UNIVERSITY OF CALIFORNIA,
IRVINE

Modulators of Th17/Treg Differentiation and Function

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Christine Pai

Dissertation Committee:
Professor David A Fruman, Chair
Professor Craig M Walsh
Associate Professor Melissa B Lodoen

2016

TABLE OF CONTENTS

LIST OF FIGURES

Page

CHAPTER 1

CHAPTER 2

CHAPTER 3

ACKNOWLEDGMENTS

I would first like to thank Dr. David A. Fruman for his support and mentorship during my doctoral studies. It was a great opportunity for me to pursue my thesis work in his lab and I now approach my research with confidence and renewed curiosity. Under his guidance, I was able to mature in my ability to think critically, which was fostered by developing a more concrete approach to scientific problems and questions. An important trait that I was able to learn in his lab was to be more patient and attentive in my experiments and communication with others. His strong commitment to making himself available at all times helped me understand the importance of establishing a strong continual dialogue and our discussions provided invaluable insight and feedback.

I would also like to thank Dr. Craig M. Walsh for his support and guidance. My interest in venturing into utilizing unfamiliar techniques for my project was wholesomely encouraged by him and I found my time in his lab to be greatly productive. Craig is always an amazingly positive mentor, with the uncanny ability to pick up on something positive, no matter how dire and dismal the current situation is.

My sincere thanks go to members of the Walsh and Fruman lab, past and present. I would like to thank Brian Weist and Jennifer Lu for their help and guidance during my early graduate years. I would also like to thank Dr. Trang Vo, Scott Lee, and Honyin Chiu for always being available to help or lend an ear. My thanks also go to Adam Idica and Dr. Sarah Lutz for their technical expertise and invaluable discussions.

I also thank Dr. Melissa Lodoen and Dr. Aimee Edinger for making themselves available to provide helpful advice and guidance, as well as for their suggestions and technical help during our joint lab meetings.

CURRICULUM VITAE

CHRISTINE PAI
16 Oakcliff Drive, Laguna Niguel CA 92677
949-824-2274 (Work), 949-910-6988 (Mobile)
paic@uci.edu

EDUCATION

2016	PhD	Immunology, UC Irvine
2008	BS	Microbiology, UC San Diego

EXPERIENCE

2013-present Graduate Researcher
UC Irvine
Dr. David A Fruman
Studies of downstream mTORC1 effectors and their role in T cell mediated autoimmunity
Collaboration with Nimbus Therapeutics: Evaluation of small molecule inhibitors for Tyk2 and ACC1/2 in treatment of autoimmune diseases.

2012-2014 Graduate Researcher
UC Irvine
Dr. Craig M Walsh
Studies of Drak2 and its role in activated $CD8^+$/$CD4^+$ T cell metabolism

2008-2011 Research Assistant
VABHS /Harvard Medical School
(Dr. Jason S Gold Lab)
Evaluated the role of Th17 cells and CCR6 blockade in colorectal cancer utilizing transgenic mouse models and human specimens.

2008-2011 Research Technician
DFCI /Harvard Medical School
(Dr. Nikhil C Munshi Lab)
Studied the role of Th17 cells and associated inflammatory cytokines in multiple myeloma patients and SCID mouse models.

2007-2008 Research Assistant
Moores UCSD Cancer Center
(Dr. Davorka Messmer Lab)
Use of Nanoparticles for tumor immunotherapy

2006-2008 Research Assistant

Moores UCSD Cancer Center
(Dr. Thomas J Kipps Lab)
Generation of a DNA vaccine using a CLL-specific antigen

2006 Summer Research Intern
The Scripps Research Institute
(Dr. Michael G McHeyzer-Williams Lab)
Study of adjuvants and vaccines in relation to memory B cells

LABORATORY SKILLS

- Assays (qPCR, ELISA, ELISPOT, Western Blot, viability, multiplex, proximity ligation)
- Metabolic studies (Seahorse, metabolomics)
- Mouse studies (transgenic strain breeding, surgery, injections, autoimmune and tumor models)
- IHC/IF (2-photon intravital imaging and sectioning)
- Flow cytometry (phosphoflow, sorting, multispectral imaging, etc.)
- Recombinant techniques (plasmid purification, bacteria culturing, etc.)
- Immune cell assays (tissue/cell isolation, stimulation, allogeneic, suppression, differentiation)
- Human/mouse specimen bank collection and culture (blood, tissue, tumor, etc.)

HONORS AND DISTINCTIONS

2015 UC Irvine Institute for Immunology Annual Fair- Top 2 Oral presentation
2010 Associate faculty member of Faculty of 1000
2007 Calit2 UCSD Summer Undergraduate Research Scholar
2006 Summer Immunology and Virology Undergraduate Fellowship, The Scripps Research Institute

PUBLICATIONS/MANUSCRIPTS

Pai C, Miao W, Kapeller R, Fruman DA. Potent and Selective Tyk2 Inhibitors Target Pathogenic Th17-mediated immune responses. In preparation.

Pai C, Miao W, Kapeller R, Fruman DA. Potent and Selective ACC Inhibitors Target Th17-mediated immune responses and Increase Treg Generation. In preparation.

Pai C, Walsh CM, Fruman DA. Context-specific function of S6K2 in helper T cell differentiation. In revision to *Journal of Immunology*.

Nandi B, Shapiro M, Samur MK, **Pai C**, Frank NY, Yoon C, Prabhala RH, Munshi NC, Gold JS. Stromal CCR6 drives tumor growth in a murine transplantable colon cancer through recruitment of tumor-promoting macrophages. *Oncoimmunology*. 10.1080

Shapiro, M., B. Nandi, **C. Pai**, M. K. Samur, D. Pelluru, M. Fulciniti, R. H. Prabhala, N. C. Munshi, and J. S. Gold. 2016. Deficiency of IL-17A, but not the prototypical Th17 transcription

factor RORγt, decreases murine spontaneous intestinal tumorigenesis. *Cancer Immunol Immunother* 65: 13–24.

C. Pai, Fracchia, K. M., and C. M. Walsh. 2013. Modulation of T Cell Metabolism and Function through Calcium Signaling. *Front Immunol* 4: 324.

Nandi, B., **C. Pai**, Q. Huang, R. H. Prabhala, N. C. Munshi, and J. S. Gold. 2014. CCR6, the sole receptor for the chemokine CCL20, promotes spontaneous intestinal tumorigenesis. *PLoS ONE* 9: e97566.

Prabhala, R. H., M. Fulciniti, D. Pelluru, N. Rashid, A. Nigroiu, P. Nanjappa, **C. Pai**, S. Lee, N. S. Prabhala, R. L. Bandi, R. Smith, S. B. Lazo-Kallanian, S. Valet, N. Raje, J. S. Gold, P. G. Richardson, J. F. Daley, K. C. Anderson, S. A. Ettenberg, F. Di Padova, and N. C. Munshi. 2015. Targeting IL-17A in multiple myeloma: a potential novel therapeutic approach in myeloma. *Leukemia*.

Prabhala, R. H., D. Pelluru, M. Fulciniti, H. K. Prabhala, P. Nanjappa, W. Song, **C. Pai**, S. Amin, Y. T. Tai, P. G. Richardson, I. M. Ghobrial, S. P. Treon, J. F. Daley, K. C. Anderson, J. L. Kutok, and N. C. Munshi. 2010. Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma. *Blood* 115: 5385–5392.

ABSTRACTS

The Role of S6 Kinase 2 in T Cell Proliferation and Differentiation. 2015 Keystone Symposia: PI 3-Kinase Signaling Pathways in Disease, poster presentation

Lack of Th17 cells does not appear to have a major impact on intestinal tumorigenesis in MIN mice. 2011 Academic Surgical Congress

Human Monoclonal Antibody Targeting IL-17A (AIN457) Down-Regulates MM Cell-Growth and Survival and Inhibits Osteoclast Development In Vitro and In Vivo: A Potential Novel Therapeutic Application in Myeloma. 2010 American Society of Hematology annual meetings

ABSTRACT OF THE DISSERTATION

Modulators of Th17/Treg Differentiation and Function

By

Christine Pai

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2016

Professor David A Fruman, Chair

Dysregulated immune responses against our own normal tissues result in autoimmunity, an often chronic, debilitating, and life-threatening group of diseases. Traditional approaches in treatment involve the deliberate induction of broad immunosuppression, which undoubtedly exposes the body to a higher risk of infection. An alternative approach would be to disrupt key steps during differentiation of selected T cell subsets, in order to offer a targeted immunosuppressive treatment. Investigating T cell differentiation in the pathogenesis and treatment of autoimmune diseases can be approached in a two-fold manner. Inflammatory T cells, specifically the Th17 subset, are a main driver in the pathogenesis of autoimmune disease, including multiple sclerosis, psoriasis, and colitis. On the opposing axis lie regulatory T cells, which modulate the immune system by maintaining tolerance to self-antigens and suppressing effector T cell proliferation.

The ability of naïve T cells to proliferate and differentiate into effector T cells depends on the mechanistic target of rapamycin (mTOR) pathway. The mTORC1 complex activates biosynthetic processes that are crucial to sustain T cell growth and effector function. The potent immunosuppressive drug rapamycin (RAP) strongly blocks CD4 T cell proliferation and effector differentiation, despite only partial inhibition of mTORC1. The ribosomal protein S6 kinase 1

and 2 are activated by mTORC1 and are highly RAP-sensitive. The first aim of this dissertation focuses on the role of S6K2 in Th17/Treg differentiation and function in autoimmunity.

Distinct metabolic processes separate the $CD4^+$ effector subsets Th17 and Treg. The metabolic signature of inflammatory Th17 cells involves a high glycolytic capacity that requires de novo synthesis of fatty acids. Conversely suppressive Tregs rely on oxidative phosphorylation, which involves beta-oxidation of extracellularly obtained fatty acids. Acetyl-CoA Carboxylase, or ACC, is a master regulator of fatty acid synthesis and oxidation. Enzymatically active ACC catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, which results in fatty acid biosynthesis and inhibition of beta-oxidation of fatty acids. Previous findings report Th17 cells to require ACC1 in their development, but that this isoform is dispensable for the induced Treg subset. Using a newly developed allosteric inhibitor of ACC1 and ACC2, the second aim of this dissertation is to evaluate the potential to target ACC enzymes using this novel inhibitor as a therapeutic strategy in the treatment of autoimmune diseases.

Chapter 1

Introduction

I got sick the way Hemingway says you go broke: "gradually and then suddenly."

-Meghan O'Rourke, author of My Struggle with Autoimmune Disease

The need for targeted immunosuppression in treating autoimmune diseases

Approximately 50 million Americans, or 1 in 5 people, suffer from autoimmune diseases. Autoimmunity is most commonly experienced as a chronic illness and is the leading cause of morbidity in women in America (1). Traditional therapies have relied on broad immunosuppressive strategies that often work well at easing disease symptoms, but at the expense of exposing patients to increased vulnerability to opportunistic infections and some serious toxicities. Rapamycin is one such FDA approved immunosuppressant, with potent ability to block inflammatory responses, but also causes a number of undesired on-target effects.

With a global, ever-increasing incidence of autoimmune diseases that include multiple sclerosis and celiac disease, there is a growing need for a more targeted immunosuppressive treatment. Advances in transplant surgery also have much to gain in assuring long-term host acceptance of donor tissues, which require immunosuppressive treatments as well. These therapies should ideally suppress only the dysfunctional inflammatory responses while sparing the rest of the otherwise healthy immune system. More recent developing immunotherapies have focused on two main concepts in targeting autoimmunity: Blocking the dysregulated inflammatory responses and/or augmenting suppressive regulatory immune responses.

$CD4^+$ T cells are an integral component of the adaptive immune system that has essential pathogenic roles in a number of autoimmune diseases. Several $CD4^+$ T cell subsets are each key players in hypersensitivity responses and also drive inflammatory pathogenesis in several diseases, including diabetes, multiple sclerosis, and graft-versus-host disease. Based on these crucial roles in autoimmunity, targeting $CD4^+$ T cell subsets has been an important component in the development of targeted immunosuppressive therapies. The focus of this thesis is elucidating regulatory pathways that are essential to inflammatory T cell subset proliferation and function, but dispensable to other subsets that provide host defense or regulate immune responses. Specifically, I have sought to identify cellular targets whose inhibition reduces the ratio of inflammatory Th17 cells to suppressive Treg cells.

$CD4^+$ T cell subsets and their role in host defense and autoimmune diseases

Naïve $CD4^+$ T cells take part adaptive immunity, an immune response that requires processing and recognition of highly specific antigens. This is in contrast to innate immunity, which involves a nonspecific immune response that offers a first line protection in the form of quick acting leukocytes (e.g macrophages, mast cells and natural killer cells) and natural physical barriers (e.g skin and digestive enzymes). Adaptive immune responses produce long-lasting protection to antigens and have the ability to respond quickly to secondary and subsequent infections. $CD4^+$ T cells bear the responsibility of orchestrating an immune response. Several distinct $CD4^+$ T cell subsets can be identified upon activation by their cytokine production and signature transcriptional factors. Each subset has unique roles in controlling infections of various nature, which can include intracellular and extracellular pathogens (Figure 1). Each $CD4^+$ T cell subset, however, is itself capable of harming the host through dysregulated responses. The following illustrates the four major subsets of

differentiated $CD4^+$ T cells (i.e. Th1, Th2, Th17, and Tregs) and their roles in both immunity and disease.

Th1 subset

The pro-inflammatory effector subset Th1 has the primary host defense role of generating immune responses against intracellular bacteria, viruses and protozoa. $CD4^+$ T cells can differentiate into Th1 by the polarizing cytokine IL-12 and then secrete the effector cytokine IFNγ (1, 2). Th1 cells can be also identified through expression of the transcription factors STAT4 and Tbet. During a Th1-type immune response, secreted IFNγ strongly activates macrophages and promotes proliferation and differentiation of $CD8^+$ T cells to help identify and clear infected cells (2). Overactivation of proinflammatory Th1 responses, however, can lead to uncontrolled tissue damage and subsequent autoimmune diseases. Undesired Th1 based inflammatory responses can also involve autoantigens, leading to breakdown of otherwise healthy tissues in the body. Type IV hypersensitivity (also known as delayed type hypersensitivity or DTH) is caused by Th1 cell proliferation induced by macrophages secreting IL-12, which leads to Th1 cells further proliferating by their own production of IL-2 and IFNγ (2). Diabetes mellitus type 1 is one such type of DTH, where beta cells of the pancreas are attacked by both $CD4^+$ and $CD8^+$ cells, which secrete effector cytokines in response to autoantibodies produced against the islet cells or insulin (3). Autoimmune gastritis is another example, where the proton pump of parietal cells is targeted by Th1 cells and can lead to digestive problems (4). The chronic inflammation can originate from either genetically inherited factors or persistent infections by *Helicobacter pylori*. The gram-negative bacteria *H. pylori* is a well-known microbe that resides in more than 50% of the world's population and has been implicated in many dysregulated autoimmune responses, as well as cancers of the digestive tract (5). In summary, Th1 immunity is primarily important

for destroying and clearing infected host cells, but overactivation of this subset leads to certain organ breakdown through excess inflammation and cytolytic T cell activity.

Th2

Th2 cells are responsible for controlling parasitic infections and most forms of inflammatory responses that result in allergies. These Th2 responses are collectively categorized as type 2 immune response and mainly involve the cytokines IL-4, IL-5, IL-9, and IL-13 (2). IL-4 is a differentiating cytokine for Th2 cells, which can be distinguished from the other subsets by expression of the transcription factors GATA3 and STAT6. A major mechanism by which Th2 cells function is through secretion of IL-4, which promotes B cell antibody class switching to IgE. IgE is a potent antibody, capable of inducing inflammation through mast cell degranulation (i.e. histamine and heparin release) (2). Though IL-4 and IgE production through Th2 mediated immune responses are important in expelling extracellular parasites (e.g. helminths), an overactive type 2 response is the primary driver of allergic conditions caused by otherwise harmless antigens. One popular medical hypothesis involving Th1 and Th2 subsets is the "hygiene hypothesis", where a dearth of exposure to natural microbes during early childhood leads to a defective development of immune tolerance (6). In this case of intracellular microbe clearance, Th1 cells play an important protective role in removing the recognized antigen. An imbalanced immune response, however, to otherwise harmless environmental antigens cause Th2 responses to dominate, leading to allergies of the skin, lungs, and gut. Th2 type autoimmune reactions are grouped into Type I hypersensitivity and include asthma, allergic rhinitis, and atopic dermatitis (7). In conclusion, Th2 cells ensure expelling of extracellular parasite infections, though an underdeveloped immune system early in life can lead to this subset to activate in response to innocuous antigens, an inappropriate

response that leads to excess inflammation and activation of unnecessary immune functions (e.g. B-cell class switching to IgE and mast cell degranulation).

Th17

Th17 cells are a proinflammatory $CD4^+$ T cell subset that have a key role in regulating mucosal surface immunity. Polarizing cytokines that promote Th17 differentiation include IL-6, IL-17, IL-21, and IL-23 (8). TGF-β is also an important differentiating factor and the identifying transcription factors are STAT3 and RORγ. Th17 cells then can produce all these differentiating cytokines, in addition to tumor necrosis factor-α (TNF-α), IL-1β, and IL-22. Specifically in the intestine, Th17 cells must constantly keep the localized immune cells and natural microbiota (both extracellular bacteria and fungi) in check by ensuring a balanced interaction at the mucosal barrier (8, 9). Indeed, loss of Th17 populations at these interfaces leads to chronic inflammation and infiltration of pathogens. Dysregulated Th17 responses have been found to be a factor of major intestinal autoimmune diseases, inflammatory bowel disease (IBD) and Crohn's disease (8, 9). Mechanistically, Th17 cells keep local microbial populations in check by releasing IL-17 and IL-22 that recruit neutrophils and promote secretion of antimicrobial peptides (8, 9). Interestingly, the Th17 subset has been found paradoxically to possess antitumor properties in certain types of cancers while promoting tumor growth in others. Several reports have attributed this curious finding to the local inflammatory milieu present and that the close interplay of Th17 cells with the interconnected Treg subset dictates the type of response to tumors (10–12). A well-characterized dysregulated Th17 immune response is their activation during viral and parasitic infections. During a mouse model of chronic viral infection, Th17 cells promote a persistent viral infection and induce a chronic demyelination disease. The IL-17 secreted causes upregulation of antiapoptotic molecules (i.e. Bcl-xl and Bcl-2), which enhances survival of cytotoxic T

cells (13). In a different model using chronic infection by *Toxoplasma gondii*, IL-27 was found to suppress the proinflammatory responses of Th17 cells (13–15). When IL-27 was knocked out in mice, the chronic infection by *T. gondii* caused severe neuroinflammation that was attributed to a prominent IL-17 response. In addition to these autoimmune disorders, rheumatoid arthritis, psoriasis, and systemic lupus erythematosus have all been found to have elevated IL-17 levels in the affected tissues (8, 9).

Treg

Tregs are a unique subset of $CD4^+$ T cells that provide a suppressive response to maintain homeostasis during infection, thus opposing chronic inflammation and autoimmunity. The essential function of Tregs is apparent in that humans deficient in the *FOXP3* gene lack this effector subset and develop a severe autoimmune disease called IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) (16). IPEX results in early death, due to a massive inflammatory response to many organs of the body, as well as multiple allergies and infections. Scurfy mice that have genetic frameshift deletion of their *Foxp3* gene lack Treg development and also develop severe autoimmunity that affects multiple organs (17). Tregs are able to reduce activation and proliferation of effector T cells by secreting IL-10 and TGFβ. Tregs are identified by expression of CD25 and the transcription factor FoxP3; in addition, these cells possess a distinct metabolic phenotype when compared to the other effector subsets (explained later). A unique feature for Tregs is that there is a naturally occurring population of Tregs at any point in the host, called nTregs. Unlike other effector T cell subsets, nTregs are generated like naïve conventional $CD4^+$ T cells in the thymus and also undergo selection by affinity interaction (18). However, Tregs can also be induced under certain conditions from naïve conventional CD4 T cells like the other subsets and are termed induced Tregs (iTregs). Though both share the same immune function, iTregs are "an

essential non-redundant regulatory subset that supplements nTreg cells, in part by expanding TCR diversity within regulatory responses" (19). While the lack or dysregulation of Tregs can cause systemic autoimmune disease (i.e. lupus), the subset also has been implicated in helping tumors evade detection. Though most tumors can elicit an immune response, the tumor infiltrating lymphocytes (TILs) often carry a higher than normal population of Tregs when comparing their portion in regular $CD4^+$ populations (20, 21). The increased prevalence of Tregs in the tumor microenvironment can be attributed to the secretion of TGFβ by the tumors themselves, which also produce a chemokine that favors trafficking of Tregs to the site (21).

Targeting the mTOR signaling network in T cells

History of mTOR

Originally discovered during a Canadian medical expedition to Easter Island in 1964, rapamycin was isolated from the bacteria *Streptomyces hygroscopicus* as an antifungal macrolide (22). After initial studies in the lab, researchers found it to be potently immunosuppressive, possessing antiproliferative properties towards lymphocytes that led to FDA approval for the use of rapamycin in preventing transplant rejection (23, 24). Genetic studies in the early 1990s led to the identification of TOR, or target of rapamycin. TOR is a serine/threonine kinase that is evolutionarily conserved across species from yeast to human. The mammalian enzyme (mTOR) exists in two separate complexes, mTORC1 and mTORC2, each that can initiate distinct cellular processes. mTORC1 is selectively and allosterically inhibited by rapamycin, though certain kinase activities remain unaffected. Rapamycin also can inhibit mTORC2 function in some cell types through inhibiting the assembly of new mTOR complexes. Interest in further utilizing rapamycin for its antiproliferative properties in cancer led to development of analogs, termed “rapalogs”. The orally active rapalogs include

temsirolimus (CCI-779) and everolimus (RAD001), which showed clinical efficacy as single agents previously in only a small number of cancers (e.g. renal cell carcinoma and mantle cell lymphoma) (25–27). The limited ability for rapalogs to treat cancer can come from several mechanisms, including that mTORC1/2 signaling possesses rapamycin insensitive downstream functions and that inhibition also removes existing negative feedback loops to PI3K and Akt.

Upstream inputs and downstream substrates of mTOR

mTORC1 in T cells acts as a sensor of both immunological (i.e. TCR stimulation, cytokines, costimulation) and environmental (i.e. growth factors, hormones, amino acids) inputs. Once activated, mTORC1 can initiate anabolic processes to support growth and proliferation (Figure 1.2). Rheb (ras homolog enriched in brain) is a small GTPase that can activate mTORC1 when bound to GTP (28). This mechanism itself is regulated by the TSC1/2 complex, which promotes GTP hydrolysis by Rheb to maintain T cell quiescence and homeostasis (29). Engagement of immunological sensors on the surface of naïve T cells results in activation PI3K, which recruits PDK1 to the plasma membrane (Figure 1.2). PDK1 is then able to activate Akt by phosphorylation, which in turn allows the later to phosphorylate and inactivate the TSC1/2 complex (30, 31). Removal of this repression allows the GTP-bound Rheb to activate mTORC1. The level of mTORC1 activity can be measured by the level of phosphorylation of its canonical downstream substrates, S6K and 4E-BP (32).

The function and targets of mTORC2 are lesser known, but do include Akt, serum- and glucocorticoid-induced kinase (SGK) and protein kinase C (PKC). (33). Activation of Akt requires phosphorylation by both PDK-1 on Thr 308, and mTORC2 on Ser 473. mTORC2 is thought to be activated by binding to PI3K lipid products and by association with ribosomes.

Upon phosphorylation by activated mTORC1, 4E-BP family members (4E-BP1, 2, 3) dissociate from eIF4E. Free eIF4E can then form the eIF4F complex, which stimulates initiation of cap-dependent translation. Activated mTORC1 also phosphorylates and activates the S6Ks, serine/threonine kinases that are known to promote cell growth (size increase). S6K1 and S6K2 share an 83% amino acid sequence homology of their kinase domain (34). S6K2 possesses a nuclear localization sequence (NLS) at the C-terminus, which keeps this isoform in the nucleus while the cell is in a quiescent state (35). Both kinases phosphorylate the ribosomal protein S6 (rpS6) at five C-terminal serine sites. There are many characterized roles for S6K, though it is not yet clear which of these is exclusively a function of S6K1, S6K2, or both kinases (Figure 1.2). Suppressing S6K1 activity has been shown to increase Akt activity through removal of a feedback loop that inhibits the PI3K pathway through phosphorylation of IRS-1 at several Ser residues (36–40). However, though S6K2 inhibition studies in cancer cells suggest an opposite role in decreasing Akt activity (see below), it is unknown in lymphocytes what role S6K1 and S6K2 may play in feedback signaling to Akt.

Sterol responsive element binding protein (SREBP1) and CAD are such proteins whose function and/or phosphorylation is promoted by S6Ks, though it still remains unknown to what degree by each kinase (41–43). SREBP and CAD are involved in lipogenesis and pyrimidine biosynthesis, respectively, and are utilized during activation and proliferation to help sustain the biosynthetic needs of the cell.

Though S6K1 and S6K2 share a high sequence homology, the conservation of two isoforms through mammalian evolution suggests that the isoforms have distinct functions. More recent research has characterized distinct biological functions for each isoform. S6K1 has been much more thoroughly studied, most likely due to its discovery a decade before S6K2.

Some studies have characterized distinct biological functions for S6K2. However, much of the characterization of this isoform has been done in cancer cell lines that often have aberrant expression and function of signaling proteins. One example is the finding that S6K2 in breast cancer cells increases Akt activity, as knockdown of S6K2 caused reduced Akt phosphorylation and cell death via the mitochondrial cell death pathway that involves the Bcl-2 family protein Bid (44).

Therefore, there exists a need to study S6K2 function in normal tissues to tease out important roles and phenotypes. Most normal tissues exhibit low S6K2 expression, though over 88% of cancer samples have comparatively higher levels that often correspond with prognostic outcome (45–47). B- and T- lymphocytes are another tissue that have elevated expression of S6K2, but this elevated expression pattern was not observed for S6K1 (48). Additionally, malignant tissues often have prominent nuclear accumulation for S6K2. In particular, S6K2 interacts directly with the transcription factor Yin Yang 1 (YY1), which is possible only through its C-terminus (49). YYI can recruit transcriptional machinery like RNA polymerase II and various transcriptional co-activators/repressors, such as DNA methyltransferases, histone deacetylases (HDAC 4), and histone- lysine *N*-methyltransferase (Ezh1/2) (50). Though S6K2 does not play a major role in cap-dependent translation, it is selectively involved in cap-independent translation of mRNAs involved in cell survival (34, 51, 52).

mTOR signaling in T lymphocytes

Genetic studies have identified roles for mTOR signaling in T cell fate decisions. By incorporating immune signals, environmental cues, and available nutrients, the complex mTOR signaling pathway can initiate transcriptional and translational networks to alter or adjust biosynthetic/energetic pathways. These steps are crucial in ensuring the T cell obtains

a proper and sustainable immune response (53). In T cell specific deletion models, partial or complete abrogation of required mTORC1 components (Rheb or raptor) or mTORC2 components (rictor) lead to deficiency in various T helper subsets. Deletion of both mTORC1 and mTORC2 diminishes Th1/Th2/Th17 differentiation, but increased Treg generation (54). Singular disruption of just mTORC1 reduces Th1/Th2/Th17 differentiation, while mTORC2 disruption reduces Th1/Th2 differentiation (54). These findings clarify that RAP has longstanding immunosuppression effects that are due not only to reduced proliferation but also to impaired T helper effector function. The role of mTORC1 in both Th1 and Th17 differentiation suggests the immunomodulatory potential of blocking mTORC1 or its downstream substrates in T cells to prevent transplant rejections and treat autoimmune disorders.

Selective effects on lymphocytes through mTORC1 inhibition

RAP is a potent and selective immunosuppressant that is used clinically to prevent transplant rejection and as a local anti-inflammatory. However, long-term systemic RAP treatment is not well tolerated, due to a broad range of on-target effects (e.g. peripheral edema, hypertension, thrombocytopenia, pneumonitis) (55). This is a main reason why RAP is not a standard treatment of autoimmune disorders, though it is sometimes prescribed off-label for example for SLE patients. One of the hallmarks of RAP activity in most cells is profound suppression of S6K phosphorylation and activity; thus there is potential in identifying S6Ks as new immunosuppressive therapeutic targets (56, 57). The greater selectivity of targeting S6Ks instead of mTORC1 and its many other substrates could reduce the potential for side effects in patients. In addition, studying S6K function could elucidate the long-standing fundamental interest in how RAP causes immunosuppression and exerts a more selective effect on lymphocytes than other cell types.

Targeting a downstream mTORC1 effector as a potential autoimmune treatment

Possible roles for S6Ks upon T cell stimulation include control of metabolic reprogramming, compensatory mechanisms in feedback signaling, and activation of key transcriptional signaling networks. In non-lymphoid cells, S6K has well-established roles in initiating anabolic processes during growth factor stimulation. Studying S6K function in lymphocytes has been difficult previously; this is due partially to perinatal lethality of double S6K1/S6K2 knockout mice, and an early assumption that both kinases have a mostly redundant function (34, 58). Moreover, until recently there were no selective chemical inhibitors of S6Ks. Two compounds that are ATP-competitive and selective inhibitors of S6K1 have been developed and are now available (LY2584702 and PF4708471) (59). In addition, the availability of an S6K2 knockout mouse has allowed for the study and characterization of S6K2 alone. It has increasingly become important to separate the functions of each kinase, as recent papers have reported unique roles for both S6K1 and S6K2 in Th17 differentiation.

S6K function in Th17 differentiation

Current knowledge of S6K function in $CD4^+$ T cell effector function put emphasis on its role in the Th17 subset. In 2012, Koyasu and colleagues found differential roles for S6K1 and S6K2 in promoting Th17 differentiation, using mainly biochemical approaches (60). Utilizing rapamycin and the p110δ inhibitor IC87114 to reduce S6K activity, S6K1 was found to inhibit Gfi1, a negative regulator of Th17 differentiation. This was attributed to upstream activation of the mTORC1-S6K1 pathway by TGFβ, which in turn increases RORγ and EGR2 expression, with the latter found to inhibit Gfi1 expression. S6K2 was found to play a distinct role in promoting Th17 differentiation by augmenting nuclear translocation of RORγ. The amount of cytoplasmic RORγ is unchanged between untreated and rapamycin-

treated T cells, but the latter showered severely reduced nuclear RORγ. This indicates a defect in the shuttling of the Th17 transcription factor into the nucleus. Transfection of embryonic kidney (HEK293T) cells with tagged RORγ and S6K2 showed direct interaction by co-immunoprecipitation. Since S6K2 but not RORγ possesses a nuclear localization sequence (NLS), the authors proposed that the interaction of these proteins increases the nuclear translocation of RORγ.

Park et al. and Sasaki et al further explored S6K1 function in Th17 cells in two recent publications (61, 62). Though both papers also found S6K1 to enhance Th17 responses, *in vivo* EAE studies performed on S6K1 knockout mice by Sasaki and colleagues found only delayed progression of clinical scores compared to wildtype mice. Notably, no published work has examined the effects of S6K2 loss of function on T cell activation and differentiation.

Metabolism of T lymphocytes

Although one might consider metabolic flux to be a static housekeeping function of cells, it is becoming apparent that specific metabolic programs are induced in distinct T cell subsets and developmental stages. For example, the metabolic status of naïve T cells is significantly different from their activated counterparts. Naïve T cells actively maintain a quiescent state through engagement of both intracellular signaling pathways and cell extrinsic signals (63), resulting in efficient use of available energy sources (64). These quiescent lymphocytes maintain a catabolic state and do not actively take up nutrients, nor do they secrete effector cytokines (65). Antigenic stimulation of mature T cells facilitates metabolic changes that support various bioenergetically demanding processes needed for rapid clonal expansion (65). It is proposed that T cells must shift from catabolic to anabolic metabolism in order to build biomass necessary for rapid proliferation, which is required for effective response to

microbial infection (66). Indeed, CD8 T cells have the capacity to divide once every 4–6 h (67), a process that is highly energy dependent. Naïve T cells appear to favor energetically efficient processes such as the tricarboxylic acid (TCA) cycle linked to the generation of ATP via oxidative phosphorylation (OXPHOS), which results in roughly 30–32 ATP units per molecule of glucose. In contrast, antigenically stimulated T cells rapidly shift to a dependence on aerobic glycolysis, a less efficient process that yields only two ATP units per molecule of glucose (68, 69). Although less efficient, activated T cells can meet their energy demands through uptake of alternative fuels such as glutamine. Furthermore, glycolytic intermediates are needed to provide building blocks for synthesis of nucleotides, proteins and lipids. Activated T cells that fail to switch metabolic processes are rendered anergic or undergo apoptosis (70), likely a consequence of failing to accommodate the specific bioenergetic demands of proliferation and differentiation (64, 71). Thus, it is clear that the metabolic status must match the needs of distinct T cell subsets and developmental stages in order for appropriate immune responses to be generated.

mTOR and T cell metabolism

With regard to the intracellular signaling involved in metabolic regulation, it has long been appreciated that the mechanistic target of rapamycin (mTOR) has a critical role in T cell metabolism (72). mTOR serves to integrate nutrient and immune signals, including the availability of amino acids and oxygen, as well as the presence of extracellular growth factors. mTOR then acts as an effector to modulate downstream cellular metabolic processes needed to meet the demands of the cell upon stimulation (73). Such processes include mRNA translational initiation via phosphorylation of S6K1 and 4E-BPs (74), and lipid biosynthesis through the transcription factor SREBP1 and the nuclear protein receptor PPARγ (31). It is crucial, however, that mTORC1 activation does not become prematurely activated until T

cells are antigenically stimulated, as the quiescent metabolic state of resting T cells appears to be important for their homeostatic proliferation (75). As detailed later, the energy sensing protein kinase AMPK acts as a master regulator of the metabolic status in resting T cells. AMPK, whose activity is determined by the AMP/ATP ratio, influences mTOR activity through the tumor suppressor tuberous sclerosis complex (76). Comprised of TSC1 and TSC2, the tuberous sclerosis complex suppresses mTORC1 activation by Rheb GTPase (77) and is crucial to maintaining homeostatic proliferation of naïve T cells. Supporting this, Yang and colleagues observed that TSC1-deficient naïve T cells possess hyperactive mTOR activity, with a resulting loss in quiescence and a predisposition to undergo apoptosis (78). Though naïve T cells do not initially require mTORC1 activity for TCR induced activation (as measured by activation markers and IL-2 production), genetic deletion of mTOR in mouse CD4 T cells yields a skewed differentiation toward induced T regulatory cells over other effector T cell subsets (79). There is also a profound delay in proliferation. In addition, mTOR has a central role in the regulation of both activated and long-lived memory T cells as its genetic deletion or pharmacologic inactivation leads to diminished $CD4^+$ memory T cell activation and function (31, 80, 81).

Metabolic States in Distinct T Cell Subsets

Naïve T cells utilize efficient oxidative metabolism to maintain their quiescent state. Conversely, once T cells are stimulated by antigen, they must quickly expand their numbers to eliminate an antigenic challenge. This metabolic program is reminiscent of the Warburg hypothesis; in which heightened glycolysis observed in cancer cells is thought to allow for rapid tumor cell proliferation. The usage of aerobic glycolysis is contrary to normal cell types, which utilize glycolysis only when oxygen is in deficit (hypoxia), which severely restricts the amount of ATP generated per molecule of glucose.

For both cancer cells and proliferating lymphocytes, glycolysis is utilized in normoxic conditions for optimal use of glycolytic byproducts that are shunted away for use as building blocks for protein, lipid, and nucleotides. The production of lactate also generates NADPH, a necessary reducing equivalent for the synthesis of long chain fatty acids. Alternatively, it has been recently proposed that the switch to aerobic glycolysis is instead necessary for commitment to effector T cell differentiation. Pearce and colleagues demonstrated that blockade of glycolysis prevented the expression of interferon gamma in activated T cells, but did not impair clonal expansion (82). Moreover, the rapid recall response observed in memory T cells, which must respond quite rapidly to antigenic rechallenge, is supported by enhanced respiratory and glycolytic capacity (83, 84). OXPHOS is required to maintain the long lived memory T cell population, while glycolysis is needed should they reactivate in order to sustain quickly initiated transcriptional programs to produce appropriate effector responses. It remains to be determined if this may reflect differential survival of unique subpopulations during clonal expansion. Regardless, distinct metabolic processes are clearly involved in providing for the energetic demands of unique T cell subpopulations, with fatty acid oxidation (FAO) and aerobic glycolysis playing significant roles.

Fatty acid oxidation

In resting T cells that circulate in the periphery, FAO is the default metabolic state, and the metabolism of these quiescent cells is characterized by a need for basal energy utilization over macromolecular biosynthesis (85). These naïve T cells utilize high energy yielding OXPHOS processes, involving β-oxidation of fatty acids and oxidation of glutamine and glucose (at equal rates) via the TCA cycle (85).

While an increase in glycolysis generally is observed in activated T cells, this is not always the case. iTregs differentiated from peripheral $CD4^+$ T cells, are a unique subset of $CD4^+$ T

cells that suppress effector T cells and are vital to immune peripheral tolerance (86). Following a lag phase heavily dependent on glycolysis and glutaminolysis (80), T cells activated in the context of extracellular signals that favor iTreg differentiation (e.g., IL-2 and TGF-β) acquire a metabolic phenotype similar to naïve T cells (87). Relying on lipid oxidation as their primary source of energy, peripherally differentiated iT_{regs} and their thymically derived nT_{reg} counterparts have intermediate to low mTOR activity (71). The distinct metabolic profile of T_{reg} can be replicated through *in vitro* culture with addition of glycolytic or mTOR inhibitors, such as 2-deoxyglucose or rapamycin, respectively (88). As with naïve T cells, PPARα and PPARγ are important for T_{regs}, serving as fatty acid sensors, and promoting Foxp3 expression in $CD4^+$ T cells activated in the presence of TGF-β (89).

Fatty acid oxidation also plays a vital role in the maintenance of memory T cell pools. Following clearance of an acute viral infection, the antiviral $CD8^+$ effector T cell pool is radically depleted, with a loss of 90–95% of virus specific $CD8^+$ T cells (90). The surviving cells in turn become long-lived memory T cells (91), possessing unique metabolic characteristics when compared with effector cells (63). Memory $CD8^+$ T cells must be able to withstand periods of both antigenic neglect and rapid antigen specific recall. This can be handled through the acquisition of increased spare respiratory capacity (SRC) which is obtained through biogenesis of mitochondria and increased glycolytic flux (84). Thus, in contrast to their effector counterparts, these long-lived $CD8^+$ T cells have significantly enhanced spare respiratory capacity. Memory $CD8^+$ T cells share an analogous metabolic profile with resting T cells and T_{regs}, primarily engaging in FAO to maintain their survival and homeostasis (92). These metabolic processes are maintained by IL-15 signaling, which facilitates the biogenesis of mitochondria and expression of CPT1A, an enzyme responsible for the rate-limiting step of FAO (84).

Glycolysis

As noted above, activated T cells switch their metabolic programing to aerobic glycolysis upon antigenic stimulation (69, 93). This may seem counterproductive, as the effective ATP output per glucose molecule taken into the cell is roughly one fifteenth of the units generated via OXPHOS (88). Instead, it has been proposed that this switch is necessary to facilitate the rapid clonal expansion required to eliminate a microbial infection (63), as well as support the commitment of activated cells to specific Th lineages. Growth factor stimulation results in enhanced uptake of glucose through the upregulation of the glucose transporter Glut1 on the surface of cells, along with increased expression of the glycolytic enzymes hexokinase and phosphofructokinase (68), processes activated in T cells by TCR ligation (94). Costimulation through CD28 leads to increased kinase activity of Akt, thereby enhancing glycolytic activity in T cells by increased Glut1 surface expression (69), and the prevention of growth factor withdrawal induced cell death (95). Supporting a crucial role for Akt in promoting metabolic changes and the survival of activated T cells, ectopic expression of an active form of Akt leads to increased rates of glycolysis and T cell survival, even in the absence of CD28 signaling (96). The converse result is seen when activated T cells are treated with a chemical inhibitor of Akt1 and Akt2, with lower glycolysis rates measured.

The AMP-dependent protein kinase AMPK serves a critical regulator of cellular metabolism, both in naïve and newly activated T cells. In resting cells, a high ratio of AMP to ATP leads to elevated AMPK activity and diminished mTOR function. TCR engagement activates LKB1 and in parallel, increases intracellular Ca^{2+} stores, both leading to an increase in the activation of AMPK (97). LKB1 phosphorylates and activates AMPK (97, 98), the latter of which can phosphorylate TSC2 and activate TSC (99). As TSC1 inhibits mTOR activity in naïve T cells through the tuberous sclerosis complex, AMPK restricts the engagement of metabolic programs associated with clonal expansion. Deletion of

the *Tsc1* gene leads to metabolic alterations in T cells, most notably, increases in glucose uptake and glycolytic flux (98). Thus, while AMPK may be associated with a quiescent T cell state, the TCR induced increase of cytosolic Ca^{2+} enhances AMPK activity. This effectively conserves ATP by inhibiting mTOR associated anabolic processes, and by promoting OXPHOS (97, 100). With time, the resulting increase in the ATP to AMP ratio leads to diminished AMPK activity, and downstream restriction of mTOR is overcome, allowing the T cell to shift its metabolism from FAO to OXPHOS.

Upon successful engagement of the TCR and the costimulation molecule CD28, the T cell begins to reprogram metabolism, utilizing aerobic glycolysis, the phosphate pentose pathway (PPP), and glutaminolysis (71). Glutamine is used as an alterative source of carbon to enter the TCA cycle since glycolytic intermediates are shuttled to biosynthetic pathways. Two transcriptions factors that coordinate metabolic status following T cell activation are Myc and HIF1α (80, 101). Induction of Myc expression in activated T cells is largely responsible for metabolic reprograming at a global gene transcriptome level, as its acute deletion inhibited glycolysis, glutaminolysis, and provoked an overall failure in cell growth and proliferation (81). Myc is also found to intersect with the mTOR pathway, as loss of Myc in activated T cells led to reduced expression and phosphorylation of the downstream mTOR targets S6 and 4E-BP (81). mTOR itself, specifically the mTOR complex 1 (mTORC1), is responsible for activating two key metabolic transcriptional targets, HIF1α and SREBP1/2. Specifically, HIF1α activates transcription of genes involved in glycolysis and glucose uptake, while SREBP1/2 activates genes encoding proteins important for the PPP and lipid biosynthesis (102). However, while SREBP activity is essential for metabolic reprograming in activated T cells (103), HIF1α appears to play a more selective role in T cell subsets such as Th17 cells (101, 104). It is likely that HIF1β serves a compensatory role in HIF1α-

deficient T cells, as loss of HIF1β leads to defective glycolysis, survival, trafficking, and function of $CD8^+$ T cells (105).

One metabolic process that diverges in Th17 cells and Tregs is their utilization of fatty acids. Th17 cells participate in *de novo* fatty acid synthesis. Creation of their own lipids provides substrate for cell membrane synthesis, subset specific signaling, and posttranscriptional modifications. Acetyl-CoA Carboxylase (ACC) is the critical enzyme that catalyzes the rate-limiting step of fatty acid synthesis through the irreversible carboxylation of acetyl-CoA into malonyl-CoA (106, 107). ACC1 performs this step in the cytoplasm, while the other isozyme ACC2 prevents the oxidation of fatty acids by further increasing malonyl-CoA levels to directly inhibit carnitine palmitoyl transferase 1 (CPT1) in the mitochondria. CPT1 shuttles cytoplasmic fatty acid into the mitochondria for ATP generation. Tregs on the other hand do not utilize de novo fatty acid synthesis, rather taking up exogenous FAs for use as a main source of fuel for oxidative phosphorylation and ATP production. This is in part due to an elevated activation of AMPK, which directly inhibits ACC1 (106, 108).

As a primary driver of autoimmune diseases, effector T cell responses offer an attractive therapeutic target whose inhibition may spare the rest of the immune system. The goal of this thesis is to evaluate two novel approaches to selectively suppress Th17 responses, while sparing or promoting suppressive iTreg generation. The feasibility of achieving these two goals is evidenced by previous genetic studies of mTOR function in $CD4^+$ T cells, where inhibition of mTORC1 blocks Th17 responses while sparing iTreg differentiation. The first goal of this thesis extends this approach to a downstream signaling component by inactivating the canonical mTORC1 substrate S6K2 in order to identify unique functions of S6K2 in Th17 differentiation. A second approach is to exploit the divergent metabolic reprograms engaged by Th17 and Treg subsets after activation (Figure 1.3). To this end, the

second goal of this thesis is to target the enzymes ACC1 and ACC2 to inhibit *de novo* fatty acid synthesis while increasing fatty acid oxidation, which accommodates Treg metabolism over Th17.

References:

1. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7: 145–173.

2. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112: 1557–1569.

3. Knip, M., R. Veijola, S. M. Virtanen, H. Hyöty, O. Vaarala, and H. K. Akerblom. 2005. Environmental triggers and determinants of type 1 diabetes. *Diabetes* 54 Suppl 2: S125–S136.

4. D'Elios, M. M., M. P. Bergman, A. Azzurri, A. Amedei, M. Benagiano, J. J. De Pont, F. Cianchi, C. M. Vandenbroucke-Grauls, S. Romagnani, B. J. Appelmelk, and G. Del Prete. 2001. H(+),K(+)-atpase (proton pump) is the target autoantigen of Th1-type cytotoxic T cells in autoimmune gastritis. *Gastroenterology* 120: 377–386.

5. D'Elios, M. M., B. J. Appelmelk, A. Amedei, M. P. Bergman, and G. Del Prete. 2004. Gastric autoimmunity: the role of Helicobacter pylori and molecular mimicry. *Trends Mol Med* 10: 316–323.

6. Renz, H., N. Blümer, S. Virna, S. Sel, and H. Garn. 2006. The immunological basis of the hygiene hypothesis. *Chem Immunol Allergy* 91: 30–48.

7. Deo, S. S., K. J. Mistry, A. M. Kakade, and P. V. Niphadkar. 2010. Role played by Th2 type cytokines in IgE mediated allergy and asthma. *Lung India* 27: 66–71.

8. Waite, J. C., and D. Skokos. 2012. Th17 response and inflammatory autoimmune diseases. *International journal of inflammation* 2012: 819467.

9. Noack, M., and P. Miossec. 2014. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev* 13: 668–677.

10. Bailey, S. R., M. H. Nelson, R. A. Himes, Z. Li, S. Mehrotra, and C. M. Paulos. 2014. Th17 cells in cancer: the ultimate identity crisis. *Front Immunol* 5: 276.

11. Martin-Orozco, N., P. Muranski, Y. Chung, X. O. Yang, T. Yamazaki, S. Lu, P. Hwu, N. P. Restifo, W. W. Overwijk, and C. Dong. 2009. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity* 31: 787–798.

12. Wu, S., K. J. Rhee, E. Albesiano, S. Rabizadeh, X. Wu, H. R. Yen, D. L. Huso, F. L. Brancati, E. Wick, F. McAllister, F. Housseau, D. M. Pardoll, and C. L. Sears. 2009. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med* 15: 1016–1022.

13. Stumhofer, J. S., A. Laurence, E. H. Wilson, E. Huang, C. M. Tato, L. M. Johnson, A. V. Villarino, Q. Huang, A. Yoshimura, D. Sehy, C. J. Saris, J. J. O'Shea, L. Hennighausen, M. Ernst, and C. A. Hunter. 2006. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nat Immunol* 7: 937–945.

14. Liu, H., and C. Rohowsky-Kochan. 2011. Interleukin-27-mediated suppression of human Th17 cells is associated with activation of STAT1 and suppressor of cytokine signaling protein 1. *J Interferon Cytokine Res* 31: 459–469.

15. Visperas, A., J. S. Do, K. Bulek, X. Li, and B. Min. 2013. IL-27, targeting antigen-presenting cells, promotes Th17 differentiation and colitis in mice. *Mucosal Immunol* 7: 625–633.

16. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27: 20–21.

17. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27: 68–73.

18. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22: 531–562.

19. Haribhai, D., J. B. Williams, S. Jia, D. Nickerson, E. G. Schmitt, B. Edwards, J. Ziegelbauer, M. Yassai, S. H. Li, L. M. Relland, P. M. Wise, A. Chen, Y. Q. Zheng, P. M. Simpson, J. Gorski, N. H. Salzman, M. J. Hessner, T. A. Chatila, and C. B. Williams. 2011. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity* 35: 109–122.

20. Gooden, M. J., G. H. de Bock, N. Leffers, T. Daemen, and H. W. Nijman. 2011. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer* 105: 93–103.

21. Oleinika, K., R. J. Nibbs, G. J. Graham, and A. R. Fraser. 2013. Suppression, subversion and escape: the role of regulatory T cells in cancer progression. *Clin Exp Immunol* 171: 36–45.

22. Vézina, C., A. Kudelski, and S. N. Sehgal. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot* 28: 721–726.

23. Houchens, D. P., A. A. Ovejera, S. M. Riblet, and D. E. Slagel. 1983. Human brain tumor xenografts in nude mice as a chemotherapy model. *Eur J Cancer Clin Oncol* 19: 799–805.

24. Martel, R. R., J. Klicius, and S. Galet. 1977. Inhibition of the immune response by rapamycin, a new antifungal antibiotic. *Can J Physiol Pharmacol* 55: 48–51.

25. Oudard, S., J. Medioni, J. Aylllon, E. Barrascourt, R. T. Elaidi, J. Balcaceres, and F. Scotte. 2009. Everolimus (RAD001): an mTOR inhibitor for the treatment of metastatic renal cell carcinoma. *Expert Rev Anticancer Ther* 9: 705–717.

26. Agarwala, S. S., and S. Case. 2010. Everolimus (RAD001) in the treatment of advanced renal cell carcinoma: a review. *Oncologist* 15: 236–245.

27. Li, S., Y. Liang, M. Wu, X. Wang, H. Fu, Y. Chen, and Z. Wang. 2013. The novel mTOR inhibitor CCI-779 (temsirolimus) induces antiproliferative effects through inhibition of mTOR in Bel-7402 liver cancer cells. *Cancer Cell Int* 13: 30.

28. Yamagata, K., L. K. Sanders, W. E. Kaufmann, W. Yee, C. A. Barnes, D. Nathans, and P. F. Worley. 1994. rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. *J Biol Chem* 269: 16333–16339.

29. Zhang, Y., X. Gao, L. J. Saucedo, B. Ru, B. A. Edgar, and D. Pan. 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol* 5: 578–581.

30. Wullschleger, S., R. Loewith, and M. N. Hall. 2006. TOR signaling in growth and metabolism. *Cell* 124: 471–484.

31. Powell, J. D., and G. M. Delgoffe. 2010. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* 33: 301–311.

32. Beugnet, A., A. R. Tee, P. M. Taylor, and C. G. Proud. 2003. Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. *Biochem J* 372: 555–566.

33. Laplante, M., and D. M. Sabatini. 2009. mTOR signaling at a glance. *J Cell Sci* 122: 3589–3594.

34. Pardo, O. E., and M. J. Seckl. 2013. S6K2: The Neglected S6 Kinase Family Member. *Front Oncol* 3: 191.

35. Savinska, L., O. Skorokhod, O. Klipa, I. Gout, and V. Filonenko. 2012. Development of monoclonal antibodies specific to ribosomal protein S6 kinase 2. *Hybridoma (Larchmt)* 31: 289–294.

36. O'Reilly, K. E., F. Rojo, Q. B. She, D. Solit, G. B. Mills, D. Smith, H. Lane, F. Hofmann, D. J. Hicklin, D. L. Ludwig, J. Baselga, and N. Rosen. 2006. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 66: 1500–1508.

37. Sun, S. Y., L. M. Rosenberg, X. Wang, Z. Zhou, P. Yue, H. Fu, and F. R. Khuri. 2005. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res* 65: 7052–7058.

38. Wan, X., B. Harkavy, N. Shen, P. Grohar, and L. J. Helman. 2007. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene* 26: 1932–1940.

39. Wang, X., P. Yue, Y. A. Kim, H. Fu, F. R. Khuri, and S. Y. Sun. 2008. Enhancing mammalian target of rapamycin (mTOR)-targeted cancer therapy by preventing mTOR/raptor inhibition-initiated, mTOR/rictor-independent Akt activation. *Cancer Res* 68: 7409–7418.

40. Zhang, J., Z. Gao, J. Yin, M. J. Quon, and J. Ye. 2008. S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2. *J Biol Chem* 283: 35375–35382.

41. Liu, X., H. Yuan, Y. Niu, W. Niu, and L. Fu. 2012. The role of AMPK/mTOR/S6K1 signaling axis in mediating the physiological process of exercise-induced insulin sensitization in skeletal muscle of C57BL/6 mice. *Biochim Biophys Acta* 1822: 1716–1726.

42. Robitaille, A. M., S. Christen, M. Shimobayashi, M. Cornu, L. L. Fava, S. Moes, C. Prescianotto-Baschong, U. Sauer, P. Jenoe, and M. N. Hall. 2013. Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis. *Science* 339: 1320–1323.

43. Ben-Sahra, I., J. J. Howell, J. M. Asara, and B. D. Manning. 2013. Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. *Science* 339: 1323–1328.

44. Sridharan, S., and A. Basu. 2011. S6 kinase 2 promotes breast cancer cell survival via Akt. *Cancer Res* 71: 2590–2599.

45. Pardo, O. E., C. Wellbrock, U. K. Khanzada, M. Aubert, I. Arozarena, S. Davidson, F. Bowen, P. J. Parker, V. V. Filonenko, I. T. Gout, N. Sebire, R. Marais, J. Downward, and M. J. Seckl. 2006. FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCepsilon, B-Raf and S6K2. *EMBO J* 25: 3078–3088.

46. Lyzogubov, V. V., D. I. Lytvyn, T. M. Dudchenko, N. V. Lubchenko, P. V. Pogrybniy, S. V. Nespryadko, A. B. Vinnitska, V. S. Usenko, I. T. Gout, and V. V. Filonenko. 2004. Immunohistochemical analysis of S6K1 and S6K2 expression in endometrial adenocarcinomas. *Exp Oncol* 26: 287–293.

47. Filonenko, V. V., R. Tytarenko, S. K. Azatjan, L. O. Savinska, Y. A. Gaydar, I. T. Gout, V. S. Usenko, and V. V. Lyzogubov. 2004. Immunohistochemical analysis of S6K1 and S6K2 localization in human breast tumors. *Exp Oncol* 26: 294–299.

48. Nardella, C., A. Lunardi, G. Fedele, J. G. Clohessy, A. Alimonti, S. C. Kozma, G. Thomas, M. Loda, and P. P. Pandolfi. 2011. Differential expression of S6K2 dictates tissue-specific requirement for S6K1 in mediating aberrant mTORC1 signaling and tumorigenesis. *Cancer Res* 71: 3669–3675.

49. Ismail, H. M., O. Myronova, Y. Tsuchiya, A. Niewiarowski, I. Tsaneva, and I. Gout. 2013. Identification of the general transcription factor Yin Yang 1 as a novel and specific binding partner for S6 kinase 2. *Cell Signal* 25: 1054–1063.

50. Caretti, G., M. Di Padova, B. Micales, G. E. Lyons, and V. Sartorelli. 2004. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. *Genes Dev* 18: 2627–2638.

51. Komar, A. A., and M. Hatzoglou. 2011. Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. *Cell Cycle* 10: 229–240.

52. Fitzgerald, K. D., and B. L. Semler. 2009. Bridging IRES elements in mRNAs to the eukaryotic translation apparatus. *Biochim Biophys Acta* 1789: 518–528.

53. Mills, R. E., and J. M. Jameson. 2009. T cell dependence on mTOR signaling. *Cell Cycle* 8: 545–548.

54. Delgoffe, G. M., K. N. Pollizzi, A. T. Waickman, E. Heikamp, D. J. Meyers, M. R. Horton, B. Xiao, P. F. Worley, and J. D. Powell. 2011. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol* 12: 295–303.

55. MacDonald, A. S., and RAPAMUNE Global Study Group. 2001. A worldwide, phase III, randomized, controlled, safety and efficacy study of a sirolimus/cyclosporine regimen for prevention of acute rejection in recipients of primary mismatched renal allografts. *Transplantation* 71: 271–280.

56. Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* 69: 1227–1236.

57. Price, D. J., J. R. Grove, V. Calvo, J. Avruch, and B. E. Bierer. 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science* 257: 973–977.

58. Pende, M., S. H. Um, V. Mieulet, M. Sticker, V. L. Goss, J. Mestan, M. Mueller, S. Fumagalli, S. C. Kozma, and G. Thomas. 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* 24: 3112–3124.

59. Tolcher, A., J. Goldman, A. Patnaik, K. P. Papadopoulos, P. Westwood, C. S. Kelly, W. Bumgardner, L. Sams, S. Geeganage, T. Wang, A. R. Capen, J. Huang, S. Joseph, J. Miller, K. A. Benhadji, L. H. Brail, and L. S. Rosen. 2014. A phase I trial of LY2584702 tosylate, a p70 S6 kinase inhibitor, in patients with advanced solid tumours. *Eur J Cancer* 50: 867–875.

60. Kurebayashi, Y., S. Nagai, A. Ikejiri, M. Ohtani, K. Ichiyama, Y. Baba, T. Yamada, S. Egami, T. Hoshii, A. Hirao, S. Matsuda, and S. Koyasu. 2012. PI3K-Akt-mTORC1-S6K1/2 axis controls Th17 differentiation by regulating Gfi1 expression and nuclear translocation of RORγ. *Cell Rep* 1: 360–373.

61. Sasaki, C. Y., G. Chen, R. Munk, E. Eitan, J. Martindale, D. L. Longo, and P. Ghosh. 2016. p(70S6K1) in the TORC1 pathway is essential for the differentiation of Th17 Cells, but not Th1, Th2, or Treg cells in mice. *Eur J Immunol* 46: 212–222.

62. Park, Y., H. S. Jin, J. Lopez, C. Elly, G. Kim, M. Murai, M. Kronenberg, and Y. C. Liu. 2013. TSC1 regulates the balance between effector and regulatory T cells. *J Clin Invest* 123: 5165–5178.

63. Michalek, R. D., and J. C. Rathmell. 2010. The metabolic life and times of a T-cell. *Immunol Rev* 236: 190–202.

64. Fox, C. J., P. S. Hammerman, and C. B. Thompson. 2005. Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol* 5: 844–852.

65. Jones, R. G., and C. B. Thompson. 2007. Revving the engine: signal transduction fuels T cell activation. *Immunity* 27: 173–178.

66. Jones, R. G., T. Bui, C. White, M. Madesh, C. M. Krawczyk, T. Lindsten, B. J. Hawkins, S. Kubek, K. A. Frauwirth, Y. L. Wang, S. J. Conway, H. L. Roderick, M. D. Bootman, H. Shen, J. K. Foskett, and C. B. Thompson. 2007. The proapoptotic factors Bax and Bak regulate T Cell proliferation through control of endoplasmic reticulum Ca(2+) homeostasis. *Immunity* 27: 268–280.

67. Van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger. 2001. Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol* 2: 423–429.

68. Vander Heiden, M. G., D. R. Plas, J. C. Rathmell, C. J. Fox, M. H. Harris, and C. B. Thompson. 2001. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol* 21: 5899–5912.

69. Frauwirth, K. A., J. L. Riley, M. H. Harris, R. V. Parry, J. C. Rathmell, D. R. Plas, R. L. Elstrom, C. H. June, and C. B. Thompson. 2002. The CD28 signaling pathway regulates glucose metabolism. *Immunity* 16: 769–777.

70. Zheng, Y., G. M. Delgoffe, C. F. Meyer, W. Chan, and J. D. Powell. 2009. Anergic T cells are metabolically anergic. *J Immunol* 183: 6095–6101.

71. Wang, R., and D. R. Green. 2012. Metabolic checkpoints in activated T cells. *Nat Immunol* 13: 907–915.

72. Delgoffe, G. M., and J. D. Powell. 2009. mTOR: taking cues from the immune microenvironment. *Immunology* 127: 459–465.

73. Heikamp, E. B., and J. D. Powell. 2012. Sensing the immune microenvironment to coordinate T cell metabolism, differentiation & function. *Semin Immunol* 24: 414–420.

74. Von Manteuffel, S. R., P. B. Dennis, N. Pullen, A. C. Gingras, N. Sonenberg, and G. Thomas. 1997. The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70s6k. *Mol Cell Biol* 17: 5426–5436.

75. Yang, K., and H. Chi. 2012. mTOR and metabolic pathways in T cell quiescence and functional activation. *Semin Immunol* 24: 421–428.

76. Ojuka, E. O. 2004. Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. *Proc Nutr Soc* 63: 275–278.

77. Fingar, D. C., and J. Blenis. 2004. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* 23: 3151–3171.

78. Yang, K., G. Neale, D. R. Green, W. He, and H. Chi. 2011. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. *Nat Immunol* 12: 888–897.

79. Delgoffe, G. M., T. P. Kole, Y. Zheng, P. E. Zarek, K. L. Matthews, B. Xiao, P. F. Worley, S. C. Kozma, and J. D. Powell. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30: 832–844.

80. Wang, R., C. P. Dillon, L. Z. Shi, S. Milasta, R. Carter, D. Finkelstein, L. L. McCormick, P. Fitzgerald, H. Chi, J. Munger, and D. R. Green. 2011. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* 35: 871–882.

81. Araki, K., A. P. Turner, V. O. Shaffer, S. Gangappa, S. A. Keller, M. F. Bachmann, C. P. Larsen, and R. Ahmed. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature* 460: 108–112.

82. Chang, C. H., J. D. Curtis, L. B. Maggi, B. Faubert, A. V. Villarino, D. O'Sullivan, S. C. Huang, G. J. van der Windt, J. Blagih, J. Qiu, J. D. Weber, E. J. Pearce, R. G. Jones, and E. L. Pearce. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153: 1239–1251.

83. Van der Windt, G. J., D. O'Sullivan, B. Everts, S. C. Huang, M. D. Buck, J. D. Curtis, C. H. Chang, A. M. Smith, T. Ai, B. Faubert, R. G. Jones, E. J. Pearce, and E. L. Pearce. 2013. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc Natl Acad Sci U S A* 110: 14336–14341.

84. Van der Windt, G. J., B. Everts, C. H. Chang, J. D. Curtis, T. C. Freitas, E. Amiel, E. J. Pearce, and E. L. Pearce. 2012. Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity* 36: 68–78.

85. MacIver, N. J., R. D. Michalek, and J. C. Rathmell. 2013. Metabolic regulation of T lymphocytes. *Annu Rev Immunol* 31: 259–283.

86. Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.

87. Michalek, R. D., V. A. Gerriets, S. R. Jacobs, A. N. Macintyre, N. J. MacIver, E. F. Mason, S. A. Sullivan, A. G. Nichols, and J. C. Rathmell. 2011. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol* 186: 3299–3303.

88. Waickman, A. T., and J. D. Powell. 2012. mTOR, metabolism, and the regulation of T-cell differentiation and function. *Immunol Rev* 249: 43–58.

89. Lei, J., H. Hasegawa, T. Matsumoto, and M. Yasukawa. 2010. Peroxisome proliferator-activated receptor α and γ agonists together with TGF-β convert human CD4+CD25- T cells into functional Foxp3+ regulatory T cells. *J Immunol* 185: 7186–7198.

90. Araki, K., B. Youngblood, and R. Ahmed. 2010. The role of mTOR in memory CD8+ T-cell differentiation, The role of mTOR in memory CD8+ T-cell differentiation. *Immunological Reviews, Immunological Reviews* .

91. Kaech, S. M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nat Immunol* 2: 415–422.

92. Pearce, E. L., M. C. Walsh, P. J. Cejas, G. M. Harms, H. Shen, L. S. Wang, R. G. Jones, and Y. Choi. 2009. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460: 103–107.

93. Brand, K., R. Netzker, U. Aulwurm, U. Hermfisse, D. Fabian, C. Weigert, D. Schaefer, and B. Hamm-Kuenzelmann. 2000. Control of thymocyte proliferation via redox-regulated expression of glycolytic genes. *Redox Rep* 5: 52–54.

94. Jacobs, S. R., and J. C. Rathmell. 2006. Lymphocyte selection by starvation: glucose metabolism and cell death. *Trends Immunol* 27: 4–7.

95. Rathmell, J. C., C. J. Fox, D. R. Plas, P. S. Hammerman, R. M. Cinalli, and C. B. Thompson. 2003. Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. *Mol Cell Biol* 23: 7315–7328.

96. Rathmell, J. C., R. L. Elstrom, R. M. Cinalli, and C. B. Thompson. 2003. Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma. *Eur J Immunol* 33: 2223–2232.

97. Tamás, P., S. A. Hawley, R. G. Clarke, K. J. Mustard, K. Green, D. G. Hardie, and D. A. Cantrell. 2006. Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca2+ in T lymphocytes. *J Exp Med* 203: 1665–1670.

98. MacIver, N. J., J. Blagih, D. C. Saucillo, L. Tonelli, T. Griss, J. C. Rathmell, and R. G. Jones. 2011. The liver kinase B1 is a central regulator of T cell development, activation, and metabolism. *J Immunol* 187: 4187–4198.

99. Shaw, R. J., M. Kosmatka, N. Bardeesy, R. L. Hurley, L. A. Witters, R. A. DePinho, and L. C. Cantley. 2004. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci U S A* 101: 3329–3335.

100. Blagih, J., C. M. Krawczyk, and R. G. Jones. 2012. LKB1 and AMPK: central regulators of lymphocyte metabolism and function. *Immunol Rev* 249: 59–71.

101. Shi, L. Z., R. Wang, G. Huang, P. Vogel, G. Neale, D. R. Green, and H. Chi. 2011. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med* 208: 1367–1376.

102. Düvel, K., J. L. Yecies, S. Menon, P. Raman, A. I. Lipovsky, A. L. Souza, E. Triantafellow, Q. Ma, R. Gorski, S. Cleaver, M. G. Vander Heiden, J. P. MacKeigan, P. M. Finan, C. B. Clish, L. O. Murphy, and B. D. Manning. 2010. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* 39: 171–183.

103. Kidani, Y., H. Elsaesser, M. B. Hock, L. Vergnes, K. J. Williams, J. P. Argus, B. N. Marbois, E. Komisopoulou, E. B. Wilson, T. F. Osborne, T. G. Graeber, K. Reue, D. G. Brooks, and S. J. Bensinger. 2013. Sterol regulatory element-binding proteins are essential for the metabolic programming of effector T cells and adaptive immunity. *Nat Immunol* 14: 489–499.

104. Dang, E. V., J. Barbi, H. Y. Yang, D. Jinasena, H. Yu, Y. Zheng, Z. Bordman, J. Fu, Y. Kim, H. R. Yen, W. Luo, K. Zeller, L. Shimoda, S. L. Topalian, G. L. Semenza, C. V. Dang, D. M. Pardoll, and F. Pan. 2011. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell* 146: 772–784.

105. Finlay, D. K., E. Rosenzweig, L. V. Sinclair, C. Feijoo-Carnero, J. L. Hukelmann, J. Rolf, A. A. Panteleyev, K. Okkenhaug, and D. A. Cantrell. 2012. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. *J Exp Med* 209: 2441–2453.

106. Wakil, S. J., and L. A. Abu-Elheiga. 2009. Fatty acid metabolism: target for metabolic syndrome. *J Lipid Res* 50 Suppl: S138–S143.

107. Wang, C., S. Rajput, K. Watabe, D. F. Liao, and D. Cao. 2010. Acetyl-CoA carboxylase-a as a novel target for cancer therapy. *Front Biosci (Schol Ed)* 2: 515–526.

108. Lochner, M., L. Berod, and T. Sparwasser. 2015. Fatty acid metabolism in the regulation of T cell function. *Trends Immunol* 36: 81–91.

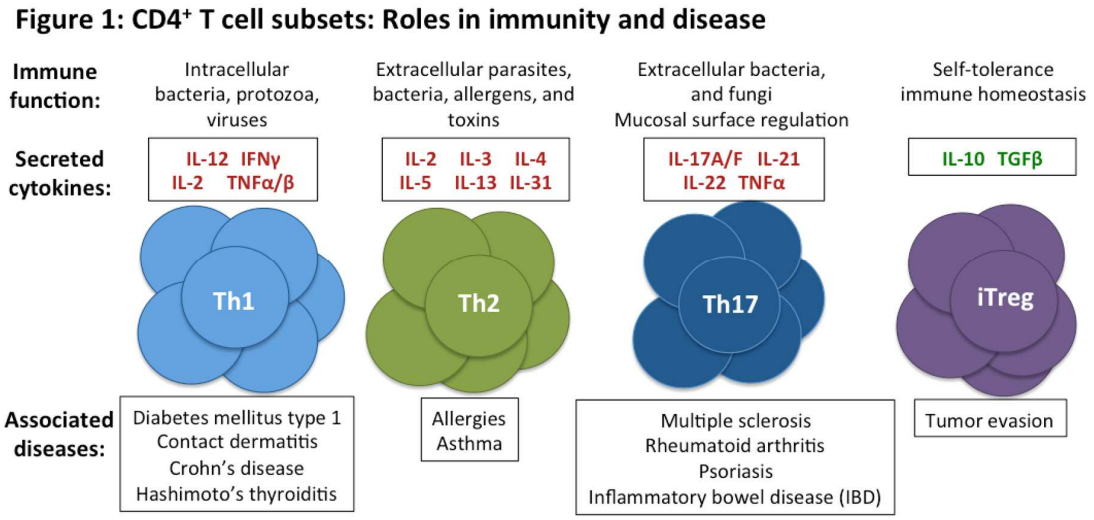


Figure 1.1: CD4$^+$ T cell effector subsets have critical roles in host immune defenses that range from intracellular viruses and bacteria to mucosal surface protection and immune homeostasis maintenance. The effector functions are carried out by cytokines secreted by each subset. The same cytokines, however, can drive autoimmune diseases through dysregulated responses. iTregs are a unique CD4$^+$ subset that produce suppressive cytokines to balance proinflammatory responses, though a drawback to these types of regulatory properties can result in tumors evading host immune responses.

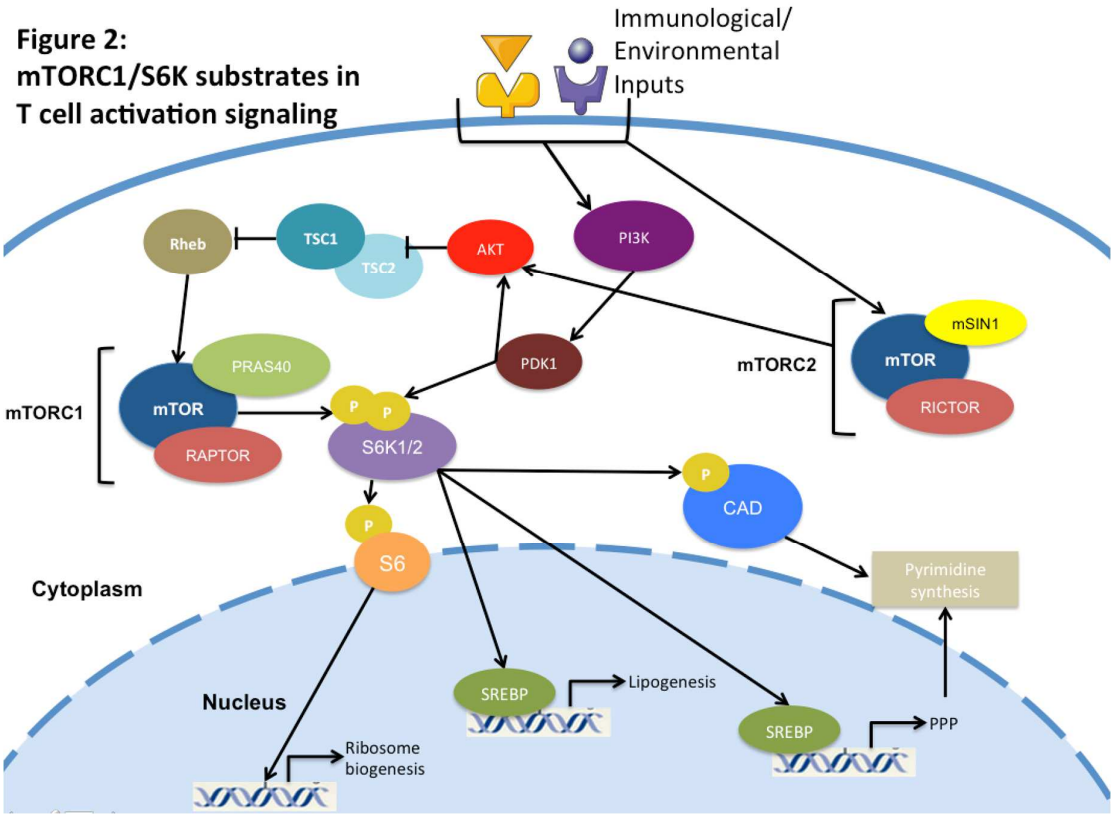


Figure 1.2: T cell activation by immunological and environment inputs result in the activation of the PI3K/Akt/mTOR intracellular signaling pathway. S6Ks are activated by PDK1 and mTORC1, resulting in phosphorylation of rpS6 and CAD. S6Ks promote SREBP activation through an indirect mechanism. The role of each S6K in activation each of these target substrates are unknown, but is required to support biosynthesis through ribosome biogenesis, lipogenesis, and pyrimidine synthesis.

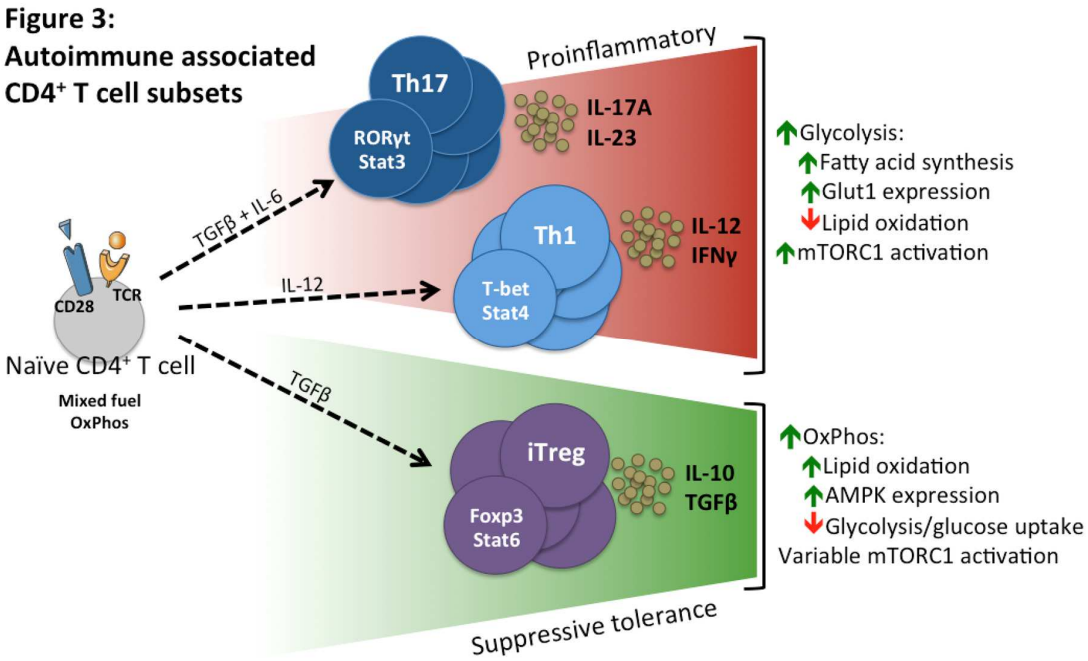


Figure 1.3: Proinflammatory and suppressive T cell subsets utilize differential metabolic programs to execute effector processes. $CD4^+$ T cell subsets Th1 and Th17 engage in glycolysis, which upon activation involves increased fatty acid synthesis and decreased lipid oxidation. mTORC1 activation also results in increased Glut 1 expression, which helps fuel glycolysis. Treg utilize OxPhos instead, increasing lipid oxidation and utilization of exogenous fatty acids. Stable AMPK expression due to mostly lower levels of mTORC1 activity suppresses glycolysis and glucose uptake.

Chapter 2:

Context-specific function of S6K2 in helper T cell differentiation

Abstract

The mammalian target of rapamycin (mTOR) is essential for helper T cell proliferation and effector differentiation, making the mTOR signaling network an attractive immunomodulatory target for autoimmune related diseases. However, direct targeting of mTOR-complex-1 (mTORC1) through the immunosuppressive drug rapamycin is poorly tolerated for long-term treatment due to systemic toxicities. Here we evaluated p70 ribosomal protein S6 Kinase 2 (S6K2), a downstream effector of mTORC1, for its role in T cell function and autoimmunity. S6K2 is a direct substrate of mTORC1, with a potential role in Th17 differentiation suggested by biochemical studies. Using a genetic approach with S6K2 knockout mice, we found that S6K2 loss reduces Th17 skewing and increases Treg differentiation *in vitro* when cultured in RPMI media. However, S6K2 was dispensable for Th17 differentiation in IMDM media. In an *in vivo* EAE model, S6K2 knockout mice did not exhibit differences in clinical score or Th17 differentiation. These results suggest that S6K2 is dispensable for Th17-driven autoimmunity and highlight how distinct experimental conditions can produce significantly different results in T cell differentiation.

Introduction

The mTOR protein is a serine-threonine kinase that forms two separate complexes, mTORC1 and mTORC2. Each complex has distinct regulation and downstream target substrates (1, 2). mTOR signaling in both naïve $CD4^+$ and $CD8^+$ T cells can be activated by engagement of a recognized antigen through the PI3K-Akt-mTOR pathway (3, 4). The complex mTORC1, defined by the regulatory-associated protein of mTOR (Raptor) subunit, regulates both bioenergetic and biosynthetic processes that are crucial for T cell growth and division (5, 6). These include protein translation, lipid biogenesis, and suppression of autophagy. Downstream of mTORC1, two directly phosphorylated substrates are ribosomal S6 Kinases (S6Ks) and eukaryotic initiation factor 4E (eIF4E) binding proteins (4E-BPs), both of which have evolutionarily conserved roles in cell growth and metabolism (1, 7, 8).

Newly activated T-lymphocytes utilize mTOR signaling to initiate transcription of metabolic regulatory genes (9), a process crucial for driving proliferation and differentiation. The ability to produce a sustainable effector program selectively requires mTORC1 activity to integrate both immune and nutrient cues. In the context of an autoimmune response, proinflammatory Th1 and Th17 subsets are highly dependent upon glucose consumption, whereas suppressive Tregs favor increased mitochondrial respiration (10, 11). Interestingly, while mTORC1 activity is required for the differentiation of both Th1 and Th17 subsets (12, 13), functionally induced Tregs do not (12, 14).

Rapamycin, a potent inhibitor of mTORC1 activity, suppresses pathology in several animal models of T cell-driven autoimmunity (15–19). Yet trials of rapamycin in human autoimmune diseases have not led to regulatory approval, perhaps due to side effects of sustained rapamycin treatment (20, 21). Targeting mTORC1 substrate pathways is a reasonable alternative that might provide a better therapeutic window.

There are two S6K isoforms, S6K1 and S6K2, both of which are expressed in T cells (22, 23). Activated mTORC1 phosphorylates S6Ks, which in turn phosphorylate the ribosomal protein S6 and other substrates involved in protein and lipid biosynthesis (7). When mTORC1 phosphorylates 4E-BPs, these proteins are released from eIF4E to promote cap-dependent translation (24). Thus, S6K and 4E-BP signaling pathways converge to regulate mRNA translation. The inhibition of mTORC1 by rapamycin results in profound suppression of S6K phosphorylation and activity (25, 26), but only partial inhibition of 4E-BP1 phosphorylation (27). However, it is unclear which mTOR functions are mediated by S6Ks during T helper (Th) cell differentiation.

Previous studies have provided limited and sometimes conflicting information about the functions of S6Ks in T cell growth, proliferation and differentiation. *S6K1*$^{-/-}$ mice have a small size phenotype but apparently normal lymphocyte function, associated with massive compensatory upregulation of S6K2 in T cells (23). One study reported that the S6K1 inhibitor PF-4708671 suppresses CD4 T cell growth and proliferation (28). However, we found that PF-4708671 has non-specific effects at the 10μM concentration used (29). Mice with a germline deletion of both *S6K1* and *S6K2* exhibit perinatal lethality, making it challenging to analyze mature immune cells (30). In the few adult double knockout mice that survive, T cell growth and proliferation is surprisingly unimpaired (29). Koyasu and colleagues used gain-of-function approaches to suggest unique roles for S6K1 and S6K2 in Th17 differentiation (13). A later study by Sasaki *et al.* (31) utilized *S6K1*$^{-/-}$ mice to show a role for S6K1 in differentiation of Th17 cells, but not other CD4^{+} T cell subsets. S6K1 was found to promote expression of Th17 associated genes (e.g. *IL17a*, *IL17f*, and *IL-23R*); however, it did not affect expression of the Th17 master transcription factor retinoic acid receptor-related orphan receptor gamma (RORγ). In addition, mice lacking S6K1 did not

show improvement of clinical disease scores in experimental autoimmune encephalomyelitis (EAE), a mouse autoimmunity model.

In this study, we investigated the specific role(s) of S6K2 in mouse T cell activation, development, and proliferation. S6K2 germline knockout mice are viable, fertile and of normal size; moreover, S6K1 expression is unchanged in S6K2-deficient T cells. We found that S6K2 knockout (*S6K2*$^{-/-}$) mice had no defects in T cell development and early CD4 T cell activation, despite a measurable decrease in overall S6K activity as determined by S6 protein phosphorylation. Interestingly, *S6K2*$^{-/-}$ cells exhibited a context-specific Th differentiation phenotype. *In vitro* experiments using RPMI culture media revealed decreased Th17 and increased Treg differentiation in *S6K2*$^{-/-}$ cells. However, in an *in vivo* EAE model of autoimmunity, *S6K2*$^{-/-}$ mice showed no difference in clinical outcome or Th17 differentiation. Re-evaluation of the *in vitro* differentiation assay conditions surprisingly showed that the Th17 differentiation defect depends on the culture medium used. IMDM medium, known to induce more robust Th17 differentiation (32), led to Th17 skewing and robust proliferation that was indistinguishable between *S6K2*$^{-/-}$ and wildtype T cells. This unexpected result indicates that S6K2 is dispensable for Th17 differentiation under optimal activation conditions. In addition, our findings suggest that the use of IMDM media for *in vitro* Th17 studies better predicts the *in vivo* response in EAE models.

Materials and Methods

Animals

All mice were kept in specific pathogen–free animal facilities and in accordance with guidelines of the University of California Institutional Animal Care and Use Committee. C57BL/6 mice were obtained from Jackson Laboratories. *S6K2*$^{-/-}$ mice were a gift from Sara Kozma.

Media and cell culture

Primary cell culture was performed in complete media consisting of RPMI or IMDM media (Gibco) supplemented with 10% FCS (Gibco), L-glutamine (Corning), ßME and antibiotics (Gibco).

Antibodies and reagents

Stimulatory anti-CD3 (2C11) and anti-CD28 (37.51), as well as neutralizing anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-1A12), anti-IL-12p40 (C17.8), and anti-IL-4 (11B11) were from eBioscience. For differentiation, recombinant cytokines were used: TGF-beta (R&D systems), mouse IL-6 and IL-12 (eBioscience). For flow cytometry analysis, surface and intracellular staining antibodies were purchased from eBioscience and Cell Signaling Technologies. For immunoblotting, rabbit antibodies specific for total and phosphorylated forms were obtained from Cell Signaling Technologies: AKT (#2965, #9267, #4058, #4685), rS6 (#4838, #2215, #2211), S6K (#9205, #2708), pCAD (#12662). For imaging, anti-Mouse RORγt (B2D) from eBioscience was used.

Cell purification

Splenic $CD4^+$ T cells depleted of natural Tregs were purified by negative selection using the EasySep Mouse $CD4^+$ T Cell Isolation Kit from Stemcell Technologies with biotinylated anti-CD25 (0.5 μg/ml) added.

Cell cycle analysis

T cells were stimulated in T-conventional (Tconv), Th17, or Treg skewing conditions for 40h and fixed with 100% ethanol. Cells were incubated with RNAse A and propidium iodine (PI) diluted in cell cycle buffer (5mM EDTA in PBS) before cell cycle analysis was performed using FACS.

Cell stimulation and differentiation

Unless otherwise stated, T cells were stimulated as Tconv, with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) from eBioscience. 10 nM of Rapamycin (LC Laboratories) and 500 nM of LY2584702 (S6K1i) (Eli Lilly & Company) were also used when indicated. When proliferation is measured, isolated T cells were prelabeled with CFSE (eBioscience). Cell stimulation cocktail (500x) from eBioscience containing PMA (phorbol 12-myristate 13-acetate), ionomycin, Brefeldin A, and monensin were used for restimulation (detection of intracellular cytokines). For Th polarization assays, isolated naïve T cells were skewed with anti-CD3 and anti-CD28 for 4 days (unless otherwise noted), restimulated for 4 hours, and harvested for flow cytometry analysis. Skewing conditions were as follows: T_H1-IL-12 (20 ng/ml) and anti-IL-4 (10 μg/ml); T_H17, TGF-β (5 ng/ml), IL-6 (20 ng/ml), anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml) and IL-2 (1 ng/ml); T_{reg}, TGF-β (5 ng/ml).

Intracellular staining for flow cytometry analysis

After restimulation with cell stimulation cocktail, cells were fixed and made permeable with BD Cytofix/Cytoperm and then stained for cell surface markers and cytokines. Intracellular staining of transcription factors (i.e. FoxP3) was done without restimulation. Gates were set appropriately with unstimulated controls and voltages were set on the basis of isotype-matched control antibodies. Data were acquired using a BD FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay

Cells were harvested after indicated stimulation periods and supernatants were collected to measure cytokine production according to manufacturer's instructions. IL-2, and IL-10 ELISA kits were obtained from eBioscience and IL-17 ELISA kit was obtained from R&D systems.

Experimental Autoimmune Encephalomyelitis

11+ week-old female wildtype and S6K2$^{-/-}$ mice were immunized with 100µg of MOG_{35-55} antigen in 200µg of CFA per mouse. Pertussis was administered i.v. on day 0 and 1 at 400ng per injection. Mice were scored on a scale of 0–5: 0-no clinical symptoms; 1-limp tail; 2-limp tail and partial hind limb paralysis; 3- limp tail and complete hind limb paralysis; 3.5-partial front or one-sided paralysis; 4- moribund; 5- death. Mice scoring 3.5 or more for two consecutive days were euthanized. Spinal cords were harvested and centrifuged through a Percoll gradient. Isolated infiltrating lymphocytes were restimulated for 4h before stained intracellularly for IFNγ and IL-17A. To analyze separately for MOG-specific $CD4^+$ T cells during disease induction, wildtype and S6K2$^{-/-}$ mice were immunized with MOG peptide without pertussis and sacrificed after 10 days. Lymph nodes were harvested for analysis of the percentage of $pS6^+$ among $CD4^+$ cells. Splenocytes were restimulated with MOG (10 and

50μg/mL), IL-23 (20ng/mL), and anti-IFNγ antibody (10μg/mL) for 3 days and then harvested for cytokine measurement in the supernatant, as well as stained intracellularly for IL-17A and FoxP3 expressing $CD4^+$ T cells.

Immunofluorescence Microscopy

$CD4^+$ T cells were harvested after 40 hours of stimulation, washed, and centrifuged. The pellets were fixed with 4% formaldehyde for 10 min at 37°C, permeabilized with 0.1% Saponin (Acros Organics) in PBS for 10 min at room temperature, blocked with 5% anti-rat serum in 1X PBS / 0.1% Saponin and stained with anti-mouse RORγT (eBioscience) for 2 hr at 37°C in PBS containing 1% BSA / 0.1% Saponin. F(ab')2 Anti-Rat IgG PE (eBioscience) were used as a secondary antibody. Specimens were further stained with DAPI (Thermo Fisher Scientific) and Rhodamine-phalloidin (Thermo Fisher Scientific). Single cell suspensions are spun onto a microscope slide by use of a cytocentrifuge Images were obtained using a Leica TCS SP8 confocal scanning microscope and LAS X core microscopy software. For statistical analysis, the Manders' Overlap Coefficient was used to measure RORγ localization in the nucleus (as determined by RORγ and DAPI staining overlap) in at least three representative fields of view for each condition.

Metabolic studies

Metabolic flux analysis was performed in complete Seahorse media (Seahorse Bioscience, 102365) supplemented with 25mM D-Glucose, 1mM Na-Pyruvate and 2mM L-glutamine.

Treg suppression assays

nTregs were sorted by $CD4^+CD25^+$ expression from combined spleen and lymph nodes of aged matched mice. nTregs were then seeded with CFSE labeled naïve $CD4^+$ T cells at 1:2

and 1:8 ratios. The wells were pre-coated with 2µg/mL of CD3 and 1µg/mL of soluble CD28 was added. After 3d of stimulation, the cells were washed and analyzed by FACS.

Statistical Analysis

Mean and SEM values were calculated from at least three independent experiments. *p* values were calculated using a two-tailed unpaired Student's t-test when determining differences between groups and the Wilcoxon signed-rank test was used to determine pairwise differences (specifically indicated in figure legends).

Results

S6K2$^{-/-}$ mice have normal T cell development and proliferation

S6K2$^{-/-}$ mice are viable, fertile, and exhibit normal development. They exhibit no evidence of autoimmunity or inflammatory conditions. Using these mice, we sought to determine the role of S6K2 in T cell development and activation. Analysis of peripheral lymphoid organs in *S6K2*$^{-/-}$ mice revealed normal percentages and ratios of $CD4^+$ and $CD8^+$ populations (Fig. 1A and 1B), and developing T cell subpopulations in the thymus were indistinguishable (Fig. 1A and 1B). Natural Treg (nTreg) populations ($CD4^+CD25^+$) were also comparable in number and percentage in the peripheral lymphoid organs (Fig. 1C). The suppressive ability of nTregs was comparable when comparing CD4+CD25+ cells sorted from WT versus S6K2$^{-/-}$ mice (Fig. S1A).

We then isolated naïve conventional T cells ($CD4^+CD25^-$) and stimulated them with anti-CD3 and anti-CD28 for 24h to assess the role of S6K2 in early T cell activation. Expression of the activation markers CD25 and CD69, and the induction of blasting as measured by forward scatter, were similar between wildtype (WT) and *S6K2*$^{-/-}$ T cells (Fig. 1D). Production of IL-2 was also unimpaired (see below).

Upon TCR engagement, the enzymes PI3K and Akt are essential for the induction of numerous signaling cascades (4). mTORC1 is a main effector downstream of PI3K and Akt, yet little is known about the roles of S6Ks in these processes. First we assessed the contribution of S6K2 to total S6K activity. Naïve T cells were stimulated for different times and analyzed by flow cytometry to measure phosphorylation of the S6 ribosomal protein (pS6). Phosphorylation of S6 at the Ser 240 and 244 residues (pS6) is S6K-dependent and a robust readout of mTORC1 and S6K activity (30, 33). This analysis revealed that *S6K2*$^{-/-}$ T cells have a reduction in the fraction of cells with high pS6 at early time points, and a delayed increase in pS6 positive cells when compared to WT T cells (Fig. 2A). Focusing on the 24hr

time point, we observed a significant reduction in both the mean fluorescence intensity (MFI) and fraction of the population positive for pS6 in *S6K2*$^{-/-}$ cells (Fig. 2B). Rapamycin was used as a positive control to show that the pS6 measurements were completely mTORC1-dependent. We also assessed the contribution of S6K1 to this response using the selective inhibitor LY2584702 (here termed S6K1i) (34). We used S6K1i instead of *S6K1*$^{-/-}$ mice because of the reported compensatory upregulation of S6K2 in such mice (23). The results showed that S6K1 inhibition strongly suppressed the activation-induced increase in pS6 in both WT and *S6K2*$^{-/-}$ T cells.

We recently demonstrated that S6Ks are surprisingly dispensable for both growth and division in activated T cells (29). Confirming these findings, *S6K2*$^{-/-}$ T cells showed comparable proliferation to WT T cells at 72hr (Fig. 2C and D). At an earlier time point of 40hr, there was no difference in cell cycle distribution between WT and *S6K2*$^{-/-}$ T cells (Fig. S1B). Addition of S6K1i to both *S6K2*$^{-/-}$ and WT T cells also did not impair their proliferation despite fully suppressing pS6 induction (Fig. 2C). Similarly, there was no difference in growth (cell size measured by forward scatter (FSC)) in WT and *S6K2*$^{-/-}$ T cells treated with or without the S6K1i inhibitor (Fig. 2E and 2F). We also tested allogeneic stimulation by mixed lymphocyte reaction and also observed no difference in proliferation or cell death (data not shown). These results show that in the absence of S6K activity, T cells are still able to grow and proliferate under neutral stimulation conditions.

Distinct signaling roles for S6K1 and S6K2

The first study of *S6K1*$^{-/-}$ mice reported that S6K1-deficient lymphocytes have a compensatory upregulation of S6K2, even under resting conditions (23). To determine whether *S6K2*$^{-/-}$ T cells have altered expression of S6K1, we used Western blots to measure total S6K1 in resting and 24h stimulated T cells. S6K1 expression in the *S6K2*$^{-/-}$ was similar

to WT T cells under basal and activated conditions (Fig. 3A and 3B). This confirms that S6K1 is not upregulated to compensate for loss of S6K2 in the *S6K2*$^{-/-}$ T cells.

In many cells, S6K1 mediates strong negative feedback on growth factor receptor signaling and the activation of PI3K and AKT (35). Consequently, inhibition of mTORC1 or S6K1 often leads to increased phosphorylation of Akt (pAkt). In accord, WT naïve T cells treated with S6K1i had a statistically significant increase in pAkt following TCR stimulation (Fig. 3C). This increase was not observed in *S6K2*$^{-/-}$ T cells, which instead showed a variable reduction in pAkt. These results are in accord with a study of breast cancer cells, which reported reduced pAkt when S6K2 was suppressed (36). We also measured phosphorylated CAD, reported to be a selective substrate of S6K1 (37, 38). CAD is essential for the *de novo* synthesis of pyrimidine nucleotides and is directly phosphorylated by S6K1 at Ser1859. Though S6K1i significantly reduced pCAD in activated WT T cells, loss of S6K2 had no apparent effect (Fig. 3A and 3B). These signaling studies support the conclusion that S6K1 and S6K2 have distinct substrates in activated T cells and contribute differently to feedback pathways.

S6K2$^{-/-}$ mice generate more Tregs and fewer Th17s *in vitro*

Although *S6K2*$^{-/-}$ T cells proliferated normally under neutral stimulation conditions, we considered the possibility that S6K2 deficiency impairs proliferation and differentiation under T helper skewing conditions. For these *in vitro* differentiation assays, highly purified naïve $CD4^+CD25^-$ T cells were labeled with CFSE and activated with under conditions that polarize cells towards Th1, Th17, or Treg differentiation. On day 4, the cells were harvested and stained for intracellular cytokines or transcription factors indicative of each subset. Though S6K2 loss did not affect skewing to the Th1 subset, the percentage of IL-17-producing cells was reduced under Th17 culture conditions when compared to WT T cells

(Fig. 4A and 4B). Conversely, *S6K2*$^{-/-}$ T cells showed an increased percentage of FoxP3+ cells when stimulated under Treg skewing conditions (Fig. 4 and 4B). Importantly, *S6K2*$^{-/-}$ T cells showed comparable proliferation to WT T cells for all three of these helper T cell subsets (Fig. 4C), indicating that altered differentiation was not linked to changes in cell division.

To assess cytokine production of both early activated and differentiated T cells, we first measured IL-2 production by ELISA using supernatants of 40h stimulated Tconv, Th17, and Treg cultures. Wildtype and *S6K2*$^{-/-}$ T cells produced similar amounts of IL-2, with minimal production for both under Th17 conditions (blocking anti-mouse IL-2 is added to Th17 skewing cultures) (Fig. S1C). Next we measured IL-10 production under Treg skewing conditions and IL-17A production under Th17 conditions, 4 days after stimulation. WT and *S6K2*$^{-/-}$ T cells produced comparable amounts of each cytokine, though there was a trend towards increased IL-10 production by *S6K2*$^{-/-}$ T cells (Fig. 4D).

S6K2 deficiency does not ameliorate EAE clinical symptoms

Drawing upon our findings of reduced Th17 and increased Treg generation *in vitro*, we tested whether *S6K2*$^{-/-}$ mice were protected from autoimmune disease in a mouse model. We chose MOG_{35-55} induced experimental autoimmune encephalomyelitis (EAE), a classical mouse model of multiple sclerosis (MS) in which Th17 cells are required and mTORC1 activity plays a prominent role (12, 13, 39, 40). The pathogenesis of EAE is strongly reinforced by a Th17/Treg imbalance (41). Surprisingly, disease onset and severity was comparable in *S6K2*$^{-/-}$ and WT mice (Fig. 5A). We also quantitated cytokine production of infiltrating lymphocytes in the spinal cord and found similar percentages of IFNγ and IL-17A producing $CD4^+$ T cells (Fig. S1D). To confirm the role of mTORC1 in our hands, we conducted a second EAE experiment comparing WT mice treated with rapamycin or vehicle.

As reported (12, 16), rapamycin treatment significantly lowered EAE scores (Fig. 5B) and reduced the percentage of CD4 T cells producing IFNγ or IL-17A in the spinal cord (Fig. 5C). Concurrently, we analyzed cohorts of WT and *S6K2*$^{-/-}$ mice that were not injected with rapamycin or vehicle. Again the absence of S6K2 did not affect EAE onset or severity (Fig. 5B), and did not affect inflammatory cytokine production in CD4^{+} T cells isolated from spinal cord infiltrates (Fig. 5C). The inability of S6K2 deficiency to reduce EAE clinical symptoms led us to reconsider our initial *in vitro* findings concerning T helper differentiation.

Because we did not detect any differences in proinflammatory responses between WT and *S6K2*$^{-/-}$ mice at the peak of disease, the EAE model was also used to characterize T effector and Treg responses *in vivo* during immune response development. Cohorts of WT and *S6K2*$^{-/-}$ mice were immunized with MOG_{35-55} without pertussis to characterize MOG specific T cells. This model provides another different type of immune response to our *in vitro* differentiation assays, since activated T cells are antigen-specific instead undergoing polyclonal T cell activation. After 10 days, the mice were sacrificed and lymph nodes were harvested to measure pS6 amounts in CD4^{+}CD44^{hi} T cells, a population enriched in antigen-activated cells. WT and *S6K2*$^{-/-}$ cells had similar pS6 as measured by both MFI and % positive (Fig. 5D). Splenocytes were restimulated with MOG peptide in cultures that included IL-23 and anti-IFNγ antibody for 3 days, to specifically measure frequencies of MOG-specific Th17 and Treg cells. Supernatants of restimulation cultures were also harvested to measure IL-17 cytokine production, which showed no difference between WT and *S6K2*$^{-/-}$ cells (Fig. S1E). Though the percentage and MFI of CD4^{+}IL17A^{+} T cells were no different, CD4^{+}FoxP3^{+} populations was significantly increased in cultures from *S6K2*$^{-/-}$ mice (Fig. 5D). In summary, our data suggest that S6K2 is not required for *in vivo* Th17 differentiation, but does seems to suppress Treg generation in this model.

The role of S6K2 in Th17 differentiation is context-specific

To understand why significant *in vitro* differences between *S6K2*$^{-/-}$ and WT T cells did not translate into altered Th17 differentiation *in vivo*, we reassessed the cell culture media used for T cell differentiation. We initially used RPMI media, which is commonly used for primary lymphocyte culture but supports very low levels of Th17 differentiation (32). Thus, we compared RPMI to IMDM, the latter a medium rich in aromatic amino acids that was shown to support robust Th17 differentiation (32). Not only did IMDM media enhance Th17 skewing in WT T cells, it also restored Th17 differentiation of *S6K2*$^{-/-}$ cells to levels equivalent to WT (Fig. 6A and Fig. S2C). In contrast to the rescue of Th17 differentiation, *S6K2*$^{-/-}$ T cells in IMDM still generated a higher percentage of FoxP3+ cells under Treg skewing conditions compared to WT.

The tryptophan metabolite 6-formylindolo[3,2-b] carbazole (FICZ) found in IMDM can enhance differentiation of Th17 cells in RPMI media (32). Therefore, we supplemented RPMI with 300 nm FICZ to see if this could account for restored Th17 differentiation in *S6K2*$^{-/-}$ T cells. Indeed, the proportion of IL-17^{+} cells was comparable between WT and *S6K2*$^{-/-}$ T cells when cultured in RPMI plus FICZ (Fig. 6C and Fig. S2A). Proliferation, however, was not restored to the robust response obtained using IMDM (Fig. S2B). To further explore these media-based phenotypic differences, we measured mTORC1 signaling output as assessed by pS6 intracellular staining. 24hr after T cell stimulation under neutral conditions, cells cultured in IMDM exhibited significantly higher mTORC1 activity compared to cells cultured in RPMI, as assessed by the MFI of pS6 as well as the proportion of pS6-high cells (Fig. 6B). This difference was observed in both *S6K2*$^{-/-}$ and WT populations.

A previous study by Kurebayashi et al. reported that S6K2 functions downstream of mTORC1 to shuttle the Th17 transcription factor RORγ to the nucleus (13). This study did not assess the role of RORγ localization in Th17 differentiation in *S6K2*$^{-/-}$ T cells. Therefore,

we measured nuclear localization of RORγ in WT and *S6K2*$^{-/-}$ T cells after 40hr of stimulation in Th17 conditions, comparing both RPMI and IMDM media. In RPMI, *S6K2*$^{-/-}$ T cells indeed showed significantly reduced RORγ nuclear localization when compared to WT T cells (Fig. S3A). Culturing cells in IMDM media resulted in significantly increased nuclear localization for *S6K2*$^{-/-}$ T cells, but did not rescue this to WT levels (Fig. S3B). WT cells cultured in IMDM did not exhibit an increase in RORγ nuclear localization compared to RPMI conditions.

Because mTORC1 activity is strongly tied to cell metabolism, we used a Seahorse metabolic flux analyzer to analyze metabolism in both WT and S6K2$^{-/-}$ cells activated in Th17, Treg, and neutral (Tneu) differentiation conditions. In these experiments, initial stimulation was done in IMDM media for Th17 conditions and RPMI for Tneu and Treg conditions. Interestingly, basal glycolysis (extracellular acidification rate (ECAR)) and oxidative phosphorylation (oxygen consumption rate (OCR)) of all three subsets were not significantly different (Fig. S4A and S4B). Rapamycin treated WT cells were used as a positive control in reducing both ECAR and OCR of Tneu stimulated cells (42).

Discussion

S6K1 and S6K2 are canonical mTORC1 substrates whose inhibition has potential to modulate T helper differentiation and treat T-cell mediated autoimmune diseases. Indeed, S6K1 knockdown reduces Th17 differentiation in both WT cells and in cells with hyperactive mTORC1 resulting from TSC1 deficiency (43). Although S6K2 is also prominently expressed in lymphocytes (22), the role of this isoform in T cells has not been investigated. Here we used S6K2$^{-/-}$ mice to assess the impact of S6K2 deficiency on T cell development,

proliferation and differentiation. Previous genetic studies of T cells lacking mTOR pathway components (i.e. mTOR, raptor, and Rheb) have generally reported no changes in early T cell development and homeostasis. Our data show similarly that S6K2$^{-/-}$ T cells develop normally and that CD4^{+} T cells have no defects in early activation. This allowed us to ensure that functional studies were unaffected by preexisting cell-intrinsic defects. Proliferation of S6K2$^{-/-}$ T cells was unimpaired under neutral stimulation conditions or when polarized to Th1, Th17 or Treg subsets. Analysis of T helper differentiation *in vitro* showed a modest but significant decrease in Th17 differentiation using RPMI media, but not in IMDM. Using MOG_{35-55}-induced EAE as an *in vivo* model of Th17-driven autoimmunity, we compared WT and S6K2$^{-/-}$ mice and observed comparable clinical scores and Th17 cell percentages in the spinal cord. Utilizing the same model to study *in* vivo Th17 and Treg responses to a specific antigen, we gated on CD4^{+}CD44^{hi} infiltrating T cells in the spinal cord and saw no change in pS6 signaling, which corresponds with our *in vitro* signaling data using IMDM. We also restimulated splenocytes with MOG and detected no difference between WT and S6K2$^{-/-}$ mice in IL-17A expression. However, there was a significant increase in CD4^{+}FoxP3^{+} populations. These findings suggest that S6K2 may play a negative role in Treg generation, but is not a key driver of Th17 differentiation or autoimmune pathology *in vivo*, at least in this EAE model. Moreover, the discordant findings using RPMI media compared to IMDM and to the EAE model emphasize the importance of using optimal *in vitro* activation conditions for assessing Th17 differentiation.

Signaling measurements indicate a lesser role for S6K2 compared to S6K1 in phosphorylation events triggered by TCR/CD3 engagement. The phosphorylation of S6 protein was modestly reduced within 24hr of activation, indicating that S6K2 contributes significantly to the overall S6K signaling output. However, S6K1 blockade with the selective inhibitor LY2584702 caused a stronger inhibition of pS6 at all time points assessed.

Furthermore, S6K1 inhibition but not S6K2 loss reduced phosphorylation of CAD, a key enzyme in *de novo* pyrimidine synthesis (37, 38). mTORC1 and S6K1 mediate negative feedback loops that suppress upstream phosphorylation of Akt in many cellular systems (35, 44, 45). In accord, S6K1i treatment of WT T cells significantly increased pAkt, indicating a removal of feedback signaling. In contrast, pAkt in activated S6K2$^{-/-}$ T cells was similar or slightly reduced compared to WT cells. We also found that S6K1 is not upregulated in the absence of S6K2, whereas a study of S6K1$^{-/-}$ mice reported massive compensatory upregulation of S6K2 in T cells (23). These distinctions further support the conclusion that S6K1 loss has greater impact on T cell biology compared to S6K2 loss. Nevertheless, the fact that S6K2 is expressed in T cells suggests that this kinase has a functional role, prompting us to assess T cell proliferation and differentiation.

S6K2$^{-/-}$ T cells became activated (based on expression of CD25 and CD69 surface markers and production of IL-2) and proliferated (assessed by CFSE dilution at 72hr and cell cycle at 40hr) to a comparable extent as WT cells. In fact, when we fully suppressed S6K function by treating S6K2$^{-/-}$ T cells with S6K1i, proliferation was unimpaired. These data agree with our recent finding that S6Ks are dispensable for lymphocyte proliferation downstream of mTORC1; instead, the 4E-BP/eIF4E axis is the main driver of lymphocyte clonal expansion (29). It is reasonable to propose that the primary role of S6Ks in T cells is to control differentiation rather than proliferation. A role for S6K1 in Th17 differentiation is supported by both gain- and loss-of-function studies (13, 43). S6K2 can promote nuclear localization of RORγT, the Th17 cell hallmark transcription factor (13), but whether this function is required for Th17 differentiation has not been determined. Therefore, we tested S6K2$^{-/-}$ CD4 T cells for the ability to differentiate into Th17 and other Th cell subsets.

Our results show that S6K2 has a context-specific role in Th17 differentiation *in vitro*. When CD4 T cells were cultured in RPMI media under Th17 polarizing conditions, S6K2$^{-/-}$

cells generated a significantly lower percentage of IL-17A$^+$ cells. This correlated with reduced nuclear localization of RORγT. This finding is consistent with the model that S6K2 and not S6K1 shuttles RORγT to the nucleus as proposed by Kurebayashi *et al*. (13). Importantly, when cells were cultured in IMDM to support more robust Th17 differentiation (32), both WT and S6K2$^{-/-}$ CD4 T cells generated higher percentages of IL-17A$^+$ cells and there was no difference between the genotypes. The IMDM condition also fully rescued the S6 phosphorylation defect in S6K2$^{-/-}$ T cells, and partially restored nuclear localization of RORγT. It is not clear how the distinct composition of RPMI and IMDM media can affect the wiring of signaling pathways, such as the S6K2-dependence of S6 phosphorylation and RORγT localization. Compared to RPMI, IMDM has increased D-glucose and L-glutamine amounts in addition to higher concentrations of inorganic salts, vitamins, and amino acids. A key difference in IMDM is the greater abundance of aromatic amino acids that are precursors of endogenous agonists for the arylhydrocarbon receptor (AhR) that drives Th17 differentiation (32). It is possible that AhR activity alters gene expression in ways that reprogram T cells to be less dependent on S6K2. In support, addition of an exogenous AhR ligand FICZ corrected the Th17 differentiation defect in S6K2$^{-/-}$ T cells.

We chose to assess Th17 differentiation *in vivo* using the MOG_{35-55}-induced model of EAE, since chemical and genetic approaches have established a role for mTORC1 in this model (12, 13, 16, 17). As expected, rapamycin treatment muted clinically apparent EAE symptoms and reduced the percentage of IL-17A$^+$ as well as IFNγ$^+$ cells in the spinal cord. However, S6K2$^{-/-}$ mice showed no difference in clinical scores or inflammatory T cells. This result suggests that selective targeting of S6K2 has limited potential for the treatment of T cell-mediated autoimmunity. Another implication of this result is that *in vitro* Th17 differentiation in IMDM, compared to RPMI, provided a better prediction of *in vivo* outcomes. Differences in media composition might underlie some of the differences reported

among various studies of specific mTORC1 pathway components in T helper differentiation (9, 12, 13).

mTOR inhibition enhances the formation of induced Tregs (12, 14), yet the relevant downstream substrates involved in this effect are not clear. Interestingly, naïve S6K2$^{-/-}$ T cells had an increased propensity to differentiate into FoxP3^{+} cells under Treg biasing conditions *in vitro*, regardless of the media used. The increase was consistent across experiments but quantitatively modest, with the percent FoxP3^{+} cells averaging 26% for WT and 35% for S6K2$^{-/-}$ T cells. In addition, the significant increase seen in Treg generation in 10 day MOG immunized S6K2$^{-/-}$ mice suggests a milder inflammatory model may benefit from inhibition of S6K2. Further studies are needed to determine whether this increase in induced Treg generation influences the severity of other autoimmune disease models and anti-tumor immunity.

The efficacy of rapamycin in the EAE model might be the result of blocking T cell clonal expansion and/or a specific effect on T helper differentiation. We initiated treatment several days after immunization to minimize effects on proliferation. *Sasaki et. al.* had previously utilized p70^{S6K1} knockout mice to show that ameliorate clinical disease scores in EAE (31). It is possible that rapamycin suppresses Th17 differentiation through a combination of S6K1 and S6K2, or through other undetermined downstream substrates. In addition, a phase I clinical trial of the S6K1 inhibitor LY2584702 revealed significant toxicity, raising concerns about the therapeutic window for targeting this enzyme (34).

There is interest in targeting S6K2 for cancer treatment, as over 88% of tumors express higher levels than corresponding healthy tissues (46–50). Selective chemical inhibitors of S6K2 have not yet been described. Should such compounds become available, the data presented here suggest that blockade of S6K2 will not be broadly immunosuppressive compared to rapamycin. Selective inhibition or conditional deletion of S6K1 and S6K2 in

various cell types will be necessary to achieve a more detailed understanding of the unique and shared functions of these kinases in different aspects of the immune response.

Acknowledgments

The authors thank Yumay Chen and Ping H. Wang (UC Irvine) for use and assistance of the Seahorse XF24 analyzer, and Lindsey Araujo, Adam K. Idica, and Sara E. Lutz for technical advice. We are grateful to Sara Kozma (IDIBELL) for providing the S6K2$^{-/-}$ mouse strain, originally generated at the Friedrich Miescher Institute.

References

1. Laplante, M., and D. M. Sabatini. 2012. mTOR signaling in growth control and disease. *Cell* 149: 274–293.

2. Cybulski, N., and M. N. Hall. 2009. TOR complex 2: a signaling pathway of its own. *Trends Biochem Sci* 34: 620–627.

3. Finlay, D., and D. A. Cantrell. 2011. Metabolism, migration and memory in cytotoxic T cells. *Nat Rev Immunol* 11: 109–117.

4. Okkenhaug, K. 2013. Signaling by the phosphoinositide 3-kinase family in immune cells. *Annu Rev Immunol* 31: 675–704.

5. Zeng, H., and H. Chi. 2013. mTOR and lymphocyte metabolism. *Curr Opin Immunol* 25: 347–355.

6. Powell, J. D., K. N. Pollizzi, E. B. Heikamp, and M. R. Horton. 2012. Regulation of immune responses by mTOR. *Annu Rev Immunol* 30: 39–68.

7. Magnuson, B., B. Ekim, and D. C. Fingar. 2012. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochem J* 441: 1–21.

8. Lawrence, J. C., and R. T. Abraham. 1997. PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem Sci* 22: 345–349.

9. Yang, K., S. Shrestha, H. Zeng, P. W. Karmaus, G. Neale, P. Vogel, D. A. Guertin, R. F. Lamb, and H. Chi. 2013. T cell exit from quiescence and differentiation into Th2 cells depend on Raptor-mTORC1-mediated metabolic reprogramming. *Immunity* 39: 1043–1056.

10. Michalek, R. D., V. A. Gerriets, S. R. Jacobs, A. N. Macintyre, N. J. MacIver, E. F. Mason, S. A. Sullivan, A. G. Nichols, and J. C. Rathmell. 2011. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol* 186: 3299–3303.

11. Shi, L. Z., R. Wang, G. Huang, P. Vogel, G. Neale, D. R. Green, and H. Chi. 2011. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med* 208: 1367–1376.

12. Delgoffe, G. M., K. N. Pollizzi, A. T. Waickman, E. Heikamp, D. J. Meyers, M. R. Horton, B. Xiao, P. F. Worley, and J. D. Powell. 2011. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol* 12: 295–303.

13. Kurebayashi, Y., S. Nagai, A. Ikejiri, M. Ohtani, K. Ichiyama, Y. Baba, T. Yamada, S. Egami, T. Hoshii, A. Hirao, S. Matsuda, and S. Koyasu. 2012. PI3K-Akt-mTORC1-S6K1/2 axis controls Th17 differentiation by regulating Gfi1 expression and nuclear translocation of RORγ. *Cell Rep* 1: 360–373.

14. Delgoffe, G. M., T. P. Kole, Y. Zheng, P. E. Zarek, K. L. Matthews, B. Xiao, P. F. Worley, S. C. Kozma, and J. D. Powell. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30: 832–844.

15. Branisteanu, D. D., C. Mathieu, and R. Bouillon. 1997. Synergism between sirolimus and 1,25-dihydroxyvitamin D3 in vitro and in vivo. *J Neuroimmunol* 79: 138–147.

16. Esposito, M., F. Ruffini, M. Bellone, N. Gagliani, M. Battaglia, G. Martino, and R. Furlan. 2010. Rapamycin inhibits relapsing experimental autoimmune encephalomyelitis by both effector and regulatory T cells modulation. *J Neuroimmunol* 220: 52–63.

17. Donia, M., K. Mangano, A. Amoroso, M. C. Mazzarino, R. Imbesi, P. Castrogiovanni, M. Coco, P. Meroni, and F. Nicoletti. 2009. Treatment with rapamycin ameliorates clinical and histological signs of protracted relapsing experimental allergic encephalomyelitis in Dark Agouti rats and induces expansion of peripheral CD4+CD25+Foxp3+ regulatory T cells. *J Autoimmun* 33: 135–140.

18. Lisi, L., P. Navarra, R. Cirocchi, A. Sharp, E. Stigliano, D. L. Feinstein, and C. Dello Russo. 2012. Rapamycin reduces clinical signs and neuropathic pain in a chronic model of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 243: 43–51.

19. Carlson, R. P., W. L. Baeder, R. G. Caccese, L. M. Warner, and S. N. Sehgal. 1993. Effects of orally administered rapamycin in animal models of arthritis and other autoimmune diseases. *Ann N Y Acad Sci* 685: 86–113.

20. Fischer, K. E., J. A. Gelfond, V. Y. Soto, C. Han, S. Someya, A. Richardson, and S. N. Austad. 2015. Health Effects of Long-Term Rapamycin Treatment: The Impact on Mouse Health of Enteric Rapamycin Treatment from Four Months of Age throughout Life. *PLoS ONE* 10: e0126644.

21. Pallet, N., and C. Legendre. 2013. Adverse events associated with mTOR inhibitors. *Expert Opin Drug Saf* 12: 177–186.

22. Nardella, C., A. Lunardi, G. Fedele, J. G. Clohessy, A. Alimonti, S. C. Kozma, G. Thomas, M. Loda, and P. P. Pandolfi. 2011. Differential expression of S6K2 dictates tissue-specific requirement for S6K1 in mediating aberrant mTORC1 signaling and tumorigenesis. *Cancer Res* 71: 3669–3675.

23. Shima, H., M. Pende, Y. Chen, S. Fumagalli, G. Thomas, and S. C. Kozma. 1998. Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. *EMBO J* 17: 6649–6659.

24. Pelletier, J., J. Graff, D. Ruggero, and N. Sonenberg. 2015. Targeting the eIF4F translation initiation complex: a critical nexus for cancer development. *Cancer Res* 75: 250–263.

25. Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* 69: 1227–1236.

26. Kuo, C. J., J. Chung, D. F. Fiorentino, W. M. Flanagan, J. Blenis, and G. R. Crabtree. 1992. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* 358: 70–73.

27. Choo, A. Y., S. O. Yoon, S. G. Kim, P. P. Roux, and J. Blenis. 2008. Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc Natl Acad Sci U S A* 105: 17414–17419.

28. Salmond, R. J., R. J. Brownlie, O. Meyuhas, and R. Zamoyska. 2015. Mechanistic Target of Rapamycin Complex 1/S6 Kinase 1 Signals Influence T Cell Activation Independently of Ribosomal Protein S6 Phosphorylation. *J Immunol* 195: 4615–4622.

29. So, L., J. Lee, M. Palafox, S. Mallya, C. Woxland, M. Arguello, M. Truitt, N. Sonenberg, D. Ruggero, and D. A. Fruman. 2016. The 4E-BP/eIF4E axis controls rapamycin-sensitive growth and proliferation in lymphocytes. *submitted* .

30. Pende, M., S. H. Um, V. Mieulet, M. Sticker, V. L. Goss, J. Mestan, M. Mueller, S. Fumagalli, S. C. Kozma, and G. Thomas. 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* 24: 3112–3124.

31. Sasaki, C. Y., G. Chen, R. Munk, E. Eitan, J. Martindale, D. L. Longo, and P. Ghosh. 2016. p(70S6K1) in the TORC1 pathway is essential for the differentiation of Th17 Cells, but not Th1, Th2, or Treg cells in mice. *Eur J Immunol* 46: 212–222.

32. Veldhoen, M., K. Hirota, J. Christensen, A. O'Garra, and B. Stockinger. 2009. Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. *J Exp Med* 206: 43–49.

33. Perez, O. D., and G. P. Nolan. 2006. Phospho-proteomic immune analysis by flow cytometry: from mechanism to translational medicine at the single-cell level. *Immunol Rev* 210: 208–228.

34. Tolcher, A., J. Goldman, A. Patnaik, K. P. Papadopoulos, P. Westwood, C. S. Kelly, W. Bumgardner, L. Sams, S. Geeganage, T. Wang, A. R. Capen, J. Huang, S. Joseph, J. Miller, K. A. Benhadji, L. H. Brail, and L. S. Rosen. 2014. A phase I trial of LY2584702 tosylate, a p70 S6 kinase inhibitor, in patients with advanced solid tumours. *Eur J Cancer* 50: 867–875.

35. Wan, X., B. Harkavy, N. Shen, P. Grohar, and L. J. Helman. 2007. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene* 26: 1932–1940.

36. Sridharan, S., and A. Basu. 2011. S6 kinase 2 promotes breast cancer cell survival via Akt. *Cancer Res* 71: 2590–2599.

37. Robitaille, A. M., S. Christen, M. Shimobayashi, M. Cornu, L. L. Fava, S. Moes, C. Prescianotto-Baschong, U. Sauer, P. Jenoe, and M. N. Hall. 2013. Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis. *Science* 339: 1320–1323.

38. Ben-Sahra, I., J. J. Howell, J. M. Asara, and B. D. Manning. 2013. Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. *Science* 339: 1323–1328.

39. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201: 233–240.

40. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6: 1133–1141.

41. Eisenstein, E. M., and C. B. Williams. 2009. The T(reg)/Th17 cell balance: a new paradigm for autoimmunity. *Pediatr Res* 65: 26R–31R.

42. Pollizzi, K. N., A. T. Waickman, C. H. Patel, I. H. Sun, and J. D. Powell. 2015. Cellular size as a means of tracking mTOR activity and cell fate of CD4+ T cells upon antigen recognition. *PLoS ONE* 10: e0121710.

43. Park, Y., H. S. Jin, J. Lopez, C. Elly, G. Kim, M. Murai, M. Kronenberg, and Y. C. Liu. 2013. TSC1 regulates the balance between effector and regulatory T cells. *J Clin Invest* 123: 5165–5178.

44. Hsu, P. P., S. A. Kang, J. Rameseder, Y. Zhang, K. A. Ottina, D. Lim, T. R. Peterson, Y. Choi, N. S. Gray, M. B. Yaffe, J. A. Marto, and D. M. Sabatini. 2011. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science* 332: 1317–1322.

45. Yu, Y., S. O. Yoon, G. Poulogiannis, Q. Yang, X. M. Ma, J. Villén, N. Kubica, G. R. Hoffman, L. C. Cantley, S. P. Gygi, and J. Blenis. 2011. Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science* 332: 1322–1326.

46. Pérez-Tenorio, G., E. Karlsson, M. A. Waltersson, B. Olsson, B. Holmlund, B. Nordenskjöld, T. Fornander, L. Skoog, and O. Stål. 2011. Clinical potential of the mTOR targets S6K1 and S6K2 in breast cancer. *Breast Cancer Res Treat* 128: 713–723.

47. Filonenko, V. V., R. Tytarenko, S. K. Azatjan, L. O. Savinska, Y. A. Gaydar, I. T. Gout, V. S. Usenko, and V. V. Lyzogubov. 2004. Immunohistochemical analysis of S6K1 and S6K2 localization in human breast tumors. *Exp Oncol* 26: 294–299.

48. Lyzogubov, V. V., D. I. Lytvyn, T. M. Dudchenko, N. V. Lubchenko, P. V. Pogrybniy, S. V. Nespryadko, A. B. Vinnitska, V. S. Usenko, I. T. Gout, and V. V. Filonenko. 2004. Immunohistochemical analysis of S6K1 and S6K2 expression in endometrial adenocarcinomas. *Exp Oncol* 26: 287–293.

49. Pardo, O. E., C. Wellbrock, U. K. Khanzada, M. Aubert, I. Arozarena, S. Davidson, F. Bowen, P. J. Parker, V. V. Filonenko, I. T. Gout, N. Sebire, R. Marais, J. Downward, and M. J. Seckl. 2006. FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCepsilon, B-Raf and S6K2. *EMBO J* 25: 3078–3088.

50. Pardo, O. E., and M. J. Seckl. 2013. S6K2: The Neglected S6 Kinase Family Member. *Front Oncol* 3: 191.

Figure legends

Figure 1: *S6K2*$^{-/-}$ mice have normal T cell compartments and CD4^{+} T cells display normal activation marker upregulation. **(A)** Flow cytometry analysis of CD4^{+} and CD8^{+} populations in the thymus, spleen, and lymph nodes. **(B)** Quantitative analysis of CD4^{+}, CD8^{+}, and CD4^{+}CD8^{+} populations in the thymus, spleen, and lymph nodes (numbers indicate millions, mean +/- SEM, n = 3 per group). **(C)** nTreg populations were analyzed by CD4^{+}CD25^{+} expression in the thymus, spleen, and lymph nodes. Average percentage mean (from total CD4^{+} populations) +/- SEM is shown below (n=3 per group). **(D)** Flow cytometry analysis of activation markers and cell size (forward scatter; FSC) of WT and *S6K2*$^{-/-}$ T cells after 24 hr of stimulation using varying concentrations of anti-CD3 under neutral conditions.

Figure 2: *S6K2*$^{-/-}$ CD4 T cells have reduced S6 phosphorylation during the first 24 hours of stimulation compared to WT, but have comparable proliferation and cell size under neutral stimulation conditions. **(A)** Histogram overlays of p-S6 comparing WT and *S6K2*$^{-/-}$ CD4 T cells during a time course of neutral stimulation. **(B)** *Left,* Graph of normalized average mean fluorescence intensities (MFIs) of p-S6 in 24hr-activated CD4 T cells, * $p < 0.05$ (n = 5). Error bars indicate SEM. *Right*, The percentage of p-S6-positive T cells was graphed for six independent experiments comparing WT and *S6K2*$^{-/-}$ CD4 T cells after 24hr stimulation. The Wilcoxon signed-rank test was used to assess p value. **(C)** CFSE and p-S6 staining of WT and *S6K2*$^{-/-}$ naïve CD4^{+} T cells unstimulated or stimulated for 3 days. (**D)** CFSE histogram overlay of WT and *S6K2*$^{-/-}$ T cells from (C). **(E)** Cell size histogram overlay WT and *S6K2*$^{-/-}$ naïve CD4^{+} T cells stimulated for 24 hours. **(F)** Average FSC MFI plotted from (E) is shown, * $p < 0.05$ (n = 3). Error bars indicate SEM.

Figure 3: S6K2 and S6K1 have distinct substrates in activated T cells and have different roles in feedback to Akt. **(A)** Immunoblot analysis of lysates from WT and *S6K2*$^{-/-}$ naïve CD4^{+} T cells unstimulated or stimulated for 24 hours. Rapamycin (+rap) or S6K1 inhibitor (S6Ki) were included during stimulation in the samples indicated. Antibodies used to probe the blots are shown on the left. **(B)** Analysis of phospho CAD and S6K1 expression from immunoblots, mean +/- SEM, n = 3. * $p < 0.05$. **(C)** Intracellular phosphoflow staining of Akt and analysis by normalized MFI, mean +/- SEM, n = 3. *$p < 0.05$.

Figure 4: Naïve *S6K2*$^{-/-}$ CD4^{+} T cells generate fewer Treg and more Th17 cells, with little effect on Th1, Th17, or Treg proliferation. **(A)** Intracellular staining of IL-17A, FoxP3, and IFNγ in WT and *S6K2*$^{-/-}$ T cells stained with CFSE for cell division tracking. Naïve WT or *S6K2*$^{-/-}$ CD4^{+} T cells were cultured in RPMI media under indicated conditions for 4 days. **(B)** Paired analysis of IL-17A and IFNγ positive cells from multiple experiments performed as in (A) (Th17: n = 4, Treg: n = 5). The Wilcoxon signed-rank test was used to assess p value. **(C)** Histogram overlays of CFSE-labeled T cells from (A). **(D)** IL-17 and IL-10 cytokine measurements by ELISA from the supernatant of WT and *S6K2*$^{-/-}$ T cells cultured in Th17 or Treg skewing conditions for 4 days (mean +/- SEM, n = 3).

Figure 5: *S6K2*$^{-/-}$ and WT mice exhibit comparable EAE development. **(A)** Clinical scores of EAE-induced WT and *S6K2*$^{-/-}$ mice (mean +/- SEM, n = 8 for each group). **(B)** Clinical scores of EAE-induced WT, WT treated with Rap or vehicle (Veh), and *S6K2*$^{-/-}$ mice (mean +/- SEM, n =3 for each group). **(C)** Graphed analysis of IFNγ and IL-17A positive CD4^{+} T cells in the spinal cord of EAE-induced mice from (B), * $p < 0.05$ (mean +/- SEM, n = 3). **(D)** pS6 (MFI and % positive) in CD4^{+} T cells from freshly harvested lymph nodes from 10 day MOG immunized WT and *S6K2*$^{-/-}$ mice (mean +/- SEM, n = 3). **(E)** CD4^{+}IL17A^{+} (% positive and

MFI) and $CD4^{+}FoxP3^{+}$ (% positive) populations in 3 day MOG restimulated splenocytes harvested from the same mice as used in (D) (mean +/- SEM, n = 3).

Figure 6: IMDM rescues Th17 differentiation in $S6K2^{-/-}$ T cells. **(A)** Intracellular staining of IL-17A and FoxP3 in WT and *S6K2*$^{-/-}$ T cells stained with CFSE. Naïve WT or *S6K2*$^{-/-}$ $CD4^{+}$ T cells were cultured under indicated conditions for 4 days in RPMI or IMDM. **(B)** Comparative flow cytometric analysis and quantitative analysis for normalized MFI of p-S6 signaling between WT and *S6K2*$^{-/-}$ T cells 24 hr of neutral stimulation, *$p < 0.05$ (mean +/- SEM, n = 3). **(C)** Intracellular staining of IL-17A in CFSE-labeled WT and *S6K2*$^{-/-}$ T cells cultured in Th17 skewing conditions for 4 days in RPMI, RPMI +FICZ, or IMDM.

Supplemental Figures

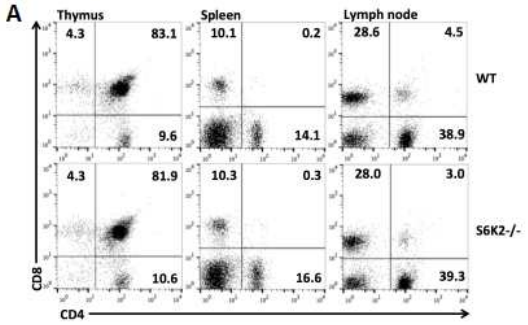
Supplemental Figure 1: Additional early activation/characterization and EAE data. **(A)** Suppressive Treg assay using isolated nTregs from WT or *S6K2*$^{-/-}$ mice. These were seeded with CFSE labeled WT naïve T cells and stimulated with CD3 and CD28 for 3 days. Data are representative of three independent experiments **(B)** Cell cycle analysis of WT and *S6K2*$^{-/-}$ CD4^{+} T cells by PI staining after 40h stimulation in Tconv, Th17, or Treg culturing conditions (mean +/- SEM, n = 3). **(C)** IL-2 cytokine measurement by ELISA in the supernatant of 40h activated WT and *S6K2*$^{-/-}$ CD4^{+} T cells in Tconv, Th17, or Treg culturing conditions (mean +/- SEM, n = 3). **(D)** Graphed Th1 and Th17 population percentages from EAE experiment 1 (Fig. 5A) by IFNγ and IL-17A cytokine expression, respectively (n = 3 for each group). Cells were isolated from the brain and gated on CD4^{+} expression. Error bars indicate SEM. **(E)** IL-17 cytokine production in the supernatant of 3 day MOG restimulated splenocytes at 10 and 50μg/mL concentrations from 10 day MOG immunized WT and *S6K2*$^{-/-}$ mice.

Supplemental Figure 2: FICZ boosts Th17 differentiation in both RPMI and IMDM **(A)** Flow cytometry analysis of IL-17A and CFSE positive cells. Naïve WT CD4^{+} T cells were stimulated under Th17 conditions for four days in RPMI and IMDM, with or without FICZ. **(B)** CFSE histogram overlay of naïve WT or *S6K2*$^{-/-}$ CD4^{+} T cells that were cultured under indicated conditions for 4 days in RPMI, RPMI +FICZ, or IMDM. **(C)** Th17 population percentages from Figure 6A and similar experiments, graphed by IL-17A cytokine expression (n = 4). The Wilcoxon signed-rank test was used to assess p value. ns = not significant ($p > 0.05$).

Supplemental Figure 3: *S6K2*$^{-/-}$ T cells show significantly reduced RORγ nuclear localization compared to WT T cells. **(A)** Immunofluorescence microscopy analysis of RORγ localization in WT or *S6K2*$^{-/-}$ CD4^{+} T cells. Cells were cultured in IMDM or RPMI for 40 hours under Th1 or Th17 differentiation conditions. **(B)** Quantification of RORγ nuclear localization from (A) using the Manders colocalization coefficient. Data represent at least 3 independent experiments, mean +/- SEM. * $p < 0.05$. For statistical analysis, 20 cells were counted in each sample.

Supplemental Figure 4: *S6K2*$^{-/-}$ and WT CD4^{+} T cells have similar metabolic profiles under various T cell skewing conditions. Naïve CD4^{+} T cells were stimulated for 40 hours under the indicated conditions. **(A)** Glycolytic rate (ECAR) **(B)** Oxidative phosphorylation rate (OCR). Data shown are representative of at least 3 independent experiments, mean +/- SEM. * $p < 0.05$.

Figure 1



B

Thymus

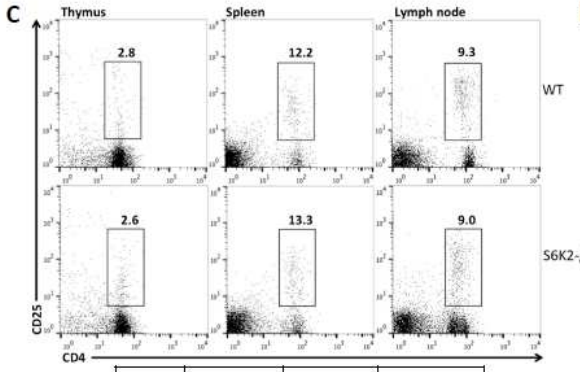
	CD4+	CD8+	CD4+CD8+
WT	10.5± 1.6	5.6± 0.6	79.3± 2.6
S6K2-/-	11.6± .52	5.9± 1.2	76.7± 3.0

Spleen

	CD4+	CD8+	CD4-CD8-
WT	15.5±1.2	9.6± 1.3	74.8± 2.4
S6K2-/-	14.4± .37	9.4± 0.9	76.2± 1.2

Lymph Node

	CD4+	CD8+	CD4-CD8-
WT	33.8± 2.6	28.2± 0.8	38.0± 2.7
S6K2-/-	31± 4.2	28± .5	40.7± 3.9



	Thymus	Spleen	Lymph Node
WT	2.5 ± .47	11.5 ± 1.3	10.1 ± .75
S6K2-/-	2.8 ± .46	11.3 ± 1.2	9.9 ± .1.9

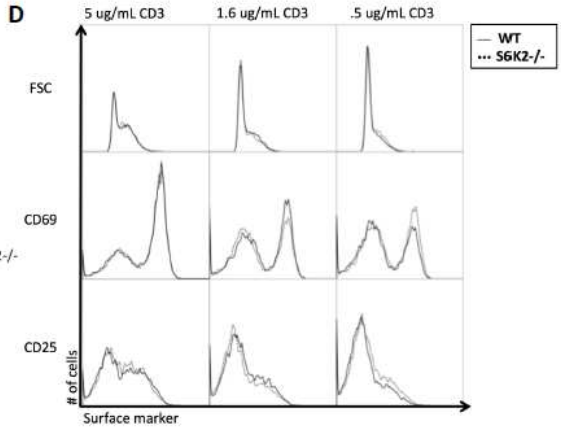


Figure 2

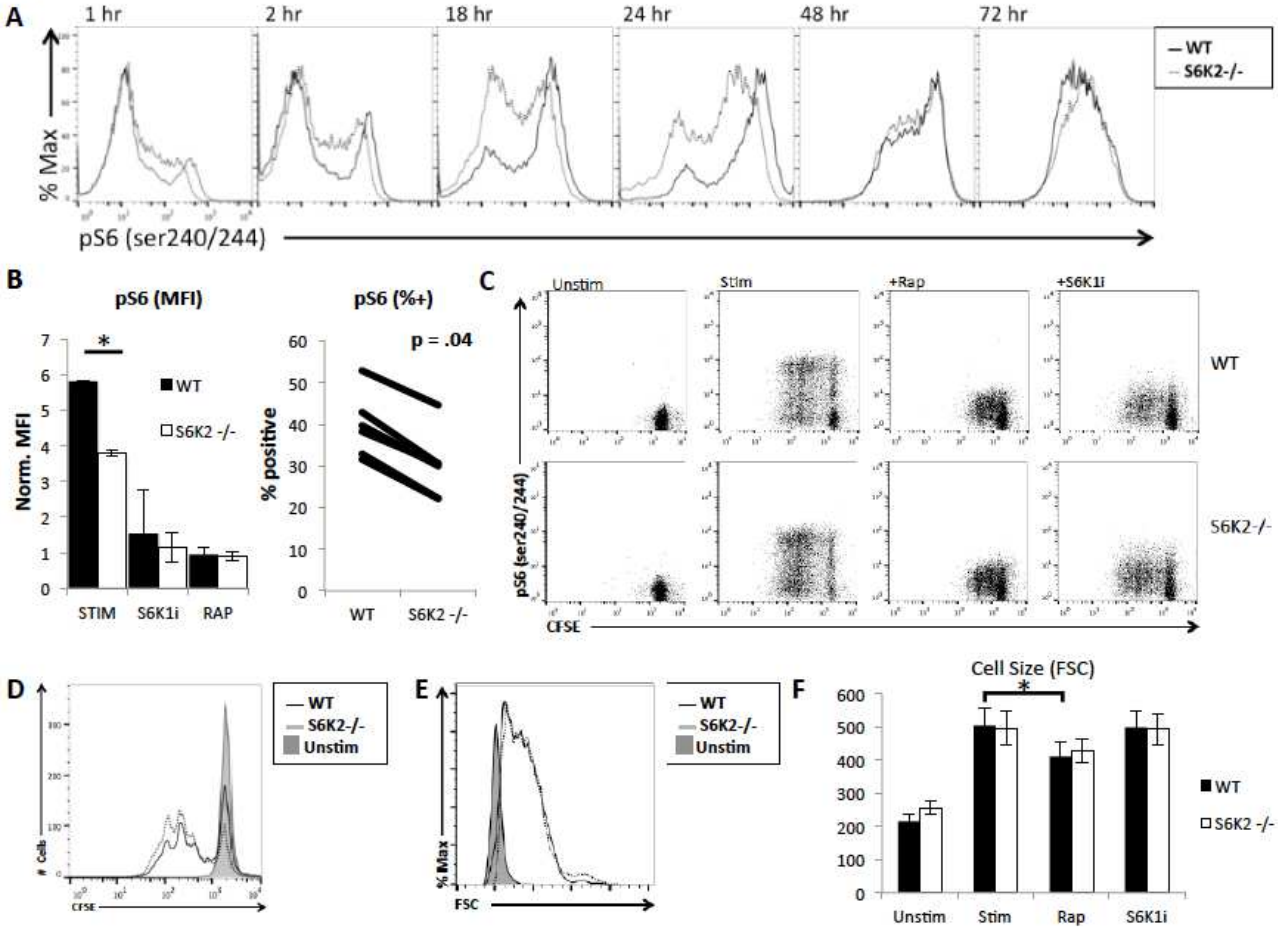

A
1 hr
2 hr
18 hr
24 hr
48 hr
72 hr
WT
S6K2-/-
% Max
pS6 (ser240/244)
B
pS6 (MFI)
pS6 (%+)
p = .04
WT
S6K2 -/-
Norm. MFI
% positive
STIM
S6K1i
RAP
C
Unstim
Stim
+Rap
+S6K1i
WT
S6K2-/-
pS6 (ser240/244)
CFSE
D
WT
S6K2-/-
Unstim
Cells
CFSE
E
WT
S6K2-/-
Unstim
% Max
FSC
F
Cell Size (FSC)
WT
S6K2 -/-
Unstim
Stim
Rap
S6K1i

Figure 3

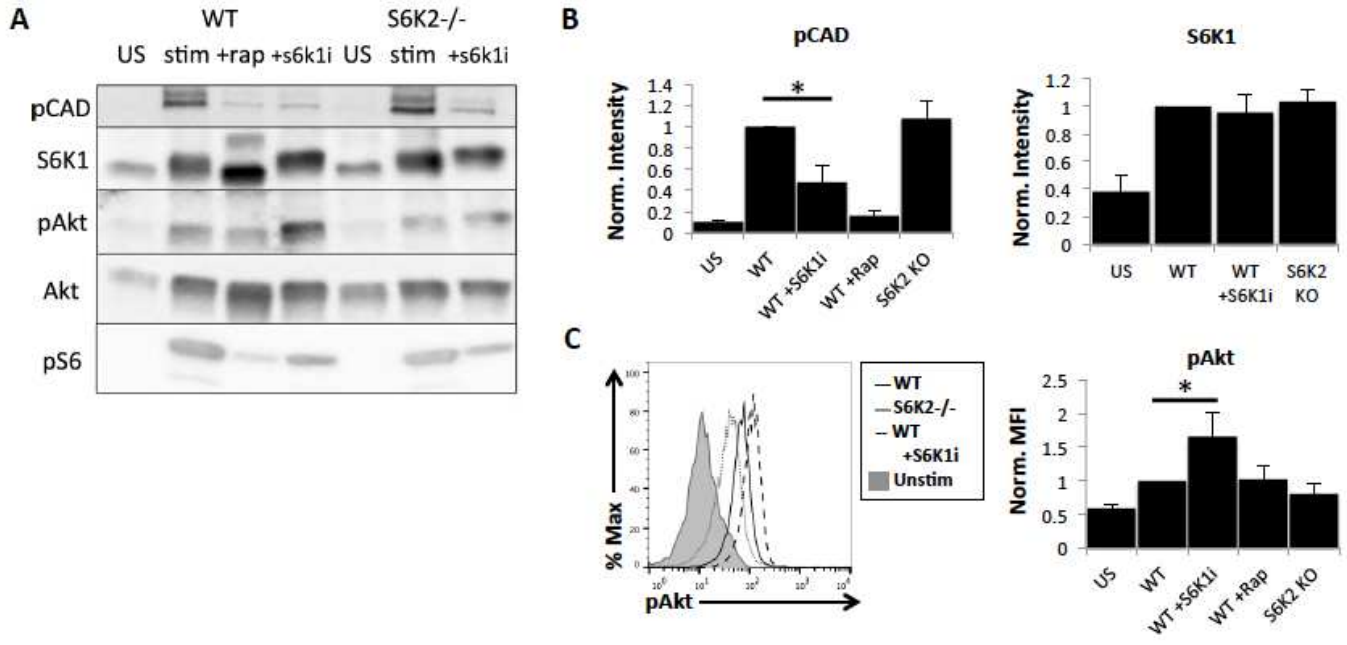

A
WT
S6K2-/-
US stim +rap +s6k1i US stim +s6k1i
pCAD
S6K1
pAkt
Akt
pS6
B
pCAD
Norm. Intensity
US
WT
WT +S6K1i
WT +Rap
S6K2 KO
S6K1
Norm. Intensity
US
WT
WT +S6K1i
S6K2 KO
C
WT
S6K2-/-
WT +S6K1i
Unstim
% Max
pAkt
pAkt
Norm. MFI
US
WT
WT +S6K1i
WT +Rap
S6K2 KO

Figure 4

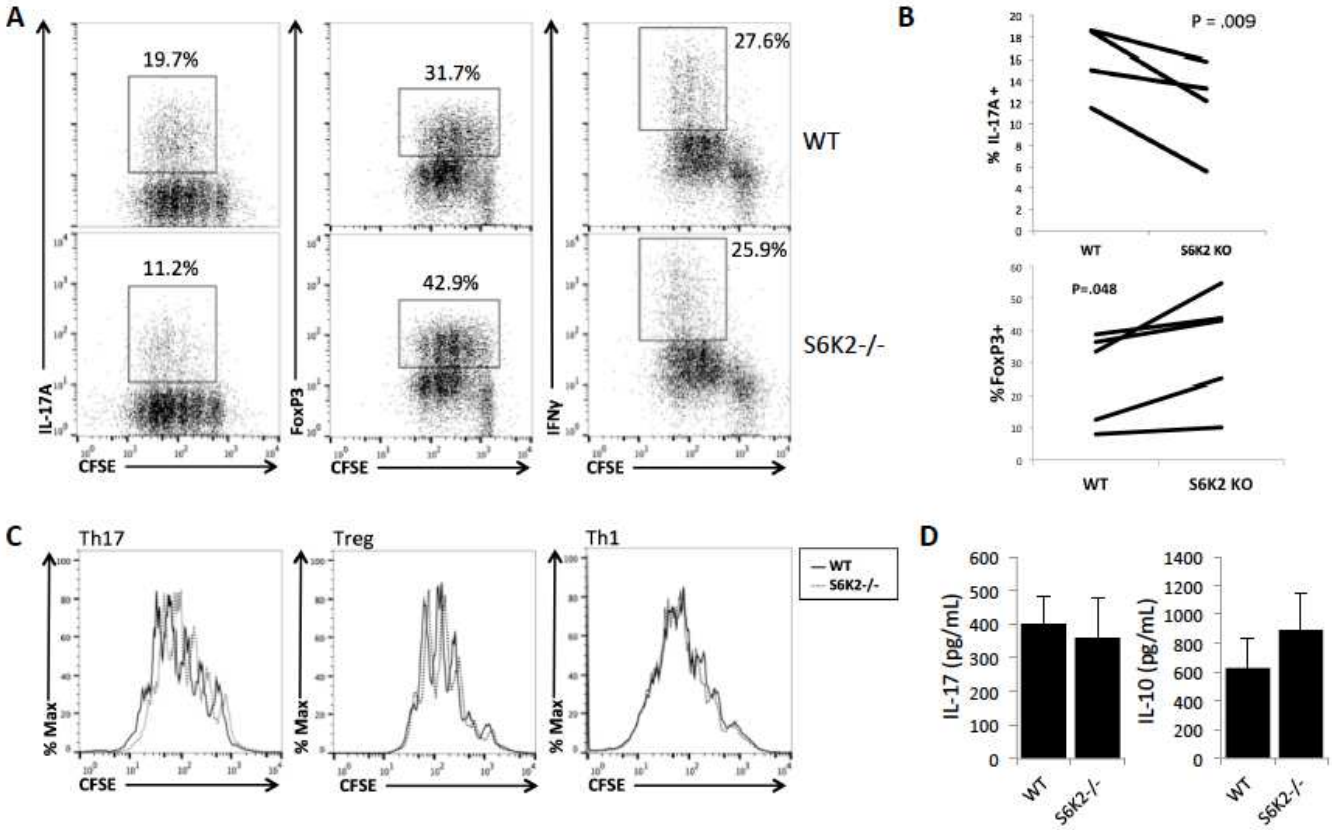

A
19.7%
31.7%
27.6%
11.2%
42.9%
25.9%
WT
S6K2-/-
IL-17A
FoxP3
IFNγ
CFSE
B
P = .009
% IL-17A +
WT
S6K2 KO
P=.048
%FoxP3+
C
Th17
Treg
Th1
WT
S6K2-/-
% Max
D
IL-17 (pg/mL)
IL-10 (pg/mL)

Figure 5

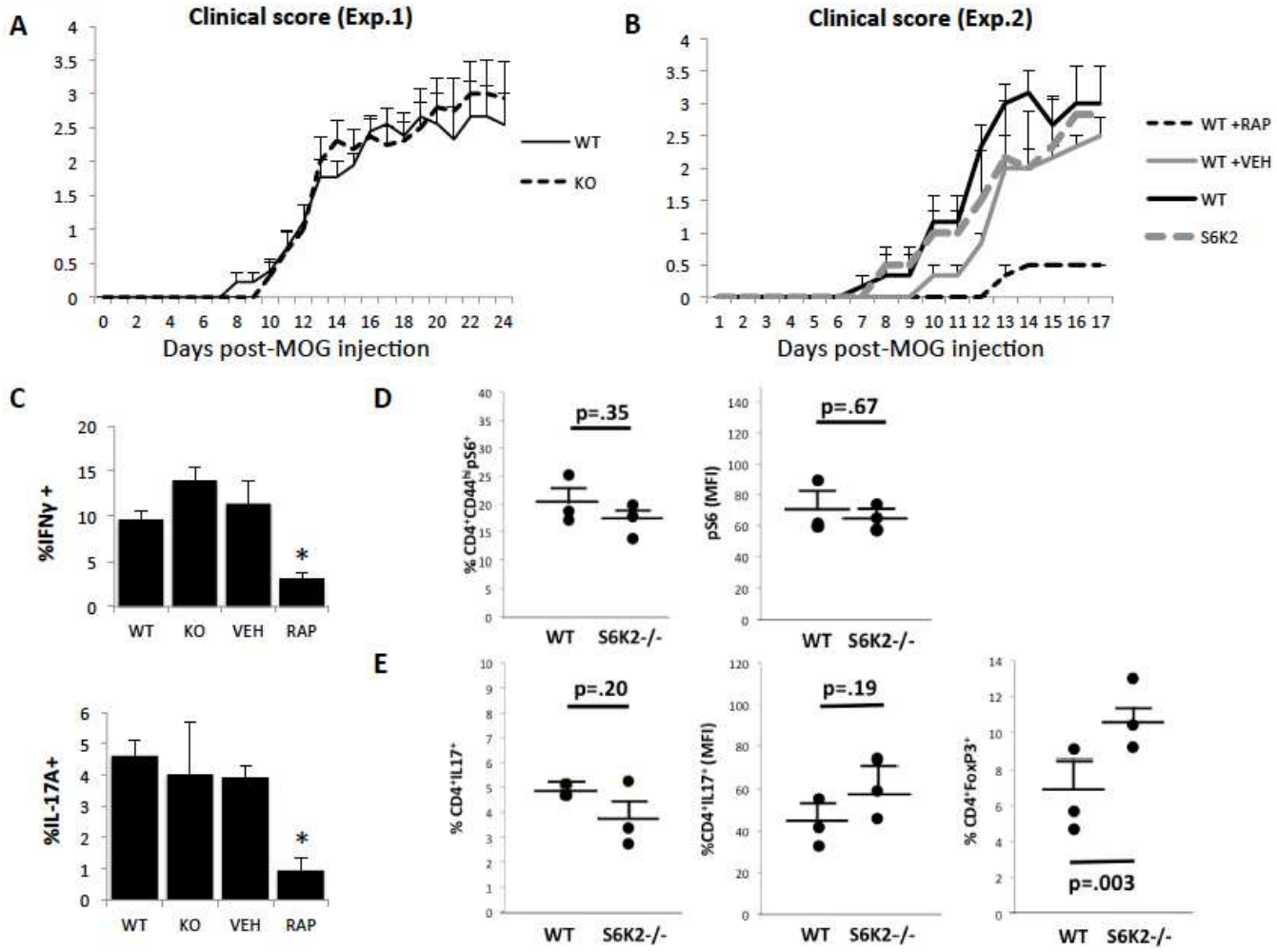
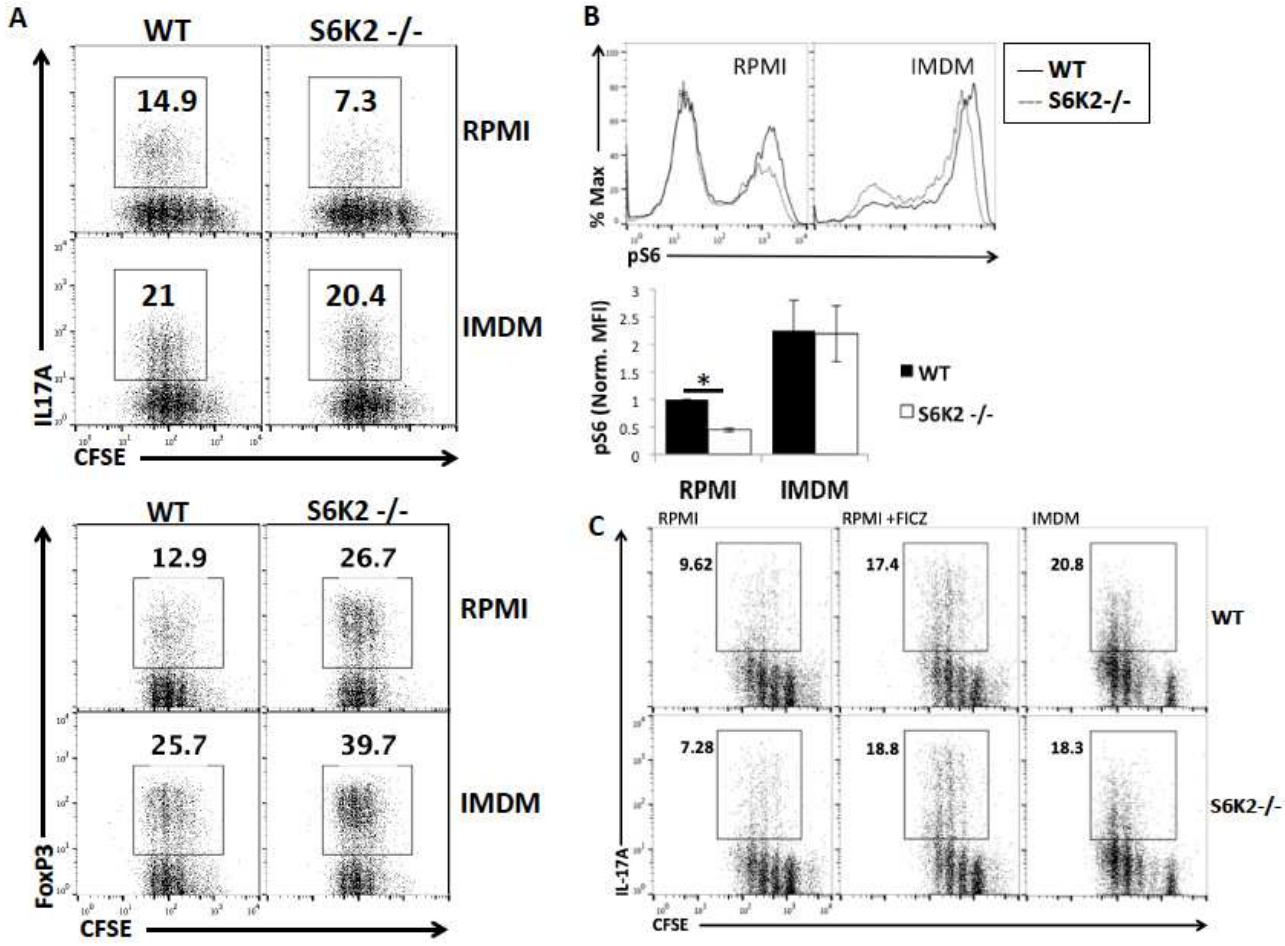
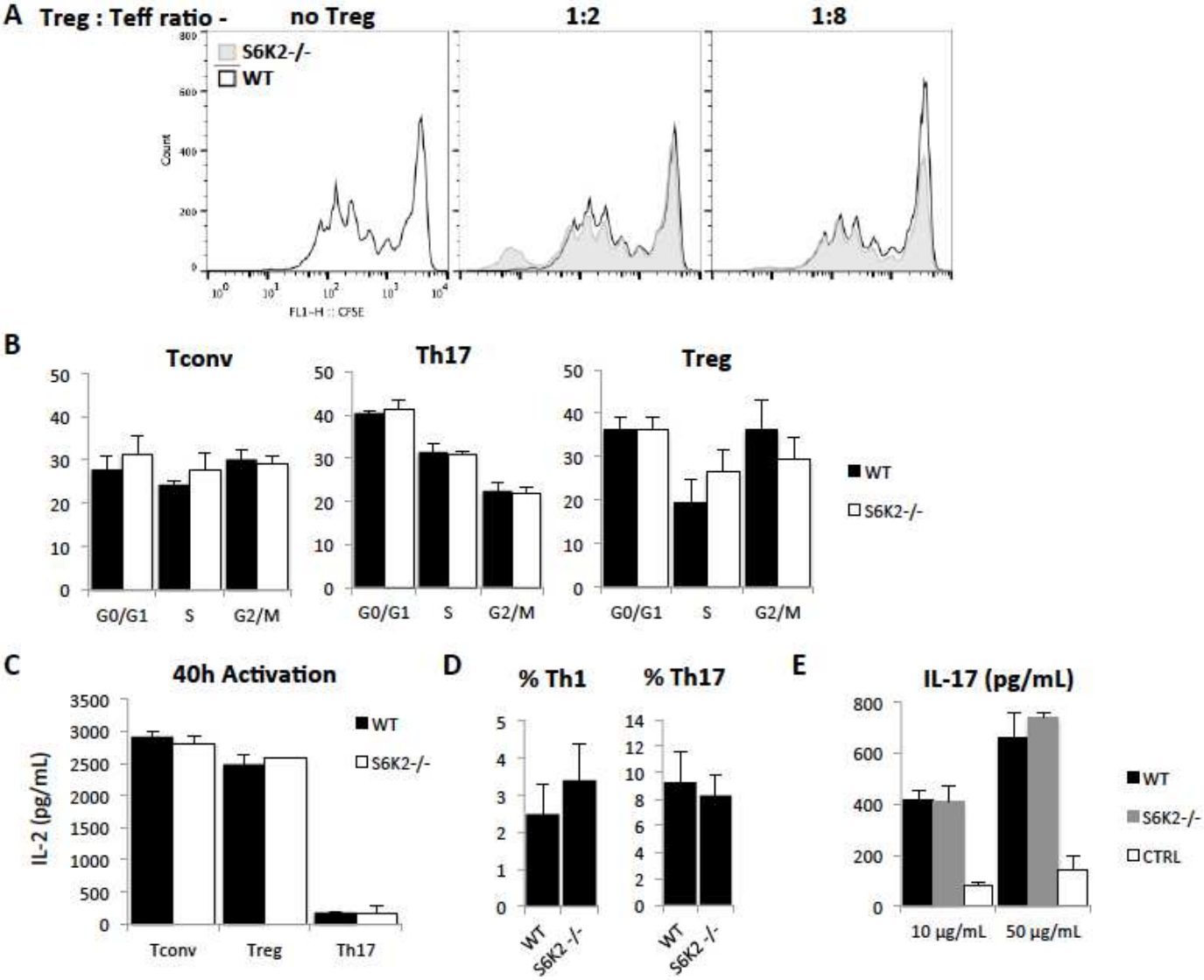

A
Clinical score (Exp.1)
Days post-MOG injection
WT
KO
B
Clinical score (Exp.2)
Days post-MOG injection
WT +RAP
WT +VEH
WT
S6K2
C
%IFNγ +
WT KO VEH RAP
%IL-17A+
WT KO VEH RAP
D
% CD4+CD44hipS6+
p=.35
WT S6K2-/-
pS6 (MFI)
p=.67
WT S6K2-/-
E
% CD4+IL17+
p=.20
WT S6K2-/-
%CD4+IL17+ (MFI)
p=.19
WT S6K2-/-
% CD4+FoxP3+
p=.003
WT S6K2-/-

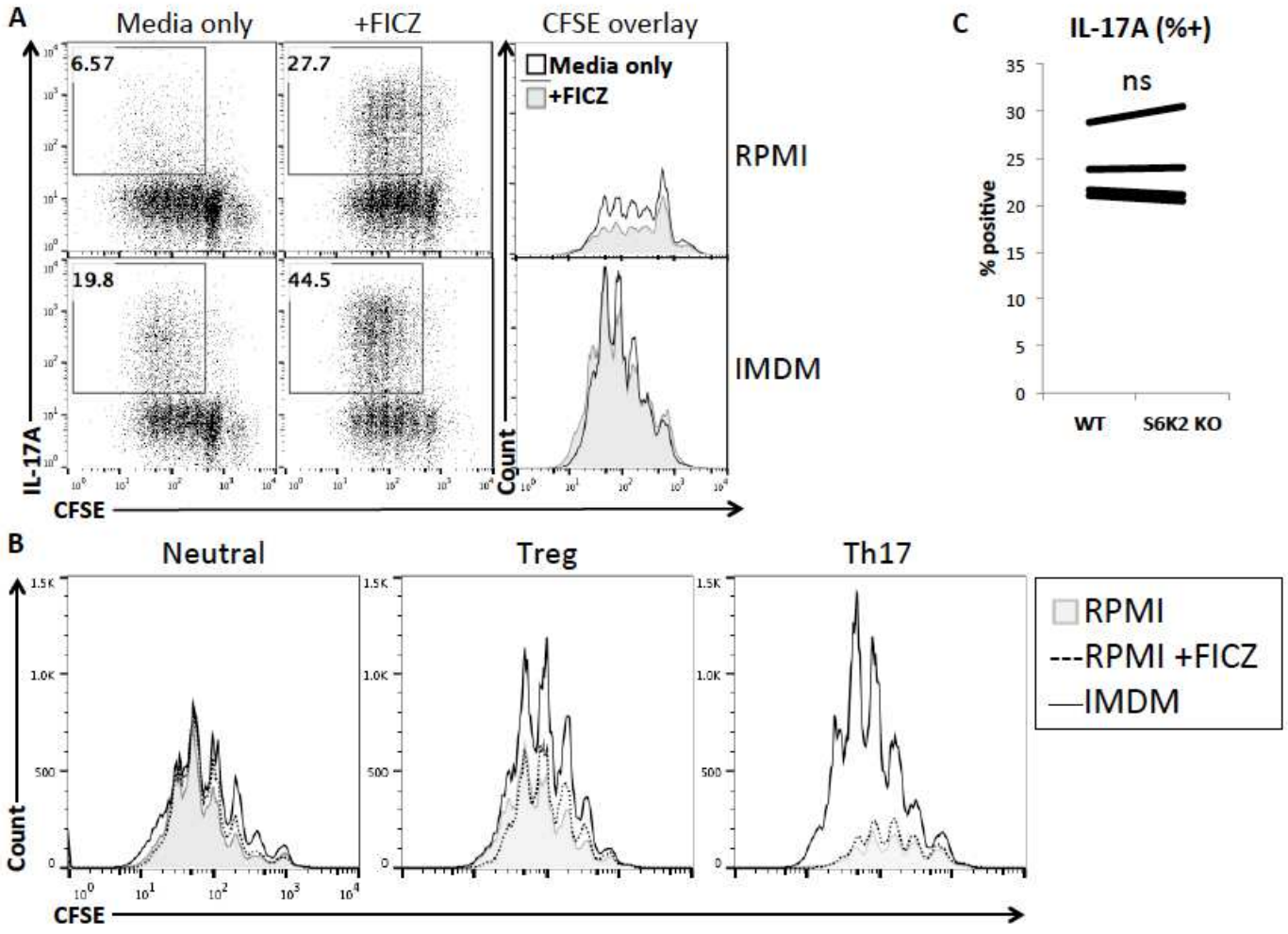
Figure 6


A
WT
S6K2 -/-
14.9
7.3
RPMI
21
20.4
IMDM
IL17A
CFSE
WT
S6K2 -/-
12.9
26.7
RPMI
25.7
39.7
IMDM
FoxP3
CFSE
B
RPMI
IMDM
WT
S6K2-/-
% Max
pS6
pS6 (Norm. MFI)
*
WT
S6K2 -/-
RPMI
IMDM
C
RPMI
RPMI +FICZ
IMDM
9.62
17.4
20.8
WT
7.28
18.8
18.3
S6K2-/-
IL-17A
CFSE

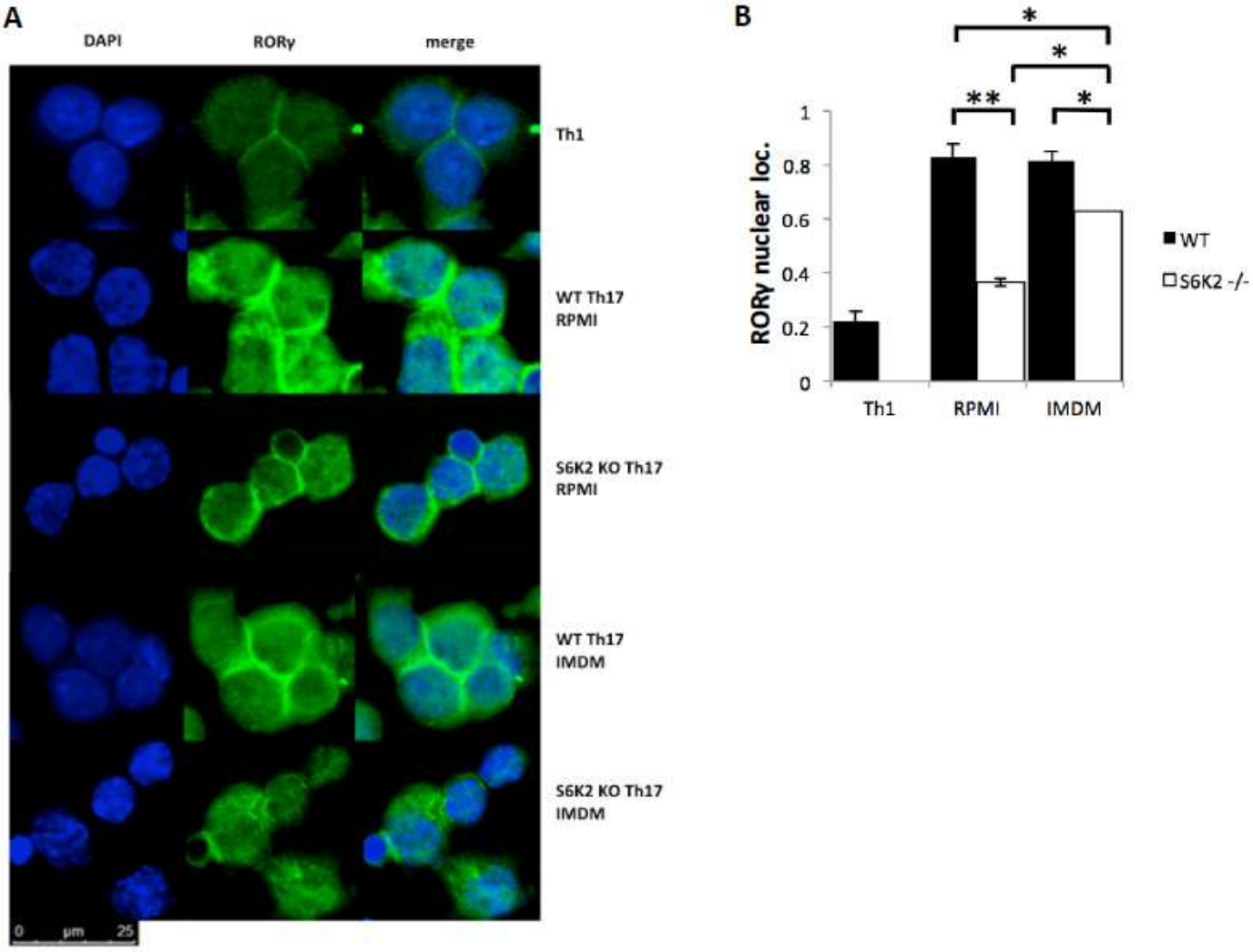
Supplemental 1



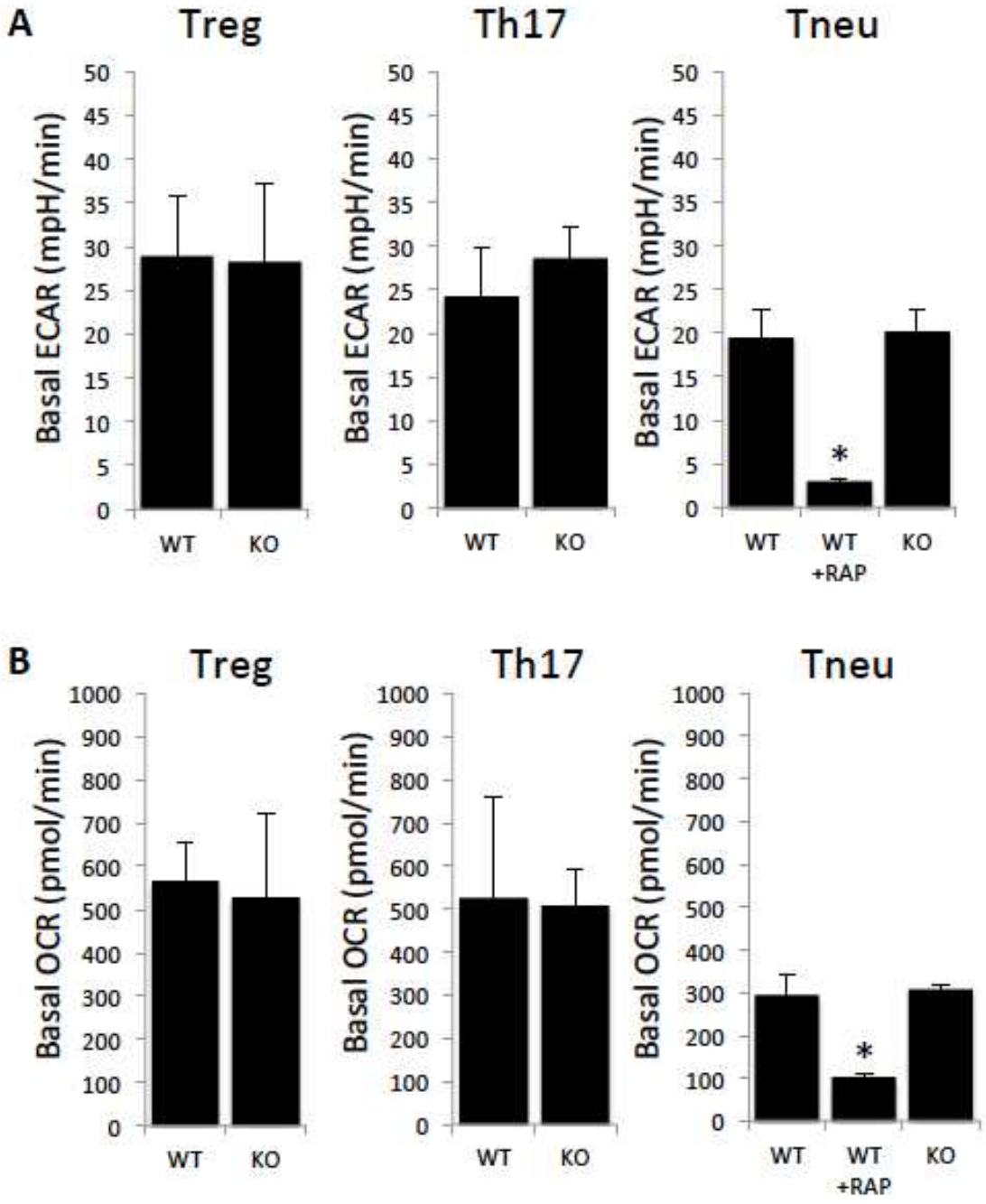
Supplemental 2



Supplemental 3



Supplemental 4



CHAPTER 3

Potent and Selective ACC Inhibitors Target Th17-mediated Immune Responses and Increase Treg Generation

Abstract

De novo fatty acid synthesis is required for effector T cell differentiation and function, providing the rapidly proliferating cells with biosynthetic precursors for cell membrane synthesis, cell signaling substrate, and posttranslational modifications. Acetyl-CoA Carboxylase, or ACC, refers to two enzymes with distinct localization and function. ACC1 catalyzes a rate-limiting step in fatty acid synthesis in the cytoplasm, whereas ACC2 inhibits β-oxidation of fatty acids in the mitochondria. T cell-specific deletion of ACC1 has established that this isozyme is required for Th17 development, but dispensable for induced Tregs. Using a potent and selective allosteric inhibitor of ACC1 and ACC2, we show significantly reduced Th17 differentiation and preferentially encourage Treg generation, under conditions when proliferation is preserved *in vitro*. Utilizing experimental autoimmune encephalomyelitis (EAE) as a mouse model for autoimmunity, we found that ACC inhibition dramatically reduced clinical scores and inflammatory cytokine production *in vivo.* Our data reinforce the idea that ACC inhibition can be used as a therapeutic strategy in the treatment of autoimmune diseases, targeting selective T effector responses while promoting Tregs.

Introduction

In an immune response, newly activated effector T cells require reprogramming of their metabolic processes to achieve sustainable growth and production of subset specific cytokines (1–3). Activated T cells are able to switch freely between fatty acid oxidation and glycolysis to meet proliferating demands and often still utilize both early in activation (4, 5). Current evidence suggests that each effector T cell subset engages distinct metabolic programs to commit to distinct lineages, a process that involves a complex integration of immunological signals and nutrient cues (3). Lipid synthesis is one such process that is essential for providing a source for membrane biosynthesis in proliferating T lymphocytes. In T cells that rely on glycolytic pathways, including cytotoxic $CD8^+$ T cells and proinflammatory $CD4^+$ T cells, *de novo* fatty acid (FA) synthesis is required to drive differentiation into these T cell subsets (1, 6). Other types of T cells like $CD8^+$ memory T cells and induced regulatory T cells (Tregs) prefer to utilize exogenous FAs as a sustaining fuel source (fatty acid oxidation, or FAO) (7). This dividing characteristic between Th1/Th17s and Tregs allows for a potential therapeutic treatment to be developed in which FA synthesis is discouraged and FAO promoted (8).

Autoimmune diseases are the result of dysregulated immune responses, often driven by inappropriate proinflammatory cytokine production from effector $CD4^+$ T cells. Th17 cells are one such proinflammatory T cell subset that has important roles in protecting mucosal barrier surfaces. However, an unwanted Th17 response towards self or foreign antigens can induce chronic inflammation, which often leads to destruction of otherwise healthy tissue. Multiple sclerosis and rheumatoid arthritis are such examples in which elevated Th17 populations secreting IL-17A recruit both innate and adaptive immune cell types, which ultimately exacerbates and progresses disease (9).

ACC is a key enzyme that determines the balance of FA synthesis and oxidation. Mammals express two isoforms, ACC1 and ACC2. Both catalyze the irreversible, ATP-dependent carboxylation of acetyl-CoA into malonyl-CoA, which is the rate-limiting step to fatty acid synthesis (10). ACC1 is localized in the cytoplasm and is required for *de novo* FA synthesis (11). ACC2 is localized in the outer mitochondrial membrane and inhibits FAO by increasing malonyl-CoA levels and allosterically inhibiting carnitine palmitoyl transferase 1 (CPT1) (12). CPT1 is the enzyme responsible for importing FA chains into the mitochondria to generate ATP.

Berod and colleagues have previously established proof of concept that ACC1/2 inhibition has potential as an immunomodulatory therapeutic (13). Using soraphen A (SorA), a naturally occurring macrolide that inhibits both eukaryotic isozymes for ACC, they found that inhibition of ACC1 inhibits Th17 differentiation and instead skews T cells towards a Treg phenotype with expression of Foxp3. Utilizing a T cell specific knockout for ACC1 (TACC1), they established that ACC1 is required specifically for Th17 differentiation. They found that additional treatment with SorA increased Treg generation when compared to the knockouts alone. Both SorA treatment and the TACC1 knockout ameliorated clinical scores in the mouse model of autoimmune disease, EAE (experimental autoimmune encephalomyelitis). In summary, their studies shows a clear potential for ACC inhibition to be used in the treatment of autoimmune diseases that are driven by Th17 cells.

Though SorA is active at nanomolar half-maximum effective concentrations (EC50: ~50nM) in cells, the drug exhibits poor pharmacological properties and is not considered a lead compound for developing a therapeutic (14). Utilizing structure-based drug design, Nimbus Therapeutics has developed a series of potent allosteric inhibitors of ACC. A set of these was characterized to correct for dysregulated fatty acid metabolism of the liver, which is known to cause the disease nonalcoholic steatohepatitis (NASH) (unpublished data). One

of these highly potent, allosteric inhibitors of both ACC isozymes was assigned Fast Track designation by the FDA and is in phase II clinical trials for treatment of NASH. However the effects of such compounds on T cells and autoimmunity have not been determined. In this study, we show that a novel synthetic ACC1/2 inhibitor has similar effects to SorA in inhibiting Th17 differentiation while promoting Treg generation *in vitro.* It also is effective *in vivo* in not only delaying EAE onset, but also in reducing overall clinical scores and incidence of the disease.

Materials and Methods

Animals

All mice were kept in specific pathogen–free animal facilities and in accordance with guidelines of the University of California Institutional Animal Care and Use Committee. C57BL/6 mice were obtained from Jackson Laboratories.

Media and cell culture

Primary cell culture was performed in complete media consisting of RPMI or IMDM media (Gibco) supplemented with 10% FCS (Gibco), L-glutamine (Corning), ßME and antibiotics (Gibco).

Antibodies and reagents

Stimulatory anti-CD3 (2C11) and anti-CD28 (37.51), as well as neutralizing anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-1A12), anti-IL-12p40 (C17.8), and anti-IL-4 (11B11) were from eBioscience. For differentiation, recombinant cytokines were used: TGF-beta (R&D systems), mouse IL-6 and IL-12 (eBioscience). For flow cytometry analysis, surface and intracellular staining antibodies were purchased from eBioscience and Cell Signaling Technologies.

Cell purification

Splenic $CD4^+$ T cells depleted of natural Tregs were purified by negative selection using the EasySep Mouse $CD4^+$ T Cell Isolation Kit from Stemcell Technologies with biotinylated anti-CD25 (0.5 μg/ml) added.

Cell stimulation and differentiation

Unless otherwise stated, T cells were stimulated as conventional T cells (Tconv), with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) from eBioscience. 20 nM of Rapamycin (LC Laboratories) was also used when indicated. When proliferation was measured, isolated T cells were prelabeled with CFSE (eBioscience). Cell stimulation cocktail (500x) from eBioscience containing PMA (phorbol 12-myristate 13-acetate), ionomycin, Brefeldin A, and monensin were used for restimulation (detection of intracellular cytokines). For Th polarization assays, isolated naïve T cells were skewed with anti-CD3 and anti-CD28 for 4 days (unless otherwise noted), restimulated for 4 hours, and harvested for flow cytometry analysis. Skewing conditions were as follows: T_H17: TGF-β (5 ng/ml), IL-6 (20 ng/ml), anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml) and IL-2 (1 ng/ml); Treg: TGF-β (5 ng/ml).

Intracellular staining for flow cytometry analysis

After restimulation with cell stimulation cocktail, cells were fixed and made permeable with BD Cytofix/Cytoperm and then stained for cell surface markers and cytokines. Intracellular staining of transcription factors (i.e. FoxP3) was done without restimulation. Gates were set appropriately with unstimulated controls (T cell subset proliferated populations were calculated with exclusion of the initial CFSE peak) and voltages were set on the basis of isotype-matched control antibodies. Data were acquired using a BD FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay

Cells were harvested after day 4 and supernatants were collected to measure cytokine production according to manufacturer's instructions. IFNγ ELISA kit was obtained from eBioscience and IL-17 ELISA kit was obtained from R&D systems.

Gene expression analysis

Total RNA was isolated using Quick-RNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions. The Tecan Infinite 200 was used to measure RNA amount and quality. RNA was then transcribed into cDNA using qScript™ XLT cDNA SuperMix (Quanta Biosciences). RT-PCR was performed with TaqMan® Universal PCR Master Mix (Life Technologies) on the StepOnePlus Real-Time PCR system (Applied Biosystems). All measured target gene transcripts were normalized to β-actin.

Experimental Autoimmune Encephalomyelitis

11+ week-old female wildtype mice were immunized with 100μg of MOG_{35-55} antigen in 200μg of CFA per mouse. Pertussis was administered i.v. on day 0 and 1 at 400ng per injection. Mice were scored on a scale of 0–5: 0-no clinical symptoms; 1-limp tail; 2- limp tail and partial hind limb paralysis; 3- limp tail and complete hind limb paralysis; 3.5- partial front or one-sided paralysis; 4- moribund; 5- death. Mice scoring 3.5 or more for two consecutive days were euthanized. Spinal cords were harvested and centrifuged through a Percoll gradient. Isolated infiltrating lymphocytes were restimulated for 4h before stained intracellularly for IFNγ and IL-17A. Splenocytes were restimulated with MOG (10 and 50μg/mL) for 3 days and then harvested for cytokine measurement in the supernatant, as well as stained intracellularly for IL-17A and FoxP3 expressing $CD4^+$ T cells.

Metabolic studies

Metabolic flux analysis was performed on 40h stimulated cells in complete Seahorse media (Seahorse Bioscience, 102365) supplemented with 25mM D-Glucose, 1mM Na-Pyruvate and 2mM L-glutamine.

Statistical Analysis

Mean and SEM values were calculated from at least three independent experiments. *p* values were calculated using a two-tailed unpaired Student's t-test when determining differences between groups and the Wilcoxon signed-rank test was used to determine pairwise differences (specifically indicated in figure legends).

Results

ACC inhibitors show potent and selective responses against Th17 differentiation

In pilot studies we compared 3 selective ACC inhibitors, added to CD4+ T cells under Th17 and Treg differentiation conditions *in vitro*. Compounds were tested at 300 nM, 1 μM, 3 μM, and 10 μM concentrations. Prior studies by Nimbus Therapeutics indicated the following IC_{50} values for the compounds, measured using isolated ACC1 enzyme in cell-free assays: 532 (NDI-011532)- 4.6 nM; 992 (NDI-010992)- 5.9 nM; 706 (NDI-011706)- 760 nM. Under Th17 differentiating conditions, all three compounds at 10 μM reduced the percentage of IL-17A+ cells (Fig. 1A). ACC inhibitors 992 and 532 exhibited a ten-fold increase in cellular potency compared to 706, which correlated with the biochemical IC_{50} values. Quantitation of replicate experiments showed that compounds 992 and 532 (1μM) significantly decreased both the percentage and numbers of IL-17A positive cells (Fig. 1B). Under Treg skewing conditions, all three compounds increased the percentage of FoxP3+ cells, compared to vehicle (Fig. 1C). However, over multiple experiments the dose dependent increase in percentage of FoxP3 positive populations did not reach statistical significance (Fig. 1D). Measuring the numbers of FoxP3 positive cells, there seemed to be less indication of increased Treg generation. Again, 992 and 532 compounds were at least ten-fold more potent than 706. We choose to focus our studies on ACC inhibitor 992 for its potency in both the Th17 and Treg differentiation studies, as well as its tissue specific selectivity.

ACC inhibition decreases *in vitro* Th17 differentiation without affecting total $CD4^+$ populations

Consistent with data from Figure 1, treatment of naïve $CD4^+$ T cells with 992 at 1μM reduced Th17 differentiation, (Fig. 2A). A 0.1% DMSO vehicle control was used for comparison and a 20nM Rapamycin treatment was added as a positive control. After four

days of culturing in Th17 skewing conditions, ACC inhibition significantly reduced both the percentage and number of IL-17A-positive cells compared to vehicle treatment (Fig. 2B). Because it was reported SorA treatment and TACC1 mice displayed a spontaneous switch to Treg when differentiated under Th17 conditions, we also intracellularly stained Th17 skewed cells for FoxP3 (13). We did not observe an increase of FoxP3 expression when the ACC inhibitor 992 was used (data not shown). Differentiated cells were also pre-labeled with the proliferation tracking dye CFSE to analyze IL-17A expression for each cell division. Both rapamycin and 992 significantly reduced Th17 differentiation throughout each cell division (Fig. 2C).

To determine the effect ACC inhibition on early activation metabolism for T cells skewed towards Th17, we measured metabolic rates using the Seahorse Metabolic Flux Analyzer. 40 hours into Th17 skewing stimulation, T cells were harvested and measured for basal glycolysis (extracellular acidification rate (ECAR)) and oxidative phosphorylation (oxygen consumption rate (OCR)). Interestingly, both ECAR and OCR were significantly reduced in ACC inhibitor-treated T cells, suggesting an effect on overall metabolism and not just glycolysis (Fig. 2D).

Late ACC inhibition *in vitro* separates its effect on overall proliferation from Th17 effector differentiation

Next we considered whether reduced generation of IL-17A+ cells was due to effects on proliferation versus differentiation. CFSE prelabeling of $CD4^+$ T cells before stimulation showed ACC inhibitor treatment reduced the percentage of IL-17A+ cells within populations at each division (Fig. 2C). However, addition of ACC inhibitor at 1μM significantly reduced the number of proliferated cells for both IL-17A positive and negative populations (Fig. 3A). This suggested an overall nonspecific inhibition of $CD4^+$ proliferation.

To separate the roles of ACC in cell cycle progression and differentiation, we modified the differentiation assay such that ACC inhibitor compounds were added to the cultures after 24 hours of initial stimulation (Fig. 3B). In cultures with 24hr addition of ACC inhibitor, the numbers of IL-17A negative cells was comparable to vehicle-treated control (Fig. 3C). In contrast, the number of IL-17A positive cells was still significantly reduced. Based on these results, we conducted all further *in vitro* experiments in a similar manner, adding the ACC inhibitor 24 hours into the four-day Th17 differentiation assay.

To determine whether the effects of ACC inhibitor were concentration-dependent, we assessed Th17 differentiation with ACC inhibitor treatment at 10nM, 30nM, 100nM, 300nM, and 1μM. Th17 differentiation assessed by percent and number of IL-17A$^+$ cells was significantly reduced over the concentration range of 100nM - 1μM (Fig. 3D). To measure other parameters of Th17 differentiation, we measured IL-17A protein in culture supernatants by ELISA. We observed a significant decrease in IL-17A cytokine production, with a concentration-dependent response from 30nM to 1μM of ACC inhibitor (Fig. 3E). Measurement of Th17-related gene transcripts by qPCR also showed reduced *Rorc* and *Il17a* expression (Fig. 3F).

ACC inhibition favors *in vitro* Treg differentiation

To assess the effect ACC inhibition on Treg differentiation, we performed *in vitro* skewing assays over 4 days and quantitated Treg generation by expression of Foxp3. At 1μM of 992, ACC inhibition increased the fraction of Foxp3+ cells in some experiments (Fig. 4A) but this increase was not significant over multiple trials (Fig. 4B). Both the total numbers and the percentages of FoxP3+ cells were unchanged to vehicle treated T cells (Fig. 4B). Following the same *in vitro* studies we performed earlier for Th17, we assessed Treg differentiation in cultures treated with ACC inhibitor 24hr after stimulation (Fig. 5A). Over

multiple experiments, late addition of 1μM 992 did significantly increase the FoxP3+ fraction, as did the positive control Rap (Fig. 5B). Interestingly, the increase in %FoxP3+ correlated with reduced numbers of proliferated FoxP3-negative cells rather than an absolute increase in FoxP3+ cells (Fig. 5C). In addition, we measured *Foxp3* transcript abundance in cells activated in Treg conditions with a range of ACC inhibitor from 10nm to 1μM. Consistent with the increase in %FoxP3+ cells, there was a significant dose-dependent increase in expression starting at 100nM of 992 (Fig. 5D).

ACC inhibition ameliorates clinical symptoms of EAE

Berod and colleagues utilized the Th17 mediated mouse autoimmune disease EAE to evaluate efficacy of pharmacological ACC inhibition *in vivo* (13). Using a SorA derivative, soraphen-S1036, they showed delayed EAE onset, as well as overall reduced clinical scores and incidence. To test the ACC inhibitor 992 in the same model, we dosed mice by oral gavage twice daily (p.o., BID) with either vehicle or 10 mg/kg of ACC inhibitor, starting day 2 post-immunization. Compared to vehicle control, mice treated with 992 showed delayed EAE onset and a highly significant reduction in clinical scores and incidence (Fig. 6A). Analysis of ACC inhibitor treated Th1 and Th17 populations in the spinal cord showed markedly reduced IFNγ and IL-17A cytokine production compared to vehicle (Fig. 6B). Isolation of cells from the spinal cords showed that ACC treatment caused a trend towards increased Treg percentages that approached significance ($p = .052$) (Fig. 6C). Notably, the percent and total number of $CD4^+$ T cells quantified in the spinal cord were comparable between both ACC and vehicle treated cohorts (Fig. 6D). In the cervical draining lymph nodes, Treg populations were significantly increased in the ACC inhibitor treated cohort (Fig. 6C). We also restimulated splenocytes with MOG at two concentrations (10μg/mL and 50μg/mL) and found the ACC inhibitor treated cohort to secrete significantly less IL-17A

compared to vehicle treated. IFNγ secretion was below the limit of detection for cells from ACC-treated mice (Fig. 6E).

Discussion

Our studies show that an ACC inhibitor with similar structure to a compound in clinical trials to treat NASH has a strong effect on Th differentiation, inhibiting Th17 production and promoting Treg generation. This potent effect was measured using *in vitro* differentiation assays, and supported by an autoimmune mouse model where pharmacologic inhibition of ACC reduced Th17 generation *in vivo* and significantly improved clinical outcome. 1μM of ACC inhibitor 992 displayed similar activity to a previous analysis of SorA in *in vitro* Th17/Treg studies. Though ACC treated T cells skewed to the Th17 subset did not spontaneously become Tregs, their IL-17 production was significantly reduced.

The effect ACC inhibition has on Th17 differentiation is not simply the result of an overall nonspecific inhibition of T cell proliferation. Overall proliferated IL-17A negative $CD4^+$ T cell populations were comparable to vehicle treatment in late inhibitor addition *in vitro* Th17 skewing experiments. Utilizing the *in vivo* EAE model, mice dosed with 10mg/kg of ACC inhibitor had dramatically less clinical symptoms than the vehicle cohort. Isolation of spinal cord infiltrating lymphocytes revealed that ACC treatment significantly reduced Th1 and Th17 cells, though overall $CD4^+$ populations did not change when compared to vehicle treatment. This preservation of T cell numbers could be explained by delayed dosing of 992, as we began ACC inhibition 2 days post MOG immunization. ACC inhibition, however, affected T cell proliferation under Treg skewing conditions. When 992 was added 24h into Treg skewing cultures, the FoxP3 negative proliferated T cell populations were significantly reduced compared to vehicle treatment. A more comprehensive look into the relationship between metabolic requirements and specific stimulation conditions such as Treg cultures would be helpful to understand their roles in cell survival and proliferation.

Th17 differentiation is accompanied by metabolic reprogramming including an increase in glycolytic rate. We found that ACC inhibition reduced both basal ECAR and OCR in cells

cultured for 40hr under Th17 conditions. It was unexpected to observe reduced OxPhos, as a previous study of ACC inhibition reported only a reduction in glycolytic rate. However, it should be considered that overall Th17 metabolism could be affected as their primary metabolic source for driving differentiation and proliferation is inhibited and OxPhos cannot be alternatively utilized. Conversely, given the lack of impaired differentiation of Tregs (which rely on FAO), it will be interesting to be determine whether OxPhos is preserved under Treg skewing conditions using either glucose or fatty acids as primary fuel. Perhaps Tregs and memory T cells that share comparable metabolic phenotypes could differentiate and function at lower rates of oxygen consumption.

Previous T cell specific ACC1 knockout mice experiments support the notion that SorA exerts its inhibitory effects on Th17 generation primarily through the inhibition of ACC1 alone, though inhibition of both ACC1/2 is required for optimal Treg generation (13). Berod *et al.* showed in a mouse model of autoimmune disease, that both TACC1 mice and mice treated with SorA have attenuated clinical symptoms compared to control wildtype mice. The effect of ACC inhibition on other immune cell types, however, has not yet been characterized in either murine or humans. It would be interesting to see how ACC inhibition affects cells that also undergo metabolic reprogramming and utilize *de novo* lipid synthesis, such as Th1 cells. In our EAE studies, we did observe significantly reduced Th1 populations from the ACC inhibitor treated cohort, both in the spinal cord and among MOG peptide responders in the spleen, as measured by $CD4^+$ T cells expressing or secreting IFNγ. Other immune cells types that utilize *de novo* lipogenesis with roles in inflammation include dendritic cells, B-lymphocytes, and other T cell subsets (e.g. cytotoxic effector CD8 cells and Th2 cells). In addition, various cancer cell lines also activate this pathway to support their rapid cell proliferation and fuel needs when under nutrient starvation. It has been shown that inhibition of ACC expression results in a major decrease in the cellular pool of palmitic acid, the end

product of fatty acid synthesis (15). This inhibition has induced apoptosis in prostate and breast cancer cells, but not in normal tissues (16, 17).

SorA treatment has been shown to render these cells more susceptible to cell death by increased reactive oxygen species (ROS) due to increased oxidation of fatty acids (by ACC2 inhibition) (18). It would be interesting to try our ACC inhibitor as an antineoplastic agent or in synergistic use with chemotherapeutics as a sensitizer.

In our studies, the targeting of ACC function as a therapeutic treatment in autoimmune disease correlates with a primary effect on Th17 differentiation and function. However, ACC inhibition seems to exert its effects not only on proinflammatory effector $CD4^+$ T cell subsets as Treg generation was spared and sometimes significantly increased, as seen in the cervical draining lymph nodes of ACC inhibitor-treated MOG-immunized mice. In conclusion, these promising results should be followed up by testing ACC inhibitors in other autoimmune models like psoriasis and colitis to realizing the potential of this approach to modulate proinflammatory metabolism for a therapeutic outcome.

References:

1. Cao, Y., J. C. Rathmell, and A. N. Macintyre. 2014. Metabolic reprogramming towards aerobic glycolysis correlates with greater proliferative ability and resistance to metabolic inhibition in CD8 versus CD4 T cells. *PLoS ONE* 9: e104104.

2. MacIver, N. J., R. D. Michalek, and J. C. Rathmell. 2013. Metabolic regulation of T lymphocytes. *Annu Rev Immunol* 31: 259–283.

3. Chang, C. H., J. D. Curtis, L. B. Maggi, B. Faubert, A. V. Villarino, D. O'Sullivan, S. C. Huang, G. J. van der Windt, J. Blagih, J. Qiu, J. D. Weber, E. J. Pearce, R. G. Jones, and E. L. Pearce. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153: 1239–1251.

4. Maciolek, J. A., J. A. Pasternak, and H. L. Wilson. 2014. Metabolism of activated T lymphocytes. *Curr Opin Immunol* 27: 60–74.

5. Gerriets, V. A., R. J. Kishton, A. G. Nichols, A. N. Macintyre, M. Inoue, O. Ilkayeva, P. S. Winter, X. Liu, B. Priyadharshini, M. E. Slawinska, L. Haeberli, C. Huck, L. A. Turka, K. C. Wood, L. P. Hale, P. A. Smith, M. A. Schneider, N. J. MacIver, J. W. Locasale, C. B. Newgard, M. L. Shinohara, and J. C. Rathmell. 2015. Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J Clin Invest* 125: 194–207.

6. Pearce, E. L., M. C. Walsh, P. J. Cejas, G. M. Harms, H. Shen, L. S. Wang, R. G. Jones, and Y. Choi. 2009. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460: 103–107.

7. Powell, J. D., and K. Pollizzi. 2012. Fueling memories. *Immunity* 36: 3–5.

8. Dang, E. V., J. Barbi, H. Y. Yang, D. Jinasena, H. Yu, Y. Zheng, Z. Bordman, J. Fu, Y. Kim, H. R. Yen, W. Luo, K. Zeller, L. Shimoda, S. L. Topalian, G. L. Semenza, C. V. Dang, D. M. Pardoll, and F. Pan. 2011. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell* 146: 772–784.

9. Lovett-Racke, A. E., Y. Yang, and M. K. Racke. 2011. Th1 versus Th17: are T cell cytokines relevant in multiple sclerosis? *Biochim Biophys Acta* 1812: 246–251.

10. Barber, M. C., N. T. Price, and M. T. Travers. 2005. Structure and regulation of acetyl-CoA carboxylase genes of metazoa. *Biochim Biophys Acta* 1733: 1–28.

11. Kim, T. S., P. Leahy, and H. C. Freake. 1996. Promoter usage determines tissue specific responsiveness of the rat acetyl-CoA carboxylase gene. *Biochem Biophys Res Commun* 225: 647–653.

12. Wakil, S. J., and L. A. Abu-Elheiga. 2009. Fatty acid metabolism: target for metabolic syndrome. *J Lipid Res* 50 Suppl: S138–S143.

13. Berod, L., C. Friedrich, A. Nandan, J. Freitag, S. Hagemann, K. Harmrolfs, A. Sandouk, C. Hesse, C. N. Castro, H. Bähre, S. K. Tschirner, N. Gorinski, M. Gohmert, C. T. Mayer, J. Huehn, E. Ponimaskin, W. R. Abraham, R. Müller, M. Lochner, and T. Sparwasser. 2014. De

novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. *Nat Med* 20: 1327–1333.

14. Shen, Y., S. L. Volrath, S. C. Weatherly, T. D. Elich, and L. Tong. 2004. A mechanism for the potent inhibition of eukaryotic acetyl-coenzyme A carboxylase by soraphen A, a macrocyclic polyketide natural product. *Mol Cell* 16: 881–891.

15. Rysman, E., K. Brusselmans, K. Scheys, L. Timmermans, R. Derua, S. Munck, P. P. Van Veldhoven, D. Waltregny, V. W. Daniëls, J. Machiels, F. Vanderhoydonc, K. Smans, E. Waelkens, G. Verhoeven, and J. V. Swinnen. 2010. De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. *Cancer Res* 70: 8117–8126.

16. Brusselmans, K., E. De Schrijver, G. Verhoeven, and J. V. Swinnen. 2005. RNA interference-mediated silencing of the acetyl-CoA-carboxylase-alpha gene induces growth inhibition and apoptosis of prostate cancer cells. *Cancer Res* 65: 6719–6725.

17. Chajès, V., M. Cambot, K. Moreau, G. M. Lenoir, and V. Joulin. 2006. Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. *Cancer Res* 66: 5287–5294.

18. Corominas-Faja, B., E. Cuyàs, J. Gumuzio, J. Bosch-Barrera, O. Leis, A. G. Martin, and J. A. Menendez. 2014. Chemical inhibition of acetyl-CoA carboxylase suppresses self-renewal growth of cancer stem cells. *Oncotarget* .

Figure Legends

Figure 1: Potent and selective ACC inhibitors. **(A)** ACC inhibitors 992, 532, and 706 were tested at a range of concentration to inhibit Th17 differentiation. 4-day Th17 skewing cultures were harvested and analyzed by FACS for IL-17A expression and proliferation by CFSE dilution. **(B)** Analysis of IL-17A percent and number positive cells from multiple experiments performed as in (A) (n=3). **(C)** ACC inhibitors 992, 532, and 706 were tested at a range of concentration to increase Treg differentiation. 4-day Treg skewing cultures were harvested and analyzed by FACS for FoxP3 expression and proliferation by CFSE dilution. **(D)** Analysis of FoxP3 percent and number positive cells from multiple experiments performed as in (C) (n=3). Mean +/- SEM. * $p < 0.05$

Figure 2: ACC inhibitor 992 significantly reduces Th17 differentiation and function. **(A)** Naïve $CD4^+$ T cells stimulated under Th17 skewing conditions for 4 days are analyzed for CFSE proliferation and IL-17A expression by FACS **(B)** Analysis of IL-17A percent and number positive cells from multiple experiments performed as in (A) (n=3). **(C)** Normalized analysis of IL-17A percent positive cells for each corresponding CFSE division from multiple experiments performed as in (A) (n=3). **(D)** Naïve $CD4^+$ T cells were stimulated for 40 hours under Th17 differentiating conditions and harvested to measure glycolytic (ECAR) and oxidative phosphorylation (OCR) rates. Data shown are representative of at least 3 independent experiments, mean +/- SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 3: 24h addition of ACC inhibitor 992 in Th17 skewing cultures still inhibits Th17 differentiation and function, but spares IL-17A negative proliferating T cells. **(A)** Naïve $CD4^+$ T cells stimulated for 4d under Th17 differentiating cultures were quantitated by IL-

17A expression. Numbers of IL-17A positive and negative populations were graphed as normalized values. **(B)** Same as (A), but with indicated inhibitors added at 24h after start of stimulation. **(C)** Flow cytometry analysis of IL-17A and CFSE positive T cells of 4d Th17 differentiated cultures with indicated inhibitors added at 24h. **(D)** Quantified analysis from multiple experiments performed as in (C), but with additional 992 concentrations. **(E)** Supernatant cultures were collected from 4d Th17 differentiation assays and measure via ELISA for IL-17 production. **(F)** 4d Th17 skewed cell cultures were harvested for qRT-PCR of *Il17a* and *Rorc* gene transcripts. Data shown are representative of at least 3 independent experiments, mean +/- SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 4: ACC inhibition supports *in vitro* Treg generation. **(A)** Naïve WT $CD4^+$ T cells were stimulated under Treg conditions for 4d and analyzed by flow cytometry for FoxP3 and CFSE positive T cells. **(B)** Analysis of FoxP3 percent and number positive cells from multiple experiments performed as in (A) (n=3). Data shown are representative of at least 3 independent experiments, mean +/- SEM.

Figure 5: 24h addition of ACC inhibitor 992 promotes Treg differentiation while inhibiting proliferation of non Treg-skewed cells. **(A)** Naïve WT $CD4^+$ T cells were stimulated under Treg conditions for 4d and analyzed by flow cytometry for FoxP3 and CFSE positive T cells. Indicated inhibitors and their concentrations were added 24h into stimulation cultures. **(B)** Analysis of FoxP3 percent and number positive cells from multiple experiments performed as in (A) (n=3). **(C)** Numbers of FoxP3 positive and negative expression of harvested T cells from (A) were graphed separately as total numbers for each population. **(D)** 4d Treg skewed cell cultures were harvested for qRT-PCR of the *Foxp3* gene transcript. Data shown are representative of at least 3 independent experiments, mean +/- SEM. * $p < 0.05$

Figure 6: The ACC inhibitor 992 delays induction of clinical EAE symptoms in mice, as well as reduce incidence and severity of disease. **(A)** Clinical scores of EAE-induced WT mice treated with vehicle or 992 day 2 after MOG immunization (mean +/- SEM, n = 8 for vehicle, n = 10 for 992). **(B)** Graphed analysis and FACS plots of IFNγ and IL-17A positive $CD4^+$ T cells in the spinal cord of EAE-induced mice sacrificed day 16 post immunization from (A). **(C)** Treg percent positive populations in the lymph node and spinal cord of mice from (A). Identified by $CD4^+CD25^+FoxP3^+$ expression. **(D)** Overall $CD4^+$ populations in the spinal cord of both cohorts from (A). **(E)** IL-17 and IFNγ cytokine production in 3 day MOG restimulated splenocytes harvested from the same mice as used in (A) (n = 5 for each cohort) (mean +/- SEM).

Figure 1

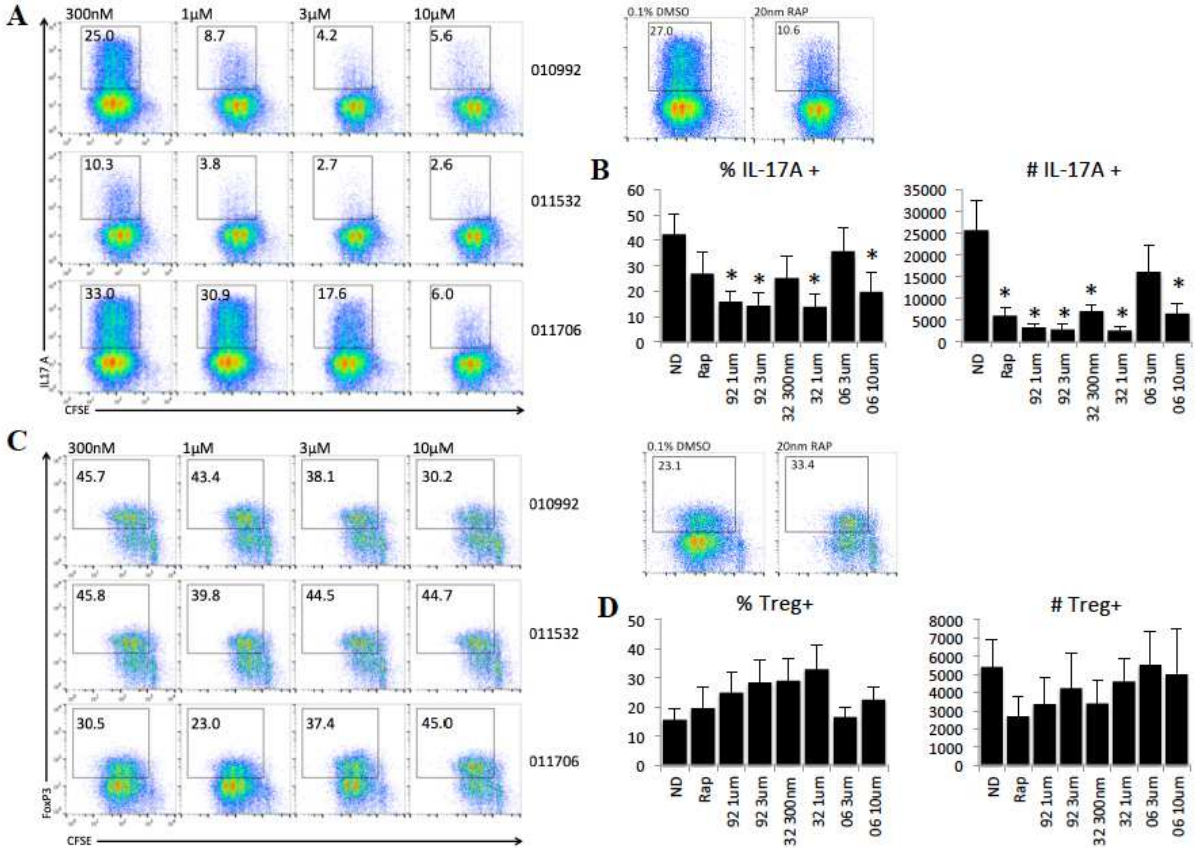

A
300nM
1µM
3µM
10µM
25.0
8.7
4.2
5.6
010992
10.3
3.8
2.7
2.6
011532
33.0
30.9
17.6
6.0
011706
IL17A
CFSE
0.1% DMSO
20nm RAP
27.0
10.6
B
% IL-17A +
# IL-17A +
ND
Rap
92 1um
92 3um
32 300nm
32 1um
06 3um
06 10um
C
45.7
43.4
38.1
30.2
010992
45.8
39.8
44.5
44.7
011532
30.5
23.0
37.4
45.0
011706
FoxP3
CFSE
0.1% DMSO
20nm RAP
23.1
33.4
D
% Treg+
# Treg+

Figure 2

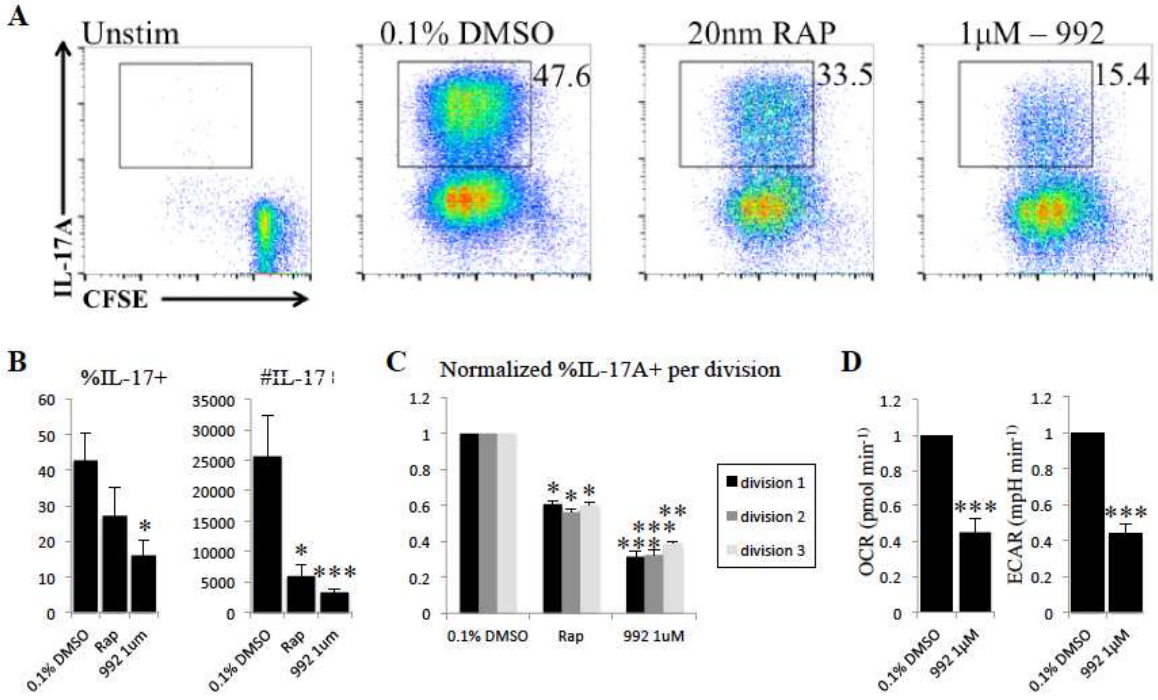

A
Unstim
0.1% DMSO
20nm RAP
1μM – 992
47.6
33.5
15.4
IL-17A
CFSE
B
%IL-17+
#IL-17+
0.1% DMSO
Rap
992 1um
C
Normalized %IL-17A+ per division
division 1
division 2
division 3
0.1% DMSO
Rap
992 1uM
D
OCR (pmol min-1)
ECAR (mpH min-1)
0.1% DMSO
992 1μM

Figure 3

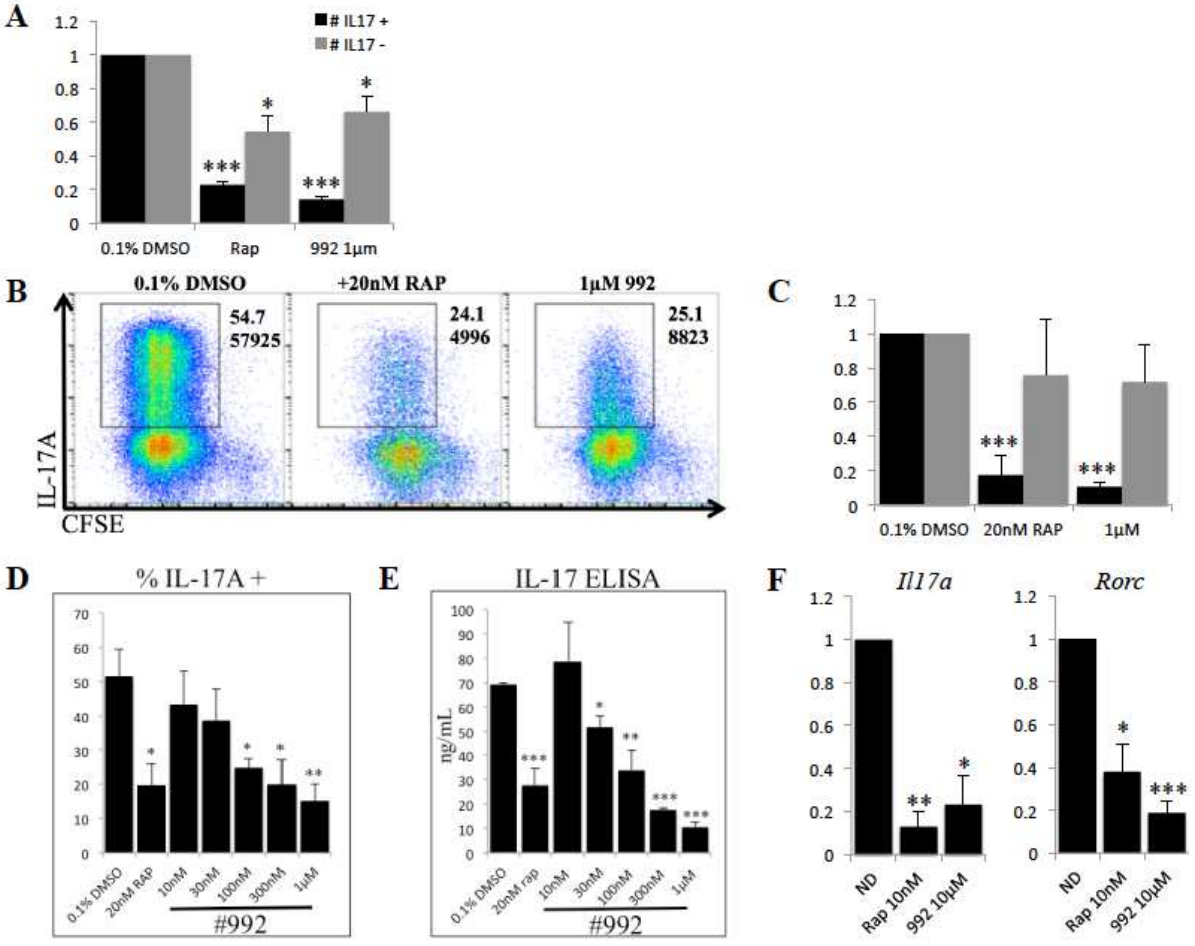

A
IL17 +
IL17 -
0.1% DMSO
Rap
992 1µm
B
0.1% DMSO
+20nM RAP
1µM 992
54.7
57925
24.1
4996
25.1
8823
IL-17A
CFSE
C
0.1% DMSO
20nM RAP
1µM
D
% IL-17A +
0.1% DMSO
20nM RAP
10nM
30nM
100nM
300nM
1µM
#992
E
IL-17 ELISA
ng/mL
0.1% DMSO
20nM rap
10nM
30nM
100nM
300nM
1µM
#992
F
Il17a
Rorc
ND
Rap 10nM
992 10µM

Figure 4

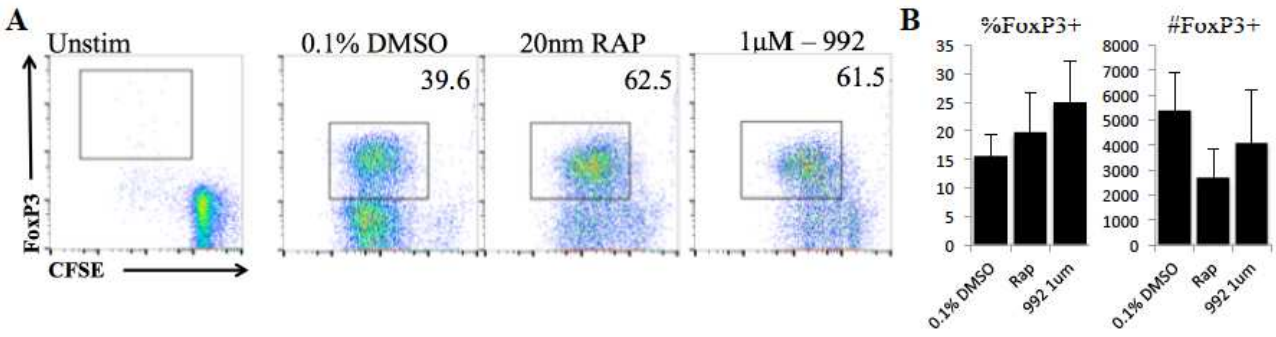

A
Unstim
0.1% DMSO
20nm RAP
1μM – 992
39.6
62.5
61.5
FoxP3
CFSE
B
%FoxP3+
#FoxP3+
35
30
25
20
15
10
5
0
8000
7000
6000
5000
4000
3000
2000
1000
0
0.1% DMSO
Rap
992 1um
0.1% DMSO
Rap
992 1um

Figure 5

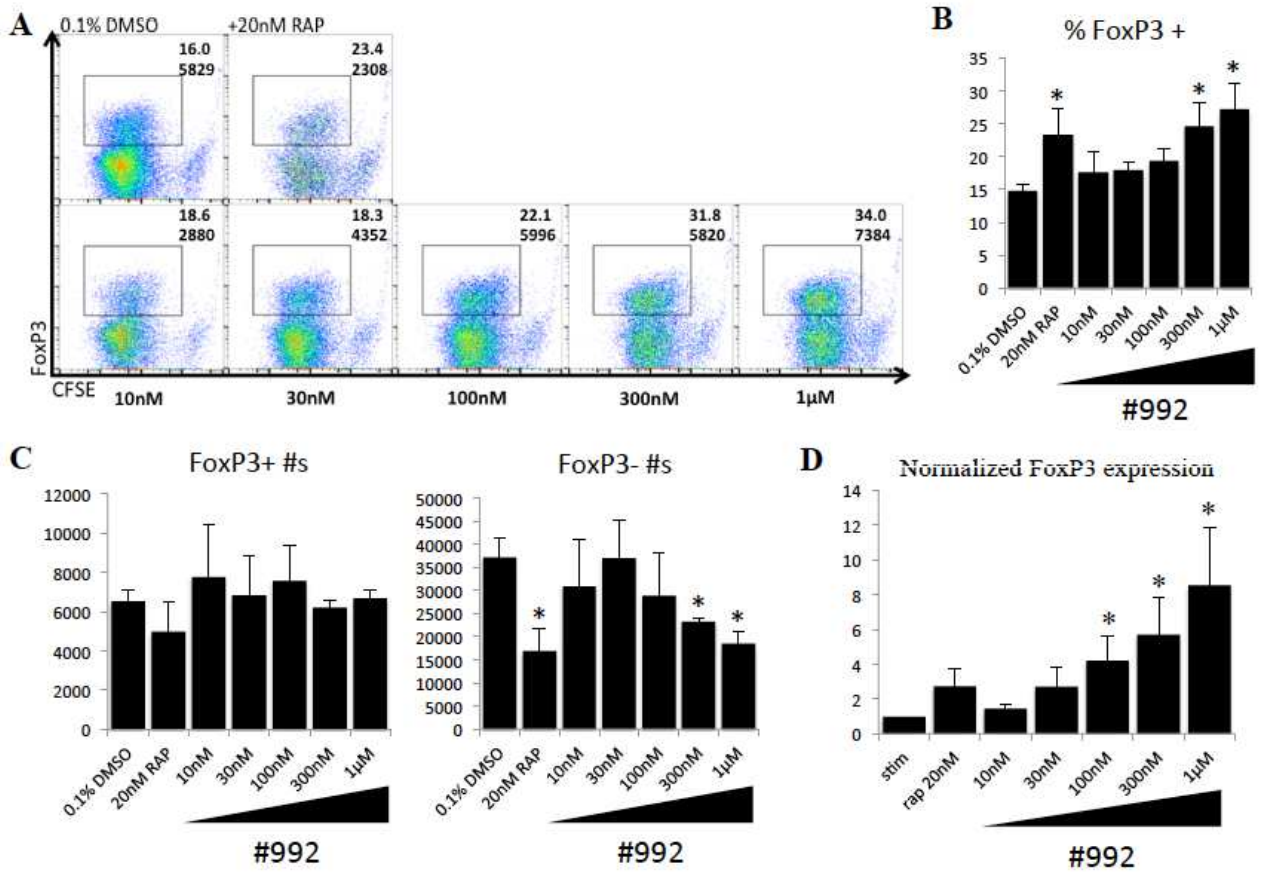
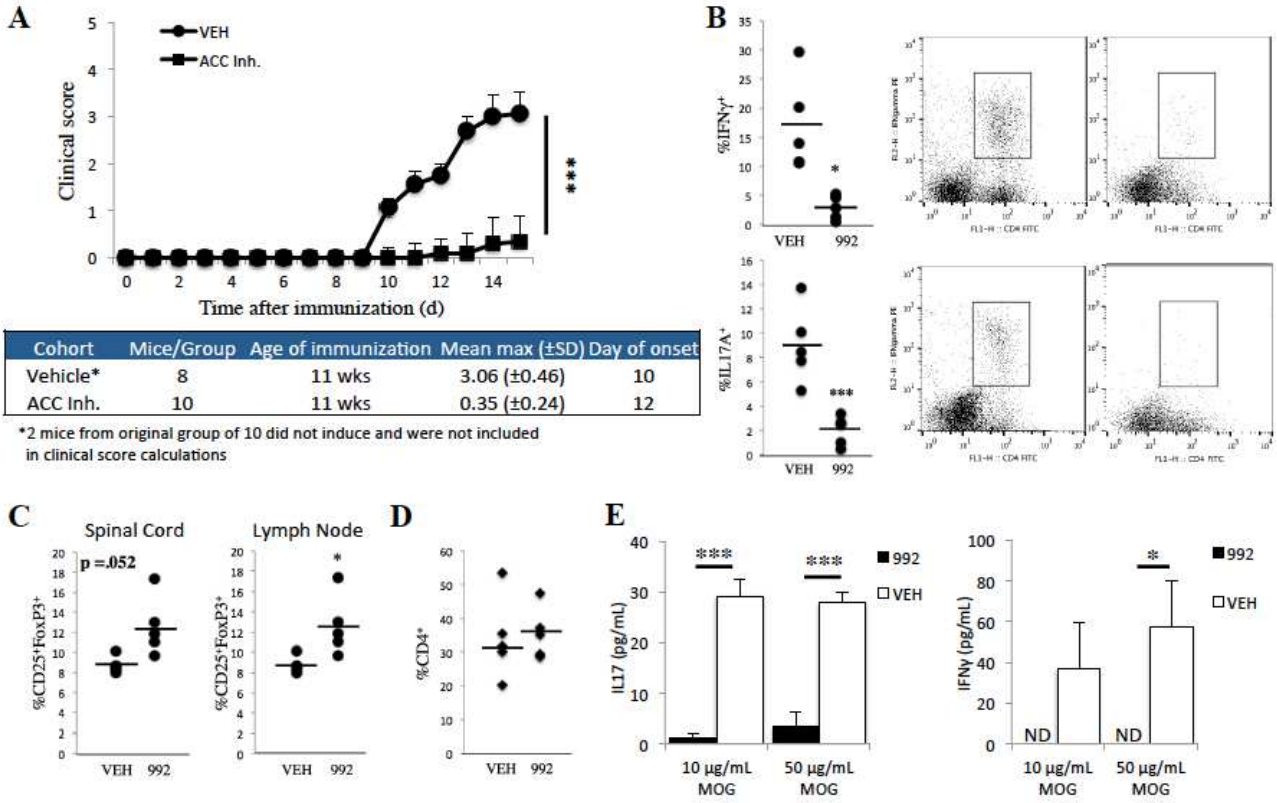

A
0.1% DMSO
+20nM RAP
16.0
5829
23.4
2308
18.6
2880
18.3
4352
22.1
5996
31.8
5820
34.0
7384
FoxP3
CFSE
10nM
30nM
100nM
300nM
1µM
B
% FoxP3 +
0.1% DMSO
20nM RAP
10nM
30nM
100nM
300nM
1µM
#992
C
FoxP3+ #s
FoxP3- #s
0.1% DMSO
20nM RAP
10nM
30nM
100nM
300nM
1µM
#992
D
Normalized FoxP3 expression
stim
rap 20nM
10nM
30nM
100nM
300nM
1µM
#992

Figure 6



Cohort	Mice/Group	Age of immunization	Mean max (±SD)	Day of onset
Vehicle*	8	11 wks	3.06 (±0.46)	10
ACC Inh.	10	11 wks	0.35 (±0.24)	12

*2 mice from original group of 10 did not induce and were not included in clinical score calculations

Chapter 4

Discussion

Autoimmune diseases are collectively a major health problem that have been increasing in prevalence, with many that consist of chronic and life-threatening symptoms. Frequently, patients suffering from autoimmune diseases only have intermittent and unspecific symptoms up until the disease becomes acute. Commonly prescribed immunosuppressants used to treat these major symptoms all involve unpleasant side effects that, when used long term, can sometimes also lead to life-threatening conditions. These side effects stem from nonspecific immunosuppression of our immune system that can lead to opportunistic infections and malignancies of otherwise healthy tissues (e.g. bone marrow and muscles like the heart). The need to develop selective immunosuppressant therapies is essential to ensure an effective therapy that can target the proinflammatory responses that cause autoimmunity, yet spare both regulatory immune responses and integral host defense systems. This type of targeted treatment could lead to a better-tolerated option that may be used long-term with less risk. The goal of this thesis was to elucidate pathways in $CD4^+$ T cells that were required for proinflammatory immune responses and could be selectively targeted to suppress autoimmunity. Specifically, we sought to selectively target Th17 responses in autoimmunity by evaluating the effects of inhibiting the mTORC1 effector S6K2. We choose S6K2 based on the potent inhibitory effect Rapamycin has on lymphocytes, as well as previous studies focused on S6Ks that suggest a role in Th17 differentiation. We also focused on the lipid metabolism enzyme ACC, as an earlier study strongly suggested inhibition of ACC to deliver a targeted immunosuppression by inhibiting Th17 differentiation and increasing Treg generation. This chapter separates our summaries and discussion for the studies and results generated for S6K2 and ACC.

S6K2 conclusions

We first hypothesized that targeting downstream effectors of mTORC1 could potentially separate its roles in proliferation and effector function in T cells. To see if S6K2 played a role in Th17 differentiation and could be used as a therapeutic target, we characterized the S6K2 knockout mouse and found normal homeostatic and proliferative T cell responses. Though we found significantly reduced Th17 differentiation and increased Treg generation in the S6K2 knockout with *in vitro* skewing cultures, this was determined to be dependent on the culturing media used. A switch from RPMI and IMDM restored the Th17 skewing to wildtype levels and this was corroborated with our EAE studies, which found no differences between knockout and wildtype cohorts.

We described a Th17 differentiation phenotype that was dependent on the *in vitro* culturing conditions. A main point that can be drawn from this observation is that preliminary characterization studies should consider and utilize all possible protocols before planning translational studies that involve *in vivo* models. The benefit to this would be to better gauge observed phenotypes with context to the given microenvironment, which could help choose a better-suited *in vivo* study. A survey of recently published journal articles involving mouse *in vitro* Th17 differentiation studies revealed a mix of protocols used that included RPMI, IMDM, or even no mention of culturing media used. Based on our own results from the *in vitro* S6K2$^{-/-}$ mouse studies, it would have been more optimal to utilize more than one *in vivo* autoimmune mouse model to better evaluate the potential for disease amelioration in S6K2$^{-/-}$ mice.

Our *in vivo* S6K2 studies showed no difference between *S6K2*$^{-/-}$ and wildtype cohorts in clinical score and inflammatory response, as IFNγ and IL-17A expressing T cells in the spinal cord were comparable populations. Though this fits with our later *in vitro* skewing

assays, the question still remains if the increased Treg population seen in *S6K2*$^{-/-}$ mice might still hold potential in alleviating autoimmune T cell responses. *S6K2*$^{-/-}$ T cells increased Treg generation in both IMDM and RPMI media, which suggests potential in testing these mice in other autoimmune models such as psoriasis.

To further explore shared and differential roles of S6Ks in lymphocytes, utilization of both mouse models and human samples could provide valuable insight. To acutely address the role of S6Ks in T cells, genetic mouse models could be generated with T cell specific deletion of S6K1, S6K2, or both. Because our findings and other suggest dispensable roles for each S6K in activated CD4^{+} T cell function and whole body S6K knockouts display perinatal lethality, an inducible CD4-cre system with deletion of total S6K activity would be helpful in understanding its complete role in T cell differentiation and function. In addition to characterizing total S6K activity, targeted S6K2 studies in which Co-IPs of S6K2 and bound proteins in the cytoplasm and nucleus of activated cells could be paired with a mouse model generated with targeted disruption of its NLS at the C-terminus domain. This would be helpful in understanding its interaction with other proteins in both cellular compartments, as well as its role in shuttling proteins in the nucleus (such as RORγ). In stark contrast to cancer related studies, S6K signaling and function has not been characterized in normal human lymphocytes. Functional studies of S6K1 and S6K2 have been generated in mostly mouse and transformed cell lines, which often does not translate cleanly to humans. Using human T cells in characterizing S6K1 and S6K2 roles with *in vitro* T cell activation signaling and differentiation assays would be key in reaffirming earlier studies done in mouse models.

As discussed in Chapter 2, others have detailed the overexpression of S6K2 in cancer, with emphasis in breast cancer (1). This is often seen along with 4EBP1 overexpression, but not S6K1. Sridharan *et al.* utilized cancer cells to show S6K2 expression contributed to an increase in TNF-induced Akt phosphorylation (2). Increase in Akt phosphorylation led to

downregulation of the proapoptotic protein Bid, which was reversed when S6K2 was silenced via knockdown. Thus, a clear case of S6K2 promoting cell survival in breast cancer cell lines has been established.

Another area of interest in cancer that could be further characterized is the interaction between S6K2 and the transcriptional regulator Yin Yang 1 (YY1) (3). S6K2 and YY1 can be detected as a complex along with mTOR in the nucleus upon serum starvation or mitogenic stimulation. The relationship between YY1 and cancer can be hypothesized through its association with the chromatin-modifying enzyme Ezh2 (4). Ezh2 has important roles in cancer, as it silences tumor suppressors (5, 6). YY1 may benefit from the NLS in the C-terminus domain of S6K2, and activation of Ezh2 by YY1 in the nucleus could be reduced by S6K2 inhibition.

Expression profiling of breast cancer patients also suggested S6K2 to be established as a clinical marker, as expression levels correlated with prognostic outcome (7). Paired with our preliminary findings that S6K2 is dispensable for basic T effector function, it would reasonable to consider S6K2 as a cancer therapy target without the potential of affecting immune responses. Also, our signaling studies showed differential Akt regulation than Sridharan and colleagues, as activated S6K2 knockout T cells do not have significantly decreased levels of phosphorylated Akt (2). In conclusion, this could indicate that S6K1 is the predominant S6K in T cell fate and function, which is correlated by the compensatory upregulation of S6K2 expression in S6K1 knockout mice but not vice versa.

ACC conclusions

Our second hypothesis was to inhibit T cell driven autoimmune responses by selectively targeting a component of proinflammatory associated metabolism. Targeting ACC proved to be effective in inhibiting Th17 differentiation while promoting Treg generation. *In vitro*

skewing cultures showed ACC inhibition to be highly significant in reducing Th17 differentiation, even when added 24 hours after start of stimulation. Later addition of ACC inhibitor did not affect overall proliferation of $CD4^+$ T cells, yet retained the capacity to selectively target Th17 differentiated cells and promote Treg generation. A main focus of this thesis was to identify a regulatory pathway that would be crucial for inflammatory T cell proliferation and function, but dispensable to other subsets that include ones that regulates immune responses. Inhibiting ACC *in vitro* at the start or 24 hours into simulation satisfied this goal, as we were able to show selectivity in significantly reducing Th17 differentiation, while promoting Treg generation. EAE studies also showed promising *in vivo* results, where mice dosed with ACC inhibitor showed delayed, as well as significantly decreased severity and incidence of clinical disease scores. In addition to decreasing both proinflammatory Th1 and Th17 cytokine production, Treg populations in the ACC inhibitor treated mice were not only spared but also significantly increased in the cervical draining lymph node. Importantly, the total percent of $CD4^+$ T cells detected in the spinal cord of the treated mice was no different than the vehicle cohort. The promising *in vivo* results corroborates with the earlier *in vitro* data to strongly suggest ACC inhibition to target only proinflammatory $CD4^+$ T cell responses, specifically the Th17 subset. In summary, though we found S6K2 to be dispensable in proinflammatory Th17 responses, lipid metabolism controlled by the enzyme ACC proved to be essential in Th17 differentiation and function.

Our ACC inhibition studies in $CD4^+$ T cells showed a selective inhibition of Th17 responses, while sparing Tregs and generally promoting their differentiation. The promising EAE data shows that lipid metabolism can be successfully targeted to inhibit proinflammatory responses and overall improve clinical disease scores. Because ACC currently cannot be selectively inhibited in only T cells, it will be important to characterize its effects in other immune cell types in addition to the other T cell subsets. Current literature

has already confirmed ACC1 to be required for $CD8^+$ T cell expansion and viability, but not function. Studies underway have begun to characterize the effect of ACC inhibition in Th2 responses, which could also lead to potential therapeutic outcome in treating type 1 hypersensitivity related diseases (unpublished data). However, if future studies do point towards limitation of ACC inhibition treatment due to side effects, it may still be of use as a topical application (e.g. eczema and psoriasis). In summary, there are a number of autoimmune disorders driven my proinflammatory $CD4^+$ T cell subsets that stand to potentially benefit from ACC inhibition, which would call for studies in additional inflammatory disease mouse models (e.g. colitis, psoriasis, collagen induced arthritis).

Targeting ACC in cancer can offer an effective strategy to selectively target cancerous cells because of their altered metabolism. The previously described Warburg effect is seen in most cancers, where glycolysis is elevated and the catabolic end product pyruvate is produced in excess (8). While most of the pyruvate is converted to lactate, it is also shunted into the TCA cycle to generate a source of acetyl-CoA. ACC enters the picture here, as acetyl-CoA is utilized for *de novo* fatty acid synthesis. Lipogenic enzymes are often overexpressed in human cancers, which includes fatty acid synthase (FASN) (9). Previous studies have shown ACC inhibition to induce apoptosis in prostate and breast cancer cells because of the depletion of palmitic acid, the end product of *de novo* fatty acid synthesis (10). Aside from treating autoimmune disorders such as NASH and multiple sclerosis, ACC could be utilized as a neoplastic agent or sensitizer to chemotherapeutics.

Concluding Remarks

Patients suffering from a variety of autoimmune diseases frequently deal with the constant threat of a dysfunctional inflammatory response. Powerful immunosuppressants are utilized to prevent many T cell mediated diseases, including transplant rejection and

inflammation of the gut. These drugs deliberately induce a compromised immune system that often results in opportunistic infections and weakened ability to detect cancer cells. Sirolimus (Rapamycin) is one such drug that has been extensively studied and can provide insight into selectively suppressing target immune cells. Although our S6K2 studies and previous studies on S6K1 have shown a dispensable role in the T cell mediated autoimmune mouse model EAE, it is important to continue elucidating downstream effectors of mTOR. It is reasonable to hypothesize that inhibiting selected downstream targets of mTOR may recapitulate the potent effects of rapamycin on lymphocytes. If this is true, then characterization of these targets may eventually lead to a selective inhibition of proinflammatory associated T cell subsets. Selectively inhibiting these same T cell subsets by targeting their metabolic processes was demonstrated by us and others in inhibiting ACC. Because certain activated immune cells like Th17 reprogram metabolism to elevate glycolysis and fatty acid synthesis, information gained from parallel research in cancer can provide immunologists with an extensive panel of metabolic enzyme targets that could be potentially used in inhibiting inflammatory responses as well. As emphasized in this dissertation, avoiding broad immunosuppression in treating autoimmunity is highly desired and the study of both downstream mTOR effectors and metabolic processes could lead to the discovery of a potent and selective immunotherapy.

References:

1. Pardo, O. E., and M. J. Seckl. 2013. S6K2: The Neglected S6 Kinase Family Member. *Front Oncol* 3: 191.

2. Sridharan, S., and A. Basu. 2011. S6 kinase 2 promotes breast cancer cell survival via Akt. *Cancer Res* 71: 2590–2599.

3. Ismail, H. M., O. Myronova, Y. Tsuchiya, A. Niewiarowski, I. Tsaneva, and I. Gout. 2013. Identification of the general transcription factor Yin Yang 1 as a novel and specific binding partner for S6 kinase 2. *Cell Signal* 25: 1054–1063.

4. Caretti, G., M. Di Padova, B. Micales, G. E. Lyons, and V. Sartorelli. 2004. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. *Genes Dev* 18: 2627–2638.

5. Kim, K. H., and C. W. Roberts. 2016. Targeting EZH2 in cancer. *Nat Med* 22: 128–134.

6. Yoo, K. H., and L. Hennighausen. 2012. EZH2 methyltransferase and H3K27 methylation in breast cancer. *Int J Biol Sci* 8: 59–65.

7. Karlsson, E., I. Magić, J. Bostner, C. Dyrager, F. Lysholm, A. L. Hallbeck, O. Stål, and P. Lundström. 2015. Revealing Different Roles of the mTOR-Targets S6K1 and S6K2 in Breast Cancer by Expression Profiling and Structural Analysis. *PLoS ONE* 10: e0145013.

8. Vander Heiden, M. G., L. C. Cantley, and C. B. Thompson. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324: 1029–1033.

9. Mashima, T., H. Seimiya, and T. Tsuruo. 2009. De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy. *Br J Cancer* 100: 1369–1372.

10. Chajès, V., M. Cambot, K. Moreau, G. M. Lenoir, and V. Joulin. 2006. Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. *Cancer Res* 66: 5287–5294.