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Unraveling Molecular Mechanisms Underlying the Development of Unconventional T Cells

Abstract

The thymus supports and guides the generation of a diverse repertoire of mature T cells from precursors derived from the bone marrow. In addition to conventional CD4 and CD8 T cells, innate-like T cells also develop in the thymus and share features of the adaptive and the innate immune system. These 'unconventional' T cells have emerging roles in tissue homeostasis and disease, but the molecular mechanisms underpinning their development remain elusive. In this study, we uncovered the roles of the molecules RAPTOR and PTEN in the thymic development of unconventional T cells. Capitalizing on genetic deletion of RAPTOR, we found RAPTOR-dependent mTORC1 signaling couples microenvironmental cues with metabolic programs to orchestrate the reciprocal development of two fundamentally distinct T cell lineages: $\alpha\beta$ and $\gamma\delta$ -T cells. Loss of RAPTOR impaired $\alpha\beta$ but promoted $\gamma\delta$ -T cell development while disrupting metabolic remodeling of oxidative and glycolytic metabolism. Mechanistically, we identified mTORC1-dependent control of reactive oxygen species (ROS) production as a key metabolic signal that, upon perturbation of redox homeostasis, impinges upon T cell fate decisions. Additionally, we showed that PTEN acts as a cell-intrinsic molecular brake for the thymic development of unconventional T cells. Our results establish mTORC1-driven metabolic signaling as a fundamental mechanism underlying thymocyte lineage choices and uncover PTEN as a cell-intrinsic molecular brake in the development of unconventional T cells.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Biomedical Sciences

Research Advisor

Hongbo Chi, Ph.D.

Keywords

gamma delta T cells, IL-17, iNKT, Innate-like T cells, PTEN, Unconventional T cells

Subject Categories

Medical Immunology | Medicine and Health Sciences

UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTOR OF PHILOSOPHY DISSERTATION

**Unraveling Molecular Mechanisms Underlying the
Development of Unconventional T Cells**

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Hongbo Chi, PhD

*A Dissertation Presented for The Graduate Studies Council of
The University of Tennessee Health Science Center
in Partial Fulfillment of the Requirements for the
Doctor of Philosophy degree from
The University of Tennessee*

in

*Biomedical Sciences: Microbiology, Immunology, and Biochemistry
College of Graduate Health Sciences*

May 2020

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DEDICATION

I dedicate this work to my parents, Rainier Bastardo and Dolores Blanco,
who inspire me and support me in going after every one of my dreams,
and to my country, Venezuela, which despite the socio-political challenges
has always remained a beacon of hope for me.

ACKNOWLEDGEMENTS

It takes a village.

I'm so incredibly thankful to the many people who have crossed my path and have inspired me, encouraged me, supported me, guided me, and made me who I am. It's the shared human experience that makes us who we are, and I'm fortunate and privileged to be living such a wonderful one — one that has empowered me to reach for the stars and to push the boundaries of knowledge in the lab.

I'm thankful to Dr. Hongbo Chi for allowing me to do science at the highest level in his lab. His mentorship, guidance, and continual support were the backbones of this work. His dedication, attention to detail, enjoyment of science, and exceptional hard work were always great motivators for me to achieve more, do more, and go the extra mile. I'm thankful for Dr. Chi's investment in making me a better scientist and fostering my personal growth every day.

I'm thankful to all members of Chi Lab, past and present, for maintaining an environment of collaboration and joy that is a catalyst for world-class science. In particular, I'm thankful to Dr. Kai Yang, for his technical training and teamwork — it was Kai who set the foundation of the projects here presented and with whom I worked extensively in projects that are now published. Additionally, though not an official member of Chi Lab, I would like to thank Dr. Cliff Guy for his willingness to work with me and try out the many ideas we had for incorporating immunofluorescence imaging into my studies — Cliff never stopped to help me in searching for ways to push the projects forward from a physiological perspective.

I would also like to express my gratitude to all my dissertation committee members, Drs. Elizabeth Fitzpatrick, Thirumala-Devi Kanneganti, Tony Marion, and Michael Whitt, for the engaging discussions we had during my committee meetings, and for their support and encouragement in finding the light at the end of the tunnel and crossing the finish line.

My training has enormously strengthened my mental strength and resilience, but, as I went through it, there were times it took a toll on my mental health. I'm thankful to my wonderful counselor NaKeshi “Nikki” Dyer and my friend and coach Dr. María Milagros Verde (Monona) for their counseling and professional advice.

I'm thankful to the University of Tennessee Health Science Center for giving me the opportunity of a lifetime, and to St. Jude Children's Research Hospital and the city of Memphis for providing me with an environment of motivation and resilience to accomplish this work — I could not have dreamed of a better place to grow and to be than this.

I want to thank my friends — all of them — for their encouragement, text messages, visits, and endless smiles. From Miami to NYC, San Diego to Indianapolis, South America to Europe, they are now spread throughout the world, but their love and friendship mean the world to me.

Finally, I give my deepest gratitude to my family, who have been my greatest cheerleaders and inspiration. I thank them for all the sacrifices they made — sacrifices that led me to this very moment when I'm writing in a language that is foreign to them, 3,800 km away from home, the final words of my dissertation for the degree of Doctor of Philosophy.

ABSTRACT

The thymus supports and guides the generation of a diverse repertoire of mature T cells from precursors derived from the bone marrow. In addition to conventional CD4 and CD8 T cells, innate-like T cells also develop in the thymus and share features of the adaptive and the innate immune system. These ‘unconventional’ T cells have emerging roles in tissue homeostasis and disease, but the molecular mechanisms underpinning their development remain elusive. In this study, we uncovered the roles of the molecules RAPTOR and PTEN in the thymic development of unconventional T cells. Capitalizing on genetic deletion of RAPTOR, we found RAPTOR-dependent mTORC1 signaling couples microenvironmental cues with metabolic programs to orchestrate the reciprocal development of two fundamentally distinct T cell lineages: $\alpha\beta$ and $\gamma\delta$ -T cells. Loss of RAPTOR impaired $\alpha\beta$ but promoted $\gamma\delta$ -T cell development while disrupting metabolic remodeling of oxidative and glycolytic metabolism. Mechanistically, we identified mTORC1-dependent control of reactive oxygen species (ROS) production as a key metabolic signal that, upon perturbation of redox homeostasis, impinges upon T cell fate decisions. Additionally, we showed that PTEN acts as a cell-intrinsic molecular brake for the thymic development of interleukin-17 (IL-17)-producing unconventional T cells. Loss of PTEN gave rise to a heterogeneous population of IL-17-producing thymocytes that included invariant natural killer T cells (iNKT17), mucosal-associated invariant T cells (MAIT17), and other unconventional T cell populations. Mechanistically, the mTORC2/FoxO1 signaling axis converged with the IL-23/STAT3 signaling pathway to mediate ROR γ t-dependent development of type 17 unconventional thymocytes in the absence of PTEN. Collectively, these results establish mTORC1-driven metabolic signaling as a fundamental mechanism underlying thymocyte lineage choices and uncover PTEN as a cell-intrinsic molecular brake for the development of thymic-resident IL-17-producing unconventional T cells.

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LIST OF ABBREVIATIONS

α GalCer	Glycosphingolipid alpha-galactosylceramide
ACK	Ammonium-Chloride-Potassium Lysing Buffer
AIRE	Autoimmune regulator
APC	Antigen presenting cell
ATP	Adenosine triphosphate
Bcl2	B-cell lymphoma 2 protein
BM	Bone marrow
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CO ₂	Carbon dioxide
cTEC	Cortical thymic epithelial cells
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DCA	Dichloroacetate
DN	Double negative (CD4 ⁻ CD8 ⁻) T cell
DNA	Deoxyribonucleic acid
dNKT	Diverse natural killer T cell
DP	Double positive (CD4 ⁺ CD8 ⁺) T cell
dsDNA	Double stranded DNA
ECAR	Extracellular acidification rate
EOMES	Eomesodermin
FAO	Fatty acid oxidation
FBS	Fetal Bovine Serum
FCCP	Fluoro-carbonyl cyanide phenylhydrazone
FoxO	Forkhead box O
FoxP3	Forkhead box P3
Gal	Galactose
GAO	Galactose oxidase
GF	Germ-free
GFP	Green fluorescent protein
GLUT1	Glucose transporter 1
GSH	Glutathione
GO	Gene ontology
GSEA	Gene set enrichment analysis
HBSS	Hanks' Balanced Salt solution
ICOS	Inducible T cell costimulatory
IEL	Intraepithelial lymphocyte
IFN- γ	Interferon gamma

IL	Interleukin
iNKT	Invariant natural killer T cell
ISP	Immature single positive (CD4 ⁻ CD8 ⁺ TCRβ ⁻) T cell
MAIT	Mucosal-associated invariant T cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
Mito	Mitochondria
MLN	Mesenteric lymph node
MR1	Major histocompatibility complex, class I-related protein 1
mTEC	Medullary thymic epithelial cell
mTOR	Mechanistic target of rapamycin
NAC	N-acetyl-L-cysteine
NFAT	Nuclear factor of activated T cells
NS	No significant
OCR	Oxygen consumption rate
Oligo	Oligomycin
OP9-DL1	Delta-like-1-expressing OP9 stromal cells
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PDK1	Phosphoinositide-dependent kinase-1
PCA	Principle component analysis
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PD-1	Programmed death-1 protein
PHTS	PTEN hamartoma tumor syndromes
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PLC	Peripheral lymph node
PLZF	Promyelocytic leukaemia zinc finger protein
PRR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
RAG	Recombination-activating gene
RAPTOR	Regulatory associated protein of mTOR
RICTOR	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute buffer
ROR	Retinoic orphan receptor
ROS	Reactive oxygen species
Rot	Rotenone
RPM	Revolutions per minute
RT	Room temperature
SEM	Standard error of the mean

SLAM	Signaling lymphocytic activated molecules
SRC	Spare respiratory capacity
STAT	Signal transducers and activators of transcription
S1PR1	Sphingosine-1-phosphate receptor 1
T-BET	T-box transcription factor
TBS	Tris-buffered saline
TCR	T cell receptor
Tc17	IL-17-secreting CD8 T cells
TEC	Thymic epithelial cells
TGF	Transforming growth factor
T _H	T helper
TMRM	Tetramethyl rhodamine, methyl ester
TNF	Tumor necrosis factor
TOR	Target of rapamycin
T _{reg}	Regulatory T cell
t _{reg}	Thymic T _{reg}
UEA1	<i>Ulex europaeus</i> agglutinin 1
WT	Wildtype
YFP	Yellow fluorescent protein

CHAPTER 1. INTRODUCTION

Overview

The immune system protects the body against pathogens, harmful substances, and cancerous cells. The two arms of the immune system, the innate and the adaptive, are comprised of heterogeneous populations of cells with distinct physiological functions and metabolic requirements (**Figure 1-1**). T-lymphocytes play a central role in adaptive immunity. Upon maturation in the thymus, naïve T cells re-circulate through the blood, lymphatic systems, and peripheral lymphoid organs (Surh and Sprent, 2008). After encountering antigen presenting cells (APC) bearing cognate antigen, and in the presence of proper co-stimulation, naïve T cells become activated and rapidly engage in metabolic remodeling to meet the demands of rapid proliferation and effector molecule production (Chang and Pearce, 2016). It is now well established that changes in the activation state of T cells are coupled to distinct metabolic profiles, which in turn control T cell function (Chapman et al., 2020).

Within the family of T cells, two fundamentally distinct lineages exist: alpha beta ($\alpha\beta$) and gamma delta ($\gamma\delta$) T cells. These T cell lineages develop from the same bone marrow-derived precursors in the thymus but diverge in their developmental trajectories after undergoing T cell receptor (TCR) rearrangement (Ciofani and Zuniga-Pflucker, 2010). The TCR $\gamma\delta$ -bearing T cells are considered unconventional in part due to the limited repertoire of TCRs they express, which is in contrast to the highly polyclonal TCR repertoire of $\alpha\beta$ -T cells (Pasman and Kasper, 2017). In addition to $\gamma\delta$ -T cells, a number of TCR $\alpha\beta$ -bearing lymphocytes are also considered unconventional as they too have limited TCR repertoires and share features of the innate immune system; these include, among others, invariant natural killer T cells (iNKT) and mucosal-associated invariant T cells (MAIT) (Godfrey et al., 2015).

Unconventional T cells are emerging as key players in diseases and tissue homeostasis, yet the precise molecular mechanisms underpinning their development remain elusive (Godfrey et al., 2015; Lantz and Legoux, 2019; Lezmi and Leite-de-Moraes, 2018). Additionally, and despite seminal advances in the field of immunometabolism, knowledge remains limited in terms of the role metabolic signaling plays in T cell lineage decisions and, in particular, the development of unconventional T cells. Hence, the main goal of this study was to unravel molecular and metabolic signaling mechanisms associated with the thymic development of unconventional T cells.

Cellular Players of the Immune System

All cells of the immune system develop from pluripotent hematopoietic stem cells that reside in the bone marrow (**Figure 1-1**). These pluripotent stem cells divide into two sets of common progenitors that together give rise to two distinct lineages of white blood cells, the myeloid and the lymphoid lineages (Bhandoola and Sambandam, 2006).

Figure 1-1. Simplified schematic of the cells of the immune system.

Pluripotent hematopoietic stem cells give rise to all cells of the immune system. In the bone marrow, these pluripotent stem cells divide into common lymphoid progenitors, which produce the lymphoid lineage, and common myeloid progenitors, which give rise to the myeloid lineage. The lymphoid lineage is comprised of natural killer (NK) cells and T- and B-lymphocytes, whereas the myeloid lineage includes neutrophils, basophils, mast cells, eosinophils, monocytes, dendritic cells, and macrophages.

Common myeloid progenitors (CMP) produce the majority of the innate immune cells, which constitute the first line of defense of the immune system (Akashi et al., 2000; Pronk et al., 2007). Though depicted as one single cell progenitor in **Figure 1-1** for simplicity, CMPs represent several different cells that are transcriptionally primed to give rise to defined myeloid cell populations (Paul et al., 2016; Perie et al., 2015).

Common lymphoid progenitors (CLP), on the other hand, give rise to the antigen-specific lymphocytes of the adaptive immune system — the more specialized arm of the immune system (Kondo et al., 1997). CLPs are also responsible for producing natural killer (NK) cells, which are considered members of the innate immune system given that they lack antigen-specificity (Fathman et al., 2011). Like CMPs, CLPs are highly heterogeneous in both function and phenotypic features (Jensen et al., 2016). Challenging evidence to the model of an absolute initial bifurcation of the myeloid and lymphoid lineages have highlighted the complexity, heterogeneity, and flexibility of the progenitors that allow for the establishment of the immune system (Adolfsson et al., 2005; Bhandoola and Sambandam, 2006; Jensen et al., 2016; Katsura, 2002).

Innate Immune Cells

Cells of the innate immune system, such as macrophages, neutrophils, basophils, and mast cells, respond rapidly to recognition of non-self signatures via signaling of germline-encoded receptors. Pattern recognition receptors (PRRs) grant these cells with the capacity to recognize nearly all non-self signatures while relying on a limited number of invariant receptors (Pasman and Kasper, 2017). These receptors recognize pathogen-associated molecular patterns (PAMPs), which are simple molecules and regular patterns of molecular structures unique to microbes (Gong et al., 2019). In this sense, the response of innate immune cells to pathogens is immediate and with no memory.

Adaptive Immune Cells

There are two major types of adaptive immune cells, namely conventional $\alpha\beta$ -T cells and B cells. Both are highly specialized white blood cells that express antigen-specific receptors: the T cell receptor (TCR) and the B cell receptor (BCR), respectively. Conventional $\alpha\beta$ -T cells use their TCR to recognize peptide antigens presented by cell-surface proteins of the major histocompatibility complex (MHC) (Neefjes et al., 2011). B cells, on the other hand, recognize antigens both in solution and on the surface of antigen-presenting cells (APCs) via their BCR (Pierce and Liu, 2010). Both cell types mount delayed immune responses that increase in kinetic speed and response amplitude upon antigen re-exposure, as a consequence of memory formation (Pasman and Kasper, 2017).

Conventional $\alpha\beta$ -T Cell Development

T cells develop in the thymus from migrant precursors that originate in the bone marrow or the fetal liver (Shortman and Wu, 1996; Zuniga-Pflucker and Lenardo, 1996). Upon entering the thymus, these immature cells undergo a differentiation program that can be followed by changes in cell-surface marker expression, proliferation status, and functionality. It is now well appreciated that the integration of both intrinsic and extrinsic signals, mediated by downstream TCR signaling and the interaction of T cell precursors with thymic stromal cells and other neighboring thymocytes, respectively, control thymic development (Ciofani and Zuniga-Pflucker, 2010; Yang et al., 2018).

The thymus is located above the heart, and it consists of multiple lobules. These lobules have a well-defined cellular architecture that provides discrete cellular microenvironments to support T cell maturation (Anderson et al., 2006; Kondo et al., 2019). Specialized stromal cells, better known as thymic epithelial cells (TECs), form the clearly defined cortical and medullary regions of the thymus (Breed et al., 2018). These regions are populated by developing thymocytes that migrate from the outer cortical region into the inner medulla region in a chemokine-dependent manner as they progress through development (Hu et al., 2015; Kwan and Killeen, 2004; Ueno et al., 2004).

The development of $\text{TCR}\alpha\beta^+$ cells is characterized by the ultimate expression of either CD4 or CD8 coreceptors, and the process can be followed by the coordinated surface expression of these coreceptors (**Figure 1-2**). Thymic lymphoid progenitors begin their developmental journey as part of the immature $\text{CD4}^-\text{CD8}^-$ double-negative (DN) precursor subset (Ciofani and Zuniga-Pflucker, 2006). This DN stage can be further subdivided into four major subsets (DN1–4) based on surface expression of the α -chain of the IL-2 receptor (CD25) and the adhesion molecule CD44, as shown in **Figure 1-2** (Godfrey et al., 1993).

DN1 cells lack CD25 expression, but express CD44. They are the most immature thymocytes, which have multi-lineage potential for the development of B cells, T cells, myeloid cells, NK cells, and dendritic cells (DC) (Ardavin et al., 1993; Matsuzaki et al., 1993; Shortman and Wu, 1996). DN2 cells express both CD25 and CD44, and constitute the next developmental stage of T cells; these cells lack B cell potential, but they are still able to develop into T cells, NK cells, and DCs (Schmitt et al., 2004; Wu et al., 1996). The DN3 stage ($\text{CD4}^-\text{CD8}^-\text{CD25}^+\text{CD44}^-$) represents a key phase of T cell development as it is at this period when thymocytes undergo TCR rearrangement, hence marking the final commitment to the T cell lineage (Capone et al., 1998; Ismaili et al., 1996). DN3 cells that successfully complete $\text{TCR}\beta$ rearrangement lose CD25 expression and become DN4 ($\text{CD4}^-\text{CD8}^-\text{CD25}^-\text{CD44}^-$) cells. Survival and proliferation signaling pathways then get activated as the cells undergo differentiation into $\text{CD4}^+\text{CD8}^+$ double-positive (DP) cells (Zeng et al., 2007). In the process of becoming DP cells, developing thymocytes rapidly transit through an immature CD8^+ single-positive (ISP) stage that is followed by the cell-surface appearance of the CD4 coreceptor (Robey and Fowlkes, 1994). DP cells then lose expression of one of the coreceptors to become either CD4^+ or CD8^+ single-positive T cells.

Figure 1-2. Stages of $\gamma\delta$ and $\alpha\beta$ -T cell development.

Simplified schematic of the developmental stages that $\gamma\delta$ and $\alpha\beta$ -T cell progenitors undergo in the thymus. Commonly used surface markers for the characterization of such developmental stages are shown underneath each cell group.

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Depending on their maturation stage, two major signaling pathways control the development of T cells: NOTCH and TCR. As early DN cells do not express a TCR, they proliferate and differentiate in a TCR-independent, NOTCH-dependent manner (Yui and Rothenberg, 2014). NOTCH receptors are large single-pass type I transmembrane glycoproteins involved in the regulation of many aspects of metazoan development and tissue renewal (Kopan and Ilagan, 2009). Four NOTCH receptors have been identified in mammals (NOTCH1–4), of which NOTCH1 has been established to be both necessary and sufficient to drive T cell development (Pui et al., 1999; Radtke et al., 1999). In terms of NOTCH ligands, two families have been reported to exist in mammals: Delta-like (DL) and Jagged (Kopan and Ilagan, 2009).

Following successful TCR gene rearrangement, survival and differentiation of developing thymocytes become TCR-dependent, and NOTCH signaling gets downregulated (Yui and Rothenberg, 2014). The process of TCR rearrangement is briefly described next.

TCR Rearrangement

Expression of the α -, β -, γ -, and δ -chains of the TCR, which combined make up the TCRs of $\alpha\beta$ - and $\gamma\delta$ -T cells, respectively, requires recombination of the variable (V), joining (J), and diversity (D) gene segments of the V domain of the corresponding TCR proteins (Raulet et al., 1991; Schatz et al., 1992). This process is catalyzed by linked recombination-activating gene 1 (RAG-1) and RAG-2 proteins (Mombaerts et al., 1992; Shinkai et al., 1993; Shinkai et al., 1992). These lymphoid-specific proteins generate site-specific DNA double-stranded breaks that are then repaired through the classical nonhomologous end-joining pathway (Matthews and Oettinger, 2009). This process ensures the generation of a diverse TCR repertoire.

The process of recombination is highly regulated and occurs at specific developmental stages. DN1 and DN2 cells have their TCR β and TCR γ loci in germline configuration (Godfrey et al., 1994; Wu et al., 1991). Rearrangement of the TCR β locus of $\alpha\beta$ -T cells starts at the DN3 stage (Livak et al., 1999). In fact, DN3 thymocytes are a mixture of small TCR β -unrearranged DN3-E (DN3a) cells and large TCR β -rearranged DN3-L (DN3b) cells (Hoffman et al., 1996; Taghon et al., 2006). Similarly, V to J rearrangements of the TCR γ locus occurs primarily in DN3 cells and can precede that of TCR β (Livak et al., 1999; Petrie et al., 1995). In contrast to TCR β rearrangement, rearrangement of the TCR α locus occurs only after thymocytes have become DP cells, at which point they undergo positive selection (Petrie et al., 1993).

$\alpha\beta$ -T Cell Development Checkpoints

β -Selection. The first checkpoint of $\alpha\beta$ -T cell development ensures that $\alpha\beta$ -lineage committed thymocytes underwent proper RAG-mediated recombination and that they express a functional TCR β -chain (Carpenter and Bosselut, 2010). This process

requires developing thymocytes to signal through their pre-TCR, which is composed of a properly rearranged TCR β -chain paired with a pre-TCR α along with a collection of proteins on the cell surface (i.e. the CD3/ ζ complex) that are involved in proximal signal transduction (Germain, 2002). Pre-TCR signaling triggers a cascade of differentiation and proliferation events that enables cells to undergo 6–8 rounds of cell division, after which it triggers the rearrangement of the TCR- α locus, giving rise to the second component of the mature $\alpha\beta$ antigen receptor (Germain, 2002). This process licenses developing thymocytes (i.e. DN3–4 cells) to progress to the DP stage (Michie and Zuniga-Pflucker, 2002; Rothenberg and Taghon, 2005).

Positive Selection. The proliferative burst originated by the pre-TCR signals during β -selection results in the establishment of a large pool of DP thymocytes. Despite the fact over 80% of total thymocytes are DP cells, only 5% of them survive this stage of development and continue their developmental process (Wang et al., 2011). This is because, once the mature TCR $\alpha\beta$ is produced, the developing thymocytes depend on TCR signaling for their survival (Yui and Rothenberg, 2014). The random nature of TCR rearrangement, and the high diversity of MHC alleles, translates into most DP thymocytes failing to productively interact with peptide:MHC ligands. Consequently, these thymocytes die of neglect (Carpenter and Bosselut, 2010). Positive selection rescues DP thymocytes from programmed cell death on the basis of the interaction of their newly formed TCR $\alpha\beta$ with self-peptide bound to MHC complexes expressed on cortical TECs (cTECs) (Ladi et al., 2006; Yui and Rothenberg, 2014).

The lifespan of DP cells during positive selection is important in shaping the TCR repertoire of peripheral T cells (Wang et al., 2011). This is because DP cells that fail to generate a productive TCR in their first round of rearrangement get to repeat rearrangement of their TCR α -chain as many times as necessary until successful, or until programmed cell death intervenes. Thus, the lifespan of a DP cell determines how many rounds of TCR α -chain rearrangement the cell can undergo, which has repercussions on the overall diversity of the organism's TCR repertoire (Guo et al., 2002). A large body of evidence supports a role for the anti-apoptotic protein Bcl-x_L in the regulation of survival of DP cells (Wang et al., 2011). In fact, several transcription factors critical for T cell development, including TCF-1 (Xie et al., 2005), E proteins (D'Cruz et al., 2010), c-Myb (Yuan et al., 2010), and ROR γ t (Sun et al., 2000), regulate survival of DP cells by means of Bcl-x_L-dependent pathways. Hence, Bcl-x_L is considered the master regulator of DP lifespan.

Positively selected DP cells mature into either CD4 or CD8 single-positive (SP) cells, which express chemokine receptors, such as CCR4 and CCR7, that allow them to migrate towards the medulla (Cowan et al., 2014; Hu et al., 2015; Ueno et al., 2004). Once in the medulla, SP thymocytes encounter a third developmental checkpoint, negative selection, which ensures T cell tolerance.

Negative Selection. Newly generated SP thymocytes are CD62L^{low}CD69^{high} semi-mature cells that are functionally incompetent and susceptible to various apoptotic signals (Takahama, 2006). Upon arrival to the thymic medulla, these developing

thymocytes undergo further maturation to become CD62L^{high}CD69^{low} mature SP thymocytes. The thymic medulla is a specialized microenvironment composed of medullary thymocytes, DCs, macrophages, and medullary TECs (mTECs) (Ladi et al., 2006). mTECs, in particular, facilitate the removal of thymocytes bearing self-reactive TCRs by, at least in part, exposing them to tissue-specific antigens in a manner dependent on the transcriptional factor autoimmune regulator (AIRE) (Giraud et al., 2012; Kyewski and Derbinski, 2004). AIRE deficiency in humans causes failure to establish central tolerance to tissue-specific antigens, resulting in autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (Takahama, 2006). Similar autoimmune diseases have been observed in mice with AIRE deficiency as well (Kuroda et al., 2005; Liston et al., 2003). Thus, AIRE⁺ mTECs are key players in the establishment of central tolerance.

Mechanisms of Central Tolerance. T cell central tolerance eliminates strongly self-reactive progenitors so that only weakly reactive T cells leave the thymus and populate the lymphoid organs. Clonal deletion, defined as the apoptotic death of T cell progenitors that have high affinity for self-antigens, is the main mechanism of negative selection for the establishment of T cell central tolerance (Palmer, 2003). Anergy and receptor editing also eliminate high-affinity self-reactive cells but to a lesser extent than clonal deletion (Hogquist et al., 2005). Anergy is a state of non-responsiveness to antigen (Hammerling et al., 1991). Receptor editing, on the other hand, refers to the process by which binding of self-antigen during development initiates secondary antigen-receptor gene rearrangement that results in the replacement of an autoreactive receptor by a self-tolerant one (McGargill et al., 2000).

Noticeably, complete deletion of self-reactive T cells is not achieved via the aforementioned mechanisms of central tolerance. A number of individual lymphocytes not only seem to escape the process of negative selection, but actually require substantial self-reactivity to carry out their function in immunity. These include natural T regulatory (nT_{reg}) cells, invariant natural killer T (iNKT) cells, natural CD8 $\alpha\alpha^+$ intraepithelial T cells that reside in the gut (nIEL), and natural T helper 17 (nTh17) cells (Stritesky et al., 2012). The development of these T cell populations is dependent on their interaction with high-affinity TCR ligands along with distinct molecular factors. Though they share common features, such as an activated/memory phenotype and regulatory function in immunity, their developmental trajectories are distinct and they can arise at distinct stages of T cell development (Stritesky et al., 2012).

Thymic Medulla. As alluded before, the medullary region of the thymus, along with its cellular players, plays a key role in the establishment of T cell central tolerance. The importance of the thymic medulla is underscored by the autoimmune diseases observed in mutant mice that present impaired medullary characteristics. Examples of this are REL-B deficient (Burkly et al., 1995; Weih et al., 1995), lymphotoxin- β receptor (LT- β R)-deficient (Boehm et al., 2003; Chin et al., 2003; Zhu et al., 2007), and tumor-necrosis factor (TNF)-receptor-associated factor-6 (TRAF6)-deficient (Akiyama et al., 2005) mice. These mutant mice present disrupted thymic structure, usually characterized by smaller medullary regions that lack mTECs; mTECs can be identified by the expression of the lectin *Ulex europaeus* agglutinin 1 (UEA1) (Kadouri et al., 2019).

Despite presenting normal positive selection, these mutant mice fail to establish central tolerance, which results in severe inflammation and/or autoimmune disease. Interestingly, some of these mutant mice also present defects in the development of regulatory T cell subsets, including NKT cells and nT_{reg}s (Hogquist et al., 2005).

Of note, development of nT_{reg} occurs in the thymic medulla (Aschenbrenner et al., 2007; Cowan et al., 2013). High-affinity TCR ligands expressed on stromal cells in the medulla mediate the process of agonist selection, which selects CD4⁺ T cell clones that recognize self-peptide:MHC-class II complexes with medium to high affinity to differentiate into Foxp3-expressing T_{reg}s (Kadouri et al., 2019). Both mTECs and DCs play important roles in this process (Cowan et al., 2013; Herbin et al., 2016; Perry et al., 2014).

Unconventional T Cells

In addition to conventional CD4⁺ and CD8⁺ $\alpha\beta$ -T cells, which require prolonged activation and differentiation to acquire effector function and also present great diversity in terms of TCR repertoire, the thymus supports the development of smaller subsets of T cells with innate-like effector functions and restricted TCRs. These so-called ‘unconventional T cells’ have an activated phenotype, which grants them with the ability to readily produce large amounts of cytokines in response to non-cognate antigen stimulation (Godfrey et al., 2015). In other words, they are not engaged by peptide:MHC complexes, yet they mount relatively immediate immune responses. These unconventional T cell subsets are conserved among mammals and include $\gamma\delta$ -T cells, iNKT cells, MAIT cells, and CD8 $\alpha\alpha^+$ IELs. There are also other $\alpha\beta$ -T cells that recognize formylated peptides presented by H2-M3, but they are not existent in humans and are not discussed here (Legoux et al., 2017).

Broadly, the generation of unconventional T cells follows two distinct developmental pathways. Most $\gamma\delta$ -T cells, and a small percentage of IELs and iNKT cells, develop from CD4⁻CD8⁻ DN thymocytes, whereas the majority of IELs and iNKT cells, along with MAIT cells and nT_{reg} cells, surge from agonist selection of CD4⁺CD8⁺ DP thymocytes (Winter and Krueger, 2019).

$\gamma\delta$ -T Cells

The first wave of T cells that appears in the thymus during fetal thymic ontogeny is composed of $\gamma\delta$ -T cells (Chien et al., 2014). The later emergence of $\alpha\beta$ -T cells is associated with a decrease in the relative proportion of $\gamma\delta$ -T cells, which ultimately comprises between ~0.5% and 16% of total CD3⁺ cells in adult human peripheral blood. In adult mice, the $\gamma\delta$ -lineage of T cells makes up ~1–4% of total T cells in the thymus and the secondary lymphoid organs. $\gamma\delta$ -T cells are much more numerous in mouse mucosal sites, however, where they can amount to be up to 40% and 70% of the

intraepithelial lymphocytes of the intestines and the skin, respectively (Goodman and Lefrancois, 1989; Gray et al., 2011).

Both $\gamma\delta$ and $\alpha\beta$ -T cells mount cytotoxic immune responses upon activation using a similar set of cytokines. Though their TCRs are comparable in terms of structure and downstream signaling pathways, they differ in their diversity and ligand recognition. $\gamma\delta$ -TCRs are not MHC-restricted, which provides for a more promiscuous recognition of ligands (Holderness et al., 2013). Thus, they can recognize and respond to unconventional antigens, including soluble, membrane-bound, and unprocessed ligands (Elliott et al., 1988; Rock et al., 1994; Steele et al., 2000). However, the diversity at the TCR γ locus is limited by no D, and only a few V/J segments, plus three functional C regions (Capone et al., 1998). In the periphery, $\gamma\delta$ -T cells have a restricted TCR diversity, which is why they are considered semi-invariant (Hayday, 2000).

The process of $\alpha\beta$ vs. $\gamma\delta$ lineage selection is mediated by the integration of multiple environmental signals in the thymus along with TCR-signaling strength (Carpenter and Bosselut, 2010; Ciofani and Zuniga-Pflucker, 2010; Haks et al., 2005; Hayes et al., 2005; Lee et al., 2014; Zarin et al., 2014). The divergence between the two lineages occurs at the DN3 stage, when the common progenitor undergoes TCR rearrangement of the β -, γ -, and δ -chains (Carpenter and Bosselut, 2010; Ciofani and Zuniga-Pflucker, 2010).

As previously discussed, the expression of a functional pre-TCR after productive rearrangement of the TCR β -chain, together with NOTCH signals, induces a proliferative burst that leads to the differentiation of progenitor cells in the $\alpha\beta$ -lineage into DP cells (Kreslavsky et al., 2012). In contrast, NOTCH signaling plays a less important role in $\gamma\delta$ -T cell development, and recent studies point to TCR signaling strength as a key mediator of $\gamma\delta$ -T lineage commitment (Haks et al., 2005; Hayes et al., 2005; Lee et al., 2014; Zarin et al., 2014). The extracellular signal-regulated kinase (ERK), early growth response-1 (EGR1), and inhibitor of DNA binding 3 (ID3) signaling axis (ERK/EGR1/ID3) has emerged as a key player in the determination of lineage commitment. In fact, studies have shown that the promotion of strong signals via induction of the helix-loop-helix protein ID3 or the mere ligand engagement by TCR $\gamma\delta$ promotes the $\gamma\delta$ -lineage fate in developing DN3 cells (Kreslavsky et al., 2008; Lauritsen et al., 2009).

Recent studies have identified *Nt5e* (CD73) as a marker of $\gamma\delta$ -T cell lineage commitment (Coffey et al., 2014). The expression of CD73 is mediated by ligand engagement of the TCR $\gamma\delta$ (Fahl et al., 2014). It is now established that CD73-expressing immature CD24⁺ DN progenitors constitute an intermediate developmental stage after commitment but before acquisition of effector fate, whereas CD73⁺CD24^{low} cells are functionally competent $\gamma\delta$ -T cells (Coffey et al., 2014).

Invariant Natural Killer T (iNKT) Cells

iNKT cells are a heterogeneous population of TCR $\alpha\beta$ ⁺ T cells that recognize lipid antigens presented by the CD1d molecule. They express an invariant TCR α -chain (V α 14-J α 18 in mice and the homologous V α 24-J α 18 in humans) paired with a semi-invariant TCR β -chain (V β 2, V β 7 or V β 8.2 in mice and V β 11 in humans) (Mori et al., 2016; Salio et al., 2014). Mouse iNKT cells are either CD4⁺ or CD4⁻CD8⁻ DN, whereas human iNKT cells also have a CD8⁺ subset. These subsets have slightly different functional properties, with human DN and CD8⁺ iNKT cells being highly cytolytic and capable of producing IFN γ , and CD4⁺ iNKT cells being able to produce both IFN γ and IL-4 and also having immunomodulatory functions (Gumperz et al., 2002; Lee et al., 2002). Furthermore, a subset of DN iNKT cells has been shown to produce IL-17 with potential roles in the pathogenicity of several diseases (Michel et al., 2007; Yoshiga et al., 2008).

The majority of iNKT cells develop from CD4⁺CD8⁺ DP cells that have randomly rearranged their TCR α locus and generated the iNKT α -chain (Egawa et al., 2005; Gapin et al., 2001), with the exception of a small number of iNKT cells that use an alternative developmental pathway starting from the DN4 stage (Dashtsoodol et al., 2017). In contrast with conventional DP precursors that are selected by MHC I/II molecules on cTECs, iNKT cell DP precursors are positively selected by CD1d-expressing DP thymocytes presenting self-lipids (Bendelac, 1995; Coles and Raulet, 2000). This DP-DP interaction provides the required lipid/CD1d ligand that, in addition to co-stimulation through members of the signaling lymphocytic activated molecules (SLAM) family of receptors and TCR signaling, licenses the iNKT precursors to continue their developmental process. The strong signaling provided by the TCR and SLAM receptors leads to high expression of the transcription factor Egr2, which binds to the promoter of *Zbtb16*, the gene encoding the promyelocytic leukaemia zinc finger protein (PLZF), and positively regulates its transcription (Krovi and Gapin, 2018). These cells then transition through an uncommitted PLZF^{high} stage before diverging into the functionally distinct iNKT1, iNKT2, and iNKT17 subsets, which are defined by the transcription factors T-BET, GATA-3, and ROR γ t, respectively. Accordingly, these iNKT subsets have cytokine capabilities similar to those of conventional T_H1, T_H2, and T_H17 cells.

The overall process of iNKT cell development in the thymus can be divided into four consecutive stages (S0–3) based on the differential expression of CD24, CD44, and NK1.1 (Benlagha et al., 2002; Pellicci et al., 2002). The least mature iNKT cells (S0) are CD24⁺. S1 iNKT cells do not express CD24 nor NKT-associated markers. S2 iNKT cells are CD44⁺NK1.1⁻ and have the capacity to leave the thymus and populate peripheral tissues, whereas S3 iNKT cells are CD44⁺NK1.1⁺ and are mostly thymus-resident (Berzins et al., 2006; Winter and Krueger, 2019). Thymic and peripheral iNKT effector populations do not exchange, so a small pool of CCR7⁺ iNKTs cells with multipotent capacities is responsible for the establishment of the effector subsets in the thymus (Wang and Hogquist, 2018).

The precise molecular mechanisms controlling iNKT cell lineage fate decisions are still not well understood, though a network of transcriptional regulators and molecular complexes has been identified to be important for iNKT cell development and effector lineage choices. The mechanistic target of rapamycin complex 1 (mTORC1) has been reported to be essential for the differentiation of iNKT1 cells (Zhang et al., 2014), while mTORC2 is important for iNKT2 and iNKT17 differentiation (Prevot et al., 2015; Wei et al., 2014). Emerging studies have indicated a role for the forkhead box O1 (FoxO1) in controlling iNKT effector lineage fate decisions by promoting iNKT1 and suppressing iNKT17 lineages (Tao et al., 2019). On the other hand, the transcriptional regulators Runx1 (Thapa et al., 2017), NKAP (Thapa et al., 2016), and BATF (Jordan-Williams et al., 2013; Miao et al., 2013), are all essential for the development of iNKT17 cells.

iNKT cells respond to lipid antigens of similar structure to glycosphingolipid alpha-galactosylceramide (α GalCer) (Pasman and Kasper, 2017). Originally, the term ‘natural killer T cells’ (NKT cells) was used to refer to CD3⁺ T cells that co-expressed proteins normally expressed on NK cells, such as NK1.1 (CD161) (Godfrey et al., 2004). However, the modern definition of iNKT cells refers only to cells specific to lipid antigens presented by CD1d molecules (Godfrey et al., 2015), which can be easily identified via flow cytometric analysis using CD1d tetramers loaded with an appropriate antigen ligand, such as synthetic KRN7000 or other α GalCer molecules.

In addition to iNKT cells, which are also known as type I NKT cells, there is a second type of NKT cells that also recognize lipids presented by CD1d molecules but that uses TCR $\alpha\beta$ -chains that do not conform with the invariant motifs used by type I NKT cells. These type II NKT cells are known as ‘diverse NKT cells’ (dNKT) (Rhost et al., 2012). Though dNKT cells represent a small subset of NKT cells in mice, they outnumber iNKT cells in humans (Iwabuchi and Van Kaer, 2019).

It is important to note too that humans express four additional structurally distinct CD1d antigen-presenting molecules: CD1a, CD1b, CD1c, and CD1e (Salio et al., 2014). Each of these CD1 proteins present divergent antigen-binding grooves with unique patterns of intracellular trafficking and tissue expression. These human-specific CD1 proteins are also known as group I CD1 molecules, whereas the mouse-and-human CD1d protein makes up the group II category of CD1 molecules.

Mucosal-Associated Invariant T (MAIT) Cells

MAIT cells represent a relatively new population of unconventional T cells with emerging roles in disease and tissue homeostasis (D'Souza et al., 2018; Xiao and Cai, 2017). They are defined by the expression of an invariant TCR α -chain (V α 19-J α 33 in mice and V α 7.2-J α 33 in humans) associated with a limited set of V β segments (V β 6, 8 in mice and V β 2, 13, 22 in humans) (Tilloy et al., 1999). MAIT cells recognize antigens in a MHCI-related protein 1 (MR1)-dependent manner, which, unlike conventional MHC molecules, presents microbial-derived vitamin B metabolites (Huang et al., 2005).

5-(2-oxopropylideneamino)6-D-ribitylaminouracil (5-OP-RU), an adduct of a key riboflavin (vitamin B2) biosynthesis pathway intermediate (5-A-RU) and a glycolysis pathway intermediate (pyruvaldehyde), is the most potent stimulatory antigen of MAIT cells (Patel et al., 2013). The identification of a potent MAIT antigen allowed for the development of antigen-loaded (5-OP-RU) MR1 tetramers that now serve as the golden standard for the identification of MAIT cells, similar to α GalCer-loaded CD1d tetramers for the identification of iNKT cells (Reantragoon et al., 2013). In contrast to the vitamin B2-derived antigens, vitamin B9 (folate)-based MR1 ligands such as 6-formyl pterin (6-FP) and its derivate acetyl 6-formylpterin (Ac-6-FP) inhibit MAIT cell activation (Yan et al., 2020).

Despite the fact that MR1 is evolutionarily conserved among mammals, mouse and human MAIT cells present several differences that go beyond TCR usage (Huang et al., 2009; Moreira et al., 2017). Mouse MAIT cells tend to be CD4⁻CD8⁻ DN, though small frequencies of CD4⁺ and CD8⁺ single-positive MAIT cells also exist (Rahimpour et al., 2015). The majority of human MAIT cells, however, are CD8⁺ single-positive (Yan et al., 2020). Further, MAIT cells are rare in mice but abundant in humans. In mice, MAIT cells are mainly found in mucosal sites, such as lungs, liver, intestine, and skin (Constantinides et al., 2019; Lantz and Legoux, 2019), at relatively low frequencies, whereas in humans they can amount to be up to 10% of peripheral blood T cells, 10% of CD8⁺ T cells, and 50% of liver T cells (Cui et al., 2015). The discrepancies between mouse and human MAIT cells have presented a challenge for the study of these cells in the context of disease, particularly as it relates to infectious diseases (Moreira et al., 2017).

MAIT cells share similar developmental trajectories with iNKT cells. In contrast to conventional T cells, which are positively selected by cTECs, both MAIT and iNKT precursor cells interact with fellow DP cells that express MR1 or CD1d molecules on their surface, respectively (Seach et al., 2013). For both MAIT and iNKT cells, this DP-DP interaction in the cortical region of the thymus generates CD24⁺CD44⁻ committed precursors (referred as S1 in MAIT development) that then cease to express CD24 (S2 in MAIT development) and transition into the medullary region in a process dependent on microRNAs (Wang and Hogquist, 2016). In the medulla, the precursors are thought to respond to environmental cues that drive their differentiation into effector subsets (S3 in MAIT development), but it is unknown what factors drive the polarization of MAIT cells (Godfrey et al., 2019).

In contrast to conventional T cells and iNKT cells, there are only two MAIT effector subsets: MAIT1 and MAIT17. These subsets produce IFN- γ and IL-17 in a T-BET and ROR γ t-dependent manner, respectively. It is unclear why MAIT cells do not produce IL-4. However, some have argued this could be related to the intensity of the TCR-MR1 interaction, which might not reach the threshold necessary of signaling strength to generate IL-4-producing MAIT2 cells (Legoux et al., 2019b). The argument is based on the observation that iNKT2 cells rely on the strongest TCR signal of all iNKT subsets during positive selection (Tuttle et al., 2018).

PLZF plays a key role in the maturation of effector subsets in both the MAIT and iNKT cell lineages, though the two cell types differ in the kinetics of their PLZF requirement. iNKT cells require PLZF during the early transitional stages of development (i.e. S1 and S2 of iNKT development, CD24⁻CD44^{+/+}NK1.1⁻) (Engel and Kronenberg, 2014), whereas MAIT cells require it at the effector stage (i.e. S3 of MAIT development, CD24⁻CD44⁺) (Koay et al., 2016). PLZF controls the expression of several transcription factors that mediate effector differentiation of MAIT and iNKT cells and that also suppresses genes of the naïve T cell program (Mao et al., 2016). In iNKT cells, TCR and SLAM costimulation promotes the expression of the transcription factor Egr2, which binds the *Zbtb16* promoter and, therefore, induces PLZF expression (Dutta et al., 2013; Tuttle et al., 2018). The signaling cascade that controls PLZF expression in MAIT cells, however, is not well understood (Legoux et al., 2019b).

The classical model of MAIT cell development, as described above, entails the full maturation of effector subsets in the thymus that then seed the peripheral organs according to their functional programs. But, similar to iNKT cells, a CCR7⁺ thymic precursors of MAIT cells that may undergo maturation and differentiation in the periphery was recently discovered (Wang and Hogquist, 2018). This finding has given rise to an alternative model of development in which mature MAIT cells are resident of the thymus and peripheral MAIT cells are derived from CCR7⁺ precursors that leave the thymus in an undifferentiated stage (Lantz and Legoux, 2019).

A couple of studies have reported the absence of MAIT cells in germ-free (GF) mice, but the underlying mechanisms for this phenomenon was unknown until recently (Koay et al., 2016; Treiner et al., 2003). Emerging studies have shown microbe-derived vitamin B2 metabolites are indispensable for the development of MAIT cells, and that these metabolites get directly transferred from mucosal sites like the gut into the thymus (Constantinides et al., 2019; Legoux et al., 2019a). These recent observations have highlighted a role for the microbiota in the development and establishment of T cells targeted to mucosal tissues. Furthermore, these studies have underscored the importance of extrinsic signals in driving the development of unconventional T cells.

Natural Intraepithelial Lymphocytes (nIELs)

Intraepithelial lymphocytes (IELs) are a heterogeneous population of T cells resident of the gut. They are embedded in the intestinal epithelial layer, where they play important functional roles that go from protective to regulatory in kind (Cheroutre et al., 2011; Sheridan and Lefrançois, 2010). How IELs carry out these functions is still debated. Two types of IELs exist: natural and induced. Natural IELs (nIELs) are the predominant type, which express CD8 $\alpha\alpha$ homodimers along with TCR $\gamma\delta$ or TCR $\alpha\beta$ (Stritesky et al., 2012); TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ nIELs are the most well studied kind. Induced IELs (iIELs), on the other hand, encompasses both TCR $\alpha\beta$ ⁺CD4⁺ and TCR $\alpha\beta$ ⁺CD8 $\alpha\beta$ ⁺ memory cells generated from naïve T cells during immune responses (Cheroutre et al., 2011).

Multiple pathways appear to exist for the development of nIELs, including both thymic precursors-derived and extrathymically produced (Stritesky et al., 2012). In the thymus, post-selection IEL precursors (IELps) can be found within the CD4⁻CD8⁻ DN thymocyte population as TCRβ⁺CD5⁺ cells (Gangadharan et al., 2006; Mayans et al., 2014; McDonald et al., 2014). A recent study revealed there are two major post-selection IELp populations in the thymus, a nascent programmed death-1-expressing (PD-1⁺) population and a T-BET⁺ population that accumulates with age (Ruscher et al., 2017). Though both IELp populations are capable of producing intestinal CD8αα⁺ IELs upon adoptive transfer, they differ in their phenotype and usage of TCR chains. PD-1⁺ IELps are enriched for self-reactive clones that are largely restricted by classical MHC molecules and that exit the thymus in a sphingosine-1-phosphate receptor 1 (S1PR1)-dependent manner. T-BET⁺ IELps, however, have no self-reactive T cells and include non-classical MHCI-restricted cells that express the receptors NK1.1 and CXCR3 along with the integrin CD103 (Ruscher et al., 2017).

mTOR Signaling Pathway

The mechanistic target of rapamycin (mTOR), formerly known as the mammalian target of rapamycin, is an evolutionarily conserved 289-kDa serine/threonine protein kinase involved in regulating cellular growth, survival, function, metabolism, and differentiation (Saxton and Sabatini, 2017). It was first discovered in the early 1990s through genetic screening of *Saccharomyces cerevisiae* for the identification of targets of rapamycin, a potent natural antifungal and immunosuppressor discovered in Rapa Nui in the early 1970s (Blenis, 2017). mTOR is now recognized as a central regulator of immune responses that senses and integrates numerous environmental signals, including nutrients and growth factors (Chi, 2012).

mTORC1 and mTORC2

mTOR exists in two highly conserved, large molecular complexes: mTORC1 and mTORC2. Though they both share the same catalytic subunit mTOR, they are distinguished by their scaffolding subunits. mTORC1 contains the regulatory associated protein of mTOR (RAPTOR) and is sensitive to rapamycin, whereas mTORC2 has the rapamycin-insensitive companion of mTOR (RICTOR) and is relatively insensitive to rapamycin under short-term treatment (Zeng and Chi, 2014).

RAPTOR/mTORC1. mTORC1 consists of the catalytic subunit mTOR, the core scaffolding components RAPTOR (Kog1) and mLST8, and the other associated components PRAS40 and Deptor (Powell et al., 2012). mTORC1 controls multiple important biological processes. The phosphoinositide 3-kinase (PI3K)-AKT pathway is central for the activation of mTORC1, which integrates signaling from both transcription factors and nutrients (Laplane and Sabatini, 2012). Upon activation, mTORC1 promotes protein synthesis by phosphorylating the translational initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase (S6K) (Hsieh et al., 2012; Thoreen et al., 2012).

mTORC1 is considered a master regulator of quiescence exit, as it induces the expression of transcription factors, such as c-MYC, hypoxia inducible factor 1 α (HIF-1 α), and sterol regulatory element-binding proteins (SREBPs), and alters anabolic and mitochondrial metabolism through transcriptional, translational, and post-translational mechanisms (Chapman et al., 2020). mTORC1 also controls lipid synthesis through SREBP1/2 transcription factors and promotes *de novo* lipid biogenesis through peroxisome proliferator-activated receptor γ (PPAR- γ)-dependent regulation (Laplane and Sabatini, 2012). mTORC1 is also involved in the regulation of cell cycling and survival by regulating cyclin D1 and c-MYC expression (Gera et al., 2004). The activation of the phosphatase and tensin homolog (PTEN) inhibits mTORC1 signaling by turning off PI3K signaling via dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Shimobayashi and Hall, 2014).

RICTOR/mTORC2. mTORC2 is comprised of mTOR, RICTOR, mLST8, and three other associated proteins, mSIN1, PROTOR, and DEPTOR. PI3K also induces mTORC2 activity, which in turn activates AKT by phosphorylating it on serine 473 (S473) (Shimobayashi and Hall, 2014). mTORC2 signaling influences cell survival, proliferation, growth, differentiation, and trafficking. Once activated, AKT phosphorylates the tuberous sclerosis complex (TSC) protein 2 (TSC2), which disables the TSC1-TSC2 complex that inhibits mTORC1. mTORC2 also contributes to quiescence exit, in part by repressing FoxO1 signaling through activation of AKT, which induces the expression of glucose transporter 1 (GLUT1), therefore, enhancing glycolytic flux (Chapman et al., 2020). Additionally, AKT promotes cell proliferation by phosphorylating the cell cycle regulatory protein p27 (Shin et al., 2002) and by indirectly inhibiting the tumor suppressor p53 (Vousden and Lane, 2007). Protein kinase C (PKC) and serum- and glucocorticoid-regulated kinase 1 (SGK1) are both downstream of mTORC2 and regulate cytoskeletal dynamics and ion transport, respectively (Chi, 2012; Zarogoulidis et al., 2014). As previously alluded, mTORC2 is insensitive to rapamycin in short-term treatment, but can still be blocked under prolonged or high dose of rapamycin treatment in a variety of cells, including CD4⁺ T cells, but not effector CD8⁺ T cells (Zeng and Chi, 2014).

Regulation of mTOR Activity in T Cells

mTOR integrates a plethora of signals that drive T cell development and function. Broadly, the activating signals of mTOR in T cells can be grouped into three categories: immune signals, environmental cues, and nutrients (Chi, 2012). Within the immune signals, antigen recognition by TCR induces mTOR activation via the activation of PI3K (Exley et al., 1994). The engagement of costimulatory receptors, such as CD28, ICOS, and OX-40, further amplifies mTOR activity (Waickman and Powell, 2012). Growth factors and immunomodulatory factors provide environmental stimuli for the activation of mTOR, which are typically accompanied by metabolic cues derived from nutrients (Chi, 2012). The GTP-loaded form of the small GTPase Ras homolog enriched in brain (RHEB) serves as a signal transducer of many of these upstream signals to mTORC1. RHEB is regulated by the GTPase-activating protein (GAP) of the TSC1-TSC2 complex.

The TSC complex integrates the respective positive and negative signals transduced from the PI3K-AKT and AMP-activated protein kinase (AMPK) pathways (Zoncu et al., 2011). When this complex is phosphorylated by AKT or ERK1/2, its GAP activity is inhibited, which allows RHEB to activate mTORC1 (Powell et al., 2012).

In comparison to mTORC1, the regulation of mTORC2 is largely understudied and less understood (Saxton and Sabatini, 2017). Growth factors are thought to be major players in the activation of mTORC2 and relatively recent studies have shown ribosomes link PI3K to mTORC2 activation (Luo et al., 2018; Oh et al., 2010; Zinzalla et al., 2011). PI3K activation by insulin or other growth factors phosphorylates PIP2 to PIP3, which binds mSin1-PH, releasing its inhibition on the mTOR kinase domain of mTORC2 (Luo et al., 2018).

mTOR in T Cell Development

mTOR has emerged as a central regulator of T cell function and fate that integrates immune signals and metabolic cues (Chi, 2012). Several studies have linked mTOR to hematopoiesis, thymopoiesis, T cell differentiation, and quiescence (Chapman et al., 2020; Chapman and Chi, 2014). Loss of mTORC1 via conditional deletion of RAPTOR in early thymocyte development induces developmental blockage of T lymphopoiesis as a consequence of reduced cell cycling and proliferation, along with increased apoptosis (Hoshii et al., 2014). Deletion of RAPTOR in later stages of T cell development does not have major developmental consequences, however (Delgoffe et al., 2009; Yang et al., 2013). Thus, mTORC1 differentially regulate T cell development.

Loss of mTORC2 via deletion of RICTOR has shown to be destructive of T cell development, leading to thymic atrophy (Chou et al., 2014; Hoshii et al., 2014; Lee et al., 2012). mTORC2-dependent regulation of T cell development differs from mTORC1, as the former is mediated by the stabilization, *de novo* synthesis, and/or post-transcriptional modification of proteins involved in thymic selection (Chou et al., 2014); these proteins include the coreceptors CD4 and CD8, pre-TCR, TCR, NOTCH, and CD147. Emerging studies have also unraveled critical functions of RICTOR in NOTCH-driven development of $\alpha\beta$ -T cells (Lee et al., 2012; Tang et al., 2012). Altogether, these studies have demonstrated discrete roles of mTORC1 and mTORC2 in thymocyte development.

mTOR and T Cell Metabolism

Metabolic remodeling of T cells is intrinsically linked to their development, activation, function, differentiation, and survival (Geltink et al., 2018). Throughout their lifespan, T cells engage on different metabolic programs to meet their bioenergetic demands. Glycolysis and oxidative phosphorylation (OXPHOS) are the two main drivers of T cell metabolism, which supply cells with the required ATP to carry out biological processes. During glycolysis, glucose gets broken down into pyruvate, in a process that is regulated by transcriptional, post-translational, and metabolic cues. This process has

relatively low ATP output, but it provides cells with important metabolic intermediates for many other metabolic pathways (Vander Heiden et al., 2009). In fact, T cells engage in aerobic glycolysis, that is the conversion of pyruvate into lactate in the presence of oxygen (also known as the Warburg effect), upon activation. This process is dependent on PI3K/AKT/mTOR signals (MacIver et al., 2013; Powell et al., 2012). A recent study further highlighted the interconnectivity of mTOR activity, glycolysis, and CD8⁺ T cell effector function as it showed mTORC1 inhibition concomitantly reduces glycolytic rate and effector functions, whereas deletion of mTORC2 enhances glycolysis and effector functions (Pollizzi et al., 2015). Thus, mTOR plays a crucial role in T cell function by controlling metabolic remodeling.

The generation of reactive oxygen species (ROS) in the mitochondria, or by NADPH oxidase expressed by CD4⁺ T cells, has been reported to be crucial in driving efficient and sustained aerobic glycolysis during CD4⁺ T cell activation (Jackson et al., 2004; Previte et al., 2017). Experiments done with the antioxidant glutathione have shown that lack of ROS accumulation inhibits T cell activation, in part by decreasing mTOR and c-Myc activity (Mak et al., 2017). Furthermore, mitochondrial ROS is critical for activation of nuclear factor of activated T cells (NFAT) and subsequent IL-2 production, both of which are drivers of T cell activation (Sena et al., 2013). Hence, ROS has emerged as an important metabolic signal for T cell activation and function.

PTEN Signaling Pathway

The phosphatase and tensin homolog gene, *Pten*, is a powerful tumor suppressor gene involved in a plethora of cellular processes, including energy metabolism (Garcia-Cao et al., 2012), cell motility (Tamura et al., 1998), genomic stability (Shen et al., 2007), and epigenome architecture (Chen et al., 2014). It was discovered in 1997 and characterized one year later as a lipid phosphatase that dephosphorylates PIP3 by removing the 3-phosphate of the inositol ring (Brandmaier et al., 2017; Maehama and Dixon, 1998), therefore, antagonizing the catalytic activity of PI3K. This antagonistic function of PTEN towards the PI3K/AKT signaling pathway contributes substantially to its polyfunctionality in cancer development, metabolism, and immunity. Nonetheless, PTEN also has lipid phosphatase-independent functions, which are observed upon its localization to the nucleus (Wang et al., 2015).

PTEN is one of the most commonly mutated tumor suppressors in human malignancies (Wang et al., 2015). Complete loss of PTEN is associated with advanced cancer and poor prognosis. Germline mutations of *Pten* are associated with a group of autosomal dominant syndromes known as PTEN hamartoma tumor syndromes (PHTS), which include Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, PTEN-related Proteus syndrome, and Proteus-like syndrome (Hobert and Eng, 2009). These syndromes are characterized by developmental disorders, neurological deficits, and an increased lifetime risk of cancer.

Regulation of PTEN

PTEN is constitutively expressed in normal tissues. Multiple molecular mechanisms control its expression and activity, which can generate a continuum of functional PTEN levels (Song et al., 2012).

Transcriptional Regulation. At the transcriptional level, EGR, PPAR γ , and p53 are the main positive regulators of *Pten* gene expression (Wang et al., 2015). These transcriptional regulators have been shown to directly bind to the *Pten* promoter region (Patel et al., 2001; Stambolic et al., 2001; Virolle et al., 2001). Conversely, mitogen-activated protein kinase kinase-4 (MAPKK4), transforming growth factor beta (TGF- β), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF- κ B), insulin-like growth factor 1 (IGF-1), the transcriptional cofactor c-Jun protooncogene, and the B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) proto-oncogene are negative regulators of *Pten* gene expression (Wang et al., 2015). Additionally, there is evidence PTEN engages in complex crosstalk with other pathways, which suggest some of the pathways controlled by PTEN can in turn regulate *Pten* transcription in a feedback loop (Wang et al., 2015).

Post-Transcriptional Regulation. Several miRNAs have been identified as promoters of tumorigenesis or metabolic disorders by downregulating PTEN expression. These include miR-17~19 cluster in lymphoproliferative disease and autoimmunity, miR-19 in leukemia and Cowden disease, miR-21 in multiple cancers, inflammation and metabolic diseases, among others (Song et al., 2012).

Post-Translational Regulation. Multiple post-translational modifications, including active site phosphorylation, ubiquitination, oxidation, and acetylation, can regulate PTEN activity too (Wang et al., 2015). In particular, phosphorylation of PTEN in its C-terminal tail determines the conformational state of PTEN and, therefore, its activity status. Additional phosphorylation of PTEN in other sites can either increase or decrease protein stability.

Protein-Protein Interactions. PTEN interacts with several proteins that influence its protein levels, conformational status, stability, and localization (Song et al., 2012). Additionally, PTEN has a 3-amino acid C-terminal region capable of binding PDZ domain-containing proteins, which may control localization of PTEN and its interaction with other proteins (Georgescu et al., 1999; Wu et al., 2000).

The PTEN/PI3K/AKT/mTOR Pathway

PTEN is the only known lipid phosphatase to antagonize PI3K activity, which is largely what makes it such an important tumor suppressor. Loss or inactivation of PTEN causes the excessive accumulation of PIP3 at the plasma membrane, which recruits and activates a subset of proteins that contain pleckstrins homology domains (PH domains) to cell membranes, such as the AKT family of proteins and phosphoinositide-dependent

kinase-1 (PDK1) (Song et al., 2012). AKT gets activated by phosphorylation at Threonine 308 (T308) and S473 by PDK1 and mTORC2, respectively (Manning and Cantley, 2007; Zoncu et al., 2011). Active AKT promotes cell survival, cell proliferation, angiogenesis, and cellular metabolism by phosphorylating downstream signaling proteins, including glycogen synthase kinase 3 (GSK3), FoxO, B cell lymphoma 2 (Bcl2) antagonist of cell death (BAD), the E3 ubiquitin protein ligase MDM2 and p27 (Manning and Cantley, 2007). AKT can also activate mTORC1 by phosphorylating TSC2, hence deactivating the TSC complex, which inhibits the activator of mTORC1 RHEB (Guertin and Sabatini, 2007). Additionally, AKT induces the inhibitory phosphorylation of the negative regulator of mTORC1 PRAS40 (Vander Haar et al., 2007; Zoncu et al., 2011).

PTEN in T Cell Development

PTEN plays fundamental roles in T cell biology. From development to function, PTEN is a key player in T cell-mediated immune responses. Deletion of PTEN in the T cell compartment causes fatal defects in central and peripheral tolerance (Suzuki et al., 2001). Mice harboring PTEN-deficient T cells suffer from mature T cell lymphomas that arise in the thymus (Hagenbeek and Spits, 2008). Although PTEN is lost early in these mice, thymocyte premalignancy does not start until mice are 6 weeks old, it then becomes stronger by 9 weeks of age, followed by metastasis after 10 weeks of age, and death by 15–20 weeks of age (Liu et al., 2010b; Xue et al., 2008). Interestingly, the development of lymphomas in the context of PTEN deficiency is mediated by developing thymocytes only, given that deletion of PTEN in mature T cells does not cause malignant transformation, though it still leads to severe multiorgan autoimmunity (Liu et al., 2010b). It has been shown that absence of PTEN allows TCR $\alpha\beta^+$ lineage thymocytes to bypass IL-7 and pre-TCR-mediated signaling, thus resulting in the survival and expansion of thymocytes that would have otherwise been deleted during β -selection (Hagenbeek et al., 2004). Heterozygous inactivation of *Pten* does not cause tumorigenesis, but still triggers lethal autoimmune disorders that are mediated by an impaired Fas response to activated lymphocytes, including self-reactive cells in the periphery (Di Cristofano et al., 1999). Additionally, it has been proposed that PTEN sets a threshold for activation that enforces the need for T cell costimulation (Buckler et al., 2006). Absence of PTEN, therefore, allows T cells to acquire effector function and produce proinflammatory cytokines in response to TCR signals alone. Altogether, these studies show PTEN plays a crucial role in the maintenance of self-tolerance and has important roles in T cell development.

Summary and Aims of Dissertation

The conserved nature of unconventional T cells highlights the importance of these unique groups of T cells for the immune system. More and more studies are revealing important functions for these cells in tissue homeostasis and diseases. However, the molecular mechanisms underpinning the fate decisions of progenitor cells towards the development of unconventional T cell lineages remain elusive. The engagement of

discrete metabolic pathways has been demonstrated to be a determining factor in T cell mediated-immune responses and a key player of T cell biology. Nonetheless, the study of metabolic signaling in T cell fate choices and, in particular, in the development of unconventional T cells has remained largely unexplored. Thus, this study was pursued with the aim of unraveling molecular mechanisms driving the development of unconventional T cells. In particular, the role of RAPTOR-dependent mTORC1 signaling in T cell fate choices and PTEN-mediated control of unconventional T cell development were explored and characterized. This work expands the field's understanding of T cell development and establishes metabolic signaling as a key mediator of T cell fate choices.

CHAPTER 2. METHODS AND MATERIALS¹

Mice

C57BL/6, *Rptor*^{flox}, *CD45.1*⁺, *Erk2*^{flox}, *Cd2-Cre*, *Rag1*^{-/-}, *Pten*^{flox}, *Il17*^{icre}, *Pten*^{+/-}, *ROSA26*^{GFP} (harboring a loxP-site-flanked STOP cassette followed by a GFP-encoding sequence inserted into the *ROSA26* locus), and *Stat3*^{flox} mice were purchased from The Jackson Laboratory. *Cd4-Cre*, *Rictor*^{flox}, *Il23*⁻, and *Foxo1*^{flox} mice have been described previously (Cua et al., 2003; Liu et al., 2010a; Paik, 2006; Shiota et al., 2006; Yang et al., 2011). Littermates (*Cre*⁻ flox/flox and *Cre*⁺ non-floxed mice) were used as WT controls. For experiments reported in **Chapter 3**, 6–10-week old mice were used unless otherwise noted, and for experiments reported in **Chapter 4**, 3–6-week old mice were used unless otherwise noted. All mice were kept in specific pathogen-free conditions in the Animal Resource Center of St. Jude Children’s Research Hospital (SJCRH). Animal protocols were approved by the Institutional Animal Care and Use Committee of SJCRH.

Bone Marrow Chimera

Bone marrow (BM) chimeras were generated by transferring a consistent number of T cell-depleted bone marrow cells from donor mice into sublethally irradiated (550 rads) *Rag1*^{-/-} recipient mice. At least 1.8×10^6 donor cells were transferred into recipient mice for the generation of full BM chimeras. For mixed BM chimeras, spike (congenitally marked *CD45.1*⁺) and donor cells (congenitally marked *CD45.2*⁺) were mixed in a 1:1 ratio for a total of at least 5×10^6 total cells, which were then transferred into recipient mice. BM cells were extracted from both posterior and anterior legs of mice by flushing the bones with Hanks’ Balanced Salt solution (HBSS). Extracted cells were then depleted of T cells by magnetic bead-based purification, using a mixture of CD4 (L3T4), CD8a (Ly-2), and CD90.2 (Thy1.2) beads in a 1:1:1 ratio. Flow-through of the magnetic columns was then normalized for equal cell numbers across all donor samples and retro-orbitally injected into recipient mice in 100 μ L of Phosphate-buffered saline (PBS). Baytril water was used as an antibiotic treatment for BM chimeras for three weeks after irradiation. Proper immune system reconstitution was verified by retro-orbital bleeding 4–6 weeks after the generation of the chimeras. Mice were analyzed 4–10 weeks after reconstitution.

¹ Portions of chapter from previously published article; final submission modified with permission of AAAS. Yang, K.*, Bastardo Blanco, D.*, Chen, X.*, Dash, P., Neale, G., Rosencrance, C., Easton, J., Chen, W., Cheng, C., Dhungana, Y., KC, A., Awad, W., Guo, X., Thomas, P., Chi, H. (2018). Metabolic signal directs the reciprocal lineage decisions of ab and gd T cells. *Science Immunology* **3**. (* Co-first authors). DOI: <https://doi.org/10.1126/sciimmunol.aas9818>.

Polymerase Chain Reaction (PCR)

PCR was used to genotype mice using primers (Invitrogen) listed on **Table 2-1**. A master mix of nuclease-free water, 1× Tsg enzyme buffer, 25 mM MgCL₂, 10 mM dNTPs (all from Lambda Biotech), allele-specific primers and Tsg enzyme was prepared. Genomic DNA purified from mouse toes, tail, or ear clips was then added to each reaction; each set of PCR reactions also had positive and negative controls. Programs used to run the reactions in thermal cyclers are listed on **Table 2-2**. To visualize the PCR products, samples were mixed with 6× DNA loading dye (Lambda Biotech) and run in 1.5% agarose gel via electrophoresis. Bands were visualized with GelRed (biotium) on a Gel Doc 2000 or a ChemiDoc Touch Imaging System (Biorad). Samples were scored according to the band size listed on **Table 2-1**.

Single Cell Suspension

Mice were sacrificed by CO₂ asphyxiation and organs (thymus, spleen, liver, lung, Peyer's patches, mesenteric and peripheral lymph nodes, which included inguinal, auxiliary, and cervical lymph nodes pooled together) were harvested. Throughout all procedures, organs were kept on ice. Unless otherwise noted, all spins were done at 2,000 RPM for 5 min at 4°C.

Thymus, Spleen, Lymph Nodes, and Peyer's Patches

Organs were placed under 70 µm nylon mesh and gently grinded with the flat end of a 3 mL syringe in 10 cm petri dishes containing 1 mL of wash buffer (HBSS + 2% Fetal Bovine Serum (FBS)). The mesh was then washed with wash buffer, and cells were collected in a 15 mL conical tube. Cells isolated from the thymus, lymph nodes, and Peyer's patches were used immediately for downstream assays, whereas splenocytes were centrifuged and resuspended in 1 mL of Ammonium-Chloride-Potassium (ACK) Lysing Buffer (Gibco) to lyse red blood cells. After 1–2 minutes at RT, the splenocytes were washed with twice as much volume of ACK lysis buffer, centrifuged, and resuspended in an appropriate volume of wash buffer for downstream assays.

Lung

Lungs were first padded on paper towels to dry up and then cut and minced with a razor blade in a petri dish. Minced tissues were then transferred into a 6-well plate containing 2–5 mL of digestion buffer (Roswell Park Memorial Institute (RPMI) buffer + 2% FBS + 1 mg/ml Collagenase IV + 0.05% DNase) and incubated at 37°C + 5% CO₂ for 45 min. After digestion, samples were harvested and filtered using a 70 µm cell strainer placed on top of a 50 mL conical tube to collect the flow-through; tissues were smashed against the filter using the bottom of a syringe. Samples were then washed with copious amounts of wash buffer, spun down, and resuspended in an appropriate volume

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

Primer Name	Forward Primer	Reverse Primer	Product Length (bp)
iCre	AGATGCCAGGAC ATCAGGAACCTG	ATCAGCCACACC AGACACAGAGATC	220
CreJAX	GCGGTCTGGCAG TAAAAACTATC	GTGAAACAGCAT TGCTGTCACTT	100
Internal control	TGAGCTGCCCCA AGAGAATAG	GTACACTGTCTG CATGGGACCA	120-130
CD4-Cre	TCTCTGTGGCTG GCAGTTTCTCCA	TCAAGGCCAGAC TAGGCTGCCTAT	~300
Raptor	CTCAGTAGTGGT ATGTGCTCA	GGGTACAGTATG TCAGCACAG	170 (loxP) 150 (wt)
ERK2	CAGAGCCAACA ATCCCAAAC	GGCTGCAACCAT CTCACA	350 (mut) 278 (wt)
Rictor	ACTGAATATGT TCATGGTTGTG	GAAGTTATTCAG ATGGCCCAGC	554 (loxP) 466 (wt)
Pten	CAAGCACTCTG CGAACTGAG	GAGTTAAGTTTT TGAAGGCAAGATGC	328 (loxP) 156 (wt)
Stat3	CCTGAAGACCA AGTTCATCTGT	CACACAAGCCAT CAAACCTCTGGTC	Upper band (loxP)
IL23a	GTGAC CTTCCAACCCT CCAGATCC	TCC TGCAGATCACA GAGCCAGC; GCAGCGCATCG CCTTCTATC	238 (wt) 307 (wt) 250 (mut)
FoxO1	GCTTAGAGCAG AGATGTTCTCA CATT	CCAGAGTCTTT GTATCAGGCAA ATAA; CAAGTCCATTA ATTCAGCACATTG	190 (KO) 149 (loxP) 115 (wt)

Table 2-2. PCR programs used to amplify genes of interest.

CreJAX	iCre, CD4-Cre, Raptor, ERK2, PTEN, Stat3, IL23a, and FoxO1	Rictor
1) 94°C for 2 min	1) 94°C for 2 min	1) 94°C for 2 min
2) 94°C for 30 sec	2) 84°C for 45 sec	2) 94°C for 30 sec
3) 60°C for 30 sec	3) 65°C for 45 sec	3) 60°C for 30 sec
4) 72°C for 1 min	-0.3°C per cycle	4) 72°C for 1 min
5) Go to 2, 31 times	4) 72°C for 5 min	5) Go to 2, 39 times
6) 72°C for 5 min	5) Go to 2, 36 times	6) 72°C for 5 min
7) 95°C for 5 min	6) 72°C for 5 min	7) 15°C for 5 min
8) End	7) 15°C for 5 min	8) End
	8) End	

of wash buffer for downstream assays. If too many red blood cells were visible, then samples were quickly ACK-lysed for no more than 1 min.

Liver

Livers were placed in 6-well plates and smashed using the flat bottom of a syringe. Homogenized tissues were then digested with 5 mL of digestion buffer (RPMI + 2% FBS + 1 mg/ml Collagenase IV) for 1 h in a 37°C + 5% CO₂ incubator. After digestion, samples were harvested and transferred into 50 mL conical tubes containing 20 mL wash buffer. Tubes were vigorously mixed to loosen cells and then spun down at 100× g for 1 min. The supernatant was passed through a 70 µm cell strainer and spun down at 600× g for 5 min. The cell pellet was resuspended in 3 mL wash buffer, and 2 mL of lymphocyte separation medium (LymphoSep, MP Biomedicals) was underlaid. The tubes were then centrifuged at 2,500 RPM for 20 min at RT with slow acceleration and brake (setting 3). Middle lymphocyte layer was then carefully extracted, washed with copious amounts of wash buffer, spun down, and resuspended in an appropriate volume of wash buffer for downstream assays.

Flow Cytometry

Flow cytometry data were acquired on LSRII or LSRFortessa (Becton Dickinson) machines and analyzed using FlowJo software (Tree Star). Unless otherwise noted, 7-Aminoactinomycin D (7AAD) or Fixable Viability Dye (FVD, eBioscience) were used to exclude dying cells from flow cytometric analyses of freshly isolated or fixed cells, respectively.

Surface Staining

For analysis of surface markers, cells were stained in FACS buffer (PBS containing 2% (wt/vol) bovine serum albumin (BSA)) for 20 min at RT or for 1 h when co-stained with tetramers. The antibodies used for flow cytometric analysis are listed on **Table 2-3**. The following tetramers were used for the identification of iNKT and MAIT cells, respectively: mouse CD1d loaded with PBS-57 and mouse MR1 loaded with 5-OP-RU; both tetramers were provided by the National Institute of Health (NIH) Tetramer Core Facility.

Intracellular Staining

For intracellular marker staining, single cell suspensions were first stained for surface markers as described above, then washed with ice-cold FACS buffer and fixed using fixation/permeabilization buffer (eBioscience) for at least 1 h at 4°C. After fixation was completed, cells were washed with 1× permeabilization buffer (eBioscience) and

Table 2-3. List of antibodies used for flow cytometric analysis.

Antibody	Clone	Manufacturer
Akt (pS473)	M89-61 (RUO)	BD Biosciences
BrdU	BU20A	eBioscience
Caspase-3, Active	C92-605	BD Biosciences
CCR6 (CD196)	R6H1	eBioscience
CCR7	4B12	eBioscience
CCR9	CW-1.2	eBioscience
CD4	RM4-5	eBioscience or Tonbo Biosciences
CD5	53-7.3	eBioscience
CD8 α	53-6.7	eBioscience or Tonbo Biosciences
CD24	M1/69	eBioscience
CD25	PC61.5	eBioscience
CD44	1M7	eBioscience or Tonbo Biosciences
CD45.1	A20	eBioscience
CD45.2	104	eBioscience
CD62L	MEL-14	eBioscience
CD69	H1.2F3	eBioscience
CD71	R17217	eBioscience
CD73	eBioTY/11.8	eBioscience
CD98	RL388	eBioscience
CD103	2E7	eBioscience
CD127	A7R34	eBioscience
FoxP3	FJK-16s	eBioscience
ID3	C-20	Santa Cruz Biotechnology
ID3	D16D10	Cell Signaling Technology
IFN- γ	FJK-16s	eBioscience
IL-4	11B11	eBioscience
IL-17A	17B7	eBioscience
IL-17F	9D3.1C8	Biolegend
Ki67	SolA15	eBioscience
ROR gamma (t)	B2D	eBioscience
p-FoxO1 (Thr24)/ FoxO3a (Thr32)	9464	Cell Signaling Technology
p-STAT3	4/p-STAT3	BD Biosciences
p-S6	D57.2.2E	Cell Signaling Technology
p-4E-BP1	236B4	Cell Signaling Technology
PTEN	A2B1	BD Biosciences
S1P1	713412	R&D Systems
T-BET	4B10	BD Biosciences
TCR β	H57-597	eBioscience or Tonbo Biosciences
$\gamma\delta$ TCR	eBioGL3	eBioscience

then stained with intracellular markers for 20 min at RT.

Intracellular Cytokine Staining/ICS Stimulation. T cell were stimulated for 4 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 1 µg/ml ionomycin in the presence of 1× monensin (GolgiSTOP, BD Bioscience) in 24- or 48-well plates, depending on cell numbers, at 37°C + 5% CO₂ (referred to as ICS stimulation). Cells were harvested, stained, and processed for flow cytometric analysis as described above.

Phospho-Antibody Staining. Cells were incubated in pre-warmed 1× Phosflow lyse/fix buffer (BD Biosciences) at 37°C for 10 min. Cells were then pelleted by centrifugation and washed twice with FACS buffer. After washing, the cells were permeabilized by adding 1 mL of ice-cold Phosflow Perm Buffer III (BD Biosciences), and incubated on ice for 30 min. The cells were washed twice with FACS buffer, stained with phospho-antibodies listed on **Table 2-3** for 1 h at RT, and then processed for flow cytometric analysis.

BrdU Incorporation Assay

Mice were injected intraperitoneally (i.p.) with 1 mg of 5-Bromo-2'-deoxyuridine (BrdU) stock 3 or 16 h before analysis and analyzed according to manufacturer's instructions (BD Biosciences). In short, cells were stained with antibodies specific for cell-surface markers for 30 min at RT, washed and resuspended in Cytofix/Cytoperm buffer for 15 min 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 µg/ml of DNase I and incubated for 1 h at 37°C. After washing with Perm/Wash buffer again, cells were stained with anti-BrdU for 20 min and processed for flow cytometric analysis.

Active Caspase-3 Staining

Cells obtained from single cell suspensions were first stained for cell surface markers as described above and then fixed and stained following manufacturer's instructions (BD Biosciences). In short, cells were fixed using BD Cytofix/Cytoperm buffer on ice for at least 1 h. Cell were then washed using BD Perm/Wash buffer, stained with active Caspase-3 antibody at RT for 30 min, and then processed for flow cytometric analysis.

Mitochondrial Staining

For measurement of ROS, mitochondrial mass, or mitochondrial potential, cells were incubated in full Click's medium containing 10 µM CM-H2DCFDA (5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; Life Technologies),

10 nM MitoTracker Deep Red (Life Technologies), or 20 nM TMRM (tetramethyl rhodamine, methyl ester; ImmunoChemistry Technologies), respectively, for 30 min at 37°C before staining surface markers as described above.

Cell Purification and Cultures

Lineage-negative WT and *Cd4^{cre}Pten^{fl/fl}* DN3 or DN3a cells were sorted on MoFlow (Beckman-Coulter) or Reflection (i-Cyt) sorters. Cell surface markers used for the identification and/or sorting of individual cell populations are listed on **Table 2-4**. DN3 cells were used for Seahorse assay and *in vitro* cultures with OP9-DL1 cells in the presence of IL-7 (0.5 ng/ml) and Flt3L (5 ng/ml) for 3–8 days. For metabolic perturbation, DN3a cells were co-cultured with OP9-DL1 cells in the presence of DCA (10 mM), galactose (0.5 mM) and galactose oxidase (GAO, 0.3 U/ml), GSH (5 mM), or NAC (20 mM) for 3–5 days. For T cell differentiation under a stromal cell-free culture condition, sorted DN3a cells were culture in DLL4 (2 µg/ml)-coated plates, in the presence of CXCL12 (100 ng/ml) or IL-7 (1 ng/ml) to promote αβ or γδ-T cell development, respectively, with or without the indicated inhibitors for 4 days.

Cell Counting

The automated Nexcelom Bioscience Cellometer Auto T4 and Auto 2000 were used to count cell numbers. Alternatively, cell suspensions were mixed with trypan blue and manually counted using a hemocytometer chamber.

$$\text{Cells per mL} = \text{Average count per square} \times \text{the dilution factor} \times 10^4$$

Quantitative Real Time PCR (qRT-PCR)

RNA was extracted from cells using the RNeasy micro kit (Qiagen). cDNA was synthesized using 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.

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).

Table 2-4. Cell surface markers used for the identification and/or sorting of populations of interest.

Cell Population	Cell Surface Markers
DP, pre-selection	TCR β ⁻ CD69 ⁻ CD4 ⁺ CD8 ⁺
DP, post-selection	TCR β ⁺ CD69 ⁺ CD4 ⁺ CD8 ⁺
DN3	CD4 ⁻ CD8 ⁻ $\gamma\delta$ TCR ⁻ TCR β ⁻ CD25 ^{hi} CD44 ⁻
DN3a	CD4 ⁻ CD8 ⁻ $\gamma\delta$ TCR ⁻ TCR β ⁻ CD25 ^{hi} CD44 ⁻ CD27 ⁻
DN3b	CD4 ⁻ CD8 ⁻ $\gamma\delta$ TCR ⁻ TCR β ⁻ CD25 ^{hi} CD44 ⁻ CD27 ⁺
DN4	CD4 ⁻ CD8 ⁻ $\gamma\delta$ TCR ⁻ TCR β ⁻ CD25 ⁻ CD44 ⁻
DN17	CCR6 ⁺ TCR β ⁺ CD127 ⁺ CD1d:PBS57-tet ⁻ MR1:5-OP-RU-tet ⁻
iNKT17	CCR6 ⁺ TCR β ⁺ CD127 ⁺ CD1d:PBS57- tet ⁺ MR1:5-OP-RU-tet ⁻
ISP	CD4 ⁻ CD8 ⁺ TCR β ⁻
MAIT17	CCR6 ⁺ TCR β ⁺ CD127 ⁺ CD1d:PBS57-tet ⁻ MR1:5-OP-RU-tet ⁺

Western Blotting

A lysing cocktail containing RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) plus cOmplete Mini, EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche) was used to obtain whole-cell lysates. Lysates were then resolved on SDS-PAGE gels and transferred into polyvinylidene difluoride membranes (Bio-Rad). Membranes were then blocked with 5% non-fat milk (Bio-Rad) in Tris-buffered saline (TBS)-T (1× TBS from Bio-Rad containing 0.1% Tween-20) for at least 1 h at RT shaking. After blocking, membranes were incubated overnight at 4°C with indicated antibodies diluted in 5% blocking buffer. Membranes were then washed three times with 0.1% TBS-T for 5 min at RT shaking, followed by staining of the appropriate Horseradish peroxidase (HRP)-conjugated secondary antibody in 5% blocking buffer for at least 40 min at RT shaking. After washing the membranes three times as described above, the bound antibodies were visualized using SuperSignal West Dura Extended Duration or SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific). The images were obtained using a LI-COR Biosciences Odyssey FC imaging system. The following primary antibodies were used: EGR1 (44D5), p-ERK (9101), ID3 (D16D10, all from Cell Signaling Technology), and β -ACTIN (AC-15 from Sigma). All primary antibodies were used at a 1:1,000 dilution, with the exception of β -ACTIN that was used at 1:20,000 dilution. Secondary antibodies were used at a 1:5,000 dilution.

Metabolic Assays

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in XF media (non-buffered Dulbecco's Modified Eagle Medium (DMEM) containing 5 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate), under basal conditions or in response to 1 μ M oligomycin (Oligo), 2 μ M fluoro-carbonyl cyanide phenylhydrazone (FCCP), or 1 μ M rotenone (Rot) using the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience), as previously described (Yang et al., 2017).

Microarray Analysis

DNase-treated RNA samples from sort-purified thymocytes were analyzed with the Affymetrix Mouse Gene 2.0 ST GeneChip array. Gene set enrichment analysis (GSEA) within hallmark gene sets (MSigDB) or canonical pathways (MSigDB c2.CP gene sets for canonical pathways) was performed as described (Subramanian et al., 2005). Differentially expressed transcripts were identified by ANOVA (Partek Genomics Suit v6.5) and the Benjamin-Hochberg method was used to estimate the false discovery rate (FDR) as previously described (Wang et al., 2013a). Lists of differentially expressed genes by $> 0.5 \log_2$ difference were analyzed using Ingenuity Pathway Analysis (IPA, Qiagen, Inc.) for the identification of canonical pathways and upstream transcriptional regulators.

Statistical Analysis of Biological Experiments

P values were calculated by two-tailed Mann-Whitney test, two-tailed unpaired Student's *t*-test, or ANOVA using GraphPad Prism software, unless otherwise noted. *P* value of < 0.05 was considered significant. All error bars represent mean and standard error of the mean (SEM).

CHAPTER 3. ROLE OF RAPTOR/mTORC1 SIGNALING IN LINEAGE DECISIONS OF ALPHA BETA AND GAMMA DELTA T CELLS²

T cells develop in the thymus, where BM-derived precursors undergo a series of well-characterized developmental processes that promote differentiation into the distinct $\alpha\beta$ and $\gamma\delta$ -T cell lineages (Yui and Rothenberg, 2014). The divergence of the two T cell lineages occurs at the DN3 stage of thymocyte development (Ciofani and Zuniga-Pflucker, 2010). Recent studies suggest signaling strength downstream of the TCR, mainly mediated by the ERK/EGR1/ID3 signaling axis, plays a key role in determining lineage choices of thymic progenitors (Hayes et al., 2005; Lee et al., 2014; Robey, 2005). However, the mechanisms linking extracellular stimuli to intrinsic TCR signaling strength remain elusive.

The kinase mTOR integrates immune signals and metabolic cues to orchestrate the function and fate of T lymphocytes (Chi, 2012). Recent studies have highlighted the involvement of mTOR signaling in thymocyte development (Lee et al., 2012; Tang et al., 2012), but whether mTOR signaling facilitates $\alpha\beta$ and $\gamma\delta$ -T cell lineage choices remains unknown. Using genetic mouse models, here we explore the role of RAPTOR-dependent mTORC1 signaling in T cell lineage choices.

Cell Metabolism and mTORC1 Activity Regulation in Thymocyte Development

To understand the metabolic requirements and regulation of developing thymocytes, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of thymic subsets were measured. OCR and ECAR act as surrogates for the measurement of cellular oxidative phosphorylation (OXPHOS) and glycolysis, respectively. In particular, thymic subsets in the late double-negative stages (DN3–DN4 stages), along with immature single-positive (ISP) and double-positive (DP) thymocytes, were evaluated at baseline and upon treatment with mitochondrial inhibitors (**Figure 3-1**). DN3 and ISP cells, which represent the first thymic stage with full T cell commitment (Carpenter and Bosselut, 2010) and the intermediate transitional stage between the DN and DP stages, respectively, showed the highest OCR at basal level (**Figure 3-1A**). Furthermore, DN3 cells displayed the greatest spare respiratory capacity (SRC) of the subsets, which is indicative of how effectively the electron transport chain can respond to energy demands (**Figure 3-1B**). As indicated by the ECAR, the glycolytic activity of thymic subsets was progressively upregulated in the transition from DN3 to ISP cells but reduced in the quiescent DP cells (**Figure 3-1C**). These data indicate developing

² Portions of chapter from previously published article; final submission modified with permission of AAAS. Yang, K.*, Bastardo Blanco, D.*, Chen, X.*, Dash, P., Neale, G., Rosencrance, C., Easton, J., Chen, W., Cheng, C., Dhungana, Y., KC, A., Awad, W., Guo, X., Thomas, P., Chi, H. (2018). Metabolic signal directs the reciprocal lineage decisions of ab and gd T cells. *Science Immunology* **3**. (* Co-first authors). DOI: <https://doi.org/10.1126/sciimmunol.aas9818>.

Figure 3-1. Developing thymocytes regulate cellular metabolism dynamically. (A) Oxygen consumption rate (OCR) in thymocyte subsets (DN3, double-negative 3 cells; DN4, double-negative 4 cells; ISP, immature single-positive cells; DP, double-positive cells) at baseline. (B) OCR in thymocyte subsets in response to oligomycin (Oligo), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone (Rot). Spare respiratory capacity (SRC) of DN3 cells is indicated. (C) Measurement of extracellular acidification rate (ECAR) in freshly isolated thymocyte subsets. Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$. Two-tailed unpaired t -test. Data are representative of at least ten independent experiments.

thymocytes undergo dynamic regulation of their metabolic activity.

Nutrient uptake and mTOR activation are closely linked to metabolic activity in T cells (Chi, 2012). Similar to the ECAR pattern observed, DN4 and ISP cells had the highest surface expression of CD98 and CD71, the corresponding amino acid and iron transporters (**Figure 3-2A**). Accordingly, these same thymic subsets had the highest phosphorylation of S6, a target of mTORC1 (**Figure 3-2B**), suggesting mTORC1 involvement in metabolic regulation of developing thymocytes.

Requirement of RAPTOR in Thymocyte Development

Generation of RAPTOR-Deficient Thymocyte Mice

To evaluate the function of mTORC1 signaling in thymocyte development, we crossed *Cd2*-Cre transgenic mice (de Boer et al., 2003) with mice carrying loxP-flanked *Rptor* alleles (*Rptor*^{fl/fl}). This genetic mouse strategy allowed us to delete RAPTOR, an obligatory component of mTORC1, in early DN cells (mice referred to as *Cd2*^{cre}*Rptor*^{fl/fl}). Western blotting confirmed deletion of RAPTOR in DN3 cells and subsequent thymocyte subgroups (**Figure 3-3A**).

Phenotypic Characterization of T Cell Development in *Cd2*^{cre}*Rptor*^{fl/fl} Mice

RAPTOR Deficiency Impairs the DN-to-DP Transition of Developing $\alpha\beta$ -T Cells. Our genetic system showed RAPTOR was required for proper T cell development. RAPTOR deficiency resulted in greatly reduced thymocyte cellularity driven by a nearly 10-fold reduction in DP cells and considerable reduction in CD4SP and CD8SP cells (**Figure 3-3B**). Correspondingly, RAPTOR deficiency caused an accumulation of DN cells, hinting at a potential blockade at the DN-to-DP transition.

The increased frequency and number of DN cells in the *Cd2*^{cre}*Rptor*^{fl/fl} mice prompted us to do detailed analysis of the thymic subsets making up the DN-to-DP transition (**Figure 1-2**). Flow cytometric analysis showed that the increased number of DN cells in RAPTOR-deficient thymocytes was driven by accumulation of DN3 cells and DN4 cells (**Figure 3-4A**). Furthermore, and in support of a role for RAPTOR in the DN-to-DP transition, frequency and number of ISP cells, the transitional stage between DN4 and DP cells, was significantly elevated too (**Figure 3-4B**).

To confirm a cell-intrinsic role for RAPTOR in the DN-to-DP transition of T cell development, we generated mixed BM chimeras consisting of congenitally marked CD45.1⁺ spike cells and CD45.2⁺ donor cells from either WT or *Cd2*^{cre}*Rptor*^{fl/fl} mice in a 1:1 ratio. T cell development in the spike and WT donor compartments proceeded normally, whereas the RAPTOR-deficient T cells had increased DN cells and ISP cells but reduced DP cells, similar to *Cd2*^{cre}*Rptor*^{fl/fl} mice (**Figure 3-4C, D**).

Figure 3-2. Nutrient transporters and mTORC1 activity are dynamically regulated in developing thymocytes.

(A) Relative surface expression of CD98 and CD71, the respective amino acid and iron transporters, on thymocyte subsets, as measured by mean fluorescence intensity (MFI) compared to the expression of DN3 cells (i.e. MFI in DN3 cells is normalized to 1). (B) Relative phosphorylation of S6, a target of mTORC1, in thymocyte subsets as measured by MFI compared to the expression of DN3 cells.

Figure 3-3. *Cd2-Cre*-mediated deletion of RAPTOR impairs $\alpha\beta$ -T cell development.

(A) Western blotting of RAPTOR in DN3 and DN4 thymocytes of WT and *Cd2^{cre}Raptor^{fl/fl}* mice. (B) Flow cytometric analysis of thymocyte subsets in WT and *Cd2^{cre}Raptor^{fl/fl}* mice along with the cellularity of total thymocytes and of each major subset. Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$. Two-tailed unpaired *t*-test. Data are representative of at least ten independent experiments. Numbers indicate percentage of cells in gates.

Figure 3-4. RAPTOR deficiency blocks the DN-to-DP transition of $\alpha\beta$ -T cell development.

(A) Flow cytometric analysis of DN1–4 subsets (gated on lineage-negative DN thymocytes) in WT and *Cd2^{cre}Rptor^{fl/fl}* mice with cellularity on the right. (B) Flow cytometric analysis (upper panels) and frequency and cellularity (lower panels) of ISP cells in WT and *Cd2^{cre}Rptor^{fl/fl}* mice. (C and D) Flow cytometric analysis of thymocyte subsets (C) or ISP cells (D) in CD45.2⁺ WT or *Cd2^{cre}Rptor^{fl/fl}* donor-derived cells (upper panels), or in CD45.1⁺ spike-derived cells (lower panels) of mixed BM chimeras. (E) Expression of CD4 and CD8 on WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells co-cultured with OP9-DL1 cells for 3 or 8 days. Data are means $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$. One-way ANOVA with Tukey's test (A), two-tailed Mann-Whitney test (B, frequency), or two-tailed unpaired t test (B, cellularity). Data are representative of at least three independent experiments. Numbers indicate percentage of cells in quadrants or gates.

To directly assess the DN-to-DP transition, we cocultured DN3 cells with OP9-DL1 stromal cells *in vitro* for 3–8 days. OP9-DL1 stromal cells have the capacity to induce a normal program of T cell differentiation from stem cells, including the generation of DP cells and CD8SP cells, and represent a well-established and simple *in vitro* system for the study of T cell development (Ciofani et al., 2006; Zuniga-Pflucker, 2004). In this context, *Cd2^{cre}Rptor^{fl/fl}* DN3 cells also showed an impaired capacity to progress to the DP stage (**Figure 3-4E**). Collectively, our results suggest RAPTOR mediates the DN-to-DP transition of developing thymocytes in a cell-autonomous manner.

To investigate the mechanistic basis for the defective DN-to-DP transition in *Cd2^{cre}Rptor^{fl/fl}* mice, we examined underlying cellular processes, such as cell death and proliferation. As measured by active caspase-3 staining, no differences in cell apoptosis were observed in any of the RAPTOR-deficient thymocyte subsets (**Figure 3-5A**). Cell proliferation, as measured by *in vivo* BrdU incorporation, was defective in RAPTOR-deficient ISP cells, but not in DN3 cells or DN4 cells (**Figure 3-5B**). It is important to note that the proliferative expansion of DN4 cells and ISP cells is known to be preceded by a period of quiescence at the DN3 stage (Carpenter and Bosselut, 2010). Notwithstanding, we found induction of intracellular TCR β (icTCR β) occurred largely normal in the DN populations, including in the TCR β -rearranged DN3b cells that are beginning the transition to becoming DP cells (**Figure 3-5C**). Our data, therefore, shows RAPTOR deficiency does not affect thymocyte survival or pre-TCR induction, but rather causes defective proliferation and differentiation of DN cells to DP cells.

Loss of RAPTOR Promotes the Development of $\gamma\delta$ -T Cells. In addition to $\alpha\beta$ -T cells, thymocyte progenitors also give rise to $\gamma\delta$ -T cells, a fundamentally distinct lineage of T cells (Carpenter and Bosselut, 2010; Ciofani and Zuniga-Pflucker, 2010). Interestingly, *Cd2^{cre}Rptor^{fl/fl}* mice presented a significant enrichment of the $\gamma\delta$ -T cell compartment (**Figure 3-6A**), which resulted in a profound change in the $\gamma\delta$ to $\alpha\beta$ -T cell thymic ratio (**Figure 3-6B**). This alteration in $\gamma\delta$ -T cell development was phenocopied by the mixed BM chimeras previously described (**Figure 3-6C**) and further confirmed using the OP9-DL1 *in vitro* coculture system, in which we cocultured DN3 cells with CD45.1⁺ spike cells in a 1:1 ratio in the presence of OP9-DL1 stromal cells (**Figure 3-6D**). These experimental systems confirmed that this phenomenon was a cell-intrinsic effect of RAPTOR deficiency in developing T cells and not a secondary consequence of the loss of DP thymocytes; this is because the CD45.1⁺ spike cells in both systems undergo normal development, hence providing a normal thymic microenvironment in terms of overall cellularity and presence of DP cells.

An interesting observation in terms of $\gamma\delta$ -T cell development was the upregulation of CD73 expression (**Figures 3-6E through G**), an established marker of $\gamma\delta$ -T cell fate commitment. *Cd2^{cre}Rptor^{fl/fl}* mice had strong enrichment of the CD73⁺ $\gamma\delta$ TCR⁺ thymic population (**Figure 3-6E, F**), which was reflected in an increased overall mean fluorescence intensity (MFI) of CD73 expression in the $\gamma\delta$ -T cell thymic compartment (**Figure 3-6G**). These data further support a role for RAPTOR in limiting $\gamma\delta$ -T cell development.

Figure 3-5. Analysis of cell death, proliferation, and intracellular TCR β (icTCR β) induction in thymic subsets of WT and *Cd2^{cre}Rptor^{fl/fl}* mice.

(A) Active caspase-3 staining in thymocyte subsets of WT and *Cd2^{cre}Rptor^{fl/fl}* mice. (B) *In vivo* BrdU incorporation in thymocyte subsets of WT and *Cd2^{cre}Rptor^{fl/fl}* mice pulsed with BrdU 3 h before analysis. (C) icTCR β staining in thymocyte subsets of WT and *Cd2^{cre}Rptor^{fl/fl}* mice. Numbers indicate percentage of cells in gates.

Figure 3-6. Loss of RAPTOR promotes $\gamma\delta$ -T cell development.

(A) Flow cytometric analysis (left) and cellularity (right) of TCR $\gamma\delta^+$ cells in WT and *Cd2^{cre}Rptor^{fl/fl}* thymocytes. (B) Ratio of TCR $\gamma\delta^+$ to TCR β^+ cells in WT and *Cd2^{cre}Rptor^{fl/fl}* thymocytes. (C) Flow cytometric analysis of the TCR $\gamma\delta^+$ compartment in CD45.2⁺ WT or *Cd2^{cre}Rptor^{fl/fl}* donor-derived cells (upper panels), or in CD45.1⁺ spike-derived cells (lower panels) of mixed BM chimeras. (D) Expression of CD4 and TCR $\gamma\delta$ on WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells (CD45.2⁺, upper panels) co-cultured with spike cells (CD45.1⁺, bottom panels) in a 1:1 ratio along with OP9-DL1 cells for 4–5 days. (E) Expression of CD73 and CD24 on CD4⁺CD8⁺TCR $\gamma\delta^+$ cells in the thymus of WT and *Cd2^{cre}Rptor^{fl/fl}* mice. (F) Cellularity of CD73⁺TCR $\gamma\delta^+$ cells in E. (G) Expression of CD73 on TCR $\gamma\delta^+$ cells in the thymus of WT and *Cd2^{cre}Rptor^{fl/fl}* mice, with MFI indicated above graphs. Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Two-tailed unpaired t test (A and F) or one-tailed unpaired t test (B). Data are representative of at least three independent experiments. Numbers indicate percentage of cells in gates or quadrants.

As assessed by Ki67 and active caspase-3 staining respectively, RAPTOR deficiency did not affect proliferation (**Figure 3-7A**) or apoptosis (**Figure 3-7B**) of TCR $\gamma\delta^+$ thymocytes. Collectively, our data suggest RAPTOR acts as a cell-intrinsic molecular modulator of $\gamma\delta$ -T cell development.

RICTOR Is Dispensable for $\gamma\delta$ -T Cell Development

RICTOR is the obligate component of mTORC2. Previous studies have already reported a role for RICTOR in $\alpha\beta$ -T cell development (Lee et al., 2012; Tang et al., 2012), but whether RICTOR was important for $\gamma\delta$ -T cell development remained unclear. Using the same crossing strategy as for the *Cd2^{cre}Rptor^{fl/fl}* mice, we crossed *Cd2-Cre* transgenic mice with mice carrying loxP-flanked *Rictor* alleles (*Rictor^{fl/fl}*) to evaluate the function of RICTOR/mTORC2 signaling in $\gamma\delta$ -T cell development. Interestingly, our studies showed RICTOR was dispensable for the development of $\gamma\delta$ -T cells, as ablation of RICTOR did not affect the frequency or cellularity of this thymocyte population (**Figure 3-8A**), or CD73 expression on TCR $\gamma\delta^+$ thymocytes (**Figure 3-8B**). Combined deletion of RAPTOR and RICTOR further reduced cellularity of total thymocytes, including $\gamma\delta$ -T cells, and it had no rescue effects in terms of CD73 expression by TCR $\gamma\delta^+$ thymocytes (**Figure 3-8A, B**). These results demonstrate RAPTOR and RICTOR have discrete roles in $\gamma\delta$ -T cell development.

RAPTOR Modulates ROS Production in Fate Decisions of DN3 Cells

The dynamic regulation of metabolic activity seen in developing thymocytes (**Figure 3-1**), paralleled by mTORC1 activity changes (**Figure 3-2**), suggested RAPTOR shaped T cell fate decisions by controlling metabolic reprogramming of DN3 cells. To test this hypothesis, we performed detailed metabolic characterization of RAPTOR-deficient DN3 cells. Compared to WT, RAPTOR-deficient DN3 cells had intact basal OCR and slightly reduced SRC (**Figure 3-9A**). Nonetheless, they showed reduced ECAR (**Figure 3-9B**), which translated to an altered OCR to ECAR ratio (**Figure 3-9C**), suggesting disrupted control of bioenergetics and an increase reliance on oxidative metabolism. Consequently, RAPTOR-deficient DN3 cells, but no other developing subgroup, had significantly increased levels of ROS (**Figures 3-9D, E**). Interestingly, RAPTOR-deficient $\gamma\delta$ -T cells also had elevated ROS production, as compared to their WT counterparts (**Figure 3-9F**). Other mitochondrial parameters, such as mitochondrial mass and mitochondrial membrane potential, as measured by MitoTracker and TMRM staining respectively, were comparable between WT and RAPTOR-deficient DN3 cells (**Figures 3-9G, H**). These results suggest RAPTOR deficiency impairs metabolic remodeling and promotes ROS production in DN3 cells.

To study the potential link between changes in ROS production and metabolic activities of DN3 cells with subsequent fate decisions, we performed *in vitro* assays to directly assess these phenomena. First, we co-cultured DN3a cells with OP9-DL1 stromal cells in the presence of two known promoters of ROS production: galactose (Gal) and

Figure 3-7. Analysis of proliferation and apoptosis in RAPTOR-deficient $\gamma\delta$ -T cells.

(A) Ki67 staining in TCR $\gamma\delta^+$ cells in WT and $Cd2^{cre}Rptor^{fl/fl}$ thymocytes. (B) Active caspase-3 staining in TCR $\gamma\delta^+$ cells in WT and $Cd2^{cre}Rptor^{fl/fl}$ thymocytes. Numbers indicate percentage of cells in gates.

Figure 3-8. Loss of RICTOR does not affect the development of $\gamma\delta$ -T cells.

(A) Flow cytometric analysis of TCR $\gamma\delta^+$ thymocytes in WT, $Cd2^{cre}Rptor^{fl/fl}$, $Cd2^{cre}Rictor^{fl/fl}$, and $Cd2^{cre}Rptor^{fl/fl}Rictor^{fl/fl}$ mice (upper panels), along with frequencies and cellularity (bottom panels). (B) Flow cytometric analysis of CD73 and CD24 expression by TCR $\gamma\delta^+$ thymocytes in WT, $Cd2^{cre}Rptor^{fl/fl}$, $Cd2^{cre}Rictor^{fl/fl}$, and $Cd2^{cre}Rptor^{fl/fl}Rictor^{fl/fl}$ mice. Data are means $\pm$ SEM. ** $P < 0.01$ and *** $P < 0.001$. One-way ANOVA with Tukey's test. Data are representative of at least three independent experiments. Numbers indicate percentage of cells in gates or quadrants.

Figure 3-9. RAPTOR modules ROS production DN3 and $\gamma\delta$ -T cells.

(A) OCR in WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells under basal conditions or in response to the indicated mitochondrial inhibitors. SRC of WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells is indicated. (B) Measurement of ECAR in WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells. (C) Ratio of OCR to ECAR in WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells. (D) Analysis of ROS production in WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells. (E) Relative MFI of ROS in thymocyte subsets of WT and *Cd2^{cre}Rptor^{fl/fl}* mice (MFI of WT DN3 cells is normalized to 1). (F) Analysis of ROS production in WT and *Cd2^{cre}Rptor^{fl/fl}* $\gamma\delta$ -T cells. (G and H) Plots of MitoTracker (G) and TMRM (H) in WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells. Data are means $\pm$ SEM. *** $P < 0.001$. Two-way ANOVA with Bonferroni's test. Data are the combination of three independent experiments (A to C) or representative/combination of two independent experiments (D to H).

galactose oxidase (GAO) (Sena et al., 2013). Gal/GAO treatment caused enrichment of the $\gamma\delta$ -T cell compartment and loss of the $\alpha\beta$ -T cell lineage (**Figure 3-10A**). Second, we treated DN3a cells in the presence of OP9-DL1 stromal cells with dichloroacetate (DCA), an inhibitor of the mitochondrial enzyme pyruvate dehydrogenase kinase (PDK). Inhibition of PDK activates pyruvate dehydrogenase (PDH), which shifts glycolysis into fueling OXPHOS in the mitochondria (Gerriets et al., 2015). Similar to Gal/Gao treatment, though not to the same extent, DCA treatment promoted $\gamma\delta$ -T cell development at the expense of $\alpha\beta$ -T cells (**Figure 3-10B**). The effects of DCA on DN3a cells were associated with increased ROS production (**Figure 3-10C**). To exclude potential effects of the pharmacological agents on the OP9-DL1 cells, we performed similar experiments in the context of a stromal cell-free system for *in vitro* differentiation of thymocytes (Tussiwand et al., 2011). The results were consistent with those of the OP9-DL1 coculture system (**Figure 3-10D**). The use of the stromal cell-free system confirmed that the pharmacological effects were mediated by changes in ROS signaling in the DN3a cells and not in the stromal cells. Finally, treatment of WT DN3a cells with the ROS scavengers N-acetyl-L-cysteine (NAC) or glutathione (GSH) diminished $\gamma\delta$ -T cell development (**Figure 3-10E**) and promoted the generation of $\alpha\beta$ -T cells (**Figure 3-10F**), further supporting a role for ROS signaling in DN3 cell fate choices.

Considering the aforementioned evidence supporting a role for ROS in DN3 cell lineage choices, we next tested whether ROS neutralization could rescue the altered fate choices of RAPTOR-deficient DN3 cells. To this end, we cocultured RAPTOR-deficient DN3 cells with OP9-DL1 cells in the presence of NAC and followed their fate decisions via flow cytometric analysis. NAC treatment significantly, though not completely, suppressed the generation of $\gamma\delta$ -T cells derived from RAPTOR-deficient DN3a cells and restored their defective $\alpha\beta$ -T cell generation (**Figure 3-11A**). The effects of NAC treatment on RAPTOR-deficient DN3a cells largely rescued the altered $\gamma\delta$ to $\alpha\beta$ -T cell ratio observed in these cells (**Figure 3-11A**). Furthermore, NAC treatment restored the impaired transition of RAPTOR-deficient DN3 cells to the DN4 stage (**Figure 3-11B**). Noticeably, NAC treatment did not improve the cellularity of RAPTOR-deficient DN3 cells (**Figure 3-11C**), suggesting that ROS regulation of DN3 lineage choices was independent of cell expansion. Altogether, these results indicate RAPTOR-dependent metabolic remodeling tunes ROS production in DN3 cells, which acts as a metabolic signal in dictating lineage choices.

RAPTOR Tunes the Strength of the ERK/EGR1/ID3 Signaling Axis

It is well appreciated that TCR signaling strength influences lineage decisions of developing thymocytes (Carpenter and Bosselut, 2010; Ciofani and Zuniga-Pflucker, 2010). Strong TCR signaling mediated by the ERK/EGR1/ID3 signaling axis favors the development of $\gamma\delta$ -T cells (Haks et al., 2005; Hayes et al., 2005). Thus, we investigated whether RAPTOR controlled the ERK/EGR1/ID3 signaling axis of DN3 cells. As compared to their WT counterparts, RAPTOR-deficient DN3 cells had elevated phosphorylation of ERK, along with increased expression of EGR1 and ID3 (**Figures 3-12A through C**). We then crossed mice with the *Erk2* gene flanked by loxP sites with

Figure 3-10. Modulation of ROS production alters fate choices of DN3 cells.

(A) Analysis of CD4 and TCR $\gamma\delta$ expression in WT DN3 cells cocultured with OP9-DL1 cells in the absence or presence of Gal/GAO for 5 days (left), along with frequencies and cellularity (right). (B) Analysis of CD4 and TCR $\gamma\delta$ expression in WT DN3 cells cocultured with OP9-DL1 cells in the absence or presence of DCA for 5 days (top), along with bar graph depicting fold change of the ratio of TCR $\gamma\delta$ ⁺ to CD4⁺ cells upon DCA treatment (bottom). (C) Analysis of ROS production in WT DN3a cells cocultured with OP9-DL1 cells in the absence or presence of DCA for 3 days, with MFI plotted in graphs. (D) Analysis of CD4 and TCR $\gamma\delta$ expression in WT DN3 cells cultured on plate-bound NOTCH ligand DLL4 in the presence of CXCL12 and treated with the indicated inhibitors (left), along with frequencies of TCR $\gamma\delta$ ⁺ cells in each condition (right). (E and F) Analysis of CD4 and TCR $\gamma\delta$ expression (E) and CD4 and CD8 expression (F) in WT DN3 cells cocultured with OP9-DL1 cells in the absence or presence of NAC or GSH for 3 days (left), along with number of TCR $\gamma\delta$ ⁺ cells (E) or DP cells (F) in each condition (right). Data are means $\pm$ SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001. Two-tailed Mann-Whitney test (A for frequency analysis), two-tailed unpaired t test (A for cellularity analysis), one-tailed unpaired t test (B), or one-way ANOVA with Tukey's test (E, F). Data are representative of at least three independent experiments. Numbers indicate percentage of cells in gates or quadrants.

Figure 3-11. RAPTOR-dependent control of ROS production impacts fate choices of DN3 cells.

(A and B) Analysis of CD4 and TCR $\gamma\delta$ expression (A) and CD25 and CD44 expression (B) in WT and *Cd2^{cre}Rptor^{fl/fl}* DN3 cells cocultured with OP9-DL1 cells in the absence or presence of NAC for 5 days, along with fold changes of the ratios of TCR $\gamma\delta$ ⁺ cells to CD4⁺ cells (A, center) and CD25⁺ cells to CD25⁺ cells (B, right), and frequencies of TCR $\gamma\delta$ ⁺ cells and CD4⁺ cells (A, right). (C) Cellularity of total cells in (B). Data are means $\pm$ SEM. * $P < 0.05$, and *** $P < 0.001$. One-way ANOVA with Tukey's test (A, B), or two-tailed unpaired t test (C). Data are representative of at least three independent experiments. Numbers indicate percentage of cells in gates or quadrants.

Figure 3-12. RAPTOR tunes the strength of the ERK/EGR1/ID3 signaling axis in DN3 cells.

(A) Immunoblot analysis of p-ERK, EGR1, ID3, and β-ACTIN in WT and *Cd2^{cre}Raptor^{fl/fl}* DN3 cells. (B and C) Flow cytometric analysis of p-ERK (B) and ID3 expression (C) in WT and *Cd2^{cre}Raptor^{fl/fl}* DN3 cells, with MFI plotted in graphs. (D) Cellularity of CD4⁺CD8⁺ DP cells from the indicated mice. Each symbol represents an individual mouse. (E) Flow cytometric analysis of CD73 and CD24 expression on TCRγδ⁺ thymocytes in the indicated mice (left), along with frequency and cellularity of CD73⁺TCRγδ⁺ cells. (F) Expression of CD73 on TCRγδ⁺ thymocytes in (E) shown as fold change (MFI on WT cells is normalized to 1). Data are means ± SEM. **P* < 0.05, and ****P* < 0.001. One-way ANOVA with Tukey's test. Data are representative of at least three independent experiments. Numbers indicate percentage of cells in quadrants.

Cd2^{cre}Rptor^{fl/fl} mice to delete ERK2 in the RAPTOR-deficient background. The resulting mice (*Cd2^{cre}Rptor^{fl/fl}Erk2^{fl/fl}*) did not show improved cellularity of DP thymocytes as compared to *Cd2^{cre}Rptor^{fl/fl}* mice (**Figure 3-12D**). However, they rescued the accumulation of CD73⁺ $\gamma\delta$ -T cells (**Figure 3-12E**) and the elevated surface expression of CD73 in $\gamma\delta$ -T cells observed in *Cd2^{cre}Rptor^{fl/fl}* mice (**Figure 3-12F**). These results suggest deletion of RAPTOR in DN3 cells leads to elevated signaling strength of the ERK/EGR1/ID3 signaling pathway, which in turn favors development of $\gamma\delta$ -T cells.

To assess whether changes in ROS levels were related to changes in signaling strength, we measured ID3 expression via flow cytometry in DCA-treated WT DN3a cells cocultured with OP9-DL1 cells. DCA, which promoted ROS production in DN3a cells (**Figure 3-10C**) and enhanced $\gamma\delta$ -T cell development at the expense of $\alpha\beta$ -T cells (**Figure 3-10B, D**), increased the expression of ID3 in WT DN3a cells after 3 days of treatment in the presence of OP9-DL1 cells (**Figure 3-13A**). This effect was accompanied by a marked increase in CD73 expression on TCR $\gamma\delta$ ⁺ cells from the cocultures of WT DN3a cells with OP9-DL1 cells (**Figure 3-13B**). Gal/GAO treatment of WT DN3a cells cocultured with OP9-DL1 cells, which had striking effects on the promotion of $\gamma\delta$ -T cell development and inhibition of the $\alpha\beta$ -T cell lineage (**Figure 3-10A**), also caused increased CD73 expression on TCR $\gamma\delta$ ⁺ cells from the cocultures of WT DN3a cells with OP9-DL1 cells (**Figure 3-13C**). Altogether, these results indicate mTORC1 activity and ROS metabolism influence TCR signaling strength and, consequently, lineage choices of DN3 cells, by impinging on the activity of the ERK/EGR1/ID3 signaling axis.

Summary

In this chapter, we evaluated the metabolic activity of developing thymocytes and uncovered a dynamic regulation of cell metabolism associated with mTORC1 activity. Using a genetic mouse system to delete *Rptor*, the obligate component of mTORC1, specifically in developing thymocytes, we explored the role of RAPTOR/mTORC1 signaling in T cell development. We discovered RAPTOR deficiency impairs the DN-to-DP transition of developing thymocytes in the $\alpha\beta$ -T cell lineage while enhancing the development of their TCR $\gamma\delta$ ⁺ counterparts. This observation was confirmed in the context of mixed BM chimeras, which circumvented the potential secondary consequences of reduced DP cellularity in *Cd2^{cre}Rptor^{fl/fl}* mice, as well as in *in vitro* coculture experiments with OP9-DL1 stromal cells. Though it was known that RICTOR, the obligate component of mTORC2, was essential for the development of $\alpha\beta$ -T cells (Lee et al., 2012; Tang et al., 2012), whether mTORC2 signaling was required for $\gamma\delta$ -T cell development remained unknown. To this end, we deleted *Rictor* in early DN cells using *Cd2*-Cre-mediated deletion and found RICTOR was dispensable for the development of $\gamma\delta$ -T cells. These results highlighted a unique role for RAPTOR in the lineage choices of DN3 cells. We then explored the effects of RAPTOR deficiency in the metabolic fitness of DN3 cells and observed disrupted control of bioenergetics and an increase reliance on oxidative metabolism, associated with elevated ROS production. *In vitro* modulation of ROS production in DN3 cells confirmed a role for RAPTOR in

Figure 3-13. Elevated ROS production increases expression of ID3 in DN3 cells and CD73 on $\gamma\delta$ -T cells.

(A) Analysis of ID3 expression in WT DN3a cells after 3 days cocultured with OP9-DL1 cells in the absence or presence of DCA. (B and C) Analysis of CD73 expression on TCR $\gamma\delta^+$ cells from coculture of WT DN3a cells with OP9-DL1 cells for 3 days in the absence or presence of DCA (B) or Gal/GAO (C). Data are representative of at least three independent experiments. Numbers indicate MFI.

controlling ROS levels, which ultimately affected fate choices of DN3 cells. Finally, we explored the relationship between RAPTOR and the ERK/EGR1/ID3 signaling axis, given that TCR signaling strength is known to govern fate choices of developing thymocytes (Carpenter and Bosselut, 2010; Ciofani and Zuniga-Pflucker, 2010) and, in particular, strong signaling strength favors $\gamma\delta$ -T cells (Haks et al., 2005; Hayes et al., 2005). Our studies indicated that mTORC1 activity and ROS metabolism tuned the activity of the ERK/EGR1/ID3 signaling axis, which controls TCR signaling strength, and, therefore, influenced lineage choices of DN3 cells. Altogether, the studies in this chapter establish mTORC1 signaling as a key mediator of T cell lineage choices and reveal ROS as an important metabolic signal in this process.

CHAPTER 4. PTEN-MEDIATED CONTROL OF UNCONVENTIONAL IL-17-PRODUCING THYMOCYTE DEVELOPMENT

In **Chapter 3**, we uncovered the fate-sorting role of RAPTOR/mTORC1 signaling in T cell development. In this chapter, we focused our efforts on unraveling the role of PTEN, an upstream regulator of mTOR signaling, in the development of unconventional TCR $\alpha\beta$ ⁺ T cells. PTEN is a lipid phosphatase that hydrolyses PIP3, thereby inhibiting the PI3K-mediated phosphorylation of PIP2. Thus, PTEN inhibits both mTORC1 and mTORC2 signaling (Chi, 2012). Additionally, PTEN possesses protein phosphatase activity and phosphatase-independent functions (Shi et al., 2012). In this way, PTEN controls a number of events that are central to T cell biology, including proliferation, survival, differentiation, energy metabolism, and cellular architecture and mobility (Chen and Guo, 2017). In fact, deletion of *Pten* in T cells results in autoimmune disorders and lymphoma (Buckler et al., 2008). Although the role of PTEN in T cell development has been studied, current knowledge of the functions of PTEN in innate-like unconventional T cells is limited.

Innate-like unconventional T cells share features of adaptive and innate immune cells. They have an activated phenotype, which grants them with the ability to mount relatively immediate immune responses in response to non-cognate antigen stimulation (Godfrey et al., 2015). These unique subsets of T cells are of critical interest as they have been shown to play important roles in tissue homeostasis and diseases (Iwabuchi and Van Kaer, 2019). However, the precise molecular mechanisms underpinning their development remain elusive. In this chapter, we explore the role of PTEN in the development of unconventional IL-17-producing TCR $\alpha\beta$ ⁺ thymocytes.

T Cell-Specific Deletion of *Pten* Promotes the Development of Unconventional IL-17-Producing Thymocytes

Generation of Mice with T Cell-Specific Deletion of *Pten*

To explore the roles of PTEN in the development of unconventional TCR $\alpha\beta$ ⁺ thymocytes, we crossed *Cd4*-Cre transgenic mice with mice carrying loxP-flanked *Pten* alleles (*Pten*^{fl/fl}). This genetic mouse model allowed us to delete *Pten* specifically in T-lineage cells (mice referred to as *Cd4*^{cre}*Pten*^{fl/fl}). Because TCR $\alpha\beta$ ⁺ lymphocytes go through a CD4⁺CD8⁺ DP stage during development (**Figure 1-2**), PTEN is deleted in all thymocytes derived from this DP population in the *Cd4*^{cre}*Pten*^{fl/fl} mice. Previous studies have confirmed deletion of PTEN in the DP population of *Cd4*^{cre}*Pten*^{fl/fl} mice and reported the development of fatal T cell lymphomas in these mice by 10-16 weeks of age (Hagenbeek and Spits, 2008). The tumorigenic effect of PTEN deletion in the T cell compartment, along with the development of autoimmune disorders, is a well-established and extensively studied phenotype (Finlay et al., 2009; Suzuki et al., 2001; Xue et al., 2008). To study the role of PTEN in the normal development of TCR $\alpha\beta$ ⁺ thymocytes,

and to avoid the defects associated with tumorigenesis, we analyzed young (4–6 weeks of age) *Cd4^{cre}Pten^{fl/fl}* mice before they developed thymoma. The frequency and cellularity of the thymic subsets in young *Cd4^{cre}Pten^{fl/fl}* mice were comparable with WT mice, suggesting T cell development is relatively normal in these mice at this age (**Figure 4-1A**).

To determine whether PTEN deletion had effects on the development of effector T cells in the thymus, we analyzed cytokine production in young *Cd4^{cre}Pten^{fl/fl}* mice. Interestingly, we found strong enrichment of IL-17A and IL-17F production (**Figure 4-1B**). This effect was specific to the IL-17 family of cytokines as no other cytokine was enriched in the thymus of *Cd4^{cre}Pten^{fl/fl}* mice (**Figure 4-1B**). These results suggest that PTEN negatively regulates the development of IL-17-producing thymocytes.

Phenotypic Characterization of IL-17-Producing Thymocytes in *Cd4^{cre}Pten^{fl/fl}* Mice

The enrichment of IL-17 production in the thymus of *Cd4^{cre}Pten^{fl/fl}* mice was striking, as this cytokine is produced at low levels by WT thymocytes under homeostasis (**Figure 4-1B**). ROR γ ⁺ is the master transcription factor that promotes IL-17 gene expression. As expected, the IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice were ROR γ ⁺ and had intermediate expression of TCR β (TCR β ^{int}) on the cell surface (**Figure 4-2A**). Additionally, they expressed other receptors associated with type 17 lymphocytes: chemokine receptor 6 (CCR6) and IL-7 receptor (CD127) (**Figure 4-2A**). Since the IL-17-producing population co-expressed CCR6 and CD127, we used these surface markers as surrogates to identify the aberrant IL-17-producing population (**Figure 4-2B**). We next assessed the expression of CD4 and CD8 coreceptors. Interestingly, the vast majority of IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice were not CD4⁺ T cells (nT_H17), but instead were a heterogeneous population of CD8⁺ SP, CD4⁻CD8⁻ DN, and CD4⁺CD8⁺ DP cells (**Figure 4-2C**). This suggests that the IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice are not classical nT_H17 cells, but rather a population of unconventional T cells.

IL-17-Producing Lymphocytes in *Cd4^{cre}Pten^{fl/fl}* Mice Reside in the Thymus

The enrichment of IL-17-producing lymphocytes in *Cd4^{cre}Pten^{fl/fl}* mice was restricted to the thymus, as no aberrant IL-17 production was observed in peripheral organs (**Figure 4-3A**). This observation prompted us to assess the surface expression of L-selectin (CD62L) and sphingosine-1-phosphate receptor 1 (S1PR1), molecules required for thymocyte egress from the thymus into peripheral tissues (Arbones et al., 1994; Matloubian et al., 2004). As compared to mature CD8SP cells and DP cells, the CCR6⁺CD127⁺ population in *Cd4^{cre}Pten^{fl/fl}* mice had markedly reduced expression of CD62L and S1PR1 expression, respectively (**Figure 4-3B**). Developing thymocytes must downregulate CD69 to allow for S1PR1 expression on the cell surface (Kimura et al., 2018; Matloubian et al., 2004). As compared to DP cells, which had the highest S1PR1 expression, the CCR6⁺CD127⁺ population in *Cd4^{cre}Pten^{fl/fl}* mice had elevated surface

Figure 4-1. *Cd4*-Cre-mediated deletion of *Pten* induces the enrichment of IL-17 production in thymus.

(A) Flow cytometric analysis of thymocyte subsets in WT and *Cd4^{cre}Pten^{fl/fl}* mice, along with frequencies (center) and cellularity (right) of each major thymocyte subset. (B) Analysis of cytokine production in thymus of WT and *Cd4^{cre}Pten^{fl/fl}* mice after ICS stimulation with frequencies (center) and cellularity (right) of each cytokine analyzed. Data are means ± SEM. ***P* < 0.01 and ****P* < 0.001. Two-tailed Mann-Whitney test (frequency) or two-tailed unpaired *t*-test (cellularity). Data are representative/combination of at least five independent experiments. Numbers indicate percentage of cells in gates.

Figure 4-2. Phenotypic characterization of IL-17-producing thymocytes.

(A) Flow cytometric analysis of classical molecules associated with type 17 TCR $\alpha\beta$ ⁺ lymphocytes in WT and *Cd4^{cre}Pten^{fl/fl}* mice after ICS stimulation. (B) Identification of IL-17-producing thymocytes as CCR6⁺CD127⁺ cells in WT and *Cd4^{cre}Pten^{fl/fl}* mice, along with frequencies (center) and cellularity (right). (C) Flow cytometric analysis of CD4 and CD8 expression on the CCR6⁺CD127⁺ thymocytes in (B) along with the mean frequency distribution of each subset (pie chart, bottom left; DN, CD4⁻CD8⁻ double-negative; DP, CD4⁺CD8⁺ double-positive; CD4SP, CD4⁺CD8⁻ single-positive; CD8SP, CD4⁻CD8⁺ single-positive), total frequencies (top right) and cellularity (bottom right). Data are means $\pm$ SEM. ** P < 0.01, *** P < 0.001 and **** P < 0.0001. Two-tailed Mann-Whitney test (frequency) or two-tailed unpaired t -test (cellularity). Data are representative/combination of at least five independent experiments. Numbers indicate percentage of cells in gates.

Figure 4-3. IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice are thymic-resident.

(A) Flow cytometric analysis of IL-17A production in peripheral organs of WT and *Cd4^{cre}Pten^{fl/fl}* mice after ICS stimulation. (B) Flow cytometric analysis of markers associated with thymic egress and tissue-residency in the indicated thymic populations in *Cd4^{cre}Pten^{fl/fl}* mice. (C) Flow cytometric analysis of IL-17 production in thymus of *Pten^{+/+}Il17^{icre}Rosa26^{GFP}* and *Pten^{+/-}Il17^{icre}Rosa26^{GFP}* reporter mice after ICS stimulation along with cellularity (right). (D) Flow cytometric analysis of IL-17A-GFP-marked lymphocytes in the peripheral organs of *Pten^{+/+}Il17^{icre}Rosa26^{GFP}* and *Pten^{+/-}Il17^{icre}Rosa26^{GFP}* reporter mice after ICS stimulation. Data are means ± SEM. Data are representative/combination of at least three independent experiments. Numbers indicate percentage of cells in gates.

expression of CD69, though not to the same extent as mature CD8SP (**Figure 4-3B**). Finally, we evaluated the expression of CD103, a mucosal homing receptor associated with tissue residency (Rahimpour et al., 2015). We found that the CCR6⁺CD127⁺ population in *Cd4^{cre}Pten^{fl/fl}* mice had high CD103 expression (**Figure 4-3B**). These results suggest that PTEN-deficient IL-17-producing thymocytes reside in the thymus.

To confirm that the PTEN-deficient IL-17-producing lymphocytes resided in thymus, and not that they were losing IL-17 expression in periphery, we used a fate-mapping system to permanently mark with green fluorescent protein (GFP) cells that express IL-17. We crossed *Il17-iCre* mice with ROSA26^{GFP} reporter mice on the *Pten^{+/-}* (germline deletion of one allele) background (mice referred to as *Pten^{+/-}Il17^{icre}Rosa26^{GFP}*). Although *Pten^{+/-}* mice had a much weaker IL-17 phenotype than *Cd4^{cre}Pten^{fl/fl}* mice (data not shown), *Pten^{+/-}Il17^{icre}Rosa26^{GFP}* mice still had slight enrichment of IL-17-producing thymocytes (**Figure 4-3C**). No major differences in frequency of peripheral GFP-tagged cells were observed between WT and *Pten^{+/-}Il17^{icre}Rosa26^{GFP}* mice (**Figure 4-3D**). These results further support that the aberrant IL-17-producing thymocyte population that emerges in the absence of PTEN is thymic-resident.

Composition of Unconventional IL-17-Producing Thymocyte Population in *Cd4^{cre}Pten^{fl/fl}* Mice

iNKT17 and Tc17 Cells

Our lab previously demonstrated that PTEN deficiency enhances the differentiation of iNKT17 cells in thymus (Wei et al., 2014). Nevertheless, iNKT17 cells did not account for the totality of the IL-17-producing thymocytes observed in *Cd4^{cre}Pten^{fl/fl}* mice (**Figure 4-4A**). Based on TCR β (**Figures 4-2A and 4-4B**) and CD3 (**Figure 4-4B**) surface expression, it was clear that the PTEN-deficient IL-17-producing thymocytes belonged to the TCR $\alpha\beta$ -lineage of T cells and were not type 3 innate lymphoid cells (ILC3, TCR β ⁻) or lymphoid tissue inducers (LTi, CD3⁻). The absence of the CD4 coreceptor and concomitant enrichment of the CD8 coreceptor among a large portion of IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice (**Figure 4-2B**) suggested that IL-17-secreting CD8 T cells (Tc17) may be enriched in this cell population. Downregulation of the T-box factors, T-box transcription factor (T-BET) and eomesodermin (EOMES), is required for CD8⁺ T cells to produce IL-17 (Intlekofer et al., 2005). Neither T-BET nor EOMES protein expression was detected in the IL-17-producing thymocyte population of the *Cd4^{cre}Pten^{fl/fl}* mice (**Figure 4-4C**). These data suggest that thymic IL-17-producing cells in *Cd4^{cre}Pten^{fl/fl}* mice share similar phenotypic characterizations as Tc17 cells.

To further characterize the population of PTEN-deficient IL-17-producing thymocytes, we next used microarrays to compare the transcriptional profiles of the non-iNKT17 fraction (CD1d:PBS57-tet⁻TCR β ^{int}CCR6⁺CD127⁺) against mature CD8SP cells

Figure 4-4. Phenotypic characterization of the IL-17-producing thymocyte population in *Cd4^{cre}Pten^{fl/fl}* mice.

(A) CD1d:PBS57 tetramer staining of the IL-17-producing thymocyte population, pre-gated as TCRβ^{int}IL-17A⁺ cells, in WT and *Cd4^{cre}Pten^{fl/fl}* mice. (B) Flow cytometric analysis of TCRβ and CD3 surface expression among CCR6⁺ thymocytes. (C) Flow cytometric analysis of intracellular T-BET and EOMES expression along with IL-17A after ICS stimulation. Data are representative of at least three independent experiments. Numbers indicate percentage of cells in gates.

(TCR β^+ CD8 $^+$ CD4 $^-$) in *Cd4^{cre}Pten^{fl/fl}* mice. Using the differentially expressed genes at the 1.5-fold change cut-offs, we performed ingenuity pathway analysis (IPA) to predict upstream transcriptional regulators. The transcription factor PLZF, encoded by the *Zbtb16* gene, was the top predicted transcriptional regulator (**Figure 4-5**). PLZF is specifically expressed in NKT and MAIT cells, but not in conventional T cells or NK cells, and it directs the acquisition of effector programs by these two unconventional T cell lineages (Cao et al., 2018; Garner et al., 2018; Kovalovsky et al., 2008; Mao et al., 2016; Savage et al., 2008). The striking enrichment of PLZF downstream target genes in the non-iNKT fraction of the PTEN-deficient IL-17-producing thymocytes prompted us to investigate whether these cells were MAIT cells.

MAIT17 Cells

MAIT cells are easily identifiable by their binding to 5-OP-RU-loaded MR1 tetramers (Rahimpour et al., 2015). Staining of PTEN-deficient thymocytes with the antigen-loaded MR1 tetramer showed that approximately 40 percent of the IL-17-producing cells in *Cd4^{cre}Pten^{fl/fl}* mice were MAIT17 cells (**Figure 4-6A**). Further, there was a nearly 20-fold enrichment of MAIT cells in the thymus of *Cd4^{cre}Pten^{fl/fl}* mice (**Figure 4-6B**). Interestingly, the enrichment of MAIT cells was specific to the MAIT17 compartment, given that nearly all MAIT cells were IL-17-producing cells (**Figure 4-6B**). These results highlight a role for PTEN in restricting the development of type 17 unconventional T cells, particularly iNKT17 cells, as previously reported, and MAIT17 cells.

We next evaluated the developmental status of the PTEN-deficient MAIT cells. Intrathymic MAIT cell development is a three-stage process that can be followed by the surface expression of CD44 and CD24 (Koay et al., 2016): CD44 $^-$ CD24 $^+$ immature cells (S1) progress into an intermediate CD44 $^-$ CD24 $^-$ stage (S2) to then become CD44 $^+$ CD24 $^-$ mature cells (S3). Remarkably, nearly all PTEN-deficient MAIT cells were mature S3 cells (**Figure 4-6C**). This observation is in contrast to PTEN-deficient iNKT cells, which are stalled in their transition from stage 2 to stage 3 (Wei et al., 2014). These results show that PTEN deficiency leads to the accumulation of mature thymic-resident MAIT17 cells.

Recent studies have highlighted the importance of the inducible T cell costimulator (ICOS) receptor in the optimal activation of MAIT cells (Koay et al., 2019; Wang et al., 2019). Signaling through ICOS also promotes the establishment of the iNKT17 effector lineage (Niu et al., 2018; Wu et al., 2014). We found nearly all PTEN-deficient MAIT cells expressed high levels of ICOS (**Figure 4-6D**). Furthermore, the total PTEN-deficient TCR β^{int} CCR6 $^+$ CD127 $^+$ population, as opposed to just the MAIT or iNKT fractions, displayed high ICOS expression (**Figure 4-6E**). These results suggest that signaling through ICOS may contribute to the establishment of the PTEN-deficient IL-17-producing thymocyte population.

Figure 4-5. Ingenuity pathway analysis (IPA) of the differentially expressed genes in the non-iNKT17 fragment of PTEN-deficient $\text{TCR}\beta^{\text{int}}\text{CCR6}^+\text{CD127}^+$ cells, as compared to mature CD8SP cells ($\text{TCR}\beta^+\text{CD8}^+\text{CD4}^-$) in $\text{Cd4}^{\text{cre}}\text{Pten}^{\text{fl/fl}}$ mice.

IPA of transcriptional regulators and ligand-dependent nuclear receptors predicted to be activated in the non-iNKT17 fragment of PTEN-deficient $\text{TCR}\beta^{\text{int}}\text{CCR6}^+\text{CD127}^+$ cells, based on the differentially expressed genes at the 1.5-fold cut-offs as compared to mature CD8SP cells ($\text{TCR}\beta^+\text{CD8}^+\text{CD4}^-$) in $\text{Cd4}^{\text{cre}}\text{Pten}^{\text{fl/fl}}$ mice.

Figure 4-6. Characterization of MAIT17 cells in *Cd4^{cre}Pten^{fl/fl}* mice.

(A) 5-OP-RU-loaded MR1 tetramer and PBS57-loaded CD1d tetramer staining of IL-17-producing thymocyte population in WT and *Cd4^{cre}Pten^{fl/fl}* mice, along with the average frequency distribution of each subset (pie chart, bottom left), total frequencies (top right), and cellularity (bottom right). (B) Analysis of cytokine production by thymic MAIT cells after ICS stimulation, along with cellularity of MAIT1 cells (IFN γ -producing MAIT cells, top right) and MAIT17 cells (IL-17A-producing MAIT cells, bottom right). (C) Flow cytometric analysis of the developmental stages of MAIT cells, along with frequencies and cellularity of each subset (S1, CD24 $^{+}$ CD44 $^{-}$; S2, CD24 $^{-}$ CD44 $^{-}$; S3, CD24 $^{-}$ CD44 $^{+}$). (D and E) Flow cytometric analysis of ICOS and CCR6 expression on thymic MAIT cells (labeled as MR1 $^{+}$, D) and TCR β^{int} CCR6 $^{+}$ CD127 $^{+}$ thymic population (E) in WT and *Cd4^{cre}Pten^{fl/fl}* mice. Data are means $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Two-tailed Mann-Whitney test (A and C for frequency) and two-tailed unpaired t -test (A through C for cellularity). Data are representative/combination of at least five (A and B) or three (C through E) independent experiments. Numbers indicate percentage of cells in quadrants or gates.

CD8 $\alpha\alpha^+$ and CD4 $^-$ CD8 $^-$ Type 17 (DN17) Cells

Although iNKT17 cells and MAIT17 cells were enriched among the PTEN-deficient IL-17-producing thymocytes, these two populations did not account for all the IL-17-producing cells (**Figure 4-6A**). To characterize the unidentified fraction of cells, we performed microarray analysis of the three subsets and compared their respective transcriptional profiles. Principal component analysis (PCA) and differential expression comparisons of the identified genes showed similar transcriptional profiles between the iNKT17 and MAIT17 subsets, but a clearly distinct transcriptome for the remaining CD1d:PBS57-tet $^-$ MR1:5-OP-RU-tet $^-$ (DN) population (**Figure 4-7A, B**). The similar transcriptional profiles shared by iNKT17 cells and MAIT17 cells in *Cd4^{cre}Pten^{fl/fl}* mice was consistent with a previous study reporting on a common transcriptomic program acquired in the thymus by these unconventional T cells (Salou et al., 2019). Gene ontology (GO) analysis of differentially expressed genes at the 1.5-fold change cut-offs in the IL-17-producing CD1d:PBS57-tet $^-$ MR1:5-OP-RU-tet $^-$ population showed these cells were enriched for genes associated with cell activation (**Figure 4-7C**). IPA analysis revealed PTEN-deficiency affected multiple canonical pathways implicated in T helper cell differentiation, activation, autoimmune diseases, immune signaling mediated by costimulatory molecules, and others (**Figure 4-8**).

Clearly, the IL-17-producing thymocyte population in *Cd4^{cre}Pten^{fl/fl}* mice was highly heterogeneous, and the CD1d:PBS57-tet $^-$ MR1:5-OP-RU-tet $^-$ fraction was markedly different from the two semi-invariant unconventional T cell populations so far identified. Next, we reassessed the expression of CD4 and CD8 coreceptors among the CD1d:PBS57-tet $^-$ MR1:5-OP-RU-tet $^-$ fraction of the IL-17-producing thymocytes. As shown in **Figure 4-9A**, these cells were mainly CD8 single-positive and CD4 $^-$ CD8 $^-$ double-negative. Within those expressing the CD8 coreceptor, we evaluated the differential expression of the CD8 α - and β - chains. Surprisingly, nearly two thirds of the CD8 $^+$ cells expressed the CD8 $\alpha\alpha$ homodimer, rather than the typical CD8 $\alpha\beta$ heterodimer (**Figure 4-9A**). The CD8 $\alpha\alpha$ homodimer does not behave as a coreceptor but instead binds to the nonclassical MHC molecule thymic leukemia (TL) antigen (Leishman et al., 2001). This is in contrast to the CD8 $\alpha\beta$ heterodimer, which is a TCR coreceptor that enhances functional avidity and that is constitutively expressed on MHC class I-restricted T cells. Therefore, we considered the CD8 $\alpha\beta$ -expressing fraction to be Tc17 cells and further investigated the CD8 $\alpha\alpha^+$ cells.

CD8 $\alpha\alpha^+$ T cells are generally considered intraepithelial lymphocytes (IEL) that have self-reactive, but not self-destructive, features, which enable them to act as a regulatory cell population that maintains tolerance in the gut (Cheroutre, 2004; Olivares-Villagomez and Van Kaer, 2018). Thymic CD8 $\alpha\alpha^+$ T cells are immature IEL precursors that become CD4, CD8 $\alpha\beta$, and CD8 $\alpha\alpha$ triple-positive (TP) progenitors before undergoing agonist selection. After selection, they lose expression of all three coreceptors and leave the thymus and migrate directly to the gut as CD4 $^-$ CD8 $^-$ DN TCR $\alpha\beta^+$ lymphocytes that display the CD5 receptor on their surface (Gangadharan et al., 2006). Upon entering the IL-15-rich environment of the intestine, CD8 $\alpha\alpha^+$ progenitors become

Figure 4-7. Microarray analysis of IL-17-producing thymocyte subpopulations in *Cd4^{cre}Pten^{fl/fl}* mice.

(A) PCA mapping of the iNKT17 (CD1d:PBS57-tet⁺, labeled as CD1d), MAIT17 (MR1:5-OP-RU-tet⁺, labeled as MR1), and CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ (labeled as DN) fractions of the IL-17-producing thymocyte population in *Cd4^{cre}Pten^{fl/fl}* mice. (B) Heat map of the transcriptional profiles of the iNKT17 (labeled as CD1d⁺), MAIT17 (labeled as MR1⁺), and CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ (labeled as CD1d⁻MR1⁻) fractions of the IL-17-producing thymocyte population in *Cd4^{cre}Pten^{fl/fl}* mice. (C) List of top 15 gene sets upregulated in the CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ fraction of the IL-17-producing thymocyte population of *Cd4^{cre}Pten^{fl/fl}* mice by gene ontology, based on the differentially expressed genes at the 1.5-fold cut-offs, as compared to the iNKT17 and MAIT17 fractions combined.

Figure 4-8. Ingenuity pathway analysis (IPA) of the differentially expressed genes in the CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ fraction of the IL-17-producing thymocyte population of *Cd4^{cre}Pten^{fl/fl}* mice.

IPA of canonical pathways enriched within the differentially expressed genes at the 1.5-fold cut-offs of the CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ fraction of the IL-17-producing thymocyte population in *Cd4^{cre}Pten^{fl/fl}* mice, as compared to the iNKT17 and MAIT17 fractions combined.

Figure 4-9. Phenotypic characterization of the CD1d:PBS57-tet⁺MR1:5-OP-RU-tet⁻ fraction of CCR6⁺CD127⁺ thymocytes.

(A) Flow cytometric analysis of CD4 and CD8α expression on CCR6⁺CD127⁺ CD1d:PBS57-tet⁺MR1:5-OP-RU-tet⁻ thymocytes in WT and *Cd4^{cre}Pten^{fl/fl}* mice (top, left), along with further characterization of CD8β expression in the CD8α⁺ (top, center) and the CD4⁺CD8α⁺ DP compartments (top, right). Frequencies (middle) and cellularity (bottom) of populations described are shown. (B) Flow cytometric analysis of markers associated with CD8α⁺ IEL precursors. Data are means ± SEM. **P* < 0.05 and ***P* < 0.01. Two-tailed Mann-Whitney test (frequency) and two-tailed unpaired *t*-test (cellularity). Data are representative/combination of at least three independent experiments. Numbers indicate percentage of cells in gates.

mature CD8 $\alpha\alpha$ ⁺TCR $\alpha\beta$ ⁺ IELs (Bruce and Cantorna, 2011). This dynamic expression of coreceptors appeared to be paralleled by the CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ fraction of the IL-17-producing thymocyte population of *Cd4^{cre}Pten^{fl/fl}* mice, which showed significantly enriched CD8 $\alpha\alpha$ ⁺, TP, and CD5-expressing DN sub-fractions (**Figures 4-9A, B**).

It was recently reported that CD8 $\alpha\alpha$ ⁺ IELs arise from two main thymic precursors that express either PD-1 (PD-1⁺T-BET⁻, type A IELp) or T-BET (PD-1⁻T-BET⁺, type B IELp) (Ruscher et al., 2017). We previously showed that the PTEN-deficient IL-17-producing thymocytes were T-BET⁻ (**Figure 4-4C**), so we next assessed PD-1 expression within the CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻CD4⁻CD8⁻ (referred to as DN17) fraction. The vast majority of DN17 cells in *Cd4^{cre}Pten^{fl/fl}* mice were PD-1⁺, consistent with a type A IELp phenotype (**Figure 4-9B**). The expression of the cytokine receptor subunit CD122 (IL-2R β ; shared with the receptor for the cytokine IL-15) is characteristic of IELps and mature TCR $\alpha\beta$ ⁺ IELs (Olivares-Villagomez and Van Kaer, 2018). Strikingly, CD122 protein expression was not detected on PTEN-deficient DN17 cells (**Figure 4-9B**). However, Sca-1 (Ly-6A), which in peripheral CD4⁻CD8⁻ DN cells has been implicated in mediating immunosuppressive functions (Holmes and Stanford, 2007; Lee et al., 2011; Zhang et al., 2002), was highly expressed (**Figure 4-9B**). Thus, these data suggest PTEN-deficient DN17 cells are not *bona fide* IELps, but rather an undescribed IL-17-producing DN population that resides in the thymus.

Cell-Intrinsic Role of PTEN in the Development of Unconventional IL-17-Producing Thymocytes

To confirm a cell-intrinsic role for PTEN in the development of IL-17-producing thymocytes, we generated mixed BM chimeras consisting of congenitally marked CD45.1⁺ spike cells and CD45.2⁺ donor cells from either WT or *Cd4^{cre}Pten^{fl/fl}* mice in a 1:1 ratio. In support of a cell-intrinsic role, only the PTEN-deficient compartment of the mixed BM chimeras (denoted as CD45.2⁺ *Pten^{fl/fl}*) had enriched IL-17 production (**Figures 4-10A through C**). Like in the *Cd4^{cre}Pten^{fl/fl}* mice, the IL-17-producing thymocytes in the mixed BM chimeras were a heterogeneous population composed of MAIT17 cells, iNKT17 cells, and other CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ IL-17-producing thymocytes (**Figure 4-10D**). These data show PTEN restrains the development of unconventional IL-17-producing thymocytes in a cell-intrinsic manner.

Cellular and Molecular Mechanisms Mediating the Development of Unconventional IL-17-Producing Thymocytes in *Cd4^{cre}Pten^{fl/fl}* Mice

Cell Survival and Death

Considering that both iNKT and MAIT cell development diverges from conventional T cells at the DP stage, and that MAIT cells use the most distal segment of

Figure 4-10. PTEN plays a cell intrinsic role in the development of IL-17-producing thymocytes

(A) Flow cytometric analysis of IL-17A production by CD45.2⁺ WT or *Cd4^{cre}Pten^{fl/fl}* donor-derived thymocytes (upper panels), or by CD45.1⁺ spike-derived cells (lower panels) of mixed BM chimeras. (B and C) Ratio of CD45.2- to CD45.1-derived IL-17A⁺ (B) and CCR6⁺CD127⁺ (C) thymocytes in the mixed BM chimeras in (A). (D) Composition of IL-17-producing thymocytes in CD45.2⁺ (upper panels) and CD45.1⁺ (lower panels) compartments in the mixed BM chimeras in (A). Data are means $\pm$ SEM. *** $P < 0.001$ and **** $P < 0.0001$. One-tailed unpaired *t*-test. Data are representative/combination of three independent experiments. Numbers indicate percentage of cells in quadrants or gates.

the V locus, which means they get selected at the late DP stage (Le Bourhis et al., 2011), we evaluated the survival of pre- and post-positive selection DP cells. First, we assessed the transcript levels of *Bcl2l1* (Bcl-x_L) and *Rorc* (ROR γ t), the two master regulators of lifespan of DP cells (Sun et al., 2000; Wang et al., 2011). Surprisingly, and opposite to what we expected, both *Bcl2l1* and *Rorc* appeared downregulated in PTEN-deficient pre-positive selection DP cells (**Figure 4-11A**). Within the post-positive selection compartment, *Bcl2l1* transcripts levels were comparable between WT and PTEN-deficient DP cells, whereas *Rorc* was sharply upregulated (**Figure 4-11A**).

Consistent with decreased transcriptional expression of *Bcl2l1* and *Rorc* at the pre-positive selection stage of PTEN-deficient DP cells, these cells presented increased caspase-3 activity as compared to their WT counterparts, and so did the post-positive selection PTEN-deficient DP cells (**Figure 4-11B**). These defects in DP cells prompted us to look at overall cell death in thymus of *Cd4^{cre}Pten^{fl/fl}* mice. No striking differences were observed among total thymocytes, however (**Figure 4-11C**). Furthermore, we evaluated the distribution of cells dying by clonal deletion (CD69^{high}CD5^{high}) or neglect (CD69^{low}CD5^{low}) and found no major apparent differences in either group (**Figure 4-11C**). Altogether, these data suggest the aberrant development of unconventional IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice is not due to enhanced survival of DP cells during positive selection.

Cell Proliferation

We next investigated the capacity of PTEN-deficient unconventional T cells to proliferate. To this end, we stained total iNKT cells and MAIT cells with Ki67 and found no significant difference in iNKT cells, but Ki67 decreased staining in PTEN-deficient MAIT cells (**Figure 4-11D**). We further characterized the proliferation status of each iNKT cell subset and observed a striking decrease in Ki67 staining among PTEN-deficient iNKT17 cells, but not in the other subsets (**Figure 4-11E**). Finally, we performed overnight *in vivo* BrdU staining and observed fewer BrdU-incorporated iNKT cells and MAIT cells among PTEN-deficient thymocytes (**Figure 4-11F**), suggesting an overall defect in cell proliferation. Together, these results indicated that the enrichment of iNKT17 cells and MAIT17 cells in *Cd4^{cre}Pten^{fl/fl}* mice was not due to elevated proliferation of these thymic subsets. On the contrary, these data suggest PTEN-deficient unconventional thymic groups have an impaired capacity to proliferate.

mTORC2/FoxO1/ROR γ t Signaling Axis

Our lab previously showed mTORC2 signaling plays a crucial role in the development of iNKT cells and, in particular, fate determination of the type 17 subset (Wei et al., 2014). Deletion of RICTOR in those studies caused a complete ablation of the iNKT17 lineage, even in the absence of PTEN, which enhanced iNKT17 generation. Provided PTEN gets deleted in the T cell lineage of *Cd4^{cre}Pten^{fl/fl}* mice starting at the DP stage and that it is at the DP stage when iNKT cells and MAIT cells diverge from

Figure 4-11. Analysis of cell survival and proliferation in *Cd4^{cre}Pten^{fl/fl}* mice. (A) Relative expression of *Bcl2l1* and *Rorc* in pre- (CD69⁻TCRβ⁻) and post- (CD69⁺TCRβ⁺) positive selection DP cells of WT and *Cd4^{cre}Pten^{fl/fl}* mice. WT is set to 1. (B) Active caspase-3 staining in pre- and post-positive selection DP cells of WT and *Cd4^{cre}Pten^{fl/fl}* mice with frequencies on the right. (C) Active caspase-3 staining in total thymocytes (left) and identification of cells dying by clonal deletion (CD69^{high}CD5^{high}) or by neglect (CD69^{low}CD5^{low}) (right) in WT and *Cd4^{cre}Pten^{fl/fl}* mice. (D and E) Ki67 staining (left) in thymic iNKT and MAIT cells (D) or in thymic iNKT subsets (E) of WT and *Cd4^{cre}Pten^{fl/fl}* mice with frequencies on the right. (F) *In vivo* BrdU incorporation in thymic iNKT and MAIT cells of WT and *Cd4^{cre}Pten^{fl/fl}* mice. Data are means ± SEM. **P* < 0.05, and ***P* < 0.01. One-tailed unpaired *t* test (A) and two-tailed Mann-Whitney test (B, D and E). Data are representative/combination of two (A) or at least three (B-F) independent experiments. Numbers indicate percentage of cells in gates.

conventional T cell development, we assessed mTORC2 activity at this critical phase of T cell development. Consistent with PTEN-mediated regulation of mTORC2 activity, we found that unstimulated PTEN-deficient DP cells had increased AKT S473 phosphorylation, which is a signature of mTORC2 activity (**Figure 4-12A**). To evaluate the involvement of RICTOR/mTORC2 signaling in type 17 unconventional T cell development in the context of PTEN deficiency, we crossed *Cd4^{cre}Pten^{fl/fl}* mice with mice carrying loxP-flanked *Rictor* alleles (*Rictor^{fl/fl}*). The progeny of these mice (*Cd4^{cre}Pten^{fl/fl}Rictor^{fl/fl}*) was deficient for both PTEN and RICTOR in the T cell lineage compartment. Deletion of RICTOR rescued the enrichment of unconventional IL-17-producing thymocytes observed in *Cd4^{cre}Pten^{fl/fl}* mice, an effect that was not restricted to the iNKT17 cell compartment only (**Figures 4-12B, C**). Noticeably, RICTOR deficiency restored the total number of MAIT cells in *Cd4^{cre}Pten^{fl/fl}* mice to WT levels (**Figure 4-12D**). Therefore, RICTOR-mediated signaling drives the expansion of unconventional IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice.

It was recently reported that FoxO1 also controls effector lineage fate decisions of iNKT cells by promoting iNKT1 but suppressing iNKT17 lineages (Tao et al., 2019). Furthermore, FoxO1 has been previously described as a T cell-intrinsic inhibitor of the ROR γ ⁺ T_H17 differentiation program (Laine et al., 2015) and it is known to be controlled by AKT-mediated phosphorylation and nuclear exclusion (Chi, 2012). This prompted us to evaluate FoxO1 signaling as a potential downstream mediator of PTEN/mTORC2-dependent control of unconventional IL-17-producing thymocyte development. As expected, we observed a marked increase in FoxO1 phosphorylation among PTEN-deficient DP cells, which was rescued by RICTOR deficiency (**Figure 4-13A**). Due to the limited breeding of *Cd4^{cre}Foxo1^{fl/fl}* mice, we generated full BM chimeras from WT and *Cd4^{cre}Foxo1^{fl/fl}* donors to rapidly expand our colony. We found *Cd4^{cre}Foxo1^{fl/fl}* full BM chimeras modestly mimicked the phenotype of *Cd4^{cre}Pten^{fl/fl}* mice in that they had a significantly enriched CCR6⁺CD127⁺/IL-17-producing thymocyte population composed of iNKT cells, MAIT cells, and other CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ cells (**Figures 4-13B, C**). Altogether, these results suggest that the involvement of the mTORC2/FoxO1 signaling axis in type 17 fate choices of iNKT cells expands beyond this particular lineage of unconventional T cells and is conserved among MAIT cells and other unconventional subsets.

IL-23/STAT3/ROR γ t Signaling Axis

The specificity of the phenotype observed in *Cd4^{cre}Pten^{fl/fl}* mice to the type 17 lineage led us to inspect the involvement of molecular players important for IL-17 production and type 17 lineage fate of $\alpha\beta$ -T cells. IL-6-dependent STAT3 activation is thought to be crucial for ROR γ t expression and for the development of T_H17 cells (Durant et al., 2010; Yang et al., 2007). To study the role of STAT3 in the development of unconventional IL-17-producing thymocytes in the context of PTEN deficiency, we crossed *Cd4^{cre}Pten^{fl/fl}* mice with mice carrying loxP-flanked *Stat3* alleles (*Stat3^{fl/fl}*). The progeny of these mice (*Cd4^{cre}Pten^{fl/fl}Stat3^{fl/fl}*) was, therefore, deficient in PTEN and STAT3 in their T cell lineage compartment. STAT3 deficiency rescued the accumulation

Figure 4-12. RICTOR-mediated signaling drives the expansion of unconventional IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice.

(A) Flow cytometric analysis of pAKT (S473) in DP (CD4⁺CD8⁺) cells of WT and *Cd4^{cre}Pten^{fl/fl}* mice with MFI plotted in graphs. (B) Flow cytometric analysis of CD127 and CCR6 expression in thymus (top left), along with 5-OP-RU-loaded MR1 tetramer and PBS57-loaded CD1d tetramer staining of the CCR6⁺CD127⁺ thymocyte population (bottom left) in WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Rictor^{fl/fl}* mice. Cellularity of each of the thymic subsets analyzed is shown (right). (C) Flow cytometric analysis (left) and cellularity (right) of IL-17-producing thymocytes in WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Rictor^{fl/fl}* mice after ICS stimulation. (D) 5-OP-RU-loaded MR1 tetramer staining (left) of total thymocytes in WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Rictor^{fl/fl}* mice, along with cellularity (right). Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. One-way (total CCR6⁺CD127⁺ population in (B), and (C, D)) or two-way (CCR6⁺CD127⁺ subsets in (B)) ANOVA with Tukey's test. Data are representative/combination of four independent experiments. Numbers indicate percentage of cells in gates or quadrants.

Figure 4-13. FoxO1 signaling is involved in the development of unconventional IL-17-producing thymocytes.

(A) Flow cytometric analysis of p-FoxO1 in DP ($CD4^+CD8^+$) cells of WT, $Cd4^{cre}Pten^{fl/fl}$, and $Cd4^{cre}Pten^{fl/fl}Rictor^{fl/fl}$ mice, with MFI plotted in graphs. (B) Flow cytometric analysis of CD127 and CCR6 expression in thymus (top left), along with 5-OP-RU-loaded MR1 tetramer and PBS57-loaded CD1d tetramer staining of the CCR6⁺CD127⁺ thymocyte population in full BM chimeras made with WT or $Cd4^{cre}Foxo1^{fl/fl}$ donor cells. Cellularity of each of the subsets analyzed is shown (right). (C) Flow cytometric analysis of IL-17 production by total thymocytes of full BM chimeras made with WT or $Cd4^{cre}Foxo1^{fl/fl}$ donor cells. Data are means $\pm$ SEM. * $P < 0.05$. Two-tailed unpaired t -test. Data are representative/combination of two independent experiments. Numbers indicate percentage of cells in gates or quadrants.

of CCR6⁺CD127⁺ cells in *Cd4^{cre}Pten^{fl/fl}* mice, including the various subsets (**Figure 4-14A**). This effect was further evidenced by the reduction of total IL-17 production among thymocytes (**Figure 4-14B**). Similar to RICTOR, the effects of STAT3 deficiency in the MAIT compartment went beyond just blocking IL-17 production, as the total number of MAIT cells was returned to WT levels (**Figure 4-14C**). These results suggest STAT3 signaling also mediates the accumulation of unconventional IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice.

As alluded before, binding of IL-6 to the IL-6R on the cell membrane of T cells activates STAT3, which then drives transcription of ROR γ t and other type 17 target genes. However, studies done by others in our lab revealed IL-6 was dispensable for the production of IL-17 by PTEN-deficient thymocytes and that so was the transcription factor ROR α (unpublished data). In addition to IL-6, IL-23 also activates STAT3 and drives the differentiation and stabilization of pathogenic T_H17 cells (Gaffen et al., 2014). Furthermore, in the context of thymic T cell development, IL-23 has been reported to promote TCR-mediated negative selection of thymocytes by upregulating ROR γ t and IL-23R (Li et al., 2014). Thus, we next assessed the involvement of IL-23 in the generation of IL-17-producing thymocytes in the absence of PTEN. For this, we crossed *Cd4^{cre}Pten^{fl/fl}* mice with IL-23-germline deleted mice (*Il23^{-/-}*) and evaluated the accumulation of IL-17-producing thymocytes in the progeny (*Cd4^{cre}Pten^{fl/fl}Il23^{-/-}*). Similar to *Cd4^{cre}Pten^{fl/fl}Stat3^{fl/fl}* mice, *Cd4^{cre}Pten^{fl/fl}Il23^{-/-}* largely rescued the accumulation of PTEN-deficient CCR6⁺CD127⁺/IL-17-producing thymocytes, including the three major subsets identified (**Figure 4-15A, B**). Interestingly, and consistent with the effects of RICTOR and STAT3 deficiency, absence of IL-23 not only blocked IL-17 production by MAIT cells, but also prevented their overall accumulation (**Figure 4-15C**). Altogether, these results suggest that the IL-23/STAT3/ROR γ t signaling axis, independently of IL-6 and ROR α , drive the development and accumulation of unconventional IL-17-producing thymocytes in the absence of PTEN.

Summary

In this chapter, we explored the role of PTEN in the development of TCR $\alpha\beta$ ⁺ cells. To circumvent the tumorigenic effects of PTEN deletion in the T cell compartment, we analyzed mice younger than 6 weeks of age, which showed no signs of tumorigenesis and presented relatively normal $\alpha\beta$ -T cell development. Unexpectedly, we found deletion of PTEN at the DP stage of $\alpha\beta$ -T cell development caused an enrichment of IL-17-producing lymphocytes that were restricted to the thymus. Interestingly, the PTEN-deficient IL-17-producing thymocytes were not conventional nT_H17 cells but rather constituted a heterogeneous population composed of iNKT17 cells, MAIT17 cells, and other unconventional T cell populations. As shown by the mixed BM chimera experiments, the effects of PTEN deficiency in the development and accumulation of unconventional IL-17-producing thymocytes were cell-intrinsic. Furthermore, the accumulation of PTEN-deficient IL-17-producing thymocytes was independent of pre-positive selection survival and post-positive selection proliferation. On the contrary, PTEN-deficient pre-positive selection DP cells had increased caspase-3 activity, an

Figure 4-14. STAT3 deficiency rescues the accumulation of unconventional IL-17-producing thymocytes in the context of PTEN deficiency.

(A) Flow cytometric analysis of CD127 and CCR6 expression in thymus (top left), along with 5-OP-RU-loaded MR1 tetramer and PBS57-loaded CD1d tetramer staining of the CCR6⁺CD127⁺ thymocyte population (bottom left) in WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Stat3^{fl/fl}* mice. Cellularity of each of the thymic subsets analyzed is shown (right). (B) Flow cytometric analysis (left) and cellularity (right) of IL-17-producing thymocytes in WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Stat3^{fl/fl}* mice after ICS stimulation. (C) 5-OP-RU-loaded MR1 tetramer staining (left) in total thymocytes of WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Rictor^{fl/fl}* mice, along with cellularity (right). Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. One-way (total CCR6⁺CD127⁺ population in (A), and (B, C)) or two-way (CCR6⁺CD127⁺ subsets in (A)) ANOVA with Tukey's test. Data are representative of three independent experiments. Numbers indicate percentage of cells in gates or quadrants.

Figure 4-15. IL-23 deficiency rescues the accumulation of unconventional IL-17-producing thymocytes in the context of PTEN deficiency.

(A) Flow cytometric analysis of CD127 and CCR6 expression in thymus (top left), along with 5-OP-RU-loaded MR1 tetramer and PBS57-loaded CD1d tetramer staining of the CCR6⁺CD127⁺ thymocyte population (bottom left) in WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Il23^{-/-}* mice. Cellularity of each of the thymic subsets analyzed is shown (right). (B) Flow cytometric analysis (left) and cellularity (right) of IL-17-producing thymocytes in WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Il23^{-/-}* mice after ICS stimulation. (C) 5-OP-RU-loaded MR1 tetramer staining (left) in total thymocytes of WT, *Cd4^{cre}Pten^{fl/fl}*, and *Cd4^{cre}Pten^{fl/fl}Il23^{-/-}* mice, along with cellularity (right). Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. One-way (total CCR6⁺CD127⁺ population in (A), and (B, C)) or two-way (CCR6⁺CD127⁺ subsets in (A)) ANOVA with Tukey's test. Data are representative of at least four independent experiments. Numbers indicate percentage of cells in gates or quadrants.

indicator of apoptosis, and both PTEN-deficient iNKT17 cells and MAIT17 cells had reduced proliferation. Studies on the molecular mechanisms associated with the accumulation of IL-17-producing thymocytes in the context of PTEN deficiency revealed that both the RICTOR/FoxO1 and the IL-23/STAT3 signaling pathways converged to drive ROR γ t-dependent development of type 17 unconventional T cells. Based on studies done by others in our lab, this aforementioned molecular control was independent of the cytokine IL-6 and the transcription factor ROR α (unpublished data). Altogether, these results reveal PTEN acts as a cell-intrinsic molecular brake for the development of unconventional IL-17-producing thymocytes.

CHAPTER 5. DISCUSSION³

Unconventional T cells have emerging roles in tissue homeostasis and disease (Iwabuchi and Van Kaer, 2019). Their conserved nature among mammals highlights the importance of these unique T cell populations in the immune system (Moreira et al., 2017). However, we are just now beginning to elucidate the molecular mechanisms mediating their development and fate decisions (Godfrey et al., 2015; Lantz and Legoux, 2019). Despite recent advances in the field of immunometabolism, it remains largely unknown what role metabolic signals play in T cell lineage decisions and, in particular, in the development of unconventional T cells. Our studies revealed that RAPTOR/mTORC1 signaling dictates the lineage choices of $\alpha\beta$ and $\gamma\delta$ -T cells by integrating metabolic activities and TCR signaling strength in developing T cells. Furthermore, we identified mTORC1-dependent control of ROS production as a key metabolic signal in this process and unraveled PTEN as a cell-intrinsic molecular brake for the development and accumulation of type 17 unconventional thymocytes. Altogether, our results point to metabolic processes and signaling as fundamental mechanisms that connect extrinsic stimuli with transcriptional events and T cell fate decisions.

An extensive body of literature highlights the role of metabolic reprogramming in controlling T cell-mediated immune responses (Chapman et al., 2020; Pearce et al., 2013; Pollizzi and Powell, 2014). Although NOTCH and IL-7 signaling have been shown to exert trophic effects in T cell development, little is known about the metabolic programs underpinning thymocyte development (Boudil et al., 2015; Ciofani and Zuniga-Pflucker, 2005). Noticeably, the adaptive immune system is characterized by the fluctuation and intertwining of cycles of quiescence and activation that accompany the developmental and functional progression of T lymphocytes throughout their lifespan (Chapman et al., 2020). Largely nondividing progenitor cells colonize the thymus, which later undergo two waves of proliferation separated by a brief period of quiescence required for TCR β -chain rearrangement at the DN3 stage (Carpenter and Bosselut, 2010). Upon arrival to the DP stage, developing thymocytes return to quiescence to undergo TCR α -chain rearrangement. Metabolic assessment of thymocyte populations revealed that DN3 and DP cells preferentially use OXPHOS, consistent with their quiescent state, whereas DN4 and ISP cells engage anabolic programs in association with their robust expansion. Moreover, we found that DN3 cells have a greater SRC in comparison to other thymic subsets, indicating that they have stronger mitochondrial capacity to produce energy in response to stress. The use of distinct metabolic programs by developing thymocytes likely fulfills the bioenergetic demands and signaling requirements of the underlying developmental processes. Importantly, these dynamic metabolic programs are associated

³ Portions of chapter from previously published article; final submission modified with permission of AAAS. Yang, K.*, Bastardo Blanco, D.*, Chen, X.*, Dash, P., Neale, G., Rosencrance, C., Easton, J., Chen, W., Cheng, C., Dhungana, Y., KC, A., Awad, W., Guo, X., Thomas, P., Chi, H. (2018). Metabolic signal directs the reciprocal lineage decisions of ab and gd T cells. *Science Immunology* **3**. (* Co-first authors). DOI: <https://doi.org/10.1126/sciimmunol.aas9818>.

with differential regulation of mTORC1 activity, suggesting a role for mTORC1-dependent control of metabolic reprogramming during T cell development.

Recent studies have highlighted the role of mTOR in thymocyte development (Hoshii et al., 2014; Lee et al., 2012; Tang et al., 2012). However, the involvement of mTORC1 signaling in $\alpha\beta$ and $\gamma\delta$ -T cell lineage choices had remained unexplored. Using genetic deletion of RAPTOR, our studies revealed that RAPTOR-dependent mTORC1 is a crucial mediator of T cell development and lineage choices. Loss of RAPTOR impaired $\alpha\beta$ but promoted $\gamma\delta$ -T cell development. Our studies using mixed BM chimeras and the OP9-DL1 cell coculture system demonstrated that this effect occurred in a cell-intrinsic manner. Additionally, RAPTOR-deficient $\gamma\delta$ -T cells expressed high levels of CD73, a marker that has been linked to TCR $\gamma\delta^+$ lineage commitment (Coffey et al., 2014), further supporting a role for RAPTOR in limiting $\gamma\delta$ -T cell development.

The RAPTOR-dependent mTOR control of $\gamma\delta$ -T cell development was independent of RICTOR, as RICTOR-deficient thymocytes presented no defects in lineage choices. However, concomitant deletion of RAPTOR and RICTOR further exacerbated the altered development of $\alpha\beta$ and $\gamma\delta$ -T cells. This genetic redundancy of mTORC1 and mTORC2 in T cell development was unique and distinct from their relationship in peripheral T cells, in which additional loss of RICTOR either ameliorates or has little impact on the phenotypes caused by RAPTOR deficiency (Yang et al., 2013; Zeng et al., 2013). These findings highlight the predominant role of RAPTOR-dependent mTORC1 signaling in T cell lineage commitment.

Considering that RAPTOR-deficient DN3 cells presented defective glycolytic rate and faulty OCR/ECAR balance, our data suggested that RAPTOR-dependent mTORC1 activation and metabolic reprogramming drove quiescence exit of DN3 cells. Additionally, RAPTOR-deficient thymocytes were less efficient in undergoing population expansion, especially during the ISP stage. Given the intricate relationship between cell proliferation and differentiation (Kreslavsky et al., 2012), such proliferative defect likely contributed to the developmental blockade and accumulation of ISP cells in *Cd2^{cre}Rptor^{fl/fl}* mice. These results suggest that RAPTOR-dependent metabolic reprogramming and quiescence exit are fundamental determinants of population expansion and lineage specification in thymocyte development.

The disruption of anabolic metabolism by genetic ablation of RAPTOR resulted in dysregulated ROS production. The elevated levels of ROS in RAPTOR-deficient thymocytes impinged upon their fate decisions as antioxidant treatment of these cells rescued, albeit incompletely, their differentiation defect. Furthermore, and similarly to the RAPTOR-associated phenotypes, pharmacological perturbation of redox homeostasis in WT thymocytes impinged upon their fate decisions. In fact, either boosting or reducing ROS production in DN3 cells had a direct impact on their fate choices toward $\alpha\beta$ or $\gamma\delta$ -T cell lineages. These results reveal a selective role for redox regulation in mediating cell fate decisions downstream of the multifunctional kinase mTORC1 and show that metabolic signaling is a decisive factor in the fate decisions of $\alpha\beta$ and $\gamma\delta$ -T cells during thymic development.

The development of $\alpha\beta$ and $\gamma\delta$ -T cells is contingent upon the fate decisions of a common bipotent thymocyte progenitor, namely the DN3 cells. TCR signaling strength has emerged as an instructional signal that determines T cell lineage fate decisions of uncommitted precursors (Haks et al., 2005; Hayes et al., 2005; Lee et al., 2014; Zarin et al., 2014). Specifically, the ERK/EGR1/ID3 signaling axis is now recognized to play a crucial role in this process, in such a way that strong signals promote the development of $\gamma\delta$ -T cells, whereas weak signals facilitate $\alpha\beta$ -T cell development (Haks et al., 2005; Hayes et al., 2005). What regulates the strength of these TCR signals has remained elusive, however. We found that loss of RAPTOR markedly promoted the activation of ERK, EGR1, and ID3 in DN3 cells, and that this effect was associated with increased development of lineage-committed $\gamma\delta$ -T cells. Additionally, increased ROS production by WT DN3 cells resulted in increased ID3 and CD73 expression. Collectively, our results suggest that mTORC1 activity and ROS metabolism impinge on the activity of the ERK/EGR1/ID3 signaling axis, therefore, influencing TCR signaling strength and the subsequent lineage choices of DN3 cells (**Figure 5-1**).

As an upstream regulator of mTOR, PTEN governs multiple cellular processes, including survival, proliferation, energy metabolism, and cellular architecture (Song et al., 2012). Selective deletion of PTEN in T cells induces the development of fatal T cell lymphomas along with the establishment of autoimmune disorders (Liu et al., 2010b; Suzuki et al., 2001). As our work with *Cd4^{cre}Pten^{fl/fl}* mice confirmed, before the onset of tumorigenesis in PTEN-deficient mice, thymocyte development proceeds largely normal with unchanged cellularity or distribution of major thymic subsets (Buckler et al., 2006). However, as our studies revealed, PTEN deficiency in developing thymocytes causes the accumulation of unconventional thymic-resident IL-17-producing lymphocytes.

In addition to the unconventional TCR $\gamma\delta^+$ T cell lineage, there are also TCR $\alpha\beta^+$ T cells that have an unconventional development and innate-like phenotype (Godfrey et al., 2015). Such unconventional lymphocytes have a limited TCR repertoire and present innate-like effector functions (Pasman and Kasper, 2017). Due to their prevalence in mice, iNKT cells are the most well-studied type of unconventional $\alpha\beta$ -T cells (Garner et al., 2018). However, the recent development of MR1 tetramers for the easy identification of MAIT cells (Rahimpour et al., 2015), along with their strong predominance in humans (Lantz and Legoux, 2018), has promoted an emerging interest in this unconventional population. Interestingly, the enrichment of IL-17-producing thymocytes in *Cd4^{cre}Pten^{fl/fl}* mice was mediated by the accumulation of a heterogeneous population of iNKT17 cells, MAIT17 cells, and a third CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ population.

Phenotypic characterization of PTEN-deficient IL-17-producing thymocytes showed that the MAIT compartment was composed of fully mature MAIT17 cells, which had a transcriptional profile that resembled that of their iNKT17 counterparts. The CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ population, however, had a markedly distinct transcriptome and presented further heterogeneity. In particular, we were able to identify at least three populations of T cells within the CD1d:PBS57-tet⁻MR1:5-OP-RU-tet⁻ fraction of the PTEN-deficient IL-17-producing thymocytes. These were Tc17 cells, CD8 $\alpha\alpha^+$ IEL-like precursors (both CD8 $\alpha\alpha^+$ and TP), and a previously uncharacterized

Figure 5-1. Schematic of RAPTOR/mTORC1-mediated control of metabolic remodeling, redox homeostasis, and TCR signal strength in T cell lineage choices. Activation of mTORC1 by TCR and NOTCH signaling orchestrates cellular metabolism of developing thymocytes. Dynamic metabolic remodeling, including the proper balance between OXPHOS and glycolysis, contributes to anabolism and cell expansion. However, dysregulated metabolism results in aberrant ROS production, which alters redox balance and impinges upon TCR signaling strength, thereby altering lineage choices of $\alpha\beta$ and $\gamma\delta$ -T cells.

thymic population of IL-17-producing CD4⁺CD8⁻ DN cells that expressed high levels of CD5, PD-1, and Sca-1, but not CD122. These results suggest a conserved mechanism controlled by PTEN for the development of type 17 unconventional T cells.

Recent studies have highlighted the role microbial metabolites play in controlling the thymic development of MAIT cells (Constantinides et al., 2019; Legoux et al., 2019a). These studies have shown that microbiota-derived metabolites are indispensable for the development of MAIT cells and that they act as cell-extrinsic facilitators of this process. Our studies done in the context of mixed BM chimeras indicated that PTEN acts as a cell-intrinsic negative regulator of the development and accumulation of IL-17-producing thymocytes, including MAIT17 cells. Therefore, our data highlights a second level of regulation for the development of MAIT cells that is cell-intrinsic.

PTEN has previously been shown to be an important regulator of proliferation of developing thymocytes, as loss of PTEN allows TCR $\alpha\beta$ -lineage thymocytes to bypass IL-7 and pre-TCR-mediated signaling in *Lck^{cre}Pten^{fl/fl}* mice (Hagenbeek et al., 2004). However, our studies in *Cd4^{cre}Pten^{fl/fl}* mice revealed pre-positive selection DP cells had reduced transcriptional expression of master regulators of DP survival, namely *Bcl2l1* (Bcl-x_L) and *Rorc* (ROR γ t), and, consequently, increased apoptosis. PTEN-deficient post-positive selection DP cells had markedly increased *Rorc* expression but, consistent with the literature (He et al., 2000), this effect did not translate to enhanced lifespan of these thymocytes. Furthermore, we found defective, not enhanced, proliferation among post-selection iNKT17 cells and MAIT cells. These results suggest PTEN plays a role in the development of innate-like IL-17-producing thymocytes that is independent of pre-positive selection survival or post-positive selection proliferation.

We next investigated molecular players downstream of PTEN mediating the development of unconventional IL-17-producing thymocytes. Consistent with previous findings from our lab (Wei et al., 2014), deletion of RICTOR caused a complete ablation of the iNKT17 lineage. Moreover, we found RICTOR was important for the development of all type 17 unconventional T cell populations, not just for the iNKT17 lineage. Our data suggested that this effect was mediated by FoxO1 signaling, consistent with evidence in the literature that FoxO1 suppresses the development of iNKT17 cells (Tao et al., 2019) and acts as a T cell-intrinsic inhibitor of ROR γ t⁺ T_H17 differentiation (Laine et al., 2015). Altogether, these results indicate a central role for the mTORC2/FoxO1 signaling axis in the activation of ROR γ t that drives the development of PTEN-deficient type 17 unconventional T cells.

In addition to the RICTOR/FoxO1/ROR γ t signaling pathway, which is known to be downstream of PTEN, we also assessed the involvement of other molecular players upstream of ROR γ t in the development of PTEN-deficient IL-17-producing thymocytes. In particular, we found STAT3 and IL-23 to be essential in the generation of type 17 unconventional thymocytes in the context of PTEN deficiency. Phosphorylation of STAT3 monomers drive their dimerization and translocation to the nucleus, where they mediate the transcription of the transcription factors ROR γ t and ROR α and the cytokines IL-17A, IL-17F, IL-21, and IL-23 (Tripathi et al., 2017; Wang et al., 2013b). The

cytokines IL-6 and IL-23 are known activators of STAT3 signaling (Rebe et al., 2013). Interestingly, experiments done by others in our lab showed that IL-6 and ROR α were dispensable for the generation of PTEN-deficient unconventional IL-17-producing thymocytes (unpublished data). Remarkably, deletion of either STAT3 or IL-23 in the context of PTEN-deficiency not only rescued the overproduction of IL-17, but also prevented the total accumulation of MAIT cells. These results suggest that the capacity of PTEN-deficient thymocytes to develop as unconventional T cells is contingent upon the activation of the IL-23/STAT3 signaling pathway.

The acquisition of innate-like effector function by unconventional T cells is dependent on the transcription factor PLZF (Cao et al., 2018). Our gene expression analyses showed that the PTEN-deficient IL-17-producing thymocytes had elevated PLZF activity. Studies in iNKT cells have shown RAPTOR-deficient iNKT cells have reduced nuclear localization of PLZF, suggesting mTORC1 promotes PLZF nuclear localization and, thereby, its activity (Yang et al., 2015). Considering the high transcriptional similarity between iNKT17 cells and MAIT17 cells, and that these unconventional lymphocytes constituted a significant fragment of the total PTEN-deficient IL-17-producing thymocyte population, it is possible that a similar mechanism of regulation occurs in the context of PTEN deficiency. However, transcriptional regulation of PLZF remained unexplored in our studies. Future work is warranted to understand the relationship between PLZF and ROR γ t, along with the RICTOR/FoxO1 and IL-23/STAT3 signaling pathways, in driving the development of type 17 unconventional T cells (**Figure 5-2**). Furthermore, it will be important to assess the physiological consequences of the accumulation of IL-17-producing thymocytes, especially as it concerns to thymic structure and medullary region homeostasis, which are disrupted in PTEN-deficient mice before the onset of disease (data not shown).

In summary, our studies revealed that thymocyte development is closely linked to dynamic regulation of metabolic programs, mTORC1 activity, and redox homeostasis, which together impinge upon T cell lineage commitment. Furthermore, we found that the tumor-suppressor PTEN is a cell-intrinsic negative regulator of the development of TCR $\alpha\beta$ ⁺ unconventional IL-17-producing thymocytes. Collectively, our studies establish mTORC1 signaling as a developmental checkpoint that links intrathymic environmental signals and metabolic programs to drive thymocyte development, and PTEN as a molecular brake for the development and accumulation of thymic-resident type 17 innate-like T cells. Future studies shall further dissect the cell context-dependent regulation and physiological effects of metabolic and redox controls in immune cell development and activation, particularly in the context of unconventional T cells.

Figure 5-2. Schematic of PTEN-mediated control of the development of unconventional IL-17-producing thymocytes.

Recognition of cognate antigen by TCR, along with ICOS costimulation, signals through RICTOR/mTORC2, which phosphorylates AKT. AKT, in turn, inhibits nuclear translocation of FoxO1 by phosphorylating it, and nuclear FoxO1 blocks RORγt-mediated transcription of IL-17. This process is controlled by the lipid phosphatase PTEN, which opposes PI3K-dependent phosphorylation of PIP2 and, therefore, activation of RICTOR/mTORC2. In parallel, engagement of IL-23 by the IL-23R, along with IL-7 stimulation, induces phosphorylation of STAT3 monomers, which allows for their dimerization and subsequent nuclear localization. In the nucleus, STAT3 mediates transcription of RORγt and the IL-17 family of cytokines. PLZF mediates the acquisition of innate-like features by unconventional T cells. However, the interrelation between PLZF and RORγt, along with the other molecular players identified, requires further investigation.

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VITA

Daniel Eduardo Bastardo Blanco was born in Cumaná, Venezuela, in 1991. In 2008, he moved to the United States to learn English at Eckerd College in St. Petersburg, FL, and, later that year, he was admitted into Elmira College in Elmira, NY, where he completed his undergraduate studies. During his time at Elmira College, Daniel conducted research in chemistry and microbiology under the advice of Dr. Dale Powers and Dr. Christine Bezotte, respectively. On the summer of 2011, Daniel was a summer undergraduate research fellow at Mayo Clinic in Rochester, MN, where he researched the effects of UV-light on the immune response of novel double transgenic mice (DR2.DQ8) in the lab of Dr. Chella David, under the advice of Dr. Ashutosh Mangalam. In 2012, he graduated summa cum laude with a bachelor's degree in Science in Biology from Elmira College and was named "Outstanding Member of the Class of 2012." In 2013, Daniel was admitted into the Integrated Biomedical Sciences Program of the University of Tennessee Health Science Center (UTHSC) in Memphis, TN, under the Microbiology, Immunology, and Biochemistry track. He joined the lab of Dr. Hongbo Chi in the Immunology Department of St. Jude Children's Research Hospital in 2014, where he conducted his dissertation studies. In Chi Lab, Daniel collaborated on several projects that explored mechanisms of immune signaling and cell metabolism that control the development, differentiation, and function of T cells. In 2017, Daniel was the winner of the Inaugural Memphis Scipreneur Challenge, and that year he became president of the UTHSC's Graduate Student Executive Council. In 2019, he was awarded the prestigious Mass Media Science & Engineering Fellowship of the American Association for the Advancement of Science for the development of science journalism skills; as part of the fellowship, Daniel temporarily moved to Milwaukee, WI, for three months to work for *Discover Magazine*. Daniel is the founding president of the Rotaract Club of Elmira College (Dist. 7120), the Rotaract Club of Cumaná (Dist. 4370), and the Venezuelan Alliance of Memphis (aka Venezolanos En Memphis). While in Memphis, Daniel has received several recognitions and awards, including being named a member of the "20 Under 30 Class of 2020" of the *Memphis Flyer*. Daniel is expected to receive his Doctor of Philosophy (Ph.D.) degree in May of 2020.

Scientific Publications

Tan, H. *, **D. Bastardo Blanco***, B. Xie, Y. Li, Z. Wu, H. Chi, and J. Peng (in press). Methods in Molecular Biology (book chapter). *Co-first authors.

Saravia, J., H. Zeng, Y. Dhungana, **D. Bastardo Blanco**, T. M. Nguyen, N. M. Chapman, Y. Wang, A. Kanneganti, S. Liu, J. Raynor, P. Vogel, G. Neale, P. Carmeliet, and H. Chi (2020). Homeostasis and Transitional Activation of Regulatory T cells Require c-Myc. *Science Advances* 6.

Yang, K. *, **D. Bastardo Blanco***, X. Chen*, P. Dash, G. Neale, C. Rosencrance, J. Easton, W. Chen, C. Cheng, Y. Dhungana, A. KC, W. Awad, X. Guo, P. Thomas,

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