UNIVERSITY OF CALIFORNIA,
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The PI3K-mTOR Pathway in Mammals: From Therapeutics to Fundamentals

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Molecular Biology and Biochemistry

by

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DEDICATION

To

Jaeseong So, Ph.D.

A wonderful father
He is the person who inspired me to pursue this degree following his footsteps
One can simply enjoy the beautiful wonders of nature
but at the same time one can try to understand what gives rise to this beauty
You have shown me that it is worth pursuing both by becoming a scientist

To my loving mother, my biggest fan and supporter

To Min-Kyung So, my sister who loves

To Jayde Kwon, Ph.D., my love forever and the most beautiful woman
I was fortunate enough to invite into my life

To David Fruman, Ph.D.

The greatest mentor I have ever had
who made all this possible by treating me as a colleague
in a field that you first paved the road
who showed me the virtue of patience and mentoring

"You don't want to get the fact wrong. The data have to be right. But the interpretation is your right. And there, everything says be bold…be bold. Make it as important as you possibly can. We are always wrong in detail because we never know enough. If you make it bold so the people will read it and pay attention that's exactly what you want. Because otherwise, you're going to spend the rest of life trying to show you're wrong. It's better to get more people involved. Because you're always wrong in detail." **–Martin Raff, M.D., MRC Laboratory, UK**

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ABSTRACT OF THE DISSERTATION

The PI3K-mTOR Pathway in Mammals: From Therapeutics to Fundamentals

By

Lomon So

Doctor of Philosophy in Molecular Biology and Biochemistry

University of California, Irvine, 2015

Professor David A. Fruman, Chair

Cells from multicellular organisms are under the control of extracellular signals to ensure the activation of certain cellular processes only in specific contexts. These extracellular signals act through intracellular signaling components that share some features with unicellular organisms such as yeast, but with additional layers of complexity and regulation. In metazoans, activation of the PI3K-mTOR signaling network is a shared response to engagement of diverse types of extracellular signals. Depending on the cell type and stimulus, activation of this pathway can promote different cellular fates including cell growth, proliferation, survival, migration and differentiation. Dysregulation of these processes is highly implicated in a variety of diseases such as cancer, diabetes, and immune disorders. Indeed, either decreased or hyperactivation of the PI3K-mTOR signaling pathway is observed in many human diseases making this network a crucial target for therapy. In this dissertation, I present a series of preclinical studies showing that indeed, pharmacological or genetic perturbation of the PI3K-mTOR pathway has an impact in the context of cancer and immunity.

Since the identification of phosphoinositide 3-kinase (PI3K) enzyme activity in

transformed cells nearly three decades ago, there has been significant advancement in the field leading to the development of a variety of novel chemical tools for therapeutic intervention. In chapter 2, I focus on the published promising finding that cancer cells with mutations in one of the most frequently mutated PI3K isoforms, p110α (encoded by *PIK3CA*), can be selectively targeted with a novel p110α-selective chemical inhibitor while preserving adaptive immune function. As it is becoming increasingly clear that an intact adaptive immune system is critical for tumor regression, these findings raise confidence that selective p110α inhibitors in cancer therapy will not be as immunosuppressive as global PI3K inhibitors currently in clinical trials.

Chapter 3 focuses on the surprising immunomodulatory effects of targeting the mammalian target of rapamycin (mTOR) downstream of PI3K with second-generation ATP-competitive mTOR kinase inhibitors (TOR-KIs). Unlike the long-known immunosuppressant rapamycin that targets mTOR through an allosteric mechanism, we observed immunoenhancing effects of TOR-KIs specifically in the context of B cell antibody class-switch recombination (CSR). I will present published work where I genetically validated that the mechanism of action of TOR-KIs on CSR is through inhibition of the mTOR complex 2 (mTORC2) signaling axis. These findings have strong implications for utilizing currently available inhibitors of the PI3K-mTOR pathway beyond cancer therapy and in modulating immunity.

Chapter 4 returns to the evolutionarily conserved fundamental role of the PI3K-mTOR pathway, which is to regulate cellular growth (mass increase). A working concept has been proposed and experimentally supported in that cells from multicellular organisms can uncouple cell growth from other cellular processes such as cell proliferation through engagement of distinct mTORC1 effectors. However, given the unique characteristics of primary lymphocytes in that they require a very long phase of growth prior to rapid divisions to produce a large number of

identical cells, I hypothesized that a common intracellular effector may coordinate both cell growth and proliferation in this cell type. This work has led to the identification of the eukaryotic translation initiation factor 4E-binding proteins (4E-BPs) downstream of mTORC1 as the critical effector in coordinating the two processes during lymphocyte activation. These results are surprising as the 4E-BPs specifically regulate only cell proliferation in other mammalian cell types. The findings highlight the amazing specificity of this pathway in coordinating cell growth to cell proliferation in a cell-type specific manner.

Despite the fundamental aspect of this research, this work has provided some major advances in the clinical context as well. The FDA-approved immunosuppressant rapamycin has long been known to have selective potency against lymphocytes although its target mTOR is ubiquitously expressed. In work presented in Chapter 4, I show that rapamycin acts upon the 4E-BPs specifically in primary lymphocytes but not other cell types, providing an explanation for its selective effects on these cells. Implications of this work beyond immunity will be discussed in Chapter 5.

In terms of basic cell biology, a fascinating aspect of this research is that during metazoan evolution, distinct cell types have adopted specialized mechanisms to utilize the PI3K-mTOR pathway for fundamental cellular processes such as growth and proliferation, in order to serve each of their roles in a multicellular system. These fundamental studies are critical as loss of proper PI3K-mTOR regulation in each cell type can ultimately lead to diseases such as cancer where cells have lost the properties to adapt to multicellularity.

Lastly, I believe that my findings support and provide more evidence towards a paradigm-shifting concept that protein synthesis can no longer be regarded as a housekeeping function that automatically pumps out proteins at the end of gene regulation. Rather, the findings strongly

argue that protein synthesis itself can be regulated and perhaps is the most important step of gene regulation given that proteins are the most important effectors that mediate cellular functions. This paradigm will be discussed and extended in the context of understanding adaptive immunity. Recent progress in understanding lymphocyte activation has mainly focused on concepts of transcriptional regulation and metabolic reprogramming. Protein synthesis in lymphocyte activation has been studied vigorously in the late 70s. The finding that cap-dependent translation is a critical step in lymphocyte activation not only rejuvenates the idea that regulation of protein synthesis plays a critical role in immunity and suggests the possibility of regulation of select transcripts that may have therapeutic potential.

CHAPTER 1:

INTRODUCTION

The first half of this introduction on PI3K is largely derived from a review article published in *the Biochemical Journal*, 442: 465-481 (2012) entitled "PI3K signaling in B- and T-lymphocytes: new developments and therapeutic advances" and will provide the background for the work presented in chapter 2. Although the dissertation is about the PI3K-mTOR in metazoan biology as a whole, I will not present literature about the role of the PI3K pathway in all the different tissues and cell types in multicellular organisms. However, in the second half of this introduction, not only the role of mTOR in immune cells will be presented (for chapter 3) but also its conserved role in controlling cellular growth in a variety of cell types in multicellular organisms will be presented as a foundation for chapters 4 and 5.

PI3K

PI3Ks are a family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol (PtdIns) and phosphoinositides (phosphorylated derivatives of PtdIns) [1–3]. Unlike yeast, whose genome encodes only one PI3K isoform (class III: Vps34) whose main role is in vesicle trafficking, the mammalian PI3Ks include eight enzymes with diverse roles in both vesicle trafficking and signal transduction. These enzymes are grouped into the categories known as class I, class II, and class III, based on substrate preference and structure. Only the class I PI3Ks have the ability to use PtdIns-4,5-bisphosphate (PtdIns-4,5-P_2) as a substrate to generate the important second messenger PtdIns-3,4,5-trisphosphate (PIP_3). Certain proteins containing a pleckstrin homology (PH) domain can specifically bind PIP_3 and be recruited to membranes where PI3K is active [4]. Hence, class I PI3K acts as a signaling hub at the plasma membrane to change the lipid composition in a way that links transmembrane receptors to the organization of multiprotein complexes, also known as signalosomes [5]. The composition of these signalosomes and the specific PH domain-containing PI3K effector proteins recruited to these assemblies varies

according to the receptor that is engaged. In most cells, the serine/threonine kinase AKT (also known as protein kinase B, PKB) is a key PI3K effector and AKT phosphorylation is a common readout of PI3K activation [6,7]. Two amino acid residues in AKT are phosphorylated in a PI3K-dependent manner: T308 by phosphoinositide-dependent kinase-1 (PDK-1), and S473 by mTORC2 (a complex of the mammalian target of rapamycin (mTOR), rictor and other proteins).

Elevation of cellular PIP_3 is transient and is controlled by lipid phosphatases [8,9]. PTEN is a 3-phosphatase that converts PIP_3 back to PtdIns-4,5-P_2. In cells lacking PTEN, basal PIP_3 amounts are increased and receptor stimulation causes exaggerated PI3K signaling. SHIP1 and SHIP2 are 5-phosphatases that convert PIP_3 to PtdIns-3,4-bisphosphate (PtdIns-3,4-P_2). This phosphoinositide can still recruit and activate PDK-1 and AKT but has other signaling functions that do not overlap with PIP_3. For example, the PH domains of TEC family kinases have high affinity for PIP_3 but not PtdIns-3,4-P_2, whereas the converse is true for the adaptor proteins Bam32, TAPP1 and TAPP2 [10].

Class I PI3Ks are further divided into two subgroups: class IA and class IB [1–3]. Class IA PI3Ks contain one of three distinct 110 kDa catalytic isoforms (p110α, p110β, or p110δ) that forms a heterodimer with one of the five regulatory (adaptor) isoforms (p85α, p55α, p50α, p85β, or p55γ). The overall structure of p110 subunits is very similar with the following domains: adaptor-binding (ABD), Ras-binding, C2, helical and lipid kinase (Figure 1.1). The class IA regulatory isoforms share a similar C-terminal half that contains two Src-homology-2 (SH2) domains that flank a coiled-coil domain (also called iSH2) that binds tightly to the p110 ABD to form the heterodimer. The N-terminal portions of p85α and p85β contain an additional Src-homology-3 (SH3) domain and a RhoGAP-homology region flanked by proline-rich motifs. Class IB PI3K is composed of a single catalytic isoform (p110γ) bound to one of two regulatory

isoforms (p101 or p84). As a general rule, class IA PI3Ks are activated by tyrosine kinase (TK)-based signals whereas class IB PI3Ks are activated by G protein-coupled receptors (GPCRs).

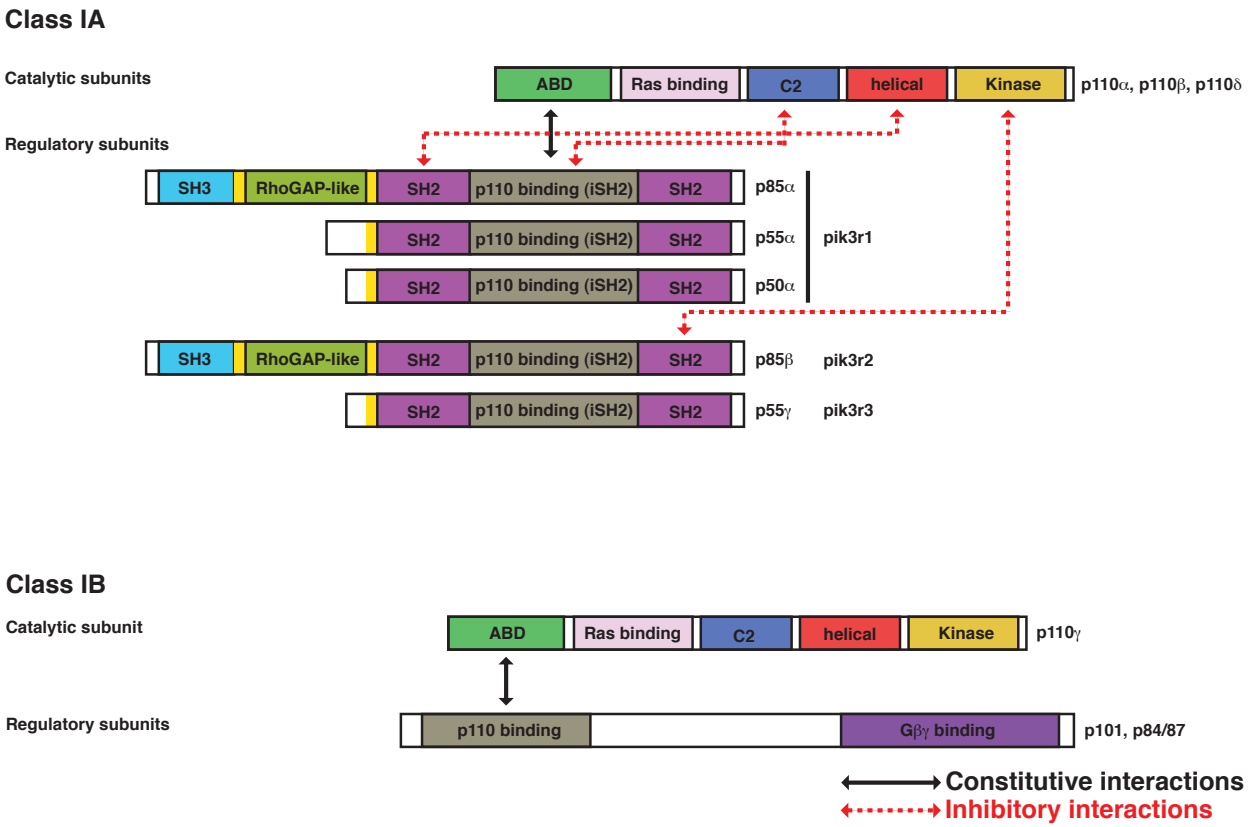

Figure 1.1. Domain structure of class IA and class IB PI3K isoforms.
Black arrows indicate constitutive interactions of the heterodimers. Dashed red arrows indicate inhibitory interactions between the regulatory and catalytic isoforms of class IA PI3K that maintain low basal activity of the enzyme. Phosphorylation of tyrosine (Y) residues on the conserved pY-X-X-M motifs on various receptors or adaptor proteins (CD19/BCAP for B cells, CD28/ICOS for T cells) recruits the regulatory isoforms via the two SH2 domains and this binding releases the inhibitory interactions. Other protein:protein interactions also contribute to class IA and IB recruitment and activation.

PI3K and Immunity

PI3K in lymphocytes

PI3K and AKT coordinate many aspects of the response to antigen receptor engagement. In B and T cells, the formation of signalosomes that drive antigen receptor-dependent Ca^{2+} mobilization is partially dependent on PI3K [5,11,12]. PIP_3 produced upon BCR or TCR clustering binds to PH domains in TEC family kinases to promote activation of phospholipase C-g and subsequent hydrolysis of PtdIns-4,5-P_2. This results in release of soluble IP_3 to initiate Ca^{2+} mobilization, and accumulation of diacylglycerol in the membrane to activate protein kinase C isoforms and GTP exchange factors for RAS. A role for PI3K upstream of RAS in lymphocytes is supported by the observation of decreased ERK phosphorylation in PI3K-deficient T and B cells stimulated through antigen receptors [13]. Activated AKT has many substrates with key roles in lymphocyte activation and trafficking. The Forkhead Box Subgroup O (FOXO) transcription factors are one key group of AKT substrates [7,14]. AKT-mediated phosphorylation inhibits DNA binding by FOXO factors and promotes their nuclear exit and cytoplasmic sequestration and degradation. AKT also can phosphorylate TSC2 and PRAS40 to increase the activity of mTORC1 (mTOR complex-1), a multifunctional signaling protein that coordinates cell growth and metabolism (see below) [7]. However, the dependence of mTORC1 activation on PI3K and AKT varies in lymphocytes according to the stimulus, which will be discussed later [15–17]. It should also be noted that MAPK signaling through RAS can also activate mTORC1 independent of PI3K activity in certain cellular contexts [18].

Various receptors on lymphocytes can engage class I PI3K. Antigen receptors, costimulatory molecules, cytokine receptors, and chemokine receptors can all trigger an increase in PIP_3 and phosphorylation of AKT. Crosslinking of the B cell receptor (BCR) leads to tyrosine

phosphorylation of the co-receptor CD19 and the adaptor protein BCAP, and each of these proteins recruits class IA PI3K through SH2 domain interactions [19–21] (Figure 1.2). T cell receptor (TCR) engagement also activates class IA PI3K but the molecular details remain to be fully established. Possible mechanisms include: binding of p85 proline-rich domains to SH3 domains of Src family kinases [22,23]; interaction of p85 Rho-GAP domains with Rac-GTP downstream of the Vav exchange factors [24–26]; and association of SH2 domains in p85 or p50 with pTyr residues in proteins associated with TCR signalosomes [27,28]. Two co-stimulatory molecules on T cells, CD28 and ICOS, recruit PI3K through pY-X-X-M motifs [29]. In the case of CD28, recruitment of PI3K appears dispensable for most of the initial costimulatory signals but is essential for CD28 function in effector T cells [19]. The role of PI3K in ICOS signaling will be discussed below. Other costimulatory molecules and cytokine receptors on B and T cells recruit and activate class IA PI3K through diverse mechanisms [30,31]. The class IB p110γ isoform is mainly activated by GPCR signals (i.e. chemokine receptors) in cells of the innate immune system such as neutrophils[32]. The subunits of heterotrimeric G proteins directly bind to p110γ and mediate enzyme activation through p101 or p84 adaptor subunits (Figure 1.1). However, the situation is more complicated in lymphocytes in that chemokines activate p110γ in T cells but p110δ in B cells [33,34]. The mechanism linking GPCRs to p110δ in B cells is not known. Furthermore, there is some evidence that TCR signaling activates p110γ, possibly via G-alpha proteins or the small GTPase RAS [35,36]. All of the class I catalytic subunits including p110γ contain RAS-binding domains (Figure 1.1), and the interaction of p110γ with RAS is required for T cell development at the b-selection stage[36].

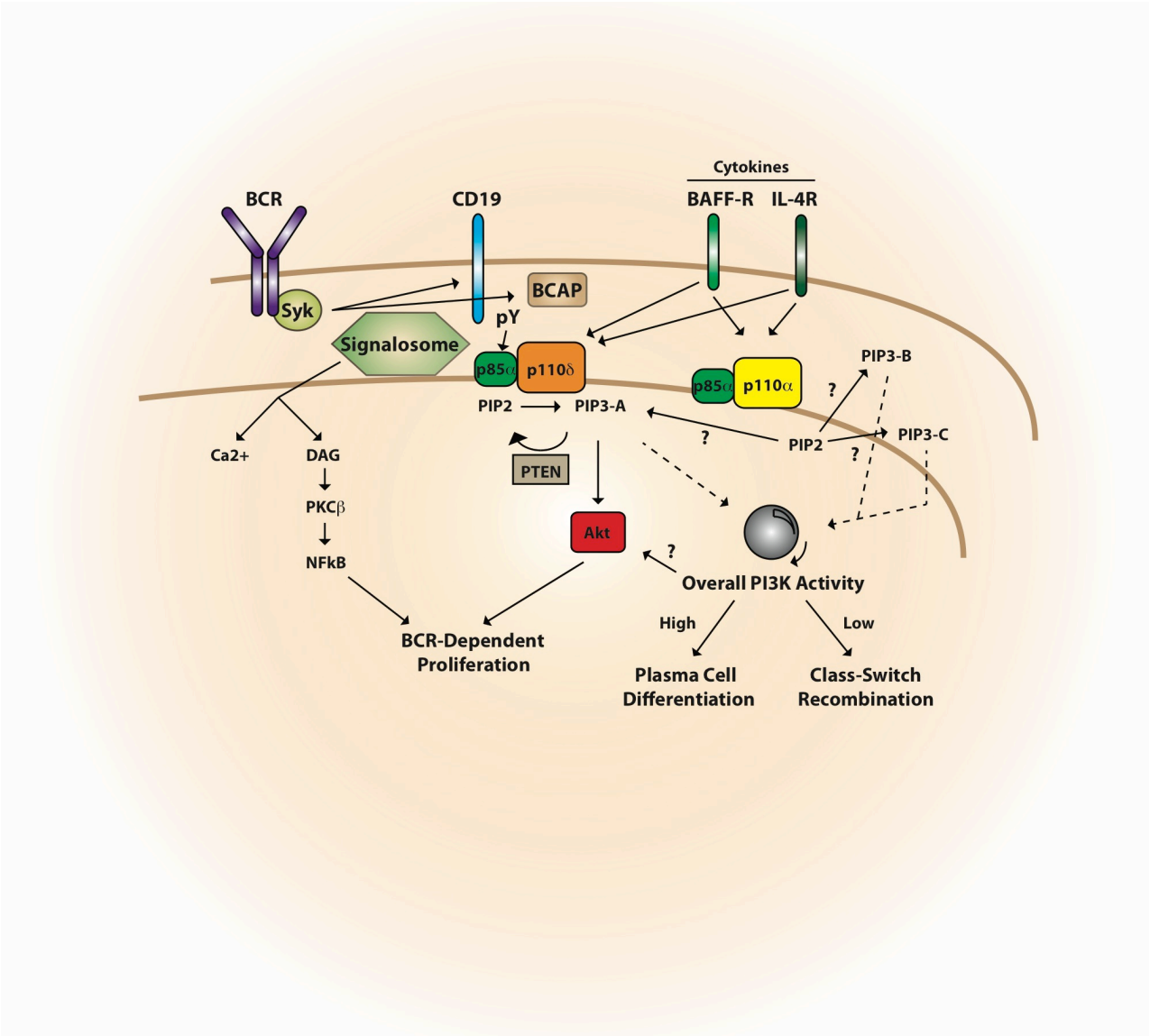

Figure 1.2. PI3K engagement in B lymphocytes and the rheostat concept.
BCR engagement triggers tyrosine (Y) phosphorylation on CD19 and BCAP to recruit PI3K dimers mainly consisting of p85α and p110δ. PI3K activation promotes signalosome assembly for Ca2+ mobilization and diacylglycerol (DAG) production, and increases activity of AKT. BCR dependent Ca^{2+} flux, AKT activation and proliferation are mainly dependent on PIP_3 pools generated by dimers of the p110δ catalytic isoform with the p85α regulatory isoform. Cytokine (BAFF and IL-4) dependent survival signals require p110α as well, which might generate distinct pools of PIP_3 as shown. *In vivo*, overall PI3K activity serves as a 'rheostat' whose signal output strength determines the nature of the response. In B cells, high PI3K activity opposes class switch recombination and promotes plasma cell differentiation. Low PI3K activity promotes class switch recombination, with p110 inhibition selectively augmenting IgE production in mice. A similar rheostat concept applies in CD4 T cells to generate the variety of different subsets required depending on the immune context.

Tools to study PI3K

Following the molecular cloning of class I PI3K isoforms in the early 1990s, PI3K signaling in lymphocytes was studied mainly using established cell lines derived from lymphoid tumors. This approach was convenient for biochemical studies because cell lines have more cytoplasm and protein content than primary T and B cells. Cell line studies also avoided the problem that primary lymphocytes die rapidly in culture and cannot easily be metabolically labeled or transfected. However, tumor cells have severe drawbacks for signal transduction research as they usually have dysregulated PI3K signaling, and cell proliferation is typically uncoupled from physiological extracellular controls. Furthermore, the PI3K inhibitors used in early experiments (wortmannin and LY294002) are non-selective compounds that inhibit all PI3K isoforms as well as mTOR and other lipid and protein kinases in cells [37–39]. A more precise understanding of PI3K signaling required better tools.

The first major technical advance was the creation of genetically modified mouse strains lacking individual class I PI3K isoforms. Germline and/or tissue-specific knockouts of the genes encoding each class I catalytic subunit, and of the genes encoding p85α/p55α/p50α and p85β, have been generated and characterized (Table 1.1). However, PI3K gene knockouts have significant limitations even when deletion is tissue-specific. One problem is that loss of one isoform of PI3K often leads to altered expression of non-targeted isoforms [40]. For example, deletion of the *Pik3r1* gene encoding p85α/p55α/p50α causes reduced expression of p110 proteins and increased expression of p85β. Deletion of individual p110 isoforms results in functional compensation of other catalytic isoforms. Hence, an important step forward was the generation of knock-in mice that express intact proteins with point mutations causing loss-of-function. This strategy was first used by Okkenhaug, Vanhaesebroeck and colleagues to generate

p110δ$^{D910A/D910A}$ mice with a point mutation inactivating p110δ kinase function [13], and has been used subsequently to generate kinase-inactive (KI) alleles of each of the p110 isoforms (Table 1.1). This strategy prevents compensatory changes in expression or function and provides a more precise model of chemical kinase inhibition. However, the knock-in strategy is still limited to some degree by the fact that the mutation can affect lymphocyte development, resulting in an altered pool of mature T or B cells. Therefore, data from knockout and knockin mice are most conclusive when combined with studies of wild-type cells treated with selective PI3K inhibitors. Of note, p110αKI mice display embryonic lethality at a stage before lymphoid precursors can be isolated.

Targeted Isoform	Approach	Studied Cell Types	References
p85α	Null	T cell	Shiroki et al. (2007)
		B cell	Suzuki et al. (1999, 2003), Donahue et al. (2004), Donahue and Fruman (2007), Hess et al. (2004)
p85α/p55α/p50α	Null	B cell	Fruman et al. (1999)
p85α/p55α/p50α	T cell specific (Lck-Cre)	T cell	Deane et al. (2007)
p85α/p55α/p50α	B cell specific (CD21-Cre)	B cell	Oak et al. (2007)
p85β	Null	T and B cell	Alcazar et al. (2009)
		T cell	Deane et al. (2004)
p85α/p55α/p50α/p85β	Conditional p85α/p55α/p50α and null p85β	T cell	Deane et al. (2007), Oak et al. (2006)
		B cell	Oak et al. (2009)
p101	Null	T cell	Janas et al. (2009)
p101/p110δ	Both null	T cell	Janas et al. (2009)
p110α	Lymphocyte specific (CD2-Cre)	B cell	Ramadani et al. (2010)
p110α/p110δ	Conditional p110α (CD2-Cre) and kinase-dead D910A knock-in p110d	B cell	Ramadani et al. (2010)
p110δ	Null	T and B cell	Clayton et al. (2002), Jou et al. (2002)
		B cell	Llorian et al. (2007), Janas et al. (2008)
		NK cell	Kim et al. (2007), Zebedin et al. (2008), Saudemont et al. (2009)
p110δ	Kinase-dead D910A knock-in	T and B cell	Okkenhaug et al. (2002), Reif et al. (2004)
		T cell	Okkenhaug et al. (2006), Garcon et al. (2008), Jarmin et al. (2008), Mirenda et al. (2007), Nashed et al. (2007), Patton et al. (2006), Liu et al. (2009), Soond et al. (2010), Sinclaire et al. (2008)
p110γ	Null or kinase-dead	T and B cell	Reif et al. (2004), Sasaki et al. (2000), Nombela-Arrieta et al. (2004), Li et al. (2000) Hirsch et al. (2000),
		T cell	Garcon et al. (2008), Alcazar et al. (2007), Martin et al. (2008), Thomas et al. (2008)
		NK cell	Tassi et al. (2007), Guo et al. (2008), Saudemont et al. (2009)
p110γ	Five point mutations (DASAA: disruption of Ras binding)	T cell	Janas et al. (2011)
p110γ/p110δ	Both null or null (p110δ)/knock-in (p110γ)	T cell	Webb et al. (2005), Swat et al. (2006), Janas et al. (2010), Ji et al (2007)
		NK cell	Tassi et al. (2007)

Table 1.1. Gene-targeted mouse strains with null, condition or knockin mutations in class I PI3K genes

The discovery and validation of selective, ATP-competitive PI3K inhibitors has been driven by efforts from both industry and academia. Although the kinase domains of class I PI3K isoforms are highly conserved, X-ray crystal structures have shown that the ATP-binding pockets have distinct topologies and flexibilities that can allow selective binding of distinct chemical structures [41]. These properties have allowed the development of compounds with good selectivity for single class I PI3K isoforms [41–43]. There are also several compounds targeting all class I isoforms with minimal off-target effects on other kinases (termed "pan-class I PI3K inhibitors"). Examples of isoform-selective and pan-class I PI3K inhibitors are shown in Table 1.2. Many of these can be purchased from commercial vendors, and most have optimized pharmacological properties to allow dosing of animals *in vivo*. In chapter 2, we use novel isoform-selective inhibitors to define the consequences of acute inhibition of p110α and other PI3K isoforms in lymphocyte function.

Targeted Isoforms	Inhibitors	Status
Pan Class I/IA	GDC-0941 XL147 ZSTK474	Basic and clinical Basic and clinical Basic and clinical
p110α	A66 MLN1117 (INK1117) BYL-719 PIK-75	Basic Clinical Clinical Basic
p110β	TGX-221, TGX-115	Basic
p110δ	IC87114 CAL-101/GS1101 CAL-263	Basic Clinical Clinical
p110γ	AS252424, AS604850	Basic
p110δ/γ dual	IPI-145	Clinical

Table 1.2. Selected PI3K inhibitors currently available for basic research or clinical trials

PI3K in mature B cells

At an early stage in B cell development, a lineage split occurs resulting in two subsets of peripheral B cells: the innate-like B-1 B cells that reside in body cavities, and B-2 B cells in blood and secondary lymphoid organs. The B-2 B cells are further divided into marginal zone (MZ) B cells that reside in the MZ of the spleen and do not circulate; and follicular (FO) B cells that recirculate through blood, lymph, and lymphoid tissues. In mouse strains lacking p85a or p110d, or in chimeric mice with AKT1/AKT2-deficient B cells, B-1 and MZ B cells are nearly absent [19,44–48]. This phenotype is similar to mice lacking CD19, a component of BCR signalosomes [49]. This suggests that a major function of CD19 is to activate PI3K and AKT to allow commitment to the B-1 or MZ lineages at key stages of B cell development. The CD19/PI3K/AKT signal appears to act through inactivation of FOXO1, since *Foxo1* deletion in peripheral B cells expands the MZ B cell population and reverses the MZ deficiency in CD19 knockouts [50].

The absence of B-1 and MZ B cells in PI3K knockout strains made it difficult to interpret whether PI3K activity is important for the function of fully developed B-1 and MZ B cells. Gold and colleagues addressed this question by utilizing a p110d-selective inhibitor IC87114 (Table 2) in immune assays using normal B-1 and MZ B cells [51]. Selective inhibition of p110δ in these cells completely inhibits AKT activation by Toll-like receptor (TLR) ligands and chemoattractants, suggesting that p110δ is the main isoform linking these extracellular signals to AKT. p110δ inhibition also suppresses chemotaxis, proliferation and antibody production by B-1 and MZ cells. Interestingly, treatment of mice with IC87114 disrupts the localization of B cells with a MZ surface phenotype ($IgM^{hi}IgD^{lo}$), suggesting that p110δ is required for chemotactic and adhesive

signals that position MZ cells in the marginal zone. B-1 and MZ cells are thought to be the major source of natural antibodies to common microbial antigens and some self-antigens. Consistent with the reduced B-1 and MZ compartments in p110δKI mice, natural antibody production is reduced. Autoantibody production is also reduced in p110δKI mice and in mice treated with p110d inhibitor. These findings suggest that selective p110δ inhibitors have potential for the treatment of autoimmune diseases driven by autoantibodies. Whether p110δ inhibition enforces B cell tolerance through anergy induction, or acts mainly by blocking proliferation and differentiation of self-reactive B cells remains to be established. Interestingly, p110δ inactivation suppresses the expansion of B-1 cells and MZ B cells that occurs in the absence of PTEN [52].

The main function of FO B cells is to produce antibodies in response to T cell-dependent antigens. These antigens can be proteins or other epitopes that are physically linked to proteins whose peptides can be recognized by T helper cells. It is well established that FO B cell function is highly dependent on class IA PI3K. B cells lacking p85α or p110δ, or expressing kinase-inactive p110δ show severely impaired signaling downstream of the BCR and a complete inability to proliferate following BCR crosslinking [19,44,45,48,53,54]. These defects are not simply due to altered development because wild-type B cells treated with IC87114 display equivalent defects [43]. A primary role of PI3K following BCR crosslinking is to promote signalosome assembly leading to diacylglycerol production, PKCβ activation, and NFκB nuclear translocation to activate gene expression (Figure 1.2) [11,14].

Class IA PI3K signals also contribute to antigen presentation by B cells [55], adhesion of T:B conjugates [56], and to differentiation of follicular helper T cells (Tfh cells,

see below). This predicts that PI3K inhibition would prevent the differentiation and function of T cells capable of delivering helper signals to B cells. It is surprising, therefore, that T cell-dependent antibody responses, while reduced, are not abolished by genetic disruption of p110δ or p85α [19,44,51,57] nor by IC87114 treatment of mice [51,58]. One potentially relevant observation is that FO B cell proliferation *in vitro* driven by CD40 ligand (CD40L) and interleukin-4 (IL-4), representing T cell helper signals, is largely intact even when PI3K is strongly inhibited [53,59]. Proliferation of FO B cells driven by TLR ligands is also partially preserved [19,44,45,53,57,59,60]. Therefore, the defects in BCR signaling might be overcome by PI3K-independent signals from T cells or TLR ligands. Further, PI3K inhibitors promote immunoglobulin (Ig) class switching under conditions where B cell proliferation is maintained (e.g. CD40L + IL-4, or LPS), whereas PTEN deletion suppresses class switching [58,61,62]. Ig class switching requires the protein AID, whose gene transcription is controlled by FOXO1 [63]. All of these observations emphasize that PI3K is not a universal "on" switch for B cell responses or for T cell help (Figure 1.2, legend). Ultimately, the balance of positive and negative effects of PI3K inhibition probably explains the retention of a reasonably robust T-dependent antibody response *in vivo*. Also of interest is the finding that p110δ inhibition or p85α deletion causes a selective elevation in basal and antigen-specific IgE production in mice [58,64]. Whether this phenomenon will affect the clinical success of p110δ inhibitors, particularly in inflammatory diseases, is a topic of interest. One of the observations in chapter 2 is that p110a-selective inhibitors do not enhance IgE production.

FO B cell survival mainly depends on the cytokines BAFF and IL-4 as well as tonic (basal) signaling through the BCR. Cytokine-dependent survival is highly

dependent on p110δ activity *in vitro* [65,66]. However, FO B cell numbers are only modestly reduced in p110δKI mice suggesting that survival signals from cytokines and/or BCR tonic signaling *in vivo* are not absolutely p110δ-dependent. A study from Rajewsky's group showed that in the absence of the BCR, a constitutively active form of p110α is sufficient to maintain FO B cell survival in the periphery [67]. The converse experiment using a conditional p110α knockout approach showed that only in the absence of both p110α and p110δ were FO B cells completely depleted [68] (Figure 1.2). These results again show the importance of dissecting PI3K signaling through both gain-of-function and loss-of-function approaches. It is likely that expression of a constitutively active p110α isoform can elevate PIP_3 beyond the physiological level, bypassing the need for p110δ engagement by tonic signals from an intact BCR. In chapter 1, I addressed this question using acute inhibition of p110α and find that selective p110α inhibitors do not significantly suppress B cell survival *in vitro* or *in vivo*.

An interesting issue raised by the p110α/p110δ-double knockout study is whether acute treatment with pan-class I PI3K inhibitors would cause a significant reduction in the peripheral B cell population, relative to selective inhibitors of individual PI3K isoforms. Given that pan-PI3K inhibitors (e.g. GDC0941) are entering clinical trials for cancer therapy, it will be important to evaluate their impact on immune homeostasis compared to more selective compounds. One might predict that selective p110d inhibitors would mainly affect the B-1 and MZ compartments without eliminating FO B cell numbers or function. Of particular interest is targeting p110α because activating mutations in the *PIK3CA* gene are very common in many solid tumors and are considered oncogenic drivers [69]. Development of a highly selective p110α inhibitor would perhaps

have the potential to selectively act on *PIK3CA* mutant tumor cells while sparing B-1 and MZ B cells and maintaining normal FO B cell function in the periphery. In chapter 2, we show that indeed this is the case for a preclinical tool that specifically targets p110α, MLN1117. When compared with the pan-PI3K inhibitor GDC0941, MLN1117 spared B cell proliferation and survival while suppressing *PIK3CA* mutant tumor growth to the same extent as GDC0941.

PI3K in mature CD4+ T cells

Resting CD4 T cells recirculate among lymphoid tissues, searching for foreign antigenic peptides presented by antigen-presenting cells (APCs), particularly dendritic cells. Antigen recognition with costimulation leads to T cell clonal expansion and differentiation into effector CD4 T cells. These are usually termed T helper cells (Th) as they provide help to other immune cells such as macrophages, B cells and CD8 T cells to orchestrate the overall immune response depending on the immune context. The functional variety of Th cells is achieved by the differentiation of naïve uncommitted CD4 T cells to distinct subsets including Th1, Th2, Th17, and T follicular helper (Tfh) cells (Figure 1.3). Distinct types of CD4 T cells with suppressive potential (Tregs) are also produced either during thymocyte selection (natural Tregs; nTreg) or upon stimulation under tolerogenic conditions (induced Tregs; iTreg). The differentiation of CD4 T cells to each subset is dictated by sensing of extracellular cues in the form of cytokines and cell contact-dependent signals from the microenvironment, with distinct outcomes depending on the immune context.

The proliferation of CD4 T cells *in vitro* driven by antibody-mediated clustering of TCR with CD28 is largely PI3K-independent [19,53,70–72]. It is likely that artificial TCR/CD28 clustering overrides certain physiological signaling requirements, as clonal expansion of CD4 T cells driven by antigen (or superantigen) and APCs is markedly suppressed by PI3K blockade, with a major role for p110δ [70–72] (Figure 1.3). Furthermore, numerous studies indicate that CD4 T cell differentiation is highly regulated by PI3K signaling [71,73,74] and its downstream effectors, particularly the AKT/FOXO axis and mTOR (discussed below).

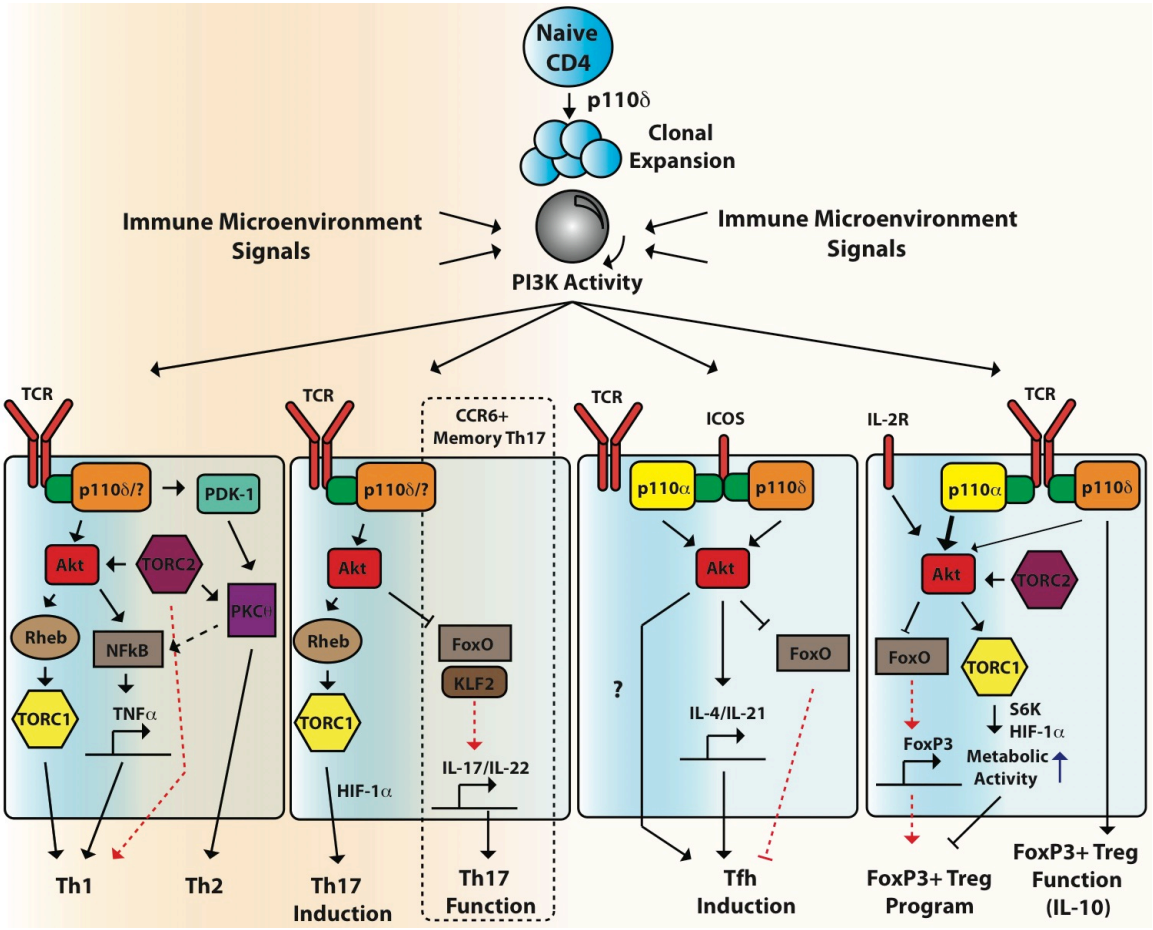

Figure 1.3. Role of PI3K and downstream effectors in the differentiation of activated CD4 T lymphocytes. Antigen encounter triggers clonal expansion of naïve CD4 T cells that is mainly dependent on p110δ. Depending on the immune microenvironment (cytokines, etc.), primed CD4 T cells differentiate into different CD4 T cell subsets (Th1, Th2, Th17, Tfh, and iTregs). TORC2-dependent AKT activation promotes Th1 differentiation through TORC1 and NFkB, and TORC2-dependent PKCq activation promotes Th2 differentiation. AKT also increases Th17 differentiation through TORC1 activation. Already established CCR6+ human memory Th17 cell function (IL-17/IL-22 secretion) also depends on PI3K/AKT activity via modulation of FOXO and its target genes including KLF2. ICOS stimulation triggers Tfh differentiation through a p110δ-dependent pathway, with a speculative role for p110α binding to ICOS. Decreased PI3K/AKT activity is required for proper iTreg induction. Isoforms other than p110δ including p110α probably suppress iTreg induction in this context. PI3K/AKT blockade results in the induction of FOXO-mediated FoxP3 expression and low TORC1 activity leads to decreased metabolic activity via S6K and HIF-1a. Established FoxP3+ Tregs also depend on p110δ signaling for proper suppressive function such as IL-10 secretion. Due to space constraints, the nucleus is not depicted where all of the genes are regulated.

Rapamycin and mTOR

Rapamycin

Rapamycin (RAP), a macrolide produced by a soil bacterium *Streptomyces hygroscopicus*, was initially isolated from a soil sample obtained from Easter Island (in its native term, *Rapa Nui*) 40 years ago by Ayerst Research Laboratories [75]. Although initially discovered as a drug that possessed antifungal activity, it was soon demonstrated that RAP showed potent immunosuppressive activity towards lymphocyte activation [76,77]. The interest in RAP's immunosuppressive activity was evoked with the discovery of FK506 by Fujisawa Pharmaceutical Laboratories where this drug was identified in a screen for natural products that inhibit IL-2 production, the major cytokine that drives T cell proliferation [78]. The similar chemical features of RAP and FK506 (distinctive hemiketal-masked α,β-diketopipecolic acid amidic component) (Fig. 1.4) provoked the interest of immunologists to examine its immunosuppressive activity. Early *in vitro* studies in lymphocytes comparing RAP and FK506 clearly showed that the mechanism of action of RAP was distinct from that of FK506 [76,79]. FK506 binds to a ubiquitously expressed intracellular receptor termed FK506 binding protein (FKBP12). It is now well established that the FK506-FKBP12 complex inhibits T cell proliferation by blocking the Ca2+-dependent phosphatase calcineurin, a critical phosphatase required for early cytokine gene transcription (e.g. IL-2) upon TCR activation [80]. Surprisingly, it was shown that although RAP binds to the same intracellular receptor FKBP12, it showed no activity against IL-2 gene transcription early in T cell activation [76]. Similar to FK506, RAP has two domains. The binding domain that interacts with FKBP12 is identical to FK506, but the effector domain that forms a composite surface with FKBP12 binds a different target

initially discovered as the target of rapamycin (TOR) protein using yeast genetics [81]. The mammalian orthologs were later isolated by five independent groups and the protein in mammals is now known as the mammalian target of rapamycin (mTOR; some investigators define the acronym as "mechanistic" target of rapamycin) [82–86].

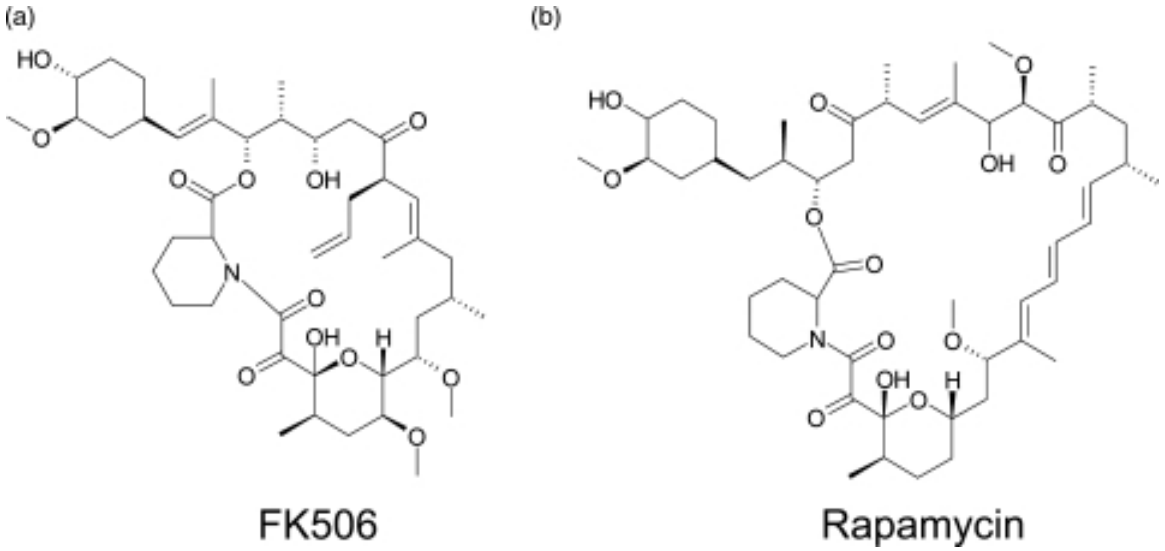

Figure 1.4. Structural comparison of FK506 and Rapamycin.

mTOR

mTOR is a serine/threonine kinase that forms two distinct complexes (mTORC1 and mTORC2). These complexes are defined by unique accessory proteins where the regulatory-associated protein of mTOR (Raptor) defines mTORC1 and the rapamycin-insensitive companion of mTOR (Rictor) defines mTORC2 (Figure 1.5) [87]. It is known that these subunits function as scaffolds for complex assembly and also for substrate binding. For example, a specific amino acid sequence called the TOR signaling (TOS) motif in mTOR substrates have been shown to interact with Raptor [88,89]. However,

whether this is universal for all mTOR substrates is still unclear, as other mechanisms exist to distinguish substrate selectivity (see below). Another subunit unique to mTORC1 is the 40 kDa Pro-rich Akt substrate (PRAS40) whereas mTORC2 contains the protein observed with Rictor 1 (PROTOR1) and PROTOR2. Both complexes share the mammalian lethal with SEC13 protein 8 (mLST8) and the DEP domain-containing mTOR-interacting protein (DEPTOR) (Figure 1.5) [87]. The detailed function of these subunits in controlling the activity of either mTORC1 or mTORC2 is partially known yet requires further investigation in the future and is not the focus of this dissertation. However, the fact that mTOR exists in these multiprotein complexes is important in the context of understanding the mechanistic action of RAP, which surprisingly remains elusive after 40 years since its discovery [90].

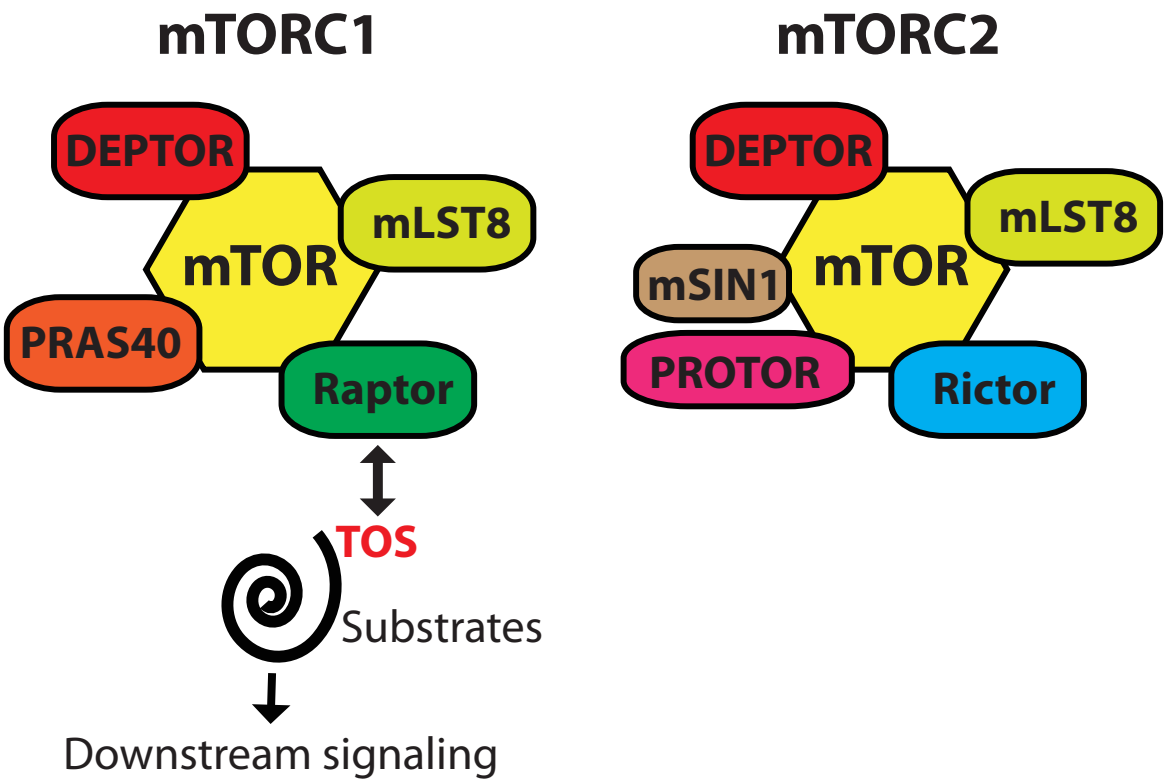

Figure 1.5. Components of mTORC1 and mTORC2
mTORC1 and mTORC2 are defined by Raptor or Rictor, respectively. Although no clear identifiable domains or motifs are found in Rictor, Raptor contains many protein-binding domains such as several HEAT repeats (also found in mTOR itself; see Figure 1.6) and WD40 domains. Some substrates of mTORC1 are recognized by Raptor through a unique amino acid sequence called the TOR signaling (TOS) motif. However, this is not the only way mTOR substrates are recruited to the active-site (see Figure 1.7 and [91]).

The immunosuppressive drug RAP inhibits the ability of mTORC1 but not mTORC2 to phosphorylate its substrates in mammals. In yeast, TOR is encoded by two genes, TOR1 and TOR2 that forms two distinct complexes as well (TORC1: composed of either TOR1 or TOR2, TORC2: composed of TOR2) where only TORC1 is RAP sensitive [92–94]. However, the precise molecular mechanism of how RAP inhibits mTORC1 activity is unclear. This is due to the fact that FKBP12-RAP does not inhibit mTORC1 kinase activity in an ATP-competitive fashion. Instead, FKBP12-RAP acts in an allosteric manner by binding to a region outside the kinase domain of mTOR, the FKBP12-RAP binding domain (FRB domain) (Figure 1.6) [95,96]. Deciphering the exact mechanism of how FKBP12-RAP allosterically inhibits mTORC1 activity has been hindered due to the lack of an atomic-level crystal structure of entire mTORC1.

Several studies have suggested that upon binding to the FRB domain, mTORC1 complex integrity is lost especially at the level of Raptor, a subunit required for substrate binding [97,98]. Although low in resolution (26A), cryo electron microscopy studies of purified human mTORC1 showed that *in vitro* incubation of purified mTORC1 with FKBP12-RAP causes rapid disassociation of the complex within hours [99]. Thus, dissociation of Raptor and preventing the binding of mTORC1 substrates seem to be a major mechanism of how treating cells with RAP abolishes phosphorylation of mTORC1 substrates. However, for some mTORC1 substrates such as the ribosomal protein S6 kinase1 (S6K1), FKBP12-RAP binding to Raptor-free mTOR completely inhibits phosphorylation of S6K1 suggesting a possible steric hindrance model of FKBP12-RAP action [99]. Recently, a major advance in the field came with a high-resolution (3.2A) atomic-level crystal structure of a partial N-terminus truncated form of mTOR in

complex with mLST8 (mTOR$^{\Delta N}$-mLST8) [91]. Purified mTOR$^{\Delta N}$-mLST8 showed similar kinase activity as mTORC1 towards its canonical substrates. According to the structure, the active site of mTOR is extremely restricted by the FKBP12-RAP binding domain (FRB) and an inhibitory helix protruding from the catalytic cleft. Superimposing the published FKBP12-RAP structure showed that upon binding, there is direct blockade of substrate recruitment as well as further restriction of the active-site (Figure 1.6) [91].

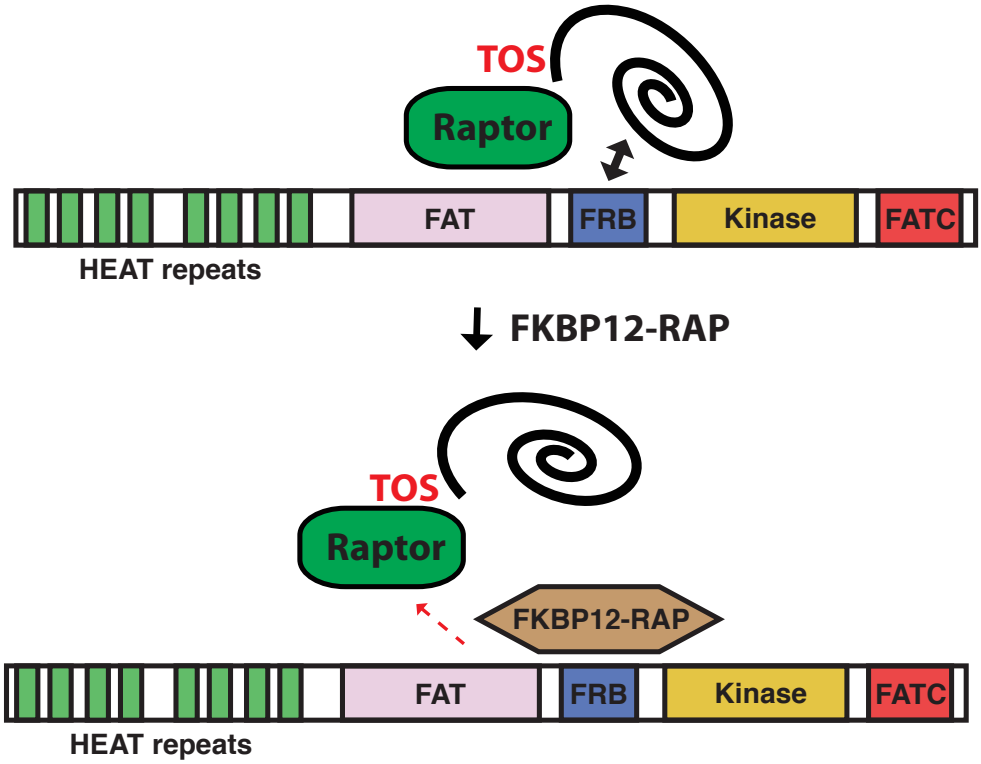

Figure 1.6. Current model of FKBP12-RAP mediated inhibition of mTORC1 activity.
FKBP12-RAP binding to the FRB domain causes disruption of Raptor-mTOR interaction thereby interfering with substrate recruitment. Purified mTORC1 is completely disassembled upon FKBP12-RAP incubation *in vitro* but whether this is also true *in vivo* is unclear. FKBP12-RAP also causes steric hindrance towards FRB mediated regulation of mTOR substrates [91,100]. This model is also illustrated in [101].

The most surprising findings about the mechanism of RAP action came with the recent discovery of second-generation mTOR kinase inhibitors that inhibit the active site (kinase domain) of mTOR in an ATP-competitive fashion (TOR-KIs) [102–106]. When TOR-KIs were compared with RAP, it was evident that in many cell types TOR-KIs inhibited the activity of mTOR kinase towards its substrates in a more complete fashion than RAP (RAP inhibits only ~25% of phosphorylation events suppressed by TOR-KIs) [107,108]. Therefore, although RAP may cause disassociation of mTORC1 and prevent substrate binding, this alone cannot explain its partial inhibition of mTORC1 revealed with the discovery of TOR-KIs. Complicating things further, it is also known that prolonged treatment with RAP in certain cell types can also inhibit mTORC2 activity by sequestering the cellular pool of free mTOR, thereby preventing mTORC2 assembly [109].

The evolutionarily conserved role of mTOR (TOR) signaling is to promote cell growth (mass increase) [110]. This is illustrated by the fact that from yeast to mammalian cells, the universal phenotype upon RAP treatment is a decrease in overall cell size. However, it has been difficult to separately study the role of mTOR in cell growth control as this process is often connected to other cell biological processes (e.g. cell proliferation, differentiation, and survival). How mTOR connects these processes is of immediate importance, as it is now known that most cancer cells have elevated mTOR activity [87,110–112]. At least in yeast cells, translation of the G1 cyclin CLN3 downstream of TOR connects cell growth to cell cycle entry. As such, forced expression of CLN3 can rescue the cells from the G1 arrest by RAP [113,114]. The situation is more complicated in mammalian cells as various extracellular factors can impinge on regulating mTOR from

upstream [87]. In addition, over the years, there has been an enormous increase in the identification of new substrates downstream of mTOR [87].

Upstream of mTOR

Unlike yeast cells where TOR activity is solely regulated at the level of nutrients, metazoan cells incorporate various extracellular factors including nutrients to activate mTOR. An evolutionarily conserved role of mTOR is to become activated by nutrients such as amino acids. There has been significant amount of research in understanding nutrient sensing mechanisms of mTOR that is outside the scope of this dissertation [115]. In addition, although intracellular growth factor signaling components such as PI3K and AKT have been linked to mTORC1 activation through AKT mediated TSC2 and PRAS40 phosphorylation [7], there are many cases where PI3K activity is disconnected from downstream mTORC1 activation where ERK/RSK dependent pathways can converge on mTORC1 activation. For instance, a BCR-ABL mediated transformed pre-B leukemic cell line lacking both p85α/β shows active mTORC1 signaling output despite complete absence of AKT phosphorylation [116]. Activation of downstream mTORC1 components in these cells was still sensitive to RAP suggesting that mTORC1 could be activated without PI3K activity. This disconnection between PI3K and mTOR activity is also seen in lymphocytes and is highlighted by the fact that genetic disruption of PI3K components and mTOR components in mice are usually distinct in terms of immune phenotypes (Figure 1.7) [16,17].

It should be noted that activation of mTOR by different extracellular factors is not simply an on or off switch similar to the PI3K rheostat concept. The ability of activated

mTOR to phosphorylate downstream substrates seems to be selective and context-dependent. This is logical for a protein such as mTOR that is ubiquitously expressed in all cell types and can sense so many different extracellular signals yet must give rise to specific cellular functions for the better of the organism as a whole.

Downstream of mTOR

The role of mTORC1 on cell growth control suggests that the signaling output converges on anabolic cellular processes. Indeed, mTORC1 activation has been linked to various anabolic processes such as lipid biogenesis [117], mitochondrial biogenesis [118], and nucleotide synthesis [119,120]. In addition, mTORC1 activation also leads to the suppression of catabolic processes such as autophagy [110,121]. However, the most studied cellular process downstream of mTORC1 is protein synthesis [122,123], which will be the major focus of this dissertation (Figure 1.7).

Two of the well-studied direct substrates of mTORC1 are the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BP1, 2 and 3) and S6Ks (S6K1 and 2) [124–127] both of which are implicated in protein synthesis. Direct phosphorylation of 4E-BPs on several residues prevents its binding to the cap-binding protein eIF4E thereby promoting the formation of the eIF4F translation initiation complex and cap-dependent translation [128]. Activation of S6K1 also promotes protein synthesis through a variety of effectors both at the level of translation initiation and elongation [123]. In hepatocytes, both S6K1 and S6K2 are required for the induction of a ribosome biogenesis transcriptional program upon re-feeding after starvation [129]. This in part required the ability of S6Ks to phosphorylate the ribosomal protein S6 as cells with a

phosphorylation-defective knockin mutant of S6 showed similar phenotypes. Interestingly, however, the defect in ribosome biogenesis was not translated into an overall translation initiation defect. Therefore, the role of S6Ks on the overall translation program seems to be specific but is still unclear. Initial studies showed that RAP only inhibited translation of certain transcripts bearing a 5' terminal oligopyrimidine (TOP) motif. Although S6Ks were suggested to regulate this specificity, this model has been refuted by studies using S6K1/2 deficient cells [130] and recent ribosome profiling studies that identified other motifs regulated by mTOR at the level of 4E-BPs [131,132].

Proteins are functional workhorses for majority of cellular processes. Thus, it is not surprising that protein synthesis regulation by S6Ks or 4E-BPs downstream of mTORC1 converge on other anabolic processes mentioned above. One example was shown recently in a Myc driven B cell lymphoma model where one isoform of the rate-limiting enzyme for nucleotide synthesis, phosphoribosyl pyrophosphate synthetase 2 (PRPS2), was under translational control by eIF4E downstream of Myc induced mTORC1 activation (Figure 1.7) [133]. It is of interest whether other anabolic processes are also regulated by mTORC1 mediated protein synthesis.

Lastly, downstream mTORC1 effectors such as S6Ks can regulate anabolic processes independent from protein synthesis as well. Activation of carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotatase (CAD), a key enzyme responsible for *de novo* pyrimidine synthesis, is increased by S6K1 directly through phosphorylation [119,120]. S6Ks are also responsible for activation of a lipid biogenesis program through activation of sterol regulatory element-binding proteins (SREBP1 and SREBP2) at the transcriptional level (Figure 1.7) [134].

As mentioned above however, activation of mTORC1 and its efficiency of substrate phosphorylation seem to be selective for a given context (Figure 1.7). One model for this selectivity for mTORC1 substrates have been proposed recently by Sabatini and colleagues where amino acid sequences surrounding the phosphoacceptor site of an mTORC1 substrate determined its sensitivity to not only nutrient status but also RAP [135]. Another possibility of differential phosphatase activity for each substrate was excluded in this study where upon TOR-KI treatment, a drug that completely inhibits all mTORC1 kinase activity, all currently known mTORC1 substrates were dephosphorylated to the same degree with similar kinetics. According to this substrate quality model, substrates with nonpolar or hydrophobic residues around the phosphoacceptor site become a high quality mTORC1 substrate and RAP-insensitive (e.g. 4E-BP1 at T37/46). In contrast, substrates harboring polar or hydrophilic residues are mainly poor quality mTORC1 substrates that are highly RAP-sensitive (e.g. S6K1 T389). However, without an atomic level crystal structure of complete mTORC1, it remains unclear as to how these amino acid differences generate substrate selectivity (Figure 1.7).

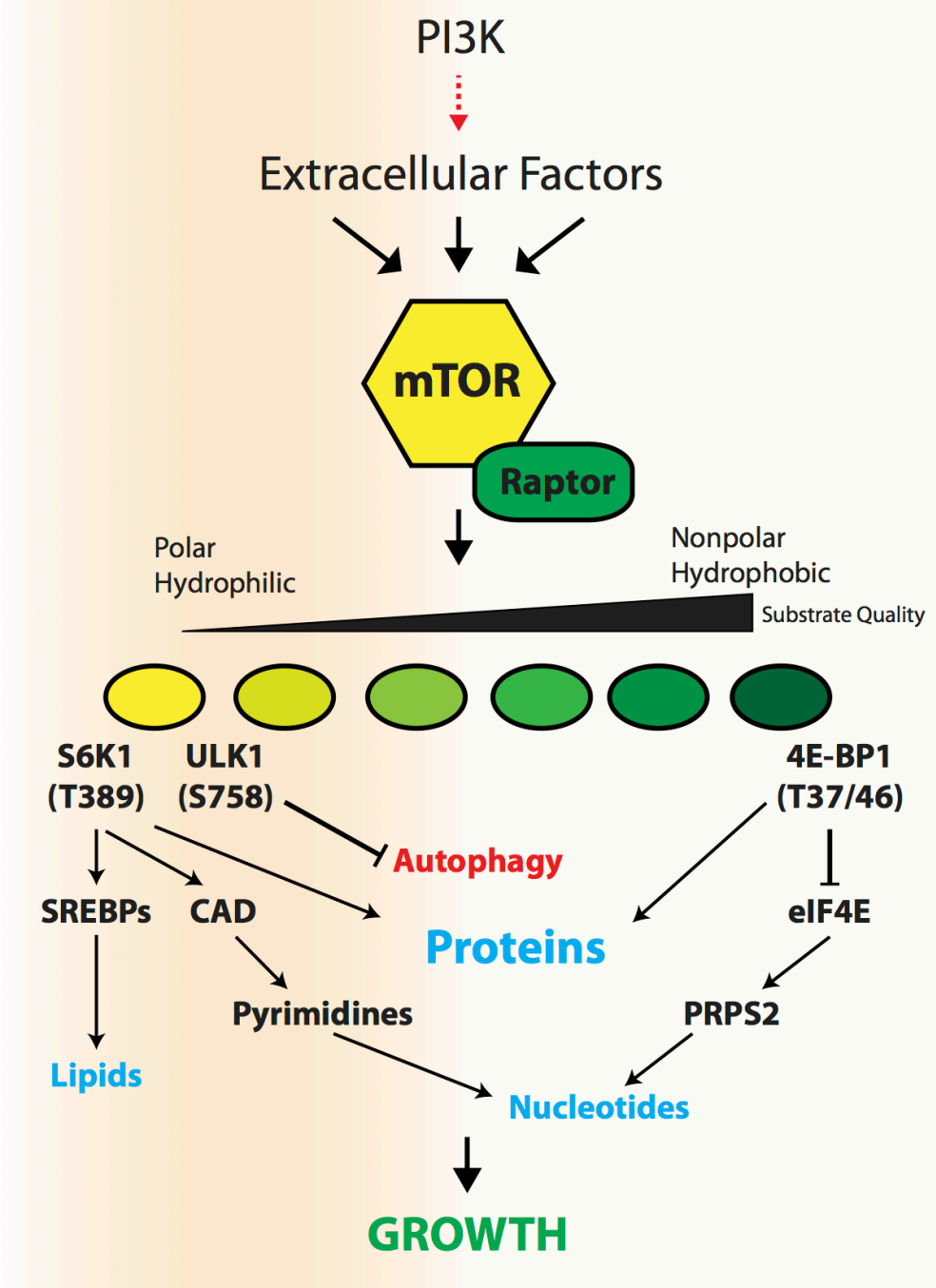

Figure 1.7. Upstream and downstream of mTORC1 and the substrate selectivity model.
Although PI3K/AKT is an upstream mTORC1 activator, the direct connection is often lost in many cell types [17,116]. Various extracellular factors can activate mTORC1 where it promotes anabolic processes (lipid biogenesis, protein synthesis, and nucleotide synthesis) and suppresses catabolic processes such as autophagy. Substrate selectivity has been observed in part determined by the amino acid sequence surrounding the phosphoacceptor site of an mTORC1 substrate (for details, see [135]). Protein synthesis is the major process upregulated by active mTORC1 but is often connected to other anabolic processes as translation of key enzymes such as PRPS2 is directly under the control of the mTORC1 effector 4E-BP/eIF4E [133].

mTOR and Immunity

Most of the components in the mTOR signaling network have been discovered using cell lines where ample amounts of material can be easily obtained for biochemical studies. However, it was the potent immunosuppressive activity of RAP in assays of primary lymphocyte function that allowed researchers to become interested in understanding this novel signaling pathway using RAP as a molecular probe [75,136]. The difficulty of genetic manipulation as well as the low amounts of material for biochemical studies of primary resting lymphocytes has hindered our understanding of this pathway in an immune cell specific context. This highlights an important point relevant to this dissertation in that the effects of RAP in inhibiting cell growth and proliferation indeed seem to be cell type specific [75]. Over two decades of research on RAP has shown particular potency of this drug in terms of a more complete G1 to S transition block on cells of lymphoid origin. Surprisingly, how this selectivity is achieved in a cell type specific manner is still unclear. With the discovery of various mTOR components and subsequent conditional knockout approaches, we are now just beginning to understand how this intricate signaling network regulates immune cell function. Although I will only focus on the role of mTOR signaling in mature lymphocytes for the purpose of dissertation, recent studies strongly suggest that mTOR is also involved in immunomodulation through regulation of other immune cell types such as NK cells, macrophages, and dendritic cells [137].

mTOR in mature T cells

The first genetic study of the role of mTOR in immune cells was through the conditional deletion of this protein in mature T cells using *CD4Cre* where the floxed mTOR gene was deleted at the double positive stage of T cell development [138]. Early studies of the potent immunosuppressive activity of RAP towards T cell activation already suggested a primary role of mTORC1 in T cell proliferation [76]. As expected, mTOR deficient T cells show a profound defect in their proliferative potential [138]. The surprising results came in the context of Th differentiation experiments. In the absence of mTOR, the default fate of an activated CD4+ T cell under neutral activation conditions was to become Tregs [138]. Further studies from the same group have identified distinct roles of mTORC1 and mTORC2 in regulating different Th subsets by genetic inactivation of either mTORC1 (initially through Rheb deletion) or mTORC2 (Rictor deletion). Rheb (a G protein where its GTP-bound state is hydrolyzed by TSC2, a GAP for Rheb; hence Rheb is a positive regulator of mTORC1 activity) deficient CD4+ T cells were initially reported to have a Th1 and Th17 differentiation defect whereas Rictor deficient CD4+ T cells showed a Th2 defect (partly shown in Figure 1.3) [139]. However, the role of the mTOR complexes in regulating the different Th subsets is still unclear, as subsequent studies using other genetic models suggest different results [140–142]. For example, deletion of Raptor or Rictor relatively early in T cell development using *LckCre* generates a different phenotype. mTORC1 deficiency by Raptor deletion in this case selectively caused a Th17 defect without affecting Th1 differentiation [141]. Similarly, Rictor deletion (mTORC2 inactivation) by *LckCre* caused a greater phenotype extending to a defect not only in Th2 but also Th1 differentiation [140]. Recently, this system has been re-examined

once more with *CD4Cre* mediated deletion of Raptor. Interestingly, unlike the initial mTORC1 inactivation model using Rheb deletion, Raptor deficient CD4+ T cells showed a defect also in Th2 differentiation [142]. These contradicting results could potentially be due to differences in T cell activation conditions. Alternatively, as a model has been proposed where mTOR acts as a master regulator that senses extracellular signals from the immunemicroenvironment [143–146], differences in serum factor composition from different labs can also result in these discrepancies. Extensive discussion about the role of mTOR in regulating Th differentiation has been reviewed elsewhere and is not the focus of this dissertation [143].

One consistent result from all the studies however is that mTORC1, but not mTORC2 [142], is critical for T cell growth and proliferation. Hence, a major hurdle is that the specific role of mTOR signaling in Th differentiation cannot be separated from the effects of mTORC1 in regulating T cell proliferation, which has been shown to be directly linked to differentiation [147] (Figure 1.8). This suggests that the mechanism of RAP acting as an immunosuppressant is mainly through inhibition of T cell proliferation and that modulation of Th differentiation may be secondary.

An important question is how mTORC1 regulates T cell proliferation. T cell (also B cells as discussed below) activation is a unique process where antigen receptor engagement results in a relatively long phase (36-44h) of pure cell growth without any evidence of S phase entry (Figure 1.8) [148]. After commitment to S phase, T cells divide rapidly where the cell cycle time has been reported to be approximately 6-7h in vitro [148,149]. Consistent with mTOR having a fundamental role in cell growth control, mTOR deletion or RAP treatment profoundly blocks this early increase in cell mass (Figure 1.8).

At least in human T cells activated by a non-specific stimulus such as PMA and ionomycin, it was shown that the delay in cell cycle entry by RAP correlated with the delay in early increase in cell mass. Once the T cells reached the same size when vehicle treated T cells entered S phase, RAP treated T cells also committed entry into the cell cycle [150]. These results suggest that RAP-sensitive biomass increase is critical for T cells to enter cell cycle. Indeed, Raptor deficient T cells have a profound defect in anabolic processes such as de novo lipid synthesis and glycolysis both at the transcriptional and post-transcriptional level [142]. However, the question of which mTORC1 effectors downstream is required for this early biosynthesis upon activation is surprisingly still unknown. This question will be the focus of chapter 4.

mTOR in mature B cells

Early studies with RAP also showed a potent immunosuppressive activity of this drug against B cell activation [77]. The first genetic model of mTOR inactivation in B cells was through a transcriptionally inactivating knockin mTOR hypomorphic mouse model where mTOR protein levels were reduced by 70% [151]. Interestingly, mTOR hypomorphic B cells displayed a defect in B cell differentiation and homeostasis. As this mouse model is hypomorphic for mTOR in all tissues, the same group further generated a conditional knockout of mTOR specifically in B cells using *CD19Cre* [152]. B cells lacking mTOR showed lower peripheral B cell numbers suggesting an intrinsic role of mTOR in regulating B cell development and/or survival. In addition, mice lacking mTOR specifically in B cells showed a defect in germinal center formation and decrease in class-switched antibody titers against a T-dependent antigen immunization. This effect on

antibody responses is most likely due to the regulation of B cell proliferation by mTOR as RAP treatment profoundly blocks the ability of B cells to proliferate by various stimuli [77].

mTORC2 also seems to play a role in B cell development and/or survival. This is not surprising given that mTORC2 is the upstream kinase required for full AKT activity that has been shown to be critical for B cell development and survival *in vivo* [153]. Boothby and colleagues investigated the effects of chronically deleting Rictor before lymphopoiesis using a *VavCre* driver mouse [154]. This resulted in the impaired development of three mature B lymphocyte subsets: follicular (FO), marginal zone (MZ), and B-1a. Similar results were obtained when Rictor was acutely deleted using a tamoxifen-inducible Cre system. The remaining B cells in the absence of Rictor also showed a defect in survival in part due to decreased AKT signaling and attenuated NFκB induction that contributed to the induction and suppression of prosurvival and proapoptotic genes, respectively. Interestingly, B cells with attenuated but not complete absence of Rictor/mTORC2 activity do not show significant survival or developmental defects. This condition was observed in our lab with a conditional deletion of the Rictor allele using *Cd19Cre* where Rictor protein levels were reduced but not completely (see chapter 3). Notably, this genetic approach to partially attenuate mTORC2 activity completely phenocopied the enhanced B cell class-switch recombination (CSR) phenotype seen with suboptimal doses of TOR-KIs both in *in vitro* assays and *in vivo* immunization with T-dependent antigens (chapter 3 and [155]). Thus, these results genetically confirm the mechanism as to how TOR-KIs can enhance one type of adaptive immune response through selective inactivation of mTORC2 activity.

Similar to T cells, B cells also initiate a long growth response followed by rapid divisions to ultimately differentiate into different effector cell fates. RAP has been known to potently block both growth and proliferation of B cells by various stimuli [77,155] suggesting again that the primary node of action of RAP is through potent inhibition of B cell proliferation even before a stage where they can differentiate into effector cells (Figure 1.8). Accordingly, high concentrations of TOR-KIs that sustain mTORC1 inhibition can inhibit CSR to the same extent as RAP that is mainly due to a block in proliferation [155]. In addition, Raptor deficient B cells show a significant proliferation defect and cannot undergo CSR [155]. However, similar to T cells, it remains unclear which effectors downstream of mTORC1 are critical for this action.

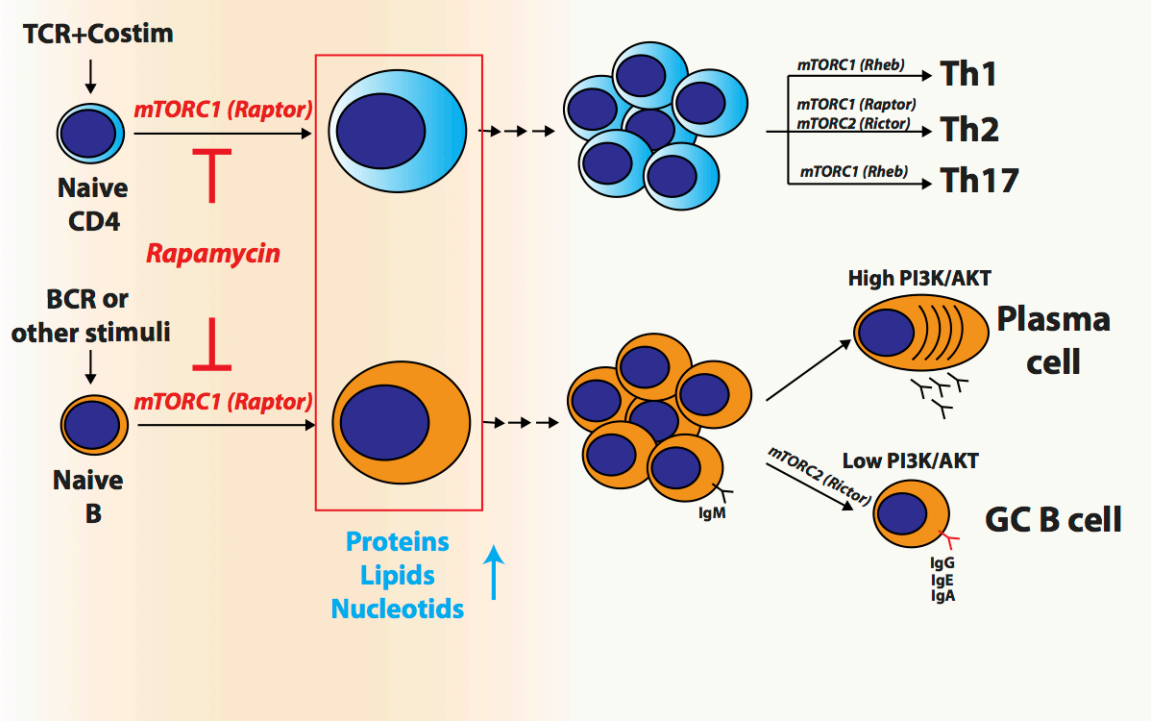

Figure 1.8. mTORC1 primarily regulates T or B cell mediated adaptive immunity through initial blastogenesis (growth) and clonal expansion (division). Naïve resting T or B cells undergo a long phase of growth known as blastogenesis upon receiving appropriate stimuli that is followed by a rapid expansion phase directly linked to effector differentiation. The blastogenesis phase is accompanied by increases in cellular biomass (proteins, lipids, nucleotides etc) where mTORC1 plays a critical role evidenced by RAP treatment and genetic loss of Raptor. The role of mTOR signaling in CD4 T cell differentiation fate is not entirely clear as different genetic models of mTORC1 or mTORC2 inactivation result in distinct differentiation phenotypes. Shown are currently available models of genetic inactivation of either mTORC1 or mTORC2 (gene deleted for each model is in parenthesis). PI3K/AKT activity has been shown to dictate B cell differentiation fate [61] where high PI3K/AKT activity promotes direct differentiation of B cells into antibody secreting plasmablasts that mainly secrete IgM. Low PI3K/AKT favors B cells to become germinal center B cells where CSR occurs. This results in the generation of higher affinity and different isotypes of antibodies. Attenuated inactivation of mTORC2 through partial deletion of Rictor in B cells result in enhanced CSR in part through lower AKT activity [155].

The Rapamycin Paradox

Studies in the Fruman lab using TOR-KIs in pre-B cell leukemia models and normal lymphocyte function have elucidated an apparent paradox as to the action of RAP [155,156]. RAP is a well-known partial mTORC1 inhibitor suggested by many studies of fibroblasts and a variety of cancer cell lines, yet when compared with minimal doses of TOR-KIs that fully block mTORC1 activity in primary lymphocytes, RAP showed a similar potency in terms of inhibition of cell growth and proliferation. This is distinct from the effects of RAP versus TOR-KIs in many other cell types where RAP consistently shows an intermediate effect on cell proliferation that is mainly attributed to its weaker effects on 4E-BP dephosphorylation [103–105,156]. These results suggest at least two possible mechanisms of action of RAP in selectively suppressing primary lymphocyte activation.

One plausible model is that due to its allosteric nature of inhibition, FKBP12-RAP inhibits a kinase-independent scaffolding function of mTOR that is critical for lymphocyte activation. Indeed, a kinase-independent function of mTOR has been reported to regulate skeletal muscle regeneration [157].

Another model would suggest that a common mTORC1 effector(s) that is equally sensitive to RAP and TOR-KIs is critical for lymphocyte growth and proliferation. An ideal candidate is the S6Ks as they are heavily implicated in cellular and organismal size control [130,158–161]. For a cell type such as lymphocytes that require a long phase of extensive cell growth prior to cell proliferation [149], it is possible that S6Ks are critical. In fact, rapid inactivation of S6K activity by RAP was observed in an IL-2 dependent T cell line even before the discovery of mTOR and was proposed to be the critical effector for

G1 to S transition of these cells [126]. On the other hand, in a similar cytokine-dependent T cell line, expression of a RAP-resistant S6K was not sufficient to restore proliferation upon RAP treatment [162]. However, after more than two decades, there has been no loss of function genetic approaches to interrogate the function of S6Ks in primary lymphocyte activation.

In this dissertation, both models mentioned above have been tested in primary resting lymphocytes (both T and B cells) and will be presented in chapter 4. Surprisingly, the data argue against either of these models, instead favoring the conclusion that the 4E-BP/eIF4E axis drives lymphocyte growth and proliferation in a RAP-sensitive manner.

Coordination of Cell Growth and Proliferation in Metazoans

Most fundamental cellular processes are conserved throughout evolution. In this last part of the introduction, I would like to discuss the concept that although key components of fundamental processes may be conserved as seen for the cell growth regulator TOR, additional layers of complexity and regulation exist that are distinct in multicellular organisms. The coordination of cellular growth to subsequent proliferation will be used as an example to illustrate this complexity, as it will be the focus of chapter 4.

Separation of Cell Growth and Proliferation in Mammals

How fundamental processes such as cell growth and proliferation are coordinated is a fascinating question yet to be answered in cell biology [163]. Classical genetic studies using the unicellular organism yeast have uncovered many aspects of this coordination,

where experimental evidence supports the existence of some kind of size checkpoint that ensures cells to divide only after they reach a certain size [164–166]. These results suggest that cell division is growth-dependent. However, the size checkpoint mechanism does not seem to be evolutionarily conserved in many types of cells from multicellular organisms.

In fact, much evidence in certain types of mammalian cells suggests that proliferation rate is independent of cell size (Figure 1.9). For example, the average cell volume of mouse embryonic fibroblasts or myoblasts lacking the mTORC1 effector S6Ks is smaller in G1 phase of the cell cycle yet they proliferate at the same rate as WT cells [130,159,160,167]. Interestingly, addition of RAP or genetic loss of raptor (thereby mTORC1 loss) did not further decrease the size of these cells [159,167]. Despite the plethora of processes that S6Ks regulate such as protein synthesis, nucleotide synthesis, and lipid biogenesis, the small cell phenotype of S6K deficient fibroblasts seems to be due specifically to a lack of S6 phosphorylation [168]. Fibroblasts with a knockin mutation of S6 lacking all five phosphorylation sites ($rpS6^{P-/-}$) exhibit the same defect in cell size that is no longer inhibited by RAP [169]. Importantly, the $rpS6^{P-/-}$ fibroblasts have a faster proliferation rate compared to WT cells suggesting again that proliferation can be growth independent.

One important question that has never been answered in this context of mTORC1 signaling is how this separation of growth and proliferation is achieved in the *in vivo* setting. For example, could there be a situation *in vivo* where activated mTORC1 preferentially signals to a particular effector for growth independent from proliferation, or vice versa? More importantly, the physiological relevance of separating the two processes in mammalian cells is unclear. One can speculate that perhaps for such organ-resident

cells in a multicellular system, an additional layer of regulation is required for maintaining the size, shape, and function of the organism as a whole [163,170,171]. Understanding this coordination in mammals is extremely important beyond its fundamental nature that stimulates our curiosity. When individual cells fail to adhere to this growth and proliferation regulation within the normal program of animal development, deleterious outcomes can occur including autoimmunity, cancer and tissue overgrowth syndromes (Figure 1.9) [172,173].

Extracellular Control of Growth and Proliferation

Separation of cell growth and proliferation in mammals would be possible if cell-to-cell signaling through extracellular factors controls the precise balance between the rate of these processes to ensure proper organ or organism size as a whole (Figure 1.9) [163,164,174]. This extracellular regulation postulation has been experimentally tested by Raff and colleagues using primary rat Schwann cells [174]. Under axon myelinating conditions, Schwann cells undergo massive growth after exiting the cell cycle, clearly suggesting that growth happens independent from the cell cycle state. However, upon injury, these quiescent cells can re-enter the cell cycle to increase in number [175]. In *in vitro* culture systems, Rat primary Schwann cells can indeed achieve this separation by different extracellular factors that are normally present together in serum. Specific addition of IGF-1 to serum-free media selectively promoted growth whereas glial growth factor (GGF) specifically promoted cell cycle entry [174]. The search for certain extracellular factors that promote the survival, growth, and proliferation of fibroblasts has a long history where Zetterberg and colleagues used 3T3 cells to show that addition of distinct

growth factors normally present in serum can promote growth or proliferation independently [176].

An unanswered question is how these extracellular factors ultimately influence the growth and proliferation of cells through intracellular signaling pathways. Another important question is whether the intracellular mediators of an extracellular response are utilized the same way across the vast variety of cell types in a multicellular organism that possess distinct functions [170]. The latter question has been addressed in the context of mTORC1 signaling in certain cell types by loss of function approaches. Sonenberg and colleagues have studied two of the well-known mTORC1 effectors S6Ks and 4E-BPs in regulating either cell growth or proliferation. As mentioned above, in fibroblasts, S6K deficiency resulted in a decrease in cell size of cells in G1 phase. However, this size decrease had no functional effect on the ability of these cells to divide, as proliferation rate was similar to WT fibroblasts [167]. Notably, the effects of mTORC1 inhibition either genetically (Raptor knockdown) or pharmacologically using TOR-KIs were largely restored in fibroblasts lacking 4E-BP1 and 4E-BP2. This restoration of cell proliferation was size independent, as mTORC1 inhibition mediated cell size decrease could not be rescued by 4E-BP deficiency [167]. These results suggest that 4E-BPs are key mediators that regulate cell proliferation independent from cell growth that seem to be mediated through S6Ks in fibroblasts. Similar results were also shown in proliferating myoblasts where S6K1 deficient myoblasts are smaller yet proliferate at a same rate as WT myoblasts [159]. The utilization of these well-known mTORC1 substrates seem to be distinct however in other cell types. For instance, using the same loss of function approach, Pende and colleagues showed that regenerating hepatocytes after partial

hepatectomy requires S6Ks for both cell growth and proliferation [177]. Whether this specificity comes from cell type difference or the specific extracellular context (liver damage) is unclear.

One example of cell growth and proliferation in response to unique extracellular stimuli has been investigated in chapter 4, where I investigated the coordination of growth and proliferation in mouse primary lymphocytes. Lymphocytes are usually in a naïve resting state *in vivo* and culturing in optimal growth media *in vitro* has no discernable effect on basal growth or proliferation. In fact, without the specific extracellular survival factors usually present *in vivo* (e.g. IL-7 for T cells and BAFF for B cells), these cells undergo progressive atrophy *in vitro* as a result of cell death [178–180]. Only upon antigen-receptor engagement, lymphocytes undergo an enormous increase in growth followed by rapid proliferation [149,181] (Figure 1.8). As sentinels of our adaptive immune system that need to rapidly eradicate an invading pathogen, lymphocytes have been proposed to specifically require coupling of growth and proliferation pathways to produce a large number of identical cells in a relatively short period of time. Interestingly, most of these activated lymphocytes are programmed to undergo cell death upon antigen clearance and only a few remain in the body as memory cells (Figure 1.9) [182–184] . Genetic deletion of Raptor or treatment with RAP potently blocks this growth and proliferation of activated primary lymphocytes suggesting that mTORC1 activation is critical downstream of the antigen receptor (Figure 1.8) [76,77,142] yet the downstream mTORC1 effector remains elusive. The data presented in chapter 4 strongly argues that unlike the organ-resident cells discussed above where growth and proliferation could be separated, both processes were coupled through a single mTORC1 effector, the 4E-BPs.

The implications of these findings in the context of basic cell biology as well as its clinic relevance will be discussed in chapter 5.

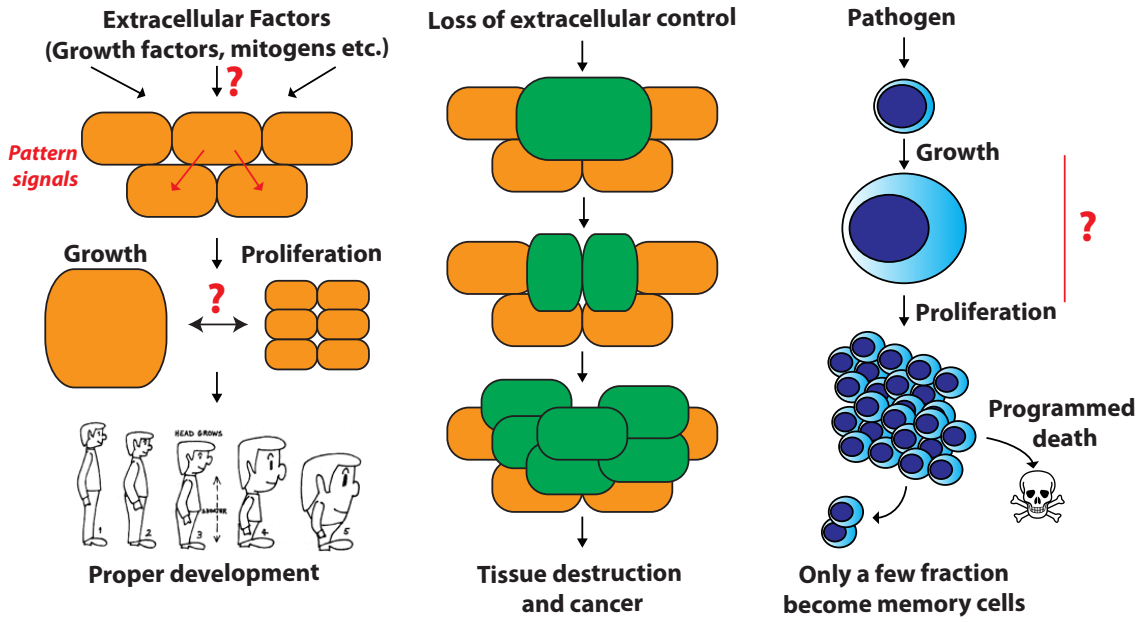

Figure 1.9. Coordination of cell growth and proliferation in a multicellular system. Organ resident cells (depicted in far left) clearly show separation of growth and proliferation control, which is thought to ensure proper size of an organ or organism as a whole. This type of separation can happen if cells are under extracellular control by various factors either in the form of growth factors, nutrients, or pattern signals that may aid in determining shape as well. Key questions as to how cells sense each extracellular factor or ultimately how growth and proliferation reaches a balance to control organ size is unknown (red question marks). An individual cell within this program may lose this adaptation to multicellularity (middle) by numerous mechanisms such as mutations in oncogenes or tumor suppressors, which will make the cell insensitive to extracellular cues. This normally results in tissue outgrowth and in late stages of cancer, these cells also develop properties to move to different areas in the body where they do not belong (known as metastasis; not shown in figure). Some mammalian cells such as lymphocytes (far right) have a unique requirement to grow and divide as fast as possible regardless of neighboring cells as in outgrowing cancer cells. However, upon clearance of the pathogen, the majority of lymphocytes never remain in the body (except for memory cells) because they are programmed to undergo cell death. How lymphocyte growth and proliferation is coordinated is unknown (red question mark) yet mTORC1 is required for both processes (see text).

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CHAPTER 2:

SELECTIVE TARGETING OF THE CANCER DRUG TARGET p110α ISOFORM (*PIK3CA*) OF PI3K PRESERVES PRIMARY LYMPHOCYTE FUNCTION

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ABSTRACT

Class IA phosphoinositide 3-kinase (PI3K) is essential for clonal expansion, differentiation, and effector function of B and T lymphocytes. The p110δ catalytic isoform of PI3K is highly expressed in lymphocytes and plays a prominent role in B and T cell responses. Another class IA PI3K catalytic isoform, p110α, is a promising drug target in cancer but little is known about its function in lymphocytes. Here we used highly selective inhibitors to probe the function of p110α in lymphocyte responses *in vitro* and *in vivo*. p110α inhibition partially reduced B cell proliferation, and diminished survival supported by the cytokines BAFF and IL-4. Selective p110δ inhibition suppressed B cell responses much more strongly, yet maximal suppression was achieved by targeting multiple PI3K isoforms. In mouse and human T cells, inhibition of single class IA isoforms had little effect on proliferation, whereas pan-class I inhibition did suppress T cell expansion. In mice, selective p110α inhibition using the investigational agent MLN1117 (previously known as INK1117) did not disrupt the marginal zone B cell compartment and did not block T cell-dependent germinal center formation. In contrast, the selective p110δ inhibitor IC87114 strongly suppressed germinal center formation and reduced marginal zone B cell numbers, similar to a pan-class I inhibitor. These findings show that selective p110α inhibitors are likely to be less immunosuppressive *in vivo* compared with p110δ or pan-class I inhibitors.

SUMMARY

Class I PI3Ks are categorized based on their ability to use phosphatidylinositol (PtdIns)-4,5-bisphosphate as a substrate to generate PtdIns-3,4,5-trisphosphate (PIP_3) [1]. A hallmark of cancer cells is an elevation in PIP_3, and targeting class I PI3K is a priority in cancer drug discovery [2–4]. Each of the class I PI3K catalytic isoforms has been implicated in tumorigenesis and/or maintenance. Of the class I PI3K catalytic isoforms, p110α has received the most attention because gain-of-function mutations in the *PIK3CA* gene encoding this enzyme are very common in human cancer [5]. Mouse models have shown that *PIK3CA* mutations can be drivers of tumorigenesis [6,7] and cell line studies have shown that *PIK3CA* mutation status correlates with sensitivity to inhibitors of p110α [8,9].

It is now appreciated that tumor growth and survival can be either restrained or promoted by cells of the immune system [10–12]. Consequently, it is important to understand how novel anti-cancer drugs impact immune cells. The ideal targeted therapy would enhance anti-tumor immunity while preserving patient immunity to infection. Two class I PI3K isoforms that are highly expressed in leukocytes, p110γ and p110δ, are known to have pleiotropic functions in a variety of immune cells [1,13]. For years there have been useful reagents to study p110γ and p110δ, including selective small molecule inhibitors and mouse strains with null mutations, conditional alleles, and kinase-inactive knockin alleles. By contrast, little is known about p110α function in the immune system even though this isoform is expressed ubiquitously. Mice with null or kinase-inactive alleles of *Pik3ca* die during embryonic development [14–16]. B cell-specific deletion of *Pik3ca* did not reveal a unique function of p110α, but suggested a redundant function with p110δ in peripheral B cell survival [17]. p110α deletion in B cells was accompanied by increased

p110β expression, potentially compensating for p110α loss [17]. Identifying the acute effects of p110α inhibition has been hindered by the absence of highly selective small molecule inhibitors.

In this study, we made use of rationally designed compounds with high selectivity for p110α relative to other PI3Ks and to other cellular kinases. The results provide the first evidence that selective p110α inhibition has a minimal effect overall on B cell and CD4 T cell function, especially when compared to p110δ inhibition. These findings support the hypothesis that p110α inhibitors in clinical trials will not strongly suppress adaptive immune function.

MATERIALS AND METHODS

Antibodies

For phospho-flow staining, rabbit antibodies specific for phosphorylated proteins were from Cell Signaling Technologies: Akt (#4060), rS6 (#5364). For flow cytometry and immunohistochemistry, anti-mouse antibodies were: CD4-PE, B220-PE, IgD-eFluor405, IgM-FITC, CD21-FITC, CD23-PE, CD24-PE, CD11d-APC, MOMA-FITC, GL7-AlexaFluor647, hCD3-APC, hCD19-PE, DyLight604-conjugated goat anti-rabbit antibody, and streptavidin-conjugated PE or APC (SA-PE/SA-APC). All flow cytometry antibody reagents were purchased from eBioscience and Biolegend. Succinimidyl ester 5-(and -6) carboxyfluorescein diacetate (CFSE), 7-amino-actinomycin D (7AAD), Fluo-3, and Fura Red were obtained from Invitrogen.

Intracellular Phosphostaining

Intracellular phosphorylation of Akt (S473) and S6 (S240/44) was performed as previously described (21). Briefly, single-cell suspensions of murine splenocytes from 8-12 week old Balb/c mouse were first treated with ACK lysis buffer to remove RBCs. A total of 10^6 cells were treated with different inhibitors for 15 minutes prior to stimulation using 10 μg/ml anti-IgM F(ab')2 (Jackson Immunoresearch) for 15 minutes in a 37 °C water bath. Cells were immediately fixed with 16% paraformaldehyde (PFA) stock solutions for a final 1.6 % concentration for 10 minutes at RT. Cells were subsequently washed, then permeabilized with ice-cold methanol for 20 minutes on ice. Before

staining, cells were washed twice in FACS buffer (PBS containing 0.5 % BSA and 0.02 % sodium azide). Unconjugated primary antibodies were added (p-Akt (S473) 1:50, p-S6 (S240/44) 1:200) for 1hr at RT. Samples were washed once with FACS buffer and an antibody mixture containing DyLight604-conjugated goat anti-rabbit antibody (1:300) and B220-PE antibody (1:200) was added for 30 minutes on ice. Specifically for pAkt (S473) analysis, the signal was amplified by using a biotin-conjugated donkey anti-rabbit antibody (1:300; Southern Biotech) for 30 min at RT before adding B220-PE and SA-APC. After staining, cells were washed once with FACS buffer and analyzed on a FACS Calibur equipped with 488 nm and 635 nm laser lines.

In vitro cell proliferation

SK-OV-3 and U87MG cell lines were obtained from ATCC. A total of 5000 cells/well in low serum media (0.2 % FBS) were seeded in triplicate wells of a 96-well flat bottom culture plate for 18h to adhere. Media was aspirated and inhibitors in 0.2 % FBS media were added to each well at the indicated concentrations. After 48h, cell viability was determined using the MTS assay (Cell Titer 96 Aqueous One solution cell proliferation assay kit; Promega) with absorbance (490nm) measured in a microplate spectrophotometer.

B cell culture

B cells were purified by negative selection using the B cell isolation kit (Miltenyi). For CFSE proliferation assays, purified B cells were labeled with 5 μM CFSE in PBS containing 2% FBS for 5 min at RT. CFSE labeled B cells were cultured for 3d in

the presence of the indicated inhibitors at a density of 10^6 cells/ml either in 24-well or 48-well plates in lymphocyte media (LCM; RPMI medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 5 mM HEPES buffer, and 50 mM 2-ME). For activation, 10 μg/ml anti-mouse IgM F(ab')2 (Jackson) with/without IL-4 (10 ng/ml) (R&D Systems) or 10 μg/ml LPS (Sigma) was used. For survival assays, purified B cells were cultured with either IL-4 (20 ng/ml) or BAFF (60 ng/ml) (Peprotech) for 2d in culture. Cells were harvested and stained for 7AAD to analyze cell death by flow cytometry. For human B cells, PBMCs were isolated using Ficoll separation from whole blood obtained from the Institute for Clinical and Translational Science (ICTS) at UCI. PBMCs were labeled with CFSE as described above and were activated with anti-IgD dextran (400 ng/ml) and human IL-4 (20 ng/ml) (R&D Systems). After 3 days, cells were harvested and stained with human CD19-PE antibody to gate on B cells.

T cell culture

RBC lysed total splenocytes from DO11.10 TCR transgenic mice were labeled with CFSE at a final concentration of 5 μM as described above. After 15 min pre-treatment with the indicated inhibitors, cells were subsequently activated using 10 nM OVA peptide 323-339 (Anaspec) for 3d in LCM. After 24h, 100 L of supernatants from each sample were collected for cytokine ELISAs. After 3d, cells were harvested and stained with a CD4-PE antibody to gate on TCR transgenic CD4 T cells. For human T cells, PBMCs were isolated from whole blood obtained from the ICTS at UCI. Monocytes and lymphocytes were then purified using a countercurrent elutriation method. On average, 80-90 % of the cells were human CD3+ based on flow cytometry.

Cells were labeled with CFSE as described above and were activated with 1 g/ml PHA (Sigma) for 3d in LCM. After 3d, cells were harvested and stained with human CD3-APC antibody to gate on total T cells.

Cytokine ELISA

Supernatants from 24h activated DO11.10 CD4 T cells were used to detect both mouse IL-2 and IFNg levels using the Ready-Set-Go ELISA kit (eBioscience). Supernatants from 24h PHA activated human T cells were used to detect both human IL-2 and IFN levels using the Ready-Set-Go ELISA kit (eBioscience).

Calcium Flux Assay

Splenocytes were stained with CD1d-biotin, SA-APC, and CD24-PE antibodies prior to loading with the calcium indicator dyes Fluo-3 and Fura Red as described previously (22). Cells were pretreated with the indicated inhibitors for 15 min at 37 °C. The baseline level of Fluo-3/Fura Red was collected for 1 min on a FACS Calibur before cells were stimulated with 10 μg/mL anti-IgM for 7 min. Cells were then stimulated with 50 ng/mL ionomycin and acquired for an additional 1 min as a positive control.

In vivo dosing of PI3K inhibitors

Wild-type 8 week-old Balb/cJ mice (Jackson Labs) were used for all experiments. MLN1117 and GDC-0941 were given by oral gavage using a sterile disposable 20G-1.5'' feeding needle (Fisher). IC87114 was delivered via intraperitoneal (i.p.) injection. For the non-immunization experiment, 2 mice per group (Vehicle, GDC-0941, and MLN1117)

were given the indicated drugs for 9d before sacrificing on day 10. For the immunization experiment, 4 mice per group were used to perform two independent studies comparing GDC-0941 or IC87114 to MLN1117 as described in the text. In all cases, the vehicle group received both vehicles used to formulate the two different drugs. Mice were treated with the drugs throughout day -1 to day 13. On day 0, all mice were immunized with NP-OVA precipitated in alum (Imject; Pierce). Drug treatment was stopped on day 13 and mice were sacrificed for collection of serum and spleens. Spleens were immediately made into single-cell suspensions for flow cytometric analysis and the rest were quickly frozen in an OCT compound (VWR) for sectioning. For TI-2 immunization, mice were immunized with TNP-Ficoll and serum was collected on day 7.

Serum ELISA

96 well NUNC MaxiSorp plates (Nalgene) were coated with 50 μL of either NP(30)BSA or NP(3)BSA (Biosearch Technologies) at a concentration of 50 μg/ml overnight at 4 °C. Serum Ig was detected with HRP-conjugated rabbit anti-mouse secondary antibodies against IgM and IgG1 (Invitrogen). Plates were developed with TMB peroxidase (eBioscience) for colorimetric detection after which the reaction was stopped with 1N sulfuric acid and read on a plate reader at 450 nm. For TNP-Ficoll immunization, plates were coated with 100 μL of TNP-BSA at a concentration of 10 g/ml for one and a half hour at room temperature. Serum Ig was detected with HRP-conjugated goat anti-mouse secondary antibodies against IgG3 (Southern Biotech). Plates were developed as described above.

Immunohistochemistry

Mouse spleens embedded in OCT medium were frozen and eight-micrometer sections were cut and mounted on Superfrost Plus slides (Fisher Scientific). Slides were fixed in acetone at -20 °C for 20 min and blocked with FACS buffer for 30 min at room temperature. Immunohistochemical staining was done with anti-mouse antibodies against IgD-eFluor405, IgM-FITC, and GL7-AlexaFluor647 (all 1:100 dilution) for 1 h at room temperature, followed by three 5 min washes in PBS. Marginal zone (MZ) B cells were identified as IgM-bright, IgD-dim cells surrounding IgM-dim, IgD-bright follicles. Germinal center B cells were identified as IgD-negative, GL7-positive. For some sections, antibodies against B220-PE and metallophilic macrophage (MOMA-1) –FITC were used as an alternative method to identify MZ B cells (B220-positive cells outside the MOMA-1 border). All images shown were acquired at either 10x or 20x magnification using Olympus Fluoview FV1000 Laser Scanning Confocal Microscope.

RESULTS AND DISCUSSION

PI3K inhibitor validation

The inhibitors used in this study are listed in Table 2.1 along with their isoform selectivity defined by *in vitro* kinase activity assays using recombinant enzymes. Two p110α-selective inhibitors with distinct chemical structure were used to minimize possible off-target effects. A66 has been studied previously in preclinical cancer models [8,18]. MLN1117, originally described by Intellikine as INK1117 [19], is currently in phase I trials for patients with advanced solid tumors (clinical trials identifier NCT01449370). To inhibit p110β we used TGX-221 [20,21]. This compound has some activity against p110δ (IC_{50} 100 nM vs. 5 nM for p110β; Table 1). As another means to inhibit p110β, I used the compound MLN1316, a dual p110α/p110β inhibitor that is highly selective relative to p110δ and p110γ (Table 2.1). IC87114 was used as a p110δ inhibitor [22–24]. To inhibit all class I isoforms, I used the two compounds GDC-0941 [25–27] and ZSTK474 [28,29]. *In vitro*, both compounds inhibit all class I isoforms with IC_{50} values between 3-75 nM with some preference for p110α and p110δ (Table 2.1). ZSTK474 is more selective than GDC-0941 with respect to mTOR (Table 2.1). The selectivity of MLN1117 compared to GDC-0941 is supported by studies of breast cancer cell lines (Figure 2.1A, B). MLN1117 inhibits AKT phosphorylation and growth in *PIK3CA* mutant breast cancer cells with IC50 values around 2 μM, while having no effect on cells lacking *PTEN*. In contrast, GDC-0941 has similar effects on cell lines with *PI3KCA* mutation or *PTEN* loss.

The selectivity of the inhibitor panel was validated using cancer cell lines previously shown to be driven primarily by p110α or p110β. SK-OV-3 (*KRAS* wild-type) has an activating *PIK3CA* mutation and was shown previously to be sensitive to A66 [8].

Compound	IC_{50} (nM)					
	PI3K Class I					
	p110α	p110β	p110γ	p110δ	mTOR	(Refs)
A66	32	>12500	3450	>1250	>5000	9
MLN1117	15	4500	1900	13900	1670	
TGX-221	5000	5	>10000	100	n.d.	26
MLN1316	10	8	780	2200	2100	
IC87114	>10000	1820	1240	70	n.d.	27
GDC-0941	3	33	75	3	580	28
ZSTK474	16	44	49	4.6	>10000	29

Table 2.1: IC50 values for inhibition of class I PI3K isoforms and mTOR

The cancer cell line U87MG lacks PTEN and is preferentially sensitive to p110β inhibition [30]. Cells were cultured for two days with titrated amounts of inhibitors before measurement of viable cell number by MTS assay (Figure 2.1C). Low serum conditions were used to increase cell dependence on endogenous PI3K activation. As expected, A66 and MLN1117 reduced growth of SK-OV-3 over a concentration range from 125 nM – 2 μM. Statistically significant effects were seen from 500 nM – 2 μM. In U87MG cells, A66 and MLN1117 had a no effect at these concentrations. MLN1316 at concentrations of 500 nM – 2 μM reduced viable cell number of both SK-OV-3 and U87MG cells. Together these data show that 1 μM concentrations of each inhibitor selectively inhibit growth and/or survival of cancer cells driven by the appropriate target, p110α or p110β. Previous studies have shown that IC87114 is specific for p110δ when cells are treated with 1 μM of this compound [24]. TGX-221 did not cause a significant effect in these experiments but showed a trend towards inhibiting U87MG cell number at 500 nM – 2 μM. Based on these considerations I used a maximum concentration of 0.5-1 μM of each inhibitor for most experiments.

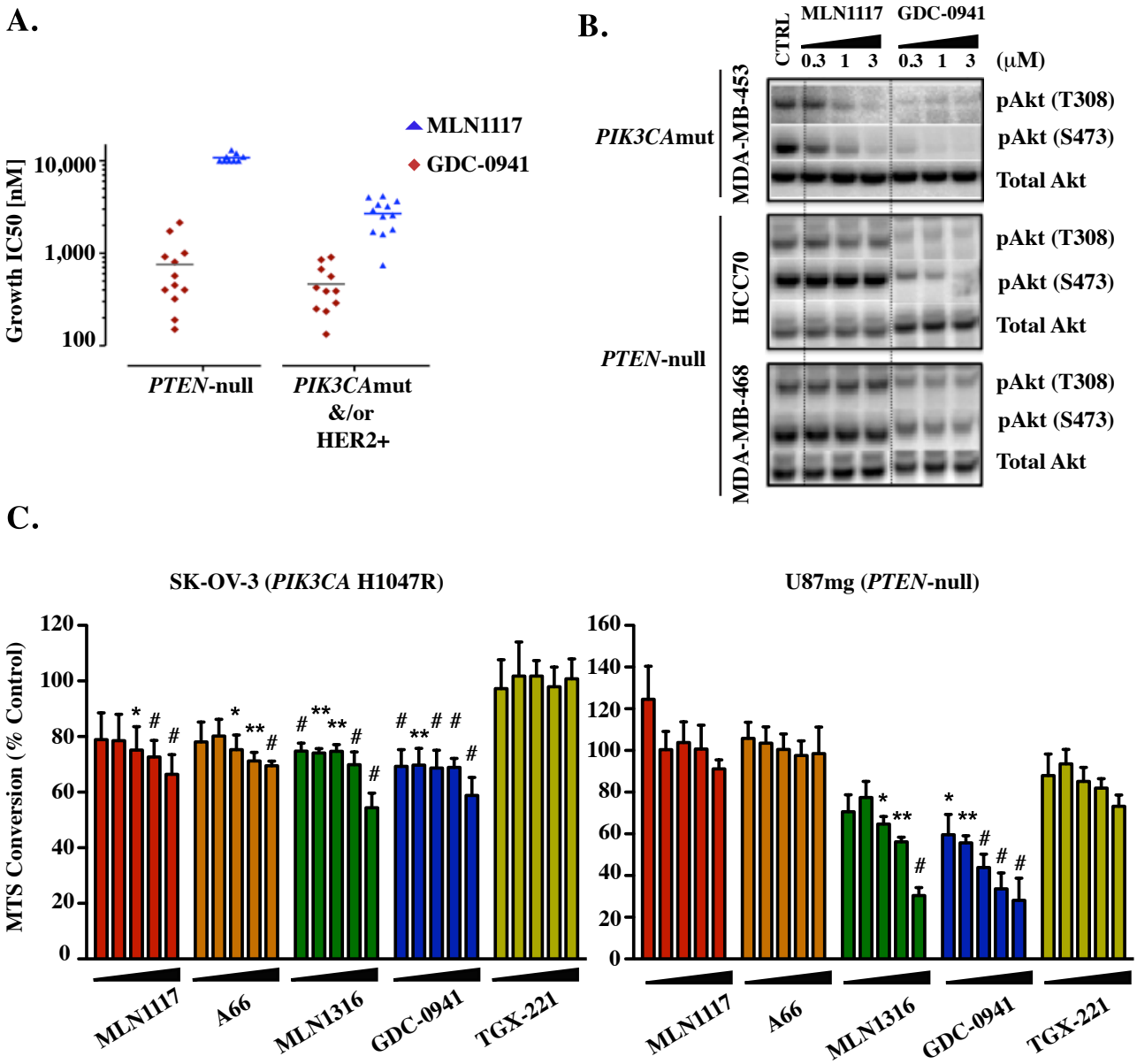

Figure 2.1. Validation of novel PI3K class IA isoform-selective inhibitors. (A) Growth IC50 values for MLN1117 and GDC-0941 in a panel of breast cancer cell lines harboring a PTEN-null mutation, compared to lines with either a p110α mutation or HER2 overexpression. (B) Phosphorylation status of Akt in response to either MLN1117 or GDC-0941 in three different cell lines harboring the mutations described above. (C) Cell lines (5000 cells/well) either harboring a constitutively activating p110α H1047R mutation (SK-OV-3) or PTEN deletion (U87MG) were cultured under low serum (0.2% FBS) conditions for 48 h in the presence of the indicated inhibitors (2-fold dilution series from right to left: 2μM, 1μM, 0.5μM, 0.25μM, 0.125μM). MTS conversion assay was used to measure viable cell number relative to vehicle-treated control (100%) (background-subtracted). Data represent mean +/- SEM of n = 3 to 5 experiments (*P<0.05, **P<0.01, #P<0.001, repeated-measures analysis of variance (ANOVA), measured versus the vehicle-treated control).

B cell proliferation and survival

The effects of inhibitors on the proliferation of purified, CFSE-labeled B cells were compared. In cells stimulated through the BCR with anti-IgM, p110δ inhibition blocked cell division nearly to the same extent as pan-class I PI3K inhibition. Figure 2.2A shows a graph of the average proliferation over multiple experiments, expressed as the percent of divided (CFSE-low) cells. Statistical analysis of normalized data showed that the effects of GDC-0941 and IC87114 were highly significant, yet there was no significant effect of 1 μM A66, MLN1117 or TGX-221. At a higher concentration (2 μM), A66 or MLN1117 did significantly suppress B cell proliferation driven by anti-IgM alone but not by anti-IgM plus IL-4 (Figure 2.2A, B). Similar results were obtained using human peripheral blood B cells stimulated with anti-human IgD and IL-4, (Figure 2.2C).

GDC-0941 caused a marked increase in B cell death, as suggested by the reduced cell recovery (data not shown) and confirmed by measuring the percentage of cells staining with DAPI nuclear dye (anti-IgM; untreated: 38 % vs GDC-0941 0.5mM: 95 %). IC87114 had an intermediate effect on B cell death (75 %) and there was only a minor effect of p110α inhibitors at the selective concentration of 1 μM.

The bacterial cell wall component lipopolysaccharide (LPS) causes polyclonal B cell proliferation through a TLR4-dependent pathway. In LPS-stimulated mouse B cells, IC87114 was the only isoform-selective inhibitor to significantly reduce the percent of divided cells (Figure 2.2D). However, under these conditions IC87114 did not block

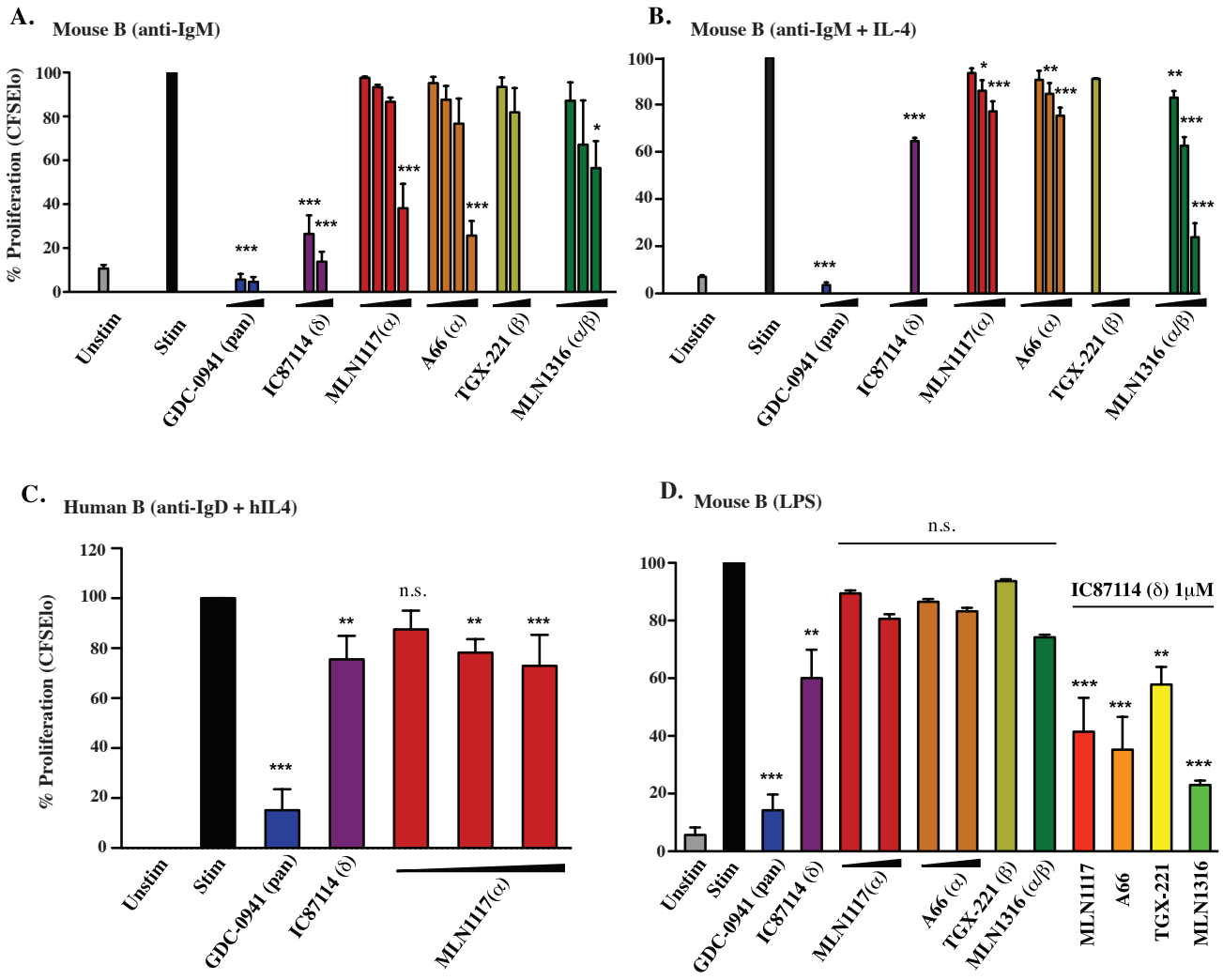

Figure 2.2. p110 is the primary PI3K isoform that contributes to B cell proliferation. **(A)** anti-IgM mediated B cell proliferation from three independent experiments was normalized to a percentage scale. Concentrations used were MLN1117 and A66 (0.25, 0.5, 1 and 2 μM), TGX-221 (0.25, 0.5 μM), MLN1316 (0.25, 0.5 and 1 μM), GDC-0941 (0.25 and 0.5 μM) and IC87114 (0.5 and 1 μM). **(B)** anti-IgM + IL4 mediated B cell proliferation from three independent experiments was normalized to a percentage scale. Concentrations used were MLN1117, A66 and MLN1316 (0.5, 1 and 2 μM), TGX-221 (0.5 μM), GDC-0941 (0.5 μM) and IC87114 (1 μM). (C) anti-IgD + IL4 mediated human B cell proliferation from three independent experiments was normalized to a percentage scale. Concentrations used were MLN1117 (0.5, 1 and 2 μM), GDC-0941 (0.5 μM) and IC87114 (1 μM). **(D)** Three independent experiments of LPS mediated B cell proliferation were analyzed in a similar manner to **(B)**. 1 μM concentration was used for all inhibitors except GDC-0941 (0.5 μM) and TGX-221 (0.5 μM). In the combination treatments, IC87114 1 μM with 1 μM of the indicated inhibitors was used. All data represent results from at least three independent experiments ((*P<0.05, **P<0.005, ***P<0.001, repeated-measures analysis of variance (ANOVA), measured versus the untreated control).

proliferation to the same degree as GDC-0941 nor did IC87114 cause death of LPS-stimulated cells (GDC-0941: 77 % vs. IC87114: 33 %). Examination of the CFSE dilution data suggested that MLN1316 measurably reduced cell division, and combining IC87114 with MLN1316 blocked cell division more fully (data not shown). These data suggest that each class IA isoform contributes to LPS-driven B cell proliferation. However, selective p110α inhibition had a negligible effect.

Next I tested the effects of PI3K inhibitors on survival of purified B cells cultured in the presence of the cytokines BAFF or IL-4. Both GDC-0941 and IC87114 blocked cytokine-dependent survival even at the low concentration of 250 nM, while GDC-0941 reduced viability below the level observed in cells cultured without cytokines or inhibitors (Figure 2.3A, B). In contrast, even at higher concentrations (1 μM) both A66 and MLN1117 caused only a minor decrease in survival. When p110α inhibitors were combined with IC87114 there was a trend towards additive suppression of survival, but this was not statistically significant. TGX-221 partially reduced B cell survival, and the dual p110α/β inhibitor MLN1316 appeared to have a greater effect especially in BAFF-treated cells.

In summary, these results of B cells *in vitro* indicate that acute p110α inhibition causes only incremental decreases in proliferation and survival. Likewise, the B cell response to LPS and survival cytokines is mediated predominantly through p110δ with lesser contributions of p110α and p110β.

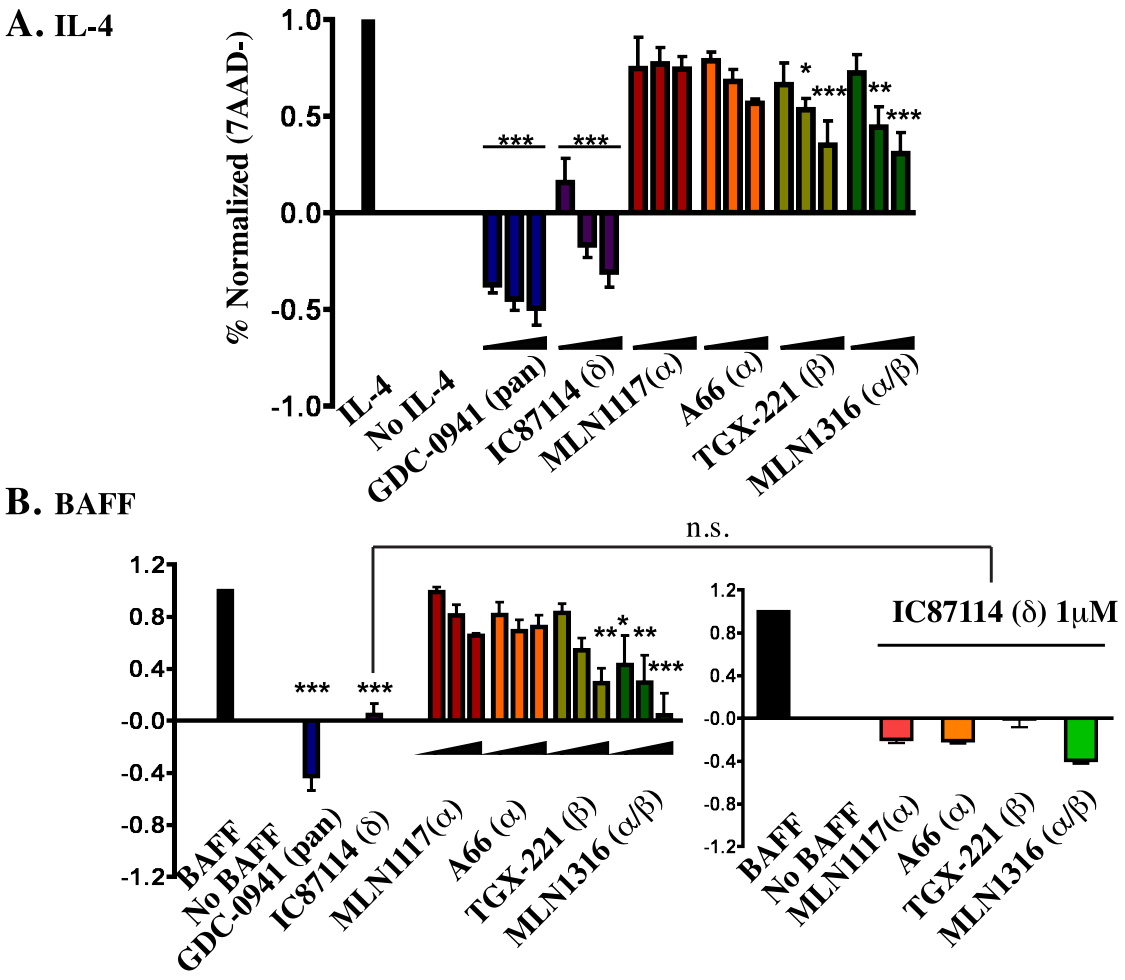

Figure 2.3. Inhibition of p110 does not significantly reduce IL-4 or BAFF mediated B cell survival. Purified B cells were cultured in B cell media containing either IL-4 **(A)** or BAFF **(B)** for 48h and cell viability was measured using 7AAD exclusion. Viable cells (%7AAD negative) were normalized for three independent experiments. For each indicated inhibitor, the three concentrations used were 0.25, 0.5 and 1 μM. **(B)** For BAFF survival experiments, single concentrations of GDC-0941 (0.5μM) and IC87114 (1 μM) were used. In the combination treatments (bottom right), IC87114 1μM with 1μM of the indicated inhibitors were used. Normalized data of the combination treatments are from the same experiments of single treatments (bottom left). All data represent results from at least three independent experiments ((*P<0.05, **P<0.005, ***P<0.001, repeated-measures analysis of variance (ANOVA), measured versus the untreated control).

T cell proliferation and cytokine secretion

Initial studies of p110δ-deficient mice showed a profound block of BCR-mediated proliferation whereas T cells from these mice proliferated relatively normally in response to co-clustering of the T cell receptor (TCR) with the costimulatory molecule CD28 [31]. Subsequent experiments using TCR transgenic T cells showed that p110δ plays a more prominent role in clonal expansion of antigen-specific CD4 T cells[32]. In addition, the p110δ-selective inhibitor IC87114 was reported to inhibit proliferation of both mouse and human T cells [24]. An important caveat is that when present at concentrations above 1μM, IC87114 inhibits proliferation of T cells with inactive p110δ. Therefore, cellular effects of IC87114 above 1μM might result from achieving a more pan-PI3K inhibition profile. Here I compared the effects of 1μM IC87114 and other PI3K inhibitors on T cell proliferation. For murine cells, I measured the proliferation of DO11.10 transgenic T cells stimulated with cognate OVA peptide in the presence of autologous splenocytes. For human cells, I used peripheral blood T cells stimulated with the polyclonal activator PHA.

As shown in Figure 2.4A, the proliferation of DO11.10 T cells in the presence of 10nM OVA peptide was only marginally reduced by individual inhibitors of class I isoforms. 1μM A66, MLN1117, TGX-221 or IC87114 did not significantly decrease the percentage of divided cells, but CFSE histogram overlays showed that each inhibitor mildly restrained cell division (data not shown). Increasing the concentration of MLN1117 or A66 to 2μM did not cause greater inhibition of cell division. Combining IC87114 with A66, MLN1117 or MLN1316 did significantly reduce the proliferative response though the overall percentage of divided cells was diminished less than 50 %.

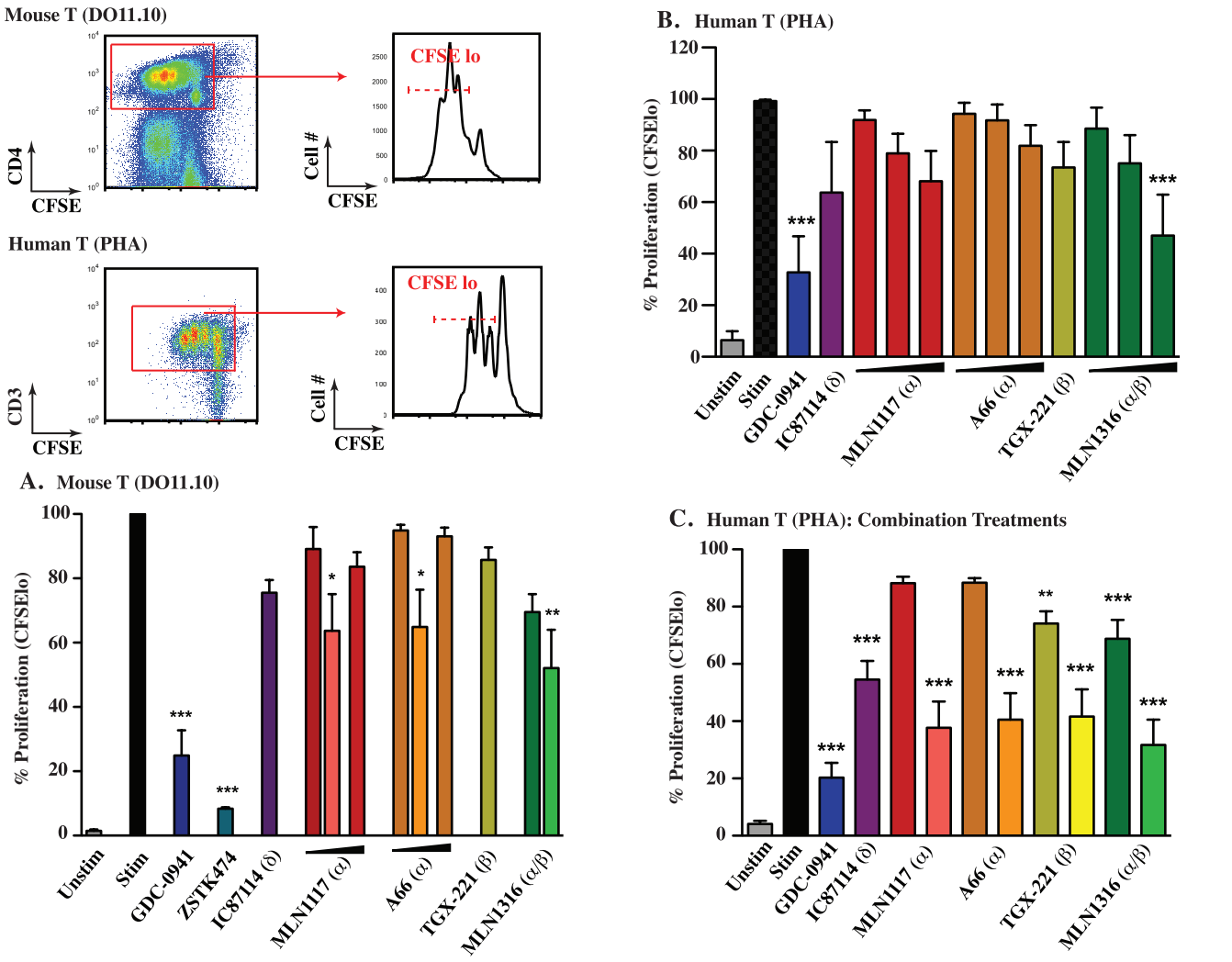

Figure 2.4. PI3K class IA isoforms have redundant functions in antigen-mediated T cell proliferation. **(A)** CFSE labeled whole splenocytes from DO11.10 TCR transgenic mouse were activated with 10 nM $OVA_{323-329}$ peptide for 3d. CD4+ T cells were gated and proliferation was normalized to a percentage scale (gating scheme is presented in the top figure). 1 μM concentration was used for all inhibitors except for MLN1117 and A66 (1 and 2μM). In the combination treatments (depicted by the lighter color next to MLN1117, A66, and MLN1316), IC87114 1 μM with 1 μM of the indicated inhibitors was used. **(B)** Human T cells were activated with 1μg/ml PHA for 3d. CD3+ total T cells were gated and proliferation was normalized to a percentage scale (gating scheme is presented in the top figure). Concentrations used were MLN1117, A66 and MLN1316 (0.5, 1, 2 μM), TGX-221 (1μM), GDC-0941 (0.5 μM) and IC87114 1μM. (C) Same experiment as in (B) with combination treatments. 1 μM concentration was used for all inhibitors except for GDC-0941 (0.5μM) and TGX-221 (0.5 μM). In the combination treatments (depicted by the lighter color next to MLN1117, A66, TGX-221, and MLN1316), IC87114 1μM with each indicated inhibitors was used. All data represent results from at least three independent experiments ((*P<0.05, **P<0.005, ***P<0.001, repeated-measures analysis of variance (ANOVA), measured versus the untreated control).

The pan-class I inhibitor GDC-0941 significantly blocked proliferation in T cells stimulated with 10nM OVA, though in most experiments the inhibition was incomplete (Figure 2.4A). The compound ZSTK474 was a more effective inhibitor of antigen-driven T cell proliferation (Figure 2.4A). Whether the greater efficacy of ZSTK474 is attributable to slightly greater inhibition of p110γ (Table 2.1) or other pharmacological differences is unclear. Together these findings indicate that multiple class I PI3K isoforms contribute to the overall function of PI3K signaling during T cell expansion. Very similar results were obtained using PHA-stimulated human T cells (Figure 2.4B, C).

The cytokine interleukin-2 (IL-2) is produced by activated T cells and acts as an autocrine and paracrine growth factor to drive proliferation. Consistent with the cell division data, selective blockade of individual PI3K isoforms partially reduced IL-2 production, whereas the response was inhibited almost completely by drug combinations or by the pan-PI3K inhibitors GDC-0941 and ZSTK474 (Figure 2.5A, B). T cell secretion of the cytokine interferon-gamma (IFNγ) seemed generally more sensitive to PI3K inhibition, with p110α and p110β inhibitors having significant effects in mouse T cells (Figure 2.5C). Nevertheless, the overall pattern for mouse IFNγ and IL-2 was comparable with IC87114 having stronger effects especially when combined with p110α and p110β inhibitors. Secretion of IFNγ by human T cells stimulated with PHA showed variability among donors but the overall pattern was similar (Figure 2.5D).

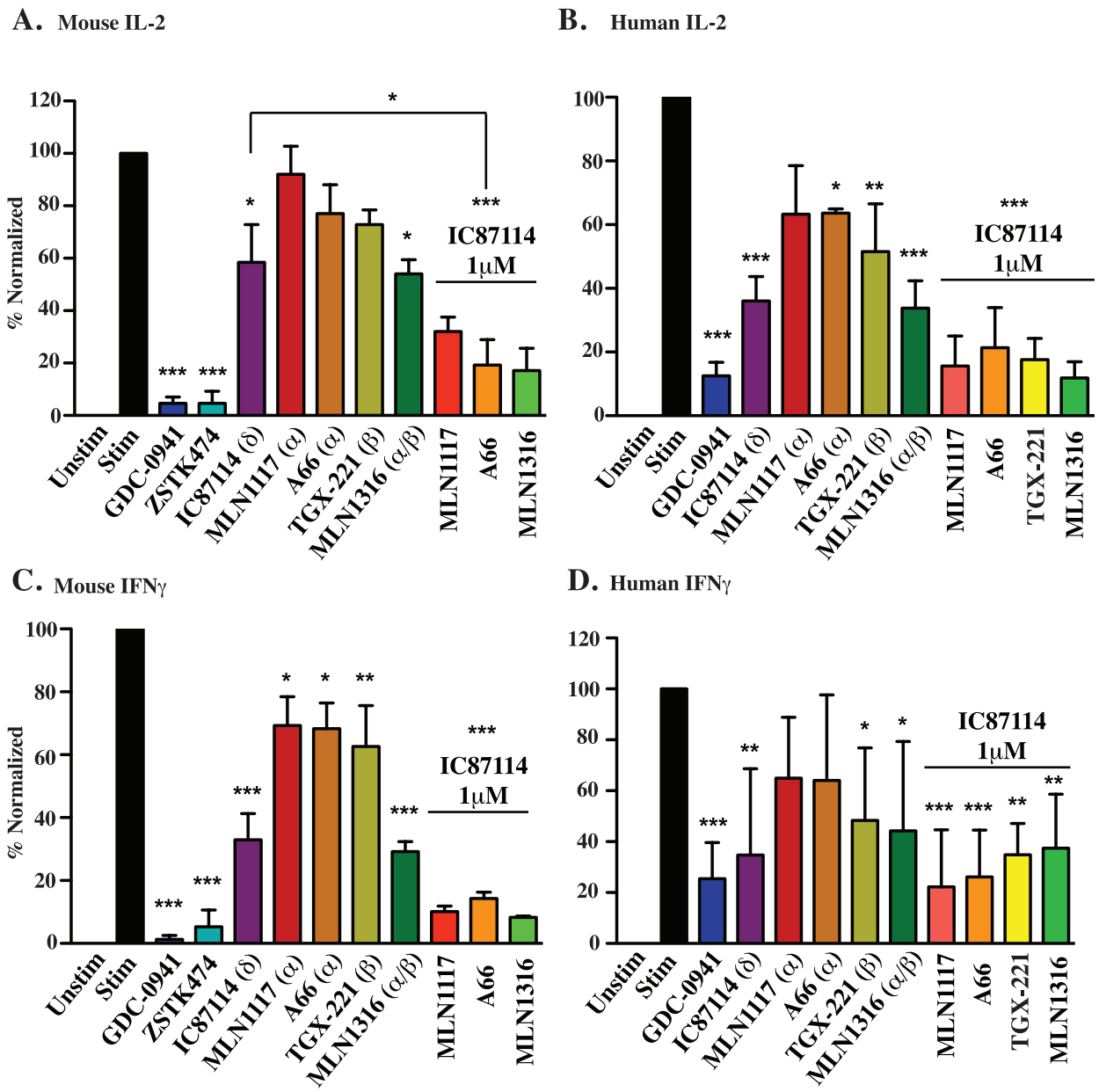

Figure 2.5. PI3K class IA isoforms have redundant functions in T cell cytokine production. IL-2 (A, B) and IFNγ (C, D) levels were measured by ELISA from the supernatants in Figure 6 after 24h activation with $OVA_{323-329}$ peptide or PHA. Cytokine levels were normalized to a percentage scale from three independent experiments. 1μM concentration for all the inhibitors except for GDC-0941, ZSTK474, and TGX-221 (0.5μM) are shown.

Lymphocyte function *in vivo*

Lastly I evaluated the effects of different PI3K inhibitors on lymphocyte subsets and function *in vivo*. For treatments with GDC-0941 or MLN1117, I used doses and formulations shown to provide anti-cancer efficacy in solid tumor xenograft models ([27] and unpublished data). For treatment with IC87114, we used a dose and treatment protocol previously shown to reduce marginal zone B cell numbers in mice[23]. Daily treatment with MLN1117, IC87114 or GDC-0941 did not alter the percentages or numbers of T cells, and did not affect the ratio of CD4 and CD8 T cells relative to vehicle-treated controls (data not shown).

Genetic inactivation of p110δ causes a large reduction in the number of marginal zone (MZ) B cells, a specialized B cell subset in the mouse spleen that responds mainly to T cell-independent antigens [31]. Similarly, pharmacological inhibition of p110δ with IC87114 *in vivo* causes aberrant localization of MZ B cells [23]. In accord, fewer MZ B cells ($IgM^{hi}IgD^{lo}$) were detected in spleen sections of mice treated with IC87114 or the pan class I inhibitor GDC-0941 (Figure 2.6A). Based on FACS-based discrimination of splenic B cell subsets, I also found that IC87114 and GDC-0941 reduced the overall percentage of MZ B cells (Figure 2.6B; MZ cells are identified as $CD21^{hi}CD23^{lo}$ by FACS). In contrast, mice treated with MLN1117 displayed no change in the percentage or localization of MZ B cells (Figure 2.6A, B). These results are consistent with a required role for p110δ but not p110α in the MZ B cell compartment.

To compare the effects of PI3K inhibitors on B cell and T cell-mediated immune responses *in vivo*, I measured antibody production in mice vaccinated with hapten-carrier conjugates. To model T cell-independent antibody responses driven by BCR crosslinking,

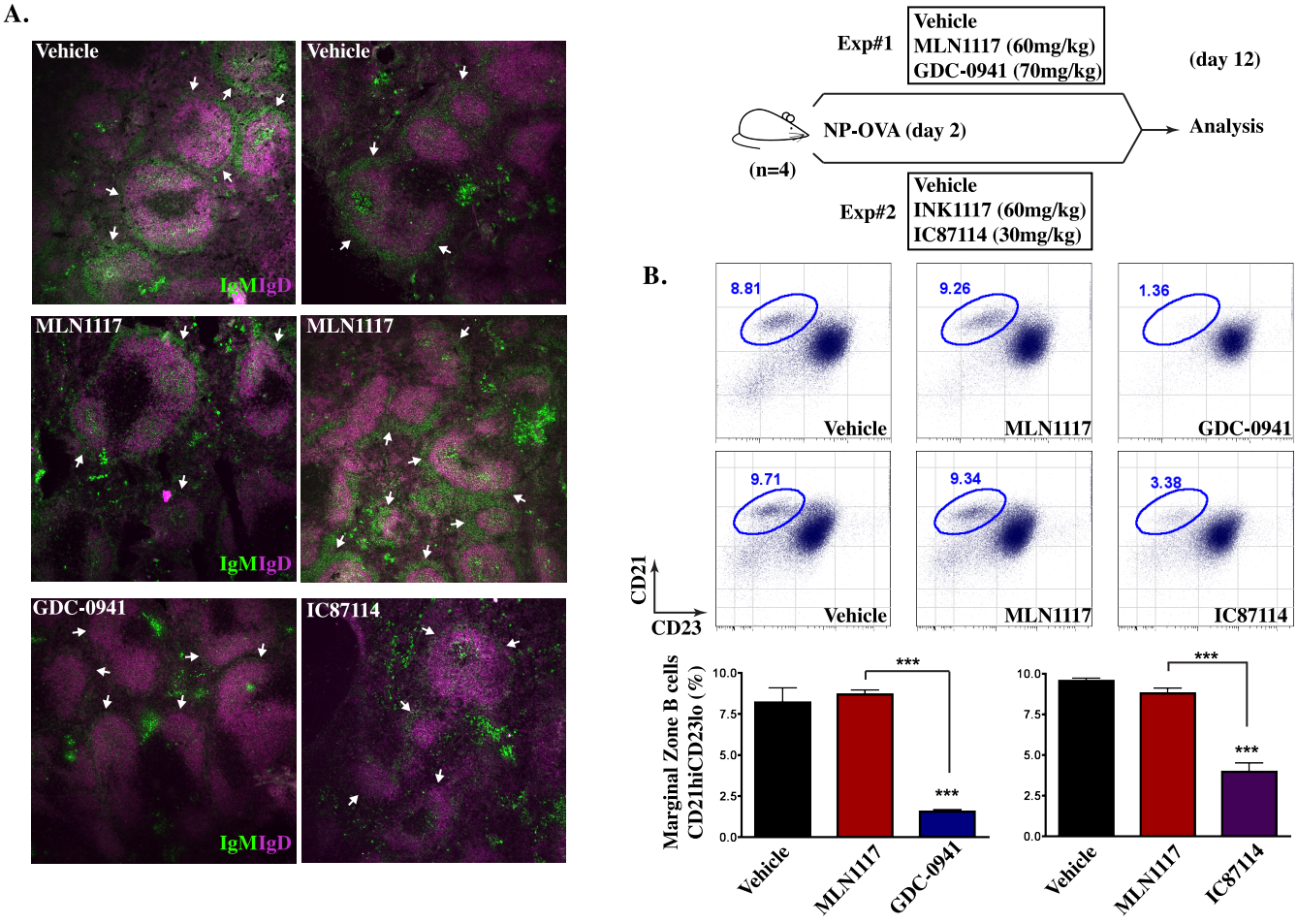

Figure 2.6. GDC-0941 and IC87114, but not MLN1117, decrease the marginal zone (MZ) B cell compartment *in vivo*. Two independent experiments (top right) were conducted where the *in vivo* effects of GDC-0941 and IC87114 compared to MLN1117 were assessed. Mice (n = 4 mice per group) were given the drugs daily for 11 days and were immunized on day 2 with a T-dependent antigen NP-OVA. **(A)** Mouse spleen sections from each treated mouse were stained with anti-IgM-FITC and anti-IgD-eFluor405 to distinguish MZ B cells ($IgM^{hi}IgD^{lo}$) from FO B cells ($IgM^{lo}IgD^{hi}$). All images are representative of multiple spleen sections from different mice (n = 4 mice per group). **(B)** Total splenocytes harvested on day 12 were stained with CD21-FITC and CD23-PE to identify MZ B cells ($CD21^{hi}CD23^{lo}$) by flow cytometry. Flow plots are representative splenocyte staining data from multiple mice (n=4 mice per group). MZ B cell frequency from four different mice is shown as a bar graph (***P<0.001, ANOVA, measured versus the vehicle-treated mice, except where indicated by brackets).

TNP-Ficoll was used as the immunogen. GDC-0941 treatment abrogated TNP-specific IgG3 production (Figure 2.7A). This indicates that the T cell-independent IgG3 response is completely PI3K-dependent. Treatment with MLN1117 at 30 and 60 mg/kg caused little reduction of TNP-specific IgG3 (Figure 2.7A). Notably, reduction of TNP-specific IgG3 at higher doses of MLN1117 (120 mg/kg) was observed, consistent with the partial reduction in cell division in B cells treated with MLN1117 before anti-IgM stimulation *in vitro*. However, 120 mg/kg is above the effective dose of MLN1117 for tumor growth inhibition (30-60 mg/kg).

To model a T cell-dependent antibody response, I used NP-OVA as the immunogen. In this case, treatment with MLN1117 (60 mg/kg) did not diminish the NP-specific IgM or IgG1 responses (Figure 2.7B). This is consistent with the minimal effect of MLN1117 on T cell proliferation *in vitro* and on B cells stimulated with anti-IgM plus IL-4. To our surprise, mice treated with IC87114 or GDC-0941 also produced normal NP-specific antibody titers and affinity (Figure 2.7B and data not shown). The absence of drug effects was not likely due to poor pharmacokinetics as both IC87114 and GDC-0941 impacted the MZ B cell compartment at these doses (Figure 2.6). Furthermore, as reported previously [33], selective p110δ inhibition with IC87114 augmented the antigen-specific IgE response (Figure 2.7B**,** lower right graph). Staining of spleen sections revealed that both IC87114 and GDC-0941, but not MLN1117, reduced the appearance of B cells with a germinal center (GC) phenotype ($IgD^{lo}GL7+$) in NP-OVA-immunized mice (Figure 2.7C, D).

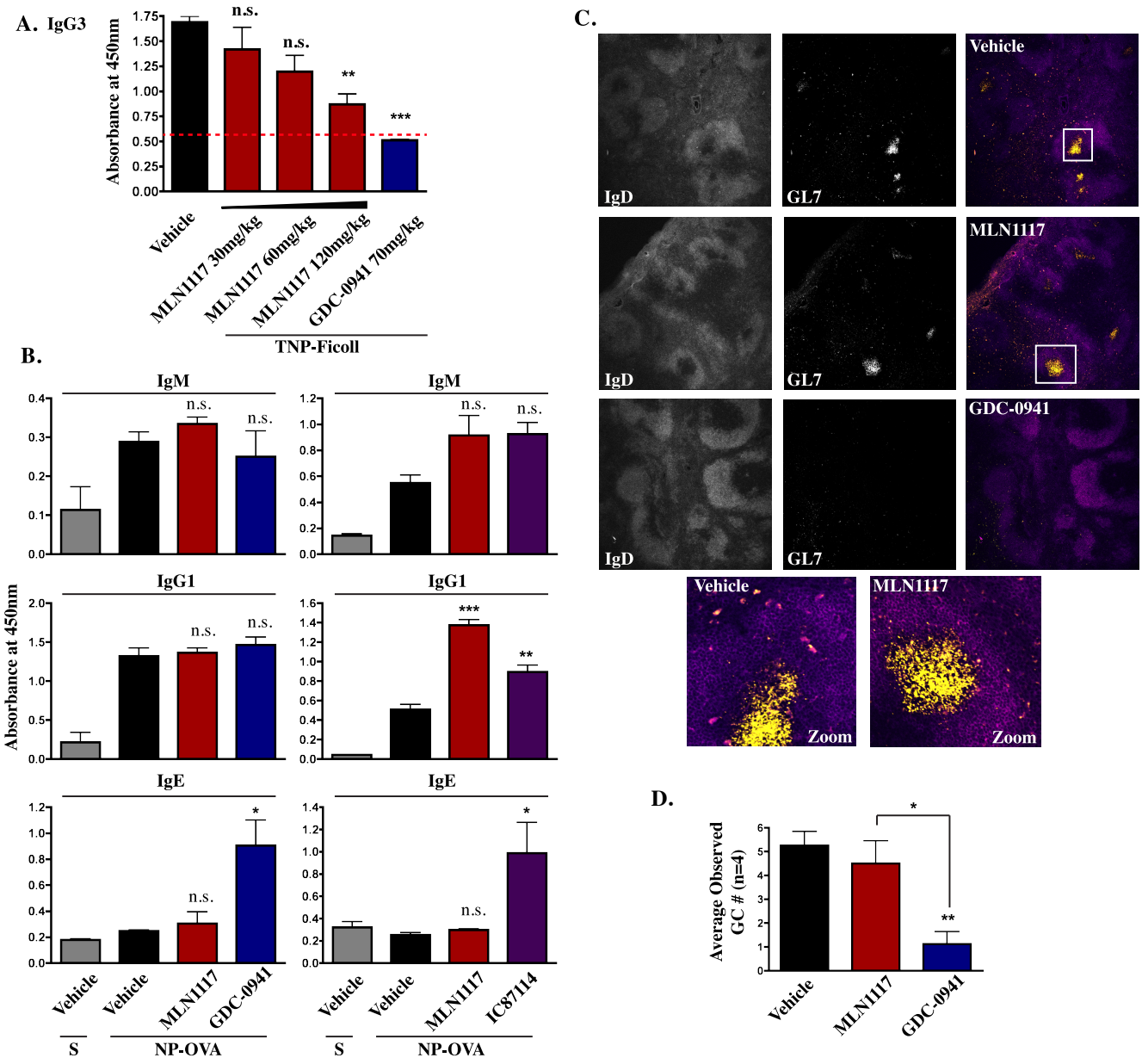

Figure 2.7. MLN1117 does not diminish the germinal center (GC) response or production of NP-specific antibody *in vivo*. **(A)** Mice (n=6 per group) were immunized with TNP-Ficoll and given the drugs at the indicated doses for 7 days. Quantification of TNP-specific IgG3 in serum was done by ELISA using TNP-BSA coated plates The dotted line indicates the lower limit of linearity of detection. (**B**) Mice (n = 4) per group were immunized with NP-OVA and nitrophenyl (NP)-specific IgM, IgG1, and IgE in serum was quantified by ELISA using NP(30)-BSA coated plates. S, sham immunized. **(C)** Spleen sections from each treated mouse were stained with anti-IgD-eFluor405 and anti-GL7-AlexaFluor647 to distinguish GCs ($IgD^{low}GL7^{+}$) within the follicles. T cell zones were distinguished by a separate CD4-FITC staining (data not shown) and were concentrated outside the follicles. **(D)** Quantification of GC numbers per mouse. Two random areas from each slide (spleen sections from one mouse on each slide, n = 4 mice per group) were observed for GCs ($IgD^{low}GL7^{+}$) and counted by two different lab members in blinded fashion (slide labels were hidden). The total GC counts per treatment group were divided by the number of mice (n = 4) to achieve the observed GC numbers per mouse for each treatment (*P<0.05, **P<0.01, ANOVA, measured versus vehicle-treated, NP-OVA-immunized mice, except where indicated by brackets). As each slide had only some sections of the spleen, the actual number of GCs per mouse would be proportionally higher.

Discussion

The data presented in this Chapter indicate that selective inhibition of p110α (and/or p110β) does not strongly impair lymphocyte proliferation or survival *in vitro*. Moreover, treating mice with the p110α inhibitor MLN1117 at doses with anti-tumor activity in preclinical models does not interfere with T cell-dependent antibody responses. In contrast, the pan-class I inhibitor GDC-0941 and the selective p110δ inhibitor IC87114 strongly suppress B cell proliferation and survival *in vitro* and impair germinal center responses *in vivo*.

The data indicate that the p110δ inhibitor IC87114 suppresses B cell survival in response to anti-IgM or BAFF, but to a lesser extent than the more broad-spectrum PI3K inhibitor GDC-0941. The finding that GDC-0941 has cytotoxic effects on B cells *in vitro* is consistent with the observation of Ramadani et al. that inactivation of both p110α and p110δ causes a nearly complete loss of mature B cells *in vivo* [17]. The apparent redundancy of p110α and p110δ in supporting B cell survival is noteworthy in light of the clinical success of the investigational agent CAL-101 (subsequently renamed GS-1101 and then given the generic name idelalisib). This selective p110δ inhibitor has shown impressive efficacy in human patients with chronic lymphocytic leukemia (CLL), and is now FDA-approved for patients with relapsed CLL, yet its primary mechanism of action seems to be disruption of the tumor microenvironment rather than direct cytotoxic effects on malignant B cells [34]. It is possible that a combined inhibitor of p110α and p110δ would have more potent anti-tumor effects in CLL and other lymphoid malignancies.

The p110δ isoform has important functions in T cell clonal expansion, differentiation and trafficking [13]. However, PI3K activation is not fully abrogated in p110δ-deficient T cells and some functional capacity is retained [32]. It is likely, therefore, that other PI3K catalytic isoforms contribute to T cell function. Some studies have suggested that the class IB isoform p110γ has overlapping functions in T cells, whereas others have disputed this claim [13]. In this study we focused on class IA isoforms, in part because available inhibitors are not sufficiently selective for p110γ. Using antigen-specific mouse T cells and PHA-activated human T cells, we found that proliferative expansion is not significantly reduced by selective inhibition of individual class IA isoforms. Moreover, the pan-class I inhibitors suppressed T cell proliferation to a greater extent than the combination of IC87114 and MLN1316. Together these findings suggest that all four class I isoforms might be engaged during the course of antigen-specific T cell activation. Interesting questions to resolve are whether the isoforms act downstream of different receptors (i.e. TCR, costimulatory, cytokine, chemokine receptors) and whether they function in a temporal order.

An apparent paradox in our data is the observation that GDC-0941 and IC87114 suppress formation of germinal centers yet they have no effect on titers of high affinity, class-switched antibodies whose production is thought to dependent on the germinal center reaction. The absence of GCs in IC87114-treated mice is consistent with previous work identifying a specific role for p110δ in the differentiation and function of T follicular-helper (Tfh) cells, a subset of CD4 T cells required for GC formation [35]. In addition, our data on NP-specific Ig production agree with previous studies showing that IC87114 does not impair class-switched antibody responses in mice [33]. Indeed, p110δ

inhibition enhances IgE production [33], a finding reproduced here. One possibility is that an extrafollicular (non-GC-based) B cell response that is Tfh-independent might be sufficient to produce antigen-specific antibody responses to protein antigens in mice treated with IC87114 or GDC-0941. Regardless, a key point is that p110α inhibition using MLN1117 *in vivo* has no measurable effect on antibody responses or GC formation. This suggests that p110α is not required for Tfh differentiation or help to B cells in general.

CONCLUSION

Many years of medicinal chemistry efforts have resulted in an expanded toolkit for probing the function of specific PI3K catalytic isoforms [36]. In this chapter, we set out to determine how selective inhibitors of p110α impact the function of T and B cells, key components of the adaptive immune system. The most important conclusion is that p110α inhibition does not significantly impair mouse and human lymphocyte proliferation *in vitro,* nor antibody responses *in vivo,* at doses demonstrating potent anti-tumor activity in preclinical models. A key implication is that selective p110α inhibitors that are in clinical trials for cancer are likely to be less immunosuppressive than pan-class I or p110-selective agents. The combined p110α/β inhibitor MLN1316 also had only modest effects on lymphocyte function, suggesting that anti-cancer compounds with this profile would likewise show minimal immunosuppression. Our results do not rule out the possibility that p110α and/or p110β inhibition can modulate lymphocyte differentiation and effector function in certain contexts. This possibility warrants further investigation using the growing assortment of PI3K isoform-selective compounds. Compounds

targeting two isoforms (e.g. p110α/δ, p110γ/δ) might also have unique effects in lymphocytes. The ability of PI3K inhibitors to enhance innate immune responses [37] also requires further investigation. A better understanding of PI3K isoforms in the immune system will improve our ability to predict and manage immunosuppression and to potentially manipulate the immune components of the tumor microenvironment.

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CHAPTER 3:

TARGETING mTORC2 WITH TOR-KIS ENHANCE B LYMPHOCYTE CLASS-SWITCH RECOMBINATION

This chapter is derived from a research paper published in *Proceedings of the National Academy of Sciences*, 111 (47): E5076-85 (2014). Jose J. Limon performed the initial experiments of TOR-KIs enhancing B cell class-switch recombination (CSR) to the IgG1 isotype both *in vitro* and *in vivo*. I am only including experiments performed by me where I validated the observation of *in vitro* B cell CSR enhancement by TOR-KIs and genetically confirmed that this enhancement was through inhibition of the mTORC2 signaling node by TOR-KIs.

ABSTRACT

The mammalian target of rapamycin (mTOR) is a kinase that functions in two distinct complexes, mTORC1 and mTORC2. In peripheral B cells, complete deletion of mTOR suppresses germinal center B-cell responses, including class switching and somatic hyper- mutation. The allosteric mTORC1 inhibitor rapamycin blocks proliferation and differentiation, but lower doses can promote protective IgM responses. To elucidate the complexity of mTOR signaling in B cells further, we used ATP-competitive mTOR kinase inhibitors (TOR-KIs), which inhibit both mTORC1 and mTORC2. Although TOR-KIs are in clinical development for cancer, their effects on mature lymphocytes are largely unknown. We show that high concentrations of TOR-KIs suppress B-cell proliferation and differentiation, yet lower concentrations that preserve proliferation increase the fraction of B cells undergoing class switching in vitro. Mechanistic investigation identified opposing roles for mTORC1 and mTORC2 in B-cell differentiation and showed that TOR-KIs enhance class switching through mTORC2 inhibition. These observations emphasize the distinct actions of TOR-KIs compared with rapamycin and suggest that TOR-KIs might be useful to enhance production of class-switched antibodies following vaccination.

SUMMARY

B cell activation by antigen leads to clonal expansion followed by differentiation into plasma cells secreting antigen-specific antibodies. Early in an immune response, some B cells differentiate rapidly into plasmablasts that secrete antibodies that are mostly IgM isotype and of low affinity. Other B cells adopt a germinal center (GC) fate and undergo class switch recombination (CSR) and somatic hypermutation (SHM). Ultimately, GC B cells that survive selection become plasma cells secreting high affinity antibodies of various isotypes, or become long-lived memory B cells.

Extracellular inputs including B cell receptor (BCR) engagement, Toll-like receptor (TLR) ligation and cytokines all activate the signaling enzyme phosphoinositide 3-kinase (PI3K) and its downstream target AKT (also known as PKB) in B cells [1]. PI3K/AKT signaling and other inputs activate the mammalian target of rapamycin (mTOR), a multifunctional kinase that promotes cell growth, division and metabolic reprogramming [1,2]. The mTOR kinase is present in two cellular complexes, mTOR-complex 1 (mTORC1) defined by the raptor subunit and mTOR-complex 2 (mTORC2) defined by rictor [3]. The classical mTOR inhibitor rapamycin forms a complex with FKBP12 that partially inhibits mTORC1 and can disrupt mTORC2 assembly upon prolonged cellular exposure. mTORC1 acts downstream of AKT and other signals to promote biosynthetic processes essential for cell growth and division. mTORC2 acts upstream of AKT by phosphorylating serine-473 in the AKT hydrophobic motif. mTORC2 and AKT function are required for subsequent phosphorylation of Forkhead Box Subgroup O (FoxO) transcription factors [4,5]. When phosphorylated, FoxO factors exit the nucleus and transcription of FoxO target genes is reduced.

Recent studies illustrate the complexity of mTOR function in B cells. Conditional deletion of the mTOR gene in mouse B cells strongly impairs proliferation and germinal center differentiation [6]. Inactivation of mTORC2 in B cells, via rictor deletion, reduces mature B cell survival and impairs antibody responses and germinal center formation [7]. At concentrations above 1 nM, rapamycin markedly impairs lymphocyte proliferation of both mouse and human B cells and suppresses antibody responses [8,9]. However, at lower concentrations that preserve B cell proliferation, rapamycin still suppresses class switching but unexpectedly promotes IgM responses that provide heterosubtypic protection from influenza [6,10]. These studies suggest that overall mTOR signaling as well as the relative activity of mTORC1 and mTORC2 control the ability of B cells to divide and to differentiate.

ATP-competitive mTOR kinase inhibitors (TOR-KIs) block activity of both mTORC1 and mTORC2 and were developed to overcome limitations of rapamycin as anti-cancer agents [11,12]. The Fruman lab reported that TOR-KIs do not block proliferation of normal mature B cells at concentrations that cause cell cycle arrest in pre-B leukemia cells [9]. However, the impact of TOR-KIs on immune function is still poorly characterized. An initial observation was made by a former graduate student Jose Limon in that when used at a selective dose that spares B cell proliferation, TOR-KIs surprisingly enhanced B cell CSR. In this study I genetically dissected mTOR signaling to understand how TOR-KIs can skew the differentiation of activated B cells. The results suggest that partial mTORC1/mTORC2 inhibition or mTORC2 deletion increase CSR, whereas selective inhibition of mTORC1 suppresses CSR.

MATERIALS AND METHODS

Mice and reagents

C57BL6 mice were bred at University of California-Irvine and used between 6-12 weeks of age. Aged mice, 16-18 months old, on a C56Bl6/SJL mixed background with a transgenic expressing GFP under CD88 promoter where kindly donated by Dr. Andrea Tenner from UCI. *Raptor*$^{fl/fl}$ mice on a C57BL6 background were obtained from Jackson Laboratories (stock number: 013138) and have been described previously (Sengupta S et al. 2010). *Rictor*$^{fl/fl}$ mice on a C57BL6 background were a generous gift from Dr. Mark Magnuson at Vanderbilt University and have been described previously (Shiota C et al. 2006). *CD19Cre* mice were obtained from Jackson Laboratories (stock number: 006368). All animals were studied in compliance with protocols approved by the Institutional Animal Care and Use Committees of the University of California-Irvine and Harvard Medical School. The active site mTORC1/2 inhibitors PP242, AZD8055, WYE-354 and Ku-0063794 were purchased from Chemdea and INK128 was obtained from Intellikine (San Diego, CA). The p110δ-selective PI3K inhibitor IC87114 and pan-PI3K class I inhibitor GDC-0941 were obtained from Intellikine. The inhibitor of AKT1 and AKT2, Akti-1/2, was purchased from Chemdea. The mTOR allosteric inhibitor, rapamycin, was purchased from LC Labs.

Primary cell culture

Mouse splenic B cells were purified by negative selection using anti-CD43 biotinylated antibody followed by incubation with anti-biotin magnetic microbeads and separation on MACS columns (Miltenyi Biotec, Auburn, CA). B cell purity was >98% as measured by FACS analysis (FACSCalibur and CellQuest Software; BD Biosciences, Mountain View, CA) using anti-B220 antibody (Biolegend). Purified B cells were seeded at a final concentration of 0.2 X 10^6/ml. For antibody-secreting cell differentiation, B cells were stimulated with 5 μg/ml LPS (Sigma) for 72 hours and for IgG1 CSR with either 3U of CD40L (gift of Dr. Paolo Casali, UCI), 1 μg/ml of anti-CD40 (HM40-3) agonistic antibody (Biolegend) or 5 μg/ml of LPS (Sigma) together with 2.5 ng/ml of mIL4 (R&D systems) for 96 hours. All B cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 1X MEM non-essential amino acids (Mediatech) and 1X sodium pyruvate (Mediatech). To assess mTORC1 activity after stimulation, cells were fixed and permeabilized using the BD Cytofix/Cytoperm buffer for 15 minutes at room temperature. Cells were subsequently washed with 0.5% Tween-20 in PBS and stained with a p-S6 (S240/244) antibody conjugated to AlexaFluor647 (Cell Signaling Technologies).

Flow cytometry, CFSE labeling and antibodies

Prior to cell surface staining, cells were incubated with TruStain fcX (Biolegend) in FACS buffer (0.5% BSA+ 0.02% NaN_3 in 1X HBSS) to block Fc receptors for ten minutes on ice. Immunophenotyping of mice were performed on splenocytes after RBC lysis. Staining with antibodies was subsequently performed also with FACS buffer and on ice for 20 minutes. Flow cytometry antibodies and other reagents used were as follows: CD4 (OKT4), B220 (RA3-6B2), IgG1 (RMG1-1), PD-1 (29F.1A12), IL-21 (FFA21), IL-17A (TC11-18H10.1) (all from Biolegend), IgD (11-26c), CD38 (90), IgM (eB121-15F9), CD21 (4E3), CD93 (AA4.1) (all from eBioscience), NP-PE (Biosearch Technologies), FAS (Jo2), CD138 (281-2), and CXCR5 (2G8) (all from BD Biosciences). CFSE labeling of B cells to track proliferation was performed as described elsewhere (30). Flow cytometric data was analyzed using Flowjo software (Treestar).

RESULTS AND DISCUSSION

High concentrations of TOR-KIs block B cell proliferation

The Fruman lab has reported previously that the TOR-KI compound PP242, when present at 100 nM, fully suppresses mTOR signaling in B cells without blocking proliferation [9]. This was surprising since the allosteric mTOR inhibitor rapamycin had only partial effects on signaling yet fully blocked B cell proliferation [9]. Our initial signaling measurements were taken 15 minutes after B cell stimulation [9], so we speculated that the effects of PP242 might be transient and wear off before the cell commits to division. To test this idea, I conducted a time course measuring phosphorylation of the ribosomal S6 protein at the serine 240/244 site (p-S6), a sensitive readout of mTORC1 activity. Consistent with the prediction, 100 nM PP242 blocked p-S6 nearly completely at 1hr after B cell stimulation but much less at 24hr and 48hr (Figure 3.1A, B). By 48hr the cells had proliferated nearly to the same extent as control vehicle-treated B cells (Fig. 3.1A, C). Increasing the concentration of PP242 to 400 nM caused sustained inhibition of p-S6 and blocked proliferation. The patterns were comparable to cells treated with rapamycin (RAP) at 10 nM. These results suggest that when used at low concentrations, the effects of TOR-KIs on B cell differentiation can be separated from the effects on proliferation. For this purpose, in subsequent B cell differentiation experiments, I used PP242 and other TOR-KIs at concentrations that minimally impact proliferation (Fig. 3.2A, D).

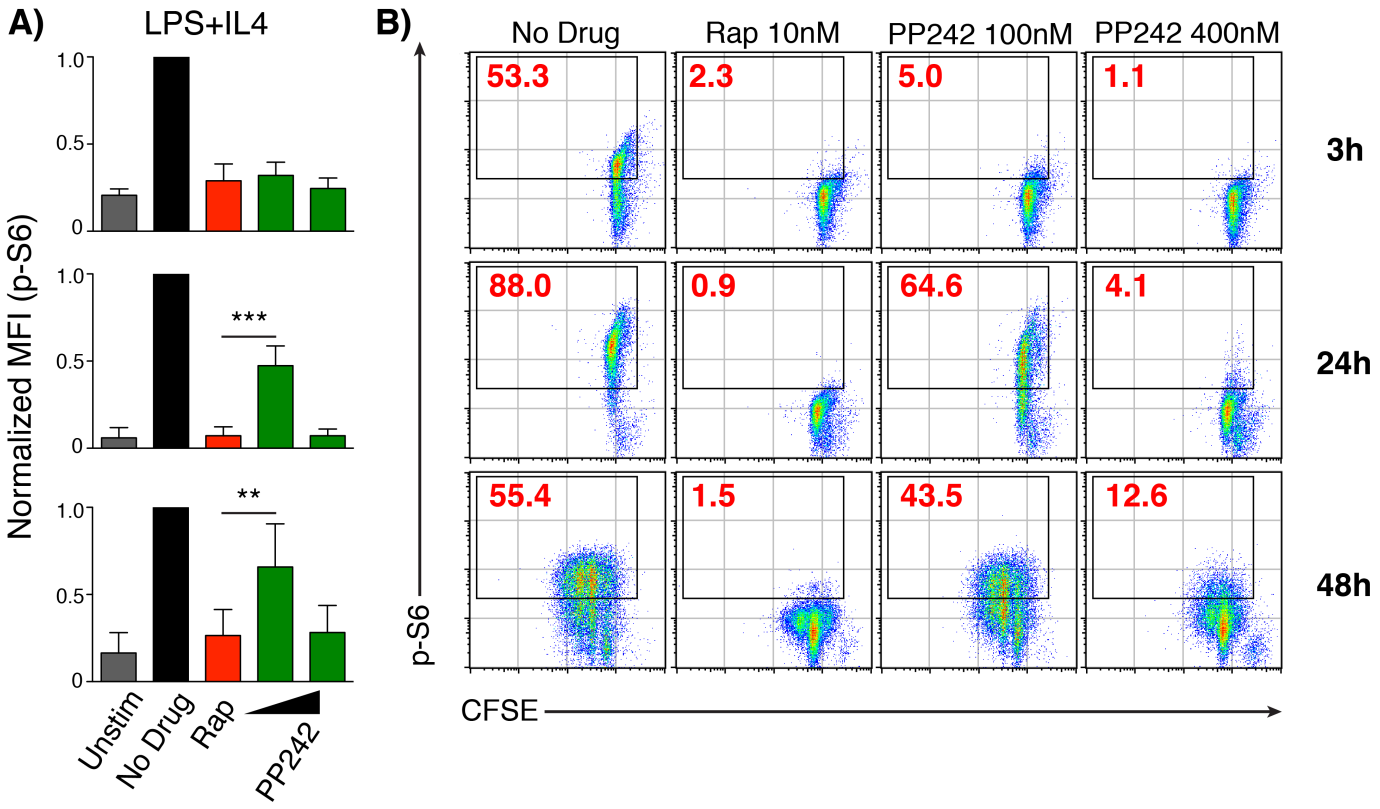

Figure 3.1. High concentrations of TOR-KIs reduce mTORC1 activity and block B cell proliferation. (A, B) Purified splenic B cells were labeled with CFSE, to track cellular proliferation, and cultured with media only or stimulated with LPS+IL4 or LPS+IL4 and inhibitors at the indicated concentrations. mTORC1 activity was assessed at the indicated timepoints by ICS for p-S6 (S240/244 site) on proliferated cells. (B) Histograph of p-S6 MFI for the indicated cell culture conditions and timepoints. The dashed red line represents the average of unstimulated samples. (C) CFSE-labeled B cells were cultured in media only or stimulated with LPS+IL4 or LPS+IL4 and inhibitors at the indicated concentrations for 48 hours. Data are representative of three independent experiments. Rap: rapamycin; 242: PP242.

TOR-KIs increase B cell isotype switching *in vitro*

To define the B cell-intrinsic effects of TOR-KIs, I assessed the differentiation of purified splenic B cells. Four different TOR-KIs were utilized that had distinct chemical structures (INK128, PP242, Ku-0063794, and AZD8055) to minimize the potential for off-target effects. Each compound increased the percentage of IgG1-switched $B220^{+}$ B cells induced by anti-CD40 plus interleukin-4 (IL4), conditions that mimic signals during a TD response and favor isotype switching to IgG1 (Fig. 3.2A, B). Direct inhibition of AKT isoforms AKT1 and AKT2 also increased the frequency of switching to IgG1 (Fig. 3.2A, B). Similar results were observed when B cells activated by lipopolysaccharide (LPS) plus IL4 were treated with INK128, PP242, Ku-0063794 or AZD8055 (Fig. 3.2C).

Next, the effect of TOR-KIs on differentiation into plasmablasts and antibody-secreting cells (ASC) following stimulation with LPS were examined. Plasmablast differentiation, as measured by cells with a $B220^{low}$ $CD138^{+}$ phenotype, was decreased in a concentration-dependent manner by TOR-KIs whereas AKT inhibition had no significant effect (Fig. 3.2D, E). Thus, TOR-KIs can directly alter B cell differentiation fate, causing increased CSR and a reciprocal decrease in plasmablast and ASC differentiation.

In contrast to the effects of TOR-KIs, rapamycin reduced both CSR and ASC differentiation in a concentration-dependent manner (Fig. 3.2). The suppression of both differentiation pathways is consistent with early studies of rapamycin action in B cells [8].

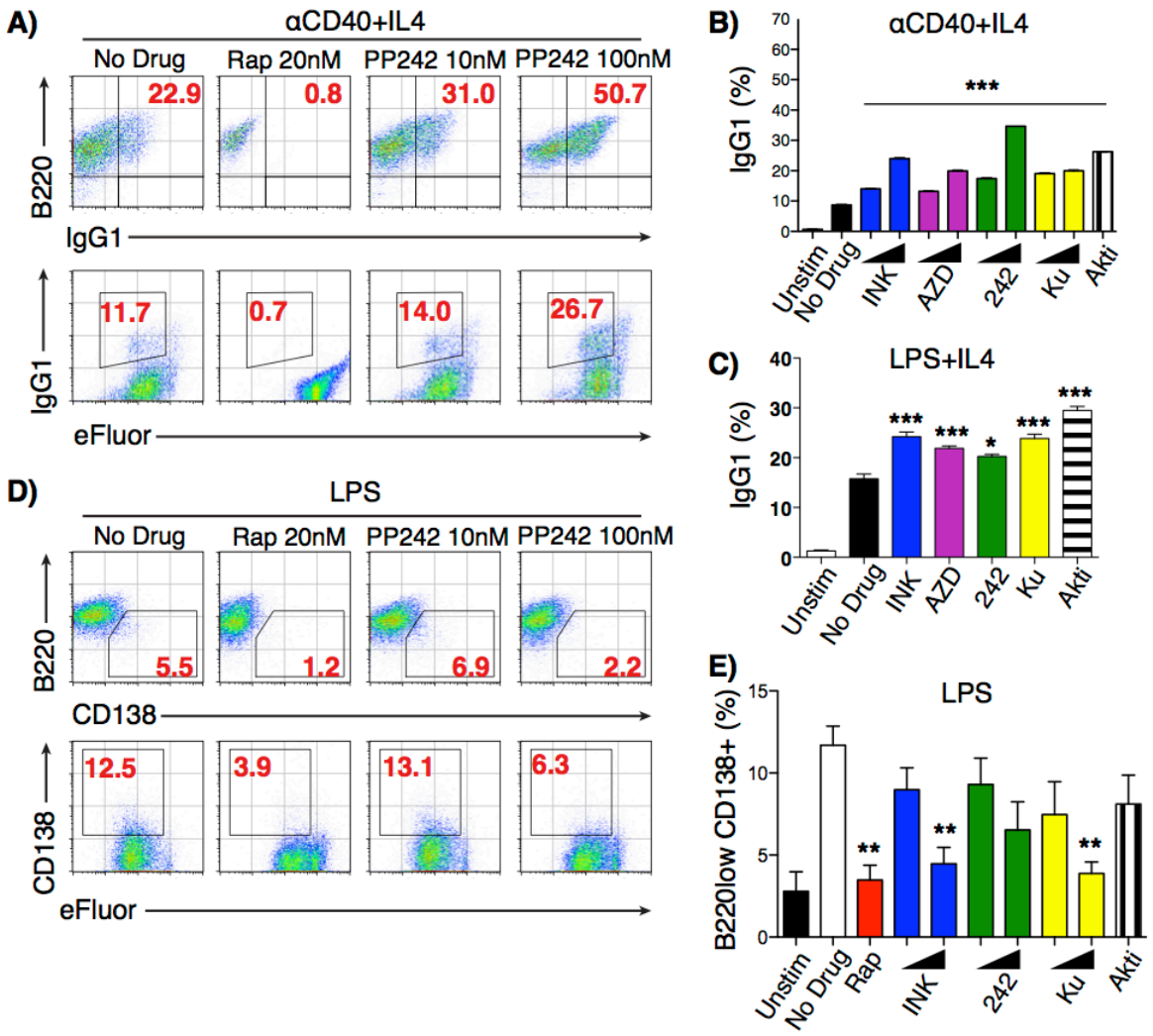

Figure 3.2. TOR-KIs increase *in vitro* B cell CSR and decrease plasmablast differentiation. (A, B) Purified B cells were cultured with media only or stimulated with either αCD40 + IL4 in the absence or presence of the inhibitors indicated. (A) Cell division history of eFluor670-labeled B cells was determined by FACS after 4 days. Other TOR-KIs in this study had similar effects on cell division at the concentrations used for differentiation experiments. (B) The percentage of live B cells that have divided at least once (based on eFluor division history) expressing surface IgG1 was determined by FACS after 4 days. (C) Switching to IgG1 was assessed as in panel B except cells were stimulated with LPS + IL4. (D) Representative FACS plots of plasmablast differentiation in purified B cells stimulated with LPS or LPS and indicated inhibitors for 3 days. B cells were labeled with eFluor 670 to track division history (bottom row). (E) Graph of the percentage of live B cells with a plasmablast phenotype determined by FACS after 3 days of stimulation as indicated in D. (*$p<0.05$, ***$p<0.01$, one way ANOVA with Tukey's Multiple Comparison Test, measured versus the No Drug sample). Rap: rapamycin; Ku: Ku-0063794; INK: INK-128; Akti: Akt inhibitor VIII; 242: PP242, AZD: AZD8055.

Inactivation of mTORC1 versus mTORC2 has opposing effects on CSR

As TOR-KIs inhibit both outputs of mTORC1 and mTORC2, it was important to understand the mechanism of CSR enhancement. As a genetic approach to assess the roles of mTORC1 and mTORC2, I used mice with conditional (floxed) alleles of *Rictor* or *Raptor* (see materials and methods). Deletion of *Rictor* using *CD19Cre* (rictor-flox/*CD19Cre*; termed rictor$^{\Delta B}$) did not significantly alter B cell subset frequencies (Fig. 3.3A). In resting mature B cells, rictor expression was not completely reduced suggesting incomplete and/or ongoing deletion (Fig. 3.4A). Consistent with reduced mTORC2 function, AKT phosphorylation at S473 was lower in B cells from rictor$^{\Delta B}$ mice (Fig. 3.4A). Importantly, this effect was specific to mTORC2 as S6 phosphorylation, a sensitive readout of mTORC1, was unaffected in rictor$^{\Delta B}$ B cells (Fig 3.4A). To address the role of mTORC1, I analyzed B cells from raptor$^{\Delta B}$ mice in which the raptor-flox allele is deleted at the transitional B cell stage using *CD21Cre*. As with rictor$^{\Delta B}$, B cell development was largely normal in raptor$^{\Delta B}$ (Fig. 3.3B). Raptor expression was also not completely reduced in resting mature B cells (Fig 3.4D) and this corresponded to a significant but not complete reduction in S6 phosphorylation in raptor$^{\Delta B}$ B cells (Fig 3.4D). A minor increase in AKT phosphorylation in raptor$^{\Delta B}$ B cells was also observed, consistent with the loss of negative feedback from mTORC1 loss (Fig 3.4D).

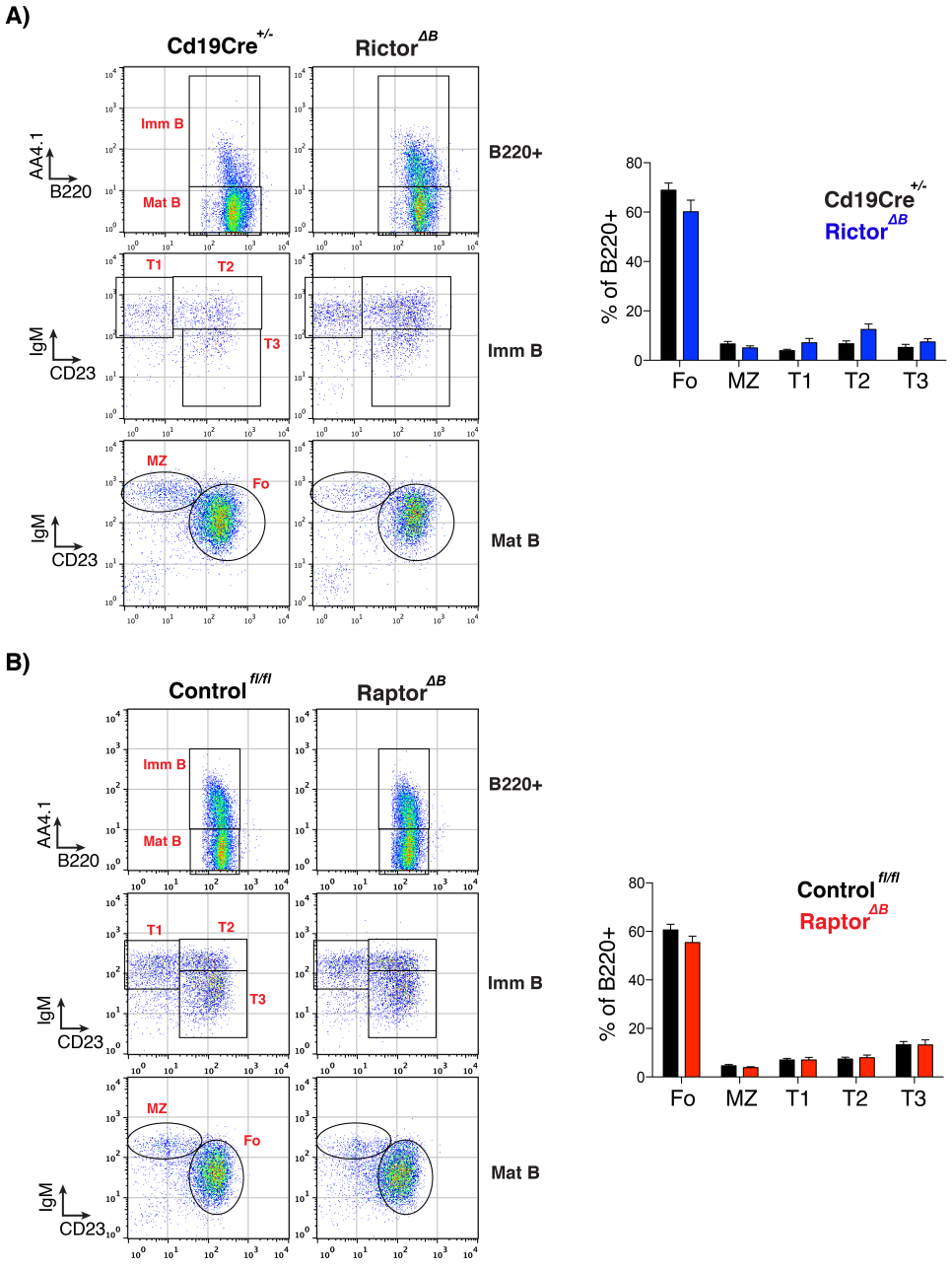

Figure 3.3. B cell subset analysis by FACS in rictor$^{\Delta B}$ and raptor$^{\Delta B}$. Splenic cells after RBC lysis were stained with appropriate antibodies to identify the major B cell subsets present in indicated mice. (A) B cell subset analysis in rictor$^{\Delta B}$ mice. Representative gating scheme to identify different B cell subsets is shown (left). Data are averaged from at least 6 different mice (right). Imm B: B220+AA4.1+ Immature B cells. Mat B: B220+AA4.1- Mature B cells. MZ: Marginal Zone B cells. Fo: Follicular B cells. T1, T2, T3: Transitional B cells. (B) B cell subset analysis in raptor$^{\Delta B}$ mice as in (A).

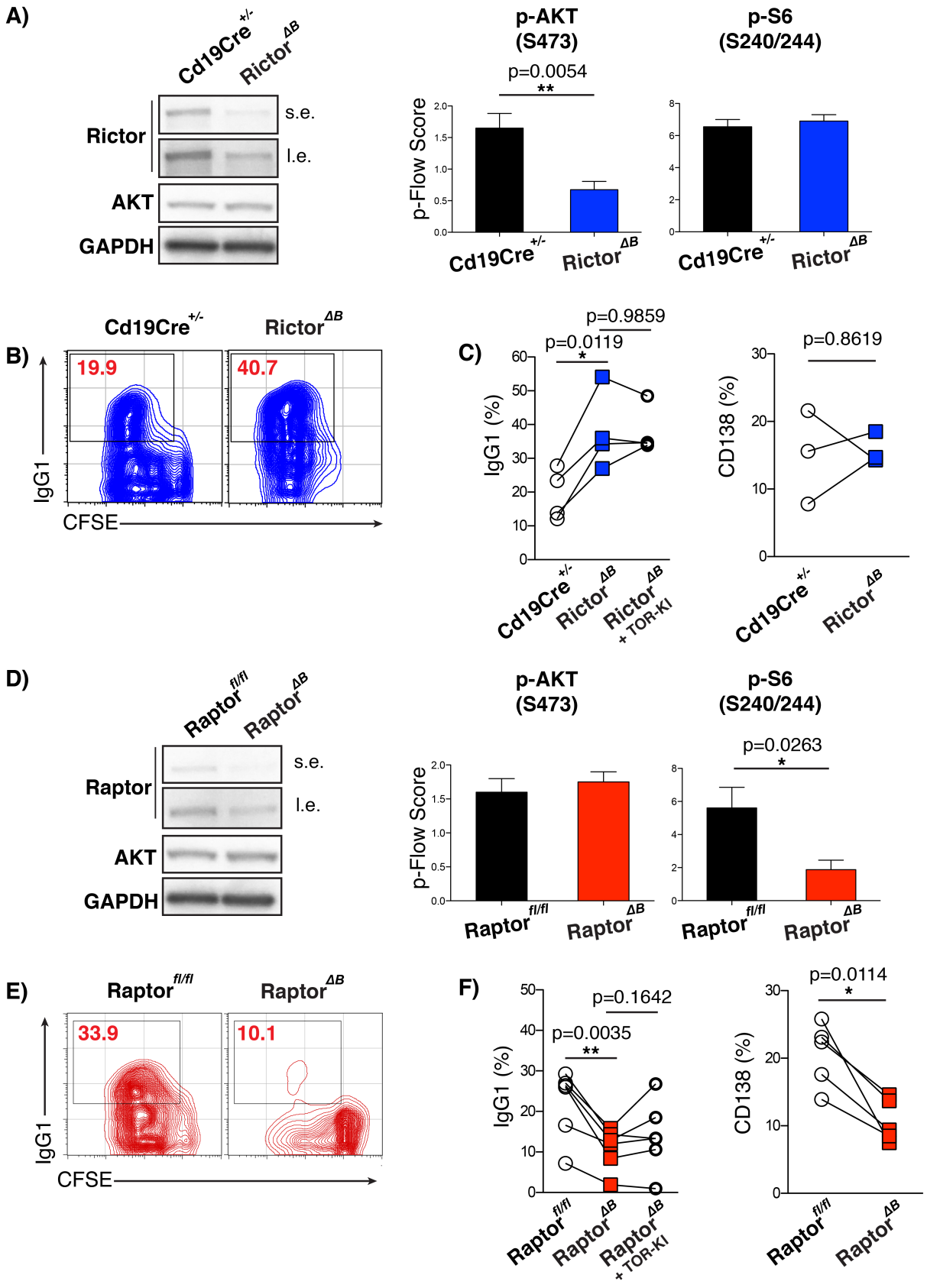
A)
Cd19Cre+/-
RictorΔB
Rictor
s.e.
l.e.
AKT
GAPDH
p-AKT (S473)
p-S6 (S240/244)
p=0.0054
**
p-Flow Score
Cd19Cre+/-
RictorΔB
B)
Cd19Cre+/-
RictorΔB
19.9
40.7
IgG1
CFSE
C)
p=0.9859
p=0.0119
*
IgG1 (%)
p=0.8619
CD138 (%)
Cd19Cre+/-
RictorΔB
RictorΔB + TOR-KI
D)
Raptorfl/fl
RaptorΔB
Raptor
s.e.
l.e.
AKT
GAPDH
p-AKT (S473)
p-S6 (S240/244)
p=0.0263
*
p-Flow Score
Raptorfl/fl
RaptorΔB
E)
Raptorfl/fl
RaptorΔB
33.9
10.1
IgG1
CFSE
F)
p=0.1642
p=0.0035
**
IgG1 (%)
p=0.0114
*
CD138 (%)
Raptorfl/fl
RaptorΔB
RaptorΔB + TOR-KI

Figure 3.4. Inactivation of mTORC1 versus mTORC2 has opposing effects on CSR.
(A) Purified B cells from either control (rictor$^{fl/fl}$) or rictor-flox/CD21Cre (rictor$^{\Delta B}$) were activated with αCD40+IL4 for the indicated times to measure changes in mTORC2 activity (pAKT-S473) and mTORC1 activity (pS6-S240/244) by Western blotting. Each signal determined by densitometry was normalized to the amount of total AKT in each sample, then normalized again to the rictor$^{fl/fl}$ time zero sample (red numbers below each lane). The arbitrary units (a.u.) of the signal was plotted as a line graph (right). (B) Purified B cells were labeled with CFSE and activated with αCD40+IL4 for 4 days. The percentage of live B cells that had divided at least once (based on CFSE division history) expressing surface IgG1 was determined by FACS after 4 days. Left: Percentage of IgG1 expressing B cells are shown for experiments where rictor$^{\Delta B}$ B cells showed an increase. Percentage of IgG1 expressing rictor$^{\Delta B}$ B cells upon low dose TOR-KI (INK128 1-5nM) treatment is also shown. Middle: Percentage of IgG1 expressing B cells are shown for experiments where rictor$^{\Delta B}$ B cells showed no phenotype. Right: For plasmablast differentiation, cells were activated with LPS for 4 days and cells expressing CD138 were determined by FACS. (C) Representative FACS plots showing the tracking of dividing B cells that express surface IgG1. (D) Western blotting was performed the same way as in (A) but with B cells from control (raptor$^{fl/fl}$) or raptor-flox/CD21Cre (raptor$^{\Delta B}$). (E) Determination of surface IgG1 or CD138 expressing raptor$^{\Delta B}$ cells that have divided at least once was determined as in (B). (F) Representative FACS plots as in (C). A paired Student's t test was used comparing either rictor$^{\Delta B}$ or raptor$^{\Delta B}$ B cells to the amount of surface IgG1 or CD138 percentage in the control B cells (rictor$^{fl/fl}$ or raptor$^{fl/fl}$). A paired Student's t test was also performed to compare the percent of divided cells that were IgG1+ between vehicle-treated rictor$^{\Delta B}$ or raptor$^{\Delta B}$ B cells to low dose TOR-KI (INK128: 1-5nM) treated B cells of each genotype.

Next I tested functional responses in B cells with partial loss of rictor or raptor. In response to anti-CD40 plus IL4, rictor-deficient B cells proliferated to a similar degree as WT whereas raptor-deficient cells proliferated less (Fig. 3.4B, E). To compare the capacity of cells to undergo class switching, we gated on divided cells and calculated the percentage of IgG1+ cells. The results showed consistently less switching in divided raptor$^{\Delta B}$ B cells compared to control (Fig. 3.4F). These findings are consistent with the effects of rapamycin on B cell division and differentiation (Fig. 3.5). In contrast, rictor$^{\Delta B}$ B cells showed significantly more switching to IgG1 than control (Fig. 3.4C). Importantly, in rictor$^{\Delta B}$ B cells, adding low dose TOR-KI (1-5nM INK128) did not further increase CSR (Fig. 3.4C).

I also measured plasmablast generation in B cells stimulated with LPS alone. raptor$^{\Delta B}$ B cells showed reduced ASC generation whereas rictor$^{\Delta B}$ B cells were similar to control (Fig. 3.4C, F). Together these genetic interventions support the conclusion that mTORC1 inhibition suppresses both CSR and plasmablast generation, whereas mTORC2 inhibition increases CSR with little effect on plasmablast generation.

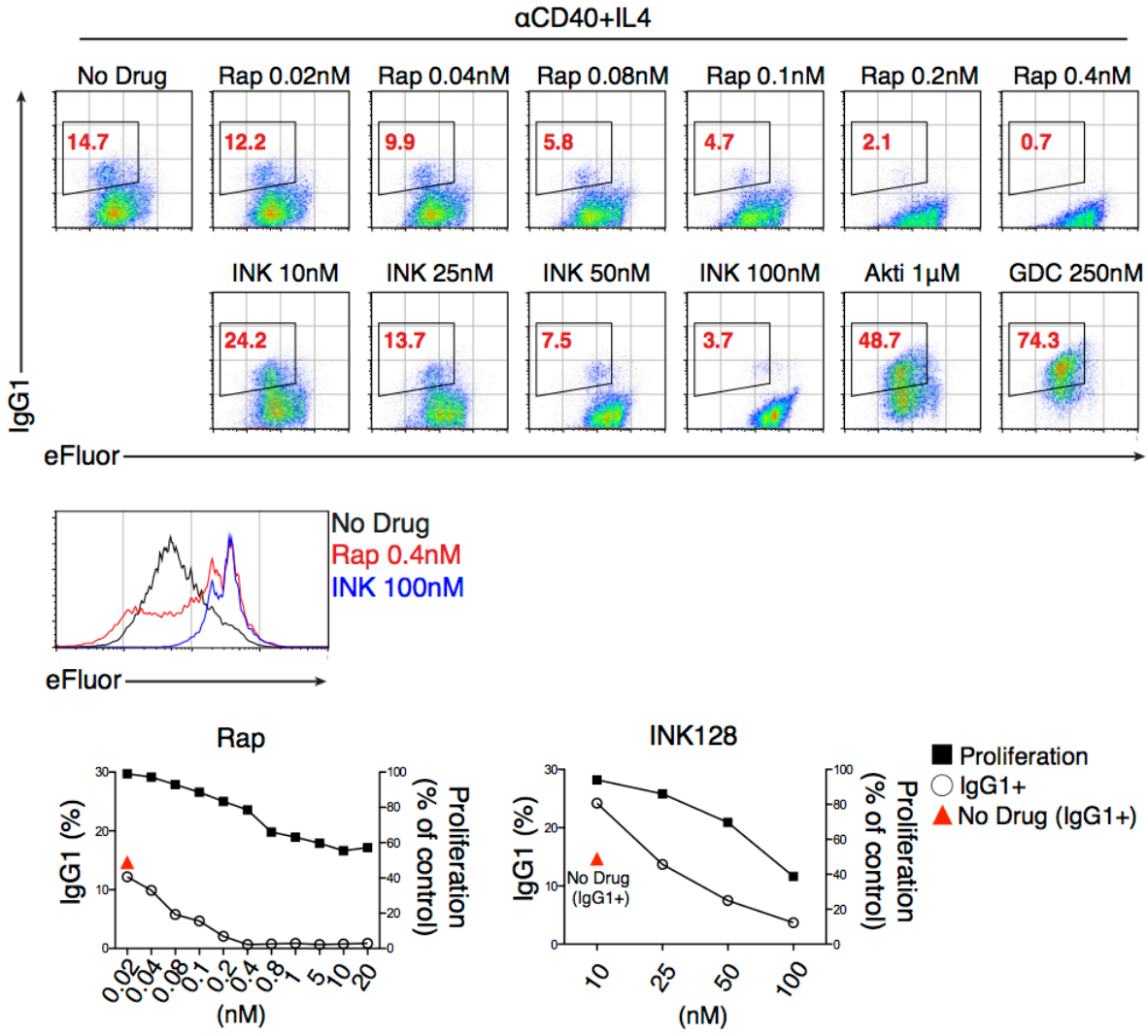

Figure 3.5. Rapamycin has a more profound effect on B cell proliferation and CSR than TOR-KIs. Purified B cells were labeled with eFluor670, to measure cell division, and cultured in media alone or stimulated with αCD40+IL4 in the presence or absence of indicated inhibitors. Representative FACS plots are shown for the different treatment conditions (upper 2 panels) and a FACS proliferation histogram for high dose rapamycin and INK128 (bottom left panel). The effects of inhibitor concentration on cellular proliferation and CSR are represented on line graphs (bottom 2 right panels). Data are representative of two independent experiments. The data was generated by another graduate student and co-author Honyin Chiu while the experiment was designed by Lomon So.

Discussion

TOR-KIs are a powerful new class of compounds that inhibit both rapamycin-sensitive and rapamycin-resistant mTOR functions. These agents not only have great promise for clinical management of cancer, but also represent new chemical tools for probing the function of mTOR kinase activity in various cell types. Here we have used a panel of chemically distinct TOR-KIs to demonstrate that mTOR kinase inhibition increases the fraction of activated B cells undergoing antibody class switching. These results were seen at TOR-KI concentrations that cause transient mTORC1/2 inhibition and only partially reduced signaling after 24hr in B cells. The effect of AKT inhibition or partial mTORC1/2 inhibition to increase CSR requires FoxO transcription factors (Figure 7 of [13]). These data support the model that mTORC2 inhibition by TOR-KIs reduces AKT activity, increasing FoxO activity to drive the enhanced class switching response in TOR-KI treated B cells. This model is consistent with previous studies showing that PI3K activity suppresses CSR through AKT-dependent inactivation of FoxO1, whereas PI3K inhibition or FoxO activation promotes CSR [14,15].

The enhanced production of class-switched antibodies by mTORC1/mTORC2 inhibition is surprising, considering the well-known immunosuppressive activity of rapamycin and the impaired survival and differentiation of mouse B cells lacking mTOR [16]. The *in vitro* studies establish the importance of using intermediate doses of competitive mTOR inhibitors that transiently inhibit both mTORC1 and mTORC2. At higher concentrations, TOR-KIs sustain mTOR inhibition and block B cell proliferation to a similar degree as rapamycin, probably through strong mTORC1 inhibition. The findings contrast with a recent report that deletion of rictor in B cells reduces survival and

proliferation, and impairs class switching [7]. It appears that these systems achieve differential efficiency of rictor deletion. Boothby and colleagues obtained efficient deletion using Vav-Cre, where rictor is deleted in all hematopoietic cells, or inducible CreER with chronic *in vivo* tamoxifen treatment that directs rictor deletion in all cell types. I used *Cd19Cre* which mediated partial deletion of rictor in B cells and partial but not complete loss of mTORC2 signaling. This allowed B cells to survive and proliferate, and led to enhanced class switching. The results we obtained with rapamycin titrations are consistent with recent evidence that mTORC1 inhibition can suppress CSR independent from proliferation. An interesting finding is that when both complexes are partially inhibited by intermediate concentrations of TOR-KIs, the effect of mTORC2 inhibition is dominant for CSR (enhancement) whereas mTORC1 inhibition is dominant for ASC generation (inhibition).

CONCLUSION

Overall, the findings emphasize that targeted inhibitors of the PI3K/AKT/mTOR pathway have important immunomodulatory effects at concentrations that do not strongly suppress lymphocyte clonal expansion. Eventually, TOR-KI treatment could be used to improve vaccine efficacy, or to boost the production of class-switched antibodies in animals for biomedical applications.

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CHAPTER 4:

COORDINATION OF CELL GROWTH AND PROLIFERATION BY 4E-BP/EIF4E IN PRIMARY LYMPHOCYTES

ABSTRACT

Cells from multicellular organisms independently regulate cell growth (mass increase) and proliferation to ensure proper size and number in different tissues. Fibroblasts differentially control growth and proliferation in part through distinct effectors of the mammalian target of rapamycin complex-1 (mTORC1), where ribosomal S6 kinases (S6Ks) promote growth and eIF4E-binding proteins (4E-BPs) regulate proliferation. However, for animal cells that need to rapidly increase in number, it has been proposed that growth and proliferation might be coordinately controlled. We assessed the roles of mTORC1 substrates in lymphocytes, a cell type that must undergo rapid expansion following antigen stimulation and where rapamycin profoundly delays both growth and proliferation. We show that mTORC1-mediated phosphorylation of 4E-BPs is required in T and B cells for both cell growth and proliferation, whereas S6Ks are dispensable. Specifically blocking the cap-dependent translation initiation complex by a mutant 4E-BP1 was sufficient to phenocopy the growth and proliferation defect seen in lymphocytes lacking raptor, an essential mTORC1 component. Surprisingly, rapamycin suppressed the 4E-BP/eIF4E axis and protein translation more strongly in primary lymphocytes than in other cell types, providing a novel mechanism to explain selective suppression of immune function by rapamycin. Thus, the 4E-BP/eIF4E axis is uniquely rapamycin-sensitive in lymphocytes and promotes clonal expansion by coordinate control of growth and proliferation.

SUMMARY

Unlike unicellular organisms (e.g. yeast) where growth rate is strictly based on nutrient availability, the growth of cells derived from multicellular organisms is limited by the availability of extracellular signals such as growth factors and patterning inputs [1]. In numerous animal cell types (e.g. fibroblasts, myoblasts, primary Schwann cells) cell growth and proliferation can be separated, a mechanism that is thought to ensure correct organ and organismal size [2–4]. Signaling by the mammalian target of rapamycin complex 1 (mTORC1) is central to these processes, as inhibition of mTORC1 reduces both cell growth and proliferation of most cells induced by multiple extracellular signals [5,6]. In fibroblasts, distinct mTORC1 effectors specifically regulate either cell growth or proliferation in a largely independent fashion. In particular, the translation initiation factor 4E-binding proteins (4E-BPs) downstream of mTORC1 regulate cell proliferation whereas the ribosomal protein S6 kinases (S6Ks) control cell size [4].

However, metazoans are composed of many different types of cells that may depend on distinct mechanisms of growth and proliferation control. Here we focus on lymphocytes, which constitute the fundamental basis of adaptive immunity. Upon activation through the antigen receptor, small resting lymphocytes undergo an enormous phase of growth (blastogenesis) followed by rapid cell proliferation known as clonal expansion [7,8]. For cells such as lymphocytes that need to rapidly increase in number in a given context, it has been proposed that cell growth and proliferation could be coupled through a common control mechanism [9]. One example was shown where extracellular signals such as cytokines could influence both growth and proliferation in a cytokine-dependent hematopoietic cell line [10]. However, there is no experimental evidence whether

a common intracellular signal exists that coordinates growth and proliferation in primary animal cells.

The FDA-approved immunosuppressant rapamycin (sirolimus) has been a valuable tool to dissect mTORC1 signaling in many cell types [11,12]. Yet, nearly 40 years after the discovery of rapamycin, it is still unclear why the cytostatic potency of this compound is greater in activated lymphocytes compared to other cell types [11]. It has been challenging in general to discern the precise mechanism of action of rapamycin, a compound that acts allosterically to inhibit phosphorylation of some but not all mTORC1 substrates [13]. For example, rapamycin weakly inhibits 4E-BP1 phosphorylation and this is considered to be an important factor that limits clinical efficacy of rapamycin and its analogs as anti-cancer agents [14–16]. Second-generation mTOR inhibitors that target the ATP-binding site (mTOR kinase inhibitors; TOR-KIs) fully block 4E-BP1 phosphorylation, correlating with greater cytostatic potency compared to rapamycin in most cell types examined [17–20]. On the contrary, we have reported that rapamycin and TOR-KIs have equivalent anti-proliferative effects in activated primary T and B cells [18,21]. These findings concur with early studies reporting the unique potency of rapamycin in blocking both cell growth and proliferation of activated primary T and B cells [22,23]. More recently, genetic depletion of the integral mTORC1 subunit raptor in T cells was found to cause a profound delay in S phase entry [24]. However, the rapamycin-sensitive effectors of mTORC1 that drive lymphocyte growth and division remain unclear.

In this study, I discovered that the 4E-BP/eIF4e signaling node downstream of mTORC1 is crucial for cell growth and proliferation in activated primary T and B cells. Unlike in other cell types studied to date, rapamycin was equally effective as TOR-KIs at

disrupting formation of the eIF4F complex and reducing protein synthesis. These results provide the first experimental evidence that in animal cells requiring rapid expansion, cell growth and proliferation are coupled through a common signaling node downstream of mTORC1. Thus, a common mTORC1 effector that is sensitive to rapamycin may explain the unique ability of this partial mTOR inhibitor to strongly block both growth and proliferation of lymphocytes.

Materials and Methods

Mice and Reagents

C57BL6 mice were bred at University of California-Irvine and used between 6-12 weeks of age. *mTOR*$^{fl/fl}$ and *Raptor*$^{fl/fl}$ mice on a C57BL6 background were obtained from Jackson Laboratories (stock number: 011009 and 013138, respectively). *mTOR*$^{KI/+}$ mice on a C57BL6 background were obtained from Lexicon Genetics and have been described previously [25]. *CD4Cre* and *CD19Cre* mice were obtained from Taconic (Model: 4196) and Jackson Laboratories (stock number: 006368), respectively. TRE-*4E-BP1*^{M} mice were obtained from Davide Ruggero (UCSF). *Rosa26-rtTA* mice were obtained from Jackson Laboratories (stock number: 006965). *S6K2*$^{-/-}$ mice were obtained from Sara Kozma (IDIBELL, Spain). Spleens from *S6K1/2*$^{-/-}$ mice were processed and shipped from Nahum Sonenberg's lab (McGill University). All animals were studied in compliance with protocols approved by the Institutional Animal Care and Use Committees of the University of California-Irvine and Harvard Medical School. The mTOR allosteric inhibitor rapamycin and the active site mTORC1/2 inhibitor MLN0128 were purchased from LC labs, and Torin1 was a generous gift from Nahum Sonenberg (McGill University).

Antibodies

For phospho-flow staining, rabbit antibodies specific for phosphorylated proteins were from Cell Signaling Technologies: p-S6 (S240/244), p-S6 (S235/236), and total S6. For flow cytometry, anti-mouse antibodies were: CD4-PE, CD8-APC, B220-PE, IgM-FITC, CD21-FITC. All flow cytometry antibody reagents were purchased from eBioscience and Biolegend. Succinimidyl ester 5-(and -6) carboxyfluorescein diacetate (CFSE) and eFluor670 were obtained from eBioscience.

Intracellular Phosphostaining and Cell Size Measurement

Activated or resting cells were fixed and permeabilized by BD Cytofix/Cytoperm buffer for 15 minutes at room temperature (RT). Cells were washed with perm/wash buffer (PBS with 0.5% Tween-20) and stained with the appropriate antibodies already conjugated with fluorophores (p-S6; 1:1000). Cells were washed once more in perm/wash and analyzed by flow cytometry. To measure size of cells, unfixed cells were directly analyzed by flow cytometry and forward light scatter was measured.

Flow Cytometry

Isolated splenocytes from mice were RBC lysed and resuspended in FACS buffer (PBS with 0.5% BSA fraction V). Surface staining with antibodies was subsequently performed also with FACS buffer and on ice for 20 minutes.

B cell culture

B cells were purified by negative selection using the B cell isolation kit (StemCell Technologies). For CFSE proliferation assays, purified B cells were labeled with 2.5μM CFSE in PBS for 5min at RT. CFSE labeled B cells were cultured in lymphocyte media (LCM; RPMI medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 5 mM HEPES buffer, and 50 mM 2-ME). For activation, 10μg/ml anti-mouse IgM F(ab')2 (eBioscience) with IL-4 (10ng/ml) (R&D Systems).

T cell culture

CD4+ T cells were purified by negative selection using the CD4 T cell isolation kit (StemCell Technologies). For CFSE proliferation assays, purified CD4 T cells were labeled with 2.5μM CFSE in PBS for 5min at RT. CFSE labeled CD4 T cells were cultured in lymphocyte media (LCM; RPMI medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 5 mM HEPES buffer, and 50 mM 2-ME). For activation, 10μg/ml anti-mouse IgM F(ab')2 (eBioscience) with IL-4 (10ng/ml) (R&D Systems).

Immunoblotting

Cells were washed once with ice-cold PBS once and lysed in RIPA buffer for 15-20 minutes on ice with occasional vortexing (every 5 minutes). After lysis, lysates were spun at maximum speed in a tabletop centrifuge in the cold room for 20 minutes to pellet the nuclei. Supernatants were collected and protein amount was quantitated using the Bradford method (Bio-Rad). Equal amounts of protein were resuspended in Laemmli

buffer and subjected to SDS-PAGE. After separation, proteins were transferred to a nitrocellulose membrane and probed with the indicated antibodies overnight in the cold room. Secondary antibody conjugated to horseradish peroxidase (HRP) was added the next day and the signal was illuminated using chemiluminescence.

4E-BP1^{M} induction in vitro

Purified B or CD4+ T cells were cultured in LCM with or without doxycycline at indicated concentrations in the figures for 6-9 hours prior to activation. In some experiments, lymphocytes were activated prior to adding doxycycline at 1μg/ml final concentration to the media.

Staphylococcal Enterotoxin B (SEB) mediated T cell activation

For *in vivo* SEB induced T cell activation, indicated genotypes of mice in groups (n=3-4) were either injected i.p. with PBS or 100μg of SEB resuspended in PBS. Cohorts of mice were sacrificed on day 1 and splenocytes were surface stained for CD4 and Vβ8 antibodies to measure the growth of CD4+Vβ8+ T cells. Another set of mice was sacrificed on day 2 and total splenocytes after RBS lysis were counted and stained for CD4+Vβ8+ T cells. The frequency of CD4+Vβ8+ T cells measured by flow cytometry was backcalculated to assess absolute cell counts, an indicator of cell proliferation or death. For in vitro T cell activation, RBS lysed total splenocytes were CFSE labeled and cultured in a U-bottom 96-well plate and SEB was added at a final concentration of 50ng/ml. Cells were analyzed on day 2, 4 and 5 to assess growth (forward scatter) and proliferation (CFSE dilution).

RESULTS AND DISCUSSION

Rapamycin and TOR-KIs inhibit lymphocyte growth and proliferation through an mTORC1 kinase substrate

The Fruman lab reported previously that in T and B cells stimulated by a variety of mitogenic signals, rapamycin blocks proliferation to a similar extent as TOR-KIs [21,26]. This was surprising considering that rapamycin has weaker anti-proliferative activity compared to TOR-KIs in fibroblasts and a variety of cancer cell lines [17,19,20,26–28]. I confirmed these results by measuring growth and proliferation of lymphocytes treated with a concentration of MLN0128 that was sufficient to sustain mTOR inhibition up to 48h (Figure 4.1A) as assessed by phosphorylated S6 at S240/244 (p-S6) (MLN0128; 50nM). At this concentration, growth of both T and B cells following antigen receptor stimulation for 24h was equally suppressed by rapamycin and MLN0128 (Figure 4.1B). When cell division was measured by CFSE dilution at 48h, rapamycin and TOR-KIs showed similar potency in inhibiting proliferation (Figure 4.1B).

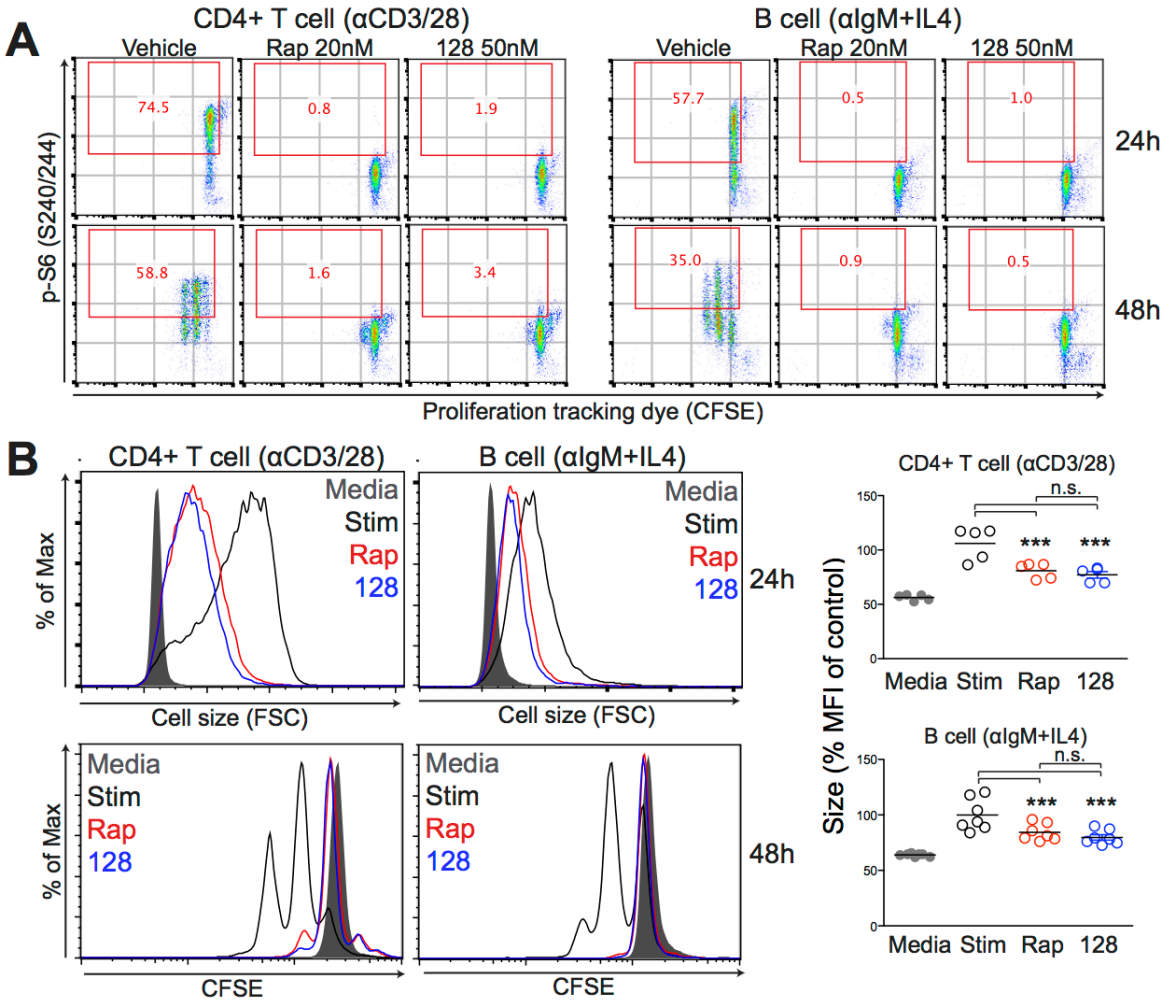

Figure 4.1. Rapamycin and TOR-KIs inhibit lymphocyte growth and proliferation to the same extent (A) Purified CD4 T (left) or B (right) cells were labeled with the cell division tracking dye CFSE and activated through the antigen receptor using crosslinking antibodies (αCD3 and αCD28 for T cells or αIgM with IL-4 for B cells) for the indicated time points. Cells were fixed/permeabilized and stained for phosphorylated S6 (p-S6) at S240/244 to check mTORC1 activity. Where indicated, cells were pre-treated with rapamycin (20nM) or MLN0128 (50nM) (B) Cell growth at 24h was measured using forward scatter analysis and the size decrease compared to control was measured for each experiment (right graph). Cell proliferation at 48h was measured using CFSE dilution analysis Results represent at least three independent experiments. Where indicated, data represent mean±S.E. of n=3-7 experiments (*, $p<0.05$;**,$p<0.01$;***,$p<0.001$, repeated-measures analysis of variance, measured versus the media control).

Since rapamycin disrupts the assembly of mTOR complexes [29–31] I considered the possibility that mTOR has important non-catalytic functions in lymphocytes that are inhibited by rapamycin. This possibility was suggested by kinase-independent mTOR functions in skeletal muscle regeneration [32]. To test this possibility, I generated a novel genetic model in which mTOR kinase activity is abolished (D2338A mutation) [25] only in T or B cells by *Cd4*-Cre and *Cd19*-Cre driver mice (Figure 4.2A, B and C). Despite the presence of mTOR protein, lymphocytes expressing kinase-inactive mTOR (mTOR-KI) displayed absence of mTORC1 kinase activity as assessed by p-S6 and phosphorylation of 4E-BP1 at T37/46 (Figure 4.2D and E). As reported previously for T- or B-cell specific mTOR knockout mice (mTOR-KO; ΔT or ΔB) [33–35], kinase-inactive mTOR (mTOR-KI) mice displayed decreases but not an absence of mature CD4 T cell and B cell frequencies in the spleen (Figure 4.2E). However, the mTOR-KI lymphocytes displayed profound reductions in growth and division that were equivalent to mTOR-KO or chemical mTOR kinase inhibition (Figure 4.3). Selective inactivation of mTORC1 through raptor deletion (Raptor-KO; ΔT or ΔB) phenocopied these defects (Figure 4.3). Together, these results show that mTORC1 mediated lymphocyte growth and proliferation is mainly driven by an mTORC1 kinase substrate.

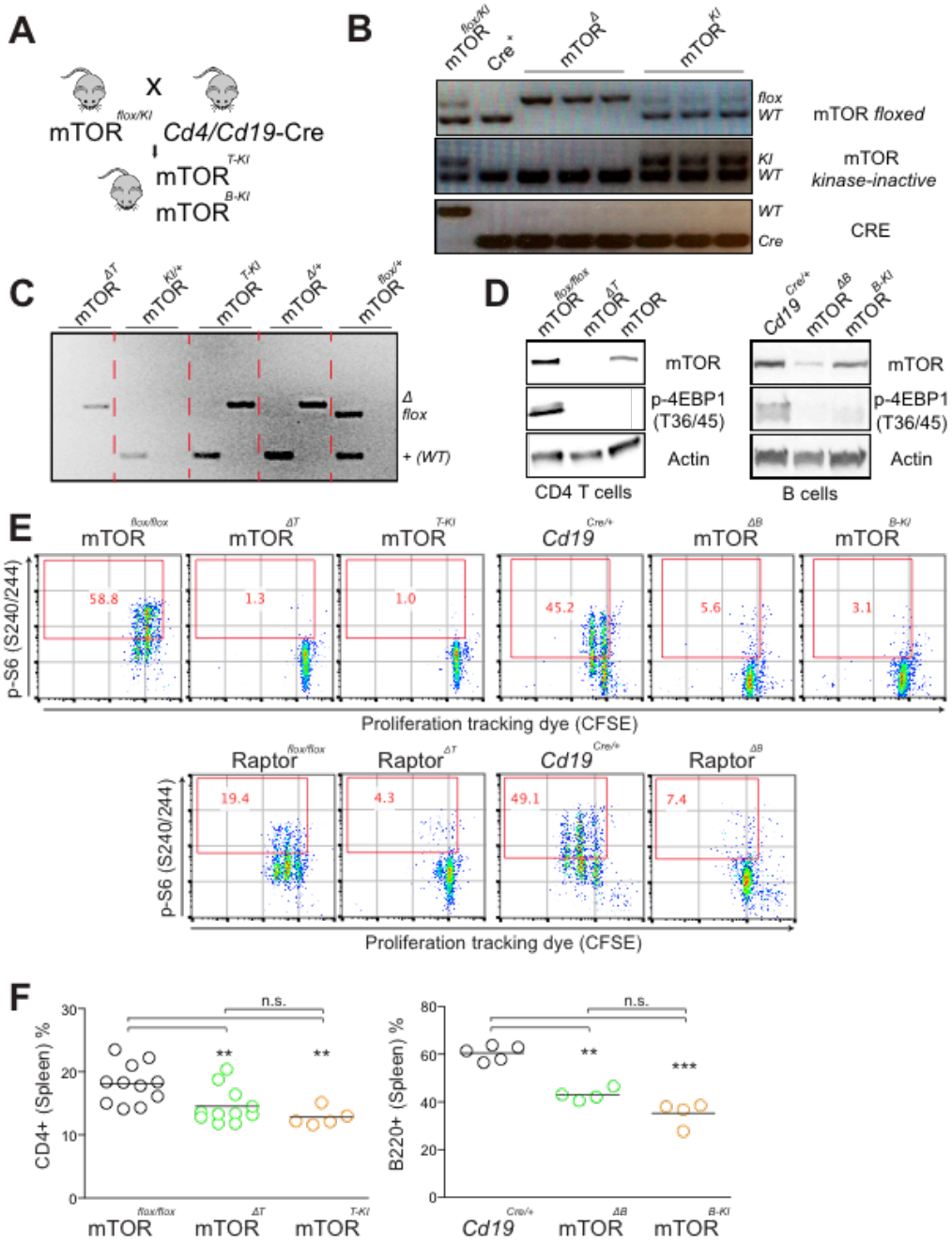

Figure 4.2. The relevant downstream mTORC1 effector in lymphocytes is an mTORC1 kinase substrate. (A) Strategy to generate T or B cell specific mTOR-KI mice. $mTOR^{KI/+}$ mice have a D2338A mutation in the mTOR kinase domain. These mice were crossed with $mTOR^{flox/flox}$ mice to generate $mTOR^{flox/KI}$. Mice were further crossed to either *Cd4*Cre or *Cd19*Cre mice to delete the floxed mTOR allele only in T or B cells (mTOR-TKI or mTOR-BKI, respectively). (B) Representative genotyping of the $mTOR^{KI}$ mice. (C) Excision PCR analysis of purified CD4 T cells from the indicated genotypes (Δ: deletion product). The KI allele with D2338A mutation is amplified by the PCR reaction as a WT (+) product. (D) Purified mTOR-TKI or mTOR-BKI CD4 T or B cells were activated for 24h and mTORC1 kinase activity was measured by Western blotting for p-4EBP1 (T37/46). (E) p-S6 (S240/244) was measured in CFSE labeled cells activated for 24 and 48h. Raptor-Δ lymphocytes were also measured for p-S6 in a separate experiment (bottom). (F) Percentages of CD4+ T cells and B220+ B cells were measured in the spleens of mice from the indicated genotypes by flow cytometry. Results represent at least three independent experiments. Where indicated, data represent mean±S.E. of n=3-11 experiments (*, $p<0.05$;**,$p<0.01$;***,$p<0.001$, repeated-measures analysis of variance, measured versus the control genotype).

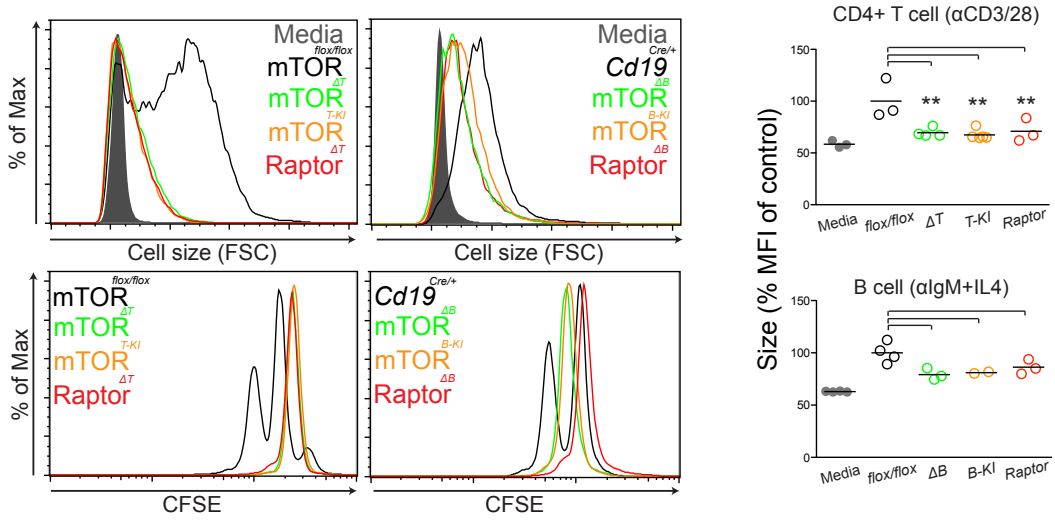

Figure 4.3. The relevant downstream mTORC1 effector in lymphocytes is an mTORC1 kinase substrate. Same analysis as in Figure 4.1 was performed for the indicated genotypes (mTOR-Δ: mTOR knockout; mTOR-KI: mTOR kinase-inactive; Raptor-Δ: Raptor knockout). For B cell genetics, *Cd19*Cre heterozygotes were used as proper controls. Results represent at least three independent experiments. Where indicated, data represent mean±S.E. of n=3-7 experiments (*, $p<0.05$;**,$p<0.01$;***,$p<0.001$, repeated-measures analysis of variance, measured versus the media control).

To extend these results to an antigen-specific system, I used the superantigen staphylococcal enterotoxin B (SEB) to activate Vβ8+ T cells *in vivo*. Within 24h, control mice had significant SEB-induced CD4 T cell growth within the Vβ8+ fraction (Figure 4.4A). After 48h, I observed an increase in absolute numbers of Vβ8+ CD4 T cells in PBS-treated control mice, indicating cell proliferation; this response was blunted equivalently in mTOR-ΔT or mTOR-KI mice (Figure 4.4B). T cell specific raptor-deficient mice showed similar defects (Figure 4.4C). This defect could not be attributed to an intrinsic impairment in early T cell activation status, as inactivation of mTOR kinase activity had no apparent effect on CD69 expression (Figure 4.4D). Similar results were obtained with *in vitro* SEB induced T cell growth and proliferation (Figure 4.4E).

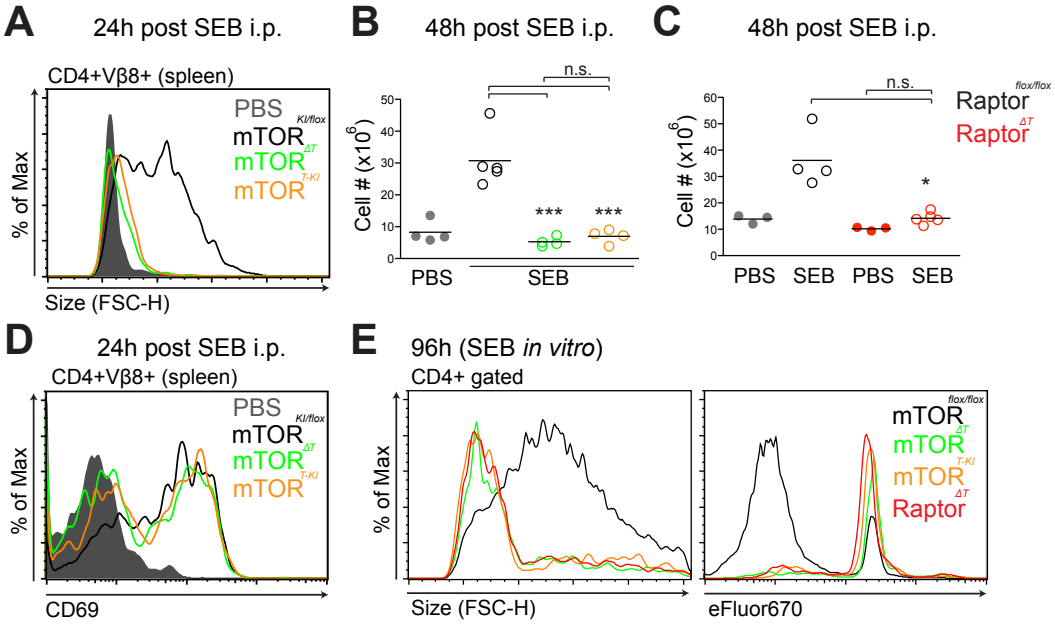

Figure 4.4. mTORC1 kinase activity is required for SEB mediated CD4 T cell growth and proliferation *in vivo* and *in vitro*. (A) Groups of 3 to 4 mice from the indicated genotypes were injected with 100μg of SEB i.p. and spleens were analyzed at 24h. Cell size increase of CD4+Vβ8+ cells was analyzed by forward scatter. (B) Cell proliferation was measured by counting total number of splenocytes and multiplying it to the percentage of CD4+Vβ8+ fraction in each sample. (C) Raptor-ΔT mice were analyzed for cell proliferation at 48h post SEB i.p. as in (B). (D) CD4+Vβ8+ cells from (A) were further analyzed for CD69 expression by surface staining at 24h post SEB i.p. (E) CFSE labeled total splenocytes from the indicated genotypes were activated with SEB for 96h. Proliferating CD4+ cells were gated to assess proliferation. Results are representative of 3 to 4 different mice samples. Where indicated, data represent mean±S.E. of n=3-4 mice per group (*, $p<0.05$;**,$p<0.01$;***,$p<0.001$, repeated-measures analysis of variance, measured versus PBS injected group).

S6Ks are dispensable for lymphocyte growth and proliferation

A universal hallmark of rapamycin treatment in all cell types is the rapid inactivation of ribosomal protein S6 kinases (S6Ks), a phenotype observed before the discovery of mTOR [36,37]. It has been proposed that the mechanism by which rapamycin inhibits T cell S phase entry is through blocking S6K function [37]. Surprisingly, there has been no loss of function genetic evidence to support that the rapid S6K dephosphorylation by rapamycin in T cells is sufficient to block proliferation. Lymphocytes from S6K1-deficient mice have apparently normal function, probably because of compensatory upregulation of S6K2 [38]. Mice lacking both S6K1 and S6K2 have a perinatal lethal phenotype but not with complete penetrance [39]. Thus, I was able to obtain splenocytes from a limited number of surviving adult mice (provided by the Sonenberg lab, McGill University). As expected, p-S6 at S240/244, a sensitive readout of S6K activity was completely absent upon T and B cell activation (Figure 4.5A). Interestingly, p-S6 at S235/236 was also completely absent suggesting (data not shown). This suggests that both S240/244 and S235/236 sites on S6 are completely S6K dependent in lymphocytes whereas in hepatocytes, the p90 RSKs are responsible for S235/236 phosphorylation through MAPK signaling [39]. Surprisingly, T and B cells lacking S6K1/2 (S6K DKO) had no significant defect in their ability to grow and proliferate (Figure 4.5B and C). Most importantly, both cell growth and division in S6K DKO lymphocytes were sensitive to rapamycin, indicating that lymphocyte growth and proliferation depend on mTORC1 outputs other than S6Ks.

Genetic knockout mouse models affecting the PI3K pathway often display developmental compensation, for example by upregulation of functionally redundant

genes [40]. To assess the acute inhibition of S6K activity in lymphocytes, I used a chemical genetic approach. I used a highly selective S6K1 inhibitor (LY2584702) [41] in S6K2 deficient lymphocytes (S6K2 KO) to acutely inhibit all S6K activity. S6K2 KO mice are viable [39] and peripheral mature T cell frequencies were unperturbed (Figure 4.6A). S6K2 knockout or S6K1 inhibitor treatment of WT cells led to a partial reduction in p-S6 in both T and B cells suggesting that both S6Ks contribute to phosphorylating the S6 protein in lymphocytes (Figure 4.6B). Treatment of S6K2-deficient lymphocytes with the S6K1 inhibitor fully suppressed p-S6 to similar levels as rapamycin (Figure 4.6B). Despite the full inhibition of S6K activity, similar to S6K DKO lymphocytes, lymphocyte growth or proliferation was unimpaired (Figure 4.6C). These results demonstrate that in the context of antigen receptor stimulation, S6K activity is dispensable for lymphocyte growth and proliferation.

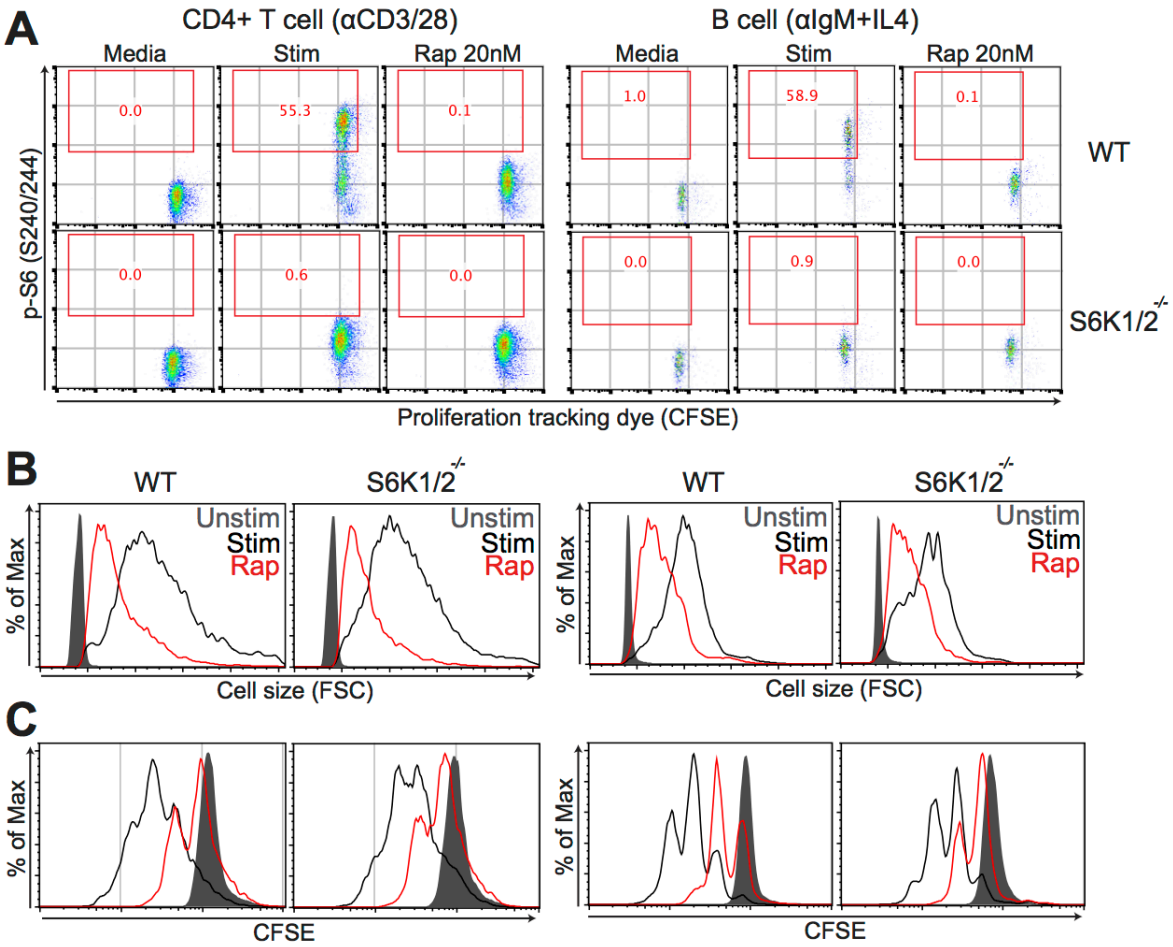

Figure 4.5. S6Ks are dispensable for lymphocyte growth and proliferation. (A) Levels of phosphorylated S6 (S240/244) were determined by intracellular staining for the indicated genotypes (WT: wild-type, S6K1/2$^{-/-}$: S6K1/2 double knockout) upon CD4 T cell (left) or B cell (right) activation (24h) through the antigen receptor using crosslinking antibodies (αCD3 and αCD28 for T cells or αIgM with IL-4 for B cells) for 24h. (B) Cell growth was determined 24h after activation by forward scatter (FSC). (C) Cell proliferation was determined by CFSE dilution analysis 72h after activation. Results represent at least two independent experiments.

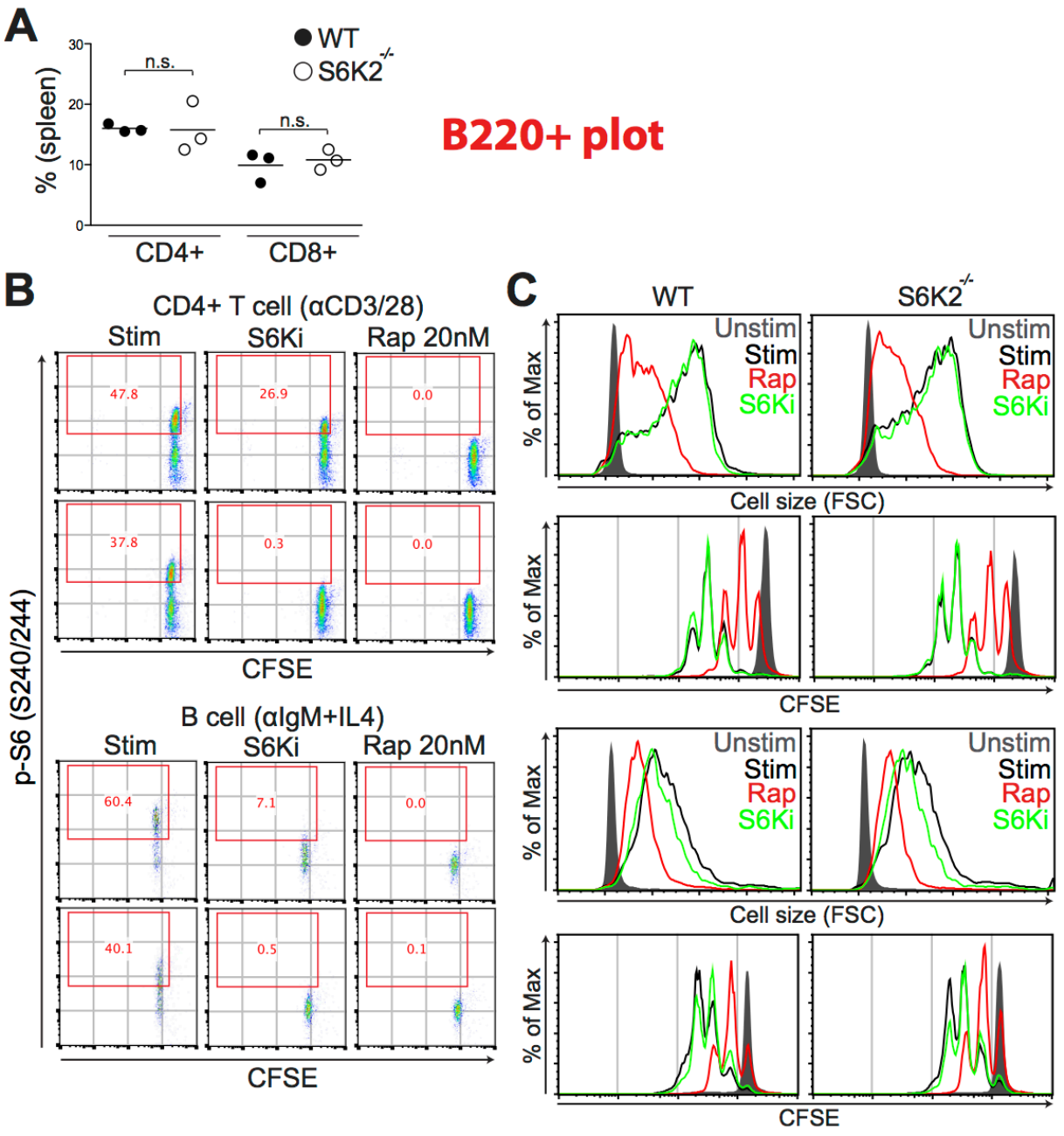

Figure 4.6. Acute inhibition of S6K activity is dispensable for lymphocyte growth and proliferation. (A) Percentages of CD4+ T cells in WT or S6K2 deficient mice ($S6K2^{-/-}$) were measured by flow cytometry. Quantification of peripheral B cell frequencies has yet to be determined (B) CD4 T (top) or B (bottom) cells labeled with CFSE were activated for 24h and S6K activity was measured by p-S6 staining in the indicated samples (S6Ki 500nM; Rap 20nM). (C) Cell growth and proliferation was measured as in Fig. 1. Results are representative of at least three independent experiments.

Specific coordination of growth and proliferation by 4E-BP/eIF4E in lymphocytes

The eukaryotic translation initiation factor 4E-binding proteins (4E-BPs) are direct kinase substrates of mTORC1 that bind to eIF4E, the rate-limiting protein in cap-dependent translation, and prevent eIF4E assembly into an active cap-binding complex, eIF4F. mTORC1 phosphorylates 4E-BPs on several sites, triggering the release of 4E-BPs.

To address the role of 4E-BPs, I used transgenic mice that express a constitutively-active form of 4E-BP1 (4E-BP1^{M}) that is expressed in a doxycycline (Dox)-inducible manner. 4E-BP1^{M} has all five mTORC1 phosphorylation sites mutated to alanines and constitutively binds to eIF4E to inhibit cap-dependent translation. These mice were crossed to Rosa26-rtTA transgenic mice that display widespread expression of the reverse tetracycline transactivator (rtTA) protein. This system allowed the expression of 4E-BP1^{M} in purified resting naïve lymphocytes prior to their activation. Upon incubation of purified lymphocytes with dox for 6h, I observed significant expression of the 4E-BP1^{M} mutant characterized by a higher molecular weight due to the FLAG-tag (Figure 4.7A). Importantly, there was no perturbation in mTOR signaling outputs such as Akt phosphorylation downstream of mTORC2 when 4E-BP1^{M} was expressed demonstrating that 4E-BP1^{M} induction does not perturb overall mTOR signaling during lymphocyte activation (Figure 4.7B). Next, I assessed the effect of 4E-BP1^{M} expression in the interaction between eIF4G and eIF4E using the cap analog 7-methyl GTP (m7GTP) sepharose bead pulldown assay. Resting naïve lymphocytes showed minimal eIF4G bound to eIF4E that was instead occupied by 4E-BP1 binding (Figure 4.7C). Upon

2h of activation, the prominent increase in eIF4G-eIF4E interaction was abolished by 4E-BP1^{M} expression (Figure 4.7C).

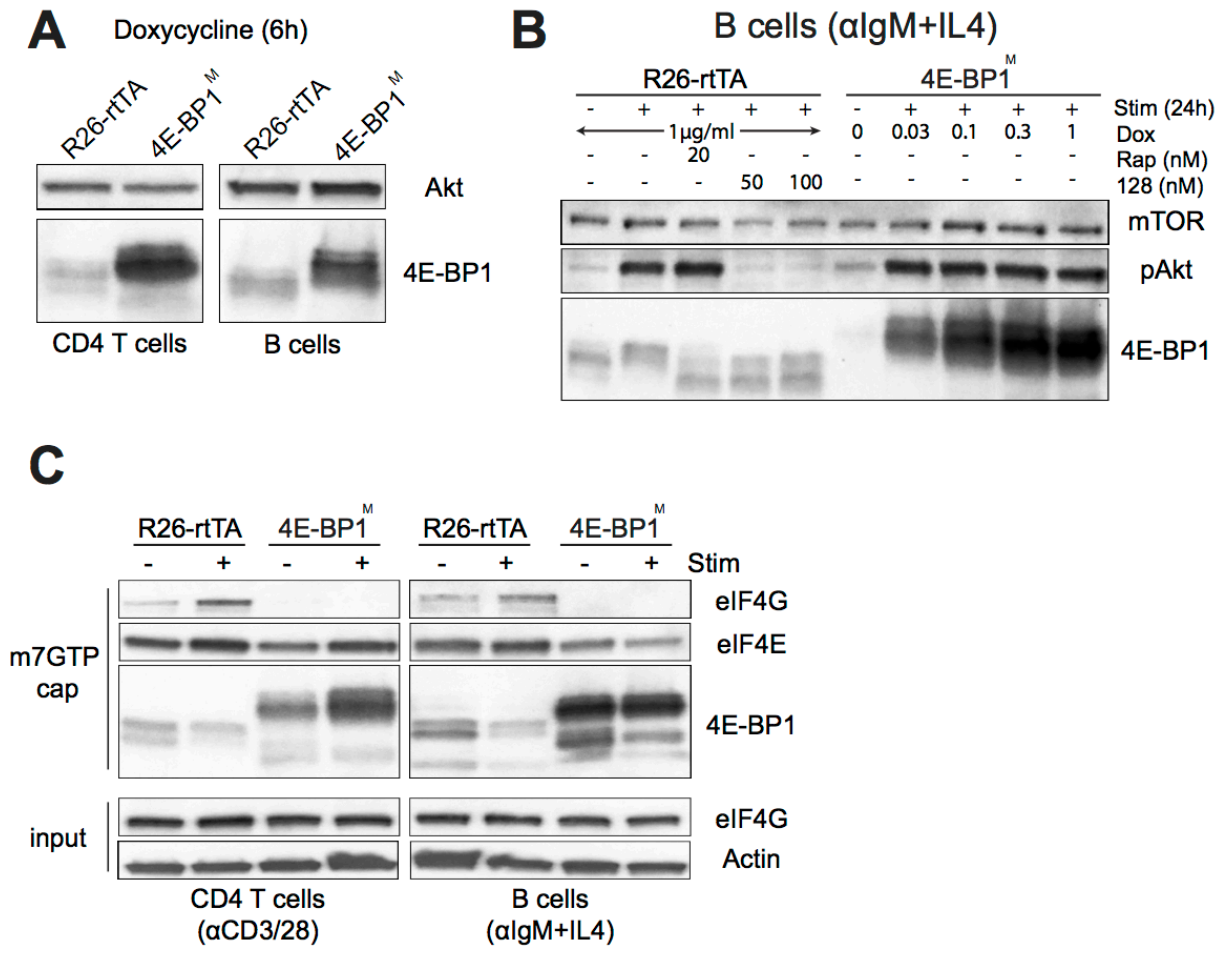

Figure 4.7. A constitutively active 4E-BP1 mutant inducible model in primary resting lymphocytes (A) Purified CD4 T or B cells were incubated with dox (1μg/ml) for 6h and subjected for Western blotting to check 4E-BP1^{M} expression. 4E-BP1^{M} in these mice is FLAG-tagged and have a higher molecular weight. (B) B cells incubated with varying amounts of dox for 6h were activated for 24h to check mTORC2-Akt signaling by Western blotting. Control samples were B cells from Rosa26-rtTA mice treated with or without dox. (C) 4E-BP1^{M} expressing CD4 T or B cells were activated for 12h and subjected to m7GTP cap pulldown. Amounts of eIF4G, eIF4E, and 4E-BP1 bound to the cap were measured by Western blotting. Total lysate (input) is shown below. Results represent at least three independent experiments.

When activated for 24h, the 4E-BP1^{M} expressing lymphocytes had a significant defect in growth that was proportional to the concentration of dox added for 6h prior to activation (Figure 4.8A). Notably, 4E-BP1^{M} -expressing lymphocytes closely phenocopied the growth and proliferation defect seen in Raptor-deficient lymphocytes (Figure 4.8B and C). Unlike the absence of mTORC1 signaling in Raptor deficient lymphocytes (assessed by p-S6 staining), 4E-BP1^{M} expression blocked proliferation independent from mTORC1 status (Figure 4.8C). The effects of 4E-BP1^{M} expression were similar to pharmacological mTORC1 inhibition by rapamycin or MLN0128 treatment (Figure 4.8D). These results indicate that specific blockade of the 4E-BP/eIF4E signaling node downstream of mTORC1 is sufficient to block both lymphocyte growth and proliferation.

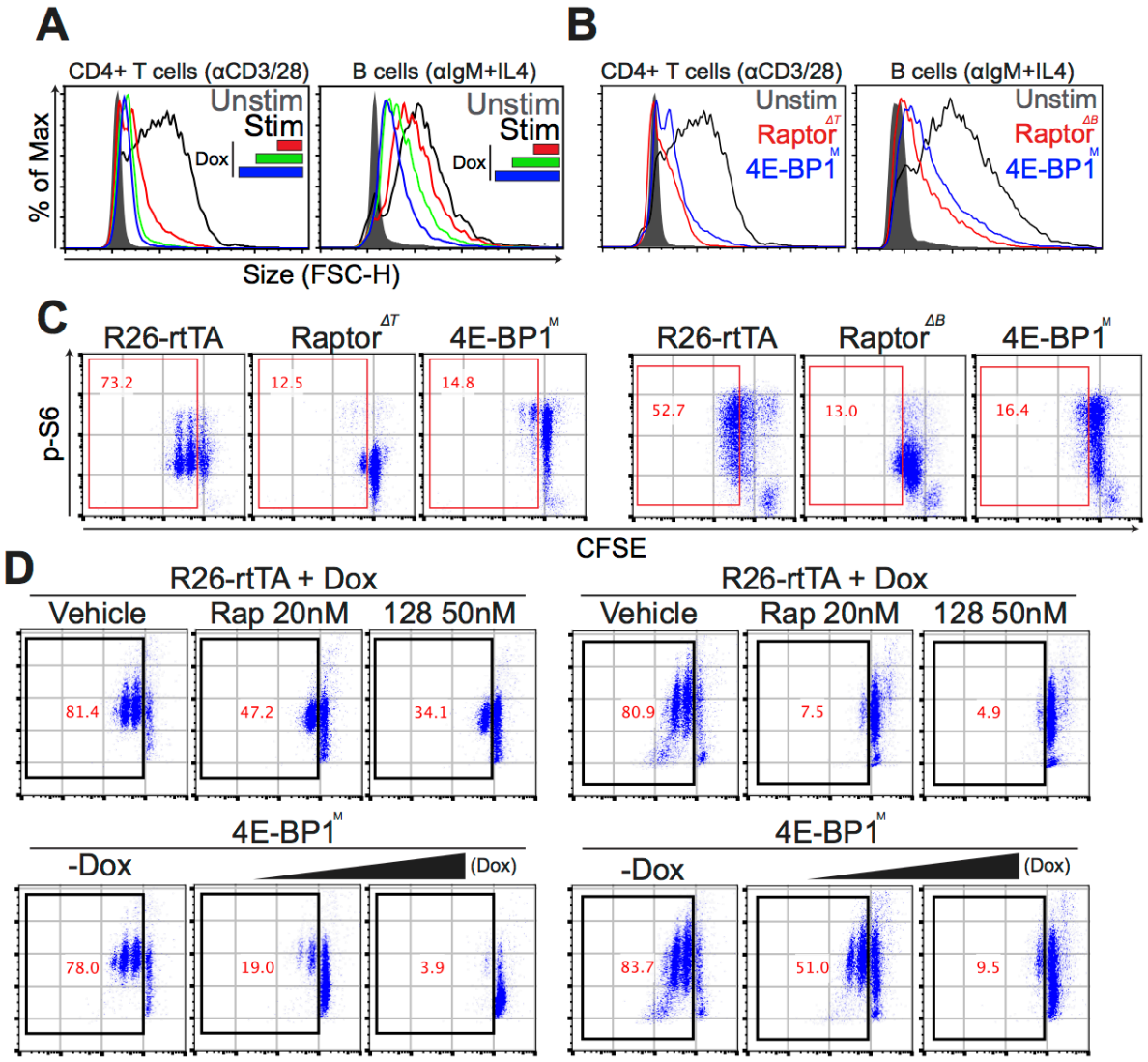

Figure 4.8. A constitutively active 4E-BP1 mutant is sufficient to phenocopy mTORC1 deficiency in lymphocytes (A) Cell growth of lymphocytes expressing 4E-BP1^{M} was measured by forward scatter. Different color coding represents different amounts of doxycycline (dox) added for 6h prior to activation. (B) Cell growth of 4E-BP1^{M} expressing lymphocytes were compared with Raptor-Δ lymphocytes. (C) Cell proliferation of 4E-BP1^{M} expressing lymphocytes were compared with Raptor-Δ lymphocytes and mTORC1 activity was measured by p-S6 staining. (D) Effects of rapamycin or MLN0128 on CD4 T (left) or B (right) proliferation at 48 or 72h were compared to 4E-BP1^{M} expressing lymphocytes by CFSE dilution analysis. Results represent at least three independent experiments.

Importantly, the effect of 4E-BP1^{M} on cell growth was specific for primary T and B cells. In 3T3 fibroblasts, induced expression of 4E-BP1^{M} suppressed proliferation, but did not reduce size or protein content (Figure 4.9). These results agree with previous reports that the 4E-BP/eIF4E axis regulates cell cycle but not size. Thus, lymphocytes uniquely utilize this pathway to control both growth and division.

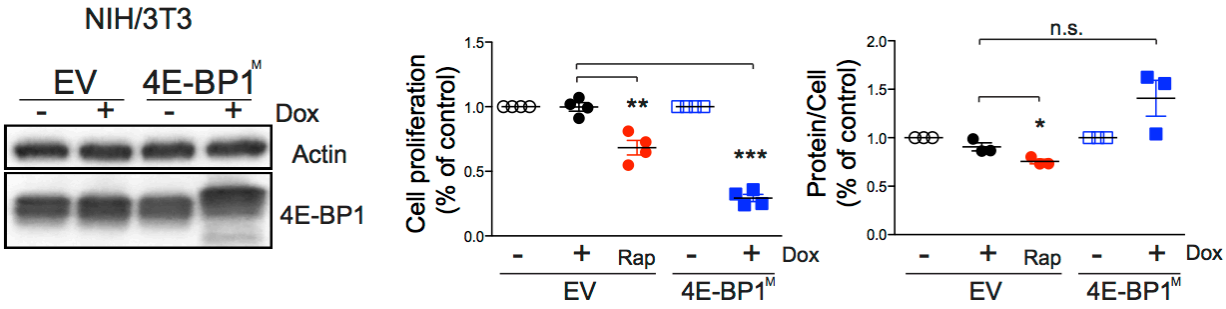

Figure 4.9. 4E-BP1^{M} specifically regulates cell proliferation in fibroblasts. Western blotting for NIH/3T3 cells engineered to express 4E-BP1^{M} upon dox addition. Cell proliferation was measured at 72h by cell counting upon 4E-BP1^{M} expression and normalized to control samples (empty vector (EV) with no dox; 4E-BP1^{M} with no dox). Total protein content per cell was measured by a standard Bradford assay and dividing it to total cell number at 72h. Results represent at least three independent experiments. Where indicated, data represent mean±S.E. of n=3-4 experiments (*, $p<0.05$;**,$p<0.01$;***,$p<0.001$, repeated-measures analysis of variance, measured versus EV with dox control). Dox-inducible 4E-BP1^{M} expressing NIH/3T3 cells were generated and selected by Sharmila Mallya. Repeat experiments for statistical analysis was performed by Miguel Palafox.

Deletion of 4E-BPs partially rescues B cell proliferation

Next, I asked whether uncoupling eIF4E from mTORC1 activity by removing 4E-BPs would be sufficient to preserve lymphocyte growth and proliferation upon mTORC1 inhibition. Using puromycin incorporation assay to assess total protein synthesis, a rapid increase in protein synthesis was observed within 2h following B cell activation that was blocked nearly completely by both rapamycin and MLN0128 (Figure. 4.10A). 4E-BP1/2 deficient B cells (4E-BP1/2 DKO) exhibited enhanced basal and activated protein synthesis rate that was less sensitive to rapamycin or MLN0128 (Figure 4.10A). This restoration in protein synthesis correlated functionally with proliferation, as DKO B cell division was partially resistant to mTORC1 inhibition (Figure 4.10B and C). It is noteworthy that B cell proliferation was partially rescued from rapamycin, suggesting that in B cells, a potential mechanism of action of rapamycin involves the 4E-BPs. This is in contrast to fibroblasts where the effect of rapamycin on proliferation seems to be independent of 4E-BPs [42]. Interestingly, DKO CD4 T cells were not rescued from any of the inhibitors (discussed below).

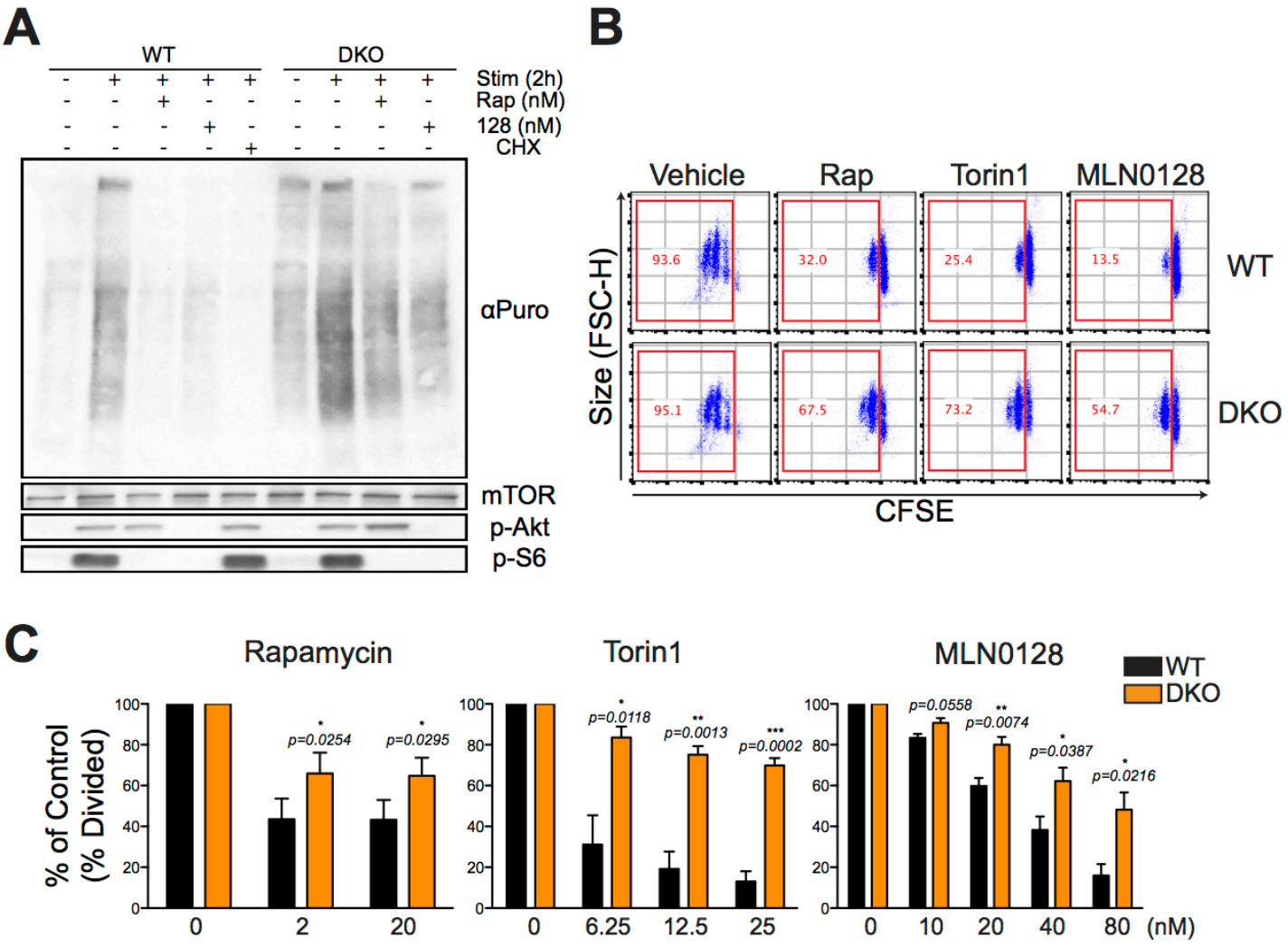

Figure 4.10. Rapamycin requires 4E-BPs to inhibit lymphocyte proliferation. (A) Puromycin incorporation assay was used to measure nascent protein synthesis in B cells (WT vs 4EBP1/2 DKO) upon activation. (B) B cells from WT or 4E-BP1/2 DKO mice were labeled with CFSE and activated for 72h to measure cell proliferation based on CFSE dilution. The percentage of cells that have divided at least once (indicated by red box) were measured. (C) Cells that have divided at least once (% divided) were normalized to vehicle treated B cells for each genotype. This was measured for a range of titrated amounts of rapamycin, MLN0128, or Torin1. Results represent at least three independent experiments. Where indicated, data represent mean±S.E. of n=3-5 experiments (*, $p<0.05$;**,$p<0.01$;***,$p<0.001$ by unpaired two sample *t*-test.

Rapamycin selectively suppresses eIF4F assembly and protein synthesis in lymphocytes

If lymphocyte growth is eIF4E-dependent and rapamycin-sensitive, then rapamycin should inhibit eIF4E function following lymphocyte activation. To directly test this, I measured eIF4G-eIF4E interaction by m7GTP pulldown upon rapamycin treatment during early activation (2h) of B cells. Rapamycin-treated samples showed a near complete displacement of eIF4G similar to resting or MLN0128 treated B cells and a similar recruitment of 4E-BP1 and 4E-BP2 to the cap (Figure 4.11A). As observed in many previous studies comparing rapamycin and TOR-KIs, rapamycin is a poor inhibitor of eIF4F assembly when compared to TOR-KIs due to its incomplete inhibition of mTORC1 activity that impinges on 4E-BP phosphorylation [17,19,20,26,43] (Figure 4.11B). To investigate a possible mechanism for the distinct rapamycin sensitivity of eIF4F assembly, I assessed the effect of rapamycin on mouse 4E-BP1 phosphorylation at T36/45 (equivalent site for T37/46 in human 4E-BP1) and on 4E-BP2 phosphorylation at T37/46. This site on 4E-BP1 is a well-known rapamycin-insensitive site. The phospho-specific antibody against T37/46 is known to recognize all three 4E-BP isoforms phosphorylated at this residue. As expected, MLN0128 treatment suppressed the activation induced increase in p-4E-BP1 and p-4E-BP2 (Figure 4.11C) whereas rapamycin had little effect on p-4E-BP1. Surprisingly, 4E-BP2 phosphorylation at T37/46 was more rapamycin-sensitive as compared to 4E-BP1 phosphorylation. This signal was completely absent in 4E-BP2 single knockout lymphocytes whereas it was the only band present in 4E-BP1 single knockout lymphocytes, confirming it to be the phosphorylated

4E-BP2 (Figure 4.11C). These results demonstrate that the reported rapamycin resistance of 4E-BP phosphorylation at the T37/46 residue applies more to 4E-BP1 than to 4E-BP2.

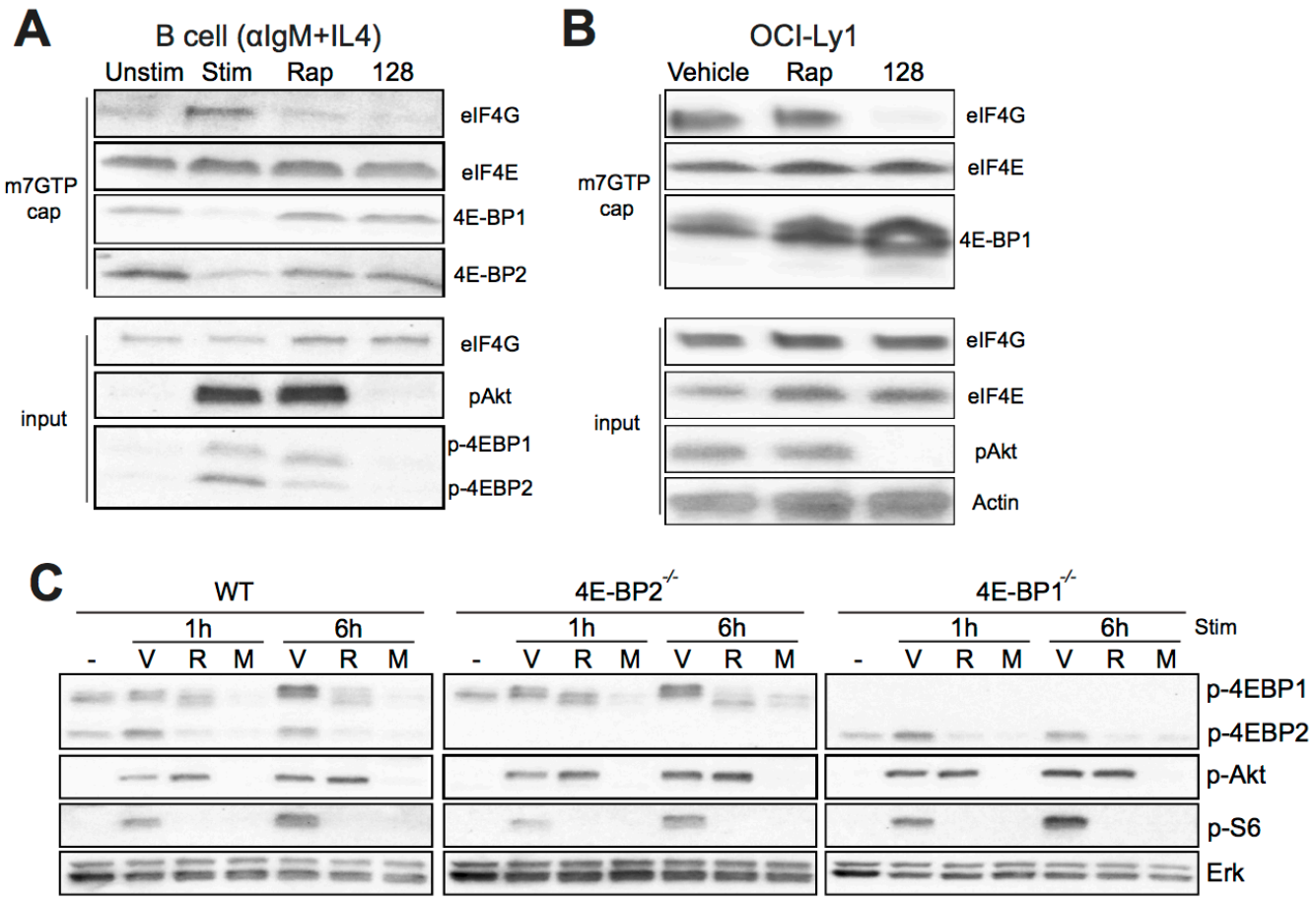

Figure 4.11. Rapamycin disrupts eIF4F complex assembly and dephosphorylates 4E-BP2 at T37/46. (A) Purified B cells were activated for 2h and subjected to m7GTP cap pulldown. Amounts of eIF4G, eIF4E, and 4E-BP1 bound to the cap were measured by Western blotting. Total lysate (input) is shown below. (B) Same cap pulldown assay as in (A) was performed on actively growing OCI-Ly1 DLBCL cell line. (C) WT, 4E-BP1 knockout (4E-BP1$^{-/-}$), or 4E-BP2 knockout (4E-BP2$^{-/-}$) B cells were activated for the indicated timepoints with or without inhibitors (V: vehicle; R: rapamycin 20nM; I: MLN0128 50nM). Phosphorylated 4E-BP1 (T36/45) and 4E-BP2 (T37/46) was measured using Western blotting. Results represent at least three independent experiments.

To check whether this differential sensitivity of 4E-BP2 was unique to primary lymphocytes, we took advantage of the diffuse large B cell lymphoma (DLBCL) cell line VAL that expresses 4E-BP2 but not 4E-BP1 as we reported previously (Figure 4.12A) [44]. Intracellular staining for p-4E-BP2 (T37/46) showed a signal that was significantly sensitive to rapamycin in VAL cells (Figure 4.12B). Another DLBCL cell line OCI-Ly1 cells were used as a control that expresses more 4E-BP1 than 4E-BP2 that was also true for other DLBCL lines such as OCI-Ly8 and SUDHL4 cells (Figure 4.12A). In OCI-Ly1 cells, the p-4EBP signal was rapamycin-insensitive whereas MLN0128 suppressed the signal (Figure 4.12C) presumably due to the signal mostly coming from the highly expressed 4E-BP1.

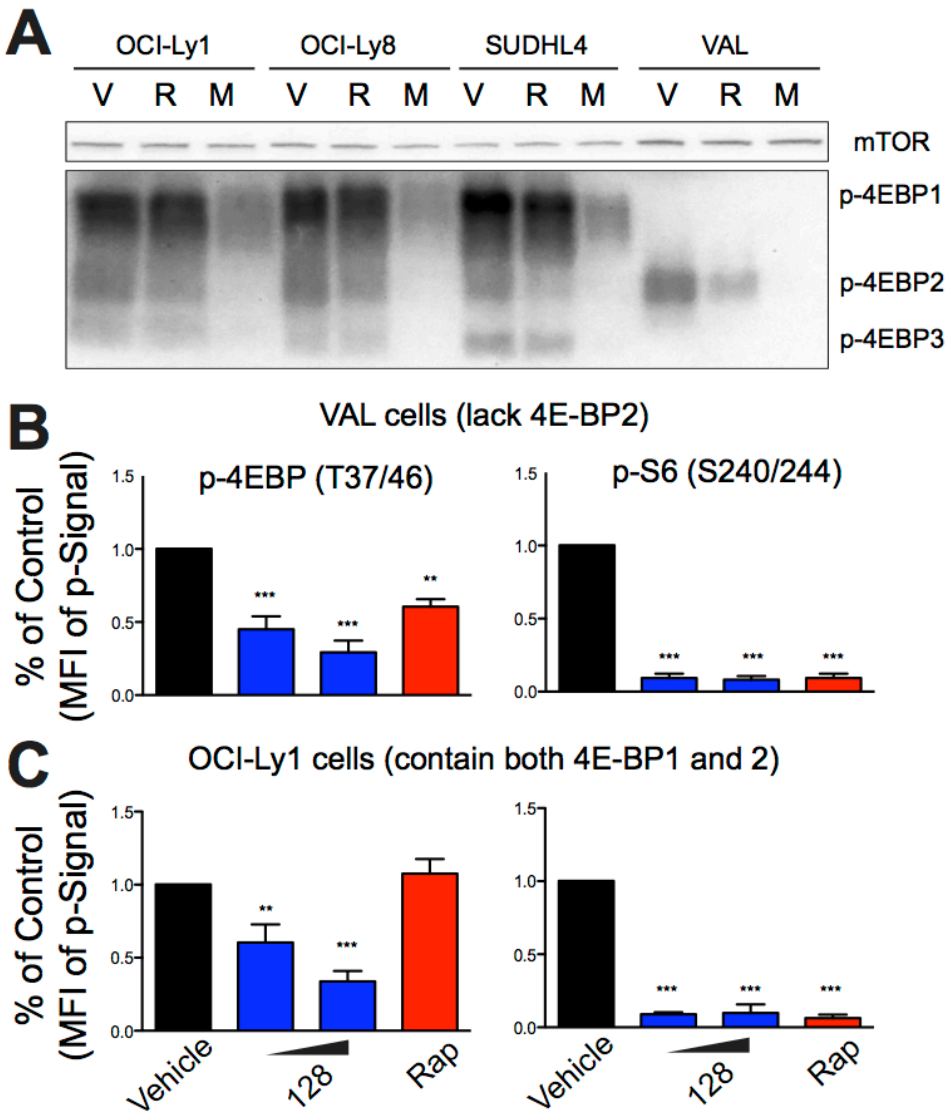

Figure 4.12. The rapamycin-sensitive 4E-BP2 phosphorylation is also observed in DLBCL lymphoma cell lines. (A) Four different DLBCL cell lines were treated with the indicated inhibitors (V: vehicle, R: rapamycin, M: MLN0128) for 2h and phosphorylated 4E-BP1, 2 and 3 at the equivalent T37/46 site was probed using Western blotting. (B and C) Two DLBCL cell lines (VAL: lack 4E-BP1; OCI-Ly1) were treated with the indicated inhibitors for 2h and fixed/permeabilized to stain for p-S6 (S240/244) or p-4EBP (T37/46). Median fluorescent intensity (MFI) for each signal was normalized to vehicle treated samples.

The differential sensitivity of mTORC1 substrates to rapamycin is encoded in part at the sequence surrounding the phosphoacceptor site[45] (see introduction for substrate selectivity model). The presence of hydrophobic or uncharged amino acids around the phosphoacceptor site for a given mTORC1 substrate makes it a good mTORC1 substrate and rapamycin-insensitive. On the other hand, mTORC1 substrates that are highly rapamycin-sensitive show a trend towards harboring hydrophilic or charged amino acids surrounding the phosphoacceptor site. Surprisingly, when the sequence surrounding the T36 site (T37 on 4E-BP2) was compared for 4E-BP1 and 4E-BP2, the -5 position to the T36 site was intrinsically different (nonpolar G in 4E-BP1; polar Q or H in 4E-BP2) and conserved through species (Figure 4.13A) possibly allowing the T37 site on 4E-BP2 to be rapamycin-sensitive relative to 4E-BP1. When two mutant forms of 4E-BP1 were generated (G31H or G31Q; -5 position G mutated to H or Q found in 4E-BP2) and expressed in MEFs that lack 4E-BP1 and 4E-BP2, a greater dephosphorylation by rapamycin was observed as assessed by the mobility shift (Figure 4.13B).

Next, the expression level of 4E-BP isoforms in primary resting lymphocytes was compared to different cell types. When compared with equal amounts of protein from MEFs, 4E-BP1 levels were significantly higher in MEFs than in lymphocytes. In contrast, lymphocytes expressed similar levels of 4E-BP2 compared to MEFs (Figure 4.13C). Thus, the ability of rapamycin to disrupt eIF4F assembly correlates with higher expression of 4E-BP2, a rapamycin-sensitive isoform, relative to 4E-BP1 in primary lymphocytes. This is also highlighted by the fact that equal amounts of 4E-BP2 were bound to eIF4E upon rapamycin treatment compared to MLN0128 treated B cells (Figure 4.11A). Together, these results suggest that eIF4E activity is required for both growth and

proliferation of lymphocytes and that rapamycin uniquely suppresses this node, possibly at the level of 4E-BP2 phosphorylation.

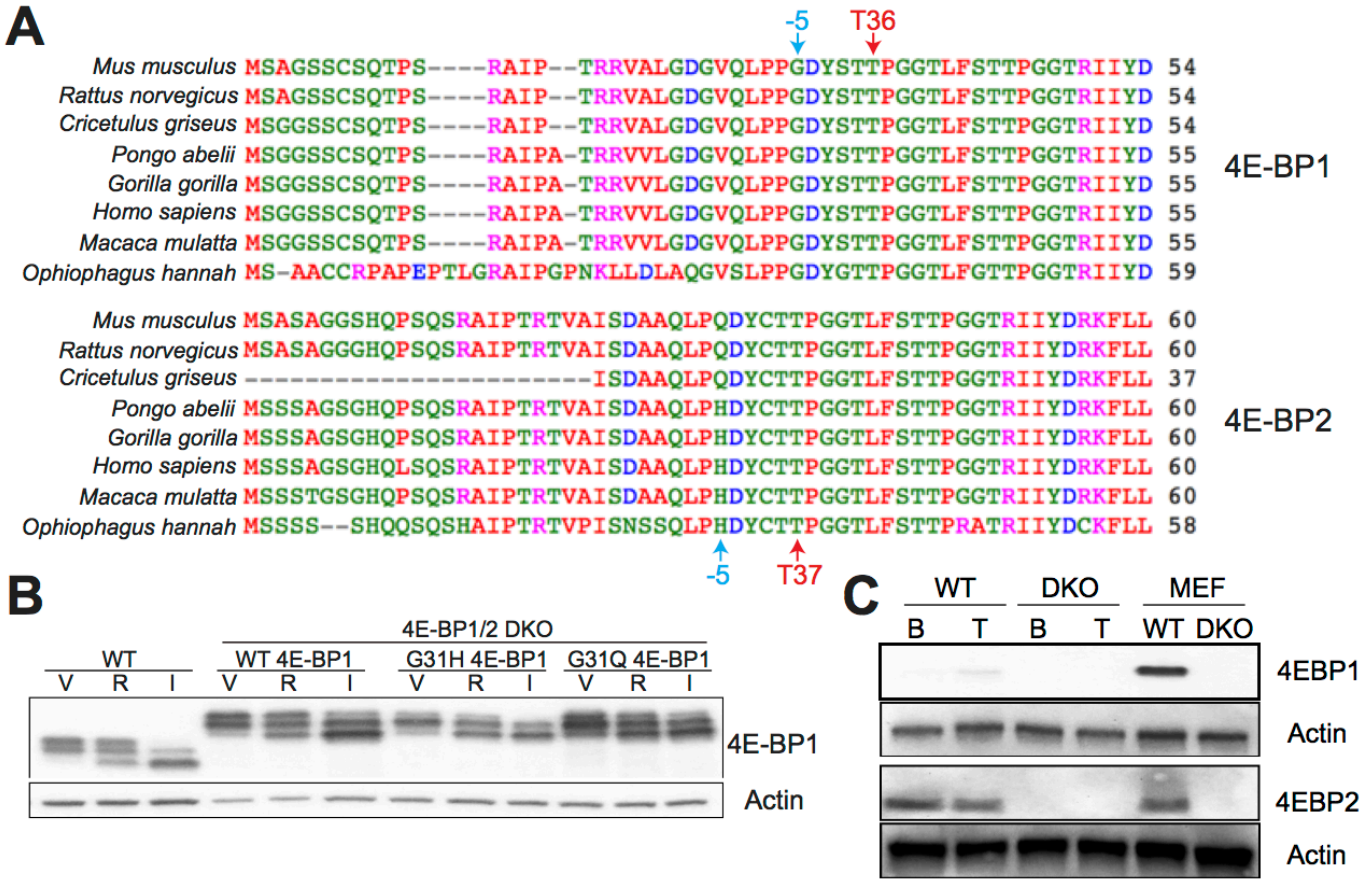

Figure 4.13. The surrounding amino acid of T37 in 4E-BP2 (T36 in 4E-BP1) is distinct from 4E-BP1 throughout evolution and determines rapamycin sensitivity. (A) Sequence alignment of 4E-BP1 or 4E-BP2 from various species. ClustalW2 software was used from EMBL-EBI (www.ebi.ac.uk). The -5 position from the T36/37 phosphoacceptor site on 4E-BP1 or 4E-BP2 (depicted in red) are indicated (depicted in light blue). (B) 4E-BP1 mutants (G31H or G31Q) were generated by site-directed mutagenesis and introduced into 4E-BP1/2 DKO MEFs. Cells were treated with the indicated inhibitors (V: vehicle; R: rapamycin 20nM; I: MLN0128 100nM) for 3h before performing a Western blot. (C) CD4 T or B cells were purified from either WT or 4E-BP1/2 DKO mice. Amount of 4E-BP1 or 4E-BP2 were compared with equal amounts of protein from either WT or 4E-BP1/2 DKO mouse embryonic fibroblasts (MEF). Results represent at least three independent experiments.

Discussion

Lymphocytes are unusual cells that undergo an extended growth phase before commitment to several rapid divisions [7,46]. The data presented here establish that 4E-BP phosphorylation is a common signaling node through which mTORC1 controls both growth and division in activated primary T and B cells. Surprisingly, despite a conserved role for S6Ks in cell size regulation [3,38,39,42,47], these kinases were dispensable for T or B cell growth as well as proliferation. This contrasts with hepatocyte regeneration in mice after partial hepatectomy, where S6Ks are important for both cell size and division [48]. The rapid and sustained activation of S6Ks that occurs following lymphocyte stimulation may drive other processes such as differentiation [49].

More than 15 years after rapamycin gained regulatory approval as an immunosuppressant, the mechanistic basis for its immune cell selectivity has remained elusive. Rapamycin slows but does not block cell cycle in fibroblasts and many cancer cell lines [11,50], and has generally weaker cytostatic effects than TOR-KIs [17,18,43]. The data show that unlike in other cell types, rapamycin is equally effective as TOR-KIs at disrupting formation of the eIF4F complex and reducing protein synthesis. Displacement of eIF4G from eIF4E correlated with prominent expression of the 4E-BP2 isoform in lymphocytes, which was more rapamycin-sensitive at the T37/46 site, a crucial priming site for 4E-BP function [51,52]. Another mechanism, which is not mutually exclusive, is that resting lymphocytes express very low levels of eIF4E [53] that can be saturated by low amounts of dephosphorylated 4E-BPs. This model may explain the observation that 4EBP1 was recruited similarly to eIF4E in cells treated with either rapamycin or MLN0128 despite greater ability of MLN0128 to prevent 4EBP1 phosphorylation (Figure

4.11A). In summary, a high ratio of rapamycin-sensitive 4E-BP2 to eIF4E together with the coupling of growth and division through eIF4E are likely mechanisms to explain why rapamycin profoundly suppresses lymphocyte blastogenesis and clonal expansion. Consistent with this model, expression of $4EBP1^{M}$ suppresses growth and division in a dose-dependent manner (Figure 4.8A).

Increased protein synthesis has long been regarded as a key step in lymphocyte activation [54]. However, selective regulation of mRNA translation has not been carefully studied in primary lymphocytes. Instead, studies of mTOR in lymphocyte regulation have focused on transcriptional and metabolic mechanisms [7,55,56]. The data raise the possibility that key regulatory events in lymphocyte activation occur at the level of cap-dependent translation downstream of mTORC1.

CONCLUSION

Overall, the findings presented in this chapter have the following significance. First, a fundamental cell biological question of how one unique mammalian cell type coordinates cell growth to proliferation has been investigated. The results are novel and surprising given that the 4E-BP/eIF4E signaling axis downstream of mTORC1 is utilized differently in lymphocytes compared to other mammalian cells. Thus, mechanisms coordinating growth and proliferation in the various cell types of a multicellular system are not universal but rather distinct according to the specific function of each cell type. The implications of this utilization of common signaling pathways such as the 4E-BP/eIF4E axis in different contexts such as oncogene-induced transformation of normal cells will be discussed further in chapter 5.

Second, the findings have highly clinical implications as they shed new light into understanding how the FDA-approved immunosuppressant rapamycin shows greater anti-proliferative potential against lymphocytes. The fact that rapamycin inhibits lymphocyte proliferation at the cap-dependent translation level suggests that downstream transcripts regulated at the post-transcriptional level could be rational targets for developing novel immunosuppressants in the future.

Lastly, I strongly believe that the data presented here will cause a paradigm shift from the current research direction of various laboratories that study lymphocyte activation at the level of transcriptional changes and metabolic reprogramming. Although a significant enhancement of transcriptional responses and a switch in metabolism is clearly observed during lymphocyte activation [7,56], the functional workhorses of a cell are eventually going to be proteins. The data that cap-dependent translation through 4E-BP/eIF4E is critical for lymphocyte activation provide a whole new avenue of research in terms of identifying novel cap-dependent transcripts important for lymphocyte growth and proliferation. It is also possible that these proteins themselves are key metabolic enzymes required for metabolic reprogramming.

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CHAPTER 5:

GENERAL DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

Overview

This dissertation has presented a unique progression of attempts to better understand the PI3K-mTOR pathway in the mammalian system. With the availability of novel pharmacological tools, I have shown promising results of selectively targeting this pathway at various points in the context of cancer and adaptive immunity. These studies were possible due to the extensive basic fundamental research on this pathway over the last three decades. However, in the last part of this dissertation, I have come back to re-visit fundamental cell biological aspects of this pathway in the context of how cells coordinate cell growth to cell proliferation, a very important question yet to be answered at least for mammalian cells.

Selective targeting of PI3K isoforms

In chapter 1, I have made use of a novel PI3K isoform-selective inhibitor for p110α (*PIK3CA*), the most frequently mutated class IA isoform in human cancer [1]. The rationale was to investigate whether selective targeting of p110α using the compound MLN1117 would be equally effective as pan-PI3K inhibitors against cancer cells that harbor *PIK3CA* mutations while preserving other important cellular functions that require PI3Ks such as adaptive immunity. It is increasingly becoming clear that the immune system must be put into consideration for cancer therapies [2,3]. Especially the importance of adaptive immunity was shown in a tumor regression mouse model where MYC could be inducibly inactivated after tumor onset. At least in this model, CD4+ T cells were absolutely required for inducing cellular senescence and also suppressing angiogenesis [3]. In addition, the importance of CD8+ T cells and NK cells in the tumor microenvironment

is well established [4–6]. In fact, although not presented in this dissertation, I was also involved in investigating the effects of this p110α selective inhibitor MLN1117 and its effects on NK cell function with the same rationale [7]. The results were clear in that the primary class IA isoform required for lymphocyte or NK cell function was the p110δ isoform and selective targeting of p110α showed minimal effects on immune cell function [7,8]. Interestingly, both MLN1117 and a pan-PI3K inhibitor (GDC-0941) that is currently in clinical trials showed equal efficacy towards inhibiting proliferation of cancer cells that harbored activating mutations in *PIK3CA*. This may suggest that activating *PIK3CA* mutations rewires their dependency for survival and growth on constitutive signaling via p110α [9,10]. One may ask how different PI3K isoforms are engaged or utilized in response to extracellular signals in normal cells. Although largely unanswered, some evidence suggest that cancer cells lacking the tumor suppressor PTEN are susceptible to p110β inhibition but not to p110α inhibition [11]. This suggests that the majority of PIP_3 production under optimal growth conditions, which is presumably balanced by expression of PTEN in normal cells, is from p110β signaling. The studies presented in chapter 2 also investigated the effects of preclinical tools that selectively target p110β or both p110α/β where the conclusion is that the major isoform that regulates lymphocyte function is p110δ. However, we cannot exclude the possibility that either p110α or p110β does contribute to PIP_3 production in lymphocytes to some extent as these isoforms are ubiquitously expressed [12]. Supporting this notion is that only with combined deletion of p110α and p110δ, FO B cells were completely depleted in mice [13]. In addition, I did consistently observe that pan-PI3K inhibition or combined p110δ and p110α/β inhibition always shows greater effects on T or B cell function compared to

single p110δ inhibition. I believe the pharmacological tools used in chapter 2 will be of great use in the future to ask how the different class IA PI3K isoforms are utilized in normal versus disease states.

Unexpected immunomodulatory effects of TOR-KIs: Same target, different effects

My scientific career started with the fortunate involvement in a project utilizing one of the very first developed second-generation TOR-KIs (PP242) and investigating its effects on normal lymphocyte function [14,15]. As mentioned above, these compounds are ATP-competitive inhibitors of mTOR that fully suppress its kinase output [16]. Given the limited clinical efficacy of the traditional mTOR inhibitor rapamycin (RAP) as an anti-cancer agent, our lab utilized PP242 in preclinical Ph+ leukemia models and showed superior efficacy of PP242 in combination with currently available therapies such as dasatinib. However, a former graduate student (Dr. Jose Limon) and myself observed an at first paradoxical finding that PP242 at anti-leukemic doses spared T or B cell proliferation compared to its complete suppression by RAP [14]. Jose followed up on these initial findings and observed another phenomenon where various TOR-KIs when used at suboptimal doses to preserve B cell proliferation, unexpectedly enhanced B cell class-switch recombination (CSR) [17]. This was in marked contrast to the long-known effects of RAP that completely suppressed CSR [18]. As TOR-KIs also inhibit mTORC2 that is the upstream kinase for AKT, the lab hypothesized that TOR-KIs enhanced CSR through inhibition of mTORC2-AKT. Indeed, I confirmed that suboptimal doses of TOR-KIs did not fully sustain inhibition of mTORC1. Next, I showed that mTORC1 activity was

absolutely required for B cells to proliferate as genetic deletion of Raptor in B cells phenocopied the effects of RAP. Thus, our initial observation that TOR-KIs did not have equal effects as RAP on B cell proliferation was due to incomplete mTORC1 inhibition. However, what about the enhanced CSR effect? Through creating B cell-specific Rictor-deficient mice, I was able to genetically confirm the observation that mTORC2-AKT is the critical node of action of TOR-KIs in enhancing CSR. Most importantly, the enhanced CSR seen in Rictor deficient B cells was not further enhanced by TOR-KIs. The master regulator of CSR, activation-induced deaminase (AID), is a well-known target of FOXO transcription factors whose activity is negatively regulated by AKT activation [19,20]. Results from Dr. Limon showing that FOXO1/2/3 triple knockout (TKO) B cells do not show enhanced CSR upon TOR-KI treatment links mTORC2-AKT activity to FOXO mediated transcriptional upregulation of AID [17]. Although TOR-KIs were initially developed to achieve better anti-cancer efficacy than RAP through full suppression of mTOR kinase activity, the results presented in chapter 3 propose a unique twist of utilizing TOR-KIs for immunomodulatory effects especially at the level of lymphocyte differentiation.

CD4+ T cells also undergo a differentiation process that can be guided by unique extracellular cytokine signature [21]. It is still not completely clear as to which mTOR complexes contribute to different CD4+ T cell fate during differentiation but our results suggest that perhaps TOR-KIs when used at right concentrations, may have immunomodulatory effects on CD4+ T cell function as well. Currently there is a great amount of interest in understanding and ultimately modulating CD4+ T cell fate to different subsets for immune therapy [21]. Most research over the last few years has

focused on the unique metabolism of different Th subsets and metabolic interventions have been proposed as therapies [22,23]. Since TOR-KIs have been shown to modulate B lymphocyte differentiation (chapter 3) and are already available tools, it will be of great interest to understand how they affect CD4+ T cell differentiation at different doses.

Understanding selective effects of RAP in lymphocytes

If TOR-KIs were developed to overcome the limited efficacy of RAP by fully suppressing mTOR kinase activity [16], one would expect that TOR-KIs used at maximal doses to inhibit mTOR would be better immunosuppressants than RAP as well. In fact, the data accumulated in our lab over many years suggest that this is not the case. Rather, our data always suggested that RAP and TOR-KIs showed similar immunosuppression in terms of *in vitro* proliferation assays in both T and B cells [14,17]. These observations are consistent with the profound anti-proliferative effects in lymphocytes that were pointed out early after RAP was discovered [24]. However, focus was shifted early on to identify key components of this novel mTOR pathway using RAP as a molecular probe. For these studies, mostly cell lines such as NIH/3T3 or HEK293T cells were used as they provided more material for biochemical assays [25–28]. With the development of TOR-KIs, it became clear that RAP showed greater anti-proliferative effects particularly in primary lymphocyte assays and our lab was in a unique position to revisit this important question of cell-type specific RAP selectivity.

Initially, at least two possible models were hypothesized in the lab that were experimentally possible to test (Figure 5.1 top). The fact that RAP was an allosteric inhibitor that causes a conformational change in mTORC1 integrity [29] allowed us to

generate a novel mouse model where unlike complete absence of mTOR, a kinase-inactive mTOR (mTOR-KI) is selectively expressed in T or B cells. The prediction was that if kinase-independent functions exist that were critical for T or B cell proliferation, mTOR-KI lymphocytes would be able to proliferate better than complete mTOR knockout lymphocytes and this residual proliferation would be RAP-sensitive. The data presented in chapter 4 argues against this model at least in the context of how I activated T or B cells *in vitro* using antibody cross-linking of the antigen receptor or through an antigen-dependent system using the superantigen Staphylococcal enterotoxin B (SEB). The second model was that given the unique phenomenon that small resting naïve lymphocytes undergo an enormous growth phase prior to proliferation, the mTORC1 effector S6Ks that are highly implicated in cell size control [30–33] would be the relevant effector downstream of mTORC1 that RAP inhibits (Figure 5.1 bottom). This is supported by the finding that rapid S6K inactivation is almost a universal hallmark of RAP treatment in cell types examined up to date [34,35]. Surprisingly, so far we have found no loss of function genetic evidence that S6Ks are critical for lymphocyte proliferation. This is probably due to the perinatal lethality phenotype of S6K1/2 deficient (DKO) mice [36]and lack of pharmacological tools to specifically inhibit S6Ks. However, due to incomplete penetrance, a few of the S6K DKO mice actually do make it to adulthood and recently there has been progress in the development of highly selective S6K inhibitors [37].I took advantage of these unique tools in chapter 4 by examining the growth and proliferative potential of S6K1/2 DKO T or B cells for the first time. To overcome potential devleopemental compensation in the S6K1/2 DKO mice, a chemical genetic approach was used to acutely inhibit all S6K activity by treating S6K2 single knockout

lymphocytes with a S6K1 selective inhibitor. The data were clear in that S6Ks were dispensable and there was another RAP-sensitive target as S6K1/2 DKO T or B cells were equally sensitive to RAP compared to WT lymphocytes. Thus, neither of our initial hypotheses was correct in terms of explaining the selective effects of RAP on lymphocytes.

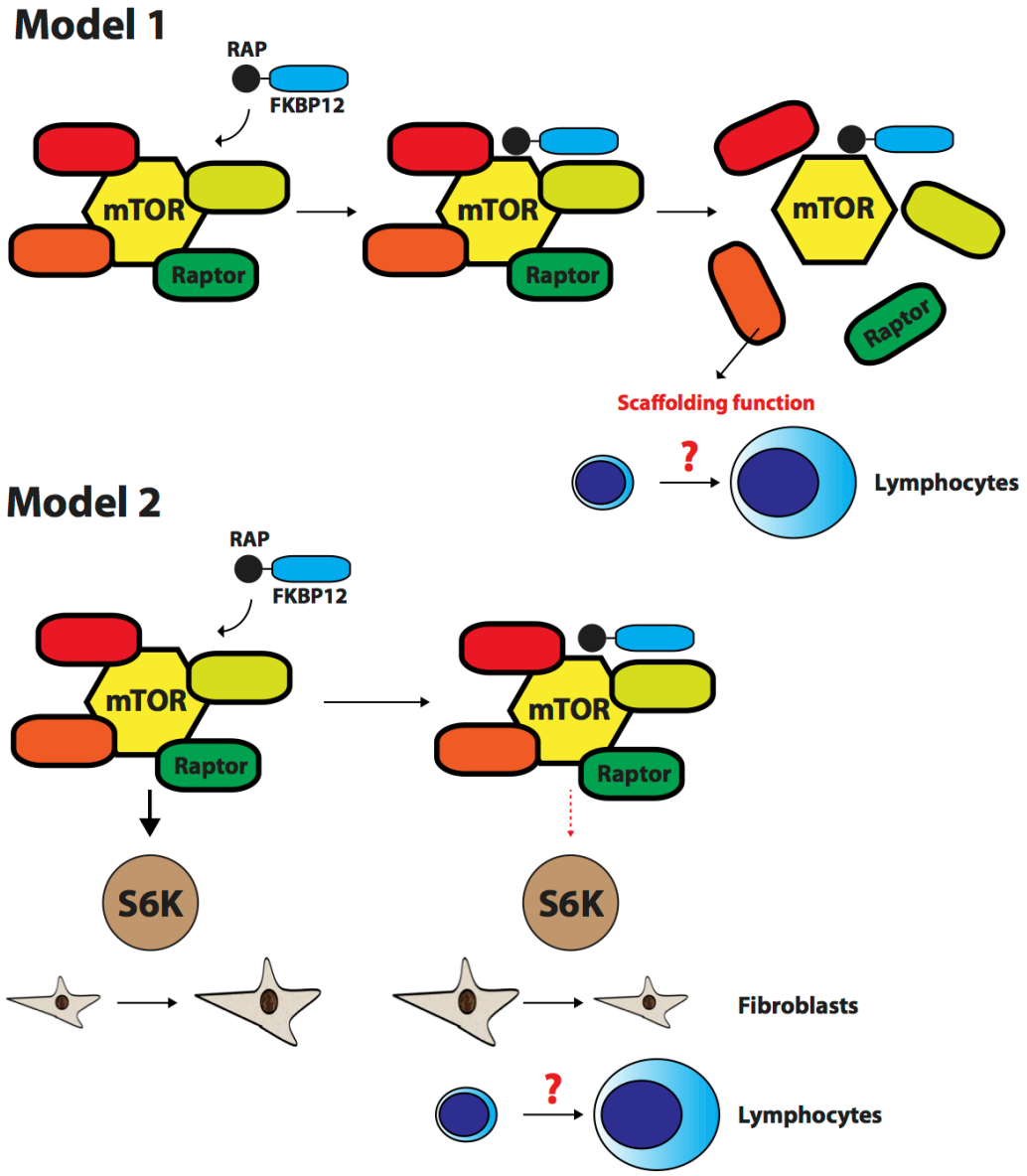

Figure 5.1. Two possible models to explain selective effects of RAP on lymphocytes. In model 1, RAP may inhibit a kinase-independent scaffolding function of mTORC1 that is critical for lymphocytes to become activated. In model 2, the highly RAP-sensitive S6Ks may play a critical role in lymphocyte growth as S6Ks have a conserved role in controlling cell size (e.g. fibroblast size is controlled at the level of S6Ks).

However, by asking this question, I discovered a completely new avenue of future research opportunities in understanding lymphocyte activation in general. In chapter 4, I used both gain-of-function and loss-of-function approaches to identify a key role for the 4E-BP/eIF4E axis in rapamycin-sensitive growth and proliferation in lymphocytes. For the gain-of-function experiments, I showed data that inducible expression of a constitutively active 4E-BP1 mutant (4E-BP1^{M}) that blocks eIF4E activity prior to T or B cell activation completely phenocopied the effects of RAP or TOR-KI treatment. In accord, the eIF4F translation initiation complex assembly was inhibited by RAP during early activation of B cells. This was a surprise as in all cell types studied to date, RAP only partially inhibits mTORC1-mediated 4E-BP1 phosphorylation and cannot displace eIF4G from binding eIF4E compared to the complete displacement of eIF4G by TOR-KIs [14,15,27,38,39]. The striking difference between the effects of RAP and TOR-KIs in disrupting eIF4F observed in many cell types probably is one of the reasons why the 4E-BP/eIF4E axis has not been considered a likely RAP-sensitive effector arm in primary lymphocytes.

In chapter 4, I further show that the ability of RAP to disrupt eIF4F correlates with its ability to dephosphorylate 4E-BP2 better than 4E-BP1. According to the substrate quality model [40] (Figure 1.7), when 4E-BP1 and 4E-BP2 sequences were aligned I observed a striking difference in the -5 position from the critical phosphorylation priming site T36 (T37 in 4E-BP2) [41]. The amino acid at this position was highly conserved throughout evolution yet differed in 4E-BP1 compared to 4E-BP2. By simple mutagenesis studies, where 4E-BP1 mutants were generated that possessed amino acids in the -5 position found in 4E-BP2 (Q or H instead of G in 4E-BP1), I showed that

the amino acid at this position could indeed change the phosphorylation sensitivity of 4E-BP1 in response to RAP treatment. Interestingly, 4E-BP2 was expressed at relatively higher levels than 4E-BP1 in primary lymphocytes compared to fibroblasts when equal amounts of proteins were analyzed. Thus, the results suggest that higher ratio of 4E-BP2 to 4E-BP1 in lymphocytes may confer RAP sensitivity in these cells. The next set of interesting experiments would be to check the higher sensitivity of the 4E-BP1 mutants to RAP actually translate into a functional effect such as cell proliferation or eIF4F complex disruption. Eventually however, the most interesting experiment would be to see if high 4E-BP2 over 4E-BP1 ratio could predict RAP sensitivity in the disease state such as cancer. It is a fact that although RAP has limited efficacy towards most cancer cells, it is clinically used for particular types of cancer such as renal cell carcinoma (RCC). Whether this correlates with high levels of 4E-BP2 is unclear, as most high throughput studies in cancer have not carefully investigated the proteome. Interestingly, there has been one study where a panel of primary breast cancer samples were analyzed for not only levels of eIF4E but also 4E-BP1 and 4E-BP2 to predict disease-free survival of patients. Notably, combining eIF4E levels with 4E-BP2 levels resulted in a better prognostic insight of cancer behavior [42]. Unfortunately, whether these patients were treated with mTOR inhibitors such as RAP was not shown.

One apparent confusion that we do not completely understand at this point is the fact that RAP not only caused equal recruitment of 4E-BP2 when m7GTP cap pulldown assays were performed but also recruited equal amounts of 4E-BP1, which should be RAP-insensitive according to the model (Figure 4.11). This might be explained in part by the differential amounts of eIF4E that different cell types express. The disruption of

eIF4G-eIF4E interaction by 4E-BPs is through a simple competition mechanism (Figure 5.2) where dephosphorylated 4E-BPs can compete with eIF4G for eIF4E binding [43]. This suggests that in cells expressing higher amounts of eIF4E than 4E-BPs, one may never efficiently inhibit cap-dependent translation even using TOR-KIs that fully dephosphorylate all 4E-BPs due to an excess of free eIF4E. The opposite is also true that cells expressing very low amounts of eIF4E to start with may be hypersensitive to mTORC1 inhibition since relatively low amounts of dephosphorylated 4E-BPs would be enough to occupy all available eIF4E. Indeed, primary lymphocytes are known to express very low amounts of eIF4E in their resting state, which increases gradually upon activation [44]. There are currently available transgenic mouse models where eIF4E levels are overexpressed using the beta-actin promoter. This would be an ideal tool to study if higher eIF4E levels in resting T or B cells can confer RAP resistance by preserving eIF4F complex assembly. During the writing of this dissertation, a study from Dr. Davide Ruggero's group at UCSF have been accepted in *Cell* where the very first loss of function genetic mouse model where eIF4E levels are reduced by 2-fold (heterozygotes) were generated. According to our hypothesis about eIF4E dosage, the prediction would be that eIF4E heterozygote lymphocytes would be hypersensitive to RAP.

Thus, low eIF4E in resting lymphocytes and higher 4E-BP2/4E-BP1 ratio may all act together to confer RAP sensitivity in lymphocytes. The important point however is that genetic blockade of the 4E-BP/eIF4E signaling axis downstream of mTORC1 was sufficient to phenocopy the growth and proliferation defect seen in mTORC1 deficient lymphocytes, suggesting that this node is critical for lymphocyte activation in general.

Rejuvenation of protein synthesis as a key research area in understanding lymphocyte activation

Lymphocyte activation is a critical process as these cells are sentinels of our adaptive immune system. Excess activation and inability to undergo programmed death upon pathogen clearance can result in severe autoimmune diseases or development of lymphomas. On the contrary, dampened activation of lymphocytes can cause immunodeficiency disorders [12,45]. Therefore, a great amount of effort has been made in understanding the molecular mechanisms of lymphocyte activation. My PhD advisor Dr. David Fruman was one of the pioneers in showing a critical role for the PI3K pathway in regulating primary B cell activation by generating the very first p85α knockout mouse [46]. These initial studies have led to the development of various PI3K inhibitors for immune therapies that I described in chapter 2. As basic fundamental research has opened up nearly a decade worth of research avenues in developing new targets for immune disorders, my findings presented in chapter 4 now open another set of research directions in potentially identifying novel targets. However, I want to emphasize not only the clinical value of these findings but also the fundamental aspect where these studies lay a new foundation for a paradigm-shift in understanding lymphocyte activation at the translational level.

This should be highlighted as most fundamental studies of lymphocyte activation have mainly focused on the following aspects: transcriptional regulation [21] and metabolic reprogramming [23,47]. Focus on transcriptional regulation during lymphocyte activation expanded with the discovery of master regulators of different Th subsets that define and are now used as biomarkers to identify these subsets. These master regulators are

transcription factors such as T-bet (Th1), GATA-3 (Th2), RORγt (Th17), and FoxP3 (Treg) that are constantly the major emphasis in highly cited reviews in this subject [21]. Indeed, there has been an expanding number of high throughout transcriptional profiling studies in understanding the entire transcriptome during T cell effector differentiation [48]. Another wave of discoveries were made in this field where different cellular metabolism was observed among different Th subsets and B cell differentiation [49,50]. These studies were possible due to recently developed high throughput metabolic flux analyzers that are easier to measure cellular metabolism compared to traditional radioactive tracer assays.

It is not my intent to downgrade the importance of the above two mainstream concepts that many laboratories still study to understand lymphocyte activation. Instead, the work presented in chapter 4 also highlights the importance of post-transcriptional regulation of the genome at the cap-dependent translation level during lymphocyte activation. In fact, it is not surprising that given the enormous increase in biomass observed during lymphocyte blastogenesis, new proteins need to be synthesized. This phenomenon has been studied since the 70s using crude approaches to inhibit global protein synthesis and correlating with a block in lymphocyte proliferation [51]. Now, we have a better understanding of the molecular machinery that regulates protein synthesis and also novel tools to identify translational targets in a high throughput fashion [52]. It is the author's hope that this work has provided a key advance in the field of lymphocyte activation by opening up new research directions. For instance, using our novel 4E-BP1^{M} genetic tool to specifically inhibit cap-dependent translation at the level of eIF4E, polysome profiling can be performed as a candidate approach to identify selective eIF4E regulated transcripts during lymphocyte activation. Whether these identified transcripts

possess unique and distinct regulatory signature at the 5' UTR as shown in other cell types [27,53] in response to TOR-KIs would be a fascinating area of future research.

Specific translational regulation by 4E-BPs in mammalian cells

Lastly, as the dissertation emphasizes the need for a cell-type specific regulation of two fundamental cellular processes; cell growth and proliferation, in a myriad of cell types in mammals, the findings in chapter 4 come back to the fundamental question of how this specificity is achieved. The data so far suggests that primary lymphocytes utilize the 4E-BP/eIF4E signaling node in a unique way compared to other cell types in that regulation of both cell growth and proliferation was dependent on this node. The fact that the same 4E-BP1^{M} construct only blocked cell proliferation where the fibroblasts continued to grow suggest that there must be differential translational regulation of certain transcripts in this cell type compared to lymphocytes. How this translational specificity is achieved is a fascinating question because it provides another layer of gene regulation. For many years, the ribosome has been regarded only as a protein producing factory without regulation. Although initially proposed by Edelman and Mauro as the 'ribosome filter' theory [48], it was recently shown experimentally that not all ribosomes are the same and Barna and colleagues have proposed an expanded version of this postulation as they defined a new term called 'specialized ribosomes' [49,50].

My findings presented in chapter 4 also have clinical relevance as translation specificity was observed in a cell-type specific manner. The results suggest that there may be ways to specifically target a subset of cells for therapy while preserving the function of other cell types. Supporting this notion, I have found that 4E-BP1^{M}

expression in normal lymphocytes has no effect on cell death suggesting that cell survival pathways are not regulated by 4E-BP/eIF4E (data not shown). Interestingly, once B lineage cells are transformed by a single BCR-ABL oncogene to produce the pre-B-ALL leukemia cell line (p190) routinely used in our lab, expressing 4E-BP1^{M} caused all cells to succumb to death (data not shown). This suggest that a rewiring event happens where p190 cell survival requires the 4E-BP/eIF4E signaling axis. These results highly correlate with previous findings from our lab where TOR-KIs that strongly suppress 4E-BP phosphorylation in p190 cells show cytotoxic effects compared to RAP treatment that only shows a cytostatic effect [14]. There are still major questions that remain to be answered but it is my hope that this dissertation has provided a novel avenue of research directions to that end.

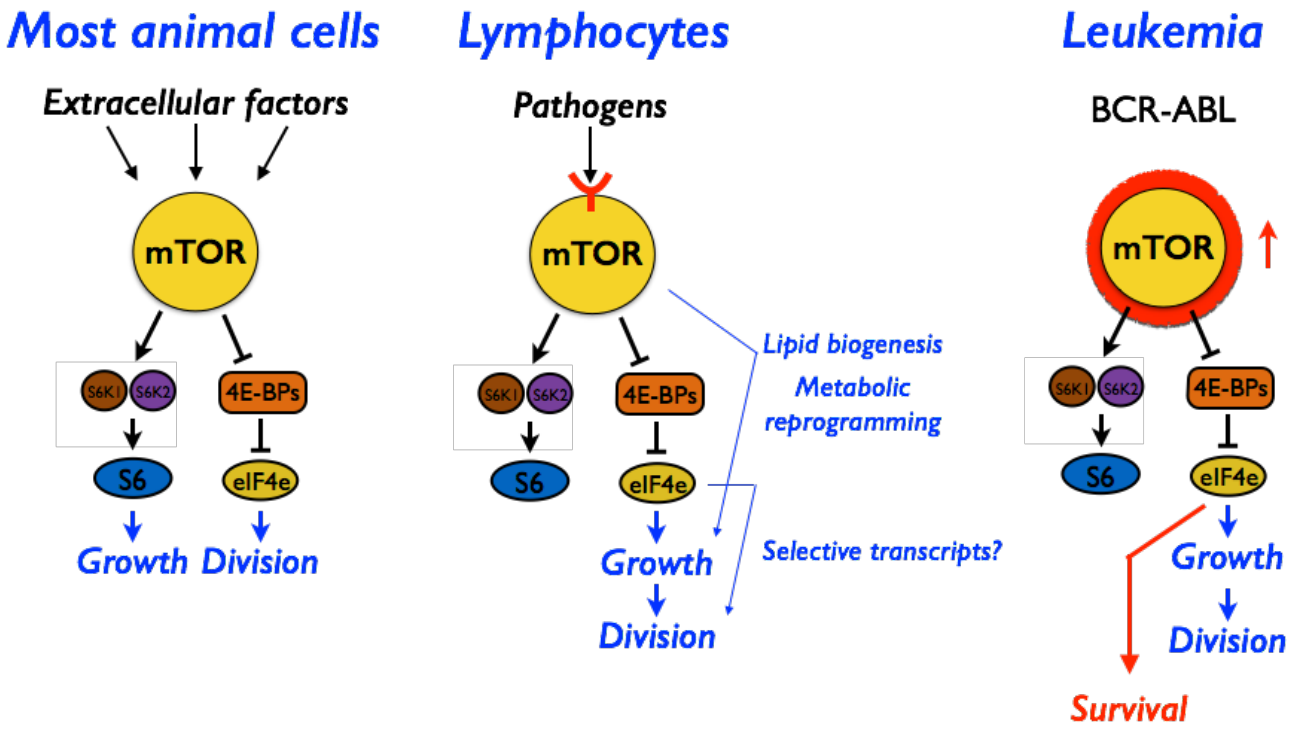

Figure 5.2. Specific translational regulation by 4E-BPs in mammalian cells. Most animal cells can separate growth and division and the 4E-BPs have been shown to mainly regulate cell division by specific translation of cell cycle related mRNAs. Lymphocytes show coordinate regulation of both cell growth and division through the 4E-BP/eIF4E axis. These results suggest that the selective repertoire of transcripts regulated at the translational level are different in lymphocytes. Upon oncogene induced transformation, lymphocytes utilize the 4E-BP/eIF4E axis to also translate cell survival related mRNAs. The nature of this rewiring is unclear but may be exploited as a unique target for anti-cancer therapy while preserving normal cell function.

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