regulation of thymic Treg development.

Materials and Methods

Mice

Sin1$^{-/-}$ mice and *Akt1*$^{-/-}$, *Akt2*$^{-/-}$ and *Akt1*$^{-/-}$*Akt2*$^{-/-}$ mice were described previously [6, 13]. $CD45.1^+$ congenic 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 prior to cell transfer. All mice were housed in the animal facilities at Yale University and all animal procedures were approved by the Yale IACU Committee.

Lymphocyte staining and flow cytometry

Cells were washed with FACS buffer (1% FBS in 1x phosphate buffered saline (PBS) with 0.1% NaN_3), incubated with indicated antibodies on ice for 30 min, then washed two more times with FACS buffer, and fixed in 1% paraformaldehyde in PBS before being analyzed with a LSRII flow cytometer (BD Biosciences). For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma) (50 ng/ml) + ionomycin (Sigma) (500 ng/ml) for 6 hours in the presence of Golgi-stop (BD Bioscience) for the last four hours. Cells were first

surface stained, fixed/permeablized with a Cytofix/Cytoperm kit (BD Bioscience), and stained with antibodies against indicated cytokines. Intracellular FoxP3 and T-bet staining were carried out according to manufacturer's instruction (EBioscience). For co-staining FoxP3 with GFP, cells were fixed by cytofix buffer (BD Bioscience), permeablized by ice-cold methanol and stained with indicated antibodies in the 1x Perm/Wash buffer (BD Bioscience).

Cell Purification and Culture

Splenocytes and lymph node cells were first stained with anti-CD4 biotin and $CD4^+$ cells were magnetically purified using a Biotin-selection kit (Stem cell). Purified CD4 T cells were stimulated with plate bound anti-CD3 (3 ug/ml; 2C11) and soluble anti-CD28 (2 ug/ml; 37N) in T cell medium (RPMI 1640, 10% FBS, 1x antibiotics, 1x non-essential amino acid and 50 μM β-mercaptoethanol). When indicated, recombinant cytokines were added into the culture: T_H1: anti-IL-4 (5 μg/ml; 11B11) and IL-12 (10 ng/ml; PeproTech); iTreg: anti-IL-4 (5 ug/ml, 11B11), anti-IFNγ (5 ug/ml; R46A2), rhIL-2 (100 U/ml, Peprotech) and indicated concentration of rhTGF-β (Peprotech); T_H17: anti-IL4 (5 ug/ml), anti-IFNγ (5 ug/ml), IL-6 (20 ng/ml, Peprotech), and indicated concentration of rhTGF-β (Peprotech). When indicated, the following inhibitors were used in this study: Rapamycin (LC laboratories); pp242 [14].

T Cell Stimulation and Immunoblotting

Naïve CD4 T cells were activated with anti-CD3/anti-CD28 antibodies in the presence of IL-2 (50 U/ml) for four days. Activated cells were then split into fresh culture medium with IL-2 (100 U/ml) and expanded for additional four to five days. Cultured T cells were rested in T cell medium without IL-2 overnight and stimulated with either plate bound anti-CD3 antibody (5 ug/ml)+anti-CD28 (2 ug/ml) for various time points. Stimulated T cells were washed with ice-cold PBS and lysed with RIPA buffer plus freshly added protease inhibitors and phosphatase inhibitors. Total cell lysates were used for immunoblot analysis. To detect S6 and Akt S473 phosphorylation following TCR stimulation, CD4 T cells were first stained with anti-CD3 (5 μg/ml) for 30 min on ice. After wash, T cells were cross-linked with anti-Hamster Ig G for 3min, fixed with Phosph-flow fix buffer I (BD Bioscience), and stained with anti-pS6 S235/236 or anti-pAkt S473 (Cell Signaling) in Phosph-flow perm/wash buffer (BD Bioscience) followed by Alex-fluor 647 conjugated anti-Rabbit IgG (Cell signaling).

T cell proliferation

Purified CD4 T cells were labeled with CFSE (3 nM) at 37C for 10min. CFSE labeled cells were stimulated with plate bound anti-CD3 and anti-CD28 as described.

Results

Sin1 is not required for the development of major thymic T cell subsets

We generated chimeric mice by transplanting E12.5 fetal liver cells from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ embryos into lethally irradiated wild type (WT) CD45.1 congenic mice [13]. Analysis of thymic T cell populations in these mice revealed that *Sin1* deficient hematopoietic stem cells gave rise to equivalent proportions of CD4/CD8 double negative (DN), CD4/CD8 double positive (DP), CD4$^+$ single positive (SP) and CD8$^+$ SP T cells as *Sin1*$^{+/+}$ cells (**Fig. 1a**). We also observed equivalent proportions of splenic CD4$^+$ and CD8$^+$ T cells in *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ chimeric mice (**Fig. 1a**). Next we measured the proportion of cytokine producing CD4$^+$ effector T cells in the periphery of unimmunized chimeric mice. We found that the proportion of IFN-γ, IL-4 or IL-17A expressing CD4$^+$ T cells in the spleen of unimmunized *Sin1*$^{-/-}$ chimeric mice was comparable to that of *Sin1*$^{+/+}$ mice (**Fig. 1b**). These data indicate that Sin1 is not required for T cell development.

We have previously shown that suppression of FoxO1 and FoxO3a transcriptional activity by Akt is dependent on Sin1 and mTORC2 in MEFs and in B cells [6, 13]. FoxO1 is a positive regulator of L-selectin (CD62L), CD127 (IL-7 receptor alpha chain, IL7r) and FoxP3 gene expression in T cells [15, 16]. Therefore we asked if *Sin1*$^{-/-}$ T cells exhibit increased expression of these FoxO1 dependent genes. CD62L expression was increased on the splenic CD4$^+$CD44^{low}CD62L$^+$ *Sin1*$^{-/-}$ T cells relative to *Sin1*$^{+/+}$ T cells (**Fig. 1c**) but CD127 expression was equivalent on *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ peripheral T cells (**Fig. 1d**).

The transcription factor FoxP3 is the master regulator of Treg development. To assess the possible role of Sin1 in Treg development we first determined the proportion of thymic Tregs in *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ chimeric mice. We observed that *Sin1*$^{-/-}$ thymocytes gave rise to 2 fold more CD25$^+$FoxP3$^+$ Treg cells when compared to *Sin1*$^{+/+}$ thymocytes (4% *Sin1*$^{+/+}$ CD4$^+$CD25$^+$FoxP3$^+$ vs. 10% *Sin1*$^{-/-}$ CD4$^+$CD25$^+$FoxP3$^+$) (**Fig. 1e**), indicating that Sin1 may be a suppressor of thymic Treg differentiation. The proportion of CD25$^+$FoxP3$^+$ T cells in the spleens of *Sin1*$^{+/+}$

and *Sin1*$^{-/-}$ chimeric mice was not significantly different (9% *Sin1*$^{+/+}$ CD4$^+$CD25$^+$FoxP3$^+$ vs. 10% *Sin1*$^{-/-}$ CD4$^+$CD25$^+$FoxP3$^+$) (**Fig. 1e**).

To determine if the Sin1 mediated suppression of thymic Treg development is cell intrinsic, we generated *Sin1*$^{-/-}$ chimeric mice containing an equivalent ratio of *Sin1*$^{-/-}$ fetal liver cells (CD45.2$^+$) and WT cells (CD45.1$^+$). There were two times more *Sin1*$^{-/-}$ CD25$^+$FoxP3$^+$ Tregs that WT Tregs (7% *Sin1*$^{+/+}$ CD4$^+$CD25$^+$FoxP3$^+$ vs. 16% *Sin1*$^{-/-}$ CD4$^+$CD25$^+$FoxP3$^+$) in the same host (**Fig. 1f**). These data indicate that Sin1 inhibits the development of thymic Treg development in a cell intrinsic manner.

Akt is a negative regulator of Treg development [17] and Akt activity is directly regulated by mTORC2 [6, 13]. Since *Sin1*$^{-/-}$ cells lack mTORC2 function and exhibit deficiencies in Akt phosphorylation and function, we hypothesized that Akt may mediate mTORC2 dependent signals to suppress thymic Treg development. To test this hypothesis we measured the proportion of thymic Treg cells in Akt deficient mice. We determined the proportion of CD4$^+$FoxP3$^+$ Treg cells in the thymus of wild type, *Akt1*$^{-/-}$ or *Akt2*$^{-/-}$ mice. We found that *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ mice had an equivalent proportion of CD4$^+$FoxP3$^+$ T cells when compared to WT mice (**Fig. 2a**). In addition, we also analyzed thymic Treg development in *Akt1*$^{-/-}$*Akt2*$^{-/-}$ fetal liver cell chimeric mice (these mice die at late embryonic stage E18-19). Consistent with the previous reports [18], we observed that thymocyte development was blocked at the DN to DP transition in *Akt1*$^{-/-}$*Akt2*$^{-/-}$ chimeric mice (data not shown). However a small number of *Akt1*$^{-/-}$*Akt2*$^{-/-}$ thymocytes were capable of developing to the CD4$^+$ SP stage. We measured the proportion of FoxP3$^+$CD4$^+$ T cells within this population of *Akt1*$^{-/-}$*Akt2*$^{-/-}$ CD4$^+$ SP cells and found that the proportion of Treg cells was similar to that observed in mice reconstituted with wild type fetal liver cells (**Fig. 2b**).

Sin1 is not required for T cell growth or proliferation

Mammalian TOR is a master regulator of cellular growth. Therefore we asked if Sin1/mTORC2 was involved in regulating T cell growth and proliferation. We found that resting lymph node and spleen CD4$^+$ and CD8$^+$ T cell size of *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ fetal liver chimeric mice was equivalent (**Fig. 3a, data not shown**). Next, we stimulated *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ T cells with anti-CD3 plus anti-CD28 and assessed T cell size and proliferation. Sin1 deficiency did not impair the blast cell growth of activated T cells (**Fig. 3b and 3c**), and CD4$^+$ T cells from *Sin1*$^{+/+}$

and *Sin1*$^{-/-}$ chimeric mice exhibited a similar activation induced proliferative capacity as determined by CFSE dilution assay (**Fig. 3d**). These data show that Sin1 is not required for T cell growth of resting or activated T cells and that Sin1 is not required for the proliferation of activated T cells.

Sin1 is not required for T_H1, T_H2, and T_H17 effector T cell differentiation *in vitro*

To test the function of Sin1 in effector T cell differentiation, we purified CD4$^+$ T cells from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ chimeric mice, activated these cells *in vitro* and differentiated these cells under T_H1, T_H2 or T_H17 polarizing conditions. *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ T cells cultured under T_H1, T_H2, or T_H17 polarizing conditions gave rise to equivalent proportions of IFN-γ (30% *Sin1*$^{+/+}$ vs. 35% *Sin1*$^{-/-}$), IL-4 (6% *Sin1*$^{+/+}$ vs. 5% *Sin1*$^{-/-}$), or IL-17 (15% *Sin1*$^{+/+}$ vs. 14% *Sin1*$^{-/-}$) expressing cells, respectively (**Fig. 4a**). We obtained the same results when we co-cultured *Sin1*$^{-/-}$ T cells with wild type congenic T cells under the same T_H polarizing conditions (data not shown) indicating that Sin1 is not required for effector T cell differentiation into the T_H1, T_H2, or T_H17 lineages.

Sin1 is required for mTORC2 dependent phosphorylation of Akt in T cells

To examine if Akt phosphorylation at the mTORC2 target sites S473 and T450 was defective in *Sin1*$^{-/-}$ T cells, resting *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ CD4$^+$ T cells were stimulated with anti-CD3 antibody and Akt S473 phosphorylation was measured. As expected, compared to unstimulated T cells, anti-CD3 stimulation induced Akt S473 phosphorylation in *Sin1*$^{+/+}$ T but failed to induce this phosphorylation in *Sin1*$^{-/-}$ T cells (**Fig. 4b**). Consistent with our previous observations in *Sin1*$^{-/-}$ fibroblasts and B cells, Akt T450 phosphorylation in *Sin1*$^{-/-}$ T cells was also deficient (**Fig. 4c**). These data show that Sin1 deficient T cells lack mTORC2 function and show defective Akt phosphorylation at the HM and TM sites.

Sin1 and mTOR differentially regulate TGF-β dependent Treg differentiation

Our observation that Sin1 deficiency promotes thymic Treg development is consistent with a current model in which mTORC2-Akt signal inhibits FoxO1 activity, which is required for Treg differentiation [10, 12]. To test if Sin1 may also inhibit the TGF-β dependent Treg differentiation of peripheral CD4$^+$ T cells, purified *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ CD4$^+$ T cells were

differentiated in the presence or absence of TGF-β. Without TGF-β *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ CD4$^+$ T gave rise to very few numbers of FoxP3$^+$ cells (1.4% vs. 1.6%) (**Fig. 5a**). In the presence of TGF-β, *Sin1*$^{-/-}$ CD4$^+$ T cells consistently gave rise to fewer FoxP3$^+$ Treg cells when compared to *Sin1*$^{+/+}$ CD4$^+$ T cells (28% vs. 40%) (**Fig. 5a**). These data are surprising since we predicted that loss of mTORC2 function would enhance Treg differentiation similar to that of *Sin1*$^{-/-}$ thymocytes. Our results raise the possibility that Sin1 may have mTORC2 independent functions that may influence TGF-β dependent Treg differentiation in the periphery.

To directly test the function of mTOR during Treg differentiation, we induced Treg differentiation of WT naïve CD4$^+$ T cells with TGF-β *in vitro* in the presence or absence of mTOR inhibitors rapamycin or pp242 [14]. Rapamycin specifically inhibits mTORC1 while pp242, a specific mTOR kinase inhibitor, targets both mTORC1 and mTORC2 [14]. We observed that rapamycin (30 nM) did not significantly change the proportion of Treg cells generated in the presence of TGF-β (untreated = 53% vs. rapamycin treated = 50%). However**,** pp242 treatment (100 nM) consistently resulted in an increase in the proportion of Treg cells generated in response to TGF-β (untreated = 53% vs. pp242 treated = 68%) (**Fig. 5b**). Both rapamycin and pp242 blocked mTORC1 dependent phosphorylation of ribosomal protein S6 while only pp242 blocked mTORC2 dependent HM site phosphorylation of Akt (**Fig. 5c**). Overall our data support a model in which inhibition of both mTORC1 and mTORC2 is necessary to promote TGF-β dependent Treg differentiation.

Discussion

In this study, we provide the first evidence examining the function of Sin1 in T cells. Our analysis of *Sin1*$^{-/-}$ fetal liver chimeric mice reveals that Sin1 is largely dispensable for the development of thymic T cells and peripheral CD4$^+$ and CD8$^+$ T cell populations. Since Sin1 is essential for mTORC2 function, our data also indicates that mTORC2 is not required for T cell development. Akt is the best characterized mTORC2 target and is required for T cell development [6, 7, 19]. *Akt1*$^{-/-}$*Akt2*$^{-/-}$ T cells show a profound block in thymic development at the DN to DP transition [19]. *Sin1*$^{-/-}$ T cells develop normally despite having a partial loss of Akt function due to impaired HM and TM phosphorylation. The dramatic difference in T cell developmental phenotypes observed in *Akt1*$^{-/-}$*Akt2*$^{-/-}$ and *Sin1*$^{-/-}$ chimeric mice indicates that functional outcomes of Akt signaling can be subdivided into HM phosphorylation independent

signals and HM phosphorylation dependent signals. Our data show that T cell development is not dependent on Akt HM phosphorylation. These findings are consistent with our previously proposed model in which mTORC2 dependent Akt HM phosphorylation is required to confer Akt specificity toward a limited subset of substrates [6]. Our data also suggest that the Akt, when activated via phosphorylation of activation loop, plays a central role for DN-DP transition, most likely by control the survival of thymic T cells.

FoxO1 is an mTORC2 dependent Akt substrate which has been shown to play a key role in regulating T cell development, homeostasis and effector cell differentiation [16, 20]. FoxO1 is required for proper expression of the genes that encode L-selectin (CD62L), interleukin 7 receptor alpha chain (CD127) and FoxP3 [15, 16, 20]. We have previously shown that Sin1 deficiency results in decreased FoxO1 phosphorylation at the Akt target sites, leading to increased FoxO1 transcriptional activity [6, 13]. Consistently, we observed an increased proportion of FoxP3 expressing nTregs in the thymus and an increased expression of CD62L expression on naive peripheral $CD4^+$ T cells in *Sin1*$^{-/-}$ chimeric mice. Surprisingly, Sin1 deficiency did not affect IL-7R expression on resting peripheral T cells. We have previously shown that in developing progenitor B cells, the mTORC2-Akt-FoxO1 signaling negatively regulates IL-7R expression [13]. IL-7R expression is suppressed in antigen activated T cells. It is possible that the loss of mTORC2 function has no effect on IL-7R expression in resting T cells because these cells normally have a very low level of Akt signaling. Mammalian TORC2 may play a more important role in suppressing IL-7R expression after T cell activation since TCR signaling strongly induces the Akt signaling pathway.

We have also determined if Akt mediates the Sin1-mTORC2 signals to regulate the development of thymic nTreg cells by examining the nTreg development in *Akt1*$^{-/-}$, *Akt2*$^{-/-}$ and *Akt1*$^{-/-}$*Akt2*$^{-/-}$ mice. We had previously used a similar experimental approach to identity Akt2 as the specific mediator of mTORC2 dependent FoxO1 regulation in B cells [13]. Disruption of Akt1, Akt2 or both Akt1 and Akt2 did not alter the proportion of $CD4^+$ thymic nTreg cells when compared to wild type mice. Therefore, it is possible that either Akt3 is the principle mediator of mTORC2 dependent FoxO1 regulation or, alternatively, FoxO1 may be inhibited by the serum & glucocorticoid dependent kinases (SGK1-3) whose activity has also been shown to be regulated by mTORC2 [21].

Since mTOR is an evolutionarily conserved regulator of cellular growth and metabolism, we investigated if Sin1 deletion may affect the size of resting peripheral T cells or activated T cell blast cell growth and proliferation. Sin1 deficiency had no effect on resting T cell growth or activation induced blast cell growth. Furthermore, Sin1 deficiency did not impair antigen receptor/co-receptor dependent T cell proliferation *in vitro*. These results contrast with those reported in mice bearing a T cell specific *rictor* deletion which show a modest defect in activation induced T cell proliferation [12, 22]. It is possible that differences in the *in vitro* T cell stimulation conditions between our assays may account for the difference in experimental results since we stimulated our T cells in the presence of plate-bound anti-CD3 antibody plus soluble anti-CD28 in the presence of exogenous IL-2.

We also explored the function of Sin1 in $CD4^+$ T helper cell differentiation. We did not observe any deficiency in the ability of *Sin1*$^{-/-}$ $CD4^+$ T cells to differentiate into T_H1, T_H2 or T_H17 effector cells. These data also differ from the results reported in *rictor*$^{-/-}$ T cells from two different groups [12, 22]. Lee and colleagues reported that Rictor deficient $CD4^+$ T cells show impaired T_H1 and T_H2 differentiation while Delgoffe and colleagues only observed a deficiency in T_H2 differentiation in *rictor*$^{-/-}$ T cells. Our results argue that Sin1 and most likely mTORC2, are not essential for $CD4^+$ T_H effector cell differentiation. However it is possible that the disparity between our data and those observed in *rictor*$^{-/-}$ T cells could be partially due to differences in the *in vitro* experimental conditions used to induce T_H cell differentiation in the three studies. Alternatively it is possible that Rictor may influence T_H cell differentiation through a mechanism which is independent of mTORC2. Analysis of the roles Rictor and Sin1 in the context of a physiologic T cell immune response should resolve these issues.

Our observation that Sin1 deficiency in T cells results in an increased proportion of thymic Treg cells is consistent with previous studies linking mTOR and FoxO transcription factors to regulatory T cell differentiation. Surprisingly however, we observed that peripheral *Sin1*$^{-/-}$ $CD4^+$ T cells gave rise to fewer $FoxP3^+$ cells when stimulated in the presence of TGF-β. The unexpected finding that *Sin1*$^{-/-}$ T cells had slightly decreased TGF-β dependent Treg differentiation suggests that Sin1 may regulate Treg development independent of mTORC2 function. It is possible that Sin1 may regulate TGF-β dependent Treg differentiation through the MAPK signaling pathway [23]. In this regard, we have recently shown that deletion of MEKK2/3, which bind to and are negatively regulated by Sin1, augments TGF-β dependent Treg

differentiation [24]. Future investigations into the role of Sin1-MAPK signaling in T cells will help elucidate the mechanism underlying this phenotype.

Conflict of Interest Statement

The authors claim no conflict of interest involved in this study.

Acknowledgements

We thank Drs. A Lorenzo and W Sessa (Yale University) for the Akt1 and Akt2 knockout mice, KM Shokat (UCSF) for providing the pp242. This work is supported in part by grant AI063348 (NIH) (to BS). ASL is a Leukemia and Lymphoma Society fellow, and XC was a recipient of Gershon and Trudeau Fellowship from Yale University.

Figure Legend

Figure 1: Sin1 is not required for T cell development.

a) $CD4^+$ and $CD8^+$ cells in the thymus (upper panel) and spleen (lower panel) of $Sin1^{+/+}$ (WT) or $Sin1^{-/-}$ (KO) chimeric mice were assessed by flow cytometry.

b) Splenic $CD4^+$ T cells from $Sin1^{+/+}$ or $Sin1^{-/-}$ chimeric mice were stimulated with PMA and ionomycin for 4 hours in the presence of Golgi-Stop and assayed for IFN-γ, IL-17A, and IL-4 expression by flow cytometry.

c) The expression of CD62L and CD44 was measured on splenic $CD4^+$ T cells from $Sin1^{+/+}$ or $Sin1^{-/-}$ chimeric mice by flow cytometry.

d) The expression of IL-7R (CD127) on splenic $CD4^+$ T cells from $Sin1^{+/+}$ or $Sin1^{-/-}$ chimeric mice was determined by flow cytometry.

e) The thymic (upper panel) and splenic (lower panel) $CD4^+FoxP3^+$ T cells in $Sin1^{+/+}$ or $Sin1^{-/-}$ chimeric mice were determined by flow cytometry.

f) The thymic $CD4^+FoxP3^+$ T cells in a chimeric mouse containing an equal proportion of WT host cells ($CD45.1^+$) and $Sin1^{-/-}$ donor cells ($CD45.2^+$) were determined by flow cytometry. Numbers in the plots depict percentages of the gated populations. All flow cytometry plots shown are pre-gated on $CD45.2^+$ $CD4^+$ $CD8^-$ cells and are representative of $Sin1^{+/+}$ (n=3) and $Sin1^{-/-}$ (n=4) fetal liver chimeric mice.

Figure 2: Loss of Akt1 and Akt2 function does not alter thymic Treg development.
a) The thymic $CD4^+FoxP3^+$ T cells in wild type (WT), *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice was assessed by flow cytometry. Graphs show the mean proportion of thymic $CD4^+CD8^-$ $FoxP3^+$ T cells from all WT (n=5), *Akt1*$^{-/-}$ (n=6), and *Akt2*$^{-/-}$ (n=6) mice analyzed. The data shown are combined from three independent experiments.
b) Fetal livers from either *Akt1*$^{-/-}$*Akt2*$^{-/-}$ (dKO) mice (n=2) or wild type (WT) mice (n=4) were used to reconstitute lethally irradiated B6.SJL mice. Ten weeks after reconstitution, donor thymic $CD4^+CD8^-$ $FoxP3^+$ T cells in WT or *Akt1*$^{-/-}$*Akt2*$^{-/-}$ mice were measured by flow cytometry. The data are from two independent experiments. Each dot in the graph represents one individual mouse, and error bars are standard deviation.

Figure 3: Sin1 is not required for T cell growth and proliferation.
a) The relative size of resting lymph node $CD4^+$ and $CD8^+$ T cells from *Sin1*$^{-/-}$ (KO) chimeric mice was measured by forward light scatter (FSC). Each chimeric mouse also contained host WT T cells, which serve as individual internal controls. WT and KO cells were distinguished by differential CD45.1 expression. Error bars indicate standard mean error and p-values were derived by two-tailed paired t-test analysis.
b) Lymph node $CD4^+$ or $CD8^+$ T cells from 3 *Sin1*$^{-/-}$ (KO) chimeric mice were stimulated *in vitro* with anti-CD3+anti-CD28 antibodies for 48 hours and relative size was determined by measuring FSC. Representative histograms of resting (shaded) and stimulated host WT (black line) or *Sin1*$^{-/-}$ (blue line) T cells from one of the three mice analyzed is shown.
c) The relative cell size of activated WT or *Sin1*$^{-/-}$ $CD4^+$ and $CD8^+$ T cells. The data shown are the average of three mice described in **b**. Error bars indicate mean error and p-values were derived using two-tailed paired t-test analysis.
d) Purified *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 for 4 days and cell proliferation was measured by CFSE dilution. Histograms shown are pre-gated on $CD45.2^+$ donor derived cells and are representative of two independent experiments.

Figure 4: Sin1 is not required for $CD4^+$ T_H effector cell differentiation.

a) Purified $CD4^+$ T cells were stimulated with anti-CD3+anti-CD28 under indicated T_H polarizing conditions and analyzed by flow cytometry. Plots shown are representative of two independent experiments.
b) *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) $CD4^+$ T cells were stimulated with anti-CD3 antibody for 3 minutes and Akt S473 phosphorylation was measured by flow cytometry. Histograms shown are pre-gated on $CD45.2^+$ donor cells and are representative of two independent experiments.
c) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ $CD4^+$ T cells were stimulated with anti-CD3 for 0, 15 or 30 minutes and Akt T450 phosphorylation was measured by immunoblotting. Total ERK2 serves as a loading control.

Figure 5: Sin1 and mTOR differentially regulate TGF-β dependent Treg differentiation.
a) *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ $CD4^+$ T cells were stimulated *in vitro* with anti-CD3+anti-CD28 antibodies in the presence or absence of 1 ng/ml TGF-β. After four days FoxP3 expressing cells were enumerated by flow cytometry. Data shown are representative of two independent experiments.
b) *Sin1*$^{+/+}$ $CD4^+$ T cells were stimulated *in vitro* with anti-CD3+anti-CD28 antibodies plus 1 ng/ml TGF-β in the presence of rapamycin (Rapa. 30 nM), pp242 (100 nM) or carrier (ctrl.). The proportion of $FoxP3^+$ cells generated after four days was measured by flow cytometry. Data are representative of two independent experiments.
c) *Sin1*$^{+/+}$ $CD4^+$ T cells were activated with anti-CD3 in the presence of indicated inhibitors and assayed for induction of S6 p-S235/S236 and Akt p-S473 by flow cytometry. Data are representative of two independent experiments.

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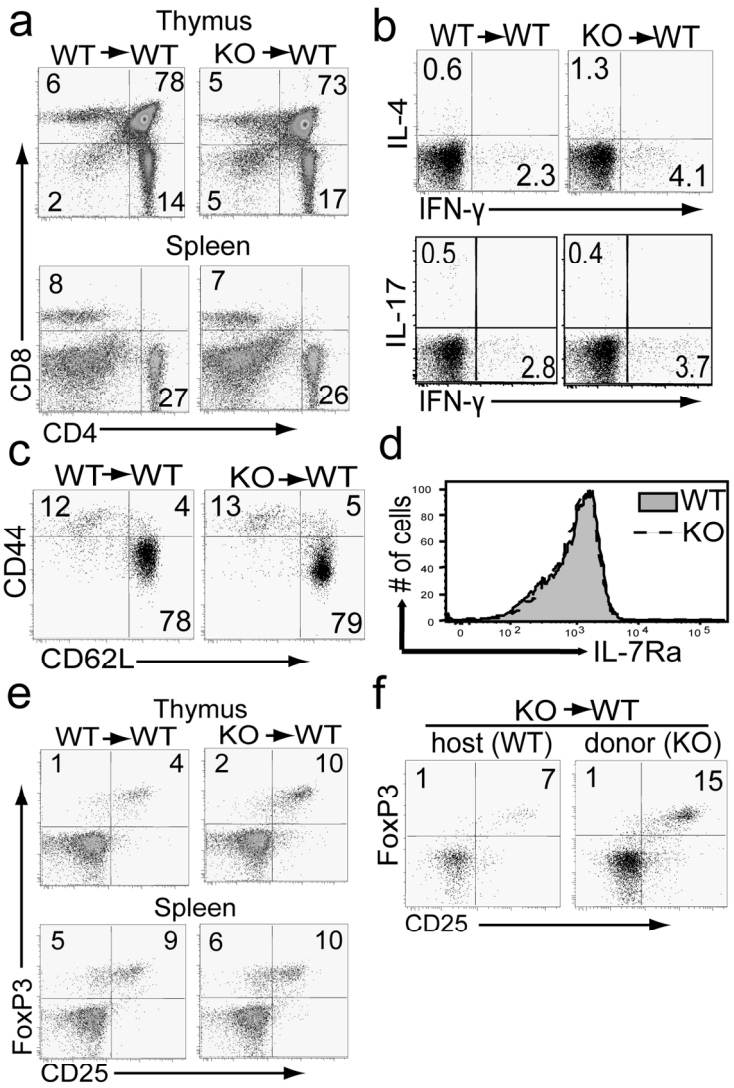

Figure 1

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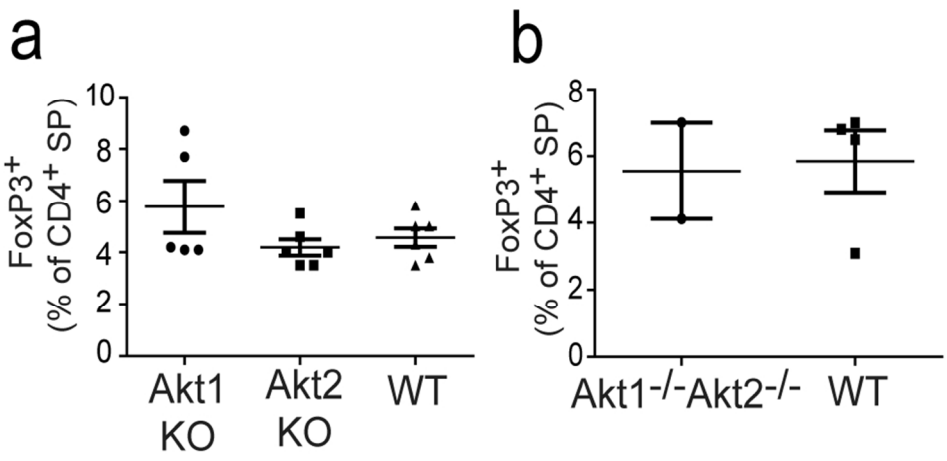

Figure 2

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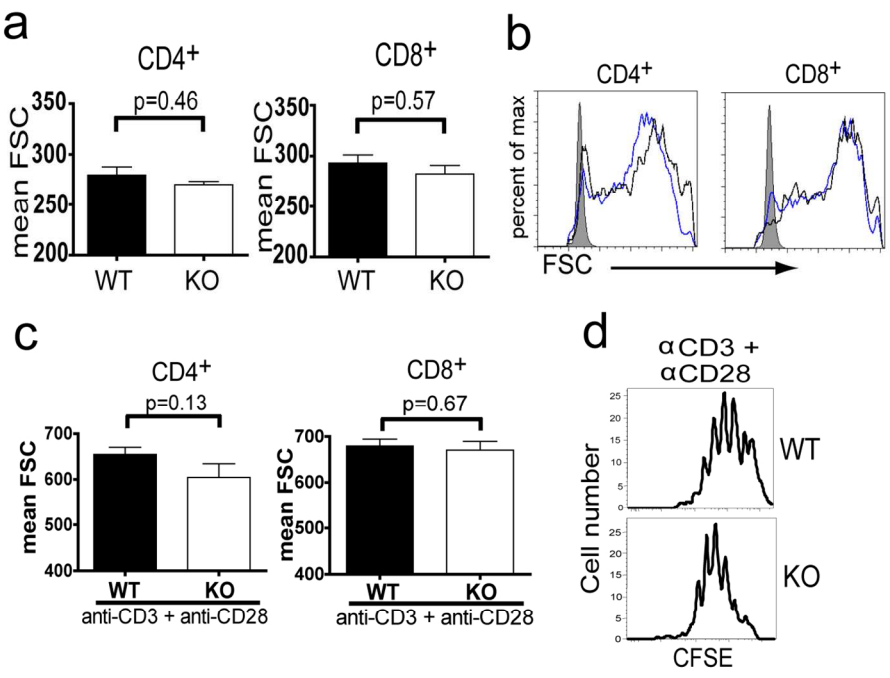

Figure 3

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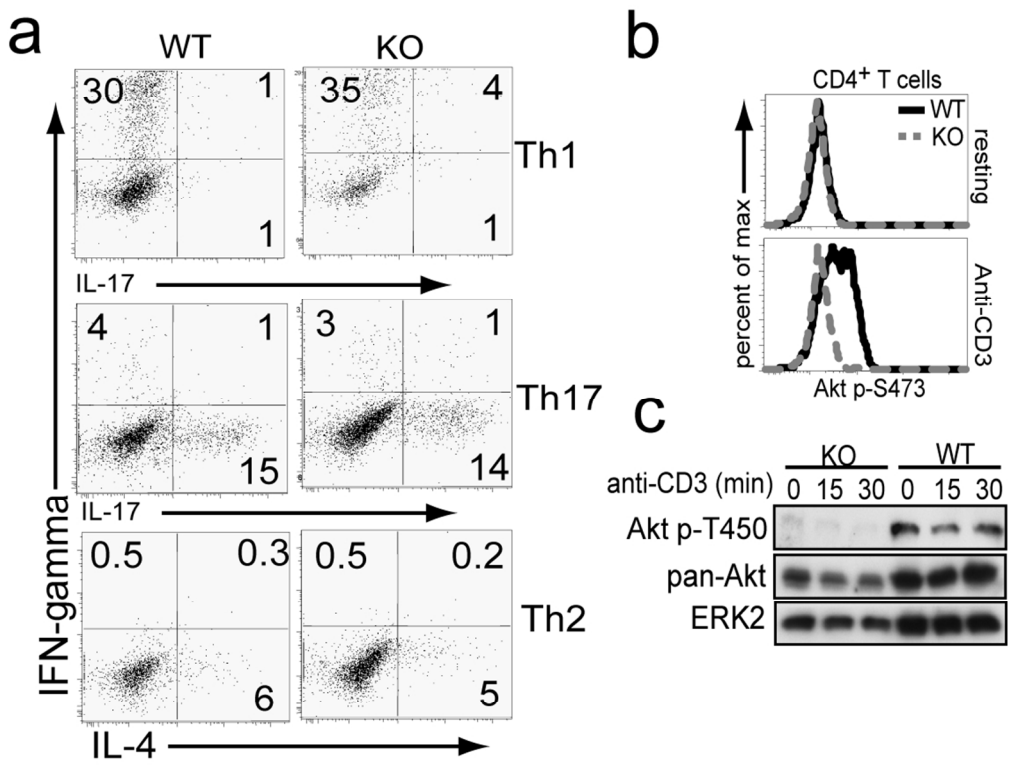

Figure 4

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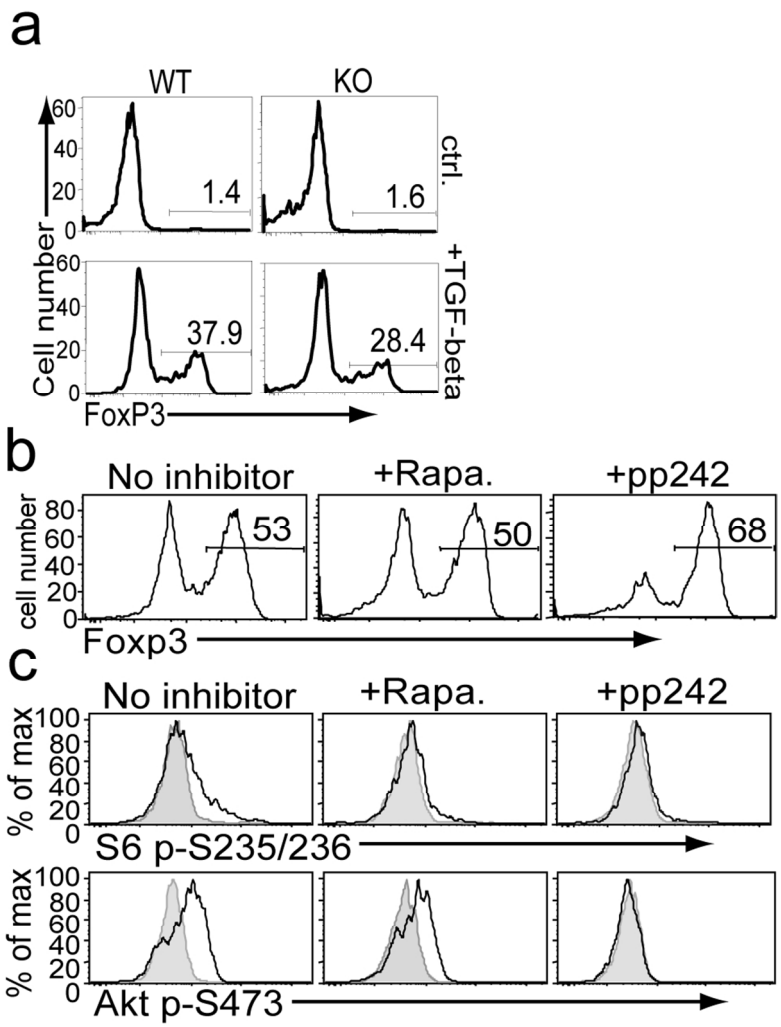

Figure 5

81x125mm (300 x 300 DPI)

Sin1/mTORC2 regulates c-Myc dependent B cell growth

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Running Title: Sin1 regulates B cell growth

Abstract

B cell clonal expansion, a process involves cell size increase and subsequent cell division, is essential for humoral immunity. It is well documented that the mechanistic target of rapamycin (mTOR) regulates B cell clonal expansion, however the precise molecular mechanism by which mTOR regulates this process is still largely unknown. Here, we show that Sin1, the essential component of the mTOR complex (mTORC)2, plays a critical role in antigen receptor mediated B cell growth regulation. Mature resting $Sin1^{-/-}$ B cells lack functional mTORC2 and exhibit smaller cell size with severely impaired blast cell growth and proliferation in response to mitogen stimulation. This Sin1/mTORC2 defect causes reduced Akt expression and diminished Akt dependent suppression of TSC1/2 and GSK3, resulting in an impaired mTORC1 activity and reduced c-Myc protein expression. Our study reveals that Sin1/mTORC2 integrates antigen receptor dependent pro-growth signals to coordinate the anabolic activities of mTORC1 and c-Myc in B cells.

Highlights

1) Sin1 regulates resting B cell size and mitogen dependent B cell growth.
2) Sin1 regulates ribosomal S6 expression through c-Myc in B cells.
3) Sin1 and mTOR regulate c-Myc stability through a GSK3 dependent mechanism.

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. 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 survival signals that is absolutely required for the growth and survival of mature B cells(2-4). The basal or tonic BCR signal activates the phosphoinisitol-3 kinase (PI3K) signaling cascade which is known to sustain mature B cell survival and growth(2). The PI3K-dependent inhibition of FoxO1 transcriptional activity is required for the pro-survival function of the tonic BCR signal but the molecular mechanism that governs resting B cell growth is still not fully defined(2).

When a resting B cell receives mitogenic stimuli via either the BCR or Toll-like receptor (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. 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(5, 6). This PI3K mediated growth supports the rapid proliferation of an mitogen stimulated B cell clone which is essential for the development of humoral immune response(5).

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase which is a key downstream effector of PI3K signaling and a master regulator of cellular growth and metabolism(7-9). Early studies show that inhibition of mTOR in B cells with the specific small molecule inhibitor rapamycin suppresses mitogen-dependent blast cell growth and inhibits cell proliferation by blocking cell cycle progression at the G1 phase(10, 11). These studies highlight the importance of mTOR in B cell mediated immune function. However, rapamycin is only a partial inhibitor of mTOR activity(12-14), so the full extent of mTOR function as well as the molecular mechanism by which the mTOR signaling is regulated in B cells remains to be fully studied. Extensive studies in the past several years demonstrate that mTOR associates with two functionally distinct multi-protein complexes called mTOR complex 1 (mTORC1), containing mTOR, raptor, mLST8 (GβL), PRAS40, and deptor, and mTORC2, containing mTOR, Rictor, Sin1, mLST8, deptor and PROTOR(15-22). Nutrients, growth factors, hormones, and energy signals activate mTORC1 which in

turn phosphorylates the translational regulators S6K and 4EBP1 leading to increased cellular protein synthesis and ribosome biogenesis(23). On the other hand, mTORC2 regulates activity of the AGC kinases PKB/Akt and SGK by phosphorylating the hydrophobic motif (HM; Ser473 of Akt1 and Ser422 of SGK1) in a PI3K-dependent manner(18, 24-26). Akt signaling promotes mTORC1 dependent cell growth by phosphorylating TSC2 which relieves the negative regulation of mTORC1 by the TSC1/TSC2 complex. Surprisingly however, mTORC2 has been shown to be completely dispensable for the Akt dependent activation of mTORC1(18, 19, 26-30). Rather, the mTORC2 dependent phosphorylation of Akt directs Akt substrate specificity towards the transcriptional regulators FoxO1 and FoxO3a(18). Additionally, mTORC2 also regulates the stability of Akt and conventional PKC in a PI3K-independent manner by phosphorylating the turn motif (Thr450 on Akt1; Thr641 on PKCβII)(14, 18, 25, 31). Unlike mTORC1, mTORC2 is less sensitive to rapamycin inhibition although chronic rapamycin treatment could inhibit mTORC2 in many types of normal and cancerous cells(13, 14).

The PI3K is an important upstream activator of mTOR(32). Signaling via the B cell receptor, cytokine receptors such as the BAFF/BlyS receptor and toll-like receptors such as TLR4 induces PI3K in B cells leading to the subsequent activation of downstream mTOR(33-35). PI3K generates phosphatidylinositol (3,4,5)-triphosphate (PIP3), which facilitates the membrane recruitment and activation of two key mediators of the PI3K signaling, PDK-1 and Akt(36, 37). Akt is activated by the PDK-1 mediated phosphorylation of Thr308 at the activation loop and the mTORC2 mediated phosphorylation of Ser473 at the HM(18, 25, 36). Once activated, Akt phosphorylates a wide range of substrates involved in the regulation of cell metabolism, growth, proliferation and survival. Akt promotes cell growth by phosphorylating and inactivating TSC2 and PRAS40, which are two important negative regulators of mTORC1(16, 17, 38-40). Akt regulates cell metabolism by phosphorylating and inactivating the glycogen synthase kinase 3 (GSK3) and controls cell survival and proliferation by inhibiting the activity of transcription factors FoxO1 and FoxO3a(41, 42). Remarkably, the disruption of mTORC2 in mouse embryonic fibroblasts, T cells, and pro-B cells does not impair the pro-growth and pro-survival activities of Akt even though Akt Ser473 phosphorylation was greatly diminished in these cells(18, 27-29, 43). Rather, the loss of Akt Ser473 phosphorylation alters the substrate specificity of Akt in these cells, specifically inhibiting the Akt-dependent phosphorylation of FoxO1 and FoxO3a but not TSC2 or GSK3(18).

We have previously shown that Sin1, an essential component of mTORC2, is required for bone marrow B cell development(29). The loss of Sin1 expression in progenitor B cells abolishes mTORC2-dependent Akt Ser473 phosphorylation and inhibits the Akt dependent phosphorylation of FoxO1. In this study, we explore the function of Sin1 and mTORC2 in peripheral B cell activation, development and survival in response to B cell mitogenic stimulation. Unexpectedly, we find that Sin1/mTORC2 plays a specific and essential role in regulating the growth and proliferation of immature and mature B cells. Quiescent, resting peripheral B cells with Sin1 deficiency are significantly smaller than wild type B cells. Upon mitogen stimulation, Sin1-deficient B cells exhibit impaired blast cell growth and proliferation. We identify the molecular mediators of Sin1-dependent B cell growth and show that Sin1, via mTORC2, regulates mTORC1 and c-Myc-dependent cell growth through an Akt-dependent mechanism. Together our data reveal that mTORC2 plays a key role in regulating B cell immunity by directly controlling the pro-growth and metabolic pathways governed by c-Myc and mTORC1.

Results

Sin1 regulates the development and growth of B cells.

Although it is known that Sin1 plays an essential role in mTOR signaling, precisely how it modulates the mTOR mediated cellular function has not been fully studied. To investigate the *in vivo* role of Sin1 in mTOR-mediated cell growth control, we generated mice that lacked Sin1 in the hematopoietic system by transplanting *Sin1*$^{-/-}$ fetal liver hematopoietic stem cells (HSC) into irradiated wild type mice as previously described(29). The recipient host mice were sub-lethally radiated in order to preserve a portion of the host hematopoietic stem cells that could gave rise to a population of wild type B cells in each recipient mice after adoptive transfer of the fetal liver HSCs.

Analysis of the *Sin1*$^{-/-}$ chimeric mice revealed that these mice had both CD45.1^{+} host wild type and CD45.1^{-} *Sin1*$^{-/-}$ donor derived splenocytes. Importantly, these mice contained populations of both host and donor B220^{+} B cells. Analysis of the CD45.1^{-}B220^{+} splenic *Sin1*$^{-/-}$ B cells revealed a significant reduction in the proportion of IgM^{hi}IgD^{low} immature/transitional B cells when compared to the CD45.1+B220+ host wild type B cells (**Fig. 1a**). Furthermore, we observed a severe reduction in number of CD21^{hi}CD23$^{int/low}$ marginal zone B cells in the *Sin1*$^{-/-}$ CD45.1^{-}B220^{+} B cells relative to the host CD45.1^{+}B220^{+} B cells (**Fig. 1b**). Despite the lack of MZ B cells in the spleens of *Sin1*$^{-/-}$ chimeric mice, we found that CD21^{int}CD23^{hi} mature follicular B cells were present in the spleen and lymph

nodes of *Sin1*$^{-/-}$ chimeric mice similar to that in the *Sin1*$^{+/+}$ chimeric mice (**Fig. 1b**). These data indicate that Sin1 is required for peripheral B cell development.

In order to explore the role of Sin1 in B cell growth we asked if Sin1 is required for the mitogen-stimulated growth of B cells. Freshly isolated splenic B cells from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ fetal liver HSC chimeric mice were stimulated *in vitro* with lipopolysaccaride (LPS) for 4 days and the relative size of the B cells was measured by FSC. We observed that the mean FSC of donor *Sin1*$^{+/+}$ B cells and wild type host B cells increased dramatically upon LPS stimulation, however this increase in FSC was severely impaired in LPS stimulated *Sin1*$^{-/-}$ B cells (**Fig. 1c**). Consistently, the mean FSC of *Sin1*$^{+/+}$ B cells was significantly greater than that of the *Sin1*$^{-/-}$ B cells under both the unstimulated and LPS stimulated conditions (**Fig. 1d**).

Next we enriched *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ splenic B cells from fetal liver HSC chimeric mice, activated these cells for 20 hours *in vitro* with anti-IgM antibody or LPS, and compared the relative cell size of the stimulated cells to that of non-stimulated resting cells by FSC. We found that anti-IgM or LPS stimulation induced the growth of *Sin1*$^{+/+}$ as well as *Sin1*$^{-/-}$ B cells relative to resting cells but the growth of *Sin1*$^{-/-}$ B cells was impaired as compared to the stimulated *Sin1*$^{+/+}$ B cells (**Fig. 2a**). We calculated the size of resting and mitogen stimulated *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ B cells using flow cytometry size standards and found that the mean cell size of anti-IgM or LPS stimulated *Sin1*$^{-/-}$ B cell blasts was significantly smaller than that of anti-IgM or LPS *Sin1*$^{+/+}$ B cell blasts, respectively (**Fig. 2b**). Additionally, the size of the resting *Sin1*$^{-/-}$ B cells was also significantly smaller than that of the resting *Sin1*$^{+/+}$ B cells (**Fig. 2b**).

To determine if Sin1 is required for mitogen-dependent B cell proliferation, splenic *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ B cells were labeled with CFSE and cultured for 72 hours in vitro with medium only, or treated with an anti-IgM antibody or with LPS, and cell proliferation was measured by CFSE dilution. Stimulation of *Sin1*$^{+/+}$ B cells with either anti-IgM antibody or LPS induced cell proliferation, however neither anti-IgM nor LPS induced the proliferation of *Sin1*$^{-/-}$ B cells (**Fig. 2c**). Furthermore, we found that the number of viable *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ B cells after 20 hrs *in vitro* culture with medium only, or stimulated with an anti-IgM antibody was significantly reduced when compared to that of *Sin1*$^{+/+}$ B cells (**Fig. 2d**). Together, these data show that Sin1 is required for the blast cell growth and proliferation of anti-IgM and LPS stimulated B cells and that Sin1 is required for the survival of resting and anti-IgM stimulated B cells.

The smaller size of *Sin1*$^{-/-}$ B cells raised the possibility that these cells may be less fit and consequently exhibit reduced survival and proliferative capacity when compared to the *Sin1*$^{+/+}$ B cells. Indeed, we found that under the unstimulated condition, the ratio of donor *Sin1*$^{+/+}$ to wild type host B cells was maintained after 4 days of in vitro culture, whereas the ratio of donor *Sin1*$^{-/-}$ B cells to host wild type B cells was significantly reduced indicating a severe defect in the survival of *Sin1*$^{-/-}$ B cells (**Fig. 2e**). Together, these data show that Sin1 is required for the growth and survival of resting splenic B cells and suggest that Sin1 may also be required for the growth of mitogen stimulated B cells.

Sin1 regulates resting B cell size.

We consistently observed that unstimulated Sin1 deficient B cells showed a smaller cell size than their wild type counterparts in our in vitro culture assays (**Figs. 1d & 2b**). These data suggest that Sin1 may regulate resting B cell size *in vivo*. We compared the relative size, as measured by forward light scatter (FSC), of the fetal liver-derived splenic B cells we found that the mean FSC of the *Sin1*$^{-/-}$ B cells was significantly less than that of the corresponding wild type host B cells (**Fig. 3a**). Additionally, the mean FSC of the fetal liver HSC derived *Sin1*$^{-/-}$ B cells was significantly less than that of the fetal liver HSC derived *Sin1*$^{+/+}$ B cells (**Fig. 3a**) suggesting that Sin1 may play an important role in regulation of B cell size.

Since mature *Sin1*$^{-/-}$ B cells lack Sin1 expression throughout their development, it is possible that the size deficiency in the mature resting *Sin1*$^{-/-}$ B cells may have originated at an earlier stage of B cell development. Therefore, we generated IL-7/OP9 stromal cell dependent pro-B cells from *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ fetal liver HSC as previously described in order to explore the role of Sin1 in the regulation of cell growth during B cell development(29). We differentiated the *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells in vitro and measured the relative size (FSC) of pre-B (IgM^-IgD^-), immature B (IgM^+IgD^-), and transitional B (IgM^+IgD^+) cells that were developed in these cultures by flow cytometry. We found that there was no significant difference in the mean FSC of *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pre-B cells (**Fig. 3b**). However, the mean FSC of *Sin1*$^{-/-}$ immature and *Sin1*$^{-/-}$ transitional B cells was significantly reduced as compared to the corresponding populations of *Sin1*$^{+/+}$ B cells. We also noted that the difference in mean FSC between *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ B cells was greatest at the transitional stage of B cell development (**Fig. 3b**).

Next, we asked if *Sin1*$^{-/-}$ pro-B cell lines would give rise to smaller B cells *in vivo*. We transplanted 5x10^6 CD45.2$^+$ *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells into sub-lethally irradiated wild type CD45.1$^+$ congenic mice. The recipient mice were sacrificed seven days later and the size of the splenic IgM$^+$ B cells that were originated from the transplanted pro-B cells was determined by flow cytometry. We found that both *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ pro-B cells give rise to a population of splenic CD45.2$^+$B220$^+$ B cells, however the pro-B cells derived *Sin1*$^{-/-}$ splenic B cells showed reduced cell size when compared to the pro-B cells derived *Sin1*$^{+/+}$ splenic B cells (**Fig. 3c**). We also observed that the mice which received *Sin1*$^{-/-}$ pro-B cells had fewer donor-derived splenic B cells than the mice that received *Sin1*$^{+/+}$ pro-B cells (**Fig. 3c**), consistent with our previous study showing that Sin1 is required for B cell development(29). Surface IgM expression was equivalent on the CD45.2$^+$B220$^+$ *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ splenic B cells thereby ruling out the possibility that the impaired growth and development of *Sin1*$^{-/-}$ B cells is due to deficient BCR expression. These data show that the *Sin1*$^{-/-}$ pro-B cells can give rise to IgM$^+$ splenic B cells which exhibit a cell growth deficiency *in vivo*.

Sin1 is required for BCR-dependent Akt signaling in B cells

Our data demonstrate that Sin1 regulates the size of resting IgM$^+$ B cells and is also required for the B cell mitogen dependent blast cell growth, proliferation and survival. The AGC kinase Akt is a well-known Sin1/mTORC2 target that is activated by B cell mitogenic signals to regulates cell growth, proliferation and survival (__). Therefore we asked if Sin1 mediates the BCR-dependent Akt signaling. We have previously shown that Sin1/mTORC2 regulates Akt protein stability(14). Therefore we measured the expression level of Akt by immunoblotting and intracellular staining in IgM$^+$ *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ B cells and found that the Akt expression level was significantly lower in *Sin1*$^{-/-}$ B cells as compared to that of *Sin1*$^{+/+}$ B cells (**Fig. 4a**). Next, we examined the role of Sin1 in mediating BCR dependent Akt signaling. *In vitro* differentiated *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ IgM$^+$ B cells were stimulated with an anti-IgM antibody for 3 minutes and Akt phosphorylation at Thr308 and Ser473 was measured by intracellular staining and flow cytometry. Anti-IgM stimulation induced Akt Ser473 phosphorylation in *Sin1*$^{+/+}$ B cells but not in the *Sin1*$^{-/-}$ B cells (**Fig. 4b**), indicating that the BCR-dependent Akt Ser473 phosphorylation is Sin1/mTORC2 dependent. Akt Thr308 phosphorylation was induced by anti-IgM stimulation in *Sin1*$^{-/-}$ B cells, however the level of Akt Thr308 phosphorylation was also reduced when compared to that of *Sin1*$^{+/+}$ B cells (**Fig. 4b**). These data indicate that the Akt signaling may be functionally impaired in *Sin1*$^{-/-}$ B cells due to the combination of reduced Akt expression and impaired Akt phosphorylation.

Akt regulates the activity of multiple substrates that may influence cell growth and metabolism (__). Therefore, we examined the phosphorylation of two important mediators of cell growth and metabolism that are downstream of Akt, glycogen synthase kinase (GSK3) and the ribosomal protein S6. We stimulated IgM$^+$ *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ B cells for 15 minutes with anti-IgM antibody and the phosphorylation GSK3α, GSK3β and S6 were assessed. BCR stimulation readily induced the phosphorylation of GSK3α Ser12, GSK3β Ser9 and S6 Ser235/236 in *Sin1*$^{+/+}$ B cells, however these phosphorylation events were impaired in *Sin1*$^{-/-}$ B cells (**Fig. 4c**). Interestingly, we noticed that the expression of S6 protein was decreased in both unstimulated and anti-IgM stimulated *Sin1*$^{-/-}$ B cells when compared to *Sin1*$^{+/+}$ B cells (**Fig. 4c**). This observation was confirmed by S6 intracellular staining and flow cytometry that showed S6 protein levels were lower in *Sin1*$^{-/-}$ relative to *Sin1*$^{+/+}$ B cells (**Fig. 4d**). Cell surface expression of IgM was equivalent between *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ B cells, ruling out the possibility that differences in BCR expression contribute to defects in Akt signaling observed in *Sin1*$^{-/-}$ B cells (**Fig. 4e**). These data show that Sin1 is mediates BCR dependent Akt signaling and indicate that Sin1 is required for the proper expression of ribosomal protein S6 in B cells.

Sin1 regulates c-Myc expression in B cells.

Our observation that Sin1 deficiency results in decreased B cell size and reduced S6 protein expression raised that possibility that Sin1 the transcription factor c-Myc, a master regulator of B cell growth and proliferation(44). Since Sin1/mTORC2 is an important mediator of PI3K signals, we asked if c-Myc might also be a target through which Sin1/mTORC2 regulates B cell growth. We first examined c-Myc protein expression and found that its level was reduced in immature and transitional *Sin1*$^{-/-}$ B cells as compared to that of *Sin1*$^{+/+}$ immature/transitional B cells (**Fig. 5a**). To identify the origin of the c-Myc expression deficiency in *Sin1*$^{-/-}$ B cells, we measured *c-myc* transcript levels in unstimulated IgM$^+$ *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ B cells and found that the level of *c-myc* mRNA was comparable in the *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ IgM$^+$ B cells (**Fig. 5b**). Furthermore, the induction of *c-myc* mRNA expression by anti-IgM antibody stimulation in *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ splenic B cells was also similar indicating that Sin1 was not required for *c-myc* mRNA expression after stimulation through the BCR (**Fig. 5c**). However, when we examined c-Myc protein levels in *in vitro*-differentiated IgM$^+$ B cells stimulated with anti-IgM antibody we found that c-Myc protein expression increased in *Sin1*$^{+/+}$ but not *Sin1*$^{-/-}$ B cells (**Fig. 5d**). These data suggest that Sin1 regulates the expression of c-Myc protein in

resting B cells and BCR stimulation-induced B cells at the level of protein translation and/or protein degradation.

C-Myc promotes cellular growth by directly regulating the expression genes involved in ribosome biogenesis(45). Since *Sin1*$^{-/-}$ B cells showed a reduced expression of ribosomal protein S6 (**Fig. 4c & 4d**), we speculated that Sin1 may regulate S6 expression and B cell growth through c-Myc. To test this possibility, we ectopically expressed a human *c-myc* cDNA in *Sin1*$^{-/-}$ B cells and determined its effect on S6 expression and cell growth. The ectopic expression of c-Myc in *Sin1*$^{-/-}$ pro-B cells significantly increased S6 expression and the cell size compared to control infected *Sin1*$^{-/-}$ pro-B cells (**Fig. 5e**). Next, we differentiated the c-Myc transduced pro-B cells *in vitro* for 7 days and asked if ectopic c-Myc expression could rescue the cell size deficiency of *Sin1*$^{-/-}$ transitional B cells. We observed that ectopic c-Myc expression increased the cell size of *Sin1*$^{-/-}$ transitional B cells when compared to empty vector infected *Sin1*$^{-/-}$ transitional B cells (**Fig. 5f**). Furthermore, we found that c-Myc rescued the expression of ribosomal protein S6 in *Sin1*$^{-/-}$ transitional B cells. Overall, these data show that Sin1 regulates B cell size and the expression of ribosomal protein S6 through a c-Myc dependent mechanism.

Sin1 regulates c-Myc protein stability.

To understand the molecular mechanism through which Sin1 regulates c-Myc expression in B cells, we cultured splenic B cells in vitro for 60 minutes with medium alone or with an anti-IgM antibody in the presence of vehicle alone, or the mTORC1 inhibitor rapamycin, or the dual mTORC1/mTORC2 inhibitor pp242. The expression of c-Myc was measured at the end of the 60 minute time point. BCR stimulation led to an up-regulation of c-Myc protein expression, which was inhibited by either rapamycin or pp242 (**Fig. 6a**). 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.

To further explore the role of mTOR in the BCR dependent induction of *c-myc* gene expression, we stimulated wild type splenic B cells with anti-IgM antibody in the presence of vehicle alone, or with rapamycin, or pp242 and measured the abundance of *c-myc* transcripts. We found that neither rapamycin nor pp242 inhibits the up-regulation of *c-myc* mRNA expression in BCR stimulated B cells (**Fig. 6b**). These data suggest that mTOR may regulate c-Myc expression by influencing c-Myc translation and/or c-Myc stability. The Akt target GSK3 has been shown to phosphorylate c-Myc

to promote the proteosomal degradation of c-Myc protein(46). Since Akt inhibits GSK3 and Akt-dependent phosphorylation of GSK3 is impaired in anti-IgM stimulated B cells which lack mTORC2 function (**Fig. 4c & 6a**), we asked if mTORC2 may regulate c-Myc protein degradation. We stimulated wild type splenic B cells with anti-IgM antibody for 2 hours to induce c-Myc protein expression and then treated the stimulated cells for 1 additional hour with cyclohexamide (CHX) alone, CHX plus pp242, or CHX plus pp242 plus the GSK3 inhibitor SB216763 (**Fig. 6c**). As expected, CHX treatment alone reduced c-Myc expression in the anti-IgM stimulated B cells when compared to the stimulated B cells without CHX treatment (**Fig. 6d**). The addition of pp242 to the CHX treated B cells resulted in a further decrease in c-Myc expression when compared to B cells treated with CHX alone indicating that mTOR activity also protects c-Myc protein from degradation. Importantly, SB216763 treatment blocked this pp242 dependent reduction in c-Myc expression in the CHX treated B cells suggesting that the GSK3 activity, which was inhibited by SB216763, may promote c-Myc degradation (**Fig. 6d**). These data suggest that mTOR may promote c-Myc protein stability through inhibiting GSK3 activity.

To further test this possibility, we stimulated wild type splenic B cells for 2 hours with anti-IgM antibody and then treated these cells with CHX, CHX+pp242 or CHX+pp242+SB216763 and measured the c-Myc protein level after 0, 10, 20, 40 or 60 minutes by intracellular staining and flow cytometry. We found that the CHX+pp242 treatment increased the rate of c-Myc degradation relative to CHX treatment alone at the 40 and 60 minute time points. Importantly, treatment with SB216763 completely blocked this pp242 dependent c-Myc degradation (**Fig. 6e**). Interestingly, pp242 and SB216763 had no effect on the rate of c-Myc protein degradation at the 10 and 20-minute time points suggesting that the rate of c-Myc degradation is mTOR and GSK3 independent at the early time points.

Finally, we asked if mTOR regulates the GSK3-dependent phosphorylation of c-Myc. Wild type splenic B cells were first stimulated for 1 hour with an anti-IgM antibody. The stimulated B cells were cultured for an additional hour with vehicle only, or with pp242, or pp242+SB216763. The pp242 treatment resulted in an increase in c-Myc phosphorylation at the GSK3 target site Thr58/Ser62 and consequently a decrease in total c-Myc expression as compared to the vehicle treated cells (**Fig. 6f**). The addition of SB216763 to the pp242 treated B cells strongly inhibited c-Myc phosphorylation at Thr58/Ser62. Consistently, the c-Myc expression was elevated to a level greater than either the

vehicle or pp242 treated B cells. These data show that Sin1/mTORC2 controls c-Myc protein stability by negatively regulating the GSK3 mediated phosphorylation of c-Myc.

Discussion

Mammalian TORC2 is a central regulator of the Akt signaling in response to growth factors but its role in cell size control has not been established in contrast to the well-characterized roles of mTORC1 in cell growth control(18, 19, 27). Mammalian TOR is a key regulator of B cell growth and immune function(10, 11, 34) yet the molecular mechanisms through which mTOR regulates B cell development and immunity remain largely unexplored. In this study we show that Sin1, an essential component of mTORC2, regulates the growth and cell size of resting and mitogen activated B cells. Sin1 promotes peripheral B cell survival and is required for the proliferation of mature B cells in response to BCR and LPS stimulation. We also show that *Sin1*$^{-/-}$ immature and transitional B cells differentiated in vitro or in vivo exhibit reduced cell size compared to wild type B cells. Mammalian TORC2 is the likely mediator through which Sin1 regulates B cell size growth. Consistently, Rictor deficient B cells, which have disrupted mTORC2 function, also exhibit similar deficiencies as *Sin1*$^{-/-}$ B cells. We further show that Sin1/mTORC2 mediates the PI3K dependent BCR signaling and promotes B cell growth by activating Akt. The mTORC2-Akt signaling axis activates mTORC1 and promotes c-Myc expression by suppressing the activity of TSC2 and GSK3 respectively. Based on our data, we present a model illustrated in **Supplemental Fig. 3** that outlines the mechanism through which Sin1 regulates B cell growth.

Interestingly, the Sin1/mTORC2 mediated B cell growth regulation is lineage specific and developmental stage dependent. We and others have previously shown that the mTORC1 dependent fibroblast cell growth is not dependent on mTORC2 nor is mTORC2 required to maintain the cell size of embryonic fibroblasts, T cells and leukemic pre-B cells(19, 26-28, 30, 47). In this study, we show that Sin1 is not required to maintain the cell size of proliferating IL-7 dependent pro-B cells nor is Sin1 required to maintain the cell size of quiescent small pre-B cells. However, Sin1 is required for cell size increase of immature and mature B cells. These results show that Sin1/mTORC2 plays a selective but important role in B cell growth regulation once a developing B cells has acquired surface BCR expression, and suggest that mTORC2 is an important mediator of BCR dependent growth signaling. Consistently, mature Sin1 deficient B cells show decreased blast cell growth when activated

through the BCR and immature *Sin1*$^{-/-}$ B cells show reduced mTORC1 dependent S6 phosphorylation after BCR stimulation.

Our data also reveal that Akt signaling is particularly dependent on mTORC2 and/or mTORC2 mediated Akt phosphorylation in immature and mature B cells. BCR stimulation fails to induce Akt Ser473 phosphorylation in Sin1 deficient B cells which is consistent with mTORC2's known role as the principle kinase that phosphorylates the Akt hydrophobic motif(18, 19, 25). However, unlike other mTORC2 deficient cell types such as fibroblasts and T cells, immature and mature Sin1 deficient B cells exhibit a greater reduction in Akt signaling that is characterized by reduced Akt expression and reduced Akt Thr308 phosphorylation when compared to wild type B cells(18, 19, 27, 28, 47). It is likely that this impaired Akt signaling contributes to the reduction in cell size and reduced blast cell growth observed *Sin1*$^{-/-}$ B cells. In this regard, *Akt1*$^{-/-}$*Akt2*$^{-/-}$ B cells also exhibit a reduction in B cell size, and show reduced mTORC1 dependent S6 phosphorylation and reduced S6 expression. The relative reduction of Akt Thr308 phosphorylation in *Sin1*$^{-/-}$ B cells is remarkable since we and others have shown that neither mTORC2 nor Akt Ser473 phosphorylation is required for the phosphorylation of Akt at T308 in MEF cells, T cells, or leukemic pre-B cells(18, 19, 26-30). This may explain why in mTORC2 deficient B cells, the Akt signaling is significantly impaired leading to decreased phosphorylation of Akt substrates TSC2 and GSK3. Consequently, the mTORC1-S6K dependent S6 phosphorylation was diminished due to the inability of Akt to suppress TSC2 in *Sin1*$^{-/-}$ B cells. Additionally, we show that mTOR regulates c-Myc expression in B cells by influencing c-Myc protein stability through an mTORC2-Akt-GSK3 dependent mechanism. Therefore mTORC2 directly regulates B cell growth by integrating the homeostatic and mitogenic growth signals to coordinate the anabolic activities of mTORC1 and c-Myc.

The remarkable reduction in Akt signaling observed in *Sin1*$^{-/-}$ B cells may be due, in part, to the reduced expression of Akt in mTORC2 deficient B cells. Akt protein stability is regulated by mTORC2(14, 31). 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(14, 31, 48). Indeed, we observe that total Akt expression is reduced approximately 3 fold in *Sin1*$^{-/-}$ IgM^{+} B cells relative to *Sin1*$^{+/+}$ IgM^{+} B cells. Additionally, mTORC2 dependent Akt Ser473 phosphorylation may also directly facilitate Akt phosphorylation at Thr308 in immature and mature B cells. The Akt hydrophobic motif phosphorylation stabilizes the C-terminal tail of Akt and creates a docking site for PDK-1, called the

PDK-1 interacting fragment (PIF) pocket(49). It is possible that, in B cells, the mTORC2 dependent Akt HM phosphorylation may facilitate Akt Thr308 phosphorylation though this PIF pocket dependent mechanism(50-52). Our data indicate that immature and mature B cells are exquisitely sensitive to the relative strength of Akt signaling and we propose that mTORC2 plays a central role in tuning the activity of Akt by integrating PI3K signals from diverse sources which include but are not limited to the BCR.

Sin1 seems to be absolutely required for the development of splenic marginal zone (MZ) B cells and peritoneal B1a B cells as seen in the *Sin1*$^{-/-}$ fetal liver HSC cell chimeric mice. PI3K dependent signaling has been implicated as a key regulator of MZ and B1 B cell development(33). B cell progenitors from mice lacking CD19 exhibit impaired PI3K signaling and defective MZ B cell development(53, 54). Therefore, our data suggest that Sin1/mTORC2 may be the principal mediator of this PI3K signal in MZ and B1 B cell development. Based on our results, the Sin1/mTORC2 signals in MZ and B1 B cell development are likely to be further transduced by the AGC kinase Akt. Consistently, B cells lacking both Akt1 and Akt2 were also found to fail to give rise to the MZ B cells (55) further supporting a role for the PI3K-Sin1-mTORC2-Akt signaling axis in the regulation of MZ B cell development.

The Sin1/mTORC2 signaling may promote MZ B cell development by regulating the Akt dependent phosphorylation and inactivation of FoxO1. FoxO1 deletion in B cells promotes the development of MZ B cells and is sufficient to restore MZ B cell development in CD19 deficient mice(56). We showed previously that the Sin1-mTORC2 dependent Akt Ser473 phosphorylation directs Akt substrate specificity towards FoxO1, which inhibits FoxO1 transcriptional activity in B cells(29). FoxO1 has emerged as a key regulator of B cell development and immune function(56-59). Since the mTORC2-Akt signaling axis integrates mitogen and growth factor signals to regulate FoxO1 activity, future studies that provide insight into the molecular mechanism of mTORC2 regulation will shed light on how B cell development and immunity is coordinated with cellular metabolism.

Acknowledgements

We would like to thank Kevan M. Shokat (UCSF) for generously providing the pp242. This work is supported in part by grant PR093728 (DoD; to B.S.). A.S.L. is a recipient of Brown-Cox Fellowship from Yale University and of a Leukemia & Lymphoma Society fellowship.

Author Contributions

A.S.L. designed and performed experiments, analyzed data and wrote the paper, O.A., X.X, and D.L performed experiments, F.T. and P.Z. provided experimental materials, B.S. designed experiments, analyzed data and wrote the paper.

Figure Legend

Figure 1: Sin1 is required for peripheral B cell development.

a) Total splenocytes from a *Sin1*$^{-/-}$ fetal liver HSC chimeric mouse were stained with the indicated antibodies and analyzed by flow cytometry. Host wild type (WT) B cells (CD45.1^{+}B220^{+}) and donor *Sin1*$^{-/-}$ (KO) B cells (CD45.1^{-}B220^{+}) were gated and analyzed for IgM and IgD expression. The proportion of host and donor IgM^{hi}IgD^{low}, IgM^{hi}IgD^{hi} and IgM^{low}IgD^{hi} splenic B cells is indicated next to each respective quadrant. The data shown are representative of three individual *Sin1*$^{-/-}$ fetal liver HSC chimeric mice.

b) The proportion of WT and KO marginal zone B cells (CD21^{high}CD23^{low}; rectangular gate) and follicular B cells (CD21^{int}CD23^{hi}; circular gate) in the spleen of the *Sin1*$^{-/-}$ chimeric mouse described in **a** are indicated next to the corresponding FACS gates. The plots shown are pre-gated on B220^{+}CD45.1^{+} host wild type (WT) or B220^{+}CD45.1^{-} donor *Sin1*$^{-/-}$ (KO) B cells and are representative of three *Sin1*$^{-/-}$ fetal liver HSC chimeric mice.

c) Total splenocytes from *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) fetal liver HSC chimeric mice were cultured in vitro with medium alone (unstimulated) or with 10 μg/ml LPS (+LPS) for 4 days. The relative size of the WT CD45.1^{+} (host) B cells and CD45.1^{-} (donor) WT or KO B cells was measured by FSC. The plots show freshly isolated splenic B cells (day 0) and splenic B cells cultured in vitro for 4 days with or without LPS. All of the plots shown are pre-gated on live, B220^{+} B cells and the numbers adjacent to each gate indicate the percent of B cells within the gate.

d) Bar graphs show the mean FSC value of unstimulated or LPS-stimulated donor-derived (CD45.1^{-}) B cells cultured in vitro for 4 days as described in panel **c**. The data shown are the average of WT (n=2) or KO (n=3) fetal liver HSC-chimeric mice and the error bars indicate standard deviation.

Figure 2: Sin1 regulates mitogen dependent B cell growth.

a) Purified *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) splenic B cells from fetal liver chimeric HSC mice were cultured in vitro with medium alone, 10 μg/ml anti-IgM F(ab')$_2$ or 10 μg/ml LPS for 21 hours and relative B cell size was measured by forward light scatter (FSC). Unstimulated cells are indicated by the shaded lines; stimulated WT cells are indicated by the grey line and stimulated KO cells are indicated by the black line. The results shown are representative of three independent experiments.
b) The bar graph shows the mean cell diameter of *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) splenic B cells cultured for 21 hours in vitro as described in panel **a**. Cell diameter was calculated from mean FSC using microbead size standards that were mixed and run with each sample of cells. The data shown are the mean of n=3 WT and n=3 KO independent cell samples. The error bars show the standard error and the data shown are from one of two independent experiments. The p-value was determined by a 2-tailed unpaired t-test.
c) WT or KO splenic B cells were obtained from fetal liver HSC chimeric mice. Cells were labeled with CFSE and cultured in vitro with medium only or with 10 μg/ml anti-IgM F(ab')$_2$ or with 10 μg/ml LPS for 72 hours. CFSE dilution in unstimulated B cells (shaded) and stimulated B cells (line) was measured by flow cytometry. The data shown are from pre-gated live, B220$^+$ B cells and are representative of n=3 WT and n=4 KO chimeric mice.
d) The cell viability of WT and KO B cells from panel **a** was determined by flow cytometry using 7-AAD dye exclusion. The bar graph shows the average percentage of live (B220$^+$7-AAD$^-$) B cells from n=3 WT and n=2 KO mice. The error bars indicate standard deviation and the p-values were determined by two-tailed unpaired t-test.

Figure 3: Sin1 regulates resting B cell size.
a) The relative size of splenic B cells from *Sin1*$^{+/+}$ (WT-->WT) or *Sin1*$^{-/-}$ (KO-->WT) fetal liver HSC-chimeric mice was measured by forward light scatter (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 are pre-gated on live, CD19$^+$ lymphocytes and are representative of n=2 WT and n=3 KO chimeric mice. The bar graph shows the mean FSC of donor-derived (CD19$^+$CD45.1$^-$) splenic B cells from WT (n=2) and KO (n=3) fetal liver HSC-chimeric mice. The error bars indicate standard deviation and the p-value was determined by 2-tailed unpaired t-test.
b) *Sin1*$^{+/+}$ (WT) and *Sin1*$^{-/-}$ (KO) pro-B cells were differentiated in vitro for 7 days on OP9 stromal cells and the mean FSC of pre-B (Gate 1), immature B (Gate 2) or transitional B (Gate 3) cells was

measured by flow cytometry. The bar graphs for each of the gated B cell populations are the average FSC measurements from 4 independent experiments with standard deviation indicated by the error bars.

c) *Sin1*$^{+/+}$ (WT-->WT) or *Sin1*$^{-/-}$ (KO-->WT) pro-B cells (CD45.2^{+}) or PBS only (mock-->WT) were adoptively transferred into sub-lethally irradiated wild type CD45.1^{+} congenic recipient mice (5x10^{6} pro-B cells/mouse). After 7 days, the recipient mice were sacrificed and the size of donor-derived WT or KO splenic B cells (CD45.2^{+}B220^{+}) was measured by flow cytometry. Surface IgM expression on the donor derived splenic B cells is also shown. The plots shown are pre-gated on live splenocytes and are representative of 4 independent experiments.

Figure 4: Sin1 regulates Akt expression and BCR dependent Akt signaling.

a) In vitro-differentiated WT or KO IgM^{+} B cells were enriched and the Akt expression was measured by immunoblotting (upper panels) and by flow cytometry following intracellular staining with a pan-Akt antibody (lower panel). For the flow cytometry analysis, Akt expression is graphed as the average Akt MFI with mean standard error of triplicate samples. The data from one of the two independent experiments is shown.

b) In vitro-differentiated *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) IgM^{+} B cells were cultured with (line) or without (shaded) 10 μg/ml anti-IgM F(ab')$_2$ for 3 minutes then fixed, permeabilized, stained with the indicated phospho-Akt antibodies for flow cytometric analysis. The data shown are representative of three independent experiments.

c) In vitro-differentiated WT or KO IgM^{+} B cells were enriched and cultured with or without 10 μg/ml anti-IgM F(ab')$_2$ for 15 minutes as indicated and then lysed and immunoblotted with the indicated antibodies for phospho- or total proteins. The data shown are representative of two independent experiments.

d) The expression of ribosomal protein S6 in WT (grey line) or KO (black line) IgM^{+} B cells was measured by intracellular staining and flow cytometry. The shaded line is the negative staining control (ctrl.). The data shown are representative of three independent experiments.

e) Surface IgM expression on in vitro differentiated Sin1 WT and KO B cells is shown. The shaded grey line is the negative staining control (ctrl.). The data shown are representative of three independent experiments.

Figure 5: Sin1 regulates c-Myc expression in B cells.

a) *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) pro-B cells were differentiated in vitro and c-Myc expression was measured in IgM^{+} B cells by intracellular staining and flow cytometry. The shaded line is the negative staining control. The data shown are representative of three independent experiments.

b) In unstimulated vitro-differentiated WT or KO IgM^{+} B cells were enriched at 4^{o}C and immediately lysed in Trizol. The expression level of *c-myc* mRNA was then measured by quantitative (q)RT-PCR. Each sample was normalized to the expression of GAPDH and the graphs shown are the average expression of triplicate experiments with standard deviation.

c) WT or KO splenic B cells (CD45.1^{-}CD43^{-}B220^{+}) were enriched from *Sin1*$^{+/-}$ or *Sin1*$^{-/-}$ fetal liver chimeric mice and cultured for 1 hour in vitro with or without anti-IgM F(ab')$_2$ and then lysed in Trizol. The expression level of c-myc mRNA was measured by qRT-PCR. Each sample was normalized to the expression of GAPDH and the graphs shown are the average expression of triplicate measurements with standard deviation. The results shown are representative of two experiments.

d) In vitro-differentiated WT or KO IgM^{+} B cells were cultured with or without with anti-IgM F(ab')$_2$ for 1 hour, then fixed, permeabilized and stained with anti-c-Myc antibody for flow cytometry analysis. The data shown are representative of two independent experiments.

e) *Sin1*$^{-/-}$ pro-B cells were infected with a control retrovirus or a retrovirus expressing human c-Myc. The expression of c-Myc or S6 in control infected (empty vector; dotted line) or *c-myc* expressing virus-infected (c-myc; solid line) *Sin1*$^{-/-}$ pro-B cells was measured by intracellular staining and flow cytometry. The relative cell size was measured by FSC. Data shown are from one of two independently derived *Sin1*$^{-/-}$ pro-B cell lines expressing human c-Myc.

f) *Sin1*$^{-/-}$ pro-B cells were infected with a control retrovirus or a retrovirus expressing human c-Myc and differentiated *in vitro* for 6 days. The relative size (mean FSC) of IgM^{+}IgD^{+} transitional B cells was measured by flow cytometry. Results from two independent c-Myc expressing KO cell lines are shown.

g) The expression S6 in *in vitro* differentiated *Sin1*$^{-/-}$ pro-B cells infected with a control retrovirus or human c-Myc retrovirus was measured by intracellular staining and flow cytometry. Data shown are representative of two independently derived *Sin1*$^{-/-}$ pro-B cells expressing human c-Myc.

Figure 6: mTORC2 regulates c-Myc protein stability

a) Wild type splenic B cells were enriched by negative selection and pretreated for 15 minutes with vehicle only (vehicle), 20 nM rapamycin or 50 nM pp242, and then cultured for an additional 60 minutes with medium only (ctrl.) or 10 μg/ml anti-IgM $F(ab')_2$. The expression level and phosphorylation status of the indicated cellular proteins was measured by immunoblotting. The data shown are representative of three independent experiments.
b) Wild type splenic B cells were pre-treated with indicated mTOR inhibitors and then stimulated with anti-IgM $F(ab')_2$ as described in panel **a**. Relative expression levels of *c-myc* mRNA was measured by qRT-PCR and normalized to the expression level of GAPDH. The data shown are the mean plus standard deviation of three measurements and are representative of two independent experiments.
c) A diagram illustrating the experimental design for panel **d**. Wild type splenic B cells were enriched by negative selection and then stimulated in vitro with anti-IgM $F(ab')_2$ for 2 hours. Cells were either untreated (vehicle), or treated with cyclohexamide (CHX), CHX+pp242 or CHX+pp242+GSK3 inhibitor SB216763 (GSKi) for 1 additional hour. Cells then were fixed, permeabilized and stained with a c-Myc antibody and analyzed by flow cytometry.
d) Splenic B cells were cultured for 2 hours with anti-IgM $F(ab')_2$ (line) or without stimulation (shaded line) and treated with vehicle only or inhibitors as indicated. The expression level of c-Myc was determined by flow cytometry. The data shown are the mean fluorescence intensity (MFI) of c-Myc staining and are representative of two independent experiments.
e) Splenic B cells were stimulated for 2 hours with anti-IgM $F(ab')_2$ and then treated with CHX+/- inhibitors for an additional 0, 10 20, 40 or 60 minutes. The expression of c-Myc was determined as described in **d**. The data shown are representative of three independent experiments.
f) Wild type splenic B cells were cultured with or without (Ctrl.) anti-IgM antibody for 1 hour. The anti-IgM stimulated B cells were then cultured for an additional hour with vehicle, pp242 or pp242+SB216763 (GSKi). Cells were lysed and the indicated phospho-proteins and total proteins were analyzed by immunoblotting. The ratio of p-Myc to total c-Myc was determined by densitometry of the corresponding bands in the blots. The data shown are representative of three independent experiments.

Supplemental Figure Legend

Supplemental Figure 1: Sin1 is required for peripheral B1 B cell development.

FACS plots showing donor derived B cells obtained from the peritoneal cavity of a *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ fetal liver HSC chimeric mice were stained for CD5 and IgM. The plots shown are pre-gated on CD45.1^{-}CD19^{+} donor B cells and are representative of n=2 *Sin1*$^{+/+}$ and n=2 *Sin1*$^{-/-}$ chimeric mice. The percentage of B1a B cells (CD5^{high}IgM^{high}) were reduced in Sin1 deficient chimeric mice when compared to WT chimeric mice (10% vs. 35% respectively).

Supplemental Figure 2: Sin1 regulates the size of resting B cells but not resting T cells.
a) The relative size of donor *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) lymph node B cells from fetal liver HSC chimeric mice was measured by forward light scatter (FSC) and compared to the size of the corresponding population of host WT lymph node B cells from the same mouse. The bar graph (right panel) shows the mean FSC of donor B cells from WT (n=2) or KO (n=3) fetal liver HSC chimeric mice. Error bars indicate standard deviation and the p-value was determined by 2-tailed unpaired t-test.
b) The relative size of splenic CD4^{+} T cells in *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) fetal liver HSC chimeric mice was measured by FSC. The histograms shown are pre-gated on the CD4^{+} T cells in the upper panel. The data shown are representative of two *Sin1*$^{+/+}$ and 2 *Sin1*$^{-/-}$ chimeric mice.

Supplemental Figure 3: A working model of Sin1/mTORC2 dependent regulation of B cell growth. Mammalian TORC2 mediates signals downstream of the BCR and TLR4 to regulate Akt activity by directly phosphorylating the Akt at Ser473 and indirectly promoting Akt Thr308 phosphorylation. Activated Akt in turn phosphorylates and inhibits TSC2 leading to increased mTORC1 signaling. Activated Akt also phosphorylates and inactivates GSK3, which in turn suppresses the GSK3 mediated degradation of c-Myc resulting in augmented c-Myc expression. The increased activity of mTORC1 and increased expression of c-Myc promote B cell growth through a mechanism which, in part, involves the regulation of S6 phosphorylation and expression by mTORC1 and c-Myc, respectively.

Materials and Methods

Mice

Sin1 knockout mice were described previously(18). 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 made by gently passing fetal liver through a 27-1/2G needle 10 times and then suspended in 1xPBS for injection. 1x10^{5}-2x10^{6} fetal liver cells were injected per recipient mouse via the tail vein. All mice were housed in the animal facilities at Yale University and all animal procedures were approved by the Yale Institutional Animal Care and Use Committee.

Pro-B cell line cultures

Pro-B cells were derived from paired *Sin1*$^{+/+}$ and *Sin1*$^{-/-}$ littermate E11.5-E12.5 embryos. The *Akt1*$^{-/-}$*Akt2*$^{-/-}$ pro-B cells were previously described(29). 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 (50U/ml; 50μg/ml) (Life Technologies) plus gentamicin (25μg/ml) (Life Technologies) plus 50μM β-mercaptoethanol (Sigma-Aldrich) supplemented with recombinant mIL-7 (10ng/ml, Peprotech or a 1:10 dilution of mIL-7 transfected 293T cell supernatant). Pro-B cell lines were differentiated in vitro by plating 30x10^{6} pro-B cells on a 10cm plate containing confluent OP9 stromal cells in RPMI 1640 medium plus 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.

Inhibitors

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

Intracellular Staining and Flow Cytometry

Single cell suspensions were first stained for surface antigens in cold FACS buffer (1x PBS pH7.4 + 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.
For the detection of intracellular antigens, cells were permeabilized with Fix Buffer 1 (BD) for 10 minutes at 37^{o}C, then washed and permeabilized for 5 minutes at room temperature with 1xPerm/Wash Buffer (BD) or Perm Buffer 3 (BD). Fixed and permeabilized cells were stained for 30 minutes at room temperature with the following antibodies diluted 1:100 in Perm/Wash buffer: anti-IgK-FITC and anti-IgM-PE (for intracellular IgH staining) (BioLegend) plus one of the following unlabeled rabbit antibodies: anti-Akt p-Thr308 (rabbit polyclonal, Cat#9275), anti-Akt p-Ser473 (587F11), anti-pan-Akt (11E7), anti-S6 p-Ser235/236 (rabbit polyclonal, Cat#2211), anti-S6 (rabbit polyclonal, Cat#9202) or anti-c-Myc (D84C12) (Cell Signaling). Cells were washed and stained with 1:500 goat-anti-rabbit-IgG-Alexa647 for 15 minutes in Perm/Wash Buffer. All cells were then washed and resuspended in FACS buffer for analysis with a FACSCalibur or LSRII (BD).

Quantitative RT-PCR

Cells were lysed in TRIzol (Invitrogen), total RNA was purified by isopropanol precipitation. Total RNA was treated with RNAse free DNAse I (Sigma) and reverse transcribed with 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).

Immunoblotting and antibodies

Cells were washed 2x with ice-cold 1xPBS and lysed in cold RIPA buffer with freshly added protease and phosphatase inhibitors. Total cell lysates were resolved by SDS-PAGE and blotted with the following antibodies: anti-Akt p-Thr308 (rabbit polyclonal, Cat#9275), anti-Akt p-Ser473 (587F11), 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-S6 p-Ser235/236 (rabbit polyclonal, Cat#2211), anti-S6 (rabbit

polyclonal, Cat#9202), anti-c-Myc p-Thr85/S62 (rabbit polyclonal, Cat#9401) and anti-c-Myc (D84C12) from Cell Signaling, anti-Sin1 (K87) (18), anti-ERK2 (381A10) (Invitrogen). Densitometry analysis was performed with a BioRad Molecular Imager Gel Doc XR system and Quantity One software (BioRad).

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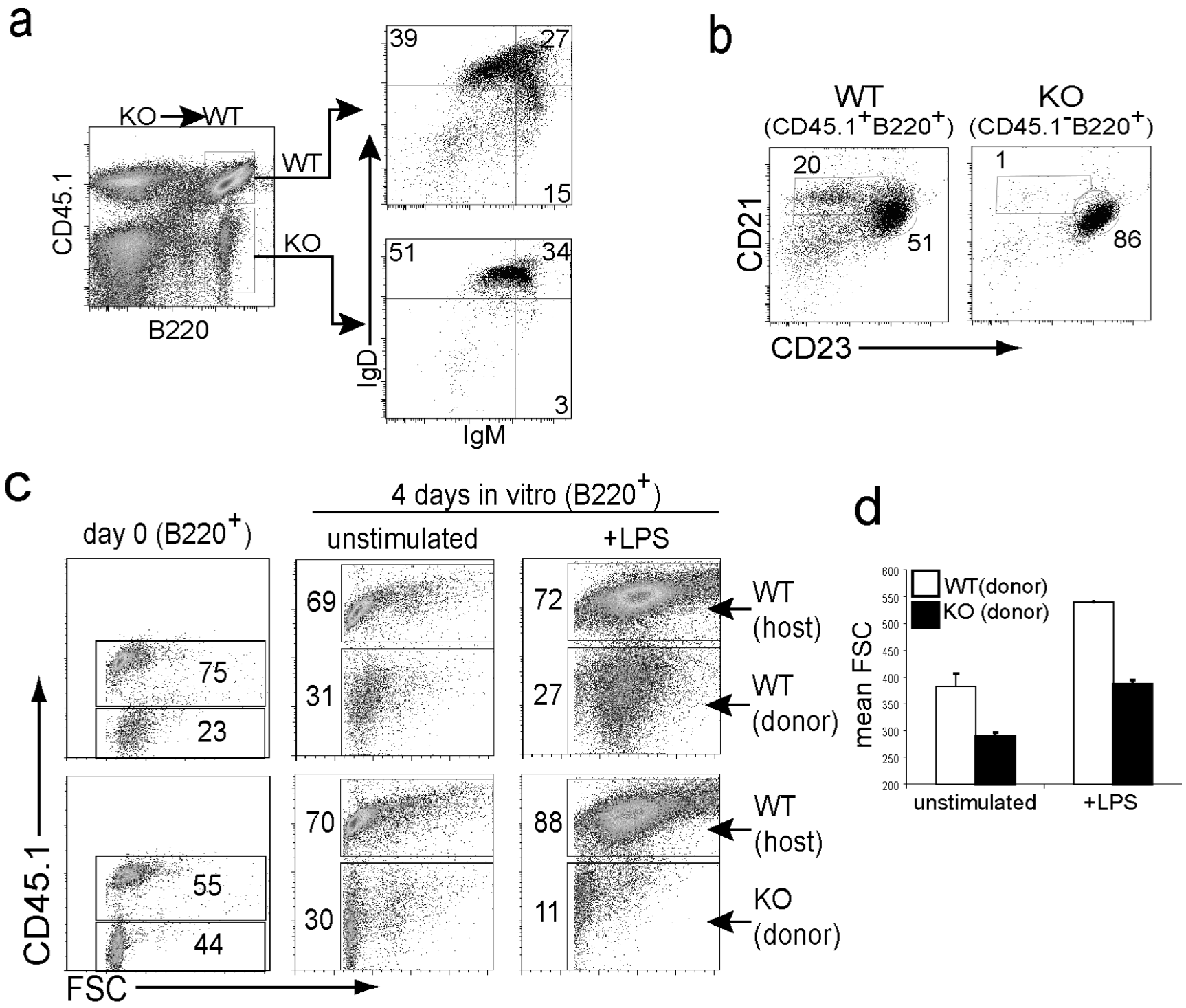
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B220
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KO
39
27
15
51
34
3
IgD
IgM
b
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KO
(CD45.1-B220+)
20
51
1
86
CD21
CD23
c
day 0 (B220+)
4 days in vitro (B220+)
unstimulated
+LPS
75
23
69
31
72
27
WT
(host)
WT
(donor)
55
44
70
30
88
11
WT
(host)
KO
(donor)
CD45.1
FSC
d
WT(donor)
KO (donor)
mean FSC
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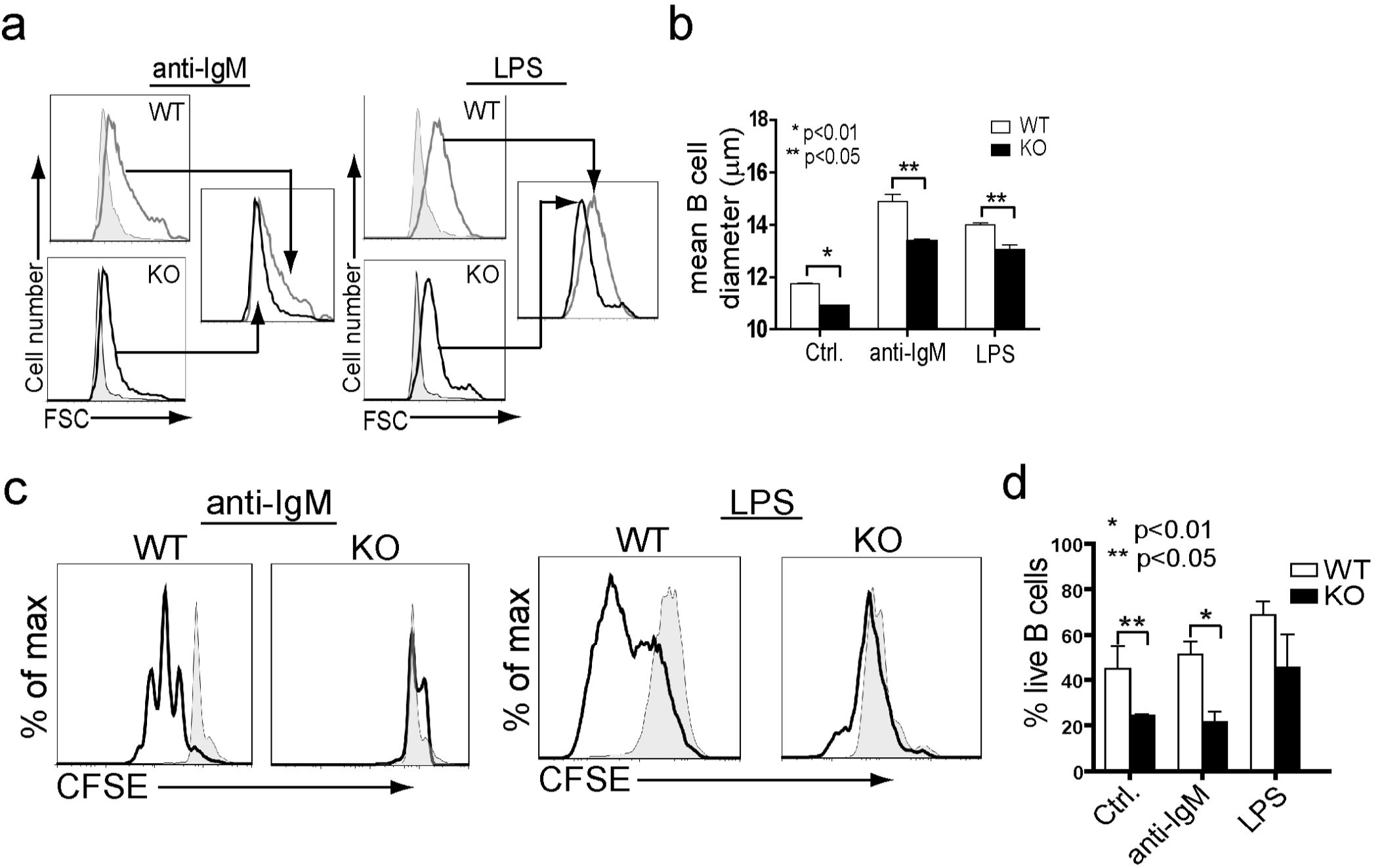
550
500
450
400
350
300
250
200
unstimulated
+LPS

Figure 1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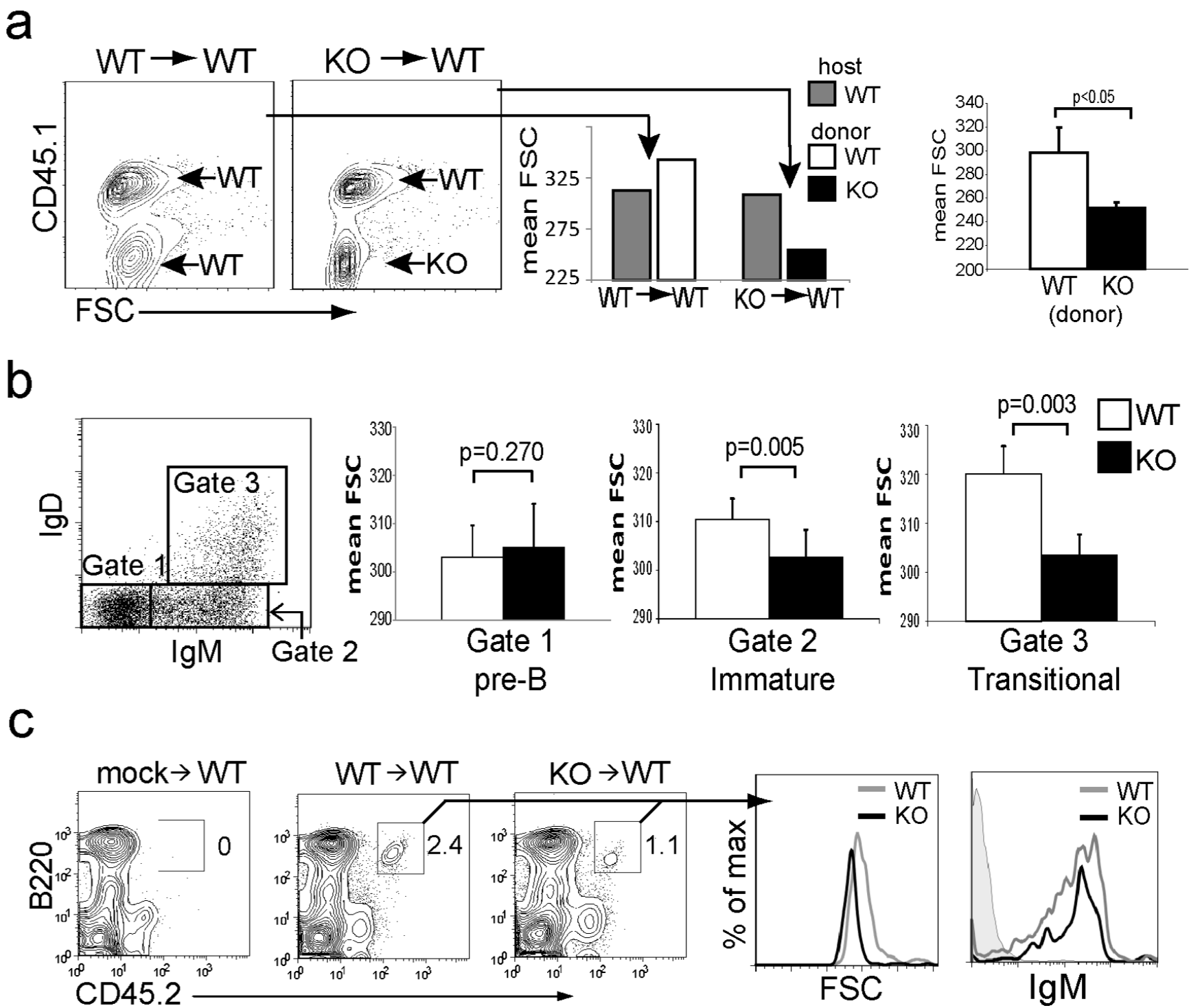
LPS
WT
KO
Cell number
FSC
b
mean B cell diameter (µm)
* p<0.01
** p<0.05
WT
KO
Ctrl.
anti-IgM
LPS
c
anti-IgM
LPS
WT
KO
% of max
CFSE
d
% live B cells
* p<0.01
** p<0.05
Ctrl.
anti-IgM
LPS

Figure 2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KO → WT
CD45.1
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WT
WT
KO
FSC
host
WT
donor
WT
KO
mean FSC
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275
225
WT → WT
KO → WT
p<0.05
340
320
300
280
260
240
220
200
mean FSC
WT
KO
(donor)
b
IgD
Gate 3
Gate 1
IgM
Gate 2
mean FSC
330
320
310
300
290
p=0.270
Gate 1
pre-B
p=0.005
Gate 2
Immature
p=0.003
WT
KO
Gate 3
Transitional
c
mock→ WT
WT →WT
KO →WT
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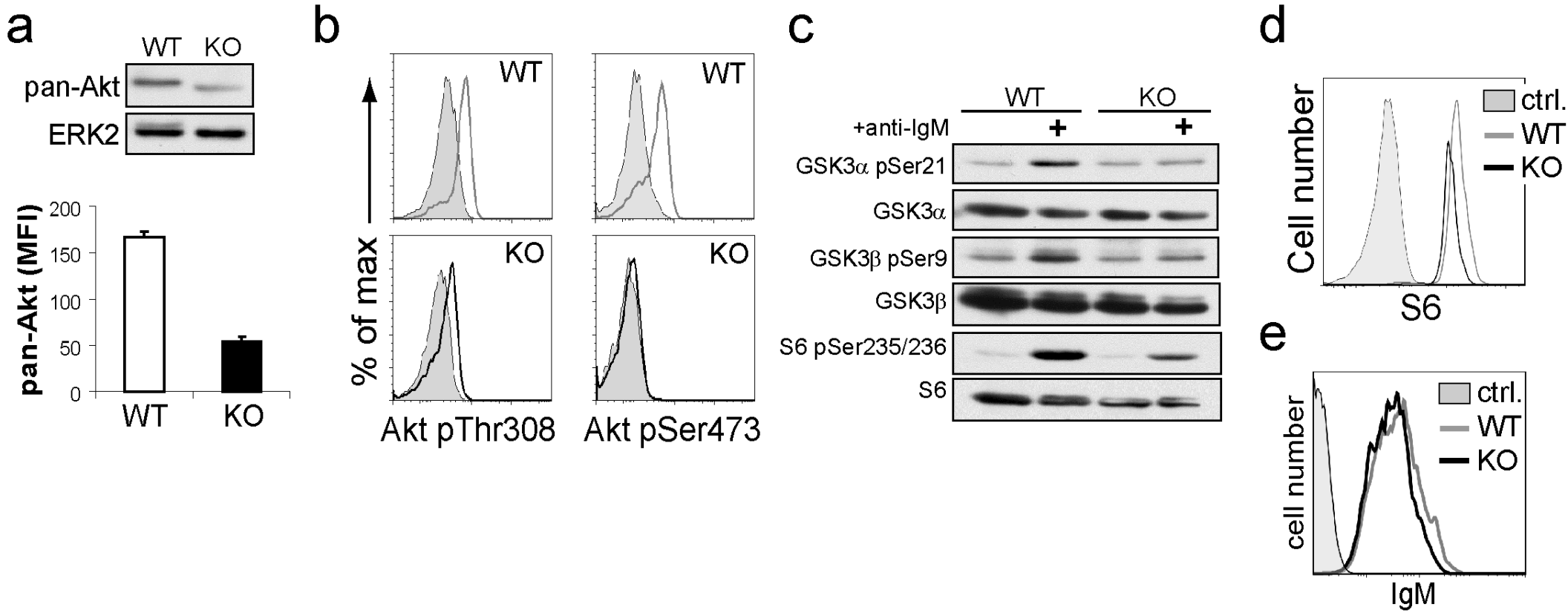
1.1
CD45.2
% of max
WT
KO
FSC
WT
KO
IgM

Figure 3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ERK2
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100
50
0
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KO
b
WT
WT
KO
KO
% of max
Akt pThr308
Akt pSer473
c
WT
KO
+anti-IgM
+
+
GSK3α pSer21
GSK3α
GSK3β pSer9
GSK3β
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S6
e
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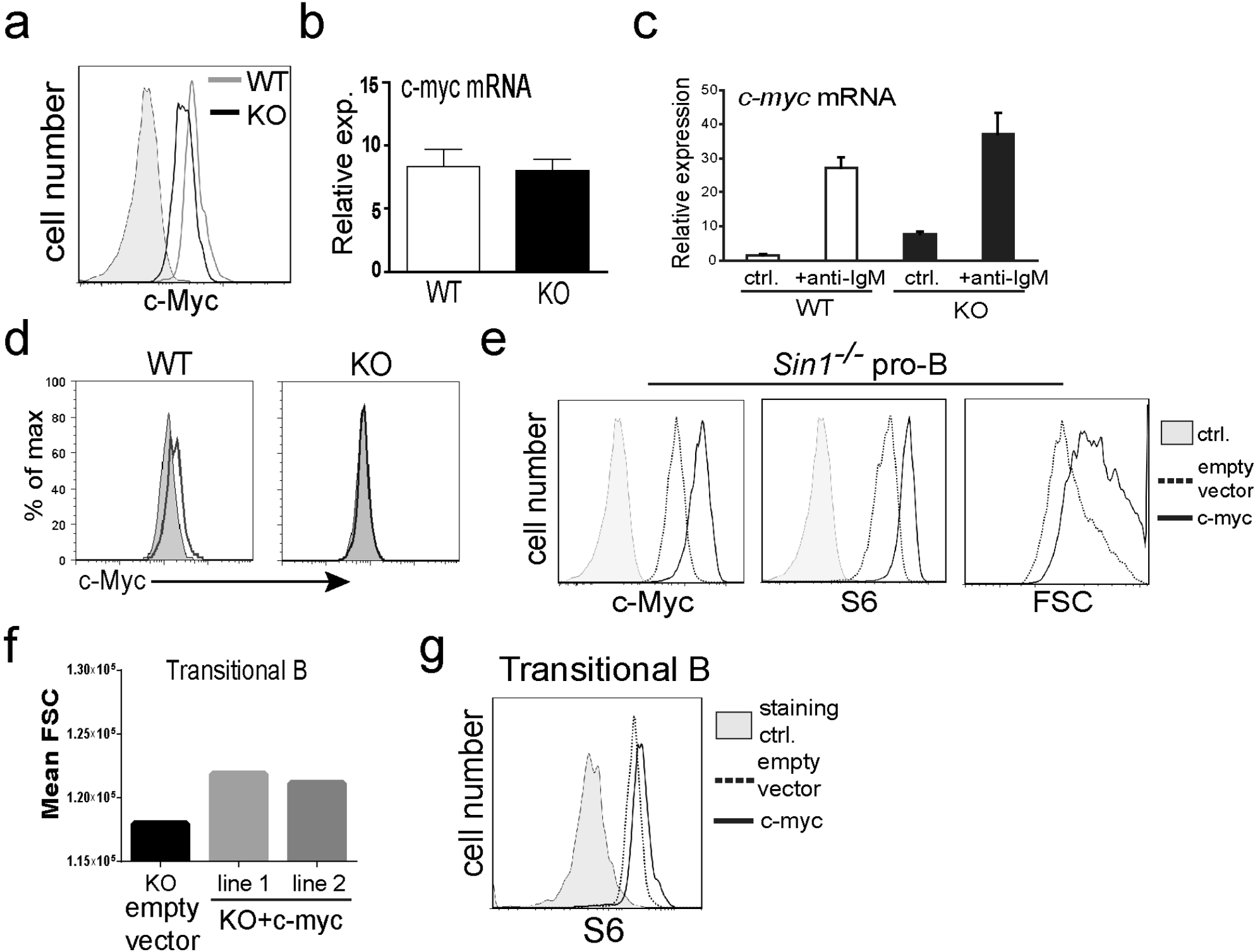
KO
IgM

Figure 4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10
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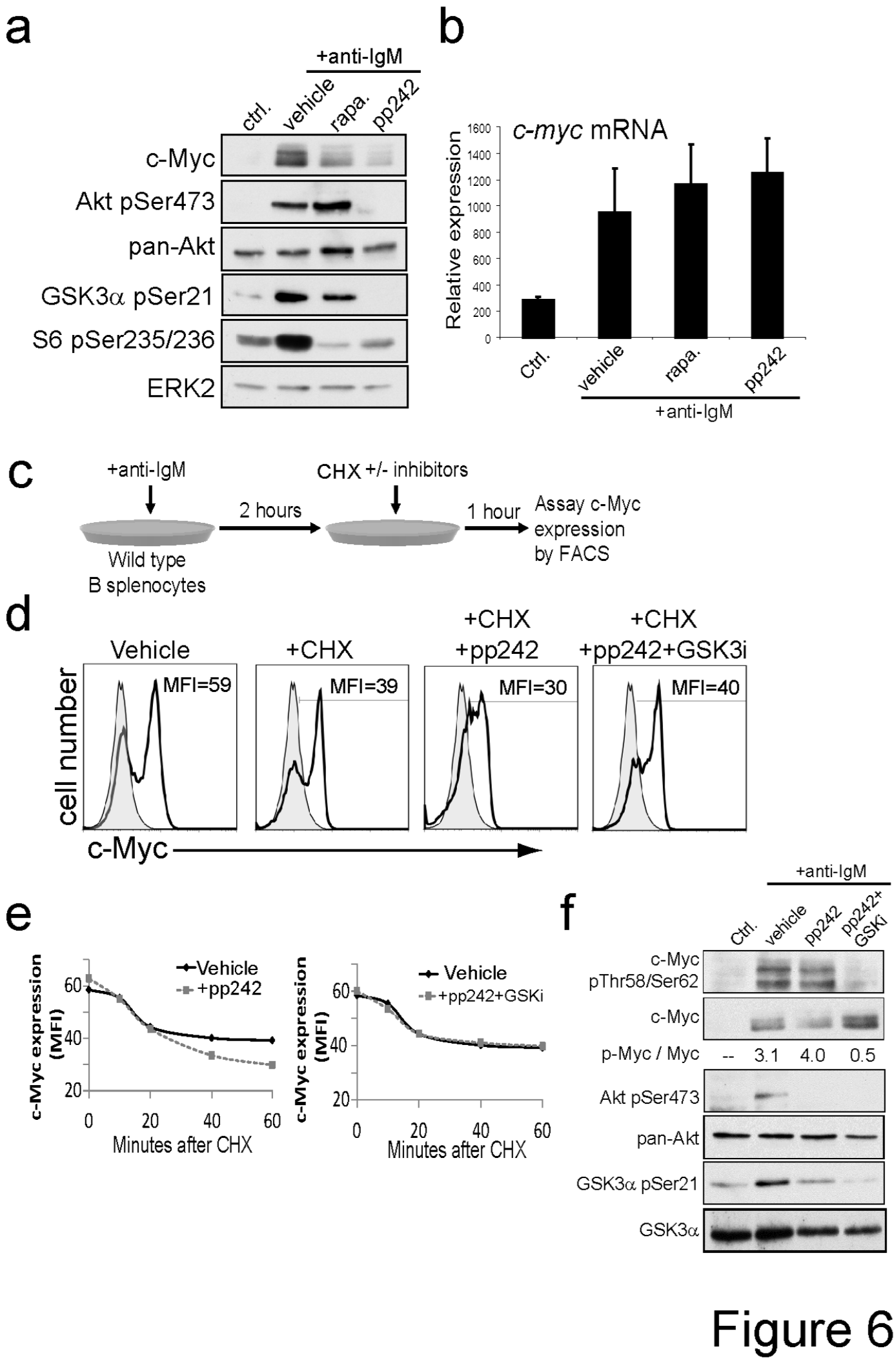
WT
KO
c
c-myc mRNA
Relative expression
50
40
30
20
10
0
ctrl.
+anti-IgM
ctrl.
+anti-IgM
WT
KO
d
WT
KO
% of max
100
80
60
40
20
0
c-Myc
e
Sin1-/- pro-B
cell number
c-Myc
S6
FSC
ctrl.
empty vector
c-myc
f
Transitional B
Mean FSC
1.30x10^5
1.25x10^5
1.20x10^5
1.15x10^5
KO empty vector
line 1
line 2
KO+c-myc
g
Transitional B
cell number
S6
staining ctrl.
empty vector
c-myc

Figure 5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ctrl.
vehicle
rapa.
pp242
c-Myc
Akt pSer473
pan-Akt
GSK3α pSer21
S6 pSer235/236
ERK2
b
c-myc mRNA
Relative expression
1600
1400
1200
1000
800
600
400
200
0
Ctrl.
vehicle
rapa.
pp242
+anti-IgM
c
+anti-IgM
2 hours
CHX +/- inhibitors
1 hour
Assay c-Myc expression by FACS
Wild type B splenocytes
d
Vehicle
+CHX
+CHX +pp242
+CHX +pp242+GSK3i
MFI=59
MFI=39
MFI=30
MFI=40
cell number
c-Myc
e
Vehicle
+pp242
+pp242+GSKi
c-Myc expression (MFI)
60
40
20
0
20
40
60
Minutes after CHX
f
+anti-IgM
Ctrl.
vehicle
pp242
pp242+ GSKi
c-Myc pThr58/Ser62
c-Myc
p-Myc / Myc
--
3.1
4.0
0.5
Akt pSer473
pan-Akt
GSK3α pSer21
GSK3α

Figure 6