The role of amino acid metabolism in the regulation of T cell fate

by

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Abstract

The study of the metabolism of the immune system is a rapidly advancing field. It is highly dynamic, and changes rapidly upon activation, and although both innate and adaptive cells undergo activation-induced metabolic changes, the changes that are associated with T cell activation have been extensively studied. Early work has identified a rapid increase in the uptake and use of glucose following T cell activation, and glycolysis followed by export of lactate, rather than TCA cycle metabolism, a phenomenon known as aerobic glycolysis. While this glycolytic phenotype has received a great deal of attention, it is accompanied by corresponding increases in amino acid metabolism. Glutamine is a non-essential amino acid which is taken up following activation to help replenish the TCA cycle during aerobic glycolysis, and leucine in an essential amino acid which is required for activation of mTOR signaling. While metabolic alterations following T cell activation were once seen as secondary to the needs of an activated cell, it is becoming increasingly clear that metabolism has a powerful ability to shape and direct the immune response. As certain patterns of metabolism have been associated with particular T cell fates, such as the previously described aerobic glycolysis in activated effector cells, or the increased reliance on oxidative metabolism on long-lived memory cells, we hypothesized that it might be possible to alter T cell immune responses by altering their use of metabolites, and particularly their use of the amino acids glutamine and leucine.

First, we assessed the role of glutamine metabolism in CD4 T cell responses. Previous research has demonstrated that the presence of glutamine in T cell culture is essential for proliferation. We recapitulated these findings, and extended them into a model of CD4 T cell polarization in which glutamine was shown to be dispensable for Th1 skewing, while essential for the development of Th2 cells *in vitro*. This was demonstrated to be due to a specific requirement for glutamine in increasing the epigenetic accessibility of the Th2 cytokine locus, which is required for the transcription and translation

of IL4 and IL13. The role of glutamine in Th2 cell development was further explored *in vivo* in a house dust mite induced asthma model, in which pharmacologic inhibition of glutamine metabolism was shown to reduce not just Th2 T cell formation, but also ILC2 and M2 macrophage polarization, while not affecting M1 macrophages or regulatory T cells, illustrating the selective effect of glutamine metabolism.

Next, glutamine metabolism was assessed in CD8 T cell memory formation. As previously stated, memory cells rely on a more oxidative metabolism than effector cells. By restricting glutamine *in vitro* in cultured CD8 T cells, we decreased the proliferation of those cells, but saw a striking change in their phenotype, reflecting a long-lived central memory-like cell. When these cells were adoptively transferred and stimulated, they were able to persist better in circulation than CD8 T cells cultured in standard media, and were better able to mount a polyfunctional cytokine response upon re-challenge. These cells also were able to mount a more vigorous anti-tumor response upon adoptive cellular therapy in an implantable tumor model. Interestingly, the only perturbation these cells were submitted to was a short-term culture in restricted glutamine media, and this was sufficient to dramatically alter their response for weeks to come, demonstrating the powerful role metabolism plays in governing the T cell response.

Lastly, we explored the role of the amino acid leucine both *in vitro* and *in vivo*. Restriction of leucine metabolism *in vitro* in CD4 T cells selectively affected Th1 cell skewing, decreasing the numbers of these cells and inducing ectopic expression of IL4 and IL13. An *in vivo* profiling of leucine transporter LAT1 expression in conditions of activation indicated that its expression is increased on activated immune cells and especially T cells in activation in viral and transplant systems, and the selective LAT1-specific depletion of cells is an effective strategy for the elimination of activated T cells in both viral and transplant models. This work on both glutamine and leucine indicates the role that metabolism plays in shaping the immune response, and demonstrates that by modulating metabolism we can powerfully alter how an immune response is mounted, beyond simply turning it on or off, but by exquisitely altering

the balance between Th1 and Th2, or effector and memory, in order to promote the exact response required for a particular situation.

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Chapter 1: Introduction

The metabolism of the immune system is highly dynamic and changes dramatically upon T cell activation

The immune system, made up of a number of cell types broadly grouped into innate and adaptive arms, is crucial for not just host defense from pathogens, but nearly every body function. As such, the regulation of this system is of crucial importance to the maintenance of health and homeostasis. Multiple layers of control have evolved to modulate the activity of such an important system, from soluble mediators[1] that set up gradients[2] to offer directionality of movement, to intricate receptor arrays which balance go/no go signals to make life or death decisions[3] to a receptor capable of discriminating between a single hydrogen bond and communicates this highly specific triggering signal intracellularly with a force-sensitive mechanical linkage[4] , each aspect of the immune system has over the course of evolution developed an array of control mechanisms that the field is still in the process of understanding. In this thesis, we propose that one such control mechanism is immune cell metabolism, and that by altering the metabolism of immune cells, it is possible to direct their development and response in very precise and intricate ways. The ability to exert this control represents a novel and extremely powerful strategy that can be applied to craft immune responses in a variety of situations, from autoimmune diseases to cancer.

Both older pioneering studies as well as recent work in the nascent field of immunometabolism have served to help better understand the metabolism of immune cells. As has become clear this metabolism is highly dynamic. Naive immune cells (and this thesis will focus mostly on T lymphocytes) have a more textbook[5] metabolism, with glucose going through the glycolytic pathway to generate pyruvate, which is then converted to acetyl-CoA by pyruvate dehydrogenase (PDH), and then enters the

mitochondria for passage through the tricarboxylic acid (TCA) cycle, generating electron donors, which are then transferred to the electron transport chain (ETC) in the mitochondrial membrane, which generates a proton motive force which can be harnessed for the production of adenosine triphosphate (ATP). This metabolism is highly optimized and efficient for making ATP, which can then go on to fuel a diverse array of cellular processes.

However, T cell receptor (TCR)-induced activation of T cells induces a number of dramatic changes to this textbook quiescent metabolism[6] . Many of these changes have been initially identified and studied in tissue culture systems as opposed to *in vivo*, and many of the alterations have been considered in the context of the culture of another highly proliferation cell type: cancer cells[7] . Cancer cells undergo rapid proliferation, but using a highly non-standard metabolism, in which glucose is taken up and sent through the glycolytic pathway but rather than pyruvate being converted to acetyl-CoA for mitochondrial respiration, it is converted to lactate by lactate dehydrogenase (LDH), and the lactate is exported from the cell. This lactate export and subsequent extracellular acidification is responsible for the characteristic yellowing of the media that occurs during cell culture. This phenomenon of aerobic glycolysis was first identified by the German biochemist Otto Warburg, and is frequently termed the Warburg Effect[8] . The phenomenon of aerobic glycolysis in T cells was first identified in 1999[9] , in a paper in which short term stimulation with the T cell mitogen concanavalin A carried out on rat thymocytes. Subsequent research focused on the role of aerobic glycolysis primarily in cancer cells, in which it was hypothesized to occur in order to facilitate the growth and production of biosynthetic intermediates for cancer cells to undergo their rapid proliferation and expansion[10]. Pioneering studies on the dynamics of T cell activation that enable this metabolic change in T cells to accompany activation were published in 2002, when the role of costimulation, or signal 2[11] , as provided by signaling through CD28, was discovered[12] . In this work, it was discovered that Akt activation that accompanies activation with anti-CD3 and anti-CD28 antibody stimulation leads to cell surface transduction of the glucose

transporter Glut1. Furthermore, this surface expression of Glut1 requires CD3 signaling accompanied by CD28 costimulation, as treatment with cytokine-containing conditioned media could not replicate these effects. Glut1 surface expression lead to an increase in glucose uptake by the activated T cells, as well as an increase in glycolysis as assessed by lactate production. Interestingly, this was the first paper to observe that, under more physiologic (lower glucose) media conditions, stimulated T cells will increase their rate of oxygen consumption for oxidative phosphorylation (OXPHOS) and use in the ETC to generate ATP. The impacts of studying immune cell metabolism in *in vitro* conditions that do not reflect the physiology found *in vivo* remain an important hurdle to the application of findings in immunometabolism.

While the role of glucose and its immediate uptake upon activation have been well studied since this initial observation, it was not long before the role of glutamine uptake became known. This thesis will go on to focus prominently on the role of glutamine in T cell metabolism, and its important role in augmenting the TCA cycle in order to promote the generation of biosynthetic intermediates for cellular proliferation and effector function, but, as with the Warburg effect, the upregulation of glutamine metabolism in aerobic glycolysis was first observed in cancer cells. In a 2007 paper, DeBerardinis and colleagues used nuclear magnetic resonance (NMR) approaches to track carbon flux through the metabolism of a proliferating glioblastoma cell line[13]. Their findings confirmed the existence of the Warburg effect as a phenomenon, but also, by tracing the incorporation of an isotopically labelled glutamine tracer, they were able to identify a role for glutamine metabolism in the anaplerotic pathways of these proliferating cancer cells. By comparing label incorporation between glucose and glutamine, these researchers were able to conclude that, while glucose was a major source of carbons for *de novo* lipogenesis, glutamine plays an important role in the generation of many biosynthetic substrates, such as nucleotides, and played an important role in replenishing the TCA cycle. The role of glutamine will be more fully discussed later, but it is key to T cell proliferation[14].

Further studies in the metabolism of cancer cells were performed that helped understand the mechanistic underpinnings of aerobic glycolysis. Work in 2008 from Christofk and colleagues sought to understand how the phenotype of aerobic glycolysis came to be in cancer cells, and identified the M2 splice isoform of the enzyme pyruvate kinase (PKM2)[15]. Their studies, utilizing a variety of mouse and human tumor models, identified expression of the PKM2 splice variant, found primarily in embryonic development rather than adult tissues as of primary expression in tumors. Knockdown of PKM2 and reconstitution with the PKM1 splice variant in their tumor models lead to tumors that relied less on aerobic glycolysis and more on OXPHOS, and lead to reduced tumorigenicity in a mouse model of lung cancer. Further studies of PKM2 in cancer cells have identified that small molecule activators of PKM2 can induce its tetramerization, and that this tetramerization can alter the behavior of cancer cells, decreasing their proliferation in hypoxic culture, leading to the inhibition of xenograft tumor growth[16] and alter the carbon flow through metabolic pathways of cancer cells, demonstrating reduced flow through biosynthetic pathways of serine, leading to an inability for cultured cancer cells to grow in the absence of serine supplementation[17]. Studies of PKM2 were continued in macrophages stimulated with LPS, with small molecule modulation of PKM2 which focused not on its enzymatic activity, but rather by its activity as a transcription factor, which can dimerize and translocate to the nucleus and work with the transcription factor Hypoxia Inducible Factor (HIF) 1 alpha, which has previously been recognized to be central to transcriptional reprogramming in the induction of glycolysis[18] . These studies found that activation of this nuclear function could be abrogated by small molecules that induce its tetramerization and in bone marrow derived macrophages this tetramerization leads to the attenuation of glycolysis and decreased inflammation as assessed by decreased IL1 beta production[18]. The special modifications of macrophage metabolism will be discussed briefly below especially as they pertain to the use of metabolic intermediates as signaling molecules for the coordination of immune response development.

In addition to the roles that aerobic glycolysis plays in the activation of T cells from an energetic perspective, this metabolic phenotype upon activation plays a very important role in the regulation of the glycolytic machinery that permits these changes in metabolism to occur. A 2013 paper from Chang and colleagues identified a role of aerobic glycolysis in cytokine production, rather than strictly as an energy source for survival and proliferation[19]. These researchers noted, similar as was previously mentioned by Frauwirth[12] , that in addition to an increase in glycolysis upon T cell activation, as measured by an increase in the extracellular acidification rate (ECAR), there is also a notable increase in the oxygen consumption rate (OCR), however it was only this increase in OCR that was required for T cell activation, as T cells cultured in galactose rather than glucose were still able to proliferate and survive. However, it was found that aerobic glycolysis was essential for cytokine production, as cells cultured in galactose had a defect in both IL2 and interferon (IFN) gamma production. The mechanism they discovered for this finding had less to do with metabolism and more to do with an alternative role for the enzyme responsible for the 6th step in the glycolytic pathway, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the commonly- used "housekeeping" gene for many protein and RNA assessment techniques. This enzyme has previously identified but underappreciated roles in mRNA binding[20] and translation regulation through its ability to bind adenylate-uridylate (AU) right regents in 3' untranslated regions (UTRs) of mRNA's such as IFN gamma and IL [21] 2 . These researchers found that, in cells cultured in galactose, which were not able to undergo aerobic glycolysis, GAPDH bind to the 3' UTR of IFN gamma mRNA, and that this binding inhibits IFN gamma expression, demonstrated both by the correlation between high GAPDH expression and low IFN gamma production as identified in infected mice, as well as retroviral expression of GAPDH was sufficient to decrease IFN gamma production[19]. These researchers also made the connection between metabolism and the control of an immune response, as galactose-cultured cells, in which aerobic glycolysis was restricted, had a less activated phenotype as determined by lower expression of the checkpoint marker programmed cell death 1 (PD1),

and when nutrient restriction was induced by coculture with EL-4 lymphoma tumor cells, cytokine production was also reduced[19] . The role of metabolic enzymes in non-metabolic processes is commonly referred to as "moonlighting,"[22] and evidence of this phenomenon can be found in a number of other metabolic pathways.

While this role of aerobic glycolysis in the promotion of IFN gamma production and activation through the modulation of GAPDH, another set of researchers sought to further define the role of aerobic glycolysis in mounting a T cell response and were able to find an alternative mechanism for its importance. Peng and colleagues sought to use a knockout system rather than culture in galactose, as galactose can still be metabolized to lactate[23] , meaning that the previously described culture method does not, in their estimation, fully abolish the aerobic glycolysis phenotype. Instead, these researchers utilized a knockout system, in which the enzyme responsible for the conversion from pyruvate to lactate, LDH, was knocked out[24] . This conversion from pyruvate to lactate is the central pathway to the Warburg effect, and upon its knockout glucose consumption as well as ECAR in these cells were reduced, and knockout cells were forced to rely more on oxidative metabolism, as OCR was dramatically increased. These metabolic changes were accompanied by a corresponding decrease in IFN gamma production, but in contrast to previous research[19] , further experiments indicated that this decrease in IFN gamma production was not the result of interaction with its 3' UTR[24] . The mechanism these researchers identified instead relied on histone acetylation, in that varying levels of the activating histone mark H3K9ac were found on a number of genes in LDH A isoform knockout cells, including IFN gamma. Supplementation of these knockout cells with acetate, or knockout of the ATP-citrate lyase enzyme, responsible for the conversion of citrate to acetyl-CoA, both lead to the restoration of IFN gamma production. Therefore, these researches identified an alternative mechanism by which aerobic glycolysis promotes T cell activation not as a direct consequence of its metabolic effects but through its role in modulating the transcription and regulation of the immune response.

Although aerobic glycolysis is crucial for the full activation and effector function of T cells, mitochondria play important and indispensable roles in this process too. While their role in the production of ATP may be limited, evidence their production of reactive oxygen species (ROS) which can act as a signaling intermediate has found to be crucial in T cell activation. Sena and colleagues studied this phenomenon in mice with a T-cell specific knockout of an ETC complex III component, the Rieske iron sulfur protein (RISP)[25] . These researchers found that following calcium influx induced by TCR triggering, there was an increased production of mitochondrial ROS (mROS), and that this was necessary for T cell activation, as RISP knockout mice were unable to produce IL2 or upregulate the activation markers CD69 and CD25 following CD3/CD28 stimulation. Furthermore, knockout cells were not able to activate and expand either *in vitro* or in *in vivo* models of lung inflammation and infection. Although the role of aerobic glycolysis in T cell activation is the most widely regarded, mitochondrial metabolism plays a crucial role as well, and by discriminating between the two, immune responses can be very carefully tailored, as will be discussed further.

T cell metabolism changes in distinct CD4 T cell subsets

Just as T cell metabolism changes throughout the course of activation, the metabolism of different T cell subsets differs dramatically between the subsets. These changes occur in order to optimize the metabolism of a particular T cell subset to its roles, with changes reflecting the need for rapid bursts of proliferation or activity, or the requirement to remain long-lived in order to mount a memory response, or the ability to become activated in a tissue environment with differing nutritional availabilities. Many of these changes are facilitated through the mammalian target of rapamycin (mTOR) kinase, which forms a central regulation node not only of metabolism but in the government of the immune response[26] . mTOR signaling is carried out through two complexes, mTORC1 and mTORC2, which are differentiated by their incorporation of distinct partners, with the regulatory-associated protein of mTOR (RAPTOR) being a component of mTORC1, and the RAPTOR-independent companion of

mTOR (RICTOR) as a part of the mTORC2 complex, with some factors being shared between the two[26,27,28]. Previous work in the Powell lab has focused on the role of mTOR signaling in T cell responses in order to define a dichotomy of signaling that extends to the metabolic perturbations induced by its signaling. Initial work in the lab focused on the signaling events associated with T cell anergy[29] , finding that inhibition of mTORC1 by the use of rapamycin treatment was sufficient to induce anergy both *in vitro* and *in vivo*, but that reversion of the anergic state *in vitro* was accompanied by increases in mTORC1 signaling as assessed by the phosphorylation of S6 kinase-1 (S6K-1)[30]. Further work by the Powell lab demonstrated that the requirement for specific mTOR complex signaling was dependent on T helper subset commitment. Using a model in which the mTOR kinase was specifically deleted in T cells, Delgoffe and colleagues demonstrated that lack of mTOR signaling lead to inhibited proliferation, although IL2 production and activation as assessed by surface expression of CD25 and CD69 remained intact[31]. It was further found that these cells were not able to differentiate into Th1, Th2, or Th17 T cells in the presence of appropriate skewing conditions, but rather polarized towards the regulatory T cell (Treg) subset. This model was further refined by subsequent studies, which, by use of T cell specific knockout of the mTORC1 regulator Rheb determined that mTORC1 signaling was required for the differentiation of Th1 and Th17 cells, but it was disposable for the development of Th2 cells, with Rheb knockout mice being resistant to the IFN gamma and IL17 driven development of experimental autoimmune encephalitis (EAE)[32]. Furthermore, when T cell specific RICTOR knockout was performed, the resulting T cells retained their competency to develop into Th1 and Th17 cells, but were incapable of mounting a Th2 mediated response, as demonstrated by a decreased response to chicken ovalbumin (OVA) adjuvanted with alum model of asthma. These findings lead to a model of T cell subset commitment based on the combination of mTOR signaling and metabolism, in which activation leads to mTOR signaling which can then modulate the metabolism of the T cell[33]. The strength of mTOR signaling

can then be informative about the progression of the immune response, leading to sufficient metabolic alterations to tailor the metabolism of the cell to the response appropriate for the setting.

The role of metabolism in making T cell fate decisions extends beyond the role of mTOR signaling. The role of glycolysis in the differentiation of CD4 T cell subsets was first identified by Michalek and collegues[34]. In this pioneering work, the metabolism of effector T cells and Tregs was compared, and it was determined that the effector subsets of CD4 T cells, Th1, Th2, and Th17 cells, are primarily glycolytic as determined by their consumption of glucose in the media, while Tregs relied primarily on mitochondrial respiration, and particularly lipid oxidation. The use of lipid metabolism, enforced by the addition of exogenous fatty acids, promoted the generation of regulatory T cells, and decreased the number of live, cytokine producing Th1, Th2, and Th1 7cells as compared to standard media under skewing conditions. Furthermore, as previous research indicated that inhibition of mTOR signaling promoted the generation of Tregs[31], these researches examined the relationship between Treg skewing and fatty acid metabolism by inhibited mTOR signaling by the use of rapamycin treatment, and noted an increase in lipid oxidation following T cell stimulation, closing the loop and demonstrating that the modulating of T cell metabolism can alter the fate decisions of activated CD4 T cells.

In addition to the role of fatty acid metabolism, other metabolic factors have been noted to be important for the discrimination between effector and regulatory T cell subsets. Studies by Klysz and colleagues identified presence of the amino acid glutamine, the metabolism and role in T cell activation will be discussed more fully later, as important for the differentiation of Th1 cells, with its absence leading to Treg polarization[35]. These *in vitro* studies, performed in media with the complete absence of glutamine, demonstrated that Th1 polarizing conditions without glutamine lead to the development of Tregs, while claiming that Th2 cells were able to polarize properly in the absence of glutamine, however evidence for this was limited to expression of Gata3 as assessed by flow cytometry. Tregs that were generated from culture in the absence of glutamine were found to be capable of proliferating in order to

reconstitute a sublethally irradiated mouse and suppressive in a mouse model of inflammatory bowel disease in Rag knockout mice. The *in vitro* mechanistic underpinning of this phenomenon was found to be decreased aKG levels in Th1 cells cultured in the absence of glutamine, and supplementation with a cell-permeable aKG derivate, dimethyl ketoglutarate (DMK), was shown to rescue Tbet expression as well as IFN gamma production from these cells[35] .

The role of glutamine in subset differentiation was further explored in a system in which the first enzyme in the pathway of glutamine metabolism, the hydrolysis of glutamine to glutamate as carried out by the enzyme glutaminase (GLS)[36], was inhibited both by use of genetic and pharmacologic means. In this study by Johnson and collegues[37] , it was determined that glutamine metabolism was rapidly upregulated upon T cell activation by use of mass spectrometry tracing experiments to assess the incorporation of an isotopically labelled glucose tracer. Activation of CD4 T cells was shown to increase intracellular glutamate and aKG levels, and while the absence of glutamine in the media prevented polarization of these cells to both Th1 and Th17 fates, GLS inhibition was only able to prevent Th17 skewing. A T cell specific knockout of GLS was shown to modulate activation of CD4 T cells, leading to reduced CD25 and CD44 induction, as well as exhibiting decreased Th17 skewing with little inhibition on Th1 cell development. This phenotype was borne out *in vivo* in an IL17 dependent model of chronic graft versus host disease (GvHD), which was attenuated in a GLS knockout setting. Curiously, assessment of GLS knockout cells in a house dust mite (HDM) induced asthma setting yielded a slight decrease in IL4 production, indicating a role of glutamine in Th2 cell development which will be more fully explored in this thesis.

In addition to its role in the promotion of the glycolytic phenotype upon activation, HIF plays an important role in the fate decisions between Th17 and Treg development. Although both Tregs and Th17 cells require transforming growth factor (TGF) beta signaling for their development, the presence of inflammatory signals at the time of polarization can help shift the balance between Th17 and Treg

cells[38]. Findings from Shi and colleagues sought to assess this important role, and first noted that, upon skewing of CD4 T cells to different subsets, HIF expression was by far highest in Th17 cells, while it was nearly absent in cells differentiated towards the Treg subset[39]. Attempts to skew HIF1a knockout CD4 T cells towards the Th17 fate led to a decrease in cells producing both IL17 and IL22 transcript, and reduced EAE clinical score when the disease was induced in HIF1a knockout mice. This was accompanied by dramatic transcriptional changes, in that microarray analysis of HIF1a knockout T cells indicated that the metabolism of these cells was dramatically different, with the upregulation in glycolytic machinery typically observed in T cell activation absent in the knockout mice. Further analysis revealed that, in keeping with previously summarized literature, that it is the activity of the glycolytic pathway (as well as mTOR signaling) that is crucial for the development of nascent Th17 cells, as inhibition of glycolysis with 2-deoxy-D-glucose (2-DG), a structural analogue of glucose that acts as a competitive inhibitor of hexokinase[40,41] and has been widely analyzed for its use as a potential chemotherapeutic agent based on its ability to induce death in cancer cells[42], as well as mTOR inhibition through the use of rapamycin, were both sufficient to inhibit the development of Th17 cells *in vitro*, and 2-DG treatment ameliorated Th17 development *in vivo* as well[39] . Further studies by Dang and colleagues added more regulatory insight into this phenomenon by analyzing the role of HIF in the signaling pathways that lead to fate decisions between the Th17 and Treg subsets[43] . This work determined that the HIF1a upregulated upon the induction of a Th17 development program occurs as a result of signal transducer and activator of transcription (STAT) 3 signaling, and that protein production of HIF1a in Th17 skewing conditions was abolished in STAT3 knockout T cells. Generation of T ell specific HIF1a knockout mice further demonstrated the requirement of HIF1a in Th17 development *in vitro* under both hypoxic and normoxic conditions, and chromatin immunoprecipitation (ChIP) experiments demonstrated that HIF1a binds to the promoter of the Th17 hallmark transcription factor ROR gamma t in order to induce its transcription, and the HIF1a and ROR gamma t combine to bind to the IL17

promoter to regulate the expression of this cytokine as well. As a further mechanism for the enforcement of the Th17 polarization as opposed to Treg development, HIF1a promotes the proteasomal degradation of FoxP3 via targeting for ubiquitination and degradation. Therefore, the balance between the Th17 and Treg subsets is intricately balanced based on the metabolic components of the cell, as well as HIF expression through multiple pathways.

However, the balance between Tregs and Th17 cells is not solely controlled by HIF1a. The role of fatty acid metabolism in the development of Tregs was demonstrated by Berod and colleagues[44] , in a system which sought to further understand the role of acetyl-CoA carboxylate (ACC) enzymes, which are responsible for the conversion of acetyl-CoA to malonyl CoA, which is essential for both the synthesis and catabolism of long chain fatty acids[45]. The researchers hypothesized that, based on the fact that the metabolic requirements of Tregs were known and distinct from other effector T cell subsets, they would be able to modulate immune responses through the modulation of the ACC isoforms. By the use of soraphen A (SorA), a polyketide isolated from *Sorangium cellulosum* and initially used as an anti-fungal, the activity of ACC isoforms could be specifically inhibited[46]. Skewing experiments carried out in the presence of this inhibitor demonstrated that Th17 cells would become polarized to a Treg phenotype, losing the expression of IL17 and gaining FoxP3 expression[44]. Use of T cell specific knockouts for either the ACC1 or ACC2 isoform demonstrated that it is the ACC1 isoform, which is typically present in tissues such as the liver and adipocytes which make lipids, to be the responsible for this effect, as ACC2 deficient cells underwent Th17 polarization normally while ACC1 deficient cells did not. Metabolic profile analysis indicated that cells that were either treated with SorA or ACC1 deficient had decreased ECAR, while ECAR was highest in the highly glycolytic Th17 cells. Tracing experiments utilizing heavy isotope labelled glucose indicated that substantial fractions of label incorporated into the fatty acid palmitate in T cells undergoing Th17 skewing, and lower incorporation into TCA cycle biosynthesis products such as amino acids. Tracing experiments in ACC1 knockout T cells lost this incorporation of

labelled glucose into fatty acid synthesis pathways, as did treatment with SorA, indicating that this was an ACC1 mediated phenomenon. Treatment with a SorA derivative *in vivo*, as well as experiments with ACC1 knockout mice, lead to a dramatic decrease in EAE severity and a delay in onset, further confirming the role of fatty acid synthesis in the development of Th17 cells[44] .

While the discussion of the metabolism of Treg cells has thus far been confined to what they are not (not glycolytic[39] , not reliant on fatty acid synthesis[44]), work by Angelin and colleagues sought to positively determine what Treg metabolism is through experiments exploring the role of the hallmark Treg transcription factor, FoxP3, on cell metabolism[47]. As it was already known that Tregs had a more OXPHOS based metabolism[34], these researchers sought to assess the mechanism for this finding and its relation to FoxP3. By retrovirally inducing FoxP3 expression, these researchers found an induction of suppressive phenotype, as well as a trend towards increased OCR, as has also been seen by other researchers inducing FoxP3 expression[48]. Microarray analysis indicated that retrovirally induced FoxP3 expression leads to dramatic gene expression changes, particularly in the downregulation of Myc targets, including multiple glycolytic enzymes. They further demonstrated a differential requirement for ETC complexes in suppressive function by using mouse models of mitochondrial defects, and determined that ETC complex I, but not ETC complex IV, was essential for Treg suppressive function[47]. As Tregs are crucial in an anti-tumor response within the tumor microenvironment (TME), further studies were undertaken to assess the role of CD4 metabolism within the tumor microenvironment. Work by Ho and colleagues[49] sought to determine what lead to T cell exhaustion within the TME, and hypothesized it might be metabolic competition between the tumor and the T cell, leading to glucose limitation. After confirming that glucose is indeed lower in tumor interstitial fluid than in blood or spleen, they noted that coculture of tumor cells with Th1 polarized CD4 T cells reduced glucose uptake of the T cells, and this lead to changes in the transcriptional program of the T cells as well as limits to their effector function, as indicated by decreased production of IFN gamma and IL2, as well as decreased

cell surface CD40L expression. By analyzing the pathways of signal transduction in these glucose-deprived CD4 T cells, it was noted that both calcium flux and nuclear NFAT localization were decreased during stimulation either in low glucose conditions or in the presence of the inhibitor of glycolysis 2-DG, leading to dramatic changes in gene expression that resulted an anergic transcriptional profile. This low glucose induced inhibition of T cell effector function was found to be overridden by overexpression of phosphoenolpyruvate carboxykinase 1 (PCK1), the enzyme responsible for the conversion of oxaloacetate to phosphoenolpyruvate, which increased CD4 T cell production of IFN gamma as well as CD40L expression in the CD4 tumor infiltrating lymphocyte (TIL) population. This led to decreased tumor growth and extended lifespans for mice treated with adoptive cellular therapy (ACT) consisting of PCK1 overexpressing CD4 T cells.

The role of CD4 T cell metabolism in subset fate determination, as well as the important roles metabolism plays on effector function, demonstrate that the metabolic fluctuations that occur in CD4 T cells upon activation and subset polarization are not merely byproducts of these processes, but are actively driving the development of the immune response. Many of these metabolic changes upon activation are controlled by the mTOR kinase signaling pathways, and manipulation of mTOR signaling has been demonstrated to be a powerful means of altering metabolism, and thus CD4 T cell subset fate decisions. By altering the metabolism of different CD4 T cell subsets at time of activation, either by withholding particular nutrients of note, or inhibiting their pathways of activation either through genetic or pharmacologic means, it is possible to dramatically shape the immune response. The role of metabolism in shaping the immune response will be further emphasized by examination of CD8 T cell responses, in that metabolic perturbation can have dramatic effects of T cell fate decisions, indicating that metabolism is not a bystander, but rather is driving processes of T cell fate determination.

CD8 T cell memory establishment and persistence are controlled by metabolism

The fate decision of CD8 T cells in regards to metabolism that has been most studied is the differentiation between effector and memory CD8 T cells. The T cell response to stimulation, such as a viral infection, is characterized by a massive proliferative burst of antigen specific cells, leading to huge clonotypic expansion of cytotoxic T cells that can target and eliminate the infection. Following resolution of infection, a massive contraction in this antigen specific CD8 T cell population occurs, leaving only a limited fraction of memory CD8 T cells[50] , which bear different surface markers and homing patterns[51] , and are primed to faster and more effective restimulation should the same infection recur. The question of what determines which specific cells of the clonally expanded population survive this contraction to become the long-lived CD8 memory T cells is an open one in the field, and while distinct patterns of transcription factor expression have been associated with memory T cell development[52] , recent findings indicate that one of the most powerful mechanisms for the development of memory CD8 T cells may be metabolism. These findings will be summarized in this section.

The initial findings that implicated metabolism in the generation of CD8 T cell memory were made by Pearce and colleagues in the setting of a tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) knockout mouse[53]. T cell specific TRAF6 knockout mice were subjected to infection with *Listeria monocytogenes* bacteria genetically engineered to express OVA (LmOVA)[54] and it was discovered that, while initial response to LmOVA infection was similar between wildtype and knockout cells, as assessed by both cytokine production and antigen specific cell percentage, TRAF6 knockout mice could not mount a memory response, and dramatically decreased percentage of antigen specific cells were found at 60 days after initial infection in the knockouts. Re-challenge experiments confirmed the lack of CD8 T cell memory induction in TRAF6 knockout mice, as these mice did not exhibit an expansion of adoptively transferred antigen specific cells following secondary stimulation, nor were these cells maintained following contraction. Microarray analysis to determine gene expression changes at 10 days

following infection were post dramatic in the expression of genes involved in metabolism, and particularly fatty acid oxidation. Fatty acid oxidation was found to increase upon the withdrawal of IL2 from cultures of transgenic CD8 T cells specific for the SIINFEKL peptide of OVA presented in the context of MHC class I (OTI), however this increase was not noted in TRAF6 knockout cells. Activation of fatty oxidation by treatment with the AMP-activated kinase (AMPK) activating drug metformin[55] was able to rescue fatty acid oxidation in TRAF6 knockout cells, and *in vivo* administration of metformin promoted the survival of both TRAF6 and endogenous antigen specific CD8 T cells through contraction in an adoptive transfer model, as well as promote survival in a tumor model in which an initial vaccination with LmOVA was given, followed 3 weeks later by challenge with an OVA-bearing EL-4 lymphoma. Treatment with rapamycin was also sufficient to promote increased memory cell formation in both wildtype and TRAF6 knockout systems, the mechanism of which will more fully discussed later.

This initial identification of the importance of fatty acid oxidation for the development of memory CD8 T cells was followed in quick succession by work that expanded on this concept to explore the mechanistic underpinning of why fatty acid oxidation was essential for memory CD8 T cell development. Work by van der Windt and colleagues identified mitochondrial metabolism, and particularly the capacity for its enhancement, as a key factor in the determination of a memory phenotype[56]. This study utilized metabolic analysis to determine that, at baseline, the OCR of memory CD8 T cells was increased as compared to effector T cells, while memory cell ECAR was decreased. However, analysis using the ATP synthase uncoupling drug carbonylcyanide p-trifluoromethoyxphenylhydraone (FCCP)[57] demonstrated that memory CD8 T cells had the largest difference between their basal and maximal OCR, a characteristic known as spare respiratory capacity (SRC). This increased SRC was further studied in an *in vitro* CD8 T cell memory model, in which OTI T cells are stimulated for 3 days and then subjected to 4 further days of culture in either IL2 or IL15, the former promoting an *in vitro* T effector phenotype, with the latter promoting more of a memory

phenotype[58]. These *in vitro* studies confirmed that memory CD8 T cells have increased OCR as SRC as compared to effector T cells[56] . These researchers next sought to determine what about IL15 signaling in culture induced a more memory-like phenotype, and determined that IL15 culture actually increased mitochondrial mass in memory CD8 T cells, a finding which was recapitulated in memory cells generated from *Listeria monocytogenes* infection. Researchers next identified the mitochondrial fatty acid transporter carnitine palmitoyltransferase 1 (CPT1) as being upregulated in IL-15 induced memory cells and that this was responsible for the increased SRC noted in these cells, and further found that overexpression of CPT1 lead to increased memory CD8 T cell persistence in the LmOVA model.

As it was observed that enhancement of fatty acid oxidation enhanced the memory phenotype of CD8 T cells, corresponding observations into the role of glycolytic activity in CD8 T cells were also made. Sukumar and colleagues sought to understand the role of glycolysis in CD8 T cell differentiation by demonstrating that overexpression of the glycolytic enzyme phosphoglycerate mutase-1 (Pgam1) promoted the generation of CD8 effector T cells as opposed to memory cells[59]. First, these researchers extended the finding that CD8 effector cells are more glycolytic than memory cells by segregating peptide-activated transgenic CD8 T cells by their uptake of the fluorescent glucose analogue 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose (2-NBDG) and subjecting them to further analysis by both transcriptional and metabolic endpoints, indicating that cells that uptake high levels of 2-NBDG have an increased ECAR, and increased expression of genes associated with an effector phenotype such as perforin and granzyme, while the low 2-NBDG uptake cells had an increased OCR/ECAR ratio, as well as increased expression of transcription factors associated with memory CD8 T cell formation, such as TCF1 and BCL6[60]. Adoptive transfer of these glycolytically distinct populations illustrated that the low 2-NBDG uptake cells were more persistent memory cells after transfer. Experiments to increase glycolytic phenotype by enforcing the expression of Pgam1 by use of retroviral transduction, lead to increased ECAR and but decreased persistence of cells in an adoptive transfer model. Conversely, *in vivo* memory

persistence as modeled by adoptive transfer of cells that received *in vitro* treatment with 2-DG to inhibit glycolysis were sufficient to promote memory CD8 T cell persistence, as well as inhibit tumor growth and improve survival in an ACT model to treat and implanted melanoma. This idea of restrictions of metabolism in culture being efficacious for the promotion of memory as well as in an anti-cancer immune response is one that will be discussed further, but these findings demonstrate the importance of mitochondrial metabolism, as induced by the restriction of glycolytic metabolism, in the promotion of a memory CD8 T cell response.

Further study of mitochondrial dynamics in the development of memory CD8 T cell responses determined that the structure of mitochondria also play an important role in their function as it relates to the promotion of a recall response. Studies by Buck and colleagues determined that the fused nature of the mitochondrial network in memory CD8 T cells is essential to their function as memory cells[61]. Following the previous observation that there is increased mitochondrial mass in IL15 induced *in vitro* memory CD8 T cells, the mitochondrial structure of effector and memory cells were assessed *ex vivo* following LmOVA infection by electron microscopy and it was found that memory cells contained a more fused mitochondrial structure, as defined by the formation of elongated tubules, as opposed to the smaller and more distinct clusters of mitochondria seen in the effector T cells. Further *in vitro* experiments sought to define this phenomenon, and determined that the T cell specific knockout of mediator of mitochondrial fusion, Opa1, lead to decreased mitochondrial organization as well as decreases in the SRC and memory formation as assessed in an adoptive transfer setting in cells deficient for this factor. Further modulation of mitochondrial dynamics by the use of drugs that promote mitochondrial fusion and inhibit mitochondrial fission promote a memory phenotype both in regards to surface receptor expression as well as metabolically, by leading to increased SRC, demonstrating that these changes in mitochondrial dynamics were key to the development of CD8 memory T cells.

Attempts to utilize this knowledge for the promotion of CD8 T cells that are more efficacious in ACT treatment was demonstrated by Sukumar and colleagues in a model in which they used the membrane potential-sensitive dye ethyl ester of tetramethylrhodamine perchlorate (TMRM)[62] to identify subsets of CD8 T cells based solely on their mitochondrial membrane potential, and demonstrated that cells with lower mitochondrial membrane potentials persist better *in vivo*, and are more effective at mounting and anti-tumor immune response[63]. Initial identification of this phenomenon was conducted by the *in vivo* activation of mice bearing transgenic CD8 T cells, and sorting based on TMRM fluorescence. These experiments determined that the low membrane potential cells resembled more of a central memory subset, with increased presence of CD44 positive CD62L positive cells. Adoptive transfer experiments assessing the *in vivo* expansion of low membrane potential cells demonstrated that they are capable of robustly expanding in lymphodepleted hosts, which was not the case of the high membrane potential CD8 T cells. RNA sequencing analysis demonstrated differential gene expression patterns between the high and low membrane potential cells, with increased expression of memory promoting factors in the low membrane potential cells, as well as global metabolic profiling revealing increased carnitine species as well as increased levels of free fatty acids, corresponding with increased CPT1a expression on low membrane potential cells. One potential key to the long-lived nature of these low membrane potential cells was identified as reduced oxidative stress, because while ROS is essential for T cell activation[25], too much can induce oxidative damage, and thus it was found that low membrane potential cells had large store of oxidized glutathione in order to combat oxidative stress, as well as reduced levels of DNA damage. Lastly, an adoptive transfer model demonstrated that at long time points, up to 300 days after transfer, low membrane potential cells persisted better and were more active, while these cells also were more activate in an anti-tumor response against a melanoma tumor. This demonstrates that not only does metabolism alter T cell

memory fate decisions, but that selection of T cell precursors based on metabolic phenotypes provides a robust strategy for the control of CD8 T cell fate.

Thus far, this summary has avoided the role of mTOR signaling in CD8 T cell fate decisions as it relates to effector or memory T cells. Although the initial finding that rapamycin could induce increased memory CD8 T cell function in TRAF6 deficient T cells, the mechanism ascribed to this was the promotion of fatty acid oxidation through AMPK mediated signaling, rather than directly relating to mTOR signaling[53]. However, a great deal of work has been done to demonstrate the crucial role of mTOR signaling in CD8 T cell fate development. Pollizzi and colleagues first identified a differential requirement for mTORC1 and mTORC2 signaling in CD8 T cell effector and memory differentiation[64]. Working in a system in which the negative regulator of mTORC1 signaling, tuberous sclerosis complex 2 (TSC2) is selectively deleted in T cells, mTORC1 activity becomes hyperactive. In these high mTORC1 cells, effector function is greatly enhanced as evidence by increased cytokine production, and this effector function was indeed found to be reliant on mTORC1 activity, as T cells deficient for the GTPase Ras homologue enriched in brain (RHEB) were incapable of mounting effector responses both *in vitro* and *in vivo*. However, the promotion of the effector response in the hyper mTORC1 TSC2 knockout mice came at the expense of a memory cell fate, as very few TSC2 knockout cells remained 30 days following adoptive transfer and *Vaccinia*-OVA infection. These studies took place in the presence of sufficient mTORC2 signaling, and thus the researchers next sought to determine the role of mTORC2 in effector T cell generation, determining through the use of an mTORC2 deficient T cell specific RICTOR knockout system that there was no defect in effector cell numbers or activation status as assessed by either surface markers or cytokine production. However, experiments with the RICTOR knockout T cells indicated that loss of mTORC2 signaling promoted a memory response, with the longer-lived RICTOR knockout T cells bearing many signs of a memory phenotype, such as increased surface expression of CD62L and CD127, as well as metabolic markers of memory, such as increased OCR and RNA expression

of CPT1a. All in all, this study demonstrates the role of mTORC1 signaling in the development of effector T cells, a role which will be further defined in an attempt to differentiate between precursors that will become effector or memory cells.

The asymmetric inheritance of cell fate determinates is an important developmental process for the differentiation of many cells[65], such as the maintenance of a stem cell population. After work in CD4 T cells that determined cell size was indicative of mTORC1 activation following activation[66], Pollizzi and colleagues next sought to apply the asymmetric inheritance of mTORC1 signaling to CD8 T cell effector or memory subset commitment fate decisions[67]. These researchers first identified that, upon initial stimulation and first division, mTORC1 activity as not equally distributed between the CD8 T cells, and correlates with the expression levels of CD8 itself. Cells that were higher in CD8 expression were also higher in mTORC1 activity, as demonstrated by increased phospho-S6 levels in these cells. These two subsets of mTORC1 high and low CD8 T cells has distinct metabolic capabilities, as the mTORC1 high cells exhibited increased ECAR and decreased OCR as well as SRC compared to the mTORC1 low cells, and the mTORC1 low cells were found to have increased mitochondrial mass as assessed by MitoTracker staining. *In vivo* assessment of the survival capacities of these different cell subsets correlated with the previously identified differences in mitochondrial metabolism, with low CD8 (and low mTORC1) cells being more persistent in adoptive transfer experiments. The mechanism behind the differential mTORC1 inheritance between daughter cells was found to be differences in mTOR translocation to the lysosome as induced by differential expression of CD98, which couples with SLC7A5 to facilitate leucine uptake in activated T cells (the role of CD98 and SLC7A5 in T cell activation will be more fully expanded upon later in this thesis). Increased CD98 expression was found to correspond with the CD8 high daughter cells, leading to increased mTORC1 activity in these more effector-like cells. As was previously described using mitochondrial membrane potential [63], this work identifies a feature of CD8 T cells predisposed to becoming memory CD8 T cells early on in the course of activation, at the time of first

division, and demonstrates that it is possible to segregate these cells for the enhancement of a memory CD8 T cell response[67].

While the studies discussed thus far have led to the straightforward and robust model of glycolysis as promoted by high mTORC1 signaling being the preferred form of metabolism for effector CD8 T cells, and enhanced mitochondrial metabolism, particularly SRC as made possible by fatty acid oxidation being the path to memory cell development, it is important to note that these findings have, for the most part, been made in the context of *in vitro* settings, and thus their application to the *in vivo* condition, while demonstrated in many cases, may not be direct. One example of the *in vivo* setting reflecting a dramatically different metabolism than *in vivo* is in regards to the use of lactate in the metabolism of immune cells. While thus far lactate production has been primarily been used as a surrogate marker for aerobic glycolysis, as measured by ECAR, Hui and colleagues sought to examine the role of lactate in *in vivo* flux of labelled carbon, and made a surprising discovery about what is widely regarded as a byproduct of aerobic glycolysis[68]. By conducting tracing experiments to identify the exchange in circulating metabolic intermediates, these researchers identified the high circulatory levels of lactate in flux, which was found to be about 2 and a half fold higher than glucose, long assumed to be the primary carbon structure in circulation. In fact, many of the metabolites that had long been considered crucial to the regulation of the immune response, such as pyruvate, alanine, and glutamine, among others, had a flux of less than 20% that of lactate. The labeling of TCA cycle intermediates malate and succinate was found to be greatest from incorporation of lactate tracer, and further analysis using a linear algebra model determined that (except in the brain) direct passage from glucose through glycolysis and then into the TCA cycle is nearly non-existent in fasted mice, while in fed mice roughly 40% of glucose entry into the TCA cycle passes through circulating lactate first. These findings demonstrate that the textbook biology of metabolic pathways is not always what is observed *in vivo*,

and that the extensive body of *in vitro* findings may not directly translate to the *in vivo* setting, thus the need for novel strategies to assess *in vivo* metabolism more directly is quite pronounced.

One such strategy to advance *in vivo* metabolic profiling in CD8 T cells was recently outline by Ma and colleagues. In this work, they used a bead isolation approach to facilitate the more rapid characterization of OTI cells stimulated with LmOVA to assess their metabolism following 3 days of *in vivo* activation[69]. These *in vivo* isolated cells were compared to either *in vitro* stimulation conditions or a T cells after a short, less than 4 hour, period of *ex vivo* culture to assess the differences in metabolism that occur as a result of differing stimulation and culture conditions. These studies indicated that the OCR of *ex vivo* isolated OTI was substantially higher than that of *in vitro* cultured cells, leading to an increased ATP production rate in these cells, fueled by their increased OXPHOS. These isolated *in vivo* cells also had substantial transcriptional and metabolic differences from *in vitro* stimulated cells, including differences in the incorporation and use of both glucose and pyruvate, including the far lower labelling of lactate from glucose identified in *in vivo* isolated OTI, and instead the incorporation of the glucose label into nucleotide and sugar synthesis, with UDP-glucose and UMP being the most enriched for labelled carbons derived from glucose in *in vivo* isolated cells as opposed to those stimulated *in vitro*. This work serves to demonstrate some of the differences between the *in vitro* and *in vivo* conditions, differences which will be discussed subsequently, and illustrate that it is important to remember that while extensive work has been done in immunometabolism, much of it has considered the *in vitro* system, rather than what may actually exist in the *in vivo* organism. As metabolism is highly plastic, it can be imagined that part of the changes witnessed are due not necessarily to reprogramming controlling activation, but also potentially due to circumstance and the impact of the nutrient milieu on metabolic changes examined. Therefore, much care must be taken to assess metabolism in its full context, and ensure that all appropriate efforts are taken to consider the circumstances surrounding the observation.

Glutamine metabolism plays an important role in T cell metabolism upon activation

Although not technically an essential amino acid, glutamine has been demonstrated time and time again to be "conditionally essential[70]" to a wide variety of cells that are undergoing a period of stress or elevated metabolic demands, such as cells of the intestinal mucosa, rapidly proliferating cancer cells, and recently activated T cells. Similar to the Warburg effect and the metabolic changes that occur upon activation, much of the work regarding glutamine metabolism in T cells has traditionally been understood in cancer metabolism[8] . Glutamine is taken up into the cell by ASCT2[71], at which point a primary fate is entry into the TCA cycle. Glutamine is converted to glutamate as well as an ammonium ion by GLS, and then can be converted to aKG by glutamate dehydrogenase (GLUD)[72]. This conversion into aKG allows the incorporation of glutamine into the TCA cycle, which, as previously discussed, is used in the metabolism of an activated T cells not for the generation of electron donors for the ETC and ATP generation, but rather for the generation of biosynthetic precursors that can be used to promote cell growth and activation. Examples of this include production of other amino acids using the nitrogen from glutamine, fatty acid synthesis, protein synthesis, nucleotide biosynthesis, and control of ROS through the generation of glutathione[73]. As previously discussed, glutamine incorporation into the metabolism of proliferating glioblastoma cells was assessed by DeBerardinis and colleagues using a NMR-based approach, and these findings indicated that glutamine is a major anaplerotic substrate for many biosynthetic pathways in the cell, including fatty acid synthesis, and the generation of oxaloacetate (OAA) produced from aKG, which replenishes the TCA cycle[13] . Other studies have contradicted this work, finding in the setting of cancer cell culture that glutamine-derived carbons are less important for the generation of cell mass and biosynthetic intermediates, and rather that these are generated by other essential and non-essential amino acids. Instead, these researchers found that glutamine was primarily used for protein synthesis, or the generation of other amino acids[74].

Although the previously described roles from glutamine have primarily been identified in the setting of cancer cells, important work has been done in T cell metabolism to identify the role of glutamine in T cell activation. Previous working describing the role of glutamine in Th1 CD4 T cells[35] and in Th17 cells as indicated by GLS knockout[37] has been summarized in an earlier portion of this chapter. Pioneering work into the role of glutamine in T cell proliferation was carried out by Carr and colleagues, who assessed the distinct amino acid requirements for this process[14]. By restricting amino acids from culture following anti-CD3 stimulation, these researchers determined that, while several both essential and non-essential amino acids were required for T cell proliferation, it was glutamine that was most impactful, as cutting glutamine in culture in half demonstrated a dramatic decrease in proliferation as assessed by tritiated thymidine incorporation, and dropping it to 10% of the standard concentration nearly completely inhibit proliferation. Glutamine free stimulation inhibited both proliferation and cytokine production robustly in these cells, and addition of other biosynthetic precursors such as glutamate, proline, or asparagine was not able to rescue this proliferative defect. Interestingly, the early activation markers CD25 and CD69 were still induced upon stimulation in glutamine- free settings. T cell activation stimulated a corresponding increase in glutamine uptake, as well as increased expression in glutamine transporters at both the RNA and protein levels. This work demonstrates the essential role that glutamine plays in T cell activation and proliferation, a role that will be more fully explored in this thesis.

In addition to its role in T cell proliferation, glutamine is important for the control of ROS, essential for T cell activation[25] but potentially dangerous, through the production of glutathione[75], which is made up of the amino acids glutamine, cysteine, and glycine[73]. Glutamine is also essential for the production of *de novo* pyrimidines, as illustrated by Beh-Sahra and colleagues[76]. In this work, the role of glutamine in the production of pyrimidine nucleotides was assessed and was found to be dependent on mTORC1 signaling, as it was inhibited both by treatment with rapamycin as well as in TSC2 knockout

cells. Swamy and colleagues found that glutamine was also essential for the production of UDP-GlcNAc, which forms the basis of the protein modification O-GlcNAc[77]. These researchers found that UDP-GlcNAc synthesis was increased upon TCR stimulation in T cells, and its levels increased almost 10-fold in the first 24 hours after activation[78]. This post-translational modification was found to be highly dynamic on T cells, but was increased dramatically following stimulation in CD4 and CD8 T cells. Using a tamoxifen-inducible knockout of the enzyme responsible for adding the O-GlcNAc modification to proteins, O-GlcNAc transferase (OGT)[77], these researchers demonstrated that proliferation and c-Myc expression were dramatically reduced, and that both glucose and glutamine in the media were required for normal O-GlcNAc levels on T cells[78]. Glutamine is also important for epigenetic modifications, serving as a cofactor for the histone and DNA demethylases[79], as well as a substrate for histone acetylation by promoting acetyl-CoA through TCA cycle metabolism[24] . These works illustrate that the amino acid glutamine has many functions in an activated T cell, and determining its exact role is highly context and situation dependent.

All of these roles for glutamine would lead one to anticipate that the targeted inhibition of any step in the pathway would have dire consequences for glutamine metabolism. However, this turns out not to be the case, as targeted GLS inhibition, while capable of inhibiting cancer cell proliferation, was found not to be sufficient to extend survival in mouse tumor models, as an adaptive response was mounted by the cancer cells in order to overcome GLS inhbition[80]. Therefore, the use of more broad-spectrum inhibitors to prevent glutamine metabolism, both in cancer and in T cells, is necessary. The next section of this chapter will discuss inhibition of the glutamine analogue 6-diazo-5-oxo-L-norleucine (DON).

Inhibition of glutamine metabolism with the use of DON and DON-derived prodrugs

DON is among the most widely-studied glutamine antagonists[81] . A structural analogue of glutamine, it was initially isolated from yeast culture broth in the 1950's, and it has subsequently been demonstrated to competitively bind the active site of a wide range of enzymes involved in the use of glutamine, and the form a covalent bond to the active site, irreversibly inhibiting the enzyme[82]. DON also can act as an inhibitor of amino acid transporters as well as transglutaminases[83]. As such, there was great enthusiasm after its discovery to examine DON clinically as an anti-cancer agent. The first study was published by Magill in 1957, in which efficacy was seen with treatment with DON as a single agent at low daily doses across a variety of tumor types[84]. While several other groups carried out subsequent studies of daily DON dosing in a variety of cancer types with indications of efficacy, its use was fairly limited until the 1980s, were its use in mouse xenograft models lead it to be reconsidered for use in the clinic[81]. These phase I studies found that toxicity was primarily found in the gastrointestinal (GI) tract and bone marrow, with higher doses leading to effects in the heart, kidneys and liver, which was more pronounced with increased exposure to drug[85]. In order to limit this toxicity, intermittent, rather than daily dosing was moved forward for a number of phase I studies in the 1980's, however no objective responses were seen with this strategy, and higher doses were found to be too toxic[81]. However, reassessment of earlier literature demonstrated that toxicity with low daily dosing was more limited as compared to more recent higher intermittent dosing studies, and were absent in studies of the GLS inhibitor CB-839[86], meaning that toxicity (and effectiveness was dependent on multiple enzymes, beyond glutaminase and was localized to glutamine depletion within the GI microenviornment[81].

Based on these findings, it was determined that there remained a therapeutic window for the use of DON in the tumor setting. However, in order to limit toxicity, a prodrug strategy was devised in which DON was modified in order to create a prodrug that would be inactive in the blood, but would be selectively activated in the TME[87]. This was done by adding acetylated lysine residues, to take

advantage of the upregulation of histone deacetylase and cathepsin L enzymes upregulated in the TME. Using this strategy, DON release was limited in other tissues such as the liver and intestine, and confined to the tumor, as well as demonstrating inhibition of tumor cell growth both in *in vitro* experiments. This drug strategy would prove to be important in a variety of treatment models. DON had initially been demonstrated to be effective in the treatment of a mouse model of cerebral malaria, in which untreated mice succumb rapidly and reproducibly between 5 and 6 days following administration of *Plasmodium berghei*, but mice treated with DON initially as late as day 5 or even the morning of day 6 were not only protected from death, but saw an improvement in clinical scoring and decreased parasitemia[88]. This was followed by the recovery of blood brain barrier integrity and decreases brain swelling, indicating that DON administration could not only treat cerebral malaria in this model, but promote the resolution of the disease.

Therefore, a novel prodrug derived from DON was designed in order to limit systemic exposure. This prodrug was designed with R groups decorating the DON structure consisting of pivaloxyl-oxy-methyl based esters, and once again demonstrated enhanced stability in plasma over DON[89], and is also advantageous due to its oral bioavailability. When this compound, called JHU-083, was tested in the cerebral malaria model previously described, it was found to enhance mouse survival similar to DON, but was also shown to reverse some of the brain pathology induced by the disease[90]. JHU-083 was next tested in a mouse model of medulloblastoma, in which it was effective at increasing cancer cell apoptosis and decreasing tumor growth and extending mouse survival with *in vivo* treatment[91]. *In vivo* treatment with JHU-083 was also shown to decrease glutaminase activity within CD11b positive cells in the brain, leading to the attenuation of inflammation induced by these cells in a mouse model of chronic social defeat stress, indicating that upon its metabolism into DON, it is still able to act by the previously understood mechanism[92].

Studies with JHU-083 culminated in recent work by Leone and colleagues, in which this DON prodrug was used *in vivo* in mouse tumor models to demonstrate that it was effective not only in inhibiting the growth of the tumor, but that inhibition of glutamine metabolism within the CD8 T cells infiltrating the tumor was effective to modulate the immune response and promote a more robust anti-tumor immune response to decrease tumor growth and enhance mouse survival[93]. Treatment of established MC38 colon cancer tumors implanted subcutaneously decreased tumor growth and extended the survival of treated mice, as well as modulated the metabolism of the tumor such that hypoxia was decreased, and both glucose and glutamine within the TME were increased, thus leading to a more favorable environment for the activation and anti-tumor response of the TIL. Experiments in with B16 melanoma cell line genetically engineered to express OVA was implanted, and mice were treated with ACT comprised of OTI cells lead to decreased tumor sizes and increased survival with JHU-083 treatment. Furthermore, combination therapy with anti-PD-1 checkpoint blockade further enhanced survival and lead to 9/10 complete rejections in the MC38 model. This phenomenon was shown to be immune-mediated, as depletion of CD8 T cells abolished drug responsiveness, and subsequent tumor challenges were rejected in an immune memory dependent manner. Closer examination of the TIL that result from JHU-083 treatment indicated that these cells were more proliferative as evidenced both by gene set enrichment analysis (GSEA) and enhanced Ki67 positivity, and more active as assessed by GSEA, surface expression of CD44 and CD69 as well as cytokine production, and were more memory-like by both surface marker expression and GSEA. This induction of a more memory-like phenotype by treatment with glutamine inhibition was extended to *in vitro* findings where DON was used to demonstrate an enhanced OCR as well as increased presence of mitochondrial proteins including CPT1a upon culture with DON. This leads to a model by with treatment with glutamine inhibition can dramatically reshape the TME, as well as the immune response in order to

promote a more sustained, memory-like response in CD8 T cells treated with glutamine inhibition with JHU-083.

Modulation of immune response by metabolism in autoimmune or autoinflammatory disease

But rather, by controlling the metabolism of cells upon activation, it is possible to exert very fine and highly directed control over the immune response. Currently, this phenomenon is perhaps best understood in cases of autoimmune or autoinflammatory diseases. By harnessing the differential metabolism that occurs both upon activation, and under pathogenic conditions of T cell activation, it is possible to very robustly inhibit a wide range of pathogenic immune responses. While these findings have been reviewed elsewhere[94], they will be briefly summarized below.

Work by Lee and colleagues demonstrated that the use of metabolic inhibition was effective in both skin and solid organ transplant models to inhibit the pathogenic immune response and preserve the lifespan of the graft[95]. In this work, the combination of 2-DG, DON, and metformin was used to inhibit cytokine production and proliferation from *in vitro* treated CD4 and CD8 T cells, as well as decrease mTORC1 activity as assessed by p-S6 staining. *In vivo* experiments in an adoptive transfer model demonstrated that this triple therapy was sufficient to inhibit the proliferation and cytokine production of cells, but enhanced the endogenous Treg population. Lastly, in models of both skin transplant and orthotopic heart transplant, in which tissue from Balb/C mice was transplanted to C57BL/6 mice, triple therapy demonstrated increased skin graft survival, and such a robust survival in the heart transplant model that all transplants survived out to 100 days post-transplantation. This indicates that treatment with metabolic inhibition is very powerful in transplant models, and can be used not to broadly inhibit the immune response, but instead to redirect it towards as more tolerogenic and healthier environment in the case of transplant, as well as in GvHD.

Graft versus Host Disease

In the case GvHD following a bone marrow transplantation (BMT), T cell metabolism is remarkably shifted. The immune response in GvHD is characterized by a constant encounter between the activated lymphocytes and their target tissue. This constant, high level of antigen engagement appears to lead to a different metabolism in the pathogenic donor cells. Their high requirement for ATP means they cannot subsist on oxidative glycolysis alone, and therefore such cells are forced to find an alternate source of fuel. This can be provided by the oxidation of fatty acids[96] . When examining the metabolic tracing of ^{13}C labelled glutamine, glucose, and palmitate in T cells from a mouse model of GVHD following allotransplant, it was found that glutamine uptake and incorporation into RNA (as ribose) and fatty acids (as palmitate) are markedly increased as compared to T cells from naïve mice. This is in contrast to the results of labelled glucose tracer incorporation, which shows no difference between ribose or palmitate incorporation, and the use of a palmitate tracer, which demonstrates decreased incorporation across the board in disease mice[97]. This indicates that fatty acids are preferentially being catabolized for oxidative phosphorylation, while glutamine is being used as an anabolic substrate, and hints at an alternative metabolism that is specific to pathogenic lymphocytes in GvHD, particularly one that is reliant on lipid oxidation for the vast ATP needs of chronically activated cells. These differences in terms of metabolic demands and reprogramming suggest that metabolic therapy might be able to selectively inhibit activation of the GvHD inducing cells.

Small molecule inhibitors of the F_1F_0 ATPase, such as BZ423 and LyC-31138, have demonstrated promise as a metabolic therapy for GvHD[96,97] . These drugs prevent the production of ATP, as well as increase the mitochondrial polarization of the pathogenically activated lymphocytes. In allotransplant BMT models of GvHD, these have been shown to rapidly induce apoptosis in the pathogenic cell population, and significantly decrease cytokine production, leading to decreased GVHD clinical score, and increased survival. These ATPase inhibitors act by inducing caspase-regulated apoptosis in the

pathogenic cells. Importantly, these agents are specific for the pathogenic cells and their rapid requirement for ATP production, and thus do not affect immunological reconstitution of the graft. Another therapeutic strategy is targeting fatty acid oxidation with etomoxir, an agent that blocks lipid metabolism at CPT1[98] . Following allotransplant, two weeks of etomoxir treatment was able to decrease clinical GvHD scores from 10 days to a month after the end of treatment. Etomoxir administration was successively able to reduce the proliferation and promote the apoptosis of GvHD inducing CD8 T cells that have divided numerous times, leaving the undivided cells alone[99] . This is advantageous, because the highly divided cells were found to be the pathogenic cell population. Targeting fatty acid metabolism is a therapeutic strategy that does not impact other cells of the immune system, such as dendritic cells or naïve T cells, and does not inhibit graft reconstitution.

Systemic Lupus Erythematosus

Another condition of pathogenic T cell activation that has been targeted with metabolic therapy is systemic lupus erythematosus (SLE). Similar to GvHD following BMT, in the case of SLE there is a constant, persistent encounter between the pathogenic lymphocyte (the pathogenesis of SLE is chiefly reliant on CD4 T cells) and its target tissue[100]. As a result, pathogenic T cells take on a chronically activated phenotype, and thus have dramatically different metabolic needs as compared to healthy tissue[101]. Similar to pathogenic T cells in GVHD, and in contrast to the lymphocytes activated under normal conditions or in the case of solid organ transplant, CD4 T cells in SLE meet their energetic needs mostly through OXPHOS. Tracer experiments with ^{13}C uniformly labelled glucose found more oxidation to CO_2 in a NZB/W mouse model of lupus, with no differences in glycolysis, or pentose phosphate pathway activity[100]. This augmented reliance on oxidative phosphorylation is coupled to increased mitochondrial polarization and mass in the T cells of SLE patients, which is caused in part by decreased mitochondrial autophagy[102] . This phenotype can be partially reversed by treatment with 3-PEHPC, a

geranylgeranyl transferase inhibitor, which is also sufficient to decrease autoantibody formation and lupus nephritis in both NZB/W and MRL/lpr mouse models of lupus[103].

Further studies have attempted to more fully characterize the metabolism of T cells in SLE. Using a triple congenic mouse model of lupus (TC), CD4 T cells were analyzed for metabolic activity *ex vivo*, and found to have increased basal metabolism prior to the onset of disease, including both increase ECAR, and OCR[104]. Pre-disease cells had decreased SRC as compared to wild type controls, and although they produced ATP at the same rate, pre-disease cells had a lower ATP charge, indicating increased energy use. To gain further insight into the metabolic differences in the lupus mice, extensive gene expression analysis was carried out. As expected, the cells from the lupus mice had higher expression of many genes in the glycolytic pathway, but unexpectedly had an increase in CPT1a. These metabolic analyses, coupled with gene expression differences, open the opportunity for metabolic therapy in the case of SLE. To capitalize on the metabolic differences found in SLE, experiments were undertaken to treat the mice with 2-DG and metformin. When these drugs are used to treat the CD4+ T cells from lupus mice *ex vivo*, they are capable of preventing the production of IFN gamma. When administered *in vivo*, 2-DG and metformin were able to decrease the ECAR and OCR to levels comparable to those of disease-free control animals. Furthermore, they do not affect naïve cells or the immune system as a whole, as circulating total antibody levels are unchanged[104] . Metabolic therapy is able to prevent disease, as there is a substantially decreased production of autoantibody and formation of pathogenic T central memory cells in the treated mice. These results rely on the synergistic effects of these two drugs, as neither was effective as monotherapy.

Although the use of combination therapy in SLE has been robustly demonstrated, recent work by Li and colleagues has sought to use a novel single agent inhibitor of glucose uptake to inhibit glycolysis, thereby inhibiting the activation of pathogenic T cells both *in vitro* and *in vivo* in mouse SLE models [105]. This novel inhibitor of glucose uptake, CG-5, was demonstrated to inhibit glycolysis in mouse

CD4 T cells in a dose dependent manner, as demonstrated by decreased ECAR, but had no effect on OCR or viability. *In vitro*, it inhibited the development of Th1 and Th17 cells, previously shown to be reliant both on glycolysis[34] and glutamine uptake[35,37], while promoting the development of Treg cells, and doing so to a far greater extent than 2-DG treatment[105]. *In vivo* treatment of the TC SLE model indicated that preventative CG-5 treatment inhibited the development of disease, as indicated by decreased anti-dsDNA antibody titers and a decreased ratio of follicular helper cells to follicular regulatory CD4 T cells. Therefore, treatment with this novel inhibitor of glucose uptake as a single agent was able to show efficacy in both inhibiting pathogenic CD4 T cell skewing, as well as preventing disease development.

The effective use of metabolic inhibition across a variety of disease settings, including skin and solid organ transplant, GvHD, and SLE demonstrate that manipulation of metabolism represents a powerful means of shaping the immune response. Crucially, by inhibiting certain pathways of metabolism, the entire response is not inhibited, but rather redirected, as indicated either by the promotion of a Treg phenotype in the studies above, or by the promotion of a memory CD8 T cell response upon promotion of mitochondrial metabolism, among other examples. Subsequent chapters of this thesis will continue to explore this theme. The inhibition of glutamine metabolism in both CD4 and CD8 T cells will be found to have distinct effects, profoundly inhibiting the Th2 response of CD4 T cells by means of epigenetic modification *in vitro*, and by inhibiting the development of type 2 immune cells (Th2 CD4 T cells, M2 macrophages, and ILC2) in a HDM asthma model *in vivo* in chapter 2. In chapter 3, the role of glutamine metabolism in CD8 T cells will be explored, where it will be demonstrated that restriction of glutamine metabolism *in vitro* promotes a more memory-like phenotype and improved CD8 T cell survival *in vivo* in both adoptive transfer models and ACT models of tumor therapy, in which this more memory-like cell promotes an anti-tumor response and improves mouse survival. Lastly, in the 4th chapter, the role of leucine metabolism in CD4 and CD8 T cells will be explored, where it will be demonstrated that in CD4 T cells, inhibition of leucine metabolism promotes a

Th2 phenotype, while in CD8 T cells, the leucine transporter LAT1 serves as a marker of activated T cells, and thus can be used to identify and specifically deplete these cells in a viral model, as well as in models of pathogenic activation such as GvHD, skin, and heart transplantation. Through this entire work, the through-line of using metabolism to control the immune response will be very evident, as it will be demonstrated that metabolism is not a consequence of activation, but rather a controlling signal, on par with antigen stimulation or soluble signals, which can be used to powerfully redirect how an immune response is carried out.

Chapter 2: The amino acid glutamine as a regulator of CD4 T cell fate

Abstract

The anabolic demands of effector T cell responses require specialized metabolic reprogramming. To this end glutamine has been recognized as playing an important role in supporting the increased metabolic needs associated with effector responses. However, it is becoming increasingly clear that metabolic reprogramming is not simply a consequence of immune cell stimulation but plays a critical role in promoting activation, differentiation, and function. To explore this further we initiated a series of experiments examining the effect of pharmacologically inhibiting glutamine metabolism and depleting glutamine on T cell activation and differentiation *in vitro*. Naïve CD4+ T cells stimulated either in restricted glutamine or in the presence of a glutamine antagonist demonstrated a marked decrease in clonal proliferative burst regardless of whether they were activated under Th1 or Th2 skewing conditions. Interestingly, upon re-challenge, whereas the Th1 skewed cells still were capable of producing their signature cytokines, Th2 skewed T cells demonstrated a marked decrease in IL-4 and IL-13 production. RNA expression analysis revealed that this inability to upregulate cytokines was mediated at the transcriptional level. Further, cell surface expression analysis revealed that this was not due to a failure of the Th2 skewed cells to become activated. Likewise, when Th2 cells were re-challenged in the absence of glutamine or the glutamine antagonist acutely, cytokine production was not inhibited. These data suggest a selective role for glutamine in Th2 T cell differentiation. In as much as alpha-ketoglutarate, a product of glutaminolysis, has been shown to influence epigenetic changes in cells, we interrogated the cells via CUT&RUN analysis for histone methylation and acetylation. Indeed, we observed increases in inhibitory histone methylation and decreases in histone acetylation specifically within the Th2 locus of cells skewed under the glutamine depleted conditions. Furthermore, by

employing cell permeable aKG, we could restore IL-13 production. Finally, by treating house dust mite antigen sensitized mice with the glutamine antagonist, we were able to markedly mitigate Th2 mediated disease in this model of asthma. Interestingly, treatment with the glutamine antagonist not only resulted in decreased T cell responses but also in a decrease in M2 macrophages and ILC2 cell responses. Overall, our studies demonstrate a selective role for glutamine metabolism in promoting Th2 responses and suggest pursuing the use of glutamine antagonists in treating Type 2 mediated inflammatory diseases.

Introduction

It is now widely understood that the metabolism of immune cells, particularly T cells, changes dramatically upon activation[6]. When T cells become activated by stimulation through the T cell receptor, and in the context of appropriate costimulation through CD28, they dramatically increase their expression of the glucose transporter Glut1 on the surface, as well as their use and uptake of glucose[12]. This glucose is utilized to carry out glycolysis, however the pyruvate produced from this process is exported from the cell as lactate, rather than being converted to Acetyl-CoA and continuing through the TCA cycle to maximize ATP generation, in a phenomenon known as the Warburg effect, which was first identified in cancer cells[8]. While this Warburg metabolism does not provide the most efficient ATP production per molecule of glucose, it provides very rapid ATP generation, as well as the regeneration of NAD+ through the conversion of pyruvate to lactate, which maintains the redox state of the T cell. Furthermore, a recently-activated T cell needs more than just ATP to become a bona-fide effector cell. Activated cells must grow, divide, and produce cytokines and other molecules to take on their effector function, requiring the use of lipids, nucleotides, and amino acids, among other biosynthetic substrates. Many of these building blocks can be provided through the continued spinning of the TCA cycle. To do this, the activated T cell increases its uptake of the amino acid glutamine, a non-essential amino acid, which can be taken up, converted to glutamate through the actions of the glutaminase enzymes, and

then enter the TCA cycle as aKG[73]. This aKG maintains the TCA cycle, and allows the generation of the biosynthetic substrates necessary for T cell growth and effector function upon activation.

The growing field of immunometabolism has been successful in identifying differences between the metabolic pathways of different subsets of T cells, with effector T cells primarily relying on glycolysis as previously described, as well as fatty acid synthesis[34]. Regulatory T cells have a metabolic program more based on fatty acid oxidation, as do memory T cells. These differences in CD4 T cell subset metabolism and commitment have been tied to the differential activity of mTORC1 and mTORC2, with mTORC1 being essential for Th1 and Th17 cell generation, mTORC2 required for Th2 cells, and the absence of mTOR leading to Tregs[26]. In this way, it would be ideal to identify a metabolic program that was specific to type 2 inflammatory T cells, such that this program could be specifically targeted in diseases like asthma. Asthma has been identified to have a metabolic component, in which a 2012 paper tested the serum lactate levels of patients with asthma and found it to be elevated over both healthy controls and patients with chronic obstructive pulmonary disease (COPD). Furthermore, treatment with dichloroacetate (DCA) to inhibit the conversion of pyruvate to lactate in a ragweed asthma mouse model carried out by these same researches decreased IL5 production by stimulated splenocytes in a dose-dependent manner[106]. This indicates that the Warburg metabolism undertaken by activated effector T cells is crucial to their function in asthma, and by inhibiting this Warburg metabolism it may be possible to alleviate disease symptoms and progression. One potential way to inhibit the metabolism of an effector cell that is carrying out Warburg metabolism, but still requires flux through the TCA cycle in order to generate biosynthetic precursors is by the inhibition of the use and uptake of glutamine, which replenishes the TCA cycle.

Glutamine is known to play an important role in T cell proliferation, and it has been shown that decreasing its concentration in culture decreases proliferation, and that ERK signaling increases glutamine uptake as well as expression of enzymes involved glutamine metabolism following

stimulation[14]. The role of CD4 T cell proliferation in skewing has been demonstrated by experiments by bird and colleagues, which used carboxyfluorescein succinimidyl ester(CFSE) dilution to assess proliferation and compared proliferation to cytokine production, determining that while IL2 could be produced at rest, IFN gamma and IL4 required passage through the cell cycle for production[107]. And while glutamine has been identified to play an important role in the development of Th1 cells, leading to Treg polarization in its absence, as well as in Th17 skewing, type 2 immune responses have previously been shown to have a special dependence on glutamine metabolism. A 2015 paper which combined side by side transcriptional analysis and metabolomics to enriched metabolic networks used this technique to examine the differences between in vitro M1 or M2 macrophage polarization, and identified a specific requirement for glutamine in M2 macrophage polarization that was absent in M1 macrophages[108]. Furthermore, based on the numerous things that glutamine does in the cell, including its role in de novo purine synthesis, post-translational glycosylation modifications[78], transcriptional regulation through its role as a cofactor for histone and DNA demethylases, and its role in redox balance through glutathione synthesis[73], it is possible that glutamine metabolism plays a role in more ways than just as being necessary for proliferation, and that its role in Th2 CD4 T cells may be similar to that of M2 macrophages. Therefore, it may be possible to inhibit Th2 responses by inhibiting the use and uptake of glutamine.

Previous research has attempted to inhibit glutamine metabolism through the use of DON, a structural analogue of glutamine that is a competitive inhibitor glutamine uptake and metabolism. Human studies undertaken to use DON as a chemotherapeutic agent showed some signs of success, however they were fraught with toxicity, particularly of the intestinal epithelium[81]. Using a novel and previously described prodrug strategy in which a compound, JHU-083, must be proteolytically cleaved to release active DON, it is possible to decrease this toxicity as well as increase bioavailability[92]. Using this prodrug strategy, it is possible to treat mice with established house dust mite induced asthma in order

to decrease Th2 T cells, M2 macrophages, and ILC2 in the lung, as well as decrease cytokine production from the T cells and ILC2 as a result of decreased antigen specific T cell presence in the lungs of treated mice. Further *in vitro* studies identify a glutamine's role not only in T cell proliferation, but specifically in Th2 (as opposed to Th1) cytokine production by an epigenetic mechanism which increases the chromatin accessibility of the Th2 locus in a glutamine-dependent manner. Through this process, it is possible to use the inhibition of glutamine metabolism not merely as a way to broadly inhibit the activity of a cell type, but to use metabolism as a specific and selective modulatory of T cell activity in order to reprogram it, and change the character of a cell by changing its metabolism.

Results

Inhibition of glutamine metabolism prevents CD4 T cell proliferation without affecting activation

First, we sought to duplicate previously published research indicating a role for the amino acid glutamine in T cell proliferation[14]. We set out parallel strategies to carry these experiments out, the first utilized an approach by which the amount of exogenous glutamine provided to our standard primary T cell culture media was titrated down. Furthermore, these experiments were carried out in media made from a base Roswell Park Memorial Institute media (RPMI) lacking glutamine, further decreasing the concentration of this amino acid. Therefore, the concentration of glutamine indicated relates only to concentration of exogenous glutamine added to the media. Transgenic 5CC7 CD4 T cells, specific for a peptide derived from pigeon cytochrome C (PCC), were labelled with the proliferation dye Cell Trace Violet (CTV), and stimulated with peptide in the indicated concentration of glutamine. When proliferation was assessed by flow cytometry after a 3 day period of culture it was found that standard culture media (with 4mM glutamine added, referred to as "4mM Gln") readily supports *in vitro* T cell proliferation (**Fig. 1a)**, it contains excess glutamine such that decreasing glutamine concentration more than 30-fold (to 125uM exogenous glutamine) has little perceptible effect on proliferation as measured

by CTV dilution (**Fig. 1b)**. Further reductions of glutamine concentration below this level decreased proliferation in a corresponding manner at day three. At the lowest concentration of added glutamine, 7.8uM, proliferation was essentially identical to the condition in which no glutamine was added to the media, either in the form of supplementation or its presence in the media (denoted as "low Gln media") (**Fig. 1a**). In this media condition, the only glutamine present in the culture is from the fetal bovine serum (FBS) added to 10% final volume. The concentration of glutamine as determined by mass spectrometry leads to a final concentration of roughly 0.1mM glutamine in the low glutamine media, 40 times less than the standard glutamine concentration in our culture media.

Similar experiments were undertaken to assess the effect of glutamine metabolism of CD4 T cell proliferation, but rather than limiting the substrate, an inhibitor of glutamine metabolism was utilized. DON is a competitive inhibitor of glutamine use, and is a structural analogue of glutamine itself[81], and experiments using DON to inhibit glutamine metabolism were carried out in standard 4mM glutamine media. DON treatment dose-dependently decreased CD4 T cell proliferation (**Fig. 1c**), with the 50uM concentration of the drug almost completely inhibiting division. Decreases in DON concentration were sufficient to increase proliferation of CD4 T cells in culture (**Fig. 1d**). Further experiments were carried out by counting plated and stimulated CD4 T cells in culture with either standard 4mM or low glutamine media at intervals over the course of culture (**Fig. 1e**), which recapitulated the previous finding of decreased glutamine in culture decreasing the proliferation of CD4 T cells. In these experiments, CD4 T cells cultured in 4mM glutamine rapidly proliferated and increased their numbers to a point far higher than those cultured in low glutamine media, however after roughly a week, the cells cultured in standard 4mM glutamine media crashed and dramatically decreased their numbers. After about a week and a half in culture, the number of cells in the low glutamine culture condition exceeded that of the standard 4mM glutamine culture. The implications of this persistence of cells cultured in low glutamine media will be addressed in chapter 3 of this thesis as it relates to CD8 T cells.

Next, we sought to assess the impact of inhibiting glutamine metabolism by decreasing glutamine content of the culture media on the activation of CD4 T cells. As previously described, increases in glutamine metabolism are an integral aspect of T cell activation, with transporters being increased upon stimulation[14]. When glutamine concentration of culture media is titrated down, the frequency CD4 T cells positive for the nuclear marker of proliferation Ki67 decreases in a concentration-dependent manner (**Fig. 2a**) to the point that there is a decrease by roughly half in the percent Ki67 positive from the standard 4mM glutamine culture media to the low glutamine media culture. Assessing Ki67 expression at a variety of timepoints beyond the standard 3 days used in the previous glutamine and DON titration experiments once again demonstrated that culture in low glutamine media led to a decrease in Ki67 staining across the board at multiple timepoints (**Fig. 2b**).

However, the decrease in the proliferation marker was not mirrored by a decrease in activation by two metrics. First, titrating down the concentration of glutamine in culture media did not decrease the activation marker CD44 (**Fig. 2c**) by substantial margin. Furthermore, when studies were undertaken to assess activation as indicated by CD44 expression at numerous timepoints, this lack of effect of low glutamine culture on CD44 expression was recapitulated (**Fig. 2d**) in that it is constant whether cells are cultured in either low glutamine media or standard 4mM glutamine culture conditions. As previously described, activation of T cells through the TCR leads to an increased activity of mTORC1, which can be assessed by flow cytometry staining for p-S6[31]. Therefore, we sought to assess p-S6 staining by flow cytometry to assess activation in conditions of restricted glutamine metabolism. Stimulation of CD4 T cells and culture for 24 hours led to an increase in the percentage of cells positive for p-S6 (**Fig. 2e**), and this increase was not inhibited by culture in low glutamine media. Interestingly, addition of high dose (50uM) DON to either standard 4mM glutamine media or low glutamine media culture was sufficient to dramatically decrease p-S6 levels, down to a level similar to the unstimulated controls. This raises the interesting point that, while for the most part the strategies of decreasing glutamine metabolism

through the use of the inhibitor and through culture in decreased media lead to phenotypically similar cells and nearly identical results, there are some differences between these two approaches that could merit further study. Most likely, the 50uM dose of DON more completely inhibits glutamine metabolism, and is more reminiscent of a condition in which glutamine is completely absent from the media, rather than simply "low." Regardless, culture in low glutamine media was not sufficient to alter p-S6 following 24 hours of culture, nor alter CD44 expression, leading to the conclusion that even though proliferation is inhibited by low glutamine cultured of CD4 T cells, activation is not.

Inhibition of glutamine metabolism in CD4 T cells does not affect Th1 skewing, but inhibits Th2 T cell skewing and cytokine production

Previous research has indicated that glutamine is essential for IFN gamma and IL4 cytokine production, through its essential role in CD4 T cell proliferation[107]. Furthermore, previous research has indicated that the presence of glutamine is essential for CD4 T cells to skew towards either Th1 or Th17, otherwise they are shunted towards the regulatory T cell (Treg) fate[35,37]. Researching comparing the metabolic and transcriptional profiles of bone marrow derived macrophages polarized towards M1 or M2 fates indicates that glutamine is required for the development of the wound repair and tissue homeostasis M2 macrophages, while it is disposable for the generation of inflammatory M1 macrophages[108]. Knowing that the exact requirements for glutamine in T cell subset commitment are unclear, we sought to determine the impact of skewing transgenic CD4 T cells to either a Th1 or Th2 subset.

Interestingly, when cells were skewed towards a Th1 fate over the course of 6 days with the addition of the cytokines IL12 and IFN gamma as well as depleting anti-IL4 antibody in culture (**Fig. 3a**) in either standard 4mM glutamine or low glutamine media, little effect on the percentage of CD4 T cells positive for the hallmark Th1 transcription factor Tbet was seen (**Fig. 3b**). Percentages of Tbet positive

cells were similar at each of the timepoints assessed: day 2, day 4, and day 6. Furthermore, when live cells that were cultured for 6 days were isolated and restimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for four hours in standard 4mM glutamine culture media to assess cytokine production, there was no decrease in the production of either Th1 hallmark cytokine, IL2 (**Fig. 3c**) or IFN gamma (**Fig. 3d**)[109]. Further, CD4 T cells that were cultured for 6 days in standard 4mM glutamine culture media with two doses of the glutamine inhibitor DON, either 500nM or 50nM, had no change in their competence to produce either cytokine. Therefore, culture in either low glutamine culture media or with decreased glutamine metabolism as a result of inhibition by DON does not affect the ability of CD4 T cells to be polarized to the Th1 subset and produce Th1 hallmark cytokines.

Next, we sought to determine what if any effects culture with low glutamine media had on skewing towards the Th2 subset. Transgenic CD4 T cells specific for PCC were once again stimulated for 6 days in either standard 4mM glutamine or low glutamine culture media, but were this time skewed towards a Th2 fate, with the addition of the cytokine IL4 and anti-cytokine antibodies against both IL12 and IFN gamma added to culture conditions (**Fig. 4a**). First, these Th2 skewed CD4 T cells were assessed for their expression of the hallmark Th2 transcription factor, Gata3[109]. When Gata3 expression was assessed by flow cytometry at day 2, day 4, and day 6 timepoints, although there were slight variations between culture in standard 4mM glutamine and low glutamine media at some timepoints, overall, there was little difference in Gata3 expression between these conditions (**Fig. 4b**).

However, things were dramatically changed when cytokine production was assessed. Following either 3, 4, or 6 days of culture in Th2 skewing conditions in either standard 4mM glutamine or low glutamine media, live CD4 T cells were isolated and their competence to produce cytokine was assessed by restimulation with PMA and ionomycin in standard 4mM glutamine culture media. When these cells are restimulated at the indicated timepoint (day 3, day 4, or day6), there is a profound decrease in the percentage of cells producing cytokine for both IL4 (**Fig. 4c**) and IL13 (**Fig. 4d**). In regards to IL4

production, it appears that decreased glutamine in the culture media delays the kinetics of cytokine production such that cells cultured for 6 days in low glutamine media resemble those cultured for just 4 days in 4mM glutamine media (**Fig. 4c**). However, cells cultured in low glutamine media are not delayed but appear to never become capable to produce IL13, even following 6 days of culture (**Fig. 4d**). This indicates that, although there is little difference in Gata3 expression in Th2 T cells skewed in the presence of low glutamine, there is a difference in cytokine production. Furthermore, it appears that this defect in cytokine production occurs as a result of the development of these cells, and not as a consequence of restricted access to glutamine during the restimulation, as restimulation was performed in standard 4mM glutamine culture media.

As previously mentioned, we had an indication that the decreases in IL4 and IL13 production following Th2 skewing in low glutamine media was a result of a process that occurs during the development of these cells as opposed to an acute effect of glutamine depletion, as the difference was still seen during restimulation in standard 4mM glutamine culture media. Therefore, we sought to assess Th2 cells that were skewed in standard 4mM glutamine, but restimulated in conditions of restricted glutamine metabolism. For the first set of experiments, cells were skewed towards the Th2 subset as previously described, live cells were isolated, and restimulated with PMA and ionomycin in either standard 4mM glutamine media, standard media in the presence of decreasing doses of DON, or in low glutamine culture media to assess cytokine production as determined by flow cytometry. First, it was noted that, regardless of stimulation in either low glutamine media or in the presence of increasing doses of DON, acute activation as indicated by surface expression of the acute activation marker CD69 was similar for all conditions (**Fig. 5a**). Furthermore, for both IL4 (**Fig. 5b**) and IL13 (**Fig. 5c**), restimulation in low glutamine media or in the presence of various concentrations of DON, including the high 50uM dose, did not inhibit cytokine production. These findings were recapitulated by experiments in which Th2 T cells that had been skewed for 6 days in standard 4mM glutamine media were

restimulated by plate bound anti-CD3 antibody and soluble anti-CD28 antibody overnight in either standard 4mM glutamine culture media, standard media in the presence of increasing doses of DON, or in low glutamine culture media. Following overnight stimulation, media was collected and cytokine production was analyzed by ELISA assay for either IL4 or IL13. Although high doses of DON (50uM) showed a slight decrease in cytokine production, there was overall very little change in cytokine production of either IL4 (**Fig. 5d**) or IL13 (**Fig. 5e**). It is interesting that slight decreases can be seen in the overnight stimulation with the highest dose of DON (50uM), as this was also the dose that was sufficient to decrease mTORC1 activity as assessed by p-S6 staining following 24 hours of treatment (**Fig 2e**). This could potentially indicate that high doses of the competitive inhibitor for longer durations are carrying out a different process or having distinct effects from restricting the availability of the amino acid. Potentially high dose DON for these longer durations is sufficient to induce cytotoxicity. Regardless, it is clear that the requirement for glutamine exhibited by Th2 cell cytokine production occurs during their skewing and development, and not as at the time of acute restimulation.

Glutamine deficiency inhibits Th2 cell development at the transcriptional level by decreasing the epigenetic availability of the Th2 cytokine locus

Due to the fact that the amino acid glutamine could be required for cytokine production in its capacity as a building block of proteins, it was important to determine whether the observed decrease in Th2 cytokine production following differentiation of Th2 cells in low glutamine media was a transcription or translation level phenomenon. Both Th1 and Th2 cells skewed in either 4mM or low glutamine media were collected and preserved in Trizol for mRNA extraction, cDNA synthesis, and quantitate PCR analysis at 2, 4, or 6 days following initial stimulation. There was little alteration in the expression of the hallmark transcription factors for either Th1 or Th2 CD4 T cells, Tbet (*Tbx21*) or Gata3 respectively[109]. Th1 cells appeared to have mildly elevated levels of *Tbx21* mRNA when cultured in low glutamine media (**Fig. 6a**), while Th2 cells cultured in low glutamine media had an early increase in

Gata3 expression at days 2 and 4, but a mild decrease in its expression at day 6 (**Fig. 6b**). Overall, the limited changes in *Gata3* levels indicate that it is unlikely that the inhibition in Th2 cell development induced by culture in low glutamine media occurs as a result of lack of expression of its hallmark transcription factor.

Next, cytokine mRNA levels were assessed, once again at the day 2, 4, and 6 timepoints. When mRNA levels for the Th1 hallmark cytokines were assessed, no consistent pattern of alteration was found. For levels of *IL2* mRNA, days 2 and 6 appeared unchanged between low glutamine culture and standard 4mM glutamine media culture, while there was a consistent elevation in *IL2* mRNA on day 4 (**Fig. 6c**). In regards to expression of *IFN gamma* mRNA, once again there was no consistent pattern of alteration, as day 2 levels were slightly elevated in low glutamine culture, day 4 levels were slightly decreased in low glutamine cultures, and day 6 levels displayed a wide spectrum of expression levels (**Fig. 6d**). However, with these slight alterations in mRNA level coupled with the lack of difference in Th1 cytokine production and the protein level (**Fig. 3c,d**) indicate that culture in low glutamine media does not substantially alter the ability of CD4 T cells to skew towards the Th1 fate.

Next, Th2 cytokine mRNA production was assessed at the same timepoints. Following an initial spike in IL4 transcription on day 2, possibly due to early transcription events[110], levels of *IL4* message were modestly down in cells differentiated in low glutamine media on day 6 (**Fig 6e**). Curiously, the point at which *IL4* expression is the lowest corresponds with the point at which the protein expression is highest as assessed by flow cytometry. Furthermore, IL13 message levels were down dramatically at all timepoints in Th2 T cells cultured in low glutamine media as compared to those cultured in 4mM glutamine (**Fig. 6f**). This finding was quite distinct from what was observed in the culture of Th1 T cells in low glutamine media, where message levels of Tbet, IL2, and IFN-gamma were increased as compared to 4mM glutamine media (**Fig. 6a,c,d**). Based on the lack of alteration in Gata3 levels, both transcriptionally (**Fig. 6b**) and based on flow cytometric analysis of protein level (**Fig. 4b**), a complete

failure to polarize to the Th2 subset seems unlikely, yet a profound defect in Th2 cytokine level at the transcriptional level was noted.

Previous research has described multiple roles for glutamine in the modulation of the epigenetic state of the cell. Alpha-ketoglutarate, derived from the conversion of glutamine to glutamate, is an important cofactor for both histone and DNA demethylases such as the Jumanji family of histone demethylases and the Tet family of DNA demethylases[111]. Furthermore, glutamine that replenishes the TCA cycle as alpha-ketoglutarate will continue through the cycle to acetyl-CoA, thereby forming new potential acetyl donors for histone acetylation events. The importance of cytosolic citrate leading to acetyl-CoA production was illustrated by work by Peng and colleagues, who demonstrated that enforced oxidative metabolism, induced by LDHA knockout lead to inhibition of Warburg physiology as illustrated by decreased glucose consumption. This led to an increase in TCA activity and OCR, which was coupled with decreased histone H3K9 acetylation, particularly at the IFN gamma promoter, leading to reduced IFN gamma production. This decrease was rescued with the addition of exogenous acetate, which was able to augment acetyl-CoA production and restore IFN gamma expression[24]. The role of aKG in epigenetic changes occurring in T cells was also demonstrated by Chisolm and colleagues, who sought to explore its role in the epigenetic remodeling events that occur downstream of IL2 signaling. These researchers determined that differences in gene expression between low IL2 and high IL2 stimulation conditions could be rescued in the low IL2 condition by addition of exogenous aKG. GSEA demonstrated that the genes induced by aKG treatment in the low IL2 condition were related to H2K27me3 histone modification and DNA methylation, and ChIP-seq examining H2K27me demonstrated that this modification was more plentiful under these conditions. These researchers went on to demonstrate changes in genomic organization that were associated with the presence of absence of aKG, further illustrating the role of this metabolite in epigenetic changes upon activation[112].

In light of this, ChIP experiments were undertaken in an attempt to determine whether skewing in decreased glutamine alters the epigenetic accessibility of the Th2 cytokine locus. When assessing the presence of both activating H3K9 acetylation and inhibitory H3K27 trimethylation at the day 3 timepoint, it was noted that there is a trend towards a less permissive epigenetic status in the Th2 cytokine locus of Th2 cells cultured in low glutamine media, indicated by an increase in the inhibitory mark at the conserved Gata3 response element (CGRE), an IL13 promoter (**Fig. 7a**) as well as a decrease in the activating mark at the IL4 internal enhancer (IL4IE) (**Fig. 7b**). Although these trends were promising, they did not reach the level of statistical significance, and thus an additional approach was attempted in order to confirm the epigenetic underpinnings of low glutamine culture leading to an inhibition in Th2 cell development. Cleavage under targets and release using nuclease (CUT & RUN) is a technique developed by the lab of Steven Henikoff and was designed as an *in situ* alternative to the traditional ChIP assay[113]. It is advantageous over traditional ChIP because it is faster, requires fewer cells as an input, and offers enhanced sensitivity. CUT & RUN is distinct from ChIP on the technical level in that the binding of the antibody to the target nuclear feature, in our case a histone modification, occurs *in situ*, as cells are gently permeabilized rather than lysed, and antibodies diffuse into the nucleus to bind. Following this, a modified nuclease capable of binding to the functional regions of the bound antibody is applied, which also diffuses into the permeabilized nuclei, and binds. Upon activation of nuclease activity with the addition of calcium, the nuclease will cleave the DNA surrounding the bound antibody while protecting the bound region itself, which is then free to diffuse back out of the cell for collection and assay. Usually, this technique is followed by high-throughput sequencing. However, as we had a limited number of regions of interest, we attempted to perform a modified CUT & RUN assay on Th2 cells cultured in either standard 4mM glutamine media or low glutamine media to assess epigenetic accessibility at the Th2 cytokine locus.

Levels of activating H3K9Ac and H3K27me3 were assessed in transgenic CD4 T cells skewing towards Th2 fate following 3 days of culture in either standard 4mM glutamine media, standard media with the addition of 0.5uM DON, or low glutamine media. As there is no equivalent "input" control to normalize against as is standard in ChIP, a delta-delta CT normalization was performed, similar to quantitative PCR, in which each condition was first normalized to a highly acetylated or methylated gene (Socs3 for H3K9Ac[114], and Gata4 for H3K27me3[115]), similar to the use of 18s ribosomal RNA as a housekeeping gene in standard eukaryotic qPCR. Next, the fold change between standard 4mM glutamine media culture and low glutamine culture or DON treatment was determined and graphed. For each of the regions assayed, IL4IE, CGRE, and a redundant set of primers for the Gata3 binding site within the IL13 promoter denoted as "Th2 locus," there was a decrease in activating H3K9Ac in both low glutamine and DON-treated culture as compared to Th2 cells skewed in standard 4mM glutamine media (**Fig 7c,d,e**). In a corresponding fashion, when the presence of the inhibitory H3K27me3 mark was assessed, it was found that culture in low glutamine media or in standard 4mM glutamine media with DON treatment increased this mark at the IL4IE, CGRE, and Th2 locus regions of interest (**Fig. 7e,f,g**). Based on the combination of the trending ChIP data, and the dramatic data obtained from CUT & RUN analysis, culture in low glutamine media or conditions of restricted glutamine metabolism such as DON treatment induce a less permissive epigenetic state within the Th2 cytokine locus, as indicated by decreased activating epigenetic marks and increased inhibitory epigenetic marks at important cytokine loci for the production of Th2 cytokines.

Next, we sought to further demonstrate the importance of glutamine metabolism and its contribution to cellular aKG through the use of aKG supplementation in an attempt to rescue cytokine production in low glutamine skewed Th2 cells. Transgenic CD4 T cells were skewed towards the Th2 subset with the addition of cytokine and blocking antibodies for 6 days following initial simulation in standard 4mM glutamine culture media or low glutamine culture media. However, a third condition

was added, in which low glutamine culture media was supplemented with dimethyl 2-oxogluterate (DMK), a cell-permeable alpha-ketoglutarate analogue (**Fig. 8a**). Treatment with DMK during the differentiation process of cells cultured in low glutamine media had little effect on the production of IL4 (**Fig. 8b**), potentially due to the fact that by the day 6 timepoint, low glutamine cultured Th2 cells have already begun producing IL4 protein (**Fig. 4c**). However, IL13 protein levels were significantly increased by DMK supplementation at the day 6 timepoint (**Fig. 8c**) as assessed by flow cytometry. The sum total of these experiments, examining the mRNA levels of Th2 cytokines, the epigenetic accessibility of the Th2 cytokine locus, and the rescue of Th2 cytokine production with the addition of DMK to low glutamine culture, leads to a model whereby naïve CD4 T cells are able to open their Th2 cytokine locus upon differentiation in the presence of sufficient glutamine levels, but the locus remains epigenetically inaccessible in the absence of glutamine, leading to abrogation of Th2 cytokine production (**Fig. 8d**).

Inhibition of glutamine metabolism in an *in vivo* model of house-dust mite induced asthma decreases Th2 T cells, as well as other type 2 immune cells

Following the studies of the role of the amino acid glutamine *in vitro* in the development of Th2 cells, studies were undertaken to in *in vivo* models to determine if the requirement for glutamine was conserved. Based on its prominence as a Th2-biased diseased that effects nearly 8% of the United States population[116], and the fact that asthma has a previously described metabolic component[106], a HDM induced asthma model was used. In this model, mice were administered two intraperitoneal injections of HDM extract one week apart, followed by two intranasal challenges with house dust mite extract, the first of which performed a week after the second IP sensitization, and the second 6 days later. Following the first intranasal challenge, mice were treated with either the antimetabolite inhibitor of glutamine, DON, or JHU-083, a DON-derived pro-drug designed to have increased bioavailability and decreased toxicity[91] (**Fig. 9a**). Following four doses, occurring every other day, mice were sacrificed, and lungs and spleens were collected for analysis (**Fig. 9b**). While only DON was used as an inhibitor of

glutamine metabolism *in vitro*, the use of the JHU-083 prodrug is restricted to the *in vivo* system, as it must have its R groups cleaved by proteases in order[89] to be converted to the active drug.

Upon assay of the lungs, a profound decreased in the percentage of Th2 T cells in the lungs of mice treated with either DON or JHU-083 were found, as indicated by a significant decrease in the percentage of lymphocytes expressing Gata3, the hallmark Th2 transcription factor as assessed by intracellular flow cytometry staining (**Fig. 9c**). However, it is not simply that glutamine inhibitor treatment decreases all T cells in the mouse, as there was no change to regulatory T cell (Treg) levels in the lungs of treated mice (**Fig. 9d**) and interestingly an enhancement in regulatory T cells in the spleens of treated mice (**Fig. 9e**), as indicated by an increased percentage of CD4+ T cells positive for both the high affinity IL2 receptor CD25 and the hallmark Treg transcription factor FoxP3 as assessed by intracellular flow cytometry staining.

In addition to the decrease in Th2 cells in the lung induced by treatment with glutamine inhibitor therapy, there was also a striking decrease in effector functions of disease. Following lung harvest and digestion, homogenates were stimulated for 4 hours with PMA and ionomycin to assess cytokine production by flow cytometry. Production of Th2 hallmark cytokines IL4 (**Fig. 10a**), IL5 (**Fig. 10b**), and IL13 (**Fig. 10c**) were significantly reduced in conditions in which glutamine metabolism was inhibited. As cytokine signals play important roles in antibody class switching, with Th2 cytokines inducing the production of particular isotypes of antibody with particular effector functions[117], we were next interested in examining the serum antibody content of house dust mite asthma mice treated with glutamine inhibitor therapy. Serum ELISA performed on plates coated with house dust mite extract and it was found that glutamine inhibitor treatment substantially decreased HDM-specific serum IgG1 (**Fig. 10d**) and IgE (**Fig. 10e**) as compared to the levels found in untreated mice. Therefore, inhibition of glutamine metabolism in mice with house dust mite induced asthma can not only decrease Th2 T cells in

the lungs, but this decrease can have a functional effect on parameters of type two immunity in these mice.

Previous work has indicated that glutamine is important not only to Th2 T cells, but also to the formation of other type 2 immune cells, especially M2 macrophages, while being dispensable to the generation of M1's[108]. This work used a single sample split across transcriptional and mass spectrometry analysis of metabolites, followed by computational network analysis in order to determine upregulated metabolic pathways in a technique referred to as CoMBI-T. This analysis determined that pathways involved in glutamine metabolism were upregulated specifically during the polarization of bone marrow derived macrophages to the M2 subset *in vitro* at both the RNA and metabolite levels. Further experiments *in vitro* confirmed that the polarization of bone marrow macrophages towards the M2 subset is indeed dependent on the presence of glutamine in the media, as indicated by failure to upregulate CD206 and RelmA in the absence of glutamine under M2 differentiation conditions. Conversely, there was no inhibition of the ability of macrophages to differentiate towards the M1 subset, as there was no defect in NOS production in the absence of glutamine in bone marrow derived macrophages differentiated in the absence of glutamine[108]. Treatment with either DON or JHU-083 lead to a decrease in the expression of M2 macrophage markers CD206 (**Fig. 11a**) and arginase (**Fig. 11b**) on the alveolar macrophages from the lungs of mice with house dust mite induced asthma. However, the M1 character of these alveolar macrophages, as assessed by their ability to produce TNF following overnight LPS stim, was not affected by the inhibition of glutamine metabolism (**Fig. 11c**).

There is also a contribution of innate lymphoid cells (ILC) type 2, particularly in the cytokine production associated with asthma[118]. Treatment with either inhibitor of glutamine metabolism, DON or JHU-083 was not found to decrease the percentage of ILC2 in the lungs of treated mice as compared to untreated mice (**Fig. 11d**), potentially because the percentage-wise findings were lost in the drastic changes occurring to numerous cell populations within the lungs of treated mice. As we were interested

in more fully assessing the role of glutamine inhibition in the formation of ILC2, we utilized a model in which intranasal IL33 was administered on three consecutive days in order to induce robust ILC2 formation in the lungs[119]. Mice were also treated every other day with DON, and harvested on 6 days after the initial administration of intranasal IL33, and lungs were removed, washed, homogenized, and stained for ILC2 content as assessed by flow cytometry. Treatment with DON in this model decreases the percentage of ILC2 in the lung (**Fig. 11e**). As this demonstrates that inhibition of glutamine metabolism is capable of decreasing ILC2 formation, we were next interested in further assessing the role of glutamine metabolism in ILC2 effector function. ILC are "cytokine factories," which are stimulated by cytokine to produce further production of soluble mediators[118], and therefore we sought to assess their production of canonical type 2 cytokines IL5 and IL13. In the HDM asthma model, treatment with either DON or JHU-083 decreases *ex vivo* IL5 (**Fig. 11f**) and IL13 (**Fig. 11g**) production from these cells as induced by stimulation with PMA and ionomycin. This work indicates that the requirement for glutamine in the formation of not just Th2 cells as found in the in vitro work, but also for other type 2 immune cells such as M2 macrophages and ILC2 is conserved in an *in vivo* asthma model.

Based on the wide variety of cellular and effector changes induced by glutamine inhibitor therapy in the house dust mite asthma model, we were next interested in determining the functional effects of this therapy. Histological analysis of the lungs indicated a dramatic decrease in cellular infiltration in the lungs of treated mice (**Fig. 12a**). Next, treated and untreated mice with house dust mite induced asthma were submitted to a test of their lung function in which airway hyper-responsiveness, which has previously been associated with asthma in mice and humans, was induced using elevating doses of nebulized methacholine. Methacholine challenge indicated that treatment with glutamine inhibitor therapy led to a slight increase in lung compliance (**Fig. 12b**) and a slight decrease in

lung resistance (**Fig. 12c**). Therefore, treatment with glutamine inhibitor therapy in a mouse model of house dust mite asthma functionally decreases the disease phenotype.

Glutamine inhibitor therapy decreases proliferation and antigen specific cell numbers in the lungs of mice with house dust mite induced asthma

Based on the demonstrated requirement for glutamine to promote T cell proliferation (**Fig. 1**), in addition to its essential role in the development of type 2 immunity *in vitro* (**Fig. 4**), the proliferation rates of the CD4 lymphocytes in the lungs of asthma mice were assessed by Ki67 staining. Treatment with either DON or JHU-083 to decrease glutamine metabolism decreased the percentage of Ki67 positive CD4 T cells in the lungs of treated mice (**Fig. 13a**) and led to a significant reduction in the percentage of actively proliferating cells in the lungs of mice in both treatments (**Fig. 13b**). This was also the case in the ILC2 in the lung, which displayed significantly reduced Ki67 staining following treatment with either DON or JHU-083 (**Fig. 13c**). To determine if this decreased proliferation resulted in fewer antigen specific cells, a class II tetramer against the major antigen in house dust mite, the DerP1 peptidase[120], was used to determine the percentage of antigen specific CD4 T cells in the lungs and spleens of treated or untreated mice. Treatment with either DON or JHU-083 decreased the tetramer positive population in the lung (**Fig. 13d**), leading to a significant decrease in the percentage of antigen specific CD4 T cells in the both the lungs (**Fig. 13e**) and spleens (**Fig. 13f**) of mice treated with glutamine inhibition therapy. This leads to a model whereby following the establishment of asthma, multiple cells of type 2 immunity including Th2 T cells, M2 macrophages, and ILC2, are established and can induce symptoms of disease. These cells will produce cytokine and take on effector function upon re-challenge (similar to asthma attack) in an untreated individual. However, upon treatment with a therapy to inhibit glutamine metabolism, the proliferation and effector function of these type 2 immune cells is profoundly inhibited, alleviating disease in an acute treatment setting (**Fig. 14**).

Discussion

This research has indicated a novel role for glutamine in the development of type 2 inflammatory responses. *In vitro* results have reiterated the known role of glutamine in the proliferation of T cells, particular in CD4 T cells, which divide less but express equivalent activation markers upon culture in either low glutamine media or when glutamine metabolism is inhibited through DON treatment. Although the requirement of glutamine in proliferation is conserved between Th1 and Th2 T cells, there is a marked difference in the requirement for this amino acid upon polarization, in that Th1 cells can produce equivalent IL2 and IFN-gamma in culture conditions with or without glutamine, but that Th2 cells display decreased IL4 and IL13 production during low glutamine culture. This decrease in Th2 cytokines is not an immediate effect of glutamine deprivation, as short-term glutamine inhibition during stimulation does not inhibit cytokine production or activation. Interestingly, extremely high doses of the inhibitor of glutamine metabolism DON were sufficient to decrease some parameters of activation and cytokine production in a short-term (4-24 hours of culture) setting such as mTORC1 activity as assessed by p-S6 (**Fig. 2e**) and Th2 cytokine production by overnight ELISA (**Fig. 5d,e**). It is possible that the extremely high dose of inhibitor used (100x the concentration used to inhibit proliferation during a 3 to 6 day culture) is leading to cell death or other deleterious effects. The prevention of cytokine production in Th2 cells cultured in low glutamine media is a transcription level event, as IL4 and IL13 mRNA are decreased in glutamine restricted culture, which is due to a decrease in epigenetic accessibility at the Th2 cytokine locus, as demonstrated by both ChIP and CUT & RUN analyses at multiple loci within the Th2 cytokine locus. When these findings are translated to an *in vivo* model of HDM asthma, the selective requirement for glutamine in type 2 immunity is conserved, where treatment to inhibit glutamine metabolism with either DON or the prodrug JHU-083 decreases Th2 T cells, M2 macrophages, and ILC2 in the lungs of treated mice while leaving splenic Treg levels unaffected, and cytokine production from the T cells and ILC2 is also decreased. Furthermore,

glutamine inhibitor therapy decreases serum HDM specific-antibody titers, reverses some of the histological changes observed in untreated mice, and improves lung function in response to methacholine challenge. Treatment decreased the antigen specific CD4 T cell population in both the lungs and spleens of mice, leading to a model in which inhibition of glutamine metabolism prevents both the formation and function of Th2 immune cells upon re-challenge in the lungs of asthmatic mice.

This requirement for glutamine specifically in type two immune cells, and most extensively studied in Th2 T cells, adds to the growing literature regarding the role of glutamine in CD4 T cell subsets. Previous research has found that glutamine is essential for Th1 T cell development, and that the absence of glutamine leads to polarization to the regulatory T cell subset, with no defect in Th2 formation[35]. However, this study failed to fully characterize the nature of the Th2 cells present, only assessing Gata3 expression by flow cytometry. Gata3 is a transcription factor that is necessary for T cell development and function of all subsets[121], and can be transiently upregulated upon activation even in Th1 cells (data not shown). Therefore, further characterization of the Th2 subset in the absence of glutamine would have likely demonstrated an inhibition similar to as seen in Th1. Furthermore, glutaminase inhibition or disruption through protein knockout has been shown to selectively decrease the formation of Th17 cells in both *in vitro* and *in vivo* settings[37]. However, the specific blockade of one step in the pathway renders a dramatically different result than a global decrease in the activity in the pathway, as T cell metabolism can be highly dynamic and bypass a single node of inhibition. Furthermore, when *in vivo* glutaminase inhibition was attempted in the house dust mite asthma model, and showed no decrease in Th2 T cells in the lung, nor in cytokine production (data not shown).

This study has identified a novel and specific requirement for glutamine in the development of type two immune responses. As type two immune responses are canonically mounted against large, extracellular pathogens, it can be expected that these prolonged immune responses are quite energetically costly. Therefore, using glutamine as a rheostat to determine the energetic state of the

host, and determine if such a response can be mounted potentially underlies the role of glutamine as an important sensing mechanism for energetic state of the host. The incorporation of this sensing mechanism into the epigenetic landscape of the developing Th2 T cell by the dependence of the accessibility of the Th2 cytokine locus being dependent on glutamine underlies the flexibility of CD4 T cell metabolism, and supports the approach that metabolic therapies can be wielded precisely to modulate the immune system, rather than serving as a simple on/off switch. This work demonstrates the requirement for glutamine in the development of type two immune responses, by promoting the epigenetic accessibility of the Th2 cytokine locus, and promoting the formation of type 2 immune cells, including antigen specific Th2 T cells, as well as the necessity for glutamine for cellular development and proliferation following re-challenge in the HDM asthma model. Furthermore, it demonstrates that metabolic therapy by inhibiting glutamine metabolism can be used to modulate immune responses and potentially inhibit undesirable type two inflammation in cases of asthma and autoinflammatory disease.

Materials and Methods

Mice

Six to eight-week-old male of female mice were used for performing all experiments in this study. Rag knockout mice bearing a transgenic T cell receptor recognizing pigeon cytochrome C peptide (5CC7) were obtained from Taconic Biosciences and maintained by breeding in house. For asthma experiments Balb/C mice were obtained from Jackson Laboratory and maintained by breeding in house. For asthma experiments involving tetramer staining, C57BL/6 mice were obtained from Jackson Laboratory and maintained by breeding in house. All mouse procedures were carried out in accordance with a protocol approved by the Johns Hopkins University Institutional Animal Care and Use Committee. No empirical test was performed for choosing sample size prior to experiments. No randomization of samples or animals was used nor were investigators blinded throughout the study.

In vitro primary T cell culture

Mice were sacrificed and spleens were isolated. Spleens were ground through 70um strainer, red blood cells were lysed with ACK lysis buffer, and cells were counted and plated in indicated media (either standard culture media containing exogenous 4mM glutamine, or low glutamine media). Transgenic CD4 T cells were stimulated by the addition of pigeon cytochrome C (PCC) peptide at a final concentration of 5uM. For Th1 skewing, IL12 was added to a concentration of 10ng/mL, IFN-gamma was added to a concentration of 2ug/mL, and anti-IL4 antibody was added to a concentration of 5ug/mL. For Th2 skewing, 10ng/mL IL4 was added to culture, as well as anti-IL12 and anti-IFN-gamma antibodies, both to a concentration of 5ug/mL. Cultures were expanded and IL2 was added to a concentration of 1ng/mL every other day. For conditions in which DON treatment was conducted, DON was added to the indicated concentration, and fresh drug to total volume was added at each culture expansion.

Flow cytometry

Cells were counted, and 2 to 3 million were plated in round bottom tubes. For tissue samples, FC receptors were blocked with anti-CD16/CD32 (Mouse BD Fc Block) (2.4G2, #553142), for 20 minutes at 4C. Surface staining was performed at 4C for 20 minutes in the presence of eBioscience Fixable Viability Dye eFluor 780 (65-0865-14). For intracellular staining, cells were fixed and permeabilized overnight at 4C with eBioscience Fixation/Permeabilization set (88-8824-00). For intracellular cytokine staining, cells were fixed in BD Fixation Permeabilization solution kit (554714) for 20 minutes at room temperature. Intracellular staining on fixed and permeabilized cells was performed for 1 hour at room temperature. For assessment of p-S6, following surface staining as described above cells were fixed with 2% formaldehyde for 15 minutes and permeabilized with ice cold 90% methanol for 20 minutes. After washing with PBS, cells were blocked in 10% FBS solution with Fc block. All experiments were

performed on a BD FACS Calibur or Celesta. Gates were determined by using unstimulated controls or isotype controls where appropriate. Data was analyzed using FlowJo software.

Enzyme-linked immunosorbent assays (ELISA)

For cytokine ELISA, cells were cultured as described above. At day 6 of culture, live cells were isolated by density centrifugation using Ficoll Paque, washed with PBS, and live cell numbers were determined by counting. One million live cells per mL of standard 4mM glutamine T cell culture media were plated in a 6 well plate coated with 3ug/mL anti-CD3 stimulatory antibody in sterile PBS (coated overnight at 4C). Cells were also stimulated with 1ug/mL anti-CD28 stimulatory antibody, which was spiked into media. Following overnight culture at 37C, media was collected and centrifuged to pellet out cells. Cell free media was frozen at -20C. Sandwich ELISA assays were performed according to manufacturer's instructions (eBioscience mouse IL4 ELISA: 88-7044 mouse IL13 ELISA: 88-7137).

For serum antibody ELISA, plates were coated overnight with HDM extract. Plates were washed, blocked, and a dilution curve of serum was applied, diluted with assay buffer. Serum was incubated on plate overnight. The next day, plates were washed, and anti-isotype (IgG1 or IgE) was incubated. Plates were washed, and secondary antibody was incubated. Following final wash, plates were developed, and absorbance was read using spectrophotometer. Results are presented as normalization to untreated mice.

Proliferation assays

Transgenic 5CC7 CD4 T cells are obtained as described above. Following cell counting, cells are labelled with Cell Trace Violet at 10e6/mL in a 1:1000 dilution of CTV for 10 minutes at 37C. Cold media is added to 15mL volume, and labelling is quenched on ice for 5 minutes. Cells are centrifuged and resuspended in appropriate media. For glutamine titration studies, cells are resuspended in either standard 4mM glutamine media or low glutamine media with indicated concentration of exogenous glutamine added.

Cells are stimulated with PCC peptide and proliferation is assessed by flow cytometry. For DON inhibitor studies, cells are resuspended in standard 4mM glutamine media and indicated concentration of DON is added. Cells are stimulated with PCC peptide and proliferation is assessed by flow cytometry.

Stimulation for cytokine production

Primary T cell culture was performed as described above. On indicated day, live cells are isolated by Ficoll density centrifugation, washed in PBS, and live cell number was determined by counting, following which 2e6 cells are plated in 200uL normal T cell media. Normal media levels of glutamine, and no DON are present at time of restimulation unless otherwise indicated by figure legend. Cells are then stimulated with PMA/ionomycin and GolgiStop for four hours, and cytokine production is assessed by flow cytometry.

RNA isolation, cDNA synthesis, and RT-PCR

Transgenic 5CC7 CD4 T cells are peptide stimulated in conditions skewed toward either Th1 or Th2 cell development (as described above) in either 4mM Gln media or low Gln media for 2, 4 , or 6 days, with expansion and addition of exogenous IL2 every other day. At indicated timepoints, cells were saved in TRIzol (Life Technologies), RNA was extracted, cDNA was made using ProtoScript II RT (New England Biolabs), and real time PCR was performed using TaqMan Universal Master Mix II (Life Technologies). Real time PCR primers and probes were obtained from Applied Biosystems for indicated genes, with 18s used as a housekeeping gene. Data are graphed as fold change of low glutamine media cells compared to 4mM glutamine media.

Chromatin Immunoprecipitation

Transgenic 5CC7 CD4 T cells are peptide stimulated in conditions skewed toward Th2 cell development (exogenous IL4, and anti-IL12 and anti-IFN gamma antibodies) in either 4mM glutamine media or low

glutamine media for 3 days, with expansion and addition of exogenous IL2 on the second day. At day 3, live cells were isolated by Ficoll density centrifugation, and 3 million cells were crosslinked in 1% PFA, lysed, sonicated, and antibodies were used to probe for histone H3K27me3 (Abcam) or histone H3K9Ac (Abcam). Antibodies were bound to magnetic beads, which were washed, isolated, and from which DNA was eluted and purified using Phenol: Chloroform: Isoamyl alcohol phase separation followed by precipitation of DNA in ethanol at -20C. DNA was subjected to quantitative PCR amplification with primers for multiple loci within the Th2 cytokine locus using Power SYBR Green master mix (ThermoFisher 4367659). Data is graphed as a percentage of input (prior to antibody binding/ pulldown) DNA.

CUT&RUN

Protocol was adapted from Skene and Henikoff[113]. Transgenic 5CC7 CD4 T cells are peptide stimulated in conditions skewed toward Th2 cell development (exogenous IL4, and anti-IL12 and anti-IFN gamma antibodies) in either standard 4mM glutamine media, standard 4mM glutamine media plus 0.5uM DON, or low glutamine media for 3 days, with expansion and addition of exogenous IL2 on the second day. At day 3, live cells were isolated by Ficoll density centrifugation, and counted. Five hundred thousand cells were washed, and bound to Concanavalin A coated beads. Cells were transferred to Lo-Bind microcentrifuge tubes, gently permeabilized with Digitonin, and incubated with 0.5ug antibodies against histone H3K27me (Abcam) or H3K9Ac (Abcam) rotating at 4C overnight. Following washing, PA-MNase was bound to antibodies for 1 hour at 4C, bead bound cells were again washed, and transferred to a metal rack in an ice bath. MNase activity was activated with the addition of 2uL 100mM CaCl2, and digestion occurred for 30 minutes on ice. Equivalent volume of 2X STOP buffer was added (prepared as described in Skene and Henikoff), and chromatin was allowed to diffuse out of cells for 30 minutes at 37C. Chromatin was collected as soluble fraction following bead binding, and chromatin was submitted to Proteinase K treatment for 1 hour. DNA was eluted and purified using Phenol: Chloroform: Isoamyl

alcohol phase separation with the addition of an equivalent volume of chloroform, followed by precipitation of DNA in ethanol at -20C. DNA was subjected to quantitative PCR amplification with primers for multiple loci within the Th2 cytokine locus using PowerSyBR master mix. Data is normalized within sample to gene with high levels of particular histone mark (Socs3 for H3K9Ac, Gata4 for H3K27me3) and fold change was graphed compared to standard 4mM glutamine media samples using delta-delta CT analysis.

House Dust Mite Asthma disease model and mouse harvest

Six to eight-week-old Balb/C mice are given two IP sensitizations of HDM (Stallergenes Greer) and two intranasal challenges, each one week apart. Two days following the first intranasal challenge, mice are dosed with DON (IP 1.8mg/mL) or JHU-083 (oral gavage 3.8mg/kg) every other day until completion of experiment. Mice are sacrificed on day 24, and lungs are macerated and stained for flow cytometry. For assessment of serum antibody titers, mice were sacrificed and cardiac puncture was performed to collect blood. Blood was allowed to coagulate at room temperature with periodic mixing for 2 hours. Coagulated blood was centrifuged at 15,000RPM for 20 minutes at 4C, serum was collected and frozen at -20C for ELISA analysis.

For assessment of cytokine production, lungs of treated and untreated mice were macerated and counted, and 2e6 cells were plated in 200uL standard T cell media in a 96 well plate. Cells were stimulated for 4 hours with PMA/ionomycin and GolgiStop. Cytokine production was assessed by flow cytometry.

Tetramer staining

Following asthma protocol, lungs were harvested, washed, macerated, and digested in 2mg/mL collagenase I in 3mL RPMI with DNAse I. Lungs were mashed through a strainer, red blood cells were lysed in ACK lysis buffer, and cells were counted. Two million lung cells were plated in 200uL of RPMI

with 10% FBS and 0.1% azide (filtered for sterility) and stained with a class II tetramer specific for the DerP1 peptidase antigen of HDM[122] bound to phycoerythrin at a 1:200 dilution. Staining was performed for 2 hours at 37C in the presence of 10nM dasatinib with Fc block. Following staining, samples were washed 2-3 times with FACS buffer, and surface staining was carried out as described above. Negative gates for tetramer binding were set according to Clip negative control tetramer staining.

rIL33 ILC2 model

Six to 8-week-old Balb/C mice were anesthetized with isoflurane, and administered 0.25ug recombinant IL33 intranasally in PBS. Recombinant IL33 treatment was performed on 3 consecutive days. Mice were treated with 1.8mg/kg DON every other day for 3 treatments, starting on the first day of rIL33 treatment. On day 6, mice were sacrificed and lungs harvested as described previously. ILC2 were characterized by flow cytometry to identify cells that were negative for lineage cocktail (CD3, Ly6C/G, CD11b, B220, TER-119) and positive for CD45, Gata3, ST2, CD44, CD25, and CD127.

Histology

At time of asthma harvest, lungs were washed, inflated with formalin, and tied off. Lungs were carefully dissected out of the chest cavity and preserved in formaldehyde overnight. Fixed tissues were trimmed, placed in processing cassettes, and dehydrated with a series of alcohol washes. Dehydrated tissue was preserved in paraffin wax, and sections were cut and mounted on glass slides. Slides were deparaffinized and rehydrated with a series of alcohol washes. Slides were washed, stained in hematoxylin, rinsed, and briefly counterstained in eosin, and washed again. Slides were mounted with coverslips and imaged by light microscopy.

Pulmonary function tests/ methacholine challenge

Lung function tests were performed as described in Collins[123]. Mice were connected to a flexi-VentTM ventilator (SCIREQ) and ventilated at 0.22mL tidal volume at 120 breaths per minute. Mice were paralyzed with an IP injection of succinylcholine, subjected to deep inhalation for 5 seconds, and returned to normal ventilation for 1 minute. Baseline measurements of respiratory system resistance, and compliance were measured. For assessment of airway hyperresponsiveness, methacholine solution was prepared fresh daily, and increasing doses (0.3 ,1, 3, 10, and 30mg/mL) of nebulized methacholine were delivered through flexi-Vent at defined intervals. Respiratory system resistance and compliance were measured following each dose of methacholine administration.

Statistical Analysis

All graphs were creased using GraphPad Prism software, and statistical analysis was performed with GraphPad Prism. A p-value less than 0.05 was considered statistically significant; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$. Error bars represent mean ± standard error of the mean.

Figures

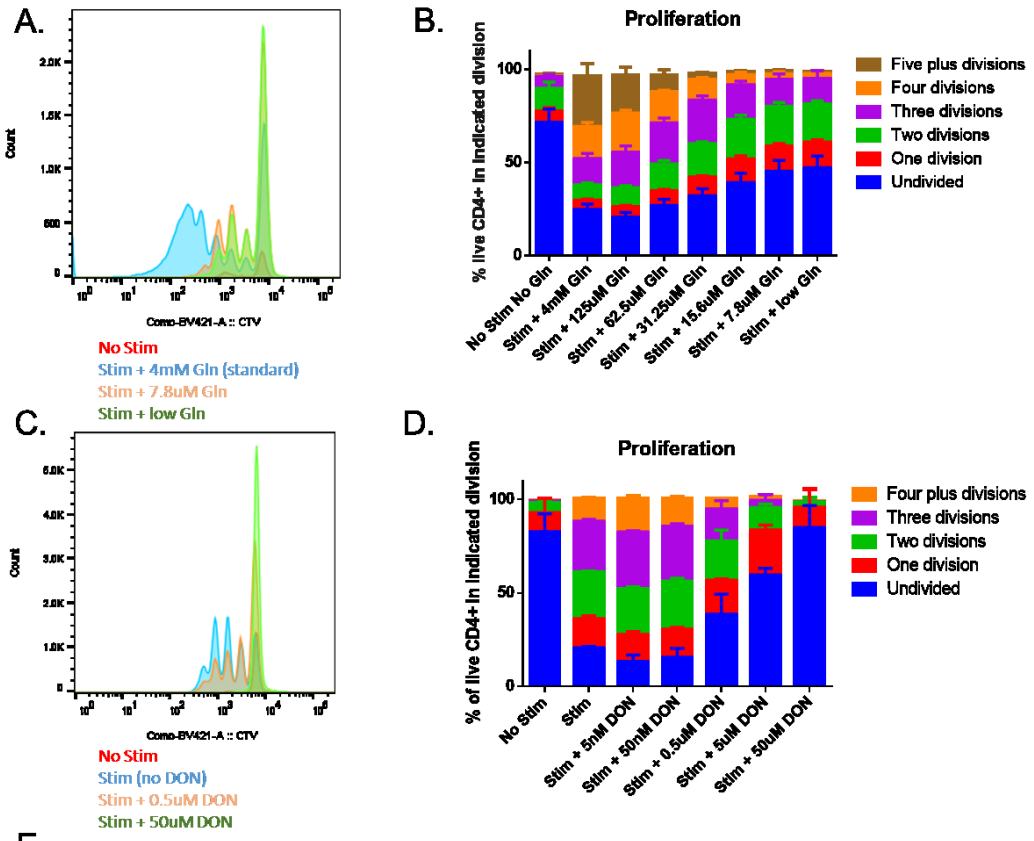

Figure 1. Inhibition of glutamine metabolism decreases CD4 T cell proliferation in a dose-dependent manner

Figure 1. Inhibition of glutamine metabolism decreases CD4 T cell proliferation in a dose-dependent manner. **(a)** CTV dilution plot of CD4 T cells stimulated and cultured for 72 hours in restricted concentrations of glutamine. **(b)** Graph of proliferation at indicated glutamine concentration. **(c)** CTV dilution plot of CD4 T cells stimulated and culture for 72 hours in indicated concentration of DON. **(d)** Graph of proliferation at indicated glutamine concentration. **(e)** Cell numbers of CD4 T cells stimulated and cultured in either standard 4mM glutamine media or low glutamine media were assessed by counting at indicated timepoints.

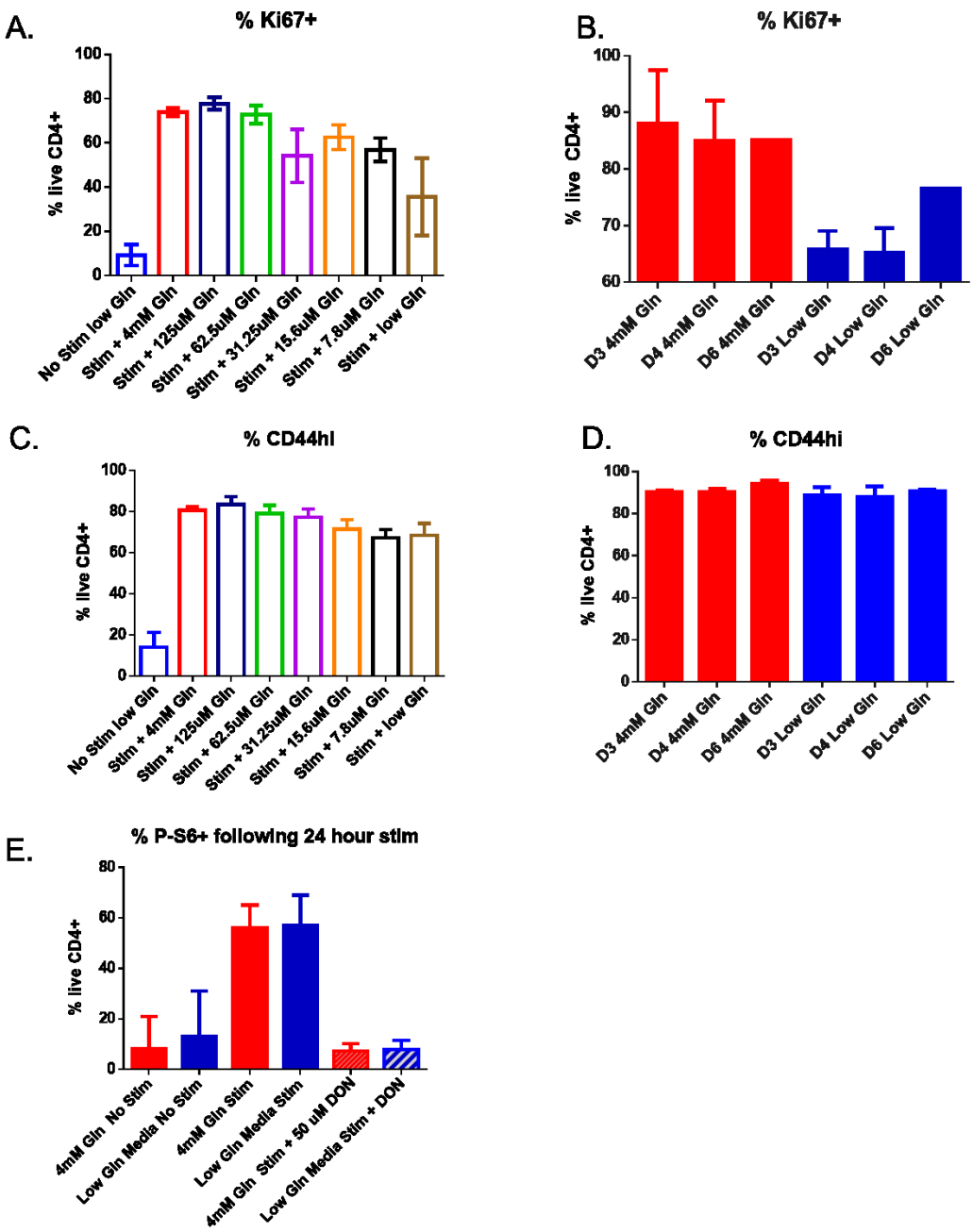

Figure 2. Decreased Ki67, but no decrease in activation or p-S6 in low Gln culture

Figure 2. Decreased Ki67, but no decrease in activation or p-S6 in low Gln culture. **(a)** CD4 T cells were assessed for Ki67 expression by flow cytometry following 3 days of stimulation and culture in indicated glutamine concentration. **(b)** Ki67 expression was assessed at indicated timepoint. **(c)** CD4 T cell were assessed for CD44 expression by flow cytometry following 3 days of stimulation and culture in indicated glutamine concentration. **(d)** CD44 expression was assessed at indicated timepoint. **(e)** mTORC1 activity as indicated by p-S6 staining was assessed by flow cytometry following 24 hour stimulation with aCD3/aCD28 antibodies in indicated conditions.

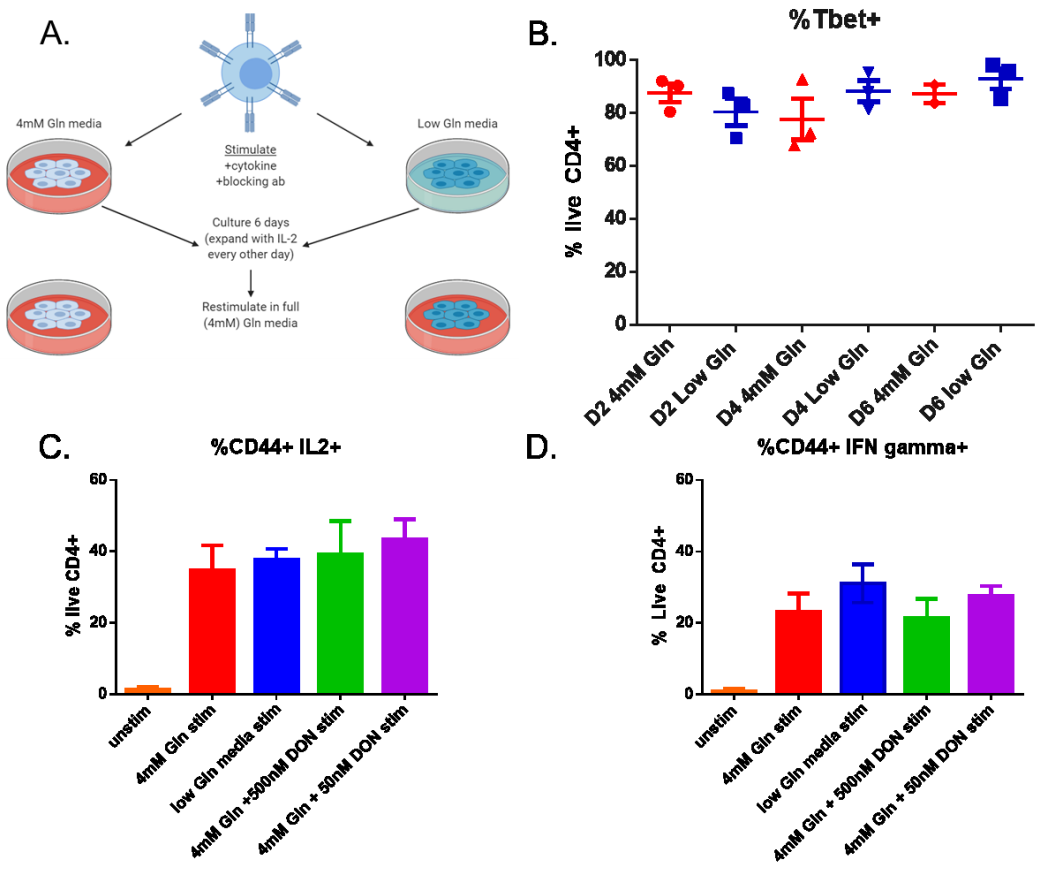

Figure 3. Skewing in low Gln media does not affect Tbet expression or Th1 cytokine production in Th1 skewed cells

Figure 3. Skewing in low Gln media does not affect Tbet expression or Th1 cytokine production in Th1 skewed cells. **(a)** experimental outline for skewing Th1 cells in the presence or absence of Gln in the media, followed by restimulation to assess cytokine production in full Gln media. **(b)** Tbet expression as assessed by flow cytometry at indicated timepoint. **(c)** on day 6, live Th1 cells were isolated from standard media, low glutamine media, or standard media plus indicated concentration of DON cultures and restimulated with PMA and ionomycin in standard media to assess IL2 production by flow cytometry. **(d)** as in (c), but assessment of IFN gamma production.

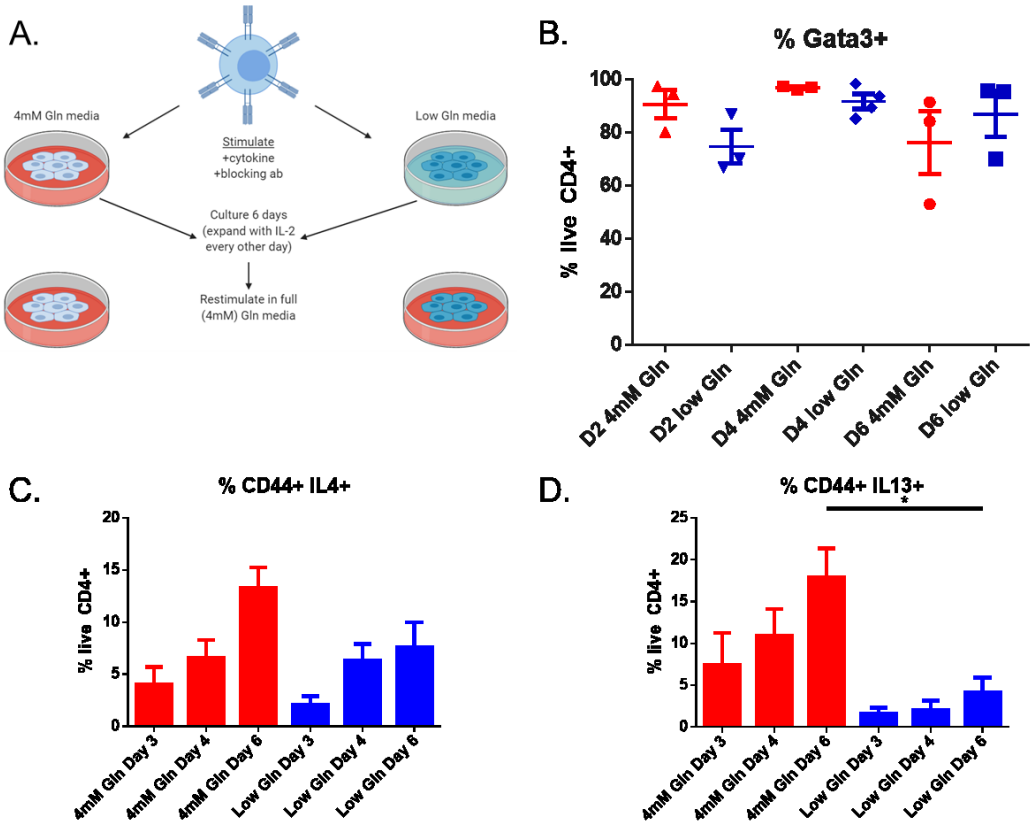

Figure 4. Skewing in low Gln media does not affect Gata3 expression, but leads to decreased cytokine production in Th2 skewed cells

Figure 4. Skewing in low Gln media does not affect Gata3 expression, but leads to decreased cytokine production in Th2 skewed cells. **(a)** experimental outline for skewing Th2 cells in the presence or absence of Gln in the media, followed by restimulation to assess cytokine production in full Gln media. **(b)** Gata3 expression as assessed by flow cytometry at indicated timepoint. **(c)** at indicated timepoints, live Th2 cells were isolated from standard media, or low glutamine media cultures and restimulated with PMA and ionomycin in standard media to assess IL4 production by flow cytometry. **(d)** as in C, but assessment of IL13 production.

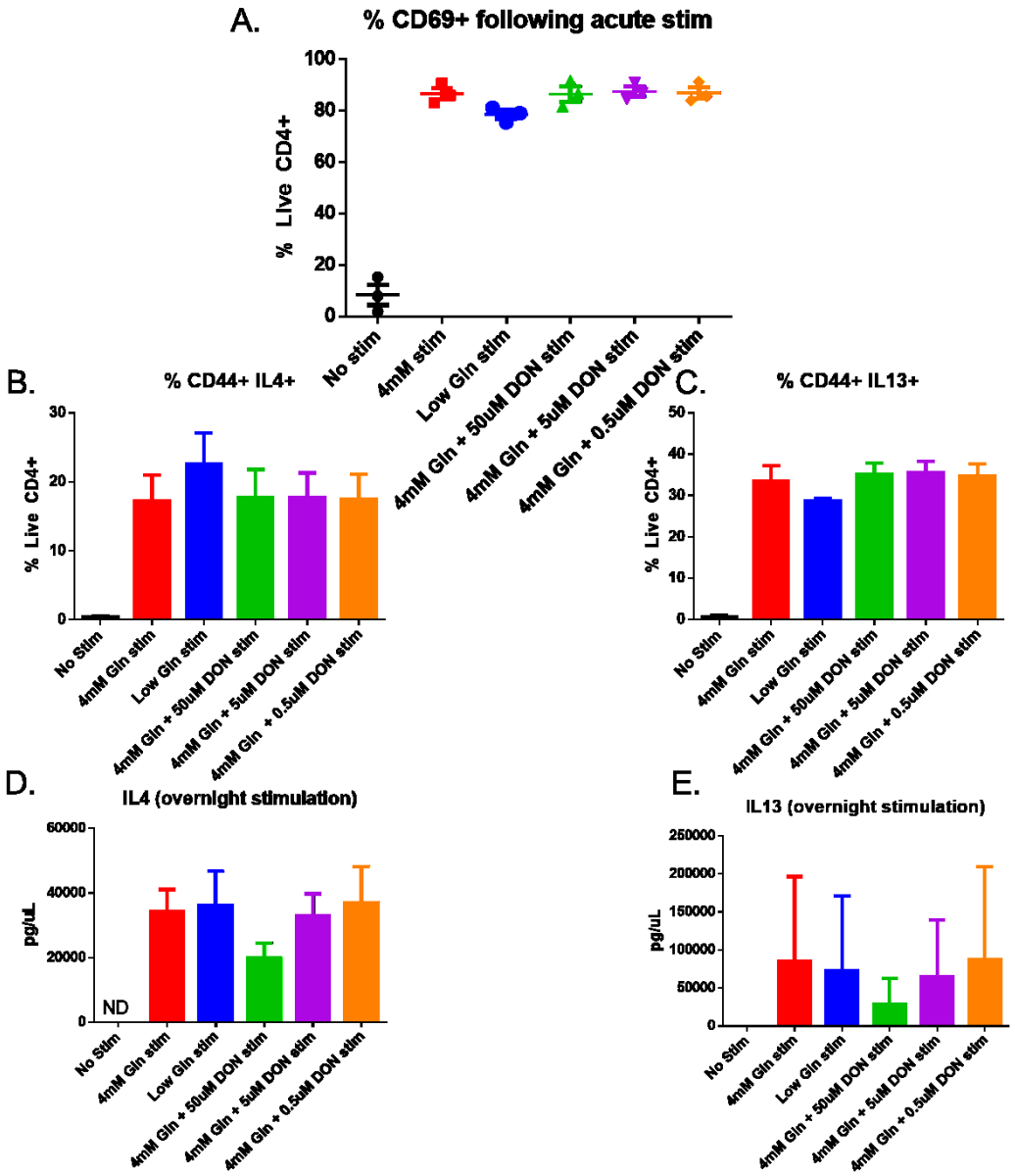

Figure 5. Deficiency in Th2 cytokine production is not an acute effect

Figure 5. Deficiency in Th2 cyotkine production is not an acute effect. **(a)** cells skewed for 6 days in standard 4mM glutamine conditions were re-stimulated with PMA and ionomycin for 4 hours and activation by CD69 expression was assessed by flow cyotmetry. **(b)** cells treated and stimulated as in (a) but with flow cytometry assessment for IL4 production. **(c)** cells treated and stimulated as in (a) but with flow cytometry assessment for IL13 production. **(d)** cells cultured for 6 days as in (a), but with overnight plate-bound anti-CD3 and soluble aCD28 stimulation followed by media collection and cytokine ELISA to assess IL4 production. **(e)** cells as in (d) but with cytokine ELISA for IL13 production

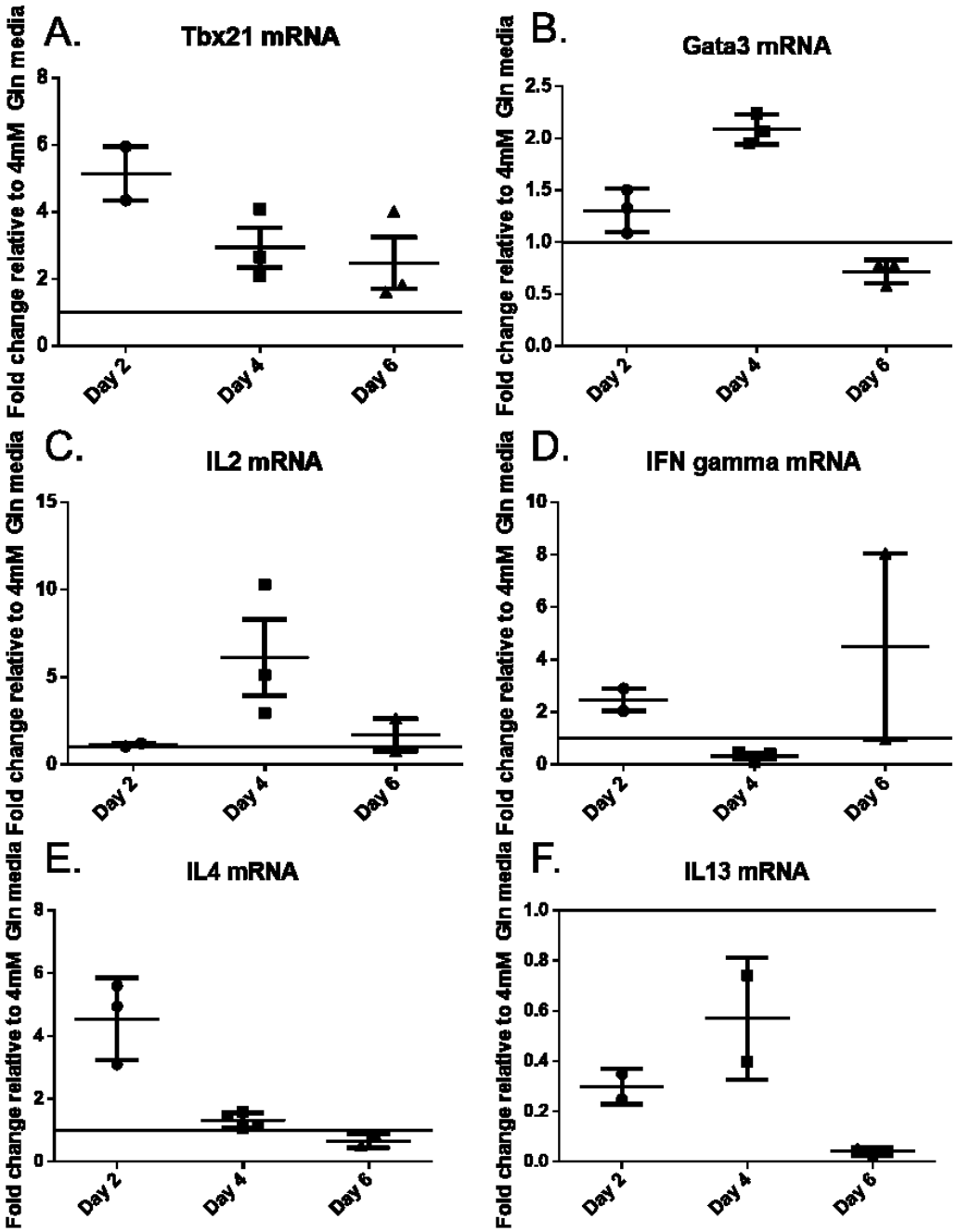

Figure 6. Th2 cytokine production is inhibited at a transcriptional level, whereas Th1 cytokine transcripts are unaffected

Figure 6. Th2 cytokine production is inhibited at a transcriptional level, whereas Th2 cytokine transcripts are unaffected. **(a)** CD4 T cells were skewed Th1 in the either standard or low Gln culture media and RNA was collected for reverse transcription and PCR analysis of *Tbx21* expression at indicated timepoint. **(b)** CD4 T cells were skewed Th2 in either standard or low Gln culture media and RNA was collected for reverse transcription and PCR analysis of *Gata3* expression at indicated timepoint. **(c)** as in (a) but analyzed for IL2 expression. **(d)** as in (a) but analyzed for IFN gamma expression. **(e)** as in (b) but analyzed for IL4 expression. **(f)** as in (b) but analyzed for IL13 expression

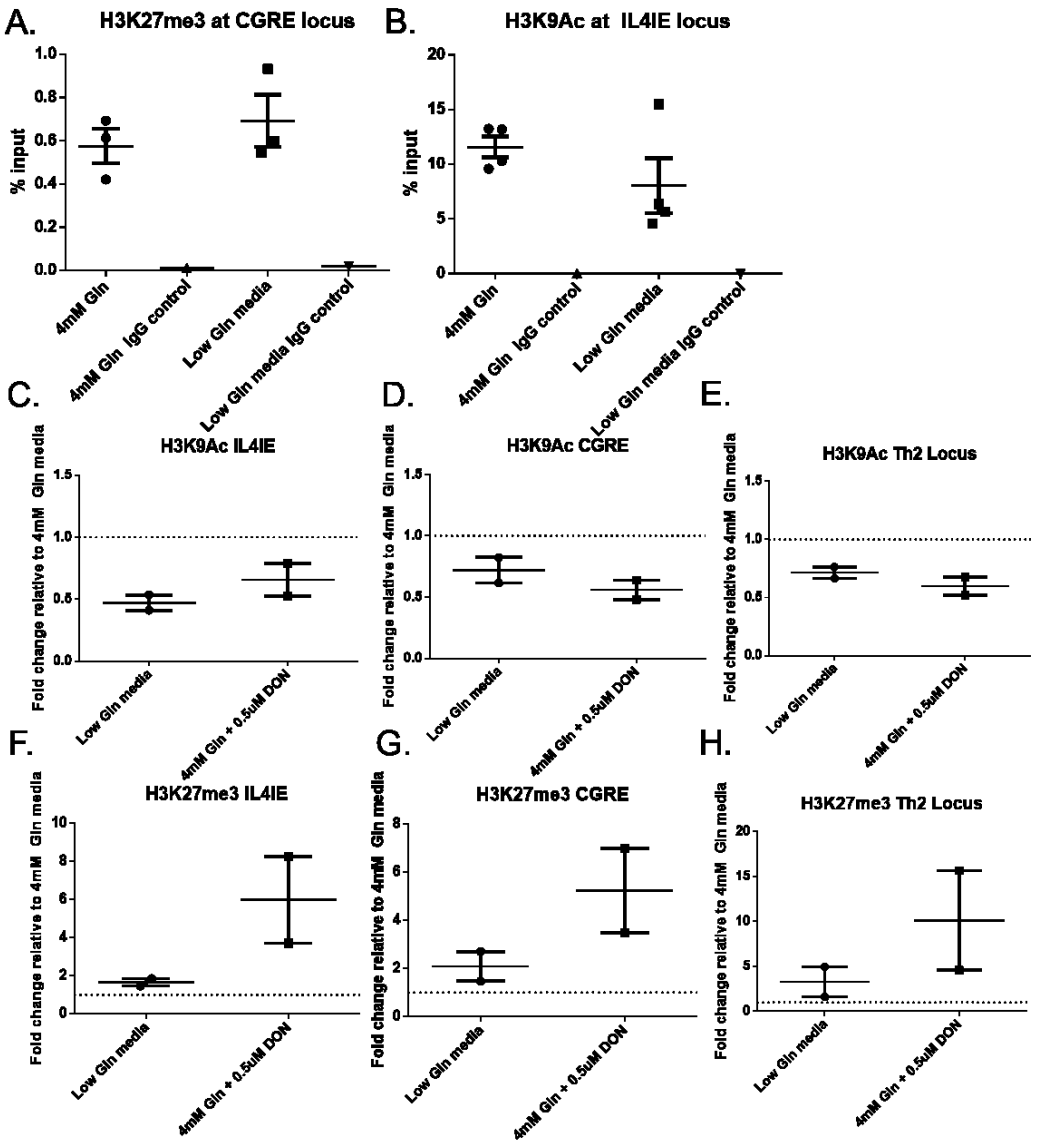

Figure 7. Low glutamine culture inhibits the increase in epigenetic accessibility of Th2 cells that typically accompanies development

Figure 7. Low glutamine culture inhibits the increase in epigenetic accessibility of Th2 cells that typically accompanies development. **(a)** ChIP analysis of day 3 cultured Th2 cells in either standard or low Gln media for histone H3K27me3 localization to element of Th2 cytokine locus **(b)** as in (a) but for ChIP for histone H3K9Ac localization. **(c)** CUT&RUN performed on Th2 cells cultured for 3 days in either low Gln media or standard media with DON treatment and analysis for histone H3K9Ac localization to IL4IE. **(d)** as in (c) but analyzed for CGRE. **(e)** as in (c) but analyzed for Th2 locus. **(f)** CUT&RUN performed on Th2 cells cultured for 3 days in either low Gln media or standard media with DON treatment and analysis for histone H3K27me3 localization to IL4IE. **(g)** as in (f) but analyzed for CGRE. **(h)** as in (f) but analyzed for Th2 locus.

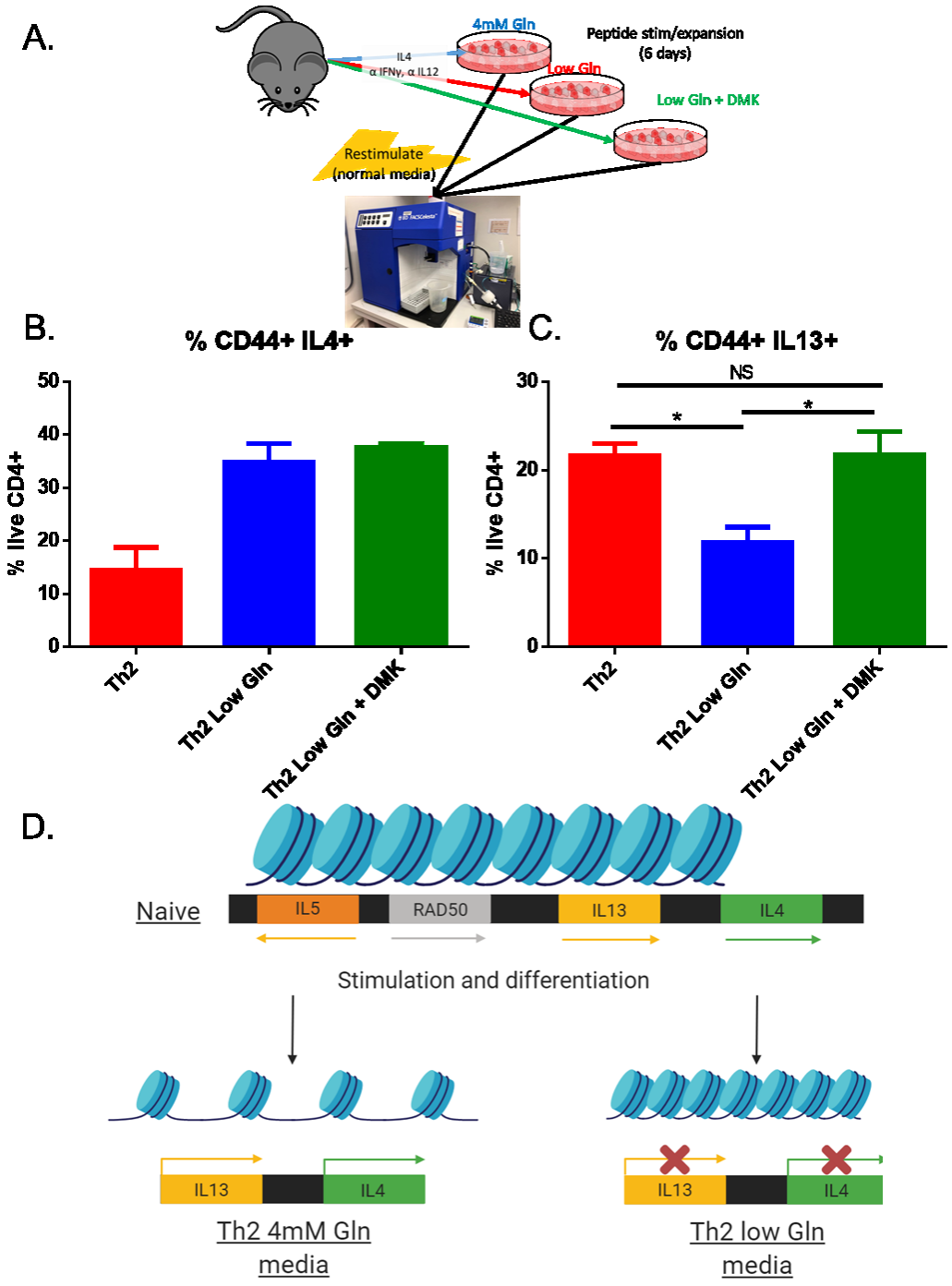

Figure 8. Supplementation in low glutamine culture with a cell permeable aKG analogue rescues Th2 cytokine production

Figure 8. Supplementation in low glutamine culture with a cell permeable aKG analogue rescues Th2 cytokine production. **(a)** experimental outline. **(b)** on day 6 of Th2 cell culture, live cells were isolated and restimulated to assess IL4 production by flow cytometry. **(c)** as in (b) but assessment of IL13 production. **(d)** model for the role of glutamine in the generation of epigenetic accessibility in Th2 cell development.

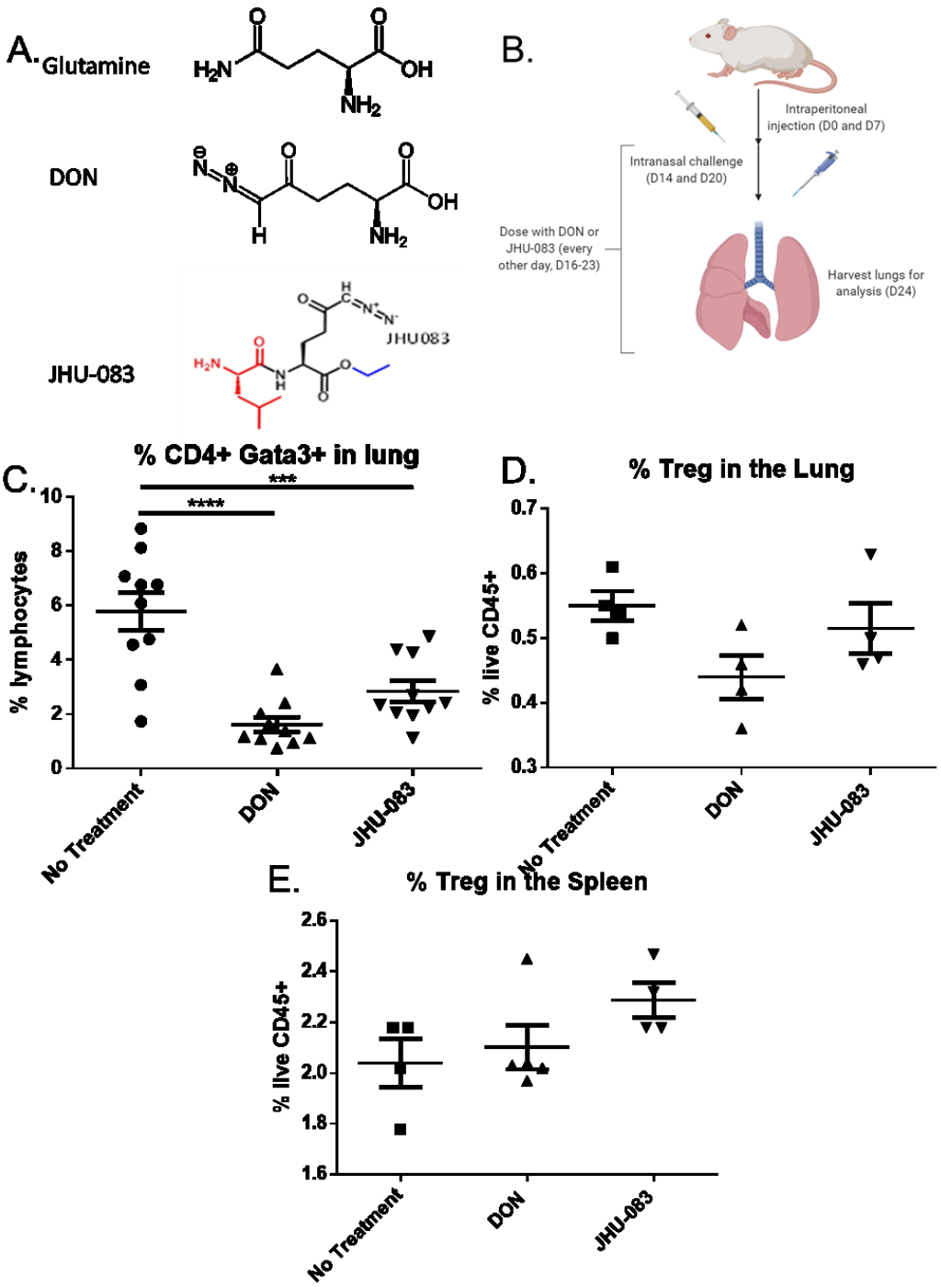

Figure 9. Treatment with a novel glutamine antagonist in vivo in a HDM asthma model decreases Th2 T cells in the lungs without affecting Tregs

Figure 9. Treatment with a novel glutamine antagonist in vivo in a HDM asthma model decreases Th2 T cells in the lungs without affecting Tregs. **(a)** structures of glutamine, DON, and JHU-083. **(b)** outline of HDM asthma model. **(c)** flow cytometric analysis of the lungs of asthma mice for the presence of Gata3+ Th2 cells. **(d)** flow cytometric analysis of the lungs of asthma mice for the presence of Tregs. **(e)** as in (d) but in the spleen

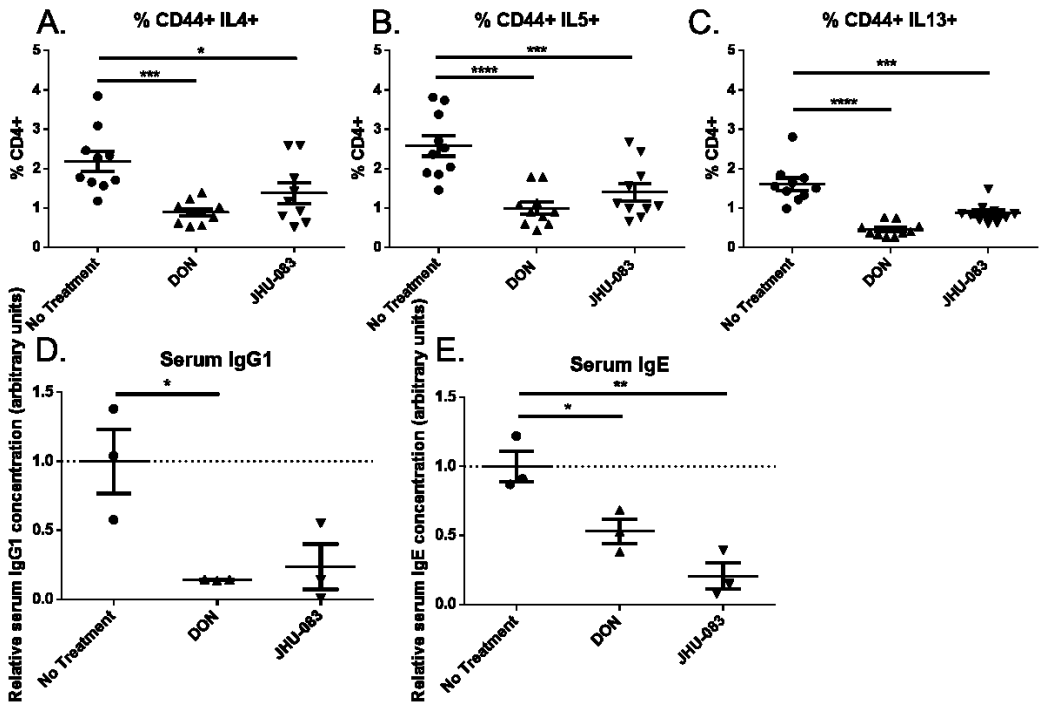

Figure 10. Glutamine inhibition decreases Th2 cytokine production in the lungs and HDM specific serum antibody titers

Figure 10. Glutamine inhibition decreased Th2 cytokine production in the lungs and HDM specific serum antibody titers. **(a)** *ex vivo* restimulation of CD4 T cells from the lungs of HDM asthma mice with PMA and ionomycin and then assessment of IL4 production by flow cytometry. **(b)** as in (a) but for IL5 production. **(c)** as in (a) but for IL13 production. **(d)** serum IgG1 titer specific for HDM as determined b ELISA and presented as relative fold change to untreated mice. **(e)** as in (d) but for IgE.

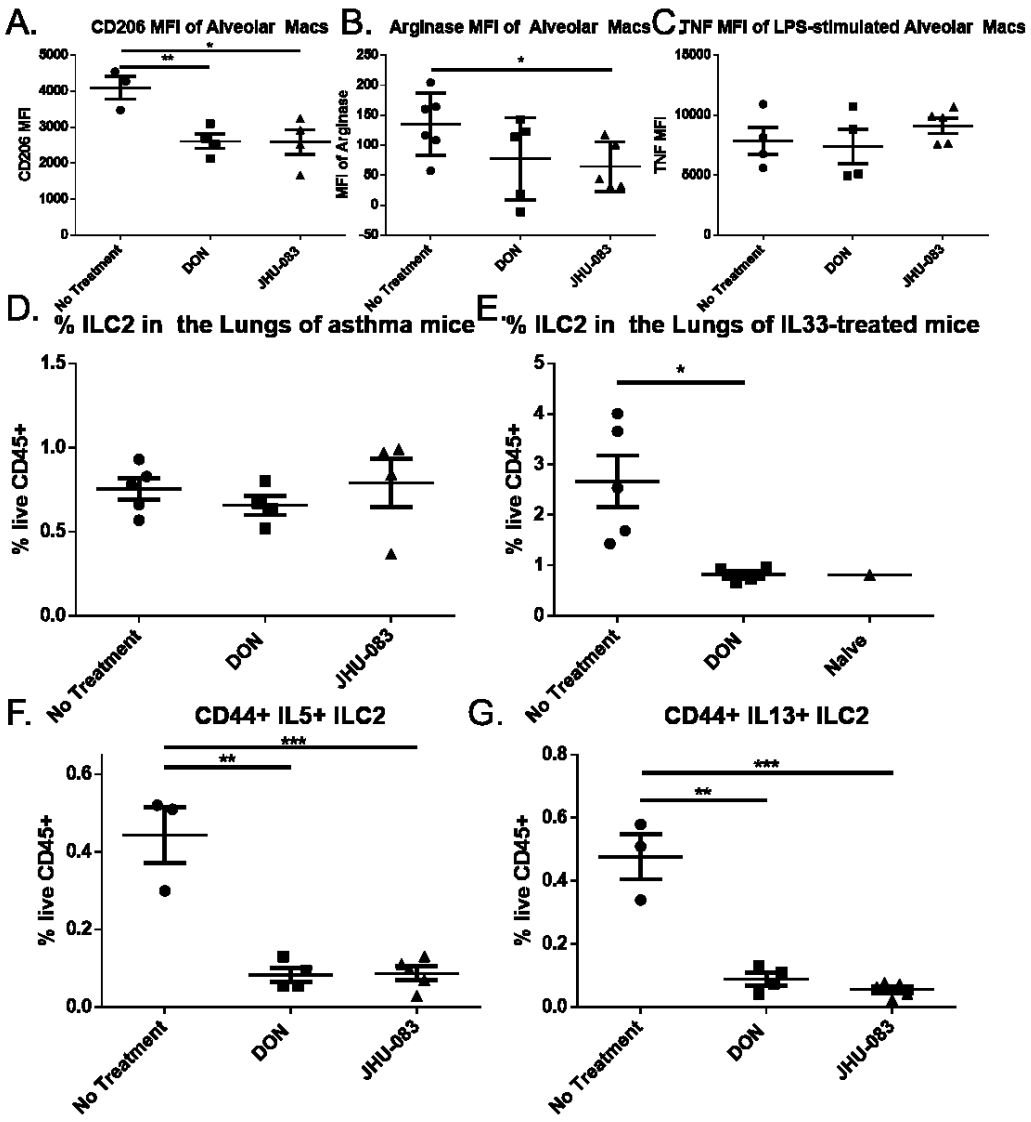

Figure 11. Type 2 immunity in the innate immune system is also decreased by glutamine inhibition in HDM asthma

Figure 11. Type 2 immunity in the innate immune system is also decreased by glutamine inhibition in HDM asthma. **(a)** alveolar macrophages from the lungs of HDM asthma mice were assessed by flow cytometry for CD206 expression. **(b)** as in (a) but for Arginase expression. **(c)** as in (a) but macrophages were stimulated overnight with LPS and TNF expression was assessed by flow cytometry. **(d)** ILC2 from the lungs of HDM asthma mice were assessed by flow cytometry. **(e)** as in (d) but in a rIL33 model of ILC2 development. **(f)** as in (d) but with 4-hour *ex vivo* PMA/ionomycin stimulation and flow cytometry analysis to assess IL5 production. **(G)** as in (f) but for IL13 production.

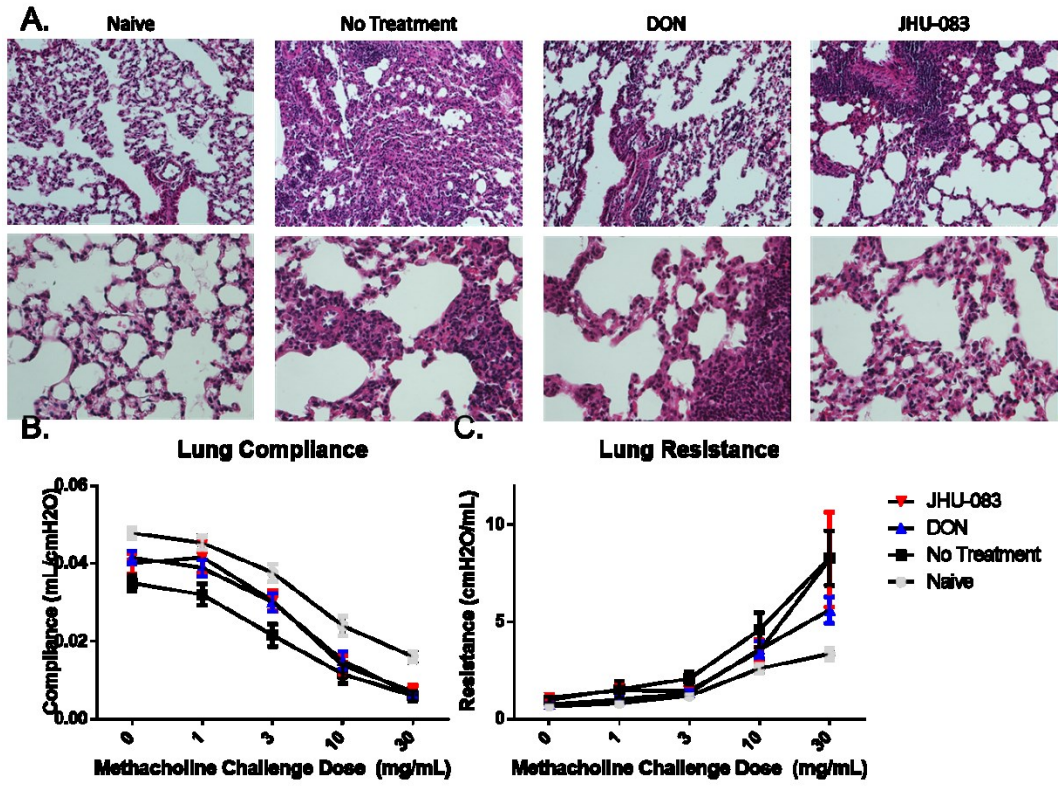

Figure 12. Treatment with glutamine inhibition decreases airway disease in HDM asthma model

Figure 12. Treatment with glutamine inhibition decreases airway disease in HDM asthma. **(a)** H&E staining of lungs of asthma mice. **(b)** lung compliance of HDM asthma mice as assessed in methacholine challenge studies. **(c)** as in (b) but for lung resistance.

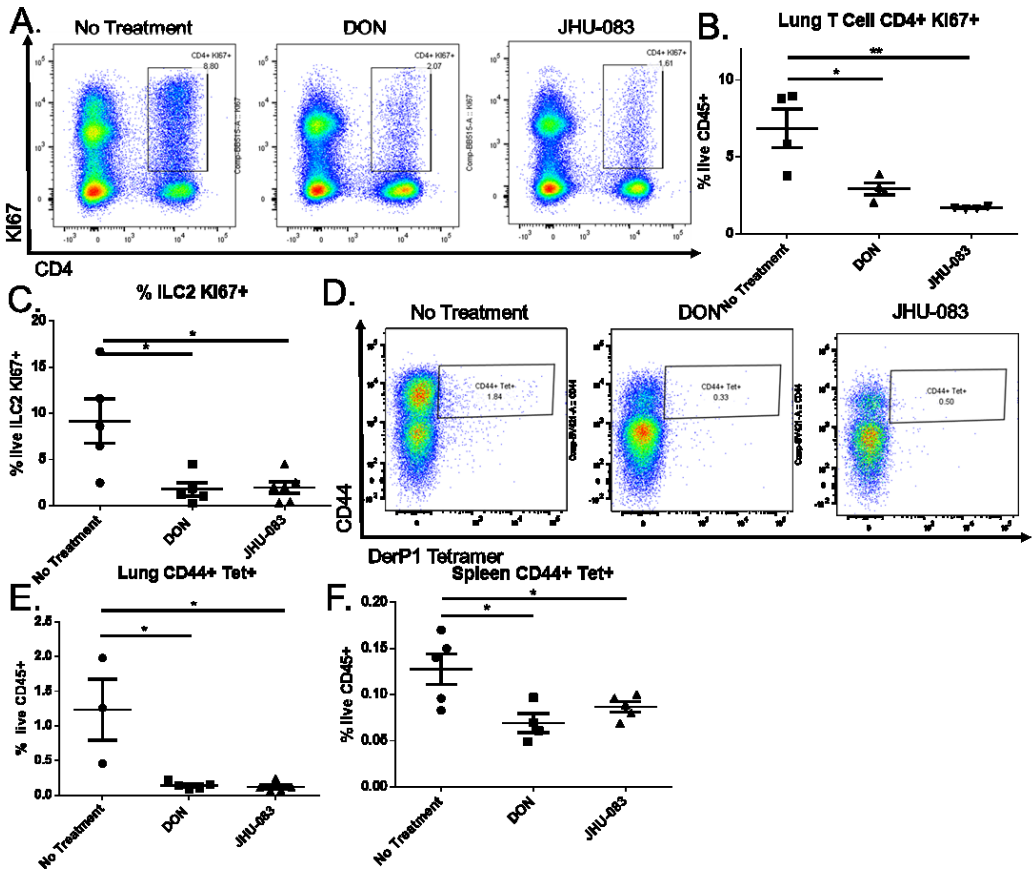

Figure 13. Decreased proliferation and antigen specific cells in the lungs of asthma mice following glutamine inhibition

Figure 13. Decreased proliferation and antigen specific cells in the lungs of asthma mice following glutamine inhibition. **(a)** example flow plots of Ki67 staining on CD4 T cells in the lungs of HDM asthma mice. **(b)** summary plot of (a). **(c)** Ki67 expression in ILC2 from the lungs of HDM asthma mice. **(d)** example flow plots of antigen specific CD4 T cells as determined by tetramer staining on CD4 T cells in the lungs of HDM asthma mice. **(e)** summary plot of (d). **(f)** antigen specific CD4 T cells as determined by tetramer staining on CD4 T cells in the spleens of HDM asthma mice.

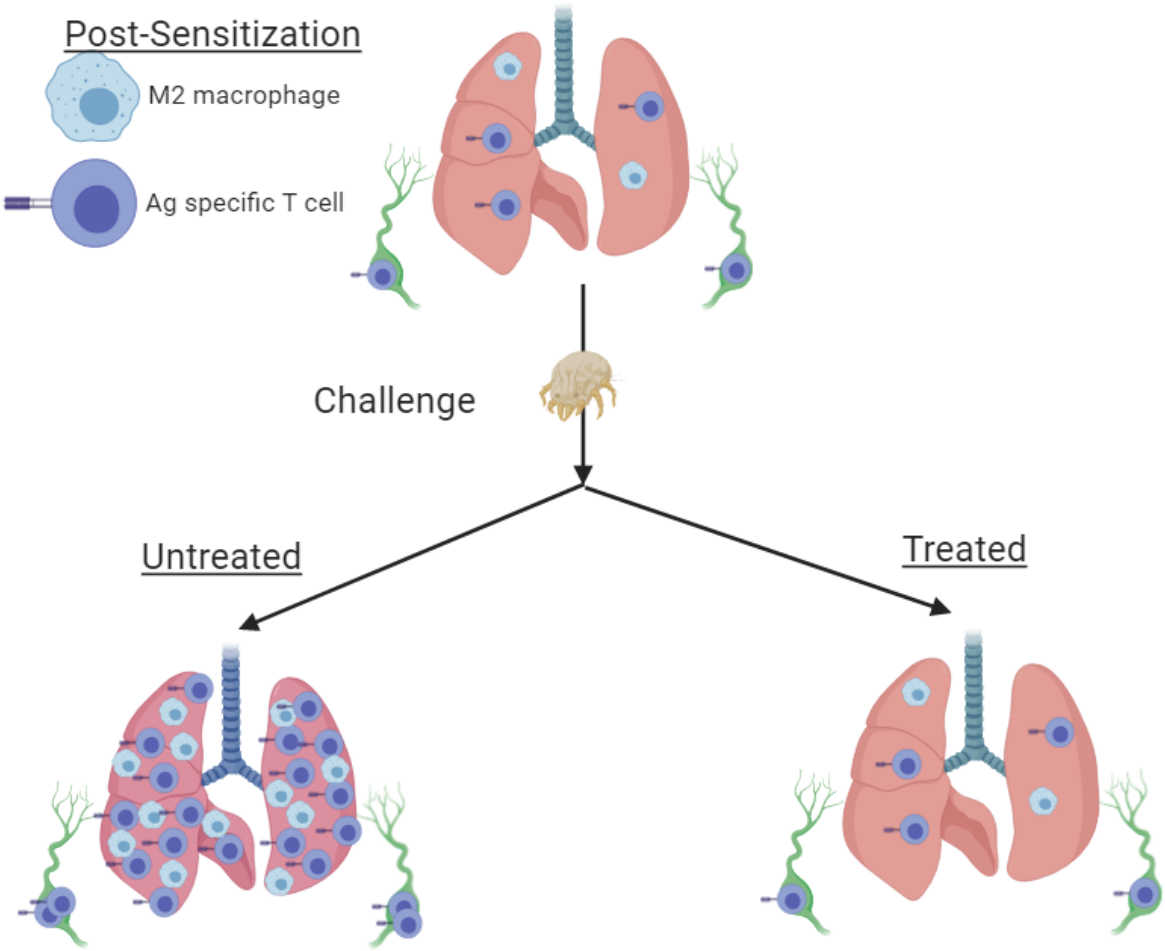

Figure 14. A model for the effects of treatment with glutamine inhibitor therapy in the HDM asthma model

Figure 14. A model for the effects of treatment with glutamine inhibition therapy in the HDM asthma model.

Chapter 3: The role of glutamine in CD8 T cell memory regulation

Abstract

As previously demonstrated, the amino acid glutamine is essential for the metabolic shifts that accompany T cell activation, including and especially the increase in proliferation. However, alterations in metabolism at the time of activation have also been shown to have important functional impacts on the character of a T cell response. In order to assess the effect of glutamine restriction on CD8 T cell responsiveness, we stimulated and cultured antigen specific transgenic CD8 T cells in either standard 4mM glutamine media, or in low glutamine culture media. Similar to previous data, it was found that culture in low glutamine media leads to decreased proliferation initially. However, although these cells were fewer in number, they bore a more memory-like phenotype *in vitro*. In an adoptive transfer model, low glutamine cultured CD8 T cells were shown to better persist in circulation, and mounted a more robust and polyfunctional response upon re-challenge. In an ACT model of tumor therapy, low glutamine cultured cells mounted a more effective anti-tumor response, leading to smaller tumors and enhanced survival. Most dramatically, all of these effects were seen in the absence of systemic therapy within the mouse, thus demonstrating the use of metabolic perturbation prior to transfer as a mechanism for enhancing response to ACT.

Introduction

The metabolic changes that a T cell undergoes upon activation serve not as a byproduct of the activation process, but rather exist to meet very important needs of the newly activated cell, thus making them intrinsic and necessary to full activation and the mounting of a robust immune response. Based on this, it is clear that metabolism is not a byproduct of stimulation, but rather serves as an important controller of T cell activation and subsequent responses. The ability of metabolism to control the CD8 T cell response has been one of the persistent through-lines in the nascent field of immunometabolism, with early work by Pearce and colleagues demonstrating that activation of fatty acid oxidation with the drug metformin was capable of increasing the memory response against LmOVA, as was inhibition of mTORC1 activity by the use of rapamycin[53]. Subsequent work identified the ability of CD8 T cells to carry out mitochondrial-based respiration, and particularly having the capacity to enhance their OCR high beyond the basal level, a quality known as SRC, was a hallmark of a good memory cell. Further, when oxidative respiration was enhanced by enforced expression of the mitochondrial fatty acid transporter CPT1, memory cell development was even further increased, leading to enhanced memory cell persistence in an LmOVA model[56]. This observation lead to the clinically applicable finding that mitochondrial activity, and mitochondrial membrane potential, can be used to discriminate between CD8 T cells mounting a rapid effector response, and those better suited to memory responses, as Sukumar and colleagues used the membrane potential dye TMRM to demonstrate that cells with lower membrane potential are phenotypically more memory-like, both in terms of surface receptor expression, and as determined by metabolic profiling[63]. These low membrane potential CD8 T cells were also better able to persist in an adoptive transfer model, indicating that metabolism is not a byproduct of activation, but rather that metabolic factors play a powerful role in determining the immune response, particularly as it relates to fate decisions made in regards to T cell memory.

Glutamine is not an essential amino acid, but plays an important role in T cell activation, both as a fuel for proliferation[14], and through its ability to enter the TCA cycle and keep it circulating in order to promote the generation of a myriad of biosynthetic intermediates necessary for T cell activation and effector function to occur[73]. However, when glutamine metabolism is restricted, the immune response is not inhibited across the board, but redirected, as was illustrated in the previous chapter, where CD4 T cell differentiation towards Th2 cells was inhibited both *in vitro* in conditions of reduced glutamine metabolism, and *in vivo* in a HDM asthma model upon treatment with either DON or JHU-083. Recently, the effect of inhibiting glutamine metabolism *in vivo* with JHU-083 was explored by Leone and colleagues. This work examined glutamine restriction in the context of tumor therapy, in which it was shown that JHU-083 treatment inhibited the growth of the tumor, and converted the TME to one which was less hypoxic, and in which there was more glucose and glutamine available to meet the nutritional needs of the TIL. Subsequent gene expression and GSEA analyses of the TIL in tumors treated with JHU-083 indicated that these cells were more proliferative, more activated, less hypoxic, and more resembled memory cells than the TIL found in untreated mice. This memory phenotype was confirmed by increased expression of CD62L, CD127, and BCL family proteins, both *in vitro* and *in vivo*[93]. Therefore, the restriction of glutamine metabolism in the context of the tumor microenvironment is an important mechanism for the promotion of a memory response. These findings were also corroborated by earlier work by Nabe and colleagues, who made similar findings, but with a phased culture approach, in which CD8 T cells were initially activated in either 2mM glutamine containing media, this media but with the addition of DON, or in glutamine depleted media, for 2-3 days before being rested in 2mM containing media prior to ACT, and it was found that glutamine restriction was also able to improve the anti-tumor response, with these cells displaying decreased PD-1 expression and increased mitochondrial health, as indicated by higher SRC and mitochondrial protein expression[124] .

As novel cancer therapies such as checkpoint blockade, and chimeric antigen receptor (CAR) T cell therapy advance further into patients and best practices are considered, the role of metabolic modulation in these therapies cannot be ignored[125,126]. As previously stated, the TME is an exceptionally metabolically hostile environment in which to mount an anti-tumor immune response, and thus all consideration must be made to modulating metabolism to promote the most potent response possible. For therapies like CAR T cell therapy and ACT, where a portion of the modulation occurs *in vitro* for the expansion of either engineered or endogenous T cells for reinfusion, the ability to optimize stimulation and expansion conditions with a mind towards promoting a more enduring, memory-like phenotype is ideal. The opportunity to enhance metabolism through additional modification of the transferred cell population, such as the addition of costimulatory domains with more metabolically preferable signaling outcomes could also serve as a powerful approach[127]. Herein, we identify glutamine metabolism in CD8 T cells as an important factor to consider, and demonstrate that initial stimulation and culture in low glutamine media, while restricting initial CD8 T cell expansion, promotes a more memory-like phenotype *in vitro*. Furthermore, when these cells are adoptively transferred, they persist longer in the blood *in vivo*, and mount a more robust response upon re-challenge. ACT with low glutamine cultured cells is sufficient to increase the anti-tumor response, leading to smaller tumors and increased survival of tumor-bearing mice. Interestingly, all of these beneficial outcomes occurred in the absence of systemic therapy, indicating that inhibition of glutamine metabolism *in vitro* at the time of stimulation was sufficient to durably promote a memory response following *in vivo* transfer and effector function, indicating that this may represent a novel and highly efficacious strategy for the promotion of an anti-tumor response with either CAR T cells or endogenous patient cells for ACT.

Results

Stimulation and culture of CD8 T cells in low glutamine media decreases proliferation, as well as cell size, and promotes a more memory-like phenotype *in vitro* and *in vivo*

In an attempt to determine in restriction of glutamine metabolism in culture alters the fate decisions of CD8 T cells in a manner it did similar to that of CD4 T cells, transgenic CD8 T cells specific to an OVA peptide presented in MHC class I, OTI, were stimulated with SIINFEKL peptide and cultured in either standard 4mM glutamine media or low glutamine culture media, as previously described. Similar to CD4 T cells, CD8 T cell culture in low glutamine media led to a decrease in CD8 T cell proliferation (data not shown), as well as a significant decrease in the percentage of CD8 T cells positive for the nuclear marker of proliferation Ki67 (**Fig. 15a**). As a reflection of their inability to blast and rapidly proliferate, CD8 T cells cultured in low glutamine media are smaller, as indicated by decreased forward and side scatter upon flow cytometry analysis following 3 days of culture (**Fig. 15b**). However, although the CD8 T cells cultured in low glutamine were fewer in number, they bore a number of phenotypic differences more resembling longer-lived memory CD8 T cells. An increased percentage of CD8 T cells cultured in low glutamine media were positive for both CD44 and CD62L after 3 days of stimulation and culture, a central memory phenotype of long-lived CD8 T cells in response to a variety of infections[128] (**Fig. 15c**). The CD8 T cells cultured in low glutamine also had increased expression of the anti-apoptotic protein BCL-2 (**Fig. 15d**), which suppresses caspase 1 activation by binding to NALP1 thereby reducing IL1 beta production inflammasome activation[129]. Low glutamine culture also significantly reduces expression of PD1 (**Fig. 15e**), a cell surface receptor which is increased upon T cell activation but also acts as a "checkpoint" to inhibit T cell responsiveness, and is frequently highjacked to inhibit T cell responses to cancer[130]. Lastly, CD8 T cells stimulated and cultured in low glutamine media displayed an increased incidence of the inhibitory histone mark H3K27me3, as evidenced by increased MFI as assessed by flow cytometry. Deposition of this inhibitory mark at loci associated with effector function

has been associated with the promotion of a memory response[131]. Taken together, this indicates that CD8 T cells stimulated and cultured in low glutamine media not only undergo decreased proliferation, but also exhibit several phenotypic changes associated with long-lived, central memory CD8 T cells.

Next, we sought to assess the long-term persistence and ability to respond to challenge of CD8 T cells cultured in low glutamine media as compared to CD8 T cells cultured in the standard 4mM glutamine media. Transgenic CD8 T cells specific for the GP33 peptide of lymphocytic choriomeningitis virus (LCMV)[132] were stimulated with cognate peptide presented in the context of whole splenocytes for 3 days, with expansion and addition of exogenous IL2 occurring on the second. On the third day, live CD8 T cells were isolated with density centrifugation, and 0.1 million live CD8 T cells were adoptively transferred to congenically distinct hosts retro-orbitally. Hosts were then infected with the LCMV-Armstrong strain of virus, which induces an acute infection that spontaneously resolves[133]. Serial cheek bleeding of mice during the course of the infection (**Fig. 16a**), as well as after its resolution, indicated that the circulating level of low glutamine cultured CD8 T cells exceeded that of the T cells cultured in the standard 4mM glutamine media (**Fig. 16 b,c,d**). Furthermore, when these cells were assessed for expression of the effector molecule KLRG-1 at 4 weeks, it was found that low glutamine cultured cells had decreased expression of this marker (**Fig. 16e**). Four weeks after the initial challenge, hosts were re-infected with a modified listeria beating the GP33 antigen against which the transferred T cells were specific. Following another 8 days, mice were sacrificed and spleens were harvested to assess recall response from the adoptively transferred CD8 T cells (**Fig. 17a**). Flow cytometry analysis revealed that a significantly higher percentage of adoptively transferred CD8 T cells were present in the spleens of mice that received CD8 T cells that had been cultured in low glutamine media as opposed to those that had been cultured in standard 4mM glutamine media (**Fig. 17b**). Furthermore, when the transferred cell population was analyzed, it was found that a significantly increased percentage of the cells that had initially been cultured in low glutamine media bore a central memory, CD44 positive, CD62L phenotype

(**Fig. 17c**). Lastly, when splenic cells were restimulated with PMA and ionomycin *ex vivo* to assess competency to produce cytokines, and especially to produce multiple different cytokines simultaneously, a property known as polyfunctionality, which is associated with higher quality effector CD8 T cells[134]. When the polyfunctionality of adoptively transferred CD8 T cells raised in either standard 4mM glutamine media or low glutamine media was assessed, it was found that low glutamine media cultured cells were more polyfunctional, with a greater percentage producing IL2, IFN gamma, and tumor necrosis factor (TNF) alpha than the standard 4mM glutamine cultured cells (**Fig. 17d**). This result was extremely striking, as the only perturbation these cells were subjected to was three days of restricted glutamine culture almost two months prior to assay. No treatment of glutamine inhibitor therapy was carried out *in vivo* in the infected mice. This finding demonstrates the robust capacity of metabolic perturbation at the time of initial activation to have dramatic effects of the fate decisions of CD8 T cells, and the powerful ability of metabolism of dramatically control the immune response.

Low glutamine culture is protective from activation-induced cell death following adoptive transfer and stimulation

The finding that cells initially cultured in low glutamine media were more persistent and found at higher percentages when adoptively transferred both in the circulating peripheral blood and in the spleens of host mice was a bit contradictory, in that restriction of glutamine metabolism decreases proliferation. However, we theorized that imparting a more memory-like phenotype by the initial restriction of glutamine metabolism at the time of stimulation might be capable of enhancing cell numbers not by promoting further division, but by enhancing long term cell survival. One key mechanism by which this function may occur is the increased expression of the anti-apoptotic protein BCL-2. The BCL-2 family of genes was initially identified as a regulator of caspase-dependent apoptosis in *C. elegans* models, and family members are localized on the mitochondrial outer membrane. BCL-2 interacts with pro-death members of the family, BAX and BID, to inhibit the actions of these pro-death

proteins and promote cell survival rather than death by apoptosis[129]. As BCL-2 levels were increased in the CD8 T cells cultured for 3 days in low glutamine media as opposed to those cultured in the standard 4mM glutamine media (**Fig. 15d**), we sought to assess the short-term survival following adoptive transfer of CD8 T cells cultured in either standard 4mM glutamine media or low glutamine media. Transgenic CD8 T cells were once again cultured for 3 days in either standard 4mM glutamine media or low glutamine media and stimulated with cognate peptide. Following expansion and addition of IL2 on day 2, and isolation of live cells by Ficoll gradient centrifugation, live CD8 T cells were labelled with the proliferation dye CTV to assess their ability to divide based on the dilution of this dye as determine by flow cytometry. These labelled CD8 T cells were then adoptively transferred retro-orbitally into congenically distinct hosts, and stimulated by infection of the host. Following a short period of activation in the hosts, 2 to 3 days, mice were sacrificed and spleens were harvested for analysis (**Fig. 18a**). Flow cytometry analysis of the spleens indicated that a significantly higher percentage of the live CD8 T cells present after a short period of expansion within an infected mouse were present following transfer with CD8 T cells initially simulated and cultured in low glutamine media as opposed to standard 4mM glutamine media (**Fig. 18b**). Further analysis of these transferred cells indicated that low glutamine cultured CD8 T cells gave rise to a significantly decreased percentage of PD1 positive cells within the transferred population (**Fig. 18c**), indicating a less exhausted phenotype induced by low glutamine stimulation and initial culture.

Next, we sought to gain further insight into the proliferative and survival capacities of transferred CD8 T cells at this early timepoint by assessing their division by CTV dilution. Paradoxically, transferred CD8 T cells that had been stimulated and cultured in low glutamine media displayed a higher mean fluorescence intensity (MFI) of CTV (**Fig. 18d**), indicating that these cells had undergone fewer divisions within the infected host in the 2-3 days following transfer. This data seems incompatible with the finding that there is an increased percentage in transferred cells arising from the low glutamine

media condition (**Fig. 18b**). We thought that increased cell death might be responsible for this discrepancy, in a model whereby the decreased percentage of transferred CD8 T cells persisting from the standard 4mM glutamine media culture conditions is a result increased proliferation, but simultaneously increased death leading to increased cell turnover of phenotypically more exhausted, higher PD1 expressing (**Fig. 18c**) cells as compared to the cells arising from low glutamine culture. In order to assess this, singlet cells withing the lymphocyte gate as drawn on the forward scatter/ side scatter plot were then gated on total CD8 positive, transferred cells (as determined by presence of congenic makers), and this total transferred population was assessed to determine what percentage of it was alive after short term restimulation within the infected host. Significantly more live cells were present in the population that had been transferred from low glutamine culture conditions (**Fig. 18e**) as compared to standard 4mM glutamine culture conditions, supporting the hypothesis that elevated expression of the anti-apoptotic protein BCL-2 (**Fig. 15d**) supports enhanced survival of cells stimulated and cultured in low glutamine media, leading to an increased persistence and more long-lived, central memory phenotype of these cells.

Adoptive cellular therapy with antigen specific CD8 T cells cultured in low glutamine media promotes anti-tumor immunity and survival

Following that findings that stimulation and culture of CD8 T cells in low glutamine media induces a more central memory like phenotype (**Fig. 15**), and upon adoptive transfer induces cells that persist for longer in both the blood (**Fig. 16**) and spleen, as well as are more polyfunctional upon rechallenge (**Fig. 17**), as well as determining that the mechanistic underpinning of this phenomenon is protection from cell death in the low glutamine cultured cells (**Fig. 18**), we next sought to assess the suitability of low glutamine cultured CD8 T cells for use in adoptive cellular therapy against a tumor. Adoptive cellular therapy (ACT) is a technique whereby endogenous cytotoxic T cells specific for an antigen, typically a tumor antigen, can be isolated from a patient, expanded *ex vivo*, and then re-infused

to the patient at increased numbers in order to enhance the patient's endogenous anti-tumor immune response[135]. This technique holds a lot of promise, but is currently held back by its expense, the time required, and the need to optimize *ex vivo* expansion conditions in order to promote the development an optimal anti-tumor cytotoxic cell that is resistant to exhaustion or any of the myriad of other mechanisms that restrain anti-tumor immunity within the tumor microenvironment, such as checkpoint inhibition[136], hypoxia, physical barriers within the tumor stroma, and inhibitory signaling pathways such as adenosine signaling[137], among others. We hypothesized that, based on previous results using DON treatment[124], initial culture in low glutamine media might promote increased anti-tumor activity and persistence of cytotoxic T cells for use in ACT. Transgenic OTI T cells were stimulated in either standard 4mM glutamine media or low glutamine media for 4 days. Male and female C57/BL6 mice were subcutaneously implanted with a B16 melanoma cell line engineered to express the cognate peptide for recognition and stimulation by OTI T cells (B16-OVA), and tumors were allowed to grow for a week to become established, at which time mice were randomized by tumor size, and CD8 T cells cultured in either standard 4mM glutamine media or low glutamine media were adoptively transferred retro-orbitally. Following transfer, tumor size was determined by measurement using calipers three times a week, until tumor size reached 20mm by 20mm, and mice were sacrificed in accordance with IACUC-approved mouse protocol (**Fig. 19a**).

Transfer of antigen specific CD8 T cells cultured in low glutamine media lead to decreased tumor volume until time of first sacrifice (**Fig. 19b**), and when tumor volume was examined on the basis of individual mice, it was once again found that transfer of antigen specific CD8 T cells initially cultured in low glutamine media led to decreased tumor sizes for longer durations prior to outgrowth as compared to transfer of cells cultured in standard 4mM glutamine media (**Fig. 19c**). Survival analysis indicates that transfer of low glutamine cultured CD8 T cells significantly prolonged survival of mice bearing B16-OVA tumors (**Fig. 19d**). However, even mice that received ACT with low glutamine cultured CD8 T cells

eventually succumbed to tumor, indicating that ACT alone was not sufficient to cure tumors in this model.

Discussion

While a previous chapter of this thesis has focused on the role of glutamine metabolism in the cell fate decisions of CD4 T cells, the present work is concerned with the modulation of glutamine metabolism as it relates to cell fate decisions in CD8 T cells. The role of metabolism in promoting a CD8 T cell memory phenotype has been well described in the literature[138] yet strategies to take advantage of these findings still require further development. Herein, we provide a strategy for the promotion of long-lived memory CD8 T cells that still maintain strong effector function: the restriction of glutamine metabolism at the time of primary stimulation and culture. Culture of transgenic CD8 T cells in low glutamine media decreases their proliferative capacity by both numbers and by a decreased percentage of Ki67 positive cells as compared to standard 4mM glutamine culture. However, when these *in vitro* cultured cells were further assessed by flow cytometry, it was discovered that they more resemble long-lived memory CD8 T cells, based on their central memory CD44 positive CD62L positive surface phenotype[128] , as well as their increased expression of the anti-apoptotic protein BCL-2[129], as well as decreased cell surface expression of the checkpoint marker PD1, and their elevated H3K27me3 histone mark (**Fig. 15**). When either standard 4mM glutamine cultured or low glutamine cultured transgenic CD8 T cells were adoptively transferred into congenically distinct host mice and stimulated, it was found that the percentage of circulating CD8 T cells comprised of the transferred population was higher when this transferred population was initially cultured in low glutamine media, even out to day 31 post-transfer (**Fig. 16**). Furthermore, when these cells were resulted four weeks post-transfer by a genetically engineered *Listeria* strain, low glutamine culture lead to an increased cell expansion, and cells that were not only more memory-like by surface phenotype but were also better effectors, as indicated by a higher percentage of polyfunctionality upon *ex vivo* stimulation to assess cytokine production (**Fig. 17**).

This finding is remarkable in that the only perturbation these cells were submitted to was initial stimulation and 3 days of culture in media with restricted glutamine. No *in vivo* treatments were conducted to enhance memory cell formation. And yet, out to over a month later, there are still profound differences in the T cell response between the cells that were initially culture in standard 4mM glutamine media and low glutamine media. This finding demonstrates the robust ability of modulation of metabolism, even for a short duration, to dramatically alter the immune response, and lends support to the idea that further attempts to modulate metabolism, for example in the setting of the anti-tumor response, may be able to promote more robust and durable immunity. Therefore, it would seem logical to consider the possibility of metabolic modulation in other settings in which a durable and long-term response is desirable, such as during vaccination. Although T cell vaccinations strategies lag behind strategies to elicit a robust B cell/ antibody response[134], the possibility of metabolic modulation, such as short-term DON treatment following vaccination in order to promote a more memory-like CD8 T cell response could help advance attempts to induce robust and durable T cell responses to vaccination.

In order to build on our initial findings, we next sought to translate these findings into a murine cancer model. A model of ACT in which transgenic CD8 T cells specific for OVA were initially stimulated and cultured in either standard 4mM glutamine or low glutamine culture media, and then were transferred to mice bearing established B16-OVA melanoma tumors was used. By assessing tumor size and mouse survival, ACT with low glutamine CD8 T cells was found to be superior to ACT with cells raised in standard culture condition (**Fig. 19**). This finding was once again remarkable, in that the only perturbation offered once again was the brief period of culture in low glutamine media, and yet a striking effect was still seen, even though treatment was not carried out in the mice. This raises the possibility of combination therapy alongside this ACT approach, as we have shown that inhibition of glutamine metabolism *in vivo* is a very potent anti-tumor strategy that simultaneously inhibits tumor cell growth and promotes effector T cell responses against the tumor by shifting the hostile TME towards

one more permissive to the immune response[93]. The combination of a more primed ACT therapy along with further metabolic enhancements provided by a systemic therapy that targets both the T cells and the tumor may yield far more positive results for the anti-tumor response.

In an attempt to further define this response, we next altered our ACT model of B16-OVA treatment such that mice were sacrificed at a defined endpoint and tumors were dissected and submitted to flow cytometry analysis in order to analyze the TIL following ACT from transgenic CD8 T cells raised in standard 4mM glutamine conditions or low glutamine media conditions. Strikingly, at this early timepoint following ACT, tumor sizes were actually slightly larger in the mice treated with cells raised in low glutamine media as opposed to standard glutamine culture, and there was a significant difference in the percentage of TIL comprised of transferred cells, with fewer coming from low glutamine ACT that standard media (data not shown). This initially seems at odds with the enhancement in tumor control and survival seen from ACT with low glutamine cultured cells in previous experiments, but these differences offer important insight into how low glutamine stimulation and culture alters the anti-tumor response. It is possible that low glutamine culture prior to adoptive transfer leads to CD8 T cells that are initially less proliferative, as the gap between standard culture and low glutamine culture in terms of contribution to circulating CD8 T cell population as assessed by serial cheek bleeding seems to widen over time (**Fig. 16**). This slight "headstart" of the standard 4mM glutamine cultured cells may partially explain the results of the TIL analysis. However, the decreased initial infiltration of low glutamine cultured ACT at the tumor site may be balanced by the increased survivability of these cells, as short term adoptive transfer experiments demonstrate that, although low glutamine cultured cells undergo fewer rounds of proliferation immediately following transfer, as evidenced by increased CTV MFI, they also survive better, as significantly more of the transferred cell population was alive following short term activation in the mouse when this transferred population was derived from low glutamine cultured cells as opposed to culture in standard 4mM glutamine conditions

(**Fig. 18**). This survivability advantage, potentially conveyed by increased expression of BCL2 in CD8 T cells cultured in low glutamine media (**Fig. 15d**) may explain the differences between the TIL analysis and the longer-term tumor survival experiments. These differences between the immediate cytotoxic advantage of the standard 4mM glutamine cultured cells for ACT and the persistence and memory-like phenotype of the low glutamine cultured cells suggests that combination therapy, where the infusion is split between both populations in order to maximize benefits, may be an important consideration for the metabolic optimization of ACT in the tumor setting.

Current ACT strategies that rely on large infusions of patient cells cultured *ex vivo* are highly resource and labor intensive, involving a great investment of time in order to produce a product for reinfusion[139]. This, coupled with the limited efficacy of these therapies, leaves a great deal to be desired, however the use of metabolism for the promotion of a more persistent cytotoxic cell to be used in ACT is an area with currently-untapped potential. Based on the findings herein, culture and expansion of patient cytotoxic T cells in glutamine restricted media could be advantageous for the promotion of a more memory-like phenotype. This potential benefit would have to be weighed against the fact that culture in low glutamine media leads to decreased proliferation, and thus additional time or input material would be required in order to achieve sufficient infusion populations. However, the previously described strategy of combining cells cultured in standard glutamine media and low glutamine media might provide the best of both words, as 4mM glutamine cultured cells can be more effector-like and help to exert a rapid (if transient) tumor control, while durable response is reinforced by the more memory-like low glutamine cultured cells. These approaches could also be combined with the use of *in vivo* therapies, both with standard checkpoint blockade such as PD1, or with further metabolic therapy, as JHU-083 mediated inhibition of glutamine metabolism in mouse tumor models has already been demonstrated by our lab to have a multi-pronged impact on the anti-tumor immune response by both

inhibiting metabolism of the tumor and thereby alleviating the metabolic restrictions within the TME, and promoting a more robust anti-tumor T cell response[93].

In conclusion, we have demonstrated that short term *in vitro* culture of CD8 T cells in glutamine restricted conditions, while decreasing the proliferation of these cells, is sufficient to induce a more memory-like phenotype, and that adoptive transfer of these cells demonstrates that they are better able to persist in circulation *in vivo*, and upon re-challenge are better able to expand and are more polyfunctional than cells from standard 4mM glutamine culture. This gives rise to a cell population that is better equipped to mount an anti-tumor immune response, and although initial infiltration of the tumor is decreased, the persistence survival of these cells is able to overcome this. The use of low glutamine culture could therefore be important from a T cell engineering standpoint for the generation of a more persistent cell population for ACT, as well as the development of vaccination strategies that are able to promote a long-lived T cell response. These findings further demonstrate the crucial role that metabolism plays on T cell fate decisions within the immune system, and how the shifting the balance of metabolism can impact these decisions to promote a more desirable T cell response.

Materials and Methods

Mice

Six to eight-week-old male of female mice were used for performing all experiments in this study. Rag knockout mice bearing a transgenic T cell receptor recognizing a peptide from OVA (OTI) were obtained from Jackson Laboratories and maintained by breeding in house. Rag knockout mice bearing a transgenic T cell receptor recognizing a the Gp33 peptide from LCMV (P14) were a gift from Dr. David Hildeman at the University of Cincinnati and were maintained by breeding in house. Congenically distinct C57/BL6 mice that served as hosts for adoptive transfer experiments were obtained from Jackson Laboratories and maintained by breeding in house. All mouse procedures were carried out in

accordance with a protocol approved by the Johns Hopkins University Institutional Animal Care and Use Committee. No empirical test was performed for choosing sample size prior to experiments. No randomization of samples or animals was used nor were investigators blinded throughout the study.

Low glutamine culture

For low glutamine culture experiments, naïve transgenic T cells specific for the SIINFEKL peptide presented in the context of MHC class I on a RAG knockout background, known as OTI, were obtained by sacrificing a mouse and collecting spleen and lymph nodes. Organs were macerated by passing through a 70uM filter, red blood cells were lysed by treatment with ACK lysis buffer, and cells were counted. Cells were stimulated by addition of peptide at a concentration of 5uM in either standard 4mM glutamine culture, or using the low glutamine culture media as outlined in chapter 2 of this thesis. Every other day, cultures were expanded and exogenous IL2 was added to account for this expanded volume.

Flow cytometry staining

Cells were counted, and 2 to 3 million were plated in round bottom tubes. For tissue samples, FC receptors were blocked with anti-CD16/CD32 (Mouse BD Fc Block) (2.4G2, #553142), for 20 minutes at 4C. Surface staining was performed at 4C for 20 minutes in the presence of eBioscience Fixable Viability Dye eFluor 780 (65-0865-14). For intracellular staining, cells were fixed and permeabilized overnight at 4C with eBioscience Fixation/Permeabilization set (88-8824-00). For intracellular cytokine staining, cells were fixed in BD Fixation Permeabilization solution kit (554714) for 20 minutes at room temperature. Intracellular staining on fixed and permeabilized cells was performed for 1 hour at room temperature. All experiments were performed on a BD Celesta. Gates were determined by using unstimulated controls or isotype controls where appropriate. Data was analyzed using FlowJo software.

Adoptive transfer

Transgenic CD8 T cells specific for a peptide from LCMV presented by MHC class I on a *Rag2* knockout background (P14) were obtained by sacrificing mouse, dissecting spleen, mashing through 70uM strainer, and lysing red blood cells with ACK lysis buffer. Cells were washed, counted, and plated in either standard 4mM glutamine media or low glutamine media. Cells were stimulated with 100ng GP33 peptide. Two days after initial stimulation, cells were expanded and exogenous IL2 was added. On the third day, live cells were isolated by Ficoll density centrifugation, washed 3 times, and counted. One hundred thousand cells were transferred retro-orbitally in 200uL HBSS into congenically distinct hosts, and hosts were infected with LCMV-Armstrong IP. At one-week intervals, circulating CD8 T cells were assessed by cheek bleeding. A lancet was used to pierce the skin of the cheek, and a small volume of blood was collected into a 1.5mL tube containing a 1:1 mixture of RPMI media plus antibiotics and 4% sodium citrate. Tubes were centrifuged, and red blood cells were lysed with ACK lysis buffer, and cells were stained and run on BD FACS Calibur. For "early" harvest, mice were sacrificed 8 days following transfer, and spleens and dissected out. Spleens were mashed through 70uM strainer and red blood cells were lysed with ACK lysis buffer. Cells were counted, and 2e6 were submitted to surface staining as previously described. For *ex vivo* restimulation to assess cytokine production, 2e6 cells were plated in standard 4mM glutamine culture media, and stimulated with 1ug GP33 peptide plus GolgiStop for 4 hours at 37C. Cells were stained for flow cytometry as previously described. For long term memory re-challenge experiments, 1 month post-transfer, hosts were re-stimulated with Listeria strain 1195 bearing the GP33 antigen via retro-orbital injection. Eight days after re-stimulation, mice were sacrificed and spleens were dissected out. Spleens were mashed through 70uM strainer and red blood cells were lysed with ACK lysis buffer. Cells were counted, and 2e6 were submitted to surface staining as previously described. For ex vivo restimulation to assess cytokine production, 2e6 cells were plated in standard 4mM glutamine culture media, and stimulated with 1ug GP33 peptide plus GolgiStop for 4 hours at 37C. Cells were stained for flow cytometry as previously described.

Short term transfer model

OTI cells were stimulated as described previously with peptide, and expanded on the second day with the addition of exogenous IL2. On the third day of culture live cells were isolated by Ficoll density centrifugation, washed, and counted. Cells were labelled with CTV in warm PBS at a 1:1000 dilution at a concentration of 10e6/mL for 10 minutes at 37C, following which staining was quenched with the addition of cold media and incubation on ice for 5 minutes. One million cells were transferred to congenically distinct hosts in 200uL HBSS via retro-orbital injection. Hosts were stimulated with IP injection of Vaccinia-OVA. After 48 hours, mice were sacrificed and spleens were harvested. Spleens were mashed through 70uM strainer and red blood cells were lysed with ACK lysis buffer. Cells were counted, and 2e6 were submitted to surface staining as previously described.

Tumor model

B16 melanoma tumors genetically engineered to express OVA were cultured in the presence of G418 selection media to preserve transgene expression, as well as ciprofloxacin for the first two passages following recovery from liquid nitrogen storage. Cells were passaged to prevent overgrowth, and allowed at least 7-10 days in culture following recovery prior to implantation in C57BL/6 mice. Two days prior to implantation, cells were split in order to ensure log phase growth at time of implantation. The day before implantation, mice were shaved by the use of electric clippers on the right flank to better visualize injection site. On the day of injection, B16-OVA cells were trypsinized, washed at least three times in phosphate buffered saline (PBS), and counted so that 200,000 cells were injected subcutaneously in 200uL of Hanks' balanced salt solution (HBSS). Seven days after tumor injection, mice were randomized by tumor size, and received adoptive cellular therapy via retro-orbital injection. Tumor sizes were measured by use of calipers three times a week, and mice were sacrificed when tumor

size reached 20cm by 20cm in accordance with the protocol approved by the Johns Hopkins Animal Care and Use Committee.

Statistical Analysis

All graphs were creased using GraphPad Prism software, and statistical analysis was performed with GraphPad Prism. A p-value less than 0.05 was considered statistically significant; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$. Error bars represent mean ± standard error of the mean.

Figures

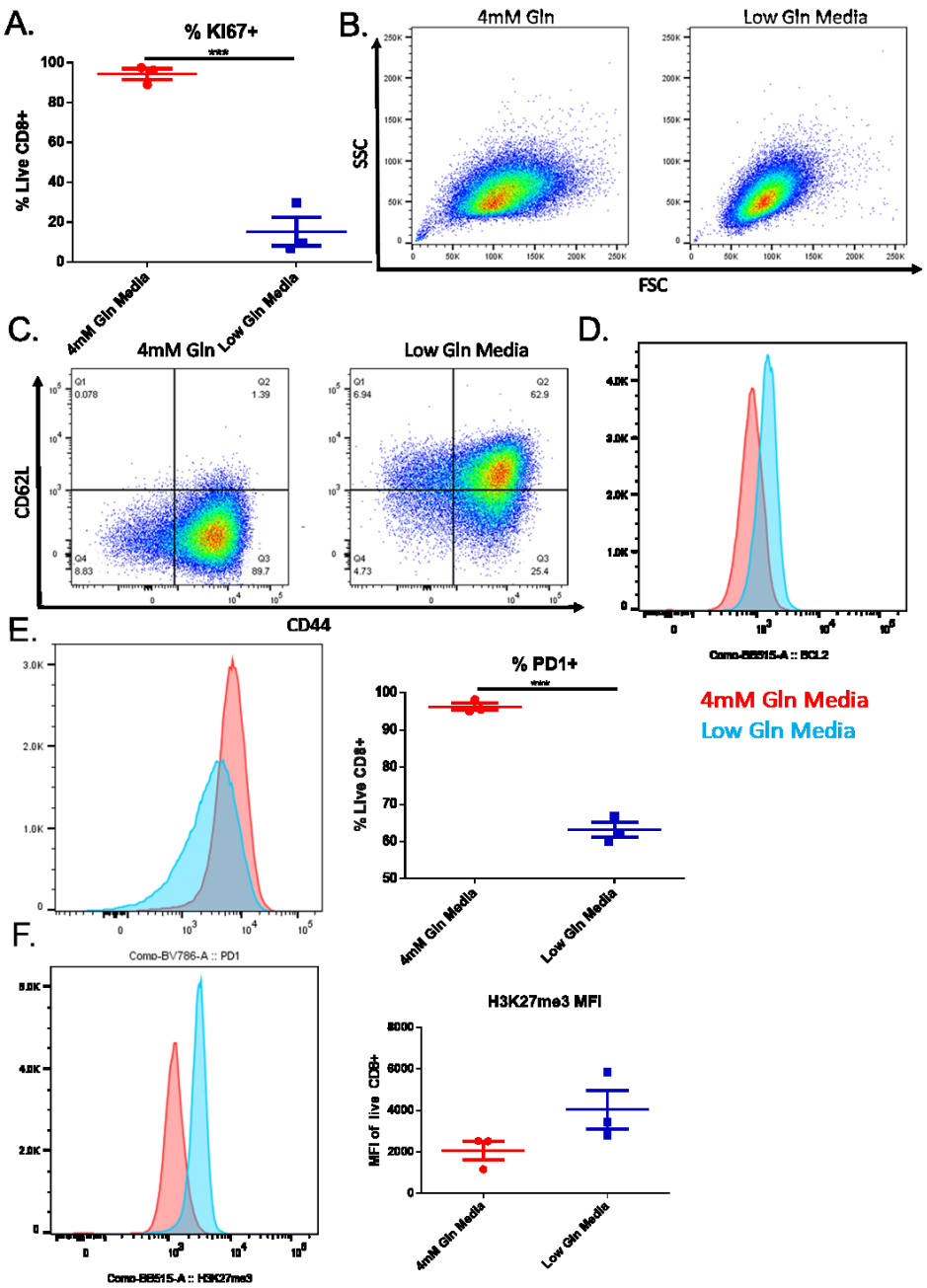

Figure 15. Culture of CD8 T cell with low glutamine decreases T cell size, and increases memory-like phenotype

Figure 15. Culture of CD8 T cells with low glutamine decreases size, and increases memory-like phenotype. **(a)** Ki67 expression of CD8 T cells cultured in standard 4mM Gln or low Gln media as assessed by flow cytometry. **(b)** CD8 T cells size as indicated by forward and side scatter. **(c)** CD44 and CD62L expression as assessed by flow cytometry. **(d)** BLC2 expression as assessed indicated by flow cytometry histogram. **(e)** PD1 expression as indicated by histogram (left) and summary graph (right). **(f)** as in (e) but with histone H3K27me3 staining

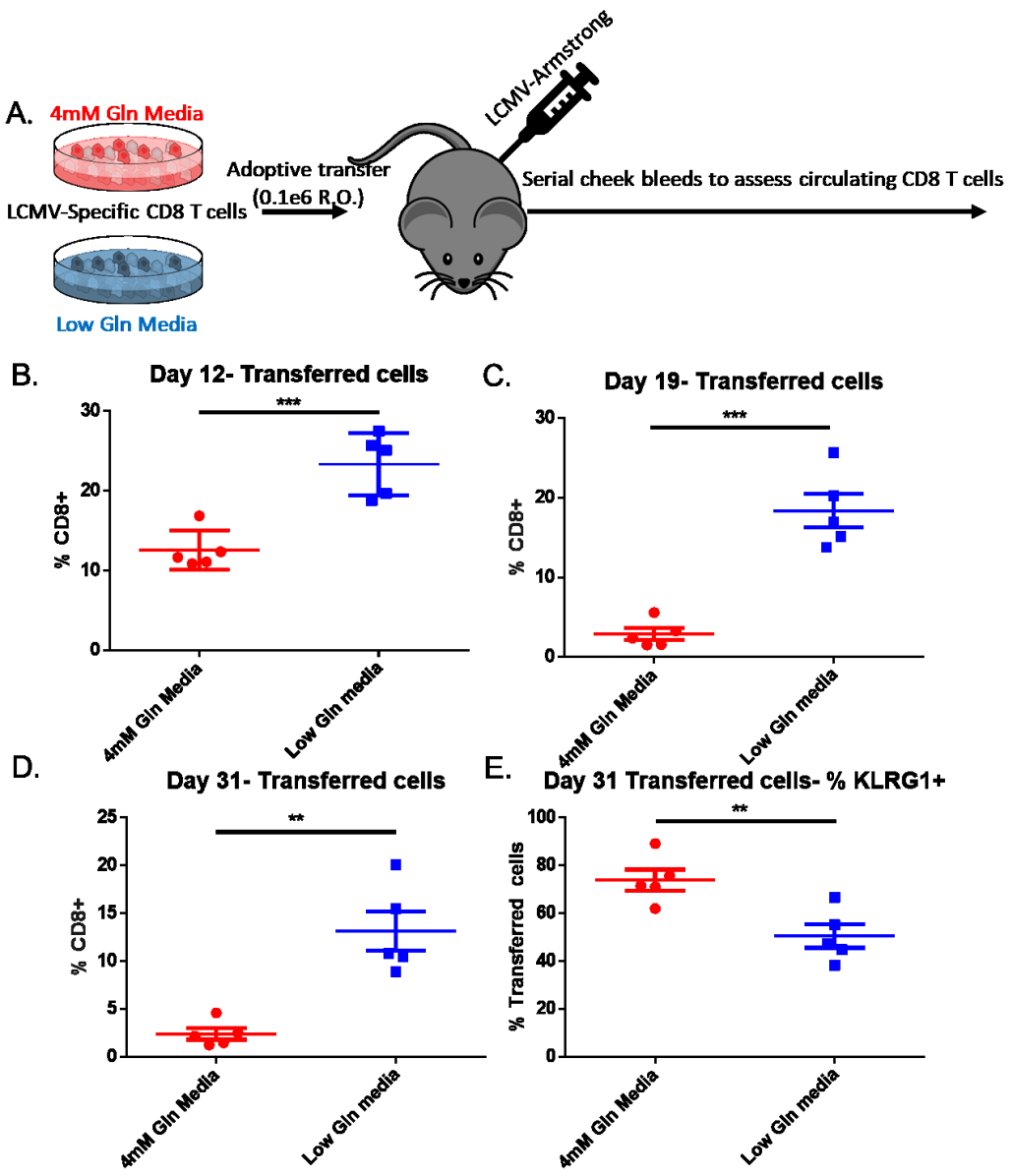

Figure 16. Adoptive transfer of CD8 T cells cultured in low glutamine media promotes a more persistent cell as assessed by serial cheek bleeding

Figure 16. Adoptive transfer of CD8 T cells cultured in low glutamine media promotes a more persistent cell as assessed by serial cheek bleeding. **(a)** experimental outline. **(b)** peripheral blood levels of transferred CD8 T cells as assessed by cheek bleeding on day 12. **(c)** as in (b) but on day 19. **(d)** as in (b) but on day 31. **(e)** KLRG1 expression on cells from (d) as assessed by flow cytometry

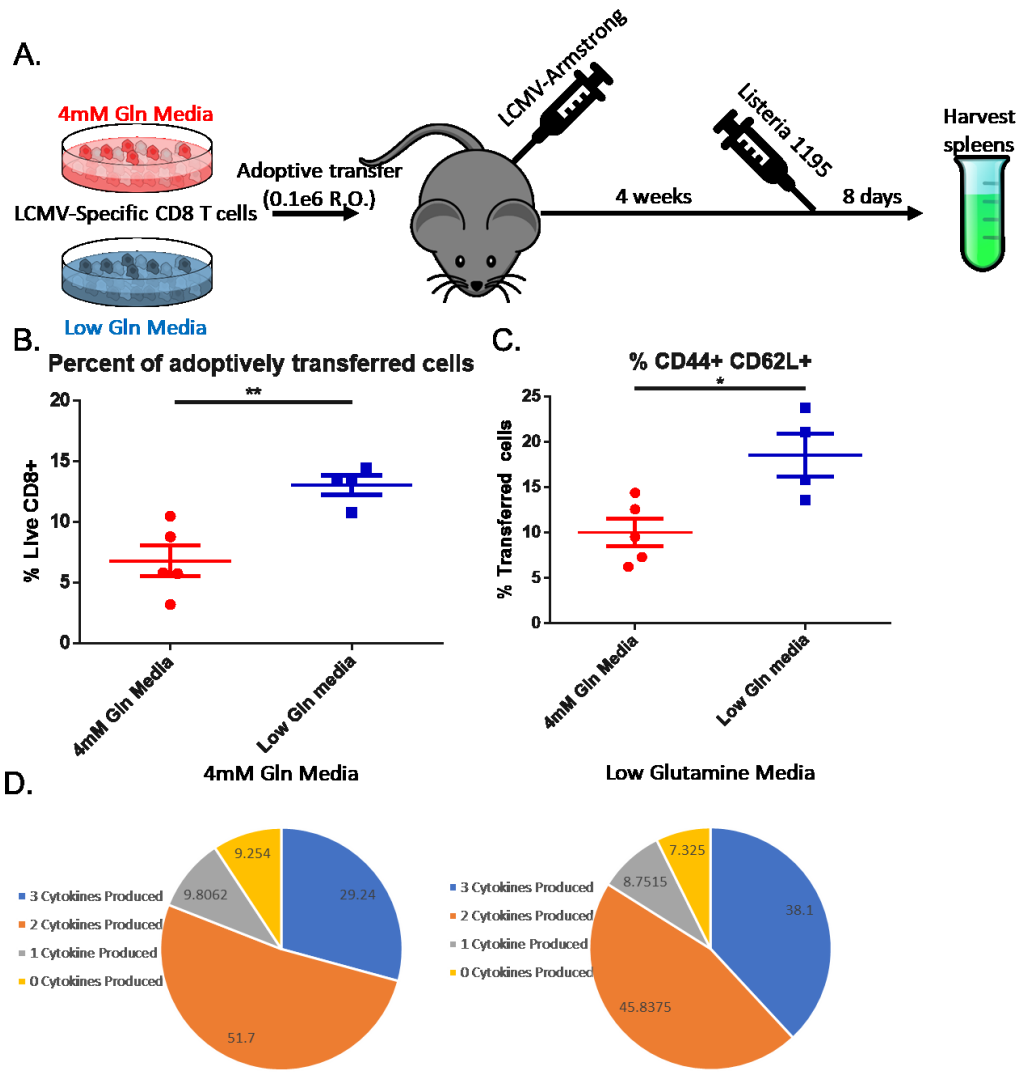

Figure 17. Adoptive transfer of CD8 T cells cultured in low glutamine media promotes a more persistent cell that is also highly active upon re-challenge

Figure 17. Adoptive transfer of CD8 T cells cultured in low glutamine media promotes a more persistent cell that is also highly active upon re-challenge. **(a)** experimental outline. **(b)** percent of transferred cells in the spleen following listeria re-challenge. **(c)** percent of transferred cells bearing CD44+ CD62L phenotype. **(d)** assessment of cytokine polyfunctionality upon *ex vivo* restimulation as assessed by flow cytometry.

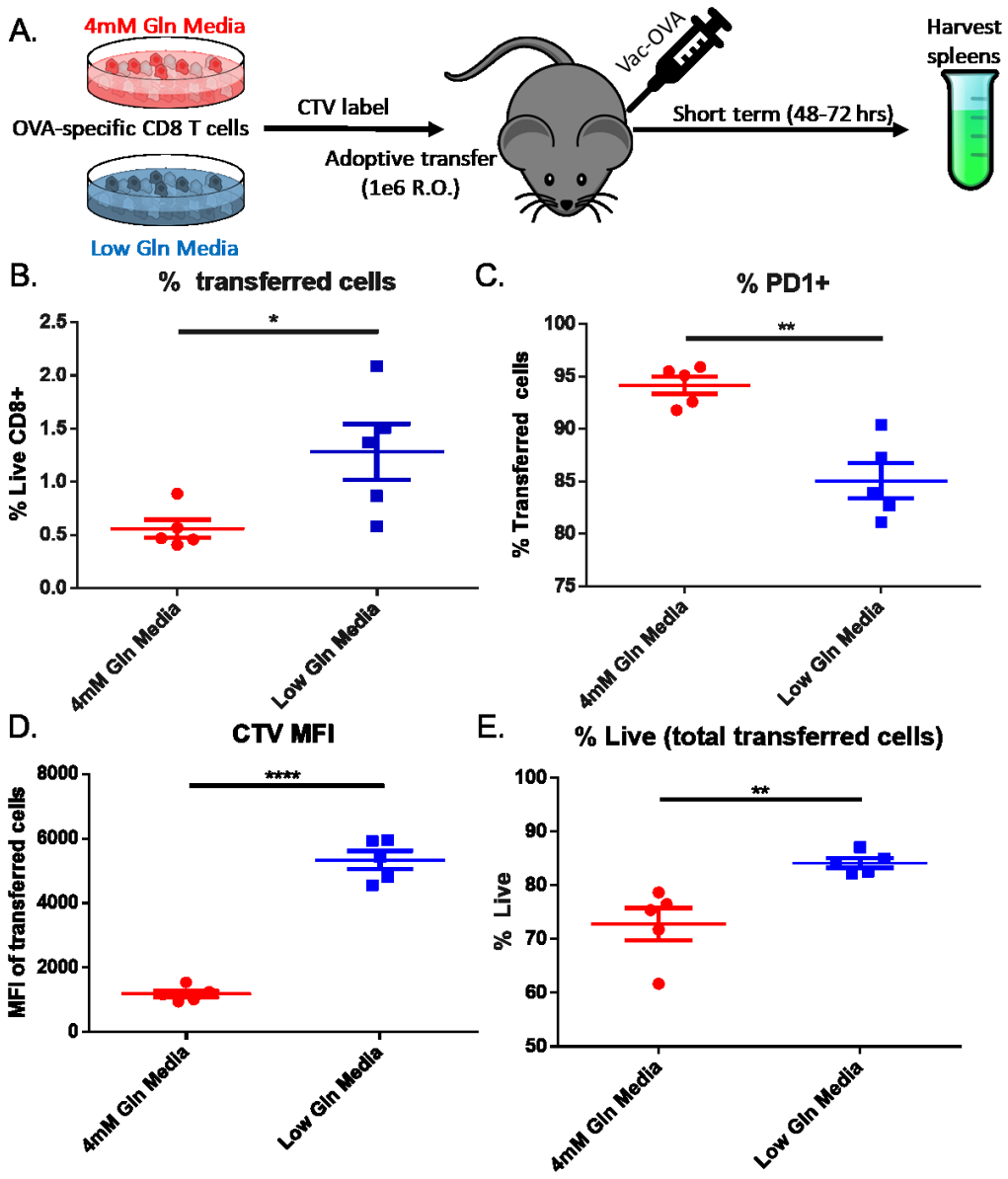

Figure 18. Low glutamine culture promotes cells with decreased sensitivity to activation-induced cell death upon adoptive transfer and stimulation

Figure 18. Low glutamine culture promotes cells with decreased sensitivity to activation-induced cell death upon adoptive transfer and stimulation. **(a)** experimental outline. **(b)** percent of transferred cells in the spleen following Vac-OVA stimulation. **(c)** percent of transferred cells expressing PD1 as assessed by flow cytometry. **(d)** as in (c) but for assessment of CTV MFI. **(e)** as in (c) but assessment of percent live cells from the total transferred population

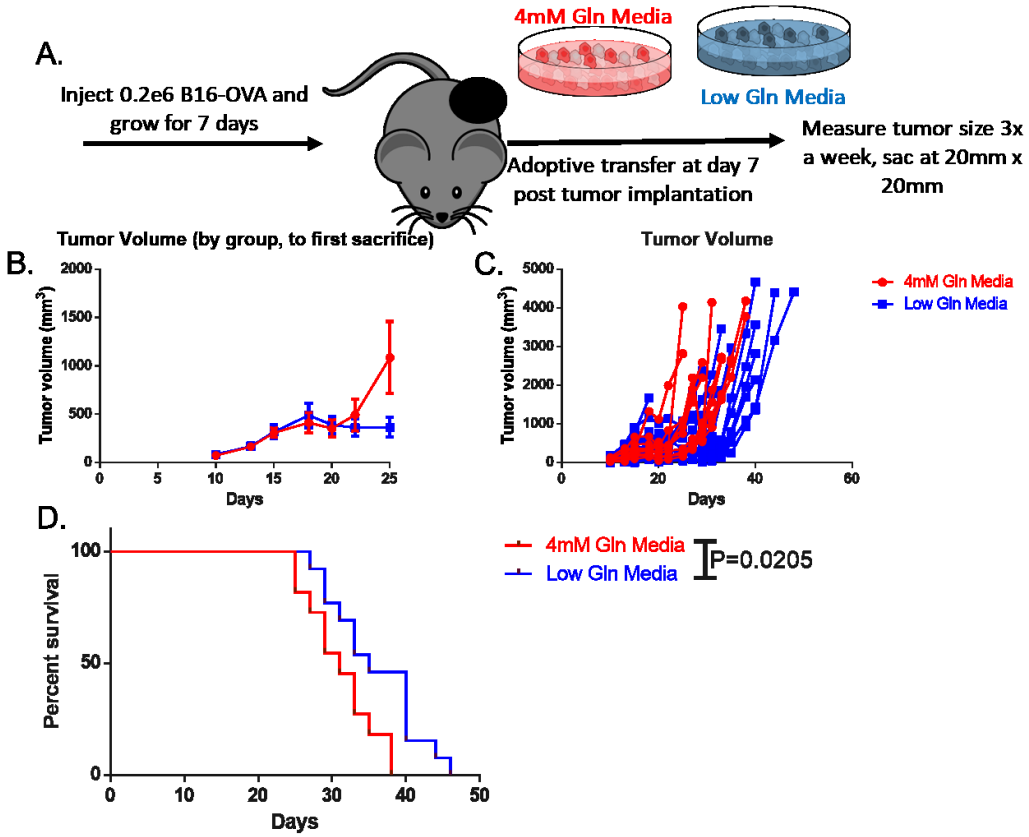

Figure 19. Adoptive cellular therapy with cells cultured in low glutamine media promotes anti-tumor immunity

Figure 19. Adoptive cellular therapy with cells cultured in low glutamine media promotes anti-tumor immunity. **(a)** experimental outline. **(b)** tumor size as assessed but measurement with calipers until time of first sacrifice. **(c)** spider plots of tumor size for individual mice. **(d)** survival plot of tumor bearing mice over time.

Chapter 4: Leucine and its role in T cell activation, as demonstrated by the targeting of leucine transport by cell-specific cytotoxic agents in viral and transplant models

Abstract

Leucine uptake through the heterodimeric large neutral amino acid transporter comprised of LAT1 and CD98 is a rapid and essential aspect of the metabolic modulation that occurs upon T cell activation. Leucine uptake permits the activation of mTORC1 signaling, which is a powerful determinant of both CD4 and CD8 T cell responses. Inhibition of leucine uptake with the highly LAT1-specific inhibitor JPH-203 *in vitro* inhibits proliferation, but also alters CD4 skewing, leading to the production of Th2 cytokines in cells polarized under Th1 conditions. *In vivo* examination of LAT1 expression indicates that, in accordance with previous research, it is robustly upregulated upon T cell activation. In models of pathogenic T cell activation such as GvHD and skin transplant, LAT1 expression is also upregulated, and targeting of pathogenically activated cells with a LAT1 specific cytotoxic drug decreases T cell activation and enhances skin graft survival in the skin transplant model. This finding identifies leucine uptake and metabolism as facilitated by LAT1 as a key controller of an immune response, and a clinically translatable target in transplant rejection systems which merits further follow-up, and further illustrates the powerful role of metabolism in dynamically shaping the immune response in a variety of settings.

Introduction

While the role of the amino acid glutamine in both CD4 and CD8 T cell activation has been discussed at length, it is not the only amino acid that plays an important role in the metabolic alterations that accompany T cell activation. Leucine, an essential amino acid, is crucial for these processes[140]. Leucine is taken up through the combination of SLC7A5, otherwise known as LAT1, and the CD98 heavy chain, also referred to as 4F2hc, and encoded by *Slc3A2*. This heterodimer forms a large neutral amino acid transporter capable of transporting leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine[141]. However, its role in leucine transport is of primary interest in the regulation of immune responses. As previously described, the mTOR signaling play crucial roles in the regulation of immune responses of both CD4 and CD8 T cells, with mTORC1 signaling being essential for the development of Th1 and Th17 responses, and mTORC2 required for Th2 responses in CD4 T cells. In CD8 T cells, mTORC1 signaling promotes a robust effector response, and its inhibition with rapamycin promoting a memory cell phenotype[26]. Amino acids, and particularly leucine, are essential for proper localization and activation of mTORC1. Leucine availability acts as an upstream signal above the Rag heterodimer proteins to induce the localization of mTOR to the lysosome, thus promoting its activation in an amino acid dependent manner[142]. Curiously, as a means of typing previous studies on the amino acid glutamine, it has been demonstrated that leucine uptake through LAT1 is dependent on cellular availability of glutamine, as the transporter is an antiporter which effluxes glutamine in exchange for leucine. Therefore, glutamine starvation has been shown to restrict mTORC1 activation (as assessed by p-S6 level) in a concentration dependent manner, with full S6 phosphorylation requiring at least 1mM glutamine in the media[143].

LAT1 expression is at undetectably low levels in unstimulated cells, but it is upregulated in an AP-1 and NFAT dependent manner rapidly following activation[144]. Work by Sinclair and colleagues demonstrated the essential role of leucine transport mediated by LAT1 in the mounting of a T cell

response[145]. Initial observations indicated that TCR stim lead to, in short order, rapid increase of amino acid uptake through System L transporters. Microarray analysis determined that upon activation a number of Slc family genes are upregulated within 4 hours, but LAT1 is among the most highly induced, with high levels of transcript at the 4 and 20-hour timepoints, and protein expression at 20 hours following TCR stimulation. Furthermore, as previously described, this process was dependent on downstream TCR signaling pathways, as treatment with the calcium signaling inhibitor Cyclosporin A inhibited *Slc7a5* mRNA expression. As a further demonstration of the essential nature of this pathway, T cell specific knockouts of LAT1 demonstrated decreased skewing to both the Th1 and Th17 subsets, with no effect on Treg generation, while a decreased generation of CD8 cytotoxic cells was noted. Upon adoptive transfer and stimulation, LAT1 knockout cells were less capable of expansion than wildtype cells. LAT1 knockout cells were also smaller and less proliferative following TCR stimulation, and produced less IFN gamma, although activation marker expression of CD25, CD44, and CD69 were equivalent. LAT1 is also essential for metabolic reprogramming upon activation, as LAT1 knockout T cells decrease their glycolysis, as evidence by decreased lactate production and less glucose uptake, as well as decreased glutamine uptake. In sum, expression of LAT1 is essential for the metabolic changes required for increased proliferation and cytokine production upon activation, and its rapid and TCR-dependent upregulation helps facilitate full T cell activation and responsiveness[145]. Furthermore, this expression of LAT1 on activated T cells may play an important role in fate decisions. Two models of CD8 T cell fate as being influence by asymmetric inheritance of factors between stimulated daughter cells (one in which mTORC1 activity is high in the daughter destined for effector fate[67], and one in which Myc is the elevated factor[146]), amino acid metabolism, as indicated by increased expression of the heterodimer partner of LAT1, CD98, was found to be upregulated on the more metabolically active (mTORC1 high or Myc high) cell, and that inhibition of amino acid metabolism either through treatment with the amino acid uptake inhibitor 2-amino-bicyclo[2.2.1]heptane-2-carboxylic acid (BCH) or by DON

treatment was sufficient to inhibit the asymmetric division[67,146]. One of the potential roles ascribed to CD98 in these systems, as part of its role in cell adhesion by interacting with ICAM-1, is helping to polarize the synapse upon activation[147].

Inhibition of LAT1 has been seen as a promising strategy in a number of settings. Owing to its important in amino acid uptake, and drawing another parallel between T cell metabolism and cancer metabolism, LAT1 is frequently upregulated on cancers. This makes its inhibition a promising tumor therapy target. Assessment of LAT1 expression in human thyroid cancer with data obtained from The Cancer Genome Atlas (TCGA) demonstrated an association between LAT1 expression and duration of patient survival, indicating that high *Slc7A5* expression was associated with decreased survival[148]. A number of inhibitors of amino acid transport through the LAT family have been developed, including the previously-mentioned BCH. However, many of these inhibitors lack specificity for LAT1 alone, and have off-target effects, especially at higher concentrations. This has fueled interest in the development of a more LAT1-specific drug. Work on a highly specific LAT1 inhibitor, JPH-203 (also known as KYT-0353) has yielded impressive results in both human and murine systems. JPH-203 treatment has been shown to robustly decrease leucine uptake in a variety of cell lines, leading to inhibited mTORC1 activity, decreased cancer cell proliferation, and inhibited tumor growth[149-151]. The inhibition of growth has been demonstrated in both human xenograft models in which tumors are implanted into athymic nude mice, as well as mouse models with an intact immune system, demonstrating that LAT1 inhibition (similar to glutamine inhibition demonstrated by Leone and colleagues with JHU-083) has effects on both the cancer cells, as well as the responding immune cells. The role that LAT1 inhibition with JPH-203 plays on T cells was indicated by studies in human CD4 T cells by Hayashi and colleagues, in which they demonstrated decreased cytokine production as assayed by media ELISA following 3 days of stimulation in the presence of 1uM JPH-203. Using a siRNA knockdown, these researchers also demonstrated that genetic perturbation of LAT1 lead to decreased leucine uptake and cytokine production[144] .

The finding that LAT1 inhibition with JPH-203 can inhibit a T cell response has been exploited in several systems in which a pathogenic T cell response leads to disease in the host. First, work by Liu and colleagues identified the role of CD98, the partner of LAT1 for transport, in a cardiac transplant model[152]. In this system, it was found that a T cell specific knockout of CD98 lead to a decreased alloresponse in mixed lymphocyte reaction (MLR) tests, and that transplant of a cardiac allograft into T cell CD98 knockout mice lead to increased graft survival. These researchers identified fewer graft infiltrating lymphocytes in the knockout mice, as well as increased Treg production. Furthermore, treatment of wildtype mice with an anti-CD98 antibody following transplant was also found to significantly increase graft survival[152]. These results were followed up by work from Nishio and colleagues, who demonstrated decreased GvHD and enhanced survival following BMT from CD98 deficient donors[153]. These researchers also identified an increased percentage of Tregs, as indicated by FoxP3 expressing CD4 T cells, and proposed this as a mechanism for decreased disease. More recently, two letters to the editor have used leucine transplant inhibition with JPH-203, rather than ablation, in models of allergic skin inflammation and T cell mediated nasal hyperresponsiveness as induced by an OVA-alum and adoptive transfer model, thereby demonstrating the role of leucine transport in T cell responses as well as for the proliferation of cancer cells[154,155].

In this work, we will expand on these previous studies showing the importance of leucine transport in T cells first by *in vitro* use of JPH-203 to demonstrate the alteration of the a CD4 T cell response. Inhibition of leucine metabolism is found to not shut down the response entirely, but rather redirect cells activated in Th1 skewing conditions towards a Th2 phenotype, with the acquisition of IL4 and IL13 production. In an attempt to assess the *in vivo* suitability of LAT1 as a biomarker of activated T cells, we confirm that LAT1 expression increases in a model of viral activation. Furthermore, we determined that LAT1 expression is also increased in transplant models of BMT inducing GvHD as well as skin transplant, and treatment with a LAT1-specific cytotoxic agent decreases T cell activation and

enhances graft survival in the skin transplant model. This promotes the idea that LAT1 inhibition or targeted deletion of LAT1 expressing cells could be a powerful metabolic modulator for conditions in which an inflammatory immune response is undesirable.

Results

In vitro studies utilizing JPH-203, a highly selective LAT1 inhibitor, indicate decreased T cell proliferation and CD4 T cell skewing to the Th1 subset

Based on the previously described role of leucine in T cell activation and particularly the requirement of leucine for mTORC1 activation[142], coupled with the advantages of JPH-203 over previous generations of leucine inhibitors,[150] we first sought to assess the role of leucine metabolism in initial T cell activation and proliferation. Transgenic 5CC7 CD4 T cells or transgenic OTI CD8 T cells were labelled with the cell proliferation dye CTV, and stimulated with their cognate peptide in the context of whole splenocyte culture. Into this culture either JPH-203 in dimethyl sulfoxide (DMSO) vehicle at indicated concentration, or a vehicle control (DMSO equivalent to the vehicle present at the highest drug concentration) was added. Following 2 days of stimulation, cultures were expanded, and supplemented with IL2 along with fresh drug (or vehicle) to the concentration of the new total volume. On day 3, cells were collected, fixed, and stained for flow cytometry (**Fig. 20a**). Analysis of CTV dilution indicates that high doses (for example, 10uM) of JPH-203 robustly decreased the proliferation of T cells, as depicted by results from the treated 5CC7 CD4 T cells (**Fig. 20b**). The lowest concentration of drug at which proliferation appeared to be inhibited was found to be 1uM in both the transgenic CD4 T cells (**Fig. 20b**) as well as the CD8 T cells (**Fig. 20c**). Flow cytometry assessment for the nuclear marker of proliferation Ki67 in CD4 T cells treated with JPH-203 indicated that high doses of drug (10uM to 4uM) robustly decreased this marker, while lower doses (500nM to 1nM) displayed little change as compared to vehicle (**Fig. 20d**). The lowest dose at which proliferation defect was observed in CTV labelling

experiments, 1uM (**Fig. 20b**) displayed some attenuation of Ki67 expression but not nearly as dramatic as higher doses. Experiments using the LAT1-specific inhibitor of leucine uptake JPH-203 clearly demonstrate that this drug is capable of inhibiting the proliferation of both CD4 and CD8 T cells.

Next, we sought to assess the effect of JPH-203 treatment on T cell activation. Examining 2 makers of T cell activation in transgenic CD4 T cells, CD44 (**Fig. 20e**) and CD69 (**Fig. 20f**) indicated that, even at high doses at which T cell proliferation is robustly inhibited and even drug-induced cytotoxicity is present (data not shown), there is still no substantial decrease in either CD44 or CD69 expression induced by inhibition of leucine metabolism with JPH-203 treatment. This parallels earlier experiments with glutamine metabolism inhibition *in vitro*, in which proliferation was robustly decreased with either culture in low glutamine media or treatment with DON but T cell activation as assessed by CD44 and CD69 staining was unaffected. These results clearly indicate that inhibition of leucine metabolism *in vitro* robustly inhibits the proliferation of CD4 and CD8 T cells, while not affecting their activation.

Previous research has demonstrated the requirement for leucine in the initiation of mTORC1 signaling[142]. Based on this, we sought to determine the effects of JPH-203 treatment on downstream phosphorylation of substates of both the mTORC1 and mTORC2 kinase complexes. Contrary to expectations, acute treatment with 1uM JPH-203, carried out by a 30-minute pretreatment prior to stimulation and a 60-minute time course, did not alter mTOR signaling (**Fig. 21a**). However, this is likely due to the fact that JPH-203 is a competitive inhibitor, and short-term exposure to the inhibitor in the context of media in which leucine is at a relatively high concentration is insufficient to display an effect. However, when Th1 cells were skewed for 6 days in the presence or absence of JPH-203, and mTOR signaling was probed by Western blot, there was a decrease noted in mTORC1 pathway signaling in Th1 cells cultured in the presence of 1uM JPH-203, indicated by decreased p-S6 and p-S6K1, as well as an increase in mTORC2 pathway signaling, as indicated by an increase in p-FoxO1 (**Fig. 21b**). Therefore, while acute treatment with a low dose of JPH-203 is not sufficient to inhibit mTORC1 signaling,

prolonged exposure to the leucine transport inhibitor appears sufficient to decrease mTORC1 and increase mTORC2 signaling activity in developing Th1 cells, potentially demonstrating a role for JPH-203 in CD4 T cell subset commitment.

The Powell lab has previously carried out research indicating the differential requirement for mTORC complex signaling in CD4 T cell subset skewing[31]. In brief, this data indicates that mTORC1 signaling is required for skewing to the Th1 and Th17 subsets, while mTORC2 signaling is required for Th2 subset commitment. In the absence of signals from either complex, CD4 T cells will polarize towards a regulatory T cell (Treg) phenotype[26]. Based on the observation of differential mTORC1 and mTORC2 signaling effects following prolonged treatment with JPH-203, we sought to assess the role of leucine metabolism in CD4 T cell subset skewing. Transgenic 5CC7 CD4 T cells 5CC7 were stimulated with PCC peptide in the context of whole splenocytes, and were polarized towards either the Th1 or Th2 subset with the addition of cytokines or cytokine blocking antibodies. For Th1 subset polarization, IFN gamma and IL12 were added, as well as an anti-IL4 antibody. For Th2 skewing, IL4 cytokine was added along with anti-IFN gamma and anti-IL12 antibodies. Cultures were also treated with 1uM JPH-203, the smallest dose of drug found to have an effect on proliferation (**Fig. 20b**). Cultures were expanded with the addition of fresh media and IL2 every other day, as well as the addition of fresh JPH-203 to 1uM in the expanded volume as appropriate. Following 6 days of expansion, live Th1 and Th2 cells were isolated by Ficoll density centrifugation, counted, and stimulated to assess the production of Th1 and Th2 hallmark cytokines through the use of PMA and ionomycin, and cytokine production was assessed by intracellular flow cytometry staining and analysis (**Fig. 22a**). Prior to stimulation, it was noted that JPH-203 treatment decreased the number of skewed cells for both Th1 and Th2, but the most dramatic decrease was seen in the Th1 subset (**Fig. 22b**), which displayed an almost 4-fold reduction, as opposed to the comparatively slight decrease in Th2 cell numbers upon treatment with JPH-203. These skewed cells were then stimulated, with cells initially skewed in standard media in the presence or absence of

treatment with 1uM JPH-203 at the time of activation, and T cell activation and cytokine production were assessed. No changes in activation by CD69 expression were noted in any condition assayed (**Fig. 22c**) nor were any defects in Th2 cytokine production of either IL4 (**Fig. 22d**) or IL13 (**Fig. 22e**) noted by Th2 cells either skewed in the presence of JPH-203 or acutely treated with the inhibitor of leucine uptake by LAT1.

However, when cytokine production by Th1 T cells was assessed, striking findings were made. Although a slight decrease in IL2 production was noted by flow cytometry in Th1 cells skewed for 6 days in the presence of 1uM JPH-203 (**Fig. 23a**), this defect was reversed when the Th1 cells were assessed for the capacity to produce IL2 following overnight stimulation with plate-bound anti-CD3 and soluble anti-CD28 antibody stimulation followed by media ELISA for cytokine production (**Fig. 23b**). Furthermore, little defect was seen in IFN gamma production either by flow cytometry (**Fig. 23c**), or by media ELISA analysis following overnight stimulation, with a trend towards increased IFN gamma production in treated cells (**Fig. 23d**). In no setting was any effect of acute (either 4 hours for the case of PMA/ionomycin, or overnight for the media ELISA assays) treatment of previously skewed Th1 CD4 T cells with 1uM JPH-203 noted. However, when the production of the Th2 hallmark cytokines IL4 and IL13 was assessed, it was found that Th1 cells skewed in the presence of 1uM JPH-203 produce statically significant amounts of both IL4 (**Fig. 23e**) and IL13 (**Fig. 23f**). No corresponding changes in cytokine production from Th2 CD4 T cells skewed in the presence of 1uM JPH-203 (for example, production of IFN gamma or increased production of IL2) were observed. Furthermore, short term treatment of previously skewed Th1 T cells with JPH-203 is not sufficient to induce the production of either IL4 or IL13, indicating that the inhibition of leucine metabolism, similar to the inhibition of glutamine metabolism in Th2 cells, plays a role in the development, rather than subsequent activation of Th1 T cells.

Next, we sought to determine the provenance of these Th1 skewed cells displaying Th2 cytokine production. Th1 cells that had been skewed for 6 days in the presence or absence of 1uM JPH-203 were restimulated with PMA and ionomycin to assess cytokine production by flow cytometry, with the addition of 1uM JPH-203 to Th1 T cells that had been skewed in its absence. By assessing live, CD4 positive T cells that had been activated (by gating on the CD44 positive population) comparison of Th1 and Th2 cytokine production was conducted. "Th1" cells in which leucine uptake inhibition had induced the production of either IL (**Fig. 24a**) or IL13 (**Fig, 24b**) demonstrated the coproduction of both IFN gamma and the indicated Th2 cytokine, as denoted by the upper right quadrant in the middle column of these example flow plots. Further analysis of coproduction indicated a significant induction of the production of IFN gamma as well as IL4 (**Fig. 24c**) and IL13 (**Fig. 24d**) occurring as a consequence of skewing towards the Th1 subset in the presence of leucine transport inhibition. Once again, this effect was not duplicated by short term treatment with 1uM JPH-203. This leads to a model whereby dueling amino acid requirements for T cell subset commitment can be identified (**Fig. 25**), with Th2 development requiring glutamine but finding leucine to be disposable, and Th1 development requiring leucine, and being unaffected by the absence of glutamine.

Identification of LAT1 as a biomarker for activated cells *in vivo* in viral infection

After establishing the essential nature of leucine uptake in T cell proliferation following *in vitro* stimulation, as well as its role in promoting CD4 T cell skewing towards the more inflammatory Th1 subset, we were interested in assessing the presence of LAT1 on different immune cell subsets *in vivo*. Previous work by the Cantrell lab had identified *SLC7A5* (LAT1) as one of the most upregulated genes after T cell receptor (TCR) stimulation, and demonstrated that a T cell specific knockout CD4 cells fail to polarize to the Th1 subset, as well as that adoptively transferred LAT1 deficient OTI are less able to persist as well as activate[145]. Therefore, we were interested in assessing LAT1 expression *in vivo* on activated CD8 T cells. A simple model was used in which mice were submitted to infection with LCMV-

Armstrong strain, which leads to an acute infection that spontaneously resolves[133]. At 8 days following infection at the height of T cell response, mice were sacrificed, and CD8 T cells specific for the major LCMV antigen, GP33, were identified by tetramer staining, and characterized by cytometric staining (**Fig. 26a**). In accordance with previous findings[132], infection with LCMV-Armstrong significantly increased the percentage of activated, CD44 positive CD62L negative CD8 T cells in the spleens of mice (**Fig. 26b**), and induced a dramatic increase in the percentage of antigen-specific CD8 T cells in the spleens of infected mice (**Fig. 26c**). When the activated, CD44 positive cell populations in either uninfected or infected mice were assessed for their expression of LAT1 as assessed by its MFI, it was found that LAT1 was significantly increased on the activated CD8 T cells in the spleens of LCMV-infected mice, and was even slightly higher on the antigen specific, tetramer positive while still being significantly increased in this population over the uninfected controls (**Fig. 26d**). Infection with LCMV-Armstrong dramatically shapes a cytotoxic T cell response, including by inducing a population of terminal effector CD8 T cells, as denoted by their expression of the cytotoxic marker KLRG1, and their lack of expression of the IL7 receptor subunit CD127[128]. These short-lived effector cells (SLEC) are dramatically increased in the spleens of mice following LCMV-Armstrong infection (**Fig. 26e**). Examination of LAT1 expression on this population indicates that it is significantly elevated in SLEC in infected mice as compared to uninfected controls, and is even slightly higher on GP33-specific SLEC, as evidenced by tetramer staining (**Fig. 26f**).

Infection with LCMV-Armstrong also induces the activation of a number of other adaptive and innate immune subsets. B cells play key roles in viral responsiveness, including in mouse models of LCMV[156]. Based on data from the Immunological Genome Project (ImmGen)[157] which indicated an increased expression of *Slc7A5* in B and NK cells upon activation, we returned to the viral model to assess the expression of both LAT1 and its partner, the CD98 heavy chain. When gating on the total B cell population in the spleens of mice 8 days after LCMV-Armstrong infection, it was found that CD98 expression, as quantified by MFI, was significantly increased over the uninfected mice (**Fig. 27a**). Next,

when CD98 expression was assessed on B220 positive plasma cells, it was found that CD98 expression was once again significantly increased on the plasma cells from the spleens of LCMV-Armstrong infected mice as opposed to the uninfected mice (**Fig. 27b**). Next, the presence of activated, B220 positive, CD43 positive B cells was assessed. It was found that LCMV-Armstrong infection in mice induced a significant increase in the percentage of activated B cells in the spleen (**Fig. 27c**), and furthermore these activated B cells displayed significantly more LAT1 expression, as determined by MFI, in LCMV-Armstrong mice as opposed to uninfected mice (**Fig. 27d**). Natural Killer (NK) cells play a crucial role in the control of viral infections by their ability to target and kill "altered self," host cells that are dysregulated and have either expressed activating markers for their own killing, or lost expression of inhibitory markers, such as MHC class I[158]. When NK cells from the spleens of LCMV-Armstrong infected or uninfected mice were assessed by flow cytometry, it was found that infected mice bore NK cells with significantly more CD98 expression as indicated by MFI than uninfected mice. Based on this data, it is clear that leucine transport, as indicated by the expression of LAT1 and its heavy chain partner CD98, is significantly upregulated on not only activated CD8 T cells in the context of viral infection, but also B and NK cells which play an important role in the immune response.

LAT1 expression on T cells in multiple models of transplant rejection

Hematopoietic stem cell (HSCT) and solid organ transplant rejection remain major health burdens. For HSCT, the incidence of GvHD, in which donor T cells derived from the graft mount an attack against host tissue, accounts for 15-30% of the deaths that occur as a result of transplant[159]. Multiple strategies have been attempted to prevent GvHD following HSCT, such as matched donor transplant and the use of immunosuppressant drugs, however there remains an unmet need for novel strategies to combat GvHD. Therefore, we sought to assess the expression of LAT1 on pathogenic T cells in a mouse model of GvHD in which radiologically depleted B5D2F1 mice received a congenically distinct transplant of bone marrow cells and purified splenic T cells (**Fig. 28a**)[160,161]. Two weeks after transplant,

mice were sacrificed and spleens were assayed by flow cytometry to assess T cell engraftment. Substantial presence of donor cells was seen within the total CD3-positive component of the spleen (**Fig. 28b**), as well as the CD4 positive (**Fig. 28c**) and CD8 positive (**Fig. 28d**) portions, as donor cells from the allogenic transplant appeared in greater frequency than reconstituted cells from the syngeneic control, due to T cell activation and expansion in the allogenic transplant[159]. When LAT1 expression was assessed by flow cytometry, it was found to be significantly higher on the allogenic transferred cell population for both the CD4 (**Fig. 28e**) and CD8 (**Fig. 28f**) fractions as compared to either the syngeneic transplant or the remaining host cells that had not been fully depleted. This indicates that in this model of GvHD, the activated and pathogenic cell population still demonstrates significant LAT1 expression.

Skin and solid organ transplant rejection also contain major unmet needs in terms of long-term treatment strategies to reduce rejection. Current therapy relies the long-term use of immunosuppressant regimens, however these strategies are suboptimal due to the wide range of associated toxicities and side effects of prolonged immunosuppressive therapy[162]. Furthermore, inhibitors of T cell activation and signaling cascades can interfere with the proper induction of tolerance, meaning therapy must be continued indefinitely. Recently, attempts have been made to modulate metabolism in an attempt to induce transplant tolerance in both skin and heart transplant models[95]. A "triple therapy" approach using DON to inhibit glutamine metabolism, 2-DG to inhibit glycolysis, and metformin, which, in addition to its role as an oral diabetes medication, also is an inhibitor of oxidative respiration at Complex I[163], was shown to be effective in decreasing T cell proliferation and cytokine production, as well as signaling through mTORC1. *In vivo* studies indicated that this triple therapy was effective at inhibiting antigen specific CD4 and CD8 T cell proliferation and cytokine production in response to viral infection, while enhancing the Treg development. When this metabolic triple therapy was utilized in mouse models of both skin and heart transplant, it was found that metabolic therapy significantly enhanced the survival of both grafts, with the orthotopic heart transplants surviving over

100 days[95]. Based on this previous success, we sought to assess the role of leucine metabolism, as determined by expression of LAT1, in models of both skin and heart transplant.

First, we assessed LAT1 expression on CD4 and CD8 T cells in a skin transplant model. In this model, skin from Balb/C mice was transplanted onto C57BL/6 mice. Ten days after transplant, mice were sacrificed, and spleen, draining, and non-draining lymph nodes were harvested and subjected to flow cytometry analysis (**Fig. 29a**). Visual inspection of skin grafts (**Fig. 29b**) indicated an immune response against the transplanted tissue leading to graft damage. Flow cytometry analysis indicated an increased percentage of splenic CD4 T cells displaying an activated, CD44 positive CD62L negative surface phenotype in mice that had received skin transplants (**Fig. 29c**). These splenic CD4 T cells also displayed increased LAT1 expression as indicated by increased MFI as compared to wildtype, un-transplanted mice (**Fig. 29d**). When the CD8 T cell compartment of the spleen was examined, it was determined that there was again an increased percentage of activated CD44 positive CD62L negative T cells in the spleens of mice that had received skin transplant as opposed to wildtype, un-transplanted mice (**Fig. 29e**). Furthermore, the spleens of mice that had received skin transplants contained an increased percentage of KLRG1 positive, CD127 negative SLEC (**Fig. 29f**), further indicating the robust activation. This robust activation was accompanied by an increased MFI of LAT1 on the CD8 T cells in the spleens of mice that had received skin transplants as opposed to those which had not (**Fig. 29g**). This indicates that, as with the GvHD model, the pathogenic immune response that leads to disease in models of skin transplant rejection is associated with an increase in LAT1 expression.

As it had previously been demonstrated that metabolic “triple therapy” was efficacious in delaying rejection in a murine heart transplant model[95], we next sought to assess LAT1 expression in this model. Hearts from Balb/C mice were orthotopically transplanted into C57BL/6, into either the cervical area or the abdomen[164]. Ten to 11 days after transplant, mice were sacrificed, and spleen, draining and non-draining lymph nodes, and the transplanted heart itself were harvested and interrogated by flow.

However, LAT1 expression was not noted to be increased in the spleens, draining lymph nodes, or in graft infiltrating lymphocytes of allogeneic heart transplant recipient mice (data not shown). It is unlikely that this is evidence of a substantial change difference in the previously noted pattern of LAT1 upregulation upon T cell activation, and it is more likely that either the day 10 timepoint or the assessment methods did not empower us to capture the characteristic upregulation of leucine transport upon T cell activation.

Inhibition of leucine uptake with JPH-203 *in vivo* has no effect in a viral model

Previously, we had used the competitive inhibition of leucine uptake through LAT1 JPH-203 *in vitro* to demonstrate the requirement for leucine uptake in T cell proliferation, but not in the upregulation of activation markers (**Fig. 20**), while demonstrating that inhibition of leucine metabolism induces a non-canonical pathway of differentiation in CD4 T cells skewed towards the Th1 subset, in that they also become competent to produce the Th2 hallmark cytokines IL4 and IL13 (**Fig. 23e,f**). This led us to consider a model of helper T cell differentiation as controlled by amino acid metabolism in which skewing towards the Th2 subset relies upon the presence of glutamine, while leucine is dispensable, while Th1 skewing requires leucine, and does not require glutamine (**Fig. 25**). We next sought to use this inhibitor *in vivo* to assess its effects on T cell responses in mice. Previous studies have characterized the use of JPH-203 in the *in vivo* setting, from its ability to inhibit the growth of human xenograft colon cancer cells in nude mice[150] and murine thyroid cancer in an immunocompetent mouse model[148] to its ability to inhibit T cell mediated inflammation in nasal and skin hyperresponsiveness[154,155]. Therefore, we sought to assess the role of leucine metabolism in an *in vivo* infection model. Transgenic OTI CD8 T cells were adoptively transferred into congenically distinct hosts via retro-orbital injection, and allowed to rest overnight. The following day, mice were infected with an engineered *Vaccinia* strain bearing an ovalbumin peptide for which OTI cells are stimulated by in the context of MHC class I[165]. Following infection, mice were treated daily with intraperitoneal injections of JPH-203 at three dose levels

selected based on previous literature. On day 7 post-transfer, mice were sacrificed and spleens were harvested for flow cytometry analysis (**Fig. 30a**).

Analysis of the spleens of infected mice indicated that treatment with JPH-203 had little effect. There was little difference in whole splenocyte counts induced by treatment, and while a slight decrease could be seen in high dose (50mg/kg) treatment, the middle and lower doses actually saw and enhancement in splenocyte numbers (**Fig. 30b**). Furthermore, there was no toxicity as a result of JPH-203 treatment, as no weight change as compared to baseline was noted in any treatment group (**Fig. 30c**). When spleens were assessed for the presence of transferred OTI cells as a percent of total CD8 population, it was found that JPH-203 treatment was insufficient to reduce transferred cell populations at any dose level (**Fig. 30d**). Furthermore, no difference was seen in the percent of transferred cells that were activated be either percent CD44 positive CD62L negative (**Fig. 30e**), percent PD1 positive (**Fig. 30f**), or percent bearing the SLEC surface phenotype of KLRG1 positive and CD127 negative (**Fig. 30g**) at any treatment level. When cytokine production was assessed by *ex vivo* simulation followed by intracellular cytokine staining for flow cytometry assay, there was no difference between treated and untreated for the production of either TNF (**Fig. 30h**) or IFN gamma (**Fig. 30i**) when graphed as either a percentage of total CD8 population (closed symbols) or as a percentage of transferred cells (open symbols). Therefore, we can conclude that treatment with JPH-203 in an *in vivo* infection model has no effect on CD8 T cell response. This result is curious, as previous studies had shown effects of *in vivo* treatment with JPH-203 at similar doses in both tumor and T cell response models. However, these studies utilized different vehicle and injection strategies, and these differences may be responsible for the lack of effect seen in the present study.

Depletion of LAT1 expressing cells with a cytotoxic agent in viral and skin transplant models decreases the immune response dramatically

Following the failure of *in vivo* inhibition of LAT1 mediated leucine by treatment with JPH-203 (**Fig. 30**) but knowing that LAT1 expression on T cells in both viral and transplant models (**Fig. 26-29**) could offer an attractive target for the modulation of immune responses, we sought an alternative strategy to target LAT1. Novel developments in the field of targeted therapies, particularly in cancer, have dramatically increased the therapeutic efficacy and decreased toxicity in many settings but more work remains to be done. However, the most recent advance has been in targeted cytotoxic therapies, which rely on prodrug strategies (such as was described with JHU-083 previously in this thesis) to couple a cell delivery vector with a cytotoxic warhead for exquisitely targeted effects only in the target tissue. Examples such as JHU-083 and Vintafolide, a conjugate of vinblastine that targets the folate receptor demonstrate that cell targeting therapies can provide highly beneficial in a variety of disease contexts[166]. Therefore, we sought to use a similar strategy targeting using LAT1 as our cell surface target, and a nitrogen mustard alkylation agent. This compound, (2S)-2-amino-3-[5-[bis(2-chloroethyl)amino]-methyl-phenyl]propanoic acid, henceforth referred to as QBS10096S, couples cytotoxicity with LAT1 recognition. To test this, we set up a more complex adoptive transfer model, in which congenically distinct transgenic CD8 T cells of two differing specificities, OVA specific (OTI) and LCMV specific (P14, specific for the GP33 antigen) were adoptively transferred into the same host. This transfer occurred at a 9:1 ratio, with mice receiving more P14 cells than OTI cells. Following transfer, OTI cells were stimulated by infection with Vac-OVA, and hosts were treated with three doses of QBS10096S every other day to deplete LAT1-expressing cells. Seven days after initial transfer and infection, circulating T cell populations were assessed by cheek bleed. Following this, P14 T cells were stimulated by infection with LCMV Armstrong strain, and mice were sacrificed a week later and spleens were harvested to assess adoptively transferred T cell content (**Fig. 31a**). This experimental design is intriguing in that, as

the OVA specific T cells are the only ones to be activated during the administration of LAT1- targeted cytotoxic therapy, they should be the only cells depleted, and the levels of the "bystander" LCMV-specific T cells should not be affected by QBS10096S treatment, either during the period of treatment or upon stimulation with LCMV in the absence of the drug.

When the transferred T cell population was assessed by cheek bleed following QBS10096S treatment, on day 7 post-transfer, it was found that the highest dose of drug, 4mg/kg, resulted in general lymphodepletion, with a substantial fraction of live CD8 T cells being depleted from the lymphocyte forward scatter/ side scatter gate (**Fig. 31b**). When the percentage of live, OVA-specific transgenic CD8 T cells was assessed, it was found that QBS10096S treatment to deplete LAT1 expressing T cells lead to a dose-dependent decrease in stimulated cell population (**Fig. 31c**). However, there was no comparable decrease in the levels of LCMV-specific transferred CD8 T cells (**Fig. 31d**), indicating that these cells were not targeted by the cytotoxic agent, as they were unstimulated and not expressing LAT1. Following LCMV stimulation, mice were sacrificed and spleens were harvested for analysis by flow cytometry. It was found that the lymphocyte population had recovered in the mice initially treated with the high dose (4mg/kg) QBS10096S (**Fig. 31e**), and that treatment had not affected LCMV-specific CD8 T cells levels, as these were similar between vehicle treated and all 3 dose levels in the spleen **(Fig. 31f**). OVA-specific CD8 T cells were still detectable in the spleens of all mice, but interestingly the lowest percentage was found in the middle, 1mg/kg QBS10096S treatment group (**Fig. 31g**). Based on this result, we were confident that QBS10096S treatment was suitable for the depletion of activated, LAT1 expressing T cells in our model systems.

Next, we sought to expand these findings from the viral model to a transplant model. As we had noted impressive activation and LAT1 expression on both CD4 and CD8 T cells from mice that received allogenic skin transplant (**Fig. 29**), we next tested QBS10096S treatment in this system. C57BL/6 mice once again received skin transplants from Balb/C mice, but transplanted mice were treated every other

day with either 1mg/kg or 0.25mg/kg QBS10096S. The 4mg/kg dose was not moved forward due to toxicity. Ten days after transplant, at the peak of immunological response, mice were sacrificed and spleens were harvested for flow cytometry analysis (**Fig. 32a**). In agreement with the viral model, 1mg/kg and 0.25mg/kg doses of QBS10096S did not display any toxicity, as there was no evidence of weight loss in treatment groups (**Fig. 32b**). Visual inspection of the grafts (**Fig. 32c**) demonstrated no dramatic differences, although the 1mg/kg dose level appeared to be slightly more robust than either the vehicle of the 0.25mg/kg dose level. Analysis of splenic CD4 T cells revealed that treatment with either dose level of QBS10096S was sufficient to decrease activation, as assessed both by the decreased percentage of splenic CD4 T cells that were CD44 positive CD62L negative (**Fig. 32d**) and a decreased percentage of PD1 positive splenic CD4 T cells (**Fig. 32e**) as compared to untreated mice, although the decrease in activation was more substantial at the higher, 1mg/kg dose level in both. However, there was no change in the overall LAT1 MFI of splenic CD4 T cells in any treatment group (**Fig. 32f**). Similar patterns were observed when splenic CD8 T cells were interrogated, as decreased activation was seen by a decrease in CD44 positive CD62L negative cells in treatment groups as compared to vehicle (**Fig. 32g**), as well as a decrease in PD1 positive cells at the 1mg/kg dose (**Fig. 32h**). A decrease in SLEC was also noted in the spleens of treated mice (**Fig. 32i**), however, similar to the splenic CD4 T cells, there was no decrease in LAT1 MFI in the splenic CD8 T cells (**Fig. 32j**). Overall, these results using a LAT1-specific cytotoxic to deplete activated T cells in both viral and transplant models indicate that this strategy holds promise moving forward for the selective targeting and depletion of pathogenically activated or autoreactive T cells in a variety of conditions. Further work remains to be done to assess the fitness of this approach for other disease states, as well as its ability to be applied to other activated immune subsets, as both B and NK cells have demonstrated robust expression of CD98 and LAT1 upon activation (**Fig. 27**).

Discussion

Leucine metabolism at the time of T cell activation is a crucial aspect of the development of an immune response. Previous research has demonstrated the necessity of leucine in mTORC1 activation[142], and the activity of the mTOR kinase complexes is key to the fate decisions of both CD4 and CD8 T cells[26,64], as reviewed extensively in the first chapter of this thesis. Leucine uptake through a transporter formed by the combination of LAT1 (*SLC7A5*) and the CD98 heavy chain is crucial for this process to occur and for these fate decisions to be made. Building on findings that CD98 knockout mice cannot mount an immune response[152], a recent paper by Doreen Cantrell's lab has demonstrated that, very soon after TCR stimulation, *SLC7A5* expression is increased at the transcriptional level, and the LAT1 protein level is detectable soon after. Furthermore, LAT1 knockout T CD4 cells are incapable of skewing towards the Th1 or Th17 subsets, while CD8 T cells were not able to fully activate or mount a durable response[145]. Based on these findings, we first sought to assess the effects of inhibition of leucine uptake *in vitro*. To do this, we relied on the competitive inhibitor of leucine uptake JPH-203[167]. JPH-203 is a tyrosine analogue which has previously been demonstrated to inhibit mTORC1 signaling as well as induce tumor cell growth inhibition as well as cell death. It is advantageous as compared to other LAT1 inhibitors such as BCH as it is more specific for LAT1 as opposed to other transporters, including other LAT1 family members[149].

First, we sought to assess the role of leucine metabolism *in vitro* in CD4 and CD8 T cell activation and proliferation. Interestingly, the results resembled what had been seen previously in the context of glutamine metabolism, in that the activation of both CD4 and CD8 T cells was preserved across all doses of JPH-203, while high doses of the inhibitor were able to potently decrease T cell proliferation (**Fig.20**). Interestingly, this occurred in the context of little alteration of mTORC1 signaling, with some evidence for a slight increase in mTORC2 signaling activity based on Western Blot analysis (**Fig. 21**). Next, we sought to assess the role of leucine metabolism in CD4 T cell subset skewing. To do this, we chose the

lowest dose of JPH-203 that induced a decrease in proliferation, 1uM, and cultured cells in Th1 or Th2 skewing conditions in the presence or absence of drug for 6 days. This set of experiments was very interesting, as we observed a slight growth defect, but no impact in cytokine production on the Th2 cells reminiscent of the lack of effect in cells skewed towards the Th1 subset in the absence of glutamine. However, we saw a profound decrease in the live cell numbers of cells skewed Th1 in the presence of 1uM JPH-203 (**Fig. 22b**). Furthermore, cells that had been polarized Th1, while still capable of expressing the Th1 hallmark cytokines IL2 and IFN gamma upon stimulation, also made IL4 and IL13 (**Fig. 23**), and this cytokine production was the result of cells co-producing Th1 and Th2 hallmark cytokines (**Fig. 24**). Previously, changes in Th2 cytokine production had been ascribed to an epigenetic mechanism in the case of glutamine restriction, as discussed previously in this thesis. We currently have no evidence that a similar epigenetic mechanism is at play here in order to enhance the accessibility of the Th2 cytokine locus in CD4 T cells skewed towards the Th1 subset in the presence of JPH-203 to inhibit leucine uptake. Further studies to assess both the Th1 and Th2 cytokine loci, potentially by utilizing a modified CUT and RUN approach, could help answer this question.

These *in vitro* studies on both leucine and glutamine lead to a model of amino acid metabolism serving as a crucial rheostat for CD4 T cell subset commitment, in which glutamine is essential for the development of Th2 T cells while leucine dispensable, and conversely leucine is essential for the development of Th1 T cells while glutamine is dispensable (**Fig. 25**). This model is very interesting because it resembles the model of mTOR complex signaling in CD4 T cell subset commitment put forward previously by our lab[26], in that Th1 and Th17 cell development requires mTORC1 signaling, Th2 development requires mTORC2 signaling, and in the absence of all mTOR signaling, cells will become polarized towards the Treg subset. This finding is especially intriguing, especially in light of the fact that T cell specific *Slc7a5* knockout was found to prevent skewing towards the Th1 and Th17 subsets[145] , however we cannot confidently attribute all observed effects here solely to impacts in mTOR complex

signaling. Inhibition of glutamine metabolism with either low glutamine media culture or the addition of DON has little effect on either mTORC1 or mTORC2 to signaling, with only extremely high doses of DON being sufficient to decrease p-S6 staining as assessed by flow cytometry (**Fig. 2e**). Interestingly, glutamine and leucine metabolism are tied directly together, as glutamine efflux is required for leucine influx through LAT1[143], and therefore combination manipulations of these two pathways might be quite efficacious. It would be interesting to assess the impacts of combination glutamine and leucine inhibition on the development and subset commitment of Th1 and Th2 T cells (for example, skewing in low glutamine culture media in the presence of 1uM JPH-203). If CD4 T cell subset commitment decisions are similar in regards to amino acid availability as they are for mTOR complex signaling, it would be expected that the inhibition of both glutamine and leucine metabolism would lead to the polarization of CD4 T cells towards the Treg fate, which could make this a very important therapy for the inhibition of autoimmune and autoinflammatory diseases, as well as promoting tolerance in transplant models.

Next, *in vivo* experiments were performed to assess the expression of LAT1 on a variety of immune cell types in different models of activation. Based on the known role of LAT1 in T cell activation[145] as well as data from ImmGen[157], we anticipated that LAT1 expression would be present on activated immune cells of the innate and adaptive systems in a variety of contexts. First, we assessed a viral model of activation, LCMV, and found that LAT1 was highly expressed on both CD4 and CD8 T cells in the spleen, and that expression was predominantly confined to the activated cell populations, such as the CD44 positive CD62L negative cells, the PD1 positive cells, and in CD8 T cells the KLRG1 positive CD127 negative SLECs (**Fig. 26**). However, leucine transporter expression was not confined to T cells, as it was also noted in activated B cell subsets including CD43 positive B cells and plasma cells, as well as NK cells during viral infection (**Fig.27**). It is highly likely that, as leucine is an essential amino acid required for mTOR signaling, which is vital for growth and development outside of the immune system

as well, that LAT1 and CD98 will be highly expressed on many other cell types, both at rest and under conditions of stress. Indeed, LAT1 is highly upregulated on many cancer types[149] and therefore efforts must be made to more fully profile the expression pattern of LAT1 in a variety of conditions, both during normal homeostasis, and in a variety of disease conditions.

Next, we began this assessment of LAT1 expression in three different transplant models: GvHD, skin transplant, and orthoptic heart transplant. A GvHD model in which congenically distinct mice were radiologically depleted prior to transfer of either syngeneic or allogenic bone marrow and purified splenic T cells indicated that the allogenic transferred cells, which are responsible for the induction of disease in this model, displayed increased LAT1 expression as compared to the syngeneic transplants, and this was the case when the bulk T cell population (as determined by CD3 positivity) was assayed, as well as individually for the CD4 and CD8 T cell fractions (**Fig. 28**). Next, a skin transplant model in which Balb/C skin sections were transplanted onto the flanks of C57BL/6 mice was tested, and again it was noted that the activated CD4 and CD8 T cells bore increased expression of LAT1 in the spleens of transplanted mice (**Fig. 29**), and that this increased expression once again coincided with a more activated T cell population. Conversely, when an orthotopic heart transplant model, in which Balb/C hearts were transplanted into the cervical region or abdomen of C57BL/6 mice, was assayed for T cell expression of LAT1, it was found that neither the T cells in the spleens nor the lymphocytes infiltrating the transplanted hearts demonstrated an increased expression of LAT1 over the un-transplanted wildtype control mice (data not shown). As LAT1 upregulation has previously been identified as a universal feature of T cell activation[145], it is likely that our system is not properly configured to capture the upregulation of LAT1 that occurs in the context of heart transplant, and therefore we moved forward with treatment strategies in other model systems.

Next, we sought to continue our studies of LAT1 *in vivo* with the use of JPH-203 to alter CD8 T cell responses in an infection model. JPH-203 has previously been demonstrated to be effective when

used in a variety of *in vivo* settings, as both an anti-cancer therapy[148,151] and as an inhibitor of T cell responses in models of T cell mediated diseases such as asthma and allergic dermatitis[154,155]. When we attempted to utilize JPH-203 *in vivo* in an adoptive transfer model of *Vaccinia* infection, we were surprised to find that almost no effects of therapy could be seen. No dose of treatment was seen to decrease the percentage of transferred cells, the activation of these cells as determined by surface phenotype, or the cytokine production of these cells (**Fig. 30**). This was astonishing, as previous literature had shown robust effects of JPH-203 when used *in vivo* at the same 50mg/kg dose level in which we treated. However, these experiments relied on both different vehicles and different injection strategies[148,154,155] (subcutaneous versus intraperitoneal), both of which may have potentially led to a "depot effect" by which more of the active drug was preserved for longer, potentially serving to extend the short half-life of JPH-203 *in vivo*[167]. The use of an inhibitor of LAT1 transport in an *in vivo* setting merits further exploration. Based on our *in vitro* work, it is likely that treatment with this agent would be able to, rather than serving as a blanket shut-down of the immune response, be capable of modulating from an inflammatory, Th1-like response towards more of the tissue repair response as promoted by Th2 cytokines, and there are a variety of settings in which the promotion of this response would be favorable, such as in wound healing or the promotion of biological scaffold growth[168]. Therefore, further work remains to be done on the use of LAT1 inhibition in our *in vivo* mouse model settings.

Next however, we turned from LAT1 inhibition to LAT1 depletion. Targeted cytotoxic therapies are a new focus of therapy for several diseases, including cancer[166], and we sought to use a LAT1 targeted cytotoxic to explore the effects of specific depletion of LAT1-expressing cells in our models. In an adoptive transfer model, we were able to co-transfer T cells of two distinct specificities and, while only activating one, demonstrate that LAT1 specific depletion affects only the activated cells, as the cells that were not activated were not targeted, and were able to expand equally following stimulation (**Fig.**

31). Although this experiment was successful, we saw pronounced lymphodepletion at the highest dose level, 4mg/kg, which led us to discontinue future studies at high dose. The finding of selective depletion is an important one, as real immune responses in real immune systems, as opposed to model systems, are complicated, and blanket inhibition would not be ideal for the vast majority of settings. Next, we sought to expand our LAT1- specific depletion experiments to treatment in the skin transplant model. Treatment did not lead to toxicity, but did lead to decreases in activation in both the CD4 and CD8 compartments of the spleen (**Fig. 32**). Curiously, we did not see any changes in LAT1 expression in either the CD4 or CD8 T cells in this experiment, but this is likely a sign that we have achieved depletion, and all of the LAT1 expressing cells have been eliminated.

While these initial studies assessing the impact of LAT1-specific depletion with QBS10096S in both viral and transplant models have shown promise, much work remains to be done to move these findings forward. Longer term studies to assess LAT1-specific depletion in the context of skin transplant will need to assess graft survival rather than early immunological endpoints to determine if depletion therapy is actually effective at extending the life of the skin graft. Further studies are also warranted to assess the efficacy of LAT1-specific depletion therapy in the other transplant models discussed herein, including heart transplant and the GvHD model. The heart transplant model was not initially selected for further studies, as there appeared to be comparatively less of an increase in LAT1 expression on the activated and tissue-infiltrating T cells present in this system. However, if this slight increase in LAT1 expression can be coupled with properly titrated and timed therapy, promotion of orthotopic heart transplant survival may be possible with LAT1-specific cytotoxic therapy. Pilot studies using QBS10096S in the GvHD model were ended prematurely due to toxicity, as treatment caused the deaths of a substantial fraction of mice (data not shown). This serves to illustrate that a more comprehensive assessment of LAT1 under a variety of conditions must be carried out, as it is possible that mortality in this model was a result of depletion of a heretofore unrecognized cell population that expressed LAT1

following the stress of radiation, or that irritated mice were more sensitive to the presence of the cytotoxic in their systems and this lead to death. Further experiments will also need to be done to test T cell LAT1 expression during homeostatic expansion, such as following transfer into a *Rag1/2* knockout mouse, because if LAT1 is expressed in this setting, depletion would be a suboptimal treatment for potential GvHD, as it would also interfere with the ability of the graft to reconstitute the immune system of the host. Therefore, a great deal of work remains in the assessment of LAT1-specific cytotoxic therapy, but there is great promise for a variety of settings.

Although our initial studies with JPH-203 were unable to show an effect, we remain interested in the effects of LAT1 inhibition, as opposed to depletion, in a variety of *in vivo* settings. Based on previous literature showing efficacy *in vivo*[154,155] coupled with our own *in vitro* work demonstrating that LAT1 inhibition is capable of reprogramming, rather than completely inhibiting an immune response, it appears that an inhibition strategy has the potential to reverse and re-wire a variety of T cell mediated diseases. The fact that LAT1 inhibition effectively prevents T cell proliferation but does not inhibit activation means that this might be a suitable strategy for the induction of T cell tolerance, for example to grafted or transplanted tissues, as the induction of tolerance is impossible without activation. Further studies that combine LAT1 inhibitor therapy with other anti-rejection therapies such as checkpoint blockade in the case of transplant may prove efficacious at inducing long-term tolerance and could cut down on the need for life-long immunosuppression regimens. Furthermore, the sequential use of LAT1-specific cytotoxic therapies followed by inhibition may be a strategy of combination therapy for example in the GvHD model, where it would be possible to deplete the early inducers of GvHD (which express LAT1 early) with initial cytotoxic therapy, followed by LAT1 inhibition in order to induce tolerance and promote long-term graft survival without the use of immunosuppression.

Further studies of LAT1 inhibition or depletion therapies must also study the role of this transporter on the non-T cells of the immune system. We have demonstrated its expression on both B

cells (including activated B cells and plasma cells) and NK cells (**Fig. 27**), and these cells play important roles in a variety of conditions. For example, the modulation of B cell responses could be extremely important in autoimmune conditions such as SLE, which anti-DNA antibody production is a key mediator of disease[169]. Previous studies have already indicated the important role of metabolism on SLE disease status, as well as the use of metabolic therapy to fight this disease in mouse models[100,104,105]. LAT1 inhibition could be combined with other approaches of metabolic therapy in order to improve control and outcomes in a number of diseases. However, as was considered with glutamine inhibitor therapy approaches, toxicity of therapy must be a concern from the outset. Leucine is an essential amino acid, and is widely utilized by a number of different cell types. In addition, its role in mTOR signaling, which is important for growth and development outside the immune system as well, means that the risk of toxicity is very real. Potentially, a pro-drug strategy as was utilized with the development of JHU-083[87] may be called for in this setting, in order to limit toxicity and confine inhibition to the specific cells against which it is desirable.

In conclusion, inhibition of LAT1 *in vitro* demonstrated a profound inhibition of T cell proliferation without affecting activation, and resulted in alterations CD4 T cell skewing such that cells polarized towards the Th1 subset in the presence of LAT1 inhibition were re-wired to produce Th2 cytokines as well. While *in vivo* studies of LAT1 inhibition were unsuccessful on a technical level, studies with QBS10096S, a LAT1-specific cytotoxic agent, was successful in depleting activated cells in both viral infection and skin transplant models, were we have shown LAT1 to be expressed. These results demonstrate the important role metabolism plays in the control of the immune response, and open the door to a range of future studies utilizing alteration of leucine metabolism, from the repolarization of immune responses to the inhibition and selective depletion of pathogenic cells in models of autoimmunity or transplant. A great deal of work remains to be done, but there is great promise in the

modulation of leucine metabolism, particularly through the inhibition or targeted deletion of the LAT1 transporter in a variety of systems.

Materials and Methods

Mice

Six to eight-week-old male of female mice were used for performing all experiments in this study. Rag knockout mice bearing a transgenic T cell receptor recognizing a peptide from OVA (OTI) were obtained from Jackson Laboratories and maintained by breeding in house. Rag knockout mice bearing a transgenic T cell receptor recognizing a the Gp33 peptide from LCMV (P14) were a gift from Dr. David Hildeman at the University of Cincinnati and were maintained by breeding in house. Congenically distinct C57/BL6 mice that served as hosts for adoptive transfer experiments were obtained from Jackson Laboratories and maintained by breeding in house. All mouse procedures were carried out in accordance with a protocol approved by the Johns Hopkins University Institutional Animal Care and Use Committee. No empirical test was performed for choosing sample size prior to experiments. No randomization of samples or animals was used nor were investigators blinded throughout the study.

Flow cytometry

Cells were counted, and 2 to 3 million were plated in round bottom tubes. For tissue samples, FC receptors were blocked with anti-CD16/CD32 (Mouse BD Fc Block) (2.4G2, #553142), for 20 minutes at 4C. Surface staining was performed at 4C for 20 minutes in the presence of eBioscience Fixable Viability Dye eFluor 780 (65-0865-14). For intracellular staining, cells were fixed and permeabilized overnight at 4C with eBioscience Fixation/Permeabilization set (88-8824-00). For intracellular cytokine staining, cells were fixed in BD Fixation Permeabilization solution kit (554714) for 20 minutes at room temperature. Intracellular staining on fixed and permeabilized cells was performed for 1 hour at room temperature.

All experiments were performed on a BD Celesta. Gates were determined by using unstimulated controls or isotype controls where appropriate. Data was analyzed using FlowJo software.

Cell trace violent proliferation and T cell activation assays

CD4 and CD8 transgenic T cells (5CC7 and OTI respectively) were obtained by sacrificing mice, and dissecting out spleens and lymph nodes. Spleens were mashed through 70uM filter, red blood cells were lysed with ACK lysis buffer, and following PBS wash, cells were counted. Following cell counting, cells are labelled with Cell Trace Violet at 10e6/mL in a 1:1000 dilution of CTV for 10 minutes at 37C. Cold media is added to 15mL volume, and labelling is quenched on ice for 5 minutes. Cells are centrifuged and resuspended in appropriate media. CTV labelled cells were stimulated with corresponding peptide and indicated concentration of JPH-203 or DMSO vehicle control. Two days after initial stimulation, cell cultures were expanded and exogenous IL2 was added. Additional JPH-203 or DMSO vehicle was added at this point to the concentration of the new volume. On day 3, cells were stained for flow cytometry and proliferation was assessed by CTV dilution.

Western blotting

Protein analysis by Western blotting was performed as described by Oh[170]. Briefly, cultured T cells were centrifuged and pellets were snap frozen in liquid nitrogen. Pellets were lysed in RIPA buffer with NaF, protease inhibitor cocktail, PMSF, sodium pyrophosphate, beta glycerophosphate, and sodium orthovanadate. Protein concentration was quantified by Bradford assay and normalized across samples. Immunoblot was performed using a standard protocol. Proteins were detected by ECL plus substrate. All images were obtained using a UVP Biospectrum500 Imaging System.

CD4 T cell skewing and intracellular cytokine stimulation/ ELISA assays

Transgenic 5CC7 mice were sacrificed and spleens were isolated. Spleens were ground through 70um strainer, red blood cells were lysed with ACK lysis buffer, and cells were counted and plated in standard 4mM glutamine T cell culture. Transgenic CD4 T cells were stimulated by the addition of PCC peptide at a final concentration of 5uM. For Th1 skewing, IL12 was added to a concentration of 10ng/mL, IFN-gamma was added to a concentration of 2ug/mL, and anti-IL4 antibody was added to a concentration of 5ug/mL. For Th2 skewing, 10ng/mL IL4 was added to culture, as well as anti-IL12 and anti-IFN-gamma antibodies, both to a concentration of 5ug/mL. Cultures were expanded and IL2 was added to a concentration of XXug/mL every other day. For the assessment of cytokine production by flow cytometry, on day 6 live cells were isolated by Ficoll density centrifugation and washed in PBS. Cells were counted, and 2e6 live cells were plated in 200uL standard 4mM glutamine culture media, and stimulated with PMA and ionomycin and Golgi-stop. Cells were stimulated for 4 hours at 37C, surface stained, fixed and permeabilized with BD fixation/ permeabilization buffer, and cytokine production was assessed by intracellular cytokine staining. For assessment of cytokine production by ELISA, live cells were isolated as described above, and one million live cells per mL of standard 4mM glutamine T cell culture media were plated in a 6 well plate coated with 3ug/mL anti-CD3 stimulatory antibody in sterile PBS (coated overnight at 4C). Cells were also stimulated with 1ug/mL anti-CD28 stimulatory antibody, which was spiked into media. Following overnight culture at 37C, media was collected and centrifuged to pellet out cells. Cell free media was frozen at -20C. Sandwich ELISA assays were performed according to manufacturer's instructions (eBioscience mouse IL2 ELISA: 88-7024, mouse IL4 ELISA: 88-7044, mouse IL13 ELISA: 88-7137 mouse IFN gamma ELISA: 88-7314).

LCMV infection to assess endogenous response

Infection with LCMV-Armstrong strain was performed as described by Joshi[128]. Briefly, 6 to 8-week-old C57BL/6 mice were infected with 2e6 plaque forming units of LCMV-Armstrong via IP injection in 200uL sterile PBS. Eight days following infection, mice were sacrificed and spleens were removed. Spleens

were mashed through 70uM strainer and red blood cells were lysed with ACK lysis buffer. Cells were washed in PBS and counted. For assessment of antigen specific CD8 T cell titers, 2e6 cells were plated in round bottom staining plates, and stained with GP33-PE tetramer for 45 minutes at room temperature in MACS buffer. Cells were washed, and surface staining was performed as described above.

Graft-versus-host disease model

BMT to induce GvHD was performed as described by Hill[160] and Cooke[161]. Briefly, bone marrow was harvested from donor C57BL/6 mice, and 5e6 bone marrow cells supplemented with 2e6 purified splenic T cells were transplanted into recipient B6DF1 mice via tail vein injection for allogenic transplant (B6DF1 donors were used for syngeneic controls). Prior to transfer, hosts were irritated with 1300cGy total body irradiation split over two fractions to reduce gastrointestinal toxicity. Fourteen days after transplant, mice were sacrificed and spleens were harvested and stained for flow cytometry as described above.

Skin transplant model (including QBS10096S treatment)

Skin transplant was performed as described by Lee[95] . Briefly, 1cm by 1cm full-thickness skin grafts were harvested from the backs of BALB/c donor mice, and fixed on the thoracic flank of recipient C57BL/6 mice with simple separate stitches. Seven days following transplant, bandages were removed and grafts were observed every day for evidence of rejected, and were considered rejected when 90% or more of the graft tissue necrosed. Ten days following transplant, mice were sacrificed and spleens, draining lymph nodes, and non-draining lymph nodes were harvested for analysis by flow cytometry as described above. For cytotoxic QBS10096S treatment, transplants were performed as above, but starting on day 3, mice were treated every other day with indicated dose of QBS10096S via IP injection until harvest on day 10.

Adoptive transfer for JPH-203 treatment model

OTI adoptive transfer and stimulation with Vac-OVA was performed as described by Pollizzi[64]. Briefly, 50,00 naïve OTI cells were isolated by negative selection and adoptively transferred retro-orbitally into congenically distinct hosts. The following day, hosts were injected with 1e6 plaque forming units of Vac-OVA. Mice were treated every other day from day 3 to day 6 with indicated dose of JPH-203 by IP injection. Seven days after initial transfer, mice were sacrificed and spleens were harvested for flow cytometry analysis as described above.

Co-adoptive transfer for QBS10096S treatment model

Naïve OTI and P14 CD8 T cells were isolated from spleen and lymph nodes by negative selection. Cells were counted and mixed in a 9:1 ratio (450,000 P14 to 50,000 OTI) and this ratio was confirmed by flow cytometry analysis. Adoptive transfer of 450,000 P14 and 50,000 OTI in 200uL HBSS was performed via retro-orbital injection into congenically distinct hosts, which were infected with Vac-OVA IP. Eight days after transfer, circulating CD8 T cells were assessed by cheek bleeding. A lancet was used to pierce the skin of the cheek, and a small volume of blood was collected into a 1.5mL tube containing a 1:1 mixture of RPMI media plus antibiotics and 4% sodium citrate. Tubes were centrifuged, and red blood cells were lysed with ACK lysis buffer, and cells were stained and assessed by flow cytometry as described above. After cheek bleeding, hosts were infected with LCMV-Armstrong via IP injection. Seven days after LCMV infection, mice were sacrificed and spleens were harvested for assessment by flow cytometry as described above.

Figures

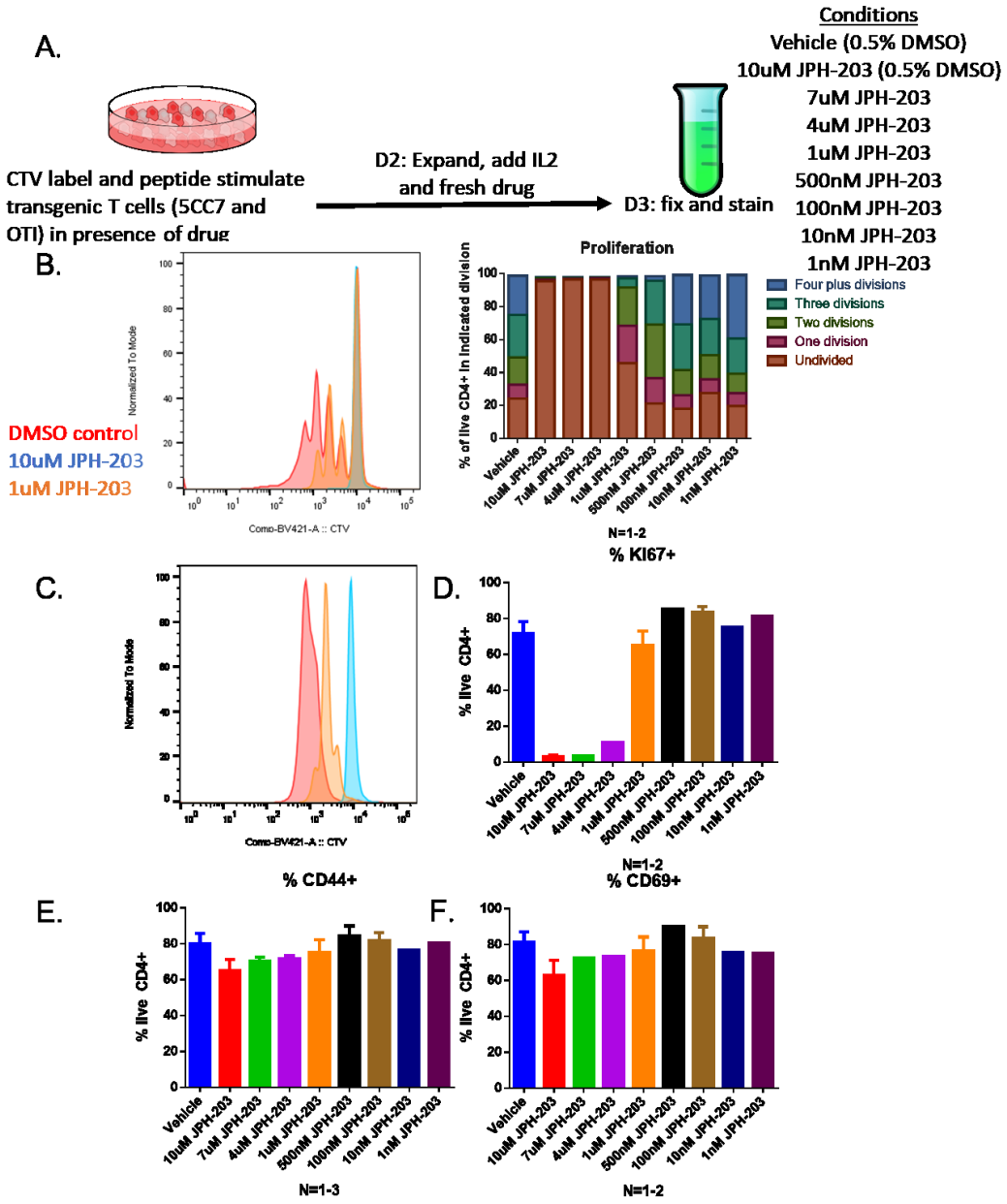

Figure 200. Inhibition of leucine metabolism in vitro inhibits T cell proliferation while only slightly affecting activation

Figure 20. Inhibition of leucine metabolism *in vitro* inhibits T cell proliferation while only slightly affecting activation. **(a)** experimental outline. **(b)** assessment of CD4 T cell proliferation as assessed by CTV dilution on flow cytometry example histogram (left) and summary graph (right). **(c)** as in (b) but with CD8 T cells. **(d)** assessment of Ki67 expression by flow cytometry. **(e)** as in (d) but for assessment of CD44 expression. **(f)** as in (d) but for assessment of CD69 expression.

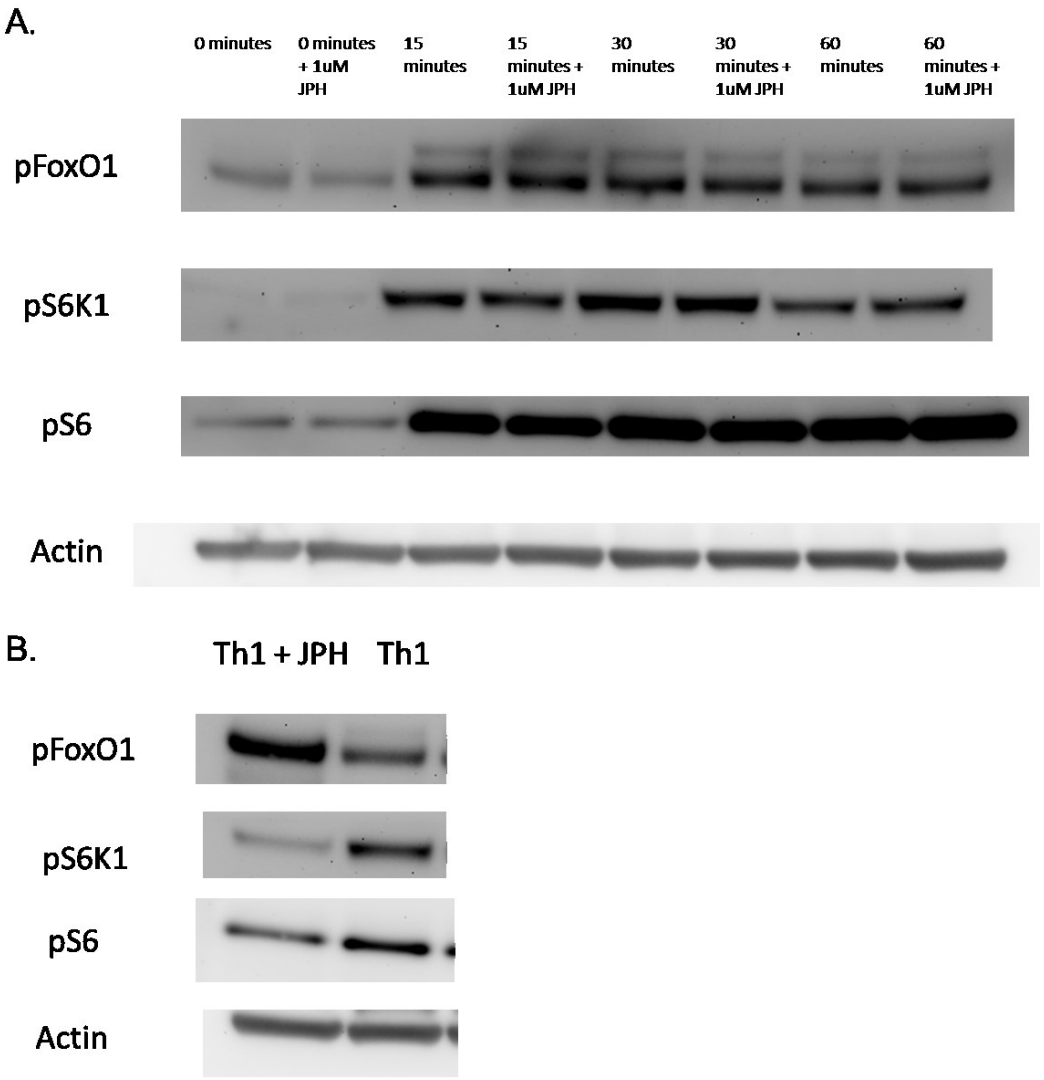

Figure 211. Inhibition of leucine metabolism inhibits mTORC1 activation while promoting mTORC2 signaling pathways

Figure 21. Inhibition of leucine metabolism inhibits mTORC1 activation while promoting mTORC2 signaling pathways. **(a)** Western Blot for acute mTOR pathway signaling with JPH-203 treatment. **(b)** Western Blot of mTOR signaling in Th1 cells skewed for 6 days in the presence or absence of JPH-203.

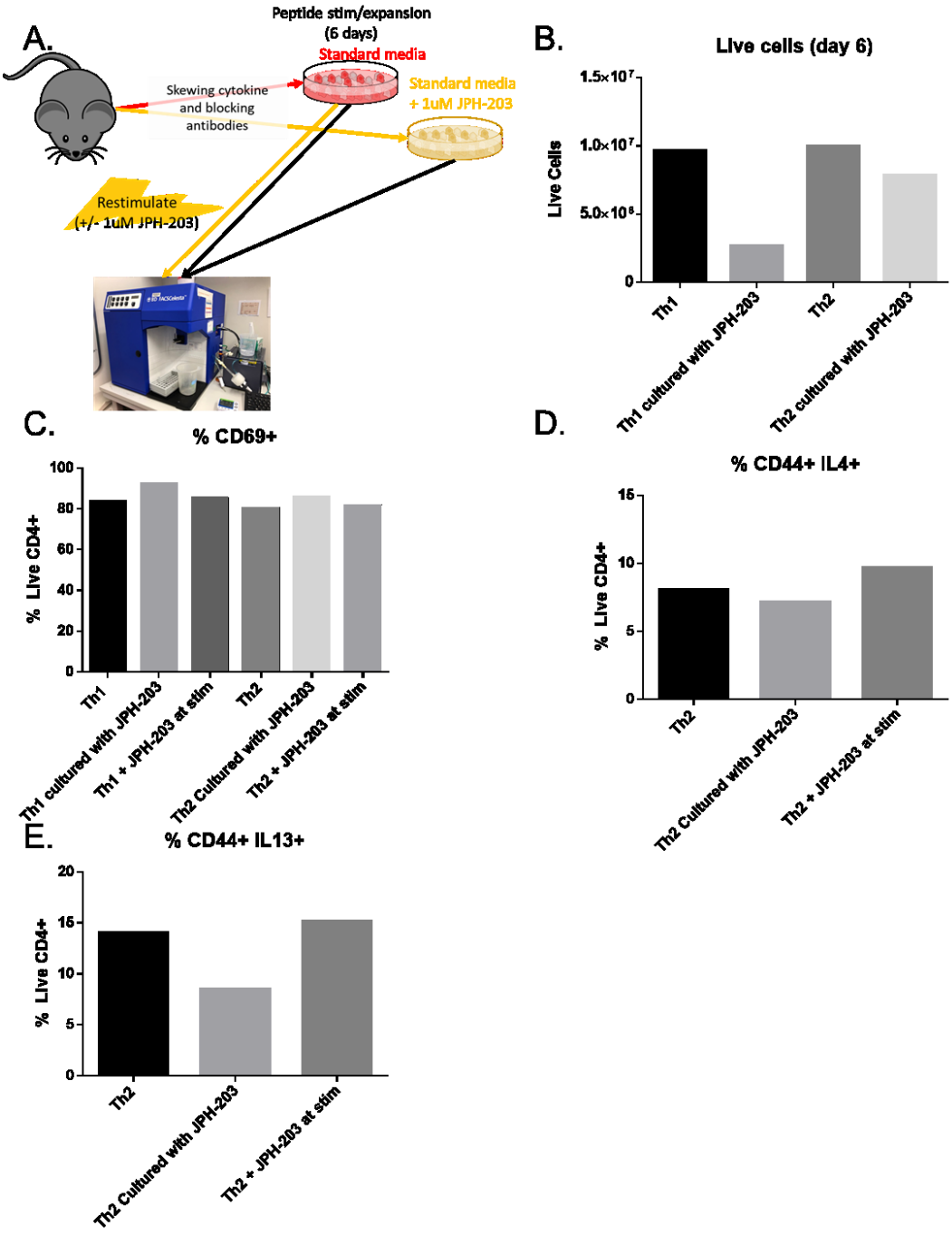

Figure 222. By blocking leucine metabolism, skewing of CD4 T cells is redirected from Th1 cytokines to Th2 cytokine production

Figure 22. By blocking leucine metabolism, skewing of CD4 T cells is redirected from Th1 cytokines to Th2 cytokine production. **(a)** experimental outline. **(b)** live cell counts following 6 days of CD4 T cell culture in the presence or absence of JPH-203. **(c)** assessment of CD69 expression by flow cytometry following CD4 T cell restimulation. **(d)** assessment of IL4 production following stimulation by flow cytometry. **(e)** as in (d) but for IL13 production.

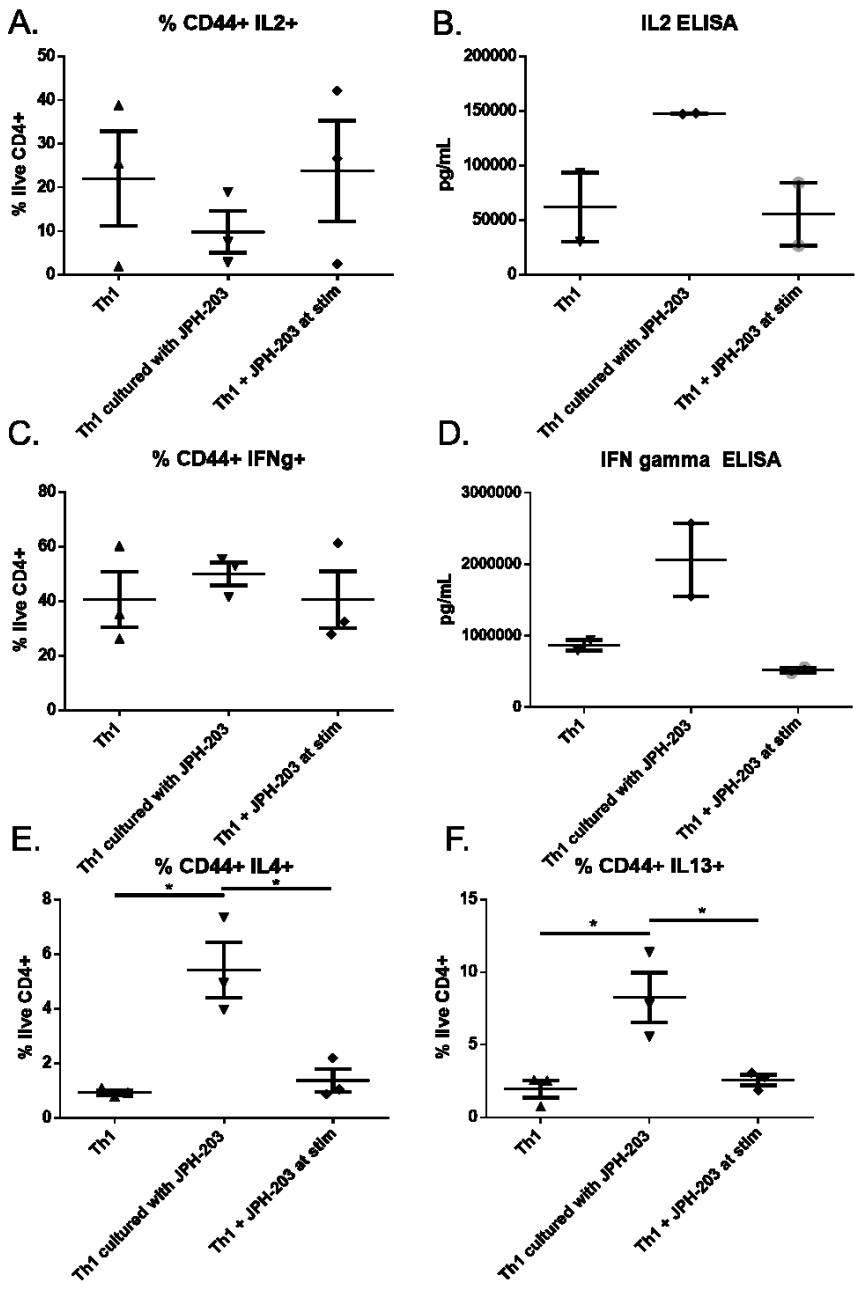

Figure 23. Inhibition of leucine metabolism imparts Th2 character to skewed Th1 T cells

Figure 23. Inhibition of leucine metabolism imparts Th2 character to skewed Th1 T cells. **(a)** stimulation of Th1 cells cultured for 6 days to assess IL2 production by flow cytometry. **(b)** stimulation of Th1 cells cultured for 6 days overnight to assess IL2 production by ELISA. **(c)** as in (a) but for IFN gamma production. **(d)** as in (b) but for IFN gamma production. **(e)** as in (a) but for IL4 production. **(f)** as in (a) but for IL13 production.

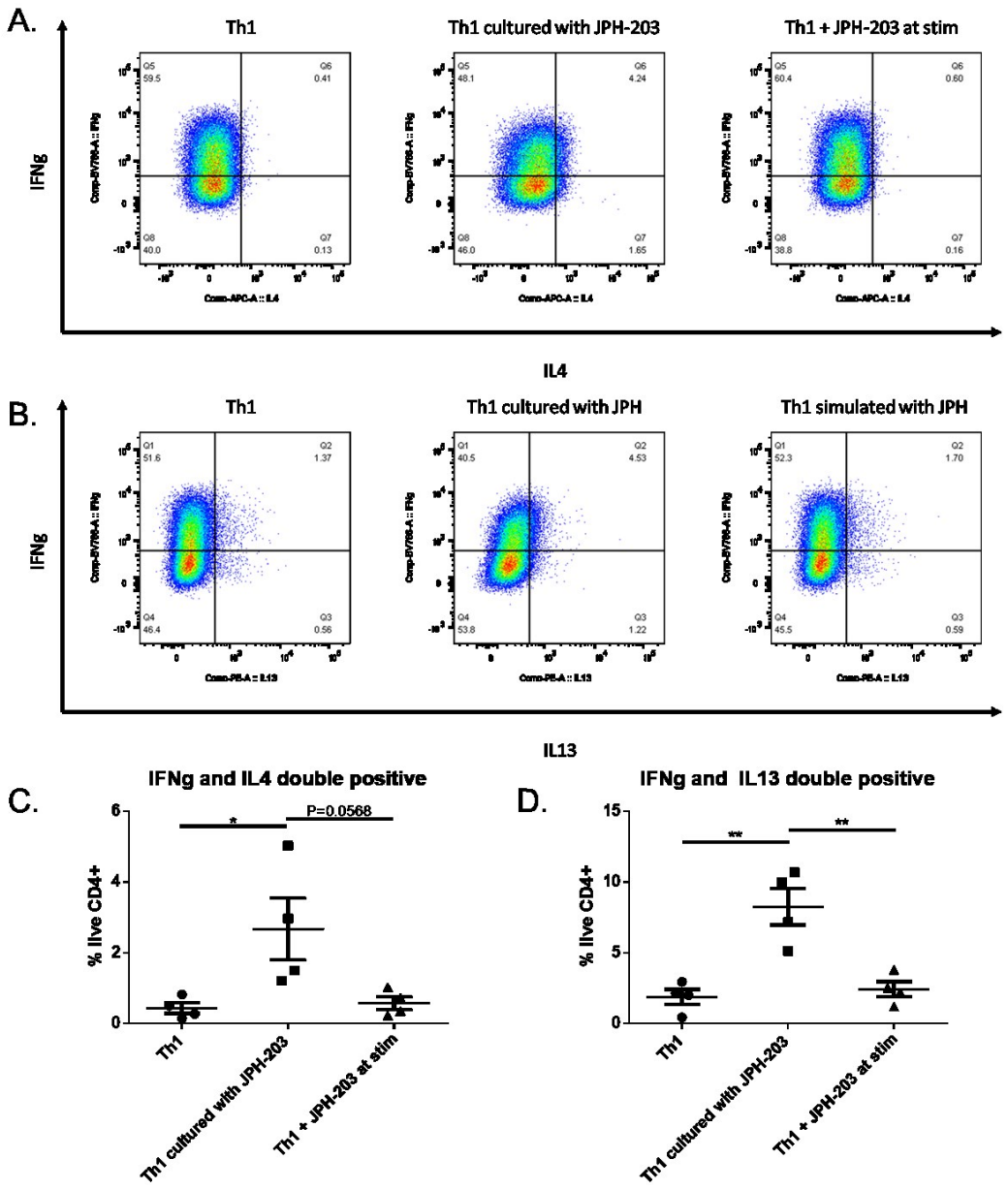

Figure 234. Th1 cells treated with JPH-203 become competent to produce both Th1 and Th2 cytokines simultaneously

Figure 24. Inhibition of leucine metabolism imparts Th2 character to skewed Th1 T cells. **(a)** flow cytometry plots of Th1 cells cultured with JPH-203 assessing IFN gamma and IL4 production. **(b)** as in (a) but for IFN gamma and IL13 production. **(c)** summary graph for cells as in (a). **(d)** summary graph for cells as in (b).

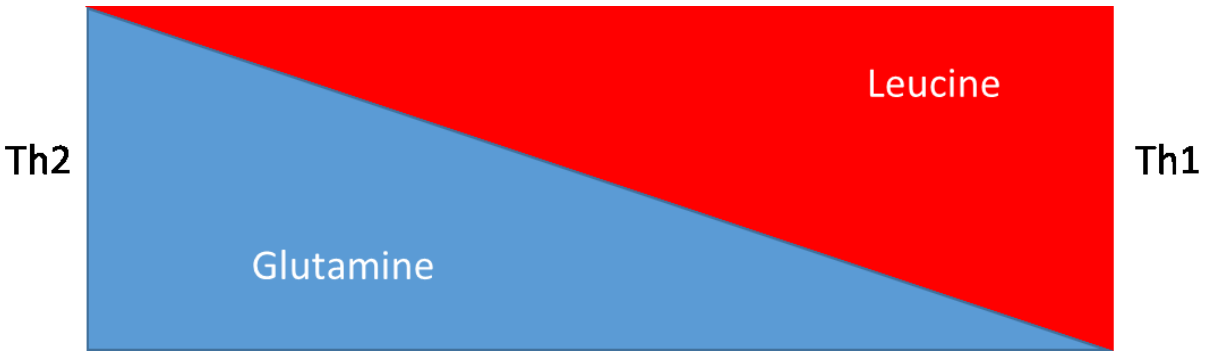

Figure 25. Model for the effect of the inhibition of amino acid metabolism on CD4 T cell skewing towards Th1 and Th2 subsets

Figure 25. Model for the effect of the inhibition of amino acid metabolism on CD4 T cell skewing towards the Th1 and Th2 subsets.

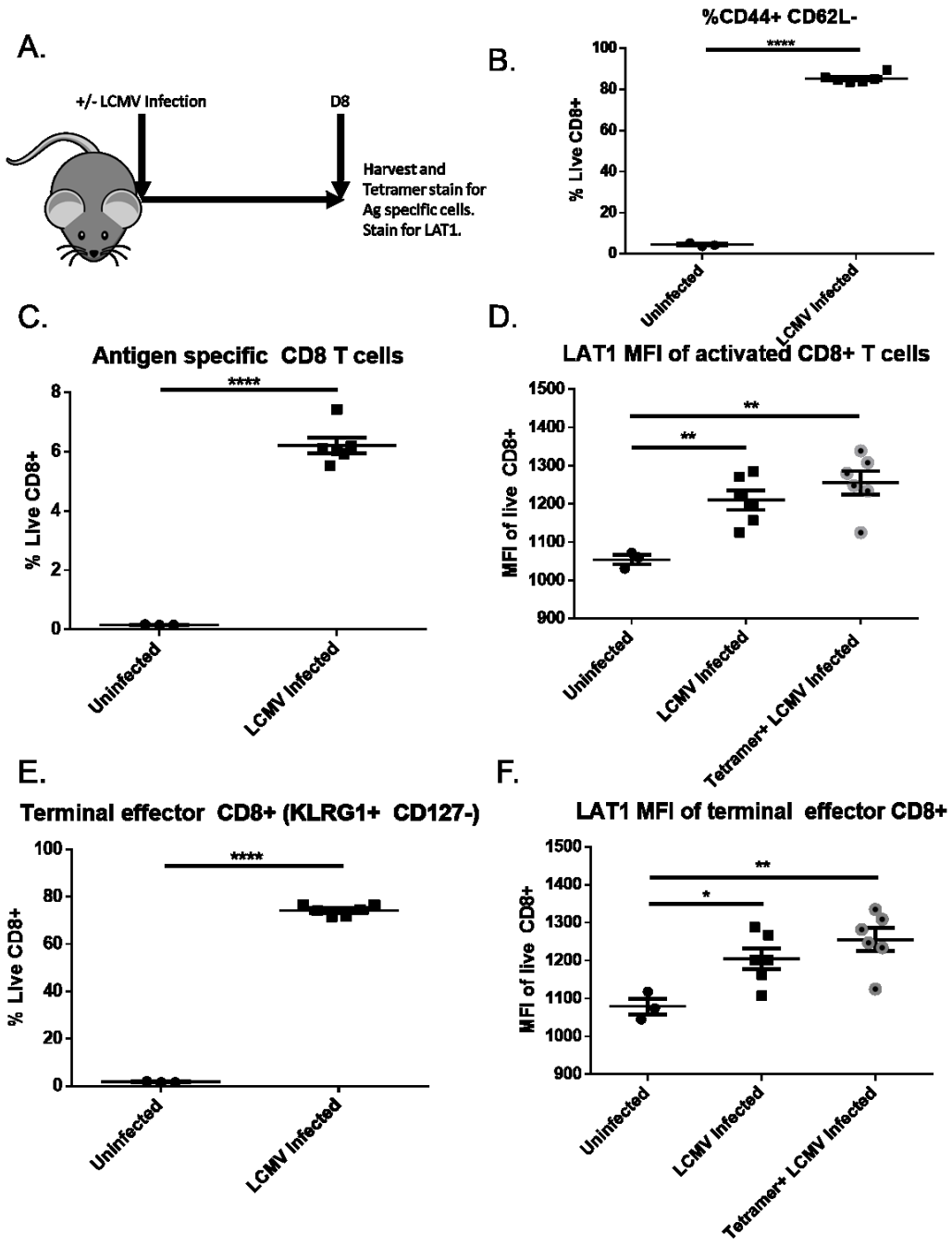

Figure 26. LAT1 (Slc7A5) is highly expressed in vivo on activated CD8 T cells

Figure 26. LAT1 (Slc7A5) is highly expressed *in vivo* on activated CD8 T cells. **(a)** experimental outline. **(b)** expression of CD44 as assessed by flow cytometry. **(c)** percent of antigen specific cells as assessed by tetramer staining. **(d)** expression of LAT1 as assessed by flow cytometry. **(e)** quantification of terminal effector phenotype. **(f)** LAT1 expression as assessed by flow cytometry in terminal effector cells.

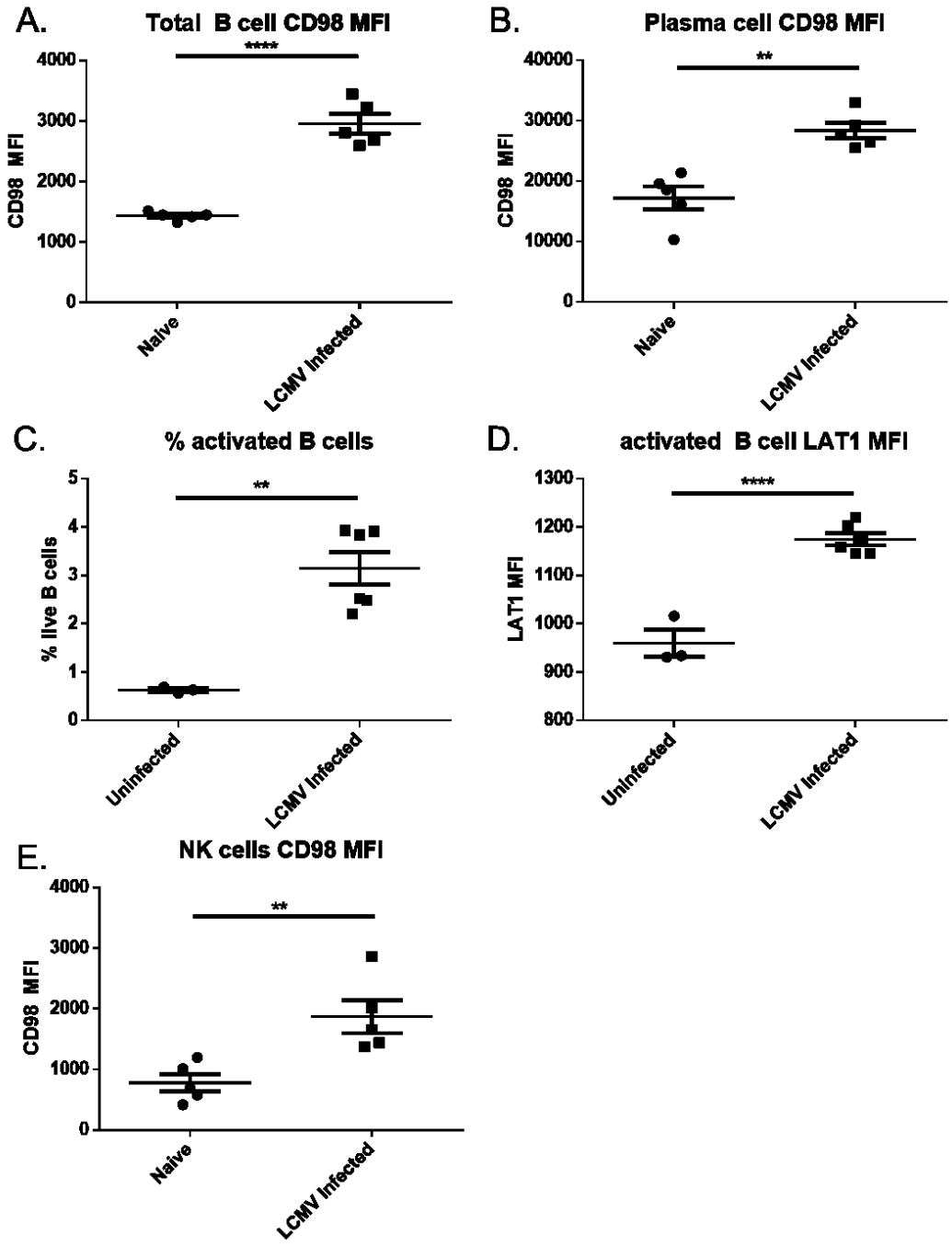

Figure 27. Leucine transporter expression is high on other activated immune cells.

Figure 27. Leucine transporter expression is high on other activated immune cells. **(a)** CD98 expression on B cells as assessed by flow cytometry. **(b)** as in (a) but for plasma cells. **(c)** percent of activated B cells as assessed by flow cytometry. **(d)** LAT1 expression as assessed by flow cytometry in activated B cells. **(e)** as in (a) but for NK cells.

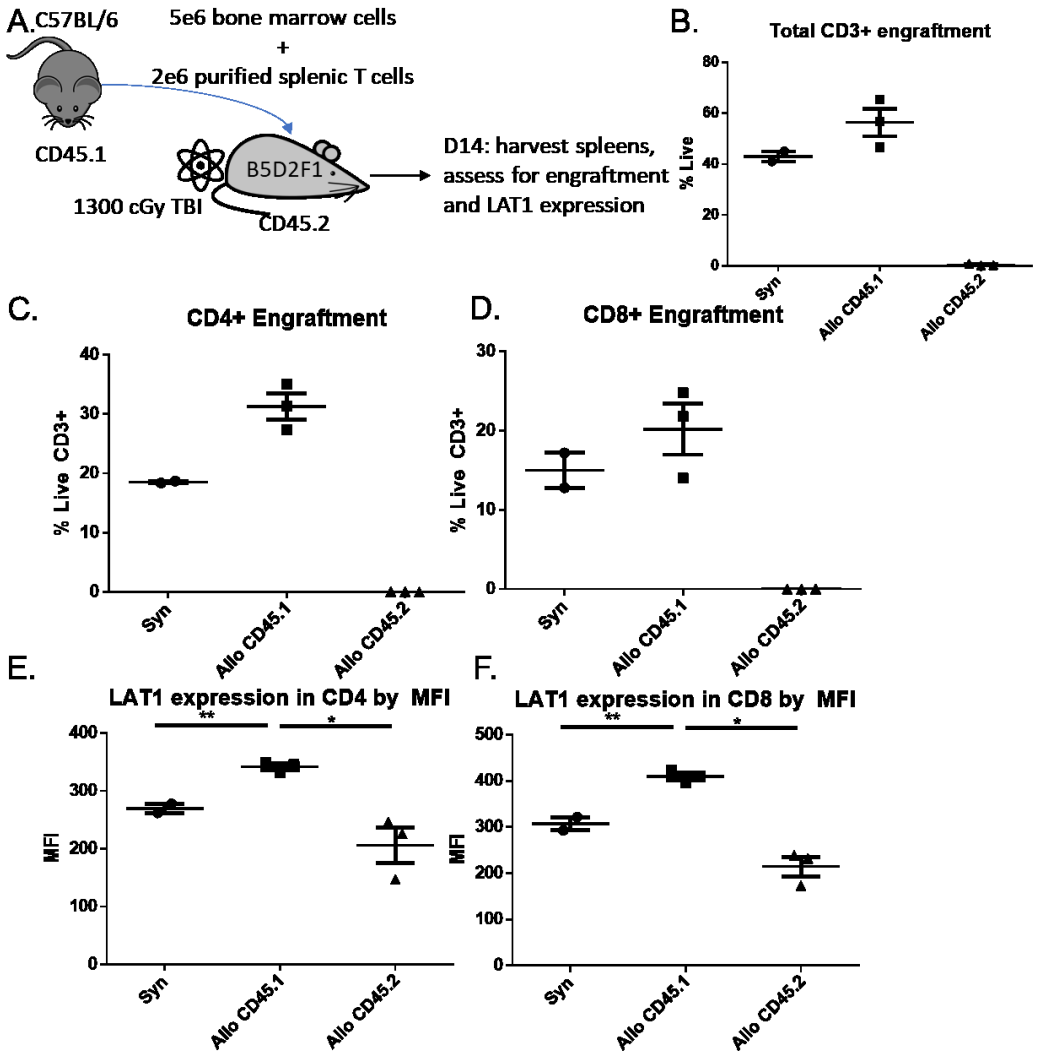

Figure 248. LAT1 expression is high on activated T cells in the context of GvHD

Figure 28. LAT1 expression is high on activated T cells in the context of GvHD. **(a)** experimental model. **(b)** total CD3 engraftment as assessed by flow cytometry. **(c)** as in (b) but for CD4 T cells. **(d)** same as (b) but for CD8 T cells. **(e)** LAT1 expression in CD4 T cells as assessed by flow cytometry. **(f)** as in (e) but for CD8 T cells.

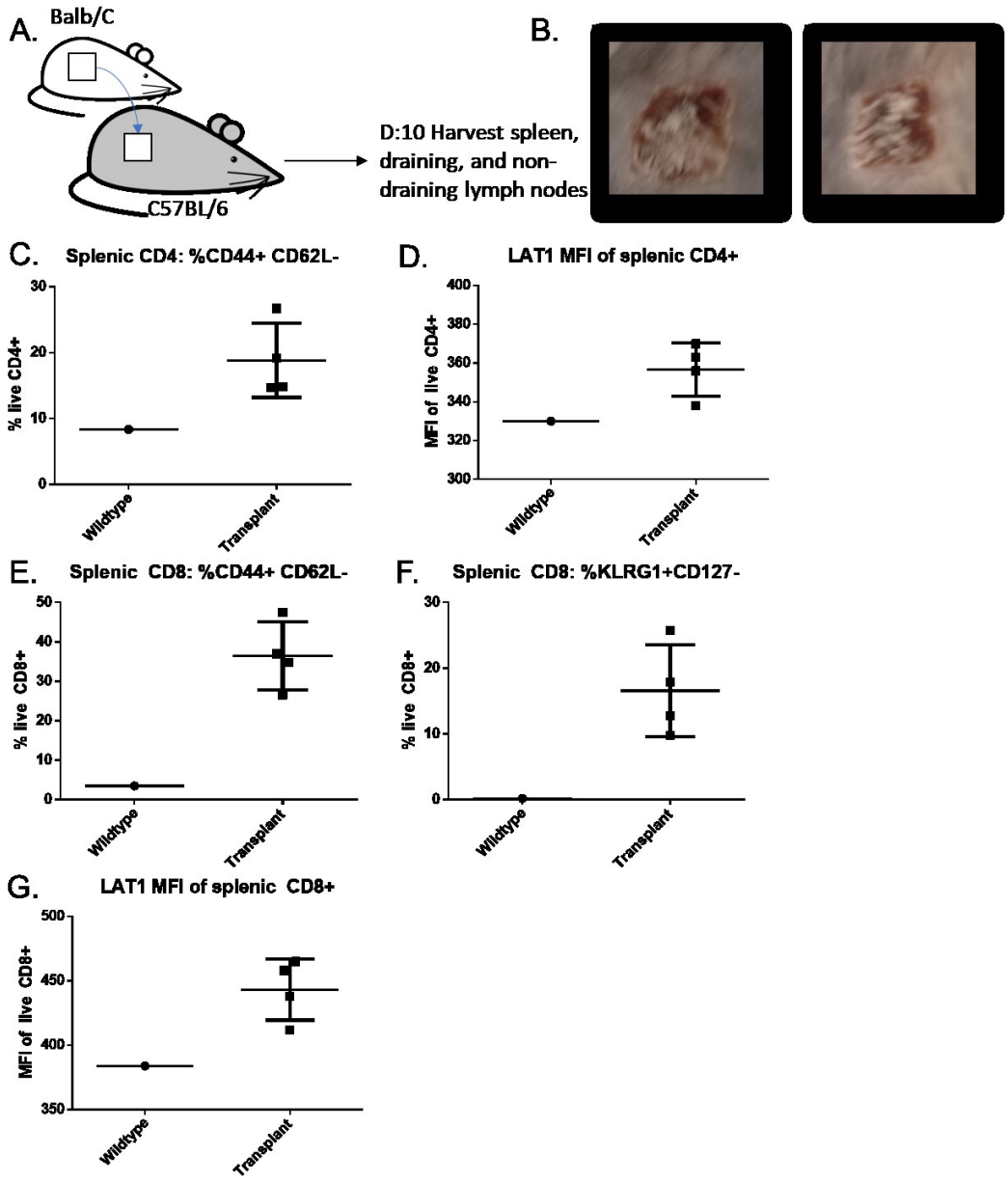

Figure 2925. LAT1 expression is high on activated T cells in the context of skin transplant.

Figure 29. LAT1 expression is high on activated T cells in the context of skin transplant. **(a)** experimental model. **(b)** photos of skin grafts. **(c)** CD44 expression on splenic CD4 T cells. **(d)** LAT1 expression of splenic CD4 T cells. **(e)** as in (c) but on splenic CD8 T cells. **(f)** quantification of terminal effector phenotype on splenic CD8 T cells. **(g)** as in (d) but on splenic CD8 T cells.

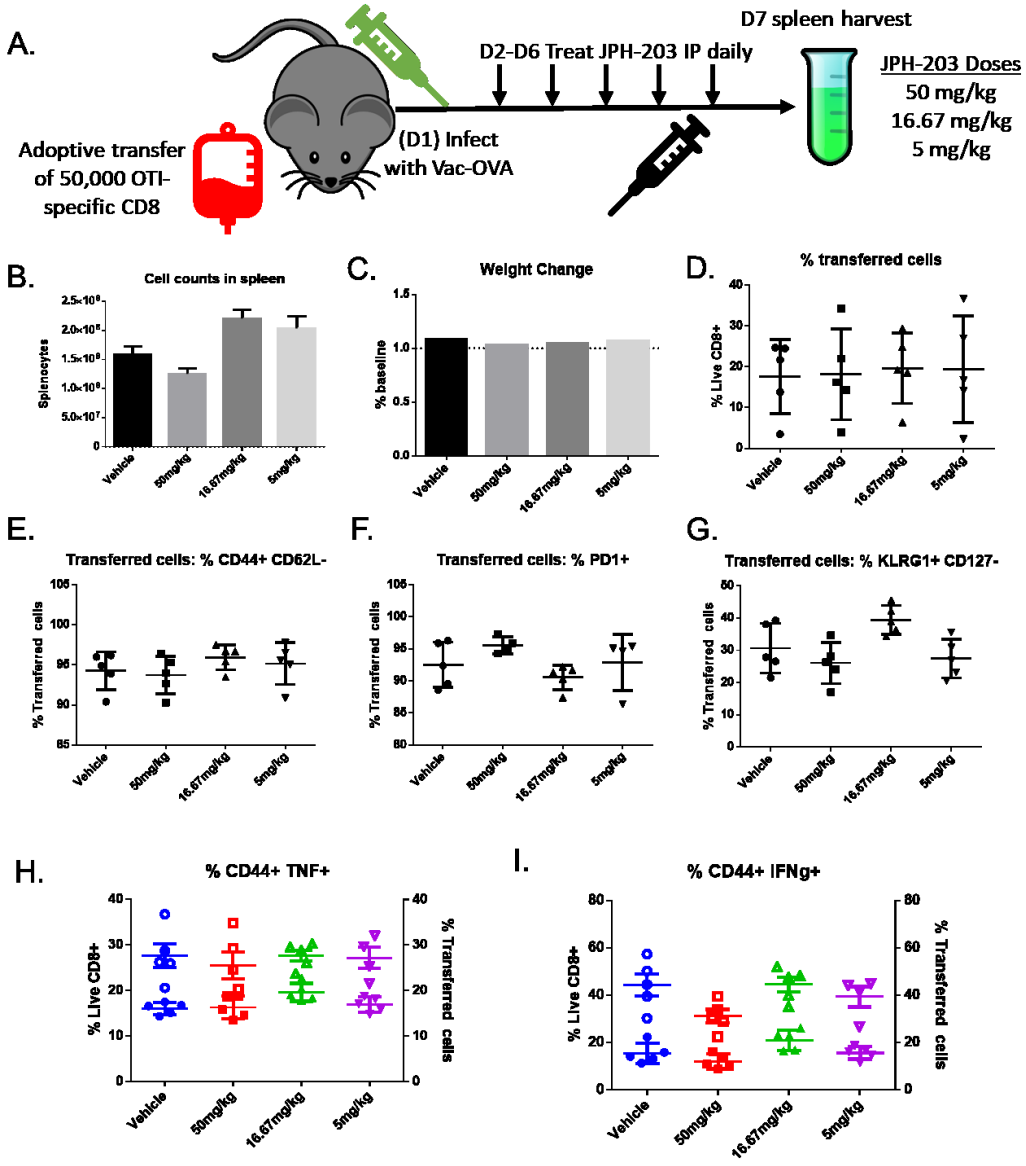

Figure 260. Inhibition of LAT1 in vivo with JPH-203 was unsuccessful in a viral model.

Figure 30. **Inhibition of LAT1 *in vivo* with JPH-203 was unsuccessful in a viral model. (a)** experimental model. **(b)** Splenocyte counts of treated mice. **(c)** weight change of treated mice. **(d)** percent of transferred CD8 T cells in treated mice. **(e)** expression of CD44 in transferred CD8 T cells. **(f)** as in (e) but for PD1. **(g)** as in (e) but for terminal effector phenotype. **(h)** production of TNF following *ex vivo* restimulation as a percent of total CD8 T cell population (filled symbols) or transferred cell population (open symbols). **(i)** as in (e) but for IFN gamma production.

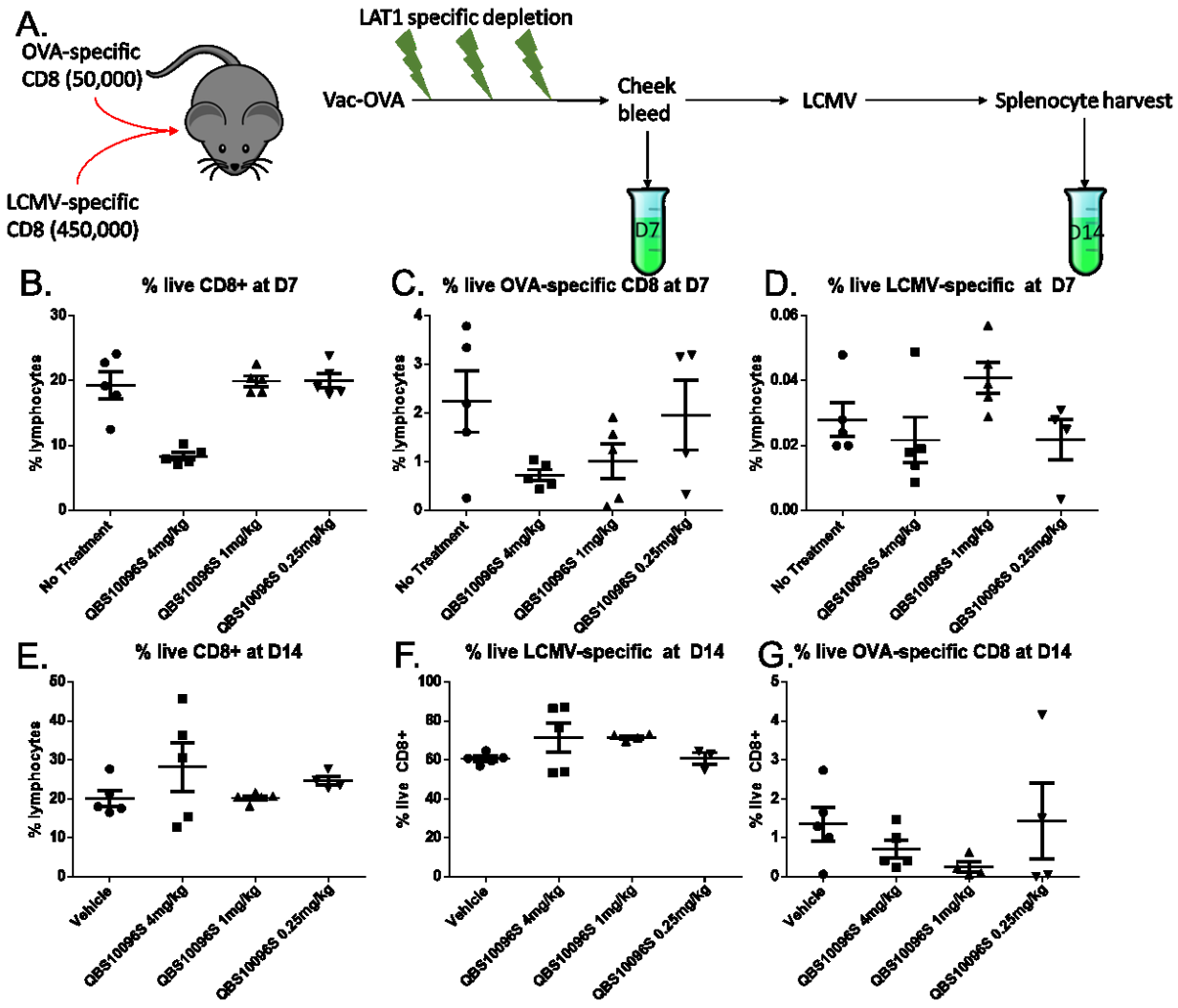

Figure 271. Depletion of LAT1 expressing cells with a targeted cytotoxic agent potently and selectively inhibits CD8 T cell

Figure 31. **Depletion of LAT1 expressing cells with a targeted cytotoxic agent potently and selectively inhibits CD8 T cell response. (a)** experimental model. **(b)** Live CD8 T cells from cheek bleed at day 7. **(c)** as in (b) but for OVA-specific cells. **(d)** as in (b) but for LCMV-specific cells. **(e)** splenic CD8 T cells at day 14. **(f)** as in (f) but for LCMV-specific cells. **(g)** as in (e) but for OVA-specific cells.

response

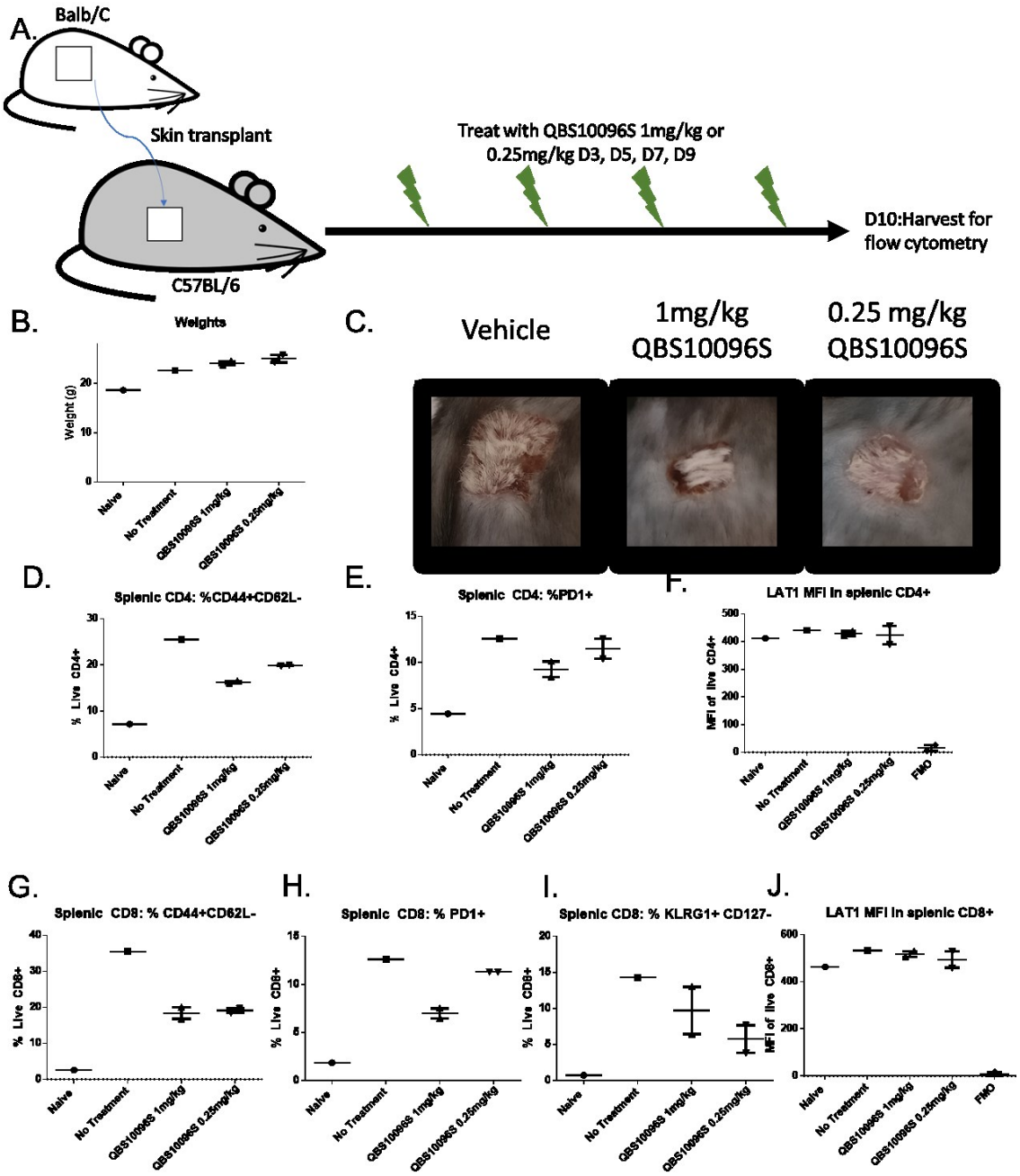

Figure 282. Depletion of LAT1 expressing cells has a moderate effect in skin transplant.

Figure 32. **Depletion of LAT1 expressing cells has a moderate effect in skin transplant. (a)** experimental model. **(b)** weights of mice at harvest. **(c)** photographs of skin grafts at time of harvest. **(d)** expression of CD44 in splenic CD4 T cells. **(e)** as in (d) but for PD1 expression. **(f)** as in (d) but for LAT1 expression. **(g)** as in (e) but for CD8 T cells. **(h)** as in (e) but for CD8 T cells. **(i)** terminal effector phenotype of splenic CD8 T cells. **(j)** as in (f) but for CD8 T cells.

Chapter 5: Conclusions and future directions

This thesis has focused on the important and impactful role that metabolism plays on the T cell immune responses. In the first chapter, the essential role of metabolism in the immune system was reviewed. TCR mediated activation induces a rapid increase in glucose uptake and increased glycolysis, even in the presence of abundant oxygen. This phenomenon, known as the Warburg effect for its discovery by Otto Warburg in his studies of cancer cells, is essential to the activation of T cells, but in order to keep the TCA cycle spinning and generate biosynthetic intermediates, the amino acid glutamine is taken up at a vastly increased rate so that it can replenish the TCA cycle to keep it spinning, as well as contribute to the biosynthesis of nucleotide precursors, protein glycosylation events, and a number of other processes within the newly activated T cell. While what are now considered the basics of immunometabolism were discovered upon activation, and were initially thought of as secondary to the processes following TCR stimulation, it soon became clear that activation did not control metabolism, but rather that metabolism controls activation. This was borne out by several studies in both CD4 and CD8 T cells that demonstrated the metabolic control of T cell differentiation and fate. In CD4 T cells, activity of the kinase mTOR has been demonstrated to play a key role in subset skewing, with both genetic and pharmacologic experiments demonstrating that Th1 and Th17 cells require mTORC1 activity for their fate commitment decisions, while Th2 cells require mTORC2 activity, and the absence of signaling from either complex leads to a Treg fate[26] . In CD8 T cells, metabolism has been tied very directly to memory generation, with increased ECAR being associated with short lived effector cells, and OXPHOS, particularly mitochondrial health indicated by parameters such as high SRC, large and highly fused mitochondrial networks, and low mitochondrial membrane potential, have been associated with memory phenotypes. Crucially, experiments in which metabolism has been modified, using both genetic and pharmacologic means, as well as experiments in which metabolic parameters are used to select

between cell populations have indicated metabolic perturbation determines cell fate outcomes, indicating that rather than being a byproduct of activation, metabolism tightly controls T cell responses and fate decisions. This theme of metabolism controlling T cell fate is the guiding one in this work. The amino acid glutamine is shown to have an important role in fate determination in both CD4 and CD8 T cells, and the amino acid leucine is shown to be crucial in CD4 T cell subset skewing. Furthermore, increases in leucine uptake, as indicated by the upregulation of its transporter LAT1 on the surface of activated cells serve as a biomarker of activated cells and in situations of pathogenic T cell activation such as GvHD or rejection of allogenic skin transplant, by which these cells can be targeted and depleted using a LAT1 specific cytotoxic agent. These findings demonstrate the crucial role metabolism plays in intricately shaping the immune response. Metabolism is not a digital on/off switch to the immune response, but rather is an analogue selector of a number of different fates, such that modulation of metabolism can be used in very precise ways to alter the immune response in order to promote the desired outcome for an immune response, a concept which will be discussed in greater depth later in this chapter.

The role of the amino acid glutamine in cellular proliferation has been widely known for some time, and it is often referred to as “conditionally essential” in proliferating cancer cells as well as T cells. In CD4 T cells, the role of glutamine in T cell proliferation was confirmed by experiments in which glutamine metabolism was restricted, either by use of the broadly specific inhibitor of glutamine metabolism DON, or by culture in restricted glutamine media inhibited T cell proliferation, however had little effect on T cell activation as assessed by CD44 expression. However, when CD4 T cells were skewed in low glutamine media in either standard 4mM glutamine media, or low glutamine media, little effect on Th1 cell skewing was observed as assessed by Tbet expression or cytokine production on either the protein or RNA levels. In contrast, CD4 T cells skewed to the Th2 subset were found to be sensitive towards glutamine restriction, exhibiting a decreased production of Th2 cytokines at both the protein

and RNA levels. The cause of this decrease in cytokine expression was determined to be reduced epigenetic availability of the Th2 cytokine locus, as glutamine derived aKG is an essential cofactor for histone demethylases, and metabolism contributes to the acetyl-CoA pool available for histone acetylation. The effects of inhibition of glutamine metabolism were next assessed in an HDM induced asthma model, with the use of both DON and a novel DON-derived prodrug JHU-083. Inhibition of glutamine in this Th2 biased model inhibited the development of type 2 immune cells, including Th2 CD4 T cells, M2 macrophages, and ILC2. Importantly, it did not inhibit the M1 function of alveolar macrophages or the development of Tregs in either the lungs or the spleen, once again illustrating the differential in glutamine requirement in Th1 and Th2 responses. This decrease in type 2 immune cells, and particularly Th2 CD4 T cells, was due to decreased proliferation, leading to fewer antigen specific cells in the lungs of HDM asthma mice as determined by tetramer staining. This leads to a model in which established asthma can be treated with glutamine metabolism inhibition at the time of an acute asthma attack, inhibiting the development and effector function of type 2 immune cells and the proliferation and accumulation of antigen specific Th2 cells. This indicates that modulation of glutamine metabolism can be used to inhibit a pathogenic Th2 response while promoting Tregs demonstrates the power of metabolism to tweak the immune response, and that restricting glutamine metabolism does not entirely inhibit the response, but instead redirects it.

Next, the role of the amino acid glutamine was assessed in CD8 T cells. As previously stated, effector CD8 T cells rely primarily on glycolytic metabolism, exhibiting a high ECAR, while memory CD8 T cells depend on mitochondrial metabolism. We attempted to expand on this dichotomy by the *in vitro* restriction of glutamine metabolism at the time of stimulation and initial culture. Culture of CD8 T cells in low glutamine media, while once again limiting T cell proliferation, also promoted a more memory-like phenotype *in vitro*, as indicated by an increased in CD44 positive CD62L positive “central memory” T cells, with increased expression of the anti-apoptotic protein BCL2 and increased histone H3K27me3,

well as decreased expression of the PD1 checkpoint marker. Thus, culture in low glutamine media, while leading to a decreased total cell output than culture in standard 4mM glutamine media, dramatically changes the quality of the cells yielded, potentially leading to a longer-lived memory cell. Next, transgenic CD8 T cells stimulated and cultured in either standard 4mM glutamine media or low glutamine media were adoptively transferred into congenically distinct mice, and stimulated in the mouse by viral infection. When circulating CD8 T cell levels were assessed by serial cheek bleeding, it was determined that culture in low glutamine media lead to a more persistent cell population. Furthermore, when cells were re-challenged by subsequent infection, it was determined that the adoptively transferred cells that were initially cultured in low glutamine media were better memory cells, expanding to a higher degree, maintaining their central memory CD44 positive CD62L positive phenotype, and were more polyfunctional in regards to their cytokine production. When a short term, 48-72 hour adoptive transfer experiment was performed, it was determined that the increased cell population yielded by the adoptive transfer of low glutamine cultured cells was due to increased cell survival as compared to cells cultured in standard 4mM glutamine media, potentially due to the increased expression of BCL-2 seen *in vitro*. Use of low glutamine cultured cells as ACT in a B16OVA tumor model was beneficial in shrinking tumor growth as well as improving the survival of treated mice as opposed to ACT with cells transferred in standard 4mM glutamine media, however short term TIL analysis indicated significantly less infiltration of the tumor by cells cultured in low glutamine media as opposed to standard 4mM glutamine media, indicating that enhanced persistence, rather than increased effector function, is the reason for increased anti-tumor responses in B16OVA mice treated with low glutamine cultured ACT. Of note in this work is that the only perturbation to which these cells were submitted was the initial short term, 3-4-day stimulation and culture in low glutamine media. No treatment was carried out in the mice after transfer in either the viral memory or tumor models. This illustrates the importance of metabolism, and particularly metabolism at the time of activation, on T cell

fate decisions. The short-term restriction of glutamine metabolism dramatically shapes the immune response, promoting the development of memory CD8 T cells, illustrating once again that metabolism can powerfully direct T cell responses, and that rather than being a byproduct of T cell activation, metabolism is the driving force behind shaping a T cell response.

In the fourth chapter of this thesis, the role of leucine in fate decisions was addressed in both CD4 and CD8 T cells. As previously described, leucine, while an essential amino acid obviously required for T cell proliferation, is also critical for mTORC1 activation[142]. Signaling pathways downstream of mTOR play essential roles in the directing of immune responses, and thus we were interested in the assessment of the role of leucine metabolism in both CD4 and CD8 T cell differentiation. First, we used the highly selective inhibitor of leucine uptake through LAT1, JPH-203, in *in vitro* experiments to demonstrate that, in keeping with previous data on both cancer cell and T cell activity with JPH-203 treatment, the proliferation of both CD4 and CD8 T cells was inhibited in a dose dependent manner with leucine metabolism was inhibited by JPH-203. However, similar to our findings in glutamine metabolism inhibition, inhibition of leucine metabolism did not globally inhibit the immune response, but rather redirected it. In this way, we noted that CD4 T cells skewed towards the Th1 subset were especially sensitive to proliferation inhibition induced by JPH-203 treatment. While cytokine production from cells skewed towards the Th2 subset was unaffected by JPH-203 treatment, the cells generated from Th1 skewing culture in the presence of the leucine uptake inhibitor were capable of producing Th1 hallmark cytokines IFN gamma and IL2, but surprisingly also produced the Th2 hallmark cytokines IL4 and IL13 at levels similar to cells that had been polarized in Th2 skewing conditions. Flow cytometry analysis revealed that the cytokine producing cells were double positive for both Th1 and Th2 cytokines, illustrating that the Th1 cells have been "re-wired" towards more of a Th2 development fate following the inhibition of leucine uptake, once again indicating the essential role of metabolism in determining cell fate decisions.

Next, we set our sights in a more translational direction, and sought to determine if the characteristic upregulation of LAT1 on activated T cells could be targeted and manipulated in a beneficial way. Previous research has demonstrated that LAT1 (*Slc7a5*) upregulation occurs very rapidly after TCR stimulation in T cells, and LAT1 expression is induced to a very high degree[145]. We first confirmed these findings in a viral model, as well as demonstrating LAT1 expression following viral infection in activated B and NK cells. Next, we assessed LAT1 expression in a variety of settings of pathogenic activation, including a BMT model of GvHD, allogenic skin transplant, and allogenic orthotopic heart transplant. We found that LAT1 was upregulated on the pathogenic, donor-derived T cells in the BMT model, and was upregulated to a higher degree than the syngeneic transplant. We also found LAT1 upregulation and expression in the skin transplant model in both CD4 and CD8 T cells obtained from the spleen and draining lymph nodes of the transplanted mice. Interestingly, we did not find the same dramatic upregulation of LAT1 expression in the heart transplant model, were we assessed T cells obtained from the spleen, draining and non-draining lymph nodes, and infiltrating cells from the graft itself. Our first attempt to capitalize on this finding was to inhibit leucine uptake in a viral infection setting with the *in vivo* administration of JPH-203. Although our dosing level and schedule was similar to previously published work, we were unable to demonstrate an effect with *in vivo* JPH-203 administration, possible due to differences in route of administration. As these *in vivo* inhibition studies were unsuccessful, we next sought to attempt an *in vivo* depletion of LAT1 positive cells by using a LAT1 specific cytotoxic agent, QBS10096S. Treatment with this cytotoxic in a co-adoptive transfer model of viral infection robustly indicated that the cytotoxic agent was highly specific for activated cells that are expressing LAT1, as it was shown to deplete the antigen specific adoptively transferred cells, while the transferred cells that were not antigen specific were unaffected, and remained to be stimulated by a subsequent infection that occurred in the absence of treatment with the cytotoxic agent. Based on this, we attempted to treat skin transplant mice with this cytotoxic agent, and demonstrated a dose

dependent decrease in T cell activation following treatment with QBS10096S. These findings demonstrate that leucine metabolism is essential in both CD4 and CD8 T cells, and that by inhibiting this metabolism we do not shut down the immune response, but rather alter it, and in contrast to our studies of glutamine metabolism, we shift the balance away from Th1 CD4 T cells, and towards the Th2. Further *in vivo* work on the expression of the leucine transporter LAT1 on both CD4 and CD8 T cells confirms that this transporter is rapidly upregulated in a variety of activated contexts, including the pathogenic activation of transplant models, and demonstrates the potential for selective LAT1 depletion to inhibit disease in these models. These findings demonstrate that modulation of leucine metabolism can be an effective strategy for the shifting of an immune response, as well as a potential translational target in transplant and other models of pathogenic T cell response.

Conclusions: the role of metabolism in the shaping of the immune response

Throughout this thesis, great attention has been paid to the fact that modulation of the immune response by the inhibition or restriction of amino acid metabolism is not a global on/off switch, but rather can be used to precisely shape the character of the immune response. While it would be easy to dismiss metabolic therapies as just the wholesale inhibition of an immune response, the reality is that things are not merely being inhibited, but fundamentally altered, and that these alterations can be therapeutically exploited in different ways. For example, the restriction of both glutamine and leucine metabolism in CD4 T cells are similar in that they do not affect activation as assessed by either CD44 or CD69 expression, and both inhibit T cell proliferation. Although these effects were similar between glutamine and leucine inhibition, the end results of these perturbations were found to be distinct, in that CD4 T cells were less capable of skewing towards the Th2 subset following inhibition of glutamine metabolism, which skewing towards the Th1 subset was inhibited in conditions of leucine metabolism inhibition, indicated by the drastic decrease in cell numbers, and that the cells that were yielded were not standard Th1 T cells, as they were also producers of the Th2 hallmark cytokines IL4 and IL13. This

illustrates that metabolism plays an important role in shaping the immune response, and that the metabolic changes that occur upon T cell stimulation are not secondary to activation, but rather play a very powerful role in controlling and shaping the result of the activation event.

The opposing fates of CD4 T cell subset commitment following restriction of either glutamine or leucine metabolism is a very interesting dichotomy. The model proposed for the modulation of CD4 T cell fate commitment by amino acid metabolism (**Fig. 25**) which illustrates the importance of leucine for Th1 polarization while it is dispensable to Th2 cell development, while glutamine is essential for Th2 polarization and is dispensable to Th1 cells, is interesting because it is reminiscent of the differential role of mTORC1 and mTORC2 signaling in CD4 T cell polarization, with mTORC1 activity being necessary for Th1 and Th17 development, mTORC2 activity being required for the development of Th2 cells, and the lack of mTOR kinase activity leading to a Treg fate[26]. There are likely some ways in which the mTOR signaling and amino acid metabolism models interact and overlap, for example there was little effect on mTORC1 activity following low glutamine media culture as indicated by flow cytometry staining for P-S6 (**Fig. 2e**), as well as is no inhibition of IL17 production following DON or JHU-083 treatment in the HDM model (data not shown), as well as limited efficacy of glutamine inhibitor therapy in a Th1/Th17 biased collagen induced arthritis (CIA) mouse model (data not shown). Furthermore, culture with either glutamine restriction (DON treatment or low glutamine media) and inhibition of leucine metabolism by treatment with JPH-203 both lead to decreased cell size as determined by flow cytometry forward scatter plots, and control of cell size is an essential role mTOR activity. However, the modulation of mTOR activity does not adequately explain all of the differences noted in CD4 T cell skewing between the restriction of glutamine and leucine. While mTORC2 activity is promoted by leucine inhibition in culture, little effect on mTORC1 is seen with the 1uM JPH-203 treatment that is sufficient to “re-wire” Th1 differentiation (**Fig. 21a**). Therefore, while mTOR activity clearly plays a clear role in the dichotomy

of CD4 T cell subset skewing following selective restriction of amino acid metabolism, there is more to the story that remains to be identified.

However, this pattern of differential amino acid requirements for CD4 T cell subset responses could play a very important role in a variety of settings. The ability to modulate the immune system between a more inflammatory, Th1 type response and a Th2 response would be a powerful manipulation for the therapeutic direction of immunity. There are a number of circumstances under which modulation of this dichotomy could be helpful, such as inhibition of a Th1 response through the restriction of leucine in inflammatory diseases such as arthritis or acute transplant rejection. Furthermore, examples of the selective inhibition of Th2 biased diseases such as asthma have clear therapeutic implications, and even the resolution of the "wound that does not heal" Th2 aspect of cancer through the inhibition of glutamine metabolism, a strategy which has already been demonstrated to be effective by Leone and colleagues[93]. Interestingly, targeted GLS inhibition in cancer was observed to be less effective than broad inhibition with DON treatment, and therefore specific inhibition of leucine uptake by LAT1 could be only scratching the surface of the possibility of the role of leucine in modulating the T cell response. Further studies examining the role of leucine by use of depleted media, similar to was done in the low glutamine culture media system may be warranted. Furthermore, a more direct assessment of the interplay between glutamine and leucine in CD4 subset commitment would also be an interesting next step. Experiments that, for example, test JPH-203 treatment in the setting of low glutamine culture media could reveal important insights in the ways these two metabolic pathways interact. Based on the model of the role of mTOR in CD4 subset commitment, it may be expected that inhibition of both glutamine and leucine metabolism may promote the development of Tregs, as occurs in the absence of both mTORC1 and mTORC2 signaling. This could potentially serve as a powerful therapeutic approach for the development of immune tolerance in transplant settings without the long-term use of immunosuppressant drugs and the side

effects associated. However, it is possible that one pathway is "upstream" of the other. As previously stated, glutamine efflux is required for leucine influx through the LAT1/CD98 transporter[143], and thus it is possible that combined inhibition of glutamine and leucine metabolism may resemble low glutamine culture. This would seem unlikely in our system, as there is still a small amount of glutamine (0.1mM roughly) in the media from the 10% FBS added, and this may facilitate leucine uptake in low glutamine culture. However, high doses of DON, which have been show previously[95] and in our hands (**Fig. 2e**) to inhibit mTORC1 signaling as assessed by p-S6 staining may act by inhibiting leucine uptake downstream of glutamine. Future studies to tease apart this pathway hold a great deal of promise for furthering our understanding of metabolic crosstalk, as well as opening up new therapeutic options for the modulation of immunity in a variety of disease contexts.

As demonstrated in chapter 2 of this thesis, glutamine plays an essential role for the development of Th2 cells and their competency to produce cytokine, and the presence of glutamine is required for the Th2 cytokine locus to open and become available for transcription. On the surface, this contrasts with previous work, for example the studies by Klysz and colleagues[35], who demonstrated the requirement for glutamine in the development of Th1 responses, and that cells polarized in the absence of glutamine take on a Treg phenotype, with the expression of FoxP3. In contrast to the research presented in chapter 2 of this thesis, those studies were carried out in the complete absence of glutamine. When these researchers carried out a glutamine titration experiment in which they added glutamine back into the media, they found that at the lowest concentration assessed, 0.4mM exogenous glutamine, the percentage of FoxP3 expressing cells was dramatically diminished (44% vs 3% by flow cytometry) and the expression of *FoxP3* mRNA was nearly absent. As we have a much lower concentration of glutamine in our low glutamine media system (roughly 0.1mM) the lack of effect of Th1 polarization in our setting is consistent with their observations. Furthermore, we did not observe FoxP3 expression in cells polarized either Th1 or Th2 in either low glutamine media settings or in the case of

low dose DON treatment (data not shown). The difference in these results demonstrates the difference between the complete absence of glutamine, and having low levels of the amino acid available, and illustrate the fine tune control of immune responses available by the modulation of metabolism. The question of why the epigenetic dependence on glutamine metabolism described herein is specific to Th2 cells as opposed to Th1 is an interesting and currently unanswered one. The role of aKG in epigenetic remodeling events in Th1 cells has been previously illustrated in work by Chisolm and colleagues, who demonstrated that glutamine derived aKG was necessary for the activation of a Th1 effector program and the associated changes in genomic structure that result from IL2 stimulation[112]. Once again, these events may require lower levels of glutamine than is required for the polarization of Th2 cells, leading to the observed differential in our system. The demonstrated requirement for glutamine in the development of Th2 responses may reflect the fact that stereotypically, Th2 responses are mounted against large, extracellular pathogens, and such responses would be expected to be long and very costly from a nutritional standpoint. Thus, the use of glutamine as a nutritional rheostat for the assessment of whether or not the host is nutritionally competent to mount such a response may be essential for ensuring that the response is successful. By tying a TCA cycle metabolite to this process, it can be ensured that this response can only be mounted by an organism healthy enough to do so, providing further evidence of the essential role of metabolism in the control of immune responses.

In chapter 3 of this thesis, the role of glutamine in the development of a CD8 T cell memory response was explored, and it was demonstrated that initial stimulation and culture in low glutamine media promotes a more long-live, memory like cell that is both more persistent in circulation and can better expand and demonstrate polyfunctional cytokine production upon re-challenge. The role of metabolism in promoting a memory CD8 T cell response has been well-illustrated and is one of the seminal findings in the nascent field of immunometabolism. Our studies demonstrating the restriction of glutamine metabolism promoting a memory CD8 T cell response contribute in that they are less

intrusive than genetic engineering approaches to introduce genes that promote OXPHOS and SRC, and thus are both simple and effective at promoting a cell that is metabolically "built to last." By promoting a long-lived memory phenotype in CD8 T cells, we are well positioned to improve recall responses in a variety of settings. One example would be as a "metabolic adjuvant" to vaccination strategies. For example, the administration of DON along with antigen and adjuvant, a strategy which would at first thought seem to be a bad idea, may actually promote a more long-lived memory response in settings of T cell-based vaccinations. It could be expected that this initial stimulation in conditions of restricted glutamine metabolism may promote the increased development of memory cells that would be better able to mount a protective response upon re-challenge. In this way, we had anticipated, both based on previous research[93,124] that inhibition of glutamine metabolism would lead to cells that are metabolically primed to do well in the nutrient depleted TME. While low glutamine ACT did promote an anti-tumor response and enhance the survival of tumor bearing mice, these effects were not reflected in the initial studies of TIL at an early timepoint. It is possible that there is a time lag in the ability of low glutamine cultured cells to be able to mount a response, as the most dramatic differences between the two groups were not seen until later in the tumor outgrowth studies. This could potentially be a result of restricted biomass in the low glutamine cultured CD8 T cells, and may be remedied by a short period of rest in standard 4mM glutamine media or other nutrient replete conditions, a strategy similar to as was used by Nabe and colleagues[124]. Another strategy would be to split the transferred cell population between the more effector-like T cells cultured in standard 4mM glutamine culture conditions in order to promote rapid tumor control, and low glutamine cultured cells in order to have sufficient memory-like cells to ensure long-term tumor control. The selection of therapy or a combination of therapies based on their metabolic properties in order to ensure the best possible response is another example of how a deeper understanding of T cell metabolism and the control that metabolism holds over immune

responses can be used to selectively modulate the immune system to promote the ideal response for a given set of circumstances.

In the 4th chapter of this thesis, the role of leucine metabolism on both CD4 and CD8 T cell subsets was explored. It was demonstrated that *in vitro* inhibition of leucine uptake through the LAT1/CD98 transporter had a selective inhibitory effect on the development of Th1 cells, and promoted Th2 cytokine production from these cells activated and cultured in Th1 skewing conditions. There are a number of settings in which this promotion of a more Th2 skewed response could be seen as beneficial, such as in enhancing wound healing or the acceptance of biological scaffolds in tissue reconstruction following injury. Further use of *in vivo* inhibition of leucine uptake through LAT1 rather than simply the cytotoxic depletion on LAT1 expressing cells would be a desirable approach moving forward, as it would allow more fine-tuned control of the response, for example short term inhibition of LAT1 transport rather than the simple depletion of a LAT1 expressing cell population. However, future studies must very carefully account for LAT1 expression in other tissues. As leucine is an essential amino acid, it can be expected that LAT1 will be expressed on a wide range of tissues. Furthermore, LAT1/CD98 can uptake a number of large, neutral amino acids in addition to leucine including isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine[141] and thus its presence on numerous tissues and the effects of its inhibition or selective targeting have the potential to be extensive. An early trial of QBS10096S mediated LAT1 specific cytotoxic depletion in the GvHD model was met with toxicity that required the early stoppage of treatment, potentially due to previously uncharacterized expression of LAT1 following irradiation. Some evidence of LAT1 expression outside of the T cell compartment exists, as we demonstrated LAT1 and CD98 expression on activated B cells and plasma cells as well as NK cells (**Fig. 27**). The inhibition of B cell metabolism has great potential to be beneficial in a variety of settings in which pathogenic B cell activity leads to disease, such as SLE, in which metabolic therapy has already been robustly demonstrated[104,105]. The combination of these existing metabolic therapies with

LAT1 inhibition widens the arsenal of treatment options available in order to more fully optimize treatment for the particular setting. Furthermore, combination strategies can be imagined to be useful solely for LAT1 targeting approaches. For example, in the setting of transplant such as BMT leading to GvHD, it would be possible to use a LAT1 specific cytotoxic therapy immediately after transplant in order to eliminate the activated and graft-specific cells, while transitioning to a LAT1 inhibitor therapy at later points to promote a Th2 response over an inflammatory Th1 response and thereby promote healing and acceptance of the graft. This model of therapy for BMT will depend on the lack of LAT1 expression on the homeostatically expanding cells that are expanding to reconstitute the immune compartment of the host, and further studies must be undertaken, for example adoptive transfer into *Rag* knockout mice, to ensure that homeostatically proliferating cells do not express LAT1. However, this expression seems unlikely, as syngeneic BMT did not lead to increased LAT1 expression in the GvHD model (**Fig. 28e,f**). Further studies will also include longer term testing for graft acceptance for example in the skin transplant model. Preliminary studies in this model have indicated that QBS10096S treatment at higher (3mg/kg and 2mg/kg) dose levels can promote graft survival over untreated mice (data not shown) but more work must be done on this. Furthermore, combination therapy with other immunomodulators such as costimulation blockade can further acceptance of grafted tissue and promote more favorable outcomes. This work further demonstrates the essential role of the essential amino acid leucine on immune responses, and how its manipulation can alter immune responses for the promotion of a more desirable outcome.

Future directions: modulation of metabolism for the control of immune responses

Throughout this thesis, the role of metabolism in shaping the immune response has been made clear. As the field of immunometabolism moves forward from the identification of metabolic alterations associated with metabolism and into attempts to proactively alter the immune response through the manipulation of metabolism, the concept of metabolism being a highly sensitive alteration will be

crucial. The use of metabolism to control immune responses and promote those beneficial for the circumstances will be a fundamental development for the field. Examples have been given for ways in which this alteration could be put to clinical use have been described, but future work must focus on the combination of multiple metabolic therapies to more precisely craft the desired response. To further these approaches, the combination of numerous metabolic strategies must be considered. Approaches like the "triple therapy" described by Lee and colleagues, which combines DON to inhibit glutamine utilization, 2-DG inhibit glycolysis, and metformin to inhibit fatty acid synthesis in order to inhibit rejection of skin and orthotopic heart transplants[95], or the combination therapy of metformin and 2-DG described by Yin and colleagues were shown to be extremely efficacious in the triple congenic model of SLE[104]. These combination therapies demonstrate that metabolic therapies could be tailored to promote the desired outcome, such as the enhancement in Tregs seen with triple therapy by Lee and colleagues[95]. These studies can further inform that tailoring. It is possible that, for example, the combination of both glutamine and leucine inhibition will act in a manner similar to as was seen in the mTOR signaling model, and inhibition of both glutamine and leucine metabolism would promote the polarization of CD4 T cells towards Tregs. Another opportunity for combinations in metabolic therapies would be in the promotion of memory CD8 T cell responses. Previous research has shown that memory CD8 T cell responses can be promoted by treatments that enhance mitochondrial respiration, or even that simply selecting cells with favorable mitochondrial properties can select for these phenotypes[63]. Perhaps combining glutamine metabolism inhibition with these approaches could yield even longer lived and more persistent memory cells, which would ensure longer lasting immunity in certain treatment settings. Further understanding and unraveling of the complex and intertwined nature of the metabolism of the immune system offers a myriad of possibilities for rationally selected therapy combinations in order to precisely engineer a desirable immune response for a particular setting and context.

In order to advance these strategies of tailoring therapy, a fuller understanding of the metabolism of the immune system and especially the crosstalk between different metabolic pathways, and the ways in which these pathways interact with each other must be sought. Some of these interactions have been discussed thus far, such as the requirement for glutamine efflux and leucine influx for mTORC1 activity[145], or the role of metabolic genes such as GAPDH in transcriptional regulation by mRNA binding activity[21]. The opportunity for crosstalk is dramatically expanded by the fact that glutamine and leucine are amino acids, which in addition to glutamine's role in replenishing the TCA cycle and leucine's status as an essential amino acid, mean that they are required for protein synthesis, as well as the myriad of other roles and biosynthetic intermediates the generation of which is dependent on them. Combine this with the fact that many other metabolites have been identified as signaling molecules, such as acetate in the promotion of a memory response[171] or succinate's role in increasing HIF signaling through stabilizing the protein[172], and the crosstalk seems almost hopelessly convoluted. However, acknowledgement and dedicated study of this interplay is essential for the furtherance of metabolic modulation as a therapeutic strategy, and this study must be conducted not in isolation but in as close to physiologic a setting as is practical, so all efforts can be taken to ensure that this delicate interplay is preserved and can be identified as best as possible.

In regards to the assessment of metabolism not just in combination but in all analyses, great care and greater emphasis must be placed on assessment under physiological conditions. Standard tissue culture media has been optimized for the rapid proliferation of cancer cells, and subsequent modifications have been made to ensure that T cells are able to rapidly proliferate as well. As such, these conditions are optimized for the rapid production of cells, and necessarily do not reflect the conditions encountered, for example, by a newly activated T cell in the spleen or lymph node. Glutamine is essential for T cell proliferation[14], and thus high levels of exogenous glutamine have been added to T cell culture medias to promote proliferation. As the serum glutamine of a C57Bl/6 mouse is

close to 700uM, our low glutamine culture approach of 0.1uM glutamine is far closer to what could be expected in circulation. Therefore, our culture approaches may far more closely approximate what may be found by a T cell at the time of activation. This is further complicated by the fact that the concentrations of glutamine in the spleen and lymph nodes may vary dramatically throughout the course of an infection, or within the intricately organized microenvironment of the lymph node. New studies using mass spectrometry approaches employing matrix-assisted laser desorption/ionization (MALDI) followed by time of flight (TOF) mass analysis approaches on tissue samples may provide a more *in situ* understanding of the metabolic microenvironment accompanying an immune response. Furthermore, glucose levels are highly increased in tissue culture media over what is found in circulation, and it has been shown that increased glucose concentration can promote aerobic glycolysis. Therefore, it has been proposed that the Warburg Effect is actually an *in vitro* artifact. While this might be an overstatement, it is clear that there is a dramatic difference between *in vitro* and *in vivo* conditions, and thus great care must be paid to ensure that the *in vivo* metabolic environment serves as the guiding light for all studies.

The differences between *in vivo* and *in vitro* conditions can lead to differences in mechanisms between these two systems. For example, the role of decreased proliferation in diminishing the antigen specific T cells of the lungs of HDM asthma mice treated with DON and JHU-083 may be more significant than the decreased epigenetic accessibility mechanism identified *in vitro* for the diminished Th2 responses in conditions of diminished glutamine metabolism. By assessing metabolism in more physiologic conditions, the field is better prepared to address these differences, and the mechanisms identified *in vitro* may be more directly applicable to *in vivo* work. By assessing things in non-physiological conditions, we are at risk of coming to the wrong conclusions, and as we add additional orders of magnitude of complexity to the systems we are analyzing, for example through attempts to manipulate multiple metabolites or metabolic pathways simultaneously, this becomes ripe for mistakes

and misinterpretations. Therefore, furthering our understanding of *in vivo* conditions is key to moving the field of immunometabolism forward, and is an essential step in increasing the translational value of studies in the field. Recent work such as that by Ma and colleagues[69] in which murine cells were rapidly isolated and submitted to labelled glucose tracing *ex vivo* for metabolomic analysis demonstrates the value of studying the metabolism of immune cells in the most physiological environment possible, and as approaches such as this are developed and moved forward, it should become more universally possible to know the *in vivo* conditions and set up experiments in the appropriate context to answer questions relevant to *in vivo* systems.

Conclusions

Throughout this thesis, the role of metabolism in shaping the immune response has been emphasized. While initial studies in immunometabolism identified metabolic changes such as aerobic glycolysis that occurred as a consequence of TCR mediated simulation, there is an evolving understanding that these changes do not merely occur as a consequence of activation, but rather are integral to its progression and the changes that must occur for an activated T cell to take on full effector function. Therefore, by altering the metabolic state of an activated cell, both at the time of activation and during its progression to a terminal fate, we can dramatically alter the cell it becomes, and the immune response that is mounted by the host. By restricting glutamine metabolism in CD4 T cells, we were able to demonstrate a selective requirement for glutamine in the development of Th2 cells, while Th1 cells were still able to polarize while cultured in low glutamine media. This selective requirement for glutamine was further demonstrated in an *in vivo* model of HDM asthma, in which glutamine restriction lead to decreased type 2 immune cells including Th2 cells, M2 macrophages, and ILC2, as well as a selective depletion in antigen specific T cells while Treg levels were unaffected. Low glutamine culture of CD8 T cells promoted a more long-lived, memory cell response both phenotypically *in vitro* and *in vivo* following adoptive transfer. Further, these cells were better able to protect mice from tumor

challenge. Inhibition of leucine uptake *in vitro* in CD4 T cells also demonstrated an alteration in CD4 T cell skewing, but this time promoting Th2 responses rather than inhibiting them. Furthermore, expression of the leucine transporter LAT1 can be used as a marker of activated cells *in vivo*, in both viral and transplant models, and targeting of these cells using a LAT1 specific cytotoxic drug works to dose-dependently decrease activated T cells in both the viral and skin transplant models.

Throughout this work, it has been demonstrated that modulation of metabolism can exert fine-tuned control over the immune response. By altering glutamine and leucine metabolism in CD4 T cells, it is possible to alter the balance between Th1 and Th2 responses. This modulation of metabolism has a great deal of therapeutic potential. The understanding of metabolism as an essential component of the immune response, in addition to antigen presentation, costimulation, and cytokine production, is an essential theme in this work, and further efforts to expand on the use of metabolism to control the immune response in beneficial ways have a great deal of potential to improve not just our understanding of basic biology, but to improve patient care in a wide range of diseases.

List of abbreviations

2-NBDG- 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose

2DG- 2-deoxy-D-glucose

ACC- acetyl-CoA carboxylase

ACT- adoptive cellular therapy

aKG- alpha-ketoglutarate

AMPK- AMP activated kinase

ATP- adenosine triphosphate

AU- Adenylate-uridylate

BCH- 2-amino-bicyclo[2.2.1]heptane-2-carboxylic acid

BMT- bone marrow transplantation

CAR- chimeric antigen receptor

CFSE- carboxyfluorescein succinimidyl ester

ChIP- chromatin immunoprecipitation

CIA- collagen induced arthritis

CPT1- carnitine palmitoyltransferase 1

CUT&RUN- Cleavage Under Targets and Release Using Nuclease

DMK- dimethyl 2-oxogluterate

DON- 6-diazo-5-oxo-L-norleucine

EAE- experimental autoimmune encephalitis

ECAR- extracellular acidification rate

ELISA- enzyme-linked immunosorbent asssay

ETC- electron transport chain

FBS- fetal bovine serum

FMO- fluorescence minus one

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

GI- gastrointestinal

Gln- glutamine

GLS- glutaminase

GLUD- glutamate dehydrogenase

GSEA- gene set enrichment analysis

GvHD- graft versus host disease

HBSS- Hanks' Balanced Salt Solution

HDM- house dust mite

HIF- hypoxia-inducible factor

IFN- interferon

ImmGen- Immunological Genome Project

IP-intraperitoneal

LDH- lactate dehydrogenase

LmOVA- Listeria monocytogenes bacteria genetically engineered to express OVA

MALDI- matrix-assisted laser desorption/ionization

MHC- major histocompatibility complex

MLR- mixed lymphocyte reaction

mROS- mitochondrial reactive oxygen species

mTOR- mammalian target of rapamycin

mTORC1/2- mammalian target of rapamycin complex ½

NK cell- natural killer cell

NMR- nuclear magnetic resonance

OAA- oxaloacetate

OCR- oxygen consumption rate

OGT- O-GlcNAc transferase

OTI- transgenic CD8 T cells specific for the SIINFEKL peptide of OVA presented in the context of MHC class I

OVA- ovalbumin

OXPHOS- oxidative phosphorylation

P14- Transgenic CD8 T cells specific for a peptide from LCMV presented by MHC class I on a *Rag2* knockout background

PBS- phosphate buffered saline

PCC- pigeon cytochrome C

PCK1- phosphoenolpyruvate carboxykinase 1

PD1- programmed cell death 1

PDH- pyruvate dehydrogenase

Pgam1- phosphoglycerate mutase-1

PKM1/2- pyruvate kinase isoforms 1 and 2

PMA- phorbol 12-myristate 13-acetate

QBS10096S- (2S)-2-amino-3-[5-[bis(2-chloroethyl)amino]-methyl-phenyl]propanoic acid

RAPTOR- regulatory-associated protein of mTOR

RHEB- Ras homologue enriched in brain

RICTOR- RAPTOR-independent companion of mTOR

RISP- Rieske iron sulfur protein

ROS- reactive oxygen species

RPMI- Roswell Park Memorial Institute media

S6K1- S6 kinase-1

SLE- systemic lupus erythematosus

SLEC- short-lived effector cells

SorA- soraphen A

STAT- signal transducer and activator of transcription

TC- triple congenic model of SLE

TCA- tricarboxylic acid

TCGA- The Cancer Genome Atlas

TCR- T cell receptor

TME- tumor microenvironment

TMRM- ethyl ester of tetramethylrhodamine percholrate

TNF- tumor necrosis factor

TOF- time of flight mass spectrometry

TSC2- tuberous sclerosis complex 2

TRAF- TNF receptor-associated factor

Treg- regulatory T cell

UTR- untranslated region

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Publications

1) Leone, R.D., Zhao, L., Englert, J.M., Sun, I.M., Oh, M.H., Sun, I.H., Arwood, M.L., **Bettencourt, I.A**., Patel, C.H., Wen, J. and Tam, A., 2019. Glutamine blockade induces divergent metabolic programs to overcome tumor immune evasion. Science, 366(6468), pp.1013-1021.

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Posters and Abstracts

Poster Presentations

1) **Bettencourt I.A** and Powell J.D. "A selective role for glutamine metabolism in the regulation of Th2 responses" Immunometabolism: Fundamentals to Prospective New Therapies, June 2019

2) **Bettencourt I.A.** and Powell J.D. "Glutamine plays an essential role in CD4 T cell proliferation and helper subset commitment" Graduate Program in Immunology Program Retreat, Baltimore MD, September 2018

3) **Bettencourt I.A.** and Powell J.D. "Targeting glutamine metabolism as a means of treating experimentally induced asthma" Keystone Symposium: Integrating Metabolism and Immunity, Dublin, Ireland, May 2017

4) **Bettencourt I.A.** and Powell J.D. "Targeting glutamine metabolism as a means of regulating T cell responses" Keystone Symposium: Immune Function and Inflammatory Disease, Banff, Alberta, Canada, February 2016