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# Pten Signaling in Regulatory T Cells and Inflammatory Disease

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# Pten Signaling in Regulatory T Cells and Inflammatory Disease

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**PTEN SIGNALING IN REGULATORY T CELLS  
AND INFLAMMATORY DISEASE**

A Dissertation  
Presented for  
The Graduate Studies Council  
The University of Tennessee  
Health Science Center

In Partial Fulfillment  
Of the Requirements for the Degree  
Doctor of Philosophy  
From The University of Tennessee

By  
Sharad Krishna Shrestha  
May 2016

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## **DEDICATION**

I dedicate this work to my parents,  
Dr. Bharat Shrestha and Sheemela Shrestha,  
and to my wife Jyoti Bastola

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## ABSTRACT

Regulatory T (Treg) cells suppress CD4<sup>+</sup> T cell responses during homeostasis and inflammation to prevent autoimmunity and other immune disorders. Although the transcriptional and epigenetic programs impacting Treg cell function have been extensively studied, the signaling and metabolic pathways underlying Treg stability and function are not fully understood. In this study, we determined the role of the phosphatase PTEN in Treg cells. We found that specific depletion of PTEN in Treg cells results in excessive T<sub>H</sub>1 and T follicular helper cells (T<sub>FH</sub>) responses, associated with elevated germinal center (GC) B cells and spontaneous development of autoimmune and lymphoproliferative disease *in vivo*. Interestingly, the exaggerated T<sub>FH</sub> and GC responses and autoimmune symptoms are suppressed when IFN- $\gamma$  expression is abrogated in mice containing *Pten*-deficient Treg cells. Thus, the uncontrolled T<sub>H</sub>1-mediated inflammation in these mice drives aberrant T<sub>FH</sub> responses and autoimmune and lymphoproliferative disease. Mechanistically, we linked PTEN to mTORC2-mediated control of transcriptional and metabolic programs that enforce Treg cell stability and function. Consistent with this notion, deletion of *Rictor*, the obligate component for mTORC2, restores Treg cell function and stability in the absence of *Pten*. Similarly, partially restoring the activity of Foxo1, a downstream transcription factor negatively regulated by mTORC2 signaling, also largely rectified the defects of PTEN-deficient Treg cells. Together, these results establish that Treg cells rely on the PTEN-mTORC2-Foxo1 axis to maintain their stability and suppressive activity in controlling T<sub>H</sub>1 and T<sub>FH</sub> cell responses.

## TABLE OF CONTENTS

<b>CHAPTER 1. INTRODUCTION .....</b>	<b>1</b>
Overview.....	1
T Cell Development, Homeostasis, and Differentiation.....	1
Differentiation of Conventional CD4 <sup>+</sup> T Cells.....	2
T <sub>H</sub> 1 cells.....	2
T <sub>H</sub> 2 cells.....	4
T <sub>H</sub> 17 cells.....	5
T <sub>FH</sub> cells.....	6
Treg Cells.....	7
Foxp3 is the master transcription factor of Treg cells.....	7
Stability of Treg cells.....	9
Heterogeneity of the Foxp3 <sup>+</sup> Treg cell population.....	10
<i>tTreg cells</i> .....	10
<i>pTreg and iTreg cells</i> .....	11
T follicular regulatory cells.....	11
mTOR Signaling Pathway.....	12
Regulation of mTOR activity.....	12
mTOR in T helper cell quiescence and differentiation.....	15
mTOR in Treg cell differentiation and function.....	17
mTOR and T cell metabolism.....	18
Phosphatase and Tensin Homolog (PTEN).....	18
Structure and regulation of PTEN.....	19
Activation of mTOR by PTEN.....	21
Regulation and roles of PTEN in T cells.....	21
Rationale and Hypothesis.....	22
Conclusion.....	22
 <b>CHAPTER 2. METHODS AND MATERIALS.....</b>	 <b>23</b>
Mice.....	23
Bone Marrow Chimera.....	23
Polymerase Chain Reaction.....	23
Single Cell Suspension.....	23
Cell Purification.....	26
Flow Cytometry.....	26
Intracellular Staining.....	26
Cell Proliferation Assay.....	28
Active Caspase-3 Staining.....	28
Active Mitochondria Staining.....	29
Immunofluorescence.....	29
Immunohistochemistry.....	29
Immunization.....	30
Serum Antibodies.....	30
Adoptive Transfer.....	30

Cell Counting.....	30
Quantitative RT-PCR.....	31
Immunoblotting .....	31
Glycolysis Assay.....	31
Gene-expression Profiling and Gene-set Enrichment Analysis.....	31
Statistical Analysis.....	32
<b>CHAPTER 3. CHARACTERIZATION OF IMMUNOLOGICAL EFFECTS MEDIATED BY PTEN SIGNALING IN TREG CELLS .....</b>	<b>33</b>
Introduction.....	33
Treg Cell-specific Deletion of PTEN Precipitates an Inflammatory Disease .....	33
Generation of Pten-deficient Treg cell mice .....	33
Phenotypic characterization of <i>Pten<sup>fl/fl</sup>Foxp3-Cre</i> mice .....	34
Disruption of immune system in <i>Pten<sup>fl/fl</sup>Foxp3-Cre</i> mice .....	34
Altered Immune Homeostasis upon Treg-specific Loss of PTEN .....	41
PTEN is essential for Treg cell-mediated immune homeostasis.....	41
Cell death and proliferation in Pten-deficient Treg cells .....	41
Uncontrolled T <sub>FH</sub> Responses and GC B Cells in Pten-deficient Treg Mice.....	41
Coordination of T <sub>H</sub> 1 and T <sub>FH</sub> Responses by PTEN Signaling in Treg Cells.....	46
PTEN Is Crucial in Maintaining the Stability of Treg Cells.....	52
PTEN Is Haploinsufficient in Treg Cells.....	60
Summary.....	64
<b>CHAPTER 4. MOLECULAR AND METABOLIC MECHANISMS OF PTEN SIGNALING.....</b>	<b>68</b>
PTEN-dependent Transcriptional and Metabolic Programs in Treg Cells.....	68
PTEN Restrains mTORC2 Activity in Treg Cells.....	72
Nuclear Retention of Foxo1 Abrogates the Effects of PTEN Loss .....	78
Summary.....	78
<b>CHAPTER 5. DISCUSSION .....</b>	<b>87</b>
<b>LIST OF REFERENCES.....</b>	<b>92</b>
<b>APPENDIX. TSC1 PROMOTES THE DIFFERENTIATION OF MEMORY CD8<sup>+</sup> T CELLS VIA ORCHESTRATING THE TRANSCRIPTIONAL AND METABOLIC PROGRAMS .....</b>	<b>119</b>
Introduction.....	119
Results.....	121
Tsc1-deficient T cells mount normal effector responses but are impaired to transition into memory cells.....	121
TSC1 deletion impairs the recall response of memory cells.....	124
A cell-intrinsic requirement of TSC1 in memory formation and function .....	128
Impaired differentiation of memory precursors in the absence of TSC1 .....	128
Tsc1-dependent gene expression programs in antigen-specific CD8 <sup>+</sup> T cells.....	133
Dysregulated mTORC1 and metabolic activities in Tsc1-deficient CD8 <sup>+</sup> T cells ..	135

Discussion.....	139
Experimental Procedures .....	141
Mice .....	141
<i>Listeria monocytogenes</i> infection .....	141
Flow cytometry .....	142
Cell purification and culture .....	142
Cell isolation and adoptive transfer .....	142
Metabolic assay.....	143
Gene expression profiling by microarray analysis.....	143
RNA, genomic DNA, and immunoblot analyses.....	143
Statistical analysis.....	143
<b>VITA.....</b>	<b>144</b>

## LIST OF TABLES

Table 2-1.	List of PCR primers used for PCR.....	24
Table 2-2.	PCR program used to amplify gene of interest.....	25
Table 2-3.	Characterization of innate and adaptive immune cell surface and intracellular markers.....	27



## LIST OF FIGURES

Figure 1-1.	Schematic representation of conventional helper T cell diversity. ....	3
Figure 1-2.	Multiple signals and differentiation stages are involved in T <sub>FH</sub> and germinal center B cell development. ....	8
Figure 1-3.	Schematic diagram showing associated protein components of two mTOR complexes, mTORC1 and mTORC2. ....	13
Figure 1-4.	mTOR signaling pathway .....	14
Figure 1-5.	mTOR signaling controls peripheral T cell fate decisions.....	16
Figure 1-6.	PTEN gene structure. ....	20
Figure 3-1.	Analysis of <i>Pten</i> mRNA and PTEN protein expression in Treg cells. ....	35
Figure 3-2.	Age-related lymphoproliferative disease seen in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ...	36
Figure 3-3.	<i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice develop spontaneous systemic autoimmune disease. ....	37
Figure 3-4.	Dysregulation of immunoglobulins isotype in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	38
Figure 3-5.	Structural integrity of the kidney in WT and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice.....	39
Figure 3-6.	Imaging analysis of kidney glomeruli of <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice.....	40
Figure 3-7.	Flow cytometric analysis of different immune cell populations.....	42
Figure 3-8.	Altered T cell homeostasis in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	43
Figure 3-9.	T <sub>H</sub> 1 immune responses are increased in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	44
Figure 3-10.	<i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice have an increased frequency of Treg cells. ....	45
Figure 3-11.	Aberrant T <sub>FH</sub> , T <sub>FR</sub> and GC B cells responses in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	47
Figure 3-12.	Analysis of T <sub>FH</sub> and GC B cells. ....	48
Figure 3-13.	Immunohistochemistry of germinal centers in WT and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	49
Figure 3-14.	Induction of germinal center responses in WT and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	50

Figure 3-15. Analysis of bone marrow-derived chimeras reveals dysregulated T <sub>FH</sub> and GC B cell responses and IFN- $\gamma$ overproduction in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	51
Figure 3-16. Generation and phenotypic analysis of <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre <i>Ifng</i> <sup>-/-</sup> mice. ....	53
Figure 3-17. Uncontrolled T <sub>FH</sub> and GC B cells in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice rescued in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre <i>Ifng</i> <sup>-/-</sup> mice. ....	54
Figure 3-18. Immunohistochemistry of germinal centers in WT, <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre, <i>Ifng</i> <sup>-/-</sup> and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre <i>Ifng</i> <sup>-/-</sup> mice. ....	55
Figure 3-19. Detection of antinuclear antibodies in WT, <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre, <i>Ifng</i> <sup>-/-</sup> and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre <i>Ifng</i> <sup>-/-</sup> mice. ....	56
Figure 3-20. Immunofluorescence analysis of glomeruli for IgG deposits in WT, <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre, <i>Ifng</i> <sup>-/-</sup> and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre <i>Ifng</i> <sup>-/-</sup> mice. ....	57
Figure 3-21. Analysis of Treg cells in WT, <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre, <i>Ifng</i> <sup>-/-</sup> and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre <i>Ifng</i> <sup>-/-</sup> mice. ....	58
Figure 3-22. Effects of <i>Pten</i> loss in Treg cells. ....	59
Figure 3-23. <i>Pten</i> deficiency impairs Treg cell stability. ....	61
Figure 3-24. Analysis of Treg cells in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre <i>Ifng</i> <sup>-/-</sup> mice and <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre Treg cell phenotypes under steady state. ....	62
Figure 3-25. <i>Pten</i> expression and immune dysregulation in <i>Pten</i> <sup>fl/+</sup> <i>Foxp3</i> -Cre mice. ....	63
Figure 3-26. Heterozygous loss of <i>Pten</i> in Treg cells is sufficient to disrupt immune homeostasis. ....	65
Figure 3-27. Detection of antinuclear antibodies in WT and <i>Pten</i> <sup>fl/+</sup> <i>Foxp3</i> -Cre mice. ....	66
Figure 3-28. Immunofluorescence analysis of glomeruli for IgG deposits in WT and <i>Pten</i> <sup>fl/+</sup> <i>Foxp3</i> -Cre mice. ....	67
Figure 4-1. The transcriptional profiles controlled by <i>Pten</i> in Treg cells. ....	69
Figure 4-2. PTEN-dependent gene expression analysis. ....	70
Figure 4-3. Ingenuity pathway analysis (IPA) of the differentially expressed genes in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre Treg cells. ....	71
Figure 4-4. PTEN-dependent metabolic programs in Treg cells. ....	73
Figure 4-5. Dysregulation of mTORC2 activity in <i>Pten</i> -deficient Treg cells. ....	74

Figure 4-6.	Analysis of Pten expression in short and long term stimulated Treg cells. ...	75
Figure 4-7.	Analysis of Treg cells in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre and <i>Pten</i> <sup>fl/fl</sup> <i>Rictor</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice under steady state.....	76
Figure 4-8.	<i>Pten</i> represses mTORC2 signaling in Treg cells to maintain immune homeostasis. ....	77
Figure 4-9.	Analysis of T <sub>FH</sub> and GC B cells in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre and <i>Pten</i> <sup>fl/fl</sup> <i>Rictor</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice under steady state.....	79
Figure 4-10.	Immunofluorescence analysis of glomeruli for IgG deposits in WT, <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre, <i>Rictor</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre and <i>Pten</i> <sup>fl/fl</sup> <i>Rictor</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice.....	80
Figure 4-11.	Immunohistochemistry of germinal centers in WT, <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre, <i>Rictor</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre and <i>Pten</i> <sup>fl/fl</sup> <i>Rictor</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre mice. ....	81
Figure 4-12.	Analysis of <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre and <i>Pten</i> <sup>fl/fl</sup> <i>Foxo1</i> <sup>AAA/+</sup> <i>Foxp3</i> -Cre mice under steady state. ....	82
Figure 4-13.	Analysis of T <sub>FH</sub> and GC B cells in <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre and <i>Pten</i> <sup>fl/fl</sup> <i>Foxo1</i> <sup>AAA/+</sup> <i>Foxp3</i> -Cre mice under steady state.....	83
Figure 4-14.	Detection of antinuclear antibodies in WT, <i>Pten</i> <sup>fl/fl</sup> <i>Foxp3</i> -Cre, <i>Foxo1</i> <sup>AAA/+</sup> <i>Foxp3</i> -Cre and <i>Pten</i> <sup>fl/fl</sup> <i>Foxo1</i> <sup>AAA/+</sup> <i>Foxp3</i> -Cre mice.....	84
Figure 4-15.	Analysis of Treg cells in <i>Pten</i> <sup>fl/fl</sup> <i>Foxo1</i> <sup>AAA/+</sup> <i>Foxp3</i> -Cre mice under steady state. ....	85
Figure 5-1.	Schematics of PTEN signaling in Treg cell functions and immune tolerance. ....	88
Figure A-1.	Normal homeostasis of CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells in <i>Tsc1</i> <sup>-/-</sup> mice under steady state. ....	122
Figure A-2.	TSC1 deficiency does not impact the effector response of CD8 <sup>+</sup> T cells...123	
Figure A-3.	The absence of TSC1 diminishes the generation of memory T cells. ....125	
Figure A-4.	Proliferation and survival of Tsc1-deficient CD8 <sup>+</sup> T cells during the contraction phase.....126	
Figure A-5.	TSC1 deficiency impairs the recall response of memory CD8 <sup>+</sup> T cells. ....127	
Figure A-6.	TSC1 promotes memory CD8 <sup>+</sup> T-cell generation through cell-intrinsic mechanisms. ....129	
Figure A-7.	TSC1 is required for the differentiation of MPECs. ....130	

Figure A-8. Intrinsic role of TSC1 in the differentiation of memory precursors. ....	131
Figure A-9. Expression of transcription factors implicated in memory T-cell differentiation. ....	132
Figure A-10. TSC1-dependent gene expression profiles in antigen-experienced CD8 <sup>+</sup> T cells. ....	134
Figure A-11. Dysregulated cell metabolism in Tsc1-deficient T cells. ....	136
Figure A-12. TSC1-dependent effects on cell size and glycolytic activity in activated <i>Tsc1</i> <sup>fl/fl</sup> CreER <sup>+</sup> OT-I <sup>+</sup> cells upon 4-OHT treatment. ....	137
Figure A-13. TSC1 deficiency leads to dysregulated mTORC1 activity and metabolism in activated CD8 <sup>+</sup> T cells. ....	138

## LIST OF ABBREVIATIONS

AHR	Airway hyperresponsiveness
AITL	Angioimmunoblastic T-cell lymphoma
ANA	Antinuclear antibodies
APC	Antigen presenting cell
Ascl2	Achaete-scute family bHLH transcription factor 2
ATP	Adenosine triphosphate
Bcl6	B-cell lymphoma 6 protein
BM	Bone marrow
BRRS	Bannayan-Riley-Ruvalcaba syndrome
BSA	Bovine serum albumin
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
CO <sub>2</sub>	Carbon dioxide
CpG	5'-C-phosphate-G-3'
CS	Cowden syndrome
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CTL	Cytotoxic T cell
CXCR5	C-X-C chemokine receptor type 5
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbent assay
FAO	Fatty acid oxidation
Foxo	Forkhead box O
Foxp1	Forkhead box P1
Foxp3	Forkhead box P3
GATA3	GATA binding protein 3
GC	Germinal center
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related protein
GO	Gene ontology
GSEA	Gene Set Enrichment Analysis
GTP	Guanosine-5'-triphosphate
H&E	Hematoxylin and eosin
HIF-1 $\alpha$	Hypoxia inducible factor 1 $\alpha$
HSC	Hematopoietic stem cells
ICOS	Inducible T-cell costimulator
IFN- $\gamma$	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin

IRF4	Interferon regulatory factor 4
iTreg	Induced Treg
JNK	c-Jun N-terminal protein kinases
KLF2	Krüpple-like factor 2
LPS	Lipopolysaccharide
MEKK4	Mitogen-Activated Protein Kinase Kinase Kinase 4
MFI	Mean fluorescence intensity
MgCl <sub>2</sub>	Magnesium chloride
MHC	Major histocompatibility complex
miRNA	microRNA
Mito	Mitochondria
MLN	Mesenteric lymph node
MMAC1	Mutated in multiple advanced cancers 1
MS	Multiple sclerosis
mTOR	Mechanistic target of rapamycin
NP	4-Hydroxy-3-nitrophenyl
OVA	Ovalbumin
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCTL-NOS	Peripheral T-cell lymphoma-not otherwise specified
PD-1	Programmed cell death protein 1
PHTS	PTEN Hamartoma Tumor Syndrome
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PLN	Peripheral lymph node
PLS	Proteus-like syndrome
PNA	Peanut agglutinin
PPAR	Peroxisome proliferator-activated receptors
PRAS40	Proline-rich AKT substrate of 40-kDa
PS	Proteus syndrome
PSGL1	P-selectin glycoprotein ligand-1
PTEN	Phosphatase and tensin homolog
pTreg	Peripheral Treg
Raptor	Regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
ROR	Retinoic orphan receptor
ROS	Reactive oxygen species
RUNX1	Runt-related transcription factor 1
SAP	Signaling lymphocyte activation molecule-associated protein
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGK1	Serum- and glucocorticoid-regulated kinase 1

SLAMF6	SLAM family member 6
SLE	Systemic lupus erythematosus
SNAIL	Zinc finger protein SNAI1
SRBC	Sheep red blood cells
SREBP	Sterol regulatory element-binding protein
STAT	Signal Transducers and Activators of Transcription
T-bet	T-box transcription factor
TBS	Tris-buffered saline
TCR	T cell receptor
TEP1	Telomerase-Associated Protein 1
T <sub>FH</sub>	Follicular helper T cell
T <sub>FR</sub>	Follicular regulatory T cell
TGF	Transforming growth factor
T <sub>H</sub>	T helper
TMRM	Tetramethyl rhodamine, methyl ester
TNF	Tumor necrosis factor
TOR	Target of rapamycin
Treg	Regulatory T cell
TSC	Tuberous sclerosis
TSDR	Treg-specific demethylated region
tTreg	Thymic Treg
ULK1	Unc-51-like kinase 1
WT	Wildtype
YFP	Yellow fluorescent protein

## CHAPTER 1. INTRODUCTION

### Overview

Regulatory T (Treg) cells, identified as the Foxp3<sup>+</sup> subset of CD4<sup>+</sup> T cells, maintain tolerance to self-antigens and prevent autoimmune diseases (Josefowicz et al., 2012; Tang and Bluestone, 2008). Continuous T cell receptor (TCR) activation is important for Treg cell function (Levine et al., 2014). TCR signaling activates the phosphoinositide 3-kinase (PI3K), AKT, and the mechanistic target of rapamycin (mTOR) pathways. The strength and duration of PI3K, AKT, and mTOR signaling modulates Foxp3 expression to control Treg cell development or differentiation (Chi, 2012). Further, the level of mTOR signaling also affects the suppressive function of Treg cells (Park et al., 2013; Wei et al., 2016; Zeng et al., 2013). Phosphatase and tensin homolog (PTEN, encoded by the *Pten* gene) is a lipid phosphatase that directly opposes PI3K signaling by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to generate phosphatidylinositol (3,4)-bisphosphate (PIP2). However, molecular pathways mediated by PTEN and its role specifically in Treg cells remain to be established. Therefore, the main goals of our study were the following:

1. Investigate the mechanism by which PTEN signaling controls the homeostasis and functions of Treg cells.
2. Identify signaling pathways and target genes involved in PTEN-mediated regulation of Treg cells.

### T Cell Development, Homeostasis, and Differentiation

T cells are white blood cells that develop in the thymus from bone marrow-derived hematopoietic stem cells (HSCs). Specifically, HSCs differentiate into multipotent progenitors capable of further differentiating into common lymphoid progenitors that give rise to T cells, B cells, and natural killer cells. Once in the thymus, lymphoid progenitors lose alternative lineage potential and commit to the T cell fate (Germain, 2002; Yang et al., 2010). The thymic microenvironment provides unique signals to T cell precursors, causing changes in transcriptional signatures that promote the expression of functional, antigen-specific TCRs. Ultimately, these precursors undergo TCR positive and negative selection and finally, they leave thymus to become mature CD4<sup>+</sup> or CD8<sup>+</sup> T cells.

Upon egress from thymus, T cells are maintained in a quiescent state. Quiescent T cells are smaller in cell size and have lower metabolic rates than activated T cells, which is discussed in greater detail below. Further, they are maintained in the G<sub>0</sub> phase of the cell cycle. Because TCR repertoire diversity is important for recognition of many pathogens, quiescence may preserve energy to limit the size of the naïve T cell pool (Yang and Chi, 2012). Several transcription factors, including Krüppel-like factor 2 (KLF2) and Forkhead box protein P1 (Foxp1), are essential for the maintenance of T cell quiescence (Buckley et al., 2001; Feng et al., 2010). The signaling molecules regulating T



cell quiescence have also been extensively studied and are discussed in other reviews (Hamilton and Jameson, 2012; Hua and Thompson, 2001; Yang and Chi, 2012). Below, we briefly discuss how the mTOR pathway regulates T cell quiescence.

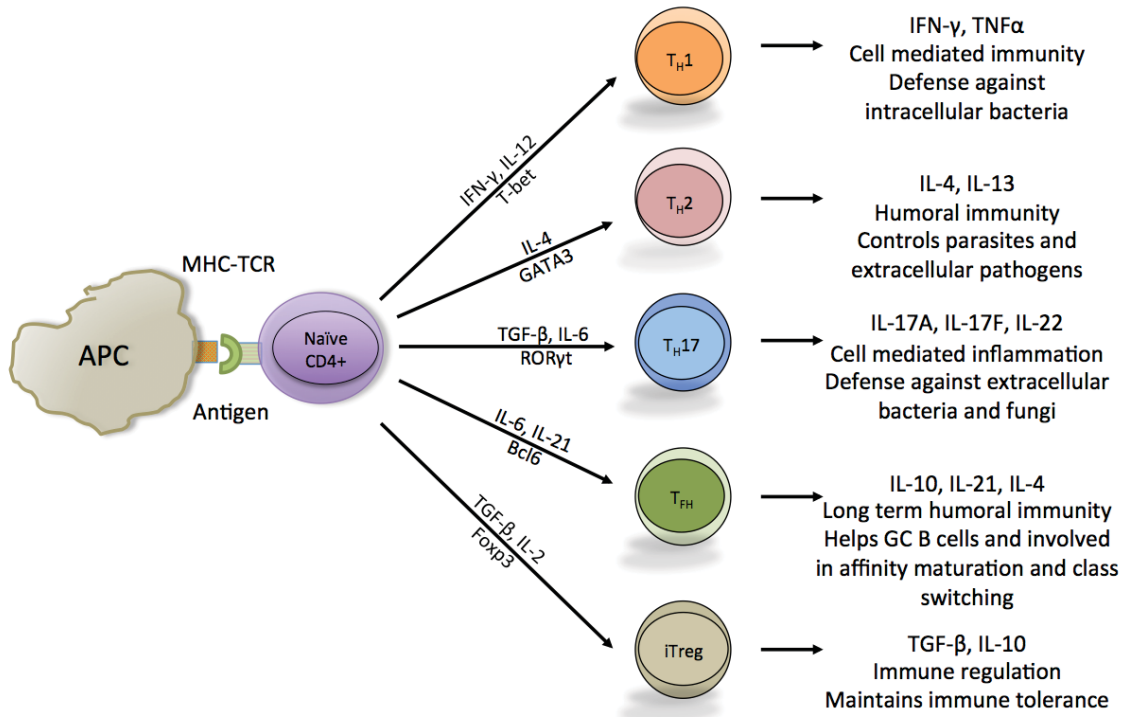
Upon receiving the appropriate activating stimuli, naïve T cells proliferate and differentiate into effector T cells. These differentiated effector populations are termed CD4<sup>+</sup> T helper (T<sub>H</sub>) cells and CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs). Three activating signals are required to drive T<sub>H</sub> and CTL differentiation. Signal 1 is generated by interaction of TCR with the cognate peptide presented in the context of the major histocompatibility complex (MHC) molecule (Banchereau and Steinman, 1998). The second signal is co-stimulation, achieved when CD28 on the T cell engages CD80 and/or CD86 on the antigen-presenting cell (APC) (Reis e Sousa, 2006). Finally, signal 3 is cytokine stimulation provided by APCs that function to orchestrate effector T cell differentiation (Curtsinger and Mescher, 2010). CD4<sup>+</sup> cells are involved in serving other immune cells, such as B cells for antibody production and activation of macrophages to promote clearance of infectious agents. CD8<sup>+</sup> T cells, on the other hand, are directly involved in killing infected host cells by releasing perforins and granzymes.

### **Differentiation of Conventional CD4<sup>+</sup> T Cells**

Naïve CD4<sup>+</sup> T cells circulate between the blood and secondary lymphoid organs. Upon receiving cognate antigen and co-stimulatory signals from APCs, naïve CD4<sup>+</sup> T cells are activated, proliferate, and can further differentiate into effector CD4<sup>+</sup> T cells. The major CD4<sup>+</sup> T helper subsets are T helper (T<sub>H</sub>) 1, T<sub>H</sub>2, T<sub>H</sub>17, and T follicular helper (T<sub>FH</sub>) cells (**Figure 1-1**). Cellular function, transcription factors and signature cytokine profiles characterize these T<sub>H</sub> subsets. The signals and transcription factors driving T<sub>H</sub> subset differentiation are discussed below.

#### **T<sub>H</sub>1 cells**

Early analysis of mouse CD4<sup>+</sup> T cell clones established the existence of two distinct T cell subsets. The first subset, called T<sub>H</sub>1 cells, predominantly secretes interferon (IFN)- $\gamma$ ; these cells also produce interleukin (IL)-2 and tumor necrosis factor alpha (TNF- $\alpha$ ) (Mosmann et al., 1986; Zhu et al., 2010). T<sub>H</sub>1 cells are primarily responsible for cell-mediated immunity via the activation of macrophages, neutrophils, and natural killer cells; they also aid CTL differentiation. T<sub>H</sub>1 cells are known to be involved in cellular immunity and help eradicate intracellular bacteria; however, overexpression of IL-12 causes excessive T<sub>H</sub>1 responses, leading to inflammation and autoimmune diseases, such as type 1 diabetes, in mice and humans (Szelachowska et al., 1997; Trembleau et al., 1995; Trembleau et al., 1999). T<sub>H</sub>1 responses are also known to be involved in the development of systemic lupus erythematosus (SLE). Elevated IFN- $\gamma$  concentrations are detected in SLE patients (Gerez et al., 1997). In mouse models of SLE, IFN- $\gamma$  is linked to exacerbated disease development, which can be significantly delayed using anti-IFN- $\gamma$  antibody (Jacob et al., 1987). Thus, excessive T<sub>H</sub>1 responses can play a



**Figure 1-1. Schematic representation of conventional helper T cell diversity.** Naïve CD4<sup>+</sup> T cells can differentiate into several different types of helper T cell subsets depending on cytokines and transcription factors present during activation.

detrimental role in autoimmune disease development, but how these responses contribute to disease development are not clear.

In addition to TCR and co-stimulatory signals, IFN- $\gamma$  and IL-12 play important roles in helping naïve T cells commit towards T<sub>H</sub>1 cells. Naïve T cells do not express IL-12 receptor inducible chain (IL-12R $\beta$ 2), which is required for activation of the signaling pathway involved in T<sub>H</sub>1 differentiation. TCR activation induces IL-12R $\beta$ 2 expression. IL-12R $\beta$ 2 expression is further stabilized by IFN- $\gamma$ , allowing IL-12R signaling to progress and ensure T<sub>H</sub>1 cell differentiation (Szabo et al., 1997). Mechanistically, IL-12R signaling is coupled to signal transducer and activator of transcription (STAT) 4 activation, which in turn promotes T-box transcription factor T-bet (encoded by *Tbx21*) expression (Szabo, Kim et al. 2000; Zhu, Yamane et al. 2010). It has been shown that T-bet-deficient naïve T cells fail to differentiate into T<sub>H</sub>1 cells, resulting in enhanced susceptibility to *Leishmania major* and other bacterial infections (Ravindran et al., 2005; Sullivan et al., 2005; Szabo et al., 2002). T-bet regulates the expression of IFN- $\gamma$  (Szabo et al., 2000; Zhu et al., 2010). Additionally, T-bet intrinsically suppresses the differentiation of other T<sub>H</sub> subsets, which are discussed below. Future studies will determine how the interplay between different T cell subsets influence T<sub>H</sub>1 responses in different disease contexts.

## T<sub>H</sub>2 cells

The second subset identified in early screens of mouse T cell clones was the T<sub>H</sub>2 population. T<sub>H</sub>2 responses are important for clearance of extracellular pathogens and parasites, such as helminths and nematodes (Finkelman et al., 1991; Sher and Coffman, 1992). In addition to producing the signature cytokine IL-4, T<sub>H</sub>2 cells produce IL-5 and IL-13 (Nelms et al., 1999; Zhu et al., 2010). T<sub>H</sub>2 cells also promote B cell antibody class-switching to generate IgG and IgE (Coffman et al., 1988; Killar et al., 1987). However, T<sub>H</sub>2-associated cytokines contribute to the induction of asthma and allergy. For instance, IL-5 causes airway hyperresponsiveness (AHR) (Cho et al., 2004; Foster et al., 1996). Moreover, excessive IL-4 and IL-5 production is linked to hyper-IgE production, eosinophil activation, and mast cell growth, which cause tissue injury. IL-13 stimulates mucus production, ultimately leading to increased airway obstruction (Danahay et al., 2002; Maddox and Schwartz, 2002). T<sub>H</sub>2-associated cytokines, therefore, act via several mechanisms to promote asthma and allergic disease pathologies.

IL-4R signaling via STAT6 and the induction of the transcription factor GATA binding protein 3 (GATA3) are required for T<sub>H</sub>2 cell differentiation. GATA3 positively regulates and maintains T<sub>H</sub>2 programming by promoting IL-4 expression, by binding to the IL-5 promoter to regulate its expression, and by supporting the continuous production of IL-13 (Zhou and Ouyang, 2003; Zhu, 2010). Moreover, T<sub>H</sub>1 and T<sub>H</sub>2 differentiation programs are antagonistic. Stable GATA3 expression inhibits T<sub>H</sub>1 differentiation, as it blocks expression of IFN- $\gamma$  (Lazarevic et al., 2013; Zhu et al., 2004). Additionally, IL-4 antagonizes IL-12R $\beta$ 2 expression to block T<sub>H</sub>1 polarization (Szabo et al., 1997). Conversely, it has been implicated that T-bet suppresses IL-4 expression (Szabo et al.,

2000). As T<sub>H</sub>2 cell-derived cytokines also help limit acute and chronic inflammation driven by excessive T<sub>H</sub>1 immune responses, there may be a potential therapeutic role for these cells in the treatment of T<sub>H</sub>1-associated diseases.

### **T<sub>H</sub>17 cells**

In addition to T<sub>H</sub>1 and T<sub>H</sub>2 cells, a third major subset of CD4<sup>+</sup> T<sub>H</sub> cells was identified. T<sub>H</sub>17 cells help provide host defense against extracellular bacteria and fungi by recruiting neutrophils and macrophages to infected tissues (Laan et al., 1999). IL-17A, IL-17F and IL-22 are the signature cytokines produced by T<sub>H</sub>17 cells (Korn et al., 2009). Excessive T<sub>H</sub>17 cell responses play significant roles in organ-specific autoimmunity. For example, excessive IL-17 levels contribute to joint destruction in a collagen-induced arthritis mouse model (Lubberts et al., 2002). T<sub>H</sub>17 responses are also harmful in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (MS) (Langrish et al., 2005), the mechanisms of which are discussed more fully below.

The differentiation of T<sub>H</sub>17 cells requires the transcription factors, retinoic orphan receptor (ROR) $\gamma$ t and ROR $\alpha$ , which regulate IL-17A and IL-17F production (Korn, Bettelli et al. 2009). The expression of ROR $\gamma$ t is controlled by the combined actions of the cytokines, transforming growth factor (TGF)- $\beta$ , IL-6 and IL-21. STAT1 signaling downstream of IL-6 and IL-21 is critical to support T<sub>H</sub>17 cell differentiation. Other transcription factors also stabilize and promote T<sub>H</sub>17 programming, including interferon regulatory factor 4 (IRF4) and runt-related transcription factor 1 (RUNX1) (Brustle et al., 2007; Huber et al., 2008; Zhang et al., 2008). The T<sub>H</sub>1 differentiation program can also suppress T<sub>H</sub>17 differentiation, as T-bet curtails T<sub>H</sub>17 commitment by inhibiting the induction of RUNX1-mediated induction of ROR $\gamma$ t (Lazarevic et al., 2013). The T<sub>H</sub>17 program is also strongly antagonized by those inducing Foxp3<sup>+</sup> T cells in the periphery, via mechanisms that involve Foxp3-mediated suppression of ROR $\gamma$ t and ROR $\alpha$  (Du et al., 2008; Ivanov et al., 2006; Zhou et al., 2008).

Compared to the other T<sub>H</sub> subsets, T<sub>H</sub>17 cells appear to be less stable. The stability of the T<sub>H</sub>17 lineage is regulated by the cytokine IL-23 (Stritesky et al., 2008). IL-23 consists of the p19 and p40 subunits. Mice deficient in p19 and p40 are protected from EAE (Cua et al., 2003). Additionally, treatment with anti-p19 and anti-p40 antibodies reduces EAE severity (Chen et al., 2006). By contrast, IL-23 overexpression exacerbates EAE (Langrish, Chen et al. 2005), thereby establishing a critical role for IL-23 in T<sub>H</sub>17-mediated disease pathologies (Awasthi et al., 2009; Thakker et al., 2007). The ability of IL-23 to promote disease may be linked to pathogenic T<sub>H</sub>17 cell differentiation. In adoptive transfer systems, T<sub>H</sub>17 cells polarized in the presence of TGF- $\beta$ 1 and IL-6 cannot drive EAE development; however, exposure to IL-23 drives autocrine TGF- $\beta$ 3 signaling that promotes T<sub>H</sub>17 pathogenicity (Lee et al., 2012). Destabilization of the T<sub>H</sub>17 lineage can also occur under conditions when TGF- $\beta$  signaling dominates over T<sub>H</sub>17-polarizing proinflammatory cytokines (e.g. IL-6), resulting in the differentiation of Foxp3<sup>+</sup> T cells over T<sub>H</sub>17 cells (Ivanov et al., 2006).

## T<sub>FH</sub> cells

T<sub>FH</sub> cells are a specialized subset of CD4<sup>+</sup> T cells that migrate to B cell follicles and provide signals to B cells, promoting germinal center reactions in secondary lymphoid organs such as spleen, lymph node and Peyer's patches (Crotty, 2011; Vinuesa et al., 2016). Germinal centers (GCs) are important sites for antibody affinity maturation mediated by B cell clonal expansion and somatic hypermutation. They also serve as sites for memory and plasma B cell differentiation. The differentiation of T<sub>FH</sub> cells from naïve CD4<sup>+</sup> T cells requires interactions with dendritic cells (DCs) in T cell areas and with B cells in B cell follicles and germinal centers, as discussed below. The lineage specific transcription factors for T<sub>FH</sub> programming are B-cell lymphoma 6 protein (Bcl6) and achaete-scute family bHLH transcription factor 2 (Ascl2) (Johnston et al., 2009; Liu et al., 2014; Nurieva et al., 2009; Yu et al., 2009). T<sub>FH</sub> cells also are characterized by the expression of chemokine receptor, CXCR5, co-stimulatory/inhibitory molecules ICOS and PD-1, and various effector molecules CD154 (also called CD40L), OX40, BTLA and CD84 (Crotty, 2011; Ma et al., 2012; Vinuesa et al., 2016). IL-21 is the primary cytokine essential for differentiation of T<sub>FH</sub> cells (Crotty, 2011; Vinuesa et al., 2016).

Uncontrolled, excessive T<sub>FH</sub> responses lead to the development of autoimmune diseases. SLE is among these diseases and is characterized by the production of autoantibodies (Craft, 2012; Linterman et al., 2009). Additionally, increased T<sub>FH</sub> frequencies have been seen in patients infected with human immunodeficiency virus (HIV) (Cubas et al., 2013; Lindqvist et al., 2012; Perreau et al., 2013). Various cancers, such as angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphoma-not otherwise specified (PCTL-NOS), also have increased T<sub>FH</sub> responses (de Leval et al., 2007; Dorfman et al., 2006; Dupuis et al., 2006; Huang et al., 2009; Ma and Deenick, 2014). Studies are currently aimed at understanding how T<sub>FH</sub> responses are exacerbated in these various diseases.

T<sub>FH</sub> cell differentiation requires interactions with both dendritic and B cells. Like other T<sub>H</sub> subsets, T<sub>FH</sub> priming is initiated when naïve T cells receive TCR and co-stimulatory signals from DCs. During priming, ICOS-ICOSL interactions and IL-6R signaling are essential to support commitment into early-T<sub>FH</sub> cells (Xu et al., 2013). These pre-T<sub>FH</sub> upregulate CXCR5 expression and downregulate CCR7 expression, allowing them to migrate to the T-B cell border and B cell follicle of secondary lymphoid tissues. Liu *et al.* has shown the transcription factor Ascl2 helps in T<sub>FH</sub> programming by upregulating CXCR5 and CXCR4 expression, and downregulating CCR7 and PSGL1 expression. As such, Ascl2 expression helps initiate T cell migration to B cell follicles and suppresses T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 differentiation (Liu et al., 2014). Further, IL-6 signaling induces B-cell lymphoma 6 protein (Bcl6) expression in pre-T<sub>FH</sub> cells, and this transcription factor is essential only for T<sub>FH</sub> differentiation among the T<sub>H</sub> subsets (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Additionally, Bcl6 represses the expression of cytokines and transcription factors essential for T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 differentiation. In support of this view, transducing CD4<sup>+</sup> T cells with a Bcl6-encoding retrovirus reduced T-bet and GATA3 expression in T<sub>H</sub>1 and T<sub>H</sub>2 polarizing conditions, respectively (Yu et al., 2009). Furthermore, overexpression of Bcl6 also reduces IFN- $\gamma$

and IL-17 production, cytokines associated with the T<sub>H1</sub> and T<sub>H17</sub> programs, respectively (Yu et al., 2009).

The second stage of T<sub>FH</sub> differentiation begins when pre-T<sub>FH</sub> interact with B cells localized in T-B cell border or B cell follicle (Crotty, 2014; Vinuesa et al., 2016). B cell-pre-T<sub>FH</sub> interactions can promote differentiation of short-lived plasmablasts, or these interactions can promote maturation of the GC reactions. GC-associated, mature T<sub>FH</sub> cells express higher levels of CXCR5 and Bcl6 than pre-T<sub>FH</sub> or other activated T<sub>H</sub> subsets. To promote mature T<sub>FH</sub> differentiation, B cells provide antigen and co-stimulatory signals via ICOS to pre-T<sub>FH</sub> cells to further drive T<sub>FH</sub> differentiation. Signaling lymphocyte activation molecule-associated protein (SAP) promotes T<sub>FH</sub> and B cells adhesion (Qi et al., 2008), which is an important process for the differentiation of T<sub>FH</sub> cells, GC B cells, memory B cells and plasma cells (Hu et al., 2013). Mechanistically, SAP binds SLAM family member 6 (SLAMF6), a molecule necessary for T<sub>FH</sub> function (Kageyama et al., 2012), and the SAP-SLAM interaction drives IL-4 production by T<sub>FH</sub> cells. Then, IL-4 and IL-21 derived from T<sub>FH</sub> promotes GC B cell survival and proliferation (Crotty, 2011; Vinuesa et al., 2016). IL-21 expressed by T<sub>FH</sub> and GC B cells also helps in plasma cell differentiation (Ozaki et al., 2004). In addition to being GC resident cells, mature T<sub>FH</sub> also have the capacity to exit the GC, where they can migrate to other B cell follicles or recirculate as a small population within the bloodstream (Shulman et al., 2013). These processes have been summarized in **Figure 1-2**.

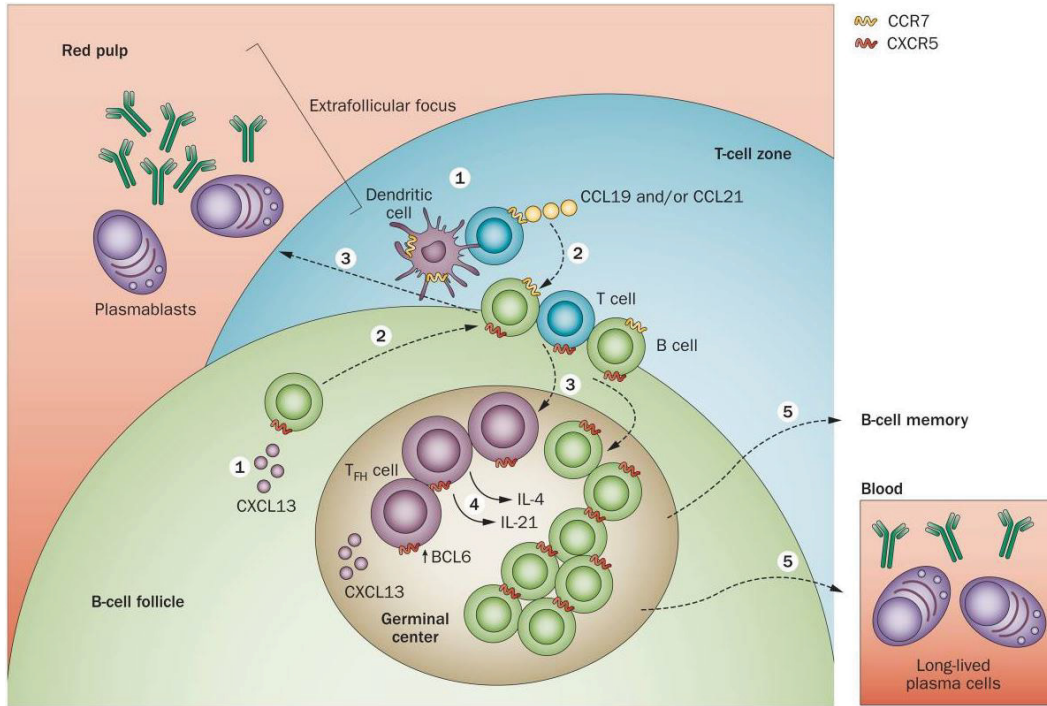
## Treg Cells

### **Foxp3 is the master transcription factor of Treg cells**

In a seminal study by Sakaguchi and colleagues, the presence of a suppressive CD4<sup>+</sup> T cell subset expressing high levels of CD25 (IL-2 receptor  $\alpha$  chain) was identified (Sakaguchi et al., 1995). These cells appeared to be distinct from effector T cells also expressing CD25, as their antigen-driven proliferation was less than conventional CD4<sup>+</sup> T cells (Hayashi et al., 2004; Thornton and Shevach, 1998). In experimental systems, the CD4<sup>+</sup>CD25<sup>+</sup> T cells were found to suppress autoreactive T cells. Further, depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells promoted organ-specific autoimmune disease, which was averted by adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> cells. These suppressive T cells are now known as Treg cells, and they play a central role in the regulation of the immune responses. These responses include the anti-tumor immunity, autoimmunity, allergic responses, graft-vs-host disease, and pathogen-induced immune responses (Josefowicz et al., 2012; Sakaguchi, 2005; Tang and Bluestone, 2008).

The forkhead/winged helix protein Foxp3 functions as a master regulator of Treg cell differentiation and is preferentially expressed in CD4<sup>+</sup>CD25<sup>+</sup> T cells in both the thymus and spleen (Ohkura and Sakaguchi, 2010). The suppressive activity of Treg cells requires continuous expression of Foxp3 (Williams and Rudensky, 2007).





**Figure 1-2. Multiple signals and differentiation stages are involved in T<sub>FH</sub> and germinal center B cell development.**

Different inducing factors are necessary for different stages of T<sub>FH</sub> cell differentiation, which ultimately help germinal center B cells to produce plasma or memory B cells. Reprinted with permission. Craft, J.E. (2012). Follicular helper T cells in immunity and systemic autoimmunity. *Nature reviews. Rheumatology* 8, 337-347.

FOXP3 mutations in humans cause a severe multi-organ autoimmune and inflammatory disorder known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Bennett and Ochs, 2001). Mutating *Foxp3* in mice also results in X-linked lymphoproliferative disease known as scurfy, which is attributed to loss of T cell tolerance (Hori et al., 2003).

Studies have indicated forkhead box O (Foxo) family transcription factors are essential for regulating the expression of *Foxp3* and thereby promoting Treg cell programming (Harada et al., 2010; Kerdiles et al., 2010; Ouyang et al., 2009; Ouyang et al., 2010; Ouyang et al., 2012). Using T cell specific deletion of *Foxo1* and *Foxo3*, Ouyang *et al.* showed that binding of *Foxo1* and *Foxo3* to the promoter region of *Foxp3* regulates *Foxp3* expression (Ouyang et al., 2010). Furthermore, Treg-specific deletion of *Foxo1* causes an inflammatory disease (Ouyang et al., 2012). Regulation of these Foxo factors is tightly controlled by the serine/threonine kinase, AKT (Calnan and Brunet, 2008; Merckenschlager and von Boehmer, 2010). Specifically, AKT-dependent phosphorylation of Foxo proteins sequesters these proteins in the cytoplasm, preventing their nuclear functions (Calnan and Brunet, 2008). Interestingly, the balance of Foxo signaling is a critical determinant of Treg cell function, since mutating the AKT phosphorylation sites of *Foxo1* and thereby driving constitutive *Foxo1* function in Treg cells also drives tissue inflammation (Luo et al., 2016).

### **Stability of Treg cells**

A fundamental issue in immunology is the interplay between effector and regulatory T cell responses. CD4<sup>+</sup>*Foxp3*<sup>+</sup> Treg cells regulate inflammatory responses and play a central role in protecting tissues from damage. Several pathways have been proposed for Treg cell-mediated control of effector T cell responses, including but not limited to deprivation of IL-2, secretion of inhibitory cytokines, and modulation of DC function via cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Sakaguchi et al., 2009; Vignali et al., 2008). The involvement of a particular mechanism may be context-dependent and may dominate in certain situations.

As noted above, *Foxp3* is a crucial transcription factor for Treg cell development and function. However, recent work has suggested 10-15% of Treg cells may lose *Foxp3* expression in certain contexts, resulting in death or instability, the latter forming ex-Treg cells (Komatsu et al., 2009; Zhou et al., 2009a). These ex-Treg cells no longer express high levels of Treg cell markers (e.g. CD25, glucocorticoid-induced TNFR-related protein (GITR), CTLA-4) and are unable to suppress effector T cell proliferation *in vitro* (Zhou et al., 2009a). This instability appears to be restricted largely to the CD25<sup>low</sup>*Foxp3*<sup>+</sup> Treg cell population, as this population has been reported to acquire effector T<sub>H</sub> cell-like functions (Komatsu et al., 2009). For example, Treg cells can produce IFN- $\gamma$  under T<sub>H</sub>1-polarizing conditions (Dominguez-Villar et al., 2011; Wei et al., 2009), and these T<sub>H</sub>1-effector phenotype Treg cells have reduced immunosuppressive function during an immune response to infection (Oldenhove et al., 2009). However, the functional consequences of IFN- $\gamma$  production by Treg cells are still unclear. Studies have



suggested that IFN- $\gamma$  production by allogeneic Foxp3<sup>+</sup> Treg cells may be beneficial in preventing experimental graft-versus-host disease (Koenecke et al., 2012), while others have termed these IFN- $\gamma$  producing cells as ex-Treg cells that enhance inflammation and thereby may contribute to autoimmunity (Zhou et al., 2009b). Defining the mechanisms involved in limiting or enhancing Treg cell stability is crucial to understanding immune system regulation, functional diversity and fate maintenance of Treg cells. Targeting these newly discovered mechanisms could provide novel therapeutic applications for disease intervention.

## **Heterogeneity of the Foxp3<sup>+</sup> Treg cell population**

Similar to conventional CD4<sup>+</sup> T cells, Treg cells are classified into different specialized subsets based upon their site of development or expression of surface molecules. Treg cells arise from the thymus (tTreg cells) or are induced in the periphery from naïve CD4<sup>+</sup> T cells (pTreg cells). *In vivo* interactions with self- and non-self antigens lead to the differentiation of tTreg cells and pTreg cells, respectively (Dhamne et al., 2013; Josefowicz et al., 2012). Naïve T cells can also be polarized into Treg cells *in vitro* (iTreg) in the presence of TGF- $\beta$  and IL-2. These subsets are discussed in greater detail below.

### ***tTreg cells***

tTreg cells differentiate from Foxp3<sup>+</sup>CD4<sup>+</sup> T cell precursors found in the thymus and comprise 5-10% of peripheral CD4<sup>+</sup> T cells. tTreg cells are essential for guarding against systemic and tissue-specific autoimmunity. In line with this idea, multi-organ autoimmunity is evident in mice that have undergone neonatal thymectomies (Nishizuka and Sakakura, 1969). This phenotype is due to the loss of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Asano et al., 1996), and the development of autoimmunity is inhibited upon transfer of CD4<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup> or CD25<sup>+</sup> T cells (Asano et al., 1996; Fowell and Mason, 1993; Sakaguchi et al., 1982).

Several signals control tTreg cell differentiation, and these signals appear to be derived from the thymic medulla (Cowan, Parnell et al. 2013). TCR recognition of MHC class II-bound peptides is essential for the development of tTreg cells, as CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells do not develop in the thymus of MHC-II-deficient mice (Fontenot et al., 2005). CD28 co-stimulation is also essential in tTreg cell differentiation, because tTreg cell numbers are decreased in CD28, CD80 and CD86 deficient mice (Salomon et al., 2000; Tai et al., 2005; Zhou et al., 2009). IL-2 is also necessary for the development of tTreg cells, as IL2R<sup>-/-</sup> mice failed to generate tTreg cells (Cheng et al., 2013; Cowan et al., 2013). Additional signals in the periphery, including continuous exposure to self-antigens, IL-2 and TGF- $\beta$ , are necessary for maintaining stability and function of tTreg cells (Li et al., 2006; Marie et al., 2005; Marie et al., 2006). Importantly, tTreg cells express similar phenotypic markers as pTreg cells, such as CTLA-4, GITR and OX40, but Helios and neuropilin-1 have been suggested as tTreg-specific markers (Dhamne et al., 2013). Furthermore, epigenetics studies have shown differences in the modifications

of 5'-C-phosphate-G-3' (CpG) in the Treg-specific demethylated regions (TSDRs) of the Foxp3 locus between tTreg cells and iTreg cells (Kim et al., 2012; Polansky et al., 2010). Specifically, the TSDRs in the Foxp3 locus of tTreg cells are mostly demethylated, whereas iTreg cells have predominately-methylated regions (Floess et al., 2007; Lal and Bromberg, 2009; Lal et al., 2009; McClymont et al., 2011).

### ***pTreg and iTreg cells***

While tTreg cells are major regulators of immune tolerance to self-antigens, pTreg cells are believed to dampen inflammatory responses to non-self antigens (Dhamne et al., 2013). For instance, pTreg cells are important for controlling inflammation at mucosal surfaces (Yadav et al., 2013). It has been reported that pTreg cells are induced *in vivo* under low antigenic conditions (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005; Thorstenson and Khoruts, 2001; Weiss et al., 2012). Similar to tTreg cells, pTreg cells express canonical markers such as CTLA-4, GITR and CD103. The lack of specific markers for pTreg cells has limited *in vivo* analyses of mechanisms driving their differentiation; so, most studies have analyzed the signals driving iTreg cell differentiation. IL-2 and TGF- $\beta$  are required for iTreg cell differentiation from naive CD4<sup>+</sup> T cells (Davidson et al., 2007; Shevach et al., 2008). Further, Foxp3 expression, survival and suppressive function of iTreg cells are maintained by TGF- $\beta$  (Nakamura et al., 2001). Interestingly, iTreg cells have unstable expression of Foxp3 and express a different gene signature than *in vivo* Treg cells (Floess et al., 2007). iTreg cells are not as suppressive and have a more limited transcriptional signature than endogenous Treg cells (Chen et al., 2003; Fantini et al., 2004; Hill et al., 2007; Wan and Flavell, 2005). Future studies will advance our understanding of similarities and differences between these Treg cell subsets.

### **T follicular regulatory cells**

T follicular regulatory (T<sub>FR</sub>) cells are a GC-resident Treg cell subset that shares phenotypic features of both tTreg cells and T<sub>FH</sub> cells. This Treg cell subset expresses CTLA-4, GITR, Bcl6, ICOS, PD-1 and CXCR5, but are functionally distinct from conventional populations. Evidence shows T<sub>FR</sub> cells originate from tTreg cell precursors (Chung et al., 2011; Crotty, 2011; Linterman et al., 2011; Vinuesa et al., 2016). Bcl6, SAP, and B cell-deficient mice lack the T<sub>FR</sub> population, while non-T<sub>FR</sub> Treg cells are represented at normal frequencies, convincingly showing that T<sub>FR</sub> cells are a distinct Treg cell subset (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011).

T<sub>FR</sub> cells in the GC center play a role in suppressing T<sub>FH</sub> cell and B cell responses, but the mechanisms for suppression functions remains unclear. Recent studies showed CTLA-4 regulate T<sub>FR</sub> cell suppressive function, as the Treg cell-specific deletion of CTLA-4 in mice leads to increased GC responses (Sage et al., 2014; Wing et al., 2014). This phenotype is at least partially attributed to diminished T<sub>FR</sub> function (Sage et al., 2014). Other means of regulating T<sub>FH</sub> and GC B cells responses by T<sub>FR</sub> cells might be through inhibitory cytokines, such as TGF- $\beta$  and/or IL-10, as it is known T<sub>FH</sub> cells are

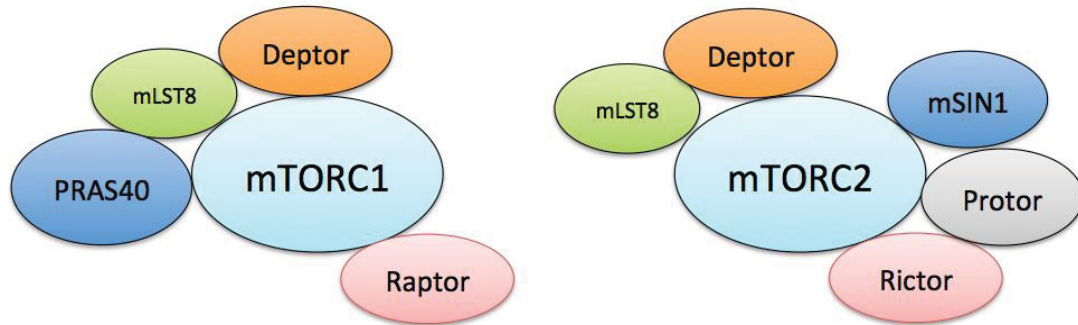
suppressed by TGF- $\beta$  and antibody production can be altered by IL-10 (Cai et al., 2012; McCarron and Marie, 2014). The molecular pathways that orchestrate the generation and function of T<sub>FR</sub> cells and the interplay with other effector cells remain to be elucidated.

### **mTOR Signaling Pathway**

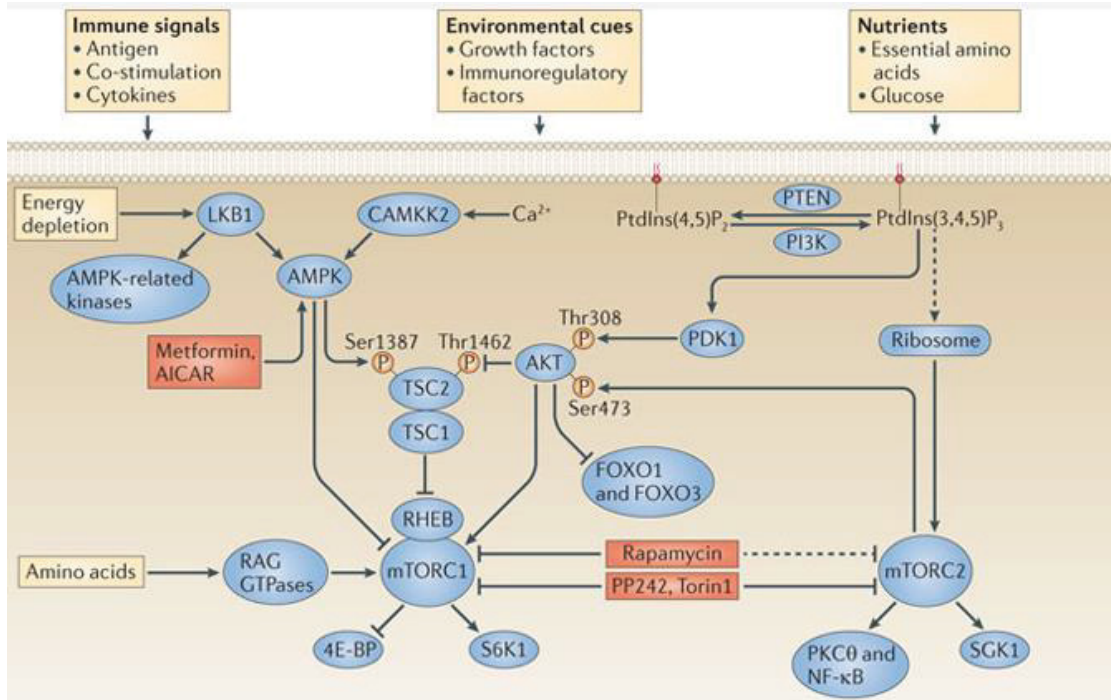
Rapamycin (also known as sirolimus) is an immunosuppressant drug widely used to prevent rejection in organ transplantation. The outcomes of studies identifying how rapamycin mediated its effects lead to the discovery of target of rapamycin 1 (TOR1) and TOR2 in yeast (Cafferkey et al., 1993; Heitman et al., 1991; Kunz et al., 1993). This protein is highly conserved across species and is also expressed in mammalian cells (Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). The mammalian mechanistic target of rapamycin (mTOR) is a serine-threonine kinase encoded as a single gene. mTOR exists in two functionally distinct, multi-protein complexes. mTOR complex 1 (mTORC1) is comprised of mTOR associated with and defined by the presence of the scaffolding protein Raptor, whereas mTORC2 is associated with and defined by the scaffolding protein, Rictor (**Figure 1-3**) (Chi, 2012; Laplante and Sabatini, 2012). mTORC1 activity is sensitive to rapamycin inhibition while mTORC2 activity is relatively insensitive to rapamycin, as much higher concentrations or long-term treatments of rapamycin are required to inhibit mTORC2 activity (Foster and Toschi, 2009).

### **Regulation of mTOR activity**

Immune signals, environmental cues and nutrients are three instructive signals that activate mTOR signaling pathway and ultimately shape T cell development and function. There are several regulators upstream of mTOR that help integrate essential signals, and enhance or inactivate the mTOR activity. One of the key negative regulators of mTORC1 function is tuberous sclerosis complex (TSC), a heterodimer consisting of TSC1 (also known as hamartin) and TSC2 (also known as tuberin). Following the activation of PI3K-AKT pathway by various receptor systems, the TSC complex is inactivated, subsequently activating mTORC1 function. TSC functions as a GTPase activating protein for Ras homolog enriched in brain (Rheb), which directly binds to mTORC1 and has reported roles in driving mTORC1 activity. Rheb needs to be in GTP bound state to help activate mTOR kinase activity. However, TSC converts Rheb to its inactive GDP bound form and negatively regulates mTOR (Chi, 2012; Laplante and Sabatini, 2012) (**Figure 1-4**). Rheb-deficient T cells have a transient reduction of TCR/CD28-induced mTORC1 activity (Delgoffe et al., 2009; Yang et al., 2013). Furthermore, disrupting the TSC1/2 complex by deleting TSC1 upregulates mTORC1 activity (O'Brien et al., 2011; Wu et al., 2011; Yang et al., 2011). However, growth factors like insulin activate mTORC1 via TSC-independent mechanisms, such as through the phosphorylation and dissociation of proline-rich AKT substrate of 40-kDa (PRAS40) from mTORC1. This process inactivates PRAS40 and subsequently activates mTORC1



**Figure 1-3. Schematic diagram showing associated protein components of two mTOR complexes, mTORC1 and mTORC2.** mTORC1 is comprised of core scaffolding protein mTOR, Raptor, mLST8 and other associated components PRAS40 and Deptor. mTORC2 consists of core protein mTOR, Rictor, mLST8 and various associated proteins mSIN1, Protor and Deptor.



**Figure 1-4. mTOR signaling pathway**

A serine/threonine protein kinase mTOR helps integrate multiple upstream signals such as immune signals, environmental cues and nutrients, thereby helping regulate crucial cell functions downstream such as cell growth, proliferation, survival, protein synthesis and transcription.

Reprinted with permission. Chi, H. (2012). Regulation and function of mTOR signalling in T cell fate decisions. Nature reviews. Immunology 12, 325-338. Courtesy of Dr. Hongbo Chi.

by allowing it to bind to its substrates (Sancak et al., 2007; Vander Haar et al., 2007; Wang et al., 2007). Throughout this dissertation, we discuss how upstream inhibitors of mTOR function control the functions of T cells and Treg cells.

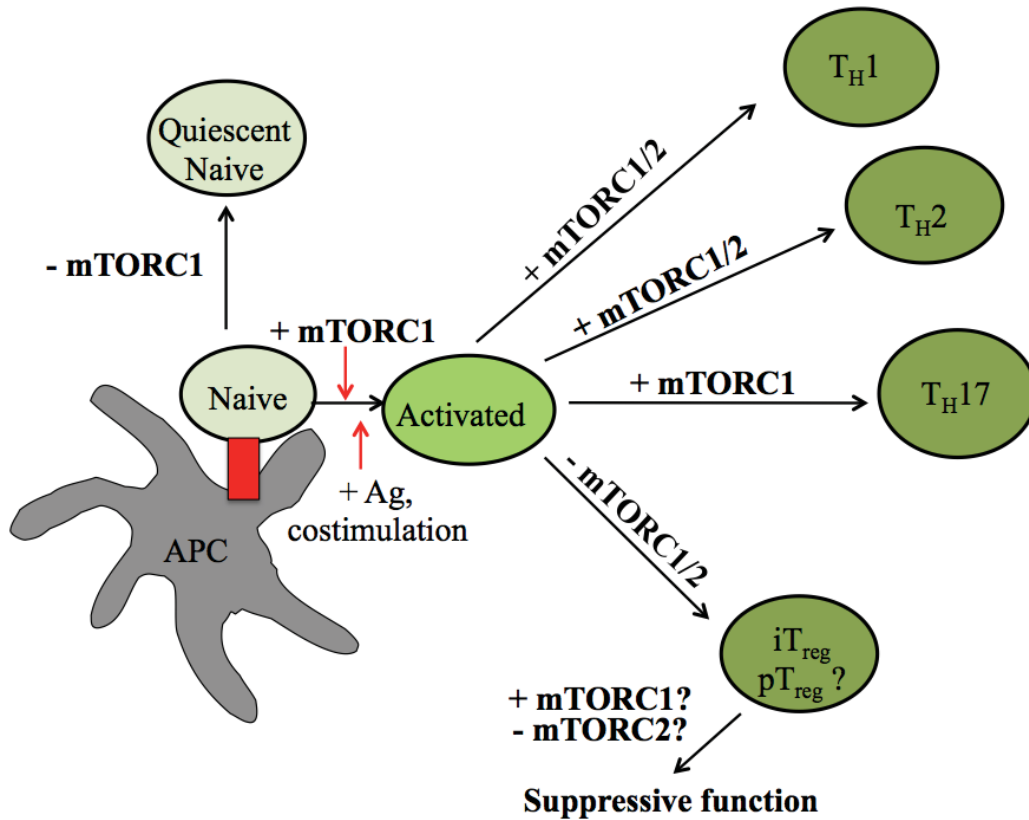
mTORC1 controls multiple downstream functions, including protein and lipid synthesis, autophagy, cell survival and metabolism. Mechanistically, active mTORC1 promotes translation and protein synthesis by phosphorylating ribosomal protein S6 kinases and eIF4e-binding protein. mTORC1 helps control lipid synthesis, which is essential for cell proliferation, through sterol regulatory element binding protein 1/2 (SREBP1/2) transcription factors, and stimulates *de novo* lipid biogenesis through peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) dependent regulation (Laplante and Sabatini, 2012). mTORC1 inhibits autophagy by phosphorylating unc-51-like kinase 1 (ULK1) (Ganley et al., 2009; Jung et al., 2009). However, there is a complex interplay between mTORC1 and autophagy, as a recent study from our lab has shown deletion of genes essential in autophagy resulted in upregulation of mTORC1 promoting defective Treg cell function (Wei et al., 2016). mTORC1 also orchestrates cellular metabolism by activating transcription and translation of hypoxia inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and c-Myc (Wang et al., 2011; Wei et al., 2016; Yang et al., 2013). Cell cycling and survival are also controlled by mTORC1 via positive regulators such as cyclin D1 and c-Myc (Gera et al., 2004). Thus, mTORC1 controls many important biological functions.

mTORC2 also plays important roles in cellular biology. mTORC2 is a crucial regulator of AKT activity via mechanisms discussed below (Sarbasov et al., 2005). In the absence of mTORC2 function (e.g. *Rictor* deletion), the AKT-dependent inhibitory phosphorylation of the Foxo transcription factors is reduced. These transcription factors are essential for expression of genes involved in cell survival, metabolism and proliferation (Calnan and Brunet, 2008). AKT is also involved in other cellular mechanisms, such as cell proliferation and metabolic regulation. Specifically, AKT promotes cell proliferation by phosphorylation of cell cycle regulatory protein p27 (Shin et al., 2002), and by indirectly inhibiting the tumor suppressor p53 (Vousden and Lane, 2007). The AKT pathway is also involved in glucose metabolism, as it is known to translocate glucose transporters such as Glut1 and Glut4 to the cell surface. This process is essential for promoting glucose metabolism, helping in T cell activation and function (Bentley et al., 2003; Boxer et al., 2006; Jessen and Goodyear, 2005; Wieman et al., 2007). Additional downstream targets activated by mTORC2 are protein kinase C (PKC) isoforms as well as serum- and glucocorticoid-regulated kinase 1 (SGK1) (**Figure 1-4**), which in turn regulates cytoskeletal dynamics and ion transport, respectively (Chi, 2012; Zarogoulidis et al., 2014). Thus, mTORC2 can influence cell survival, proliferation, growth, differentiation, and trafficking.

### **mTOR in T helper cell quiescence and differentiation**

In addition to maintaining T cell homeostasis, mTOR actively participates in the highly complex process of T cell differentiation (**Figure 1-5**). mTORC1 and mTORC2





**Figure 1-5. mTOR signaling controls peripheral T cell fate decisions.** mTOR signaling pathway is involved in promoting differentiation of effector helper T cell subsets, and inhibits the programming of induced regulatory Treg cells. Modified with permission. Chapman, N.M., and Chi, H. (2014). mTOR Links Environmental Signals to T Cell Fate Decisions. *Frontiers in immunology* 5, 686. Courtesy of Dr. Nicole Chapman and Dr. Hongbo Chi.

serve different roles in regulating T cell differentiation and function (Chapman and Chi, 2014). Quiescent naïve T cells have low metabolic and energy demands, which is correlated with low mTORC1 signaling. *Tsc1*<sup>-/-</sup> naïve T cells have excessive mTORC1 activity, which leads to metabolic dysregulation that promotes spontaneous cell growth, increased cell size, overt cellular activation, and cell proliferation. (O'Brien et al., 2011; Wu et al., 2011; Yang et al., 2011). Increased apoptotic death induced by TCR stimulation occurs in *Tsc1*<sup>-/-</sup> naïve T cells and is rescued upon anti-apoptotic protein Bcl2 overexpression (Yang et al., 2011). Thus, mTORC1 activity is restrained to regulate T cell homeostasis.

Furthermore, experiments involving mTOR, Rheb, Raptor and Rictor-deficient T cells have shown the importance of mTOR signaling pathway in T cell clonal expansion (Delgoffe et al., 2009; Yang et al., 2013). Loss of Rheb diminishes T<sub>H1</sub> and T<sub>H17</sub> differentiation, but promotes T<sub>H2</sub> polarization (Delgoffe et al., 2011). Absence of Raptor-mTORC1 signaling results in impaired T<sub>H1</sub> or T<sub>H2</sub> generation (Yang et al., 2013; Zeng et al., 2013). In contrast, loss of Rictor-mTORC2 results in defective T<sub>H2</sub> but not T<sub>H17</sub> differentiation. The role of mTORC2 in T<sub>H1</sub> generation is controversial and requires further investigation (Delgoffe et al., 2011; Lee et al., 2010).

mTOR signaling is also involved in the maintenance and differentiation of CD8<sup>+</sup> T cells (Araki et al., 2009; Li et al., 2011). Briefly, deletion of *Tsc1* during the late stages of thymocyte development results in altered CD8<sup>+</sup> T cell homeostatic proliferation (Wu et al., 2011; Yang et al., 2011; Zhang et al., 2012). Loss of Raptor-mTORC1 function results in impaired effector CD8<sup>+</sup> T cell responses *in vivo* (Yang et al., 2013). Recently, Pollizzi *et al.* has linked two different complexes of mTOR, mTORC1 and mTORC2 to different phases of CD8<sup>+</sup> T cell programming. Briefly, *Tsc2* deficiency enhances mTORC1 activity and is associated with terminal differentiation of effector CD8<sup>+</sup> T cells and impaired differentiation into memory CD8<sup>+</sup> T cells. Additionally, T cell specific deletion of *Rheb* caused inhibition of effector differentiation of CD8<sup>+</sup> T cells, while *Rictor*-deficient T cells have heightened memory CD8<sup>+</sup> T cell differentiation and responses (Pollizzi et al., 2015). Robust memory CD8<sup>+</sup> T cell generation in spleen and blood upon rapamycin treatment has also been reported, linking mTOR signaling to CD8<sup>+</sup> T cell programming. (Sowell et al., 2014).

### **mTOR in Treg cell differentiation and function**

The presence and magnitude of mTOR signaling also influence Treg cell function. We recently found that Raptor-mTORC1 signaling is an essential regulator of metabolic programming necessary for Treg cell function (Yang et al., 2013; Zeng et al., 2013). By contrast, Treg cell function is largely normal in cells lacking mTORC2 activity (Zeng, Yang et al. 2013). Park *et al.* demonstrated that the mTORC1 signaling threshold maintains Treg cell stability and function. Briefly, the absence of *Tsc1* in Treg cells causes dysregulated immune homeostasis. *Tsc1*-deficient Treg cells are unstable and acquire an effector T<sub>H17</sub>-like phenotype (Park et al., 2013). These studies highlight the critical role of mTORC1 signaling in Treg cell function. However, the inhibition of both



mTORC1 and mTORC2 complex is necessary for the generation of iTreg cells (Delgoffe et al., 2009; Delgoffe et al., 2011), suggesting that mTOR signaling is a complex regulator of Treg cell biology.

Upstream signals modulate mTOR signaling to control Treg cell function. For example the sphingosine-1-phosphate receptor 1 (S1PR1)-mTOR axis controls the programming of Treg cells in the thymus and periphery, thereby limiting the suppressive function of Treg cells *in vivo* and *in vitro* (Liu et al., 2009; Liu et al., 2010). Autocrine signaling of the hormone leptin modulates Treg cell proliferation via the leptin receptor. It has been reported that leptin strongly induces mTORC1 activity in Treg cells. Additionally, transient mTORC1 inhibition and loss of either leptin or its receptor subsequently promotes anti-CD3/28-induced Treg cell proliferation. Interestingly, the expression of leptin and leptin receptor by Treg cells is regulated by mTORC1 activation, because short-term rapamycin treatment suppresses their expressions. Therefore, the leptin-mTOR pathway dynamically regulates energy metabolism essential for Treg cells to control self-tolerance (Procaccini et al., 2010).

### **mTOR and T cell metabolism**

Distinct metabolic programs exist throughout different stages of T cell development. Quiescent naïve T cells engage oxidative phosphorylation (OXPHOS) or fatty acid oxidation (FAO) to generate adenosine triphosphate (ATP) (Fox et al., 2005; Pearce and Pearce, 2013; van der Windt and Pearce, 2012; Wang et al., 2011). Activated T cells require greater energy demands to rapidly grow and proliferate, which is mediated primarily by aerobic glycolysis (MacIver et al., 2013; Vander Heiden et al., 2009). Furthermore, T<sub>H1</sub>, T<sub>H2</sub> and T<sub>H17</sub> cells rely upon glycolysis, whereas Treg cells engage fatty acid oxidation (Michalek et al., 2011a; Zeng and Chi, 2013). Extensive crosstalk between metabolic programming and the mTOR signaling pathway is crucial for T cell fate decisions (Buck et al., 2015). Briefly, rapamycin treatment in activated T cells blocks c-Myc expression as well as glycolytic activity (Shi et al., 2011; Wang et al., 2011). Although naïve T cells experience comparatively low metabolic activity, increased mTORC1 activity in *Tsc1*<sup>-/-</sup> naïve T cells is linked to the upregulation of genes related to glucose metabolism (Yang et al., 2011). Upon TCR activation, *Tsc2*<sup>-/-</sup> T cells are highly glycolytic (Michalek et al., 2011b). One of the downstream targets of mTORC1, HIF-1 $\alpha$ , plays a crucial role in glycolysis. Interestingly, HIF-1 $\alpha$ <sup>-/-</sup> mice have increased Treg cells, pointing to an inhibitory role for glycolytic metabolism in Treg cell function (Shi et al., 2011). Furthermore, a recent study from our lab suggested that dysregulated glycolytic activity can impair Treg cell stability (Wei et al., 2016). We further explore the link between glycolytic metabolism and Treg cell function in this project.

### **Phosphatase and Tensin Homolog (PTEN)**

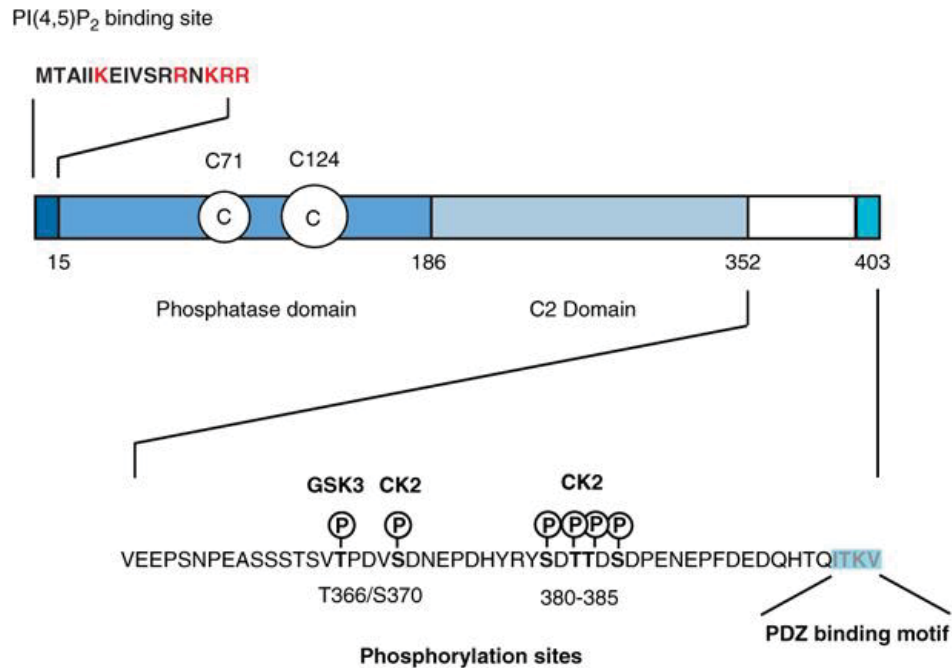
The *Pten* gene was identified in 1997 as a tumor suppressor and is also known as Telomerase-Associated Protein 1 (TEP1) and Mutated in Multiple Advanced Cancers 1

(MMAC1) (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997). This gene is located in long (q) arm of chromosome 10 at position 23.3 and is frequently mutated in a large number of human cancers (Chalhoub and Baker, 2009). In humans, germline mutations in PTEN are associated with several cancers and developmental disorders. PTEN Hamartoma Tumor Syndrome (PHTS) is a spectrum of disorders caused by loss of function of PTEN. PHTS includes Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), PTEN-related Proteus syndrome (PS), and Proteus-like syndrome (PLS) (Hobert and Eng, 2009). Additionally, heterozygous loss of PTEN function or expression is known to cause autoimmune disease and cancers (Salmena et al., 2008; Song et al., 2012). Even nearly 20 years after its discovery, most PTEN-related research studies have focused on its tumor suppressor functions. Little is known regarding the role of PTEN in autoimmune disorders, specifically within Treg cells, which we aim to characterize in this project.

### **Structure and regulation of PTEN**

PTEN is composed of 403 amino acids and has five distinct functional domains, which are the N-terminal region PIP2 binding domain, a phosphatase domain for enzymatic activity, a C2 domain for membrane recruitment, a carboxy-terminal tail thought to maintain stability and a PDZ-binding domain for protein interactions (**Figure 1-6**) (Georgescu, 2010; Leslie et al., 2008; Song et al., 2012). The function of PTEN is regulated through various mechanisms. Epigenetic changes via DNA promoter methylation modulate *Pten* expression at the transcription level. The proteins zinc finger protein SNAI1 (SNAIL), c-Jun N-terminal protein kinases (JNK), and MYC and Mitogen-Activated Protein Kinase Kinase Kinase 4 (MEKK4) are well-known suppressors of *Pten* transcription, among many others. In contrast, PPAR $\gamma$  and the p53 pathway upregulate *Pten* transcription. The NOTCH1 pathway, which plays important roles in T cell development and Treg cell function (Asano et al., 2008; Charbonnier et al., 2015; Deftos and Bevan, 2000), both positively and negatively regulates *Pten* transcription in a context-dependent manner (Salmena et al., 2008; Song et al., 2012).

PTEN expression is also controlled at the post-transcriptional level. First, endogenous, non-coding RNAs known as microRNAs (miRNAs) are essential regulators of PTEN expression. For instance, miR-19, miR-21, miR-26a and the miR-17~92 cluster, and miR-153 suppress PTEN expression (Huse et al., 2009; Meng et al., 2007; Olive et al., 2009; Song et al., 2012; Wu et al., 2013; Xiao et al., 2008). Additionally, post-translational phosphorylation events regulate the function of PTEN. The phosphorylation of several C-terminal residues (S365, T366, T382, S385, and T388) of PTEN is associated with its localization and stability (Bermúdez Brito et al., 2015; Salmena et al., 2008). In the context of diseases, the aberrant regulation of PTEN can occur at the genetic level. Allelic loss and mutations within different exons causes functional inactivation of PTEN. The loss of single PTEN allele is often seen in sporadic cancers, whereas homozygous loss is often correlated with advanced cancer and metastasis (Hollander et al., 2011; Pezzolesi et al., 2007; Song et al., 2012). We discuss the T cell-specific control of PTEN later in this chapter.



**Figure 1-6. PTEN gene structure.** Structure of 403 amino acids long PTEN gene with five different functional domains. Reprinted with permission. Leslie, N.R., Batty, I.H., Maccario, H., Davidson, L., and Downes, C.P. (2008). Understanding PTEN regulation: PIP<sub>2</sub>, polarity and protein stability. *Oncogene* 27, 5464-5476.

## Activation of mTOR by PTEN

Many signals, including TCR, co-stimulatory, and cytokines, induce PI3K signaling in T cells (Chapman and Chi, 2014; Chi, 2012). PI3K phosphorylates the membrane lipid, PIP2, to generate PIP3. One function of PIP3 is to modulate protein localization by recruiting proteins with pleckstrin homology domains to the plasma membrane. PDK1 and AKT are two such proteins activated downstream of PI3K. Mechanistically, PDK1 phosphorylates AKT T308, and phosphorylation of AKT S473 by mTORC2 promotes maximum catalytic function of AKT. Active AKT drives cell survival, proliferation and cellular metabolism, in part by phosphorylation of the Foxo family proteins. AKT also activates mTORC1, which is a central regulator of cell metabolism, growth, proliferation and survival as discussed above (**Figure 1-4**) (Chi, 2012). TCR/CD28-induced mTORC1 is activated via a PI3K-PDK1-dependent, AKT-independent pathway in activated CTLs and Treg cells (Finlay et al., 2012; Wei et al., 2016). Further, we found upregulation of the PI3K-PDK1 pathway is correlated with reduced Treg cell stability and function (Wei et al., 2016). Additionally, PI3K signaling is positively linked to mTORC2 activation, because PIP3 appears to directly activate mTORC2 kinase activity (Gan et al., 2011; Liu et al., 2015). Because PTEN controls the regulative balance of PIP3 available for driving PDK1, AKT, mTORC1, and mTORC2 function, PTEN might also be an essential regulator of Treg survival, proliferation, stability or suppressive function.

## Regulation and roles of PTEN in T cells

While PTEN expression is crucial for its function as a tumor suppressor, PI3K activation depends on downregulation of PTEN. Therefore, T cell responses, including proliferation and survival, depend on temporal instances of low PTEN expression. Studies have shown that TCR stimulation is sufficient to inhibit PTEN expression (Bensinger et al., 2004; Locke et al., 2009; Walsh et al., 2006), thus activating the PI3K pathway. However, CD28 costimulatory signals require PTEN (Buckler et al., 2006). It is also known that the expression of *Pten* is higher in Treg cells than in effector T cells (Bensinger et al., 2004; Zeiser et al., 2008). These results suggest that PTEN expression is a critical determinant of T cell functions.

Conditional gene knockout and germline knockout approaches have been used to study the tumor suppressor function of PTEN. Initially, the role of PTEN in T cells was analyzed in *Pten*<sup>+/-</sup> mice, because complete disruption of *Pten* results in early embryonic lethality (Di Cristofano et al., 1998; Suzuki et al., 1998). These *Pten*<sup>+/-</sup> mice develop CD4<sup>+</sup> T cell lymphoma and autoimmunity (Di Cristofano et al., 1999). To understand the intrinsic role of PTEN, *Pten* was conditionally deleted within the T cell compartment (Suzuki et al., 2001). Analyses of these mice demonstrated that the regulation of PIP3 levels by PTEN is important for T-cell development and T-cell responses to activation and apoptotic-inducing signals. The analysis of T-cell conditional knockout mice revealed the loss of PTEN signaling during T cell lineage programming drives preferential T<sub>H</sub>2 differentiation (Buckler et al., 2008). Given the evidence that PTEN has

a profound impact on many aspects of T cell development and autoimmunity, one might predict that the Treg cell population would be altered in *Pten* conditional knockout mice. However, by using tissue-specific conditional deletion or transgenic mice, several groups have found that Treg cells appear to develop normally and their suppression ability seems to be intact in the absence of *Pten* ( Buckler et al., 2008; Walsh et al., 2006). Additionally, *Pten*-deficient Treg cells tend to be hyperproliferative to IL-2 signaling alone due to the inability to activate PI3K (Walsh et al., 2006). In summary, studies have shown loss of PTEN function in T cells disrupts immune tolerance, but there is a need for further study to elucidate the role of PTEN specifically in Treg cells.

### **Rationale and Hypothesis**

Although previous studies suggest PTEN is an important regulator of T cell selection and cell fate decisions, (Buckler et al., 2008; Walsh et al., 2006), those studies provided little direct evidence for a cell-intrinsic role for PTEN in Treg cell function. Therefore, we generated mice with a Treg-specific deletion of *Pten* to circumvent the potential influence of *Pten* deletion within multiple T cell populations. Using this system, we also minimize the undesired Cre-mediated toxicity (Schmidt-Supprian and Rajewsky, 2007). The purpose of the study was to investigate the requirement of PTEN in the homeostasis and functions of Treg cells. We hypothesized that the loss of *Pten* in Treg cells would result in defective immune regulation.

### **Conclusion**

Much of the recent emphasis has been placed on the roles of transcription factors that program Treg lineage stability and functional diversity, but how immunological signals are sensed and integrated by Treg cells to impinge upon distinct effector responses remains obscure. The data presented in this dissertation support a role for the PTEN-mTORC2 axis in regulating Treg cell stability and suppressive functions that dampen  $T_H1$  and  $T_{FH}$  responses linked to systemic autoimmunity. Our results presented in Chapters 3 through 5 have led to a better understanding of how PTEN orchestrates Treg cell stability and functional diversity.

Additionally, another project presented in Appendix (**Figures A-1 to A-13**) discusses how mTORC1 controls the function of memory T cells. The findings establish the role of the upstream mTORC1 regulator, TSC1 in immune signaling and cell metabolism necessary to orchestrate memory  $CD8^+$  T-cell differentiation and function.

## CHAPTER 2. METHODS AND MATERIALS\*

### Mice

*Pten*<sup>fl/fl</sup>, CD45.1<sup>+</sup>, *Rag1*<sup>-/-</sup>, *Ifng*<sup>-/-</sup> and Rosa26<sup>GFP</sup> (harboring a loxP-site-flanked STOP cassette followed by the GFP-encoding sequence inserted into the Rosa26 locus) mice were purchased from the Jackson Laboratory. *Rictor*<sup>fl/fl</sup>*Foxp3*-Cre mice have been described (Zeng et al., 2013). *Foxp3*<sup>YFP-Cre</sup> mice were a gift from Dr. A. Rudensky (Rubtsov et al., 2008) and *Foxo1*<sup>AAA/+</sup>, from Dr. M.O Li (Ouyang et al., 2012). *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice were analyzed at 10–12 weeks of age unless otherwise noted using age and sex-matched *Foxp3*-Cre mice as controls (referred to as WT). All mice were housed in a specific pathogen-free facility in the Animal Resource Center at St. Jude Children's Research Hospital. Animal protocols were approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

### Bone Marrow Chimera

Bone marrow (BM) chimeras were generated by transferring  $7 \times 10^6$  T-cell–depleted bone marrow cells into sublethally irradiated (5 Gy) *Rag1*<sup>-/-</sup> mice, followed by reconstitution for at least 2.5 months. Baytril water was used as an antibiotic treatment for BM chimeras for three weeks after irradiation.

### Polymerase Chain Reaction

Polymerase chain reaction was used to genotype mice using primers (Invitrogen) listed in **Table 2-1**. A master mix of nuclease-free water, 1X Tsg enzyme buffer, 25 mM MgCl<sub>2</sub>, 10 mM dNTPs (all from Lamda Biotech), allele-specific primers and Tsg enzyme was prepared. Genomic DNA purified from mouse toe, tail, or ear clips was then added to each PCR reaction. Each set of PCR reactions also contained a positive and a negative control. Programs used to run the reaction in the thermal cycler are listed in **Table 2-2**. The samples were mixed with 6X DNA loading dye (Lamda Biotech) and loaded onto 1% agarose gel. Bands were visualized with GelRed (Biotium) on a Gel Doc 2000 (Biorad). The samples were scored according the band size listed in **Table 2-1**.

### Single Cell Suspension

Mice were sacrificed by CO<sub>2</sub> asphyxiation and organs (spleen, mesenteric lymph and peripheral lymph nodes that included inguinal, auxiliary and cervical lymph nodes)

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\*Chapter 2 adapted with permission. Shrestha, S., Yang, K., Guy, C., Vogel, P., Neale, G., and Chi, H. (2015). T<sub>reg</sub> cells require the phosphatase PTEN to restrain T<sub>H1</sub> and T<sub>FH</sub> cell responses. *Nature immunology* 16, 178-187.

**Table 2-1. List of PCR primers used for PCR.**

<b>Primer Name</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Product Length (bp)</b>
<i>Foxp3</i> <sup>YFP</sup> -Cre	AGGATGTGAGGGA	TCCTTCACTCTGAT	346
	CTACCTCCTGTA	TCTGGCAATTT	(knockin)
	GCGTATGATCAGT	CTGCCCTCCTAGA	423 (wild-
	TATGCCTGTGTGG	TCATATCATCTGCC	type)
<i>Pten</i>	CAAGCACTCTGCG	AAGTTTTTGAAGG	328 (loxP)
	AACTGAG	CAAGATGC	156 (wild- type)
IFN- $\gamma$	AGAAGTAAGTGGA	AGGGAAACTGGGA	320
	AGGGCCCAGAAG	GAGGAGAAATAT	(mutant)
	TCAGCGCAGGGGC	ATCGACAAGACCG	260 (wild-
	GCCCGGTTCTTT	GCTTCCATCCG	type)
Rictor	ACTGAATATGTTC	GAAGTTATTCAGA	554 (loxP)
	ATGGTTGTG	TGGCCCAGC	466 (wild- type)

**Table 2-2. PCR program used to amplify gene of interest.**

<b>PTEN and IFN-<math>\gamma</math></b>	<b>Foxp3<sup>YFP-Cre</sup> and Rictor</b>
94°C for 2:00 min	94°C for 2:00 min
94°C for 0:45 min	94°C for 0:30 min
65°C for 0:45 min	60°C for 0:30 min
- 0.3°C per cycle	72°C for 1:00 min
72°C for 1:00 min	Goto 2, repeat 39 times
Goto 2, repeat 41 times	72°C for 5:00 min
72°C for 5:00 min	15°C for 5:00 min
15°C for 5:00 min	End
End	



were harvested. The procedures here onwards were done in sterile conditions under the hood. Organs were placed in 10-cm petri dishes containing 1 mL of wash buffer (HBSS + 2% FBS). 70- $\mu$ m nylon mesh was laid over the organs, and the flat end of a 3 mL syringe was used to gently grind the organs to create single cell suspensions. The mesh was washed with wash buffer, and the cells were collected in a 15-mL conical tube. Cells isolated from the lymph nodes were used immediately for downstream assays. Splenocytes were centrifuged and then resuspended in 1 mL of ACK lysis buffer (Gibco). After 1-2 minutes, the cells were washed, centrifuged and resuspended in appropriate volume of wash buffer.

### **Cell Purification**

Single cell suspensions from pooled spleen and peripheral lymph nodes were stained with magnetic L3T4 CD4<sup>+</sup> T cell isolation microbeads (Miltenyi Biotech) for 15 minutes at 4°C. The cells were subsequently washed and resuspended in wash buffer and filtered through a MACS MS column (Miltenyi Biotech) for positive selection of CD4<sup>+</sup> T cells. The enriched cells were washed and stained with the appropriate surface markers for 30 minutes in 4°C. Cells were then washed, and naïve and Treg cells were sorted using a MoFlow (Beckman-Coulter) or Reflection (i-Cyt) fluorescence-based cell sorter based on the surface markers listed in **Table 2-3**.

### **Flow Cytometry**

For analysis of surface markers, cells were stained in FACS buffer (PBS containing 2% (wt/vol) bovine serum albumin (BSA)). The following antibodies were used for flow cytometry analysis: anti-CD4 (RM4-5), anti-CD8 $\alpha$  (53-6.7), anti-TCR $\beta$  (H57-597), anti-CD69 (H1.2F3), anti-CD25 (PC61.5), anti-CD44 (1M7), anti-CD62L (MEL-14), anti-CD45.1 (A20), anti-CD45.2 (104), anti-PD-1 (J43), anti-GL7 (GL-7), anti-CD95 (15A7), anti-ICOS (C398.4A), anti-GITR (DTA-1), anti-CD19 (1D3), anti-CXCR3 (CXCR3-173), anti-MHCII (M5/114.15.2), anti-CD11b (M1/70), anti-CD11c (N418), anti-Ly6G (RB6-8C5; all from eBioscience). CXCR5 was stained with biotinylated anti-CXCR5 (clone 2G8) followed by streptavidin-conjugated PE (both from BD Biosciences). Flow cytometry data were acquired on the LSRII or LSR Fortessa flow cytometer (BD Biosciences) and analyzed using Flowjo software (Tree Star).

### **Intracellular Staining**

For intracellular marker staining, single cell suspensions were resuspended in FACS buffer containing antibodies for surface markers and incubated for 30 minutes in 4°C. The stained cells were then washed with ice-cold FACS buffer and fixed using fixation/permeabilization buffer (ebioscience, catalog # 005123-43). One hour later, cells were washed with 1X permeabilization buffer (ebioscience, catalog # 008333-56) twice and then stained with intracellular markers for 20 minutes at 4°C. Anti-Foxp3 (FJK-16s),

**Table 2-3. Characterization of innate and adaptive immune cell surface and intracellular markers.**

<b>Cell Population</b>	<b>Cell Markers</b>
Naïve T cells	CD62L <sup>+</sup> CD44 <sup>-</sup>
Activated T cells	CD44 <sup>+</sup> CD62L <sup>-</sup>
Treg cells	CD4 <sup>+</sup> TCRβ <sup>+</sup> YFP <sup>+</sup> or CD4 <sup>+</sup> TCRβ <sup>+</sup> Foxp3 <sup>+</sup>
T <sub>FH</sub>	CD4 <sup>+</sup> CXCR5 <sup>+</sup> PD-1 <sup>+</sup> or CD4 <sup>+</sup> CXCR5 <sup>+</sup> ICOS <sup>+</sup> or CD4 <sup>+</sup> CXCR5 <sup>+</sup> Bcl6 <sup>+</sup>
GC B cells	CD4 <sup>-</sup> CD19 <sup>+</sup> GL7 <sup>+</sup> CD95 <sup>+</sup>
T <sub>FH</sub>	CD4 <sup>+</sup> CXCR5 <sup>+</sup> PD-1 <sup>+</sup> YFP <sup>+</sup>
T <sub>H1</sub>	CD4 <sup>+</sup> TCRβ <sup>+</sup> IFN-γ
T <sub>H17</sub>	CD4 <sup>+</sup> TCRβ <sup>+</sup> IL-17 <sup>+</sup>
T <sub>H2</sub>	CD4 <sup>+</sup> TCRβ <sup>+</sup> IL-4 <sup>+</sup>
B cells	CD4 <sup>-</sup> CD19 <sup>+</sup>
DC	MHCII <sup>+</sup> CD11c <sup>+</sup>
Neutrophils	Ly6G <sup>+</sup> CD11b <sup>+</sup>

anti-T-bet (4B10), anti-IRF4 (3E4), anti-IFN- $\gamma$  (XMG1.2), anti-IL-4 (11B11), and anti-IL-17 (17B7) antibodies were all from eBioscience. Anti-CTLA4/CD152 (UC10-4B9) and anti-Blimp1 (3H2-E8) were purchased from Biolegend and Thermo Scientific, respectively. Cells were then washed and analyzed by flow cytometry.

For intracellular cytokine staining, T cells were stimulated for 4 h with 50ng/ml PMA plus 1  $\mu$ g/ml ionomycin in the presence of 1X monensin (GolgiSTOP, BD Bioscience) in 48-well plates at 37°C + 5% CO<sub>2</sub>. Cells were harvested, stained, and processed for flow cytometric analysis as above.

For phospho antibody staining, cells were incubated in pre-warmed 1X Phosflow lyse/fix buffer (BD Biosciences) at 37°C for 10 minutes. Cells were then pelleted by centrifugation and washed once with FACS buffer. After washing, the cells were permeabilized by adding 1 mL of ice-cold Phosflow Perm Buffer III (BD Biosciences), and incubated in ice for 30 minutes. The cells were washed twice with FACS buffer, stained with antibodies specific for as p-STAT3 (4/P-STAT3), PTEN (A2B1), and Bcl6 (K112-91; all from BD Biosciences) for 1 h at room temperature, and processed for flow cytometric analysis.

### **Cell Proliferation Assay**

Mice were injected intraperitoneally (i.p) with 1mg of 5-Bromo-2'-deoxyuridine (BrdU) stock 16 hours before analysis. Single cell suspensions were prepared from spleen, peripheral and mesenteric lymph nodes and thymus. Cells were stained with antibodies specific for cell-surface markers for 30 minutes at 4°C, washed and resuspended in Cytofix/Cytoperm buffer for 15 minutes on ice. Cells were then washed with Perm/Wash buffer, incubated in Cytoperm Plus buffer for 10 min on ice, washed again with Perm/Wash buffer and resuspended in Cytofix/Cytoperm buffer. After 5 min incubation on ice, cells were washed with Perm/Wash buffer, resuspended in PBS containing 300  $\mu$ g/ml of DNase I and incubated for 1 h at 37°C. After washing with Perm/Wash buffer, cells were stained with anti-BrdU (BD Pharmingen) and processed for flow cytometric analysis. All buffers and additional reagents described in this protocol were obtained from BD Biosciences (552598).

### **Active Caspase-3 Staining**

Cells obtained from single cell suspension were first stained with cell surface markers for 30 minutes at 4°C deg, washed and then fixed using BD Cytofix/Cytoperm buffer on ice for 20 minutes. Cells were washed twice using BD Perm/Wash buffer and stained with active Caspase-3 at room temperature for 30 minutes and then processed for flow cytometric analysis. All buffers and additional reagents described in this protocol were in the active caspase-3 apoptosis kit (550941) purchased from BD Pharmingen.

## Active Mitochondria Staining

For staining mitochondria, lymphocytes were incubated for 30 min at 37 °C with 10 nM MitoTracker Deep Red (Life Technologies) or 20 nM TMRM (tetramethyl rhodamine, methyl ester; ImmunoChemistry Technologies) after staining surface markers. ROS were measured by incubation with 5 µM MitoSOX Red (Life Technologies) after staining surface markers. The samples were then analyzed by flow cytometry.

## Immunofluorescence

For cryosections, kidneys and MLNs were freshly frozen in OCT embedding medium (Ploysciences, Inc). 10-µm-thick cryosections were fixed with cold acetone for 5 min before rehydration in Tris-buffered saline (TBS). Nonspecific binding was blocked by incubation in TBS containing 2% BSA and 5% normal donkey serum for 30 min before incubation with primary antibodies (2 µg/ml) overnight at 4 °C. Slides were washed for 15 min in TBS before incubation with Alexfluor568-conjugated goat anti-mouse antibody (1 µg/ml; A11031; Life Technologies), Alexfluor568-conjugated donkey anti-goat antibody (1 µg/ml; A11057; Life Technologies), Cy5-conjugated donkey anti-goat antibody (1 µg/ml; 705-175-147; Jackson ImmunoResearch), or Alexfluor488-conjugated streptavidin (1 µg/ml; S11223; Life Technologies) for 1 h at room temperature. Slides were washed for 15 min in TBS before mounting in Vectashield hard set with DAPI (Vector Laboratories). Fluorescence images were acquired using a Zeiss Axiovert200M and 20X EC Plan-NeoFluar objective, detected using a Cascade II EMCCD camera (Photometrics) and analyzed using Slidebook software (3i Intelligent Imaging Innovations). Large image composites were acquired with a Nikon Ti-E inverted microscope with 20× CFI Plan Apochromat Lambda objective and iXon DU897 EMCCD camera, using NIS-Elements software.

Visualization of ANA antibodies was performed by staining fixed Hep-2 slides (MBL). Specifically, serum samples were applied to the slide and incubated for 2 h at room temperature followed by 15 min washing in TBS. Bound murine antibodies were detected using Alexfluor568-conjugated goat anti-mouse antibody (1 µg/ml; A11031; Life Technologies) for 1 h at room temperature, and Alexafluor488-conjugated phalloidin (A12379; Life Technologies) was used to visualize F-actin, and nuclei were detected using DAPI. The anti-CD3 (sc-1127 (M-20)) antibody was purchased from Santa Cruz, Biotinylated Peanut Agglutinin (PNA) from Vector Laboratories (B-1075), and IgD (558597) from BD Biosciences.

## Immunohistochemistry

For paraffin sections, the spleen, kidney and Peyer's patches were fixed by immersion in 10% (vol/vol) neutral buffered formalin solution. Fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin, and the clinical

signs of autoimmune diseases were analyzed by an experienced pathologist (Dr. Peter Vogel), blinded to sample identity.

### **Immunization**

For experiments involving antigen-induced T<sub>FH</sub> and GC B cell response, antigen for immunization was prepared by mixing NP<sub>14</sub>-OVA (14 molecules of NP linked to OVA; Biosearch Technologies) and 10% KAl(SO<sub>4</sub>)<sub>2</sub> dissolved in PBS at a ratio of 1:1, in the presence of LPS (*Escherichia coli* strain 055:B5; Sigma). The solution was set to pH 7, and NP-OVA (100 µg) and LPS (10 µg) precipitated in alum was injected intraperitoneally as described (Kang et al., 2013).

Alternatively, a fresh preparation of  $1 \times 10^9$  PBS-washed  $1 \times 10^9$  SRBCs from Colorado Serum Company (31112) were injected intravenously to induce a robust splenic GC response (Cato et al., 2011).

### **Serum Antibodies**

100µl of blood from WT and *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice was collected in 1.5ml tubes and placed at room temperature for 30 minutes. The tube containing the blood was then centrifuged in 1000g for 10 minutes. The supernatant was then used for autoantibodies (dsDNA) and immunoglobulin subclasses detection assays with kits from Alpha Diagnostic International (5110) and Millipore (MGAMMAG-300K), respectively.

### **Adoptive Transfer**

For adoptive transfer, sorted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3-YFP<sup>+</sup> cells from *Foxp3*-Cre and *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice (CD45.2<sup>+</sup>) were transferred to the congenically marked (CD45.1<sup>+</sup>) recipients. Seven days after the transfer, mice were euthanized for the analysis of Foxp3 and CD25 expression.

### **Cell Counting**

The automated Nexcelom Bioscience Cellometer Auto T4 was used to count cell numbers. Alternatively, the cell suspension was mixed with trypan blue and counted using hemocytometer chamber.

Cells per ml = the average count per square x the dilution factor x  $10^4$

## Quantitative RT-PCR

RNA was extracted from cells using the RNeasy micro kit (Qiagen), and cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen). An ABI 7900 Real-time PCR system was used for quantitative PCR, with primer and probe sets from Applied Biosystems. The probes were (*Pten*, Mm00477208\_m1), *Ifng* (forward - TCGAATCGCACCTGATCACTA; reverse – GGGTTGTTACCTCGAACTTG and probe – CATCCTTTTTTGCTTTACTGTTGCTGAAGAAG).

Results were analyzed with SDS 2.1 software and in Graphpad prism. The cycling threshold value of the endogenous control gene (*β-actin*) was subtracted from the cycling threshold value of each target gene to generate the change in cycling threshold (DeltaCT). The expression of each target gene is presented as the fold change relative to that of wild-type unstimulated samples using the delta delta CT method (Menon et al., 2007).

## Immunoblotting

For immunoblotting, the immunoprecipitates or whole-cell lysates were resolved on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Bio-Rad) and blocked using 5% non-fat milk (Bio-rad) in TBST (1X TBS from Bio-rad containing 0.1% Tween-20). The membranes were incubated with various antibodies diluted in 5% Bio-Rad blocking buffer and washed using 0.1% TBST. The bound antibodies were visualized with Horseradish peroxidase (HRP) conjugated antibodies against mouse or rabbit IgG by using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific). The images were obtained using film.

Primary antibodies used were the following: anti-p-S6 (Ser235/236) (2F9, Cell Signaling Technology), anti-p-4E-BP1 (Thr37/46) (236B4, Cell Signaling Technology), anti-p-Foxo1 (Ser256) (9461, Cell Signaling Technology), anti-p-AKT S473 (D9E, Cell Signaling), anti-PTEN (138D6; Cell Signaling Technology) and anti-β-actin (AC-15; Sigma). The primary antibody dilutions were 1:1,000 (phospho-antibodies and PTEN antibody) and 1: 2,000 (β-actin antibody).

## Glycolysis Assay

Treg cells were stimulated with plate-bound anti-CD3-CD28 for 6 h, and glycolytic flux was measured by detritiation of [<sup>3</sup>H]-glucose, as described (Shi et al., 2011).

## Gene-expression Profiling and Gene-set Enrichment Analysis

DNase-treated RNA samples from sort-purified WT or PTEN-deficient Treg cells were analyzed with the Affymetrix HT MG-430 p.m. GeneTitan peg array, and

expression signals were summarized with the robust multi-array average algorithm (Affymetrix Expression Console v1.1). Lists of genes differentially expressed by 1.5-fold or more were analyzed for functional enrichment using the Ingenuity Pathways (<http://www.ingenuity.com>). GSEA within canonical pathways was performed as described (Subramanian et al., 2005). The microarray data have been deposited into the GEO series database ([GSE63625](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63625)).

### **Statistical Analysis**

*P* values were calculated with Student's *t*-test (GraphPad Prism). *P* < 0.05 was considered significant. All error bars represent mean and s.e.m.

## CHAPTER 3. CHARACTERIZATION OF IMMUNOLOGICAL EFFECTS MEDIATED BY PTEN SIGNALING IN TREG CELLS\*

### Introduction

We and others have shown a central role for mTOR in T cell-mediated immune responses (Chi, 2012; Powell et al., 2012). As mTOR is activated by diverse upstream signals and serves multifactorial roles in T cell responses, multiple mechanisms actively enhance or suppress mTOR signaling (Chi, 2012). For instance, loss of the tumor suppressor TSC1 aberrantly upregulates mTORC1 activity and disrupts T cell quiescence, homeostasis and functions (Yang et al., 2011). As a pluripotent molecule, PTEN antagonizes PI3K activity and thus has been shown to inhibit both mTORC1 and mTORC2 complexes (Chi, 2012); Deletion of *Pten* in T cells leads to the development of leukemia and autoimmunity (Liu et al., 2010; Suzuki et al., 2001). PTEN also possesses nuclear functions independent of PI3K-AKT activity (Song et al., 2011). Although PTEN has been implicated in the homeostasis and abundance of Treg cells (Bensinger et al., 2004; Zanin-Zhorov et al., 2012), previous studies have demonstrated *Pten*-deficient Treg cells maintain their suppressive activity *in vitro* (Walsh et al., 2006). Therefore, the functional impacts and molecular pathways of PTEN in Treg cell-mediated immune homeostasis and function remain to be established.

To investigate the *in vivo* functions and mechanisms of PTEN in Treg cells, we developed a mouse model to delete *Pten* selectively in Treg cells. Treg-specific loss of *Pten* was sufficient to induce a systemic, lupus-like autoimmune and lymphoproliferative disease. This disorder was associated with excessive T<sub>FH</sub> and GC B cell responses, as well as exuberant IFN- $\gamma$  production and T<sub>H</sub>1 reactions. Importantly, deletion of IFN- $\gamma$  considerably rectified T<sub>FH</sub> and autoimmune responses, indicating that PTEN signaling in Treg cells coordinately regulates T<sub>H</sub>1 and T<sub>FH</sub> responses.

### Treg Cell-specific Deletion of PTEN Precipitates an Inflammatory Disease

#### Generation of *Pten*-deficient Treg cell mice

To better understand the role of PTEN in the homeostasis and functions of Treg cells *in vivo*, we crossed mice carrying loxP-flanked *Pten* alleles (*Pten*<sup>fl/fl</sup>) with Foxp3<sup>YFP-Cre</sup> (Foxp3-Cre) mice (Rubtsov et al., 2008) to generate mice in which the *Pten* conditional alleles are deleted specifically in Treg cells (referred to as

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\*Chapter 3 reprinted with permission. Shrestha, S., Yang, K., Guy, C., Vogel, P., Neale, G., and Chi, H. (2015). T<sub>reg</sub> cells require the phosphatase PTEN to restrain T<sub>H</sub>1 and T<sub>FH</sub> cell responses. *Nature immunology* 16, 178-187.



*Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice). PTEN was deleted efficiently at both at the mRNA and protein levels in Treg cells but not in other T cell subsets (**Figure 3-1**).

### Phenotypic characterization of *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice

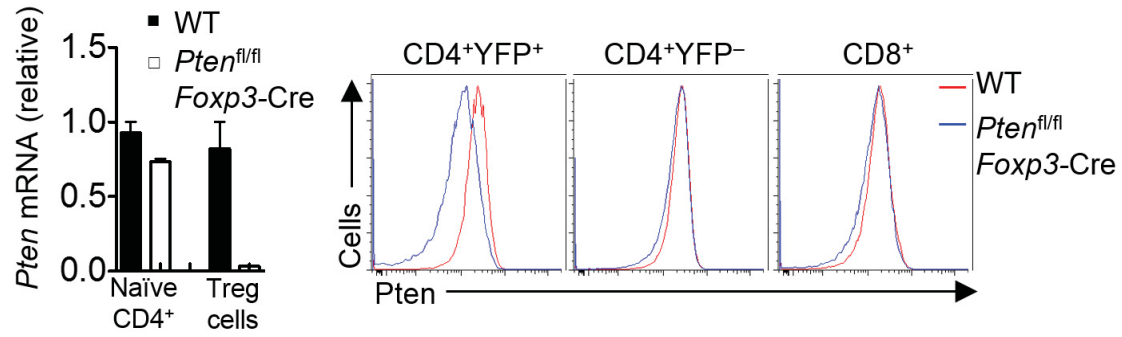
*Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice were phenotypically indistinguishable from littermate controls (*Foxp3*-Cre mice, designated as WT) from birth to 2-3 months of age. However 4-5-months-old *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice spontaneously developed cervical lymph node hyperplasia at. At this age, the *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice also displayed modest splenomegaly (**Figure 3-2A**).

Next, we wanted to assess if there was a global inflammatory response in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice. Routine histological examination by H&E staining was conducted in groups of mice at different age intervals. Most of the organs in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice had multifocal inflammation (unpublished observations). Interestingly, the number and size of reactive follicles in Peyer's patches were greater in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice than WT mice (**Figure 3-2B**). This finding of Peyer's patch abnormality and the spontaneous development of lymphadenopathy in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice indicated an ongoing lymphoproliferative disease, although it was not lethal and no signs of malignancies were identified. The affected mice were able to survive more than a year (unpublished observations).

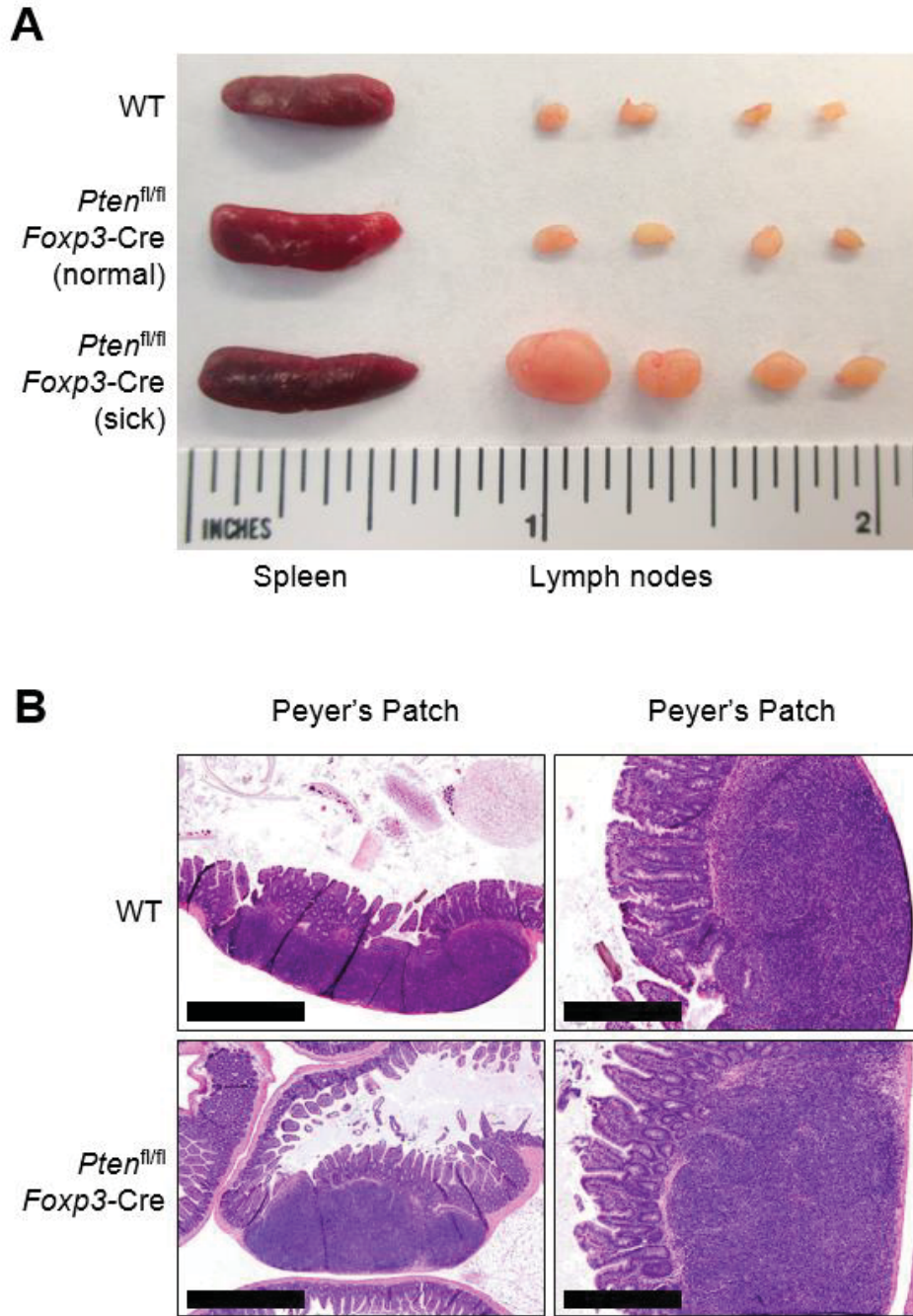
### Disruption of immune system in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice

To clarify if the pathology of *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice was related to the development of a systemic autoimmune disease rather than lymphoma, we quantified total antibody and autoantibody activity in the serum of WT or *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice. The amounts of anti-dsDNA and anti-nuclear antigen (ANA) autoantibodies in the serum were measured using ELISA and binding to fixed Hep-2 slides, respectively. We found significantly increased titers of circulating anti-dsDNA and ANA antibodies in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice compared with WT (**Figure 3-3A and B**), indicative of autoimmune reactions. Furthermore, upon examination of mouse immunoglobulin isotypes, we found considerably elevated titers of serum IgG2a/c and IgG2b isotypes in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice. In contrast, IgG1 and IgG3 titers were reduced, while IgM and IgA titers were comparable between WT and *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice (**Figure 3-4**).

Lymphocytic infiltration leading to predominant renal nephritis is frequently associated with clinical manifestations of systemic or non-organ specific autoimmune diseases. We therefore examined the glomerular architecture of the kidney in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice. To our expectation, the size and cellularity of the glomeruli were greatly increased in the kidney from *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice, indicative of glomerulonephritis (**Figure 3-5**). Furthermore, immunofluorescence imaging revealed prominent IgG deposits in the kidney glomeruli of *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice (**Figure 3-6**). In summary, these results highlight that loss of *Pten* in Treg cells results in the

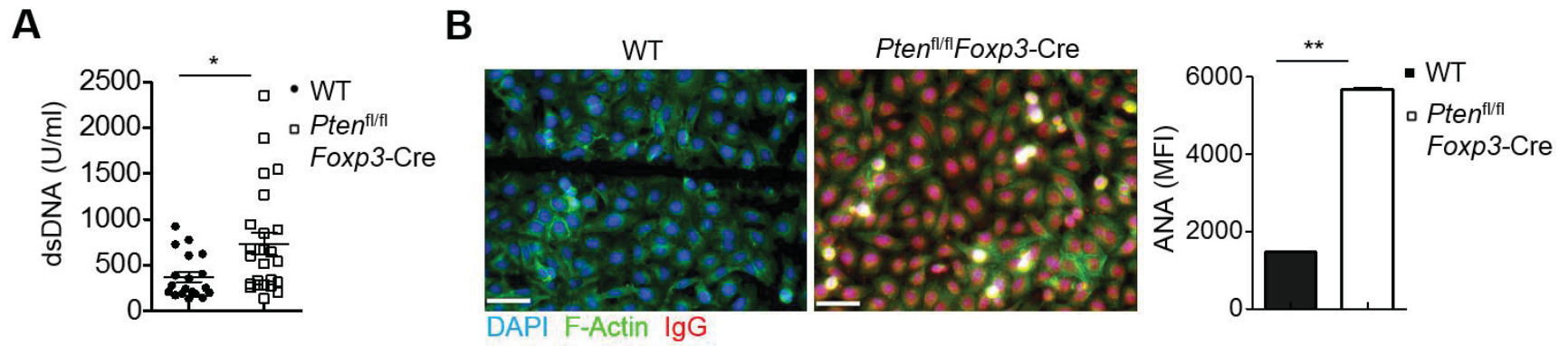


**Figure 3-1. Analysis of *Pten* mRNA and PTEN protein expression in Treg cells.** Real-time PCR analysis of *Pten* mRNA expression in Treg and naïve CD4<sup>+</sup> T cells (left) and histogram from flow cytometry analysis showing PTEN protein expression in CD4<sup>+</sup>YFP<sup>+</sup>, CD4<sup>+</sup>YFP<sup>-</sup> and CD8<sup>+</sup> T cells from the spleen of WT and *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice (right). Results were normalized using  $\beta$ -actin housekeeping gene and expressed as fold change for real-time PCR.



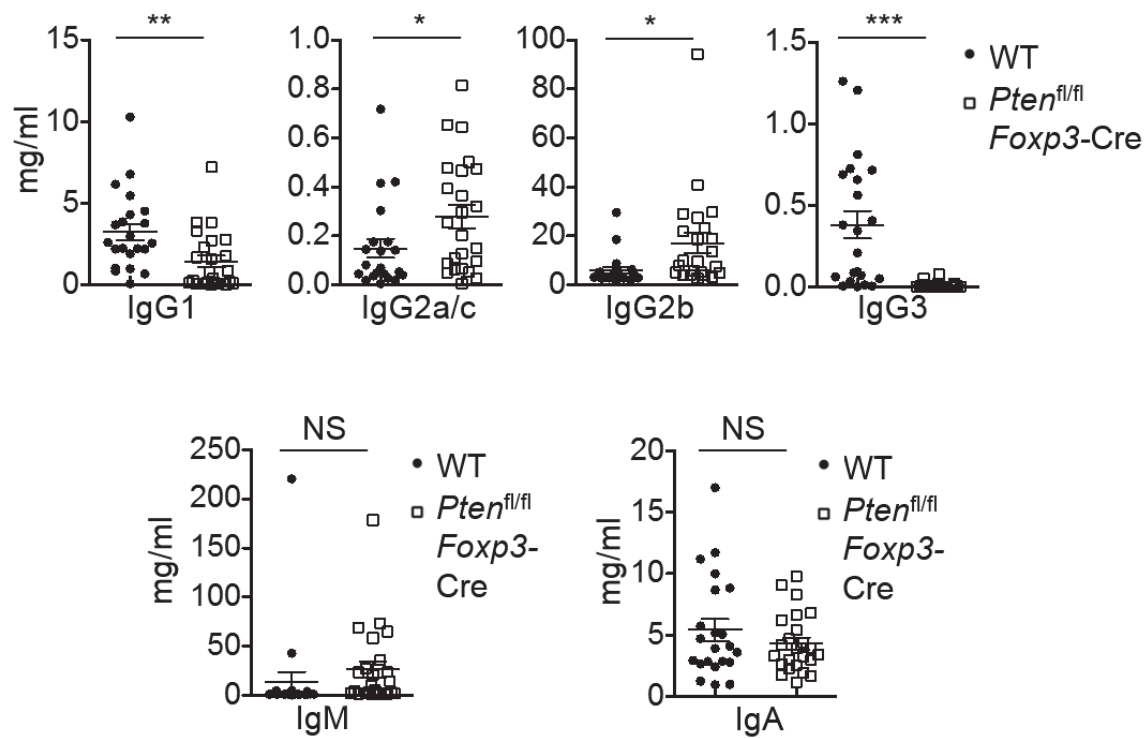
**Figure 3-2.** Age-related lymphoproliferative disease seen in *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice.

(A) Images of spleen and peripheral lymph nodes from WT (upper, ~5 months old), *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice prior to the development of lymphoproliferative disease (middle, ~2.5 months old), and *Pten*<sup>fl/fl</sup>*Foxp3-Cre* with lymphoproliferative disease (lower, ~5 months old). (B) Hematoxylin and eosin (H&E) staining of Peyer's patches in the intestine of WT and *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice (magnification: left, x4; and right, x20).



**Figure 3-3. *Pten<sup>fl/fl</sup>Foxp3-Cre* mice develop spontaneous systemic autoimmune disease.**

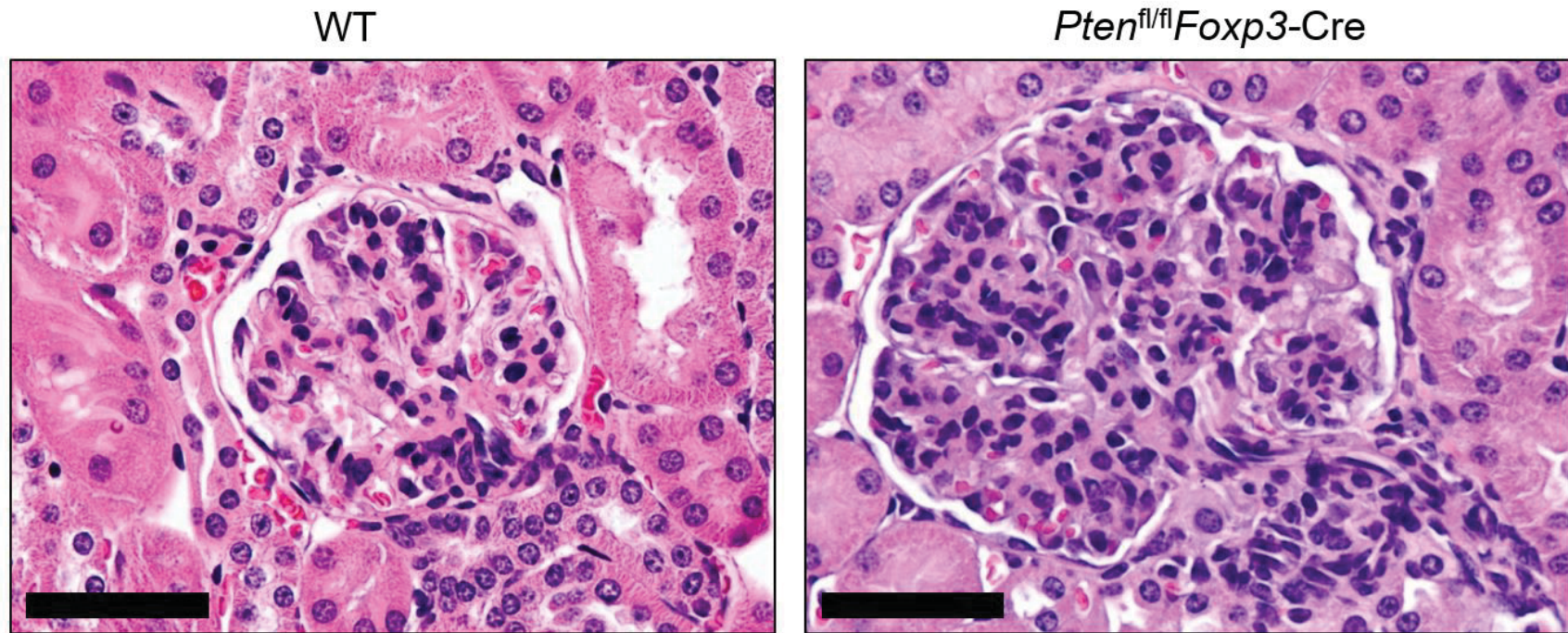
(A) Quantification of dsDNA-specific IgG in the serum of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* (2-6 months old; WT, n = 19; *Pten<sup>fl/fl</sup>Foxp3-Cre*, n = 24). (B) Immunofluorescence image (scale 20  $\mu$ m) and quantification of fluorescent intensity (right) of serum ANA IgG autoantibodies detected with fixed Hep-2 slides. Data are mean  $\pm$  s.e.m. \*P < 0.05 and \*\*P < 0.001.



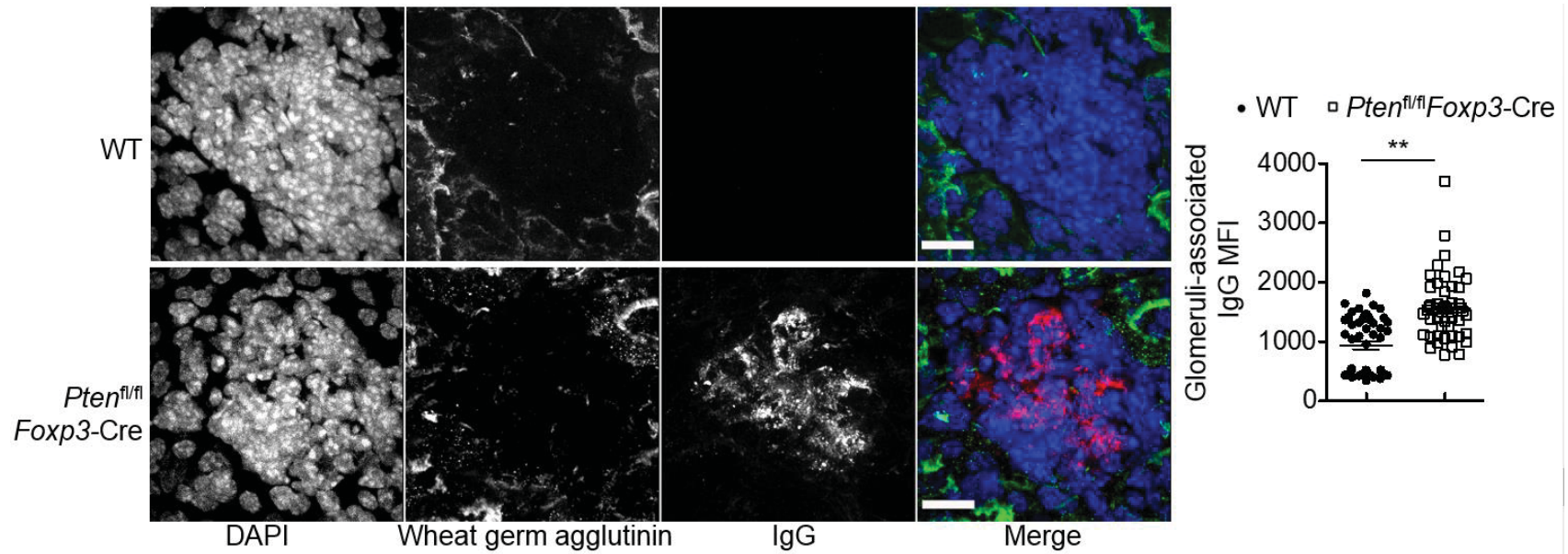
**Figure 3-4. Dysregulation of immunoglobulins isotype in *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice.**

Quantification of IgG subclasses, IgM and IgA in the serum of WT and *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice (the IgG2a binding antibody shows cross-reactivity with IgG2c). Data are mean  $\pm$  s.e.m. NS, not significant; \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001.





**Figure 3-5. Structural integrity of the kidney in WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.** Representative images of kidney glomerulus sections stained with H&E stain (magnification: x60, scale bar 50  $\mu$ m). The size and cellularity of the glomeruli of *Pten<sup>fl/fl</sup>Foxp3-Cre* mice (right) were greatly increased in the kidney, indicative of glomerulonephritis.



**Figure 3-6. Imaging analysis of kidney glomeruli of *Pten<sup>fl/fl</sup> Foxp3-Cre* mice.** Immunofluorescence images (left) and quantification (right) of kidney glomerulus sections showing IgG deposits (scale 20  $\mu$ m) in WT and *Pten<sup>fl/fl</sup> Foxp3-Cre* mice. Data are mean  $\pm$  s.e.m. \*\*P < 0.001.

development of a systemic autoimmune disorder characterized by aberrant antibody responses.

### **Altered Immune Homeostasis upon Treg-specific Loss of PTEN**

Disturbances in homeostasis cause T cells to upregulate effector functions, and this processes can contribute to autoimmune manifestations (Theofilopoulos et al., 2001). The development of the autoimmune and lymphoproliferative disease prompted us to examine whether homeostasis of the immune system was altered in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. In our following analyses, we used mice at a young age, prior to the development of the lymphoproliferative disease.

#### **PTEN is essential for Treg cell-mediated immune homeostasis**

In the steady-state environment, WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice had comparable numbers of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, B cells, dendritic cells, and neutrophils (**Figure 3-7**). However, the numbers of activated CD62L<sup>lo</sup>CD44<sup>hi</sup> effector/memory T cells in the CD4<sup>+</sup> and CD8<sup>+</sup> compartments were increased in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice (**Figure 3-8**). Moreover, CD44<sup>hi</sup> cells from these mice expressed elevated levels of IFN- $\gamma$  (**Figure 3-9A**). Similarly, expression of CXCR3, the signature chemokine receptor of T<sub>H</sub>1 cells, was also elevated (**Figure 3-9D**). In contrast, IL-17 and IL-4 production was largely normal (**Figure 3-9B and C**). Thus, T cells from *Pten<sup>fl/fl</sup>Foxp3-Cre* mice were spontaneously activated *in vivo*, with a propensity to differentiate into the T<sub>H</sub>1 phenotype. Despite severe autoimmune diseases, *Pten<sup>fl/fl</sup>Foxp3-Cre* mice had increased percentage and numbers of Foxp3<sup>+</sup> Treg cells in the spleen and lymph nodes (**Figure 3-10A**).

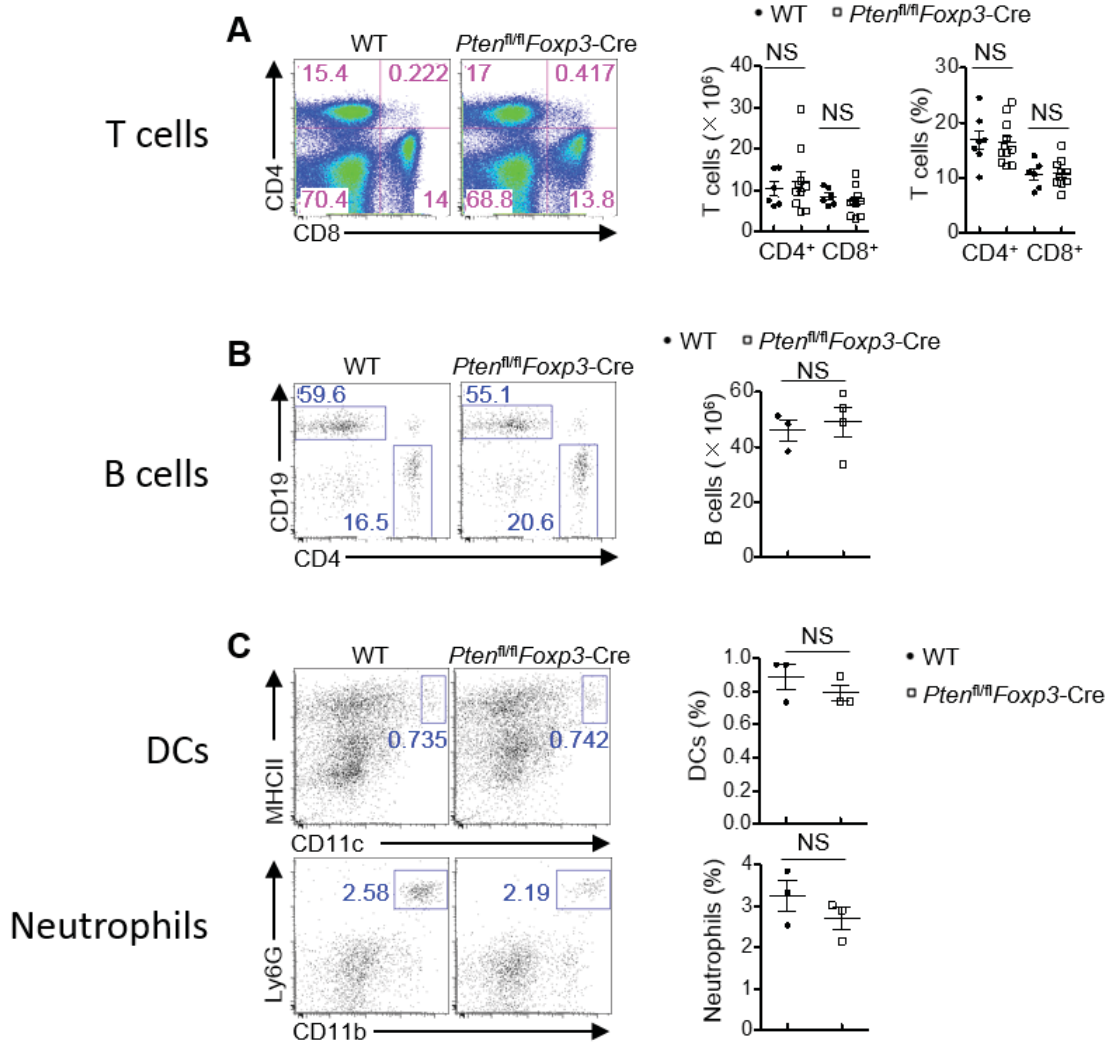
#### **Cell death and proliferation in Pten-deficient Treg cells**

To explore the underlying basis for the increased Treg cellularity, we used caspase-3 staining and BrdU incorporation assays to measure Treg cells apoptosis and proliferation, respectively. Treg cells from WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice had comparable caspase-3 staining (**Figure 3-10B**), but *Pten<sup>fl/fl</sup>Foxp3-Cre* Treg cells had more BrdU incorporation than WT cells (**Figure 3-10C**), indicative of an elevated rate of proliferation. Therefore, *Pten* deficiency causes an increased Treg cellularity and proliferation.

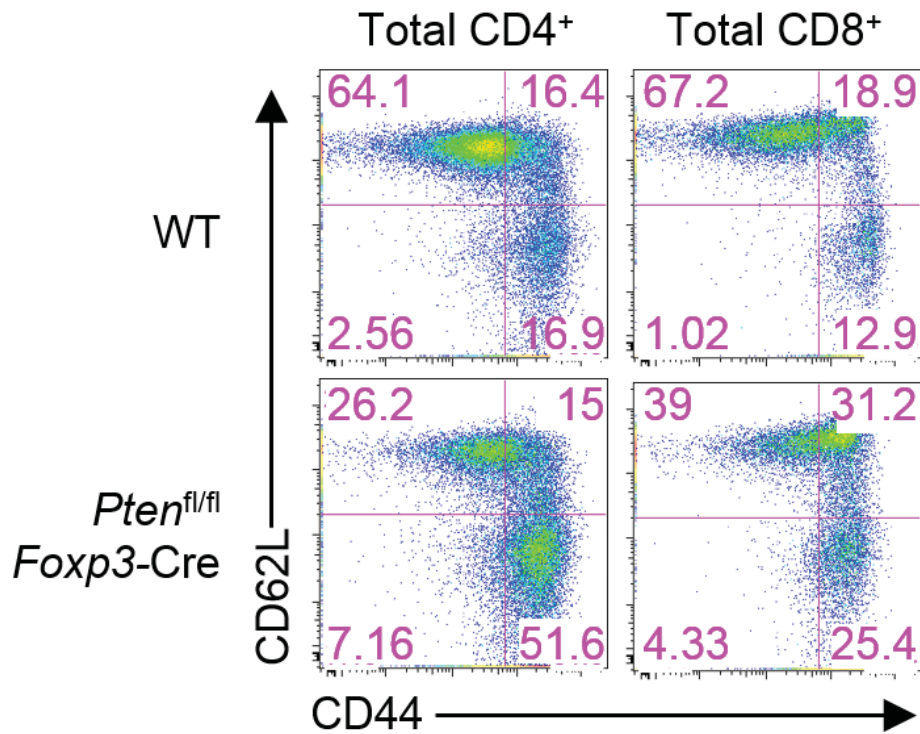
#### **Uncontrolled T<sub>FH</sub> Responses and GC B Cells in Pten-deficient Treg Mice**

Systemic lupus erythematosus (SLE), the prototypical systemic autoimmune disease, is characterized by the heterogeneity of the underlying T cell responses. Aside from the roles of the conventional effector (e.g. T<sub>H</sub>1 and T<sub>H</sub>17) and regulatory responses, recent studies demonstrate a crucial role of T<sub>FH</sub> cells in the overproduction of pathogenic autoantibodies and tissue damage in SLE (Crotty, 2011; Linterman et al., 2009).



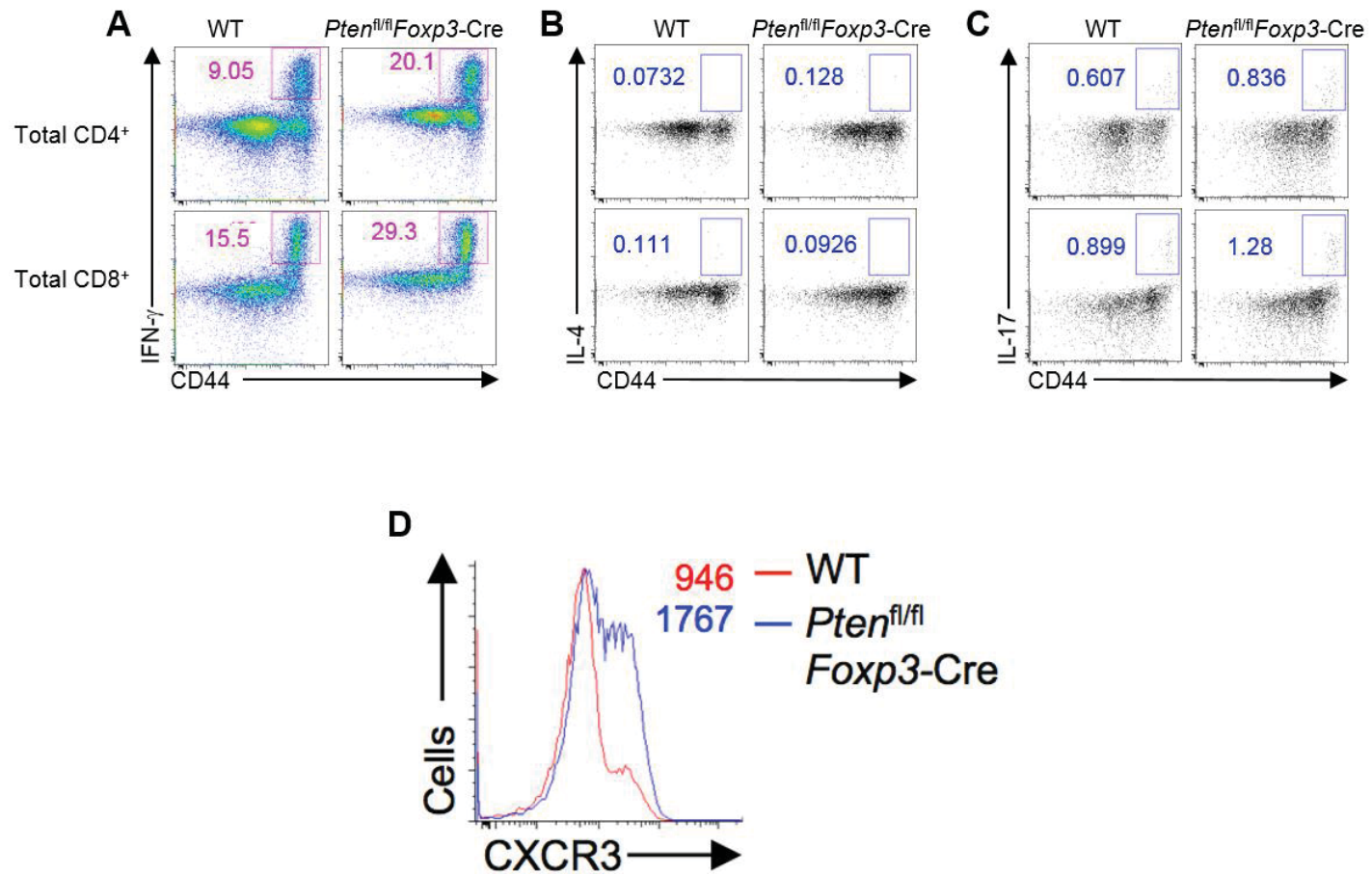


**Figure 3-7. Flow cytometric analysis of different immune cell populations.** (A) Analysis and quantification of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (B) B cells, (C) conventional dendritic cells (MHCII<sup>+</sup>CD11c<sup>+</sup>) and neutrophils (Ly6G<sup>+</sup>CD11b<sup>+</sup>) in the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Numbers indicate percentage of cells in quadrants or gates. Data are mean  $\pm$  s.e.m. NS, not significant.



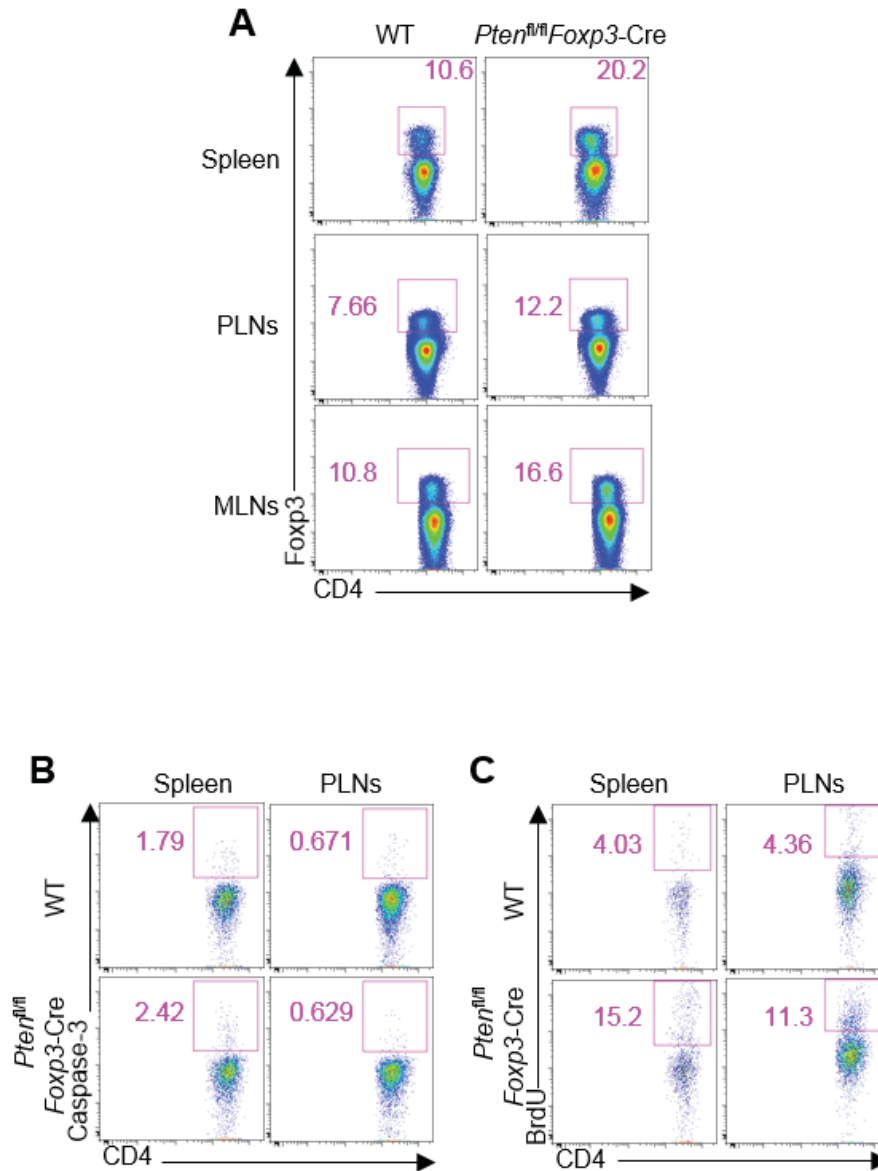
**Figure 3-8. Altered T cell homeostasis in *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice.**

Expression of CD62L and CD44 on WT and *Pten*<sup>fl/fl</sup>*Foxp3-Cre* splenic T cells. Numbers indicate percentage of cells in quadrants or gates. *Pten*<sup>fl/fl</sup>*Foxp3-Cre* mice have a higher frequency of activated CD44<sup>hi</sup>CD62L<sup>lo</sup> and a lower frequency of naïve CD44<sup>lo</sup>CD62L<sup>hi</sup> T cells. Numbers indicate percentage of cells in quadrants or gates.



**Figure 3-9. Th1 immune responses are increased in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.**

Expression of (A) IFN- $\gamma$ , (B) IL-4 and (C) IL-17 in CD4<sup>+</sup> and CD8<sup>+</sup> T cells of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice after *in vitro* stimulation for 4 h with phorbol 12-myristate 13-acetate (PMA) plus ionomycin in the presence of monensin. Numbers indicate percentage of cells in quadrants or gates. (D) Expression of CXCR3 on CD4<sup>+</sup> T cells from the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Numbers in the graph indicate mean fluorescence intensity of CXCR3.



**Figure 3-10. *Pten<sup>fl/fl</sup>Foxp3-Cre* mice have an increased frequency of Treg cells.** (A) Flow cytometric analysis of Treg cells in WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* splenic CD4<sup>+</sup> T cells. (B) Caspase-3 activity and (C) BrdU incorporation in Treg cells from the spleen or peripheral lymph nodes (PLN) at 16 h after BrdU injection in WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Numbers indicate percentage of cells in quadrants or gates.

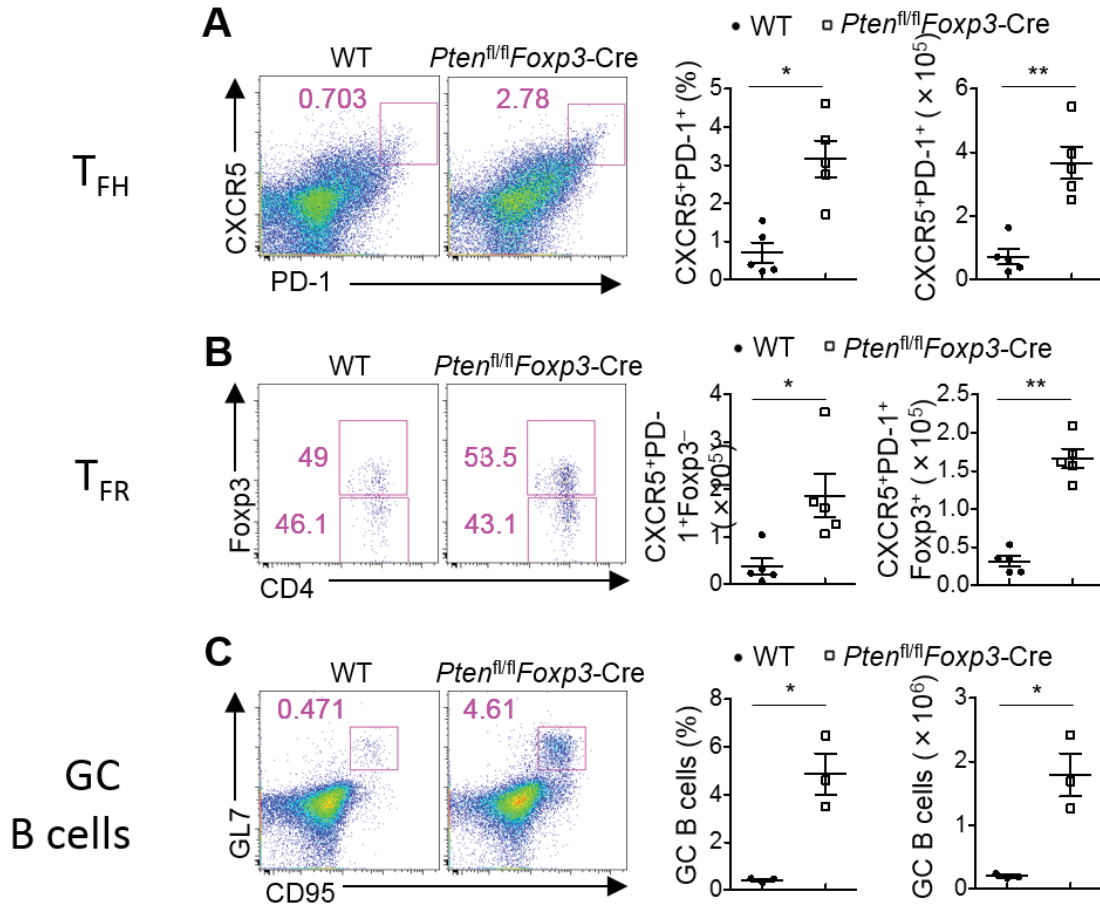
The development of SLE-like symptoms in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice prompted us to examine whether the T<sub>FH</sub> response was altered in these mice. Under steady state, only a small percentage of WT splenic CD4<sup>+</sup> T cells were stained positive for the T<sub>FH</sub> signature molecules, CXCR5 and PD-1, but the CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> population was greatly expanded in the spleen and MLNs of *Pten<sup>fl/fl</sup>Foxp3-Cre* mice (**Figures 3-11A and 3-12A**). Similar changes were noticed for CXCR5<sup>+</sup> cells expressing the T<sub>FH</sub>-associated co-stimulatory molecule ICOS and the transcription factor Bcl6 (**Figure 3-12B and C**). The CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup> cells can be further divided into immunostimulatory T<sub>FH</sub> and immunoregulatory T<sub>FR</sub> cells, as indicated by the absence or presence of Foxp3 expression, respectively (Chung et al., 2011; Linterman et al., 2011). Both subsets were increased in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice (**Figure 3-11B**). Consistent with the increase of CXCR5<sup>+</sup>PD-1<sup>+</sup> cells, the spleen and MLNs of *Pten<sup>fl/fl</sup>Foxp3-Cre* mice contained 3-fold more GC B cells, denoted by the expression of GC signature markers GL7 and CD95 (**Figures 3-11C and 3-12D**), even though total B cell numbers were largely unaltered (**Figure 3-7B**). Moreover, immunohistochemistry showed that the spleen and MLNs of *Pten<sup>fl/fl</sup>Foxp3-Cre* mice contained considerably more and larger peanut agglutinin (PNA)-positive GCs than did their WT counterparts (**Figure 3-13A and B**). These results revealed spontaneous T<sub>FH</sub> and GC formation in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.

We next determined whether *Pten* deficiency in Treg cells affects T<sub>FH</sub> and GC responses after T<sub>FH</sub>-inducing immunization. After immunization with sheep red blood cells (SRBCs), a strong protein antigen, the formation of T<sub>FH</sub> cells and GC B cells was greatly enhanced in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice compared with WT mice (**Figure 3-14A**). We observed a similar finding after challenging WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice with a T cell-dependent antigen, NP-OVA precipitated in alum and LPS (**Figure 3-14B**). We conclude that deletion of *Pten* in Treg cells results in enhanced T<sub>FH</sub> and GC reactions both under steady state and upon immunization.

### Coordination of T<sub>H1</sub> and T<sub>FH</sub> Responses by PTEN Signaling in Treg Cells

We next investigated whether the increased T<sub>FH</sub> response in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice was a cell-autonomous defect. To this end, we generated mixed bone marrow (BM) chimeras by reconstituting alymphoid *Rag1<sup>-/-</sup>* mice with a 1:1 mixture of *Pten<sup>fl/fl</sup>Foxp3-Cre* CD45.2<sup>+</sup> (donor) and CD45.1<sup>+</sup> (spike) BM cells (denoted as *Pten<sup>fl/fl</sup>Foxp3-Cre:CD45.1<sup>+</sup>*), and as a control, a mixture of WT and CD45.1 cells (denoted as WT:CD45.1<sup>+</sup>). The frequency of T<sub>FH</sub> cells was considerably increased in both the donor and spike-derived populations in the *Pten<sup>fl/fl</sup>Foxp3-Cre:CD45.1<sup>+</sup>* chimeras, as compared with the frequency of T<sub>FH</sub> cells in the WT:CD45.1<sup>+</sup> chimeras (**Figure 3-15A**). Additionally, *Pten<sup>fl/fl</sup>Foxp3-Cre:CD45.1<sup>+</sup>* chimeras had augmented GC B cells (**Figure 3-15B**). Thus, *Pten* deficiency in Treg cells results in a dominantly acting effect on the T<sub>FH</sub> and GC responses.

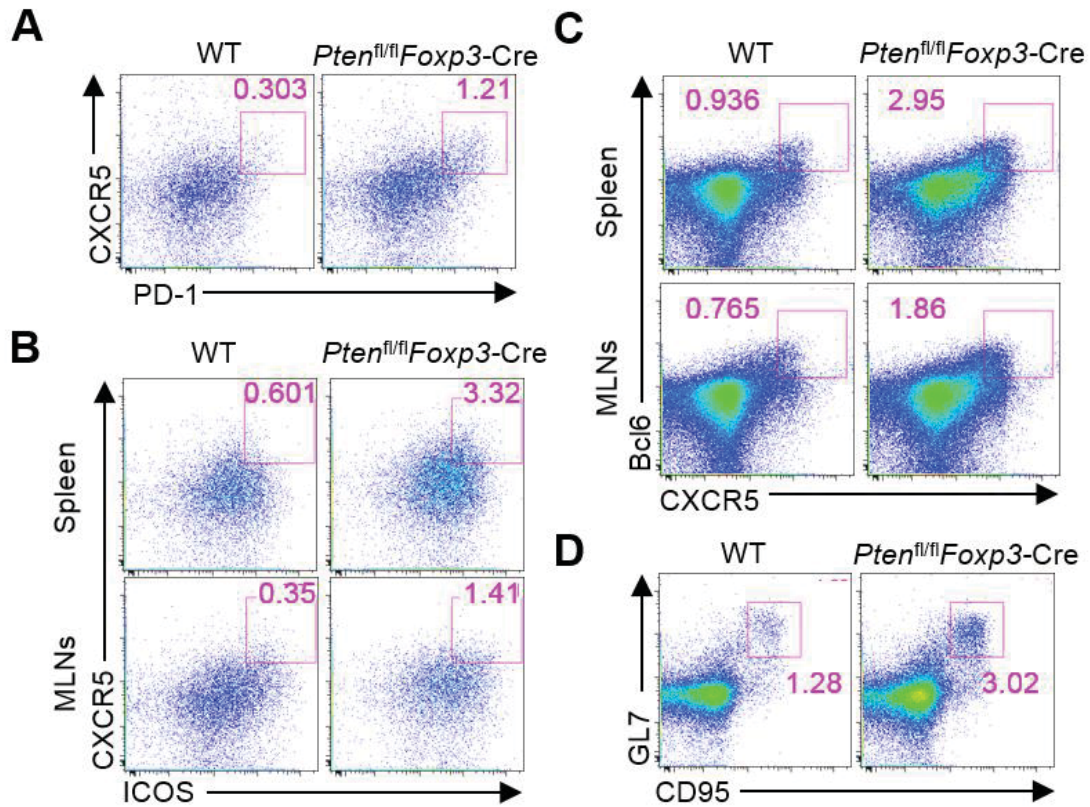
The T<sub>H1</sub> cytokines such as IFN- $\gamma$  have been implicated in potentiating T<sub>FH</sub> responses (Lee et al., 2012a), although opposing evidence also exists (Nakayamada et al., 2011; Ray et al., 2014), suggesting a context-dependent effect. We therefore explored



**Figure 3-11. Aberrant T<sub>FH</sub>, T<sub>FR</sub> and GC B cells responses in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.**

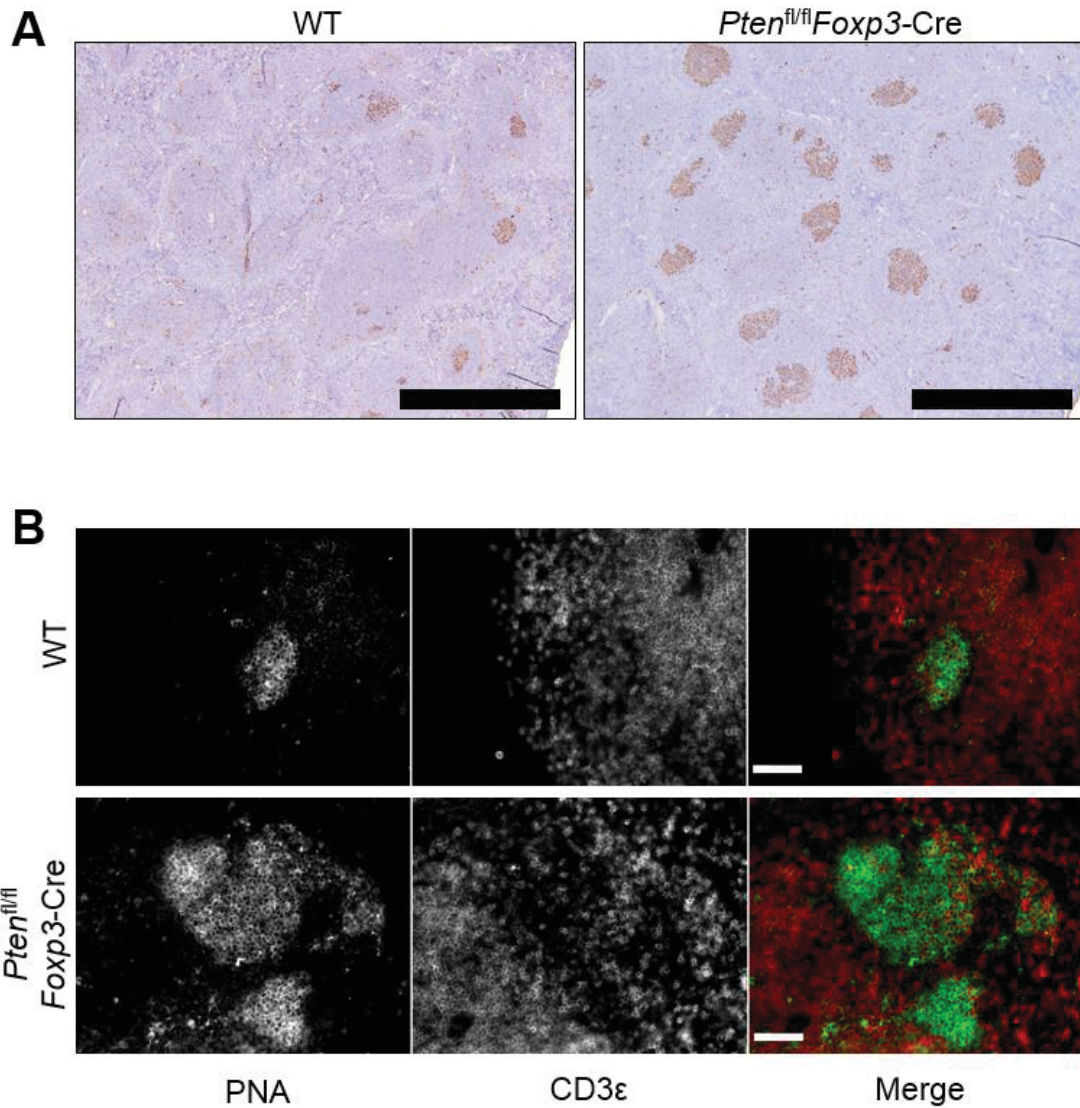
(A) Left, flow cytometry analysis of T<sub>FH</sub> cells (CXCR5<sup>+</sup>PD-1<sup>+</sup>) cells (gated on CD4<sup>+</sup>TCRβ<sup>+</sup> cells) in the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Right, the frequency and numbers of T<sub>FH</sub> cells. (B) Left, analysis of conventional T<sub>FH</sub> (CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>Foxp3-YFP<sup>-</sup>) and T<sub>FR</sub> cells (CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>Foxp3-YFP<sup>+</sup>) in the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. (C) Left, analysis of GL7<sup>+</sup>CD95<sup>+</sup> GC B cells (gated on CD19<sup>+</sup> B cells) in the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Right, the frequency and numbers of GC B cells. Numbers indicate percentage of cells in quadrants or gates. Data are mean ± s.e.m. \*P < 0.05 and \*\*P < 0.01.





**Figure 3-12. Analysis of T<sub>FH</sub> and GC B cells.**

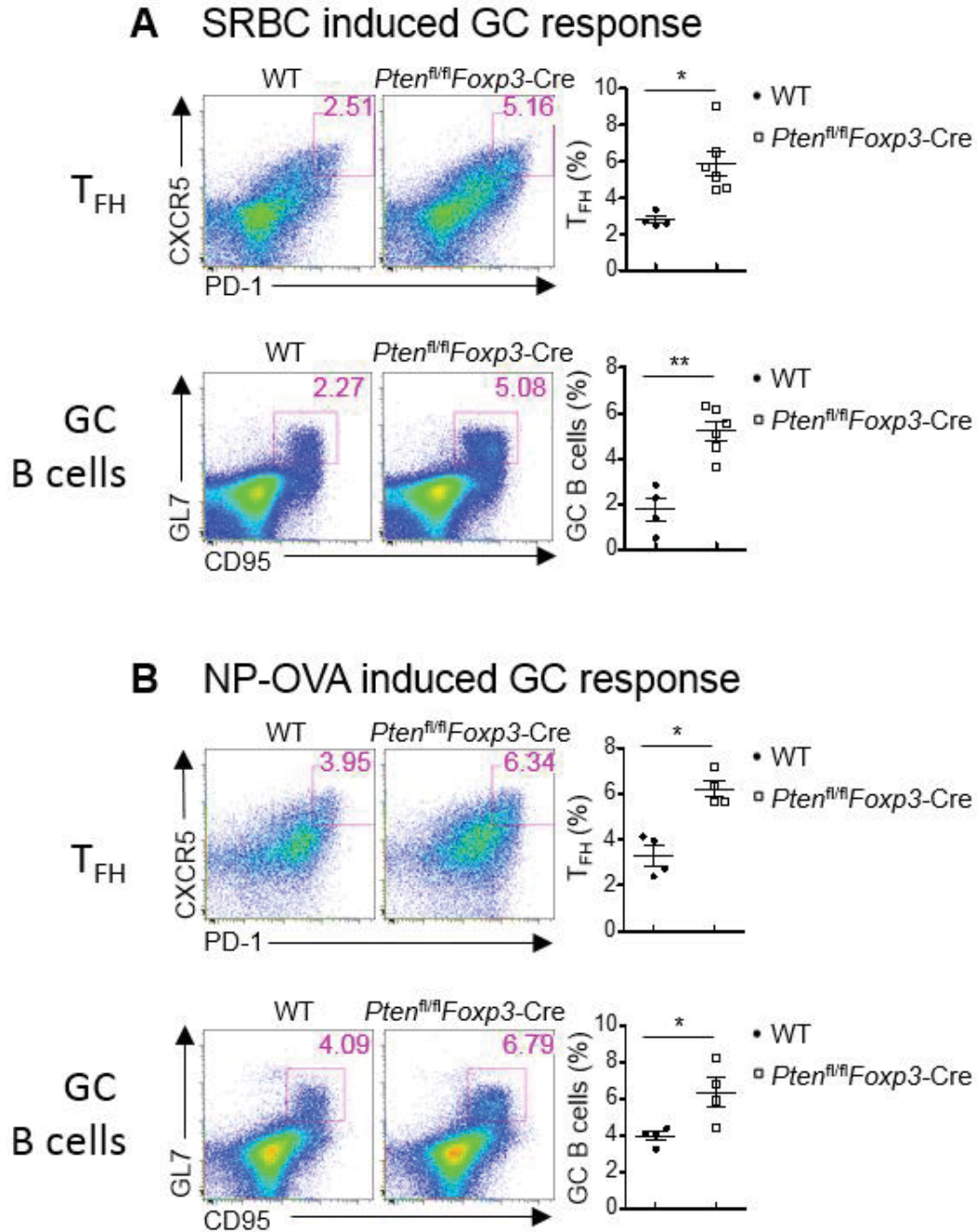
(A) Flow cytometry of CXCR5 and PD-1 expression (gated on CD4<sup>+</sup>TCRβ<sup>+</sup> cells) in the MLNs of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Flow cytometry of (B) CXCR5 and ICOS and, (C) CXCR5 and Bcl6 expression (gated on CD4<sup>+</sup>TCRβ<sup>+</sup> cells) in the spleen and MLNs of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. (D) Flow cytometry of GL7 and CD95 expression (gated on CD19<sup>+</sup> cells) in the MLNs of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.



**Figure 3-13. Immunohistochemistry of germinal centers in WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.**

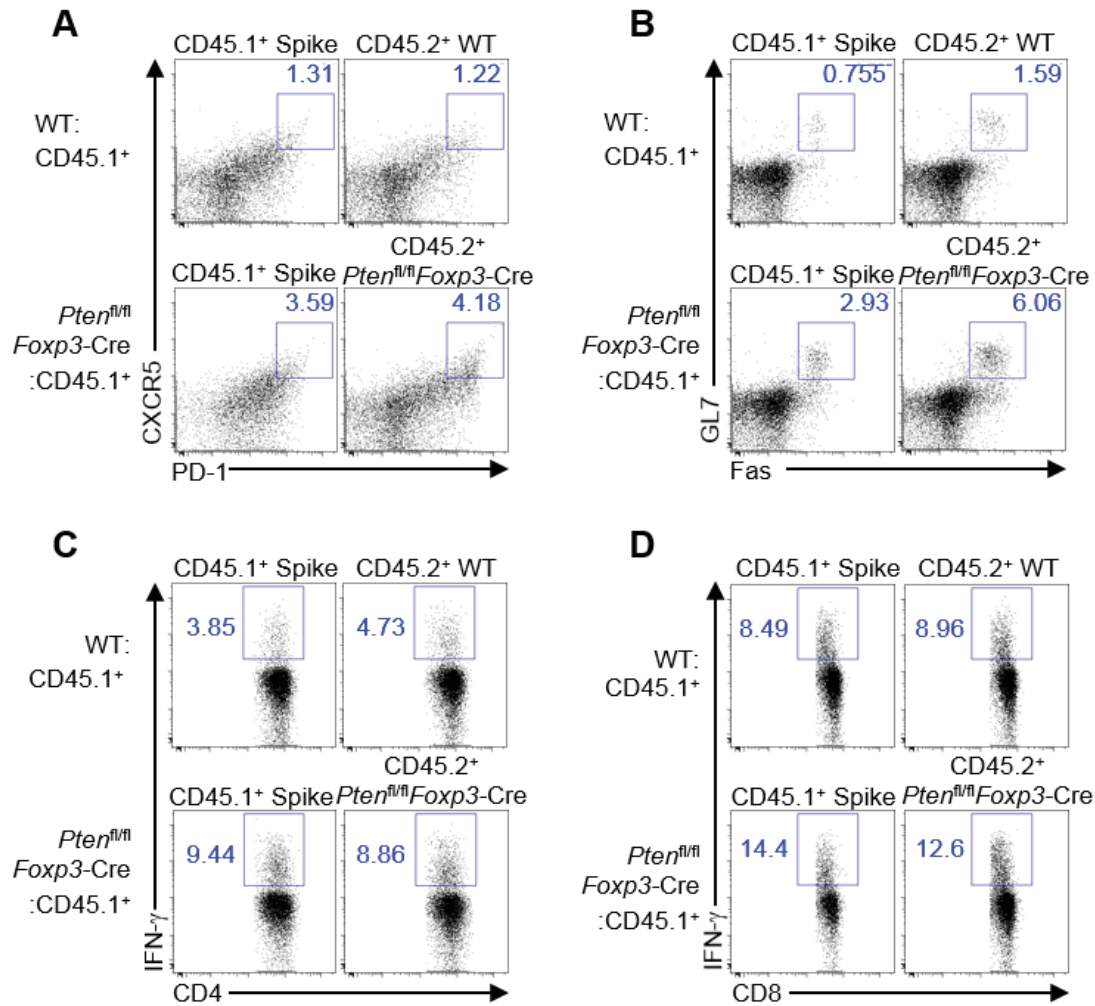
(A) Germinal center (brown) defined by peanut agglutinin (PNA) staining of spleen sections of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice (magnification, x4; scale bars, 1mm). (B) Immunofluorescence of germinal center in MLN sections of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice defined by the staining of CD3 (red, marking T cell zones) and PNA (green) (scale 60 μm).





**Figure 3-14. Induction of germinal center responses in WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.**

Analysis and quantification of T<sub>FH</sub> and GC B cells in the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice immunized with (A) SRBCs and (B) NP-OVA 7 days previously. Numbers indicate percentage of cells in quadrants or gates. Data are mean ± s.e.m. \*P < 0.05 and \*\*P < 0.01.



**Figure 3-15. Analysis of bone marrow-derived chimeras reveals dysregulated T<sub>FH</sub> and GC B cell responses and IFN- $\gamma$  overproduction in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.** Sublethally irradiated *Rag1<sup>-/-</sup>* mice were reconstituted with a 1:1 mix of CD45.1<sup>+</sup> bone marrow (BM) cells with either CD45.2<sup>+</sup> WT or *Pten<sup>fl/fl</sup>Foxp3-Cre* BM cells. Following reconstitution, the mixed chimeras were analyzed for (A) T<sub>FH</sub>, (B) GC B cells and intracellular staining of IFN- $\gamma$  in (C) CD4<sup>+</sup> and (D) CD8<sup>+</sup> T cells. Numbers indicate percentage of cells in quadrants or gates.

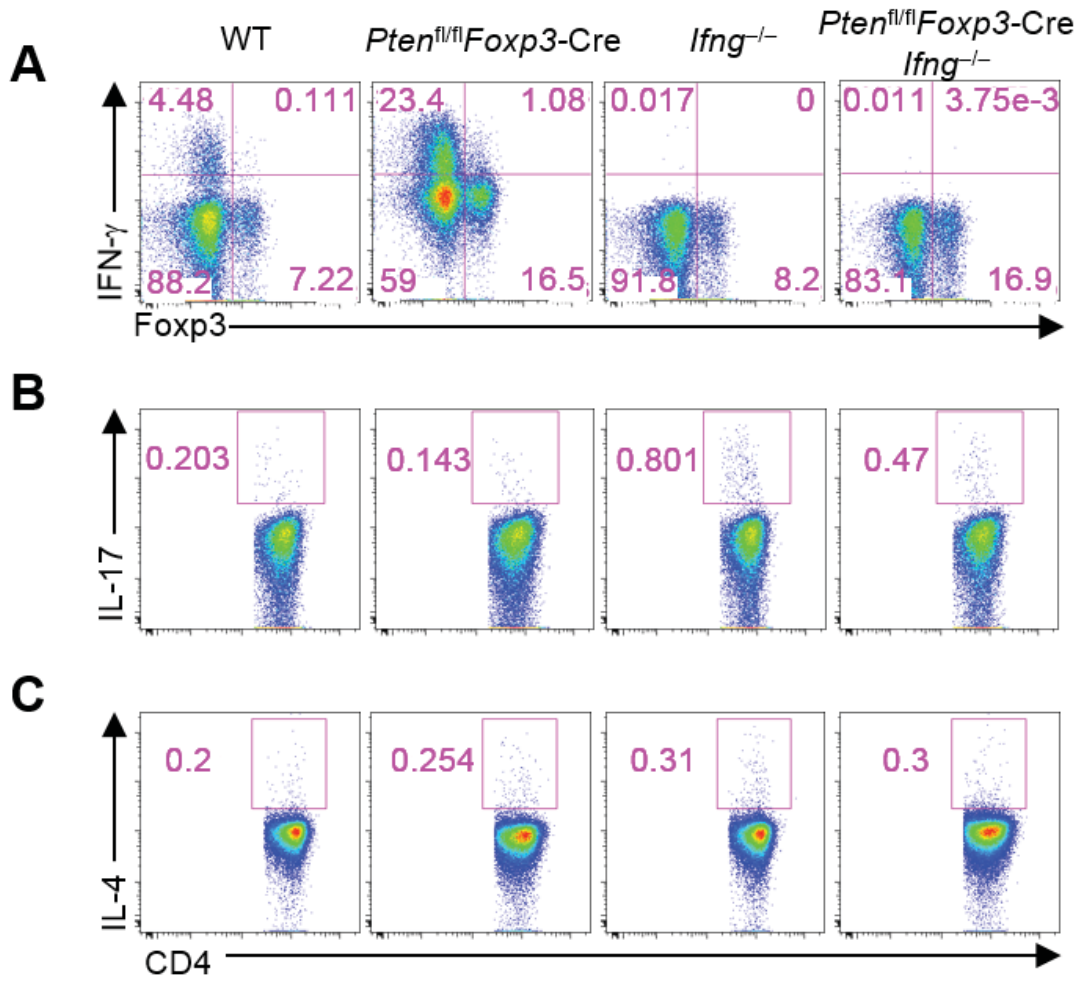
whether dysregulated cytokine production was observed in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre:CD45.1<sup>+</sup>. In the *Pten*<sup>fl/fl</sup>*Foxp3*-Cre:CD45.1<sup>+</sup> mixed chimeras, IFN- $\gamma$  production from CD4<sup>+</sup> and CD8<sup>+</sup> T cells was enhanced irrespective of the source of donor cells (**Figure 3-15C and D**).

To determine the functional effects of the augmented IFN- $\gamma$  production, we crossed *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice with *Ifng*<sup>-/-</sup> mice to generate *Pten*<sup>fl/fl</sup>*Foxp3*-Cre *Ifng*<sup>-/-</sup> double knockout mice (**Figure 3-16A**). Deletion of IFN- $\gamma$  did not exert strong effects on the production of IL-17 and IL-4 from the conventional CD4<sup>+</sup> T cells (**Figure 3-16B and C**). However, IFN- $\gamma$  deficiency substantially blocked the elevated frequencies of T<sub>FH</sub> and GC B cells (**Figure 3-17A and B**) and the increased formation of GCs in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice (**Figure 3-18**). Moreover, the increased production of serum ANA antibody and the deposition of IgG in the kidney glomeruli of *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice were essentially rectified in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre *Ifng*<sup>-/-</sup> mice (**Figures 3-19 and 3-20**). Thus, the increased production of IFN- $\gamma$  in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice largely accounts for the exacerbated T<sub>FH</sub>, GC, and autoimmune responses, thereby highlighting the crucial role of PTEN signaling in Treg cells to coordinate T<sub>H1</sub> and T<sub>FH</sub> reactions.

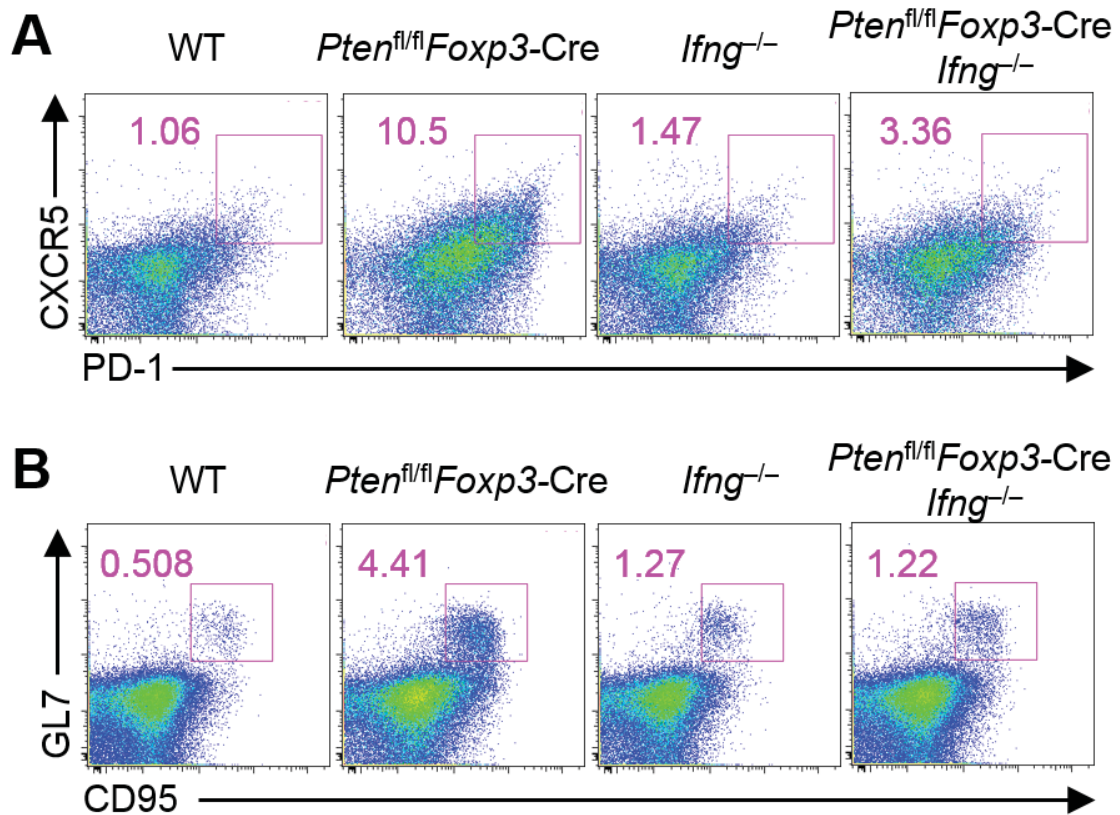
### PTEN Is Crucial in Maintaining the Stability of Treg Cells

Despite the crucial role of IFN- $\gamma$  overproduction in disrupting immune homeostasis in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice, deletion of IFN- $\gamma$  did not affect the phenotype of increased Treg cellularity in these mice (**Figure 3-21A**). We therefore explored the direct effects of *Pten* deficiency on the homeostasis and functionality of Treg cells. As compared with WT counterparts, Treg cells from *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice showed higher expression of CD44 and CD69 but lower levels of CD62L, indicating an elevated level of activation (**Figure 3-22A**). We next examined Treg-selective effector molecules. *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells showed increased levels of ICOS and PD-1, and to a lesser extent, GITR, whereas the expression of CTLA4 was largely normal (**Figure 3-22B**). In sharp contrast to the elevated expression of activation markers and effector molecules, the expression of CD25, a signature molecule of Treg and activated T cells, was markedly downregulated in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells, corresponding to the expansion of the Foxp3<sup>+</sup>CD25<sup>-</sup> population (**Figure 3-22C**). As compared with Foxp3<sup>+</sup>CD25<sup>+</sup> cells, the Foxp3<sup>+</sup>CD25<sup>-</sup> population expressed lower levels of Foxp3, as reported previously (Komatsu et al., 2009). Consistent with this observation, Blimp1, a transcription factor implicated in CD25 downregulation in CD8<sup>+</sup> T cells (Shin et al., 2013), was upregulated in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells (**Figure 3-22D**). These results indicate that *Pten* deficiency in Treg cells results in dysregulated expression of multiple Treg activation and phenotypic molecules.

The lineage stability of Treg cells is a matter of considerable interest and debate (Bailey-Bucktrout et al., 2013; Komatsu et al., 2014; Miyao et al., 2012; Rubtsov et al., 2010; Tsuji et al., 2009; Zhou et al., 2009b), but the signaling mechanisms involved are largely unexplored. To determine whether *Pten* deficiency affects the stability of Treg



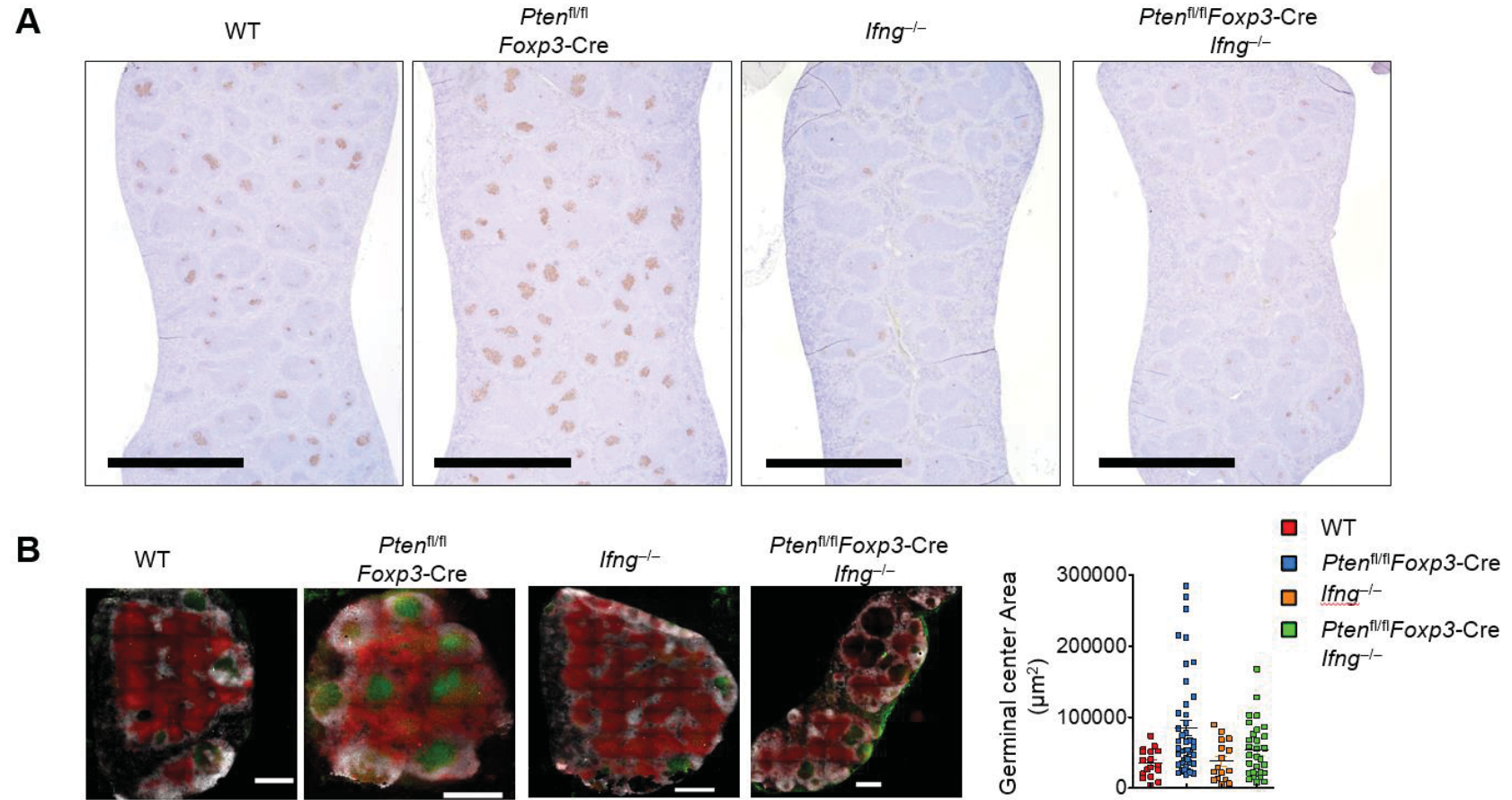
**Figure 3-16. Generation and phenotypic analysis of *Pten*<sup>fl/fl</sup>*Foxp3-Cre* *Ifng*<sup>-/-</sup> mice.** Cytokine production of (A) IFN- $\gamma$ , (B) IL-17 and (C) IL-4 by CD4<sup>+</sup> T cells from WT, *Pten*<sup>fl/fl</sup>*Foxp3-Cre*, *Ifng*<sup>-/-</sup> and *Pten*<sup>fl/fl</sup>*Foxp3-Cre* *Ifng*<sup>-/-</sup> mice after in vitro stimulation for 4 h with PMA plus ionomycin in the presence of monensin. Numbers indicate percentage of cells in quadrants or gates.



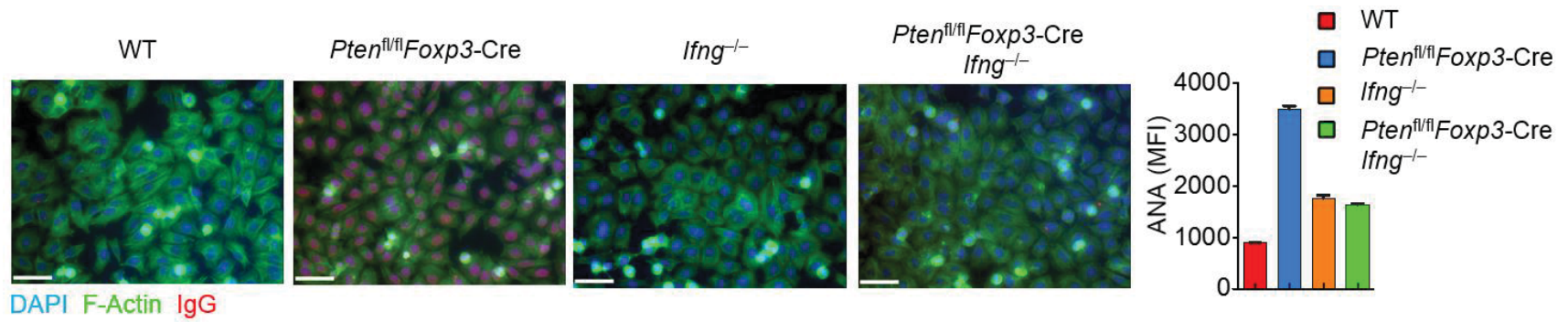
**Figure 3-17. Uncontrolled T<sub>FH</sub> and GC B cells in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice rescued in *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice.**

Analysis of (A) T<sub>FH</sub> and (B) GC B cells in the spleen of WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice. Numbers indicate percentage of cells in quadrants or gates.

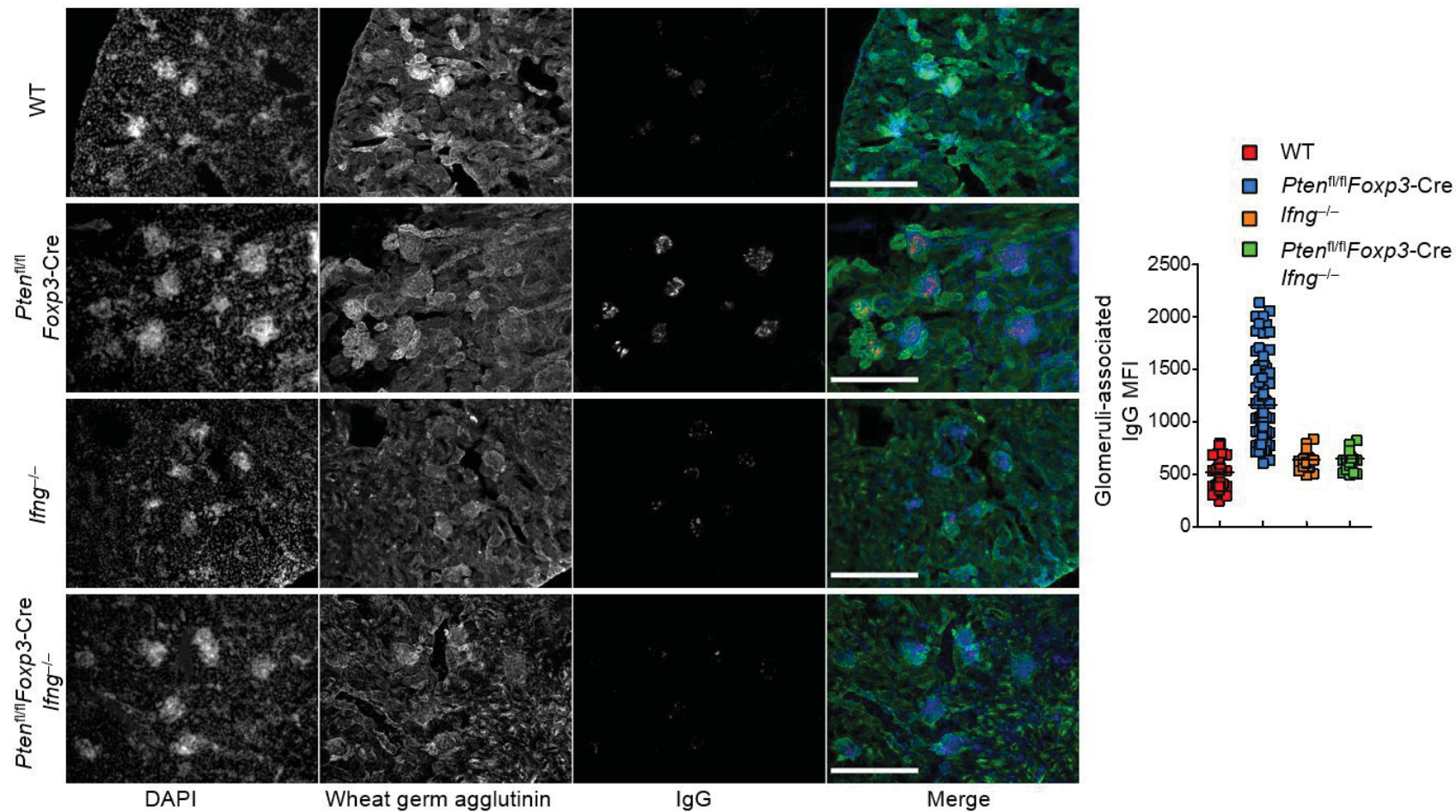




**Figure 3-18. Immunohistochemistry of germinal centers in WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice.** (A) PNA staining of spleen sections (magnification, x2; scale bars, 2mm) and (B) immunofluorescence and quantification of MLN sections for the staining of CD3 (red), PNA (green) and IgD (white) (scale 500  $\mu\text{m}$ ) from WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice.



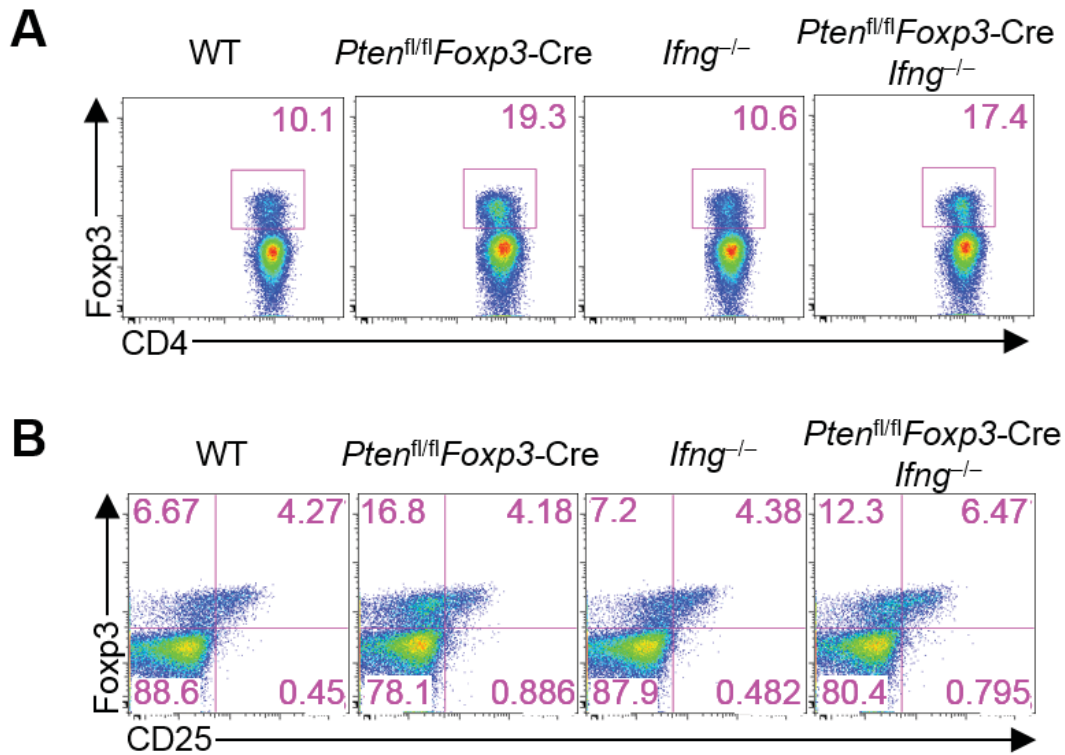
**Figure 3-19. Detection of antinuclear antibodies in WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice.** Representative images and quantification of fluorescent intensity of ANA IgG autoantibodies detected with Hep-2 slides in the serum from WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice. (Scale 60  $\mu$ m).



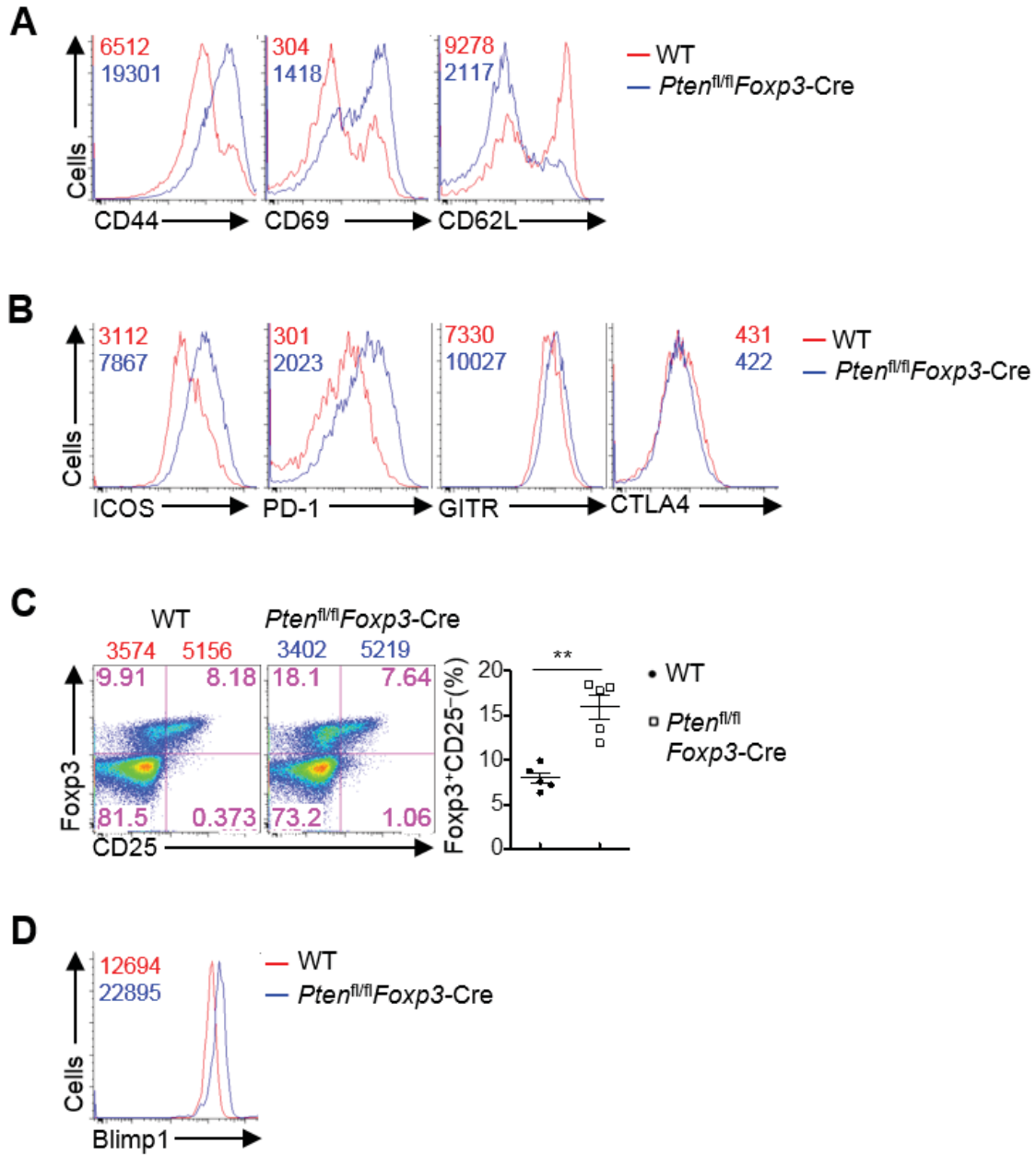
**Figure 3-20. Immunofluorescence analysis of glomeruli for IgG deposits in WT, *Pten<sup>fl/fl</sup> Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup> Foxp3-Cre Ifng<sup>-/-</sup>* mice.**

Representative images of immunofluorescence imaging of kidney sections showing IgG deposits (scale 300  $\mu$ m), and quantitative analysis (right) from WT, *Pten<sup>fl/fl</sup> Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup> Foxp3-Cre Ifng<sup>-/-</sup>* mice.





**Figure 3-21. Analysis of Treg cells in WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice.**  
 Flow cytometry analysis of (A) Treg (CD4<sup>+</sup>Foxp3<sup>+</sup>) cells and expression of (B) CD25 and Foxp3 (gated on CD4<sup>+</sup>TCRβ<sup>+</sup> cells) in the spleen of WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice. Numbers indicate percentage of cells in gates.



**Figure 3-22. Effects of *Pten* loss in Treg cells.**

(A) Expression of CD44, CD69 and CD62L and (B) ICOS, PD-1, GITR and CTLA4 in Treg cells from the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice, with mean fluorescence intensity (MFI) plotted above graphs. (C) Expression of CD25 and Fopx3 (gated on CD4<sup>+</sup>TCRβ<sup>+</sup> cells) in the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice; numbers above graphs indicate MFI of Fopx3 in CD25<sup>-</sup> and CD25<sup>+</sup> subsets. Right, quantification of Fopx3<sup>+</sup>CD25<sup>-</sup> cells. Numbers indicate percentage of cells in quadrants or gates. (D) Expression of Blimp1 in splenic Treg cells of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Numbers above graphs indicate MFI of Blimp1. \*\**P* < 0.001. Data are mean ± s.e.m.

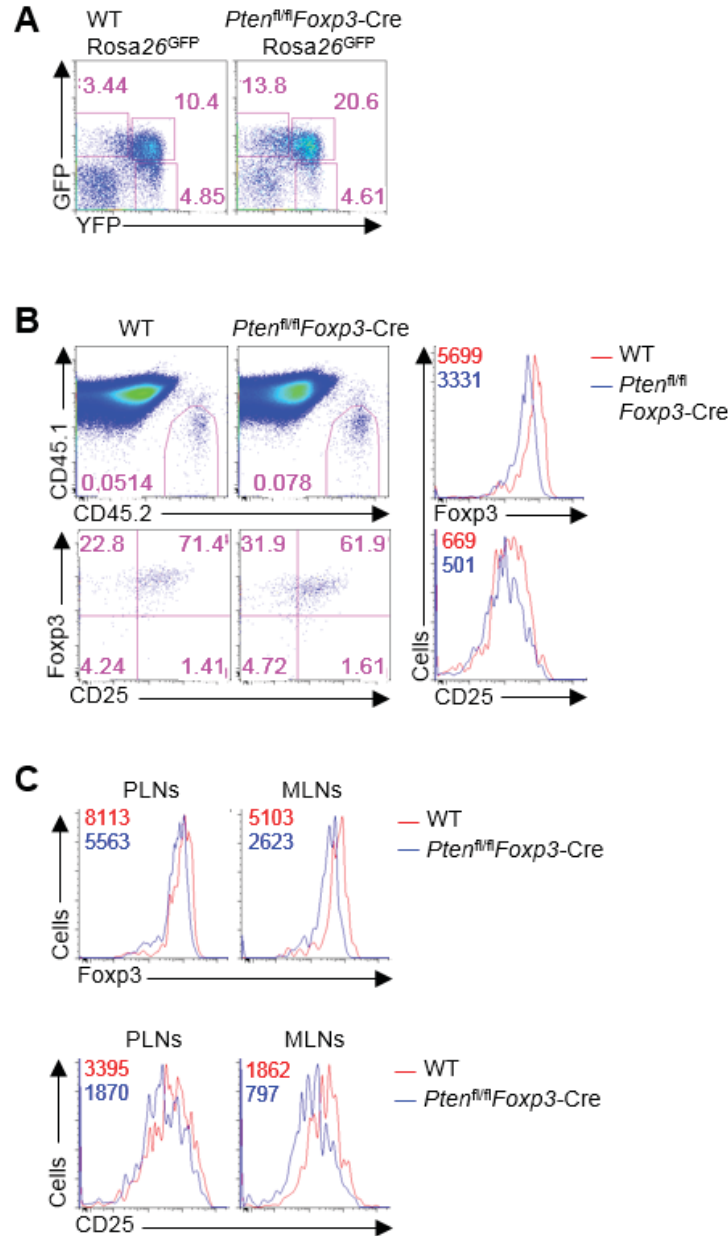
cells, we crossed *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice with a Cre recombination-sensitive reporter allele, in which the ubiquitously expressed ROSA26 locus contains a loxP site-flanked STOP cassette followed by the gene encoding green fluorescent protein (GFP) (Zeng et al., 2013). In this lineage tracing system, *Foxp3*-Cre-mediated excision of the floxed STOP cassette results in constitutive, heritable expression of GFP, even for those “ex-Treg” cells that have lost *Foxp3* expression (the GFP<sup>+</sup>YFP-*Foxp3*<sup>-</sup> population). *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice contained a notable accumulation of the GFP<sup>+</sup>YFP-*Foxp3*<sup>-</sup> population (**Figure 3-23A**), indicating the preferential loss of *Foxp3* expression upon *Pten* deletion. Additionally, Treg cells from *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice had increased expression of IFN- $\gamma$ , whereas IL-17 expression was largely unaltered (**Figure 3-24A and B**).

Moreover, *Pten*-deficient Treg cells upregulated signature molecules characteristic of T<sub>H</sub>1 and T<sub>FH</sub> cells, including CXCR3 and T-bet, and CXCR5 and Bcl6, respectively, whereas expression of IRF4 and p-STAT3, which are required for Treg cell-mediated suppression of T<sub>H</sub>2 and T<sub>H</sub>17 cells (Chaudhry et al., 2009; Zheng et al., 2009), remained unchanged (**Figure 3-24C**). Consistent with these observations, *Pten*-deficient Treg cells contained an expanded T-bet<sup>+</sup>CXCR3<sup>+</sup> population that is important for the regulation of type 1 inflammation (**Figure 3-24D**) (Koch et al., 2009). Because the development of this Treg cell subset is dependent upon IFN- $\gamma$  signaling (Koch et al., 2009), we examined whether excessive IFN- $\gamma$  production in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice was involved in the dysregulation of T-bet and CXCR3. IFN- $\gamma$  deficiency had a partial rescue effect on the expansion of T-bet<sup>+</sup>CXCR3<sup>+</sup> population in *Pten*-deficient Treg cells (**Figure 3-24D**), indicating that the augmented expression of T-bet and CXCR3 upon loss of *Pten* is largely cell intrinsic, not simply secondary to the overproduction of IFN- $\gamma$ . Finally, to directly test the role of PTEN in maintaining Treg stability, we sorted *Foxp3*<sup>+</sup>CD25<sup>+</sup> cells from WT and *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice and transferred them into congenic mice (CD45.1<sup>+</sup>). The expression of *Foxp3* and CD25 was downregulated in *Pten*-deficient Treg cells in various organs examined, as compared with WT cells (**Figure 3-23B and C**). These complementary approaches indicate that *Pten* deficiency results in a loss of Treg stability.

Notably, despite the strong effects of IFN- $\gamma$  at disrupting immune homeostasis, deletion of IFN- $\gamma$  in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice did not affect the spontaneous development of the *Foxp3*<sup>+</sup>CD25<sup>-</sup> population (**Figure 3-21B**). Thus, the enhanced IFN- $\gamma$  expression from *Pten*-deficient Treg cells is associated with the loss of Treg cell stability, but this cytokine is unlikely to be the main driver of instability.

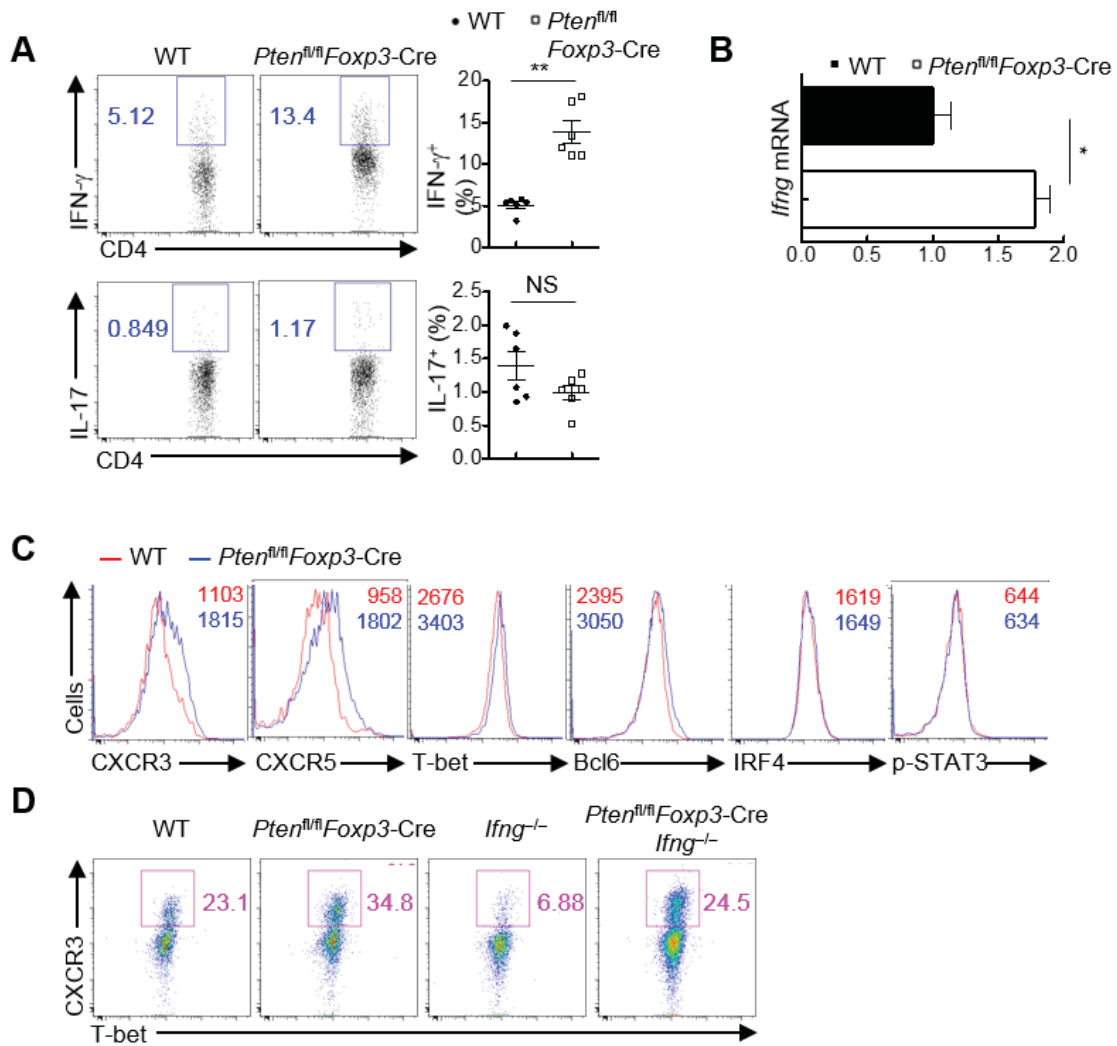
### PTEN Is Haploinsufficient in Treg Cells

Given the potent effects of PTEN signaling in Treg cells, we asked whether partial loss of PTEN function in Treg cells is physiologically relevant, by analyzing *Pten*<sup>fl/+</sup>*Foxp3*-Cre mice. As expected, *Pten*<sup>fl/+</sup>*Foxp3*-Cre Treg cells, but not other T cell subsets, had partial reduction of PTEN expression at both the mRNA and protein levels (**Figure 3-25A and B**). Remarkably, heterozygous loss of *Pten* resulted in the expansion

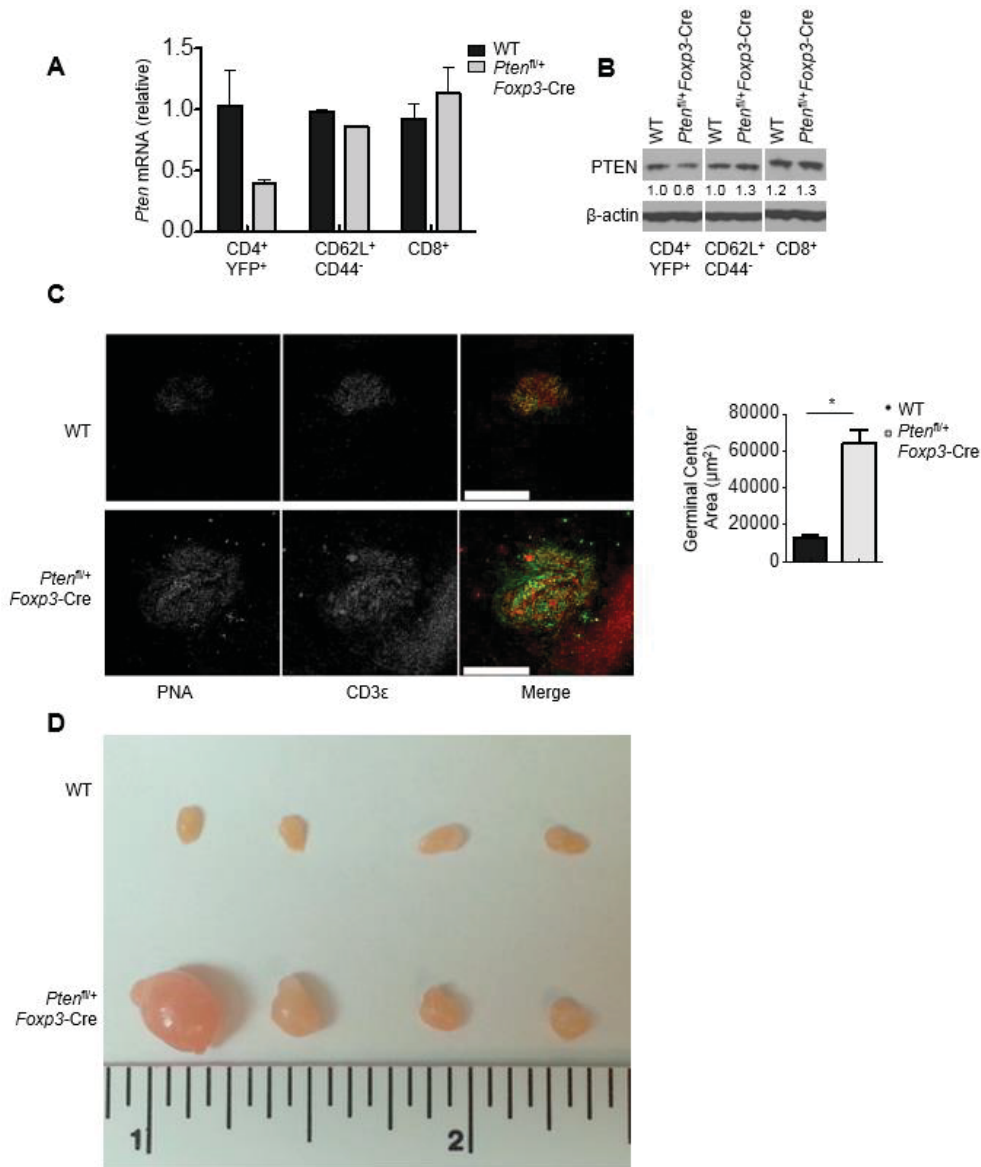


**Figure 3-23. *Pten* deficiency impairs Treg cell stability.**

(A) Foxp3-YFP and GFP expression in CD4<sup>+</sup> T cells from *Pten*<sup>+/+</sup> Foxp3-Cre Rosa26<sup>GFP</sup> and *Pten*<sup>fl/fl</sup> Foxp3-Cre Rosa26<sup>GFP</sup> mice. (B) Left, donor cell percentages (top) and Foxp3 and CD25 expression (bottom) in WT and *Pten*<sup>fl/fl</sup> Foxp3-Cre Treg (CD45.2<sup>+</sup>) cells transferred into CD45.1<sup>+</sup> recipients. Right, expression of Foxp3 and CD25 from donor-derived WT and *Pten*<sup>fl/fl</sup> Foxp3-Cre T cells; numbers above graphs indicate MFI. (C) Analysis of Foxp3 (top) and CD25 (bottom) expression in PLNs and MLNs of CD45.1<sup>+</sup> recipients after transferring WT and *Pten*<sup>fl/fl</sup> Foxp3-Cre Treg (CD45.2<sup>+</sup>) cells. Numbers above graphs indicate MFI. Numbers indicate percentage of cells in quadrants or gates.



**Figure 3-24. Analysis of Treg cells in *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice and *Pten<sup>fl/fl</sup>Foxp3-Cre* Treg cell phenotypes under steady state.** (A) Expression of IFN- $\gamma$  and IL-17 (right, quantification of IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup> producing cells in Treg cells) (B) Analysis of *Ifng* RNA in Treg cells of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. (C) Expression of CXCR3, CXCR5, T-bet, Bcl6, IRF4 and p-Stat3 in Treg cells from the spleen of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. (D) Analysis of CXCR3 and T-bet expression in Treg cells in the spleen of WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Ifng<sup>-/-</sup>* and *Pten<sup>fl/fl</sup>Foxp3-Cre Ifng<sup>-/-</sup>* mice. Data are mean  $\pm$  s.e.m. \*P < 0.05 and \*\*P < 0.001.



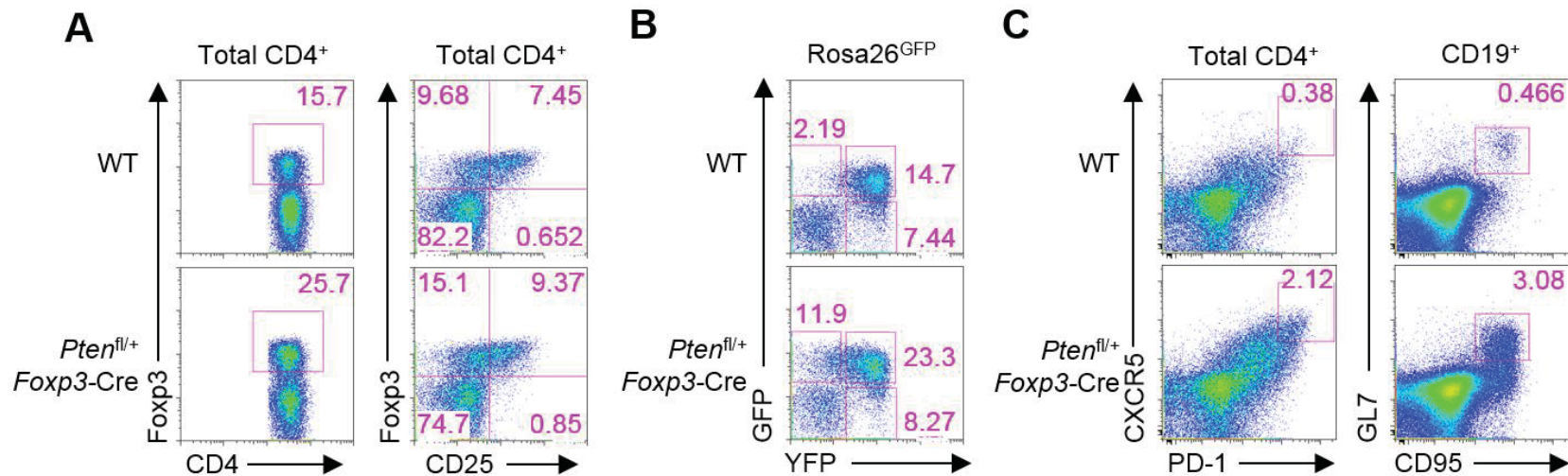
**Figure 3-25. *Pten* expression and immune dysregulation in *Pten*<sup>fl/+</sup>*Foxp3-Cre* mice.** Analysis of (A) *Pten* mRNA and (B) protein expression in Treg, naïve CD4<sup>+</sup> (CD62L<sup>+</sup>CD44<sup>-</sup>), and CD8<sup>+</sup> T cells from WT and *Pten*<sup>fl/+</sup>*Foxp3-Cre* mice. Numbers below the PTEN lanes in (B) indicate band intensity relative to that of β-actin. (C) Immunofluorescence of spleen sections of WT and *Pten*<sup>fl/+</sup>*Foxp3-Cre* mice for the staining of CD3 (red) and PNA (green) showing germinal center (scale 60 µm). Right, quantification of germinal center area. (D) Images of peripheral lymph nodes from WT and *Pten*<sup>fl/+</sup>*Foxp3-Cre* mice (~5 months old) with lymphoproliferative disease. Data are mean ± s.e.m. \*P < 0.05.

of Treg cells, associated with the considerable downregulation of CD25 expression (**Figure 3-26A**). *Pten*<sup>fl/+</sup>*Foxp3*-Cre mice also showed a notable accumulation of GFP<sup>+</sup>YFP-*Foxp3*<sup>-</sup> population in the lineage tracing system (**Figure 3-26B**). Moreover, these mice had higher frequencies of CXCR5<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub> cells and GL7<sup>+</sup>CD95<sup>+</sup> GC B cells (**Figure 3-26C**), and a greater abundance of GCs in the spleen (**Figure 3-25C**). These findings prompted us to examine whether heterozygous loss of *Pten* in Treg cells caused autoimmunity and lymphoid hyperplasia as observed in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice. *Pten*<sup>fl/+</sup>*Foxp3*-Cre mice displayed significantly elevated titers of circulating ANA antibodies (**Figure 3-27**) and augmented IgG deposits in the kidney glomeruli (**Figure 3-28**), indicative of systemic autoimmunity. Furthermore, *Pten*<sup>fl/+</sup>*Foxp3*-Cre mice spontaneously developed lymphadenopathy (**Figure 3-25D**). These results demonstrate that PTEN is haploinsufficient in Treg cells.

### Summary

In this chapter, we used conditional knockout mice with *Pten* specifically deleted in Treg cells to explore its functional relevance *in vivo*. We have highlighted Treg-specific loss of *Pten* results in higher frequency of activated CD44<sup>hi</sup> T cells and Treg cells. Additionally, excessive T<sub>FH</sub> responses (CXCR5<sup>+</sup>PD1<sup>+</sup>CD4<sup>+</sup> T cells) and spontaneous formation of germinal centers (detected with PNA staining and immunofluorescence assay) was also seen in steady state. Increased T<sub>FH</sub> and germinal center reactions were also evident in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre after using SRBC or NP-OVA immunization. Loss of *Pten* in Treg cells was sufficient to induce the development of systemic autoimmune and lymphoproliferative disease, as determined by histological analyses, autoantibody (dsDNA and ANA) detection, and immunofluorescence of IgG deposits in kidney. Furthermore, deletion of IFN- $\gamma$  considerably blocked such immune dysregulation, indicating the coordinated control of T<sub>H1</sub> and T<sub>FH</sub> responses by PTEN signaling in Treg cells (investigated using *Pten*<sup>fl/fl</sup>*Foxp3*-Cre *Ifng*<sup>-/-</sup> double knockout mice). Using lineage tracing and *in vivo* transfer models, we elucidated the underlying mechanisms by showing that deletion of *Pten* resulted in instability of Treg cells, featured by decreased expression of CD25 and *Foxp3* expression. Finally, we presented evidence that PTEN is haploinsufficient in Treg cells. Together, the findings shown in this chapter have established the necessity of PTEN signaling in Treg cells for normal immune tolerance.

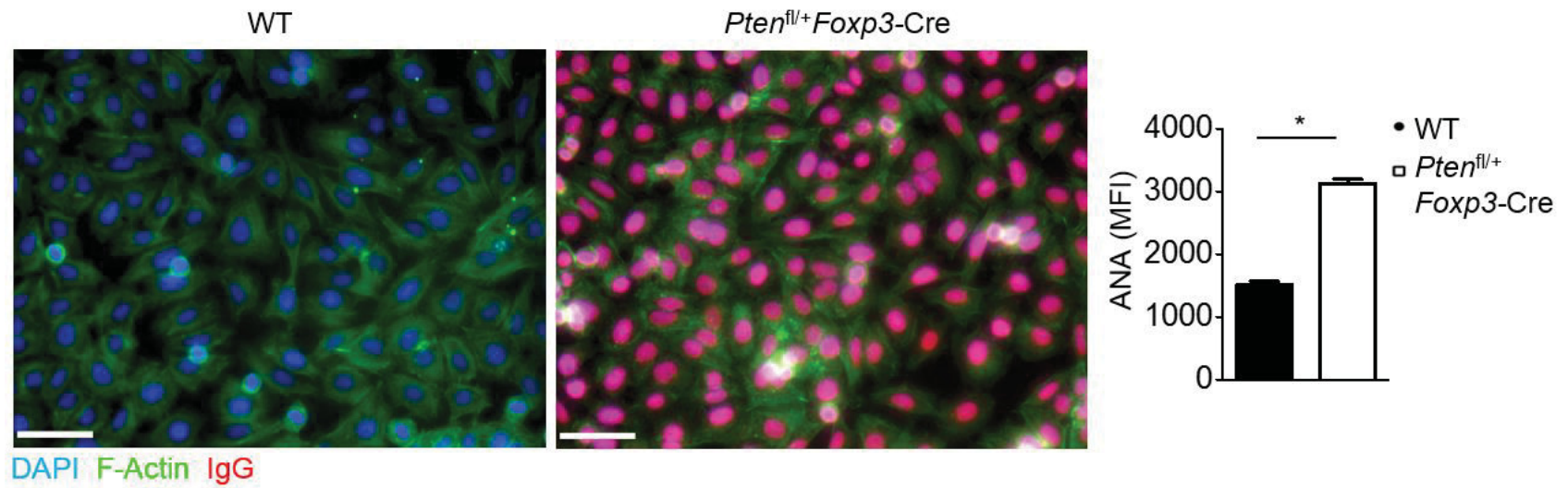




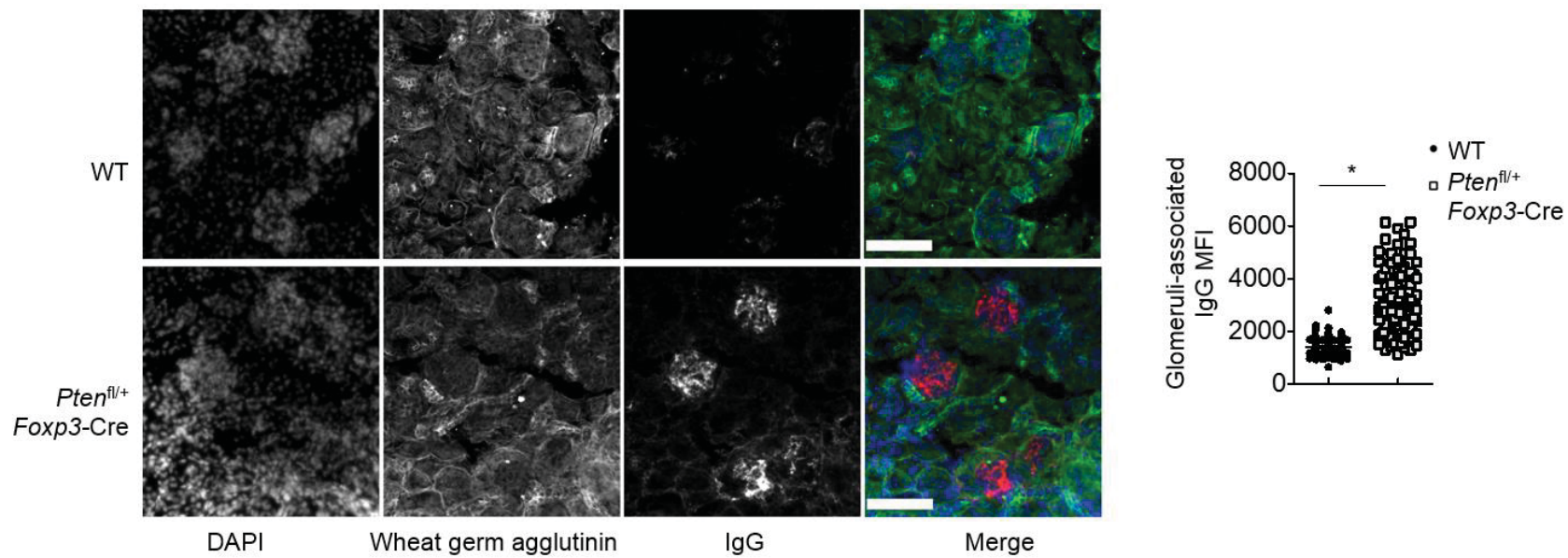
**Figure 3-26. Heterozygous loss of *Pten* in Treg cells is sufficient to disrupt immune homeostasis.**

(A) Flow cytometry analysis of Foxp3 and CD25 expression in WT and *Pten<sup>fl/+</sup> Foxp3-Cre* splenic CD4<sup>+</sup> T cells. (B) Foxp3-YFP and GFP expression in CD4<sup>+</sup> T cells from *Pten<sup>fl/+</sup> Foxp3-Cre Rosa26<sup>GFP</sup>* and *Pten<sup>fl/+</sup> Foxp3-Cre Rosa26<sup>GFP</sup>* mice. (C) Analysis of CXCR5<sup>+</sup>PD-1<sup>+</sup> cells (gated on CD4<sup>+</sup>TCRβ<sup>+</sup> cells) and GL7<sup>+</sup>CD95<sup>+</sup> GC B cells (gated on CD19<sup>+</sup> B cells) in the spleen of WT and *Pten<sup>fl/+</sup> Foxp3-Cre* mice. Numbers indicate percentage of cells in gates.





**Figure 3-27. Detection of antinuclear antibodies in WT and *Pten<sup>fl/+</sup>Foxp3-Cre* mice.** Representative images (scale bars, 60  $\mu$ m) and quantification of fluorescent intensity (right) of serum anti-ANA IgG detected with fixed Hep-2 slides in WT and *Pten<sup>fl/+</sup>Foxp3-Cre* mice. Data are mean  $\pm$  s.e.m. \*P < 0.001.



**Figure 3-28. Immunofluorescence analysis of glomeruli for IgG deposits in WT and *Pten<sup>fl/+</sup> Foxp3-Cre* mice.** Immunofluorescence (left) and quantification (right) of IgG deposits in kidney glomerulus sections (scale bars, 100  $\mu$ m) from WT and *Pten<sup>fl/+</sup> Foxp3-Cre* mice. Data are mean  $\pm$  s.e.m. \*P < 0.0001.

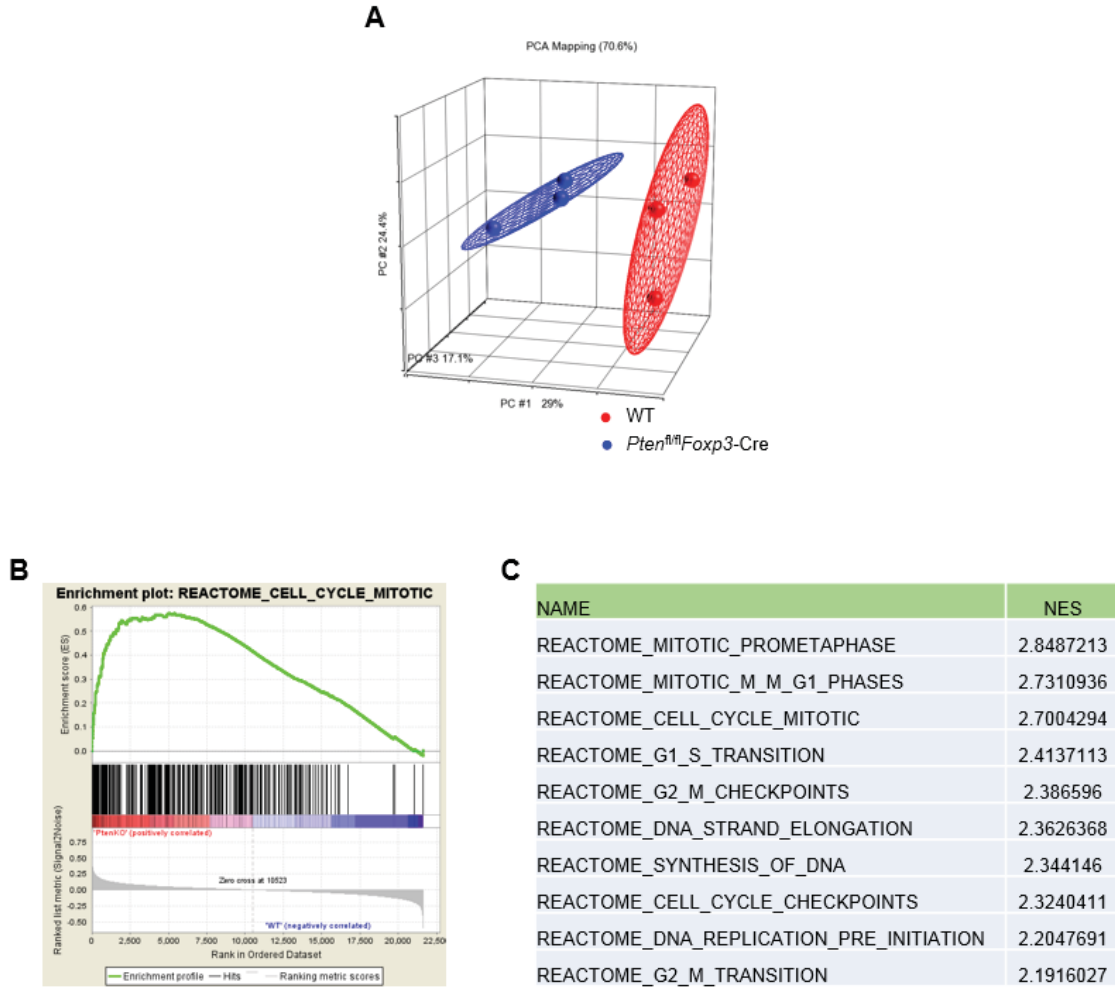
## CHAPTER 4. MOLECULAR AND METABOLIC MECHANISMS OF PTEN SIGNALING\*

In this chapter, we aimed to characterize the mechanisms underlying *Pten*-deficient Treg cell dysfunction and stability described in Chapter 3. Mechanistically, *Pten* deficiency results in dysregulated transcriptional and metabolic programs, including the balance between glycolytic activity and mitochondrial fitness. Further, mTORC2, not mTORC1 activity, is upregulated by *Pten* deletion in Treg cells. Depletion of Rictor-mTORC2 activity is sufficient to restore the functional abnormalities observed in *Pten*-deficient Treg cells. Additionally, retention of mTORC2 downstream target Foxo1 in the nucleus seems to abrogate the effects of PTEN loss in Treg cells. Our studies presented in this chapter establish a crucial role of the PTEN-mTORC2 axis in mediating Treg cell stability and homeostasis of the immune system.

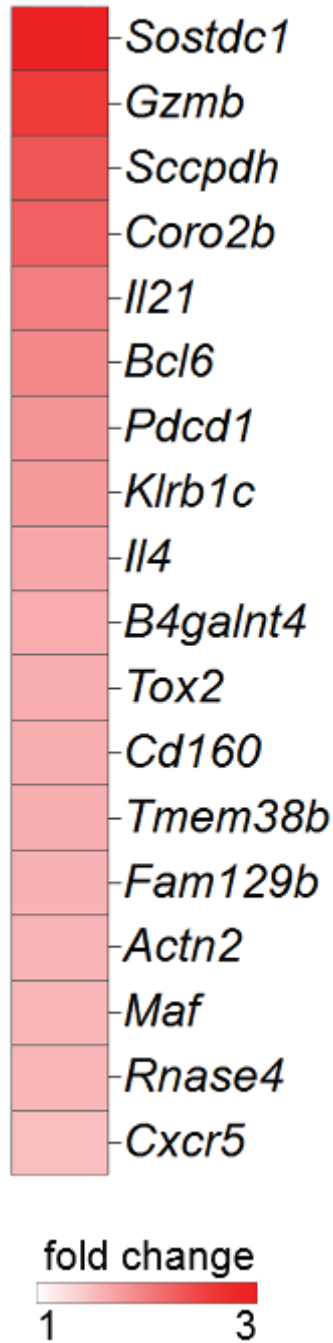
### PTEN-dependent Transcriptional and Metabolic Programs in Treg Cells

To explore PTEN-dependent molecular mechanisms in Treg cells, we next used microarrays to compare the transcriptional profiles between WT and *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells. *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells contained a total of 498 probes (representative of 352 unique genes) with a greater than 1.5-fold difference, including 212 upregulated and 286 downregulated probes. Principal component analysis (PCA) showed the clear distinctions between WT and *Pten*<sup>fl/fl</sup>*Foxp3*-Cre samples (**Figure 4-1A**). Consistent with the dysregulated stability, loss of *Pten* failed to restrain the transcription of genes involved in T<sub>FH</sub> differentiation, including *Gzmb*, *Il21*, *Bcl6*, *Pdcd1*, *Il4*, *Maf* and *Cxcr5* (Crotty, 2011; Wang et al., 2014) (**Figure 4-2**). To identify key networks regulated by PTEN, we performed gene-set enrichment analysis (GSEA) (Subramanian et al., 2005) to compare gene expression profiles of WT and *Pten*-deficient Treg cells. Remarkably, the top-ten, upregulated gene-sets in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells were all associated with cell cycle pathways (**Figure 4-1B** and **C**). To examine PTEN-dependent canonical pathways in Treg cells, we next performed the ingenuity pathway analysis (IPA) system by interrogating the differentially expressed genes at the 1.5-fold cut-offs. As shown in **Figure 4-3**, *Pten* deficiency affected multiple canonical pathways implicated in autoimmune diseases, helper T cell differentiation, and immune signaling mediated by transcription factors, co-stimulatory molecules and cytokines. Gene ontology (GO) analysis of these differentially regulated genes also showed that *Pten*-deficient Treg cells significantly upregulated groups of genes involved in autoimmune diseases, such as autoimmune thyroid disease and SLE, as well as in cell cycle regulation (Supplementary Data 1 – Gene Ontology Analysis of *Pten*-deficient Treg Mice). The transcriptome analysis therefore highlights an important role of PTEN in the regulation of immune response and cell cycle pathways.

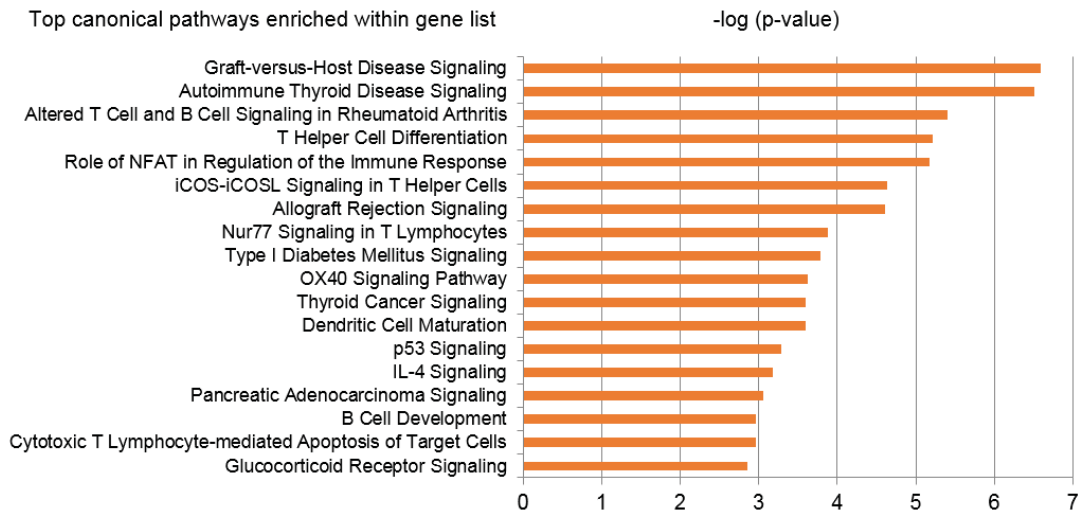
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\*Chapter 4 reprinted with permission. Shrestha, S., Yang, K., Guy, C., Vogel, P., Neale, G., and Chi, H. (2015). T<sub>reg</sub> cells require the phosphatase PTEN to restrain T<sub>H</sub>1 and T<sub>FH</sub> cell responses. *Nature immunology* 16, 178-187.



**Figure 4-1. The transcriptional profiles controlled by *Pten* in Treg cells.** (A) PCA mapping of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* Treg cells. (B) GSEA reveals the cell cycle mitotic pathway as one of the most extensively upregulated pathways in *Pten<sup>fl/fl</sup>Foxp3-Cre* Treg cells. (C) The list of top 10 gene sets upregulated in *Pten<sup>fl/fl</sup>Foxp3-Cre* Treg cells by GSEA. NES, normalized enrichment score.



**Figure 4-2. PTEN-dependent gene expression analysis.**  
 Heat map of expression of T<sub>FH</sub> genes related upregulated in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice, relative to the WT mean ( $\geq 1.5$ -fold).



**Figure 4-3. Ingenuity pathway analysis (IPA) of the differentially expressed genes in *Pten<sup>fl/fl</sup>Foxp3-Cre* Treg cells.**

IPA of canonical pathways controlled by *Pten* in Treg cells, differentially expressed genes at the 1.5-fold cut-offs.



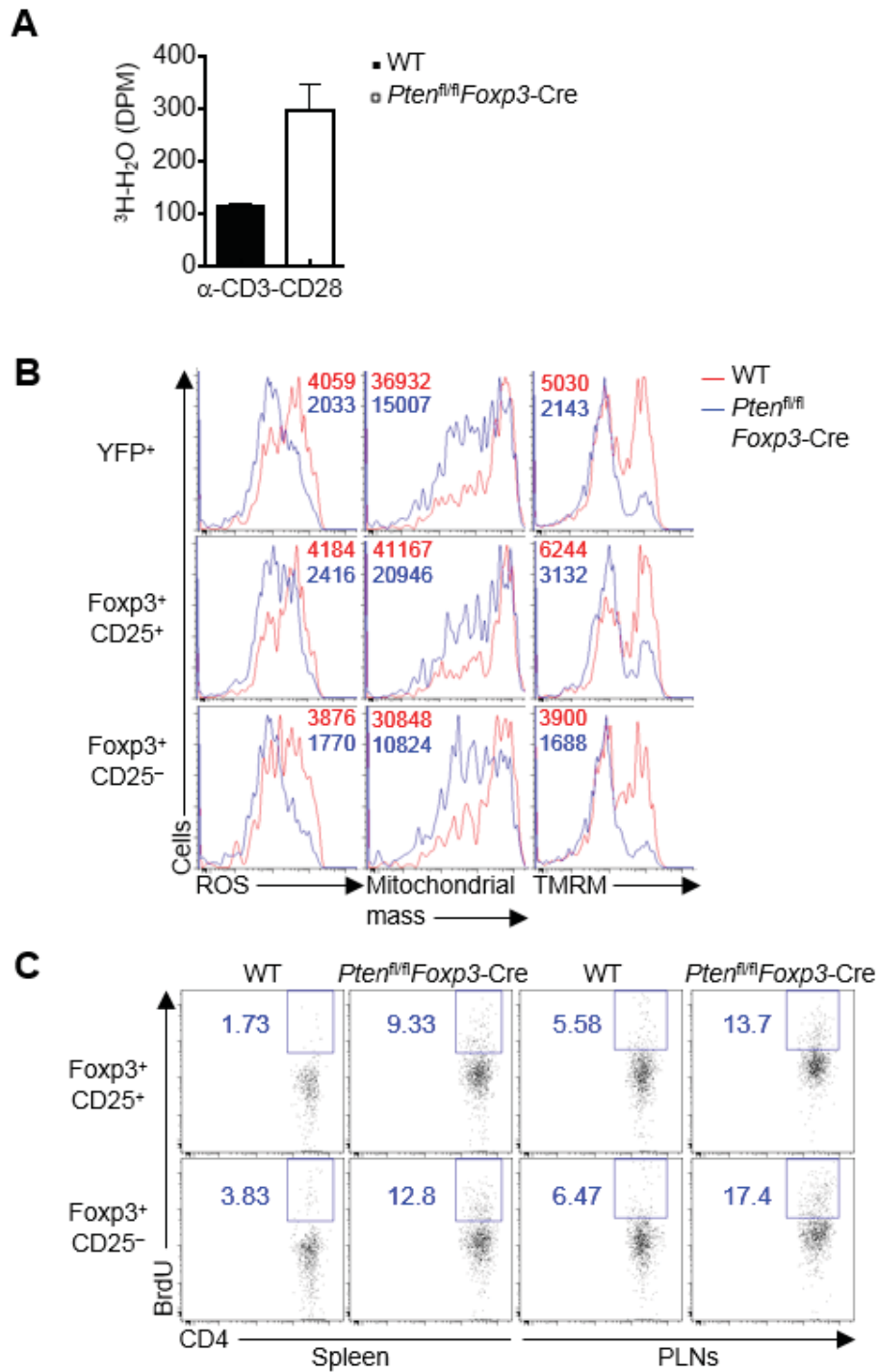
Cellular metabolic programs, especially glycolysis and mitochondrial oxidative phosphorylation, play an important role in shaping Treg cell generation and function (Michalek et al., 2011a; Shi et al., 2011), although the molecular mechanisms are unknown. As compared with WT Treg cells, Pten-deficient Treg cells more strongly upregulated glycolysis after TCR/CD28 stimulation (**Figure 4-4A**). We next examined whether *Pten* deficiency affects mitochondrial fitness by measuring multiple parameters associated with mitochondria homeostasis and functions. Pten-deficient Treg cells showed considerable reductions of reactive oxidative species (ROS), mitochondrial mass, and mitochondrial membrane potential, indicative of impaired mitochondrial fitness (**Figure 4-4B**). Notably, the reductions of these parameters was more prominent in *Pten*-deficient Foxp3<sup>+</sup>CD25<sup>-</sup> Treg cells than Foxp3<sup>+</sup>CD25<sup>+</sup> Treg cells (**Figure 4-4B**), indicating that impaired mitochondrial function is associated with Treg cell instability. Moreover, the Foxp3<sup>+</sup>CD25<sup>-</sup> Treg subset had modestly increased BrdU incorporation than Foxp3<sup>+</sup>CD25<sup>+</sup> cells (**Figure 4-4C**). Altogether, these data establish that PTEN signaling links immune response gene expression, cell cycle progression, and metabolic balance between glycolytic activity and mitochondrial fitness in Treg cells.

### PTEN Restrains mTORC2 Activity in Treg Cells

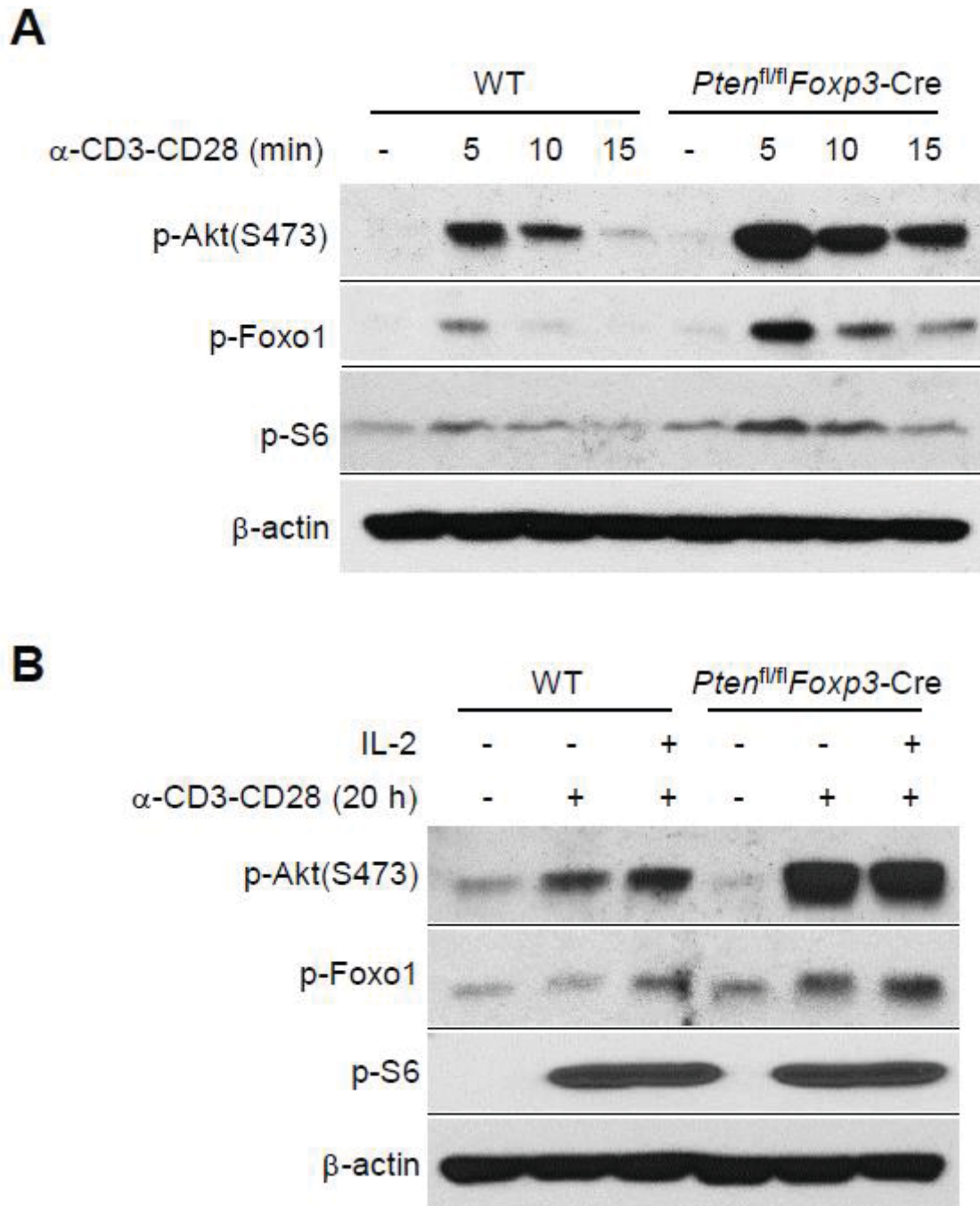
We next determined PTEN-dependent biochemical mechanisms in Treg cells by examining mTORC1 and mTORC2 activities, as both are under the control of PTEN in multiple cell types (Chi, 2012). In response to short-term anti-CD3-CD28 stimulation (5-15 min), *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells exhibited slightly elevated phosphorylation of the mTORC1 target S6, as compared with WT Treg cells (**Figures 4-5A and 4-6A**). In contrast, *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells exhibited much stronger activation of mTORC2 signaling than WT cells, as indicated by the enhanced phosphorylation of AKT at Ser473 and of the AKT substrate, Foxo1 (**Figure 4-5A**). We next determined the effects of stimulating Treg cells with anti-CD3-CD28 for a longer time (20 h), in the presence or absence of IL-2. Both WT and Pten-deficient Treg cells responded to these stimuli with robust phosphorylation of S6 and 4EBP1, indicative of comparable activation of the mTORC1 pathway under these conditions (**Figures 4-5B and 4-6B**). In contrast, *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells exhibited much stronger activation of mTORC2 signaling than WT cells (**Figure 4-5B**). Therefore, loss of PTEN results in preferential activation of mTORC2 signaling in Treg cells.

To determine the contribution of mTORC2 activity to the phenotypes of *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice, we generated mice bearing Treg cells with a double deletion of *Pten* and the mTORC2 obligate adaptor *Rictor* (termed *Pten*<sup>fl/fl</sup>*Rictor*<sup>fl/fl</sup>*Foxp3*-Cre mice), since Rictor is an essential regulator of mTORC2 activity in Treg cells (Zeng et al., 2013). In contrast to the phenotype observed in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice, Treg cells in *Pten*<sup>fl/fl</sup>*Rictor*<sup>fl/fl</sup>*Foxp3*-Cre mice exhibited normal abundance and CD25 expression (**Figure 4-7**). Other Treg cell markers analyzed were also considerably rescued by the deletion of Rictor from *Pten*<sup>fl/fl</sup>*Foxp3*-Cre Treg cells (**Figure 4-8A**). Additionally, T cells in *Pten*<sup>fl/fl</sup>*Rictor*<sup>fl/fl</sup>*Foxp3*-Cre mice exhibited largely normal distribution of naïve and effector/memory phenotypes (**Figure 4-8B**). Also, the spontaneous development of T<sub>FH</sub> and T<sub>H1</sub> cells and GC B cells observed in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice was blocked by

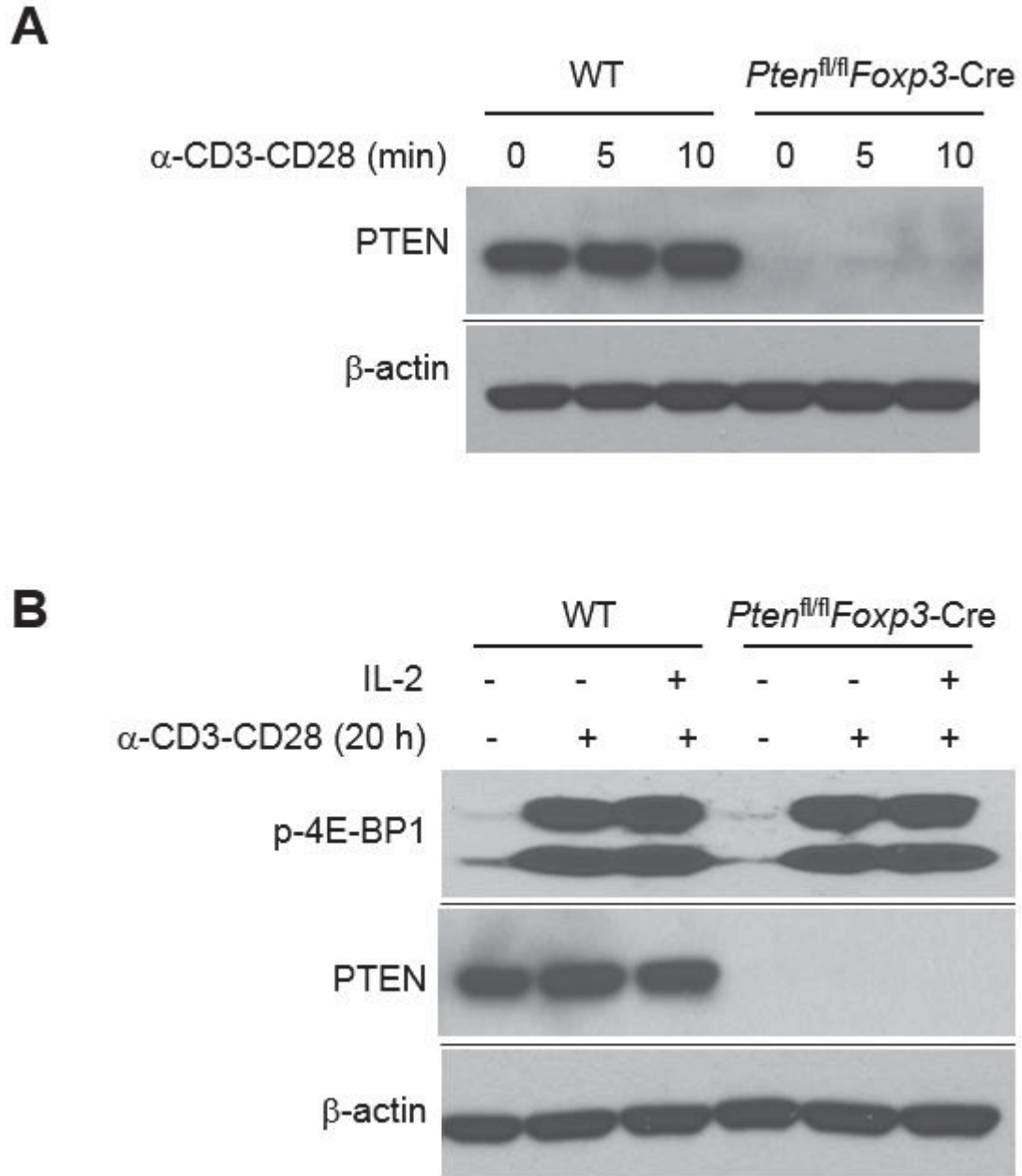




**Figure 4-4. PTEN-dependent metabolic programs in Treg cells.** (A) Glycolytic activity of Treg cells stimulated with  $\alpha$ -CD3-CD28. (B) ROS production, mitochondrial mass and mitochondrial membrane potential in Treg cells of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Numbers above graphs indicate mean fluorescence intensity (MFI). (C) BrdU incorporation in Treg subsets of WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. Numbers indicate percentage of cells in gates.

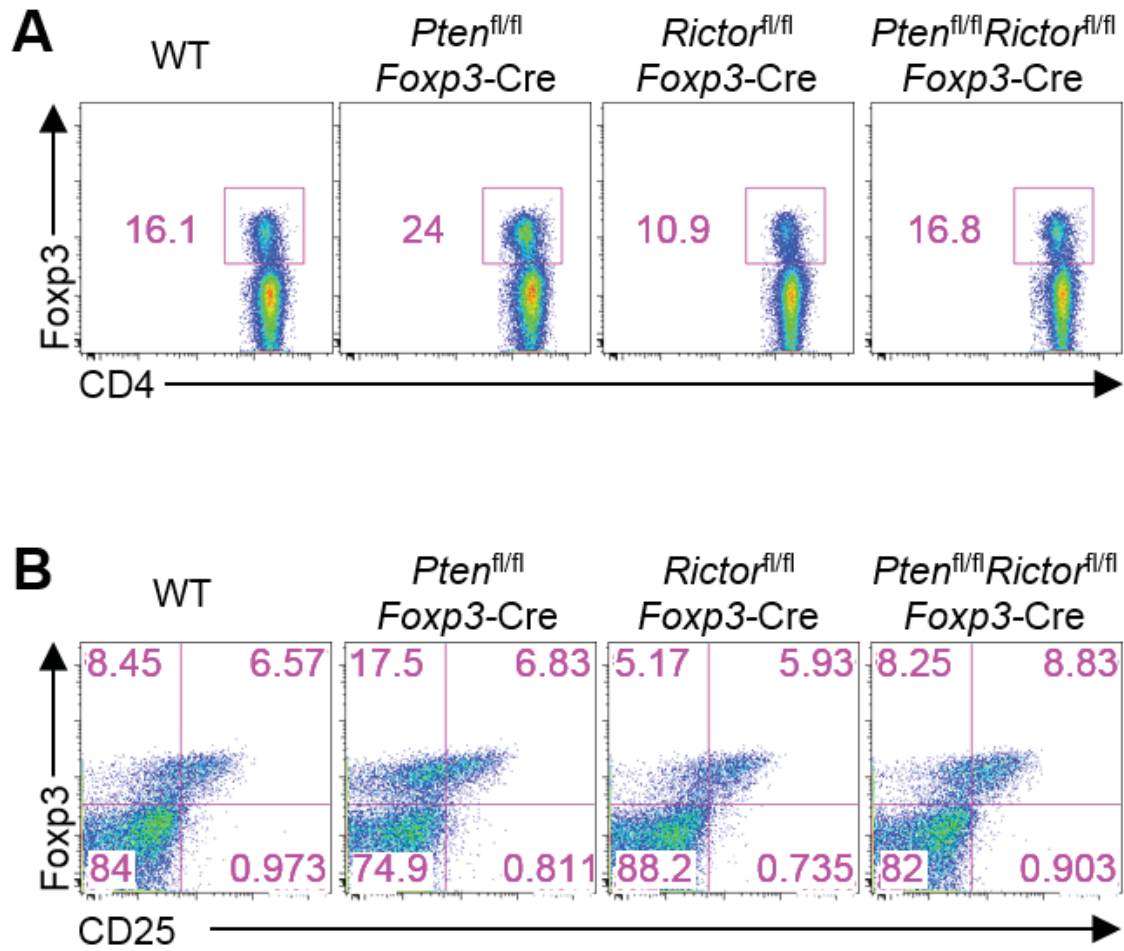


**Figure 4-5. Dysregulation of mTORC2 activity in *Pten*-deficient Treg cells.** Immunoblot analysis of (A) p-Akt(S473), p-Foxo1 and p-S6 in resting and short-term-stimulated and (B) long-term-stimulated Treg cells isolated from WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.

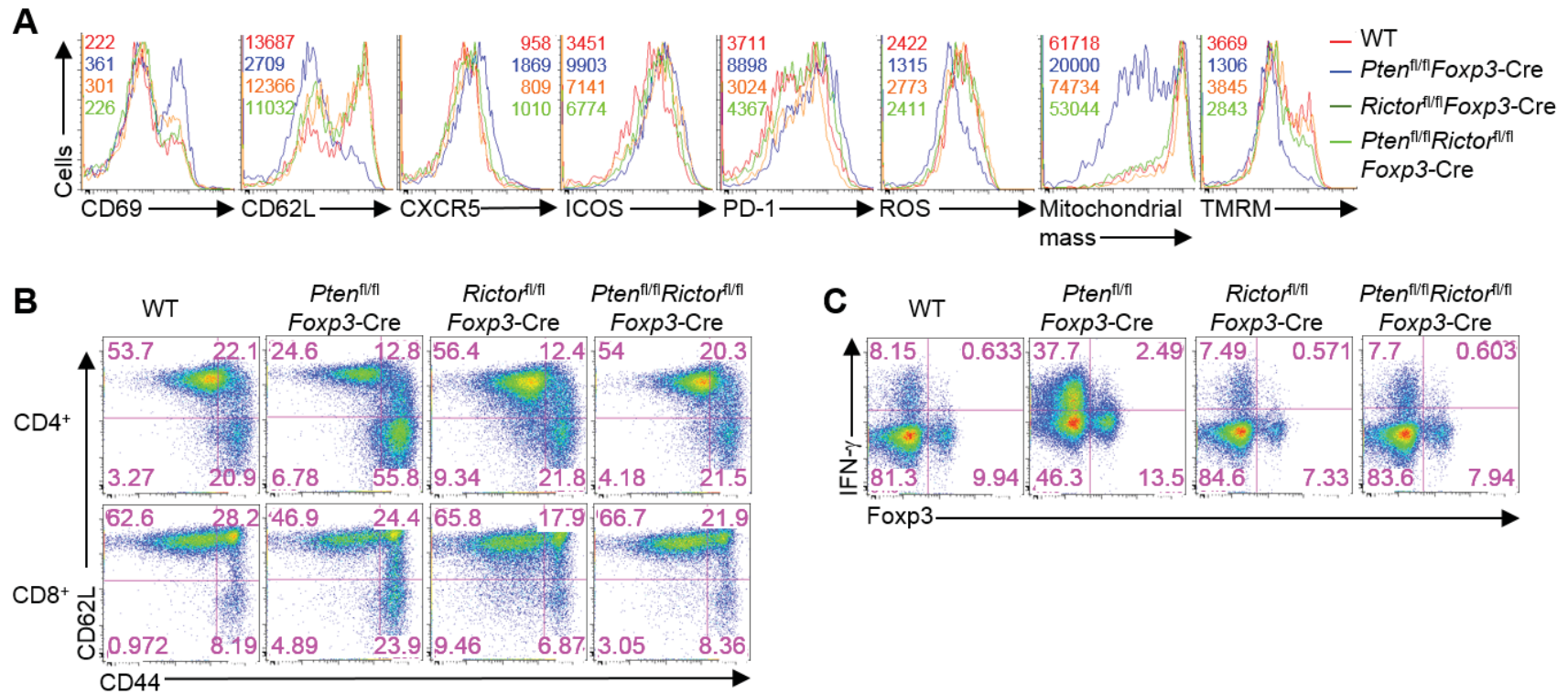


**Figure 4-6. Analysis of Pten expression in short and long term stimulated Treg cells.**

(A) *Pten* expression in resting and short-term stimulated Treg cells and (B) phosphorylation of 4E-BP1 and *Pten* in resting and long-term stimulated Treg cells isolated from WT and *Pten<sup>fl/fl</sup>Foxp3-Cre* mice.



**Figure 4-7. Analysis of Treg cells in  $Pten^{fl/fl}Foxp3-Cre$  and  $Pten^{fl/fl}Rictor^{fl/fl}Foxp3-Cre$  mice under steady state.**  
 Flow cytometry analysis of WT,  $Pten^{fl/fl}Foxp3-Cre$ ,  $Rictor^{fl/fl}Foxp3-Cre$  and  $Pten^{fl/fl}Rictor^{fl/fl}Foxp3-Cre$  mice for (A) Foxp3 and (B) CD25 expression in CD4<sup>+</sup> T cells. Numbers indicate percentage of cells in gates.



**Figure 4-8. *Pten* represses mTORC2 signaling in Treg cells to maintain immune homeostasis.**

(A) Expression of CD69, CD62L, CXCR5, ICOS and PD-1, and measurements of ROS, mitochondrial mass, and mitochondrial membrane potential (TMRM) in Treg cells from the spleen of WT, *Pten*<sup>fl/fl</sup>*Foxp3-Cre*, *Rictor*<sup>fl/fl</sup>*Foxp3-Cre* and *Pten*<sup>fl/fl</sup>*Rictor*<sup>fl/fl</sup>*Foxp3-Cre* mice. (B) Flow cytometry analysis of WT, *Pten*<sup>fl/fl</sup>*Foxp3-Cre*, *Rictor*<sup>fl/fl</sup>*Foxp3-Cre* and *Pten*<sup>fl/fl</sup>*Rictor*<sup>fl/fl</sup>*Foxp3-Cre* mice for CD62L and CD44 expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells and, (C) cytokine production of CD4<sup>+</sup> T cells.

*Rictor* deletion (**Figures 4-9 and 4-8C**). Moreover, unlike *Pten<sup>fl/fl</sup>Foxp3-Cre* mice, *Pten<sup>fl/fl</sup>Rictor<sup>fl/fl</sup>Foxp3-Cre* mice did not contain IgG deposits in the kidney glomeruli (**Figure 4-10**), and exhibited normal GC numbers in the spleen and MLNs (**Figure 4-11A and B**). Therefore, despite the plethora of pathways identified to mediate PTEN functions in various cells (Chi, 2012), PTEN acts in a mTORC2-dependent manner in Treg cells to impinge upon immune homeostasis and tolerance.

### Nuclear Retention of Foxo1 Abrogates the Effects of PTEN Loss

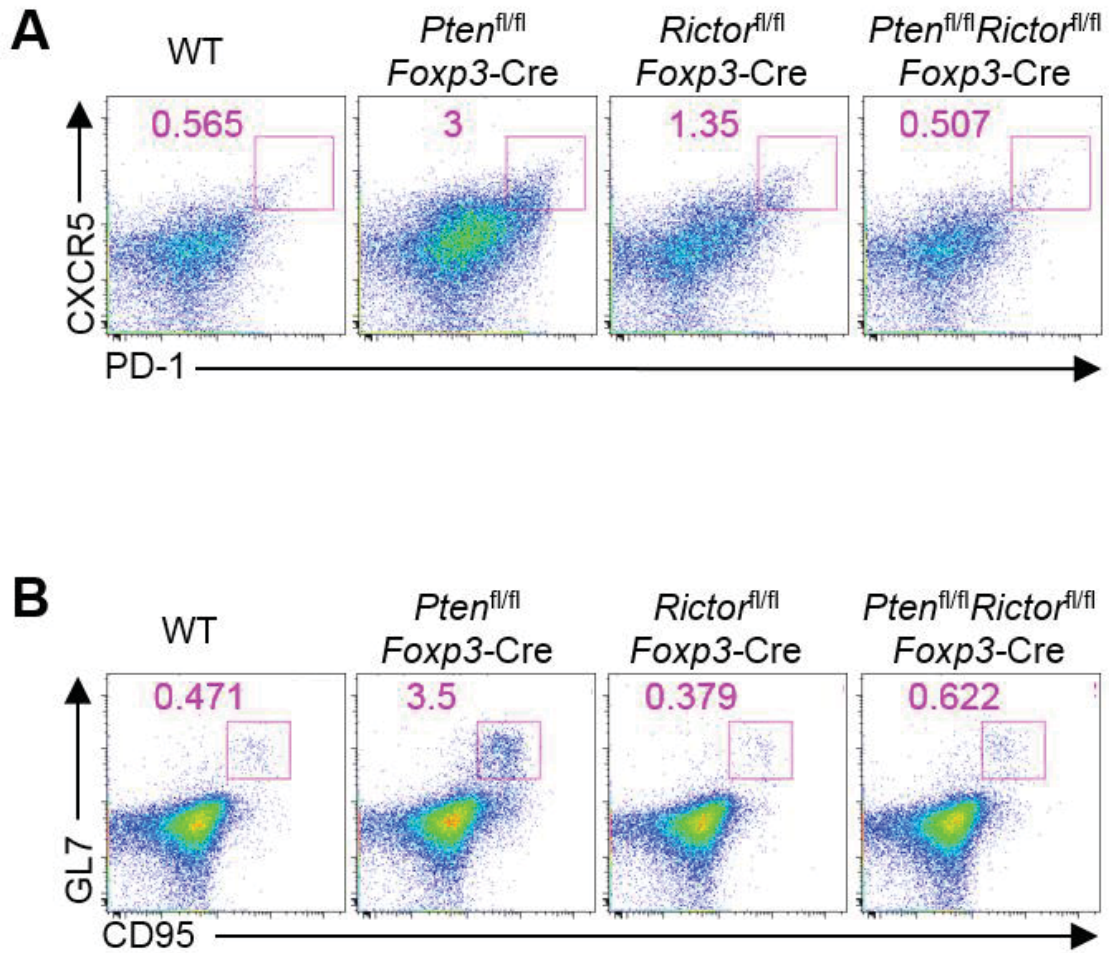
Activation of AKT by mTORC2 leads to phosphorylation of transcription factor Foxo1 that interacts with Foxp3 and is critical for Treg cell lineage maintenance (Harada et al., 2010; Kerdiles et al., 2010; Ouyang et al., 2009; Ouyang et al., 2010; Ouyang et al., 2012). To determine the contribution of mTORC2-AKT-mediated inhibition of Foxo1 activity to the phenotypes of *Pten<sup>fl/fl</sup>Foxp3-Cre* mice, we crossed *Pten<sup>fl/fl</sup>Foxp3-Cre* mice with those expressing the one allele of Foxo1<sup>AAA</sup> mutant to generate *Pten<sup>fl/fl</sup>Foxo1<sup>AAA/+</sup>Foxp3-Cre*. This mutant is constitutively localized to the nucleus, as it lacks the inhibitory AKT phosphorylation sites (Ouyang et al., 2012). Importantly, the Foxo1<sup>AAA</sup> heterozygous mice were selected for analysis, as homozygous mice are prone to wasting disease (Luo et al., 2016).

In preliminary experiments, ectopic expression of Foxo1<sup>AAA</sup> ameliorated several phenotypic characteristics, such as the altered distribution of naive and effector or memory and increased IFN- $\gamma$  production seen in T cells from *Pten<sup>fl/fl</sup>Foxp3-Cre* mice (**Figure 4-12A and B**). Additionally, expression of Foxo1<sup>AAA</sup> substantially dampened the aberrant T<sub>FH</sub> and GC B cell responses of *Pten<sup>fl/fl</sup>Foxp3-Cre* mice (**Figure 4-13**). Moreover, no visible lymphadenopathy (unpublished observations) and rectified ANA levels (**Figure 4-14**), indicates absence of lymphoproliferative disease in *Pten<sup>fl/fl</sup>Foxo1<sup>AAA/+</sup>Foxp3-Cre* mice. Interestingly, the high frequency of CD4<sup>+</sup>YFP<sup>+</sup> and CD25<sup>+</sup>YFP<sup>+</sup> Treg cells were still seen in *Pten<sup>fl/fl</sup>Foxo1<sup>AAA/+</sup>Foxp3-Cre* mice (**Figure 4-15**). Therefore, despite Foxo1 being a downstream target of mTORC2-AKT, the data presented above indicates alternative downstream targets control Treg cell cellularity and stability, which needs to be further studied.

### Summary

In this chapter, we highlighted PTEN orchestrates transcriptional programs and the metabolic balance between glycolysis and mitochondrial fitness to support Treg cell function. Phenotypic observations of abnormalities we characterized in Chapter 3 due were attributed to dysregulated activation of the mTORC2-mediated pathway, as generation of *Pten<sup>fl/fl</sup>Rictor<sup>fl/fl</sup>Foxp3-Cre* mice ameliorated those defects. Further, these defects were linked to excessive inactivation of Foxo1, because expression of the AKT-insensitive FoxoAAA mutant also alleviated several features of disease seen in *Pten<sup>fl/fl</sup>Foxp3-Cre* mice. However, the higher frequency and instability of Treg cells still remained consistent, which indicated involvement of additional alternative targets

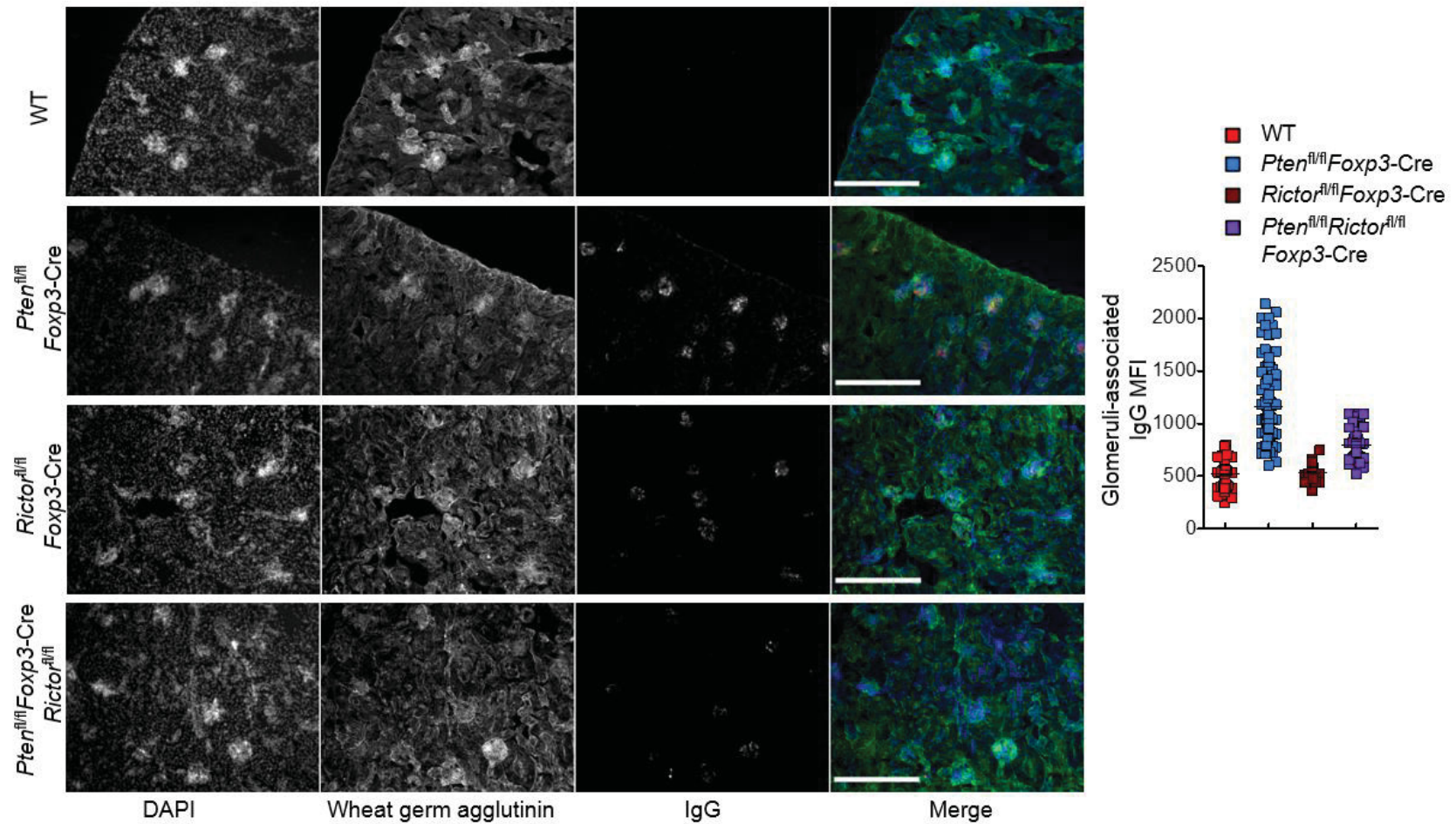




**Figure 4-9. Analysis of T<sub>FH</sub> and GC B cells in  $Pten^{fl/fl}Foxp3-Cre$  and  $Pten^{fl/fl}Rictor^{fl/fl}Foxp3-Cre$  mice under steady state.**

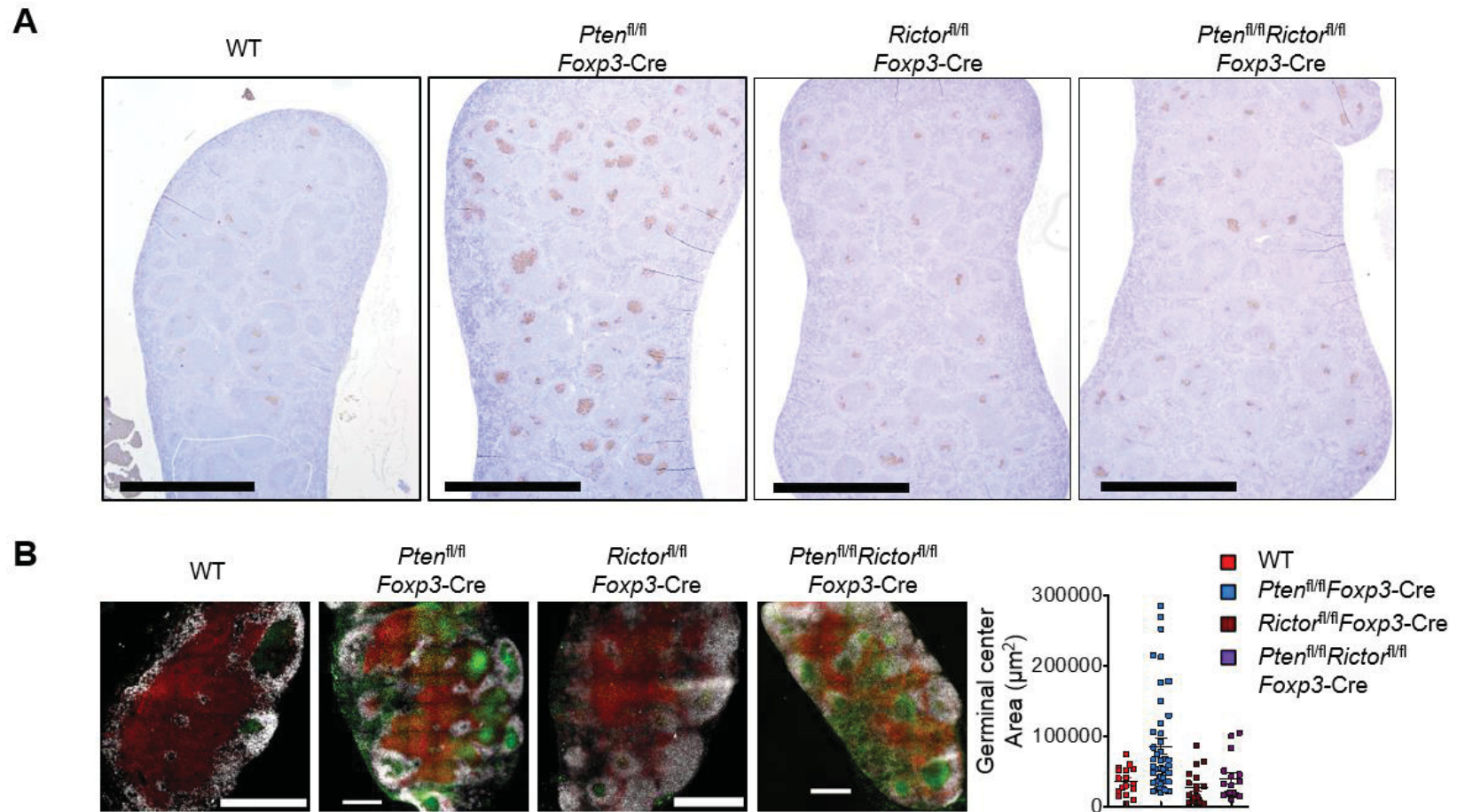
Flow cytometry analysis of WT,  $Pten^{fl/fl}Foxp3-Cre$ ,  $Rictor^{fl/fl}Foxp3-Cre$  and  $Pten^{fl/fl}Rictor^{fl/fl}Foxp3-Cre$  mice for proportions of (A) T<sub>FH</sub> cells in CD4<sup>+</sup> T cells and (B) GC B cells in total B cells. Numbers indicate percentage of cells in gates.





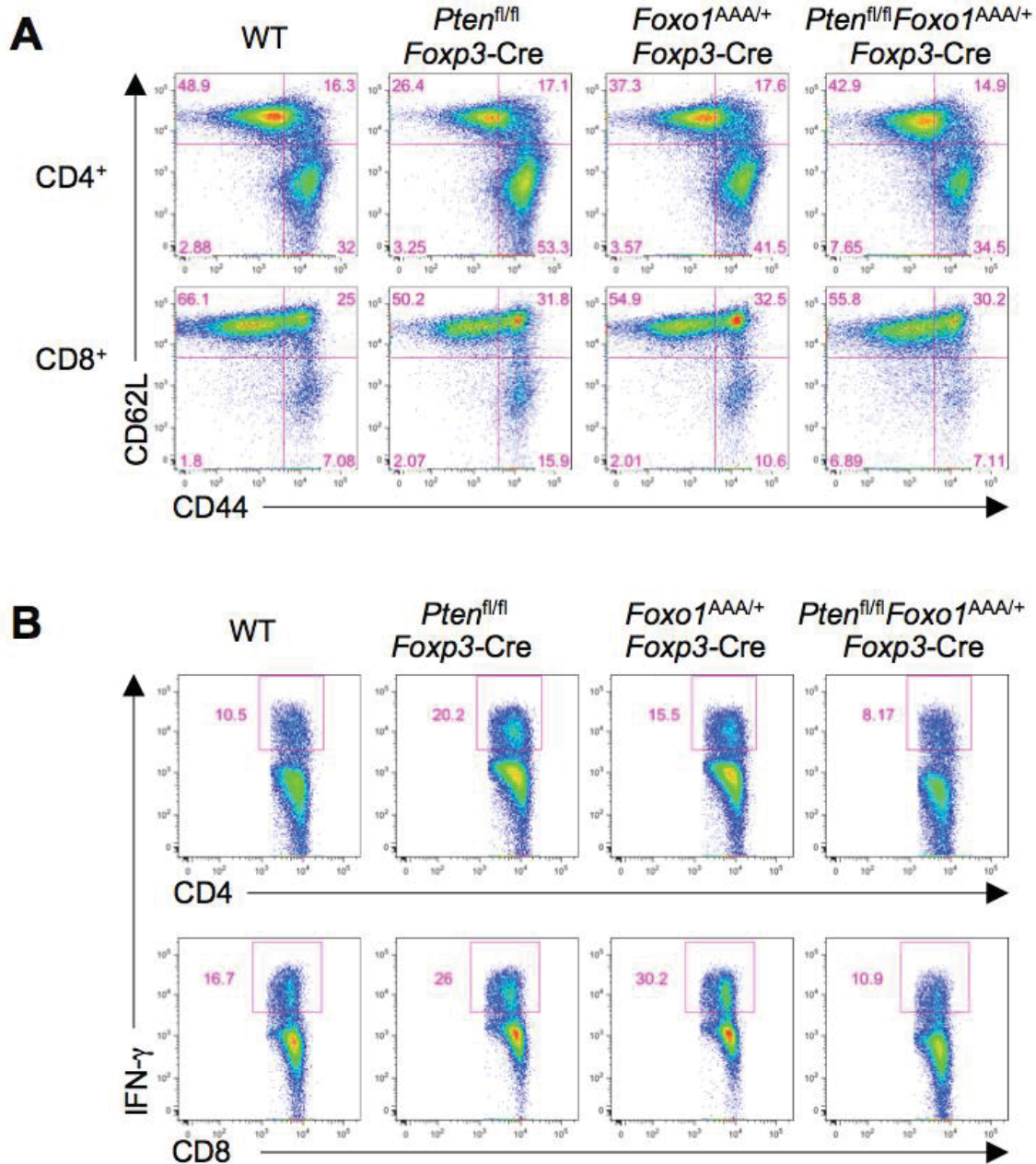
**Figure 4-10. Immunofluorescence analysis of glomeruli for IgG deposits in WT, *Pten*<sup>fl/fl</sup> Foxp3-Cre, *Rictor*<sup>fl/fl</sup> Foxp3-Cre and *Pten*<sup>fl/fl</sup> *Rictor*<sup>fl/fl</sup> Foxp3-Cre mice.**

Imaging (left) and quantification (right) of IgG deposits in kidney sections of WT, *Pten*<sup>fl/fl</sup> Foxp3-Cre, *Rictor*<sup>fl/fl</sup> Foxp3-Cre and *Pten*<sup>fl/fl</sup> *Rictor*<sup>fl/fl</sup> Foxp3-Cre mice (scale bars, 300  $\mu$ m).



**Figure 4-11. Immunohistochemistry of germinal centers in WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Rictor<sup>fl/fl</sup>Foxp3-Cre* and *Pten<sup>fl/fl</sup>Rictor<sup>fl/fl</sup>Foxp3-Cre* mice.**

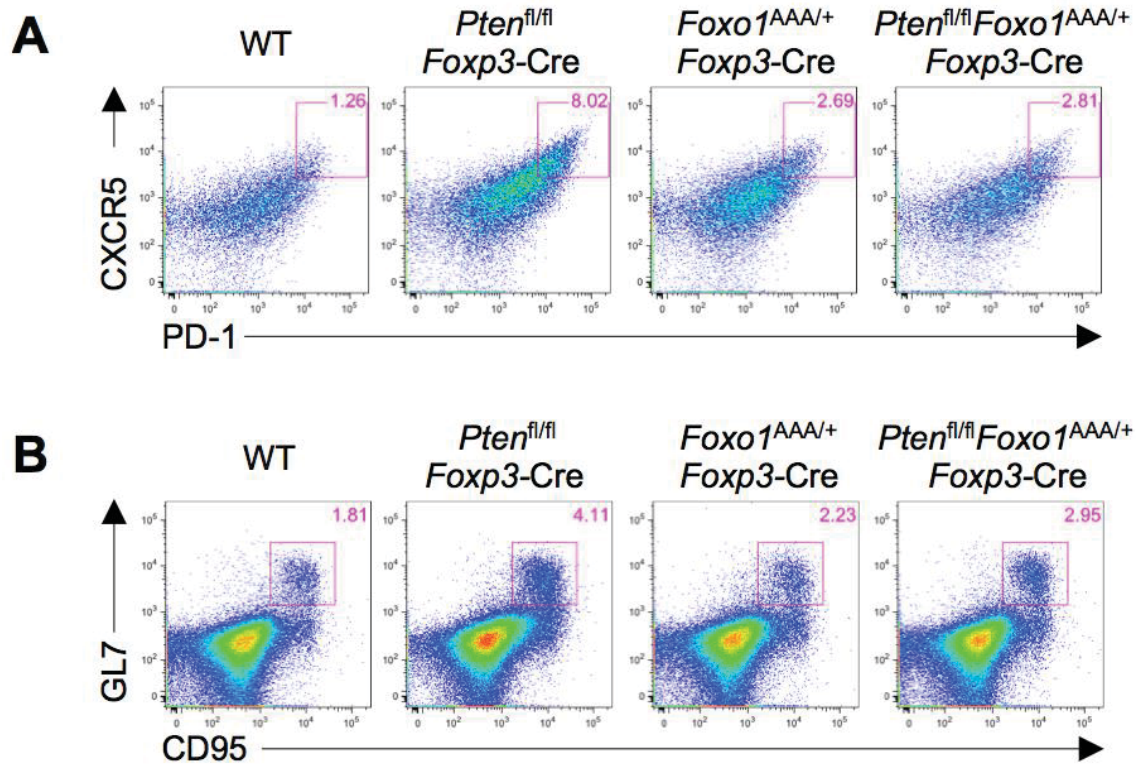
(A) PNA staining of spleen sections (magnification, x2; scale bars, 2mm) and (B) immunofluorescence and quantification of MLN sections for the staining of CD3 (red), PNA (green) and IgD (white) (scale 500  $\mu\text{m}$ ) from WT, *Pten<sup>fl/fl</sup>Foxp3-Cre*, *Rictor<sup>fl/fl</sup>Foxp3-Cre* and *Pten<sup>fl/fl</sup>Rictor<sup>fl/fl</sup>Foxp3-Cre* mice.



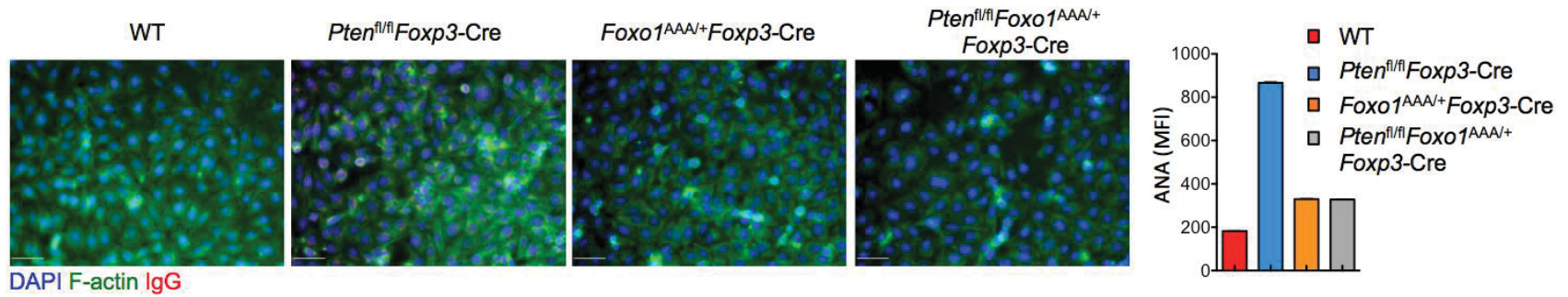
**Figure 4-12. Analysis of *Pten*<sup>fl/fl</sup> *Foxp3-Cre* and *Pten*<sup>fl/fl</sup> *Foxo1*<sup>AAA/+</sup> *Foxp3-Cre* mice under steady state.**

(A) Flow cytometry analysis of WT, *Pten*<sup>fl/fl</sup> *Foxp3-Cre*, *Foxo1*<sup>AAA/+</sup> *Foxp3-Cre* and *Pten*<sup>fl/fl</sup> *Foxo1*<sup>AAA/+</sup> *Foxp3-Cre* mice for CD62L and CD44 expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells and, (B) IFN- $\gamma$  production of by CD4<sup>+</sup> and CD8<sup>+</sup> T cells.



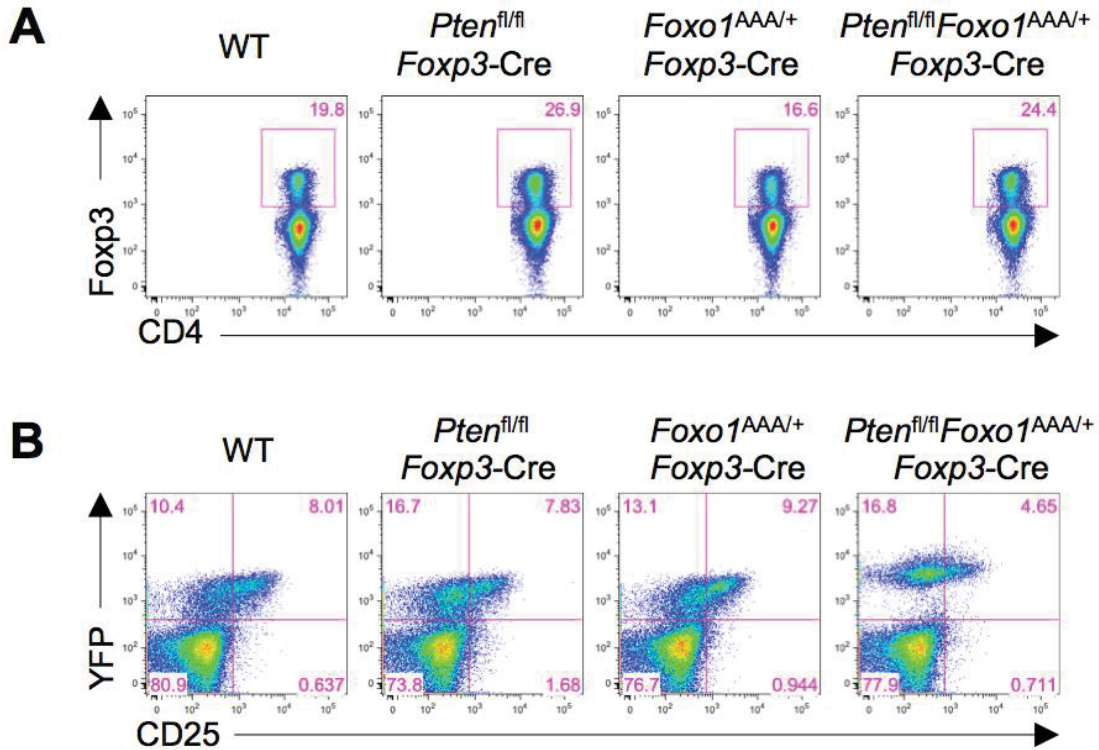


**Figure 4-13. Analysis of T<sub>FH</sub> and GC B cells in *Pten<sup>fl/fl</sup> Foxp3-Cre* and *Pten<sup>fl/fl</sup> Foxo1<sup>AAA/+</sup> Foxp3-Cre* mice under steady state.**  
 Flow cytometry analysis of WT, *Pten<sup>fl/fl</sup> Foxp3-Cre*, *Foxo1<sup>AAA/+</sup> Foxp3-Cre* and *Pten<sup>fl/fl</sup> Foxo1<sup>AAA/+</sup> Foxp3-Cre* mice for proportions of (A) T<sub>FH</sub> cells in CD4<sup>+</sup> T cells and (B) GC B cells in total B cells. Numbers indicate percentage of cells in gates.



**Figure 4-14. Detection of antinuclear antibodies in WT, *Pten*<sup>fl/fl</sup>*Foxp3-Cre*, *Foxo1*<sup>AAA+/+</sup>*Foxp3-Cre* and *Pten*<sup>fl/fl</sup>*Foxo1*<sup>AAA+/+</sup>*Foxp3-Cre* mice.**

Representative images (scale bars, 50 μm) and quantification of fluorescent intensity (right) of serum anti-ANA IgG detected with fixed Hep-2 slides in WT, *Pten*<sup>fl/fl</sup>*Foxp3-Cre*, *Foxo1*<sup>AAA+/+</sup>*Foxp3-Cre* and *Pten*<sup>fl/fl</sup>*Foxo1*<sup>AAA+/+</sup>*Foxp3-Cre* mice.



**Figure 4-15. Analysis of Treg cells in *Pten*<sup>fl/fl</sup> *Foxo1*<sup>AAA/+</sup> *Foxp3*-Cre mice under steady state.**

Flow cytometry analysis of WT, *Pten*<sup>fl/fl</sup> *Foxp3*-Cre, *Foxo1*<sup>AAA/+</sup> *Foxp3*-Cre and *Pten*<sup>fl/fl</sup> *Foxo1*<sup>AAA/+</sup> *Foxp3*-Cre mice for (A) Foxp3 and (B) CD25 expression in CD4<sup>+</sup> T cells. Numbers indicate percentage of cells in gates.

downstream of mTORC2-AKT pathway for Treg cell programming. To summarize, we have established a new paradigm that the interplay between immune signaling and metabolic activity links Treg cell stability. At the molecular levels, PTEN orchestrates Treg stability and functional diversity by impinging upon the metabolic balance and mTORC2 signaling.



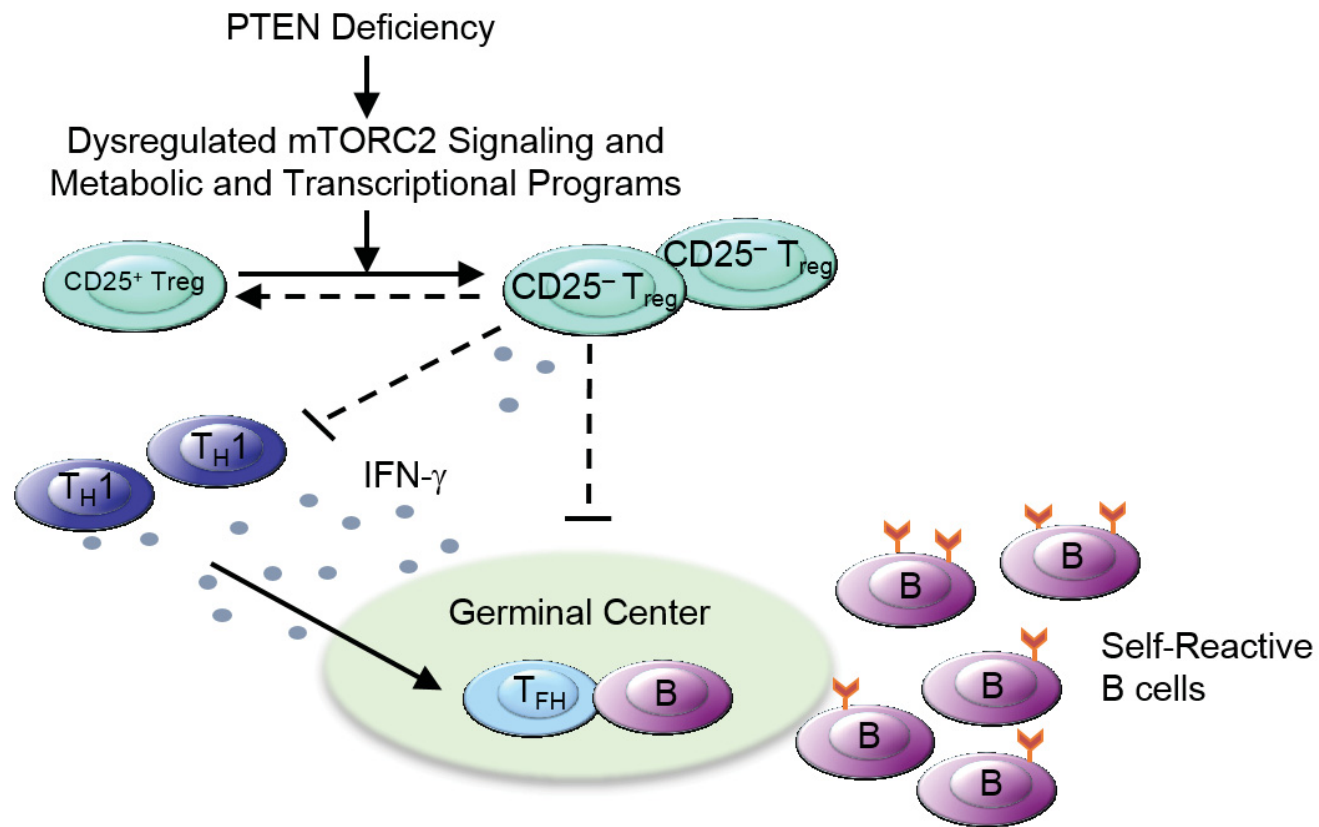
## CHAPTER 5. DISCUSSION\*

Much emphasis has been placed on the transcriptional and molecular pathways underlying effector T cell diversity and plasticity (Zhu et al., 2010). In contrast, how these distinct effector T cell responses are controlled by extrinsic mechanisms is poorly understood. Moreover, despite the emerging evidence for the adoption of T<sub>H</sub>-specific transcription factors by Treg cells to suppress the corresponding effector responses (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009), we have little understanding about the signaling mechanisms in Treg cells that program and coordinate these suppressive activities. Our studies identify PTEN as an important molecular pathway in Treg cells that coordinately regulates T<sub>FH</sub> and T<sub>H1</sub> responses (**Figure 5-1**). Our findings are consistent with the results reported by our colleagues, Huynh *et al.* (Huynh et al., 2015). In particular, loss of *Pten* in Treg cells results in exacerbated T<sub>FH</sub> and GC responses and the loss of immune tolerance and tissue homeostasis. Using genetic approaches to ablate IFN- $\gamma$  function, we have revealed the hierarchy between Treg cell-mediated control of T<sub>H1</sub> and T<sub>FH</sub> responses, with the production of the T<sub>H1</sub> signature cytokine IFN- $\gamma$  a prerequisite for the potentiation of T<sub>FH</sub> responses *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice. Furthermore, the repression of T<sub>H1</sub> and T<sub>FH</sub> responses is associated with the active maintenance of Treg cell stability mediated by PTEN signaling. At the molecular levels, PTEN controls the transcriptional program and metabolic balance in Treg cells, and signals mainly via inhibition of mTORC2 activity. Further, our data suggests PTEN-dependent mTORC2 inhibition is important to maintain the appropriate balance of Foxo1 function in Treg cells. Our studies therefore establish that the PTEN-mTORC2 signaling axis acts as a central pathway to enforce Treg cell stability and further links it to the control of T<sub>H1</sub> and T<sub>FH</sub> responses. Loss of this control mechanism is detrimental to immune homeostasis and tolerance, highlighting the physiological significance of this new control mechanism.

The lineage stability of Treg cells remains contentious (Bailey-Bucktrout et al., 2013; Komatsu et al., 2009; Komatsu et al., 2014; Miyao et al., 2012; Rubtsov et al., 2010; Tsuji et al., 2009; Zhou et al., 2009b) but is an issue of utmost importance, as it directly impinges upon Treg cell-mediated control of health and disease and therapeutic strategies (Sakaguchi et al., 2013). Part of the controversies stem from the limited understanding of the underlying molecular processes, despite the extensive research on the regulation of Foxp3 itself. Using lineage tracing and adoptive transfer systems, we show that *Pten*-deficient Treg cells are more prone to lose Foxp3 expression *in vivo*. However, *Pten* deficiency results in apparently hyper-activated Treg cells, as evidenced by increased cycling, upregulation of activation and phenotypic markers, and dysregulated expression of IFN- $\gamma$  and other T<sub>H1</sub> and T<sub>FH</sub> signature molecules. Consistent

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\*Chapter 5 reprinted with permission. Shrestha, S., Yang, K., Guy, C., Vogel, P., Neale, G., and Chi, H. (2015). T<sub>reg</sub> cells require the phosphatase PTEN to restrain T<sub>H1</sub> and T<sub>FH</sub> cell responses. *Nature immunology* 16, 178-187.



**Figure 5-1. Schematics of PTEN signaling in Treg cell functions and immune tolerance.**

Loss of PTEN in Treg cells dysregulates mTORC2 signaling and metabolic and transcriptional programs, leading to the disrupted stability of Treg cells (as evidenced by the downregulation of CD25 expression, as well as other abnormalities not depicted here). Associated with loss of Treg cell stability and the ensuing T<sub>H1</sub> responses and IFN- $\gamma$  production is the aberrant induction of T<sub>FH</sub> responses, spontaneous formation of germinal center responses, and development of autoimmune and lymphoproliferative disease.

with the hyper-activation of Treg cells, *Pten* deletion results in marked loss of CD25 expression, which is normally downregulated in Treg cells after *in vivo* activation and proliferation (Gavin et al., 2002).

While representing only a minor proportion of Foxp3<sup>+</sup> Treg cells under steady state, the Foxp3<sup>+</sup>CD25<sup>-</sup> population likely makes an important contribution, via conversion or selection, to the generation of ex-Treg cells under inflammatory or lymphopenic environment. Therefore, our studies highlight that the stability of Treg cells under steady state requires active enforcement by PTEN, which functions, at least in part, by preventing overt Treg cell activation and CD25 downregulation.

Both mTOR complexes plays an important role in maintenance of Treg cells, as dysregulated mTORC1 signaling results in unstable Treg cells giving rise to T<sub>H</sub>17-like effector T cells (Park et al., 2013), suggesting a dominant role of the PI3K-PDK1 pathway on Treg stability and function (Wei et al., 2016). Our results showing instability of Treg cells and phenotypes associated with T<sub>H</sub>1 cells due to loss of PTEN is mTORC2-mediated. As PIP3 has been linked to direct activation of mTORC2 (Gan et al., 2011; Liu et al., 2015), the results we obtained seem to be independent of PDK1-associated activation.

The generation of T<sub>FH</sub> cells, the specialized providers of help to B cells, requires unique transcriptional programs and is antagonized by T<sub>FR</sub> cells (Chung et al., 2011; Linterman et al., 2011). However, T<sub>FR</sub> cells can also promote antigen-specific high-affinity B cell responses (Linterman et al., 2011) and influenza-specific GC reactions (Leon et al., 2014). Extensive crosstalk also exists between the differentiation of T<sub>FH</sub> cells and other effector lineages (Crotty, 2011), although the involvement of Treg cells in these processes remains largely unexplored. Here we show that PTEN signaling in Treg cells is crucial in the repression of T<sub>FH</sub> responses, and this is further linked to Treg cell stability and Treg cell-mediated control of T<sub>H</sub>1 responses. Strikingly, loss of PTEN in Treg cells results in spontaneous T<sub>FH</sub> differentiation and GC formation, and the development of SLE-like autoimmune symptoms. These immune defects are associated with dysregulated expression of many genes involved in T<sub>FH</sub> and T<sub>FR</sub> responses in *Pten*-deficient Treg cells. Furthermore, the uncontrolled T<sub>FH</sub> responses in *Pten*<sup>fl/fl</sup>*Foxp3*-Cre mice are dependent upon the T<sub>H</sub>1 signature cytokine IFN- $\gamma$ , as deletion of IFN- $\gamma$  considerably rectifies the dysregulated T<sub>FH</sub> response and autoimmune disease. Notably, the relationship between T<sub>H</sub>1 and T<sub>FH</sub> cells is complex and remains controversial. For instance, whereas IFN- $\gamma$  is essential in driving T<sub>FH</sub> cells in the *Roquin*<sup>san/san</sup> autoimmunity model (which has a point mutation in the *Roquin* gene) (Lee et al., 2012a), it is dispensable for the differentiation of T<sub>FH</sub> cells induced by viral infection (Ray et al., 2014). Further, T-bet induction dampens rather than enhances the T<sub>FH</sub> differentiation program (Nakayamada et al., 2011). Our studies demonstrate that T<sub>FH</sub> and T<sub>H</sub>1 responses are coordinately regulated by Treg cells, with the T<sub>H</sub>1-mediated IFN- $\gamma$  production being a main driver of T<sub>FH</sub> and GC reactions. However, we cannot conclude the direct contribution of the exacerbated T<sub>H</sub>1 response, independent of T<sub>FH</sub> cells, to the autoimmune and lymphoproliferative phenotypes.

As one of the most frequently mutated tumor suppressor genes, PTEN is implicated in the regulation of multiple cancer types. Selective deletion of PTEN in T cells results in rapid development of T cell leukemia, as well as the loss of immune tolerance (Suzuki et al., 2001). More recent studies unveil that these two effects can be dissociated, as they are derived from abnormalities in the thymus and periphery, respectively (Liu et al., 2010). PTEN is also implicated in the regulation of effector T cell responses. Deletion of *Pten* in activated T cells enhances effector responses but does not lead to autoimmunity or cancer (Soond et al., 2012). miRNAs targeting PTEN expression are also implicated in shaping T<sub>FH</sub> responses (Kang et al., 2013). In Treg cells, PTEN expression is maintained at a higher level than conventional T cells (Zeiser et al., 2008). PTEN restrains the abundance of Treg cells but is largely dispensable for Treg cell suppressive activity *in vitro* (Walsh et al., 2006). We found that deletion of *Pten* in Treg cells leads to the exacerbated effector T cell responses and loss of immune tolerance and tissue homeostasis. *Pten*-deficient Treg cells dominate over WT cells in mediating T<sub>FH</sub> and GC responses, which are mediated, at least in part, by dysregulated IFN- $\gamma$  expression. Moreover, PTEN functions in a haploinsufficient manner, in that heterozygous loss of *Pten* is sufficient to disrupt Treg cell and immune homeostasis. These results together establish PTEN signaling in Treg cells as a unique regulator of immune system homeostasis and function.

How does PTEN function in Treg cells at the molecular levels? Our microarray, metabolic and immunological assays reveal the important role of PTEN in linking cell cycle and metabolic machineries and the expression of immune effector genes in Treg cells. Given the opposing effects of glycolytic and mitochondrial metabolism on Treg cells (Michalek et al., 2011a; Shi et al., 2011), the disrupted balance between these activities in *Pten*-deficient Treg cells is likely an important contributor to the phenotypic alteration, a notion that we are currently investigation. Of note, despite the metabolic functions of PTEN and PI3K signaling identified in non-immune cells, the underlying mechanisms are highly complex and poorly understood (Garcia-Cao et al., 2012). For instance, PTEN can either potentiate or inhibit mitochondrial activity (Garcia-Cao et al., 2012; Li et al., 2013), and the metabolic functions of PTEN are associated with both mTORC1-dependent and independent activities (Garcia-Cao et al., 2012; Song et al., 2011; Tandon et al., 2011). Indeed, various effector pathways downstream of PTEN have been identified, including PI3K-AKT, mTORC1, mTORC2, and PI3K-AKT-independent nuclear functions (Chi, 2012; Garcia-Cao et al., 2012; Newton and Turka, 2012; Shen et al., 2007; Song et al., 2011). It is therefore unexpected that our biochemical and genetic rescue experiments have identified mTORC2 signaling as a dominant mechanism to mediate PTEN functions in Treg cells. Emerging evidence highlights a crucial role of mTOR and metabolic signaling in Treg cells, which was believed to mainly function as a negative regulator of Treg cells (Delgoffe et al., 2009). However, recent genetic studies indicate that both loss and gain of mTORC1 activity are detrimental to Treg-mediated suppressive function and immune tolerance (Park et al., 2013; Wei et al., 2016; Zeng et al., 2013). In contrast, the physiological significance and regulatory mechanisms of mTORC2 are much less clear. Here our results have established the PTEN-mTORC2 axis as a central determinant of Treg cell stability and Treg cell-mediated control of effector T cell responses. Mechanistically, this signaling pathway likely orchestrates and

coordinates both the metabolic and transcriptional programs, such as those mediated by Foxo1 and Blimp1 (Ouyang et al., 2012; Shin et al., 2013), in impinging upon Treg stability and functions.

In line with this idea, ectopic expression of Foxo1<sup>AAA</sup>, which is insensitive to phosphorylation downstream of AKT-mTORC2, seems to alleviate several *Pten*-deficient Treg cell phenotypes, it failed to rectify the higher frequency of Treg cells. This demonstrates that additional and alternative downstream targets might regulate Treg cell programming. We note that the loss of Treg cell stability also results from Treg cell-specific deletion of neuropilin-1 and Foxo1 (Delgoffe et al., 2013; Ouyang et al., 2012), although the cellular phenotypes and effects on immune homeostasis are rather distinct from the *Pten*<sup>fl/fl</sup>*Foxp3*-Cre model. Since the effects on T<sub>FH</sub> and humoral responses in the other genetic models have not been described, further work is required to explore the crosstalk between these molecular pathways in Treg cells.

Briefly, studying the downstream signaling pathway Foxo1 further to expand its role during the loss of PTEN in Treg cells would provide valuable insights regarding production of autoantibodies, programming of T<sub>FH</sub> and T<sub>FR</sub> cells and Treg metabolism. Additional studies would include dissecting mechanistic role of PTEN in controlling T<sub>H1</sub> responses and T<sub>FR</sub> cells, if and how mTOR or PI3K inhibitors would restore function and stability in PTEN-deficient Treg cells. Furthermore, examination of additional pathways such as SGK1, an enzyme downstream of mTORC2 that promotes Foxo1 phosphorylation, would be insightful.

In summary, our study has unveiled the interplay between the PTEN-mTORC2 signaling axis and the transcriptional and metabolic regulation in Treg cells as a new mechanism for maintaining Treg cell stability and regulating Treg cell-mediated repression of T<sub>H1</sub> and T<sub>FH</sub> responses. Given the potent effects of selective *Pten* deficiency in Treg cells on immune tolerance and tissue homeostasis, the identification of this signaling axis provides a new target for therapeutic intervention of systemic autoimmune and lymphoproliferative diseases.

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## APPENDIX. TSC1 PROMOTES THE DIFFERENTIATION OF MEMORY CD8<sup>+</sup> T CELLS VIA ORCHESTRATING THE TRANSCRIPTIONAL AND METABOLIC PROGRAMS\*

### Introduction

Memory CD8<sup>+</sup> T cells play an important role in protective immunity and have the capability to mount robust recall responses upon reexposure to tumor or pathogen-derived antigens. Recent studies have revealed developmental pathways and transcriptional programs important for the generation of long-lived memory cells (Kaech and Cui, 2012). Antigen-activated CD8<sup>+</sup> T cells rapidly expand and generate heterogeneous populations of effector cells, namely short-lived effector cells (SLECs) and memory-precursor effector cells (MPECs) (Joshi et al., 2007; Sarkar et al., 2008). SLECs (CD127<sup>lo</sup>KLRG1<sup>hi</sup>) exhibit strong cytotoxicity with high expression of perforin and granzymes and are predisposed to cell death, whereas MPECs (CD127<sup>hi</sup>KLRG1<sup>lo</sup>) display the increased potential to survive and further differentiate into mature memory CD8<sup>+</sup> T cells. The fate decision between SLECs and MPECs is important for effector functions and memory differentiation and is shaped by a number of transcription factors. For instance, transcription factors Eomes and Bcl6 promote the generation of MPECs, whereas Blimp1 and T-bet drive the transcriptional programs for SLEC differentiation (Kaech and Cui, 2012).

T cells dynamically reprogram cellular metabolism to fulfill the bioenergetics and biosynthetic requirements for their survival, proliferation, and differentiation (MacIver et al., 2013a; Pearce et al., 2013; Wang and Green, 2012). Naïve and memory T cells use catabolic metabolism via oxidative phosphorylation, especially fatty acid oxidation, to produce ATP for their survival. In contrast, antigen-stimulated T cells switch to anabolism to support their rapid proliferation through up-regulating expression of genes involved in multiple metabolic pathways, including glycolysis, fatty acid and cholesterol biosynthesis, and amino acid transport (Kidani et al., 2013; Sinclair et al., 2013; Wang et al., 2011; Yang et al., 2013). Emerging studies indicate that distinct metabolic pathways contribute to the fate decisions of effector and memory T cells. For instance, the increased glycolytic metabolism promotes effector T-cell generation (Sukumar et al., 2013), whereas oxidative phosphorylation and mitochondrial spare respiratory capacity facilitate memory T-cell differentiation (Pearce et al., 2009; van der Windt et al., 2012). Recent studies have also identified transcriptional regulators of cell metabolism that promote effector T-cell differentiation, including HIF1 and IRF4 (Doedens et al., 2013; Finlay et al., 2012; Man et al., 2013; Yao et al., 2013). In contrast, how cell metabolism

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\*Appendix reprinted with permission Shrestha, S., et al. (2014). "Tsc1 promotes the differentiation of memory CD8<sup>+</sup> T cells via orchestrating the transcriptional and metabolic programs." Proceedings of the National Academy of Sciences of the United States of America 111(41): 14858-14863.

is regulated by immune signaling pathways in effector and memory T-cell differentiation remains unclear.

mTOR signaling has been implicated in the control of effector and memory T-cell differentiation (Araki et al., 2009; Li et al., 2011; Rao et al., 2010). Inhibition of mTOR signaling by rapamycin promotes the generation of MPECs and their subsequent differentiation into memory T cells upon acute lymphocytic choriomeningitis virus (LCMV) infection (Araki et al., 2009). *In vitro* treatment of effector cells with rapamycin also enhances the developmental potential of memory cells through increasing the expression of Eomes at the expense of T-bet (Rao et al., 2010). Moreover, rapamycin enhances the ability of homeostatic proliferation-induced memory CD8<sup>+</sup> T cells against tumor challenge via regulating the expression of Eomes and T-bet (Li et al., 2011). Despite these studies linking mTOR signaling to the regulation of memory T-cell differentiation, the upstream regulators of mTOR remain unresolved. Notably, deletion of *Pten* does not cause significant defects in memory formation in LCMV infection (Hand et al., 2010). Moreover, whether mTOR or the canonical activator AKT impacts memory T-cell differentiation via metabolic pathways or other pathways such as cell migration is unclear (Finlay and Cantrell, 2011). Of note, AKT regulates the differentiation and function of effector CD8<sup>+</sup> T cells via orchestrating the transcriptional program instead of cellular metabolism (Macintyre et al., 2011). Therefore, the upstream and downstream mechanisms for mTOR-dependent regulation of memory generation remain to be defined.

Tuberous sclerosis 1 (TSC1), a negative regulator of mTORC1 signaling (Laplane and Sabatini, 2012), has been implicated in T-cell homeostasis and anergy (O'Brien et al., 2011; Wu et al., 2011; Xie et al., 2012; Yang et al., 2011). We and others have demonstrated that deletion of *Tsc1* in naïve T cells disrupts their quiescence and survival and dampens T-cell-mediated primary responses to bacterial infection (O'Brien et al., 2011; Wu et al., 2011; Yang et al., 2011). Additionally, Pollizzi *et al.* has recently shown the importance of *Tsc2*, the gene encoding TSC2 that forms heterodimer with TSC1, in CD8<sup>+</sup> T cell programming. Specific deletion of *Tsc2* in CD8<sup>+</sup> T cells by using *Cd4-Cre* mice resulted in terminal differentiation of effector cells, which demonstrated increased glycolytic activity and were unable to undergo further programming into the memory phase (Pollizzi et al., 2015). To circumvent the crucial requirement of TSC1 in naïve T-cell homeostasis, we developed a mouse model to specifically delete *Tsc1* in antigen-experienced CD8<sup>+</sup> T cells. We describe here that TSC1 plays a critical role in promoting the differentiation and function of memory CD8<sup>+</sup> T cells in *Listeria monocytogenes* (LM) infection. In contrast to the deletion of *Tsc1* in naïve T cells, mice with *Tsc1* deficiency in antigen-experienced CD8<sup>+</sup> T cells retained normal effector responses but were markedly impaired in the recall response to antigen reexposure. Mechanistically, *Tsc1* deficiency diminished the generation of MPECs while promoting the development of SLECs in a cell-intrinsic manner. The functional genomic analysis indicated that TSC1 coordinated gene expression programs underlying immune function, transcriptional regulation, and cell metabolism. Furthermore, *Tsc1* deletion led to excessive mTORC1 activity and dysregulated metabolism, including glycolysis and oxidative phosphorylation, in response to IL-15 stimulation. These findings establish a

previously unappreciated role of TSC1 in linking immune signaling and cell metabolism to orchestrate memory CD8<sup>+</sup> T-cell development and function.

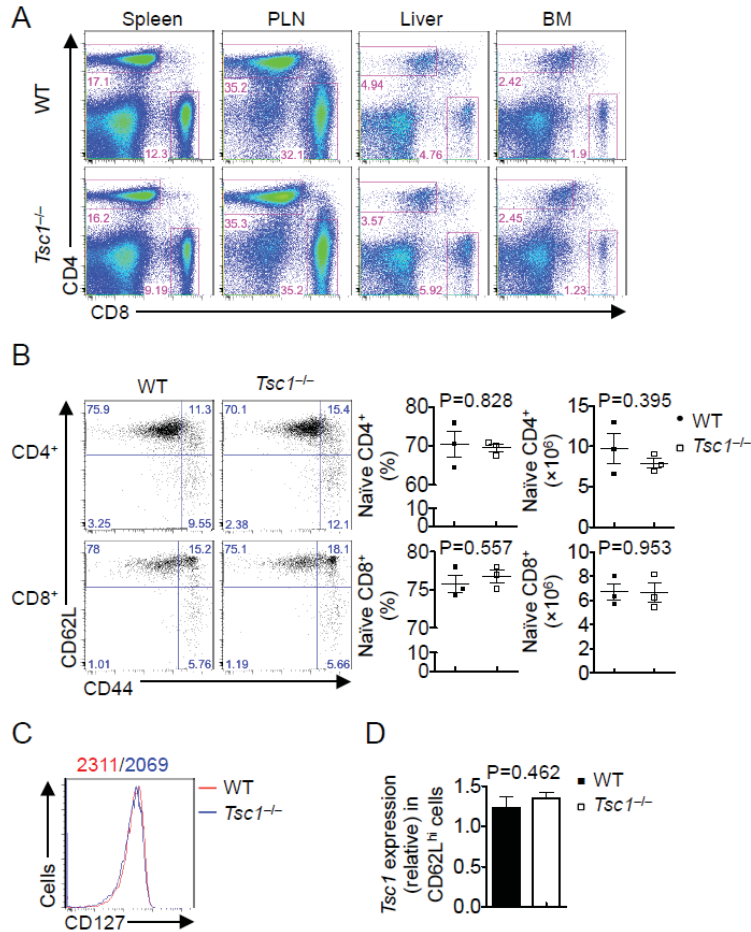
## Results

### Tsc1-deficient T cells mount normal effector responses but are impaired to transition into memory cells

TSC1 is essential to maintain the quiescence of naïve T cells. T-cell-specific deletion of *Tsc1* in mice via *Cd4*-Cre impairs naïve T-cell survival, homeostasis, and primary immune responses (O'Brien et al., 2011; Wu et al., 2011; Yang et al., 2011). Hence, this mouse model impedes the investigation of TSC1 functions in antigen-specific effector and memory responses. To this end, we crossed mice carrying the floxed *Tsc1* alleles (*Tsc1*<sup>fl/fl</sup>) with those expressing the Cre recombinase under control of the Granzyme B promoter (*Gzmb*-Cre) (Jacob and Baltimore, 1999) to specifically delete *Tsc1* in antigen-experienced CD8<sup>+</sup> T cells (called “*Tsc1*<sup>-/-</sup> mice” here). Under steady state, *Tsc1*<sup>-/-</sup> mice had normal distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (**Figure A-1A**). Homeostasis of these T cells was also undisrupted, as indicated by the normal expression of CD62L, CD44, and CD127 (**Figure A-1B and C**), consistent with the absence of *Tsc1* deletion in naïve CD8<sup>+</sup> T cells (**Figure A-1D**).

To examine whether *Tsc1* deficiency affects effector responses, we challenged wild-type *Gzmb*-Cre-negative *Tsc1*<sup>fl/fl</sup> mice (called “WT mice” here) and *Tsc1*<sup>-/-</sup> mice with *L. monocytogenes* expressing the chicken ovalbumin (LM-OVA), and assessed antigen-specific CD8<sup>+</sup> T cells in various organs at day 9 postinfection (p.i.), the peak phase of effector cell expansion. Real-time PCR results showed that *Tsc1* genomic DNA and mRNA were both effectively deleted (**Figure A-2A and B**). However, the frequency and number of OVA-specific CD8<sup>+</sup> T cells were similar between WT and *Tsc1*<sup>-/-</sup> mice in the various organs examined (**Figure A-2C**). Also, *Tsc1*<sup>-/-</sup> effector cells in the spleen and liver showed slightly increased proliferation and largely normal death, as indicated by BrdU incorporation and active caspase-3 staining, respectively (**Figure A-2D and E**). To measure the ability of antigen-specific CD8<sup>+</sup> T cells to produce cytokines, we restimulated splenocytes from infected mice with the OVA<sub>257-264</sub> [comprised of amino acids Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu (SIINFEKL)] peptide *ex vivo*. WT and *Tsc1*<sup>-/-</sup> splenocytes had similar frequency and number of antigen-specific T cells producing IFN- $\gamma$  and TNF- $\alpha$  (**Figure A-2F**). Thus, loss of TSC1 in antigen-experienced CD8<sup>+</sup> T cells does not significantly affect effector responses.

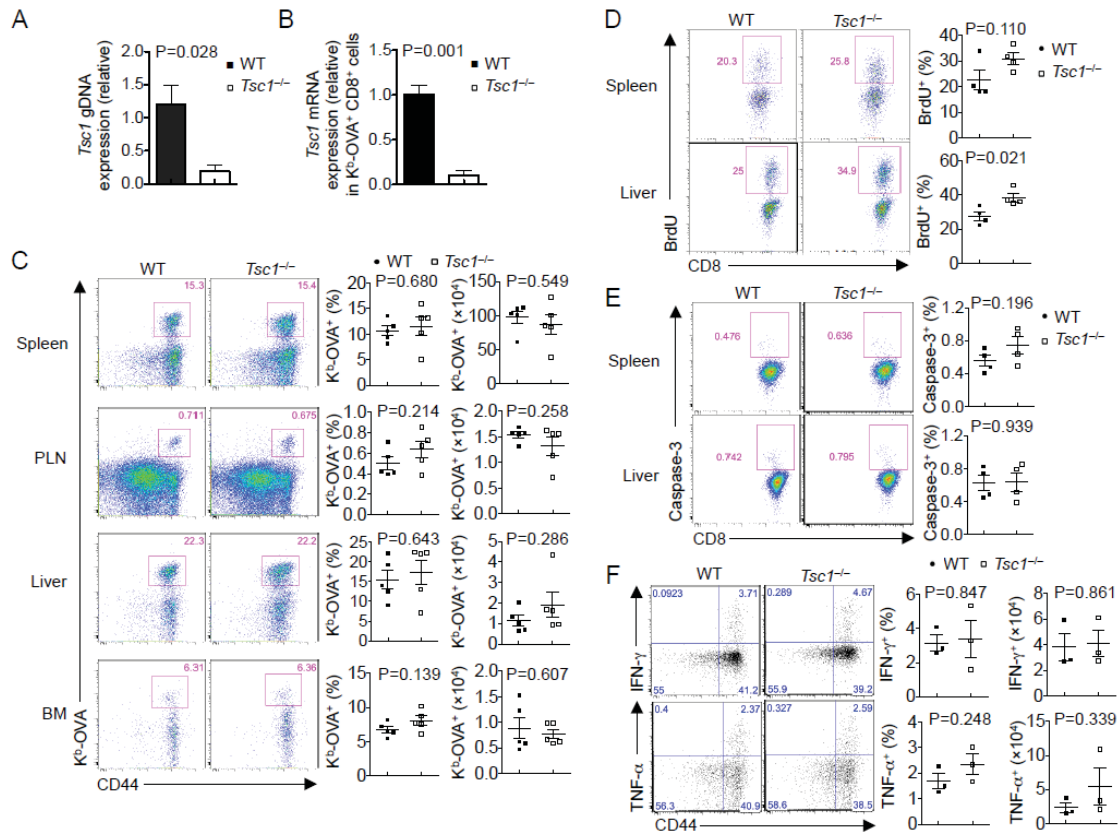
After the rapid expansion of antigen-experienced CD8<sup>+</sup> T cells, the majority of effector cells undergo cell death, whereas only a small subset survives and differentiates into memory cells (Kaech and Cui, 2012). We investigated whether *Tsc1* deficiency impairs the transition from effector to memory T cells by following the dynamics of OVA-specific T cells in the blood of WT and *Tsc1*<sup>-/-</sup> mice infected with LM-OVA. After clonal expansion, OVA-specific T cells in WT mice underwent the contraction phase,



**Figure A-1. Normal homeostasis of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in *Tsc1*<sup>-/-</sup> mice under steady state.**

(A) Flow cytometry of conventional T cells in the spleen, peripheral lymph nodes (PLNs), liver, and bone marrow (BM) from WT and *Tsc1*<sup>-/-</sup> mice. (B) Expression of CD62L and CD44 (Left) and the frequency (Center) and number (Right) of naïve CD4<sup>+</sup> and CD8<sup>+</sup> (CD62L<sup>hi</sup>CD44<sup>lo</sup>) T cells. (C) Expression of CD127 on CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice. (D) Real-time PCR analysis of *Tsc1* mRNA expression in naïve CD8<sup>+</sup> (CD62L<sup>hi</sup>CD44<sup>lo</sup>) T cells from WT and *Tsc1*<sup>-/-</sup> mice. Data are representative of two independent experiments and are presented as the mean ± SEM.





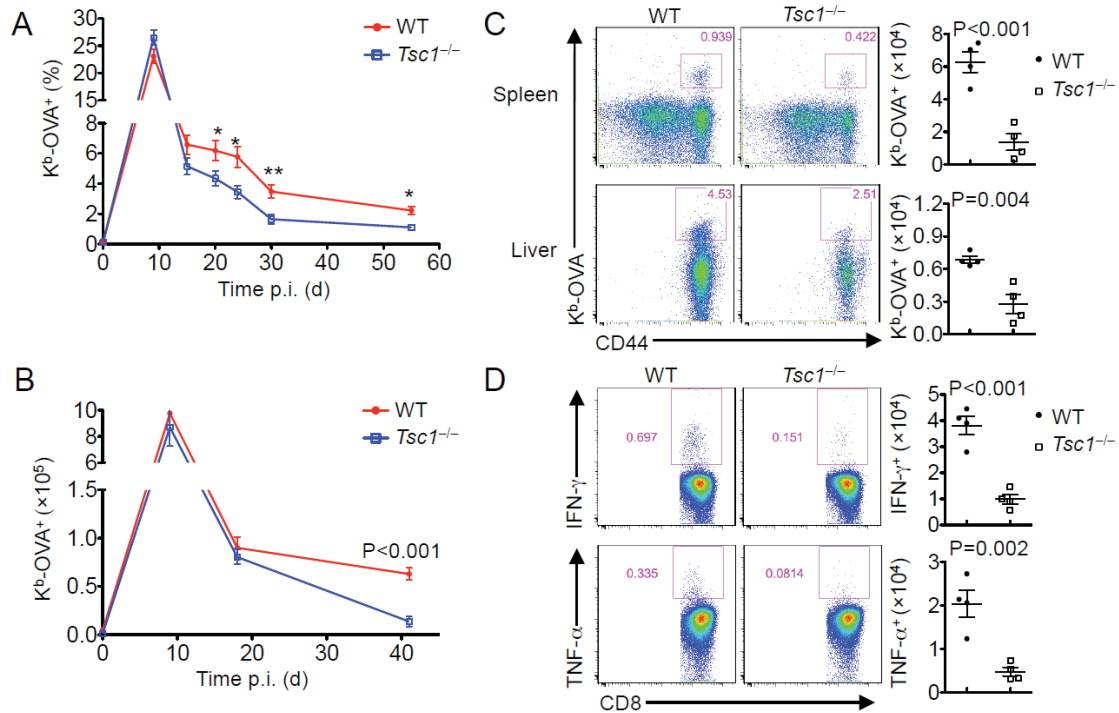
**Figure A-2. TSC1 deficiency does not impact the effector response of CD8<sup>+</sup> T cells.** WT and *Tsc1*<sup>-/-</sup> mice were infected with LM-OVA and analyzed at day 9 p.i. (A and B) Real-time PCR analysis of *Tsc1* gDNA (A) and mRNA expression (B) in K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells at day 9 p.i. (C) Representative flow cytometry plots of CD8<sup>+</sup> T cells (Left) and the frequency (Center) and number (Right) of K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells. (D) BrdU staining of K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells in the spleen and liver of WT and *Tsc1*<sup>-/-</sup> mice at day 9 p.i. (Left) and the frequency of BrdU<sup>+</sup> cells (Right). (E) Active caspase-3 staining of K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells in the spleen and liver of WT and *Tsc1*<sup>-/-</sup> mice at day 9 p.i. (Left) and the frequency of active caspase-3<sup>+</sup> cells (Right). (F) At day 9 p.i., splenocytes from WT and *Tsc1*<sup>-/-</sup> mice were restimulated with the SIINFEKL peptide for intracellular cytokine staining of IFN- $\gamma$  and TNF- $\alpha$ . Shown are representative flow cytometry plots of CD8<sup>+</sup> T cells (Left) and the frequency (Center) and number (Right) of IFN- $\gamma$ <sup>+</sup> and TNF- $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells. Data are representative of two independent experiments (B–F) or one experiment (A; n = 3 mice per group) and are presented as the mean  $\pm$  SEM.

followed by the formation of long-lived memory cells (**Figure A-3A**). Consistent with the data described above, the frequency of *Tsc1*<sup>-/-</sup> OVA-specific T cells was largely normal at day 9, but the decline was much greater than that of WT cells (**Figure A-3A**). Therefore, loss of TSC1 resulted in a progressive defect in the generation of memory T cells. To further address this question, we measured OVA-specific T cells in the spleen of WT and *Tsc1*<sup>-/-</sup> mice at different time points. At day 18 p.i., when *Tsc1*<sup>-/-</sup> splenocytes had yet to show significant reduction of antigen-specific CD8<sup>+</sup> T cells (**Figure A-3B**), these cells already up-regulated caspase-3 activity (**Figure A-4A**). In contrast, *Tsc1*<sup>-/-</sup> cells showed largely normal proliferation at this stage, as indicated by Ki-67 expression and BrdU incorporation (**Figure A-4B**). Moreover, at day 41 p.i., the number of OVA-specific CD8<sup>+</sup> T cells was significantly reduced in the spleen and liver of *Tsc1*<sup>-/-</sup> mice (**Figure A-3B and C**). We next examined cytokine production by memory CD8<sup>+</sup> T cells at day 41 via restimulating splenocytes with the OVA peptide *ex vivo*. Consistent with the reduced number of memory CD8<sup>+</sup> T cells, *Tsc1*<sup>-/-</sup> mice had fewer antigen-specific cells producing IFN- $\gamma$  and TNF- $\alpha$  than WT mice (**Figure A-3D**). Therefore, TSC1 depletion in antigen-experienced CD8<sup>+</sup> T cells impairs the transition from effector responses to memory formation.

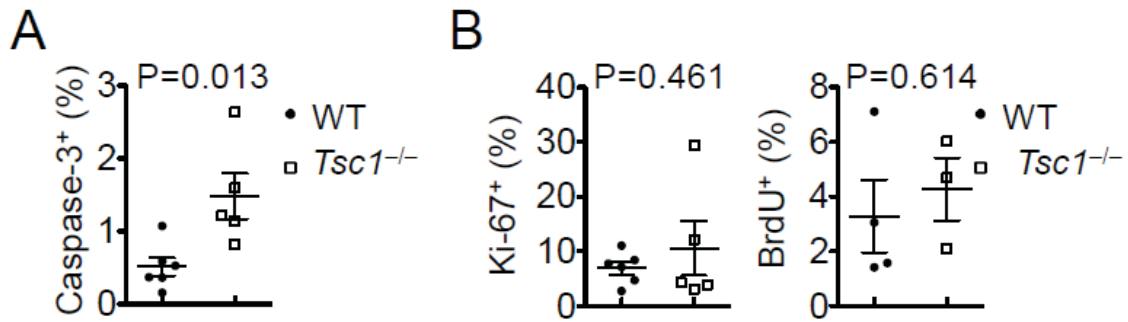
### **TSC1 deletion impairs the recall response of memory cells**

One hallmark of memory T cells is their ability to mount accelerated recall responses to antigen reexposure. To examine the role of TSC1 in this process, we used LM-OVA to rechallenge WT and *Tsc1*<sup>-/-</sup> mice at day 35 after primary infection. At day 4 after the secondary infection, WT mice mounted robust recall responses in the blood, spleen, and liver (**Figure A-5A**). In contrast, the frequency and number of OVA-specific CD8<sup>+</sup> T cells were significantly reduced in *Tsc1*<sup>-/-</sup> mice (**Figure A-5A**). We next assessed cytokine production of WT and *Tsc1*<sup>-/-</sup> splenocytes after restimulation with the OVA peptide *ex vivo*. *Tsc1*<sup>-/-</sup> mice had reduced frequency and number of T cells producing IFN- $\gamma$  and TNF- $\alpha$  compared with WT mice (**Figure A-5B**). Therefore, *Tsc1* deficiency impairs the recall response of memory cells.

One potential caveat of the experimental system above was that the defective recall response of *Tsc1*<sup>-/-</sup> memory T cells could be ascribed to the uneven numbers of memory T cells in WT and *Tsc1*<sup>-/-</sup> mice before rechallenge. To circumvent this possibility, we sorted OVA-specific memory T cells from WT and *Tsc1*<sup>-/-</sup> mice (CD45.2<sup>+</sup>) at day 41 p.i. and adoptively transferred equal numbers of WT and *Tsc1*<sup>-/-</sup> memory T cells into naïve recipients (CD45.1<sup>+</sup>), followed by LM-OVA infection 24 h later. At day 5 p.i., we assessed the frequency of donor-derived cells in various organs. The frequency of *Tsc1*<sup>-/-</sup> donor cells was significantly reduced compared with that of WT donor cells in all organs examined (**Figure A-5C**). We conclude that TSC1 is required for the recall response of memory cells upon antigen reexposure.

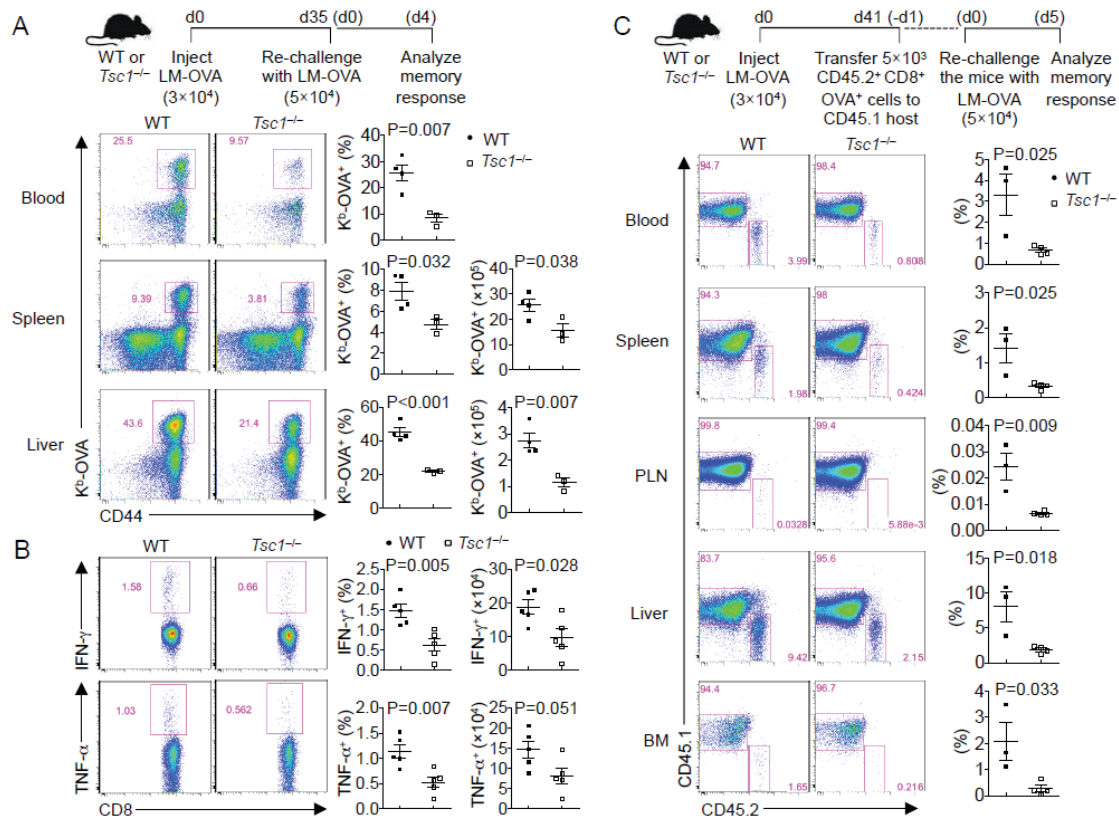


**Figure A-3. The absence of TSC1 diminishes the generation of memory T cells.** (A) The frequency of K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells in the blood from WT and *Tsc1*<sup>-/-</sup> mice infected with LM-OVA at the indicated time points was determined by flow cytometry (\**P* < 0.05, \*\**P* < 0.01). (B) Number of K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells in the spleen from WT and *Tsc1*<sup>-/-</sup> mice at days 0, 9, 18, and 41 p.i. (C) Flow cytometry of CD8<sup>+</sup> T cells (Left) and the number of K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells (Right) in the spleen and liver from WT and *Tsc1*<sup>-/-</sup> mice at day 41 p.i. (D) Splenocytes from WT and *Tsc1*<sup>-/-</sup> mice at day 41 p.i. were restimulated with the SIINFEKL peptide for intracellular cytokine staining of IFN-γ and TNF-α. Shown are representative flow cytometry plots of CD8<sup>+</sup> T cells (Left) and the number (Right) of IFN-γ<sup>+</sup> and TNF-α<sup>+</sup> CD8<sup>+</sup> T cells. Data are representative of three independent experiments and are presented as the mean ± SEM.



**Figure A-4. Proliferation and survival of *Tsc1*-deficient CD8<sup>+</sup> T cells during the contraction phase.**

Flow cytometry of active caspase-3<sup>+</sup> (**A**), Ki-67<sup>+</sup> and BrdU<sup>+</sup> (**B**) in K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells in the spleen from WT and *Tsc1*<sup>-/-</sup> mice at day 18 p.i. Data are representative of two independent experiments and are presented as the mean ± SEM.



**Figure A-5. TSC1 deficiency impairs the recall response of memory CD8<sup>+</sup> T cells.** (A) WT and *Tsc1*<sup>-/-</sup> mice were infected with LM-OVA and rechallenged with LM-OVA 35 d later. The recall response of memory CD8<sup>+</sup> T cells was examined at day 4 after secondary infection. Shown are representative flow cytometry plots of CD8<sup>+</sup> T cells (Left) and the frequency (Center) and number (Right) of K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells. (B) At day 4 after the secondary infection, splenocytes from WT and *Tsc1*<sup>-/-</sup> mice were isolated and restimulated with the SIINFEKL peptide for intracellular cytokine staining of IFN-γ and TNF-α. Shown are representative flow cytometry plots of CD8<sup>+</sup> T cells (Left) and the frequency (Center) and number (Right) of IFN-γ<sup>+</sup> and TNF-α<sup>+</sup> CD8<sup>+</sup> T cells. (C) Memory CD8<sup>+</sup> T cells (CD45.2<sup>+</sup>) sorted from WT and *Tsc1*<sup>-/-</sup> mice at day 41 p.i. were transferred into naïve CD45.1<sup>+</sup> recipients. At 24 h, the recipients were challenged with LM-OVA and analyzed 5 d later for CD45.1 and CD45.2 staining of CD8<sup>+</sup> T cells (Left) and the frequency of CD45.2<sup>+</sup> donor cells (Right). BM, bone marrow; PLN, peripheral lymph nodes. Data are representative of two independent experiments and are presented as the mean ± SEM.

## A cell-intrinsic requirement of TSC1 in memory formation and function

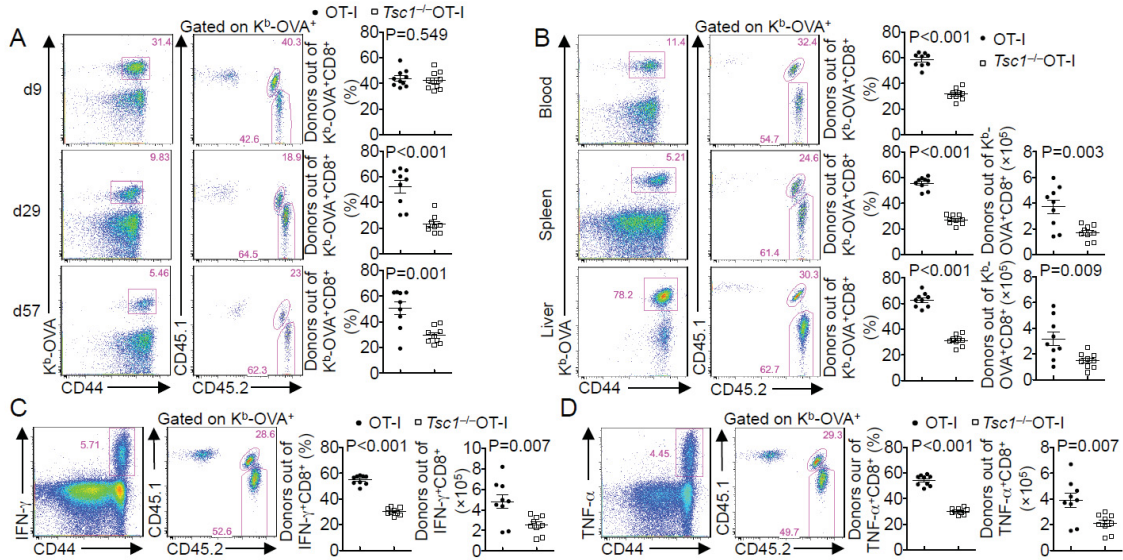
Given the role of TSC1 in the homeostasis of naïve T cells (O'Brien et al., 2011; Wu et al., 2011; Yang et al., 2011), it remains possible that the T-cell receptor (TCR) repertoire of *Tsc1*<sup>-/-</sup> T cells could be altered and contributed to the defects observed above. We therefore crossed *Tsc1*<sup>-/-</sup> mice onto the TCR-transgenic background (OT-I) in which CD8<sup>+</sup> T cells expressed a SIINFEKL peptide-specific TCR. We mixed congenically marked naïve OT-I and *Tsc1*<sup>-/-</sup>OT-I cells at a 1:1 ratio and cotransferred them to WT recipient mice, followed by LM–OVA infection. This adoptive transfer system also allowed us to exclude the effects of potential *Tsc1* deletion in cells other than CD8<sup>+</sup> T cells. Consistent with the normal effector responses observed in *Tsc1*<sup>-/-</sup> mice (**Figure A-2**), the frequencies of OT-I and *Tsc1*<sup>-/-</sup>OT-I cells were comparable at day 9 p.i. (**Figure A-6A**). However, at day 29 and 57 p.i., the percentage of *Tsc1*<sup>-/-</sup>OT-I cells was substantially lower than that of OT-I cells (**Figure A-6A**), indicative of a defect in the generation of memory cells. Upon LM–OVA rechallenge, the frequencies of *Tsc1*<sup>-/-</sup>OT-I cells were also significantly lower than those of OT-I cells in various organs examined (**Figure A-6B**). Moreover, among IFN- $\gamma$ - or TNF- $\alpha$ -producing cells in the recipient mice, the majority of them were derived from OT-I instead of *Tsc1*<sup>-/-</sup>OT-I donor cells (**Figure A-6C and D**). These results establish a cell-intrinsic role of TSC1 in promoting the formation and function of memory CD8<sup>+</sup> T cells.

## Impaired differentiation of memory precursors in the absence of TSC1

Aside from rapid expansion, effector cells differentiate into diverse subsets with distinct potentials for the formation of memory T cells: MPECs (CD127<sup>hi</sup>KLRG1<sup>lo</sup>) and SLECs (CD127<sup>lo</sup>KLRG1<sup>hi</sup>) (Joshi et al., 2007; Sarkar et al., 2008). To investigate whether TSC1 deficiency affects the differentiation of MPECs and SLECs, we examined the expression of CD127 and KLRG1 on WT and *Tsc1*<sup>-/-</sup> effector cells at day 9 p.i. We found that *Tsc1*<sup>-/-</sup> OVA-specific CD8<sup>+</sup> T cells had markedly reduced frequency and number of MPECs, whereas the frequency and number of SLECs were increased (**Figure A-7A**). Similar findings were observed in *Tsc1*<sup>-/-</sup>OT-I T cells in the adoptive transfer system (**Figure A-8A**). Moreover, to exclude the potential role of TSC1 in cell survival (**Figure A-4A**), we crossed *Tsc1*<sup>-/-</sup> mice with mice expressing a Bcl2 transgene in lymphocytes (labeled as Bcl2-Tg) (Yang et al., 2011). Following LM–OVA infection, *Tsc1*<sup>-/-</sup> Bcl2-Tg T cells showed reduced MPECs but increased SLECs, compared with the Bcl2-Tg counterparts (**Figure A-8B**). Taken together, these results reveal an intrinsic effect of *Tsc1* deficiency on the fate decisions between MPECs and SLECs.

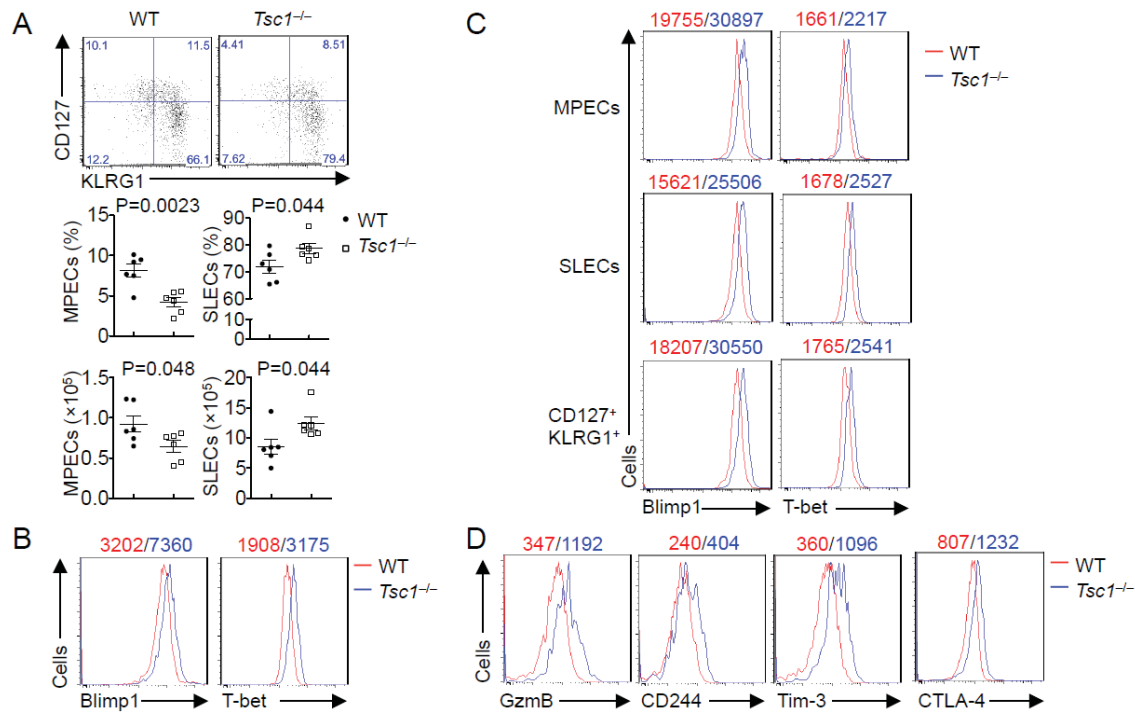
The differentiation of activated CD8<sup>+</sup> T cells into MPECs and SLECs is controlled by the differential expression of specific transcription factors, with Eomes and Bcl6 important in promoting the differentiation of MPECs (Kaech and Cui, 2012). The expression of these transcription factors in antigen-specific CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice at day 9 p.i. was largely comparable (**Figure A-9**). However, TSC1 deficiency considerably enhanced the expression of Blimp1 and T-bet, which are crucial





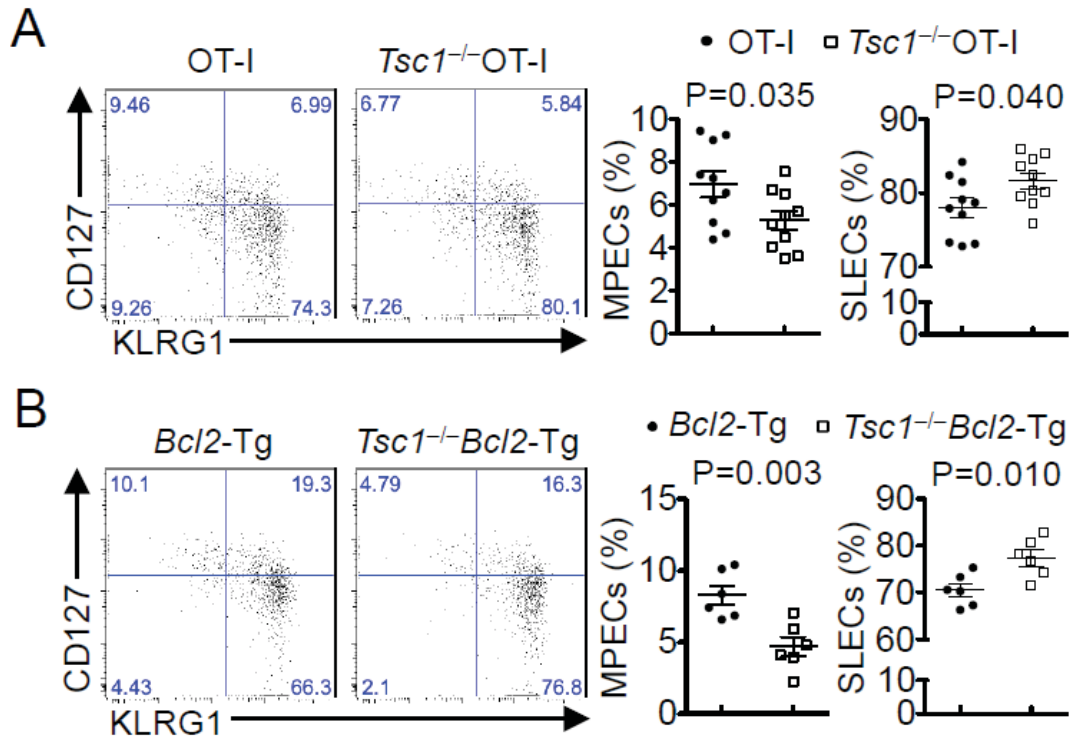
**Figure A-6. TSC1 promotes memory CD8<sup>+</sup> T-cell generation through cell-intrinsic mechanisms.**

Naïve CD8<sup>+</sup> T cells from OT-I<sup>+</sup> (CD45.2<sup>+</sup>) and *Tsc1*<sup>-/-</sup> OT-I<sup>+</sup> (CD45.1.2<sup>+</sup>) mice were isolated, mixed at a 1:1 ratio, and transferred to CD45.1<sup>+</sup> recipients, followed by LM-OVA infection 1 d later. **(A)** The frequencies of OT-I<sup>+</sup> (CD45.2<sup>+</sup>) and *Tsc1*<sup>-/-</sup> OT-I<sup>+</sup> (CD45.1.2<sup>+</sup>) donor T cells out of the total K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> cells in the blood at days 9, 29, and 57 p.i. were analysed by flow cytometry. **(B–D)** At day 57 p.i., mice were rechallenged with LM-OVA and analyzed 3 d later by flow cytometry. *(Right)* The frequencies and numbers of OT-I<sup>+</sup> (CD45.2<sup>+</sup>) and *Tsc1*<sup>-/-</sup> OT-I<sup>+</sup> (CD45.1.2<sup>+</sup>) out of the total K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells from the blood, spleen, and liver **(B)** or out of splenic IFN- $\gamma$ <sup>+</sup> **(C)** or TNF- $\alpha$ <sup>+</sup> **(D)** CD8<sup>+</sup> T cells after restimulation with SIINFEKL peptide. Data are representative of three independent experiments and are presented as the mean  $\pm$  SEM.

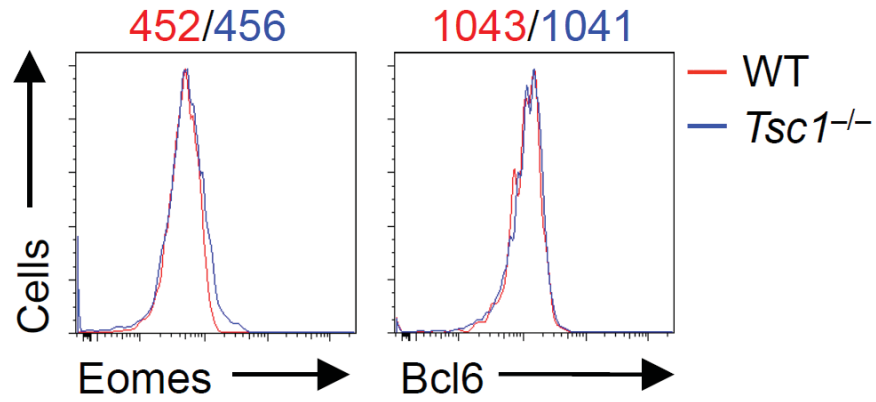


**Figure A-7. TSC1 is required for the differentiation of MPECs.**

(A) Flow cytometry of CD127 and KLRG1 (*Top*) on K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice at day 9 p.i., and the frequency (*Middle*) and number (*Bottom*) of MPECs (CD127<sup>hi</sup>KLRG1<sup>lo</sup>) and SLECs (CD127<sup>lo</sup>KLRG1<sup>hi</sup>). (B) Intracellular staining of Blimp1 and T-bet in splenic K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice at day 9 p.i. (C) Intracellular staining of Blimp1 and T-bet in MPECs, SLECs, and CD127<sup>hi</sup>KLRG1<sup>hi</sup> cells in splenic K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice at day 9 p.i. (D) Flow cytometry of GzmB, CD244, Tim-3, and CTLA-4 at day 9 p.i. in K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice. Mean fluorescent intensity (MFI) is presented above the plots (WT, red; *Tsc1*<sup>-/-</sup>, blue; B–D). Data are representative of three independent experiments and are presented as the mean ± SEM.



**Figure A-8. Intrinsic role of TSC1 in the differentiation of memory precursors.** (A) Naïve CD8<sup>+</sup> T cells from OT-I<sup>+</sup> (CD45.2<sup>+</sup>) and *Tsc1*<sup>-/-</sup> OT-I<sup>+</sup> (CD45.1.2<sup>+</sup>) mice were isolated, mixed at a 1:1 ratio, and transferred to CD45.1<sup>+</sup> recipients, followed by LM-OVA infection 1 d later. Flow cytometry of CD127 and KLRG1 expression and frequencies of memory-precursor effector cells (MPECs) and short-lived effector cells (SLECs) from OT-I<sup>+</sup> (CD45.2<sup>+</sup>) and *Tsc1*<sup>-/-</sup> OT-I<sup>+</sup> (CD45.1.2<sup>+</sup>) donor T cells out of the total K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells at day 7 p.i. (B) Flow cytometry of CD127 and KLRG1 and frequencies of MPECs and SLECs in K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells from *Bcl2*-transgenic (Tg) and *Tsc1*<sup>-/-</sup> *Bcl2*-Tg mice at day 8 p.i. Data are representative of two independent experiments and are presented as the mean ± SEM.



**Figure A-9. Expression of transcription factors implicated in memory T-cell differentiation.**

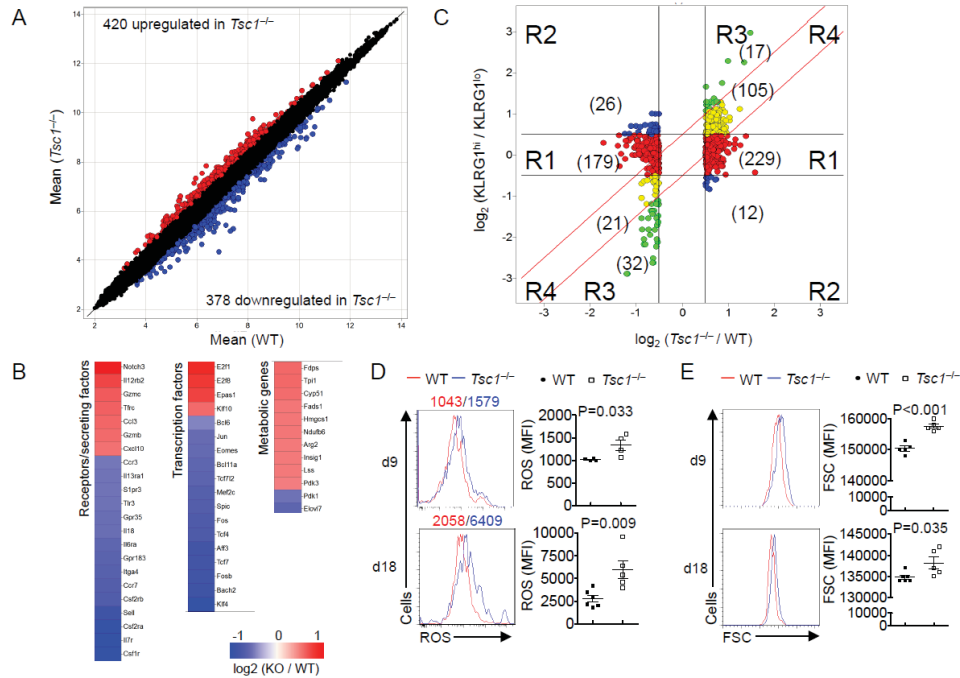
Intracellular staining of Eomes and Bcl6 in splenic  $K^b$ -OVA<sup>+</sup> CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice at day 9 p.i. Data are representative of three independent experiments.

factors for the development of SLECs and cytotoxic T-lymphocyte functions (Kaech and Cui, 2012) (**Figure A-7B**). The altered expression of Blimp1 and T-bet was observed in subdivided populations including MPECs, SLECs, and CD127<sup>+</sup>KLRG1<sup>+</sup> cells (**Figure A-7C**), highlighting a direct effect of TSC1 deficiency on these transcription factors. Moreover, *Tsc1*<sup>-/-</sup> CD8<sup>+</sup> T cells had elevated expression of Granzyme B and immune inhibitory receptors CD244 (2B4), Tim-3, and CTLA-4 (Blackburn et al., 2009; Jin et al., 2010; Wherry, 2011) (**Figure A-7D**). Therefore, consistent with the impaired memory development and MPEC formation, TSC1 deficiency enhances the expression of effector-promoting transcription factors Blimp1 and T-bet and multiple immune inhibitory receptors.

### Tsc1-dependent gene expression programs in antigen-specific CD8<sup>+</sup> T cells

We performed functional genomics to identify additional pathways controlled by TSC1 by comparing global gene expression profiles of WT and *Tsc1*<sup>-/-</sup> OVA-specific CD8<sup>+</sup> T cells sorted from mice at day 9 p.i. *Tsc1*<sup>-/-</sup> cells contained a total of 420 increased probes and 378 decreased probes by greater than 0.5 log<sub>2</sub> fold change between the comparison (**Figure A-10A**). The heat maps in **Figure A-10B** showed that *Tsc1*<sup>-/-</sup> CD8<sup>+</sup> T cells differentially expressed multiple genes (with log<sub>2</sub> fold change > 0.5) associated with the differentiation of effector and memory CD8<sup>+</sup> T cells, including receptors and secreted factors (*Il12rb2*, *Gzmb*, *Sell*, and *IL7r*) and transcription factors (*Tcf7*, *Bach2*, and *Klf4*). Additionally, *Tsc1*<sup>-/-</sup> CD8<sup>+</sup> T cells had altered expression of genes involved in various metabolic pathways, including glycolysis (*Tpi1*), cholesterol synthesis (*Fdps*, *Hmgcs1*, and *Insig1*), and fatty acid synthesis (*Fads1* and *Elovl7*) (**Figure A-10B**). Therefore, TSC1 coordinates the expression of immune response and metabolic genes in antigen-specific CD8<sup>+</sup> T cells.

Given the effects of TSC1 on the differentiation of MPECs and SLECs, it remains possible that the altered gene profiles of *Tsc1*<sup>-/-</sup> cells were simply secondary to the disrupted ratios of MPECs and SLECs. To address this issue, we compared TSC1-dependent targets with the genes differentially expressed in KLRG1<sup>hi</sup> and KLRG1<sup>lo</sup> CD8<sup>+</sup> T cells in the public database (Hess Michelini et al., 2013). Out of 798 probes with a log<sub>2</sub> fold change > 0.5 in *Tsc1*<sup>-/-</sup> cells, 621 were matched to this database and were included for further analysis (Supplementary Data 2 – Tsc1 Datasheet). These TSC1-dependent targets were partitioned into four distinct clusters that differed in their relationship to the specific gene targets in KLRG1<sup>hi</sup> cells (**Figure A-10C**). A salient feature was that the majority of TSC1-dependent targets (408 out of 621 probes) fell into cluster 1 (red circles), in which their expression was comparable between KLRG1<sup>hi</sup> and KLRG1<sup>lo</sup> cells (<0.5 log<sub>2</sub> fold change). Further, cluster 2 (blue circles) contained 38 probes that showed the opposite direction of change in expression, and cluster 3 (green circles) contained 49 probes that were differentially expressed in both types of comparisons, but to a greater extent in KLRG1<sup>hi</sup> cells compared with *Tsc1*<sup>-/-</sup> cells. Only cluster 4 (yellow circles, 126 probes) contained the target genes with equal magnitude of change (>0.5 log<sub>2</sub> fold change) in both *Tsc1*<sup>-/-</sup> and KLRG1<sup>hi</sup> cells, thus representing concordant changes in both types of cells. Overall, ~80% of all TSC1-dependent gene targets are independent of



**Figure A-10. TSC1-dependent gene expression profiles in antigen-experienced CD8<sup>+</sup> T cells.**

(A) Scatterplot comparison of global gene expression profiles between WT and *Tsc1*<sup>-/-</sup> K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells sorted from mice at day 9 p.i.; transcripts with >0.5 log<sub>2</sub> fold change are highlighted (WT, *n* = 4; *Tsc1*<sup>-/-</sup>, *n* = 5). (B) Heat maps of differentially expressed genes in K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells (with >0.5 log<sub>2</sub> fold change), with genes upregulated or downregulated in *Tsc1*<sup>-/-</sup> cells shown in red and blue, respectively. (C) Comparison of expression changes in *Tsc1*<sup>-/-</sup> versus WT cells with those in KLRG1<sup>hi</sup> versus KLRG1<sup>lo</sup> cells. The TSC1-dependent target genes (>0.5 log<sub>2</sub> fold change) were partitioned into four main clusters, shown and colored by regions (R1–R4). Numbers within parentheses indicate the number of probes within each subregion. (D and E) ROS production (D) and cell size (E) of splenic K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> T cells from WT and *Tsc1*<sup>-/-</sup> mice at day 9 and 18 p.i. Data are representative of one experiment (A–C) and two independent experiments (D and E) and are presented as the mean ± SEM.



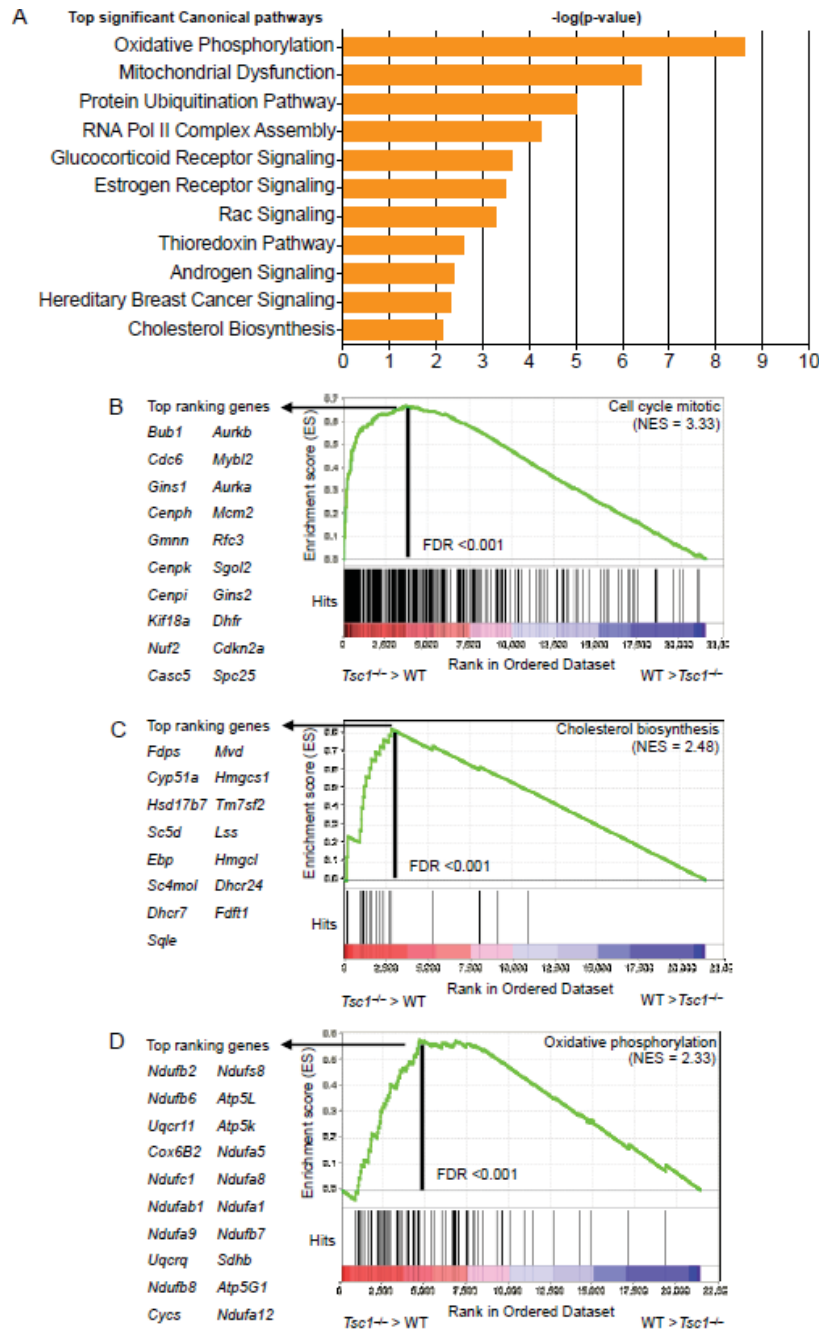
KLRG1 expression (clusters 1–3), indicating a distinct gene expression program controlled by TSC1.

We next used the ingenuity pathway analysis (IPA) system to analyze canonical pathways controlled by TSC1 in activated CD8<sup>+</sup> T cells by investigating the differentially expressed genes with a false discovery rate (FDR) < 0.1. **Figure A-11A** shows the top 11 canonical pathways affected by *Tsc1* deficiency with P < 0.01, including oxidative phosphorylation and mitochondria dysfunction. Finally, to identify key networks regulated by TSC1, we did a gene-set enrichment analysis to compare the gene expression profiles of WT and *Tsc1*<sup>-/-</sup> K<sup>b</sup>-OVA<sup>+</sup> CD8<sup>+</sup> cells. This unbiased approach identified multiple pathways with significant enrichment, including cell cycle, cholesterol biosynthesis, and oxidative phosphorylation, all of which were up-regulated in *Tsc1*<sup>-/-</sup> CD8<sup>+</sup> T cells (**Figure A-11B-D**).

Given that oxidative phosphorylation was affected by *Tsc1* deficiency in both GSEA and IPA analysis, we examined mitochondrial reactive oxygen species (ROS) production, which is associated with oxidative phosphorylation (Pearce et al., 2013). *Tsc1*<sup>-/-</sup> CD8<sup>+</sup> T cells showed increased ROS production at day 9 and 18 p.i. (**Figure A-10D**). Moreover, *Tsc1*<sup>-/-</sup> CD8<sup>+</sup> cells had larger cell sizes than WT cells (**Figure A-10E**), indicative of increased cell growth. Altogether, these data indicate that TSC1 coordinates diverse pathways involved in immune function, transcriptional regulation, and cell growth and metabolism.

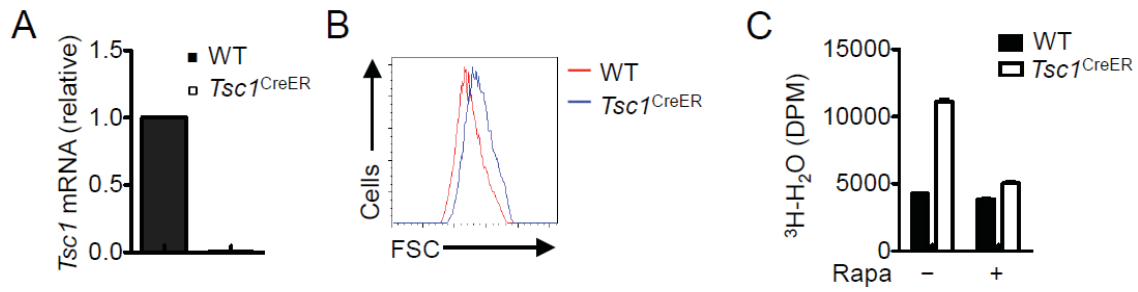
### Dysregulated mTORC1 and metabolic activities in *Tsc1*-deficient CD8<sup>+</sup> T cells

The cytokines IL-2 and IL-15 are critical for the development and maintenance of CD8<sup>+</sup> effector and memory T cells, respectively (Mitchell et al., 2010). To understand upstream and downstream mechanisms underlying TSC1-dependent memory T-cell differentiation, we used an *in vitro* culture system that mimics the programs of effector and memory T-cell differentiation (van der Windt et al., 2012; Weninger et al., 2001) and examined the effect of acute deletion of *Tsc1* in this system. To this end, we crossed *Tsc1*<sup>fl/fl</sup> with *Rosa26-Cre-ER*<sup>T2</sup> (CreER) and OT-I transgenic mice. We sorted naïve CD8<sup>+</sup> T cells from *Tsc1*<sup>+/+</sup> CreER<sup>+</sup> OT-I and *Tsc1*<sup>fl/fl</sup> CreER<sup>+</sup> OT-I mice and activated them with OVA and IL-2 in the presence of 4-hydroxytamoxifen (4-OHT) for 4 d, followed by an additional culture in either IL-2 or IL-15. The deletion of *Tsc1* after 4-OHT treatment was confirmed by real-time PCR analysis (**Figure A-12A**), which was also reflected in the increased cell size (**Figure A-12B**). IL-2 and IL-15 induce distinct effects on T-cell growth and metabolic activities (Cornish et al., 2006; van der Windt et al., 2012), although how these metabolic programs are regulated is poorly defined. We therefore examined oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which denote mitochondria respiration and glycolysis, respectively, in cells cultured with IL-2 or IL-15. Consistent with previous observations (van der Windt et al., 2012), IL-15 mediated a stronger effect on OCR than IL-2 did, whereas IL-2 but not IL-15 potentially elevated ECAR activity (**Figure A-13A and B**). Importantly, *Tsc1* deficiency markedly enhanced ECAR and OCR in cells cultured with IL-15, but effects were very



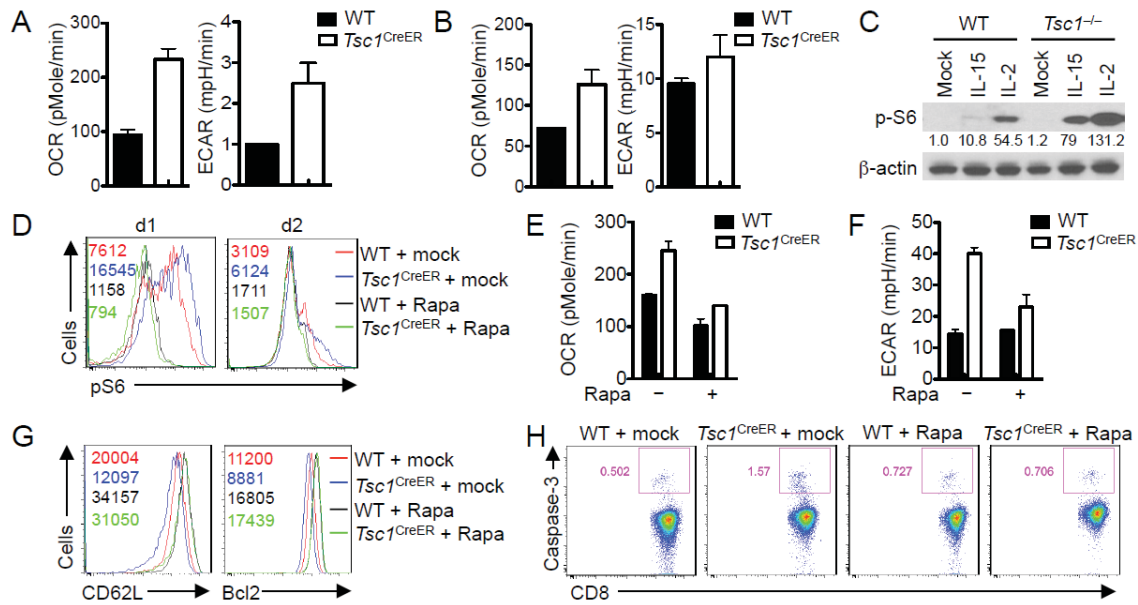
**Figure A-11. Dysregulated cell metabolism in *Tsc1*-deficient T cells.**

(A) Ingenuity pathway analysis of canonical pathways controlled by *Tsc1* in  $K^b$ -OVA<sup>+</sup> CD8<sup>+</sup> T cells sorted from mice at day 9 p.i. (FDR < 0.1). (B–D) Gene set enrichment analysis reveals the overrepresentation of signature genes in cell-cycle mitotic (B), cholesterol biosynthesis (C), and oxidative phosphorylation (D) in *TSC1*-deficient CD8<sup>+</sup> T cells at day 9 p.i. (n = 4–5 mice per genotype). The middle part of the plots shows the distribution of the genes in each gene set (“Hits”) against the ranked list of genes. The lists on the left show the top genes in the leading edge subset. NES, normalized enrichment score.



**Figure A-12. TSC1-dependent effects on cell size and glycolytic activity in activated *Tsc1*<sup>fl/fl</sup> CreER<sup>+</sup> OT-I<sup>+</sup> cells upon 4-OHT treatment.**

Naïve CD8<sup>+</sup> T cells from *Tsc1*<sup>+/+</sup> CreER<sup>+</sup> OT-I<sup>+</sup> (WT) and *Tsc1*<sup>fl/fl</sup> CreER<sup>+</sup> OT-I<sup>+</sup> (*Tsc1*CreER) mice were stimulated with OVA in the presence of IL-2 and 4-OHT for 4 d, followed by analysis of *Tsc1* mRNA expression (A), cell size (B), and glycolytic activity after IL-15 stimulation for 24 h in the absence or presence of rapamycin (Rapa) (C). Data are representative of two independent experiments.



**Figure A-13. TSC1 deficiency leads to dysregulated mTORC1 activity and metabolism in activated CD8<sup>+</sup> T cells.**

(A and B) Naïve CD8<sup>+</sup> T cells from OT-I<sup>+</sup>*Tsc1*<sup>+/+</sup> CreER<sup>+</sup> (WT) and OT-I<sup>+</sup>*Tsc1*<sup>fl/fl</sup> CreER<sup>+</sup> (*Tsc1*<sup>CreER</sup>) mice were stimulated with OVA in the presence of IL-2 and 4-OHT for 4 d, and live cells were isolated and cultured with IL-15 or IL-2 for 5 d. OCR and ECAR were measured in IL-15 (A) and IL-2–cultured cells (B). (C) Phosphorylation of S6 in WT and *Tsc1*<sup>CreER</sup> cells activated with OVA and then stimulated with IL-2 or IL-15 for 18 h. Numbers below lanes indicate band intensity relative to that of β-actin (loading control). (D) Flow cytometry of S6 phosphorylation in WT and *Tsc1*<sup>CreER</sup> cells activated with OVA and then stimulated with IL-15 for 1 and 2 d in the absence or presence of rapamycin (Rapa). (E and F) OCR (E) and ECAR (F) of WT and *Tsc1*<sup>CreER</sup> cells activated with OVA and then stimulated with IL-15 for 24 h in the absence or presence of rapamycin. (G and H) Flow cytometry of CD62L, Bcl2 (G), and caspase-3 activity (H) in WT and *Tsc1*<sup>CreER</sup> cells activated with OVA and then stimulated with IL-15 for 2–3 d in the absence or presence of rapamycin. Data are representative of three independent experiments and are presented as the mean ± SEM.

modest in cells cultured with IL-2 (**Figure A-13A and B**). Thus, *Tsc1* deletion results in dysregulated metabolic activity during IL-15–mediated differentiation of memory T cells.

Moreover, compared with *Tsc1*-sufficient controls, *Tsc1*-deficient T cells up-regulated mTORC1 activity, as indicated by the increased phosphorylation of the ribosomal protein S6. Notably, the extent of up-regulation over control cells was much more profound in IL-15–stimulated conditions (~7.5-fold) compared with IL-2–stimulated conditions (~2.5-fold) (**Figure A-13C**). Furthermore, treatment of IL-15–stimulated cells with rapamycin considerably blocked the dysregulated mTORC1 (**Figure A-13D**), OCR (**Figure A-13E**), and ECAR activities (**Figure A-13F**), as well as T-cell glycolytic activity (**Figure A-12C**). Therefore, excessive mTORC1 activation in the absence of TSC1 drives the metabolic dysregulation. Moreover, compared with IL-2, IL-15 has more of a stringent requirement for TSC1 functions in actively suppressing mTORC1 and mTORC1-dependent metabolic activities.

Associated with dysregulated metabolism, *Tsc1*-deficient cells down-regulated CD62L and Bcl2 expression (**Figure A-13G**) but up-regulated caspase-3 activity (**Figure A-13H**). Importantly, rapamycin treatment restored the excessive activity of mTORC1 and the dysregulated expression of CD62L, Bcl2, and caspase-3 activity (**Figure A-13G and H**). Therefore, TSC1 links mTORC1 activation, cell metabolism, and immune regulation.

## Discussion

In this study, we reveal an important function of TSC1 in promoting the differentiation and function of memory CD8<sup>+</sup> T cells in bacterial infection. By developing a mouse model to ablate *Tsc1* specifically in antigen-experienced CD8<sup>+</sup> T cells, we found that TSC1 is dispensable for effector responses at the expansion phase, but is essential for the formation of memory CD8<sup>+</sup> T cells and their recall responses to antigen reexposure in a T-cell–intrinsic manner. Mechanistically, deficiency of TSC1 dampens the differentiation of MPECs, associated with the impaired transcriptional and metabolic programs. In particular, loss of TSC1 results in excessive mTORC1 activity and dysregulated glycolytic and oxidative metabolism in response to IL-15 stimulation. These findings provide new mechanistic insight into T-cell memory formation and function and highlight that modulation of TSC1 function is a potential strategy to improve the quality and quantity of memory responses.

Emerging studies highlight a pivotal role of mTOR signaling in the fate decisions of effector and memory T cells (Araki et al., 2009; Hand et al., 2010; Li et al., 2011; Rao et al., 2010), but the upstream regulators of mTOR remain unresolved. Notably, mTOR can be activated by AKT-independent pathways in effector CD8<sup>+</sup> T cells (Finlay et al., 2012; Macintyre et al., 2011), and deletion of *Pten* does not cause significant defects in memory formation (Hand et al., 2010). Here, we circumvented the requirements of mTOR signaling in naïve T-cell homeostasis (O'Brien et al., 2011; Wu et al., 2011; Yang et al., 2011) and immediate TCR activation (Yang et al., 2013), by specific ablation of

*Tsc1* in antigen-experienced CD8<sup>+</sup> T cells. Although *Tsc1* deficiency elevates mTORC1 activity, this does not significantly impact the generation of effector CD8<sup>+</sup> T cells at the peak phase of primary responses. Instead, TSC1 function is essential to ensure proper differentiation between MPECs and SLECs, which is a prerequisite for the subsequent generation of memory T cells (Kaech and Cui, 2012). Specifically, *Tsc1* deficiency suppresses the formation of MPECs and reciprocally promotes the formation of SLECs. These effects were observed in multiple independent systems including direct challenge, adoptive transfer of OT-I cells, and Bcl2-Tg backgrounds, thereby highlighting a direct role of TSC1 in cell fate decisions. Accordingly, *Tsc1*-deficient antigen-specific CD8<sup>+</sup> T cells have increased expression of Blimp1 and T-bet, which likely suppress the function of transcription factors required for memory differentiation (Kaech and Cui, 2012). Additionally, *Tsc1* deletion up-regulates inhibitory receptors associated with T-cell exhaustion (Blackburn et al., 2009; Jin et al., 2010; Wherry, 2011). Moreover, *Tsc1* deficiency impairs the expression of genes involved in T-cell trafficking, such as L-selectin (encoded by *Sell*) and CCR7, which are required for the migration to central-memory T cells in the T-cell areas of secondary lymphoid organs (Finlay and Cantrell, 2011). These results indicate that TSC1 promotes memory T-cell responses, in part, by impinging upon the transcriptional programs required for memory precursor differentiation.

Metabolic programs are dynamically regulated to match the differentiation and function of T cells (MacIver et al., 2013; Pearce et al., 2013; Wang and Green, 2012). For the fate decisions between effector and memory CD8<sup>+</sup> T cells, glycolytic and lipid synthetic metabolism promote effector T-cell generation (Kidani et al., 2013; Sukumar et al., 2013), whereas oxidative phosphorylation and mitochondrial activity facilitate memory development (Pearce et al., 2009; van der Windt et al., 2012). From the microarray and bioinformatics analyses, we found that *Tsc1*<sup>-/-</sup> antigen-specific CD8<sup>+</sup> T cells exhibit elevated expression of metabolic genes involved in glycolysis, lipid synthesis, and oxidative phosphorylation. Moreover, *Tsc1*-deficient T cells show enhanced glycolytic and oxidative phosphorylation rates *in vitro* that are closely linked with IL-15-mediated signaling. Although the increased glycolysis upon *Tsc1* deletion is in agreement with impaired development of memory cells (Sukumar et al., 2013), the effect of the increased oxidative phosphorylation remains to be established. Notably, the IPA analysis indicates that *Tsc1* deficiency impinges upon both oxidative phosphorylation and mitochondria function. Further, loss of TSC1 results in excessive production of mitochondrial ROS. These findings indicate that TSC1 functions to ensure proper regulation of the metabolic programs, the disruption of which may contribute to impaired memory T-cell differentiation.

A recent study investigated the role of TSC2 in CD8<sup>+</sup> T cell function. T cell-specific deletion of *Tsc2* (referred to as *Tsc2*<sup>-/-</sup>) increased glycolytic activity in CD8<sup>+</sup> T cells, and *Tsc2*-deficient CD8<sup>+</sup> T cells were unable to differentiate into memory CD8<sup>+</sup> T cells (Pollizzi et al., 2015). However, differences in terms of effector function exist in between *Tsc1*<sup>-/-</sup> and *Tsc2*<sup>-/-</sup> mice. The potential mechanism for this difference might due to cell death processes, as Bcl2 expression and activation-induced cell death are not affected in *Tsc2*<sup>-/-</sup> mice, unlike *Tsc1*<sup>-/-</sup> mice. Additionally, presence mTORC2 activity in



*Tsc2*<sup>-/-</sup> mice resulted in potent effector CD8<sup>+</sup> T cell function that was reduced in *Tsc1*<sup>-/-</sup> mice (O'Brien et al., 2011; Pollizzi et al., 2015; Wu et al., 2011; Yang et al., 2011).

We and others have previously demonstrated a key role of TSC1 in the establishment of naïve T-cell quiescence (O'Brien et al., 2011; Wu et al., 2011; Yang et al., 2011). Although the exit from quiescence in response to TCR stimulation is essential for proper T-cell activation and effector responses (Yang et al., 2013), how memory T cells reestablish a quiescent state is unclear. The increased cell size and dysregulated metabolic activities of *Tsc1*-deficient T cells are consistent with an impaired quiescence state of memory T cells. Additionally, gene expression profiling indicated that *Tsc1*-deficient CD8<sup>+</sup> T cells down-regulate the expression of transcription factors required for the maintenance of cell quiescence including *Bach2* and *KLF4* (Roychoudhuri et al., 2013; Tsukumo et al., 2013; Yamada et al., 2009). Therefore, the failure to reestablish quiescence in *Tsc1*-deficient memory T cells is the likely basis for their poor recall responses to antigen rechallenge. This is reminiscent of the observation that abrogated quiescence of naïve T cells is associated with the defective initiation of primary immune responses (Yang et al., 2011).

In summary, TSC1 functions as a critical controller of the generation and function of memory T cells. TSC1 regulates the formation of memory T cells through shaping the fate decisions between MPECs and SLECs and by orchestrating the transcriptional and metabolic programs. Modulation of TSC1 functions in T cells represents a potential strategy to enhance the quantity and quality of memory T cells against infections and tumors.

## Experimental Procedures

### Mice

*Tsc1*<sup>fl/fl</sup>, CD45.1 and T-cell receptor-transgenic OT-1 mice were purchased from the Jackson Laboratory. *Rosa*<sup>26</sup>-Cre-ER<sup>T2</sup> (CreER) mice have been described previously (Yang et al., 2011). *GzmB*-Cre mice were kindly provided by Drs. J. Jacob and M. Li. All mice were backcrossed to the C57BL/6 background for at least 8 generations. Animal protocols were approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

### *Listeria monocytogenes* infection

For the study of primary immune response, mice were intravenously infected with  $3 \times 10^4$  colony-forming units (CFU) of LM-OVA. For the analysis of secondary immune response, mice were rechallenged with  $5 \times 10^4$  CFU of LM-OVA at day 35 or more after primary infection. The recall responses were determined 4-6 days after the secondary infection.

## Flow cytometry

For analysis of surface markers, cells were stained in PBS containing 2% (wt/vol) BSA, with anti-CD4 (RM4-5), anti-CD8 $\alpha$  (53-6.7), anti-TCR $\beta$  (H57-597), anti-CD44 (1M7), anti-CD62L (MEL-14), anti-KLRG1 (2F1), anti-CD127 (A7R34), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD244.2 (eBio244F4) (eBioscience) and Tim-3 (215008) (R&D Systems). OVA peptide (SIINFEKL)-loaded mouse H-2K<sup>b</sup> tetramer was from Baylor Tetramer Production Facility. BrdU labeling and active caspase-3 staining were performed according to the manufacturer's instructions (BD Biosciences). For intracellular staining, anti-Eomes (Dan11mag) and anti-Ki-67 (20Raj1) were purchased from eBioscience, anti-T-bet (4B10) and anti-Gzmb (GB11) from Biolegend, anti-Bcl2 (N46-467) from BD Biosciences, and anti-Blimp-1 (3H2-E8) from Thermo Scientific. For intracellular cytokine staining, CD8<sup>+</sup> T cells were stimulated for 5 hours with OVA257–264 (SIINFEKL) in the presence of monensin before being stained according to the manufacturer's instructions (BD Biosciences). ROS production was measured by incubating T cells for 30 min at 37°C with 10  $\mu$ M CM-H2DCFDA (5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; Invitrogen) after staining of surface markers. Staining with antibodies to S6 phosphorylated at Ser235 and Ser236 (D57.2.2E; Cell Signaling Technology) was done after fixing cells with Phospho Lyse/Fix Buffer, followed by permeabilization with Phosflow Perm Buffer III (BD Biosciences). Flow cytometry data were acquired on an LSRII or LSR Fortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar).

## Cell purification and culture

Sorted naïve T cells (CD8<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup>) from OT-1<sup>+</sup> *Tsc1*<sup>+/+</sup> CreER<sup>+</sup> and OT-1<sup>+</sup> *Tsc1*<sup>fl/fl</sup> CreER<sup>+</sup> mice were cultured *in vitro* in Click's medium (plus  $\beta$ -mercaptoethanol) supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin and stimulated with 10  $\mu$ g/ml SIINFEKL peptide and 100 U/ml IL-2 for 4 days in the presence of 0.5  $\mu$ M 4-OHT. Ficoll-purified live CD8<sup>+</sup> T cells were then stimulated with IL-15 (50 ng/ml) or IL-2 (50 U/ml) in the presence or absence of rapamycin (50 nM).

## Cell isolation and adoptive transfer

For adoptive transfer, naïve CD8<sup>+</sup> T cells from OT-I<sup>+</sup> (CD45.2<sup>+</sup>) and *Tsc1*<sup>-/-</sup> OT-I<sup>+</sup> (CD45.1.2<sup>+</sup>) were transferred to CD45.1<sup>+</sup> recipients. One day after transfer, mice were infected with  $3 \times 10^4$  cfu of LM-OVA for the primary response and  $5 \times 10^4$  CFU of LM-OVA for the recall response. OVA<sup>+</sup> CD8<sup>+</sup> T cells were sorted from the spleen of WT or *Tsc1*<sup>-/-</sup> mice (CD45.2<sup>+</sup>) at day 41 p.i. and transferred into congenically marked (CD45.1<sup>+</sup>) recipients. One day after the transfer, mice were infected with  $5 \times 10^4$  CFU of LM-OVA and used for the analysis of secondary CD8<sup>+</sup> T-cell responses at days 4–6.

## Metabolic assay

The bioenergetic activities of the extracellular acidification rate and oxygen consumption rate pathways were measured using the Seahorse XF24-3 Extracellular Flux Analyzer per the manufacturer's instructions (Seahorse Bioscience). The glycolytic flux assay was determined by measuring the detritiation of [3-3 H]-glucose, as described (Shi et al., 2011).

## Gene expression profiling by microarray analysis

RNA samples from freshly isolated OVA<sup>+</sup> CD8<sup>+</sup> cells from WT (n = 4 after the exclusion of one sample due to abnormal *Gapdh* 3'/5' ratio and based on principal component analysis) and *Tsc1*<sup>-/-</sup> mice (n = 5) were analyzed with the Affymetrix HT MG-430 PM GeneTitan peg array, and expression signals were summarized with the robust multi-array average algorithm (Affymetrix Expression Console v1.1). Lists of differentially expressed genes by ANOVA (FDR < 0.1) were analyzed for functional enrichment using the Ingenuity Pathways (www.ingenuity.com). GSEA within canonical pathways was performed as described (Subramanian et al., 2005).

## RNA, genomic DNA, and immunoblot analyses

Real-time PCR analysis was performed with primers and probe sets from Applied Biosystems, as described (Liu et al., 2009). Genomic DNA (gDNA) was purified from the sorted naïve CD8<sup>+</sup> (CD8<sup>+</sup>CD62L<sup>hi</sup>CD44<sup>lo</sup>) T cells using a DNeasy blood and tissue kit from Qiagen. *Tsc1* gDNA was quantified by real-time PCR normalizing with nuclear intron of *β-globin* gDNA with the following primers: *Tsc1* forward 5'-GTCACGACCGTAGGAGAAGC-3', *Tsc1* reverse 5'-GAATCAACCCACAGAGCAT-3', and *β-globin* forward 5'-GAAGCGATTCTAGGGAGCAG-3', *β-globin* reverse 5'-GGAGCAGCGATTCTGAGTAGA-3'. Immunoblots were performed as described previously (Liu et al., 2010), using the following antibodies: p-S6 (2F9; Cell Signaling Technology) and *β-actin* (AC-15; Sigma).

## Statistical analysis

*P* values were calculated with Student's t-test (GraphPad Prism). *P* values of less than 0.05 were considered significant. All error bars represent the SEM.

## VITA

Sharad Shrestha was born on 1984 in Kathmandu, Nepal. He finished his undergraduate studies in biotechnology from St. Cloud State University, Minnesota in 2008. His interest in T cell immunology led him to join Dr. Hongbo Chi's Lab in the Department of Immunology at St. Jude Children's Research Hospital as a Research Technologist in 2008. After working with Dr. Chi for three years, he decided to expand his knowledge in immunology and enrolled in the Integrated Program in Biomedical Sciences at the University of Tennessee Health Science Center for graduate study in 2011. He remained in Dr. Chi's lab and conducted research related to the mechanistic target of rapamycin (mTOR) signaling pathway in regulatory T cells and memory CD8<sup>+</sup> T cells. He is expected to receive his Doctor of Philosophy degree from the University of Tennessee in May 2016.

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