

**METABOLIC REGULATION OF T LYMPHOCYTES
IN INFECTION AND CANCER**

By

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To my family, thank you for supporting me unconditionally

Abstract

T lymphocytes are key elements of the adaptive immunity that support effector and regulatory functions against immune challenges. Upon T cell activation, key signaling pathways including Phosphoinositide 3-kinase (PI3K)/Akt pathway drive downstream metabolic reprogramming of increased glucose metabolism through glycolysis, a critical process to support optimal T cell activation, differentiation and proliferation. Additionally, activated Akt signaling as well as increased nutrient uptake positively regulate mTORC1 activity, which not only further supports T cell activation and growth, but also attributes to sustained and increased protein synthesis of Hypoxia Inducible Factors (HIFs) for enhancement of glucose metabolism and glycolysis. Together, these signaling pathways are required to escalate and sustain the effector metabolic programs for optimal immune responses against challenges including infection and cancer.

Increased glucose uptake is preferentially catabolized and excreted as lactate through glycolysis, rather than entering the tricarboxylic acid (TCA) cycle to be oxidized to CO₂. Commonly known as the Warburg effect, why T cells utilize this rewired metabolism as part of their normal physiological processes remains unresolved. Using a mouse strain with lactate dehydrogenase (LDHA) deficiency in T cells, we showed that while LDHA-mediated glycolysis was dispensable for regulatory T cells (Tregs) in chronic viral infection and tumor, it was essential for conventional T cell functions in an oncogene-driven spontaneous breast cancer model. Enhanced HIF expression through T cell-specific ablation of Von Hippel-Lindau (VHL) further resulted in significantly improved anti-tumor immunity. These

results suggested distinctive modes of metabolic dependency for different subsets of T cells, illustrating a key role of LDHA-mediated glycolysis in supporting conventional T cells, but not Tregs, in immune challenging environments.

Glucose also supports cell growth through *de novo* synthesis of amino acids, which are critical nutrients for anabolism. Using a mouse model with T cell specific-depletion of Phosphoglycerate dehydrogenase (PHGDH), we found that PHGDH was a critical enzyme for supporting optimal T cell activation and differentiation in environments with restricted serine. In a nutrient-replete environment, PHGDH-mediated *de novo* serine synthesis through glucose was dispensable. However, PHGDH-deficient CD8 T cells became auxotrophic for exogenous serine for their proper activation, expansion and effector molecule expression. The defects were associated with reduced Akt and mTORC1 signaling, which was dependent on serine, but not its downstream metabolites. The inability to sustain optimal metabolic signaling resulted in reduced numbers and effector function of antigen specific CD8 T cells, as shown by diminished expression of Granzyme B (GzmB) in a model of bacterial infection. These results suggested a critical role of serine, which was conditionally essential for CD8 T cell signaling and functionality.

Proper metabolic reprogramming is key to support activation of distinct subsets of T cells, which depend not only on fluctuating nutrient availability, but also the optimal and sustained T cell signaling. Our findings revealed the importance of glucose and serine metabolism in supporting T cell functions, and further substantiates the current understanding of how metabolism of nutrients could further feedback to support signaling regulation for optimal T cell responses.

Biographical Sketch

Amy Shyu was born in Urbana, Illinois and grew up in Taipei, Taiwan. She received her Bachelor of Science in Pharmacy from the National Taiwan University and received her Certificate of Pharmacist in 2012. She then attended the School of Engineering in the University of Pennsylvania for Master of Science in Biotechnology. Postgraduate, she worked as an associate scientist at the Novartis Institute of Biomedical Sciences developing Chimeric Antigen Receptor T cell therapies for multiple diseases including Acute Lymphoblastic Leukemia (ALL), Chronic Lymphocytic Leukemia (CLL), and Multiple Myeloma (MM). In 2016, Amy moved to New York City to pursue her PhD studies at Louis V. Gerstner, Jr Graduate School of Biomedical Sciences at Memorial Sloan Kettering Cancer Center. Following rotations in in the laboratories of Dr. Joseph Sun and Dr. Alexander Rudensky, she joined the laboratory of Dr. Ming Li. Amy has worked on multiple projects centered around metabolic regulations of T cells in the context of infections and cancer, with the goal to translate her studies for improvement of patient benefit and health.

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List of Abbreviations

3PG	3-phosphoglycerate
3PHP	3-phosphohydroxypyruvate
3PS	3-phosphoserine
4EBP	eIF4E Binding Protein
Acetyl-CoA	Acetyl-coenzyme A
ACL	Adenosine triphosphate citrate lyase
ACT	Aspartate kinase-Chorismate mutase-TyrA
AIRE	Autoimmune regulator Antibody
ALL	Acute Lymphoblastic Leukemia
α -KG	Alpha-ketoglutarate
ALL	Acute lymphoblastic leukemia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Anti-	Antibody
ANOVA	Analysis of Variance
Akt	Protein kinase B, PKB
AP-1	Activator protein 1
APC	Antigen presenting cells
ASB	Allosteric substrate binding
ASCT	Alanine/serine/cysteine/threonine transporters
ATF4	Activating Transcription Factor 4

ATP	Adenosine 5' triphosphate
B16-OVA	B16-Ovalbumin
b-actin	Beta-actin
Bcl6	B cell lymphoma 6
CAR	Chimeric antigen receptor
CASTOR	Cytosolic arginine sensor for mTORC1 subunit
CCR7	CC-chemokine receptor 7
C-MYC	Cellular MYC
CD	Cluster of differentiation
CD40L	CD40 ligand
CD62L	L-selectin
CD127	IL-7 Receptor
CDK	Cyclin-dependent kinases
CH ₂ -THF	5,10-methylenetetrahydrofolate
CLL	Chronic Lymphocytic Leukemia
CTD	Control diet
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTV	Cell Trace Violet
CXCR	C-X-C motif chemokine receptor
DAG	Diacylglycerol
DC	Dendritic cells
DM-aKG	Dimethyl alpha-ketoglutarate
ECAR	Extracellular acidification rate

EIF4E	Eukaryotic Translation Initiation Factor 4E
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
EOMES	Eomesodermin
FAS-L	FAS ligand
Foxo	Forkhead Box Subgroup O
Foxp3	Fork-head box P3
FSC	Forward side scatter
G6PD	Glucose-6-phosphoate dehydrogenase
GADS	GRB2-related adaptor downstream of Shc
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA-binding protein 3
GATOR	GTPase activating proteins toward Rags
GCN2	general control nonderepressible 2
Glu	Glutamate
GLUT	Glucose transporter
GM	Geometric mean
GRB2	Growth factor receptor-bound protein 2
GSK-3b	Glycogen Synthase Kinase 3 Beta
GzmB	Granzyme B
γ C	Common gamma chain
HEV	High endothelial venules
Het	Heterozygous

HK	Hexokinase
HIF	Hypoxia Inducible Factor
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IACUC	Institutional Animal Care and Utilization Committee
ICOS	Inducible costimulatory
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILTC1	Type1 innate-like cytotoxic T lymphocytes
IP ₃	Inositol 1,4,5-trisphosphate
IP ₃ R	IP ₃ receptor
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ITAM	Immunoreceptor tyrosine-based activation motifs
JAK	Janus kinase
JMJC	Jumonji C
KLRG1	Killer cell lectin-like receptor subfamily G member 1
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAMP2	Lysosome-associated membrane protein 2
LAT	Linker for activation of T cells
Lck	lymphocyte-specific tyrosine kinase

LCMV	Lymphocytic choriomeningitis virus
LDHA	Lactate dehydrogenase
LE	Long exposure
LKB1	Liver kinase B1
LmOVA	Listeria monocytogenes expressing chicken ovalbumin protein
LRS	Leucyl-tRNA synthetase
LT- α	Lymphotoxin- α
MAPK	Ras mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MDHFD2	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase
MDM2	Mouse double minute 2 homolog
MDSC	Myeloid derived suppressor cells
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
MiTF/TFE	Microphthalmia-associated transcription factors
mLN	Mesenteric lymph nodes
MM	Multiple Myeloma
MMTV	Mouse mammary tumor virus
MTEC	Medullary thymic epithelial cells
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
MYC	Myelocytomatosis
NAD ⁺	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor- κ B
NIH	National Institutes of Health
NRF2	Nuclear factor erythroid 2-related factor 2
OCR	Oxygen consumption rate
OVA	Ovalbumin
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PDK1	Phosphatidylinositide-dependent protein kinase 1
PFU	Plague-forming units
pLN	Peripheral lymph nodes
PHGDH	Phosphohydroxyglycerate dehydrogenase
PEP	Phosphoenolpyruvate
PHD	Prolyl-hydroxylase
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol (3,4,5)- trisphosphate
PLC γ 1	Phospholipase C gamma 1
PKC	Protein kinase C
PKM2	Pyruvate kinase isoform M2

PMA	Phorbol myristate acetate
pMHCs	peptide-MHC complexes
PPP	Pentose phosphate pathway
PRAS40	Proline-Rich AKT substrate of 40kDa
PRR	Pattern recognition receptors
PSAT1	Phosphoserine aminotransferase 1
PSPH	Phosphoserine phosphatase (PSPH)
PTEN	Phosphatase and tensin homologue
pTregs	Peripherally derived Tregs
PyMT	Polyoma virus middle T-antigen
RAPTOR	Regulatory protein associated with mTOR
Rheb	RAS homologue enriched in brain
RICTOR	Rapamycin insensitive companion of mTOR
ROR- γ t	Receptor-related orphan receptor- γ t
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S1PR1	Sphingosine-1-phosphate receptor 1
S6K1	p70S6 Kinase 1
SAM	S-Adenosyl methionine
SAMTOR	S-adenosylmethionine sensor upstream of mTORC1
SAT	System A transporters
SE	Short exposure
SEM	Standard error of the mean

SD	Serine deficient
SGD	Serine glycine deficient diet
SGK	Serum glucocorticoid- induced protein kinase
sgRNA	Single-guide RNA
SH2	Src-homology-2
SHMT	Serine hydroxymethyltransferase
SLC	Solute carrier family
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SKI	Sloan Kettering Institute
SOS	Son of Sevenless
SREBP	sterol responsive element binding protein
SSP	Serine synthesis pathway
STAT	signal transducer and activator of transcription
TAA	Tumor-associated antigens
T-bet	T-box transcription factors
TCA	Tricarboxylic acid cycle
Tcm	Central-memory T cells
TCM	T cell medium
TCR	T cell antigen receptor
Teff	Effector T cell
Tem	Effector-memory T cells
TET	Ten-eleven translocation
TF	Transcriptional factors

Tfh	T follicular helper cell
TGF- β	Transforming growth factor beta
Th	Helper T cell
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor–related apoptosis–inducing ligand
Treg	Regulatory T cells
TSC	Tuberous Sclerosis
tTregs	Thymic regulatory T cells
Trm	Resident Memory T cell
TSA	Tissue-restricted antigens
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
ULK1	Unc-51-like autophagy activating kinase 1
VEGF-A	Vascular endothelial growth factor A
VHL	Von Hippel-Lindau
ZAP70	Zeta activated protein 70 kDa

Chapter 1: Introduction and Overview

1.1 T cell overview

The immune system comprises of organs, cellular players, and chemicals that are vital for the defense against pathogen invasions and neoplastic cell growth, while maintaining tolerance to healthy self. The innate immunity recognizes conserved pathogen patterns, while the adaptive immunity holds the capability not only for antigen-specific recognitions, but also for the formation of efficient memory responses against prior encountered pathogens. As pivotal components of the adaptive immune system, T lymphocytes exert both effector and regulatory functions in response to immunological challenges. In the current view, T cells can be broadly dissected into the $\alpha\beta$ T cell lineage and the $\gamma\delta$ T cell lineage, which contain distinct types of T cell antigen receptors (TCRs) early during thymic T cell development. The $\alpha\beta$ T cell lineage can be further categorized into CD8 and CD4 T cells based on the glycoprotein co-receptor expression. CD8 T cells exert cytotoxic functions that allow direct killing of intracellular pathogen-infected or cancerous cells, while CD4 T cells can be further classified into CD4 helper T cells and CD4 regulatory T cells to jointly support regulation of host defense and self-tolerance. Together, these cellular players orchestrate optimal immunological responses to maintain host health and fitness.

1.1.1 Conventional T cells

Cytotoxic CD8 T cells and helper CD4 T cells are the two major subsets of immune cells that constitute the conventional T cell population. They express TCRs that undergo somatic V(D)J gene rearrangements, random nucleic acid addition and excision at gene junctions, as well as pairing of the α and β chains that generates a combinatorial diversity as high as $\sim 2 \times 10^{19}$ (1), allowing vast capability to recognize unique antigens when presented as peptide noncovalently bound together with the major histocompatibility complex (MHC). These T cells are matured in thymus, where they undergo positive and negative selection to retain cells with moderate reactivity, while eliminating cells that are self-reactive. Cytotoxic, or “killer” CD8 T cells, produce cytolytic granules and small signaling proteins such as cytokines to directly kill or recruit other immune cells to mount an effective immune response. Helper CD4 T cells can be further delineated into Th1, Th2, Th17 types of cells, which acts as key mediators in diverse settings including infection, allergy and autoimmunity. Through cytokine, chemotactic cytokine (chemokine) production or direct interactions, these CD4 T cells help activate CD8 T cells, B cells and myeloid cell lineages for their optimal responses.

1.1.2 Regulatory T cells

The concept of T cell suppression was initially implicated by the finding that neonatal thymectomy leads to autoinflammatory diseases in mice (2). It was later unveiled that among the naïve CD4 T cells, a population that receives strong TCR signaling with high reactivity to self can differentiate into thymic Treg cells (tTregs) (3,4). Additionally, activation of naïve CD4⁺ T cells under suboptimal conditions

can as well induce differentiation of Treg cells (5,6). Characterized by transcription factor Fork-head box P3 (*FOXP3*), Treg cells maintain immune homeostasis by suppressing self-destructive immune responses.

The transcription factor *FOXP3* is critical for Treg cell development and function (7,8,9): mice deficient of *Foxp3* expression, also known as the scurfy mice, develop lethal T cell-driven systemic autoimmune diseases; humans with loss-of-function mutations in *FOXP3* lack Treg cells, and develop the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome manifested with multiorgan autoimmune diseases (10,11,12). Thymic Treg cells are selected by high-affinity “tissue-specific” antigens expressed at low level by the thymic epithelial cells, and exit the thymus in a resting state characterized by high expression of the lymph node-homing molecule CD62L (13). Treg cells depend on the cytokine Interleukin 2 (IL-2) as well as STAT5 signaling downstream of IL-2 receptor for their development and suppressive function (14). Following antigen reencountering, resting Treg cells are activated and migrate to peripheral tissues to promote self-tolerance and limit the effector T cell responses (15). Treg cells also robustly expand in the context of chronic inflammatory diseases such as cancer and chronic infection (16,17).

1.1.3 Maintenance of T cell tolerance

Given the diversity of TCRs, T lymphocytes possess immensely large capabilities to recognize different types of antigens. The ability to mount effector immune responses and elicit memory responses long post initial pathogen

encounter suggests the high potency of T cell-mediated responses. Inversely, this also indicates the importance of having regulatory mechanisms in place to prevent host tissue damage by improper immune activation and attacks.

To maintain proper immunological tolerance, which is the prevention of immune responses against self-antigens, the immune system has evolved multiple mechanisms broadly segregated as the central tolerance and the peripheral tolerance. Central tolerance, or the negative selection, eliminates T cells that are highly reactive to self-antigens. This occurs mainly in the medullary of the thymus, where medullary thymic epithelial cells (mTEC) express tissue-restricted antigens (TSA) driven by the transcription factor autoimmune regulator (AIRE) (18). TSA presentation through the mTECs or dendritic cells in the form of peptide-MHC complexes can be recognized by single positive thymocytes expressing either CD8 or CD4 co-receptors, and the ones that react with high affinity in the thymus will be deleted, a process known as the “clonal deletion” to limit the population that are self-reactive (19). Alternatively, self-reactive thymocytes can undergo “receptor editing” to reduce the affinity (20), or “clonal diversion” and differentiate into Treg cells (21).

Although multiple mechanisms are set in place to prevent the development of self-reactive T lymphocytes, their full elimination through central tolerance is not guaranteed. Peripheral tolerance mechanisms thus serve to further regulate tolerance particularly for T lymphocytes whose specificity exists towards antigens that were not expressed in the thymus. In cell-intrinsic regulations, T cells may induce a hyperresponsiveness state known as “anergy” upon exposure with self-

antigens with suboptimal activation conditions, or undergo apoptotic cell death under chronic stimulation (22,19). In cell extrinsic regulations, Treg cells serve as a pivotal player that actively monitors and prevents abnormal activation or expansion of lymphocytes. Multiple mechanisms have been reported for the immune-suppression mediated by Treg cells. First of all, Treg cells can suppress the proliferation and cytokine production of lymphocytes through cell direct contact (23). With high expression of CD25, or the IL-2 receptor alpha chain, Treg cells possess high affinity for IL-2 and can compete with CD8 T cells for it (4,24). Treg cells may also secrete immunosuppressive cytokines including Transforming growth factor beta (TGF- β), IL-10 and IL-35 (25,26). Additionally, Treg cells may deliver negative signals including cyclic AMP and adenosine through CD39 and CD73 (27,28,29); as well as CTLA4-mediated induction of indoleamine 2,3-dioxygenase (IDO) in dendritic cells (DCs) to increase the conversion of tryptophan to kynurenine, an inhibitory metabolite for conventional T cells whilst promoting induced Treg cell differentiation (30). Mechanisms of suppression likely differ in different tissue sites for immune homeostasis. Together, these diverse mechanisms act to ensure T cell tolerance at steady state.

1.1.4 T cell activation, differentiation and memory

Post successful T cell development, naïve T cells exit the thymus to enter blood circulation and recirculate between secondary lymphoid organs, blood and lymph constantly in search for their cognate antigens. Naïve T cells are

characterized by high expression of lymph node-homing receptors L-selectin (CD62L), CC-chemokine receptor 7 (CCR7) and low expression of CD44; their homeostasis depends on the common gamma chain (γ C) family of cytokine IL-7 through the Janus kinase (JAK) 1 and 3, signal transducer and activator of transcription (STAT) 5 and phosphoinositide 3-kinase (PI3K)–Akt (31), while other γ C family of cytokines including IL-15 and IL-2 can also promote their proliferation (32).

Once the naïve T cells encounter specific antigens presented on antigen presenting cells (APCs) as peptide-MHC complexes that can be recognized by their unique TCRs, which allow antigenic peptide recognitions presented either on MHC class I or class II by different APCs, they are activated to become effector T cells that undergo massive clonal expansion with escalated effector functions. Naïve T cells exhibit condensed chromatin and express little to no RNA for effector molecules such as cytokines IFN- γ , IL-4, tumor necrosis factor (TNF) and perforin (33). The activation of T cells induces robust changes transcriptionally, epigenetically, and metabolically. With proper signals through TCR, co-stimulation and cytokine milieu that will be discussed later, quiescent T cells undergo drastic remodeling of chromatin structure for enhanced accessibility that allows expression of activation associated genes (34). Furthermore, activation induces subsequent clonal proliferation and functional differentiation. Enhanced proliferation is initiated as quiescent T cells exit the G₀ phase of cell cycle, further going through the processes of interphase, mitosis and cytokinesis that are tightly regulated by the ordered expression cyclin-dependent kinases (CDKs), cyclins,

and CDK inhibitors that act as negative regulators to complete cell divisions (35,36). CD8 T cells, compared to CD4 T cells, require a shorter antigen exposure time to trigger the activation program; CD8 T cells also exhibit a shorter cell division cycle that allows more rapid proliferation than CD4 T cells (37,38).

Depending on the nature of antigens encountered, CD4 T cells can further differentiate into various lineages including Th1, Th2, Th17 helper cells, T follicular helper cells (Tfh) and peripherally derived Tregs (pTregs) (39,40) under distinct cytokine polarization (Figure 1.1). Th1 helper cells, characterized by the transcription factor T-box transcription factors (T-bet) and cytokines IFN- γ and TNF- α , are vital in the protection against intracellular pathogen infections. Th2 helper cells are characterized by the transcription factor GATA-binding protein 3 (GATA3) and cytokines IL-4, IL-5 and IL-13, and are important in the responses against extracellular pathogens, such as helminth worms. Th17 helper cells are characterized by the transcription factor retinoic acid receptor-related orphan receptor- γ t (ROR- γ t) and cytokine IL-17, and play a vital role against certain extracellular bacteria and fungi. Tfh cells are characterized by the transcription factor B cell lymphoma 6 (BCL-6) and IL-21, they reside in the germinal center that serve to provide B cell help for germinal center development, B cell maturation for high affinity antibody production (41). Lastly, pTregs, together with tTregs as mentioned prior, act to keep effector T cell responses in check to prevent excessive immunological responses and autoimmunity (40). On the other hand,

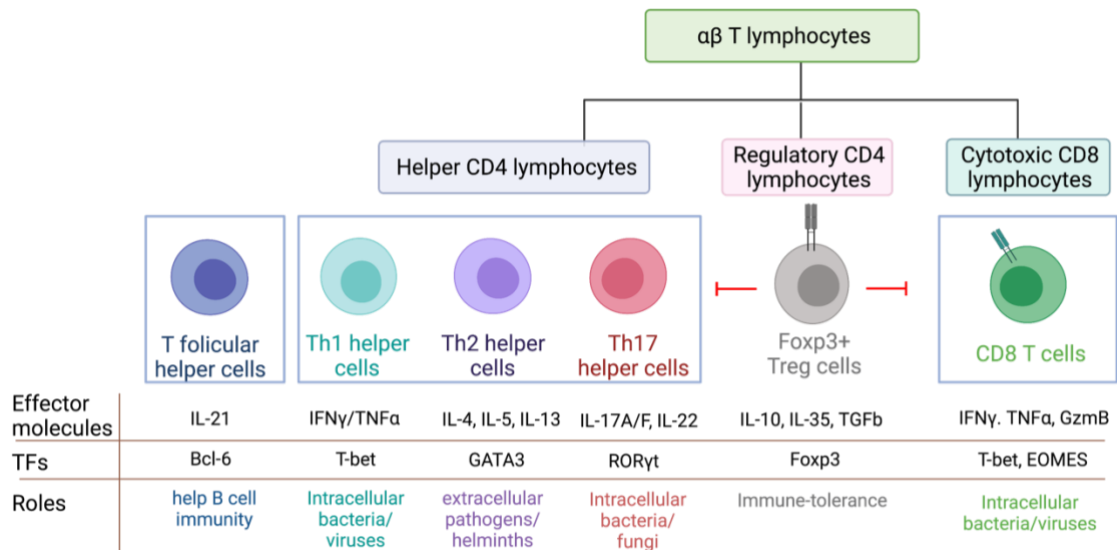


Figure 1. 1 Types of T cell lymphocytes

T lymphocytes are pivotal components of the immune system for host protections through multiple defense mechanisms against diverse immune challenges. Under different pathogenic challenges, naïve T cells can differentiate into conventional CD8 and CD4 T cells that are characterized by expression of key cytokines and transcriptional factors (TFs). On the other hand, regulatory T cells can functionally inhibit and modulate conventional T cell responses to maintain tolerance and prevent autoimmunity.

effector CD8 T cells are cytotoxic and exert direct killing to eliminate cells infected by intracellular pathogens like bacteria and virus. The killing process includes inflammatory cytokines IFN- γ and TNFs, as well as a calcium-dependent release of lytic granules containing effector molecules granzymes and perforin. Perforin forms transmembrane pores in the target cell membrane, and granzymes can be released to lyse target cells that further trigger apoptotic cell death. Last but not least, activated CD8 T cells and some effector CD4 Th1 cells may express FAS ligand (FAS-L) that binds to FAS expressed on target cells, which triggers apoptosis through activation of caspases (42).

A typical primary T cell response may take up to 7-15 days for its peak response, accompanying pathogen eradication and resolution of inflammation. Once the infection has been cleared, the immune responses enter a contraction phase, in which the majority (~90%) of effector T cells are eliminated through apoptosis. However, some of the effector T cells can differentiate into long-lived memory T cells with ~8-15 years of half-life (43,44,45). Compared with naïve T cells, memory T cells are poised for activation due to distinct epigenetic landscapes that were “primed” during the initial activation, allowing faster dynamics for gene re-expression and have less stringent requirements for subsequent activation. Maintained through IL-7 and IL-15 for their survival and cell-renewal (46), memory T cells are antigen-independent and can be further differentiated into subsets of central-memory T cells (T_{cm}), effector-memory T cells (T_{em}), and resident-memory T cells (T_{rm}). T_{cm} cells exhibit the functional capability of higher proliferation potential, express high levels of IL-7 receptor (CD127), adhesion

marker CD62L, high levels of the chemokine/homing receptor C-C chemokine receptor type 7 (CCR7), and low levels of the surface marker killer cell lectin-like receptor subfamily G member 1 (KLRG-1). These surface markers allow them to home to secondary lymphoid organs and bone marrow. On the contrary, Tem cells are characterized by enhanced effector functional potential but less proliferation, generally expressing low levels of CD62L, CD127, and CCR7 with high levels of KLRG1 that allow preferential trafficking to the nonlymphoid organs and peripheral tissues. Trm cells are characterized by tissue-resident markers CD103, CD49a and CD69, allowing *in situ* responses to reinfection. Particularly, memory T cells that reside in nonlymphoid tissues exhibit increased proliferation potential and much more rapid effector response (33,47). The capability to launch a stronger immune response in a much-shortened time frame upon antigen re-exposure through immunological memory provides protection against reinfections, serving as the bases for vaccination.

1.1.5 T cell-mediated immunity in infection

The immune system serves to protect against threats of external invading pathogens as well transformed malignancies from self-tissues; the resulting immune responses are critically important for effective clearance of diseases while minimizing collateral damage. Both the innate and adaptive immunity cooperate for optimal responses, and T cells are critical components of the adaptive immunity that respond to disturbance of host homeostasis.

Cytotoxic CD8 T cells and Th1 helper cells are key subsets of T cells important for the type 1 immunity, both secreting key IFN- γ for the proinflammatory effects that could stimulate the oxidative burst and intracellular phagocytosis by macrophages and neutrophils (48,49,50,51), intracellular killing of microbes, as well as upregulation of MHC molecules for antigen presentation (52,53), and vessel dilation for recruitment of more innate immune cells. This elicits an inflammatory cascade escalated by the induction of other cell types including endothelial cells, keratinocytes, and fibroblasts (48) to secrete more proinflammatory cytokines such as TNFs and chemokines that jointly create an inflammatory environment to counter intracellular infections. For cytotoxic CD8 T cells, the response is dependent on the recognition of peptides derived from pathogens presented on MHC class I molecules of infected cells. Mutations in genes coding for proteins that transport MHC I to the cell surface, such as TAP1 and TAP2, impair CD8+ T cell development and function (54). Defects in effector molecules can as well compromise the optimal responses against clearance of pathogens (55,56). In the context of chronic infections, multiple studies have identified CD8 T cell dysfunctions, where chronic exposure of antigens can result in decreased proliferation potential and effector function of antigen specific T cells, leading to the exhaustion of CD8 T cells. Dysfunction and loss of Th1 cell responses may also occur in chronic infections (57), resulting in the inability to clear pathogens (58).

Type 2 immunity, supported by Th2 helper cells and type 2 innate lymphoid cells, is the dominant inflammatory responses against parasites such as helminths.

Type 2 cytokines, as discussed earlier, can activate B cells for high titers of antibody production, support their class-switching from the immunoglobulin G (IgG) to IgE (59, 60), as well as tissue repair in the infection resolution phase (61). Type 2 immunity is often characterized by eosinophilic and basophilic tissue infiltration together with mast cell degranulation that is dependent on the cross-linking of surface-bound IgE, and excessive Th2 cytokine production is also associated with the development of allergy (62).

Th17 cells are dominated by the production of proinflammatory IL-17 family cytokines (IL-17A and IL-17F) and IL-22. These cytokines protect against extracellular bacteria and fungi through promoting the accumulation of immune cells including macrophages, neutrophils and lymphocytes to help clear such pathogens (63, 64). However, Th17 cells have also been implicated in many autoimmune diseases, including rheumatoid arthritis, Crohn's disease, psoriasis and type 1 diabetes (65, 66, 67, 68). Interestingly, Th17 cells also exhibit a high degree of plasticity, allowing them to trans-differentiate into Th1, Treg cells, and Tfh cells (69), complicating their roles in diseases.

Last but not the least, in addition to the critical role of Treg cells in maintaining tolerance at steady state, Treg cells also control immune responsiveness to infectious pathogens ranging from bacteria and virus to worms and fungi. These roles can be positive or negative depending on the disease context (70, 71). In the Herpes simplex virus (HSV) acute infections, Treg cell depletion leads to increased viral load, reduced cytokines and cell trafficking to sites of infection (72). In the LCMV chronic viral infections, the accompanied

expansion of Treg cells provides an immune-evasion mechanism for virus (73). Additionally, Treg cells may alter the quality of CD8 T cell responses, as shown by capability of Treg cells to increase the avidity of primary CD8+ T cell responses by destabilizing low-affinity T cell-DC interactions in mice following *Listeria monocytogenes* infection (74). Treg cells can also promote memory responses in a murine *Leishmania* infection model through blocking the sterile eradication of the pathogen, thereby providing long-term antigen persistence for the maintenance of memory responses (75). Based on the unique dynamics and niches of infectious pathogens, Treg cells are involved in nearly all infections studied, regulating immune responses leading to differentiating outcomes from healthy to enhanced pathogenesis.

1.1.6 T cell-mediated immunity in cancer

Cancer cells are transformed cells of self-origin. The high similarity of these malignant cancer cells to host tissue makes it challenging for therapeutic development. In the beginning of twentieth century, it was first hypothesized by Paul Ehrlich that the immune system can recognize neoplastic cells generated from self. This led to later development of the “cancer immunosurveillance hypothesis”, describing how tumor cells can express “neoantigens” recognized and targeted by the immune system that trigger the rejection as foreign tissue (76, 77). However, tumor cells are capable of evading the immune system through various complex interactions and mechanisms, and reports have suggested that immune system have dual roles in tumor-elimination and tumor-protection (78). T

lymphocytes, a large proportion of the infiltrating immune cells, are critically important in supporting the dynamic interactions in tumor development.

Generally, the presence of tumor infiltrating lymphocytes correlates positively with patient prognosis (79, 80, 81). The protective role of T cells against cancer development is further supported by the *Tcrb*^{-/-}*Tcrd*^{-/-}, and athymic (nude) genetic mouse models developing more carcinogen-induced tumors and spontaneous cancer than wild-type mice (82, 83). In most cancer cells, mutations or translocations exist to promote malignance cell survival, while generating tumor-associated antigens (TAAs) or neoantigens that do not exist or are less commonly expressed in normal tissues. A number of TAAs have been reported (84). These TAAs and neoantigens can be recognized by T cells; although the mechanisms are not fully resolved, cytotoxic CD8 T cells are the core component of the defense. Overexpression of these antigens in the context of inflammatory signals like type I IFNs in cancer allow antigens to be taken up and cross-presented by CD8 α DCs as peptide-MHC class I complexes on the surface. This process further activates and triggers CD8 T cell-mediated destructions through perforins, granzymes (85), FAS-L, tumor necrosis factor–related apoptosis–inducing ligand (TRAIL), as well as IFN- γ , which play a critical anti-tumor effect by inhibiting tumor cell proliferation and angiogenesis, or by activating macrophages for tumor cell engulfment (86, 87). The importance of these anti-tumor mechanisms has been implicated through genetic knockout mouse models, resulting in increased susceptibility to carcinogen-induced or spontaneous tumors (88,89,90). It was further reported that T cells capable of killing melanoma cells can also kill normal melanocytes,

suggesting that tumor immunity is a form of autoimmunity (91). However, the elimination of normal counterparts doesn't always occur in parallel to tumor cell killing, indicating alternative mechanisms of regulation for further investigation.

CD4 T cells, on the other hand, are increasingly recognized for their roles in cancer (92). Activated CD4 T cells can secrete IL-2 to support CD8 responses, or indirectly help CD8 T cells through CD40 ligand mediated maintenance of proinflammatory dendritic cells (93). Th1-polarized CD4 helper T cells can secrete effector cytokines $TNF\alpha$ and $IFN-\gamma$ to mediate direct anti-tumor effect (94), as well as providing help to enhance B cell responses and antibody titers against tumor antigens (95). The roles of other T helper subsets including Th2, Th17 and Tfh in tumor remain inconclusive, varied in different tissues and models.

T cells may also promote cancer development. For instance, $\gamma\delta$ T cells have been shown to promote cancer progression by inhibiting anti-tumor immunity and promoting tumor angiogenesis (96). CD4 Treg cells are generally thought to promote local immunosuppression of anti-tumor immune effector responses in the tumor microenvironment. The ratio of Treg cells to T effector cells (Teff) are elevated in growing tumors (97), and low Treg/Teff ratio in human patients is associated with improved prognosis (98,99). Along these lines, the ablation of Foxp3+ Treg cells leads to significantly slower tumor growth accompanied by enhanced effector T cell responses (100). Last but not the least, immunoediting can occur in tumors, which is heavily mediated by $IFN\gamma$ -producing CD8 T cells in the tumor microenvironment (101). $IFN\gamma$ -producing CD8 T cells may trigger tumor genetic instability, induce neoantigen downregulation, or trigger selective

outgrowth of tumor cells with defects in the IFN γ signaling pathway including IFNGR1, IFNGR2, JAK2 and IRF1 (102) that exhibit enhanced aggressive growth or resistance to therapies.

Persistent antigen exposure in cancer, however, has demonstrated exhaustion phenotypes that could dampen T cell responses similarly as in chronic infections (103). This happens through a variety of mechanisms, including T cell intrinsic inhibition through the expression of negative receptors, programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4). Signaling through these molecules serve as a “break” to reduce T cell functions and potential collateral damages to host. Exhaustion hinders effective immunity in the context of chronic infections and cancer, and immune checkpoint blockade therapies including anti-PD1 and anti-CTLA4 to unleash T cell responses against tumors have shown great promises in multiple cancer types with greater immunogenicity, including melanoma, renal cell carcinoma, prostate cancer and ovarian cancer. (86, 104, 105, 106). Additionally, cancer cells may also exhibit cell extrinsic inhibitory mechanisms including competition with T cells for nutrient usage, and generation of toxic metabolites that are immunosuppressive (107).

Lastly, given the increased understanding of T cell responses in mediating anti-tumor immunity, strategies utilizing T cells are becoming more appealing therapeutic approaches. In addition to the immune checkpoint blockade mentioned above, therapeutic vaccines utilize tumor associated antigens (TAAs) presented on dendritic cells to activate naïve T cells for further differentiation and expansion to defend against tumors (108). In recent years, adoptive T cell therapies have

emerged as another promising type of cancer immunotherapy. In one approach, tumor infiltrating lymphocytes (TILs) are isolated and expanded *ex vivo* in conditions favoring their persistence, and select for antigen-specificity before reinfusing into patients. Another type of therapy, known as chimeric antigen receptor (CAR) T cells, utilize peripheral T cells genetically modified to express an antigen receptor specific for a cancer cell surface protein. CAR proteins consist of an extracellular domain recognizing antigen and an intracellular signaling domain such as CD3- ζ , CD28 and 4-1BB to fully activate T cells. These strategies have shown preliminary success in liquid tumors such as acute lymphoblastic leukemia (ALL), but not as effective in solid tumors. Additional engineering strategies are currently in development to enhance functionality and persistence of engineered T cells (109).

1.2 T cell signaling

1.2.1 Optimal stimulatory signals

Primary T cell signaling requires the integration of three important signals for optimal activation: Signal 1, TCR signaling upon antigen recognition that drives naïve T cell activation. Signal 2, co-stimulatory ligand engagement of CD28 receptor. Signal 3, cytokine stimulation for polarization of distinct T cell subsets (Figure 1.2). The outcome of these inputs drive T cell development, homeostasis, activation and differentiation (110, 111), which is context-dependent and highly

coordinated. The process involves multiple evolutionarily conserved signaling molecules to incorporate environmental cues for the development of diverse immune responses.

1.2.1.1 Signal 1: TCR signaling

TCR acts as the antigen detector exhibiting specificity for a particular antigen. Conserved gene recombination mechanisms generate vast diversity for the TCR repertoire (112). TCR can be activated upon the recognition of its cognate antigen in the peptide-MHC (pMHC) complex format with its extracellular portion (or other stimulus such as anti-TCR crosslinking). The binding is further strengthened by the binding of co-receptors, CD4 and CD8. The cytoplasmic tail of CD4 or CD8 co-receptors recruit the Src kinases leukocyte-specific tyrosine kinase (Lck) to the TCR complex, and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3 δ -, γ -, ϵ -, and ζ -chains (113). Phosphorylated CD3 ITAMs further recruit the Syk family kinase Zeta activated protein 70 kDa (Zap70) via Src-homology-2 (SH2)-domain interactions (114). Zap70 resides in the cytoplasm and is autoinhibited prior to TCR activation. The binding to ITAMs allow Zap70 recruitment to the plasma membrane where it is no longer autoinhibited and stabilized by Lck phosphorylation to sustain its active conformation (Figure 1.3). Activated Zap70 can further propagate TCR signaling through phosphorylation of its downstream target linker for activation of T cells (LAT), a membrane-associated scaffolding protein (115). LAT contains four Zap70

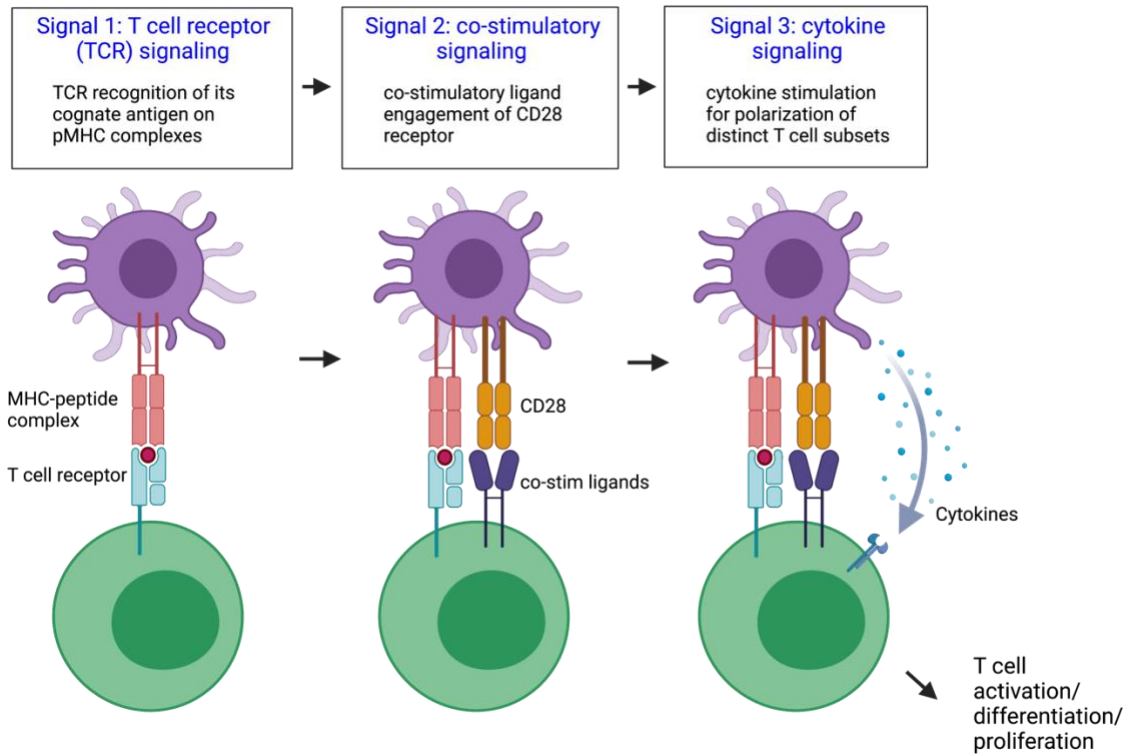


Figure 1. 2 Critical signals for proper T cell activation

Primary T cell activation requires successful integration of three distinct signals delivered in sequence. (1) TCR activation through antigen recognition on peptide-MHC (pMHC) complexes, (2) co-stimulation, and (3) cytokines. The presence of all three signals lead to successful T cell activation, differentiation and robust clonal expansion. TCR activation in the absence of signal 2 and 3 leads to hyporesponsive state and the development of anergic T cells.

phosphorylation sites that recruit adaptor proteins phospholipase C gamma 1 (PLC γ 1), growth factor receptor-bound protein 2 (GRB2), and GRB2-related adaptor downstream of Shc (GADS) (116). The recruitment of multiple adaptor proteins forms a super complex known as the LAT signalosome, which regulates multiple downstream signaling pathways. PLC γ 1 recruitment through the SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) can provide for calcium and Ras mitogen-activated protein kinase (MAPK) pathway activation, which is mediated by the hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2) to generate the secondary messengers inositol 1,4,5-trisphosphate (IP $_3$) and diacylglycerol (DAG). DAG can further activate protein kinase C (PKC) and extracellular signal-regulated kinases (ERK) in the plasma membrane (117), which activates the nuclear translocation of key transcription factors activator protein 1 (AP-1) and nuclear factor-kB (NF-kB) (118, 119). IP $_3$, on the other hand, binds to IP $_3$ receptor (IP $_3$ R) that causes the release of calcium from the endoplasmic reticulum (ER), resulting in the influx of extracellular calcium through channels in the plasma membrane (120), which is important for the activation of nuclear factor of activated T cells (NFAT) (121). Additionally, the recruitment of Son of Sevenless (SOS) to LAT via GRB2 leads to activation of the Ras/MAPK pathway, which is also critical for diverse signaling inputs that activates transcription factors to support T cell activation.

In addition to the generation of IP $_3$ by hydrolysis, PIP2 can be phosphorylated by the phosphoinositide 3-kinase (PI3K) to generate

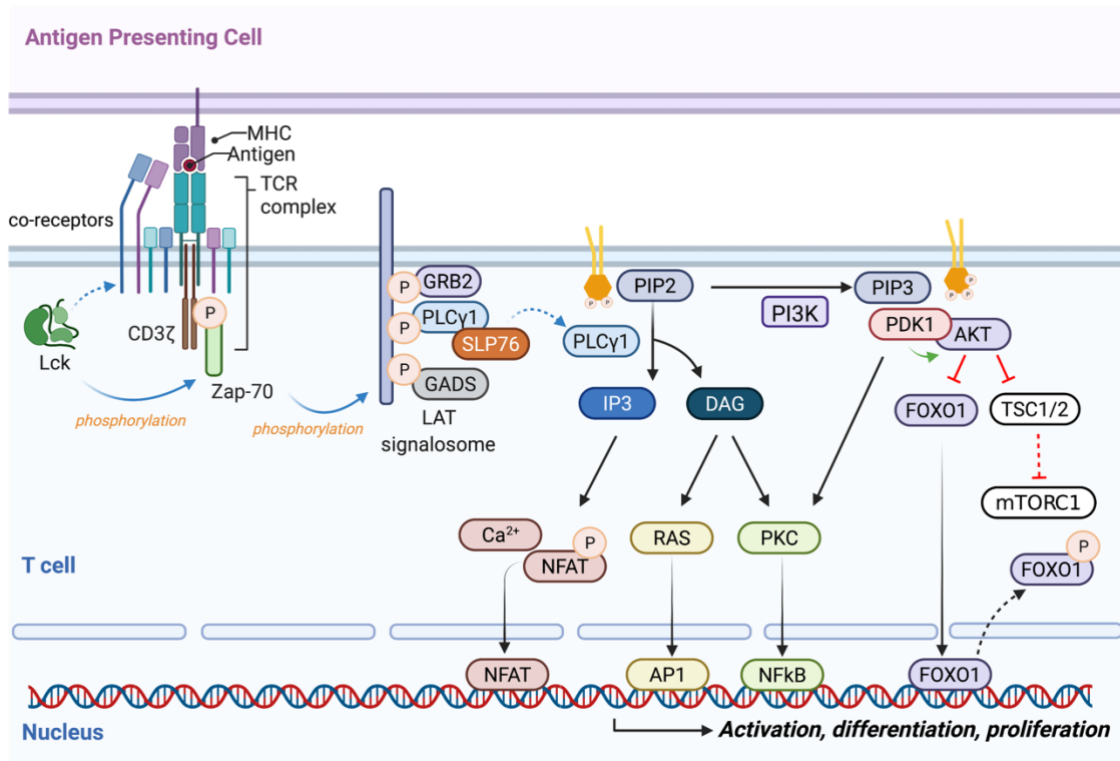


Figure 1. 3 Overview of the major TCR signaling pathways.

TCR signal transduction is activated when TCR binds to pMHC complexes by antigen presenting cells (APCs) presenting its cognate antigen and stabilized by co-receptor binding. Lck is then recruited by co-receptors to phosphorylate ITAMS on CD3 chains, further recruiting and activating ZAP70. ZAP70 recruits and phosphorylates the transmembrane adaptor protein LAT, leading to the formation of LAT signalosome that consists of multiple downstream signal transducer proteins. Major downstream pathways include DAG/Ras/ERK/AP-1 pathway, PKC- dependent NF- κ B pathway, Ca²⁺- dependent activation of NFAT, and PI3K/Akt/Foxo1 pathways.

phosphatidylinositol (3,4,5)- trisphosphate (PIP3) that gets recruited by SLP76 and LAT adapter proteins to the complex (122). The resulting secondary messenger PIP3 provides docking sites for downstream effectors phosphatidylinositide-dependent protein kinase 1 (PDK1) and Akt (Protein kinase B) that can be further phosphorylated by PIP3. PDK1 supports activation of NF- κ B through PKC; Akt regulates downstream signaling effectors including Foxo1, TSC1 and Glycogen Synthase Kinase 3 Beta (GSK-3 β) (123), whereas PDK1 and Akt phosphorylation can both activate mechanistic target of rapamycin complex 1 (mTORC1) to support downstream cell anabolism and metabolic reprogramming of T cells (124). Thus, TCR signaling is amplified by multiple effector proteins that attribute to downstream successful T cell responses.

1.2.1.2 Signal 2: Co-stimulation

Successful outcome of TCR stimulation is dependent on the presence of secondary signals, co-stimulation, which further regulates T cell differentiation, function and survival (125). When TCR signaling occurs in the absence of co-stimulation, T cells result in a nonresponsive or anergic state that become refractory to restimulation. CD28 is known as the most prominent co-stimulatory receptor in T cells for the delivery of co-stimulation signals. Constitutively expressed on T cell surfaces, CD28 can interact with its binding partners CD80 (B7-1) and CD86 (B7-2) expressed by APCs, leading to robust signal transduction. Ligation of CD28 with its binding partners induces phosphorylation of its cytoplasmic motifs, YMNM and PYAP, which are required for the localization of

CD28 at the immunological synapse and the recruitment of downstream proteins like PI3K, PKC, RAS, ERK (126). In addition to CD28, other co-stimulatory molecules such as OX40, 41BB and ICOS, which are members of the tumor necrosis factor receptor family, have been reported (127). On the other hand, co-inhibitory molecules can further fine tune T cell responses. For instance, CTLA4 competes with CD28 for the same binding molecules, and the ligation induces phosphorylation of the cytoplasmic tail of CTLA4 that further recruit phosphatases to dephosphorylate membrane-proximal effectors. Binding of programmed cell death protein ligand 1 (PD-L1) to PD-1 induces inhibition in a similar manner (128); these molecules thus regulate the amplitude and duration of T cell responses.

1.2.1.3 Signal 3: Cytokine stimulation

Lastly, a third signal known as cytokines along with antigen and co-stimulation are required for optimal T cell polarization and functions. CD8 T cells stimulated with TCR and co-stimulatory signals but absent with cytokine support fail to develop optimal effector functions, demonstrating the importance of signal 3 to propagate productive responses (129). The types of inflammatory cytokines that are often produced by APCs present in the tissue microenvironment determines the differentiation of both CD4 T helper cells and cytotoxic CD8 T cells.

For CD8 T cells, IL-12 and Type I IFN (IFN α/β) are major sources of signal 3 that determine the optimal IFN- γ production and cytolytic functions, as demonstrated in multiple viral, bacterial and transplant models (130). IL-12 can

further regulate memory formation through a gradient expression of the T-bet transcription factor (131) while repressing Eomesodermin (EOMES) (132). These cytokines can come from APCs including dendritic cells and help from other innate immune cells, as well as CD4 helper cells to facilitate the development of T cell-mediated cytotoxic immunity (133).

For CD4 T cells, Th1 cell differentiation is critically dependent on the presence of IL-12 and IFN- γ , promoting signaling of STAT4 and T-bet upregulation (134) to enhance IFN- γ production through a positive feedback loop that also suppresses other T helper subset differentiation such as Th2 and Th17 (135). For Th2 cells, both IL-4 and IL-2 cytokines are important for Th2 differentiation. IL-4-induced STAT6 promotes transcription factor GATA3 that can suppress T-bet transcription and Th1 differentiation (136), while Coordinated activity of IL-2 mediated STAT5 activation with GATA3 is required to induce activation of the effector cytokine gene locus IL-4/IL-13 (137). Th17 differentiation involves multiple major signaling cytokines including IL-6, IL-21, IL-23 and TGF β , which jointly activate STAT3 to promote ROR γ t expression and IL-17 production (138). On the other hand, TGF- β is also the critical cytokine for commitment of induced Treg cells that are developed in the periphery post antigen encounter. TGF- β induces smad2 and smad3, which are both known to induce Foxp3 (139); along with STAT5-induced downstream to IL-2 signaling, these key cytokines support the differentiation of peripheral Treg cell lineage.

1.2.2 Master signaling regulations

Several signaling pathways downstream of T cell activation influence T cell fate, differentiation, proliferation and survival. These signaling transduction pathways tightly coordinate with the three signals to allow integration of antigen types as well as environmental cues for optimal functional responses. Critical signaling pathways for T cell regulation are discussed below (Figure 1.4).

1.2.2.1 The PI3K/Akt/Foxo1 signaling axis

The PI3K signaling network is evolutionarily conserved to support many cellular events, including cell proliferation and survival, cytoskeletal remodeling, migration, and trafficking of intracellular organelles depending on the cell type and stimulus; these diverse roles have also been implicated in many studies for support of T cells. There are four classes of PI3K; the p110 δ class IA catalytic subunit and the p110 γ class IB catalytic subunit are studied most in immunology since these proteins are preferentially expressed in leukocytes (140). The Class IA PI3Ks are activated downstream of TCR and CD28 signaling, which acts as a signaling hub at the plasma membrane to change the lipid composition in order to link transmembrane receptors to the organization of multiprotein complexes post activation.

PI3K can further recruit effector molecule PDK1 and its substrate Akt through phosphorylation at the Thr308 site (141). Activated Akt can further phosphorylate the Forkhead Box Subgroup O (Foxo) transcription factors, leading to their nuclear exclusion and inhibition (142). Foxo1 downstream targets includes

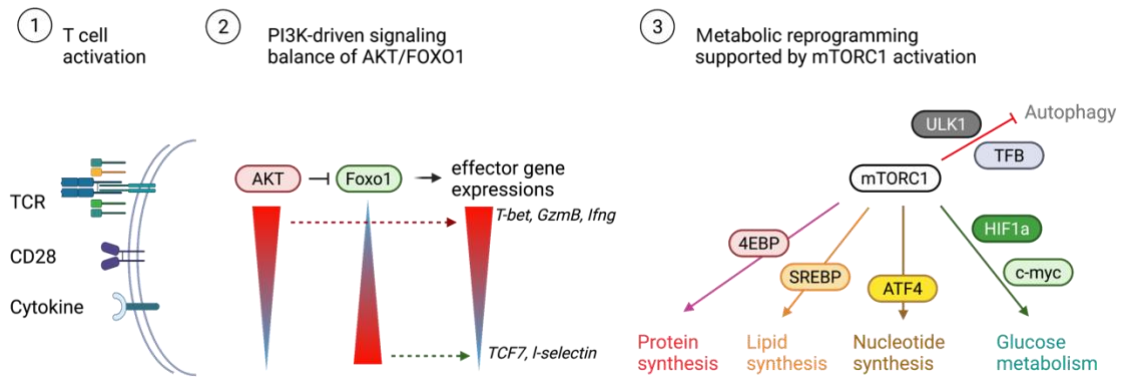


Figure 1. 4 T cell activation and master signaling regulations

Downstream of T cell activation, multiple signaling pathways are upregulated to support optimal T cell responses. Enhanced Akt signaling intensity supports effector functions through inhibition of Foxo1, as well as increased mTORC1 activation that leads to metabolic reprogramming to support cell growth and proliferation that T cells require for rapid division.

the transcription factor TCF7 and the chemokine receptor CCR7, which have pivotal roles in regulating central memory T cell differentiation and trafficking. Abrogation of Foxo1 in CD8 T cells preferentially favor the development of short-lived effector cells (SLECs) over memory precursor effector cells (MPECs), illustrating how Akt/Foxo1 signaling activity can determine the fate of memory differentiation in CD8 T cells (143, 144).

Additionally, Akt can also phosphorylate Tuberous Sclerosis (TSC) 2 and Proline-Rich Akt substrate of 40kDa (PRAS40), which are both negative regulators of mTORC1, the master growth regulator. Thus, Akt can signal to increase the activity of mTORC1 that coordinates with cell growth and downstream metabolism (145). Another negative regulation is the inhibition of GSK-3 β through Akt. GSK-3 β can further cross-regulate PI3K/Akt amongst other signaling pathways to regulate downstream cell growth and metabolism (146).

Both the Akt/Foxo1 and mTORC1 (discussed later) signaling axis positively regulate conventional CD8 and CD4 T cell differentiation (147). Interestingly, PI3K signaling is also a critical regulator for Treg cells (148). It was first illustrated by studies in mice with a kinase-inactive knocked-in form of p110 δ (p110 δ D910A), which results in a less suppressive phenotype of Treg cells in secondary lymphoid organs, suggesting that p110 δ activity is important for Treg cell function in the periphery (149). However, Treg cells also exhibit reduced level of PI3K activity compared to conventional T cells, which is critical for maintenance of their normal suppressive function (150). Constitutive activation of Akt can also represses the development of thymic Treg cells (151), while deletion of Foxo1 in Treg cells

reduces Treg cell function manifested in multiorgan inflammation (152). These studies suggest that fine-tuned PI3K activity is critical for development and function of Treg cells. Collectively, PI3K/Akt/Foxo1 signaling axis is critical for T cell functions in a cell-context dependent manner. Increased intensity of Akt supports CD8 effector phenotypes as opposed to memory differentiation, whereas Treg cells require reduced Akt signaling strength as well as higher and sustained levels of Foxo1 activity.

1.2.2.2 The mTOR signaling regulation of T cells

mTOR, or the mechanistic target of rapamycin, is an evolutionarily conserved serine/threonine protein kinase that exists as two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both mTORC1 and mTORC2 contain scaffolding proteins in addition to mTOR, which are regulatory protein associated with mTOR (RAPTOR) and rapamycin insensitive companion of mTOR (RICTOR), respectively (153). mTOR integrates environmental signals including the availability of nutrients, growth factors, energy and oxygen level, thus acts as a critical sensor to control cell anabolism and downstream metabolism.

Both mTORC1 and mTORC2 includes several key downstream targets. Upon activation, mTORC1 supports cell anabolism through upregulation of protein, lipid, nucleotide synthesis as well as glucose metabolism (153). Two key effectors downstream of mTORC1 include p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP). Phosphorylation of S6K1 promotes mRNA translation through initiation through eIF4B (154). Phosphorylation of 4EBP dissociates it from

Eukaryotic Translation Initiation Factor 4E (eIF4E) to further promote 5' cap-dependent mRNA translation (155). Both effects positively support protein synthesis. Additionally, growing cells require increase supply of lipids and nucleotides for their cell division and proliferation. mTORC1 positively regulates *de novo* lipid biosynthesis through the sterol responsive element binding protein (SREBP) transcription factors that upregulate fatty acid synthesis-related genes (156). *De novo* nucleotide synthesis is also modulated by mTORC1, which increases the Activating Transcription Factor 4 (ATF4)-dependent expression of methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD2) to support purine synthesis (157), as well as carbamoyl-phosphate synthetase (CAD) for *de novo* pyrimidine synthesis (158). Finally, mTORC1 increases glucose metabolism through translation of transcription factor Hypoxia Inducible Factor 1 Subunit Alpha (HIF-1 α) and another downstream target Myelocytomatosis oncogene (MYC). Cellular-MYC (C-MYC) is rapidly induced post T cell activation, and functions as a universal amplifier of gene expression (159). Both C-MYC and HIF-1 α enhance glucose metabolism, which further promotes carbon flux through the oxidative pentose phosphate pathway (PPP) and the serine synthesis pathway (SSP), both utilizing glucose-derived carbon to generate other metabolites and co-factors such as serine and nicotinamide adenine dinucleotide phosphate (NADPH) essential for rapid proliferation and growth (160). Surprisingly, it has been shown that acute loss of C-MYC, but not HIF-1 α , dramatically suppresses the initial metabolic reprogramming post T cell activation (161). Within the nutrient rich environment, mTORC1 also supports cell growth by suppressing autophagy and lysosome

biogenesis through unc-51-like autophagy activating kinase 1 (ULK1) and microphthalmia-associated transcription factors (MitF/TFE), respectively (162, 163).

mTORC2, on the other hand, can promote proliferation and survival by phosphorylation of multiple downstream targets. One key target is Akt, the key effector downstream of the PI3K pathway, allowing indirect regulation of Foxo, mTORC1 and GSK-3 β . mTORC2 also regulates other targets that are members of the AGC (PKA/PKG/PKC) family of protein kinases. These targets modulate downstream cellular function such as the actin cytoskeleton through PKC, as well as serum glucocorticoid-induced protein kinase (SGK) for ion transport and cell survival. (164).

Under steady state, inhibitory molecules including phosphatase and tensin homologue (PTEN), tuberous sclerosis 1 (TSC1) and liver kinase B1 (LKB1) keep mTOR activity in check. Both the mTORC1 and mTORC2 are activated within minutes of TCR activation, which correlates with duration of the interaction (165, 166). Both signal 1 and 2 critically integrates through mTOR to facilitate downstream cell division and growth post activation. For instance, CD28-mediated co-stimulation can shape the mTOR activity through positive regulation of the PI3K/Akt activation to sustain TCR activation (167, 168); whereas PD1 co-inhibitory receptor can downregulate mTOR for immune tolerance (169).

In particular, mTOR critically supports T cell immune responses by regulation at multiple levels. First, fine-tuned activity of mTORC1 is vital for naïve T cell homeostasis signaled through IL-7; enhanced mTORC1 activity due to loss of

TSC1 impairs their homeostasis and survival (170, 171, 172). T cells deficient of mTOR fail to differentiate into Th1, Th2 and Th17 post activation, with a skewed preference towards FoxP3 Treg cell differentiation (173). mTORC2 regulates Th1 and Th2, but not Th17 differentiation (174, 175). On the other hand, constitutive activation of mTORC1 supports a terminally differentiated CD8 effector phenotype at the expense of memory population. Reduced mTORC1 activity favors memory differentiation, but these memory-like CD8 T cells suffer from metabolic defects when rechallenged with bacteria infection. Interestingly, reduced mTORC2 activity through RICTOR deficiency enhance CD8 T cell memory differentiation without affecting effector functions (176, 177). Notably, mTORC2 may limit T cell memory generation through its downstream target Akt activation that dampens Foxo1 activation, negatively impacting key receptors CCR7, L-selectin and IL-7R (178,179, 180). Overall, these data suggest critical signaling regulations through mTOR as well as downstream metabolic modulation to support T cell differentiation and anabolic growth.

1.2.2.3 The hypoxic inducible factor (HIF) axis

HIF is a heterodimeric transcription factor comprised of a constitutively expressed HIF-1 β subunit (also known as the aryl hydrocarbon receptor nuclear translocator, or ARNT), and an α -subunit (either HIF-1 α or HIF-2 α). Both HIF-1 and HIF-2 are tightly regulated at the protein level, which are typically stabilized in the absence of oxygen. In immune cells, HIF expression and stabilization can be triggered not only by hypoxia, but also by various stimuli as well as pathological

stresses associated with leukocyte activation and inflammation, such as Th1 cytokines (181). In addition to serving as an oxygen sensor, the activation of HIF plays a critical role in the regulation of immune cell metabolic function (182,183).

Activation downstream of TCR signaling activates a cascade of pathways including the induction of HIF-1 α (184). Similar to tumor cells, HIF-1 α drives the expression of several glycolytic enzymes that shift the cells to glycolysis-dominant metabolism in T cells (185, 186, 187). T cells deficient of HIF-1 α exhibit reduced expression of GLUT1, a glucose transporter, as well as many downstream glycolytic genes (188). In CD4 T cells, this is accompanied by reduced glycolysis and dampened Th17 differentiation in CD4 T cells in a STAT3- and ROR γ t-dependent manner, with preferential polarization into CD4⁺ FoxP3⁺ T cells (189). HIF-1 α also sustains the inflammatory functions of human Th17 cells (190), and its overexpression through deletion of its negative regulator Von Hippel-Lindau (VHL) leads to dysfunctional Treg cells (191), suggesting a key role of HIF-1 α activity for the balance between Th17/Treg cell differentiation.

In CD8 T cells, HIF-1 α was found to be critical for their rejection against tumors and chronic viruses, presumably due to the inability of T cells to conduct proper metabolic upregulation to support effector state and functions, such as G α mB and IFN- γ (192,193). Deletion of the HIF-1 β gene in CD8⁺ T cells also impairs the expression of cytolytic effector molecules perforins and granzymes, as well as the L-selectin that is involved in the migration of activated T cells to nonlymphoid tissues (194). On the contrary, deletion of VHL results in increased expression of costimulatory receptors and cytolytic molecules (193). Deficiency of

three oxygen-sensing prolyl-hydroxylase enzymes (PHD1, PHD2, and PHD3), negative regulators of HIF, promotes T cell effector function against tumor (195). This effect is mediated in part through HIF-1 α 's downstream target Vascular endothelial growth factor A (VEGF-A), as VEGF-A deletion in CD8 T cells demonstrated enhanced effector functions (196). Overall, modulation of the HIF axis affects T cell subsets differentially, demonstrating a crosstalk between signaling and metabolism to alter functional outcome.

1.2.2.4 The C-MYC signaling pathway

T cell survival, differentiation and expansion are also dependent on a critical transcription factor part of the Myelocytomatosis (MYC) protein family, cellular-myc (C-MYC). Transcriptional induction of C-MYC occurs post engagement of TCR and IL-2 signaling (197, 198). Both its mRNA and protein expression are digitally switched on post T cell activation, allowing the antigen stimulus strength to determine frequency of C-MYC-expressing T cells. The quantity of C-MYC can be further fine-tuned with cytokine signaling (198). C-MYC is subjected to constant proteasomal degradation mediated by GSK-3 β phosphorylation, resulting in a short half-life. The sustained expression is thus critically dependent on amino acid availability and the rate of protein synthesis (198, 199, 200).

The MYC protein family coordinates the global metabolic reprogramming, a critical feature of T cells that will be discussed later. In premalignant cells, MYC proteins are often turned on as drivers of tumorigenesis to support cellular biosynthesis through glucose, lipid and amino acid metabolism (201). Additionally,

MYC can also regulate amino acid transport in order to meet the proliferation demand for essential amino acids. Oncogenic MYC can selectively upregulate the solute carrier family (SLC) 7 member 5 (SLC7A5) and SLC43A1 to sustain amino acid uptake, which further provide a feed-forward loop to sustain transcription of MYC through attenuation of the general control nonderepressible 2 (GCN2)-Activating Transcription Factor 4 (ATF4)-Eukaryotic Translation Initiation (EIF) 2a axis (202). In addition, MYC also cooperates with the HIF-1 α axis or ATF4 response to support upregulation of *de novo* amino acid synthesis including the serine synthesis pathway (SSP) (203), an important pathway for proliferating cells. Besides cellular anabolism, serine catabolism through Serine hydroxymethyltransferase 2 (SHMT2) for the generation of glycine and one-carbon units is also positively regulated by MYC, as MYC can upregulate SHMT2 to promote serine catabolism to generate glycine and one-carbon unit (204) to support *de novo* nucleotide synthesis. In T cells, C-MYC upregulates expression of the amino acid transporter System-L transporter Slc7a5, and the deletion of the transporter hinders C-MYC induction of subsequent biosynthetic programs (205). Importantly, deficiency of C-MYC cripples the ability of T cells to undergo robust metabolic reprogramming that will be discussed later, and is associated with global changes of T cell transcriptome and dampened proliferation *in vivo* (161). C-MYC-dependent genes not only include genes in cell cycle progression and ribosomal biogenesis, but also those that are directly involved in glucose uptake, glycolysis, glutamine oxidation, polyamine synthesis (161). These data suggest the critical signaling role of C-MYC may be a result of direct regulation of metabolic genes, or

indirectly through feedback of general cell cycle regulation and protein synthesis. C-MYC also exhibits a role in controlling mitochondrial ribosome biogenesis, which was shown by a report through proteomics profiling (206). Overall, these data suggest how C-MYC signaling plays a pivotal role for regulation of genes relevant to nutrients uptake and metabolism, which cross regulate to support cellular growth and function.

1.3 Metabolic reprogramming of T cells

Metabolism is a network of biochemical reactions to sustain the functions of a living organism. In the immune system, distinct T cell subsets possess different metabolic requirements for their functional demands. Conventional T cell activation leads to its exit from quiescence, and is accompanied by dramatic metabolic reprogramming of glucose uptake and utilization primarily through glycolysis to support their clonal expansion (207). This rewired metabolism of increased fermentation from glucose to lactate that occurs even in the presence of sufficient oxygen to support oxidative phosphorylation, is similar to the observation in aberrant proliferating cancer cells known as the “Warburg effect” or “aerobic glycolysis”. This rewired metabolism for T cells part of their normal physiological process is dependent on the activation of several signaling regulators, including PI3K/Akt/mTOR, HIF, as well as C-MYC (208). Particularly, glucose is the preferred nutrient for T cells that lies at the center of this metabolic switch. Glucose-

derived carbons can be utilized in different metabolic pathways to support cell growth either through catabolism or anabolism (209). Increased glucose uptake promotes T cell differentiation and proliferation (210). In the absence of glucose, T cell functions are impaired (210, 211, 212), even in the presence of alternative carbon sources like glutamine (213, 214), suggesting the pivotal role of glucose as an essential nutrient for T cells.

1.3.1 Glucose catabolism: aerobic glycolysis

The catabolism of glucose can occur through the breakdown of carbons for the generation of high energy molecules adenosine 5' triphosphate (ATP). The processes that are involved in glucose catabolism includes glycolysis, which breaks down the six-carbon unit glucose into two three-carbon units pyruvate. This is followed by either anaerobic fermentation of pyruvate to lactate in the absence of oxygen, or the aerobic respiration involving pyruvate oxidation to carbon dioxide through the tricarboxylic acid cycle (TCA cycle, also known as the Krebs cycle or the citric acid cycle). The latter is further coupled with oxidative phosphorylation (OXPHOS) mediated by the electronic transport chain to generate ATP in the mitochondria. Thus, pyruvate is a pivotal point for a cell to commit to two different downstream metabolic pathways.

In T cells, aerobic glycolysis is part of the normal activation process (Figure 1.5). The direct effects of aerobic glycolysis include rapid cytosolic ATP synthesis (215) and maintenance of redox-balance through the regeneration of electron acceptors, nicotinamide adenine dinucleotide (NAD⁺) (216). This is mainly

supported by the enzyme lactate dehydrogenase (LDHA), which converts pyruvate to lactate with the regeneration of one NAD⁺ per pyruvate to fuel the consumed NAD⁺ in upstream glycolysis. This is critical in maintaining the redox-neutral state to facilitate continuous glycolysis. On the other hand, if pyruvate were fluxed through the TCA cycle and OXPHOS, a total of 38 molecules of ATP can be generated. Thus, it has been a long-standing question of why aerobic glycolysis takes place when the energy generation per glucose for cells is not as efficient.

Many studies have since focused on this unique phenomenon of aerobic glycolysis dependence in fast proliferating cells including cancer cells and T cells. One widely discussed hypothesis is that aerobic glycolysis supports increased flux of glucose as sources of carbon, which can be further utilized for anabolic requirements to support proliferation through biomass generation, as well as influencing cellular activity such as signaling or gene regulation (217, 218). Nevertheless, the avenues that lead to biosynthesis from glucose would lead to absence of lactate fermentation, which contradicts the original observation where most carbon atoms were secreted in the form of lactate (219).

Glycolytic enzymes and their metabolic intermediates can also modulate T cell responses. Reduced level of glycolytic intermediate phosphoenolpyruvate (PEP) dampens calcium-NFAT signaling and subsequent T cell activation (212), while the glycolytic enzyme Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can bind and post-transcriptionally suppress *Ifng* mRNA translation in T cells when glycolysis is reduced (210). LDHA-mediated glycolysis has also been reported to support Th1 helper cell differentiation through an epigenetic mechanism (220),

these data suggest the multifaceted role of glucose utilization in T cells to support their proper activation and functions.

Importantly, since lactate produced by aerobic glycolysis can be actively secreted out of cells by monocarboxylate transporters (MCTs), LDHA-catalyzed reaction thus support rapid cytosolic ATP synthesis, fast carbon disposal and redox-balance (221). More recently, our group has demonstrated that PI3K signaling is positively supported by LDHA-mediated bioenergetics to sustain a positive feedback circuit for both CD8 and CD4 T cell-mediated immunity (222, 223), with deficiency in LDHA significantly attenuated their expansion and differentiation in part through dampened PI3K-dependent phosphorylation of AKT and enhanced activity of the downstream substrate Foxo1 activity. Supplementation of exogenous ATP was able to rescue PI3K-mediated PIP3 generation as well as the defected Akt/Foxo1 signaling. These data suggest that intriguingly, cytosolic ATP may be limiting in activated T cells for their signaling, while LDHA plays an essential role for fueling the bioenergetics for optimal T cell responses (Figure 1.5).

On the other hand, mixed results have been reported for Treg cells. It has been suggested that Treg cells are more reliant on mitochondria glucose and fatty acid oxidation instead of glycolysis (224, 225). Unconstrained glycolysis or enforced glucose transporter GLUT1 expression can lead to decreased Foxp3 expression, reduced murine Treg cell suppressive activity, and aberrant expression of inflammatory cytokine IFN- γ (226, 227), whereas Foxp3 expression in human Treg cells is promoted by glycolysis (228). In other reports, Treg cells

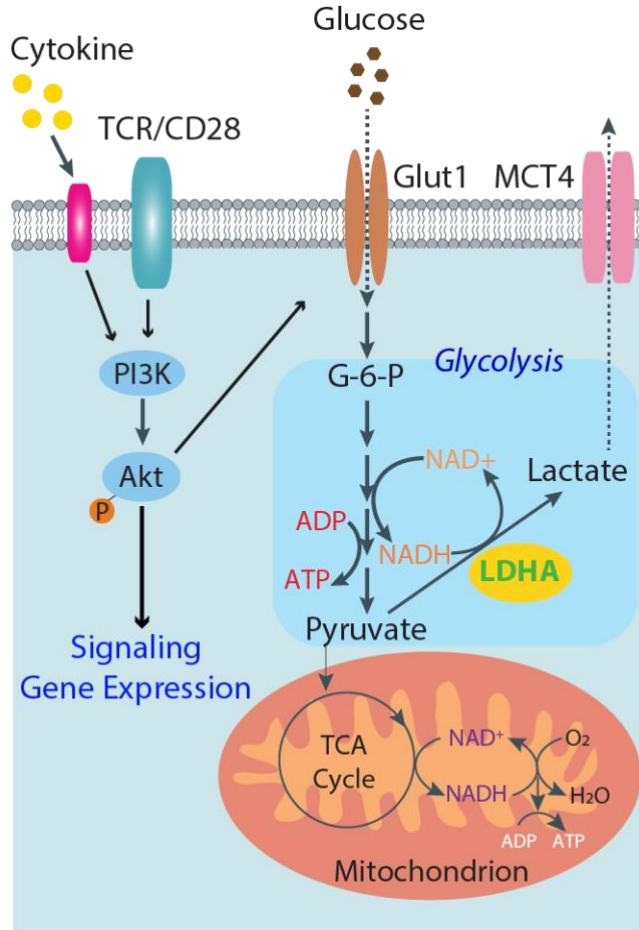


Figure 1. 5 Signaling and metabolic regulations of T cells.

T cells undergo rapid metabolic reprogramming post activation. TCR, co-stimulation and cytokine activate signaling pathways including PI3K/Akt that induces glucose uptake. Most glucose is catabolized by LDHA to lactate through aerobic glycolysis and excreted via monocarboxylate transporters (MCT) 4, but not oxidized in the mitochondria.

undergo reverse glycolysis by converting lactate to pyruvate in a high-lactate environment *in vitro* (229); yet, intratumoral Treg cell expansion is supported by both glycolysis and fatty acid metabolism (230), while migration of activated Treg cells to inflammatory sites is glycolysis-dependent (231). Overall, current studies suggest that Treg cells may have selective dependency on glycolysis to support of their functions in different disease and tissue context, which remains a field for further investigation.

1.3.2 Glucose anabolism: *de novo* synthesis of serine and nucleotides

Glucose can donate its carbons to construct macromolecules necessary for cell growth and proliferation through glucose anabolism. Various molecules can be synthesized from glucose in T cells. Additionally, aerobic glycolysis from glucose generates metabolic intermediates that are used for macromolecule synthesis and biomass to support cellular proliferation and function, which is also a key part of glucose anabolism.

First of all, glucose can donate its 6-carbon to support the biosynthesis of Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is synthesized in Golgi Apparatus and Endoplasmic Reticulum (ER) for the synthesis of glycans and protein glycosylation (232). Glycans regulate various aspects of T cells, including glycosylation of multiple T cell surface receptors that are involved in their development in thymus, co-stimulation, co-inhibition, and cytokine receptors, thus affecting the balance between immunoreactivity and immunotolerance (233). For instance, reduced glycosylation of PD1 coinhibitory receptor limits its expression,

resulting in enhanced immunity (234). Glycosylation of TCRs can modulate their threshold of activation by regulating the proximity amongst each other; loss of galectin-binding to TCRs lead to heightened immune response and autoimmune diseases (235, 236). Lastly, T cells adhesion and trafficking through their adhesion molecule, selectins, can be altered with modulation of different glycans (Kawashima). Binding of L-selectin (CD62L) on lymphocytes with endothelial cells in the high endothelial venules (HEV) is critical for their tethering and rolling (237); improper glycosylation due to deficient glycosyl-transferase can cause defective homing (238).

Glucose can also donate 5-carbon to support the biosynthesis of ribose-5-phosphate (R-5-P) that is the precursor for *de novo* nucleotide biosynthesis, which is pivotal to support the increased biomass in fast proliferating cells. Precursors for this pathway can come from the pentose phosphate pathway (PPP) (239), one-carbon metabolism pathway shunted from glycolytic flux, as well as different amino acids (240). *De novo* serine synthesis is another pathway that can be attributed from glucose directly through glycolytic intermediate 3-phosphoglycerate (3-PG). Synthesized serine and its downstream metabolites including glycine and one-carbon units are critical to support nucleotide synthesis for cell expansion. Deficiency in both extracellular as well as *de novo* synthesized serine severely impacts T cell proliferation (241). Another critical component of cell proliferation, the cellular membrane lipids, can also be synthesized from serine through ceramide for both sphingolipids and phospholipids. The process of serine synthesis also generates alpha-ketoglutarate (α -KG), which is not only a key

anaplerotic intermediate for TCA cycle, but also a co-factor that regulates DNA and histone methylation through α -KG-utilizing dioxygenases including the Jumonji C (JMJC) family members and the ten-eleven translocation (TET) methylcytosine hydroxylases (242). Lastly, although glucose has been reported to synthesize alanine through pyruvate transamination by the enzyme alanine aminotransferase, or the glutamate-pyruvate transaminase (GPT), T cells produce low levels of alanine post activation, and extracellular alanine is required for T cells to exit quiescence as well as for subsequent protein synthesis (243).

Glucose can donate 2 carbons to support synthesis of acetyl-coenzyme A (acetyl-CoA). As acetyl-CoA is compartmentalized, glucose-derived pyruvate can enter the mitochondria to synthesize downstream metabolite citrate, which is then shunted out into the cytosol for conversion back to cytosolic acetyl-CoA through the enzyme ATP-citrate lyase (ACL). ACL-dependent production of acetyl-CoA not only supports lipogenesis for proliferating cells, but also affects gene expression through histone acetylation (244, 245). Glucose-derived acetyl-CoA has been shown to exhibit major impacts on histone acetylation and gene expression in multiple cancer cell lines (246). In T cells, LDHA-mediated glycolysis maintains high concentrations of acetyl-CoA to enhance histone acetylation for the transcription of *Irfg*, which is critical for their effector functions (220).

1.3.3 Cross-talk between signaling and metabolism

The metabolic reprogramming of activated T cells often occurs through the “top-down” fashion, where the ligation of activating receptors positively modulates

signaling transduction pathways like PI3K/Akt, further promoting gene transcription of metabolic regulators like mTORC1 and C-MYC. This enhanced activity leads to amplified metabolism including glucose uptake and utilization, escalated amino acid uptake and synthesis, augmented lipid metabolism, as well as nucleotide biosynthesis. Thus, signaling is capable of regulating metabolic activity from top-down, and upregulated metabolic pathways jointly contribute to the biomass increase that a proliferating cell critically needs (208).

On the other hand, increasing evidence shows that intracellular metabolite and nutrient levels are capable of providing feedback and modulating signaling reciprocally in a “bottom-up” fashion, further shaping cell fate and function. First, metabolite can serve as co-factors for enzymes involved in signaling pathways, as illustrated by the regulation of α -KG-utilizing dioxygenases to support DNA and histone modification. Furthermore, metabolites can directly bind to proteins to regulate its activity. For instance, glucose deprivation leads to reduced cellular energetics at the ATP level and enhanced oxidative stress by reactive oxygen species (ROS); both can be sensed through adenosine monophosphate (AMP)-activated protein kinase (AMPK), an important regulator of cellular activity. Particularly, enhanced level of AMP can displace ATP binding to AMPK to yield allosteric activation (247). Low ATP level and high ROS level both engage AMPK to inhibit the master growth regulator mTORC1, leading to reduced cell anabolism including mRNA translation, protein synthesis and reduced proliferation as cellular outcome. Other than inhibiting anabolism, AMPK can also promote catabolism such as autophagy through ULK1 (248), as well as fatty acid oxidation and

glycolysis to support energy-stressed cells, which also fuels the synthesis of NADPH to support oxidative stress. Loss of this energy sensor in T cells leads to decreased mitochondria respiration and cellular ATP levels with limited metabolic adaptation regardless of glucose availability, thus restraining the optimal response against infections (249).

1.3.4 mTORC1 as a sensor to support metabolic reprogramming

Activation of mTORC1 supports metabolic reprogramming critical for T cells. As a unique complex, mTORC1 incorporates the sensing of both growth factor and amino acid availability into regulation for cell anabolism (Figure 1.6). In the growth factor sensing axis, the tuberous sclerosis complex (TSC) 1 and 2 serve as negative regulators of mTORC1. Upon binding of growth factors to receptor tyrosine kinases (RTKs), the PI3K/Akt signaling pathway is activated, and Akt phosphorylates TSC2 to inhibit the TSC complex by inducing its release from lysosome, allowing activation of mTORC1 by GTP-loaded RAS homologue enriched in brain (Rheb), a positive regulator of mTORC1. In conditions of high AMP/ATP ratio when cellular energy level is low, AMPK is activated to positively regulate TSC2, which can then inhibit mTORC1 activity (153).

In the nutrient sensing axis, amino acid availability can be sensed by various units in the mTORC1 complex. Prior reports demonstrated that amino acids could promote mTORC1 activation through the Rag family of GTPases, which consists of heterodimers RagA/B or RagC/D that are anchored to the lysosomal surface by the Ragulator complex (250), and are essential components in the amino acid-

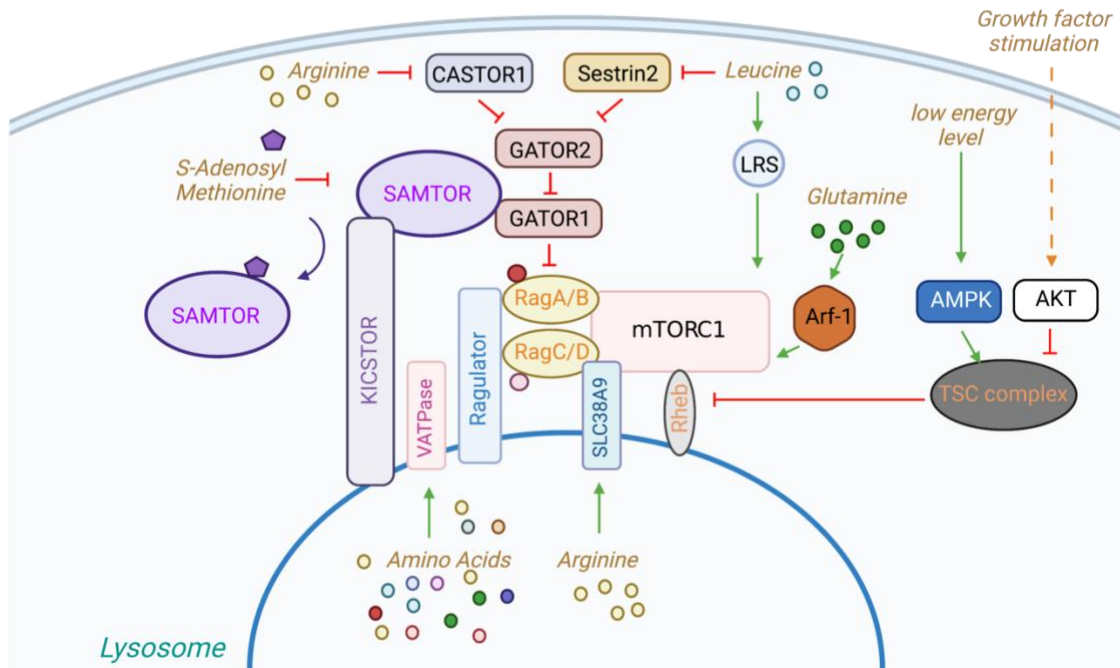


Figure 1. 6 Activation of mTORC1 complex integrates sensing of growth factor and amino acid availability.

Growth factors are sensed by TSC complex, which relieves the inhibitory effect on mTORC1 complex. Amino acids can be sensed by V-ATPases and Rag GTPases for optimal activation of mTORC1 complex. Individual amino acids can also be sensed; the presence of leucine, arginine, and S-adenosymethionine (SAM) can be sensed by cytosolic and lysosomal sensors, converging on the activation of Rag GTPases that facilitates the recruitment of mTORC1 onto lysosomal surface and downstream activation by Rheb GTPase. Glutamine can also activate mTORC1 in a Rag- GTPases independent fashion.

sensing pathway upstream of mTORC1 (251, 252). When cells are starved of amino acids, mTORC1 is dispersed in the cytoplasm. In the presence of amino acids, Rag GTPases becomes activated in either GTP-RagA/B or GDP-RagC/D form, allowing binding to raptor of the mTORC1 complex that further gets recruited to the lysosomal surface where it interacts with and be activated by Rheb GTPases. The vacuolar H⁺-adenosine triphosphatase (v-ATPase) is another critical component located on the lysosomal surface for amino acid sensing—amino acid addition strengthens its interaction with the Ragulator complex, which is necessary for optimal mTORC1 translocation and activation (253).

Recent studies have also uncovered additional signaling cascades to mTORC1 activation dependent on individual amino acids or metabolites. The presence of leucine can activate the leucyl-tRNA synthetase 1(LRS), an enzyme involved in attaching leucine to its tRNA, to translocate to the lysosomal surface and activate Rag GTPases that further enhances mTORC1 activity (254). Alternatively, leucine can also promote GTPase activating proteins toward Rags 2 (GATOR2)-mediated inhibition of GATOR1, a negative regulator of mTORC1, thus promoting subsequent mTORC1 activation (255). Arginine directly binds the cytosolic arginine sensor for mTORC1 subunit 1 (CASTOR1) and CASTOR2. Similar to leucine, this binding induces dissociation from GATOR2, which can inhibit GATOR1 that leads to mTORC1 activation (256, 257). S-adenosylmethionine (SAM), a metabolite of the methionine cycle, can bind to the S-adenosylmethionine sensor upstream of mTORC1 (SAMTOR); the binding reduces SAMTOR's interaction with GATOR1 and KICSTOR, abrogating the

inhibitory effects on mTORC1 activity (258). Glutamine can induce mTORC1 lysosomal translocation and activation through Arf1 in a Rag GTPases-independent manner (259). In addition to the nutrients mentioned above, multiple individual amino acids including alanine, arginine, asparagine, glutamine, histidine, leucine, methionine, serine, threonine, and valine, can promote mTORC1 activity in the context of acute starvation (260). Overall, current literature suggests nutrient sensing is primarily orchestrated through the mTORC1 complex that allows signal integration of both intracellular and extracellular cues to support downstream cellular functions.

1.4 Summary

T lymphocytes are key mediators of immune activity in diseases ranging from infections to cancer. Optimal T cell activation coupled with downstream signaling transduction leads to its robust metabolic reprogramming to support growth and effector functions; proper control of metabolism is essential to ensure optimal control of T cell-immunity with minimized collateral damage. This is critically supported by glucose metabolism, an integral component for T cell activation. Currently, our understanding of how glucose metabolism regulates T cell responses are incompletely understood. An in-depth understanding will not only reveal novel mechanisms of T cell regulation, but also unravel new therapeutic strategies of metabolic targeting in immunological disorders.

Chapter 2: Glucose metabolism in T cell immunity

2.1 Introduction

Enhanced glucose metabolism through glycolysis is tightly linked and necessary for optimal T cell activation and functions. This chapter will explore the role of glucose metabolism and glycolysis through genetic mouse models in the contexts of infections and tumors to assess the role of these metabolic pathways in distinct T cell subsets.

2.1.1 VHL/HIF regulation and glycolysis axis

Under normoxia conditions, HIF transcription factors including HIF-1 α and HIF-2 α can be hydroxylated in the presence of oxygen, iron and 2-oxoglutarate by oxygen-dependent prolyl-4-hydroxylases (PHDs). Hydroxylation of HIF- α creates a binding site for VHL, an E3 ubiquitin ligase complex, for its polyubiquitination and targeting of HIFs to proteasome for its rapid degradation (261, 262). Under hypoxic conditions or in the presence of TCR activation, HIF- α is stabilized and translocated into the nucleus, binding to its dimerization partner HIF-1 β and enhances the transcription of HIF target genes. In the presence of tumors, where hypoxia microenvironment often promotes HIF-1 α overexpression, its transcription program supports downstream gene expression of angiogenesis and enhanced glycolytic metabolism (263). Thus, HIF-1 α is often considered as an oncogene, while VHL is considered as a tumor suppressor. In the context of activated T cells, both HIF-1 α and HIF-2 α are stabilized post T cell activation even in normoxia, and

many of the HIF target genes are critical for the metabolic reprogramming of T cells to support downstream T cell functions, including glucose metabolism and glycolytic enzymes. Indeed, VHL-deficient CD8 T cells displayed elevated glycolysis and activation-associated receptors, which correlated with enhanced control of persistent viral infection and altered memory differentiation towards effector-like phenotypes (193, 264). Ectopic expression of HIF-2 α , but not HIF-1 α , led to increased cytotoxic differentiation and cytolytic functions (265). These findings suggest the critical role of VHL/HIF axis regulation to support optimal T cell functions. However, how this axis regulate T cell anti-tumor function remains incompletely understood.

2.1.2 T cell metabolic fitness in cancer

The tumor microenvironment is comprised of complex interactions between endothelial cells, stromal cells, extracellular matrix, tumor cells and immune cells; the outcomes of these interactions play a critical role in tumor development. Interestingly, both activated T cells and tumor cells undergo similar metabolic reprogramming that are highly dependent on aerobic glycolysis. Thus, it is often postulated that T cells undergo metabolic competition with tumor cells within the hostile tumor microenvironment. Although primed in nutrient-rich lymph tissues, T cells enter tumor microenvironment, which is often hypoxic with limited oxygen and glucose. Hypoxia can lead to upregulation of PD-L1 expression in myeloid derived suppressor cells (MDSCs) and tumor cells (266). Enhanced PD-L1 expression further engages with PD1 receptor that signals to divert glycolytic dependence of

T cells to fatty acid metabolism, which is associated with a reduction in the activation and cytotoxicity of CD8 lymphocytes. (267), Additionally, hypoxia can further trigger tumor cells to adapt to glycolytic metabolism, competing with infiltrating T cells for the rapid consumption of glucose to support cancer progression (268). Glucose availability also regulates T cell polyfunctionality through the methyltransferase EZH2 expression (269). Key nutrients including glutamine, serine and arginine, which are critical to support both T cell functions and cancer cell growth, are all subjected to local depletion as a result for nutrient competition and utilization. However, glucose remains one of the most consistently reduced metabolites in tumor microenvironment (212, 270, 271)

Besides limiting access to nutrients, tumor microenvironment exhibits additional mechanisms that regulate T cell metabolic fitness. For instance, the accumulation of immune-suppressive metabolites including adenosine and tryptophan catabolite kynurenine can inhibit cytotoxic T cell functions while supporting the immunosuppressive Treg cell compartment, (229, 272, 273). Furthermore, high lactate concentrations secreted by tumor cells can further inhibit CD8 T cell glycolysis due to impeded lactic acid export (274). Collectively, present data illustrate various means of metabolic restrictions against T cells to mount an effective immune response in the tumor microenvironment due to altered T cell metabolism, which remains a field of active research.

2.1.3 MMTV-PyMT mouse model

To characterize the complex roles of metabolism in support of T cell immunity in the tumor microenvironment, we focused our studies on the murine MMTV-PyMT spontaneous breast cancer model, in which the mouse mammary tumor virus (MMTV) promoter drives expression of the polyoma virus middle T-antigen (PyMT) in mouse mammary luminal epithelial cells (275, 276). This model closely recapitulates human disease progression in the four stages of breast cancer: hyperplasia, adenoma, early-stage carcinoma, and late-stage carcinoma; this model thus allows comprehensive studies throughout early cancer cell transformation and later development.

2.2 Results

2.2.1 A critical role of LDHA-mediated glycolysis in supporting anti-cancer T cell functions

Glucose is the key nutrient to support the rewired glycolytic metabolism in T cells, which is mediated by LDHA. We sought to assess how cell intrinsic deficiency of LDHA-mediated glycolysis may impact T cell functions in tumor. LDHA-mediated bioenergetics is critical for fueling PI3K/Akt signaling pathway to support optimal functions of CD8 T cells in infections, and CD4 T cells in autoimmunity (222, 223). While many reports suggested inhibition of T cell glycolysis attributes to increased tumor progression, there is currently no direct genetic evidence, and the role of LDHA-mediated glycolysis in supporting anti-tumor immunity is unresolved. To assess the role of LDHA in mediating immunity

against cancer, we utilized a mouse strain that allowed conditional deletion of LDHA crossed with CD4cre strain, which allowed T cell-specific deletion of LDHA. (hereon referred to as 4creLDHA^{fl/fl}). Mice with LDHA-deficient T cells (4creLDHA^{fl/fl}) were further bred to MMTV-PyMT mice on the C57BL/6 background to assess the effect of T cell-LDHA in mediating anti-tumor immunity, hereafter referred to as the 4creLDHA^{fl/fl}PyMT mice (Figure 2-1A), which deleted LDHA in both conventional T cells and TCRβ⁺ type1 innate-like cytotoxic T lymphocytes (ILTC1s, 277). Mammary tumor growth was monitored on a weekly basis to compare tumor growth in the 4creLDHA^{fl/fl}PyMT mice with their WT littermates, LDHA^{fl/fl}PyMT.

We first observed that there was a significantly accelerated tumor growth in the 4creLDHA^{fl/fl}PyMT mice compared to their WT littermates (Figure 2-1B), suggesting that LDHA positively supported T cells in their functions for anti-tumor immunity. Phenotyping the PyMT tumors by flow cytometry analysis revealed a significant reduction of infiltrating TCRβ⁺ cells (Figure 2-1C, 2-1D), decreased CD8⁺ and CD4⁺ T cells (Figure 2-1E, 2-1F, 2-1G). Both tumor-infiltrating CD8 and CD4 T cells were similarly impacted by LDHA deficiency, as shown by unaltered CD8/CD4 ratio (Figure 2-1H).

PD1 co-inhibitory receptor serves as an antigen-experienced activation marker and exhaustion marker (Figure 2-2A). The infiltrating conventional CD8 T cells had much reduce expression of PD1 marker (Figure 2-2B) that allowed assessment for both T cell activation and exhaustion. These data suggested that

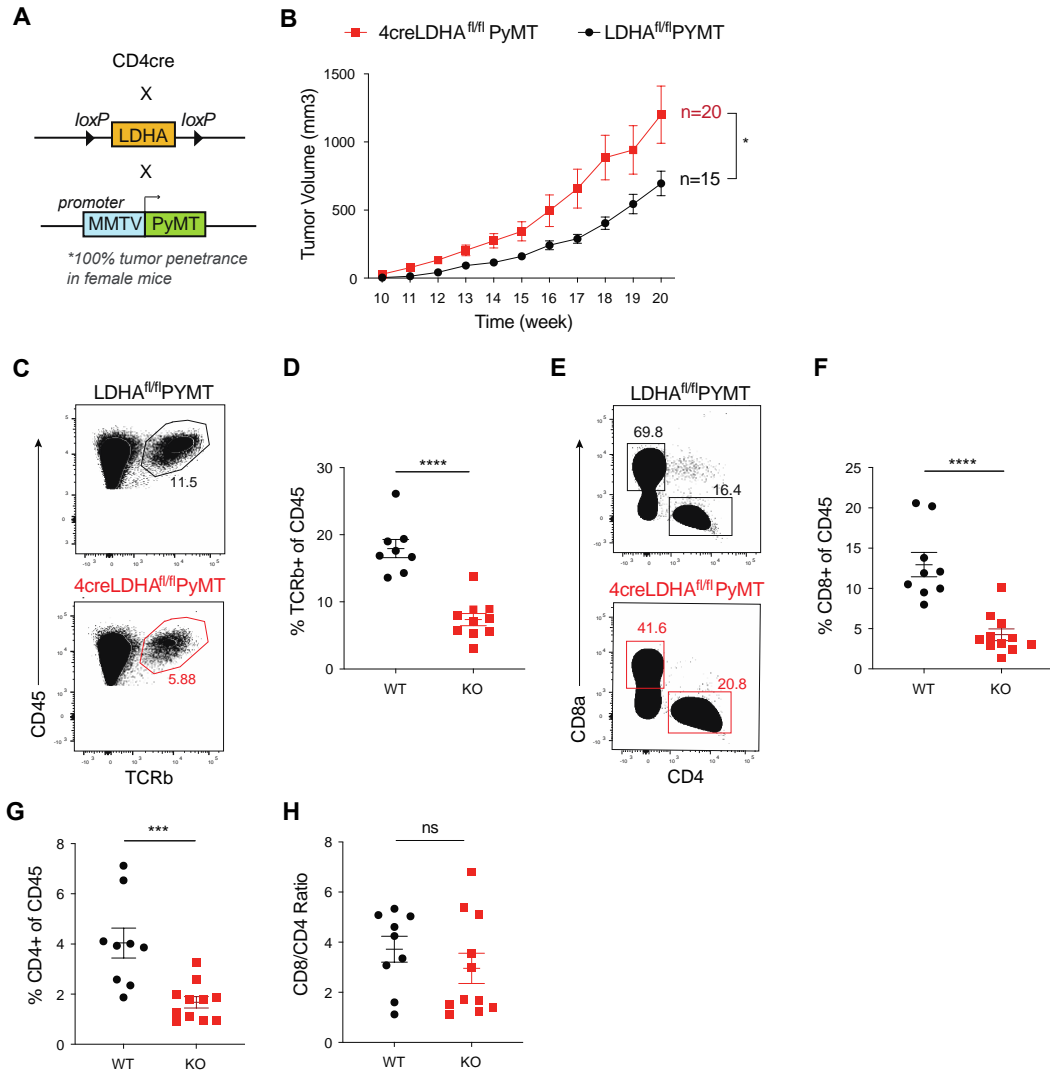


Figure 2. 1 LDHA supported T cell-mediated anti-tumor immunity
 (A) A schematic depicting the mouse breeding strategy crossing CD4cre to LDHA^{fl/fl}, which was further bred to PyMT for tumor development in female mice .
 (B) Weekly tumor volume measurement in LDHA^{fl/fl}PyMT (WT) and 4creLDHA^{fl/fl}PyMT (KO) mice. (C)(D) Flow cytometric analysis of %TCRβ+ cells in tumors. (E)(F) Flow cytometric analysis of %CD8+, (G) %Foxp3-CD4+ T cells of total CD45+ cells and (H) CD8/CD4 ratio in PyMT tumors.

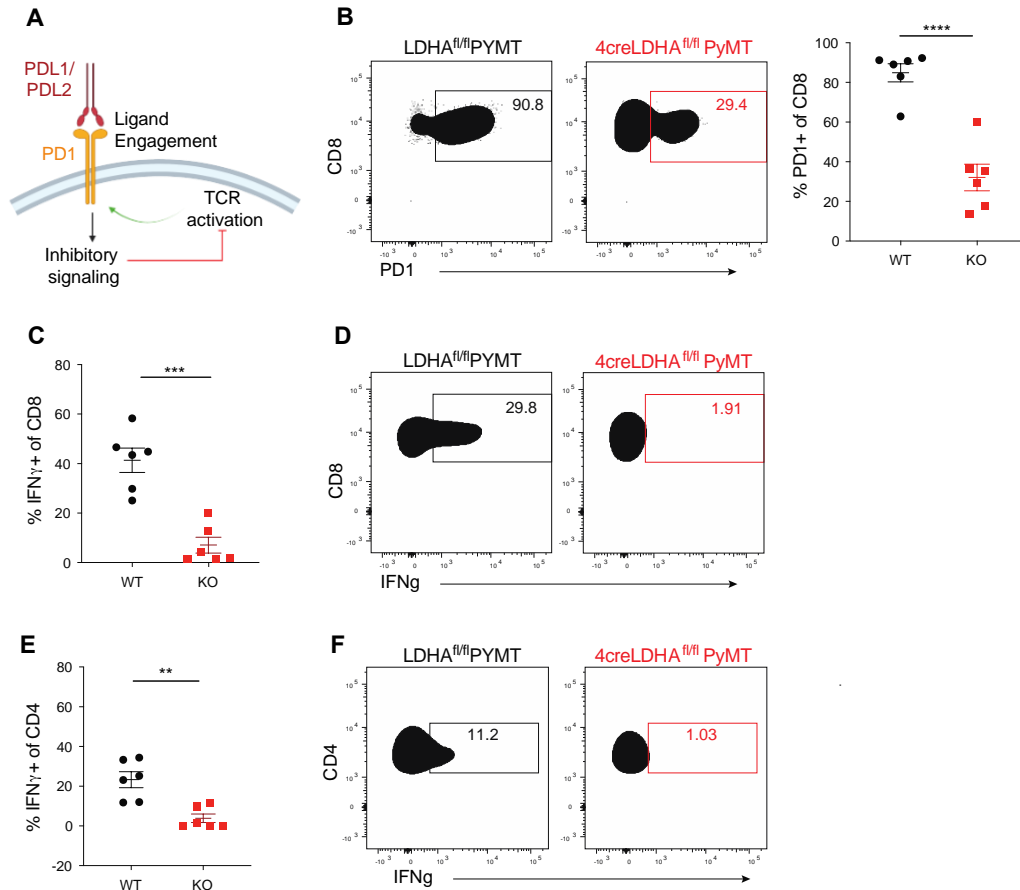


Figure 2. 2 T cells deficient of LDHA displayed dysfunctional activation and cytokine production.

(A) A schematic of the role of program death 1 (PD1) in T cells. Upon ligand engagement, PD1 can transduce inhibitory signals to mediate TCR inhibition. (B) Flow cytometric analysis of PD1 expression on intratumoral CD8 T cells in LDHA^{fl/fl}PyMT (WT) and 4creLDHA^{fl/fl}PyMT (KO) mice. (C)(D) IFN- γ production from intratumoral CD8 T cells and (E)(F) CD4 T cells from LDHA^{fl/fl}PyMT (WT) and 4creLDHA^{fl/fl}PyMT (KO) mice stimulated 4hour ex vivo with PMA/Ionomycin/GolgiStop.

the infiltrating CD8 T cells may be less activated due to LDHA deficiency and were unable to sustain the metabolic reprogramming required for T cell activation. Further assessment through the *ex vivo* stimulation with phorbol myristate acetate (PMA) and ionomycin showed significantly decreased production of IFN- γ in both the CD8+ and CD4+ T cell compartment (Figure 2-2C - 2-2F). Previous studies had indicated the function of LDHA in supporting gene transcription through acetylation of the *Ifng* gene locus, therefore supporting CD4 T cell effector function (220). This data suggested similar regulation of IFN- γ for intratumoral CD8 and CD4 T cells, which was critical for the defense against tumor cells.

To assess if different subsets of T cells were affected in the PyMT tumor model, we investigated the ILTC1 population. ILTC1s are characterized by expression of NK1.1+ marker, tissue resident markers CD49a+ and CD103, as well as high levels of cytolytic granules granzyme B (GzmB); they have been reported to seed early in the tumors to attribute to cancer immunosurveillance (277). We found that there was a significant decrease of the frequency of TCR β + ILTC1s expression in the tumor, and a relative increase of TCR $\gamma\delta$ + ILTC1s that were not targeted by genetic deletion by CD4cre (Figure 2.3A-B). Currently, there is very limited understanding of how LDHA impacts unconventional cytolytic T lymphocytes. This data suggested that LDHA may be important for either the survival or function for the TCR β + ILTC1 population to maintain its cancer immunosurveillance role in the PyMT tumor model, which may attribute to the accelerated PyMT tumor growth observed early in tumor development.

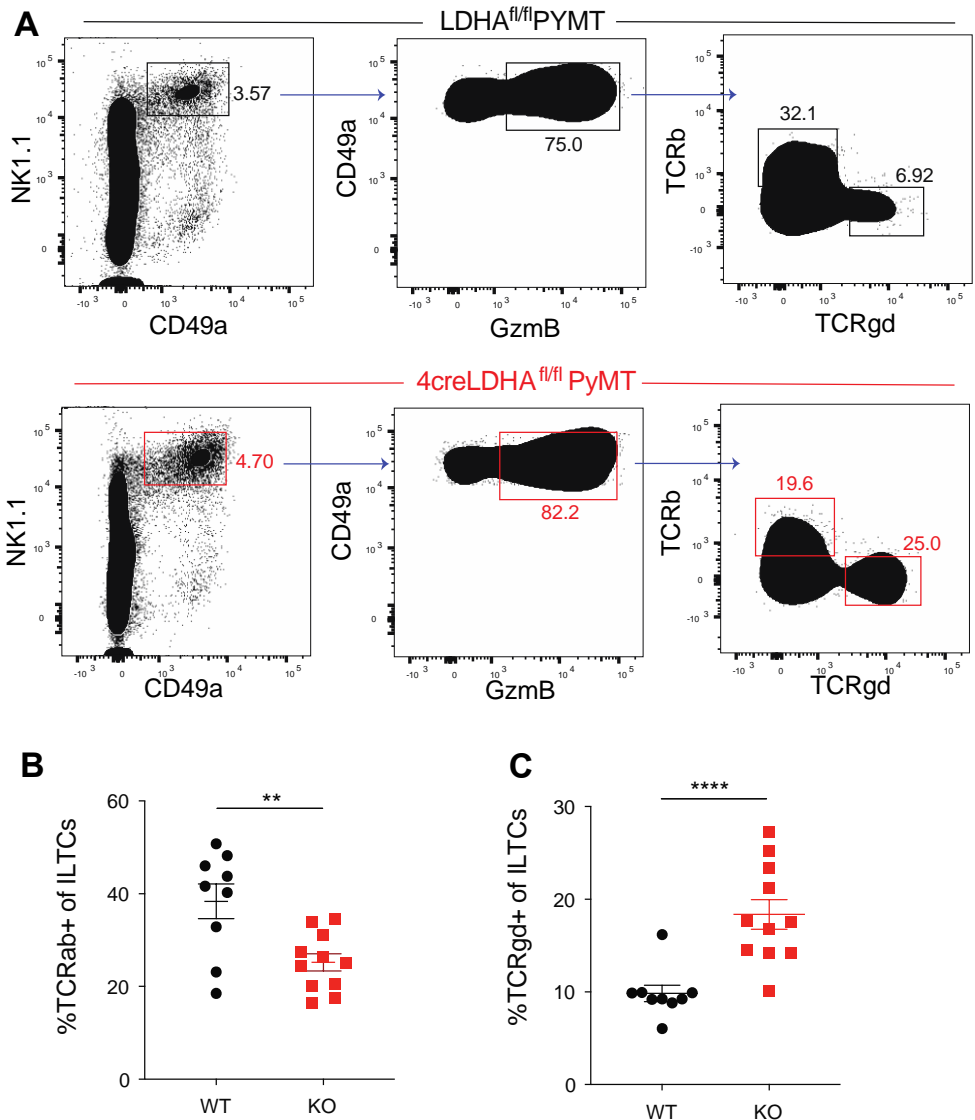


Figure 2.3 The population of Intramural TCR $\alpha\beta$ + Innate-like Type 1 T lymphocytes (ILTC1s) population were reduced by LDHA deficiency. (A) Flow cytometric gating for ILTC1s in PyMT tumors from LDHA^{fl/fl}PyMT (WT) and 4creLDHA^{fl/fl}PyMT (KO) mice: CD49a⁺ and NK1.1⁺ double positive cells were gated amongst Live CD45⁺ cells, NK1.1⁺CD49a⁺ cells were further gated on its positive expression of GzmB, and further gated on TCR β or TCR $\gamma\delta$. (B)(C) Quantification of flow cytometric analysis of TCR β + ILTC1s and TCR $\gamma\delta$ + ILTC1s from PyMT tumors in WT and KO mice.

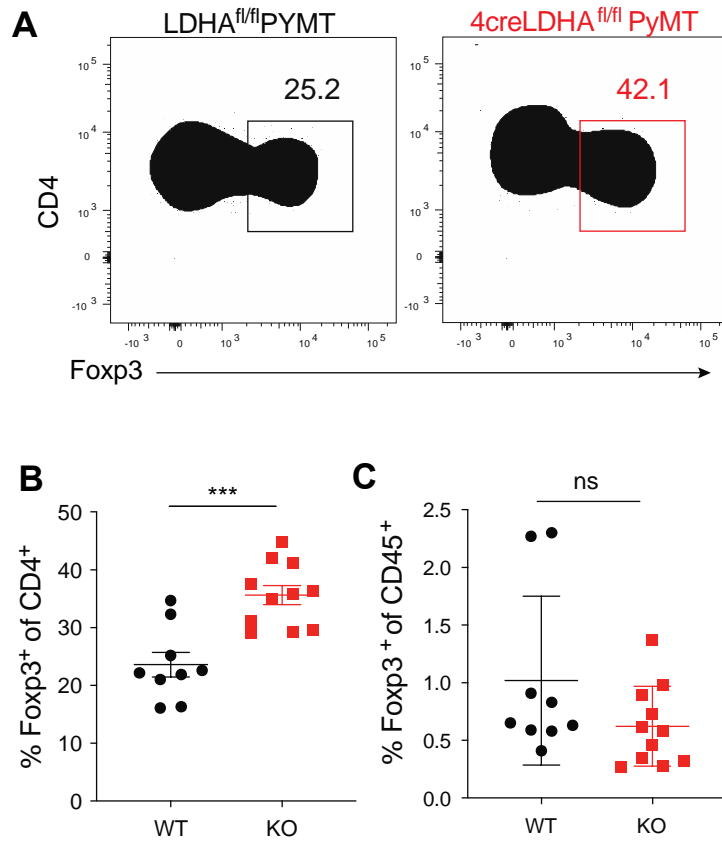


Figure 2. 4 Frequency of Treg cells was unaltered in total immune population by LDHA deficiency.

(A) Flow cytometric analysis of CD4⁺ Foxp3⁺ Treg cells in PyMT tumors from LDHA^{fl/fl}PyMT (WT) and 4creLDHA^{fl/fl}PyMT (KO) mice. (B) Quantification of Treg cells out of total CD4 T cells and (C) total immune (CD45⁺) population.

Treg cells have been reported to play a role in suppressing anti-tumor immunity. Given the mixed reports of Treg cell utilizing glycolysis for their functions, we wondered if LDHA-mediated glycolysis impacted Treg cell functions in the PyMT tumors. Since Treg cells were also targeted by CD4cre, 4creLDHA^{fl/fl}PyMT (KO) mice served as a great tool for this question. Surprisingly, we observed a proportional increase of Treg cells within total T cell compartment in the PyMT tumor (Figure 2-4A, 2-4B), whereas the total population within the immune cells did not change (Figure 2-4C). This suggested to us that Treg cells were not affected by LDHA-deficiency, and that the proportional increase of Treg cells may be a result of decreased conventional T cell compartment, also indicating that Treg cells may depend on different metabolic regulations than conventional T cells for their survival, migration to tumor and the subsequent immune functions intratumorally.

2.2.2 Regulatory T cells exhibit less dependency on LDHA-mediated glycolysis

Our observation of the distinct effects of LDHA-deficiency on conventional T cells and Treg cells in the PyMT tumor model prompted us to further study metabolic regulations in different subsets of T cells. Particularly, we were interested to study if Treg cells have different metabolic dependencies than conventional T cells. There have been numerous reports on whether Treg cells depend on glycolysis, which remains somewhat controversial. To investigate if LDHA-mediated glycolysis supported Treg cell responses, we generated Treg cell-

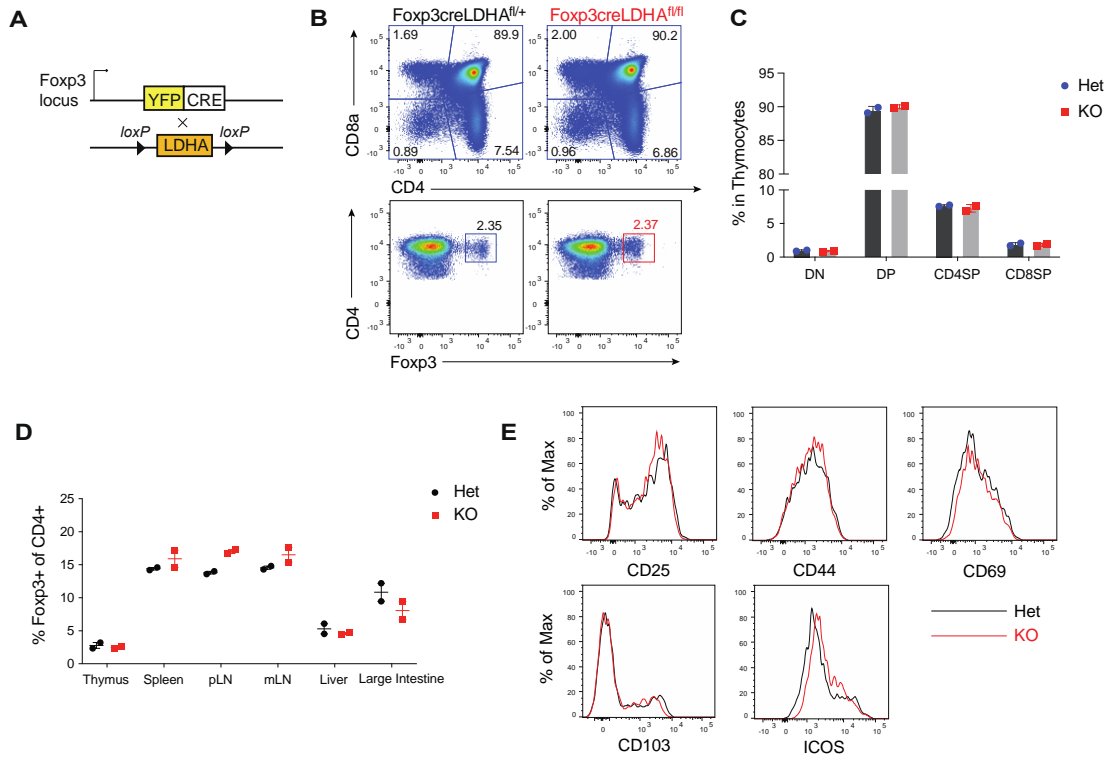


Figure 2. 5 Treg cells with LDHA deficiency showed comparable steady state phenotype with WT.

(A) Schematic showing the breeding scheme generating Foxp3creLDHA^{fl/fl} mice, Foxp3creLDHA^{fl/+} littermates were used as controls. (B) Flow cytometry analysis of CD4 and CD8 expression in thymocytes (upper panel) and Foxp3 expression in CD4+CD8- T cells (lower panel) from Foxp3creLDHA^{fl/+} (Het) and Foxp3creLDHA^{fl/fl} (KO). (C) Frequencies of CD4-CD8- (DN), CD4+CD8+ (DP), CD4+CD8- (CD4SP), and CD4-CD8+ (CD8SP) thymocytes. (D) Frequencies of Treg cells in thymus, spleen, peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), liver and large intestine in het and KO animals. (E) Expression of Treg cell activation markers CD25, CD44, CD69, CD103 and ICOS on Treg cells from mLN of het and KO animals.

specific LDHA-deficient mouse model by crossing *Ldha^{fl/fl}* mice with *Foxp3Cre* mice (Figure 2.5A). *Foxp3CreLDHA^{fl/+}* het littermates were used as controls to compare with *Foxp3CreLDHA^{fl/fl}* mice. Mice with LDHA-deficient Treg cells had normal T cell development and Treg cell compositions in thymus (Figure 2.5 B-C), secondary lymphoid organs and nonlymphoid organs such as liver and large intestine at homeostasis (Figure 2.5D), with similar surface activation marker expression including CD25, CD44, CD69, CD103 and ICOS (Figure 2.5E). Using CD25 as a surface marker for Treg cells, we isolated Treg cells (CD25+) and conventional CD4 T cells (CD25-) from lymph nodes and spleen for *in vitro* culture activated for 3 days, in which we observed comparable LDHA induction (Figure 2.6A-B). However, Seahorse extracellular flux analysis showed that compared to day 3- activated WT CD4 T cells differentiated in Th0 and Th17 conditions, which had been shown to be highly glycolytic, Treg cells isolated and cultured *in vitro* for three days displayed both low levels of glucose-induced extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in a glycolysis stress test, suggesting that *in vitro* cultured Treg cells may be less glycolytic compared to Th0- and Th17- differentiated CD4 T cells for their metabolic and biogenetic demands (Figure 2.6 C-D).

To investigate how LDHA-deficient Treg cells behave upon immune challenges *in vivo*, we utilized the clone 13 (c13) Lymphocytic choriomeningitis virus (LCMV) model that leads to chronic viral infection (Figure 2.7A). In this model, Treg cells proliferate extensively and peak at day 16 (73). *Foxp3creLdha^{fl/fl}* mice and respective littermates were infected with LCMV c13 to assess Treg cell

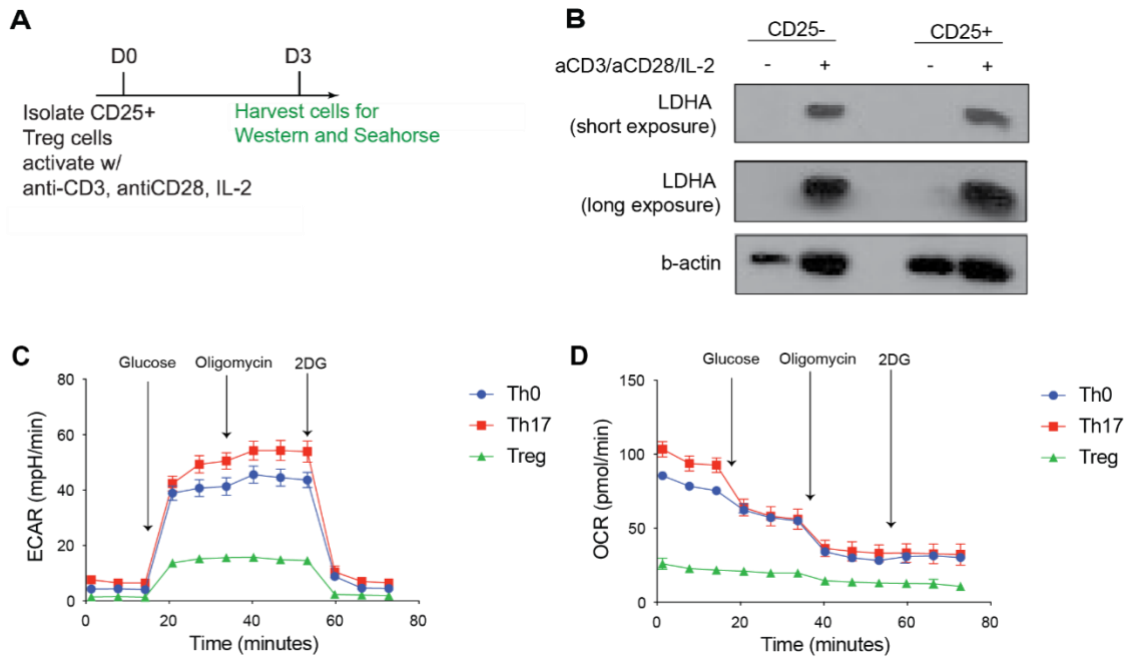


Figure 2. 6 Treg cells displayed reduced ECAR and OCR level in a glycolysis stress test compared to *in vitro* differentiated Th17 cells.

(A) Experimental schematic describing *in vitro* T cell activation for western blot and seahorse experiment. (B) LDHA expression in activated CD25+ Treg cells; CD4+ CD25- conventional T cells were used as a control. (C) Extracellular acidification rate (ECAR) and (D) Oxygen consumption rate (OCR) of Th0, Th17 and Treg cells activated *in vitro* in a glycolysis stress test.

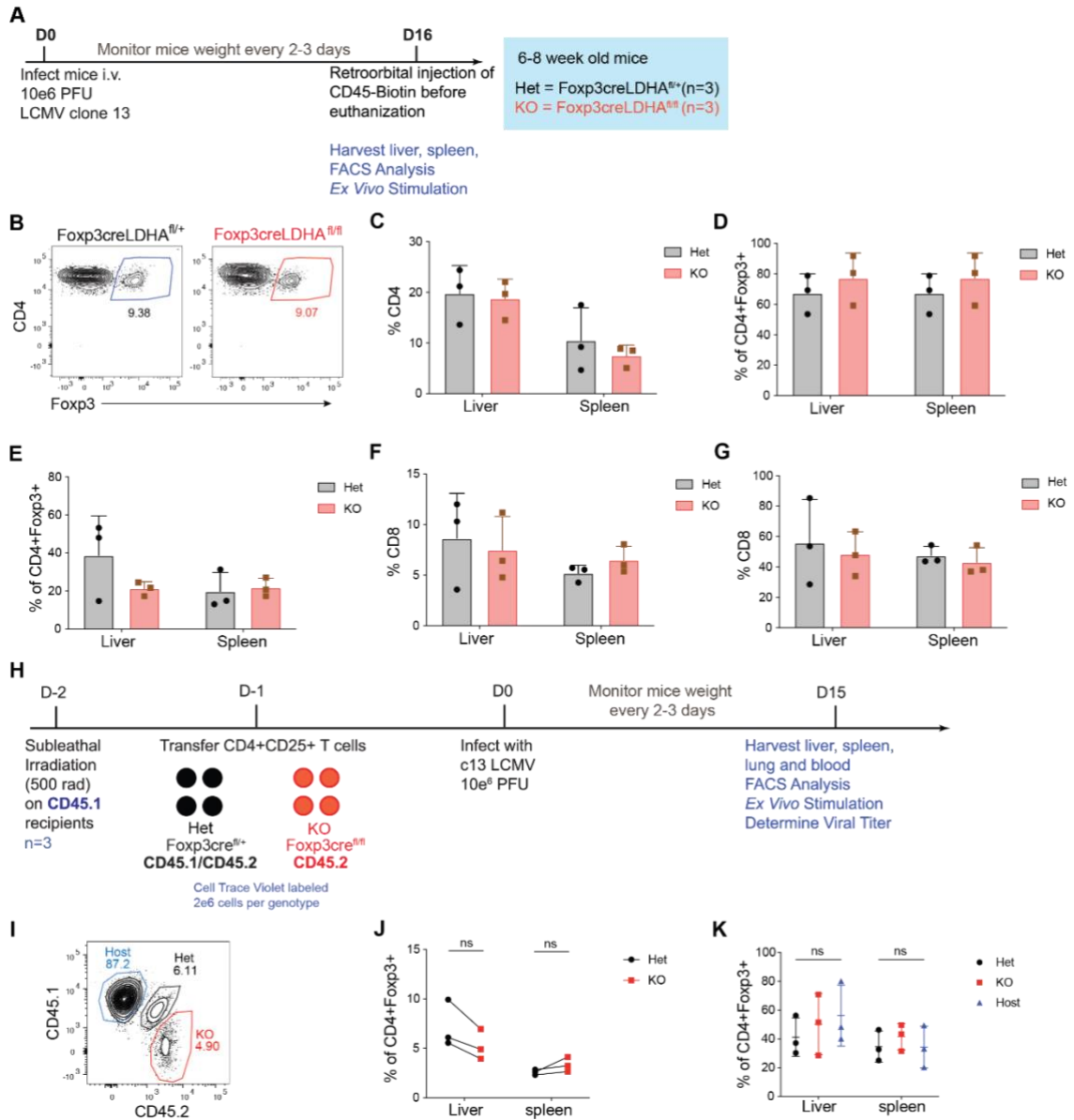


Figure 2. 7 LDHA-deficient Treg cells exhibited comparable frequencies in a LCMV c13 chronic infection model.

(A) Experimental schematic for LCMV c13 infection. (B)(C) Flow cytometric analysis of Treg cell population in CD4⁺ T cells. Representative graph shown from liver. (D) Quantification of tissue resident Treg cells by CD45-biotin antibody injected intravenously 5min prior to animal euthanization. (E) Frequencies of Vβ5⁺ Treg cells and (F) Gp33⁺ antigen specific CD8 T cells. (G) Frequencies of cytokine IFN-γ producing CD8 T cells from infected livers and spleens post peptide stimulation for 4hour. (H) Schematic of Het (CD45.1/CD45.2) and KO (CD45.2) Treg cell co-transfer experiment into WT (CD45.1) recipient host. (I) Representative figure of co-transferred Treg cells gated on live TCRβ⁺CD4⁺Fxp3⁺ cells. (J) Quantification of Treg cell frequencies and (K) Vβ5⁺ Treg cells in infected livers and spleens.

responses, and a CD45 antibody was injected intravenously to differentiate between circulating and tissue resident Treg cells. Interestingly, we observed that Treg cells defective of LDHA displayed comparable expansion with WT Treg cells in liver at D16 (Figure 2.7B), with no significant differences between circulating (CD45+) and tissue resident (CD45-) Treg population, suggesting that Treg cells was not dependent on LDHA for its optimal expansion (Figure 2.7C). To further assess if LDHA regulates expansion of thymus-derived antigen-specific Treg cells, we took advantage of the V β 5⁺ Treg cells responsive to endogenous retroviral superantigens, which were reported to expand upon chronic LCMV infection, promoting pathogen persistence (73). Similar to the bulk population of Treg cells, antigen-specific V β 5⁺ Treg cells did not display significant expansion differences compared to V β 5⁺ Treg cells in the cre positive heterozygous littermates (Figure 2.7D). Given that Treg cells are known to inhibit antigen-specific T cell responses, we assessed the LCMV viral antigen specific CD8⁺ T cells by analyzing the GP33⁺ CD8 T cells, an antigen-specific population for LCMV. GP33⁺ CD8 T cells showed comparable population in both *Foxp3creLdha^{fl/fl}* mice and their het littermates (Figure 2.7E), as well as similar levels of IFN- γ production upon *ex vivo* stimulation with mixed LCMV peptides (Figure 2.7F). This suggested that numbers of LCMV antigen-specific T cells and their cytokine producing capacity were not significantly altered by the suppression of Treg cells that were deficient of LDHA.

To assess if LDHA-deficient Treg cells have proliferation disadvantage in a competitive setting, we co-transferred LDHA-deficient Treg cells and WT Treg cells marked by distinct congenic markers one day before LCMV c13 infection of WT

recipient mice (Figure 2.7G). We observed that in the co-transfer experiment, LDHA-deficient Treg cells had slight but insignificant decrease in expansion compared to the WT Treg cells in the same host (Figure 2.7H-J). These data collectively suggested that Treg cells were not dependent on LDHA for their optimal expansion in the chronic viral infection model.

The functionality of Treg cells can be investigated through an *in vivo* suppression assay by co-transferring Treg cells with conventional CD4 effector (CD4+CD25-Foxp3-) T cells into Rag1^{-/-} recipient mice deficient of adaptive immunity. Transfer of effector T cells into Rag1^{-/-} recipient mice exhibit homeostatic proliferation. If transferred in the absence of Treg cells, conventional T cells can drive pathogenic inflammation accompanied with drastic weight loss, immunopathology and lethality. To investigate if the suppressive function of Treg cells were affected by LDHA-deficiency, we conducted the *in vivo* suppression assay and monitored weight change of recipient mice (Figure 2.8A). Interestingly, mice transferred with LDHA- deficient Treg cells displayed slight but insignificant increase of weight loss compared to mice receiving WT Treg cells together with conventional CD4 T cells (Figure 2.8B). This was accompanied by comparable proportion of Treg cells in both mLN and spleen (Figure 2.8C-D). A small but insignificant increase of activating and proliferating CD4+Foxp3- conventional T cells, as shown by markers CD44 and ki67, were observed in mLN and spleen (Figure 2.8E). Lastly, we observed that although the survival of recipient mice was not significantly different (Figure 2.8F), the data trended towards a lower survival

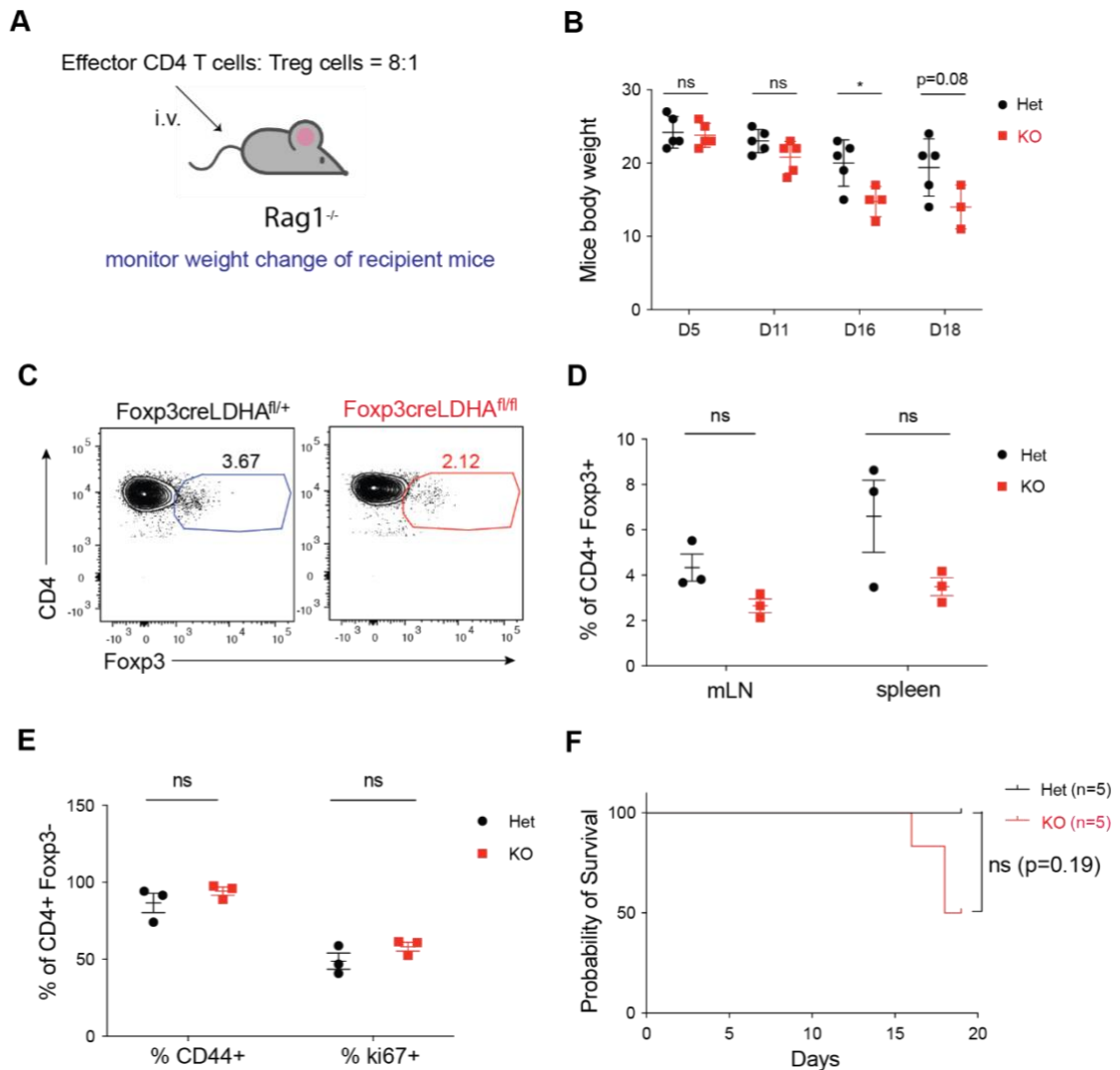


Figure 2.8 LDHA-deficient Treg cells exhibited similar phenotypes as their het control Treg cells in an *in vivo* suppression assay.

(A) A schematic showing co-transfer of Het or KO Treg cells together with conventional effector CD4 T cells into Rag1^{-/-} immunodeficient host animals. (B) Mice body weight post transfer. (C)(D) Flow cytometric analysis of transferred Treg cells at day 20 post transfer. (E) Activation and proliferation marker expressed on transferred conventional CD4 T cells in mLNs. (F) Survival curve of recipient animals.

in mice transferred along with LDHA-deficient Treg cells. These data suggested that in this *in vivo* suppression assay where Treg cells may need to exhibit more aggressive proliferation to regulate the transferred effector T cells, LDHA-mediated glycolysis—although not critical, could be more important to support Treg cell proliferation and suppressive functions.

Lastly, intratumoral Treg cells have been shown to have a negative impact on anti-tumor immunity (278, 279, 280, 281). Tumor cells and conventional T cells both actively engage in aerobic glycolysis and competition for nutrients, while Treg cells were thought to be less dependent on glucose and more metabolic advantageous in the high lactate tumor microenvironment (210, 229). To characterize whether Treg cell response in tumor microenvironment were altered by LDHA, we used the B16-OVA melanoma transplant model (Figure 2.9 A), a model highly populated by Treg cells (282). We observed an insignificant difference of tumor growth transplanted in either *Foxp3creLdha^{fl/fl}* mice or respective littermates (Figure 2.9 B). These data collectively suggested that LDHA-mediated glycolysis was not as critically important for Treg cell survival, expansion and suppressive functions compared to its significance for conventional T cells. While numerous reports have demonstrated the importance of LDHA in supporting conventional T cell functions, here we demonstrate that distinct types of T cells have different modes of metabolic reliance using genetic knockout models to show the independence of Treg cells on LDHA-mediated glycolysis.

2.2.3 Enhanced HIF through VHL deficiency drives enhanced anti-tumor immunity

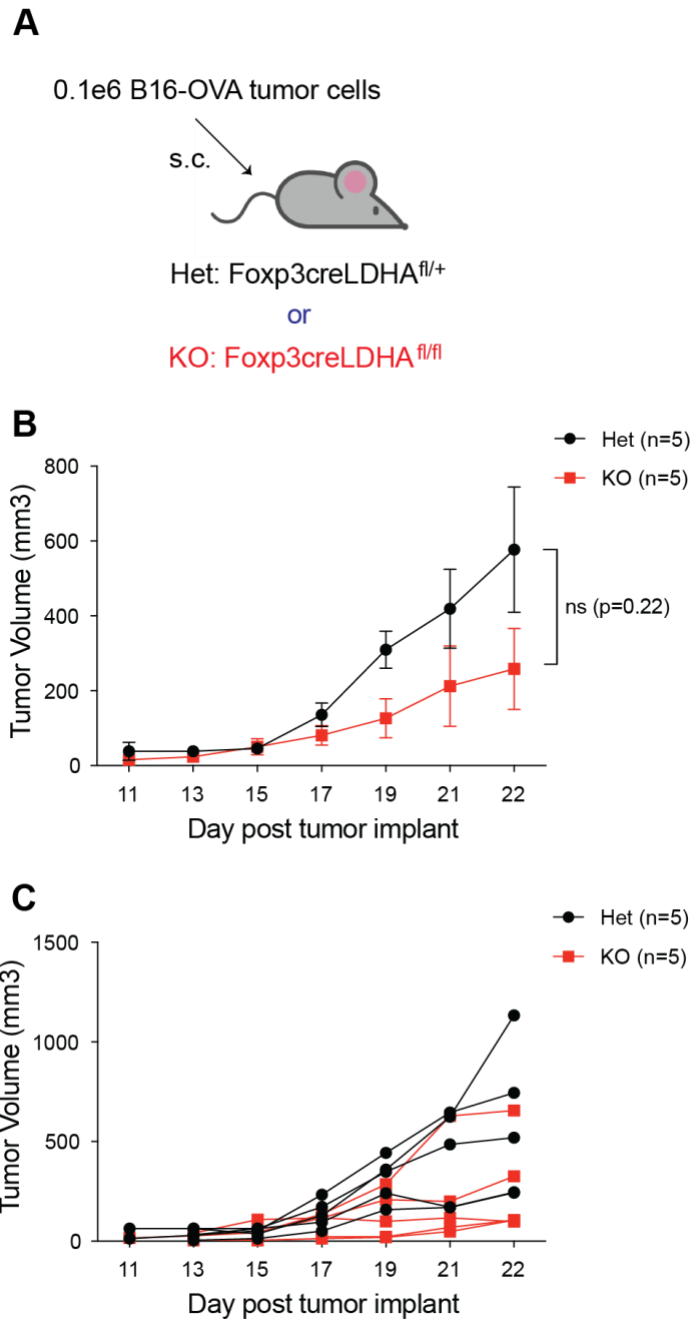


Figure 2. 9 *Foxp3creLDHA^{fl/+}* (Het) and *Foxp3creLDHA^{fl/fl}* (KO) animals demonstrated comparable B16-OVA tumor growth challenged subcutaneously (s.c.).

(A) Experimental schematic for het and KO animals challenged with B16-OVA melanoma tumor cells. (B) Average tumor growth curve from Het and KO animals post tumor transplant. n=5 for het and KO. (C) Tumor growth curve from individual het and KO animals post tumor transplant.

Increased glucose uptake promotes activated T cell proliferation and differentiation in part through upregulated glycolysis. To investigate whether enhanced glucose metabolism in T cells may impact anti-tumor immunity, we utilized the VHL^{fl/fl} mice bred with the dlck (distal Lck) cre, allowing specific deletion of VHL in the T cell compartment post positive selection in the thymus (283), known as the dlckcreVHL^{fl/fl} mice. Deletion of VHL, the negative regulator of HIF transcription factors, can increase their expression to drive enhanced glycolytic activity and decreased OXPHOS dependence (193). We further crossed the dlckcreVHL^{fl/fl} mice to the MMTV-PyMT mice, which allowed us to investigate the effect of VHL deficiency in conventional T cells in the PyMT tumor model. Strikingly, we observed a significantly augmented anti-tumor immunity in the dlckcreVHL^{fl/fl}PyMT mice compared to its WT littermates (Figure 2.10A), suggesting an increased anti-tumor functionality of T cells. To assess if this anti-tumor effect was dependent on either the CD8 or CD4 T cell compartment, we further generated CD8 and CD4 T cell specific deletion of the VHL gene by crossing to the CD8cre and Thpokcre (*Thpok* is also known as *Zbtb7b*) mice, respectively. CD8cre allowed deletion in peripheral CD8 T cells (284), while Thpokcre contained a *Thpok* silencer inserted into a *Cd4* enhancer–promoter locus to drive Cre expression in mature CD4⁺ T cells (285). CD8creVHL^{fl/fl} mice and ThpokcreVHL^{fl/fl} mice were further bred with MMTV-PyMT mice. Interestingly, neither CD8creVHL^{fl/fl}PyMT mice nor ThpokcreVHL^{fl/fl}PyMT mice demonstrated protection against PyMT tumor development (Figure 2.10B-C). This suggests to us that elevated anti-tumor immunity mediated

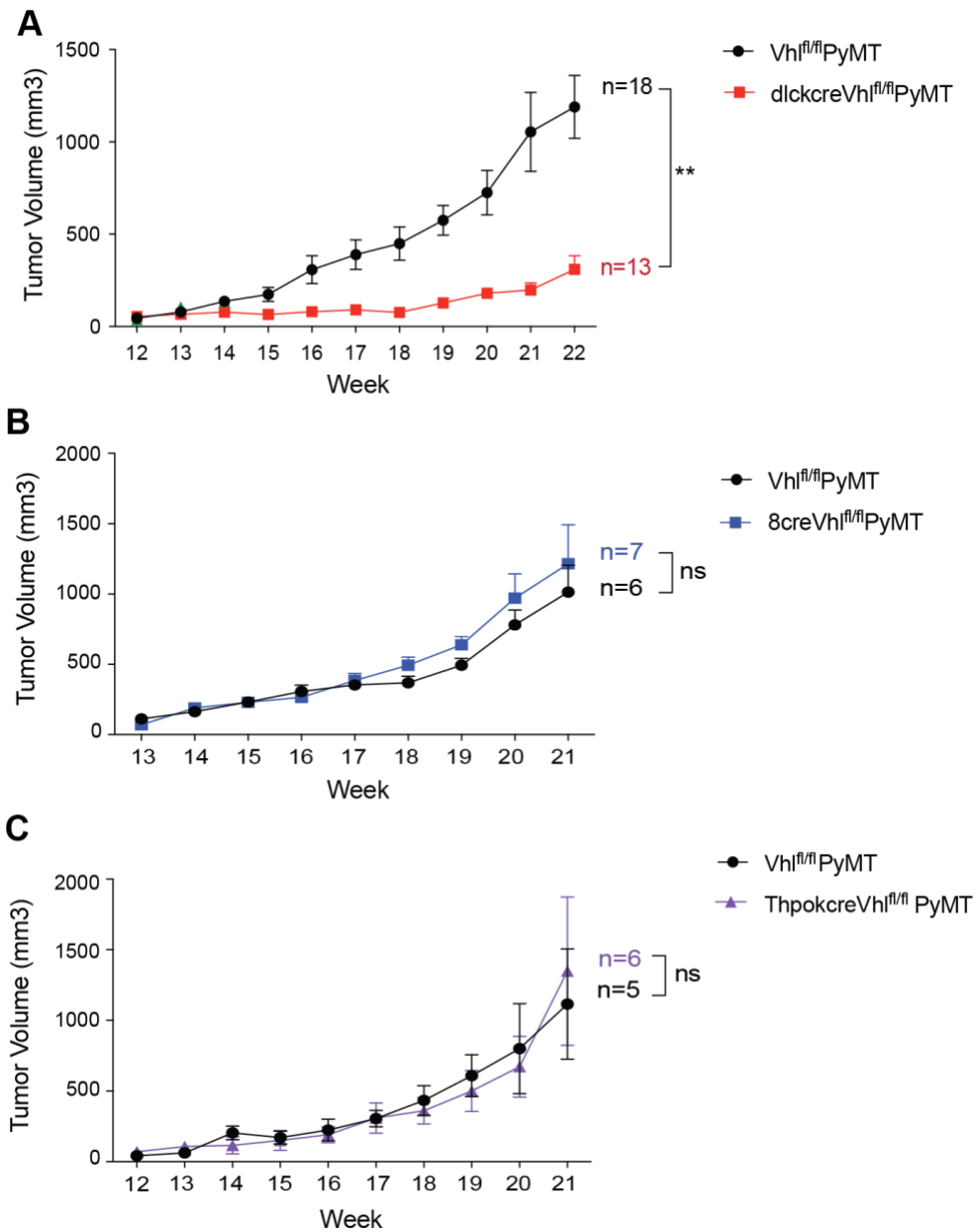


Figure 2. 10 dlckcreVhl^{fl/fl}PyMT mice exhibited significantly reduced tumor growth.

(A) Tumor growth from dlckcreVhl^{fl/fl}PyMT mice, which had VHL-deficiency in mature T cell compartment. (B) Tumor growth from 8creVhl^{fl/fl}PyMT mice, which had VHL-deficiency in CD8 T cell compartment. (C) Tumor growth from ThpokcreVhl^{fl/fl}PyMT mice, which had VHL-deficiency in CD4 T cell compartment.

by T cells through VHL deficiency required both CD8 and CD4 compartment to achieve maximum activity.

We further sought to investigate the mechanism behind enhanced anti-tumor immunity that we had observed in the *dlckcreVHL^{fl/fl}PyMT* mice (hereon referred to as the VHL KO). We hypothesized that the enhanced glucose metabolism supported increased glycolysis mediated by LDHA, which allowed better metabolic fitness of T cells in the tumor microenvironment. To test this hypothesis, we further bred *dlckcreVHL^{fl/fl}PyMT* mice with *LDHA^{fl/fl}* mice to generate *dlckcreVHL^{fl/fl}LDHA^{fl/fl}PyMT* mice. Surprisingly, monitoring tumor growth in the *dlckcreVHL^{fl/fl}LDHA^{fl/fl}PyMT* (hereon referred to as the VHL LDHA DKO) mice showed an insignificant difference of tumor growth compared to the VHL KO tumor mice (Figure 2.11A). This suggested to us that there may be alternative mechanisms mediated by VHL deficiency that was independent of LDHA to support anti-tumor T cell immunity.

To assess the functional changes of VHL-deficient T cells in the PyMT tumor, we assessed the composition of intra-tumoral immune cells. While T cell compositions amongst total CD45+ immune cells were comparable in both spleen and draining lymph node (Figure 2.11B-C), a significant decrease of the proportion of CD8 T cells out of total infiltrating CD45+ immune cells was observed in both the VHL KO and VHL LDHA DKO tumor mice (Figure 2.11D), whereas no significant conventional Foxp3- CD4 T cell population changes were found (Figure 2.11E). When normalized to per gram of tumor, however, there was no significantly different number of CD8 or Foxp3-CD4 T cells across WT, VHL KO, and VHL

LDHA DKO tumors (Figure 2.11F). This was presumably attributed by the size of tumors, since smaller tumors have a higher composition of CD45+ immune infiltrating cells.

To further decipher the subset of CD8 T cells that accounted for the proportional decrease amongst total immune cells, we assessed the unconventional cytotoxic lymphocytes ILTC1s. Because VHL mice did not express NK1.1 marker, we used alternative markers, including low levels of CD5 and high expression levels of CD200R1, CD103 and GzmB to identify the population (data unpublished). There were no significant differences of the ILTC1 population out of total immune cells across WT, VHL KO and VHL LDHA DKO tumors (Figure 2.11G), suggesting that the proportional changes of CD8 T cells we had previously observed were more likely due to changes of conventional CD8 T cells.

Conventional CD8 T cells can differentiate into distinct types of memory cells, which can traffic, reside-in and patrol peripheral tissues to facilitate protective immune roles against pathogens and tumors. VHL-deficiency had been shown to drive an increased proportion of long-lived effector memory T cells in acute viral infection (264). Interestingly, we observed an insignificant but increased trend in expression of tissue-resident markers, CD49a and CD103, on both VHL KO and VHL LDHA DKO CD8 T cells (Figure 2.12A). The data preliminarily suggested that compared to WT T cells, VHL KO and VHL LDHA DKO CD8 T cells could have altered differentiation to favor tissue-residency for protection against tumors.

Furthermore, in the tumors, conventional CD8 T cells could impact cancer cell survival through various mechanisms, including effector molecules IFN- γ

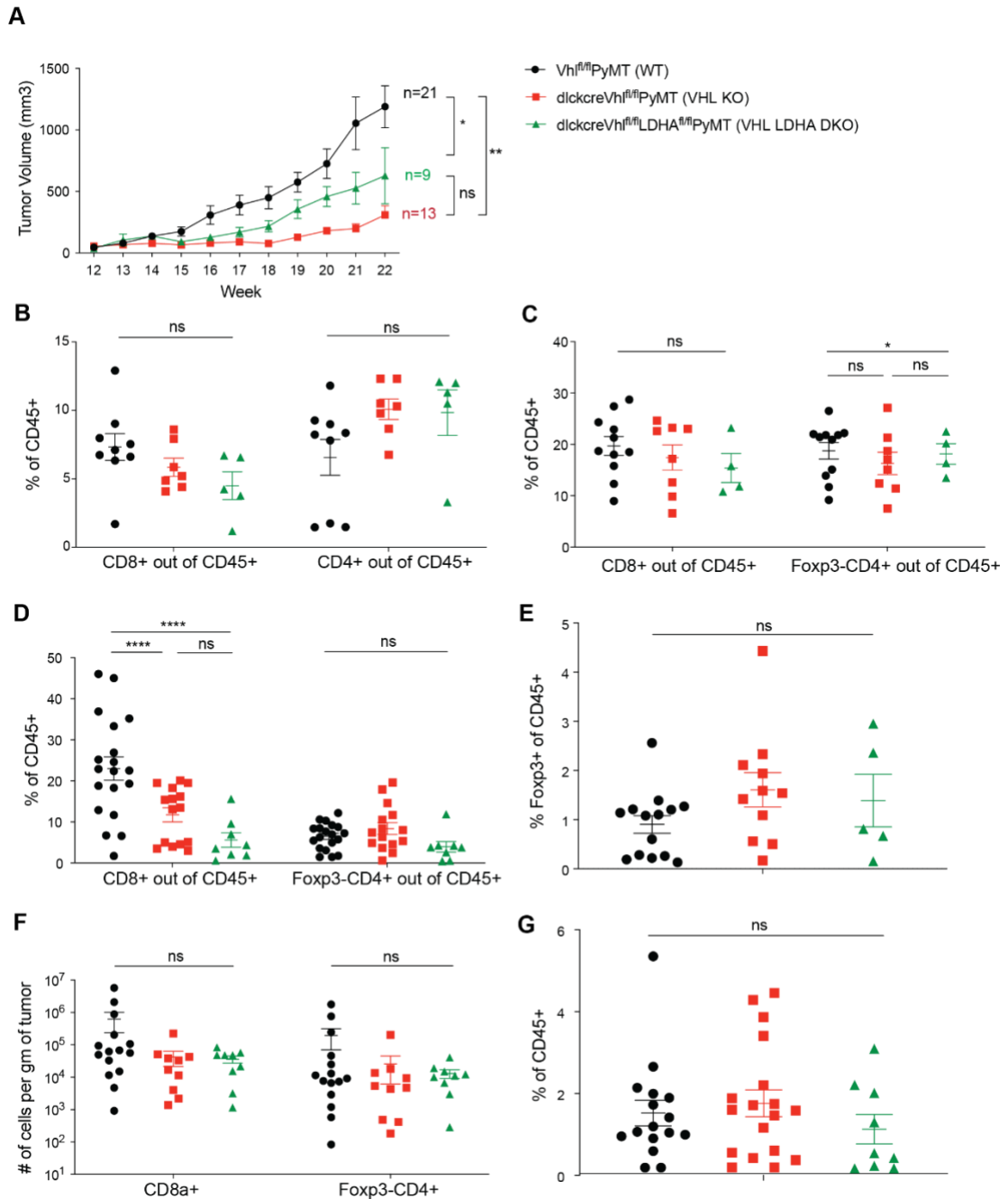


Figure 2. 11 VHL-deficient T cells controlled PyMT tumor growth independent of LDHA.

(A) Tumor growth in WT, dlckcreVHL^{fl/fl}PyMT (VHL KO) and dlckcreVHL^{fl/fl}LDHA^{fl/fl}PyMT (VHL LDHA DKO) mice. (B) Frequencies of T cells in spleen, (C) tumor draining lymph node, and (D) tumor. (E) Foxp3+ Treg cells in tumor. (F) Number of T cells normalized to per gram of PyMT tumor. (G) Frequencies of CD5^{low}CD200R1^{high} ILTC1s out of total CD45+ immune cells in tumor.

and GzmB. IFN- γ not only can act directly on tumor cells as a cytotoxic cytokine, but can also stimulate proinflammatory phenotypes of other infiltrating immune cells (286). To assess if enhanced tumor immunity was mediated by increased IFN- γ cytokine, we assessed the IFN- γ production from intratumoral CD8 T cells subjected to *ex vivo* stimulation. Interestingly, we saw a significant decrease of IFN- γ production from CD8 T cells in both VHL KO and VHL LDHA KO tumor mice compared to WT tumor mice (Figure 2.12B). Given that LDHA has been shown to support IFN- γ gene expression, the low IFN- γ expression from VHL LDHA DKO T cells aligned with previous reports. However, our data suggested that enhanced glucose metabolism may not necessarily support enhanced IFN- γ cytokine production.

GzmB is another key effector molecule that cooperates with perforin, an effector molecule that forms pores in membranes of target cells. This further leads to successful delivery of granzymes into cytosol for cleavage of critical intracellular contents (85). Granzymes has also been shown to play a critical role in supporting CD8 T cell mediated anti-tumor immunity. GzmB inversely correlates with PD1 (Cencioni et al 2017), which can transcend suppressive signaling with the impairment of cytokine and perforin production when engaged with its ligand (287). We hypothesized that VHL-deficiency may drive enhanced cytolytic molecules like GzmB to support the anti-tumor function of CD8 T cells. Analysis of intra-tumoral conventional CD8 T cells showed a significantly decreased population of PD1⁺GzmB⁻ (Figure 2.12C), a subset potentially exhausted and dysfunctional. On the other hand, PD1⁻GzmB⁺ CD8 T cell population was significantly increased in

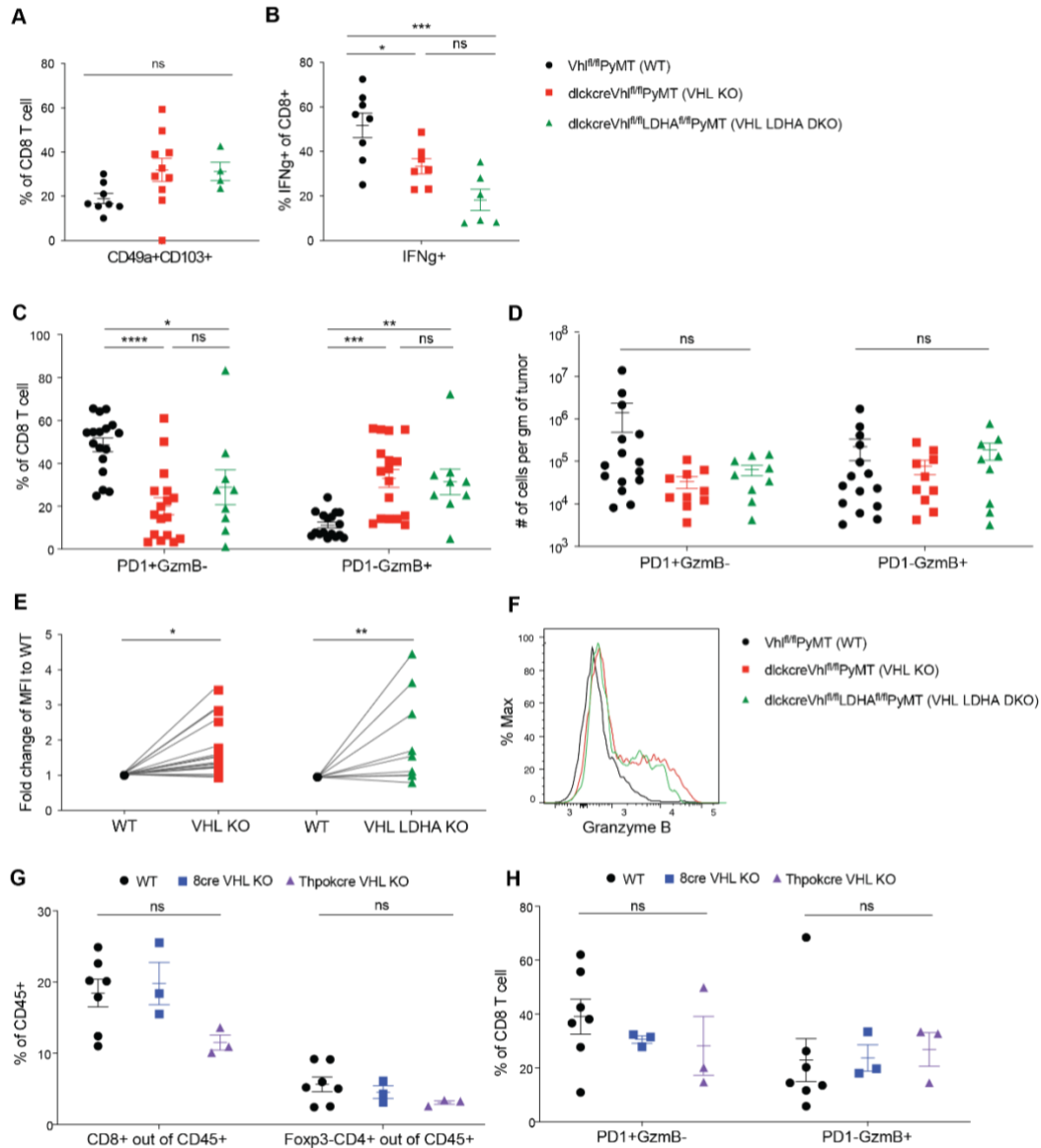


Figure 2. 12 VHL-deficient CD8 T cells exhibited enhanced expression of cytolytic granule granzyme B.

(A) CD49a+CD103+ expression amongst conventional CD5+CD8+ T cells (B) IFN- γ production from intratumoral CD8 T cells stimulated with PMA/Ionomycin/GolgiStop *ex vivo*. (C) Expression of PD1 and GzmB markers in CD8 T cells, and (D) normalized to number of cells per gram of PyMT tumor. (E) Mean Fluorescent intensity (MFI) fold change of VHL KO and VHL LDHA KO CD8 T cells to their WT littermates. (F) Representative graph of GzmB MFI from VHL KO and VHL LDHA KO CD8 T cells. (G) Frequency of conventional CD8 and CD4 T cells out of WT, 8creVhl^{fl/fl}PyMT and ThpokcreVhl^{fl/fl}PyMT tumor mice. (H) PD1 and GzmB marker expression on CD8 T cells from 8creVhl^{fl/fl}PyMT and ThpokcreVhl^{fl/fl}PyMT tumor mice.

both tumors with VHL KO and VHL LDHA DKO T cells, suggesting that a more functionally active population may be responsible for the tumor phenotype we had observed (Figure 2.12C). While the number of PD1-GzmB+ CD8 T cells per gram of tumor was not significantly different compared to WT (Figure 2.12D), we observed an increase of GzmB MFI, which may have resulted in increased functional killing capacity on a per cell basis for both VHL KO and VHL LDHA DKO T cells (Figure 2.12E-F). As expected, the altered CD8 compartment and the functional changes of CD8 T cells marked by PD1-GzmB+ subsets were not significantly altered in tumors from both CD8creVHL^{fl/fl}PyMT and ThpokcreVHL^{fl/fl}PyMT mice (Figure 2.12G-H), suggesting that VHL deficiency in both CD8 and CD4 T cells were required to elicit this functional phenotype change we observed.

Lastly, we set forth to assess how CD4 T cells may be altered in the VHL KO and VHL LDHA DKO tumor mice. In both models, we found comparable Treg cell population out of total infiltrating immune cells. While CD4 T cells did not express GzmB, we observed a trend of reduced PD1 expression in the VHL KO and VHL LDHA DKO mice. This piece of data suggested that VHL-deficiency may also reduce the population of exhausted CD4 T cells in the PyMT tumors, which could have offered more direct CD4 help to support CD8 T cell functions in the VHL KO and VHL LDHA DKO tumors for optimal anti-tumor immunity.

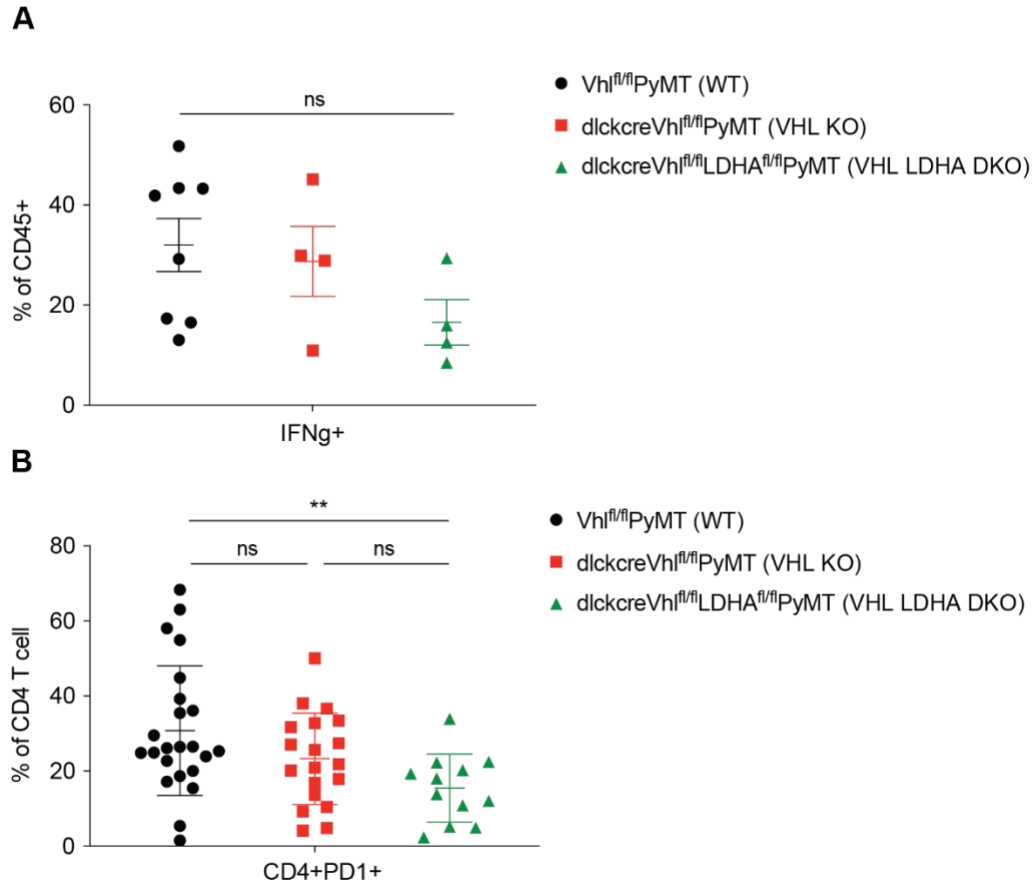


Figure 2. 13 Characterization of VHL-deficient CD4 T cells in the PyMT tumors.

(A) IFN- γ production from intratumoral Foxp3-CD4 conventional T cells stimulated with PMA/Ionomycin/GolgiStop for 4 hours *ex vivo*. (B) PD1 marker expression from VHL KO and VHL LDHA DKO CD4 T cells.

2.3 Discussion

T cell is a key player in the adaptive immune system important for host defense against immunological challenges. Upon antigen encountering, T cells undergo rapid metabolic reprogramming to sustain the differentiation, proliferation and effector functions needed against invading pathogens or malignant cells. This metabolic reprogramming is largely dependent on glucose, a major macronutrient essential for robust T cell responses. Our data showed a critical role for glucose metabolism through glycolysis to support conventional T cells, but not Treg cells for their optimal functions in various diseases. Enforced glucose metabolism presumably through a LDHA-independent manner can further escalate the anti-tumor immunity in a spontaneous breast cancer model, highlighting the significance of glucose metabolism in supporting T cells.

Glycolysis is critically supported by the key enzyme LDHA, which mediates the lactate fermentation from pyruvate in parallel of NADH oxidation to maintain redox balance. Upon T cell activation, LDHA is induced downstream of C-MYC and HIF axis, attributing to enhanced lactate fermentation. Although at a lower ATP-production per glucose, aerobic glycolysis allows a redox-neutral manner with higher rate of cytosolic ATP generation to support cellular demands (221). LDHA-mediated glycolysis not only supports T cell proliferation, cytokine production through epigenetic regulation (220), but also fuels bioenergetics that sustain a positive feedback for signaling pathways like PI3K/Akt axis. PI3K/Akt activation is critical for expression of effector gene expression such as granzyme B molecules.

Additionally, Akt signaling reduces Foxo1 signaling, which downregulates markers including L-selectin for exit from lymphoid tissues to sites of infections (288). Thus, LDHA is important for proper T cell differentiation, migration and functional responses (222, 223). Blocking LDHA through inhibitors or genetic ablation limits the aforementioned T cell responses. Similarly, increased lactate concentration in the microenvironment limits LDHA-mediated conversion from pyruvate to lactate, further inhibiting T cell glycolysis by reducing the NAD⁺/NADH ratio, as well as decreasing proliferation supported by the *de novo* serine synthesis branched off from glycolytic intermediate 3-PG (289). These data highlight the role of LDHA in supporting T cell functions, however, less is known about LDHA-mediated functions of T cells in the tumor microenvironment, which is intriguing given the similar metabolic dependency for tumor cells on glycolysis. Our data suggested that LDHA was critical for T cell functions in tumor. Reduced conventional CD8 and CD4 T cell population may be attributed by defective T cell migration to tumor, as well as in situ proliferation within the tumor. In addition to reduced population, intratumoral CD8 and CD4 T cells were also characterized by reduced IFN- γ production, which may be a result of decreased *Irfg* locus acetylation that resulted in reduced gene transcription (220). These data suggested that LDHA support conventional T cell responses through multiple aspects and was critical for T cell-mediated antitumor responses.

In various murine tumor models including the PyMT tumor, a type 1 innate-like cytotoxic T lymphocyte (ILTC1) population had been described (277). This population was characterized as high levels of cytolytic granule-expressing

population with tissue residency markers that were seeded early during transformation of premalignant cells. We were surprised to find that these TCR β ⁺ ILTC1s also depended on LDHA, and depletion of LDHA significantly reduced this population in the PyMT tumors. Future studies with ILTC1-specific cre-lox models will be instrumental to assess whether LDHA would disrupt their cytolytic functions for cancer immunosurveillance, and whether this would result in differential tumor growth.

It has long been a consensus in the field that conventional T cells are heavily dependent on aerobic glycolysis. However, mixed results have been reported for Treg cell metabolism. We therefore utilized a murine model of Treg-specific LDHA-ablation to assess Treg cell dependency on LDHA-mediated glycolysis in steady state and immunological challenges. Intriguingly, we found that LDHA-deficient Treg cells and WT Treg cells exhibited comparable phenotypes at steady state. In challenged states including chronic infections where Treg cells undergo expansion, the population of LDHA-deficient Treg cells and WT Treg cells did not show significant differences, indicating their proliferation was not dependent on LDHA. In another model of B16-OVA tumor transplant model in which Treg cells accumulate and attribute to suppression of T cell anti-tumor immunity, the outcome of tumor burden was not significantly different between animals with WT Treg cells or LDHA-deficient Treg cells. This was consistent with the low ECAR observed in WT Treg cells post *in vitro* activation, suggesting lower dependence of Treg cells on glycolysis than conventional T cells. However, in the *in vivo* transfer assay where Treg cells were critical for suppressing conventional T cell-mediated colitis,

we observed slight but insignificant decrease of survival in mice with LDHA-deficient Treg cells. Due to transfer into immune-deficient host, Treg cells may need to undergo more extensive and faster proliferation than the models previously described, which may explain an enhanced dependency of LDHA by Treg cells in this *in vivo* suppression model. Overall, data suggested that Treg cells had less reliance on LDHA and glucose metabolism than conventional T cells, and were primarily depend on other forms of metabolism for their function, survival and proliferation. These features presumably also allowed Treg cells to accumulate in microenvironments that may be much more suppressive for conventional T cells (274), due to limited glucose nutrient and enhanced lactate production that could hinder T cell glycolysis, while Treg cells were minimally affected.

HIF is one of the key pathways for upregulation of glycolytic enzymes post T cell activation. Targeting its negative regulator VHL allows enhanced HIF-1 α and HIF-2 α expression, as well as expression of glucose transporters and glycolytic enzymes (193). VHL deficiency leads to enhanced CD8 T cell effector responses to persistent antigens in chronic viral infection, as well as enhanced CD8 T cell effector memory phenotype in acute infections (264). However, less is known of how VHL deficiency-mediated increase of T cell glycolytic metabolism may support T cell responses against cancer. Using the spontaneous MMTV-PyMT breast cancer model, we found that VHL-deficiency in mature T cells led to significantly reduced PyMT tumor growth. Interestingly, tumors with VHL-deficient CD8 T cells or VHL-deficient CD4 T cells alone exhibited similar growth as WT tumors, suggesting to us that enhancing glycolytic metabolism in both CD8 and CD4 T cells

may be required for efficient anti-tumor immunity to manifest. The reduced population of infiltrating T cells in tumors from both VHL KO and VHL LDHA KO mice may be a direct consequence of low immune infiltration at smaller tumor stage, or that enhanced cancer-immunosurveillance from VHL-deficient CD8 T cells were effective at restraining tumor growth even with reduced T cell population. Surprisingly, LDHA deficiency did not significantly revert the reduced tumor growth phenotype observed in the VHL KO tumor mice, potentially attributed by the high levels of GzmB expression observed in both VHL KO and VHL LDHA DKO tumor mice. Both effector molecules including IFN- γ and granzymes contribute to T cell-mediated anti-tumor immunity (290 , 291). Moreover, LDHA-mediated bioenergetics has been shown to fuel PI3K/Akt pathway for CD8 T cell effector functions (222); since Akt is critical for expression of effector molecules including granzymes and perforins (292), it is thus intriguing that VHL LDHA DKO tumor mice retained high GzmB expression. HIF stabilization through VHL-deficiency leads to increased expression of effector molecules in CD8 T cells including GzmB, perforin, as well as many key T cell migratory and activation receptors (193, 194, 196). This suggest that HIF overexpression may independently sustain enhanced cytolytic molecule expression through Akt independent mechanism, such as the PDK1-mTORC1-HIF pathway (194) that contribute to anti-tumor immunity. Alternatively, glycolytic enzymes other than LDHA may sustain augmented glucose metabolism that could fuel and support the enhanced functions of both VHL KO and VHL LDHA KO CD8 T cells. Another possibility to consider is that the VHL and LDHA allele deletion may not have occurred in the same cells. If the

deletion efficiency varied, with some T cells becoming VHL KO and others LDHA KO, we may see VHL KO T cells predominantly populate the tumors, with the enhanced anti-tumor immunity as a result of VHL-mediated increase in GzmB expression. Thus, it will be important in the future to assess the deletion efficiency of VHL and LDHA in these VHL KO and VHL LDHA DKO T cells. Lastly, since HIF stabilization also impacts T cell activation, migration, and transcription factor expression, it would be interesting to see how these gain-of-function T cells are regulated. For instance, they may become more effector memory-like or tissue-resident, and become more dependent on cytokines IL-15 and TGF β (293). This was supported by our observation of an increased trend of tissue resident markers CD49a and CD103 expressions on both VHL KO and VHL LDHA DKO T cells in PyMT tumors. Future studies of whether they would depend on CD122 (the shared IL-2 and IL-15 receptor β -chain) or TGF β receptor RII for their functions would be an interesting area of investigation.

Another interesting key phenotype is the reduced expression of exhaustion marker PD1 on CD8 T cells. PD1 serves as a marker of antigen-experienced or exhaustion for T cells, which is typically mutually exclusive with expression of effector molecules on T cells in chronic infection or cancer. We observed a significantly reduced population of CD8 T cells expressing PD1 and a trend of reduced population of PD1-expressing CD4 T cells. CD4 T cells can support anti-tumor immunity through a variety of mechanisms; including the support for B cells antibody production against tumor, activation of APCs through cytokines in a CD40-CD40L dependent fashion to enhance their IL-12 cytokine expression and

co-stimulation receptors for indirect support of CD8 T cells (294), direct help for CD8 T cell immunity through secretion of cytokines like IL-2, or direct cytotoxicity mediated through cytokines IFN- γ and TNF- α (92). Our data suggested potential coordination between both VHL-deficient- CD8 and CD4 T cells is required to achieve enhanced anti-tumor immunity. It is thus plausible that enhanced CD4 T cell help through VHL deficiency elevated CD8 T cell functions or sustained their activation with less exhaustion. Notably, diminished expression of PD1 marker was likely an effect independent of HIF regulation (193), suggesting other uncharacterized VHL substrates subjected to post-translational modification of protein hydroxylation regulation could be responsible for such phenotype; future studies identifying downstream substrates directly regulating PD1 may be of interest for new therapeutic strategies. Importantly, both the PD1 and granzyme B expression difference were only seen in dlckcre-mediated deletion of VHL (impacting mature T cells), but not Thpokcre (CD4 T cells) or CD88cre (CD8 T cells), implicating enhanced cytolytic functions and reduced exhaustion are dependent jointly on CD8 and CD4 T cells.

In summary, our data demonstrated how glucose metabolism through glycolysis affected functions of distinct T cell subset in homeostasis and disease challenges. Conventional T cells were dependent on glucose metabolism primarily fluxed through glycolysis, and LDHA-mediated glycolysis was critical for supporting T cell-mediated anti-tumor immunity. Intriguingly, while Treg cells upregulated LDHA upon activation, they were not as dependent on LDHA-mediated glycolysis for their suppressive function and proliferation both at steady

state and diseases. Enforced glucose metabolism through HIF stabilization attributes to enhanced anti-tumor immunity through increased cytolytic granule expression, which was dependent jointly on mature CD8 and CD4 T cells. Future studies identifying HIF-dependent and independent mechanisms through alternative VHL substrates will be instrumental for the understanding of metabolic and post-translational regulation of T cells, as well as for delineating their anti-tumor functions.

2.4 Experimental Procedures

Mice

C75BL/6 CD8cre (stock # 008766), C75BL/6 Cd4Cre (stock # 022071), B6.129 VHL^{f/f} (stock # 012933) were purchased from Jackson Laboratory. B6.129 Foxp3-YFP-cre was generated in the laboratory of A. Y. Rudensky (295), and Foxp3-YFP-cre was used as littermate controls. C75BL/6 LDHA^{f/f} was generated in the laboratory of M. O. Li (220, 222, 223). All in vivo mouse experimental procedures were performed under Sloan Kettering Institute (SKI) Institutional Animal Care and Utilization Committee (IACUC)-approved protocols. Mice were housed in designated specific pathogen-free animal facilities in ventilated cages with at most five animals per cage, provided with food and water.

Tumor measurement and analysis

Mammary tumor growth from female PyMT tumor mice were measured for cumulative tumor burden on a weekly basis for six months. Cre-positive and Cre-negative littermates were housed together to control for experimental variability. Experimental animals were euthanized between 20-24 weeks of age; tumors and peripheral lymphoid organs were dissected and subject to flow cytometry for cellular phenotype.

Antibodies

Antibodies against CD127-PE (Clone A7R34; Cat. # 1 2-1271-82), KLRG1-PE-Cy7 (Clone 2F1; Cat. # 25-5893-82), Foxp3-efluor450 (Clone FJK-16s; Cat. # 48-5773-80), PD1(CD279)-FITC (Clone RMP1-30; Cat. # 11-9981-82) were purchased from eBioscience. Antibodies against CD44-PerCP-Cy5.5 (Clone IM7; Cat. # 65-0441-U100), CD8a-PE-Cy7 (Clone 53-6.7; Cat. # 60-0081- U100), TCRb-FITC (Clone H57-597; Cat. # 35-5961-U100), IFN-g-APC (Clone XMG1.2, Cat. # 20-7311-U100), IFN-g-FITC (Clone XMG1.2, Cat. # 35- 7311-U100) and IFN-g-PE-Cy7 (Clone XMG1.2, Cat. # 60-7311-U100), Ghost Dye Red 780 (Cat. # 13-0865-T100) were purchased from Tonbo. Antibodies against CD4-BV510 (Clone RM4-4; Cat. # 740105), CD4-BV786 (Clone GK1.5; Cat. # 563331), CD45-BV605 (Clone 30-F11; Cat. # 563503), TCRb-BV711 (Clone H57-597; Cat. # 563135), CD8a-BV650 (Clone 53-6.7; Cat. # 563234), ki67-BV650 (Clone B56; Cat. # 563757), CD49a-BV711 (Clone Ha31/8; Cat. # 562113), CD103-BV786 (Clone M290; Cat. # 564322) were purchased from BD Biosciences. Antibodies against Granzyme B-PE-TexasRed (Clone GB11; Cat. # GRB17) were purchased from

Thermo Fisher Scientific. Antibodies against TCR V β 5⁺-PE (Clone: MR9-4; Cat. # 139504) were purchased from Biolegend. Fluorescent-dye-labeled tetramers H-2Db-GP33 BV421 was obtained from NIH. Polyclonal rabbit anti-LDHA antibody (Cat. #2012S) were purchased from Cell Signaling Technology.

Antibodies for in vitro T cell culture

Monoclonal anti-mouse CD28 (Clone 37.51; Cat. # BE0015-1), monoclonal anti-human CD3 (Clone OKT-3; Cat. # BE0001-2) and monoclonal anti-mouse CD3e (Clone 145-2C11; Cat. # BE0001-1) were purchased from BioXCell.

In vitro T cell differentiation

In vitro T cell experiments were performed by using primary mouse T cells isolated from lymph nodes and spleens of male and female mice cultured at 37°C with 5% CO₂ in T cell medium (TCM): RPMI-1640 media (Media Preparation Core, SKI) supplemented with 1000X b-mercaptoethanol (Invitrogen), 100X penicillin-streptomycin (Gimini Bio), 100X glutamine (Media Preparation Core, SKI) and 10% heat inactivated FBS (Sigma). For CD4⁺ Treg and conventional CD4 T cell differentiation assays, CD4⁺CD25⁻ Treg cells were isolated from Foxp3creLdha^{fl/+} or Foxp3CreLdha^{fl/fl} mice with positive selection kit (EasySep, Cat. 18783), the flow-through was then subjected to a naïve CD4 negative isolation kit (EasySep, Cat. 19765) for CD4⁺CD25⁻ T cells. Purity was validated by flow cytometry and was over 95%. For *in vitro* T cell activation, 1e6 T cells were cultured in 24-well plates pre-coated with 5 µg/ml anti-CD3, and 2 µg/ml soluble anti-CD28

in TCM. For Th0 polarization condition, no additional cytokines or antibodies were added. For Th17 polarization condition, naïve CD4+ T cells were cultured with the addition of 2ng/ml rhTGF-b1, 10 ng/mL IL-6, 10 ng/ml IL-1b, 10 ng/ml IL-23, 10 µg/ml anti- IFN-g, 10 µg/ml anti-IL-4, and 10 µg/ml anti-IL-2. For Treg polarization condition, CD4+ CD25+ T cells were cultured with the addition of 200 U/ml rhIL-2 (NIH).

Seahorse XF Glycolysis Stress test

Experiments were performed on Agilent Seahorse XF96 bioanalyzer. *In vitro* three-day activated Th0, Th17 and Treg cells were plated onto XF96 microplates (1.5×10^5 cells/well) that had been pre-coated with 0.01% Poly-L-lysine solution. The plates were quickly centrifuged to immobilize cells. Cells were rested in the seahorse XF Assay Medium (Agilent, Cat. 102353-100) in the presence of 1mM Glutamine in a non-CO2 incubator for 30 min before the assay. Glycolysis associated parameters were determined by Seahorse XF Glycolysis Stress test kit with three injections: (1) 10 mM glucose; (2) 1 µM oligomycin; and (3) 50 mM 2-DG.

Immunoblotting procedures

Prior to immunoblotting, cell lysates were heated for 5 minutes at 95 degree Celsius and centrifuged for 30 seconds at 14000g. Lysates were then resolved via SDS-PAGE gel (9%) in each lane without sample dilution. Gel was then immediately transferred to nitrocellulose membrane using the Trans-Blot Turbo

Transfer System from Bio-Rad. Membrane was then Blocked in either 5% milk or Intercept blocking buffer for 1 hour. Primary antibodies were blotted overnight at 4 degree Celsius. Membrane was then washed three times with PBST for 5 minutes. Secondary antibodies were blotted for 1 hour at room temperature. Membrane was then washed three times with PBST for 5 minutes. For readout using chemiluminescence, results were developed using film in the dark room.

LCMV clone 13 infection

Frozen aliquot/vial of LCMV clone 13 (stored at -80°C) was thawed, 6 to 8-week-old *Foxp3CreLdha^{f/f}* mice and their wild-type littermates were inoculated intravenously with 2×10^6 plaque-forming units (PFUs). At day 16, Treg cells and conventional T cell responses were assessed, and $V\beta 5^+$ Treg cells were enumerated in target organs including spleen and liver and kidney. CD45-biotinylated antibody was injected intravenously 5min before euthanasia of infected mice to distinguish between circulating and tissue Treg cells. For co-transfer experiments, wild-type recipient mice were sublethally irradiated for 500 rad two days prior to infection. One day later, congenically marked LDHA-deficient and control $V\beta 5^+$ Treg cells were sorted, and 5×10^6 cells from each group were transferred per wild-type recipient mice. The next day, mice were infected with clone 13 LCMV. Treg cells and conventional T cell responses were analyzed at day 16 post-infection.

Adoptive transfer of T cells for in vivo suppression assay

Naïve CD4⁺ T cells were purified from C57BL/6 mice expressing high expression of CD45Rb marker, and co-transferred together with 0.2e⁶ WT or LDHA-deficient CD4⁺ Foxp3⁺ Treg cells into *Rag1*^{-/-} mice. Treg cells and conventional CD4 T cells were transferred at an 1:8 ratio. After T cell reconstitution, mice were monitored weekly for the weight changes and signs of disease. Mice were euthanized three weeks after T cell transfer, activation and differentiation of CD4 T cells were further analyzed.

B16-OVA transplant model

B16 melanoma cell lines engineered to express the model OVA antigen was obtained from the laboratory of Morgan Huse (MSKCC). Cells were thawed from frozen vial (stored at -80°C), cultured for one passage in RPMI-1640 medium with 10% heat inactivated fetal bovine serum, and 0.1e⁶ cells per mice were subcutaneously inoculated into Foxp3CreLDHA^{fl/fl} mice and their het littermates. Tumors become palpable one week post injection, and were monitored for progression three times per week. Mice were euthanized three weeks after inoculation of tumors.

Flow Cytometry

For the PyMT tumor analysis, tumors were dissected and digested for 60 min in 37 degreeC with shaking at 225 rpm. Tumors were then grinded and filtered through 70 µm mesh to make single cell suspension. Cells were concentrated using 44% and 66% percoll overlay. Cells were then incubated with specific

antibody cocktails for 10 min at 4°C in the presence of anti-FcγR to block FcγR binding. Dead cells were excluded by Ghost Dye™ Red 780 or DAPI staining. For intracellular staining, cells were fixed for 20 minutes and stained in fix/perm buffer at room temperature for 20 minutes before flow cytometry. All samples were acquired with an LSR II flow cytometer (Becton, Dickinson) and analyzed with FlowJo software.

Stimulation for cytokine assessment

For stimulation of primary T cells *ex vivo*, cells were collected and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 1 μM ionomycin (Sigma) in the presence of GolgiStop (BD Biosciences) for 4 hours. Cells were then collected for flow cytometry.

Statistical Analysis

Statistical tests were performed with paired, unpaired t tests or one-way Anova with Tukey's multiple comparison tests. A value of $p < 0.05$ was considered statistically significant. All error bars represent mean \pm standard error of the mean (SEM). All Statistical analysis were performed in Prism 9.

Chapter 3: Serine metabolism in T cell immunity

3.1 Introduction

Upon T cell activation, multiple metabolic pathways including amino acid metabolism are increased to provide sufficient supply for cell growth and function. In particular, serine is an amino acid of interest that positions at the central node of cellular biosynthesis given its roles to support protein, lipid, and nucleotide synthesis. Proliferating cells are in high demand for extracellular serine, while increased serine synthesis is also one of the metabolic changes. In this chapter, we explore the role of serine in supporting T cell activity through genetic ablation of the serine synthesis pathway (SSP) together with diet manipulation.

3.2 The multifaceted roles of serine

Serine is a nonessential amino acid important to support multiple biosynthetic pathways in proliferating cells (Figure 3.1). First of all, serine is a precursor for nonessential amino acids glycine and cysteine. Produced from serine by the serine hydroxymethyltransferase (SHMT) 1/2 reaction, glycine can support downstream synthesis of porphyrins— a structure critical for iron binding, as well as for synthesis of nucleotides and glutathione to support antioxidant regulation (296, 297). *De novo* synthesis of nucleotides is dependent on the one-carbon unit transfer (298). The conversion of serine to glycine generates one-carbon units to tetrahydrofolate for the synthesis of 5,10-methylenetetrahydrofolate (CH₂-THF).

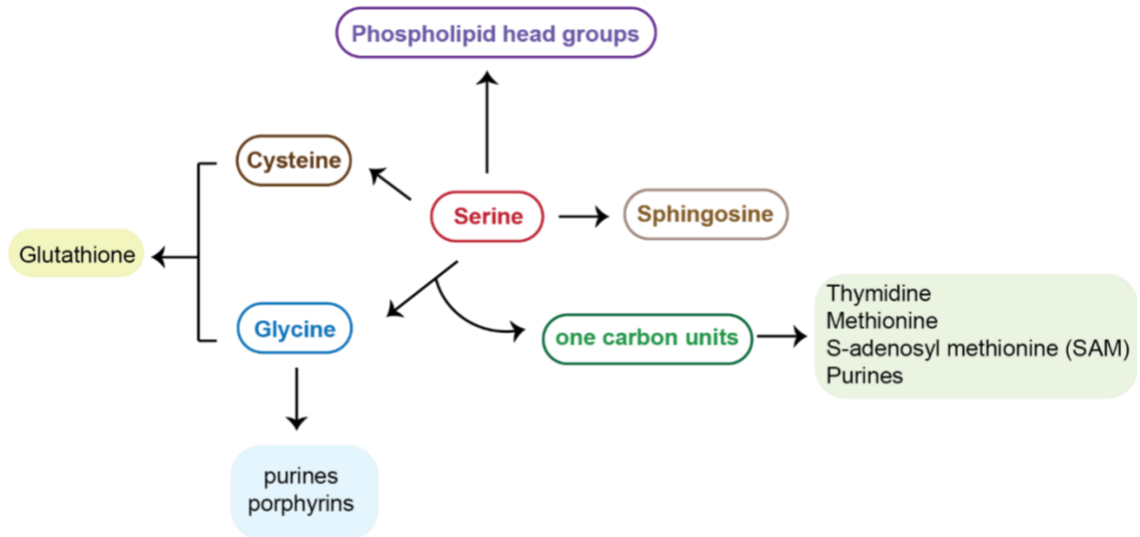


Figure 3.1 Serine supports multiple biosynthetic pathways in proliferating cells.

Serine serves as precursors for other non-essential amino acids, supports nucleotide biosynthesis, co-factors for gene regulations, as well as sphingosine and phospholipids generation. The multifaceted roles of serine position it as one of the most important non-essential amino acids.

CH₂-THF serves as a precursor for folate species for purine synthesis and thymidine, which are important to support *de novo* synthesis of nucleotides. Folates also support the methionine cycle for the conversion of homocysteine to methionine, promoting the generation of S-adenosylmethionine (SAM), which serves as the methyl donor to support both DNA and histone methylation for gene regulation. In addition, serine can be incorporated into ceramide for the synthesis of cell membrane lipids through sphingolipid, as well as supporting phospholipid generation as phospholipid head group. Last but not least, serine supports protein synthesis that is the building block for optimal cell mass growth and anabolism.

With such multifaceted roles to support cellular synthesis and functions, sufficient serine availability becomes crucial for proliferating cells. Indeed, multiple studies have emphasized serine dependence in proliferating cancer cells (299, 300, 301, 302), in which depletion of serine limits their optimal growth. Activated T cells, which also proliferate rapidly extensively, are also critically dependent on serine for their optimal expansion (241). Serine depletion may result in altered redox balance, reduced survival, decreased proliferation, diminished synthesis of lipids, as well as defective mitochondrial fatty oxidation. The involvement of serine in these biosynthesis pathways emphasize its important biological relevance.

3.3 Two sources of serine: dietary and *de novo* synthesis

Cells can acquire nonessential amino acids through exogenous uptake, degradation of cellular proteins, and intrinsic cellular synthesis. For most proliferating cells that require an increased supply of serine to support cellular

growth, the two main serine sources come from either the uptake through extracellular environment, or *de novo* synthesis by cells through the serine synthesis pathway (SSP) (Figure 3.2). As a small and neutral amino acid, exogenous serine can be transported through three systems. Two out of the three systems are sodium-dependent symporters, which include the alanine/serine/cysteine/ threonine transporters ASCT1 and ASCT2 (encoded by *SLC1A4* and *SLC1A5* respectively) (303), and the system A transporters SAT1 and SAT2 (encoded by *SLC38A1* and *SLC38A2*, respectively). The third system is the alanine/serine/cysteine (ASC) antiporter system (304, 305). Upregulation of serine transporter expression is important for the functions of growing cells. Indeed, overexpression of these transporters in rapidly proliferating cells has been observed: *SLC1A4* and *SLC1A5* are upregulated in various tumor tissues (306, 307); *SLC1A5* is vital for T cells that require rapid amino acid uptake, and deficiency of this transporter hinders inflammatory Th1 and Th17 cell responses (308).

One of the other main sources of acquiring serine lies in cells' *de novo* synthesis from glucose through SSP (Figure 3.2). SSP is the exclusive method for intracellular serine synthesis in non-photosynthetic organisms, which is also an important process to support serine concentration in animals (309, 310). SSP utilizes the glycolytic intermediate, 3-phosphoglycerate (3PG) converted from glucose in three steps. PHGDH acts as the first and rate-limiting step that converts 3PG into 3-phosphohydroxypyruvate (3PHP) through NAD⁺-dependent oxidation; 3PHP is then converted to 3-phosphoserine (3PS) in a glutamate (Glu)-dependent

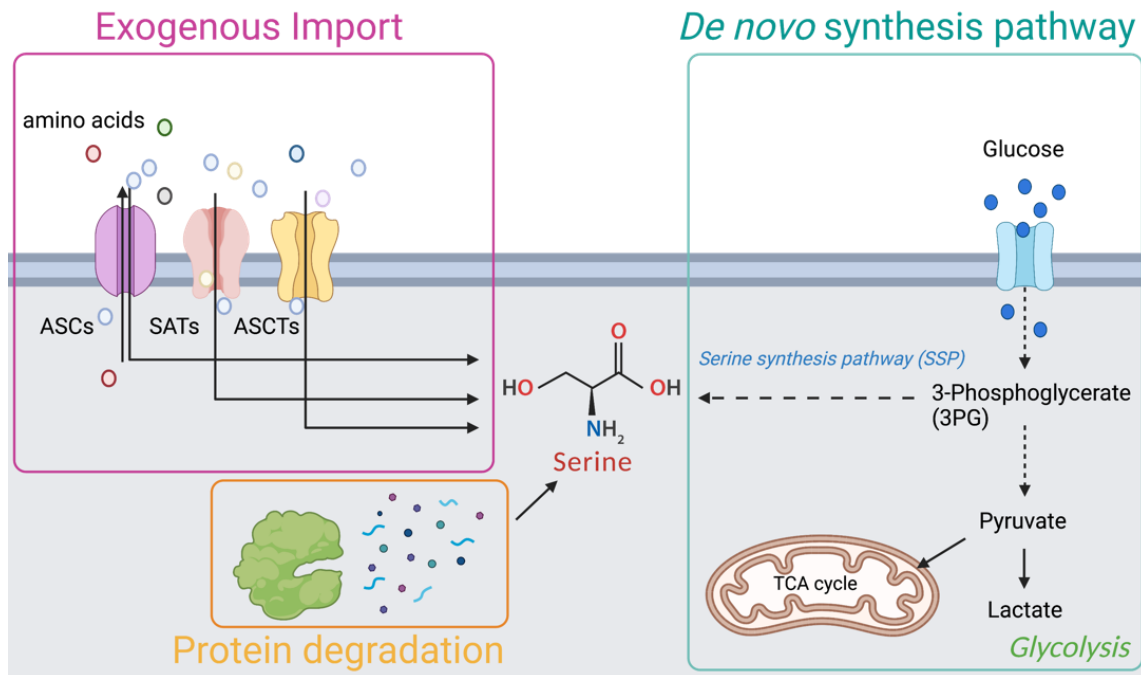


Figure 3. 2 Multiple sources of serine support cellular functions.

Cells can acquire serine through intracellular protein degradation, exogenous import and *de novo* synthesis. Exogenous import of serine from extracellular environment involves transport through alanine/serine/cysteine/threonine transporters (ASCTs), system A transporters (SATs), and alanine/serine/cysteine (ASC) antiporter system. *De novo* synthesis pathway shunts glycolytic intermediate 3-phosphoglycerate (3-PG) into the serine synthesis pathway (SSP) to synthesize serine in a three-step process.

transamination reaction through Phosphoserine aminotransferase 1 (PSAT1), generating α -KG as a byproduct. In the last step, Phosphoserine phosphatase (PSPH) catalyzes the hydrolysis of 3PS to generate serine (Figure 3.3).

PHGDH becomes particularly important in conditions or niches of limited serine, such as the brain (311). A mouse deficient of PHGDH exhibited reduced levels of L-serine in the brain (309), which further reduced the concentration of neurotransmitter D-serine that can be converted from L-serine in neurons. These observations were closely linked to the neurological disorders manifested in human patients with *PHGDH* mutations, which were characterized by symptoms including mild developmental delay, defects in myelination, and juvenile onset of seizures (310). However, given that extracellular serine is not always limiting, it has been postulated that *de novo* serine synthesis may have alternative roles. For instance, the transamination reaction downstream of PHGDH by PSAT1 which converts glutamate to α -KG could further fuel the TCA cycle (312), or regulate cell differentiation through epigenetic mechanisms (313). Another possibility that has been proposed is to meet the enhanced demand for nucleotide synthesis through serine support of the folate cycle (298).

T cells that rapidly proliferate upon encountering their cognate antigens upregulate SSP enzymes post activation. Interestingly, it was shown that exogenous serine attribute to roughly 70% of intracellular serine compared to glucose-derived serine using isotope tracing, suggesting that dependence of extracellular serine is more dominant when serine is not limiting (241). In a model of OVA-expressing listeria infection (LmOVA) with transgenic OT1 CD8 T cells,

serine-glycine deficient diet (SGD) limited their proliferation. Reduced proliferation was rescued through glycine and one-carbon units *in vitro*, implying that serine supports T cell proliferation through *de novo* nucleotide synthesis. It was also shown in the same studies that serine from both exogenous and *de novo* synthesis attributed minimally to methionine in T cells. A later report illustrated that either dietary restriction of serine, PHGDH enzyme knock-down, or the combination of both reduced expansion of antigen-specific CD8 T cells (314), but the impact of serine deficiency beyond proliferation remains unknown.

3.3 Regulation of the Serine Synthesis Pathway (SSP)

De novo serine synthesis is often turned on in environments of limited serine availability. Increased SSP can be dependent on general control nonderepressible 2(GCN2)- Activating Transcription Factor 4 (ATF4) regulation, pyruvate kinase isoform M2 (PKM2) and histone methyltransferase G9A-dependent activation of the three enzymes (302, 315, 316). In the context of tumors, which comprises of transformed and rapidly proliferating cancer cells, SSP enzyme expressions can be amplified through activation of oncogenes such as *KRAS*, *MYC*, *MDM2*, and *NRF2* (300, 302, 317, 318) to support growth and survival. Additionally, deprivation of glucose or glutamine leads to upregulation of all involving enzymes of SSP dependent on MYC transcription, which is critical for downstream redox balance and nucleotide biosynthesis (318).

In T cells, SSP enzyme expressions are increased post T cell activation (241). The enzymes of SSP are also regulated on multiple levels, including

feedback inhibition and substrate inhibition (Figure 3.3). Since the last enzymatic step mediated by PSPH is a nonreversible dephosphorylation, substrate inhibition is less pronounced. Serine, on the other hand, can act to feedback regulate the activity of PHGDH and PSPH (319), and PHGDH is particularly interesting as the first and rate limiting step of SSP. All PHGDH enzymes contain substrate binding domain and nucleotide binding domain. Type I PHGDH, including mammalian PHGDHs, contain additional regulatory domains. In some, but not all organisms, this binding can lead to reduced enzyme catalytic activity. The allosteric substrate binding (ASB) domain is responsible for substrate inhibition mediated by PHP, whereas the Aspartate kinase-Chorismate mutase-TyrA (ACT) domain is responsible for serine-mediated feedback inhibition. The current available reports also suggested that the sensitivity of serine for the regulation of PHGDH varies greatly across organisms. (320)

Serine has also been shown to regulate activities of other enzymes. At physiological concentrations, serine is an allosteric activator of PKM2 (321), which catalyzes the last step of glycolysis. When cells are starved of serine, PKM2 activity is reduced, slowing down the rate of glycolysis to support diversion of the glycolytic intermediate 3PG into SSP, therefore supporting more *de novo* serine synthesis. Additionally, reduced amino acid availability can be sensed through the eIF2 α kinase GCN2. GCN2 phosphorylates eIF2 α , which increases translation of the integrated stress response (ISR) transcription factor ATF4 at the expense of global mRNA translation. ATF4 can further increase expression of stress response genes including enzymes in the SSP pathway (315). Interestingly, while serine

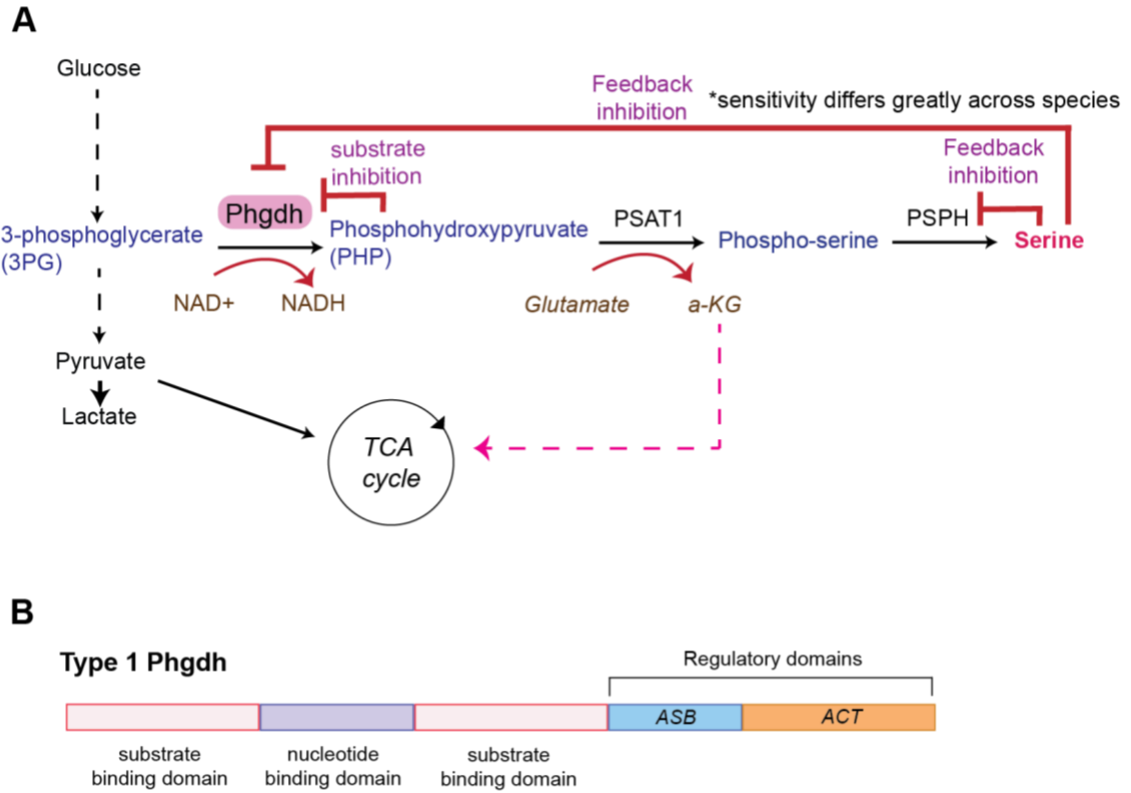


Figure 3.3 *De novo* synthesis of serine is regulated at multiple levels.
 (A) Glycolytic intermediate 3-PG enters the serine synthesis pathway (SSP) for *de novo* serine synthesis. Enzymes in the pathway are subjected to substrate and feedback inhibition to regulate the serine synthesis. (B) Type 1 PHGDHs, including human and murine PHGDH enzymes, contain substrate binding domain, nucleotide binding domain, as well as regulatory domains including allosteric substrate binding (ASB) domain and Aspartate kinase-Chorismate mutase-TyrA (ACT) domain. In some type 1 PHGDHs, ACT allow binding of serine to regulate enzyme activity.

starvation triggers SSP induction through GCN2-ATF4, ATF4 induction is diminished in conditions of both serine depletion and PHGDH inhibition due to blockade of general protein translation, suggesting a coordinated feedback of cellular stress responses intricately regulated by serine availability (322).

3.4 Crosstalk between nutrients and signaling pathways

As discussed in the previous chapter, signaling and metabolism dynamically cross-regulate each other to facilitate cellular activity in response to changes in the environment and cellular status. The important roles of two key signaling pathways, PI3K/Akt and mTORC1, lie at the center of such intersections. Understanding how the cross-regulation occurs and integrates between pathways will be instrumental for T cell biology and could improve our capabilities for future therapeutics design.

Upon T cell activation, PI3K/Akt serves as one of the vital signaling pathways to support cell growth and metabolic reprogramming, including the so-called “Warburg effect”. Whether metabolic feedback regulates PI3K/Akt pathway remains unresolved. Recent studies have unveiled some of these potential “bottom-up” regulations. For instance, LDHA-mediated glycolysis induced by PI3K/Akt pathway supports proper redox balance and ATP production that positive feedback to support optimal Akt/Foxo1 phosphorylation (222, 223). PI3K/Akt activation also promotes the pentose phosphate pathway (PPP) through inhibiting the E3 ligase TRIM21-mediated degradation of Glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of glycolysis. Reciprocally, PPP

metabolites reinforce Akt phosphorylation by negatively regulating the expression of PHLDA3, an inhibitor of Akt that competes with Akt binding and translocation to membrane lipids (323). These reports suggest the presence of multiple feedback regulatory mechanisms in place that critically sustain optimal metabolic signaling, which remains a field of active study.

mTORC1 acts as another critical nutrient sensor that regulates metabolic activity through direct sensing of amino acid availability. Amino acid deprivation limits mTORC1 activation and thus reduces T cell activity (153). Leucine, arginine, methionine, and glutamine can individually bind to different protein complexes part of the mTORC1 complex to positively modulate its activity (324). Methionine, in particular, is an interesting amino acid that can be sensed by the S-adenosylmethionine sensor upstream of mTORC1 (SAMTOR) protein. Methionine serves as a precursor of S-Adenosyl methionine (SAM). In addition to inducing mTORC1 activation through the binding of SAM to SAMTOR (258), methionine-synthesized SAM serves as a co-factor for one-carbon metabolism. The presence of SAM critically regulates the methylation of DNA, RNA, proteins, and lipids through SAM-dependent methyltransferases (325), acting as another key example of how metabolite level can further regulate gene expression for downstream signaling activity. Interestingly, while serine has been proposed as a critical amino acid for multiple cellular processes to support growth, how serine may feedback to regulate signaling pathways still remains incompletely understood. Given the critical dependence of T cells on serine for their functions, we set out to investigate how serine may regulate T cell signaling in support of their immune responses.

3.5 Results

3.7.1 PHGDH-deficient CD8 T cells are quantitative and qualitative distinct in serine restricted environment

In response to pathogens and encountering of antigens, naïve T cells are primed, and antigen-specific T cells become activated through various T cell signaling in combination with nutrients and growth factors to support optimal effector T cell responses. As discussed in previous chapters, effector T cells can further differentiate into SLECs and MPECs. When pathogens are cleared, effector T cells contract and form memory cells that allow the mounting of robust immune responses when the same pathogens are encountered again in the future. Importantly, nutrient availability is critical for driving optimal T cell responses. To investigate how two distinct sources of serine may impact CD8 T cell responses, we took advantage of a mouse strain that allows conditional deletion of PHGDH, crossed with the CD4cre, which allows specific ablation of PHGDH in the T cell compartment (hereon referred to as 4crePhgdh^{fl/fl}, Figure 3.4A). First, to validate that PHGDH is efficiently deleted through the cre-lox system, we isolated naïve CD8 T cells from WT and 4crePhgdh^{fl/fl} mice cultured *in vitro* for 24hour stimulated with anti- CD3/CD28 and IL-2. Upon activation, WT naïve CD8 T cells upregulated their expression of PHGDH in both conditions with or without serine, whereas 4crePhgdh^{fl/fl} T cells did not express PHGDH (Figure 3.4B), confirming the successful deletion of PHGDH enzyme in the T cell compartment.

At steady state, 4crePhgdh^{fl/fl} mice did not display any lethality or phenotypic differences compared to their littermates. To assess CD8 T cell responses to the

responses to acute challenge of bacteria pathogens, we utilized a model of intracellular bacterial infection with *Listeria monocytogenes* expressing chicken ovalbumin protein (LM-OVA) as a model antigen. Mice were placed on either control diet (CTD) or serine-glycine deficient diet (SGD) at least two weeks prior to disease challenge. At day 7, we harvested the livers and spleens of infected animals to assess T cell responses in nonlymphoid and lymphoid organs (Figure 3.4C). We observed that in the acute infection-challenged states for animals placed on control diet, WT and 4crePhgdh^{fl/fl} had comparable frequency of TCRβ+, CD8 and CD4 T cells out of total immune cells (% CD45) with unaltered CD8/CD4 ratio in spleen; whereas in 4crePhgdh^{fl/fl} animals placed on serine-glycine deficient diet, we observed a significant reduction of total TCRb+, CD8, CD4 T cells, suggesting that the number of T cells were only compromised when both the dietary sources and *de novo* synthesized serine were restricted (Figure 3.4D-E). In liver of day 7 of LM-OVA challenged animals, we observed a reduced proportion of CD8 T cells in serine-glycine deficient diet condition (Figure 3.4D), suggesting that dietary serine restriction may have a larger effect in certain organs like liver, or that restricted serine may also affect CD8 T cells' ability to traffic to nonlymphoid organs.

To investigate the effect of serine on antigen-specific T cell responses, we assessed the H-2Kb- OVA+ Teff cells isolated from day 7 Lm-OVA infected mice (Figure 3.4F-H). We found that OVA+ Teff cells from both WT and 4cre-Phgdh^{fl/fl} mice treated with control diet had comparable expansion, suggesting that in the

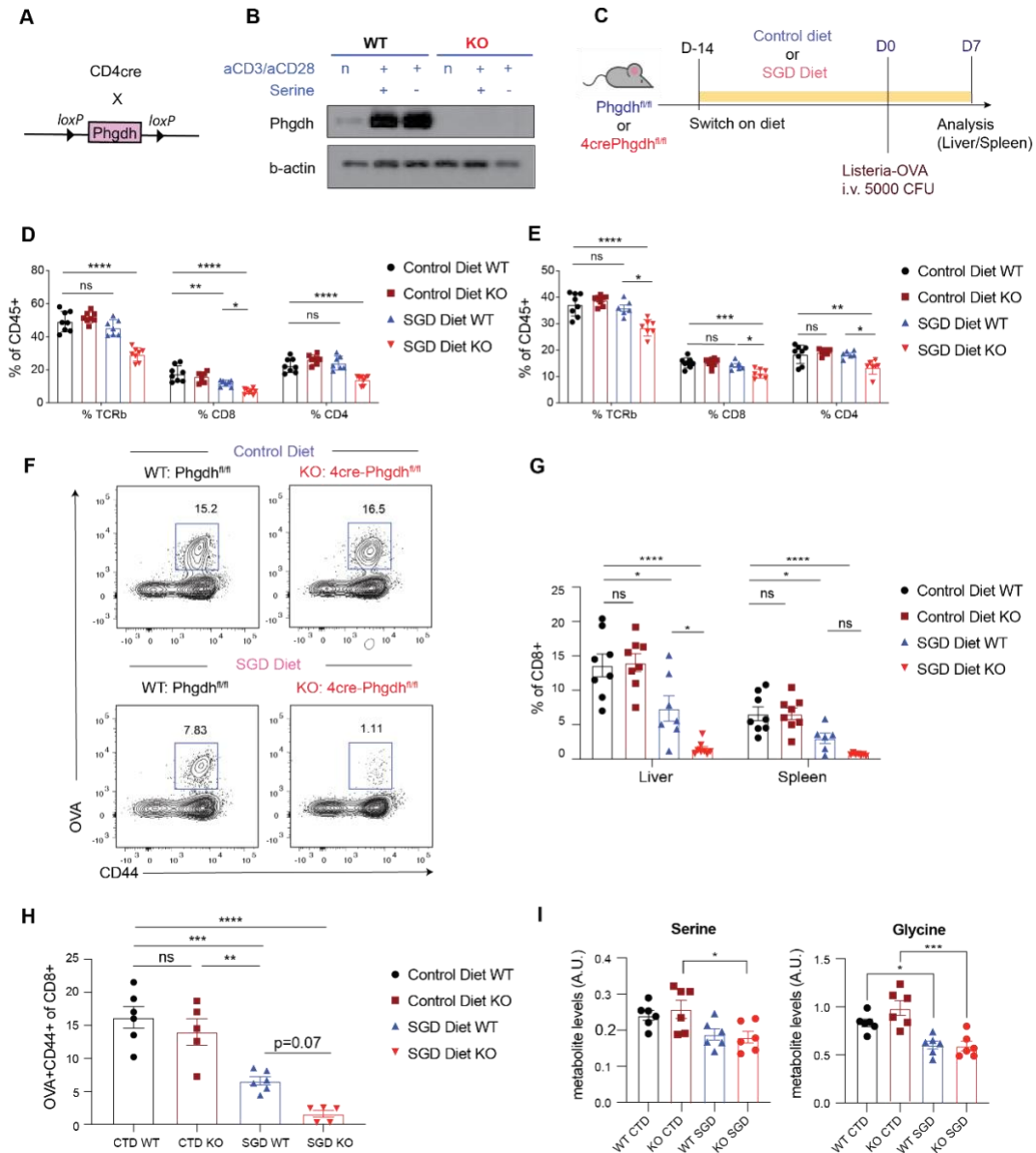


Figure 3. 4 PHGDH-deficient T cells exhibited defective expansion when treated with serine-glycine deficient (SGD) diet

(A) Schematic illustrating mouse breeding for T cell-specific PHGDH deficiency. (B) Naïve CD8 T cells isolated from Phgdh^{fl/fl} (WT) and 4cre-Phgdh^{fl/fl} mice activated *in vitro* for 24hr to assess PHGDH expression. (C) Schematic for Listeria-OVA infection. (D) Frequencies of TCRb⁺ T cells, CD8 T cells and CD4 T cells in livers and (E) spleens. (F) Representative diagrams of OVA+CD44⁺ population out of CD8⁺T cells in infected livers from WT and 4cre-Phgdh^{fl/fl} mice. (G) Frequencies of OVA+CD44⁺ of total CD45⁺ immune population in livers, spleens and (H) blood. (I) Relative serine and glycine concentration in serum of WT and 4cre-Phgdh^{fl/fl} mice treated with either control diet (CTD) or serine-glycine-deficient diet (SGD) measured with mass spectrometry.

normal physiological conditions, dietary serine concentration was sufficient to support optimal CD8 T cell expansion, whereas *de novo* serine synthesis was dispensable. Nevertheless, in WT animals treated with serine-glycine deficient diet, we observed roughly a two-fold compromised expansion of OVA+ Teff cells, which suggested to us that optimal dietary serine concentration was critical to support optimal CD8 T cell expansion. In 4crePhgdh^{fl/fl} animals treated with serine-glycine deficient diet, we saw a significantly diminished population of OVA+ Teff cells in livers, spleens, and blood. The reduction of relative serine and glycine levels was validated by analysis of blood from both WT and 4crePhgdh^{fl/fl} animals placed on serine-glycine deficient diet that were significantly lower than animals placed on control diet (Figure 3.4I). Overall, the results suggested to us that dietary serine and *de novo* serine synthesis work as two redundant pathways to fuel antigen-specific T cell proliferation.

To investigate if two sources of serine may phenotypically affect T cell functions, we assessed the expression of cytolytic molecule Granzyme B (GzmB) in OVA+ Teff cells (Figure 3.5A-B). Interestingly, despite the reduced expansion of WT T cells placed on serine-glycine deficient diet, OVA+ CD8 T cells expressed comparable levels of GzmB as WT and 4crePhgdh^{fl/fl} T cells placed on the control diet. This result suggested to us that neither environmental restricted levels of serine nor *de novo* serine synthesis deficiency alone was critical for optimal CD8 T cell effector molecule expression, revealing a functional redundancy for two pathways to acquire serine for supporting optimal T cell responses. Surprisingly, we observed reduced GzmB molecule expression in 4crePhgdh^{fl/fl} T cells treated

with serine-glycine deficient diet (SGD diet), implicating that antigen-specific T cells exhibited defective effector molecule expression in the absence of *de novo* serine synthesis while subjected to SGD diet.

Post priming, antigen-experienced conventional T cells can differentiate into SLECs, which are CD127^{low}KLRG1^{high}; or MPECs, which are CD127^{high}KLRG1^{low}. The optimal expression level of T-bet is critical for SLEC differentiation, accompanied by higher levels of GzmB expression (131). Through assessment of markers CD127 and KLRG1, we observed a significantly decreased population of SLECs that was only present in 4crePhgdh^{fl/fl} T cells treated with SGD diet, displaying a qualitatively distinct phenotype than control diet treated animals as well as WT T cells in animals treated with SGD diet (Figure 3.5C-D). Moreover, we confirmed in our observations that higher levels of GzmB expression were present in OVA+ SLECs over OVA+MPECs (Figure 3.5E), suggesting that 4crePhgdh^{fl/fl} T cells primarily differentiated into a less effector-like population. Overall, our data suggested that 4cre-Phgdh^{fl/fl} T cells treated with SGD diet comprised of CD8 T cells with reduced effector phenotype, as shown by compromised differentiation of higher-GzmB expressing population SLECs and total GzmB reduction. The reduced effector function only manifested when both sources of serine were limited. These results suggested to us that serine affects antigen-specific CD8 T cell responses in two levels: Dietary serine restriction alone leads to a quantitative reduction of CD8 T cell expansion, whereas lack of *de novo synthesis* in combination with SGD diet leads to both qualitative and quantitative differences of CD8 T cells.

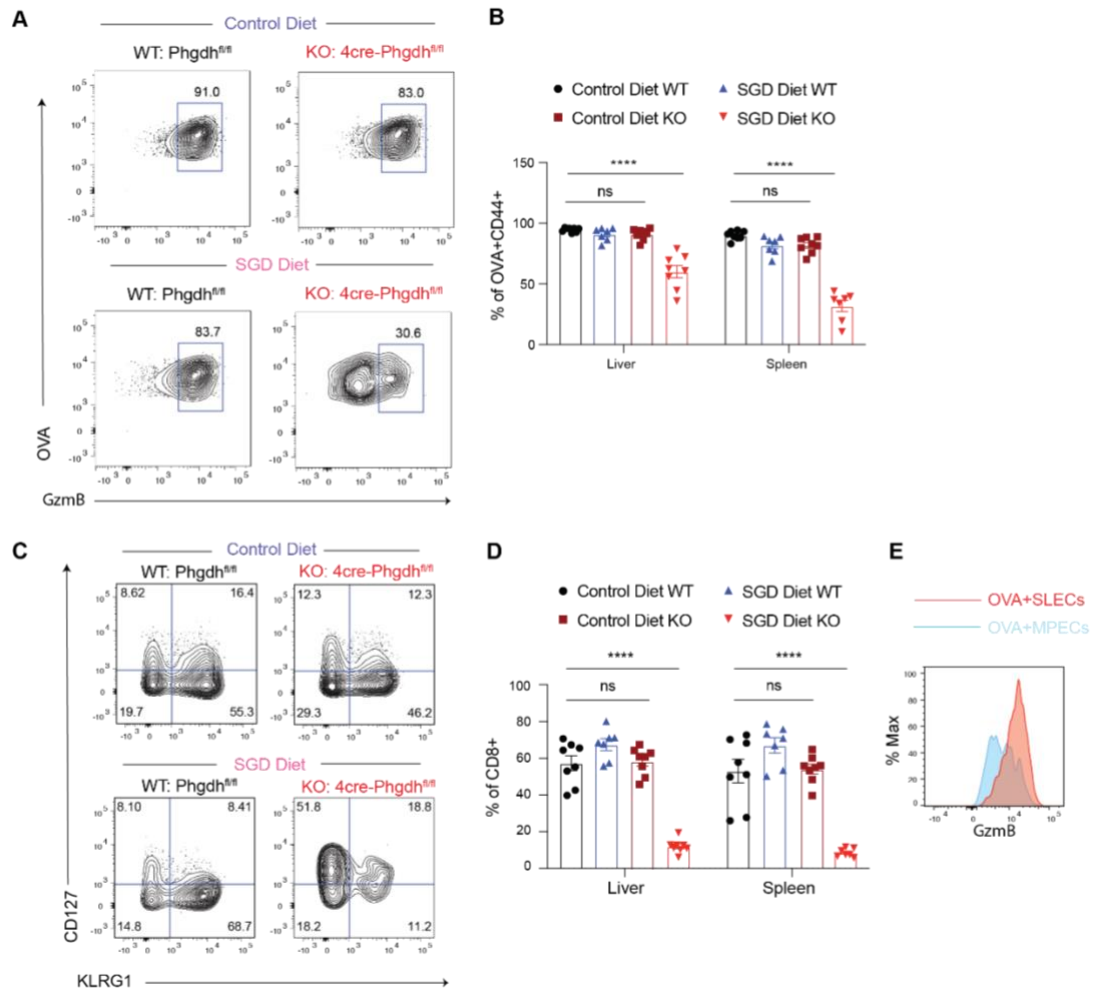


Figure 3. 5 PHGDH-deficient T cells displayed qualitatively distinct phenotypes compared to WT T cells when fed with serine-glycine deficient diet.

(A) Flow cytometric analysis of GzmB expression in OVA+CD44+ CD8 T cells. (B) Frequencies of GzmB⁺ in OVA+CD44+ CD8 T cells. (C) Flow cytometric analysis of MPECs (CD127^{high}KLRG1^{low}) and SLECs (CD127^{low}KLRG1^{high}). (D) Frequencies of SLECs in OVA+CD44+ CD8 T cells in livers and spleens of WT and 4cre-Phgdh^{fl/fl} mice treated with either control diet or serine-glycine-deficient diet. (E) GzmB expression levels in MPECs and SLECs population from OVA+ antigen specific WT CD8 T cells.

3.7.2 PHGDH-mediated de novo serine synthesis supports T cells in a HCC tumor model

To assess if PHGDH-deficient T cells may function differently in their anti-tumor activity, we utilized a liver tumor model resembling human hepatocarcinoma (HCC) using hydrodynamic tail-vein injections of a transposon vector expressing MYC and luciferase (MYC-lucOS) that was linked to three model antigens: SIYRYGL (SIY), SIINFEKL (SIN; OVA 257-264), and OVA 323-339, together with a vector expressing SB13 transposase required for the integration of the transposon-based vector into genomic DNA of the hepatocytes, and a CRISPR/Cas9 vector targeting Trp53 with a single-guide RNA (sgRNA) (326, Figure 3.6A). WT and 4crePhgdh^{fl/fl} mice were placed on the control diet or serine-glycine deficient diet two weeks prior to hydrodynamic injections, and were kept on the diet for the duration of the experiment. On day 4 post injection, we observed equivalent luciferase expression in livers measured by bioluminescence imaging in both WT and 4crePhgdh^{fl/fl} mice, indicating similar injection efficiency and expression levels. On day 17 post injection, WT livers showed a significant reduction of the luciferase signal, suggesting successful clearance of luciferase and antigen-expressing hepatocytes. Interestingly, while WT and KO mice showed no significant difference when placed on the control diet, livers from 4cre-Phgdh^{fl/fl} mice maintained on the serine-glycine deficient diet displayed sustained high signals of luciferase, indicating a defect in the clearance of antigen-expressing cells (Figure 3.6B-C). Analysis of livers from WT and 4crePhgdh^{fl/fl} mice at day 17 showed a significant decrease of OVA+ CD8 T cells in 4crePhgdh^{fl/fl} mice placed

on the serine-glycine deficient diet (Figure 3.6D-E). These antigen-positive cells demonstrated reduced GzmB expression (Figure 3.6F-G). Interestingly, while the WT mice exhibited a higher proportion of SLECs in SGD diet condition than CTD diet, which could be the diet effect on transformed cells that led to different expression MPEC/SLEC differentiation pattern, we observed a significant decrease of SLECs population in KO CD8 T cells compared with WT in SGD, while there were no differences in CTD diet treated animals (Figure 3.6 H-I). Overall, these results closely resembled the quantitative and qualitative different cellular phenotypes from the listeria-OVA infection results we had previously observed.

Transformed hepatocytes can progressively develop into HCC tumors in livers of injected mice. To assess if mice with PHGDH-deficient T cells in restricted serine environment may lead to increased tumor incidence, we maintained the injected mice on serine-glycine deficient diet and monitored the mice weekly for luciferase signals and tumor formation. Interestingly, while none of the WT placed on SGD diet developed tumors, there was an increased tumor incidence in the 4cre-Phgdh^{fl/fl} mice (Figure 3.7A). Livers from mice that developed tumors manifested multiple tumors (Figure 3.7B) that impacted their survival. Analysis of livers demonstrated a markedly low OVA-specific T cell population in two out of the three 4cre-Phgdh^{fl/fl} mice compared to WT mice (Figure 3.7C-D). While all of these OVA-specific T cell populations were PD1 high, indicating they were activated and antigen-experienced, only the OVA+ T cells from 4cre-Phgdh^{fl/fl} mice that developed tumors had low GzmB expression (Figure 3.7 E-G), suggesting their defective functionality. Overall, we observed an increased cumulative tumor

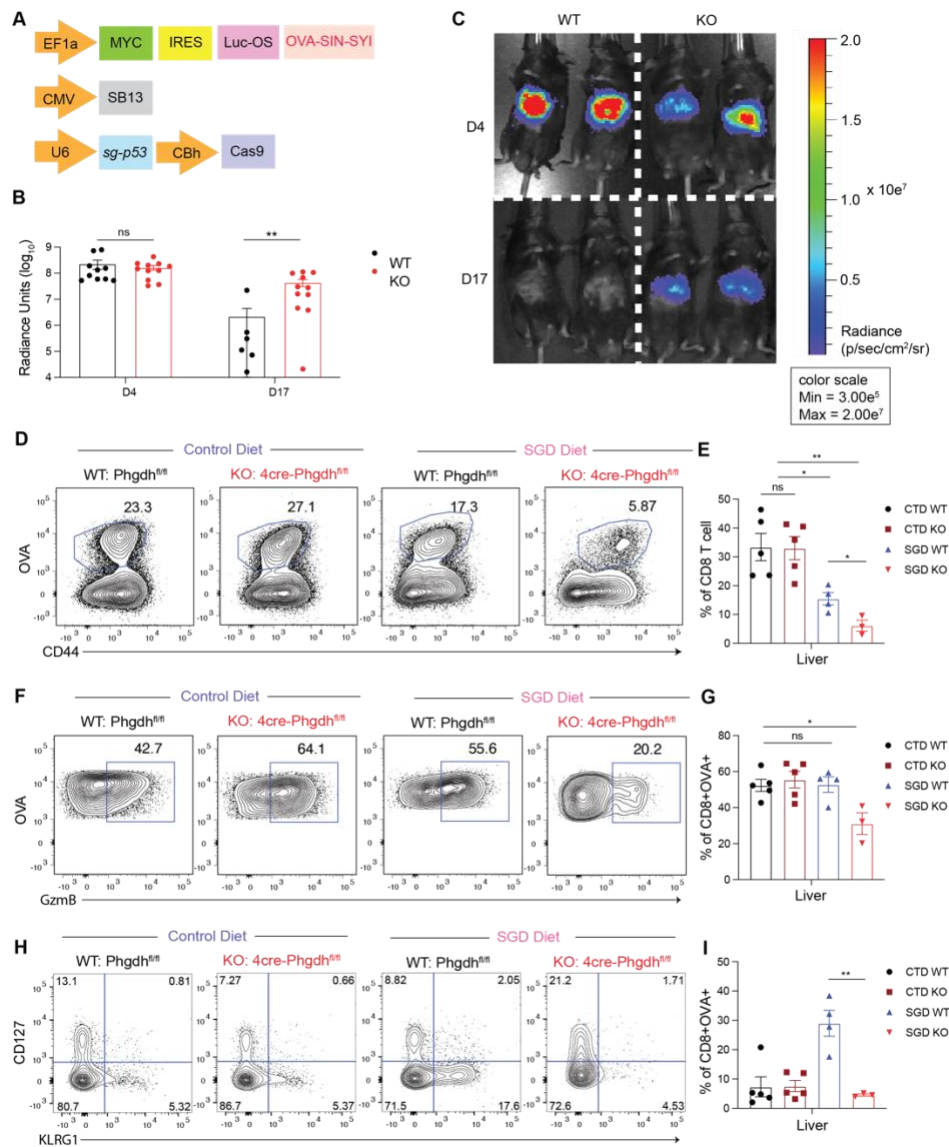


Figure 3.6 4cre-Phgdh^{fl/fl} mice showed delayed clearance of antigen-expressing hepatocytes in a hydrodynamic injection-induced hepatocellular carcinoma model.

(A) Schematic illustration of vectors injected into WT and 4cre-Phgdh^{fl/fl} mice. Mice were placed on CTD diet or SGD diet two weeks prior to injection and maintained throughout the experiment. (B) Luciferase signal at D4 and D17 from WT and 4cre-Phgdh^{fl/fl} (KO) mice treated with SGD diet. (C) Representative images of luciferase signal in WT and KO mice treated with serine-glycine deficient diet. Images were taken with IVIS imaging system. (D)(E) Flow cytometric analysis of OVA+ CD8 T cells, (F)(G) GzmB expression in OVA+ CD8 T cells, and (H)(I) SLEC (CD127-KLRG1+) populations of OVA+CD8 T cells in livers of animals at day 17.

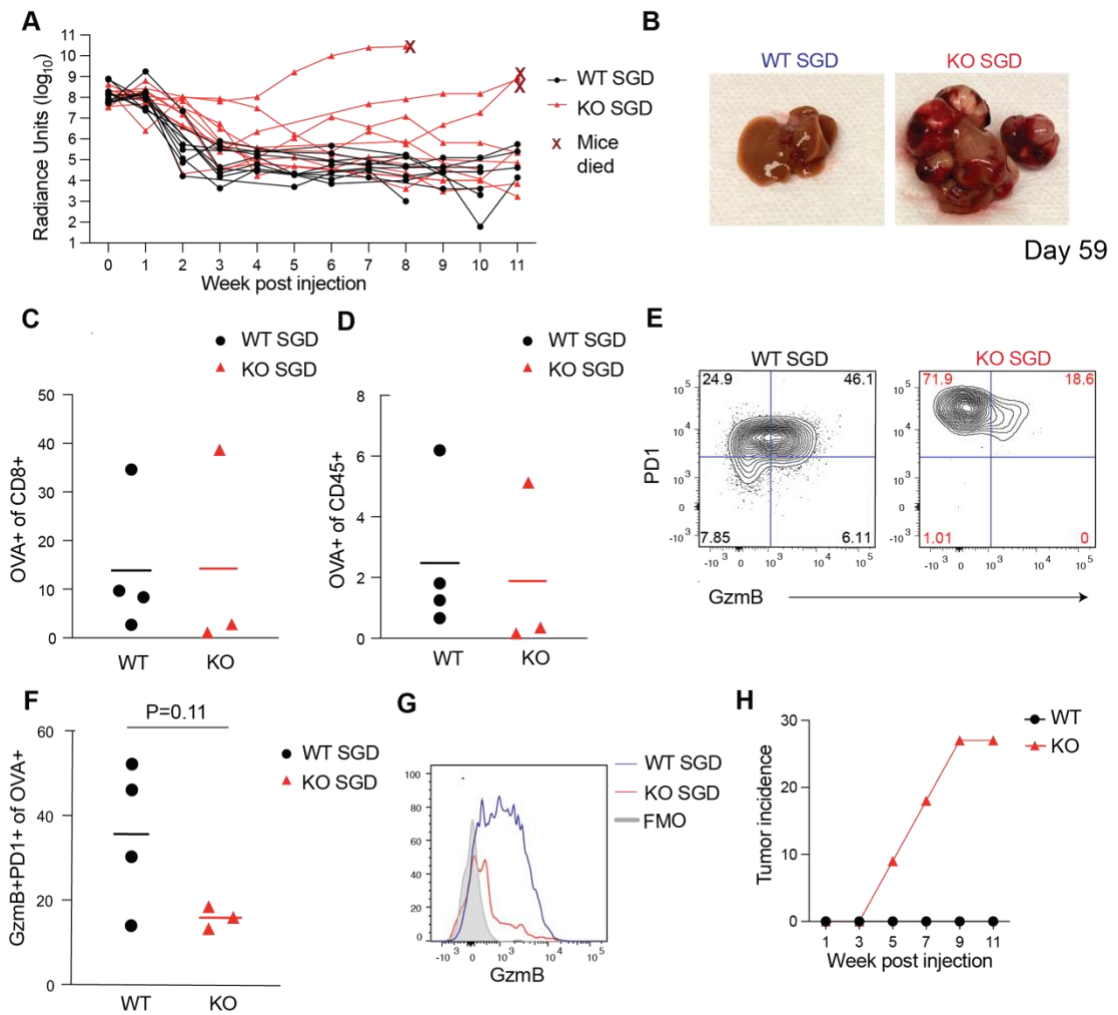


Figure 3. 7 Mice with PHGDH-deficient T cells had increased tumor incidence in serine-glycine deficient diet.

(A) Development of tumors measured by bioluminescent signals. (B) Representative liver images from 4cre-Phgdh^{fl/fl} mouse placed on diet, only KO animals had developed tumors and none of the WT mice controls developed tumors. (C) Flow cytometric analysis of OVA-specific CD8 T cells in livers from WT and 4cre-Phgdh^{fl/fl} (KO) mice out of CD8 T cells and (D) total CD45+ immune cells. (E)(F) PD1+GzmB+ expression level of OVA-specific T cells. (G) Representative expression level of GzmB with FMO controls. (H) Cumulative tumor incidence in WT and 4cre-Phgdh^{fl/fl} (KO) mice.

incidence in 4cre-Phgdh^{fl/fl} mice (Figure 3.7H) that were characterized by reduced effector molecule expression. These results suggested the importance of PHGDH in supporting CD8 T cells and their anti-tumor activity when exogenous serine was limited.

3.7.3 PHGDH-deficient T cells become auxotrophic for exogenous serine

To gain more mechanistic insights of the distinct phenotypic changes we observed in PHGDH-deficient CD8 T cells with SGD diet, we isolated naïve CD8 T cells from WT or 4cre-Phgdh^{fl/fl} (KO) mice for *in vitro* cultures. Naïve CD8 T cells were activated in the presence of anti-CD3/CD28 and IL-2 for optimal TCR, co-stimulation and cytokine signaling (Figure 3.8A). We first investigated the kinetics of PHGDH expression (Figure 3.8B). Activated WT CD8 T cells displayed a time-dependent increase of PHGDH expression in the first 24-hour post activation; the induction of PHGDH was more prominent in the absence of serine, suggesting potential regulation to compensate for exogenous serine deficiency. Expansion deficiency was robustly recapitulated in a three-day *in vitro* culture, where WT and KO T cells proliferated to comparable extent in the presence of sufficient serine in culture medium (Figure 3.8C). In the absence of serine, WT T cells displayed significantly reduced cell expansion numbers, whereas KO T cells were unable to proliferate at all. Indeed, using Cell Trace Violet (CTV) staining, we saw that WT and KO T cells in the presence of serine robustly proliferated as readout through CTV dilution, whereas KO T cells were unable to proliferate in the absence of exogenous serine (Figure 3.8D).

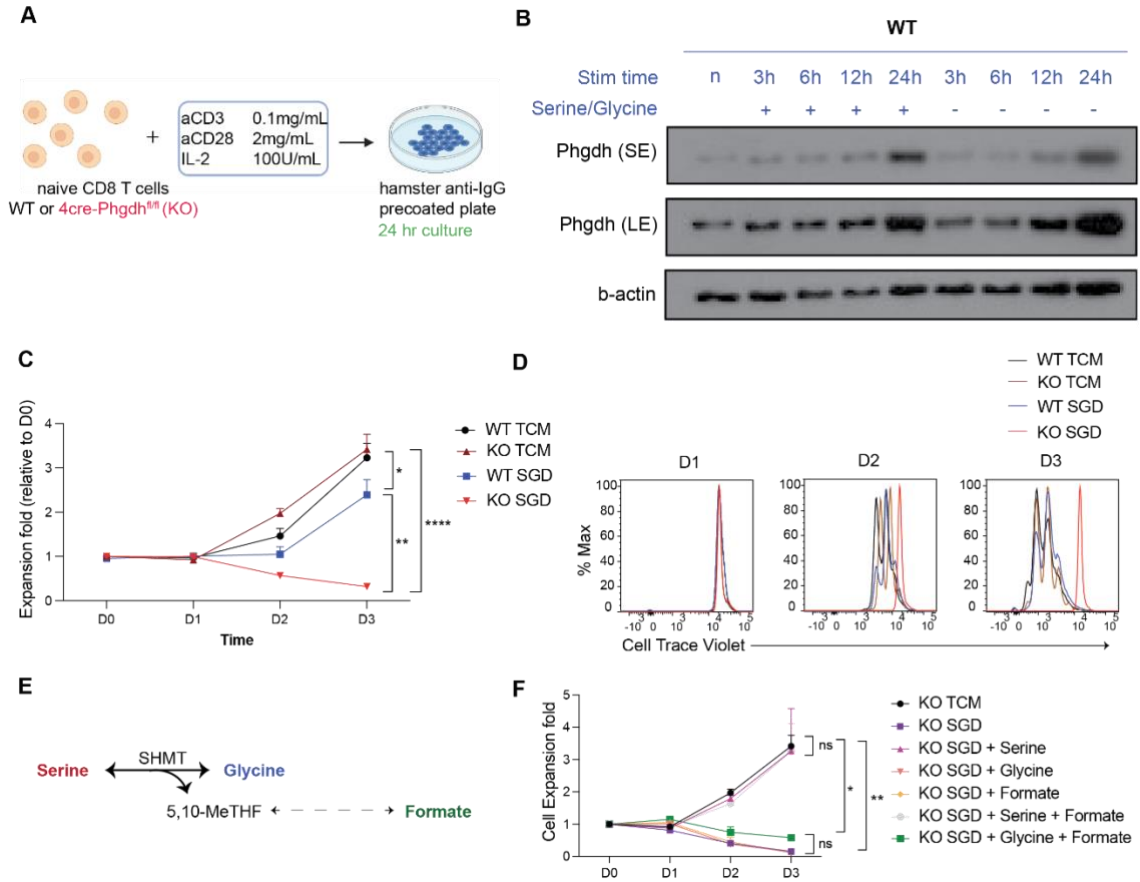


Figure 3. 8 Serine, but not its downstream metabolites glycine and formate, supported PHGDH-deficient CD8 T cell expansion.

(A) Schematic of *in vitro* CD8 T cell culture. (B) The kinetics of PHGDH induction in WT CD8 T cells. (C) Expansion fold of WT and KO T cells cultured in TCM (T cell medium) or SGD (serine glycine deficient medium). SE: short exposure; LE: long exposure (D) Three-day culture of Cell Trace Violet-labeled of WT and KO T cells cultured in TCM or SGD. (E) Illustration of serine and its metabolism to downstream metabolites glycine and formate. (F) Expansion fold of KO T cells cultured three days with various different supplements.

Serine can be utilized downstream to synthesize glycine through the enzyme SHMT. In addition, serine also serves as the precursor of one-carbon units, which is ultimately synthesized as formate to support *de novo* nucleotide biosynthesis for cell growth and proliferation (Figure 3.8E). To assess if defective proliferation could be rescued by glycine and formate downstream of serine, we supplemented serine, glycine, formate alone, or various combinations in a three-day *in vitro* culture of KO T cells. Surprisingly, KO T cells were only able to proliferate in conditions with serine supplementation, but not formate and glycine (Figure 3.8F). This result suggested to us that serine, but not its downstream metabolite glycine or formate, was critical for the optimal expansion of CD8 T cells.

Given that KO T cells in the absence of serine did not proliferate at all, we hypothesized that defects may have already manifested during their early activation phase. Flow cytometry analysis showed that upon activation, CD8 T cells blast and increase in size, as indicated by their forward side scatter (FSC). We found that KO CD8 T cells in the absence of serine (serine deficient medium, or SD) did not blast and remained small (Figure 3.9A-B). Furthermore, KO T cells displayed reduced viability in the absence of serine, suggesting a critical role of serine in maintaining cell fitness (Figure 3.9C). The level of activation marker CD25 was significantly lower in KO T cells cultured in the absence of serine (Figure 3.9D-E). Furthermore, similar to our *in vivo* LmOVA observation, Day 3 cultured KO T cells in the absence of serine displayed reduced expression of GzmB, suggesting compromised differentiation of PHGDH-deficient CD8 T cells in environments without exogenous serine (Figure 3.9F-G).

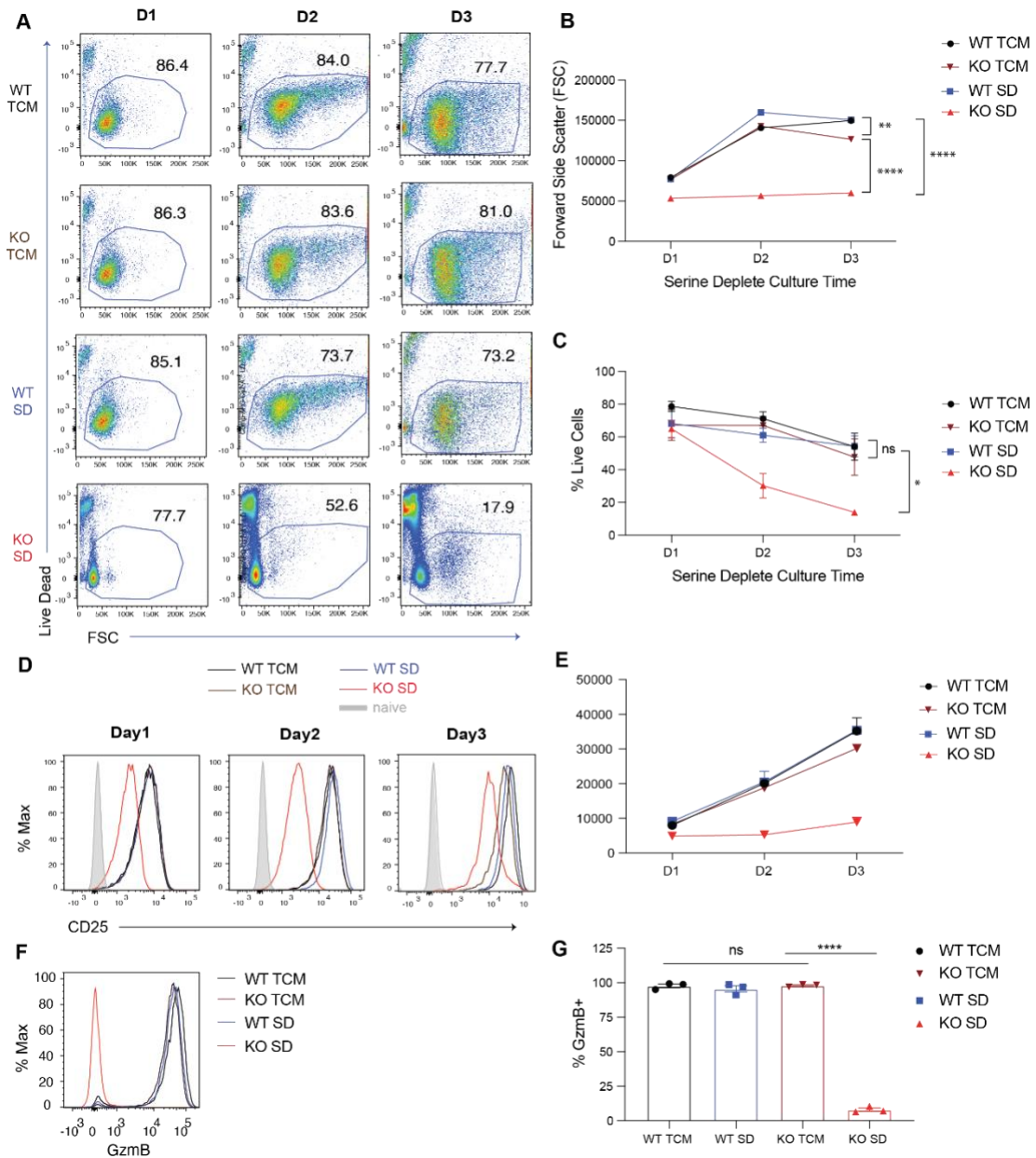


Figure 3. 9 PHGDH-deficient CD8 T cells exhibited reduced blasting, viability and differentiation.

(A) Representative graphs of forward side scatter (FSC) in three-day cultured WT and KO T cells in TCM and SD (serine deficient medium). (B) Quantification of WT and KO CD8 T cell FSC. (C) Viability of WT and KO CD8 T cells. (D)(E) CD25 activation marker expression of WT and KO CD8 T cell. GzmB expression of WT and KO CD8 T cells at day 3 post activation. (F) Representative histogram and (G) quantification of GzmB expression level in WT and KO CD8 T cells at day 3 post activation.

Since PHGDH deficiency was only restricted to the T cell compartment, serine can still be *de novo* synthesized by other compartments. In human, 75% of serine is *de novo* synthesized in kidney (320). Thus, even under serine-glycine deficient diet, serine concentration would not be completely deficient *in vivo*. It has been shown that serine concentration in mouse plasma ranges from 10-20 mg/L; serine-glycine deficient diet reduces 50-70% of the concentration of serine in mouse plasma (300, 311). To investigate how different concentrations of exogenous serine limit optimal T cell functions, we conducted *in vitro* cultures with serine concentrations titrated for WT and KO T cells (Figure 3.10A-B). We observed that while serine reduction did limit the ability of WT T cells to expand, KO T cells were much more dependent on exogenous serine for their optimal proliferation. Interestingly, while KO T cells displayed a serine dose-dependent effect on the expression of GzmB, WT T cells displayed comparable levels of GzmB regardless of exogenous serine availability (Figure 3.11) Although *in vitro* cultured T cells displayed different kinetics of marker expression and we were unable to assess KLRG1, GzmB can also be used as a SLEC marker readout, (Figure 3.5E). These results suggested that KO T cells become auxotrophic for exogenous serine necessary for their optimal proliferation and differentiation.

3.7.4 Serine is required for optimal activation, differentiation and signaling of CD8 T cells

Given the cascade of defective functional responses we observed in the PHGDH-deficient T cells in the absence of exogenous serine, we hypothesized

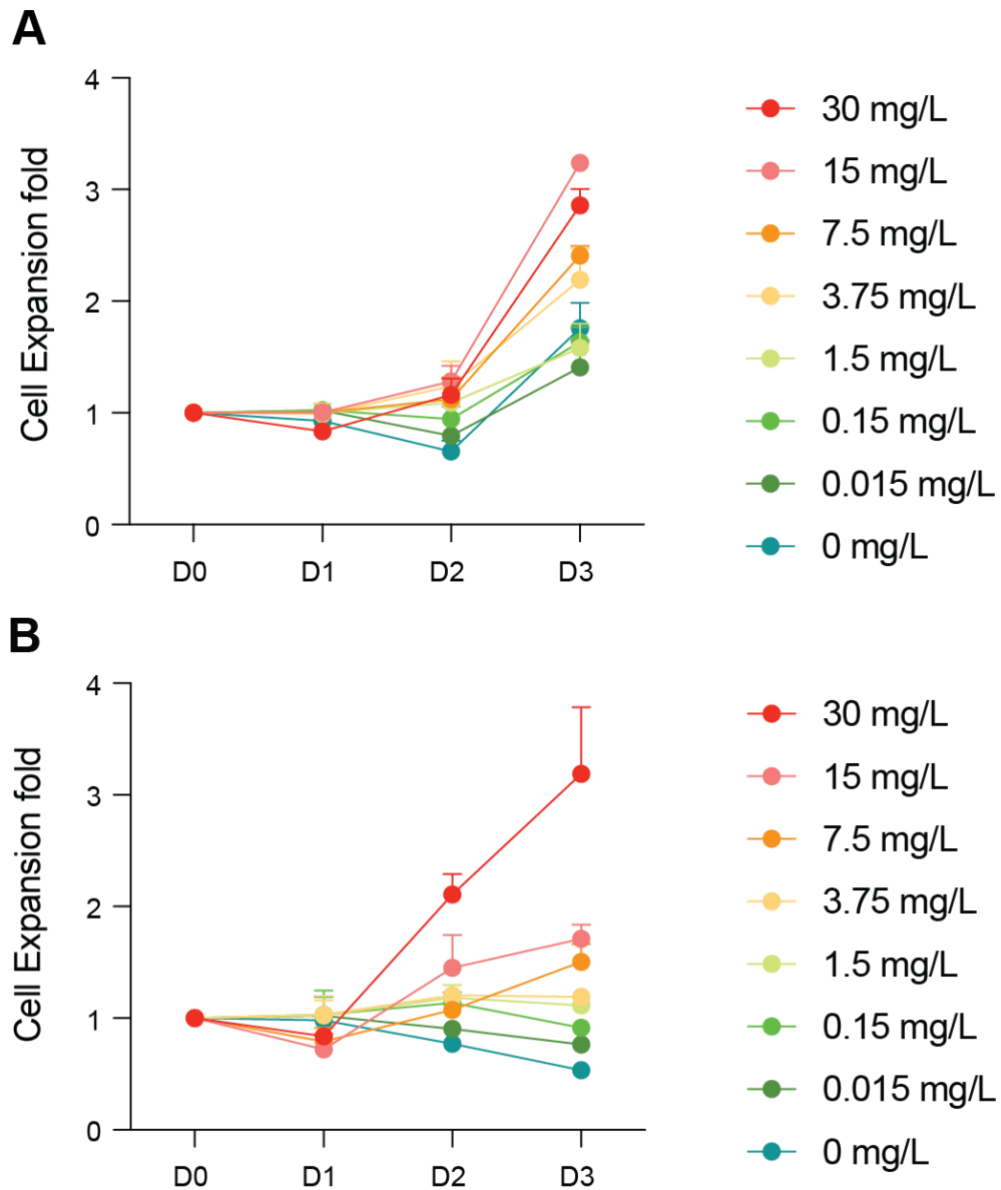


Figure 3. 10 PHGDH-deficient T cells were auxotrophic for exogenous serine.

(A) Expansion fold of WT and (B) KO CD8 T cells cultured in various amounts of serine for a three-day *in vitro* culture.

that defects may take place in the initial state of T cell activation. Optimal signaling is required to not only initiate and but also sustain CD8 T cell activation and differentiation. Akt/Foxo1 and mTORC1 are both key signaling effectors downstream of T cell activation to support differentiation of SLECs and MPECs, as well as positively regulating effector molecule expression (Figure 3.12A). We thus hypothesized that Akt/Foxo1 signaling may be altered by limited serine availability, which could reflect the phenotypes we observed *in vivo*. To assess if there were defective signaling responses of Akt/Foxo1 in PHGDH-deficient CD8 T cells when exogenous serine was limited, we assessed the phosphorylation signals 20hour post activation (Figure 3.12B-E). Phosphorylation signals can be viewed from two perspectives: the frequency of activated CD8 T cells, as shown by % positive population (Figure 3.12B-C); and the activation level or signal strength amongst activated CD8 T cells, as shown by the median fluorescent intensity (MFI) of the signal positive population (Figure 3.12D-E). We observed that while comparable proportions of CD8 WT T cells and KO T cells were positive for p-Akt signaling post activation, WT T cells remained insensitive to the fluctuation of serine availability for their activation level amongst pAkt+ CD8 T cells, as read out by MFI. Importantly, KO CD8 T cells were dependent on serine level for optimal activation status, as demonstrated by the reduction of MFI when serine level in culture was increasingly limited. Interestingly, by overlaying histograms, we can clearly see that at the concentration of 15mg/L of serine in culture, well within the range of *in vivo* serine concentration in mouse plasma, WT and KO CD8 T cells exhibited comparable levels of pAkt activation (Figure 3.12F). However, at 3.75 mg/L of

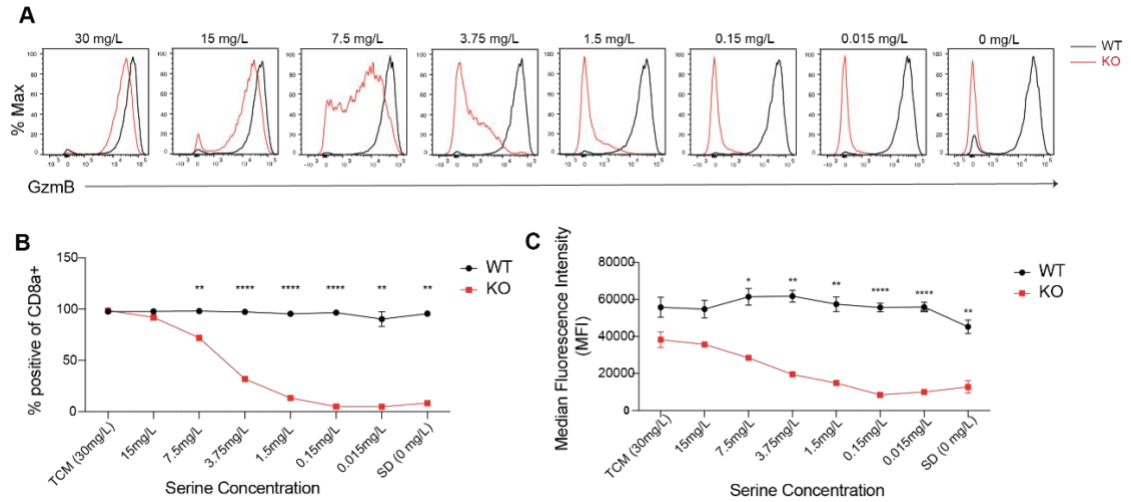


Figure 3. 11 PHGDH-deficient CD8 T cells were dependent on exogenous serine for optimal effector molecule expression at D3 post activation.

(A) Representative histograms of GzmB expression on WT and KO CD8 T cells cultured in various different concentration for three days. (B) Frequencies of Granzyme B expression in WT and KO T cells cultured in various serine concentration for three days. (C) MFI of Granzyme B expression in WT and KO T cells cultured in various serine concentrations for three days.

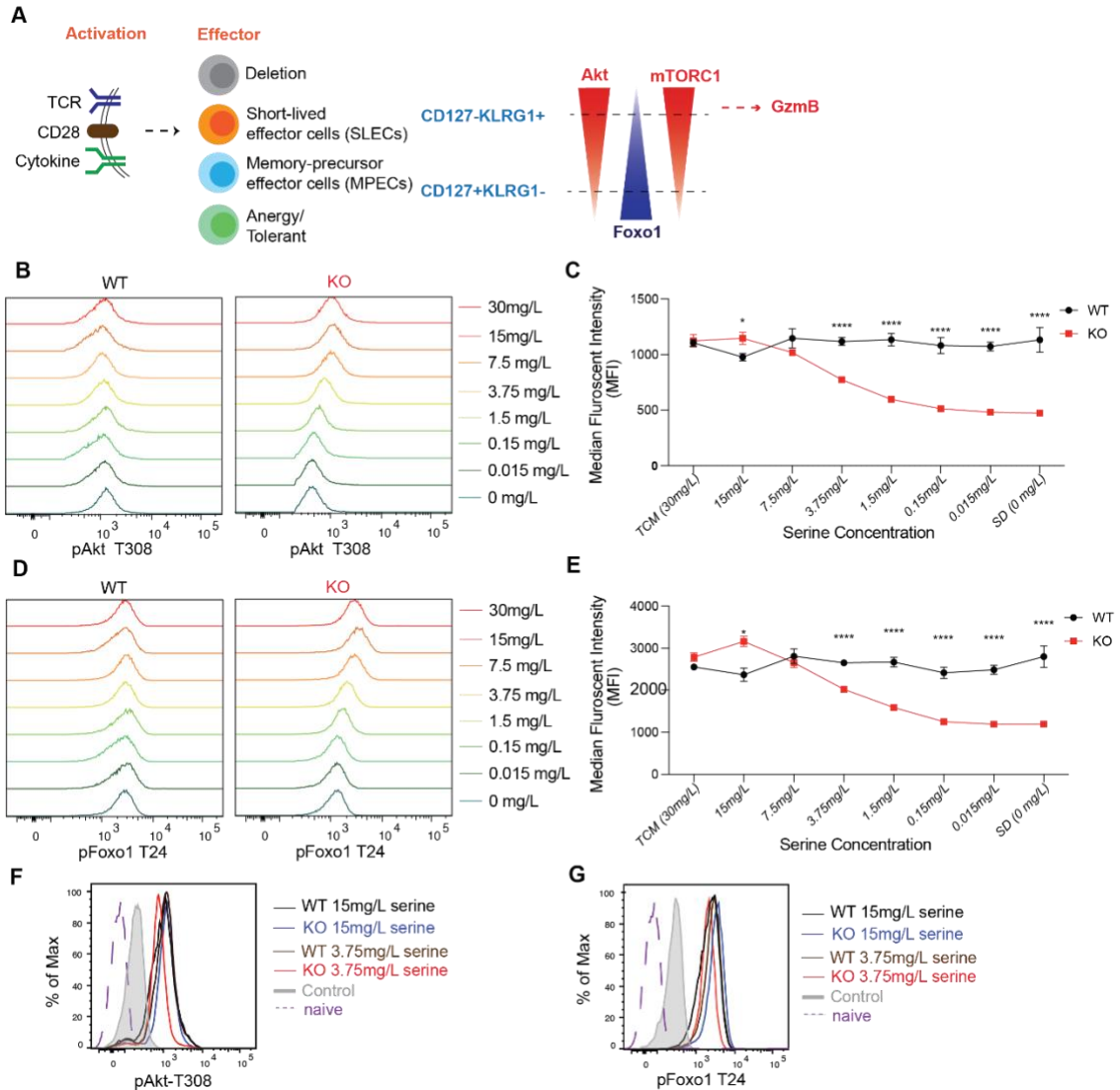


Figure 3. 12 Exogenous serine was important to support Akt/Foxo1 signaling in a dose-dependent manner for PHGDH-deficient CD8 T cells. (A) Illustration of Akt/Foxo1 signaling for balance of downstream effector T cells and effector molecule expression. Optimal strength of signaling determines the differentiation of effector cells into SLECs or MPECs. (B) Representative histograms of and (C) quantification of MFI of pAkt (T308) in various serine concentrations 24hour post activation. (D) Representative histograms of and (E) quantification of MFI of pFoxo1 (T24) in various serine concentrations 24hour post activation. (F) Representative histograms overlaid of pAkt T308+ WT and KO CD8 T cells cultured in 15mg/L or 3.75 mg/L serine, illustrating range of serine relevant to *in vivo* CTD and SGD diet treatment. (G) Representative histograms overlaid of pFoxo1 T24 WT and KO CD8 T cells cultured in 15mg/L or 3.75 mg/L serine, illustrating range of serine relevant to *in vivo* CTD and SGD diet treatment.

serine in culture, which was within the reduction range of serine concentration *in vivo* when treated with SGD diet, we found a significant decrease in pAkt MFI in KO CD8 T cells, but not in their WT counterparts. Similarly, we observed that while WT and KO T cells showed comparable pFoxo1 positive population independent of serine availability, the MFI of KO T cells, but not WT T cells, was dependent on exogenous serine concentration (Figure 3.12G). Reduced pFoxo1 signal indicates augmented Foxo1 signaling presumably due to decreased signaling inhibition from Akt, which was required for proper SLEC differentiation *in vivo*. These data suggested to us that serine level was critical for regulating optimal Akt/Foxo1 activation level in PHGDH-deficient CD8 T cells.

To assess if signaling transduction may be inherently defective in PHGDH-deficient CD8 T cells, we acutely stimulated naïve CD8 T cells isolated from WT and KO animals for 5 minutes (Figure 3.13A). Proximal TCR signaling marked by phospho-LAT (Figure 3.13B), downstream signaling marked by PI3K-dependent Akt phosphorylation and phospho-Foxo1 (Figure 3.13C-E) showed comparable levels between WT and KO T cells either with or without serine (Figure 3.13A). Suggesting that in the absence of serine, PHGDH-deficient naïve CD8 T cells do not exhibit acute signaling defects during the initial stimulation phase, and the signaling defects were not an inherent characteristic.

As discussed previously, the metabolic rewiring towards glycolytic metabolism is critical for T cells to engage in necessary activation and differentiation. It has been shown that the enhanced glycolysis through LDHA generates cytosolic ATP to support PI3K/Akt signaling. Defects of such process

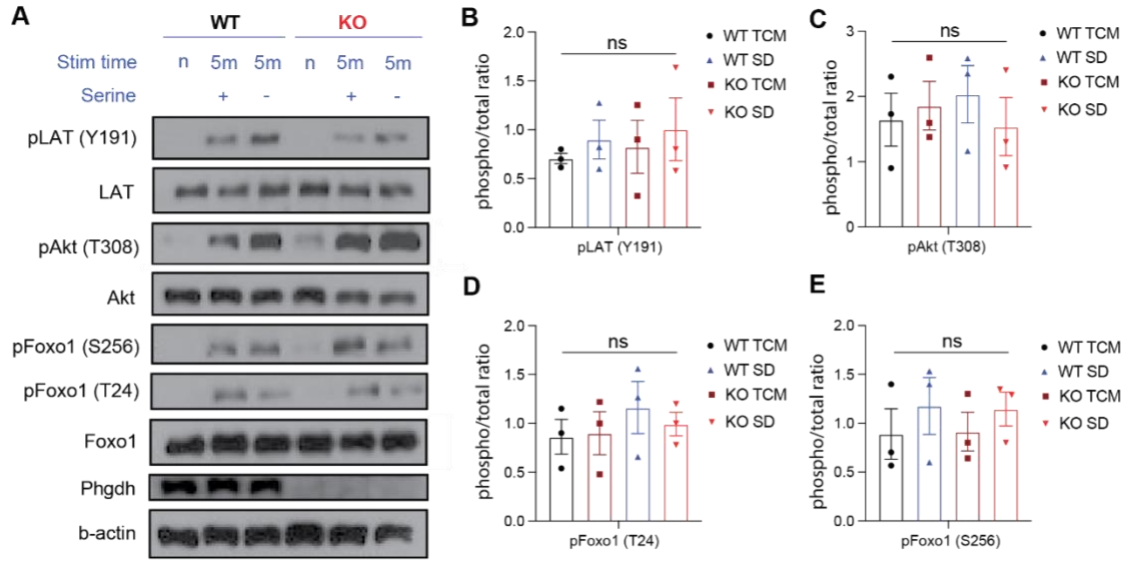


Figure 3. 13 Acute signaling was not affected by serine deficiency in PHGDH-deficient CD8 T cells.

(A) Immunoblotting of acute aCD3/aCD28 crosslinking stimulated TCR signaling in WT and KO T cells. (B) Quantification of pLAT (Y191), (C) pAkt (T308), (D) pFoxo1 (T24) and (E) pFoxo1 (S256) levels normalized to total protein.

leads to reduced pAkt signaling, and increased pFoxo1 signaling as a consequence (222, 223), which could result in reduced activation and differentiation aligned with our observations (Figure 3.9). Thus, we hypothesized that defective Akt/Foxo1 signaling we found could be an indirect effect resulting from defective activation and induction of LDHA-mediated glycolysis. LDHA is a direct target of C-MYC; since C-MYC-LDHA axis is critical for such metabolic reprogramming to glycolysis in T cells (159, 208), we investigated how serine restriction may affect C-MYC signaling in T cells. Indeed, we observed a dose-dependent effect for serine in PHGDH-deficient CD8 T cells for their C-MYC upregulation (Figure 3.14A-B). Interestingly, we observed a bell-shaped pattern for WT T cells, in which the culture conditions with low serine concentrations displayed a lower C-MYC expression than in culture conditions with complete serine deficiency. In concentrations ranging from 0.15mg/L to 3.75mg/L of serine for WT T cells, C-MYC expression was much reduced; this increased heterogeneity could be a result due to competition for serine before *de novo* synthesis caught up, or potential feedback mechanisms that inhibited *de novo* serine synthesis that was necessary to fuel optimal C-MYC signaling. However, no feedback inhibition has been documented for murine versions of PHGDH (320). Furthermore, reduced C-MYC expression in PHGDH-deficient CD8 T cells under serine-deficient culture condition was unable to be rescued by individual downstream serine metabolites, glycine and formate, while the combination of glycine, and formate led to a slight increase of C-MYC expression (Figure 3.14C). This result aligned with previous

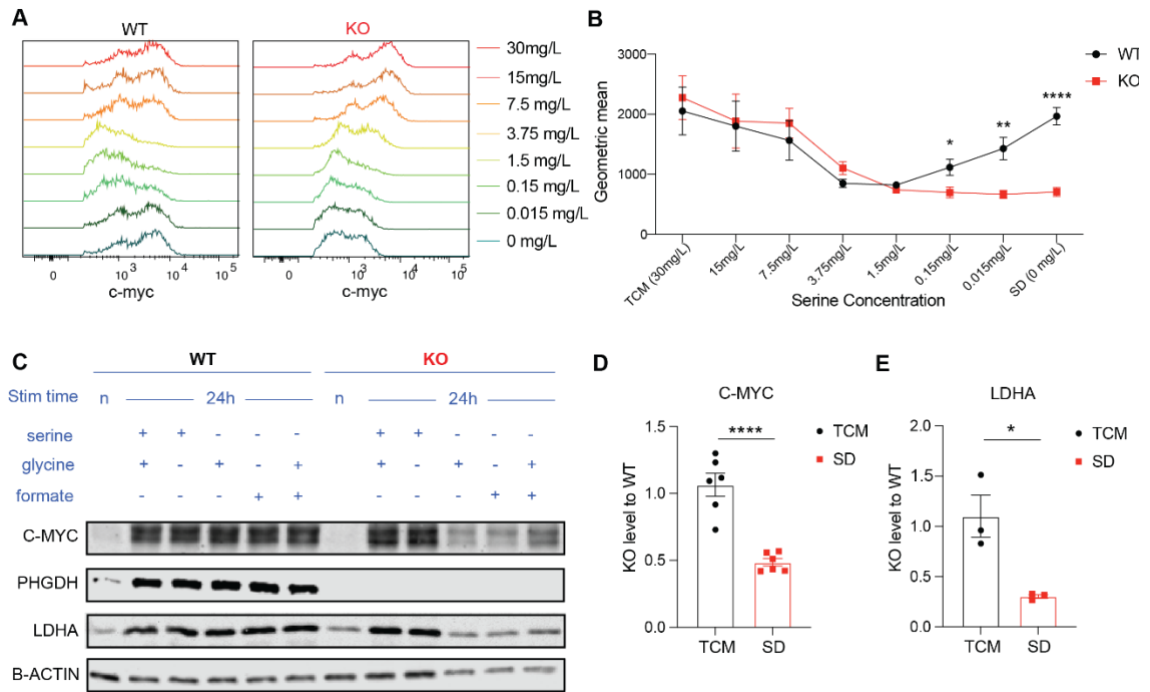


Figure 3. 14 Serine supported optimal C-MYC signaling and induction of downstream glycolytic enzyme LDHA

(A) Representative histograms of C-MYC expression in WT or KO CD8 T cells 24hour post activation in various serine concentrations. (B) Quantification of geometric mean (GM) of C-MYC expression in WT or KO CD8 T cells 24hour post activation in various serine concentrations. (C) Immunoblotting of C-MYC, PHGDH and LDHA in naïve or 24hour-activated CD8 T cells supplemented with serine, glycine or formate. b-actin was used as a loading control. (D) Expression level of C-MYC and (E) LDHA in KO CD8 T cells relative to WT.

observations that deficiency of serine was the key cause of phenotypes we saw (Figure 3.8F). Complete serine deficiency resulted in a significant reduction of C-MYC and LDHA expression (Figure 3.14 D-E) only in KO T cells, supporting our hypothesis that KO T cells may not undergo proper metabolic switch to support their proper functions, including Akt/Foxo1 signaling for differentiation.

mTORC1 signaling is another key regulator to support activated effector CD8 T cells for SLEC differentiation and optimal effector T cell functions (Figure 3.12A). More importantly, mTORC1 positively regulates C-MYC to support metabolic reprogramming in activated T cells (159). Given the well-documented role of mTORC1 in sensing nutrient availability for integration of downstream cell signaling and functions (153), we set forth to investigate how serine availability may impact mTORC1 signaling as read out by phosphorylation of its downstream substrate S6 protein (pS6). Interestingly, we observed that at 24hour post activation, mTORC1 activation displayed a bell-shaped curve of serine concentration dependency for their pS6+ population similar to C-MYC expression, which was particularly prominent in WT T cells (Figure 3.15A-B). In cultures with sufficient serine supplement or complete serine depletion, WT CD8 T cells were both optimally activated with comparable levels of pS6+ cells. However, in low serine concentrations ranging from 0.15 mg/L to 3.75 mg/L, we observed a reduction in pS6+ cells, suggesting that these cells were not activated with optimal mTORC1 signaling level. Since the activation of pS6 is digitally switched on according to the phosphorylation pattern we had observed, the result indicated that

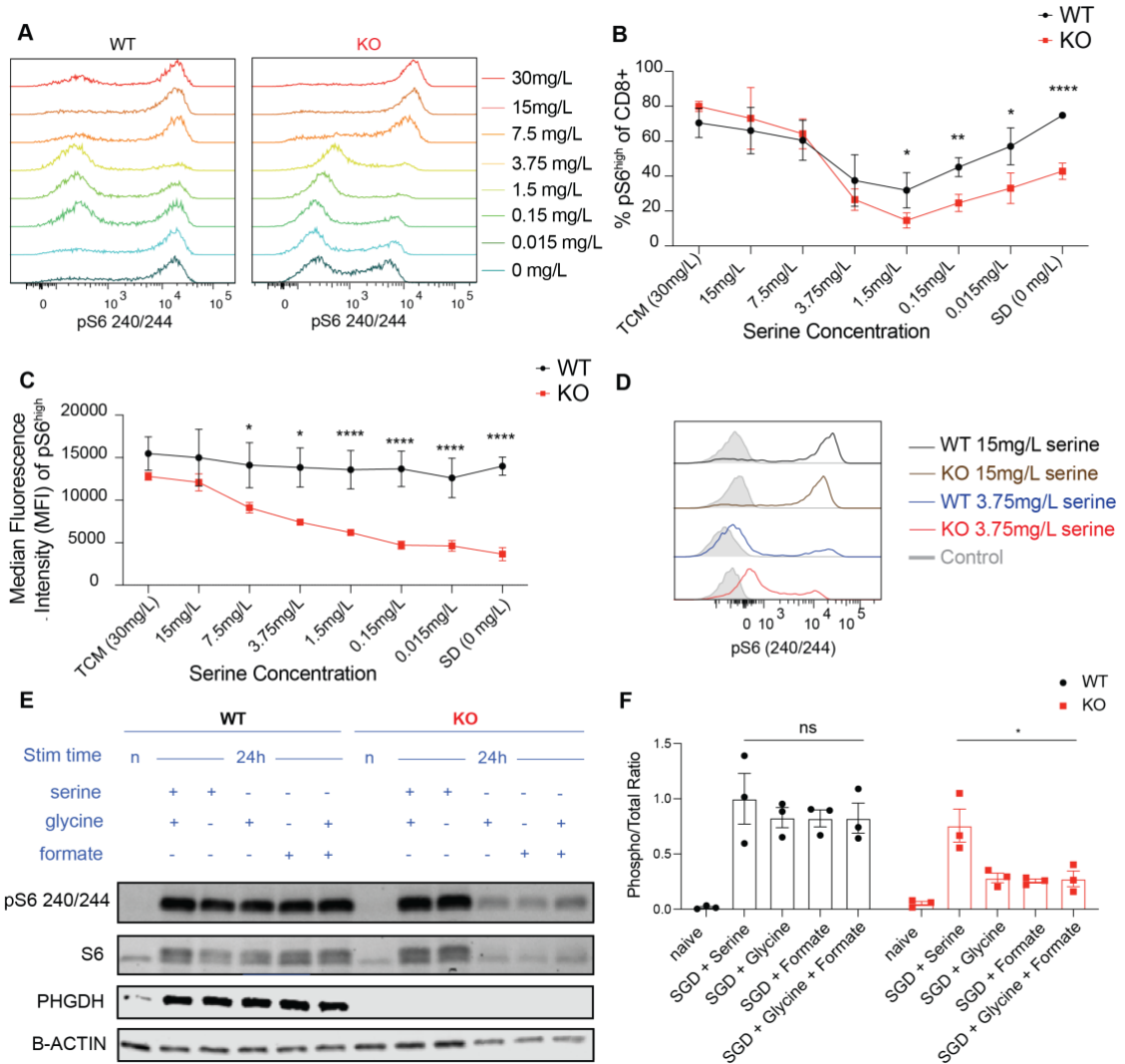


Figure 3. 15 Serine served as a critical mediator to support optimal mTORC1 signaling

(A) Representative histograms of pS6 240/244 expression, (B) frequencies of pS6 240/244 high population, and (C) MFI of pS6 240/244 high population in WT or KO T cells activated for 20hour in various serine concentrations. (D) Representative histograms of pS6 240/244 expression in WT and KO CD8 T cells cultured in 15mg/L or 3.75 mg/L serine, illustrating concentrations relevant to *in vivo* CTD and SGD treatment. (E) Immunoblotting of pS6 240/244, S6 protein, C-MYC, PHGDH and LDHA 24hour post activation supplemented with serine, glycine or formate. b-actin was used as a loading control. (F) Normalization of pS6 signal to S6 total protein signal with various different treatments.

there was heterogeneity in the activated T cell population particularly when serine was low but not depleted, manifested as a “pS6^{high}” and a “pS6^{low}” population, respectively (Figure 3.15A-B). Given the lower serine concentration that was available, the heterogeneity may be a result of competition between WT T cells, where cells that had initially uptake more serine were activated to optimal levels at the expense of pS6^{low} cells. KO CD8 T cells, on the other hand, displayed a serine dose-dependent effect on pS6⁺ activation, which was as expected since KO CD8 T cells become auxotrophic for exogenous serine for optimal functions (Figure 3.15 A-B). Readout of MFI in the pS6⁺ CD8 T cell population further showed that exogenous serine was dispensable for optimal mTORC1 activation strength in WT CD8 T cells, whereas KO CD8 T cells displayed dose-dependency on exogenous serine for their optimal signaling strength amongst mTORC1 (pS6)-activated T cells (Figure 3.15C). Intriguingly, when we compared WT and KO CD8 T cells cultured in limited serine (3.75mg/L), we found that despite similarly reduced frequency of pS6^{high} CD8 T cells, KO T cells still displayed reduced levels of MFI in pS6^{high} CD8 T cells, indicating that *de novo* synthesis of serine was critical at this suboptimal concentration of serine to support and sustain the optimal mTORC1 signaling of CD8 T cells for their activation (Figure 3.15D). In the complete absence of serine, we observed a significant reduction of pS6 signaling when normalized to total S6 protein. This effect, similar to C-MYC, was unable to be rescued by glycine and formate, indicating that reduced mTORC1 activation in KO T cells under serine deprivation was independent of downstream metabolites, glycine, and formate. These observations, together with reduced Akt signaling,

suggested that the defective signaling responses could be the driver for the qualitatively distinct phenotype we had previously observed for KO CD8 OVA+ Teff cells *in vivo*.

To further assess the potential effect caused by reduced mTORC1 signaling, we looked at its downstream targets CD98, an amino acid transporter, and CD71, the transferrin receptor (Figure 3.16A-C). These markers are nutrient receptors critical for T cell survival, proliferation, and also serve as a T cell activation marker (327). Since mTORC1 is the master regulator for cell growth and anabolism, we also looked at cell size by FSC readout (Figure 3.16D-E). 24hour post activation, we observed a similar bell-shaped pattern for both CD98 and CD71 expression, as well as cell size change in WT CD8 T cells with decreasing serine concentrations, whereas KO CD8 T cells showed a serine dose-dependent expression pattern for both markers and cell size. The reduced populations of CD71^{high} and CD98^{high} in WT T cells in low, but not serine deficient culture condition again supported a model of potential competition that resulted in heterogeneity of receptor expression, where low mTORC1 population may lead to low nutrient transporter expression, giving it less advantage to compete with WT T cells that had higher mTORC1 expression and higher nutrient transporter expression, ultimately becoming the population that could activate, blast, and grow into larger sizes in a serine limiting environment. These observations also suggested the importance of PHGDH-mediated *de novo* synthesis and exogenous serine supplement to cooperate for optimal downstream T cell signaling, particularly in environments with restricted or fluctuating serine concentration.

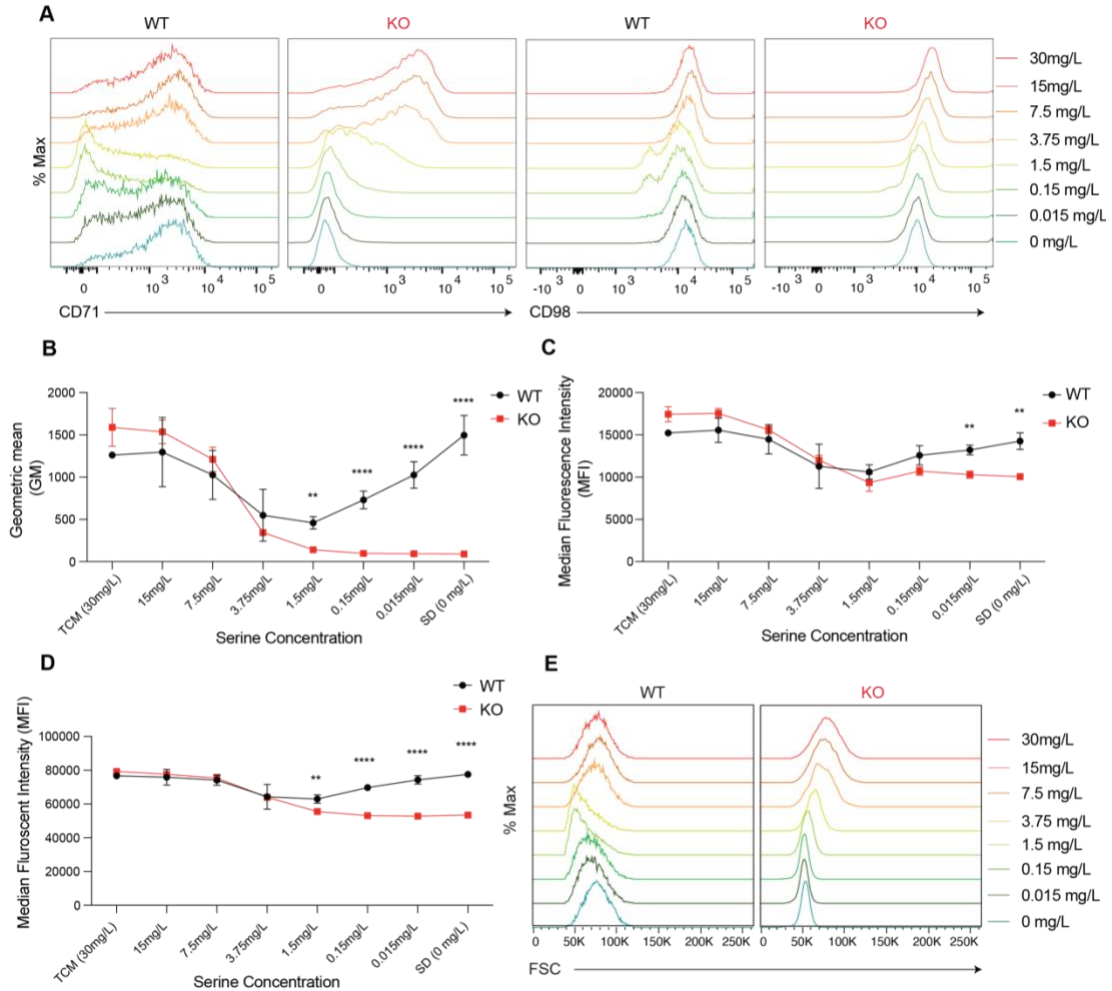


Figure 3. 16 Expression level of mTORC1 targets were sensitive to exogenous serine concentrations for PHGDH-deficient CD8 T cells.

(A) Representative histograms of CD71 and CD98 in WT and KO CD8 T cells cultured with various serine concentrations 20hour post activation. (B) Quantification of CD71 expression level in 20hour activated WT and KO CD8 T cells using geometric mean. (C) Quantification of CD98 expression level in 20hour activated WT and KO CD8 T cells using median fluorescent intensity. (D) Cell size change using FSC as read out in 20hour activated WT and KO CD8 T cells. (E) Representative histograms of FSC in WT and KO CD8 T cells cultured with various serine concentrations 20hour post activation.

Lastly, to observe if defective signaling was sustained at the later activation phase, we looked at phosphorylated signaling at day 3 post activation (Figure 3.17A-B). We noticed that KO CD8 T cells cultured with 15 mg/L of serine showed a slight but insignificant decrease in pAkt, pFoxo1, pS6, and C-MYC expression, this was presumably caused by gradual depletion of serine in the culture that was not replenished *in vitro*. KO CD8 T cells cultured in 3.75 mg/L serine, however, displayed significantly reduced expression of pAkt, pFoxo1, and C-MYC, while pS6 also showed a reduced level that was statistically insignificant. Readout of cell size showed that by day 3, WT CD8 T cells displayed comparable cell sizes across different serine concentrations, whereas KO CD8 T cell size was dependent on exogenous serine concentration (Figure 3.17C-D). This result preliminary suggested that in WT CD8 T cells, *de novo* serine synthesis ultimately caught up to support serine for cell function and growth as exogenous serine levels were depleted (as shown by comparable FSC across different serine concentrations), whereas PHGDH-deficient CD8 T cells were unable to adapt to the exogenous environment of limited serine. Although at later time points the signaling defects could be secondary or compounded effects from all the defects previously described, our results suggested that metabolic signaling effectors including Akt, mTORC1, and C-MYC were observed to have lower expression early during activation and remained low, thus unable to sustain optimal CD8 T cell effector responses.

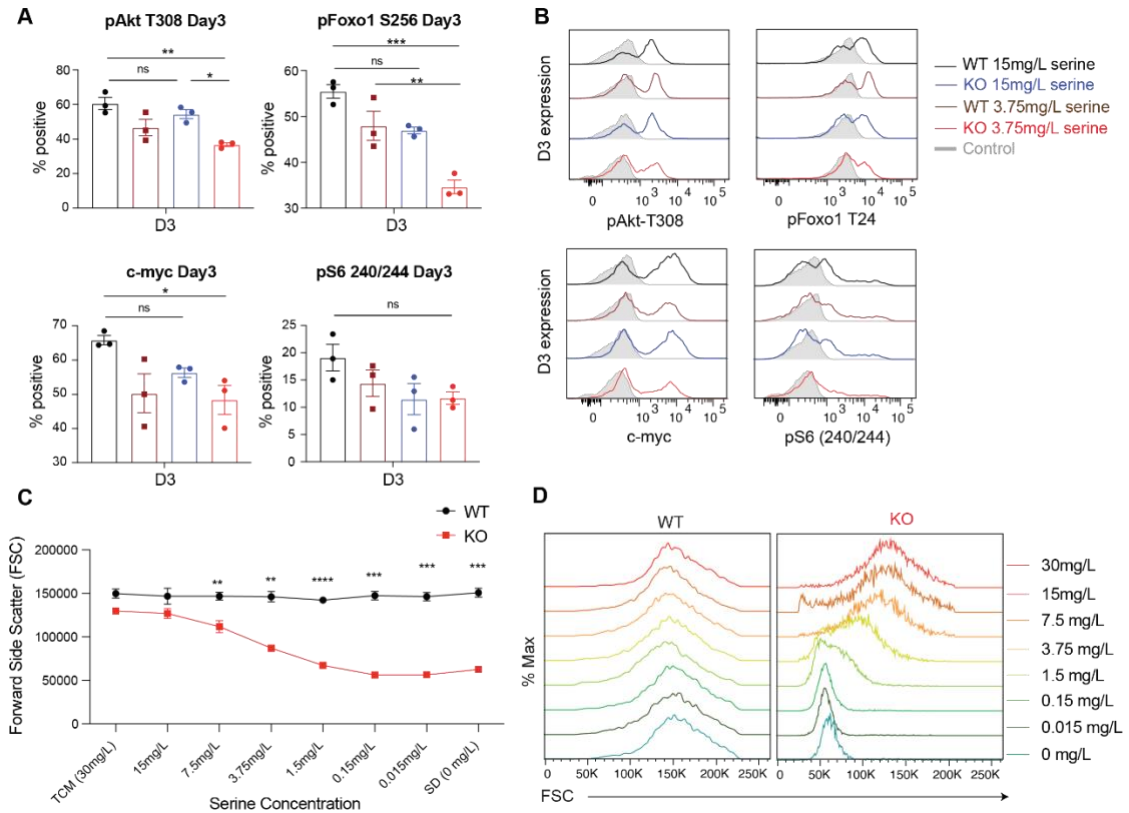


Figure 3. 17 Defective signaling was sustained in PHGDH-deficient CD8 T cells with restricted exogenous serine supply.

(A) Expression level of pAkt T308+, pFoxo1 T24+, C-MYC and pS6 240/244+ in WT and KO CD8 T cells at day 3 post activation. (B) Representative histograms of pAkt T308+, pFoxo1 T24+, C-MYC and pS6 240/244+ in WT and KO CD8 T cells cultured in 15mg/L or 3.75mg/L serine. (C) Cell size measured by FSC in WT and KO CD8 T cells at day 3 post activation. (D) Representative histograms of D3 activated WT and KO T cells in various serine concentrations.

3.7.5 Serine, but not its downstream metabolites, directly activates mTORC1 signaling

There have been increasing reports on the roles of metabolites as regulators for their upstream signaling activity. Our results suggested that serine, but not glycine and formate, attributes to the mTORC1 signaling. However, the fates of serine extend beyond these two metabolites. To investigate if serine itself, or other downstream metabolites and byproducts of serine biosynthesis pathway could attribute to optimal mTORC1 signaling, we supplemented key downstream metabolites and synthesis pathway byproducts of serine into the culture medium when activated naïve WT or KO CD8 T cells *in vitro*. Glycine serves as the immediate downstream metabolite of serine, while formate, a precursor for nucleotide biosynthesis, is further downstream of serine utilization for one-carbon units (Figure 3.8E). Phosphoserine aminotransferase 1, or PSAT1, is the enzyme downstream of PHGDH for serine biosynthesis that also converts glutamate to alpha-ketoglutarate (a-KG, Figure 3.3A). a-KG has been described previously to positively regulate mTORC1 activation (328). Furthermore, serine has also been implicated in downstream functions to support redox balance through the synthesis of cysteine and further supporting glutathione production (Figure 3.1). Lastly, serine restriction has been reported to deplete the production of lipids including ceramide, fatty acid oxidation, as well as TCA cycle intermediates including acetyl-CoA (329). Thus, we selected potential downstream metabolites that were evidenced to be depleted, which could be candidates to positively signal the mTORC1 and C-MYC pathway. To our surprise, 24 hour post activation, only

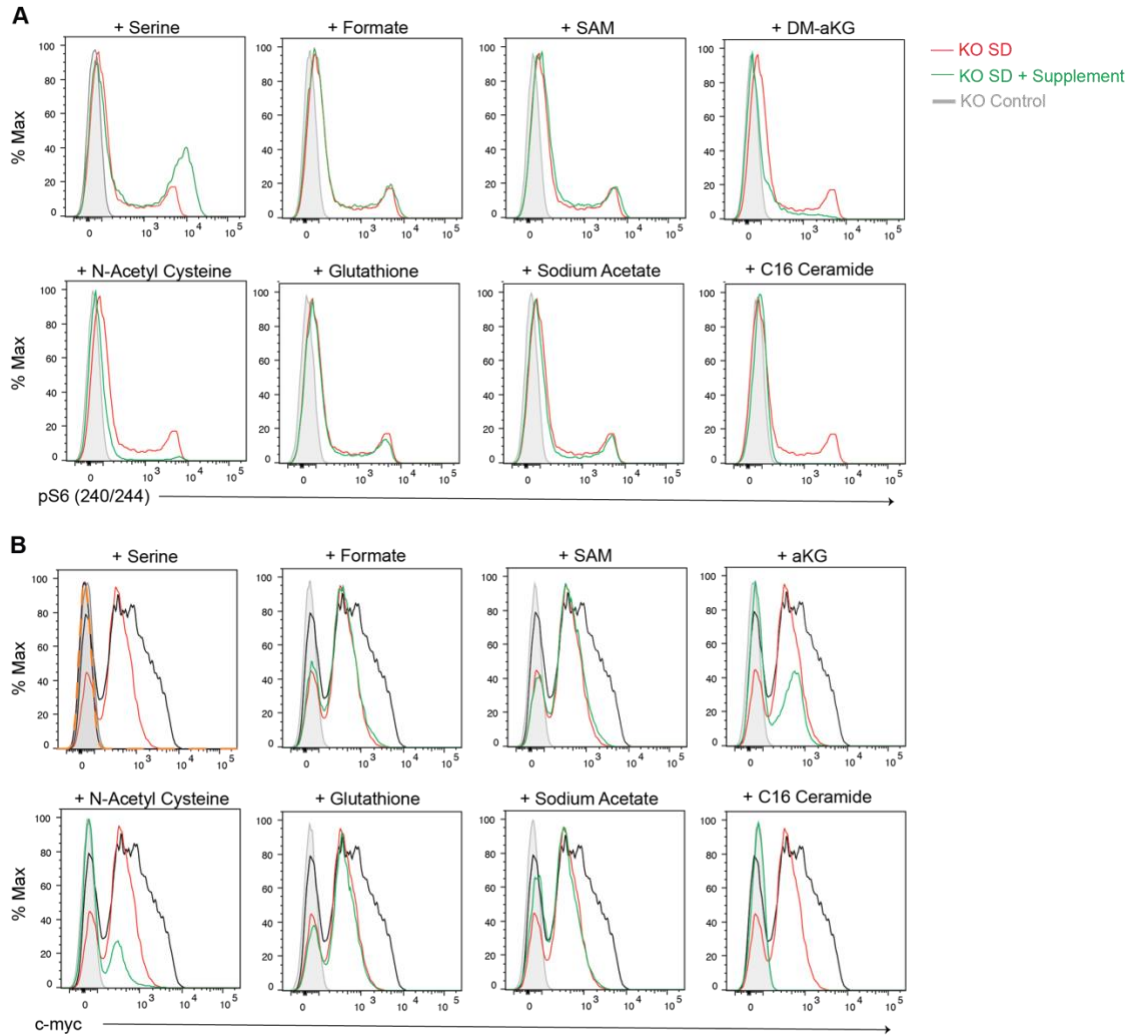


Figure 3. 18 Serine directly supported mTORC1 signaling and C-MYC expression.

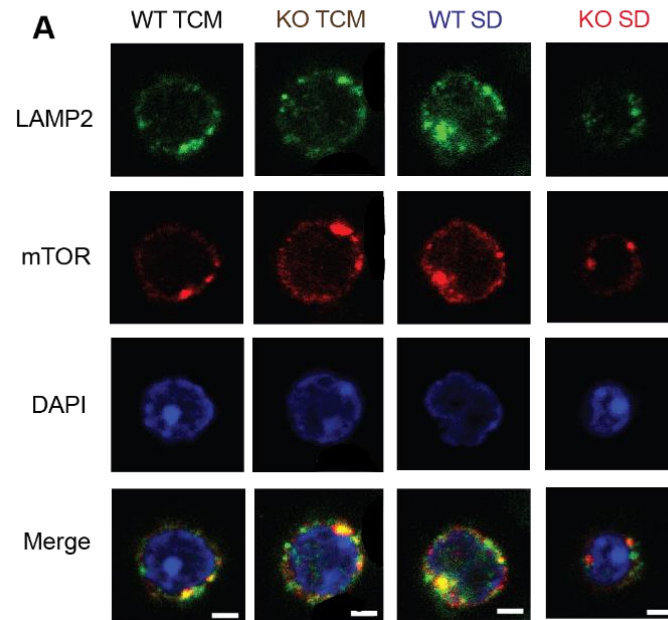
(A) Expression level of pS6 240/244 and (B) C-MYC in KO CD8 T cells cultured in serine deficient medium supplemented with serine and its downstream metabolite. pS6 240/244 and C-MYC level were assessed 20hr post activation via phospho-flow. DM-aKG: dimethyl alpha-ketoglutarate (cell permeable)

serine, but not its downstream metabolites, was able to optimally activate mTORC1 and its downstream target C-MYC (Figure 3.18A-B). This suggested to us a mechanism where serine is directly sensed by the mTORC1 complex, presumably through the amino acid sensors RagA and RagB that support optimal mTORC1 activation.

To test our hypothesis, we assessed mTOR complex localization to the lysosomal surface in the presence or absence of serine. Using LAMP2 as a lysosomal marker, we observed similar colocalization of mTOR and LAMP2 in WT T cells with or without exogenous serine, as well as KO T cells with serine in culture. In KO T cells cultured in medium without serine, we observed a significantly reduced level of colocalization (Figure 3.19). Interestingly, the culture medium contained multiple amino acids that were known to activate mTORC1 individually, but our data implied that cumulative serine was critical for optimal mTOR localization to the lysosome for downstream activation and signaling. Overall, the results suggested to us a mechanism where mTORC1 can act as a sensor for serine, which becomes conditionally essential in environments with fluctuating serine for T cells. To our knowledge, this is the first report that mTORC1 can act as a serine sensor, further depicting the importance of serine as a metabolite that can regulate signaling.

3.8 Discussion

Enhanced amino acid uptake and synthesis are key features of activated T cells. Increased supply of amino acids not only support synthesis of proteins,



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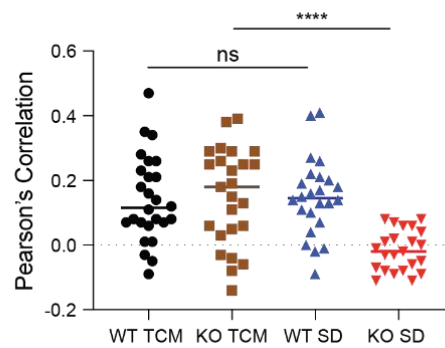


Figure 3. 19 Cumulative serine was critical for optimal mTOR complex activation and colocalization with lysosomes.

(A) Representative immunofluorescent images taken with confocal microscopy of WT and KO T cells cultured for 20hour in TCM or SD medium. Scale bar indicates 2 μ m size. (B) Quantification of colocalization between mTOR and LAMP2 in WT and KO T cells.

but also other critical processes including nucleotide synthesis, redox control and epigenetic regulation to support a proliferating T cell. Particularly, serine is an amino acid that exhibits multiple functional roles in supporting cellular biosynthesis for nucleotides, lipids, and proteins. While previous studies centered on its role in facilitating T cell expansion (241, 314), we were able to investigate additional functions of serine using diet manipulation and genetic knockout models. Intriguingly, we observed that while PHGDH-competent cells exhibited reduced expansion of antigen-specific CD8 T cells, which was consistent with prior reports for serine being a biosynthetic precursor for cell biomass generation; T cells defective of PHGDH with limited dietary serine showed a qualitatively different phenotype manifested with reduced effector differentiation, as shown by reduced SLEC population and lower GzmB expression in the Lm-OVA bacterial infection model. The reduced effector-like cellular phenotype was recapitulated in the HCC model when KO animals were placed on the SGD diet, demonstrating increased tumor incidence. While the SGD diet likely also affected tumor formation intrinsically, our results strongly suggested that *de novo* synthesis of serine was critical for T cells to mediate cancer immunosurveillance when exogenous serine was limiting. Although one out of the three KO animals that had tumors exhibited higher OVA+ antigen-specific T cells, this may have been complicated by potential differences of liver antigen load within developing tumors, or increased serine concentration locally that the animal may have compensated with *de novo* serine synthesis from other cell types like tumor cells after being placed on SGD for an extended period. While monitoring of serine concentration in the local liver

compartment was technically challenging, it has been shown that serine concentration fluctuates based on multiple factors, including diet, circadian rhythm, and different tissue environment (330), the fluctuations may be limiting for T cell functions that attributed to differential disease outcome. Further *in vitro* data showed an early defect post T cell activation in mTORC1 and Akt/Foxo1 pathways, suggesting that serine was an important regulator for these key signaling T cell activation pathways critical for T cell activation and differentiation to support their responses against infection and cancer. Interestingly, defective mTORC1 and C-MYC signaling pathway can be rescued by serine, but not its downstream metabolites; cumulative serine was necessary to support mTORC1 localization to lysosomes for subsequent activation despite the presence of additional amino acids known to activate mTORC1 through its nutrient-sensing role, suggesting a direct role of sensing by serine for support of optimal mTORC1 signaling. These data implied the role of serine extending beyond a nutrient for cell building blocks, but a regulatory role for signaling transduction to support cellular functions.

In addition to the defective mTORC1 pathway, we also observed a reduction in key metabolic regulator C-MYC in PHGDH-deficient T cells with limited serine. Reduced C-MYC upregulation could be attributed from either defective upstream mTORC1 activation pathway, or protein translation defect. Data from previous studies demonstrated that exogenous serine restriction in combination with PHGDH knockdown impeded the proliferation of multiple tumor cell lines in part through a global protein synthesis defect (322). In our results, we saw a reduced C-MYC upregulation post T cell activation (Figure 3.14C), as well as reduced total

S6 protein levels (Figure 3.15E). These data suggested preliminarily that newly synthesized proteins may be reduced due to limited serine availability, although individual proteins may be subjected to differential regulations of protein half-life and serine content. Interestingly, C-MYC protein contains 14.4% of serine, which was the highest composition of all amino acids, potentially supporting its sensitivity in a reduced serine environment. However, normalization of phosphorylated protein indicated reduced mTORC1 activation in PHGDH-deficient T cells absent of serine (Figure 3.15E), suggesting to us that mTORC1 signaling was still one of the key defects that functioned either upstream or in parallel with C-MYC for dampened metabolic reprogramming (as read out by upregulation of glycolytic enzyme LDHA) and signaling to attribute to the defective CD8 T cell responses.

Both our data and prior reports demonstrated the critical importance of serine to support cellular functions in multiple aspects. Given the significance of serine as a key nutrient for cells, it is reasonable for regulating mechanisms to exist for *de novo* serine synthesis. Past literature had suggested that SSP can be regulated by both mTORC1 and C-MYC, which are both positive regulators of the rewired metabolic program post T cell activation (157, 203). Interestingly, in our studies with titrating serine levels of culture, we observed that culturing with 3.75 mg/L of serine led to reduced frequencies of pS6^{high} population even in WT CD8 T cells with intact *de novo* serine synthesis pathway. The reduced pS6^{high} population was recovered when cells were cultured in a complete serine-deficient medium. Since mTOR localization to the lysosome and subsequent activation was dependent on optimal serine level (Figure 3.19), it was plausible that at this serine

concentration, T cells were inadequately supported by mTOR activation to sustain the metabolic switch essential for their functions. As a result, activated WT T cells may need to compete for exogenous serine. Some T cells may have gained access to more exogenous serine uptake presumably due to stochastically expressed transporters at higher levels during early T cell activation. These T cells could thus not only sustain the metabolic switch, but also initiate a positive feedback loop to upregulate nutrient transporters for more macronutrient and amino acid uptake including serine, eventually able to activate cells to optimal mTORC1 levels at the expense of other “loser” CD8 T cells. High mTORC1 can lead to enhanced C-MYC expression, both contributing to enhanced metabolic reprogramming including glucose metabolism, glycolysis and PI3K/Akt signaling through LDHA to sustain optimal T cell activation and functions (Figure 3.20). Such competition and heterogeneity did not exist in environments of high serine or complete serine deficiency. In a high serine environment, optimal signaling for both PHGDH-competent and PHGDH-deficient CD8 T cells was sufficient through exogenous uptake. When grown in complete serine deficiency, WT T cells could exhibit much less heterogeneity since no serine was available to compete; although it may take a longer period, the cells could ultimately upregulate and depend on *de novo* synthesis to reach the cumulative serine necessary for optimal mTORC1 activation and downstream biosynthesis for growth. This was supported by WT T cells cultured in the serine-deficient medium resulting in reduced proliferation, but no qualitative phenotype changes like GzmB expression. On the other hand, KO T cells were unable to compensate for the deficiency of serine, leading to inadequate

mTORC1 signaling for the subsequent metabolic switch and cell activation (Figure 3.21).

At an organism level, serine levels could fluctuate and become limiting in different tissues and organs due to multiple factors such as dietary habits, environmental and host conditions. The limited but not depleted serine likely attributes to the heterogeneity we observed, which could be more relevant to *in vivo* physiological conditions. While the “winner” cells could engage in a positive feedback of metabolic activation program in limited serine environment as discussed earlier, the “loser” cells may only rely on other means of serine acquisition, such as autophagy that may be insufficient for their competition in the absence of mTORC1 and C-MYC-mediated metabolic reprogramming. The “winner” cells may further polarize their metabolic differences with positive feedback, ultimately proliferate to become the antigen-specific T cells we observed *in vivo*—but at a lower frequency, whereas the loser cells may die off as they become more disadvantaged (Figure 3.22). This model could potentially explain why WT T cells in the SGD diet *in vivo* displayed quantitative but not qualitative phenotypes. Along these lines, it is worth noting that even though WT T cells cultured in 3.75mg/L of serine resulted in reduced frequency of pS6^{high} population, the MFI of these pS6^{high} population was still higher than KO T cells. This suggested that PHGDH-mediated *de novo* synthesis of serine was still critical for optimal pS6 activation when exogenous serine was limited, further supporting the *in vivo* qualitatively distinct phenotype of KO CD8 T cells in infection and cancer models when placed on SGD diet. Although serine plasma concentrations reported in the

past were at a higher range than 3.75mg/L, even under SGD diet manipulation (300); it remains possible that in local tissue environment, serine concentrations may be lower, thus limiting serine access to T cells and their subsequent signaling.

In conclusion, our data unveiled new roles of serine that not only extended beyond as building blocks for cell growth, but also as signaling molecules to regulate T cell activation and differentiation. Optimal serine was necessary to support mTORC1, C-MYC, and Akt/Foxo1 signaling pathways, which are all critical for downstream T cell metabolic reprogramming and effector differentiation. Limited serine in an exogenous environment could trigger heterogeneity and competition within the WT T cell population. The “winner” population allowed positive feedback of metabolic reprogramming that grew out at the expense of the “loser” population. Within the “winner population”, *de novo* synthesis-mediated serine was still required to coordinate with exogenous serine to support optimal T cell signaling particularly when serine was limited, which was sensed at the level of mTORC1 to reinforce the feedback loop. These observations escalate our understanding of the cross-regulation of metabolites and signaling.

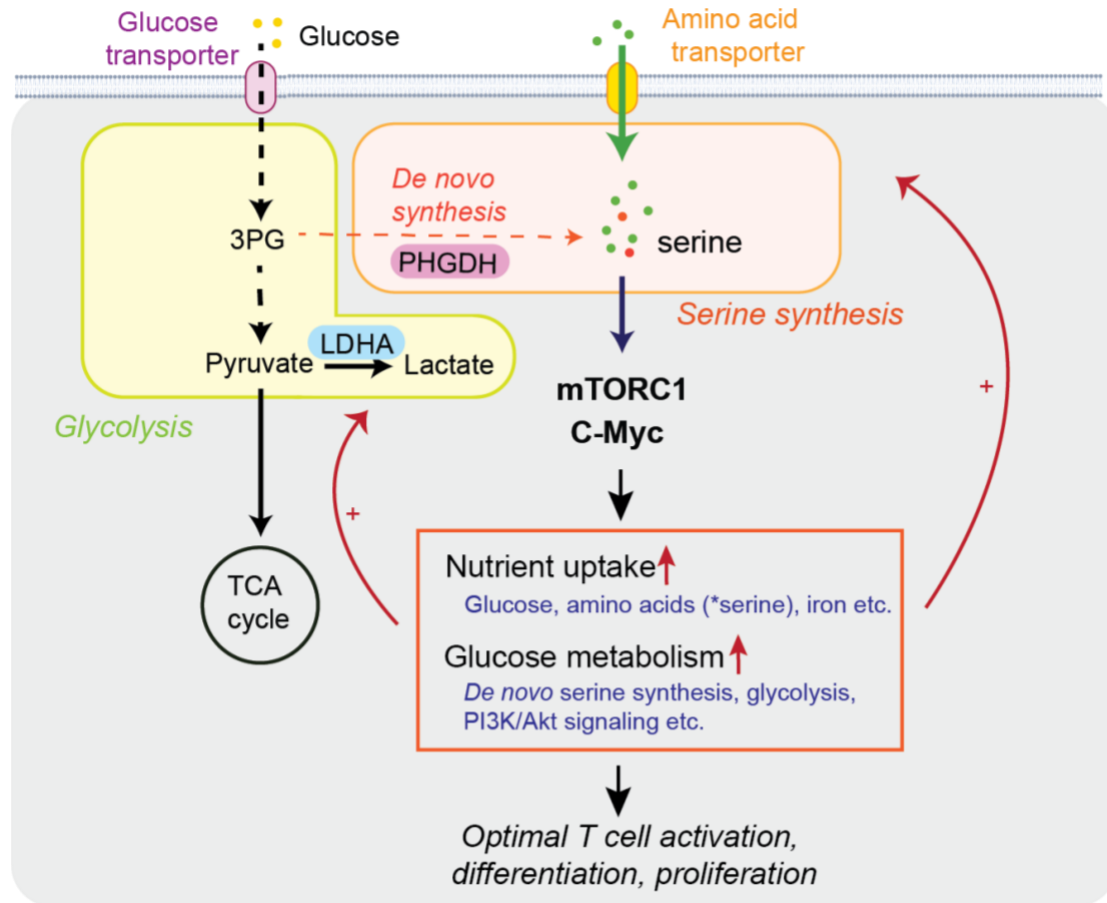


Figure 3. 20 Cumulative serine sensed by mTORC1 and C-MYC support positive feedback for the metabolic reprogramming necessary for optimal T cell function

Model illustrating key importance of cumulative serine to support optimal T cell functions through a positive feedback. Exogenous serine and *de novo* synthesized serine coordinate to support cell function sensed at the level of mTORC1. Both mTORC1 and C-MYC contribute to the metabolic switch, including the upregulated amino acid and glucose transporter expression, synthesis of serine and glucose metabolism primarily through LDHA-mediated glycolysis. Optimal activation of mTORC1 and C-MYC positively feedback to support such metabolic switch for optimal T cell signaling and downstream activation, differentiation and proliferation. In the absence of sufficient serine to mediate mTORC1 and C-MYC activation, cells were unable to undergo such metabolic switch, leading to defective signaling to mediate T cell functions.

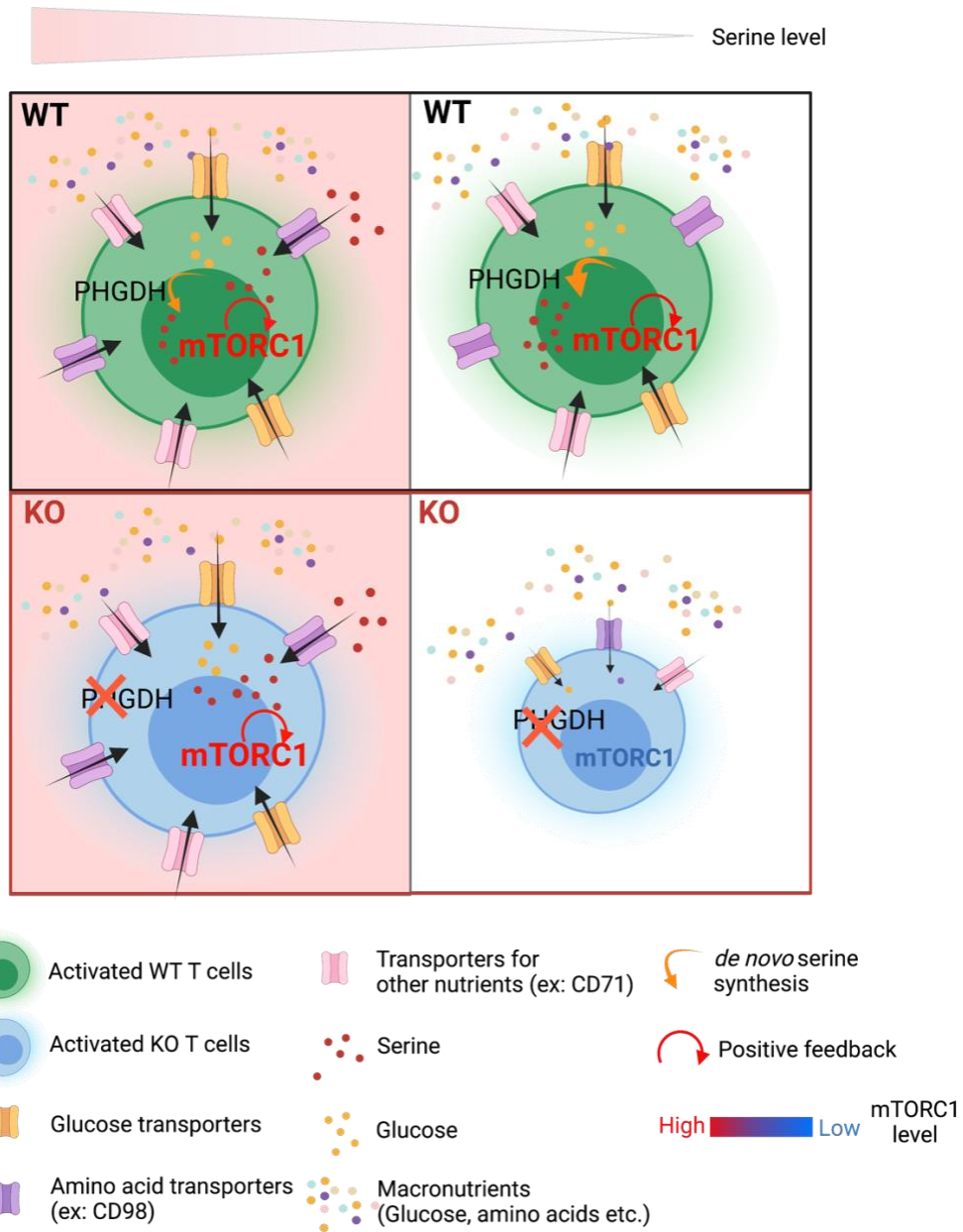


Figure 3. 21 Exogenous serine and *de novo* synthesis exhibit redundancy for support of optimal signaling and feedback for metabolic switch

In environments with high serine, exogenous serine uptake was sufficient for both WT and KO T cells. However, when exogenous environment became depleted of serine, *de novo* synthesis of serine in WT T cells may still support optimal signaling, fueling the positive feedback for cell activation in a “mTORC1^{high}” state; whereas in KO T cells, suboptimal signaling led a “mTORC1^{low}” state, resulting in the inability to support metabolic switch and feedback.

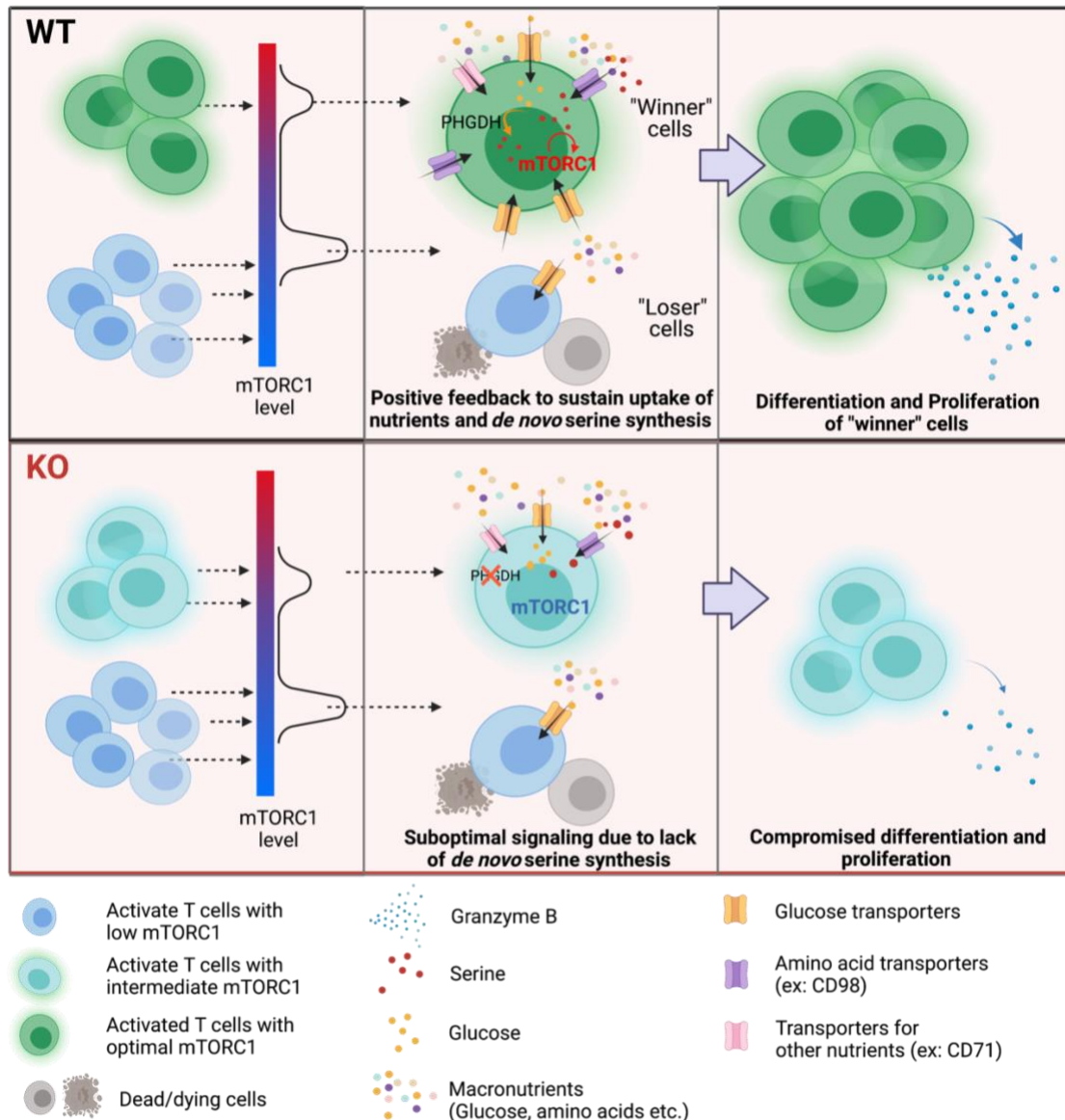


Figure 3. 22 In limited serine environment, T cells exhibit heterogeneity in mTORC1 signaling that is overcome by both exogenous serine uptake and *de novo* serine synthesis.

When T cells were activated in environment with limited exogenous serine, T cells exhibited reduced frequencies of population with high mTORC1 activity. Potentially due to competition for exogenous serine. Serine was required for optimal mTORC1 activation to engage in metabolic reprogramming that also supported *de novo* serine synthesis, ultimately facilitating a positive feedback for “winner” cells to compete over “loser” cells at the population level, with outgrowth of winner cells that exhibited competent differentiation and expression of effector molecules like GzmB. KO T cells exhibited less polarized heterogeneity, compromised differentiation as well as proliferation due to lack of *de novo* serine synthesis support.

3.9 Experimental Procedures

Mice

C75BL/6 Cd45.1 (stock # 002014), C75BL/6 Cd4Cre (stock # 022071), C75BL/6 OT-I (stock# 003831) were purchased from Jackson Laboratory. Mice carrying a floxed allele of *Phgdh* (B6N-*Phgdh*^{tm1a(KOMP)wtsi}) were obtained from the Korea Mouse Phenotyping Center (KPMC), which were crossed with CD4^{cre} mice to delete PHGDH in T cells. All in vivo mouse experimental procedures were performed under Sloan Kettering Institute (SKI) Institutional Animal Care and Utilization Committee (IACUC)-approved protocols. *Listeria monocytogenes*-OVA and LCMV (clone Armstrong) infection conformed to all relevant regulatory standards. Mice were housed in designated specific pathogen-free animal facilities regarding normal and infected conditions in ventilated cages with at most five animals per cage, provided with food and water. In all experiments comparing wildtype to LDHA-deficient mice, healthy, sex- matched, and age-matched mice were used (male and female, 8-14 weeks of age unless otherwise stated). No sex differences in the T cell phenotype were noted.

Antibodies

Antibodies against CD127-PE (Clone A7R34; Cat. #1 2-1271-82), KLRG1-PE-Cy7 (Clone 2F1; Cat. # 25-5893-82), CD71-FITC (Clone R17217; Cat. # 11-0711-82), CD98-PE (Clone RL388; Cat. # 12-0981-83), Foxp3-eFluor450 (Clone FJK-16s;

Cat. # 48-5773-80), CD69-PE (Clone H1.2F3; Cat. # 12-0691), PD1(CD279)-FITC (Clone RMP1-30; Cat. # 11-9981-82) were purchased from eBioscience. Antibodies against TNFa-PE (Clone MP6-XT22; Cat. # 506306) were purchased from Biolegend. Antibodies against CD44-PerCP-Cy5.5 (Clone IM7; Cat. # 65-0441-U100), CD62L- PE-Cy7 (Clone MEL-14; Cat. # 60-0621-U100), CD8a-PE-Cy7 (Clone 53-6.7; Cat. # 60-0081- U100), TCRb-FITC (Clone H57-597; Cat. # 35-5961-U100), IFN-g-APC (Clone XMG1.2, Cat. # 20- 7311-U100), IFN-g-FITC (Clone XMG1.2, Cat. # 35- 7311-U100) and IFN-g-PE-Cy7 (Clone XMG1.2, Cat. # 60-7311-U100), CD25-APC (Clone PC61.5; Cat. #20-0251-U100), Ghost Dye Red 780 (Cat. # 13-0865-T100) were purchased from Tonbo. Antibodies against CD4-BV510 (Clone RM4-4; Cat. # 740105), CD4-BV786 (Clone GK1.5; Cat. # 563331), CD45-BV605 (Clone 30-F11; Cat. # 563503), TCRb-BV711 (Clone H57-597; Cat. # 563135), CD71-BV605 (Clone C2; Cat. # 563013), CD98-BV510 (Clone H202-141; Cat. # 744980), CD8a-BV650 (Clone 53-6.7; Cat. # 563234), CD69-APC-Cy7 (Clone H1.2F3; Cat. # 561240) were purchased from BD Biosciences. Antibodies against Granzyme B-PE-TexasRed (Clone GB11; Cat. # GRB17) were purchased from Thermo Fisher Scientific. Fluorescent-dye-labeled tetramers H-2Db- NP396 Alexa 647, H-2Db-GP33 BV421 and H-2Kb-OVA APC were obtained from NIH.

Antibodies for in vitro T cell culture

Monoclonal anti-mouse CD28-biotin (Clone 37.51; Cat. # 13-0281-81) and monoclonal anti-mouse CD3e-biotin (Clone 145-2C11; Functional Grade; Cat. # 36-0031-85) were purchased from Invitrogen. Monoclonal anti-mouse CD28

(Clone 37.51; Cat. # BE0015-1), and monoclonal anti-mouse CD3e (Clone 145-2C11; Cat. # BE0001-1) were purchased from BioXCell. AffiniPure Goat Anti-Armenian Hamster IgG (H+L) (Cat. # 127-005-099) was purchased from Jackson ImmunoResearch Laboratories.

In vivo diet manipulation

Purified control amino acid diet (5CC7) and diet lacking Ser/Gly (5BJX) (WF Fisher and Sons) were formulated as previously described (299, 300). 6-12 weeks old mice were maintained on diets for at least 2 weeks before initiating experiments. Diets were maintained throughout the experiment. For Lm-OVA memory challenge experiments, mice were switched back to normal chow post D7 of primary infection, and switched back to deficient or control diet 2 weeks before memory rechallenge.

Serine/glycine concentration measurement

Serine and glycine concentration measurement were done in collaboration with Dr. Lydia Finley and Dr. Julia Brunner (SKI). Briefly, 100 μ L of blood was collected from tail of mice of different diet treatment or genotypes, coagulate on ice for 30 minutes for 20 μ L serum collection. 1 mL ice-cold 80% methanol containing 2 μ M deuterated 2-hydroxyglutarate(d5-2HG) was incubated with serum overnight at -80 degree Celsius to extract metabolites. The next day, lysates were collected and centrifuged at 21,000g for 20 minutes for protein removal. The protein-free supernatants were dried in an evaporator (Genevac EZ-2 Elite), and incubated with 40 mg ml⁻¹ methoxyamine hydrochloride (Sigma) in pyridine (ThermoFisher)

for 2 h at 30 degree Celsius. Metabolites were then derivatized with 80 μ l of MSTFA (ThermoFisher) with 1% TCMS (ThermoFisher) and 70 μ l ethyl acetate (Sigma). Analysis of samples were done with Agilent 7890A Gas Chromatograph coupled to Agilent 5977C mass selective detector in splitless mode using constant helium gas flow at 1 ml/min. The following ions were quantified: d5-2HG 254m/z; serine 306m/z; glycine 276m/z. Peaks were assessed with MassHunter software v.B.08 (Agilent) and normalized to internal standards (d5-H2G) as well as protein content of triplicate samples.

Listeria Monocytogenes-OVA (LM-OVA) infection

Frozen aliquot/vial of LM-OVA (stored at -80°C) was thawed and 200 μ l of bacteria was added into 5 ml of sterile brain heart infusion (BHI) media. Bacterial culture was incubated 3.5 to 4 hours at 37 °C shaking at 225 rpm. Bacterial OD600 was monitored and ready when reached between 0.1-0.2 (OD600 = 0.1 is equal to 1×10^8 bacteria/ml). CD4Cre $Phgdh^{fl/fl}$ and $Phgdh^{fl/fl}$ mice were intravenously injected with 5×10^3 colony-forming units (CFU) of LM-OVA. Acute antigen-specific CD8+ T cell responses were assessed on day 7.

Antibodies for phospho-flow cytometry, immunoblotting and immunofluorescence

Monoclonal rabbit anti-pT308 Akt (Clone D25E6; Cat. # 13038S), polyclonal rabbit anti-pS256 Foxo1 (Cat. # 9461S), polyclonal rabbit anti-pT24 Foxo1/pT32 Foxo3a (Cat. # 9464S), polyclonal rabbit anti-pS235/S236 S6 (Cat. # 2211S), monoclonal rabbit anti-PAN Akt (Clone C67E7; Cat. # 4691L), monoclonal mouse anti-PAN

Akt (Clone 40D4; Cat. #2920), monoclonal rabbit anti-Foxo1 (Clone C29H4; Cat. # 2880S), monoclonal mouse anti-Foxo1 (Clone D8T1S; Cat. # 97635S), polyclonal rabbit anti-LDHA (Cat. # 2012S), monoclonal mouse anti-b-actin (Clone 8H10D10; Cat. # 3700S), polyclonal rabbit anti-LAT (Cat. # 9166S), polyclonal rabbit anti-pT191 LAT (Cat. # 3584S), monoclonal rabbit anti-c-myc (Clone D84C12; Cat. # 5605S), monoclonal rabbit anti-EGR1 (Clone 44D5; Cat. # 4154S), monoclonal mouse anti-pT389-p70 S6 Kinase (Clone 1A5; Cat. # 9206S), polyclonal rabbit anti-LDHA antibody (Cat. #2012S), polyclonal rabbit anti-p70 S6 Kinase (Cat. # 9202), monoclonal mouse anti-S6 Ribosomal Protein (Clone 54D2; Cat. # 2317S), polyclonal anti-pS240/244 S6 Ribosomal Protein (Cat/ # 2215S), monoclonal anti-mTOR (Clone: 7C10; Cat. # 2983), horseradish peroxidase (HRP) anti-rabbit IgG (Cat. # 7074), horseradish peroxidase (HRP) anti-mouse IgG (Cat. # 7076) were purchased from Cell Signaling Technology. Polyclonal rabbit anti-Phgdh (Cat. # 14719-1-AP), goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488 (Cat. # A11034) were purchased from Thermo Fisher Scientific. Donkey anti-mouse IgG polyclonal antibody (IRDye® 680RD; Cat. # 926-68072), donkey anti-rabbit IgG polyclonal antibody (IRDye® 800CW; Cat. # 926-32213) were purchased from LI-COR Biosciences. Rat monoclonal anti-LAMP2 (Clone GL2A7; Cat. # ab13524) was purchased from AbCAM.

Immunoblotting reagents

Precision Plus Protein™ Dual Color Standards (Cat. # 1610374) were purchased from Bio-Rad. Amersham™ Protran® Premium Western blotting membranes, nitrocellulose membrane (Cat. # 10600003) were purchased from Thomas Scientific. Tris-HCl 1M, pH 8.8 buffer (Cat. # T1088) were purchased from Teknova. Tris 1M pH 6.8 buffer was purchased from Thermo Scientific. 10x Tris/Glycine/SDS (Cat. # 1610732), 10x Tris/Glycine buffer (Cat. # 1610734), 30% Acrylamide/Bis Solution, 37.5:1 (Cat. #1610158) were purchased from Bio-Rad. TEMED LICOR Intercept (PBS) blocking buffer (Cat. # 927-70001) were purchased from LI-COR Biosciences. Immobilon western chemilum HRP substrate (Cat. # WBKLS0100), TEMED (Cat. # T9281), ammonium persulfate (Cat. # 248614) were purchased from Millipore Sigma.

Immunoblotting procedures

Prior to immunoblotting, cell lysates were heated for 5 minutes at 95 degree Celsius and centrifuged for 30 seconds at 14000g. Lysates were then resolved via SDS-PAGE gel (8-10%) in each lane without sample dilution. Gel was then immediately transferred to nitrocellulose membrane using the Trans-Blot Turbo Transfer System from Bio-Rad. Membrane was then Blocked in either 5% milk or Intercept blocking buffer for 1 hour. Primary antibodies were blotted overnight at 4 degree Celsius. Membrane was then washed three times with PBST for 5 minutes. Secondary antibodies were blotted for 1 hour at room temperature. Membrane was then washed three times with PBST for 5 minutes. For readout using chemiluminescence, results were developed using film in the dark room. For

readout using fluorescence, membrane was washed in PBS for five minutes then detected by Odyssey Clx imaging system.

Flow Cytometry

Tissues from spleens and livers were grinded and filtered through 70 µm mesh to make single cell suspension. Cells were then depleted of erythrocytes by hypotonic lysis. Cells were incubated with specific antibody cocktails for 10 min at 4°C in the presence of anti-FcγR to block FcγR binding. Dead cells were excluded by Ghost Dye™ Red 780 or DAPI staining. For tetramer staining, PE-/APC-/BV421 conjugated H-2Kb-OVA, H-2Db-GP33 and H-2Db-NP396 tetramers (NIH) was used and incubated for 30 min at 4°C. For phospho-signal flow cytometry, spleens were isolated and immediately meshed into 4% PFA solution for 15 min at room-temperature followed by 1 hour permeabilization with fix/perm buffer at 4°C. Cells were then washed and incubated with primary phospho- for 1 hour, and secondary-antibodies for 30minutes in Perm buffer before flow cytometry. All samples were acquired with an LSR II flow cytometer (Becton, Dickinson) and analyzed with FlowJo software.

In vitro T cell activation

In vitro T cell experiments were performed using primary mouse T cells isolated from lymph nodes and spleens of male and female mice cultured at 37°C with 5% CO₂ in RPMI-1640 media deficient of serine, glycine, glucose (Teknova; Cat. # R9660) supplemented with 1000X b-mercaptoethanol (Invitrogen), 100X penicillin-

streptomycin (Gimini Bio), 100X L-glutamine (Media Preparation Core, SKI), HEPES buffer (Media Preparation Core, SKI), D-(+) glucose (Sigma-Aldrich), glycine (Sigma-Aldrich), 10% heat inactivated dialyzed FBS (Fisher Scientific), and serine (Sigma-Aldrich) of desired concentration. When indicated as T cell medium (TCM), serine concentration is 30mg/L. Naïve (CD62L⁺ CD44⁻) CD8⁺ T cells were isolated from spleens and lymph nodes of *Phgdh^{fl/fl}* or *CD4CrePhgdh^{fl/fl}* mice using the EasySep™ Mouse Naïve CD8⁺ T Cell Isolation Kit (Cat. #19858) from STEMCELL Technologies. Cells were isolated in FACS buffer with 2% heat inactivated Fetal Bovine Serum purchased from Thermo Fisher Scientific (Cat. # 16140063). Purity was validated by flow cytometry and was over 95%. For *in vitro* T cell activation shorter than 24 hr, 1×10⁶ naïve CD8⁺ T cells were cultured per well in 24-well plates pre-coated with goat anti-american hamster IgG (H+L) overnight at 4 degree Celsius (1:50 in PBS), and 2 µg/ml soluble anti-CD28 and 100 U/ml rhIL-2 (NIH) in medium unless specific conditions were mentioned. For *in vitro* T cell activation longer than 24 hr, either 0.5×10⁶ naïve CD8⁺ T cells were cultured per well in 24-well plates, or 0.2 ×10⁶ naïve CD8⁺ T cells were cultured per well in 96-well plates. For cells cultured with inhibitors, MG132 (Sigma, M8699-10mg) was used at 10µM concentration, and GSK3β Inhibitor (IM-12, EMD Millipore, 361565-10MG) was used at 3 µM concentration. Inhibitors were added in the beginning of T cell activation for four hours. Cells were collected either for flow cytometry, phospho-flow, immunoblotting or re-stimulation. For flow cytometry, cells were collected and washed in FACS buffer prior to staining. For phospho-flow, cells were fixed in 4% Formaldehyde for 15min and fixed in Fix/Perm buffer

for 1 hour before staining. For immunoblotting, cells were collected and lysed in RIPA lysis buffer on ice for 15 min, centrifuged at 15000g for 10min, supernatants were then collected and added with 6x SDS reducing buffer.

In vitro T cell culture rescue

For rescue experiments, formate (formate acid 0.5 μ M), s-adenosyl methionine (SAM, 50 μ M), dimethyl-alpha-ketoglutarate (3.5 mM), N-acetyl-cysteine (10 mM), reduced glutathione (GSH, 1 mg/L), sodium acetate (20 mM), C16 ceramide (50-100 μ M) were purchased from Sigma and added into the medium at the start of the culture.

Stimulation for cytokine assessment

For stimulation of primary T cells from *in vitro* culture, cells were collected and stimulated with phorbol myristate acetate (PMA), ionomycin and GolgiStop for 4 hours. For *ex vivo* stimulation of T cells from Lm-OVA infected animals, cells were stimulated with 10 μ g/L of OVA peptide (SINFEKL). Cells were then collected for flow cytometry.

Acute anti-CD3/CD28 cross-linking

In acute cell stimulation experiments, 1 million cells were used in each group. Cells were labeled with biotinylated anti-CD3 and anti-CD28 for 30 min at 4°C. Then, cells were pre-warmed for 2 min in a 37°C water bath and acute streptavidin-biotin

cross-linking was performed for an additional 5 min. Cell lysates were prepared and subjected to immunoblotting.

HCC tumor induction and imaging

Mice were placed on CTD or SGD diet at least two weeks prior to start of tumor induction and were maintained on diet throughout the experiment. Liver tumors were induced in 6-12 weeks old mice by performing hydrodynamic tail-vein injections of a transposon vector expressing MYC together with a highly immunogenic version of luciferase (pT3-EF1a-MYC-lucOS) that was linked to three model antigens: SIYRYYGL (SIY), SIINFEKL (SIN; OVA257-264), and OVA323-339; a vector expressing SB13 transposase (CMV-SB13), which was required to integrate the transposon-based vector into the hepatocyte genomic DNA, and a CRISPR/Cas9 vector expressing a single-guide RNA (sgRNA) targeting Trp53 (px330-sg-p53; ref. 31). Mice were kept on modified diet and monitored weekly by the IVIS imaging system.

Immunofluorescence

Naïve CD8 T cells were activated for 20 hours *in vitro*, starved for 1.5 hours in RPMI without amino acids, and stimulated with prior culture conditions (same as the 20hour culture medium) for 5min. Cells were centrifuged and plated on coverslips, then rinsed with PBS and fixed with 4% paraformaldehyde for 15 min. The slides were rinsed with PBS, and cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After rinsing with PBS, the slides were incubated with

primary antibodies in 5% goat donkey serum/ 2% mouse serum for 3 h, washed three times with PBS, and incubated with secondary antibodies for 1 h. Slides were washed three times with PBS and mounted on glass coverslips using ProLong Gold (Invitrogen). Images were acquired on a Leica-Upright Point scanning confocal microscope with a 63× oil objective and zoom of 3. Colocalization analysis was done with Imaris software, and at least 25 different fields were randomly selected per sample.

Statistical Analysis

Statistical tests were performed with paired, unpaired t tests or one-way Anova with Tukey's multiple comparison tests. A value of $p < 0.05$ was considered statistically significant. All error bars represent mean \pm standard error of the mean (SEM). All Statistical analysis were performed in Prism 9.

Chapter 4: Discussion and Perspectives

4.1 Introduction

Metabolic rewiring of T cells post activation is an essential feature to support increased cellular demands for effective immune responses. Failure to do so weakens the ability to counter invading pathogens and cancerous cells. How metabolism regulates T cells is thus increasingly recognized for its significance to improve optimal immunological responses. The work in this dissertation demonstrated how glucose-mediated glycolysis and serine biosynthesis, two key pathways for support of T cell functions, critically regulate T cell responses in infections and cancer. Furthermore, our data demonstrated the cross-regulation between metabolite and signaling, suggesting the multifaceted roles of metabolites beyond usage for biosynthesis. This chapter will focus on the discussions and implications from work presented in the dissertation, which deepens our understanding for metabolic pathway regulation and future therapeutic design.

4.2 Glucose metabolism as a key regulator for T cell responses

Glucose is the predominant fuel source for energy and biosynthesis in most living organisms. At the cellular level, glucose is the final substrate taken up through glucose transporters to fuel catabolism or anabolism. In T cells, glucose is

transported through high-affinity receptor Glucose transporter 1 (GLUT1) (331), phosphorylated by hexokinase (HK), which then traps glucose in cells for glycolysis to generate pyruvate. In most nonproliferating and terminally-differentiated cells, pyruvate is fully oxidized through the TCA cycle, whereas fast proliferating T cells oxidize pyruvate into lactate instead. This is similar to the “Warburg effect” observed in tumor cells (332), in which glucose is primarily utilized to generate cytosolic ATP despite the presence of sufficient oxygen and higher ATP production efficiency through OXPHOS.

Glucose plays a crucial role in shaping T cell responses. Naïve T cells remain quiescent until encountering its cognate antigen presented by APCs, which drive T cell activation in the presence of supporting cytokines. As critical mediators of the adaptive immune response, T cells are dependent on optimal activation to support defense against invading pathogens and transformed self. This activation is tightly linked with metabolic reprogramming, which is essential to fuel the demand of energy to support T cell differentiation, effector functions and proliferation (333, 334). Unlike naïve cells and memory T cells that depend mostly on OXPHOS, activated T cells upregulate nutrient transporters for glucose and amino acids (199, 211), as well as enzymes for glycolysis and mitochondria ATP synthesis through mTORC1, C-MYC, and HIF signaling axes (161, 335, 336). Activated T cells heavily utilize both glucose and glutamine (161, 337), and particularly enhance their glucose catabolism in the form of lactate through aerobic glycolysis. Glycolysis supports rapid cytosolic ATP energy generation, while glycolytic metabolites can be shunted for glucose anabolism to support other

metabolite generation, such as nucleotide synthesis and amino acids for cellular usage.

Insufficient glucose supply or blocking of glycolysis prevents appropriate T cell responses. Glucose deprivation reduces CD8 T cell effector cytokine production (338). Blocking glycolysis through T cell culture in galactose or genetic ablation of LDHA leads to reduced T cell effector function and inhibition of *de novo* serine synthesis (210, 220, data unpublished), illustrating key dependence of glucose and this rewired metabolism in supporting T cell for host defense. Of all challenging disease contexts, tumor microenvironment is particularly suppressive given multiple inhibitory mechanisms evolved to prevent T cell infiltration and effector functions. As both tumor cells and T cells exhibit this glycolytic switch, tumor cells may compete for glucose, as well as produce excessive amounts of lactate with a low pH microenvironment that inhibits glycolysis in T cells. It has thus been a long-standing question of how glycolysis affects T cell functions in tumors that may impose active metabolic stress and competition.

In our findings, we discovered that LDHA-mediated glycolysis was critical for the presence of T cells and their effector functions in a mouse spontaneous breast PyMT tumor model. These data indirectly implicated that despite similar metabolic dependencies on glucose-fueled glycolysis, T cells lack the flexibility to switch to other metabolisms for their functional responses, while PyMT tumors can depend on metabolisms other than LDHA-mediated glycolysis for their growth (data unpublished). This data also suggested that the LDHA inhibitors currently in development were more likely to dampen T cell anti-immunity, as opposed to their

desired effects on cancer cells. One other recent study demonstrated that NK cells, a cytotoxic lymphocyte population part of the innate immune system, were also dependent on LDHA for their anti-tumor functions (339). Thus, the use of these inhibitors likely would hinder the desired cytotoxic lymphocyte-mediated effector functions against tumors, and may only be more therapeutically beneficial in tumors that were highly glycolytic with low immune infiltration.

On the other hand, increased HIF stabilization leads to enhanced glycolytic metabolism in CD8 T cells (193). VHL-deficient CD8 T cells are more capable of controlling persistent viral infections than WT cells, but at the expense of increased immunopathology. This led us to hypothesize that enhanced glucose metabolism through HIF stabilization mediated by VHL deletion could present a potential strategy to enhance functional capacities by breaking the T cell tolerance against tumors. Indeed, VHL-deficient T cells led to striking protection against tumor growth in PyMT mice, and were characterized by increased cytolytic granule expression and reduced exhaustion marker PD1. The tumors with infiltrating VHL-deficient T cells remained significantly smaller than tumors from WT mice, suggesting an active elimination of malignant cells by VHL-deficient T cells. Reduced PD1 expression may spare VHL-deficient T cells from PD1 ligation-mediated inhibition, with enhanced GzmB for successful tumor cell killing before its escape and outgrowth. Notably, the effect was dependent on both CD8 and CD4 T cells, suggesting this enhanced function required coordination between CD8 and CD4 T cells for manifestation. Mice with CD8-specific VHL deficiency lost the GzmB phenotype and anti-tumor effect, suggesting that intrinsic upregulation

of CD8 T cell glycolytic metabolism was not sufficient. Alternatively, enhanced co-stimulatory molecule expression on both CD8 and CD4 T cells may be necessary to support anti-tumor immunity. Enhanced co-stimulation had been shown to elicit CD4 anti-tumor functions through IFN- γ expression (340). While we did not observe increased cytokine expression from CD4 T cells, increased CD4 T cell help from co-stimulation may enhance the gain of CD8 T cell function that we observed. CD4 helper T cells have also been shown to promote and regulate effector memory differentiation (341). Future analysis to understand the differentiation state of VHL-deficient CD8 and CD4 T cells may help dissect further molecular mechanisms.

Our reports thus far have substantiated the dependency of conventional T cells on glucose for their functions in cancer. Interestingly, different subsets of T cells engage in distinct metabolic dependencies. In our studies, we found that Treg cells were less dependent on LDHA-mediated glycolysis for their cell function and proliferation, which was in concordance with previous studies on how enforced glycolytic metabolism dampened Treg cell stability (191). In another recent report, glucose uptake correlated with reduced suppressive function and murine Treg lineage instability in the tumor (342). These data, together with our observations, further supported how Treg cells may withstand and accumulate at a higher frequency in tumor microenvironments that typically exhibit higher levels of lactate, an inhibitory metabolite that could be produced by tumor glycolysis, whereas conventional T cells may be much more sensitive to. Lactate may be further utilized by Treg cells through LDHB-mediated mechanisms that convert lactate to pyruvate

for TCA cycle oxidation (229). Interestingly, although LDHA is the primary isoform expressed in T cells (220), studies have shown that LDHB may replace functions of LDHA for conversion from pyruvate to lactate (343). While conventional T cells are dependent on LDHA for proper immune responses (220, 222, 223), Treg cells may have the flexibility to adapt to either LDHA or LDHB functions for the generation of electron acceptor NAD⁺ in scenarios that require fast proliferation and NAD⁺ for cytosolic redox balance, or directly utilize lactate for generation of intermediates necessary for proliferation (342). Deletion of MCT1 that facilitates lactate uptake is required for Treg cells in tumors, but not in the periphery, which also suggests that Treg cell metabolism may operate with flexibility depending on the tissue microenvironment (342). Importantly, our data in the *in vivo* suppression model suggested that in conditions where Treg cells were forced to proliferate at a higher extent, LDHA may still be important to support Treg cell functions. However, in other scenarios like tumors or chronic infections where Treg cells may not be required to proliferate as much, LDHA-mediated glycolysis may be dispensable. These flexibilities and adaptations thus position Treg cells in a different metabolic dependency than conventional T cells.

4.3 Serine as a conditionally essential amino acid for T cell functions

Serine is a key metabolite for proliferating cells due to its multifaceted roles in supporting protein, nucleotide, and lipid synthesis. It is often considered a

conditionally essential amino acid because it may not be *de novo* synthesized in sufficient quantity to meet the cellular demands (344). Activated CD8 T cells expand with a doubling time as short as 8hr during their clonal expansion (345), requiring enhanced nutrient supplement to support biomass generation and daughter cell production. Indeed, serine has been shown to be a critical metabolite for optimal CD8 T cell expansion through one-carbon metabolism supporting *de novo* nucleotide synthesis (241), regulating CD8 T cell proliferating capacity. However, less is known about the roles of serine in supporting early T cell activation and signaling events.

T cell activation is tightly linked with metabolic reprogramming to a glycolytic-dependent cell state primarily through signaling pathways including PI3K/Akt and mTORC1 activation; failure to do so through blocking the activation of signaling pathways lead to a reduction in T cell effector functions and proliferation (346). Our data suggest that serine itself, but not its downstream metabolites, may be sensed early post activation that attributes to optimal Akt/Foxo1 and mTORC1 signaling for effector response. Cumulative serine was required for optimal mTORC1 activation; T cells cultured with low amounts of serine (3.75mg/L) supply possess significantly lower frequencies of T cells that were digitally switched to become “mTORC1 high” as shown by higher pS6 levels. Intriguingly, PHGDH-deficient CD8 T cells in low serine medium had even lower MFI within the “mTORC1 high” population, suggesting signaling requires coordination of both sources of serine particularly when serine levels became depleted in starvation or disease. While the specific molecular interacting targets

for serine still required further investigation, our data suggested that the mTORC1 complex was directly involved in the sensing process. mTORC1 is the master regulator of cell anabolism, integrating the availability of amino acids and growth factor sensing to support cellular metabolic reprogramming. To date, multiple sensors have been reported that integrate the availability of specific amino acids into mTORC1 signaling activation, including arginine, leucine, and glutamine (Figure 1.6). Both arginine and leucine can bind to upstream protein complexes CASTOR1 and Sestrin2, respectively, to dampen their inhibitory effects on positive regulator GATOR2, thus positively support mTORC1 activity. It would be interesting to see if serine was sensed through similar mechanisms by disrupting GATOR2 complex to further locate specific molecular mechanisms of how serine was sensed by mTORC1.

SSP and exogenous sources of serine exhibit redundancy for support of T cells. However, *de novo* serine synthesis pathway becomes essential in serine-limiting environments to coordinate with exogenous sources for support of optimal signaling, which was supported by our data. Optimal balance of Akt/Foxo1 signaling is critical for effector T cell differentiation and migration to sites of immunological challenges. Direct downstream targets of Foxo1 include *Il7ra*, which encodes IL-7R α (CD127), a subunit of the key survival receptor for naive T cells; and *Klf2*, which further controls the expression of *Sell* that encodes L-selectin and sphingosine-1-phosphate receptor 1 (S1pr1). Both of these receptors are important for the entry and exit of lymphocytes from lymphatic tissues (178, 347, 348). Downregulation of these receptors is critical for effector T cells migration to

peripheral tissues. Enhanced Akt signaling post T cell activation leads to phosphorylation of Foxo1 by Akt that reduces the activity and downstream Foxo1 target expression, while Akt signaling targets such as T-bet and Granzyme B are critical for CD8 differentiation and functions. Interestingly, sustained and optimal PI3K/AKT signaling is dependent on LDHA-mediated bioenergetics through glycolysis, a potential explanation for the “Warburg effect” observed in T cells. LDHA is downstream of C-MYC and HIF1a, two targets that can be positively regulated by mTORC1. In our data, we observed reduced upregulation of LDHA post activation, potentially attributed from dampened mTORC1 activity and downstream C-MYC regulation. It is plausible that reduced Akt/Foxo1 signaling was an indirect effect from reduced mTORC1 signaling and dampened downstream C-MYC/LDHA axis. Reduced mTORC1 and C-MYC activity may lead to a defective metabolic switch to glycolysis-dependent activated T cells, leading to the inability to sustain bioenergetics and the feedback regulation of optimal Akt signaling. It has been reported that both mTORC1 and C-MYC can positively regulate *de novo* synthesis of serine (157, 203) through SSP enzymes as well as glycolytic enzyme LDHA (349, 350). Thus, reduced supply of serine and the resulting defective signaling amplified a negative feedback loop as cells become unable to mediate their activation and functions, while sufficient serine from dietary and *de novo* sources supported the positive signaling feedback for T cells even in scenarios with limited serine supply. In *in vivo* scenarios where serine becomes limiting, competition likely results in heterogeneity further polarized by these positive and negative feedbacks, ultimately selecting the “winner” populations that

were more fit and could successfully engage in the metabolic reprogramming for cell activation and functions.

In summary, our findings revealed a new aspect of serine serving not only as metabolites to support cell growth, but also through signaling activation to support optimal cellular responses in T cells for host protection against diseases like infection and cancer. In environments where serine levels were not limited, *de novo* synthesis was dispensable for CD8 T cell activation, differentiation, and growth. Nevertheless, when exogenous serine was limited, such as in contexts where the host exhibited limited dietary intake of serine in malnutritional states, or in physiological compartments such as the cerebrospinal fluid where serine was more limited (311), serine becomes a key nutrient that is critically dependent on *de novo* synthesis for not only cellular anabolism, but also early activation and signaling events to mount an effective T cell immune response. These data expanded the role of serine as a conditionally essential amino acid, and further substantiate our understanding of how metabolites and signaling cross-regulate each other.

4.4 Future research and indications

The local nutrient level is subjected to fluctuation contributed by multiple factors, including daily dietary intake, feeding time and pattern, subsequent digestion and absorption by the host, as well as different tissue compartments for

an individual cell's usage. Malnutrition, including both underweight and overweight, has increasingly been recognized as a global health burden (351, 352). In particular, undernourished children suffer from increased mortality dying from common infectious diseases; this was often accompanied by impaired functions in both innate and adaptive immunity, implying a potential link for immune-deficiency (353). Nutrient metabolites can act as direct immune stimuli (354), while nutrient availability largely determines immune cell functions to meet the energy requirement of activated immune cells for anabolism and catabolism in response to immunological challenges. For instance, low protein diet leads to defects in the homeostatic proliferation and protective functions of memory CD8 T cells against viral reinfections (355). Furthermore, deficiencies in specific nutrients and metabolites may further influence T cell metabolism and downstream functions: glucose and cellular energy level can be sensed by AMPK (356), while individual amino acids leucine, arginine, and glutamine can be sensed and integrated into mTORC1 signaling to further regulate cellular metabolism (357). Thus, nutrient metabolite levels serve as a critical determinant for optimal cellular functions.

Our studies demonstrated that glucose and serine act as two key nutrients that support T cell responses through cell proliferation, differentiation, and effector function. Glucose is the key nutrient for generation of energy and biosynthetic molecule build-up, while glucose-derived serine synthesized *de novo* and extracellular serine jointly support protein, lipids, and nucleotide synthesis through the one-carbon metabolism pathway. Dietary restrictions that leads to reduced levels of glucose and serine may negatively impact the ability of T cells to mount

appropriate responses against infections and cancers. Currently, inhibitors targeting LDHA and PHGDH for cancer therapies aim to hinder their proliferation, altering cytosolic redox balance and cell anabolism. However, this may very well dampen T cell immunity as an unwanted consequence, which could be considered instead for the treatment of autoimmune diseases in future studies. Enforced glucose metabolism, on the other hand, serves as a promising strategy for cell therapy optimization. Currently, engineered CAR T cells co-delivered with modified HIF unit insensitive to VHL degradation showed promise with increased anti-tumor efficacy (265). Future studies incorporating T cell metabolism as modulators to improve immune potency may serve as the basis for next-generation therapeutics.

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