



Investigating Amino Acid Regulation in T cells

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presented by Sheila Johnson

candidate for the degree of Doctor of Philosophy and hereby
certify that it is worthy of acceptance.

Signature SK Dougan
Typed name: Prof. Stephanie Dougan

Signature Alison Ringel
Typed name: Prof. Alison Ringel

Signature Ana C. Anderson
Typed name: Prof. Ana Anderson

Signature Marcia Haigis
Typed name: Prof. Marcia Haigis

Date: 7/31/2025

Investigating Amino Acid Regulation in T cells

A Dissertation presented

by

Sheila Johnson

to

The Graduate School of Arts and Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Chemical Biology

Harvard University

Cambridge, Massachusetts

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Investigating Amino Acid Regulation in T cells

Abstract

Amino acids are fundamental to cellular homeostasis and have emerged as critical regulators of T cell activation, differentiation, and effector function. Despite their importance, the specific requirements and temporal dynamics of amino acid utilization in distinct T cell subsets remain incompletely understood. This dissertation investigates how modulation of amino acid availability and transporter function influences CD4⁺ and CD8⁺ T cell responses in contexts of autoimmunity and tumor immunity. In CD4⁺ T cells, we identify asparagine as a conditionally essential amino acid, necessary for optimal activation, proliferation, and differentiation. Asparagine depletion impaired these processes *in vitro* and attenuated disease severity in a mouse model of experimental autoimmune encephalomyelitis, highlighting its potential as a therapeutic target. In CD8⁺ T cells, we uncover a regulatory role for the amino acid exporter Slc43a2. Genetic ablation of Slc43a2 enhances T cell activation, effector function, and proliferation *in vitro* and promotes tumor control *in vivo*. These findings suggest that Slc43a2 limits nutrient acquisition and metabolic fitness during T cell activation. Together, this work demonstrates how manipulation of amino acid availability and transport can differentially modulate T cell subsets and presents novel strategies for immunotherapeutic intervention.

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Science has been a lifelong inspiration and driver of curiosity. Research has provided an avenue to pursue this curiosity, offering deep fulfillment while also presenting challenges that test both patience and perseverance. The gratification of discovery and problem-solving is accompanied by setbacks and failures. True success in science requires resilience, the capacity to persist, adapt, and grow in the face of obstacles. I could not have developed or sustained this resilience without the steadfast support of my advisors, lab mates, and my family. I am deeply grateful for this journey and for the work that has emerged from it. It is my hope that this modest contribution to the scientific body of knowledge will, in some way, advance the broader mission of science: to deepen understanding and to enhance the lives of people everywhere.

CHAPTER 1: INTRODUCTION

OVERVIEW

Amino acids are fundamental monomeric constituents of the cell and serve as the primary building blocks of life. Among the most versatile biomolecules, amino acids fulfill a wide range of cellular functions. Several studies have investigated amino acid utilization in cell types that exhibit a high nutrient demand, such as T cells. However, some questions remain regarding the relevance of specific amino acids and processes involved in amino acid homeostasis in select T cell subsets. This thesis will focus on how amino acids and their regulatory mechanisms contribute to T cell physiology and pathology. In this introduction, I will provide a brief overview of T cell function and discuss how different amino acid fates influence T cell activation, differentiation, and effector activity. Furthermore, I will discuss cellular mechanisms of amino acid homeostasis. While this introduction does not encompass all amino acid-mediated mechanisms of T cell regulation, I will discuss a select subset of amino acids that are highlighted in this thesis.

T CELL ACTIVATION AND DIFFERENTIATION

T cells are a central component of the adaptive immune system, responsible for recognizing and responding to specific pathogens. T cells undergo several intrinsic changes to execute this effector process (Chi et al., 2024; Raynor et al., 2024). Following thymic development, T cells emerge as naive cells that display a metabolically quiescent phenotype and primarily circulate throughout the lymphatic system (Chapman et al., 2019). When an immunological challenge such as infection occurs, naive T cells that express a T cell receptor (TCR) specific for a peptide epitope of the pathogen recognize this peptide when it is presented on major histocompatibility complex class (MHC) molecules on antigen presenting cells (APCs), thereby receiving the first signal for their activation program (Hwang et al., 2020). Simultaneously, co-stimulatory

molecules such as CD80 or CD86, expressed on APC surface bind to the CD28 receptor on the T cell to initiate the second signal for T cell activation (June et al., 1987; Greenwald et al., 2005; Chen et al., 2013). Together, these two signals activate multiple signaling cascades resulting in the induction of key transcription factors including NFAT and AP-1, among others (Hwang et al., 2020). The third signal for activation is mediated by cytokines secreted from the APC and the T cell in a paracrine and/or autocrine manner. These cytokines bind to their corresponding receptors on the T cell surface and further enhance the activation program (Liang et al., 2021; Jacobs et al., 2010; Rollings et al., 2018; Sinclair et al., 2013). All together these factors orchestrate the transcriptional and metabolic reprogramming required for cells to efficiently exit quiescence and undergo cell growth, proliferation and differentiation into various subsets (Shah et al., 2021; Chi et al., 2024; Chen et al., 2013).

After TCR stimulation and upregulation of various external and internal signaling pathways, naive T cells differentiate into distinct T cell states/subtypes (Sun et al., 2023). Naive CD8⁺ T cells can differentiate into effector and memory cells. Effector CD8⁺ T cells, also known as cytotoxic T lymphocytes (CTLs), eliminate target cells through the release of perforin and granzyme to target cells, such as cancer and virally infected host cells (Kaech et al., 2007). In contrast, memory CD8⁺ T cells play a key role in long-term immunity by mounting rapid and robust responses upon re-exposure to their specific antigen (Kaech et al., 2003). Naive CD4⁺ T cells differentiate into various subsets including Th1, Th2, regulatory T cells (Tregs), follicular helper T cells (Tfh), Th17, Th9, Th22, and CD4⁺ CTLs among others (Cenerenti et al., 2022). Each subtype exhibits distinct expression of surface molecules, cytokines, and key transcription factors (Geginat et al., 2014; Luckheeram et al., 2012).

Activated T cells undergo dynamic changes in their metabolism through several mechanisms, including an increase in nutrient uptake and metabolism, lipid synthesis, and

mitochondrial activity (Raynor et al., 2024). Due to the dynamic metabolic states of T cell activation, expansion, and effector function, a controlled amount of nutrients is required at different stages. Among the various nutrients required to sustain these dynamic metabolic demands, amino acids play a critical role in the licensing and maintenance of proper T cell activation.

ROLES OF AMINO ACIDS

In mammalian systems, there are 20 amino acids that are classified as proteinogenic amino acids (Ren et al., 2013; Ling et al., 2023; Wu et al., 2009). These amino acids are further categorized based on the chemical structure and biophysical properties (Ren et al., 2013; Wu et al., 2009). The chemical structure of an amino acid consists of a central carbon atom bonded to 4 different groups: an amino group, a carboxyl group, a hydrogen atom, and a side chain (Ren et al., 2013). Amino acids are categorized as either essential or non-essential. Essential amino acids cannot be synthesized endogenously and must be obtained from the diet, whereas non-essential amino acids can be synthesized endogenously. Some amino acids have garnered the classification of conditionally essential in specific cell types, particularly when the proteins responsible for endogenous production are expressed at low levels (Ren et al., 2013). The fundamental role for amino acids is to serve as the monomeric units for protein synthesis, however, other mechanisms by which these molecules contribute to the complex systematic function of cells have been elucidated (Ren et al., 2013; Chandel et al., 2021; Chen et al., 2024). In addition to serving as the building blocks for proteins, amino acids can operate as indicators of nutrient status and serve as intermediates in critical cellular pathways (**Figure 1.1**).

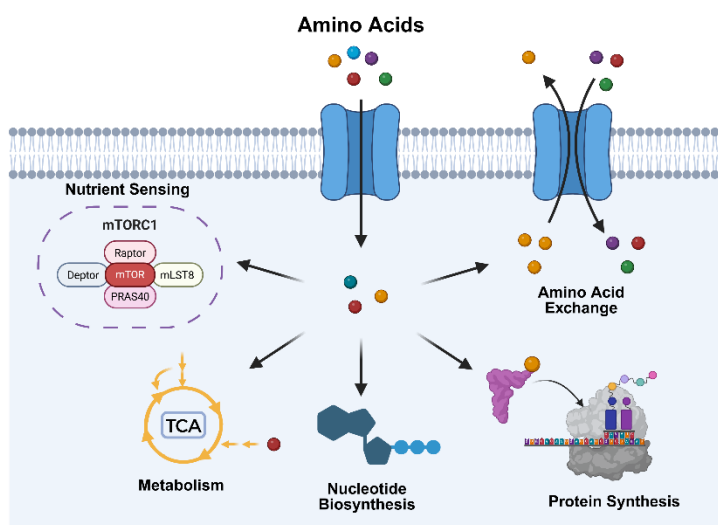


Figure 1.1. Amino acid fates in the cell. Amino acids can participate in nutrient sensing through interactions with various components of the mTOR complexes. Amino acids and their catabolites can participate in several metabolic pathways to support energy production and cellular growth. They can also act as substrates and intermediates for the biosynthesis of additional macromolecules, including nucleotides. A fundamental role of amino acids is to serve as monomers for protein synthesis. Additionally, specific amino acids can act as exchange factors to promote input of many other amino acids, thus maintaining homeostatic levels of all amino acids required for optimal cellular function.

Amino acids and nutrient sensing

Another significant and incredibly regulated role of amino acids is their ability to serve as indicators of nutrient status. This process of intracellular nutrient sensing is primarily mediated by the mechanistic target of rapamycin (mTOR), a central coordinator of cellular metabolism and growth (Panwar et al., 2023; Takahara et al., 2020). mTOR integrates external and internal signals to coordinate cellular states of anabolism or catabolism and amino acids function as a prominent signal for its activity. (Wulschleger et al., 2006; Sengupta et al., 2010; Saxton et al., 2017; Sancak et al., 2008; Kim et al., 2008; Bar-Peled et al., 2012; Dyachok et al., 2016). When amino acids are present at sufficient levels mTOR is activated. Once activated, mTOR

complexes phosphorylate many proteins that promote anabolic processes such as protein synthesis, lipid synthesis, and nucleotide biosynthesis, thereby supporting cell growth, proliferation, and survival (Panwar et al., 2023; Holz et al., 2005; Düvel et al., 2010; Ben-Sahra et al., 2013; Robitaille et al., 2013).

Studies have elucidated specific amino acid sensing mechanisms (Takahara et al., 2020). For example, leucine binding to leucyl-tRNA synthetase (LRS) induces its translocation to mTOR complex and subsequently promotes activity of mTOR (Choi et al., 2017; Han et al., 2012; Han et al., 2012; Choi et al., 2017; Kim et al., 2017). Furthermore, leucine can promote mTOR activity by binding to Sestrin 2, a negative regulator of mTORC1 function (Wolfson et al., 2016; Saxton et al., 2016; Kim et al., 2015; Chantranupong et al., 2014). In a similar manner to leucine, arginine and methionine metabolite, S-adenosylmethionine (SAM), interact with mTOR complex proteins to promote its activation (Chantranupong et al., 2016; Saxton et al., 2016; Xia et al., 2016; Gu et al., 2017).

Amino acid sensing influences T cell activation and differentiation

Nutrient sensing through mTOR is a critical driver of T cell activation. Downstream of T cell receptor signaling is the initiation of the phosphoinositide 3-kinase (PI3K) pathway. PI3K activity inhibits negative regulators of mTOR, thus enhancing mTOR localization to lysosome and subsequent activation. PI3K signaling also activates the transcription factors nuclear factor- κ B, Rel, and Jun, which act on the *interleukin-2* (IL-2) promoter to induce IL-2 expression (Hwang et al., 2020). IL-2 signaling in T cells stimulate the expression of the transcription factor Myc, a key regulator of cellular metabolism in proliferating T cells (Wang et al., 2011). Myc activity promotes upregulation of Slc7a5, an amino acid transporter responsible for large neutral amino acid uptake (Marchingo et al., 2022). Proteomic studies exploring the effects of single gene

ablation of Myc or Slc7a5 demonstrate similar protein changes and nutrient deficiencies, likely indicating that a primary function of Myc is to drive Slc7a5 expression and increase amino acid import (Marchingo et al., 2020). Furthermore, mTOR and Myc activity synergizes to promote a feedforward loop to support nutrient acquisition (Chi et al., 2012). Given the short half-life of Myc, sustained Myc activity is supported by mTOR mediated deactivation of translation inhibitor eukaryotic translation initiation factor 4E-binding protein 1 (Hann et al., 1984). Myc induced upregulation of amino acid transporters promotes intracellular accumulation of amino acids to continuously support mTOR activation.

Interestingly, mTOR and amino acids can also impact T cell differentiation trajectories. In the case of CD4⁺ T cell differentiation into lineage subtypes Th1, Th2, and Th17, mTOR activation is critical (Delgoffe et al., 2011; Chi et al., 2012). Ablation of mTOR activity promotes the differentiation of CD4⁺ T cells to Tregs (Delgoffe et al., 2011; Chi et al., 2012). Additionally, varying levels of mTOR activity can influence CD8⁺ T cell differentiation into terminally differentiated or memory cell states (Pollizzi et al., 2015; Chen et al., 2023). The connection between mTOR and amino acids and their role in directing T cell differentiation states was highlighted in the analysis of daughter cells generated after T cell activation (Chang et al., 2007). Verbist et al., revealed that during the first division of an activated T cell, Myc is asymmetrically distributed between the dividing cells. Asymmetric distribution of amino acids, their transporters, and mTOR was correlated with c-Myc expression (Verbist et al., 2016). This bifurcation in nutrients, their transporters, and signals during asymmetric division has been implicated as a potential explanation for the determination of effector and memory cell states after activation (Kaeck et al., 2012; Gräbnitz et al., 2023).

Amino acid metabolism

Amino acid metabolism encompasses anabolic and catabolic processes that support cellular energy production, biosynthesis, and signaling. When not incorporated into proteins or specialized molecules, amino acids can be exported or deaminated, yielding free ammonia and carbon backbones (Torres et al., 2023; Chandel et al., 2021). These byproducts feed into multiple metabolic pathways. For example, ammonia can be recycled for nucleotide and amino acid biosynthesis (Spinelli et al., 2017), while excess amounts are excreted via the urea cycle due to their cytotoxicity (Wu et al., 2009; Wu et al., 1995; Adeva et al., 2012). The carbon backbones are used to generate key fuels like ATP, glucose, and fatty acids (Bender et al., 2012; Torres et al., 2023), as seen in branched-chain amino acid (BCAA) and glutamine catabolism, which contribute to acetyl-CoA production and TCA cycle activity (Green et al., 2015; Pietrocola et al., 2015; Yoo et al., 2020). The carbon backbone can also contribute to the synthesis of several non-essential amino acids and serve as an anaplerotic substrate and intermediate for nucleotide biosynthesis (Yoo et al., 2020; Yang et al., 2017; DeBerardinis et al., 2007). Beyond energy and biomass production, amino acid-derived metabolites are used as precursors for cofactors and signaling molecules (Rhoads et al., 2009; Wu et al., 1998; Ling et al., 2023).

Amino acids in T cell metabolism

Studies investigating effects of amino acid depletion via media, chemical, or genetic manipulation have revealed critical nodes of metabolic regulation and function in T cells. For example, glutamine and its metabolites have been extensively investigated in the context of T cell activation and differentiation. Some of the earliest studies investigating the requirement of glutamine in peripheral blood mononuclear cells (PBMCs) demonstrated that glutamine is important for T cell activation and proliferation. These effects were attributed to its role as a

metabolic precursor for glutathione production, which is crucial for managing ROS generated during T cell activation (Horig et al., 1993; Robinson et al., 1993; Roth et al., 2002).

The role of the glutamine catabolism pathway, glutaminolysis, is critical for T cell activity. However, studies reveal that specific T cell subtypes display diverse responses to glutaminolysis pathway modulation (Yang et al., 2021). For example, CD8⁺ T cells and Th1 cells display enhanced cytokine production and effector function *in vivo* following chemical or genetic inhibition of glutamine catabolizing enzyme, glutaminase (GLS). However, extended inhibition of GLS in Th1 cells promoted an exhausted phenotype, illustrated by increased T cell immunoglobulin and mucin domain 3 (TIM-3), PD-1, and LAG3 expression (Johnson et al., 2018). An additional study established that T cells can compensate for glutaminolysis inhibition by utilizing other anaplerotic sources such as acetyl-CoA and oxaloacetate to fuel the TCA cycle (Leone et al., 2019). In contrast to Th1 cells, Th17 cells demonstrated poor effector function due to GLS inhibition, and no compensatory mechanisms were observed. Authors suggest that reduced glutamine-derived glutamate blocks glutathione production, which is needed to curtail ROS generated during activation and differentiation of Th17 cells (Johnson et al., 2018). These studies show that different T cell subtypes display distinct metabolic requirements for optimal activation and effector function. These findings present a question as to whether other non-essential amino acids and their downstream metabolites display enhanced essentiality in specific T cell subsets and/or differentiation states.

In addition to glutamine, several other non-essential amino acids—such as alanine, arginine, aspartate, and asparagine— and their metabolites, play roles in T cell activation, differentiation, and effector function (Ma et al., 2024). While these amino acids can be synthesized from a downstream metabolite of glucose or glutamine, some studies have identified specific T cell subtypes and conditions where these amino acids are conditionally

essential. Alanine, for instance, is synthesized from pyruvate through transamination. However, in the presence of glucose-sufficient media, extracellular alanine remains essential for CD4⁺ T cell growth and proliferation. Studies show that a lack of extracellular alanine impairs T cell exit from quiescence following T cell stimulation, likely due to low levels of alanine biosynthesis gene, glutamate-pyruvate transaminase (Ron-Harel et al., 2019). This finding showed that alanine is critical for CD4⁺ T cell size increase upon activation, however to what extent activation marker expression is impacted in T cells due to alanine withdrawal in CD4⁺ and CD8⁺ T cells remains to be determined.

Arginine has also been described as a conditionally essential amino acid in murine T cells. It plays a role in supporting CD3 ζ expression during activation and supports Th1-mediated antiviral activity by serving as a precursor for polyamine synthesis (Zea et al., 2004; Puleston et al., 2021; West et al., 2023). Arginine utilization in the polyamine synthesis pathway is differentially regulated in different Th17 subtypes. Pathogenic Th17 cells metabolize arginine to polyamines, while non-pathogenic Th17s synthesize and accumulate arginine (Puleston et al., 2021; Wagner et al., 2021). Disruption of the polyamine biosynthetic pathway induced a Treg-like state in pathogenic Th17 cells and reduced autoimmunity in a murine model of multiple sclerosis (Wagner et al., 2021).

Aspartate is a glutamine-derived non-essential amino acid that exhibits a critical role in proliferation and maintenance of mitochondrial function (Bailis et al., 2019; Wu et al., 2021a). This amino acid serves as a substrate in the malate-aspartate shuttle, which contributes to the regeneration of the nicotinamide adenine dinucleotide (NAD⁺) pool for glycolysis, and it provides a mechanism for electron transport into the mitochondrial matrix. Aspartate also promotes mitochondrial homeostasis. Wu et al. identified that T cells from rheumatoid arthritis (RA) patients display reduced NAD⁺ and supraphysiological production of TNF-alpha, likely due to

enlarged endoplasmic reticulum (ER). RA-sourced naive T cells that were treated with aspartate or underwent a mitochondria transfer, yielded reduced ER and expression of ER stress signature genes. The proposed mechanism suggests aspartate-generated NAD⁺ promotes ADP-ribosylation of BiP, a subunit of ER sensor GRP78/BiP. This modification of BiP supports its binding to inositol-requiring enzyme 1 α (IRE1) and thus inhibits IRE1 induction of ER expansion and subsequent TNF α production. (Wu et al., 2021a) These findings indicate that healthy, aspartate-producing mitochondria are crucial for maintaining T cell homeostasis. It is interesting that aspartate is conditionally essential for T cells exhibiting mitochondrial dysfunction, however, there remains questions as to whether endogenous production alone is sufficient to maintain T cell function in all CD4⁺ T cell subtypes and to what extent extracellular aspartate is essential for CD4⁺ T cell activation and proliferation.

Asparagine is a non-essential amino acid that has garnered recent interest in CD8⁺ T cell biology. Asparagine can be synthesized from aspartate via asparagine synthetase (ASNS). One study has designated asparagine as a conditionally essential amino acid for T cell activity, given its positive interaction with LCK, a kinase a part of the TCR signaling process (Wu et al., 2021b). Conversely, other studies report that extracellular asparagine restriction promotes CD8⁺ T cell differentiation and function (Gnanaprakasam et al., 2023; Fernández-García et al., 2022). Asparagine restriction during naive CD8⁺ T cell activation promoted the generation of more effector-like CD8⁺ T cells that display enhanced proliferation and IFN γ expression. Adoptive transfer of T cells generated in asparagine deficient conditions resulted in enhanced tumor cell clearance and survival (Gnanaprakasam et al., 2023). It is interesting that timing of extracellular asparagine administration can influence CD8⁺ T cell differentiation trajectory. It has

yet to be determined whether this phenomenon is also evident in CD4⁺ T cells, and to what extent extracellular asparagine is required for its activation and effector functions.

Methionine is an essential amino acid, and its metabolites have exhibited contrasting effects in T cell function. An essential non-proteinogenic function of methionine is its conversion to SAM, which acts as a cofactor in reactions involving histone and RNA methylation. Depletion of extracellular methionine decreases H3K79me2 in CD8⁺ T cells (Bian et al, 2020) and histone H3K4 methylation in Th17 cells (Roy et al., 2020), thus imposing a block on their activity and differentiation respectively. Paradoxically, exogenous supplementation of SAM has been associated with negative effects on T cell function. Human CD8⁺ T cells treated with SAM for an extended period *in vitro*, exhibited reduced proliferation and IFN- γ expression. In addition, the frequency of PD-1⁺ CD28⁻ and TIM3⁺CD28⁻ populations increased after 7 or 14 days of extended culture with SAM treatment, and these cells displayed enhanced expression of Tox and Tbet, indicating the promotion of an exhausted phenotype. (Hung et al., 2021). These results show that the presence of metabolites of essential amino acids may serve as positive or negative regulators of T cell activity at different times during the T cell life cycle.

MECHANISMS OF AMINO ACID HOMEOSTASIS

Given their requisite roles in many intracellular reactions, amino acid homeostasis is crucial for optimal cellular function. Under normal physiological conditions amino acids are in constant flux, being transported across the plasma membrane, incorporated into protein or specialized molecules, or catabolized to generate energy units. However, in conditions of amino acid surplus, depletion or imbalance, cells employ several compensatory mechanisms to restore homeostasis (Bröer et al., 2022). The regulatory process involves the coordinated control of nutrient deprivation signaling and amino acid transport activity.

Regulation of amino acids during nutrient deprivation

In conditions of amino acid depletion, cells initiate several mechanisms to replenish the amino acid pool and reduce cellular demand for protein synthesis. A key signaling pathway that is activated in cellular states of amino acid deprivation or imbalance is the general control non-derepressable 2 (GCN2)/ activating transcription factor 4 (ATF4) axis. The GCN2/ATF4 pathway ultimately blunts global translation while selectively enhancing expression of ATF4, a transcription factor that orchestrates the cellular response to amino acid stress (Hinnebusch et al., 2005; Vatter et al., 2004). Once expressed, ATF4 functions as a transcriptional regulator that promotes amino acid transporters and non-essential amino acid biosynthesis enzyme expression. This coordinated transcriptional response promotes both increased amino acid import and intracellular synthesis (Harding et al., 2003; Lange et al., 2008). For example, CD8⁺ T cells activated in asparagine deficient conditions, upregulated ATF4 and NRF2 to promote ASNS expression and nucleotide synthesis respectively (Gnanaprakasam et al., 2023). This coordinated response to nutrient deprivation promoted CD8⁺ T cell inflammation and antitumor function (Gnanaprakasam et al., 2023).

Transporter mediated regulation of amino acids

A key aspect of amino acid homeostatic regulation is amino acid transporter activity. The study of amino acid transporters in mammalian cells has been an area of active research for over half a century (Christensen, 1966). Early investigations have characterized transporter sequences and identified potential amino acid substrates (Christensen, 1979). Further studies significantly advanced our understanding of substrates and co-substrate conditions required for optimal transport function (Palacín et al., 1998; Nakanishi et al., 2011; Alexander et al., 2021).

Mammalian amino acid transport consists of a large array of membrane proteins with varying substrate specificities, tissue and subcellular localizations, co-substrate dependencies, and mechanism of action (Alexander et al., 2021).

Amino acid transporters not only function as net transport mechanisms, like other nutrient transporters (i.e. glucose transporters), but many amino acid transporters serve as obligatory amino acid exchangers (Palacín et al., 1998; Meier et al., 2002; Chillarón et al., 1996; Alexander et al., 2021). Transporters that participate in this process typically consist of antiporters, which mediate the reciprocal transport of amino acids across the plasma membrane by coupling the inward movement of one amino acid with the outward movement of another. Other transport mechanisms include symporters and uniporters (facilitated diffusers), which respectively transfer two amino acids in the same direction or a single amino acid unidirectionally (Alexander et al., 2021). Many antiporters and symporters require ions as co-substrates such as Na^+ , H^+ , or Cl^- , to facilitate transport activity and maintain electrochemical gradients (Alexander et al., 2021). Extensive studies of substrate specificity, co-substrate dependency, and transporter molecular structure have enabled the categorization of transporters into families, culminating in the development of a detailed transporter family compendium (Alexander et al., 2021).

Cells utilize antiporters and symporters to quickly normalize amino acid levels when there is an intracellular amino acid imbalance. In a model of osteosarcoma, antiporter exchangers ASCT2 (Slc1a5) and LAT1(Slc7a5) play key roles in maintaining amino acid balance under conditions of fluctuating metabolic demand (Bröer et al., 2016). ASCT2 is an importer of glutamine, which serves as an exchange substrate for LAT1 antiporter activity, thereby facilitating import of neutral amino acids required for cell growth. (Nicklin et al., 2009). Interestingly, ASCT2 ablation did not alter the proliferative capacity of the cancer cells. In fact,

ASCT2 depletion resulted in upregulation of rescue symporters, SNAT1 and SNAT2 (Slc38a1 and Slc38a2), which work as sodium dependent net importers of neutral amino acids, including glutamine (Bröer et al., 2016). Increased SNAT1 and SNAT2 activity was sufficient to maintain intracellular amino acid levels necessary for exchange activity of LAT1 in ASCT2 ablated cells, thus sustaining cell growth (Bröer et al., 2016).

Given the diverse effects of amino acids and their metabolites in T cells, it is important that fine-tuned mechanisms are employed to control their intracellular amino acid levels. In response to TCR stimulation, T cells exhibit dynamic regulation of transporter expression. A prominent example includes the increased expression of transporters, Slc7a5 and Slc3a2, which form a heterodimeric complex to facilitate neutral amino acid import (Sinclair et al., 2013). These transporters are crucial for sustained amino acid acquisition and contribution to protein synthesis, mTOR signaling, and metabolism. In a similar manner to some cancer cells, CD8⁺ T cells also demonstrate compensatory transporter upregulation under conditions of impaired amino acid transport. For example, mouse CD8⁺ T cells treated with ASCT2 inhibitor, V9302, exhibited consistent glutamine import via upregulation of rescue transporter, Slc6a14 (Edwards et al., 2021). It remains an open question whether additional compensatory transport systems operate within CD8⁺ or CD4⁺ T cells. Furthermore, the characterization of the T cell transporter proteome remains incomplete, leaving many potential regulators of amino acid import in T cells unexplored.

CONCLUSION

Both non-essential and essential amino acids play crucial roles in metabolism, proliferation, and effector function in a variety of T cell subtypes. An important revelation of these studies is that amino acid metabolic pathways and regulatory mechanisms of importance in one T cell subtype

or differentiation state do not apply to others. Therefore, various amino acid dependencies and homeostasis mechanisms have yet to be characterized in many T cell subtypes and differentiation states.

We sought to address gaps in knowledge in amino acid dependencies in CD4⁺ T cell activation and amino acid regulatory mechanisms in CD8⁺ T cell activity. In chapter 2, we explore non-essential amino acid dependencies in CD4⁺ T cells over the course of their activation, differentiation, and autoreactivity *in vivo*. Our results point to a specific CD4⁺ T cell dependency on extracellular asparagine to maintain optimal CD4⁺ T cell function. We used two orthogonal approaches to modulate extracellular asparagine levels *in vitro* and *in vivo*, and determined the consequence of this modulation in the context of *in vitro* CD4⁺ T cell activation and pathogenic Th17 mediated induction of experimental autoimmune encephalomyelitis in murine mouse models.

In chapter 3, we aimed to fill a critical knowledge gap by identifying essential mediators of T cell nutrient uptake and signaling through an *in vivo* metabolic CRISPR screen. This approach revealed guide RNAs targeting transporters and receptors that either enhanced or reduced T cell accumulation within the tumor microenvironment. Among the gene targets more abundantly represented in tumor-infiltrating T cells, the amino acid transporter *Slc43a2* emerged as the second most enriched. Given this prominent representation, we investigated the role of *Slc43a2* in CD8⁺ T cell biology more closely.

In conclusion, amino acids are multifaceted components of the cell that mediate many critical pathways related to T cell activation, differentiation, and function. Extensive research has focused on understanding the factors that mediate amino acid uptake and utilization, thus revealing ways to enhance cellular energetics and improve immune therapies. However, many modulators of amino acid metabolism in T cell subsets remain unidentified. In this dissertation, I

set out to investigate understudied nodes of amino acid regulation in CD8⁺ and CD4⁺ T cells in contexts of the tumor immunity and autoimmunity.

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CHAPTER 2: DEPLETION OF EXTRACELLULAR ASPARAGINE IMPAIRS SELF-REACTIVE T CELLS AND AMELIORATES AUTOIMMUNITY IN A MURINE MODEL OF MULTIPLE SCLEROSIS

Peter Georgiev^{1,2,3#}, Sheila Johnson^{2,3#}, Kiran Kurmi², Song-Hua Hu^{2,3}, SeongJun Han^{1,2,3}, Dillon Patterson^{1,3}, Thao H. Nguyen^{1,3}, Linglin Huang³, Dan Liang^{1,3}, Naomi Goldman^{1,3}, Thomas Conway^{1,3}, Hannah Creasey^{1,3}, Jared Rowe^{1,2,3,4}, Marcia C. Haigis^{2,3*}, and Arlene H. Sharpe^{1,2,3*}

1. Department of Immunology, Blavatnik Institute, Harvard Medical School, Boston, Massachusetts 02115, USA.
2. Department of Cell Biology, Blavatnik Institute, Harvard Medical School, Boston, Massachusetts 02115, USA.
3. Gene Lay Institute of Immunology and Inflammation of Brigham and Women's Hospital, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA.
4. Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02215, USA.

* Co-senior and co-corresponding authors

Co-first authors

This chapter is adapted from a draft of a manuscript currently in submission.

ATTRIBUTIONS

Peter Georgiev (Figure 2.1-2.6, S2.1-2.4) and Sheila Johnson (Figure 2.1-2.2, 2.4, S2.1) designed and completed experiments and data analysis presented in this chapter. Kiran Kurmi performed heavy labeled tracing experiments and analysis (Figure 2.3). Song-Hua Hu performed western blot and Linglin Huang performed RNAseq analysis (Figure 2.2).

ABSTRACT

Amino acids play critical roles in the activation and function of lymphocytes. Here we show that the non-essential amino acid, asparagine, is essential for optimal activation and proliferation of CD4⁺ T cells. We demonstrate that asparagine depletion at different time points after CD4⁺ T cell activation reduces mitochondrial membrane potential and function. Furthermore, asparagine depletion at specific time points during CD4⁺ T cell differentiation reduces cytokine production in multiple CD4⁺ T cell subsets. In an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE), myelin oligodendrocyte-specific pathogenic T helper 17 cells differentiated under Asn-deficient conditions exhibited reduced encephalitogenic potential and attenuated EAE severity. In a model of EAE induced by active immunization, therapeutic depletion of extracellular Asn significantly reduced disease severity. These results identify asparagine as a key metabolic regulator of the pathogenicity of autoreactive CD4⁺ T cells and suggest that targeting asparagine metabolism may be a novel therapeutic strategy for autoimmunity.

INTRODUCTION

Naive T cell activation and clonal expansion are bioenergetically demanding processes that require substantial changes in energy and substrate utilization (Chapman et al., 2020; Klein Geltink et al., 2018; MacIver et al., 2013). Following T cell receptor (TCR) stimulation, activated T cells increase their metabolic activity and shift towards the use of anabolic pathways such as aerobic glycolysis, fatty acid synthesis, and mitochondrial biogenesis (Chapman et al., 2020; Klein Geltink et al., 2018; MacIver et al., 2013; Warburg et al., 1927; Buck et al., 2015). This metabolic transition depends on the uptake of extracellular nutrients and is enabled by the rapid upregulation of nutrient transporters (Wei et al., 2017; Zhang et al., 2014). Among these, the uptake of exogenous amino acids is important for sustaining intracellular biosynthetic processes including nucleotide biosynthesis, ATP generation, and nascent protein synthesis (Zhang et al., 2014; Kelly et al., 2020; Sinclair et al., 2013).

Amino acids are fundamental building blocks for the synthesis of nascent proteins but have additional functions during T cell activation. Amino acids serve as substrates for nucleic acid biosynthesis, support post translational modifications of proteins and are critical for maintaining redox balance (Zhang et al., 2014; Kelly et al., 2020; Sinclair et al., 2013). The reliance of activated T cells on the non-essential amino acids glutamine, serine, alanine, and arginine is well established (Carr et al., 2010; Geiger et al., 2016; Ma et al., 2017; Ron-Harel et al., 2019). For example, activated T cells critically depend on uptake of serine, which provides glycerol and carbon units for de novo nucleotide biosynthesis and one-carbon metabolism (Ma et al., 2017). Similarly, uptake of alanine is required for T cell proliferation and the efficient exit from quiescence following TCR stimulation (Ron-Harel et al., 2019). Extracellular asparagine (Asn) has recently garnered attention as a nutrient important for CD8⁺ T cell differentiation and function, however its role in helper CD4⁺ T cell responses has not been explored (Hope et al.,

2021; Wu et al., 2021; Chang et al., 2025; Gnanaprakasam et al., 2023; Fernandez-Garcia et al., 2022). In fact, a number of extracellular amino acid dependencies have yet to be characterized in the specific context of CD4⁺ T cell function. To address this gap, we assessed the requirement for extracellular non-essential amino acids during CD4⁺ T cell activation and proliferation. Through this analysis, we uncovered a critical role for extracellular Asn in supporting CD4⁺ T cell responses.

In this study, we demonstrate that CD4⁺ T cells depend on extracellular Asn for optimal proliferation, activation, and differentiation into helper cells, despite upregulating the Asn-generating enzyme Asn synthetase (ASNS). The absence of extracellular Asn impairs TCR-induced metabolic reprogramming and results in dysfunctional mitochondria. Our findings reveal that early or delayed Asn depletion during the course of experimental autoimmune encephalomyelitis (EAE) attenuates disease severity. In an adoptive transfer model of EAE, pathogenic T helper 17 cells differentiated in the absence of extracellular Asn accumulate poorly in the central nervous system (CNS) and exhibit defects in protein synthesis and metabolic fitness ex vivo. Collectively, our results suggest that extracellular Asn bioavailability is a key metabolic checkpoint that regulates the functional responses of CD4⁺ T cells.

RESULTS

Extracellular asparagine (Asn) is essential for optimal activation and proliferation of CD4⁺ T cells

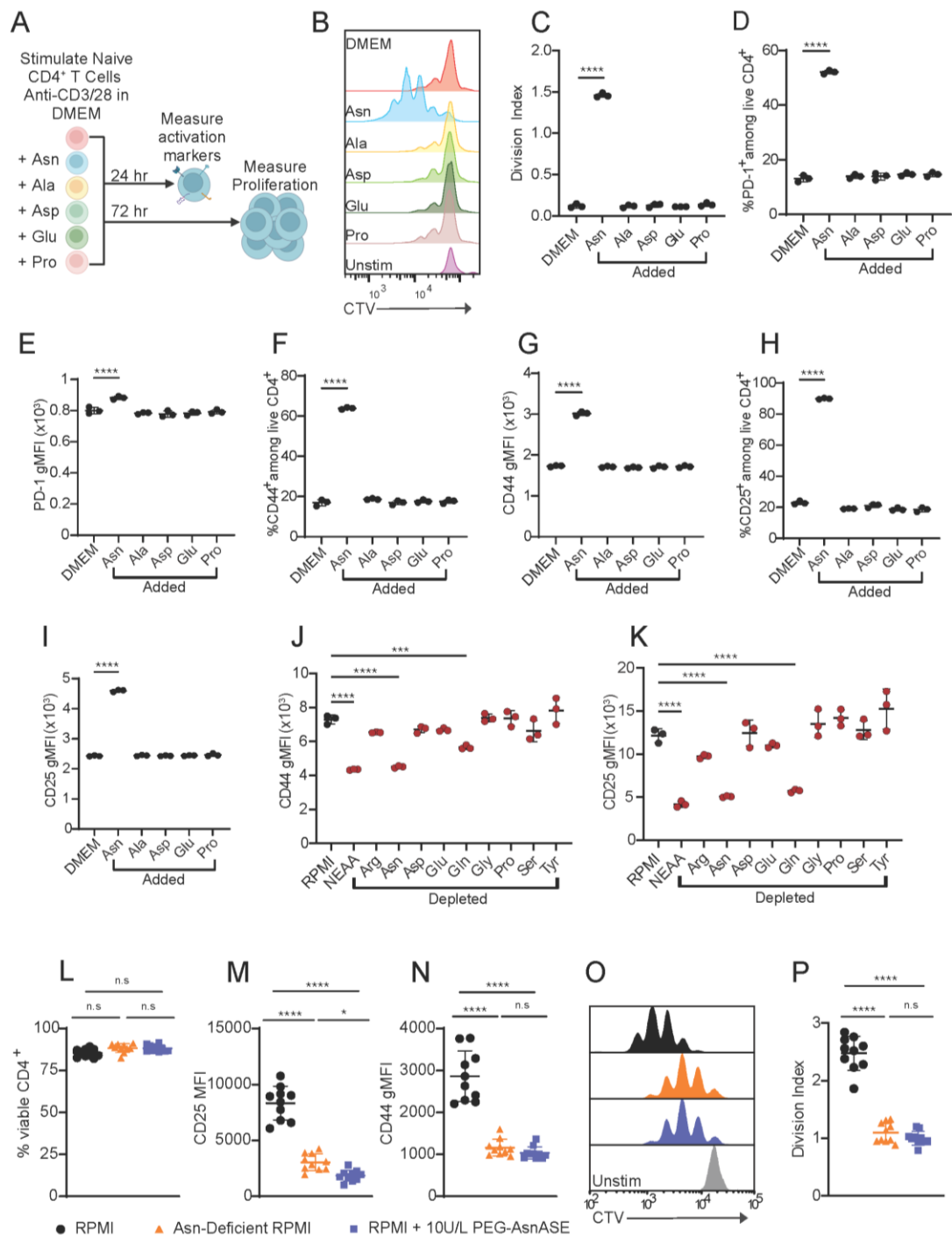
To investigate the requirement for non-essential amino acids in CD4⁺ T cell activation, we performed experiments under culture conditions where amino acids were individually added or withdrawn from media. For single amino acid addition experiments, naive CD4⁺ T cells were activated on plates coated with anti-CD3 and anti-CD28 antibodies and cultured in DMEM

media with glutamine. Non-essential amino acids (NEAA) lacking in standard DMEM formulation, but present in the conventional RPMI formulation, were individually added. After 24 hours of stimulation in these culture conditions, only the addition of Asn was sufficient to fully activate CD4⁺ T cells and promote expression of canonical activation markers (**Figure 2.1A, D-I**). After 72 hours of activation in these culture conditions, only Asn addition led to significant proliferation, as measured by CTV dye dilution (**Figure 2.1A-C**). To assess the essentiality of NEAA for activation and proliferation of naive CD4⁺ T cells, we activated naive CD4⁺ T cells in RPMI lacking nine NEAA or RPMI depleted of each non-essential amino acid for 24 hours or 72 hours. Depletion of Asn or glutamine resulted in reduced expression of activation markers CD44, CD25, and PD-1, and phenocopied the effects seen when all nine NEAA were depleted (**Figure 2.1J-K, S2.1A**). The expression of transferrin iron receptor, CD71, was also reduced under conditions of Asn, arginine, and glutamine depletion after 24 hours of activation (**Figure S2.1B**). However, Asn deprivation resulted in the greatest defect in expression of all the activation markers. In a similar manner, the frequency of CD44 and PD-1 positive cells was subtly reduced in conditions where either Asn or all NEAA were depleted (**Figure S2.1C-D**). However, no significant difference was detected in the frequency of CD25 positive cells when cultured under single amino acid depletion conditions (**Figure S2.1E**). Interestingly, Asn deprivation alone significantly reduced the frequency of CD71 positive cells after 24 hours of activation (**Figure S2.1F**).

We also sought to explore the effects of single NEAA depletion on CD4⁺ T cell proliferation (**Figure S2.1G**). After 72 hours of activation, we observed that NEAA, arginine, glutamine, asparagine, and tyrosine deficient conditions resulted in a substantial defect in proliferative capacity compared to other NEAAs (**Figure S2.1H**). Among the conditions that

Figure 2.1 (following page). Asparagine is critical for early activation and proliferation of CD4⁺ T cells. (A) Schematic of experimental design. Naive CD4⁺ T cells were stimulated for either 24 hours or 72 hours with plate-bound anti-CD3 and anti-CD28 mAbs in DMEM media with glutamine or DMEM media with glutamine supplemented with 0.38 mM asparagine (Asn), 0.38 mM alanine (Ala), 0.15 mM aspartate (Asp), 0.13 mM glutamate (Glu), or 0.17 mM proline (Pro). (B) Representative flow cytometry histogram depicting CTV dye dilution in naive CD4⁺ T cells following 3 days of stimulation with plate-bound anti-CD3/CD28 mAbs in DMEM media with glutamine or DMEM media with glutamine supplemented with the indicated amino acids. (C) Quantification of division index in B. (D-I) Quantification of the proportions of CD4⁺ T cells expressing the cell surface activation markers PD-1 (D), CD44 (F) CD25 (H) as well as expression levels of each respective marker on a per cell basis (E,G,I) following 24 hours of stimulation with plate-bound anti-CD3/CD28 mAbs in DMEM media with glutamine or DMEM media with glutamine supplemented with 0.38 mM Asn, 0.38 mM Ala, 0.15 mM Asp, 0.13 mM Glu, or 0.17 mM Pro. (J-K) Expression levels of activation markers CD44 (J), CD25 (K) following 24 hours of stimulation with plate-bound anti-CD3/CD28 mAbs in RPMI lacking the indicated individual amino acids shown in red. Non-essential amino acids (NEAA) include asparagine (Asn), aspartate (Asp), glutamate (Glu), proline (Pro), arginine (Arg), glutamine (Gln), glycine (Gly), serine (Ser), and tyrosine (Tyr). (L) Quantification of the proportions of viable CD4⁺ T cells following 24 hours of stimulation with plate-bound anti-CD3/CD28 mAbs in complete RPMI (RPMI), Asn-deficient RPMI or RPMI with 10 IU/L PEGylated-asparaginase (PEG-AsnASE) added at the start of culture. (M-N) Expression level of CD25 (M) and CD44 (N) on a per cell basis following 24 hours of stimulation with plate-bound anti-CD3/CD28 mAbs under the same conditions as in L. (O) Representative flow cytometry histograms showing CTV dye dilution in naive CD4⁺ T cells following 3 days of stimulation with plate-bound anti-CD3/CD28 mAbs in RPMI, Asn-deficient RPMI or RPMI with 10 IU/L PEG-AsnASE added at the start of culture. Grey histogram represents unstimulated control. (P) Quantification of division index in O. Each dot represents cells obtained from an individual animal (L-N, P). Results are shown as mean $\pm$ SD (C-N, P) and are representatives of 2 independent experiments (B-K, O) or pooled from 2 independent experiments (L-N, P). Not significant (n.s), *p < 0.05, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Dunnet's multiple comparison test (C-K) or Tukey's multiple comparison test (L-N, P).

Figure 2.1 (Continued).



promoted sub-optimal division indices, cells under Asn deficient conditions displayed the lowest viability after 72 hours (**Figure S2.1I**). These results show that Asn is essential for CD4⁺ T cell activation in these culture conditions.

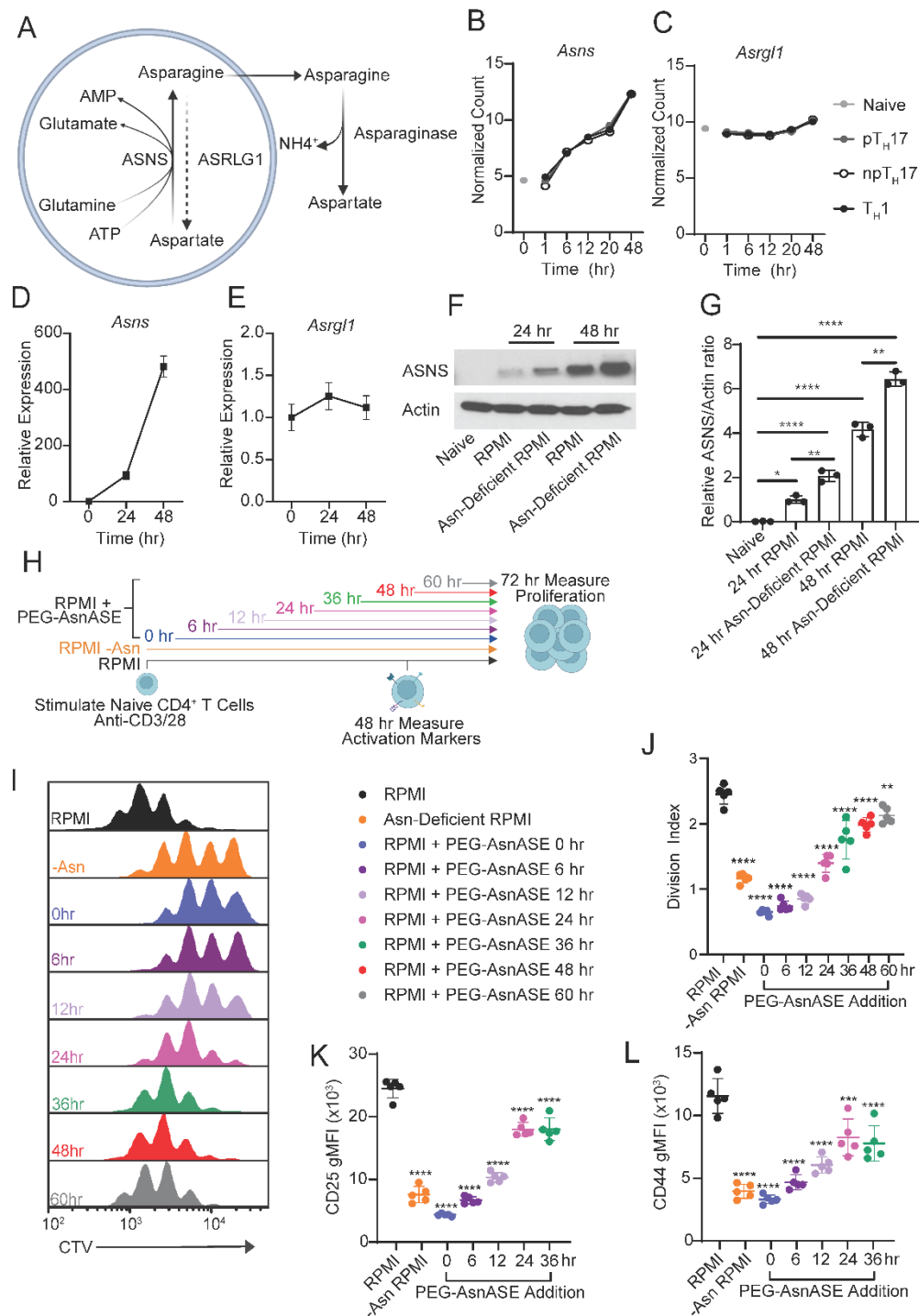
To further evaluate the extent to which extracellular Asn depletion affects CD4⁺ T cell activation and proliferation, we employed two orthogonal approaches to deplete Asn. We activated naive CD4⁺ T cells in media treated with PEGylated asparaginase (PEG-AsnASE), an enzyme that catabolizes asparagine into aspartate and ammonia, or in custom-formulated RPMI media specifically lacking Asn. Viability did not significantly differ between complete or Asn-deficient media conditions (**Figure 2.1L**), however, cells cultured in Asn-deficient media displayed reduced expression and frequencies of CD25, CD44, and PD-1 positive cells after 24 hours in culture (**Figure S2.1J-M**). These results were mimicked with PEG-AsnASE treated media. In contrast, at longer culture periods after TCR stimulation, cells demonstrated reduced viability after 72 hours in Asn-deficient media (**Figure S2.1P**). Proliferation was also impaired by the depletion of Asn after 3 days of TCR stimulation (**Figure 2.1O-P**). In sum, CD4⁺ T cells exhibit a requirement for extracellular Asn at early stages of activation.

Asparagine is described as a non-essential amino acid and thus can be produced endogenously. Considering the essentiality of extracellular Asn during the early stages of CD4⁺ T cell activation, we next explored whether CD4⁺ T cells lack the intracellular machinery needed for endogenous production of Asn (**Figure 2.2A**). To evaluate the levels of Asn metabolism proteins present in CD4⁺ T cells, we used a publicly available bulk RNA-seq dataset consisting of naive CD4⁺ T cells differentiated *in vitro* under T helper 1 (TH1), non-pathogenic T helper 17 (npTH17), and pathogenic T helper 17 (pTH17) polarizing conditions for 1, 6, 12, 20 or 48 hours (**Figure 2.2B-C**) (Thakore et al., 2024). These data suggest that the Asn synthesizing enzyme, asparagine synthase (*Asns*), increases in expression upon activation under all polarizing

Figure 2.2 (following page). Extracellular asparagine is needed for sustained CD4⁺ T cell activation and proliferation at later stages following initial activation.

(A) Schematic of Asn metabolism in mammalian cells. (B-C) Bulk RNA-seq analysis showing expression of *Asns* (B), and *Asrgl1* (C) in naive CD4⁺CD62L⁺CD44⁻ T cells at baseline (gray dot) or following stimulation with anti-CD3/CD28 mAbs under T helper 1 (T_H1), pathogenic T helper 17 (pT_H17) and non-pathogenic T helper 17 (npT_H17) conditions for 1, 6, 12, 20 and 48 hours. Results are shown as average (n=3 for each condition, per time point). (D-E) qPCR analysis showing the expression kinetics of *Asns* (D) and *Asrgl1* (E) over time in naive CD4⁺ T cells at baseline or stimulated with anti-CD3/CD28 mAbs for 24 and 48 hours. (F) Western blot analysis of ASNS protein expression in CD4⁺ T cells activated with anti-CD3/CD28 mAbs in either RPMI or Asn-deficient RPMI at 24 or 48 hours after activation. Naive CD4⁺ T cells are shown as controls. (G) Densitometry analysis of F showing the relative *Asns* to actin ratio. (H) Schematic of experimental design. Purified naive CD4⁺ T cells were activated *in vitro* with plate-bound anti-CD3/CD28 mAbs in complete RPMI media (RPMI), Asn-deficient RPMI, or RPMI with 10 IU/L PEGylated-asparaginase (PEG-AsnASE) added at 0, 6, 12, 24, 36, 48 and 60 hours. (I) Representative flow cytometry histogram showing cell trace violet (CTV) dye dilution in naive CD4⁺ T cells following 3 days of stimulation. (J) Quantification of division index in I. (K-L) Quantification of the gMFI of CD4⁺ T cells expressing CD25(K) and CD44(L) following 2 days of stimulation with plate-bound anti-CD3/CD28 mAbs in complete RPMI, Asn-deficient RPMI or RPMI with 10 IU/L PEG-AsnASE added at 0, 6, 12, 24, 36 hours. Each dot represents cells from an individual animal (J-L). Results are shown as average (n=3 per condition, per time point) (B-C) or mean ± SD (D-E, G, J-L) and are representative of 2 independent experiments (D-G, I-L). non-significant (n.s), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Dunnet's multiple comparison test (J-L) or Tukey's multiple comparison test (G).

Figure 2.2 (Continued).



conditions, whereas the asparagine catabolizing enzyme, *Asrg1*, expression demonstrates little change upon activation. We validated these findings in CD4⁺ T cells activated under non polarizing conditions (**Figure 2.2D-E**) using quantitative PCR (qPCR). To confirm that the observed transcriptional changes correspond to protein expression levels, we assessed ASNS protein expression by western blotting (**Figure 2.2F**). The changes in ASNS protein levels reflected the transcriptional changes of *Asns*. Notably, when naive CD4⁺ T cells were activated in Asn-limited media, there was greater production of ASNS compared to cells cultured in Asn sufficient media (**Figure 2.2G**). Together, these results show that activated CD4⁺ T cells possess the enzymatic machinery to generate Asn in both Asn-depleted and Asn-replete conditions.

Given that upregulation of ASNS allows CD8⁺ T cells to function in the absence of extracellular Asn (Hope et al., 2021; Gnanaprakasam et al., 2023) and our findings showing that ASNS is upregulated in CD4⁺ T cells upon TCR activation in the presence and absence of Asn (**Figure 2.2F-G**), we next investigated whether endogenous Asn could compensate for the lack of extracellular Asn and promote CD4⁺ T cell function at 24 and 48 hours after TCR stimulation. To interrogate when extracellular Asn is needed for CD4⁺ T cell activation and proliferation, we depleted extracellular Asn using PEG-AsnASE at different time points following activation, including 6, 12, 24, 36, 48 and 60 hours post TCR activation (**Figure 2.2H**). Depletion of extracellular Asn significantly inhibited CD4⁺ T cell proliferation, even when PEG-AsnASE was added to cultures at 48 hours post TCR activation (**Figure 2.2I-J**). The anti-proliferative effects of PEG-AsnASE treatment were statistically significant at time points corresponding to increased ASNS expression, including 24 and 36 hours after TCR activation (**Figure 2.2D and 2.2F-G**). In addition, depletion of extracellular Asn at various time points after TCR activation impaired the activation of naive CD4⁺ T cells, reducing the proportions of CD4⁺ T cells

expressing activation-associated markers (**Figure S2.2A-C**). Expression levels of these markers were also reduced in the absence of Asn and never fully recovered to levels observed in control conditions (**Figure 2.2K-L**). These results suggest that extracellular Asn availability is not only limiting early during TCR activation, but also at later stages, despite increased ASNS levels.

Extracellular Asn is a building block for protein synthesis following T cell activation

Because amino acids act as fundamental building blocks for the synthesis of nascent proteins, we reasoned that deficits in the activation and proliferation of CD4⁺ T cells upon Asn deprivation might result from decreased protein synthesis (Kelly et al., 2020). To measure levels of protein synthesis in anti-CD3/anti-CD28 activated CD4⁺ T cells, we used fluorescently labeled O-propargyl-puromycin (OPP), an alkyne analog of puromycin which is directly incorporated into the C-terminus of translating polypeptide chains (**Figure 2.3A**). Naive CD4⁺ T cells stimulated in Asn-deficient RPMI for 24 hours exhibited a significant deficit in nascent protein synthesis, similar to levels observed in unstimulated controls and control cells treated with the protein synthesis inhibitor cycloheximide (**Figure 2.3B-C**). Adding Asn back to Asn-deficient RPMI largely restored protein synthesis (**Figure 2.3B-C**). These results demonstrate a requirement for Asn in tRNA charging to support nascent polypeptide synthesis during the activation of naive CD4⁺ T cells.

Next, we conducted a time course activation study using ¹⁵N₂-Asn to assess the contribution of extracellular Asn to total CD4⁺ T cell protein content (**Figure 2.3D**). LC/MS analysis of hydrolyzed total protein fractions from naive CD4⁺ T cells at baseline, 24 hours, and 48 hours post TCR stimulation revealed that nearly 50% of Asn from proteins was ¹⁵N₂ labeled after 24 hours and roughly 75% of Asn was labeled by 48 hours (**Figure 2.3E**). Collectively, our

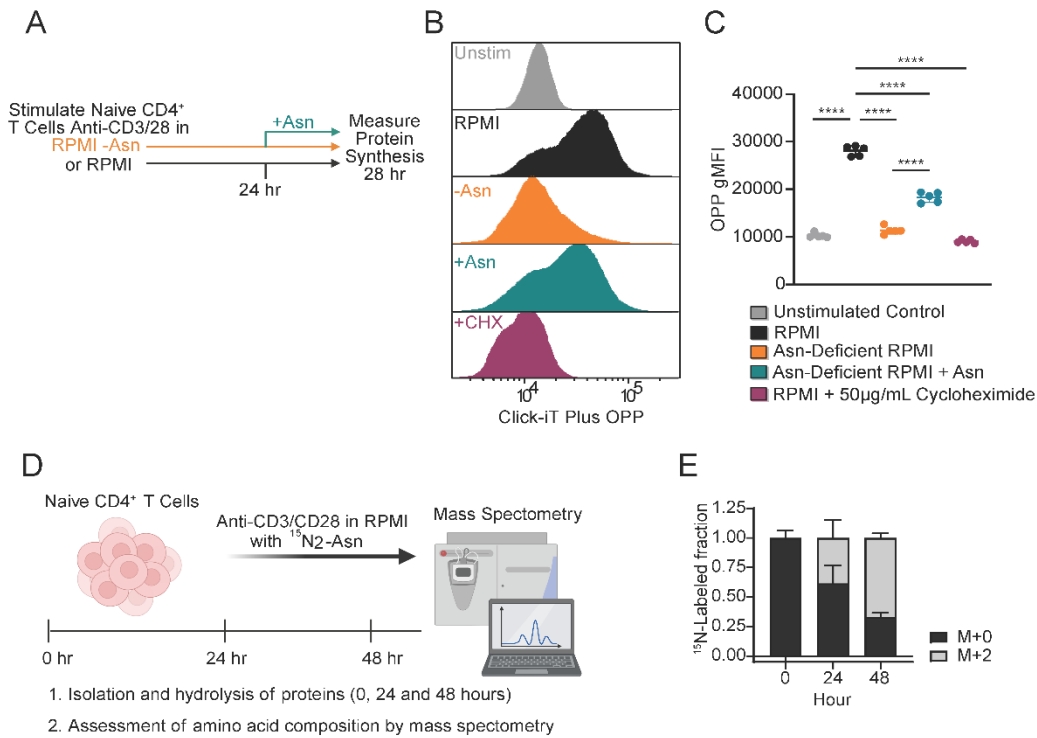


Figure 2.3. Asparagine is taken up by CD4⁺ T cells and incorporated into their proteome.

(A) Schematic of experimental design. Purified naive CD4⁺ T cells were activated *in vitro* with plate-bound anti-CD3/CD28 mAbs in complete RPMI media (RPMI) or Asn-deficient RPMI for 24 hours. At 24 hours Asn was added to cells stimulated in Asn-deficient media at a final concentration of 0.38 mM for 4 hours. At 28 hours protein synthesis was measured using the O-propargyl-puromycin (OPP) probe. As a positive control T cells cultured in RPMI were treated with 50 μg/mL of the protein synthesis inhibitor cycloheximide (CHX). (B) Representative flow cytometry histogram showing mean fluorescent intensity (MFI) of the nascent protein synthesis reporter Click-iT OPP. Naive CD4⁺ T cells are shown as controls. (C) Quantification of Click-iT OPP gMFI. (D) Experimental design for measuring the incorporation of heavy labeled ¹⁵N₂-Asn into proteins following naive CD4⁺ T cell activation. Naive CD4⁺ T cells were stimulated with plate-bound anti-CD3/CD28 mAbs in Asn-deficient media reconstituted with 0.38 mM ¹⁵N₂-Asn for 24 or 48 hours. (E) Quantification of the ¹⁵N₂-Asn labeled fraction in the T cell proteome. Results are shown as mean ± SD (C and E) and are representative of at least two independent experiments (B-C, E). ****p < 0.0001, one-way ANOVA with Tukey's multiple comparison test (C).

studies demonstrate that Asn is taken up by CD4⁺ T cells and plays a requisite role in protein synthesis.

Asparagine depletion impairs metabolic reprogramming associated with CD4⁺ T cell activation

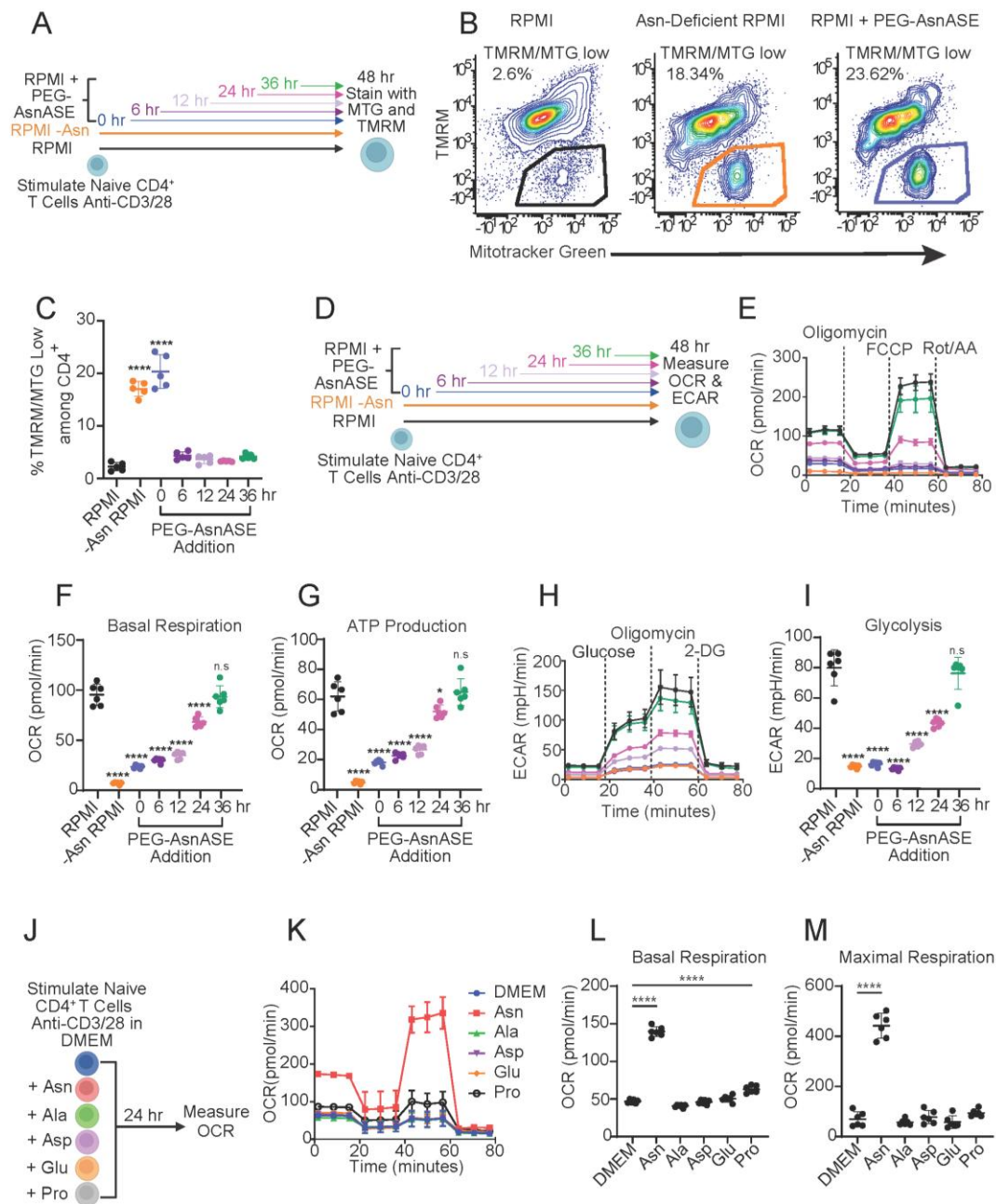
Following TCR activation, T cells rapidly upregulate aerobic glycolysis and mitochondrial biogenesis to support the bioenergetic demands required for clonal expansion (Chapman et al., 2020).¹ Because nutrient uptake and metabolic reprogramming are highly coordinated processes, (Klein Geltink et al., 2018; Wei et al., 2017) we next investigated whether extracellular Asn availability affects CD4⁺ T cell bioenergetics. To probe the functional state of mitochondria under conditions of Asn deprivation, we utilized the metabolic dyes Tetramethylrhodamine methyl ester (TMRM) to label active mitochondria with intact membranes and mitotracker green (MTG) to assess mitochondrial mass. (**Figure 2.4A**). TMRM and MTG fluorescence were decreased following Asn deprivation or treatment with PEG-AsnASE (**Figure S2.3A-B**). In addition, a population of depolarized mitochondria displaying high MTG staining (referred to as TMRM/MTG low) emerged (**Figure 2.4B**). TMRM/MTG low cells have previously been shown to mark dysfunctional mitochondria in exhausted T cells. (Yu et al., 2020) The proportion of TMRM/MTG low cells increased in the absence of extracellular Asn (**Figure 2.4B-C**), suggesting that depletion of extracellular Asn supports the accumulation of depolarized mitochondria with impaired fitness.

Given our results showing that asparagine deprivation results in the accumulation of dysfunctional mitochondria, we next sought to understand how Asn availability affects overall T cell metabolic reprogramming by measuring mitochondrial respiration and glycolytic flux

Figure 2.4 (following page). Extracellular asparagine depletion impairs CD4⁺ T cell bioenergetics upon activation.

(A) Schematic of experimental design. Naive CD4⁺ T cells were stimulated for 48 hours with plate-bound anti-CD3/CD28 mAbs in either complete RPMI media (RPMI), Asn-deficient RPMI or RPMI treated with 10 IU/L PEGylated-asparaginase (PEG-AsnASE) added at 0, 6, 12, 24, or 36 hours and stained with Mitotracker green (MTG) and Tetramethylrhodamine methyl ester (TMRM). (B) Representative flow cytometry contour plots depicting TMRM and MTG in CD4⁺ T cells. (C) Quantification of proportion of TMRM/MTG low cells. (D) Schematic of experimental design. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in naive CD4⁺ T cells stimulated for 48 hours with plate-bound anti-CD3/CD28 mAbs in either complete RPMI media (RPMI), Asn-deficient RPMI or RPMI with 10 IU/L PEG-AsnASE added at 0, 6, 12, 24, or 36 hours. (E) OCR under mitochondrial stress test conditions. (F) Quantification of basal respiration and (G) ATP production. (H) ECAR under glycolysis stress test conditions. (I) Quantification of glycolysis. (J) Schematic of experimental design. OCR was measured in naive CD4⁺ T cells stimulated for 24 hours with plate-bound anti-CD3/CD28 mAbs in DMEM with glutamine or DMEM with glutamine supplemented with 0.38 mM of either asparagine (Asn), alanine (Ala), aspartate (Asp), glutamate (Glu), or proline (Pro). (K) OCR under mitochondrial stress test conditions. (L) Quantification of basal respiration and (M) maximal respiration. Each dot in panels C, F-G, and I represent cells obtained from an individual animal. Results are shown as mean $\pm$ SD and are representative of at least 2 independent experiments. non-significant (n.s), * $p < 0.05$, **** $p < 0.0001$, one-way ANOVA with Dunnet's multiple comparison test.

Figure 2.4 (Continued).



(**Figure 2.4D**). Oxidative phosphorylation (OXPHOS), as measured by the oxygen consumption rate (OCR), was significantly decreased in the absence of extracellular Asn under mitochondrial stress test conditions (**Figure 2.4E**). PEG-AsnASE treatment at 6, 12 or 24 hours post TCR activation led to decreased OCR and ATP production, while PEG-AsnASE treatment at 36 hours post activation did not blunt mitochondrial respiration (**Figure 2.4E-G**). These results suggest that continuous Asn availability during the early stages of TCR activation is necessary to promote mitochondrial respiration. Under glycolysis stress test conditions, depletion of extracellular Asn similarly reduced the extracellular acidification rate (ECAR), resulting in decreased glycolysis (**Figure 2.4H-I**). These findings indicate that extracellular Asn availability determines the extent of TCR-induced glycolytic flux and mitochondrial respiration during CD4⁺ T cell activation. Complementary studies using DMEM media supplemented with Asn showed a significant increase in OCR under mitochondrial stress test conditions, resulting in heightened basal respiration and maximal respiration (**Figure 2.4J-M**). In contrast, DMEM media supplemented with alanine, aspartate, glutamate, or proline failed to sufficiently increase OCR under mitochondrial stress test conditions (**Figure 2.4J-M**). These results suggest that extracellular Asn is crucial for mitochondrial respiration following T cell activation.

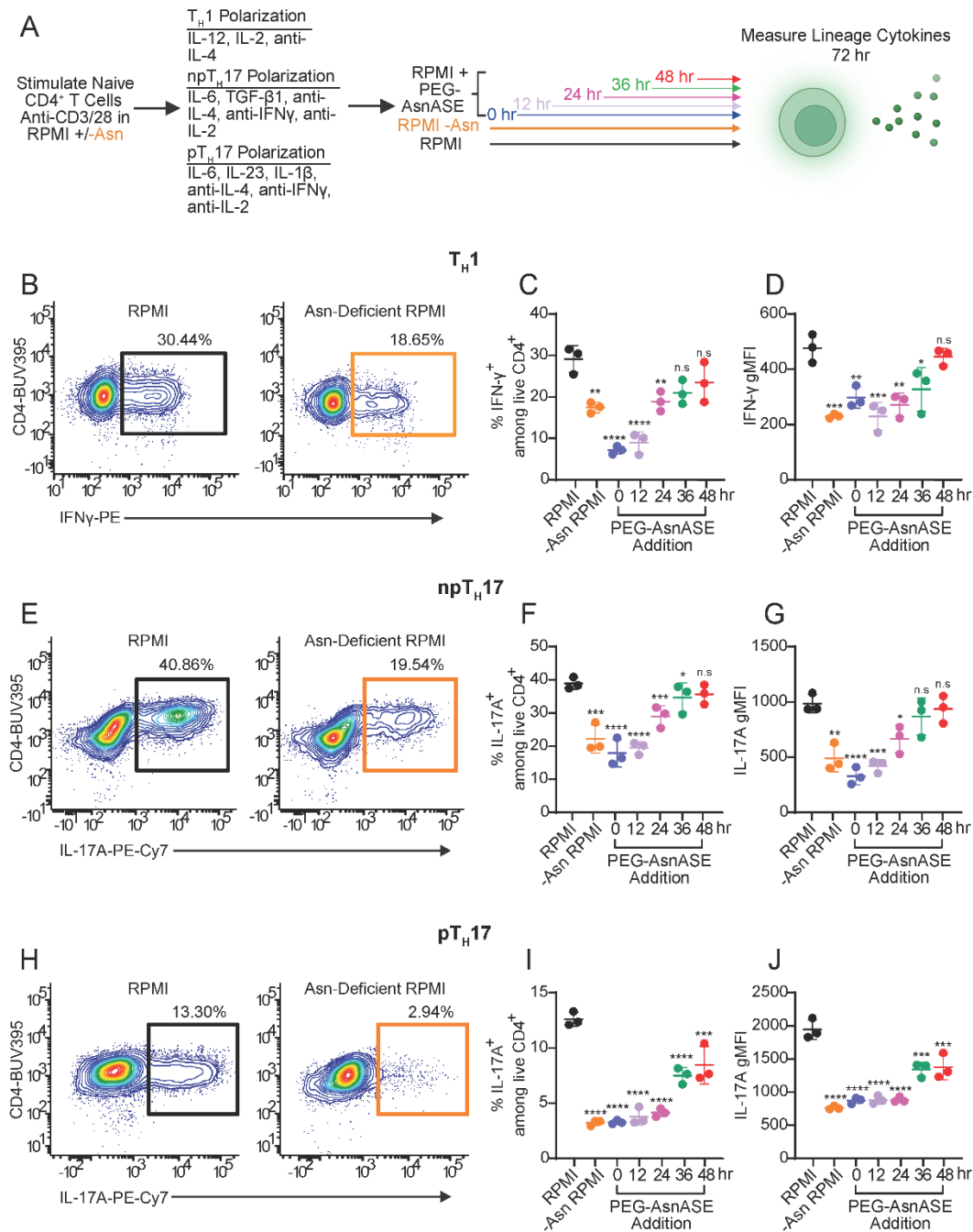
Asparagine depletion reduces CD4⁺ helper T cell lineage-specific cytokine production

Given the key roles of mitochondrial respiration and amino acids in the specification of distinct helper T cell lineages (Klysz et al., 2015; Johnson et al., 2018; Puleston et al., 2021; Delgoffe et al., 2009; Lee et al., 2010), we next tested whether extracellular Asn availability influences the differentiation of naive CD4⁺ helper T cells into distinct subsets. We differentiated naive CD4⁺ T cells under TH1, npTH17, or pTH17 polarizing conditions, depleting Asn at the initiation of culture or at 0, 12, 24, 36 and 48 hours after activation (**Figure 2.5A**). Asn depletion impaired

Figure 2.5 (following page). Asparagine deficiency reduces lineage-specific cytokine production in CD4⁺ T helper subsets.

(A) Schematic of experimental design. Purified naive CD4⁺ T cells were activated *in vitro* with plate-bound anti-CD3/CD28 mAbs under T helper 1 (T_H1), pathogenic T helper 17 (pT_H17), and non-pathogenic T helper 17 (npT_H17) conditions in either complete RPMI (RPMI) media, Asn-deficient RPMI or RPMI with 10 IU/L PEGylated-asparaginase (PEG-AsnASE) added at 0, 12, 24, 36 or 48 hours. On day 3 cells were restimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin for intracellular staining. (B) Representative flow cytometry contour plots depicting intracellular staining of IFN- γ in T_H1 differentiation conditions in RPMI or RPMI without Asn. (C) Quantification of the proportions of IFN γ -producing CD4⁺ T cells under T_H1 differentiation conditions. (D) Quantification of IFN- γ gMFI as shown in C. (E) Representative flow cytometry contour plots depicting intracellular staining of IL-17A in npT_H17 differentiation conditions in RPMI or RPMI without Asn. (F) Quantification of the proportions of IL-17A-producing CD4⁺ T cells under npT_H17 differentiation conditions. (G) Quantification of IL-17A gMFI as shown in F. (H) Representative flow cytometry contour plots depicting intracellular staining of IL-17A in pT_H17 differentiation conditions in RPMI or RPMI without Asn. (I) Quantification of the proportions of IL-17A-producing CD4⁺ T cells under pT_H17 differentiation conditions. (J) Quantification of IL-17A gMFI as shown in I. Each dot in panels C-D, F-G, and I-J represents cells obtained from an individual animal. Results are shown as mean $\pm$ SD and are representative of at least 2 independent experiments. non-significant (n.s), *p < 0.05 **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Dunnet's multiple comparison test.

Figure 2.5 (Continued).



the differentiation of all helper subsets tested, whether it was depleted at the initiation of each culture or at specific time points following T cell activation by using PEG-AsnASE (**Figure 2.5B-J**). Naive CD4⁺ T cells differentiated under TH1 polarizing conditions exhibited reduced intracellular expression of the TH1-defining cytokine IFN- γ and a reduced proportion of IFN- γ -expressing T cells (**Figure 2.5C-D**). Asn depletion similarly affected npTH17 and pTH17 cells in the early stages of activation, as intracellular IL-17A expression levels were significantly reduced within both subsets at 0, 12, and 24 hour timepoints (**Figure 2.5F-G, I-J**). Notably, pTH17 intracellular IL-17A production was specifically affected by PEG-AsnASE treatment in the later timepoints of differentiation compared to npTH17 in these culture conditions (**Figure 2.5F-G, I-J**). This finding may point to differences in nutrient requirements as naive CD4⁺ T cells differentiate into distinct subsets. These results suggest that Asn depletion impairs the production of lineage-defining cytokines in CD4⁺ helper T cell subsets.

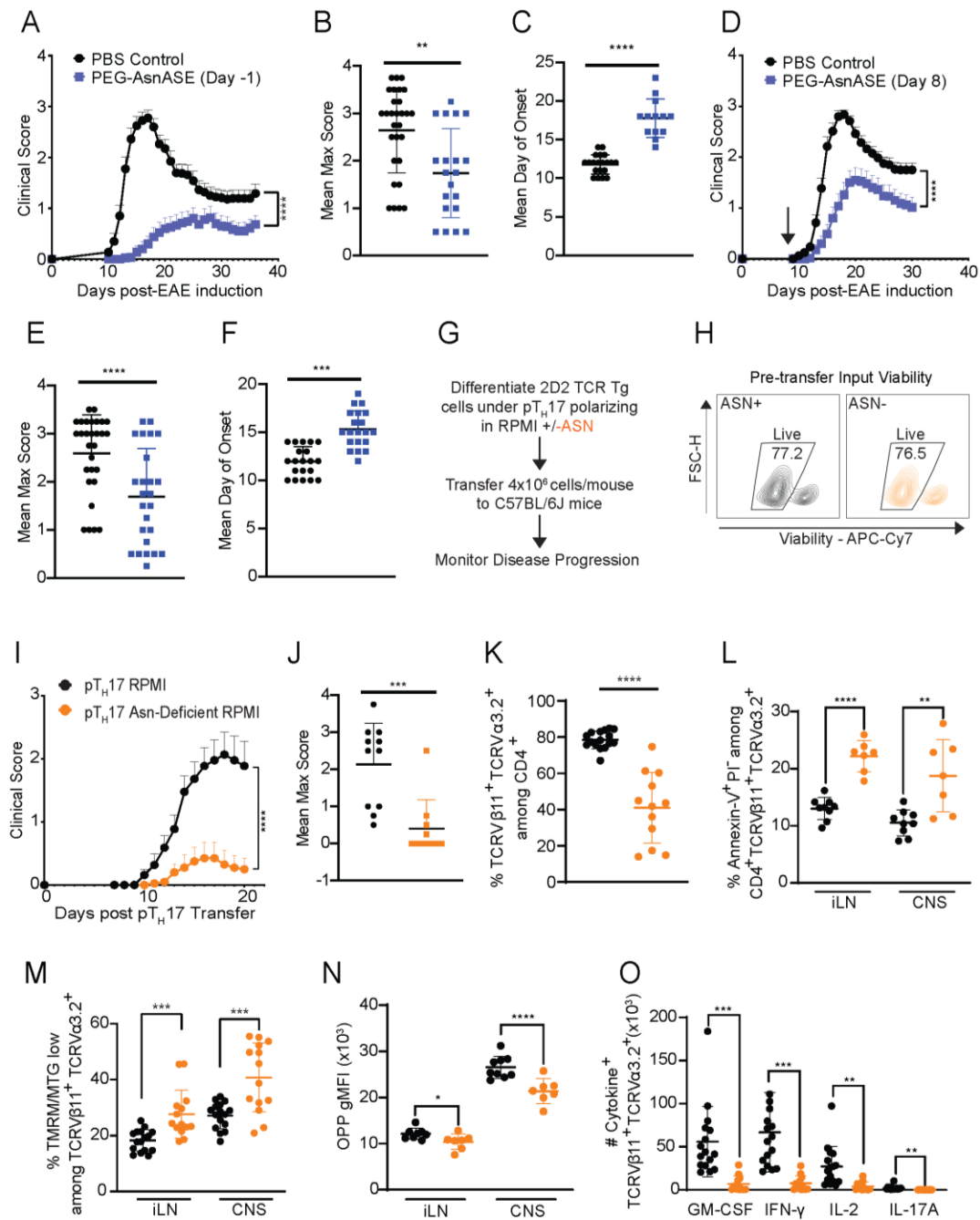
Asn depletion ameliorates the severity of experimental autoimmune encephalomyelitis

We hypothesized that by exploiting the dependency of CD4⁺ T cells on extracellular Asn by systemically depleting Asn bioavailability, we could modulate the severity CD4⁺ T cell mediated pathologies, such as experimental autoimmune encephalomyelitis (EAE). To do this, we first prophylactically administered a single dose of PEG-AsnASE to mice one day before inducing EAE through immunization with an emulsion of myelin oligodendrocyte glycoprotein (MOG) peptide, MOG_{35–55}, in complete Freund's adjuvant (CFA) followed by administration of pertussis toxin. Strikingly, while PBS treated controls developed EAE peaking around day 16, PEG-AsnASE treated mice exhibited significantly milder disease with delayed onset (**Figure 2.6A-C**). To evaluate the therapeutic potential of PEG-AsnASE in a more clinically relevant scenario, we delayed PEG-AsnASE treatment until day 8 after active immunization with MOG_{35–55}

Figure 2.6 (following page). Asparagine deficiency ameliorates experimental autoimmune encephalomyelitis (EAE).

(A) Mice were treated with a single dose of 25 IUs of PEGylated-asparaginase (PEG-AsnASE) or PBS i.p. one day prior to immunization with MOG₃₅₋₅₅/CFA and pertussis toxin (PTX) to induce EAE and monitored daily for signs of disease. (B) Quantification of the average maximal EAE scores in PBS vs. PEG-AsnASE treated mice. (C) Quantification of the mean day of onset in PBS vs. PEG-AsnASE treated mice. (D) EAE was induced by immunization with MOG₃₅₋₅₅/CFA and pertussis toxin (PTX) and scored daily for disease. Mice were treated with a single dose of 25 IUs of PEG-AsnASE or PBS i.p on day 8 of active EAE. (E) Quantification of the average maximal EAE scores in PBS vs. PEG-AsnASE treated mice. (F) Quantification of the mean day of onset in PBS vs. day 8 PEG-AsnASE treated mice. (G) Schematic of experimental design. Pathogenic T helper 17 (pT_H17) cells were differentiated from naive CD4⁺ FoxP3⁻ T cells from 2D2 TCR transgenic mice in RPMI media with or without Asn and viable 2D2 cells were adoptively transferred (4x10⁶/mouse) into 10-week-old C57BL/6J female recipients to induce EAE. Mice were monitored daily for disease. (H) Representative flow plot displaying percentage of viable pT_H17 polarized 2D2 cells in sufficient and deficient conditions prior to transfer. (I) Daily EAE scores. (J) Quantification of the average maximal EAE scores in mice receiving pT_H17 cells generated in the presence or absence of Asn. (K) Quantification of the proportions of CNS-infiltrating Vβ11⁺Vα3.2⁺ 2D2 pT_H17 cells at peak of EAE (pT_H17 RPMI n=16, pT_H17 Asn-deficient RPMI n=12). (L) Quantification of the proportions of Vβ11⁺Vα3.2⁺ 2D2 pT_H17 cells actively undergoing apoptosis (Annexin-V⁺PI⁻) in the CNS and inguinal lymph node at peak of EAE. (M) Quantification of the proportions of TMRM/MTG low 2D2 pT_H17 cells in the CNS and inguinal lymph node at peak of EAE. (N) Quantification of OPP gMFI in 2D2 pT_H17 cells in the CNS and inguinal lymph node at peak of EAE. (O) Quantification of the absolute numbers of the indicated cytokines expressed by 2D2 pT_H17 cells in the CNS at peak of EAE. Results are shown as mean ± SEM (A, D, I) or mean ± SD (B-C, E-F, J-O) and are pooled (A-F, I-K, M, O) or a representative of at least two independent experiments (H, L, N). Each dot represents an individual mouse (B-C, E-F, J-O) *p < 0.05 **p < 0.01, ***p < 0.001, ****p < 0.0001 two-way ANOVA (A, D & I) and Student's t test (B-C, E-F, and J-O).

Figure 2.6 (Continued).



(**Figure 2.6D**). Remarkably, a single dose of PEG-AsnASE was sufficient to attenuate disease severity and delay onset, similar to prophylactic PEG-AsnASE treatment, resulting in a substantially milder disease (**Figure 2.6D-F**). These results suggest that therapeutic Asn depletion has the potential to be an immunosuppressive strategy to target CD4⁺ T cell-mediated autoimmune pathologies.

pTH17 cells generated in the absence of extracellular Asn are poorly encephalitogenic and exhibit deficits in protein synthesis and mitochondrial fitness in vivo

Since systemic Asn depletion reduces severity of EAE and pTH17 cells are key mediators of EAE, we next investigated how Asn depletion affects the pathogenic potential of pTH17 cells *in vivo*. We generated pTH17 cells from TCR(V β 11*V α 3.2⁺) transgenic 2D2 mice, which express a TCR specific for myelin oligodendrocyte glycoprotein, using either standard RPMI or Asn-deficient RPMI media and compared their capacity to induce EAE *in vivo*. We adoptively transferred equal numbers of viable 2D2 pTH17 cells into Asn-sufficient WT C67BL/6J hosts and monitored mice for development of EAE (**Figure 2.6G-H**). 2D2 pTH17 cells generated in Asn-deficient RPMI induced a milder disease compared to 2D2 pTH17 cells generated in RPMI (**Figure 2.6I-J**). Consistent with these observations, both the proportions and absolute numbers of 2D2 pTH17 cells from Asn-deficient cultures were significantly reduced in the CNS of recipient mice at the peak of EAE (**Figure 2.6K, S2.4A-B**). Furthermore, 2D2 pTH17 cells generated in Asn-deficient RPMI exhibited increased apoptosis, as determined by Annexin V, in the CNS and inguinal lymph nodes (iLN) at peak EAE (**Figure 2.6L, S2.4C**). Moreover, 2D2 pTH17 cells generated in Asn-deficient RPMI exhibited reduced MTG and TMRM gMFI in the CNS and iLN (**Figure S2.4D-E**). There was an enrichment of TMRM/MTG low 2D2 pTH17 cells

in the iLN and CNS when differentiated in the absence of Asn (**Figure 2.6M**), consistent with the deleterious effects of PEG-AsnASE on mitochondrial function in *in vitro* activated CD4⁺ T cells (**Figure 2.4B-C**).

We next assessed the ex vivo protein synthesis capability of 2D2 pTH17 cells isolated from the iLN and CNS, using the OPP probe. Both iLN and CNS-infiltrating 2D2 pTH17 differentiated in the absence of Asn exhibited a significant decrease in OPP gMFI at peak of EAE, indicating reduced protein synthesis (**Figure 2.6N**). The absolute numbers of pathogenic cytokine-producing 2D2 pTH17 cells generated in Asn-deficient cultures also were reduced in the CNS at peak EAE (**Figure 2.6O**), further showing the negative impact of Asn deficiency on pTH17 cell protein synthesis capability. Taken together, these results suggest that the deprivation of extracellular Asn during pTH17 cell differentiation leads to deficits that reduce the pathogenic potential of autoreactive T cells.

DISCUSSION

Over the past two decades there have been significant advances in our understanding of the metabolic processes involved in T cell function and lineage commitment. This has increased interest in the potential of modifying T cell responses using targeted metabolic perturbations (MacIver et al., 2013; Patel et al., 2017; Corrado et al., 2022). To meet the metabolic demands of activation and clonal expansion, T cells increase uptake of essential metabolic nutrients that fuel macromolecule biosynthetic processes. It is now appreciated that limiting availability of glucose (Araujo et al., 2017; Cham et al., 2005; Jacobs et al., 2008), glutamine (Carr et al., 2010; Nakaya et al., 2014), alanine (Ron-Harel et al., 2019), leucine (Ananieva et al., 2016), methionine (Roy et al., 2020) and arginine (Geiger et al., 2016; Rodriguez et al., 2007; Choi et al., 2009) can lead to deficits in T cell activation and function. Our studies extend this

understanding by revealing that extracellular Asn availability is essential for optimal activation and proliferation of helper CD4⁺ T cells. This dependency is tightly linked to protein synthesis. By targeting this metabolic dependency, we show that Asn depletion can be used to ameliorate disease severity during autoimmunity driven by pTH17 cells. This is effective whether extracellular Asn is depleted prophylactically or later during active EAE. Our observations suggest that Asn deprivation could potentially restrict the function of pathogenic effector T cells in inflammatory and autoimmune disorders.

Our results build upon recent reports demonstrating the importance of extracellular Asn in the activation and proliferation of CD8⁺ T cells (Hope et al., 2021; Wu et al., 2021; Chang et al., 2025; Gnanaprakasam et al., 2023; Fernandez-Garcia et al., 2022). Consistent with our observations, extracellular Asn has been shown to be critical for TCR-induced activation, proliferation and metabolic reprogramming of naive CD8⁺ T cells (Hope et al., 2021; Wu et al., 2021). Although upregulation of ASNS enables CD8⁺ T cells to function in the absence of extracellular Asn, ASNS-expressing CD8⁺ T cells activated in Asn-deficient media exhibit significantly lower activation, proliferation and effector molecule production compared to Asn-sufficient media (Hope et al., 2021). Interestingly, pharmacological inhibition of ASNS activity only modestly decreases CD8⁺ T cell function, suggesting that newly synthesized Asn has a lower impact than extracellular Asn on the initiation of CD8⁺ T cell responses (Wu et al., 2021). Our results also demonstrate that CD4⁺ T cells require continuous extracellular Asn for optimal activation and proliferation, despite upregulating ASNS. The relative expression levels of ASNS and timing of Asn depletion also can influence the differentiation states of CD8⁺ T cells (Gnanaprakasam et al., 2023; Fernandez-Garcia et al., 2022). While Asn depletion early during the differentiation of CD8⁺ T cells favored the maintenance of an effector phenotype (Gnanaprakasam et al., 2023; Fernandez-Garcia et al., 2022), depletion of extracellular Asn late

during CD8⁺ T cell activation promoted polarization towards a central memory phenotype (Fernandez-Garcia et al., 2022). We find that Asn depletion at early stages of T helper subset polarization inhibits lineage-defining cytokine production. However, further studies are needed to examine the requirement of Asn during later stages of activation and differentiation, as well as its role in supporting the longevity of CD4⁺ helper T cell responses. In addition, it remains to be determined whether the generation of memory CD4⁺ T cells or their recall responses similarly depend on extracellular Asn availability.

How does Asn depletion impair the proliferation and functional activity of T cells? In this work, we demonstrate that Asn functions as a proteinogenic amino acid, thereby promoting CD4⁺ T cell proliferation and lineage-defining cytokine production. These results are consistent with observations in mammalian cell systems, which highlight the essentiality of Asn in the setting of glutamine deprivation (Krall et al., 2016; Pavlova et al., 2018). Studies have shown that simultaneous glutamine and Asn depletion cripples T cell activation and proliferation, similar to the effects seen when ASNS-deficient T cells are deprived of extracellular Asn (Hope et al., 2021). Asn essentiality in glutamine deficient conditions is also reflected by their shared role as amino acid exchange factors. In a cell model of liposarcoma, Asn and glutamine export promoted import of amino acids crucial for one-carbon metabolism and mTOR activation (Krall et al., 2016). Since both mTOR activation and one-carbon metabolism are upregulated after TCR stimulation, it is possible that Asn-mediated amino acid exchange activity contributes an additional functional role in supporting CD4⁺ T cell activation and proliferation. Our work shows that Asn availability is important for the maintenance of mitochondrial mass and membrane potential, and its depletion promotes accumulation of depolarized mitochondria with a phenotype similar to those observed in exhausted CD8⁺ T cells (Yu et al., 2020). Given that Asn can directly bind to and enhance the activity of LCK (Wu et al., 2021), a key TCR signaling

kinase, it is possible that Asn might also interact with other proteins related to mitochondrial respiration. Future studies determining the interactome of Asn in activated T cells will be crucial for uncovering novel regulatory functions of Asn beyond protein synthesis.

In this study, we observed that CD4⁺ T cells rely on extracellular Asn to promote optimal expression of activation markers after 24 hours of stimulation. In fact, most of our single amino acid depletion experiments revealed that Asn deprivation resulted in the greatest defect in frequency and expression of the markers investigated. Among the activation markers explored, CD71 frequency was the most impacted due to Asn deprivation. This finding aligns with a previous study revealing that extracellular asparagine deprivation affects CD71 expression and intracellular iron levels in cancer cells lacking ASNS, however no direct mechanism of this phenomenon was uncovered, and it was not determined whether other nutrient limitations result in a CD71 defect (Jiang et al., 2023). Our work sheds more light on the nutrient restriction conditions mediating this defect, given that we observed that Asn deficiency specifically affects the frequency of cells that upregulate this receptor and is not a response of general amino acid deficiency. As previously mentioned, Asn interacts with LCK to promote its kinase activity, thus Asn could potentially demonstrate additional protein interactions with regulators of CD71 (Wu et al., 2021).

Our work has shown that asparagine restriction limits the production of lineage defining cytokines, and the greatest deficiency was observed in the pathogenic TH17 subset. Interestingly, studies have linked iron deficiency to reduced pathogenicity of TH17 cells (Li et al., 2021; Voss et al., 2023). In fact, authors revealed that TH17 cells specifically rely on CD71 mediated iron uptake to promote their differentiation (Li et al., 2021). Given our observations showing that pTH17 cells are especially dependent on asparagine to maintain IL17A production throughout the entire differentiation period, and Asn deprivation specifically mediates CD71

deficiency in CD4⁺ T cells, these findings may point to a specific nutrient regulatory axis that could be perturbed in diseases primarily mediated by TH17 pathogenicity. In future, it would be interesting to explore in the context of pTH17 differentiation and effector function, the effect of extracellular Asn modulation on CD71 kinetics and intracellular iron accumulation.

Our finding that Asn deprivation can ameliorate the severity of EAE, even after the priming of CNS-reactive CD4⁺ T cells, warrants further investigation in models of autoimmunity and immune dysregulation. It would be interesting to explore whether therapeutic asparaginase could be utilized to prevent the induction or severity of colitis or delay the spontaneous development of diabetes in NOD mice mediated by islet-reactive CD4⁺ and CD8⁺ T cells. Given our results showing that CD71 frequency is reduced specifically in Asn deficient conditions, it would be interesting to explore the effects of asparaginases in disease states where autoreactive T cells are dependent on iron or display enhanced iron uptake. For instance, a study observed that autoreactive CD4⁺ T cells from lupus-prone mice display enhanced iron uptake and increased expression of CD71 (Voss et al., 2023). In addition, patients with systemic lupus erythematosus (SLE) harbored CD4⁺ T cells with high CD71 expression and increased frequency of TH17 cells within its CD4⁺ T cell pool (Voss et al., 2023). Future studies should investigate whether targeted delivery of asparaginase impacts disease severity of SLE and other autoimmune diseases, without compromising anti-pathogen immune response essential for the host.

Although the therapeutic potential of asparaginase is interesting to explore in the context of autoimmunity, it is important to consider possible ramifications upon its administration. Classically, asparaginase has been used to treat acute lymphoblastic leukemia, however, pancreatitis is a complication observed among a portion of those treated (Müller et al., 1998; Skipper et al., 2023; M'harzi et al., 2022). A detailed mechanism has yet to be clearly defined,

however, it has been suggested that administration of asparaginase depletes serum asparagine levels and inhibits protein synthesis in tissues with enhanced protein production and secretion, including the pancreas (Müller et al., 1998; M'harzi et al., 2022). Asparaginase associated pancreatitis and hypersensitivity has been correlated with a few genetic risk factors, and its incidence is associated with increasing number of administered doses (Skipper et al., 2023; Liu et al., 2016; Albertsen et al., 2019). Thus, it is important to identify additional biomarkers or genetic indicators that denote individuals that are hypersensitive to asparaginase and optimize schedule of administration for those who may be predisposed to asparaginase mediated toxicity.

In summary, our studies reveal that extracellular Asn availability represents a metabolic vulnerability for the activation and differentiation of naive CD4⁺ T cells, largely due to the requirement for Asn in sustaining protein synthesis. Therapeutic Asn depletion by targeted asparaginase treatment may provide a conceptually novel strategy for autoimmunity.

MATERIALS AND METHODS

Mice

Wild-type (WT) C57BL/6J and 2D2 T cell receptor (TCR) transgenic female mice were purchased from the Jackson Laboratories. 8–12-week-old female mice were used for all experiments. All mice were maintained under the guidelines and policies set by the Harvard Medical School Standing Committee on Animals and the National Institutes of Health. All mouse protocols were approved by the Harvard Medical Area Standing Committee on Animals.

Isolation and activation of murine CD4⁺ T cells

Naive CD4⁺CD62L⁺CD44⁻ T cells were isolated from mouse spleens using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) in accordance with the manufacturers' protocols. Following isolation, naive CD4⁺ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (Thermo Fisher Scientific) at a concentration of 4 µg/mL using a 96-well flat-bottom plate (10 x 10⁴ cells/well). For proliferation studies, naive CD4⁺ T cells were labeled with cell trace violet dye (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Cells were subsequently cultured in standard RPMI-1640 supplemented with 10% heat inactivated FBS, 10 mM HEPES, 0.05 mM 2-mercaptoethanol and 1% penicillin-streptomycin or Asn-deficient RPMI (Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS, 10 mM HEPES, 0.05 mM 2-mercaptoethanol and 1% penicillin-streptomycin. In some studies, standard RPMI media was treated with 10 IU/L of PEGylated asparaginase (PEG-AsnASE) to remove Asn and used as an additional control. In studies in which amino acids were depleted, RPMI without amino acids (US Biological Sciences) was supplemented with 10% heat inactivated FBS, 10 mM HEPES, 0.05 mM 2-mercaptoethanol and 1% penicillin-streptomycin, with pH adjustment to 7.3, and specific amino acids were added depending on desired amino acid composition. Concentrations for each added amino acid were based on Thermo Fisher Scientific RPMI Formulation (<https://www.thermofisher.com/us/en/home/technical-resources/media-formulation.114.html>). All amino acids apart from threonine (Thermo Fisher Scientific) were purchased from Sigma-Aldrich. For additional activation studies, DMEM with glutamine (Thermo Fisher Scientific) was supplemented with 10% heat inactivated FBS and 1% penicillin-streptomycin and individual non-essential amino acids depending on desired condition. Prior to surface, intracellular and metabolic dye staining, cells were transferred to a 96 well V-bottom plate.

CD4⁺ helper T cell differentiation and intracellular staining

Naive CD4⁺CD62L⁺CD44⁻ cells were isolated from mouse spleens using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) in accordance with the manufacturer's protocols. Following isolation, naive CD4⁺ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (Thermo Fisher Scientific) at a concentration of 4 µg/mL in a 96-well flat-bottom plate (10 x 10⁴ cells/well) and cultured in either RPMI, Asn-deficient RPMI or RPMI treated with 10 IU/L PEG-AsnASE. In some studies, 10 IU/L PEG-AsnASE was added to cultures at 0, 6, 12, 24, 36 hours following stimulation. Naive CD4⁺ T cells were differentiated into distinct helper T (T_H) cell subsets using the following polarization conditions and recombinant proteins and antibodies: For T_H1 differentiation: 10 ng/mL IL-12 (Peprotech), 5 ng/mL IL-2 (Peprotech) and 10 µg/mL anti-IL-4 (Biolegend Clone 11B11). For non-pathogenic T_H17 conditions: 20 ng/mL IL-6 (Peprotech), 2 ng/mL TGF-β1 (Peprotech), 10 µg/mL anti-IFN-γ (Biolegend Clone XMG1.2), 10 µg/mL anti-IL-4 (Biolegend Clone 11B11) and 10 µg/mL anti-IL-2 (Biolegend Clone JES6-1A12). For pathogenic T_H17 conditions: 20 ng/mL IL-6 (Peprotech), 10 ng/mL IL-23 (R&D Systems), 10 ng/mL IL-1β (Peprotech), 10 µg/mL anti-IFN-γ (Biolegend Clone XMG1.2), 10 µg/mL anti-IL-4 (Biolegend Clone 11B11) and 10 µg/mL anti-IL-2 (Biolegend Clone JES6-1A12). On day 3 cells were re-stimulated using a 1X eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) for 4 hours at 37°C and transferred to a 96 well V-bottom plate for intracellular staining. Cells were then washed two times using a 1X cell stain buffer solution (Biolegend) and stained with CD4 (Biolegend Clone RM4-5) and a 1:2000 fixable viability stain 780 (BD) for 30 minutes on ice, followed by two washes using 1X cell stain buffer. Intracellular staining was performed using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD) according to the manufacturer's instructions. The following antibodies were diluted at 1:200 in 1X Perm buffer and used for intracellular staining: IL17A (Biolegend Clone TC11-18H10.1) and IFN-γ (Biolegend Clone

XMG1.2) Acquisition was performed on a FACSymphony cytometer with DIVA software (BD), and data were analyzed using FCS Express Software (De Novo).

Surface/Intracellular Staining and Flow Cytometry

Primary mouse cells were isolated from spleen and central nervous system (CNS) including brain and spinal cord. Single-cell suspensions were incubated with 1:100 TruStain FcX™ (Biolegend) 1X DPBS solution for 15 minutes at room temperature to block Fc receptors. Viability was assessed using a fixable viability stain 780 (BD Biosciences) at a 1:1000 dilution in 1X DPBS for 20 minutes on ice followed by one wash in cell stain buffer (Biolegend). For surface staining, cell suspensions were incubated using cell stain buffer (Biolegend) and brilliant stain buffer (BD) at a 1:1 ratio for 30 minutes on ice in the dark followed by 2 washes with cell stain buffer (Biolegend). Cells were then resuspended in a 1X stabilizing fixative (BD) solution. Intracellular staining was performed using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD) according to the manufacturer's instructions. The following antibodies were used: anti-CD4 (Biolegend, RM4-5), CD25 (Biolegend, PC61), CD3 (BD Biosciences, 145-2C11), CD69 (Biolegend, H1.2F3), CD44 (Biolegend, IM7), CD71 (Biolegend, RI7217), PD-1 (Biolegend, 29F.1A12), anti-Foxp3 (Ebioscience, FJK-16s), anti-IL17A (Biolegend, TC11-18H10.1), IFN γ (Biolegend, XMG1.2), TCR β (BD Biosciences, H57-597), CD8 (BD Biosciences, 53-6.7), CD45 (BD Biosciences, 30F11), Ki-67 (BD Biosciences, B56), GM-CSF (Biolegend, MP1-22E9), IL-22 (Biolegend, Poly5164), IL17F (Biolegend, 9D3.1C8), Tbet (Biolegend, 4B10). Annexin-V staining was done using the FITC Annexin V Apoptosis Detection Kit I (BD) in accordance with the manufacturer's instructions. Acquisition was performed on a FACSymphony cytometer with DIVA software (BD), and data were analyzed using FCS Express Software (De Novo).

Mitochondrial and metabolic dye staining

Naive CD4⁺CD62L⁺CD44⁻ cells were isolated from mouse spleens using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Following isolation naive CD4⁺ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (Thermo Fisher Scientific) at a concentration of 4 µg/mL in a 96-well flat-bottom plate (10 x 10⁴ cells/well) and cultured in either RPMI, Asn-deficient RPMI or RPMI treated with 10 IU/L PEG-AsnASE. For Mitotracker™ green (Thermo Fisher Scientific) and tetramethyl rhodamine, Methyl Ester, Perchlorate (Thermo Fisher Scientific) staining, cells were subsequently incubated at 37°C for 30 minutes in 200 µL of prewarmed RPMI media containing 100 nM MTG and TMRM followed by two washes in 1X DPBS. Viability and cell surface staining was performed as described above.

Seahorse analysis

The Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were evaluated under mitochondrial and glycolysis stress test conditions, respectively, following the manufacturer's instructions and protocols, utilizing a XFe96 Extracellular Flux Analyzer (Agilent). One day prior to the measurement, an Agilent Seahorse XFe96 Sensor Cartridge (Agilent) was hydrated with HPLC grade water in a CO₂-free incubator. On the subsequent day, the solution was replaced with XF Calibrant (Agilent), and the cartridge was maintained in a 37°C CO₂-free incubator for a minimum of two hours. Naive CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 mAbs (Thermo Fisher Scientific) at a concentration of 4 µg/mL in a flat-bottom 48-well plate for 2 days, using either RPMI, Asn-deficient RPMI, or RPMI treated with 10 IU/mL PEG-AsnASE at 0, 6, 12, 24, 36 hours following stimulation. After 48 hours, CD4⁺ T cells were enumerated and transferred to a poly-D-lysine-coated Seahorse XF96 tissue culture microplate

(Agilent) at a density of 100,000 cells/well. Seahorse XP RPMI or DMEM medium (Agilent), comprising, 2 mM L-glutamine, and 1 mM sodium pyruvate, was used for assessment of ECAR and 10 mM glucose was added. In some experiments, asparaginase (AsnASE) from *E. coli* (Sigma-Aldrich) was injected first for a final volume of 10 mM under mitochondrial stress test conditions. In additional experiments, naive CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 mAbs at a concentration of 4 µg/mL in a flat-bottom 6-well plate for 1 day supplemented with either asparagine, alanine, glutamate, aspartate, or proline at a 0.38 mM final concentration. After 24 hours, CD4⁺ T cells were processed as described above.

Chemicals

¹⁵N₂-L-Asn hydrate [Chemical Formula H₂*NCOCH₂CH*(NH₂)COOH:H₂O] was acquired from Cambridge Isotope Laboratories Inc (Cat # NLM-3286-0) with a documented purity ≥ 98% as determined HPLC. For tracing studies, ¹⁵N₂-L-Asn hydrate was dissolved in Asn-deficient RPMI media at a final concentration of 0.38 mM. All PEG-AsnASE experiments were performed using pegaspargase (Oncaspar, Shire Pharmaceuticals, Lexington, MA), an FDA-approved PEGylated form of *E. coli* asparaginase.

Immunoblotting

Equal numbers of cells were washed once with 1X DPBS and lysed by adding 1X SDS sample buffer (Sigma) with subsequent boiling for 15 minutes. The resulting cell extracts were clarified via centrifugation at 13,000 x g, separated through SDS-PAGE, and then transferred to nitrocellulose membranes (Bio-Rad) using electrophoresis. Membranes were then immersed in Tris-buffered saline (TBST) buffer with 3% (w/v) bovine serum albumin (BSA) for a 30-minute blocking period followed by incubation with ASNS primary antibody (Cell Signaling Technology)

diluted in blocking buffer overnight at 4°C. Membranes were washed three times for 1 hour and subsequently incubated with anti-mouse IgG HRP-conjugated secondary antibody (Thermo Fisher Scientific) for 1 hour at RT, followed by 3 washes and visualization using the Western Lightning ECL Pro Chemiluminescence Substrate (PerkinElmer).

Bulk RNA sequencing analysis

The original data was obtained from GSE206607(Thakore et al., 2024) and reanalyzed to examine expression of Asn metabolism-related genes including *Asns* and *Asrgl1* in helper T cells differentiated *in vitro* under T helper 1 (T_H1), non-pathogenic T helper 17 (npT_H17), and pathogenic T helper 17 (pT_H17) polarizing conditions. Reads were aligned to the mm10 genome using Tophat followed by duplicate removal and htseq counts were used for the generation of gene count tables. Read counts were normalized using the DESeq2 package.

Real time PCR

Total DNA from CD4⁺ T cells was extracted using the DNA Micro kit (Qiagen). DNA was quantified using the Qubit™ dsDNA Quantification Assay Kits (Thermo Fisher Scientific). cDNA was subsequently synthesized using the iSCRIPT kit (BioRad). Quantitative PCR analysis was conducted using the SYBR Green Fast Mix (Quanta BioSciences) on a LightCycler 96 Instrument (Roche). Primers used: *Asns*: F: 5'- GATCTTCATCGCACTCAGACA-3', R: 5'- CCTCTGCTCCAC CTTCTCT-3'; *Asrgl1*: F: 5'- GATACTTTCCCCATGTCCTGTG-3', R: 5'- TTGGCTTACGCAACC TCTAC-3'. DNA concentrations were within the linear range of the primers.

OPP Protein Synthesis Assay

Naive CD4⁺CD62L⁺CD44⁻ cells were isolated from mouse spleens using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Following isolation, naive CD4⁺ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (Thermo Fisher Scientific) at a concentration of 4 µg/mL in a 96-well flat-bottom plate (20 x 10⁴ cells/well) and cultured in either RPMI or Asn-deficient RPMI. After 24 hours, 0.38 mM Asn was added to samples cultured in Asn-deficient RPMI and cells were cultured for an additional 4 hours. For detection of nascent protein synthesis, the Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit (Thermo Fisher Scientific) was used in accordance with the manufacturer's protocols. As a positive control, some cell suspensions were treated with 50 µg/mL cycloheximide (Sigma Aldrich) for 30 minutes at 37°C to block protein synthesis. Cell suspensions were then resuspended in 200 µL of a 1X stabilizing fixative (BD) solution followed by flow cytometry assessment for OPP fluorescent intensity using a FACSymphony cytometer with DIVA software (BD).

Isolation of protein and hydrolysis into amino acid monomers

Cell pellets were resuspended in 200 µL of lysis buffer containing 2% SDS, 150 mM NaCl, 50 mM Tris (pH 8.5), proteinase inhibitor mix (Roche), 5 mM DTT, and incubated on ice for 10 minutes followed by incubation at 60°C for 45 minutes. After cooling to room temperature, iodoacetamide was added to each sample for a final concentration of 14 mM, and the samples were incubated for an additional 45 minutes. The treated samples were then mixed with a solution consisting of 3 parts ice-cold methanol, 1 part chloroform, and 2.5 parts H₂O followed by centrifugation at 4000 x g for 10 minutes. The top layer was then removed and 3 parts of ice-cold methanol were added, followed by centrifugation at 4000 x g for 5 minutes. Following removal of the top layer, the samples were mixed with 3 parts of ice-cold acetone, vortexed, and

centrifuged at 4000 x g for 5 minutes. The pellet was then washed with 2 mL of ice-cold acetone and stored at -80°C prior to chemical hydrolysis. The protein pellet obtained was resuspended in 6 N HCl/acetic acid (50:50, 100 µL) and subjected to heating at 95°C for 1 hour. The resulting aqueous solution was diluted into a mixture of 40% acetonitrile, 40% methanol, and 20% water and analyzed by LC-MS.

Induction of EAE

EAE was induced by immunization of 10-week-old female C57BL/6J mice with the Hooke Kit™ MOG₃₅₋₅₅/CFA Emulsion PTX in accordance with the manufacturer's instructions. Briefly, mice were acclimated in our animal facility for at least 7 days prior to immunization. For EAE induction, mice were immunized with 200 µg of antigen (MOG₃₅₋₅₅) in emulsion with complete Freund's adjuvant (CFA) in both flanks followed by administration of 120 ng pertussis toxin (PTX) on the day of immunization and following day. Mice were monitored for signs of clinical disease and scored following the Hooke scoring system

(<https://hookelabs.com/services/cro/eae/MouseEAEscoring.html>). For analysis of cellular infiltrates in the CNS, brain and spinal cords were isolated at the peak of disease. Prior to CNS collection, mice were perfused with 1X DPBS and brains and spinal cords were mechanically dissociated through a 70 µm nylon cell strained followed by digestion with Collagenase D (Sigma-Aldrich) for 20 minutes in a 37°C shaker. Digests were then filtered through a 70 µm strainer, resuspended in a 30% Percoll/DPBS solution and overlaid over a 70% Percoll gradient for mononuclear cell isolation. Following centrifugation at 800 x g for 30 minutes at room temperature, lymphocytes in the interface were collected, washed with RPMI media, and stimulated using a 1X eBioscience™ Cell Stimulation Cocktail plus protein transport inhibitors (Thermo Fisher Scientific) for 4 hours at 37°C. Cells were then centrifuged at 600 x g for 3

minutes followed by resuspension in cell stain buffer (Biolegend) for subsequent flow cytometry staining.

Pathogenic T_H17 differentiation for EAE induction and adoptive transfer

Naive CD4⁺CD62L⁺CD44⁻ cells were isolated from the spleens of female 2D2 TCR transgenic mice using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) in accordance with the manufacturer's protocols. 2x10⁶ naive CD4⁺ T cells were then stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (Thermo Fisher Scientific) at a concentration of 4 µg/mL in a 48 well flat-bottom plate and cultured for 3 days in either RPMI media or Asn-deficient RPMI media under pathogenic T_H17 polarizing conditions: 20 ng/mL IL-6 (Peprotech), 10 ng/mL IL-23 (R&D Systems), 10 ng/mL IL-1β (Peprotech), 10 µg/mL anti-IFN-γ (Biolegend Clone XMG1.2) and 10 µg/mL anti-IL-4 (Biolegend Clone 11B11). Cell suspensions were then rested for 2 days in the absence of TCR stimulation using either RPMI media or Asn-deficient RPMI media containing 20 ng/mL IL-23 (R&D Systems). After two days of rest, cells were restimulated with plate-bound anti-CD3 and anti-CD28 mAbs (Thermo Fisher Scientific) at a concentration of 4 µg/mL in a flat bottom 6 well plate for two days, followed by two washes in 1X DPBS. Following counting a small aliquot of cells to determine viability by flow cytometry, 4x10⁶ T viable cells from each respective culture condition was transferred by intravenous injection into C57BL/6J female recipient mice to induce EAE. Mice were monitored for signs of clinical disease and scored following the Hooke scoring system.

Statistical Analysis

Statistics were computed with GraphPad Prism 9 software (GraphPad Software) using unpaired Student's T-test for comparisons between two groups, one-way ANOVA followed by Tukey's or

Dunnets multiple comparison when comparing three or more groups, or Two-way ANOVA for multiple comparisons within groups. Graphs containing EAE clinical scores represent mean values with error bars representing the Standard Error of the Mean (SEM). Unless noted otherwise, all other data are represented as mean $\pm$ SD. P-values are denoted in figures as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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CHAPTER 3: INVESTIGATING THE EFFECTS OF AMINO ACID TRANSPORTER MODULATION ON T CELL FUNCTION

ATTRIBUTIONS

The experiments in this chapter were performed by Sheila Johnson (Figure 3.1-3.5). Jared H. Rowe and Ilaria Elia conducted the CRISPR screens with assistance from Martin W. LaFleur (Figure 3.1). Sarah Weiss conducted bioinformatics analysis (Figure 3.1). Conghui Yao and Yan Yi Chen assisted with retro-orbital injections and James Han and Lydia Raines assisted with tumor injections (Figure 3.5). Peter Georgiev, James Han, Ayantu Temesgen, and Lydia Raines assisted with tumor and tumor-draining lymph node isolation experiments (Figure 3.5).

INTRODUCTION

The tumor microenvironment (TME) is a complex system that is composed of a diverse repertoire of cell types that exchange metabolites, nutrients, and collectively shape a landscape conducive to tumor progression and immune modulation (Visser et al., 2023). A central aspect of the TME is the sequestration of essential nutrients critical for optimal tumor and antitumor function (Vitale et al, 2019; Srivastava et al., 2010; Bodac et al., 2021). Tumor cells undergo metabolic rewiring that produces a high demand for nutrients such as glucose and amino acids to sustain enhanced glycolytic metabolism and proliferation (Do et al., 2025; Elia et al., 2021). Given the proliferative and highly glycolytic nature of immune effector cells, there is a resulting competition for these key nutrients (Yu et al., 2023; Elia et al., 2021).

For instance, conditioned media derived from the B16-OVA melanoma cell line display reduced glutamine and leucine levels relative to complete media (Elia et al., 2022). Glutamine and leucine are critical for CD8⁺ T cell activation and function (Swamy et al., 2016; Ananieva et al., 2016). These amino acids not only function as proteinogenic substrates but also participate in key signaling pathways and support tricarboxylic acid (TCA) cycle activity and downstream electron transport chain (ETC) function in both CD8⁺ T cells and tumor cells (Ananieva et al.,

2016; Chen et al., 2024). This is one example of many that demonstrates the complex interplay between tumor and immune cells, due to their similar propensity for growth and expansion.

To support their metabolic demand, tumor cells not only upregulate metabolic enzymes to process key extracellular nutrients, but also enhance the expression of nutrient transporters, including SLC2A1, SLC1A5, SLC7A5, SLC7A11, and SLC6A14 (Amann et al., 2009; et al., 2024; Nakanishi et al., 2011). These transporters promote amino acid uptake and exchange activity. Because activated T cells display many similar nutrient requirements as tumor cells upon activation, T cells also accommodate their nutrient demand through modulations of transporter expression. For example, amino acid transporters such as Slc1a5 and slc7a5 are expressed at significantly higher levels in cytotoxic lymphocytes (CTLs) compared to naive cells (Howden et al., 2019). Despite the known upregulation of these transporters in both tumor and immune cells, the specific functional roles of many individual transporters remain poorly defined within the context of the tumor-immune interface.

Metabolic transporters serve as key players in a complex system that maintains homeostatic levels of nutrients and solutes via mechanisms of substrate influx and efflux (Alexander et al., 2021; Bröer et al., 2022). Classification of metabolic transporters has been underway for decades and many substrates and structures have been elucidated (Palacín et al., 1998). Although some transporters have defined substrate specificities with varying degrees of binding affinity, many transporters and respective substrate specificities have yet to be fully characterized (Palacín et al., 1998; Alexander et al., 2021). An additional layer of complexity surrounding metabolic transport systems are differences in substrate transport direction. Some transporters function as uniporters, symporters, or antiporters, in which substrates are transported individually in one direction, two substrates in the same direction, or two substrates in opposing directions, respectively (Palacín et al., 1998). Every cell type contains its own array

of different nutrient transport systems that ultimately promote a net flux of desired nutrients to support its specific energy demands.

Knowing that transporters are an integral part of metabolic crosstalk and nutrient acquisition, we sought to understand their role in the context of T cell and tumor interaction. To gain a better understanding of the potential role of transporters in CD8⁺ T cells in the TME, an *in vivo* CRISPR screen targeting both defined and unexplored transporters was performed. From this screen an understudied transporter, namely Slc43a2, was identified. This chapter describes my studies to investigate this transporter in the context of CD8⁺ T cell antitumor function.

RESULTS

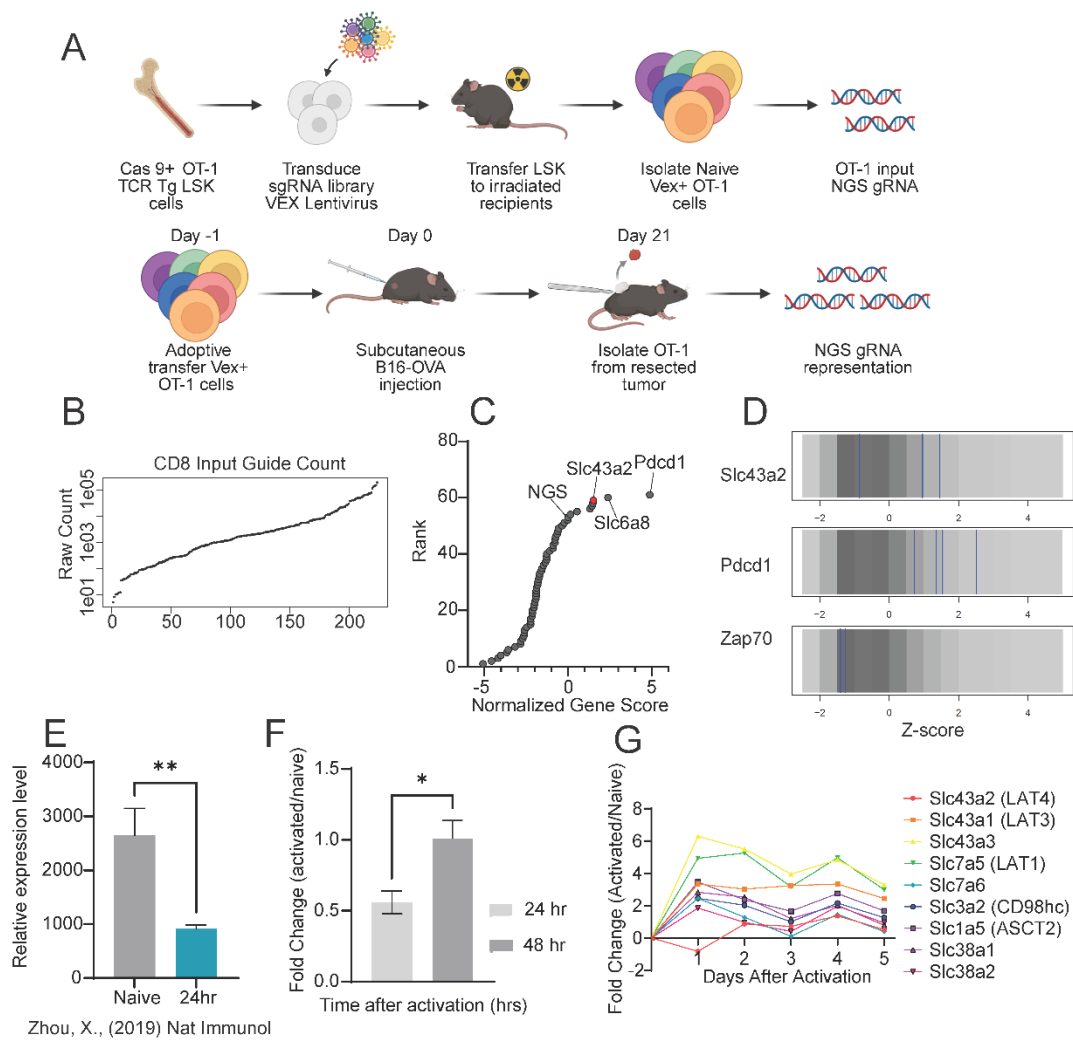
Development of an in vivo CHIME screen of metabolite transporters in CD8⁺ T cells.

To identify metabolic transporters of consequence in CD8⁺ T cells in the context of the TME, an *in vivo* CRISPR screen was employed. A CRISPR/Cas9 bone marrow delivery system, CHimeric IMmune Editing (CHIME), was used to target multiple metabolic transporters in hematopoietic progenitor cells from Cas9 expressing OT-I TCR transgenic mice (**Figure 3.1A**). CHIME enables the studies of gene function in naive T cells without genetically modifying them post differentiation *ex vivo* (Lafleur et al., 2019a; Lafleur et al., 2019b). The hematopoietic progenitor cells used for this screen consisted of Lineage- Sca-1⁺ c-Kit⁺ (LSK) cells that were transduced with lentivirus containing violet excited GFP (Vex) and a pool of gRNAs targeting 56 metabolic transporters and receptors of varying substrates/ligands including amino acids, nucleotides, organic compounds, monocarboxylate acids, neurotransmitters, sugars, polyamines, and others. This gRNA library contained 3 guides per targeted gene, 20 non-targeting controls, and gRNAs targeting genes that are known to inhibit or improve T cell

Figure 3.1 (following page). *In vivo* metabolic CRISPR screen reveals overrepresented transporters in tumor infiltrating lymphocytes.

(A) Schematic describing the CRISPR screen design. (B) Log10 NGS read counts of each gRNA. (C) Rank of genes from transporter targeted screen based on gRNA abundance normalized to input. (D) Plots indicating Z-score of each guide for Slc43a2 and controls Zap70 and Pdcd1. Individual guides targeting Slc43a2, Pdcd1, and Zap70 are represented by a blue line. Grey represents the background distribution of the library. (E) RNA seq data from Zhou et al., 2019 indicating expression level of Slc43a2 in naive and 24 hour activated OT-1 CD8⁺ T cells. (F) Relative fold change in expression of Slc43a2 in CD8⁺ T cells activated for 24 and 48 hours relative to naive state. Fold change was calculated using quantitative polymerase chain reaction (qPCR) Ct values. (G) qPCR analysis detailing the fold change in Slc43a2 and related transporters over the course of multiple days of CD8⁺ T cell activation. Results shown as mean $\pm$ SD (E), mean $\pm$ SEM (F), or mean (G) and are representatives of 2 independent experiments (F-G). *p < 0.05, **p < 0.01 Student's t test (E-F)

Figure 3.1 (Continued)



function in the context of the TME, including Zap70, CD28, and Pdc1. Two hundred thousand transduced cells were transferred to each irradiated mouse.

In vivo CHIME screen identifies potential regulators of T cell antitumor function

After immune reconstitution (~8 weeks) naive OT-1 CD8⁺CD44^{low} Vex⁺ cells were isolated and sorted via fluorescence-activated cell sorting (FACS) and transferred to C57BL/6 mice with a different congenic marker (**Figure 3.1A**). The following day mice were injected with syngeneic B16 melanoma tumor cells displaying the ovalbumin peptide, thus promoting the T cell-tumor antigen specific response. On day 21, tumors were resected from mice and tumor infiltrating OT-1 cells were isolated via FACS sorting based on its distinct congenic marker. Genomic DNA isolated from a portion of cells was processed for next generation sequencing (NGS) and subsequent STARS analysis to determine the relative gRNA frequency of the reconstituted pool. To determine if any guides were selected against during immune reconstitution, the number of raw sequencing reads were plotted for each guide (**Figure 3.1B**). None of the targeted genes from the screen fell below the inflection point of the plot, which suggests that the targeted guides were relatively well represented (**Figure 3.1B**).

To account for differences in gRNA abundance observed post-screen, gRNA frequency was normalized to input gRNA abundance (**Figure 3.1C**). Following our expectations, gRNAs targeting internal controls were properly represented in the resulting screen. Zap70 was the second most underrepresented guide in the pool while gRNAs targeting Pdc1 (gene encoding PD-1) was the most represented in the pool. Of the targets of interest, gRNA targeting Slc6a8 and Slc43a2 were the most represented (**Figure 3.1C**). A critical role for creatine transport via Slc6a8 has already been described in the context of CD8⁺ T cell antitumor function (Di Biase et al., 2019; Samborska et al., 2022), however the role of Slc43a2 has been minimally studied in

mammalian systems, and even less so in immune-related cell types, leading to our focus on Slc43a2.

Slc43a2 displays dynamic expression kinetics in CD8⁺ T cells

Slc43a2 encodes an amino acid transporter, LAT4. Earlier studies have proposed that this transporter has a substrate preference for branched chain amino acids (leucine, isoleucine, and valine), methionine, and phenylalanine (Bodoy et al., 2005). It has been classified as a uniporter that transports one substrate in an ion independent manner and in some tissues, it primarily functions as an exporter of amino acids. Considering its representation in the screen, we sought to gain a better understanding of its expression kinetics throughout CD8⁺ T cell activation. To determine the expression of Slc43a2 at varying stages of T cell activation, we utilized the Immgen RNA-seq dataset detailing the relative expression of genes in various immune cell types, and a published RNA-seq dataset exploring activation of OT-1 T cells stimulated with anti-CD3 and anti-CD28. Based on the Immgen dataset, the relative expression level of Slc43a2 is the highest in the naive state and after LCMV antigen exposure, transcript levels decrease. CD8⁺ T cells in the memory differentiated state contain the lowest level of Slc43a2 transcripts. Based on work from Zhou et al., naive OT-1 T cells contain a higher level of Slc43a2 transcripts compared to OT-1 cells activated for 24 hours (**Figure 3.1E**). To verify the expression levels of Slc43a2, naive C57BL/6 CD8⁺ T cells were activated on plates coated with anti-CD3 and anti-CD28 and intracellular RNA content was collected at 24 and 48 hours and processed for downstream quantitative polymerase chain reaction (qPCR) experiments. Results show that after 24 hours of activation, Slc43a2 transcript levels decreased, however, after 48 hours, Slc43a2 levels increased (**Figure 3.1F**). An extended time-course experiment was performed in which transcript levels of Slc43a2 and additional transporters with similar substrate specificities

were measured (**Figure 3.1G**). In line with previous observations, Slc43a2 levels decreased upon activation, however nearly all the other transporters increased upon activation. After 48 hours, Slc43a2 transcript levels increased in a manner similar to other transporters (**Figure 3.1G**). Taken together, this data indicates that Slc43a2 displays a unique dynamic expression pattern after activation.

Slc43a2 limits CD8⁺ T cell activation

Upon activation, CD8⁺ T cells upregulate their transporters, which ultimately can support the nutrient demand necessary for optimal activation and proliferation. Since Slc43a2 was overrepresented in the screen and it primarily functions as an amino acid exporter in intestinal epithelial tissues (Rajendran et al., 2020), we hypothesized that Slc43a2 ablation may increase the net flux of nutrients inside the cell and support CD8⁺ T cell function. To determine the effects of Slc43a2 expression modulation on CD8⁺ T cell function, we utilized a retrovirus system to introduce shRNA targeting Slc43a2 transcripts (**Figure 3.2A**). To do this, we isolated naive CD8⁺ T cells from spleens and/or lymph nodes of C57BL/6 mice using magnetic bead separation and seeded cells on plates coated with anti-CD3 and anti-CD28 in complete RPMI with IL-2 and IL-12. After 24 hours of activation, cells were collected and processed for spinfection with retrovirus containing shRNA and green fluorescent protein (GFP). Two days post spinfection, cells were FACS-sorted based on GFP expression and a portion were collected for qPCR analysis to verify knock down of Slc43a2 and the remaining fraction of cells were cultured in IL-7 for two days (**Figure 3.2A**). Quantification of RNA content revealed an 80% reduction in Slc43a2 transcripts. After 2 days, cells were restimulated via plates coated with anti-CD3 and anti-CD28 and analyzed 24 hours later. Upon restimulation, we observed increases in CD44 and IFN- γ expression in Slc43a2 ablated T cells, however, granzyme B

levels did not change (**Figure 3.2B-G**). In addition, the frequency of Ki67 positive cells was higher in Slc43a2 ablated cells compared to control (**Figure 3.2H-I**). All together our findings imply that Slc43a2 ablation provides a boost during CD8⁺ T cell activation.

Slc43a2 limits proliferation and effector function of activated CD8⁺ T cells

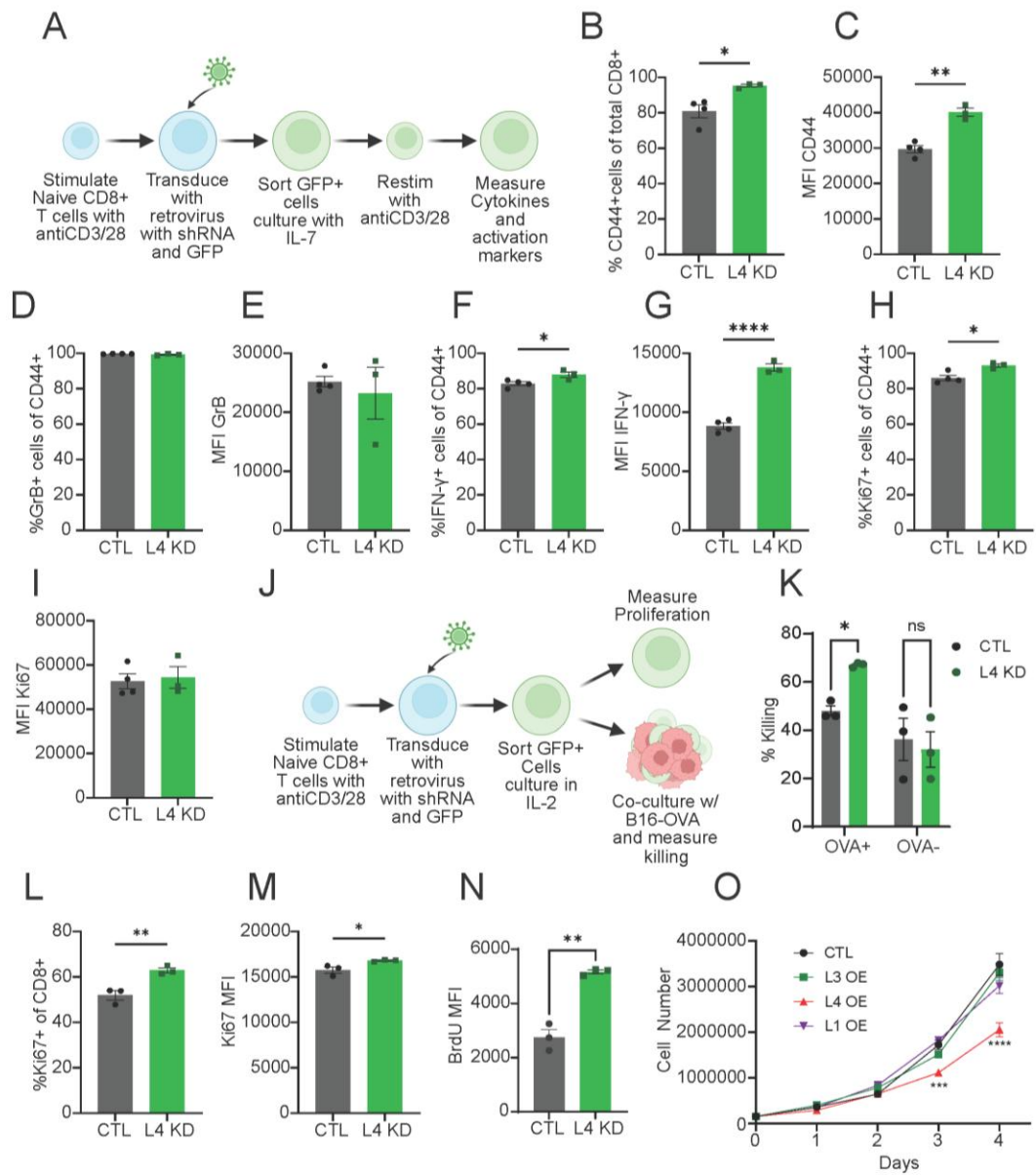
Considering our observations of Slc43a2 ablation benefiting CD8⁺ T cell activation, we next determined how Slc43a2 affected T cell cytotoxicity and proliferation. To do this, we isolated naive OT-1 CD8⁺ T cells from spleen and/or lymph nodes from mice using magnetic bead separation and stimulated and transduced them in a similar manner described above. GFP⁺ cells were collected via FACS sorting and cultured overnight (**Figure 3.2J**). The following day, the cells were co-cultured with B16-OVA melanoma tumor cells for 24 hours, after which remaining tumor cells were measured. Slc43a2 ablation provided a boost in effector killing function in an antigen specific manner (**Figure 3.2K**). We also explored the effects of Slc43a2 ablation on CD8⁺ T cell proliferation. Slc43a2 KD cells were acquired as previously described and cultured in RPMI with IL-2. Slc43a2 ablated cells expressed a higher percentage of Ki67⁺ cells and enhanced Ki67 expression compared to control (**Figure 3.2L-M**). To further assess proliferative capacity, Slc43a2 ablated cells were pulsed with bromodeoxyuridine (BrdU) for 1.5 hours prior to analysis and demonstrated enhanced uptake of the nucleoside analog (**Figure 3.2N**).

Seeing that Slc43a2 ablation promotes an enhanced proliferative phenotype, we next determined if Slc43a2 overexpression would result in a converse effect. We transduced Jurkat cells with overexpression vectors that promote LAT family transporter expression. Of all the overexpressed transporters, only Slc43a2 was sufficient to induce a proliferation defect

Figure 3.2 (following page). Slc43a2(LAT4) limits CD8⁺ T cell activation and proliferation.

(A) Schematic detailing method of Slc43a2 ablation (L4 KD) and restimulation. (B) Quantification of the proportion of CD8⁺ T cells expressing CD44. (C) Expression level of CD44 on a per cell basis following 24 hours of restimulation. (D) Quantification of the proportion of CD8⁺ CD44⁺ T cells expressing Granzyme B (GrB). (E) Expression level of GrB on a per cell basis following 24 hours of restimulation. (F) Quantification of the proportion of CD8⁺ CD44⁺ T cells expressing IFN- γ . (G) Expression level of IFN- γ on a per cell basis following 24 hours of restimulation. (H) Quantification of proportion of CD8⁺ CD44⁺ cells expressing Ki67. (I) Expression level of Ki67 on a per cell basis following 24 hours. (J) Schematic detailing method of LAT4 ablation and subsequent effector and proliferation analyses. (K) Quantification of proportion of B16 tumor cells with or without OVA presentation killed after co-culture with CTL or L4 KD T cells for 24 hours. (L) Quantification of proportion of CD8⁺ T cells expressing Ki67. (M) Expression level of Ki67 on a per cell basis. (N) Quantification of bromodeoxyuridine (BrdU) intracellular level in CD8⁺ T Cells. (O) Growth curve of Jurkat cells overexpressing LAT1, LAT3, and LAT4. Results are shown as mean $\pm$ SEM and are representatives of 2 independent experiments (B-I, K-O). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 Student's t test (B-I, K-N) Two-way ANOVA with Dunnet's multiple comparison test (O)

Figure 3.2 (Continued).



(**Figure 2O**). These results suggest that Slc43a2 limits the effector and proliferative capacity of activated T cells.

Slc43a2 ablation promotes CD8⁺ T cell proliferation and survival in late stages of in vitro culture

The CRISPR screen revealed that Slc43a2 guides were overrepresented in tumors that developed over the course of three weeks. Considering the extended period in which Slc43a2 overrepresentation was observed, we next tested if there were any long-term benefits of Slc43a2 ablation on CD8⁺ T cell function *in vitro*. To probe this, we generated Slc43a2 ablated T cells in a similar manner aforementioned. Once positively transduced cells were identified we cultured cells up to 12 days post activation (**Figure 3.3A**). Slc43a2 ablation resulted in a higher frequency of viable cells after extended culture, compared to controls (**Figure 3.3B**). In addition, Slc43a2 ablated cells exhibited a higher frequency of TCF1 expression, which is an indicator of stemness (**Figure 3.3C-D**). We also evaluated CD8⁺ T cell proliferation, twelve days after activation, by pulsing CD8⁺ T cells with BrdU for 1.5 hours prior to analysis. Slc43a2 ablated cells demonstrated enhanced frequency and uptake of the nucleoside analog, indicating that Slc43a2 ablation promotes proliferation at later stages of CD8⁺ T cell life cycle (**Figure 3.3E-F**).

Slc43a2 modulation alters amino acid levels and salvage and de novo nucleotide synthesis intermediates

Since Slc43a2 is described as an amino acid transporter, we sought to understand the metabolic consequence of its modulation. We generated Slc43a2 ablated, and control cells as previously described, cultured them in complete RPMI for 6 hours and subsequently cells were added to quenching solution. This process allowed for the termination of metabolic processes

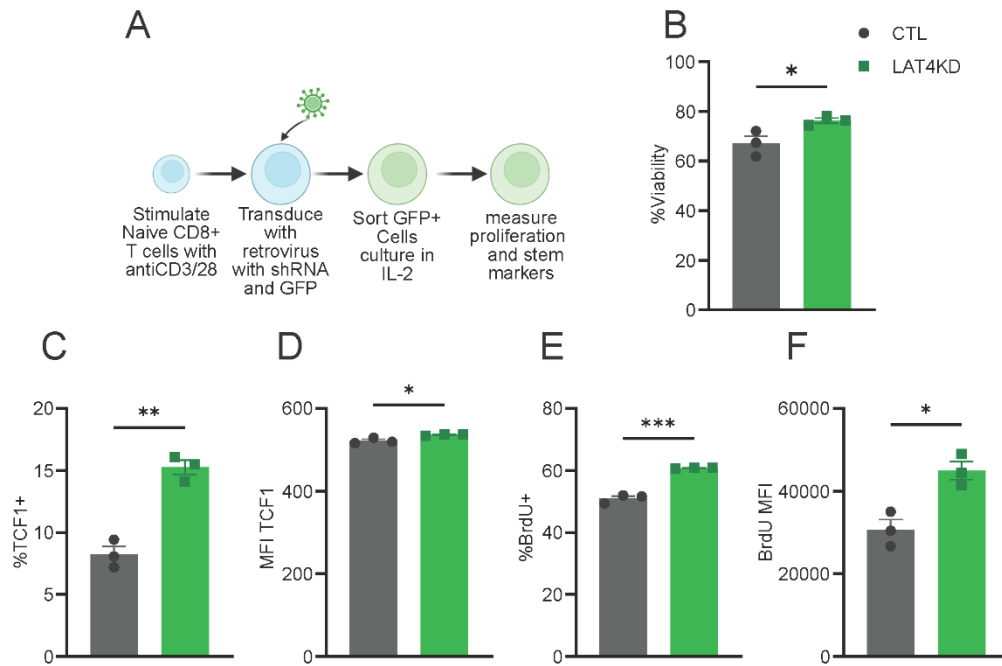


Figure 3.3. Slc43a2(LAT4) ablation promotes enhanced survival and proliferation at later stages.

(A) Schematic of experimental method of Slc43a2 ablation (LAT4KD) and subsequent analysis 12-days post initial activation. (B) Quantification of viable cells 12 days post initial activation. (C) Quantification of proportion of CD8⁺ T cells expressing TCF1 12 days post activation. (D) Expression level of TCF1 on a per cell basis. (E) Quantification of proportion of CD8⁺ T cells with BrdU incorporation 12 days after activation. (F) Measurement of intracellular level of BrdU incorporation. Results are shown as mean $\pm$ SEM and are representatives of 2 independent experiments (B-F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Student's t test (B-F)

while maintaining cell membrane structure. Metabolites were extracted from quenched cell pellets and prepared for pHILIC liquid chromatography–mass spectrometry (LC-MS) analysis. Data analysis identified metabolites that are upregulated and downregulated due to Slc43a2 ablation (**Figure 3.4A-B**).

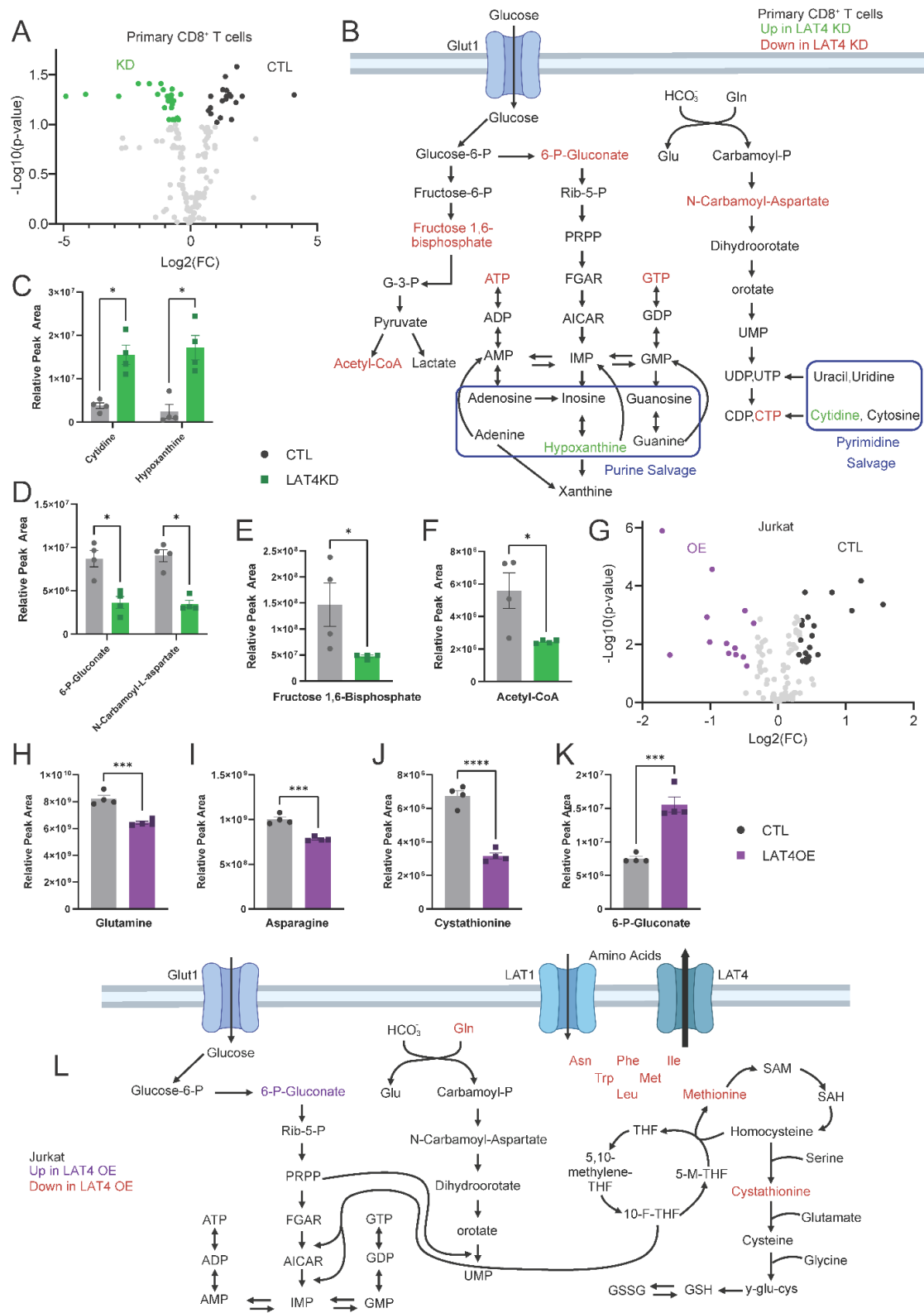
Interestingly, two related metabolic pathways were inversely represented due to Slc43a2 ablation (**Figure 3.4B**). Hypoxanthine and cytidine, which are nucleobases part of the purine and pyrimidine salvage pathway, were upregulated with Slc43a2 ablation (**Figure 3.4B-C**). Conversely, metabolites part of the de novo nucleotide synthesis pathway, such as N-carbamoyl-aspartate and 6-P-gluconate, were downregulated with Slc43a2 ablation (**Figure 3.4B, D**). Additional metabolites that were altered include fructose 1-6 bisphosphate and acetyl-CoA, which are metabolic intermediates of the glycolytic and TCA cycle respectively (**Figure 3.4B, E-F**). All the metabolic pathways in which intermediates have been altered due to Slc43a2 ablation are connected to cell growth and proliferation. However, steady-state metabolomics alone are not sufficient to indicate the rate of metabolic flux within these pathways. Thus, these results help identify metabolic pathways that can be further interrogated through metabolic tracing studies to determine if and to what extent Slc43a2 ablation alters the metabolic flux through these pathways.

Considering that Slc43a2 functions as an exporter in some cell types, and its over expression results in reduced proliferation in Jurkat cells, we sought to determine the metabolic status associated with this state. To this end, we utilized a Jurkat cell line transfected with a vector designed to overexpress Slc43a2 cDNA. After positive selection and validation, cells were cultured for 6 hours in complete medium and subsequently harvested for metabolite extraction and LC-MS (**Figure 3.4G**). Upon analysis, we observed that all known substrates of

Figure 3.4 (following page). Steady-State metabolomics of T cells with Slc43a2(LAT4) modulation.

(A) Volcano plot showing distribution of metabolite fold changes in activated CD8⁺ T cells with and without Slc43a2 ablation (KD) after 6 hours in RPMI. Metabolites in green are upregulated in LAT4 KD CD8⁺ T cells and metabolites in gray are upregulated in CTL CD8⁺ T cells. Significant changes were determined by the following threshold, fold Change>1.25, p value <0.1, FDR correction (B) Metabolic map of significant hits from experiment A. Metabolites in green are upregulated in LAT4 KD CD8⁺ T cells and metabolites in red are downregulated in LAT4 KD CD8⁺ T cells. (C) Relative peak areas of cytidine and hypoxanthine in CTL and LAT4 KD cells. (D) Relative peak areas of 6-P-gluconate and N-carbamoyl-aspartate in CTL and LAT4 KD cells. (E) Relative peak areas of fructose 1-6-bisphosphate in CTL and LAT4 KD cells. (F) Relative peak areas of acetyl-CoA in CTL and LAT4 KD cells. (G) Volcano plot showing distribution of metabolite fold changes in Jurkat T cells with and without Slc43a2 overexpression (LAT4OE) after 6 hours in RPMI. Metabolites in purple are upregulated in LAT4OE Jurkat cells and metabolites in gray are upregulated in CTL Jurkat. Significant changes were determined by the following threshold, fold Change>1.25, p value <0.1, FDR correction. (H) Relative peak areas of glutamine in CTL and LAT4OE cells. (I) Relative peak areas of asparagine in CTL and LAT4OE cells. (J) Relative peak areas of cystathionine in CTL and LAT4OE cells. (K) Relative peak areas of 6-P-gluconate in CTL and LAT4OE cells. (L) Metabolic map of significant hits from experiment G. Metabolite in purple is upregulated in LAT4 OE cells and metabolites in red are downregulated in LAT4 OE cells. Fold Change>1.25, p value <0.1, FDR correction. Results are representatives of 2 independent experiments. *p < 0.05, ***p < 0.001, ****p < 0.0001 Student's t test (C-F, H-K)

Figure 3.4 (Continued).



LAT4 were reduced in LAT4 overexpression T cells (**Figure 3.4L**). Interestingly, amino acids glutamine, asparagine, and tryptophan were also reduced in these cells due to LAT4 overexpression (**Figure 3.4H-I, L**).

Additional metabolites not previously connected to LAT4 transport activity but altered in this context include cystathionine and 6-P-gluconate (**Figure 3.4J-L**). Reduced levels of cystathionine may indicate a reduction in glutathione production, thus rendering cells more susceptible to oxidative stress and resulting survival and/or proliferation defects. Interestingly, 6-P-gluconate, a metabolic intermediate of de novo nucleotide synthesis pathway, was elevated in Slc43a2 OE cells yet downregulated in Slc43a2 KD cells (**Figure 3.4B, L**). Since Slc43a2 upregulation and downregulation results in contrasting proliferative phenotypes, it may suggest that de novo nucleotide synthesis is a metabolic pathway of consequence in states of amino acid deprivation.

Slc43a2 limits CD8⁺ T cell tumor control

To validate the CRISPR screen, we sought to understand the effects of Slc43a2 ablation *in vivo*. Our screen deleted Slc43a2 in OT-1 LSK cells that subsequently differentiated into naive OT-1 T cells. To replicate this process, we isolated naive CD8⁺ T cells from the spleens and lymph nodes of OT-I mice and nucleofected them with a ribonucleoprotein (RNP) complex containing Cas9 and gRNA targeting Slc43a2 (**Figure 3.5A**). After 6 days in culture with IL-7, live cells were FACS sorted. A portion of the cells were collected for gDNA isolation and processed to determine the relative percentage of inserts and deletions (indel) in the Slc43a2 gene region. Our nucleofection method resulted in 90% indel formation. Thus, we moved forward with this method for downstream *in vivo* experiments.

To ascertain the effects of Slc43a2 ablation on CD8⁺ T cells effector function *in vivo*, we transferred naive OT-I nucleofected cells into C57BL/6 mice via retro-orbital injection and the following day mice were implanted with B16-OVA melanoma tumor cells subcutaneously (**Figure 3.5A**). Tumors were measured every 2-3 days to determine differences in tumor control. As expected, tumors grew robustly in mice that received no T cell transfer (**Figure 3.5B-D**). Mice that received control OT-1 T cells demonstrated some tumor clearance, but tumors outgrew at a rate that was not significantly different from the no T cell transfer group (**Figure 3.5B-D**). Interestingly, mice that received Slc43a2 ablated T cells exhibited the most clearance and their tumor growth was significantly different from the mouse group that did not receive control T cells. By recording tumor growth up until the experimental endpoint for each mouse, we observed that mice treated with Slc43a2 ablated cells exhibited more tumor clearance and enhanced survival compared to mice treated with CTL T cells. (**Figure 3.5E**). Taken together, our data implies that Slc43a2 limits CD8⁺ T cell mediated tumor control.

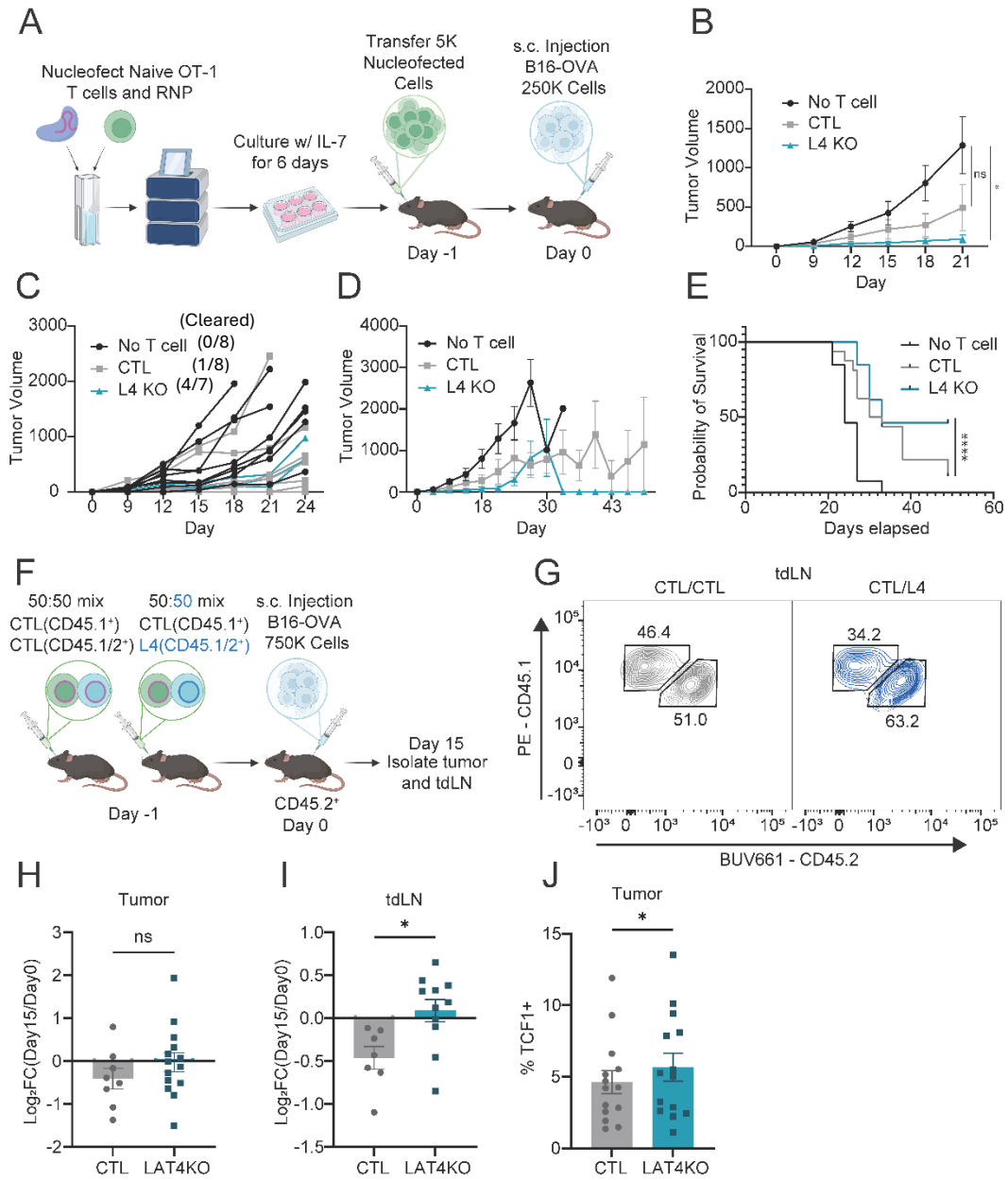
Slc43a2 ablation promotes T cell accumulation in tdLN

Given our results revealing a trend of enhanced tumor control with Slc43a2 deletion, we next investigated the T cell intrinsic effects of Slc43a2 ablation *in vivo*. We performed a co-transfer experiment in which both control and Slc43a2 ablated cells were co-transferred into mice and B16 OVA tumor cells were implanted one day later (**Figure 3.5F**). The co-transfer experiment consisted of congenically distinct populations of naive OT-1 T cells that were nucleofected with either Slc43a2(LAT4) gRNA or CTL nontargeting gRNA (**Figure 3.5F**). After 6 days in culture with IL-7, live cells were sorted and 5,000 cells from each population were mixed and transferred into congenically distinct mice. On day 15, tumors and tumor draining lymph nodes (tdLNs) were resected and lymphocytes were isolated and processed for flow cytometry

Figure 3.5 (following page). Slc43a2 (LAT4) ablation enhances CD8⁺ T cell accumulation and antitumor function *in vivo*.

(A) Schematic detailing method of Slc43a2 knock-out (L4 KO) in naive OT-1 CD8⁺ T cells and subsequent subcutaneous (s.c) injection of B16-OVA melanoma tumor cells. (B) Average tumor growth curves showing tumor volume in mice after adoptive transfer of naive OT-1 CD8⁺ T cells (n=8), naive OT-1 CD8⁺ T cells with LAT4 ablation (n=7), or no transfer of CD8⁺ T cells (n=8). (C) Tumor growth curves of individual mice with no T cell transfer, naive OT-1 T cell transfer, and naive OT-1 LAT4 KO transfer from experiment in B. (D) Extended average tumor growth curve showing tumor volumes of mice up to experimental end point from experiment B. (E) Combined Kaplan-Meier survival curve from experiment B and an additional independent experiment. (F) Schematic representing *in vivo* competitive co-transfer experiment consisting of congenically distinct LAT4 ablated (L4) naive OT-1 CD8⁺ T cells and control (CTL) naive OT-1 CD8⁺ T cells. (G) Representative contour plot of relative frequency of each transferred population from the tumor draining lymph node (tdLN) 15 days after tumor initiation. (H) Collective fold change of frequencies of transferred populations from the tumor normalized to input. (I) Collective fold change of frequencies of transferred populations from the tdLN normalized to input. (J) Frequency of transferred TCF1⁺ CD8⁺ T cells from the tumor. Results are shown as mean $\pm$ SEM and are representatives of 2 independent experiments (B-D, G) or pooled from 2 independent experiments (E, H-J). Not significant (ns), *p < 0.05, ****p < 0.0001 Two-way ANOVA with Tukey's multiple comparison test (B) Log rank test (E) Student's t test (H-J)

Figure 3.5 (Continued).



analysis (**Figure 3.5F**). By comparing the frequencies of congenically distinct CTL T cell and Slc43a2 ablated T cell groups, we observed that the two populations did not significantly differ in frequency in the tumor (**Figure 3.5H**). However, there was a significant difference in frequency between the two populations in the tdLN (**Figure 3.5G, I**). This frequency difference in the tdLN may indicate that a larger pool of cells was present at the initial priming stage and this increased pool may provide an explanation for the sustained tumor growth control observed in the single transfer experiments. If Slc43a2 ablation results in a greater pool in the tdLN it may serve as a consistent source of effector T cells over the course of tumor progression and thus promote enhanced tumor control. Upon comparing the frequency of markers expressed in the T cell populations found in the tumor, we identified a difference in the frequency of TCF1 positive cells (**Figure 3.5I**). This is of great interest because this result coincides with our *in vitro* experiments showing that T cells cultured for extended periods with Slc43a2 ablation also display a higher frequency of TCF1 positive cells (**Figure 3.3C**). All together our results indicate that Slc43a2 ablation promotes accumulation of CD8⁺ T cells upon antigen exposure, which may support the trend in enhanced tumor growth control.

DISCUSSION

CD8⁺ T cells are key mediators of antitumor function and thus require an extensive supply of nutrients to carry out antitumor functions (Raskov et al., 2021; Raynor et al., 2024; Giles et al., 2023). Many studies have investigated the essentiality of various metabolites that help fulfill CD8⁺ T cell nutrient requirements including amino acids, glucose, and lipids (Raynor et al., 2024; Yu et al., 2023). Extracellular nutrients can be acquired through various methods including endocytosis and transmembrane transporters (Charpentier et al., 2021; Wang et al., 2020; Ren et al., 2017; Palm et al., 2017) Although these mechanisms of nutrient import have

been identified, there is still a lack of characterization of proteins that mediate the transport process. In efforts to better understand the functional consequence of these transporters and receptors in the regulation of CD8⁺ T cell activity, we performed an *in vivo* metabolic CRISPR screen including known genes for transporters involved in CD8⁺ T cell activation as well as many understudied genes involved in carbohydrate, amino acid, lipid, hormone, and solute transport and signaling. One consideration in this screen and many other screens that seek to understand metabolic dependencies of transporters is that many transporters have overlapping substrate specificities. Thus, removal of a single transporter may elicit little to no effect due to transport function redundancy. This is reflected in our screen, given that the absence of known critical transporters such as Slc7a5 and Slc3a2 were moderately represented, likely stemming from the fact that other transporters with similar substrate specificities were still present. An additional potential contributor to minute differences in gRNA abundance among the selected targets is the process of transporter compensation. For example, in a model of osteosarcoma, removal of amino acid transporter, Slc1a5, promoted expression of transporters Slc38a1 and Slc38a2 (Broer et al., 2019). Thus, it would be interesting to perform single cell-omic studies with transporter modulation to carefully characterize compensatory mechanisms and possibly reveal metabolic transporter co-dependencies.

Previous studies have investigated Slc43a2 in other cell types found in the TME, including T regulatory (Treg) cells and cancer cells. Interestingly, cancer cells benefit from the presence of Slc43a2 whereas other cells display reduced proliferation with its expression. For example, Bian et al., observed that treatment of tumor cells with LAT transporter inhibitor, BCH, reduced tumor growth and authors imply that targeting Slc43a2 could serve as a viable anti-cancer therapy. However, BCH targets all LAT transporters, including Slc7a5, which is one of primary neutral amino acid importers highly expressed in many mammalian cancers (Bian et al.,

2020; Lee et al., 2025; Nakanishi et al., 2011). Conversely, when assaying multiple human cancer cell lines for SLC43A2 dependency using the DepMap database, we see that the majority of cancer cell lines display a proliferation benefit when SLC43A2 is depleted (Arafah et al., 2025). This is consistent with our observations showing that Slc43a2 ablation promotes murine CD8⁺ T cell proliferation, and Slc43a2 overexpression results in a proliferation defect in Jurkat cells. A study from Chidley et al., revealed that both Slc43a2 and paralog Slc43a1 CRISPRa induction decreases intracellular accumulation of neutral amino acids leucine, phenylalanine, and valine in complete media conditions in K562 chronic myelogenous leukemia cells (Chidley et al., 2024). In line with this, we observed that overexpression of SLC43A2 in Jurkat cells resulted in reduced steady state levels of branched chain amino acids, methionine, phenylalanine, and asparagine. An additional study, exploring Notch signaling in Treg cells cultured in IL-2 deficient conditions, suggests that Slc43a2 amino acid transporter activity promotes T regulatory cell (Treg) survival. Treg function primarily relies on mitochondrial fatty acid oxidation (FAO), and the presence of amino acids can increase mTOR function and reduce FAO (Michalek et al., 2011). Thus, Slc43a2 ablation may serve as a negative regulator of Tregs because it promotes higher amino acid levels and mTOR function and restricts proper Treg activity and survival. Since authors did not provide a direct mechanism of Slc43a2 function in the study, it would be interesting to more carefully analyze the transport function of Slc43a2 in Treg and other immune cell types with a range of proliferative and glycolytic demands and correlate proliferative need and Slc43a2 expression levels.

In this study, we observed that Slc43a2 depletion promotes CD8⁺ T cell activation marker expression and proliferation *in vitro*. Many of our observations revealing the benefit of Slc43a2 ablation were conducted in preactivated CD8⁺ T cells. Because the greatest expression of Slc43a2 is observed in naive cells, it would be interesting to further test the effects of Slc43a2

ablation in naive cells and evaluate its activation and metabolic reprogramming. Moreover, assessing the rate of amino acid uptake over the course of naive CD8⁺ T cell activation in the presence or absence of Slc43a2 would shed light on the CD8⁺ T cell specific amino acid flux. In addition to amino acid tracing studies, metabolic tracing studies exploring glucose and hypoxanthine metabolic fates would be interesting to investigate, considering the steady state changes observed in both salvage and de novo nucleotide metabolites.

Other cell types also have been subjected to Slc43a2 genetic modifications and subsequent intracellular amino acid measurements. These studies show that different cell types accumulate different amino acids. For example, Slc43a2 ablation in intestinal epithelial cells promoted the accumulation of intracellular methionine and leucine, whereas in K562 erythroleukemia cells, SLC43A2 overexpression resulted in intracellular depletion of previously defined Slc43a2 substrates and novel substrates, proline and tyrosine (Rajendran et al., 2020; Chidley et al., 2024). This variation in amino acid transport may point to species specific substrate preferences for Slc43a2 transport.

It is interesting that cell types that require a large amount of nutrients express a transporter that functions primarily as an exporter of preferred substrates. Since essential amino acids cannot be synthesized intracellularly, it may be challenging to rationalize the presence of an essential amino acid exporter. One possible explanation for the presence of an exporter in a proliferating cell is that transporters can function in a network of many other transporters that all work together to produce a nutrient net flux benefit. For example, in HeLa cells, amino acid transporter Slc1a5 promotes glutamine import, and this import activity is essential for Slc7a5 antiporter function in which glutamine export promotes the import of neutral amino acids (Nicklin et al., 2009). This exchange activity has also been described for asparagine but the transporters have not yet been defined (Krall et al., 2016). An additional transporter network examining

transporters colocalized in proximal kidney tubule and small intestinal membranes, was examined more closely in *Xenopus* oocytes (Ramadan et al., 2007). Ramadan et al., revealed that TAT1 (Slc16a10) effluxes aromatic amino acids that are subsequently imported by the neutral obligatory amino acid antiporter LAT2-4f2hc (Slc7a8 and Slc3a2), which ultimately promotes the export of neutral amino acids (Bröer et al., 2023; Ramadan et al., 2007). Thus, in the case of CD8⁺ T cells, it would be interesting to identify a transporter network involving Slc43a2 by performing a transmembrane protein-targeted proteomics experiment. Careful characterization of T cell transporter proteomes could reveal novel areas of cell regulation and downstream candidates for drug targets.

Our observations showing that Slc43a2 limits CD8⁺ T cell tumor control reveal a potential therapeutic approach that could be further exploited. Given its localization, Slc43a2 could serve as an ideal target for small molecule therapies or biologics. In addition, in future it would be interesting to see if there is a synergistic effect of Slc43a2 ablation with anti-PD-1 administration. As previously mentioned, a complete characterization of CD8⁺ T cell transmembrane transporter proteome has yet to be completed, however, it would be of great benefit to identify additional transporters that may be limiting for T cell antitumor function and subsequently exploited for therapeutic gain. However, given the propensity for cancer and T cells to demonstrate similar nutrient dependencies and likely transporter function, it may be challenging to target a negative regulator of CD8⁺ T cells without enhancing tumor progression upon perturbation. In summary, are results show that Slc43a2 limits T cell proliferation and survival, and its ablation promotes CD8⁺ T cell antitumor immunity.

METHODS AND MATERIALS

Cell lines

Platinum-E cells were selected in 1 µg/ml puromycin and 10 µg/ml blasticidin (Thermo Fisher Scientific; cat. A11139-03). B16 cells expressing chicken ovalbumin (B16-OVA) used for *in vivo* experiments, and Platinum-E cells were cultured in DMEM (Thermo Fisher Scientific; cat. 11965-118) supplemented with 10% FBS (Corning; cat. 35-010-CV), 100 units/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific; cat. 5140-122). B16-OVA with a GFP marker and B16 with a RFP marker were generated by retroviral transduction as previously described (Drijvers et al., 2021) and used for *in vitro* experiments. The cell line was grown in RPMI-1640 (Thermo Fisher Scientific; cat. 11875-119) supplemented with 10% FBS (Corning; cat. 35-010-CV), 100 units/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific; cat. 5140-122), 10 mmol/l HEPES (Thermo Fisher Scientific; cat. 15630-130), 1 mmol/l sodium pyruvate (Thermo Fisher Scientific; cat. 11360-070), and 55 mmol/l 2-mercaptoethanol (Thermo Fisher Scientific; cat. 21985-023). The Jurkat cell line was acquired from ATCC (cat. TIB-152) and was transduced with lentivirus containing the pLenti-CMV-blast plasmid with cDNA for LAT transporters and violet excited GFP (VEX).

Mice

8-week-old WT C57BL/6J (cat. 000664) and OT-1 (C57BL/6J-Tg(Tcra/Tcrb)1100Mjb/J) (cat. 003831) mice were purchased from The Jackson Laboratory or bred in our animal facility. These mice were used to generate congenically marked (CD45.1/2) OT-1 mice. All mice were maintained in a pathogen-free facility and used according to institutional and National Institutes of Health guidelines.

CD8⁺ T cell isolation and culture

Primary naive CD8⁺ T cells were purified from spleens and or lymph nodes of WT (C57BL/6) or OT-1 mice using a naive CD8⁺ T cell isolation kit (Miltenyi Biotec; cat. 130-096-543). The isolated naive CD8⁺ T cells were activated for 24 hours at 37°C in plates coated with 2 µg/ml anti-CD3 (clone 145-2C11; Bio X Cell; cat. BE0001-1) and 2 µg/ml anti-CD28 (clone 37.51; Bio X Cell; cat. BE0015-1) in the presence of 100 units/ml IL-2 (R&D Systems; cat. 202-IL-050) and 10 ng/ml IL-12 (PeproTech; cat. 210-12-50 mg). CD8⁺ T cells were cultured in RPMI-1640 (Thermo Fisher Scientific, cat. 11875-119) supplemented with 10% FBS (Corning; cat. 35-010-CV), 100 units/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific; cat. 15140-122), 10 mmol/l HEPES (Thermo Fisher Scientific; cat. 15630-130), 1 mmol/l sodium pyruvate (Thermo Fisher Scientific; cat. 11360-070), and 55 mmol/l 2-mercaptoethanol (Thermo Fisher Scientific; cat. 21985-023).

shRNA knockdowns

Platinum-E cells were transfected with pMKO.1 plasmids containing a GFP selection marker and specific shRNA. Transfection was achieved by using Fugene 6 (VWR/Promega; cat. E2691) and DNA mixture at a 1:3 DNA:Fugene ratio in Opti-MEM I reduced serum medium (Life Technologies; cat. 31985-062) for 6 hours. Retroviral supernatants were collected after 24 and 48 hours transfections and filtered through 45 µm filters. Naive CD8⁺ T cells were isolated using a naive CD8αβ T-cell isolation kit (Miltenyi Biotec; cat. 130-096-543) and activated on plates coated with 2 µg/ml anti-CD3 and 2 µg/mL anti-CD28 in the presence of 100 units/ml IL-2 (R&D Systems; cat. 202-IL-050) and IL-12 (PeproTech; cat. 210-12-50 mg) for 24 hours at 37°C prior to transduction. Transductions were performed by spinfecting T cells at 807 x g for 90 minutes at 37°C in presence of the retroviral supernatants and polybrene (Santa Cruz, cat. Sc-255611). The cells were incubated at 37°C, in 5% CO₂ for 2-4 hours before washing off the virus. The T

cells were expanded for 48 hours in RPMI containing 100 units/ml IL-2 (R&D Systems; cat. 202-IL-050) and 10 ng/ml IL-12 (PeproTech; cat. 210-12-50 mg). Transduced CD8⁺ T cells were selected by FACS sorting based on GFP expression using a BD Aria II or Sony SH800S cell sorter.

RNA isolation and quantitative real-time PCR

Total RNA was isolated with the Quick-RNA MiniPrep Kit (Genesee Scientific; cat. 11-327). RNA (500 ng) was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Biorad; cat. 1708891). The analyzed genes were: Slc43a2 (Fw: ACAAGTATGGTCCAAGGAAGC; Rv: GCGATAAAGATGAGCACAGAGA), Slc43a1 (Fw: CCTATGCGTCTTCTGCCATC; Rv: CCAGTTGAGAGCACAGTTCA), Slc43a3 (Fw: ATCATCATTGCCTTCACCTCT; Rv: TCTGTAGGTTGGTGATAAGGAAC), Slc7a5 (Fw: GTTCACGTCCTCAAGACTGTT; Rv: GGCGTACATCAGCCTCAT), Slc7a6 (Fw: ACCTGTGATCCTCCATATGTG; Rv: CGTGAATGTGTCTTGTACTCGT), Slc3a2 (Fw: ACCTCACTCCCAACTACCA; Rv: CATTCATCAGCTTTCCACATC), Slc1a5 (Fw: CCTCTCATCTACTTCCTCTTCAC; Rv: CCATTCTTCTCCTCTACACACTTC), Slc38a1 (Fw: GAGTCTCACAAACAGCCATCT; Rv: CCAAGATTCCACTGCCCAT), and Slc38a2 (Fw: CATGGCTAATACTGGAATTGCTC; Rv: CCTTATGTCCCAACTGTTTCGTA). The relative levels of gene transcripts were compared to the control gene RPL-19 (Fw: AAGCCTGTGACTGTCCATTC; Rv: GATCCTCATCCTTCTCATCCAG) and were determined by quantitative real-time PCR using SYBR Green PCR Master Mix (Quanta BioSciences; cat. 101414-270). Amplification was performed at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Flow cytometry

CD8⁺ T cells were harvested from culture and pelleted by centrifugation at 400 x g for 5 minutes and resuspended in staining buffer (PBS containing 1 % FBS and 2 mM EDTA) or Cell Staining buffer (Biolegend, 420201). Single-cell suspensions of tumor infiltrating lymphocytes were prepared by mechanical dissociation and resuspended in staining buffer or Cell Stain Buffer (Biolegend, 420201). Cells were stained with APC/Cy7 LIVE/DEAD fixable near-IR dead cell stain kit (Thermo Fisher Scientific; cat. L34976) and Fc Block (for *in vivo* flow experiments) for 20 minutes and were subsequently washed and stained with the cell surface antibodies indicated below. For intracellular staining, cells were washed twice after cell surface staining and fixed using eBioscience™ Fcγ3 / Transcription Factor Staining Buffer Set (Invitrogen, 00-5523-00). Cells were subsequently washed with perm buffer twice and stained with intracellular antibodies. After intracellular staining, cells were washed twice with a perm buffer and finally resuspended in cell staining buffer for flow cytometry analysis. Cell Surface Antibodies: BUV661 anti-mouse CD45.2 (BD Biosciences; cat. 741516); BUV395 anti-mouse CD3 (BD biosciences; cat. 563565); BUV805 anti-mouse CD8 (BD biosciences; cat. 612898); BUV395 anti-mouse TCRβ (BD biosciences; cat. 742485); BUV496 anti-mouse CD4 (BD Biosciences, cat. 612952); PE anti-mouse CD45.1 (BioLegend; cat. 110708); BV605 anti-mouse CD62L (BioLegend; cat.104438), APC anti-mouse CD44 (BioLegend; cat. 515403). Intracellular Antibodies: BV421 anti-mouse Ki-67 (BioLegend; cat. 652411); APC anti-mouse IFNγ (BioLegend; cat. 505810); APC anti-mouse IFNγ (BioLegend; cat. 505809); FITC anti-mouse GzmB (BioLegend; cat. 515403); PerCP/Cy5.5 anti-mouse Ki67 (BioLegend; cat. 652424); PE-Cy7 anti-mouse Bcl-2 (BioLegend; cat. 633512); AF488 anti-mouse TCF1 (BD biosciences; cat. 567018). Flow cytometry was performed on BD LSR II and BD FACS Symphony machines. Flow analyses were performed using FlowJo 10.6.1 software (TreeStar). All flow antibodies

were used at 1:100 dilution. UltraComp beads (Thermo Fisher Scientific; cat. 01-2222-42) were used for compensation.

BrdU

Naive CD8⁺ T cells were isolated from spleen (and lymph nodes) and transduced as described above. One and a half hours prior to assay endpoint BrdU was added to cells at a concentration based on the Phase-Flow™ Alexa Fluor® 647 BrdU Kit (Biolegend, cat. 370704). Cells were stained with APC/Cy7 LIVE/DEAD fixable near-IR dead cell stain for viability and cell surface antibodies: BV421 anti-mouse CD8 (BioLegend, 126629) and BV510 anti-mouse CD25 (BioLegend; 102041). Subsequent fixation and DNase treatment and intracellular BrdU staining were carried out based on kit protocol. Prior to analyzing by flow cytometry, cells were stained with 7-AAD for 15 minutes. Stained samples were analyzed using BD LSRII or BD Symphony flow cytometers.

Killing assay

For the killing assay, 15,000 tumor cells were plated in 96-well plates and transferred to a 37°C incubator to let them attach. Twenty-four hours later, a test plate was counted and OT-1 CD8⁺ T cells that were previously activated and transduced, as described above, 4 days prior to plating were seeded on top of the tumor cells using a 1:5 ratio (CD8⁺ T cells: tumor cells). Antigen-specific killing was evaluated by co-culturing pre-activated CD8⁺ T cells with B16-OVA cells while non-antigen-specific killing was evaluated by co-culturing pre-activated CD8⁺ T cells with B16 cells not expressing OVA. All assays were performed after co-culture for 24 hours. For killing assays, plates were trypsinized, tumor cells and CD8⁺ T cells were resuspended in PBS (Invitrogen, cat. 14190-250) and analyzed by flow cytometry to determine the numbers of

cancer cells and T cells. The percentage of killing was calculated by the following formula:
number of (1-(B16-OVA+ or - WT live/ (number of B16-OVAWT+ or - total (no T cell))) × 100%.

Cell Quenching

Fifty-ml falcon tubes with 60% methanol (Fisher Scientific, cat. A456-500) and 10 mM ammonium acetate (Sigma-Aldrich, cat. A1542-250G) were precooled in a dry ice-ethanol bath (−40 °C) for 20 minutes. CD8⁺ T cells in culture were collected and transferred to 15 ml falcon tubes and pelleted by centrifugation (430 x g, 3 minutes, 4 °C) to remove excess of medium. Medium was aspirated by using a vacuum line and the remaining cell pellet was immediately added to the 50 ml falcon tube containing 5 ml of the quenching solution at −40 °C. Cells were pelleted by centrifugation (1288 x g, 30 seconds, 4 °C) and the supernatant was removed by quickly inverting the tube. The cell pellet was washed a second time with quenching solution (−40 °C). After the second wash, the cell pellet was stored at −80 °C. Jurkat cells were collected and transferred to 2 ml Eppendorf tubes and pelleted by centrifugation (600 x g, 3 minutes, 4 °C). Pellets were resuspended in 1 ml of ice-cold 150 mM sodium chloride solution and pelleted by centrifugation (600 x g, 3 minutes, 4 °C). Cell pellets were quenched in liquid nitrogen and stored at −80 °C.

Metabolite extraction and liquid chromatography-mass spectrometry analysis

Metabolites were extracted from CD8⁺ T cell pellets by adding 800 µl 60% methanol (Fisher Scientific, cat. A456-500). After addition of 500 µl chloroform (Sigma-Aldrich, cat. C2432), samples were vortexed for 10 minutes at 4°C and then spun at 17,000 x g for 10 minutes at 4°C. Jurkat cell metabolites were extracted with 80% methanol and vortexed for 10 minutes at 4°C and then spun at 17,000 x g for 10 minutes at 4°C. The upper water-methanol phase

containing polar was transferred into a fresh tube and dried down in a Vacufuge plus speed-vac at 4°C. The lower phase was also dried down in a Vacufuge plus speed-vac at 4 °C and proteins were resuspended in 0.2M NaOH and heated at 90°C for 15 minutes. Dried down metabolites were resuspended based on protein concentration. The protein concentration was determined using the Pierce BCA Protein Assay Kit Assay (ThermoFisher, cat. 23227). The metabolite extract was separated using an iHILIC-(P) Classic column (2.1 μ m, 150 mm $\times$ 2.0 mm I.D., The Nest Group) coupled to a Thermo Scientific Vanquish U-HPLC system. The autosampler and column oven were held at 4°C and 25°C, respectively. The iHILIC-(P) Classic column was used with buffer A (0.1% ammonium hydroxide, 20 mM ammonium carbonate) and buffer B (100% acetonitrile). The chromatographic gradient was run at a flow rate of 0.150 ml/minute with the following linear gradient: 0–23 minutes from 95% to 5% B; 23–25 minutes hold at 5% B; 25-25.5 minutes transition to 95% B at 0.20 mL/minute; 25.5-32.5 minute at 95% B; and 32.5-33 minutes at 95% B with 0.15 mL/minute flow rate. The mass spectrometer was operated in full scan, negative ion model. Mass spectrometry detection was carried out on a Q Extractive HF-X orbitrap mass spectrometer with a HESI source. Mass spectrometry acquisition was conducted in full scan mode over a mass range of 70–1000 m/z, with a resolution of 60,000 at 200m/z, an AGC target of 1e6, and a maximum injection time of 100 ms. For metabolite feature extraction and quantification TraceFinder software (ThermoFisher) was used. GraphPad Prism 10 software was used for statistical analysis.

Nucleofection

Naive CD8⁺ T cells were isolated from OT-1 mice using the naive CD8⁺ T cell isolation kit (Miltenyi Biotec; cat. 130- 096-543). RNP formation was induced by incubating Alt-R Streptococcus pyogenes Cas9 Nuclease (IDT, cat.1081059) and gRNA (Synthego

CRISPRrevolution) for 10 minutes at room temperature. Afterwards, naive cells and RNP complexes were combined in cP3 buffer (Lonza, cat. V4XP-3024) and electroporated with the 4-D Nucleofector (Lonza). Nucleofected cells were cultured in IL-7 for 6 days.

In vivo adoptive transfer

For *in vivo* tumor growth experiments, 5,000 nucleofected OT-1 cells were transferred to C57BL/6J mice via retroorbital injection. The following day 250,000 B16-OVA melanoma cells were injected into the flank subcutaneously. Tumor growth was measured every 2-3 days using calipers and calculated using $=((4\pi)/3)*((d/2)^2)*(D/2)$ where D is the longer diameter and d is the shorter diameter. For *in vivo* co-transfer experiments, naive OT-1 CD8⁺ T cells were isolated from the spleen and lymph nodes from congenic marked 45.1 and 45.1/.2 mice. The use of congenic marked mice allows us to distinguish, through flow cytometry, adoptively transferred CD8⁺ T cells from endogenous CD8⁺ T cells and distinguish between CTRL and specific knock-out. OT-1 CD8⁺ T cells were nucleofected as described above. To evaluate whether specific genetic manipulation affects the capacity of CD8⁺ T cells to infiltrate the tumor, 5,000 OT-1 CD8⁺ LAT4KO 45.1/.2 T cells and 5,000 OT-1 CD8⁺ CTL 45.1 T cells were mixed and adoptively transferred in C57BL/6J female WT mice prior to tumor implantation. Additionally, 5,000 OT-1 CD8⁺ CTL 45.1/.2 T cells and 5,000 OT-1 CD8⁺ CTL 45.1 T cells were mixed and transferred to C57BL/6J female WT mice to account for any variation due to congenic markers. Input frequencies were determined on the day of adoptive transfer using flow cytometry. The day after, 750,000 B16-OVA tumor cells were injected in the flank subcutaneously. Fifteen days after tumor implantation, tumor and tdLN were isolated and dissociated. Tumors were dissociated using gentleMACS dissociator program 1.1 subjected to collagenase treatment in a 37C shaking incubator for 30 minutes. Tumors were then subjected to gentleMACS dissociator

program 3.2. TIL was isolated from tumor using the Lymphocyte Separation Medium (MPbiomedicals, cat. 0850494X). Collected TIL and dissociated tdLN lymphocytes were processed for downstream flow cytometry analysis.

Quantification and statistical analysis

Statistical analyses were performed in GraphPad Prism 10. A two-tailed unpaired Student's t test was used for comparison of two unpaired groups. A two-way ANOVA with Sidak's multiple comparison test was used for comparison of multiple groups. Error bars display standard error of mean (SEM) unless otherwise noted. p values are noted as *p < 0.05; **p < 0.01; ***p < 0.001.

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CHAPTER 4: CONCLUSION

DISCUSSION

Amino acids are essential for maintaining optimal cellular function and have emerged as key regulators of T cell activity in both physiological and pathological contexts (Wang et al., 2020; Sinclair et al., 2013). Due to their diverse roles in supporting critical cellular processes, cells employ multiple mechanisms to ensure amino acid homeostasis. (Bröer et al., 2022) This balance is maintained through modulation of transporter activity, metabolic pathways, and protein synthesis. In response to changes in extracellular nutrient availability and signaling cues, certain cell types, such as cancer cells and T cells, activate distinct amino acid regulatory programs to meet their metabolic demands. Cancer cells, for instance, often harbor oncogenic mutations that hyperactivate signaling pathways to drive uncontrolled proliferation (Jones et al., 2009; Pylayeva-Gupta et al., 2011). Similarly, T cells rely on T cell receptor (TCR) signaling to induce analogous growth-promoting pathways that support clonal expansion, cytokine production, and effector functions (Hwang et al., 2020, Shah et al., 2021; Ma et al., 2024).

While it is well established that immune cells and other proliferative cells require these nutrients to support their metabolic functions, the specific amino acid requirements of distinct T cell subsets remain incompletely characterized. Furthermore, the temporal dynamics of nutrient dependency across the course of T cell activation have yet to be fully elucidated. Additionally, strategies that modulate amino acid availability by targeted manipulation of transporter expression, and the subsequent impact on T cell effector function, have not been comprehensively investigated. Thus, we sought to investigate these understudied aspects in CD4⁺ and CD8⁺ T cell biology in the contexts of tumor immunity and autoimmunity.

This dissertation focused on exploring how modulating amino acid utilization and transport affects specific T cell subsets. In chapter 2, we examined the functional relevance of non-essential amino acids during CD4⁺ T cell activation. We used a range of methods to

modulate extracellular amino acid levels and ascertain the extent to which individual amino acids influence CD4⁺ T cell activation, proliferation, and cytokine production. We identified asparagine as a non-essential amino acid that is conditionally essential for CD4⁺ T cells to maintain optimal activation and function. In chapter 3, we sought to understand the consequence of transporter modulation in CD8⁺ T cells and determine if ablation of Slc43a2 transporter enhances CD8⁺ T cell effector function in the context of tumor immunity. Utilizing genetic perturbation strategies, we defined the limitations imposed by this exporter on CD8⁺ T cell metabolism, proliferation, and effector function.

Asparagine is essential for optimal CD4⁺ T cell function

We determined that asparagine is critical for optimal CD4⁺ T cell function and activation. This was demonstrated by activating CD4⁺ T cells in DMEM media lacking specific amino acids, and supplementing individual non-essential amino acids one at a time. These experiments revealed that asparagine alone was sufficient to promote CD4⁺ T cell proliferation and activation in these culture conditions. To further evaluate the extent of amino acid dependency, we performed experiments in conditions where individual non-essential amino acids were depleted from the media. Among the non-essential amino acids, asparagine depletion most significantly impaired the expression of key activation markers CD25, CD44, PD-1, and CD71. In addition, the frequency of activation markers CD44, PD-1, and CD71 were particularly sensitive to asparagine deficiency. We further confirmed the requirement for asparagine to promote expression and optimal proliferation using an orthogonal approach of asparagine depletion. Culturing T cells with pegylated asparaginase resulted in activation and proliferation defects comparable to those observed in custom RPMI media lacking asparagine. Subsequent qPCR and immunoblotting analyses revealed that CD4⁺ T cells express the endogenous proteins

needed to synthesize asparagine after 24 hours of activation. However, despite this intrinsic synthetic capability, CD4⁺ T cells exhibit an extracellular requirement for asparagine to sustain early activation.

To investigate the temporal requirement for extracellular asparagine in CD4⁺ T cell activation, proliferation, and differentiation during later stages of activation, we treated T cells with asparaginase at defined time points following T cell stimulation and assessed activation and proliferation parameters at 48 and 72 hours post-stimulation. Our results demonstrate that continuous availability of extracellular asparagine is required for maximal proliferation, expression of activation markers, and production of the lineage defining cytokine IL-17A in pathogenic Th17 cells. Further analyses revealed a significant defect in overall protein synthesis, as evidenced by reduced incorporation of an O-propargyl-puromycin (OPP) probe and confirmed by heavy labeled amino acid tracing experiments.

Building on the observation that asparagine deprivation impairs protein synthesis and leads to sub-optimal differentiation, we sought to identify the impact of asparagine depletion *in vivo* in the context of CD4⁺ T cell-mediated autoimmune pathology. To this end, we administered pegylated asparaginase to mice prior to induction of EAE using MOG₃₅₋₅₅/CFA and pertussis toxin to induce experimental autoimmune encephalomyelitis (EAE). Mice treated with pegylated asparaginase exhibited a significant reduction in disease severity, indicating that systemic depletion of asparagine can attenuate pathological CD4⁺ T cell responses. These findings reveal a potential therapeutic approach for targeting other pathologies driven by autoreactive CD4⁺ T cell activity.

Slc43a2 limits CD8⁺ T cell function

Amino acids are critical mediators of the cellular growth process, making regulatory mechanisms that control amino acid uptake and utilization crucial for highly proliferating cells such as cancer and CD8⁺ T cells (Hwang et al., 2020, Shah et al., 2021; Ma et al., 2024; Jones et al., 2009; Pylayeva-Gupta et al., 2011). Proliferation is a crucial aspect of CD8⁺ T cell effector function, given that their clonal expansion allows for the generation of a sufficient pool of effectors capable of mounting effective immune responses against pathogens or malignant cells. To support this proliferation, T cells need to upregulate nutrient uptake mechanisms, including the modulation of surface transporter and receptor expression (Wang et al., 2020).

In chapter 3, we aimed to identify key mediators of T cell nutrient acquisition and signaling through an *in vivo* metabolic CRISPR screen. This approach enabled the identification of guide RNAs targeting nutrient transporters and receptors that either enhanced or diminished T cell accumulation within the tumor microenvironment. Among the positively enriched gene targets in tumor-infiltrating T cells, *Slc43a2*, an amino acid transporter, emerged as the second most highly represented gene. Given its representation we investigated the functional role of *Slc43a2* in CD8⁺ T cell biology. Expression analysis revealed that *Slc43a2* is dynamically regulated following T cell receptor (TCR) engagement—initially downregulated during early activation, with expression increasing at later stages. Previous studies suggest that *Slc43a2* may function as an amino acid exporter; thus, we hypothesized that its early downregulation facilitates increased intracellular amino acid retention, thereby supporting biosynthetic and growth-promoting pathways.

To further investigate the functional consequences of sustained *Slc43a2* ablation on T cell biology, we employed several genetic perturbation strategies. Specifically, we utilized viral transduction to either knock down *Slc43a2* in primary activated CD8⁺ T cells or overexpress

SLC43A2 in Jurkat T cells. Slc43a2 ablation in primary CD8⁺ T cells led to enhanced expression of activation markers and interferon- γ upon restimulation. Moreover, Slc43a2 downregulation promoted increased proliferation and effector function in activated CD8⁺ T cells. Extended culture under conditions of Slc43a2 loss resulted in elevated expression of TCF1 and increased BrdU incorporation, indicating enhanced proliferative potential and long-term fitness. In contrast, overexpression of SLC43A2 in Jurkat T cells impaired cell growth and was associated with decreased intracellular levels of branched-chain amino acids, phenylalanine, and methionine. Collectively, these findings suggest that modulation of Slc43a2 influences nutrient availability and supports enhanced effector function, proliferation, and persistence of T cells.

Considering that Slc43a2 expression limits the canonical function and proliferative capacity of CD8⁺ T cells *in vitro*, we next investigated whether these effects are seen *in vivo*. To this end, we genetically deleted Slc43a2 in naive primary CD8⁺ T cells and evaluated their capacity to control tumor growth. Slc43a2-deficient T cells exhibited enhanced tumor control and improved overall survival in recipient mice. Furthermore, in competitive adoptive transfer experiments, where both control and Slc43a2-ablated cytotoxic T lymphocytes (CTLs) were co-transferred into the same host, we observed comparable frequencies of each population within the tumor. However, a significantly higher frequency of Slc43a2-deficient T cells was observed in the tumor-draining lymph node (tdLN). These findings support a model in which Slc43a2 downregulation enhances T cell survival and expansion during the priming phase in the tdLN, thereby generating a larger reservoir of activated T cells capable of sustaining antitumor responses upon tumor challenge.

Given the limited understanding and available tools specific to Slc43a2 function in T cells, there is a critical need to develop T cell-specific knockout models. This need is underscored by the finding that global deletion of Slc43a2 results in neonatal lethality (Guetg et

al., 2015). A conditional, T cell–specific Slc43a2 knockout model would enable investigation of the transporter's role during the early priming phase of T cell activation and facilitate direct assessment of amino acid flux changes following TCR stimulation. In parallel, the development of tools to measure Slc43a2 protein expression and functional activity across distinct T cell subsets and activation states would be extremely valuable.

A comprehensive characterization of amino acid transporters in T cells remains incomplete, as current research has largely focused on highly expressed transporters, such as Slc7a5, Slc3a2, and Slc1a5 (Wang et al., 2020). However, these transporters are also highly expressed in tumor cells, so therapeutic strategies targeting them could have comparable effects on both T cells and malignant cells. Therefore, identifying transporters with differential expression profiles or functional roles between immune and malignant cells is of great interest. Future work should prioritize the systematic characterization and quantification of surface transporter expression across diverse immune and tumor cell types. Such efforts may reveal context-specific metabolic vulnerabilities that can be selectively targeted to enhance antitumor immunity while minimizing effects on normal cells.

FINAL THOUGHTS

T cell biology is complex and dynamic, making this field both fascinating and challenging to completely characterize. Given the critical roles T cells play in both health and disease, it is important to continue advancing our understanding of T cell functions, mediators, and regulatory systems. Amino acids serve as one of many fuel sources and mediators of T cell function. This dissertation provides further insight into T cell amino acid utilization and regulation by exploring specific transporter and amino acid dependencies in CD8⁺ T cells and CD4⁺ T cells respectively. These findings are significant and have important future implications, however many questions remain. Are there additional transporters in the T cell proteome that can be modulated to

enhance T cell function? Are there other methods of transporter modulation that could improve T cell function in other disease settings? Do specific CD4⁺ T cell subsets have different amino acid dependencies? My hope is that this work, along with future studies, will contribute to a deeper understanding of T cell fuel utilization and regulation, and inspire novel approaches to improve therapeutic outcomes.

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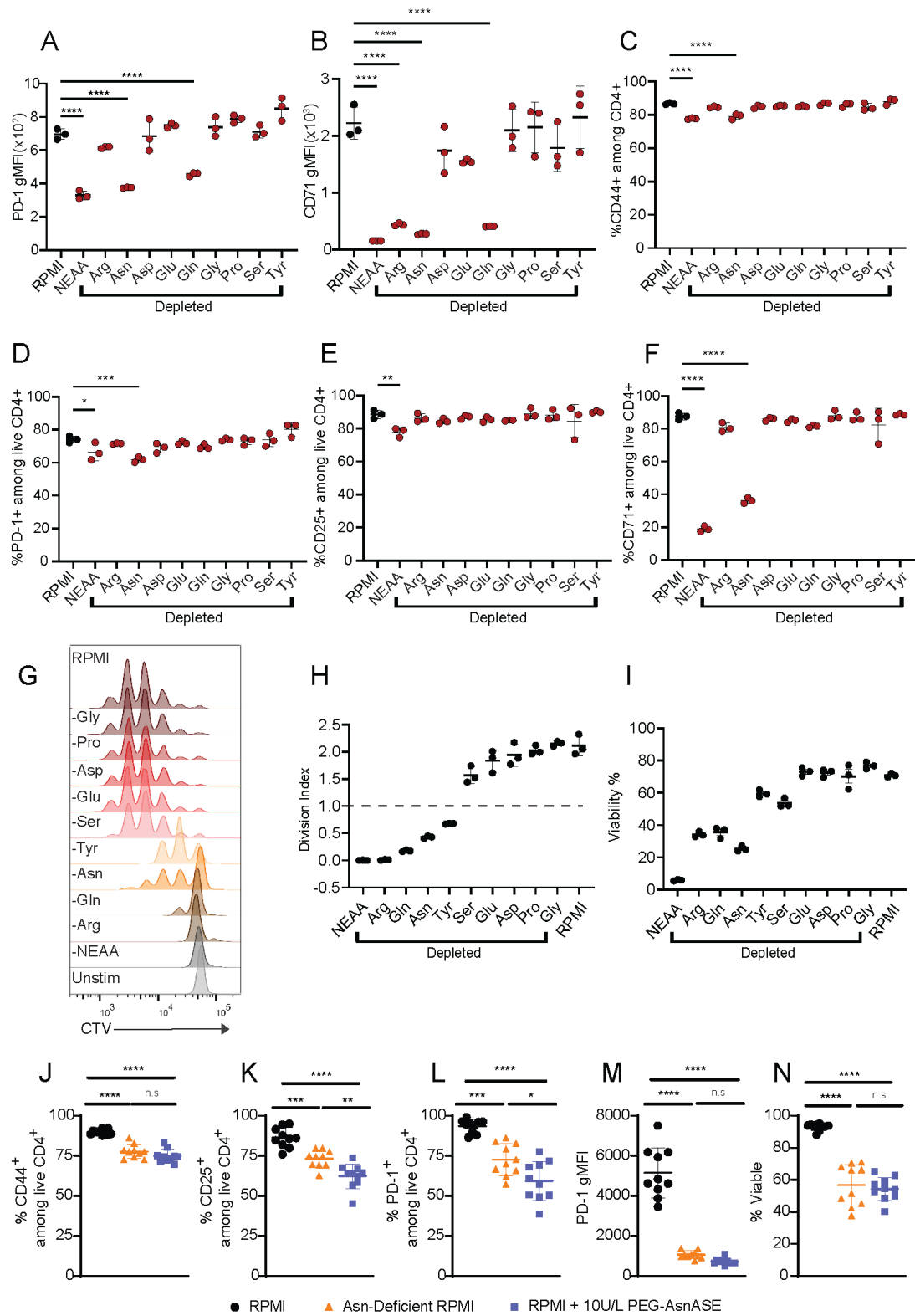
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APPENDIX: SUPPLEMENTARY DATA TO ACCOMPANY CHAPTER 2

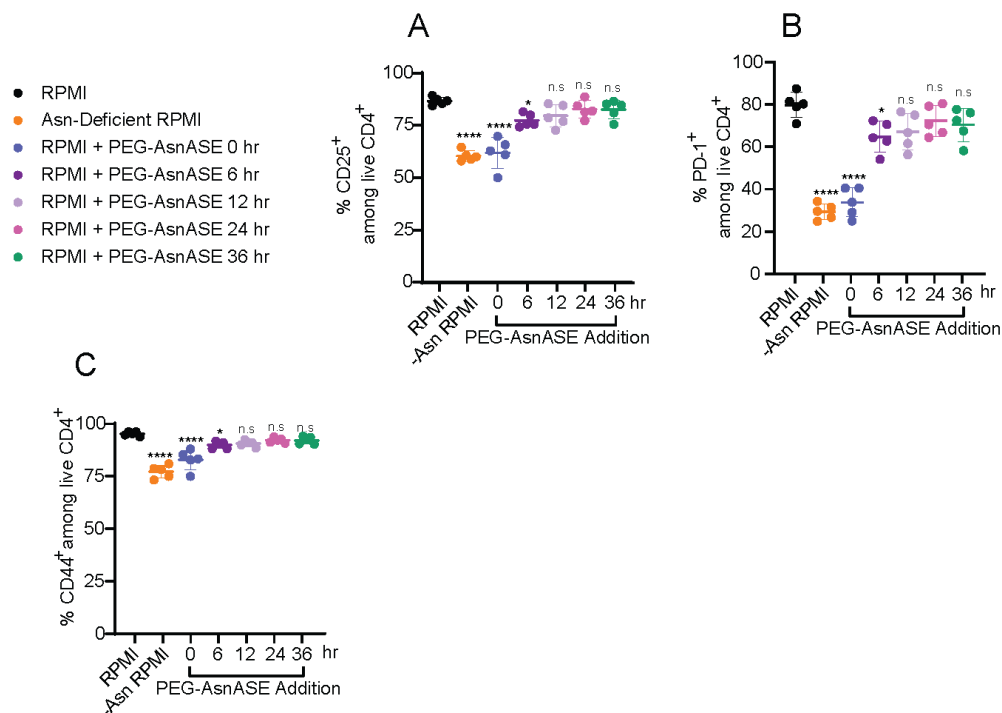
Supplemental Figure 2.1 (following page). Extracellular asparagine is essential for CD4⁺ T cell activation. (Related to Figure 2.1) (A-F) Frequency and expression levels of the activation markers following 24 hours of stimulation with plate-bound anti-CD3/CD28 mAbs in RPMI lacking the indicated individual amino acids shown in red. Non-essential amino acids (NEAA) include asparagine (Asn), aspartate (Asp), glutamate (Glu), proline (Pro), arginine (Arg), glutamine (Gln), glycine (Gly), serine (Ser), and tyrosine (Tyr). Expression level of PD-1 (A) and CD71(B). Quantification of the proportions of CD4⁺ cells expressing cell surface activation markers CD44(C), PD-1(D), CD25 (E), and CD71(F). (G) Representative flow cytometry histogram depicting CTV dye dilution in naive CD4⁺ T cells following 3 days of stimulation with plate-bound anti-CD3/CD28 mAbs in RPMI or RPMI lacking the indicated individual amino acids. (H) Quantification of division index in G. (I) Quantification of the proportions of viable CD4⁺ T cells in each respective culture condition after 3 days of stimulation with plate-bound anti-CD3/CD28 mAbs in RPMI or RPMI lacking indicated amino acids. Quantification of the proportions of CD4⁺ T cells expressing cell surface activation markers CD44 (J), CD25 (K), and PD-1 (L) as well as expression levels PD-1 (M) on a per cell basis following 24 hours of stimulation with plate-bound anti-CD3/CD28 mAbs in complete RPMI (RPMI), Asn-deficient RPMI or RPMI with 10 IU/L PEGylated-asparaginase (PEG-AsnASE) added at the start of culture. (N) Quantification of the proportions of viable CD4⁺ T cells in each respective culture condition after 3 days of stimulation with plate-bound anti-CD3/CD28 mAbs in RPMI, Asn-deficient RPMI or RPMI with 10 IU/L PEG-AsnASE added at the start of culture. Results are shown as mean $\pm$ SD and are pooled from 2 independent experiments (J-N) or a representative of 2 independent experiments (A-I). non-significant (n.s), * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Turkey's multiple comparison test (J-N) or Dunnet's multiple comparison test (A-F).

Supplemental Figure 2.1 (Continued)



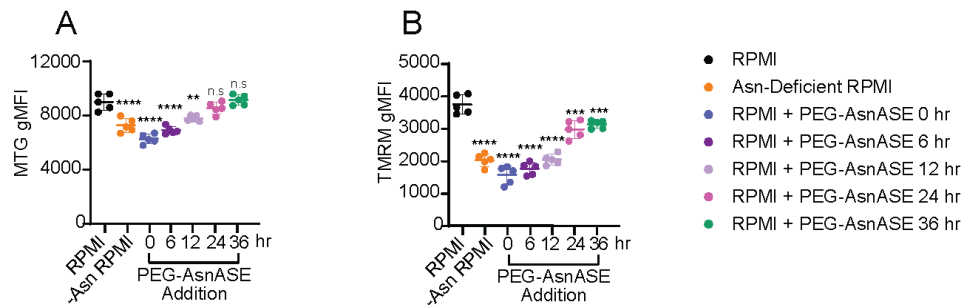
Supplemental Figure 2.2. Expression of CD4⁺ T cell activation proteins in asparagine sufficient and deficient conditions. (Related to Figure 2.2)

(A-C) Quantification of the proportions of CD4⁺ T cells expressing CD25 **(A)**, PD-1 **(B)**, and CD25 **(C)** following 2 days of stimulation with plate-bound anti-CD3/CD28 mAbs in complete RPMI, Asn-deficient RPMI or RPMI with 10 IU/L PEGylated-asparaginase added at 0, 6, 12, 24, or 36 hours. Each dot represents cells obtained from an individual animal (A-C). Results are shown as mean $\pm$ SD and are representative of 2 independent experiments non-significant (n.s), * $p < 0.05$, **** $p < 0.0001$, one-way ANOVA with Dunnet's multiple comparison test.



Supplemental Figure 2.3. Asparagine deprivation impairs mitochondrial function in CD4⁺ T cells. (Related to Figure 2.4)

(A-B) Naive CD4⁺ T cells were stimulated for 48 hours with plate-bound anti-CD3/CD28 mAbs in either complete RPMI media (RPMI), Asn-deficient RPMI or RPMI treated with 10 IU/L PEGylated-asparaginase (PEG-AsnASE) added at 0, 6, 12, 24, or 36 hours. **(A)** Quantification of mitotracker green (MTG) gMFI. **(B)** Quantification of tetramethyl rhodamine methyl ester (TMRM) gMFI. Results are shown as mean $\pm$ SD and are representative of at least 2 independent experiments. non-significant (n.s), **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Dunnet's multiple comparison test.



Supplemental Figure 2.4. Asparagine deprivation impairs pT_H17 function in a model of induced EAE. (Related to Figure 2.6)

(A) Pathogenic T helper 17 (pT_H17) cells were differentiated from naive CD4⁺ FoxP3⁻ T cells from 2D2 TCR transgenic mice in RPMI media with or without Asn. Viable pT_H17 polarized 2D2 cells were adoptively transferred (4x10⁶/mouse) into 10-week-old C57BL/6J female recipients to induce EAE. Mice were scored daily for signs of disease. **(B)** At peak EAE (Day 16), 2D2 cells were isolated from the CNS and inguinal lymph (iLN) node and analyzed. Quantification of the absolute numbers of central nervous system (CNS)-infiltrating Vβ11⁺Vα3.2⁺ 2D2 pT_H17 cells at peak of EAE (pT_H17 RPMI n=16, pT_H17 Asn-deficient RPMI n=12). **(C)** Quantification of the proportions of Vβ11⁺Vα3.2⁺ 2D2 pT_H17 cells that have undergone apoptosis (Annexin-V⁺PI⁺) in the CNS and iLN node at peak of EAE. **(D-E)** Mitotracker green (MTG) and tetramethyl rhodamine methyl ester (TMRM) gMFI in iLN and CNS infiltrating TCRVβ11⁺TCRVα3.2⁺ CD4⁺ T cells at peak of EAE. Each dot represents an individual mouse (B-E). Results are shown as mean ± SD and are pooled (A-B) or a representative of at least 2 independent experiments (C-E). non-significant (n.s), *p < 0.05 **p < 0.01, ****p < 0.0001, two-way ANOVA (A), and Student's t-test (B-E).

