Aus dem Lehrstuhl
für Hals-Nasen-Ohren-Heilkunde
Prof. Dr. Christopher Bohr
der Fakultät für Medizin
der Universität Regensburg

The Glutamine Synthetase in lymphocytic leukemia cells and non-malignant T lymphocytes

Inaugural – Dissertation
zur Erlangung des Doktorgrades der Medizin

der
Fakultät für Medizin
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vorgelegt von
Andreas Marco Siegmund

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1. Introduction

Tumor cells display an increased metabolic rate to sustain proliferation. Among various pathways, glutamine metabolism enables macromolecular synthesis in proliferating tumor cells (1). Blocking this essential metabolic process is a relatively new therapeutic strategy (2). However, it should be noted that other rapidly dividing cells also rely on glutamine metabolism. Activated T cells exhibit a similar metabolic phenotype compared to cancer cells (3).
In this thesis, our goal was to obtain a more comprehensive understanding of glutamine metabolism in both human T lymphocytes and leukemia cells. Additionally, we investigated whether another amino acid (glutamate) could compensate for glutamine deprivation.

1.1 Tumor cells

1.1.1 Characteristics of cancer cells

Characteristics that are observed in tumor cells and thought to drive the development and progression of cancer were summarized in the “hallmarks of cancer”. These hallmarks were first proposed by Douglas Hanahan and Robert A. Weinberg in 2000. The concept originally included features such as evading apoptosis and overcoming growth restriction, thereby sustaining proliferation as well as angiogenesis (4). Since then, the concept has been further developed and refined. In 2011, Hanahan and Weinberg proposed additional emerging hallmarks, including the accelerated and, in some respects, altered metabolism. Deregulated metabolism is a prerequisite to provide the necessary energy and biomolecules for their proliferative capacity. Furthermore, the ability of tumors to evade the immune system was added (5).
It can thus be stated that tumor cells find various ways to divide and grow uncontrollably (**Figure 1.**). Effective therapies have been derived from the specific metabolic changes in tumors. A notable example is the use of asparaginase, an enzyme responsible for the conversion of the amino acid asparagine into aspartic acid and ammonia. This enzyme plays a crucial role in the treatment of acute lymphoblastic leukemia (ALL) (6).

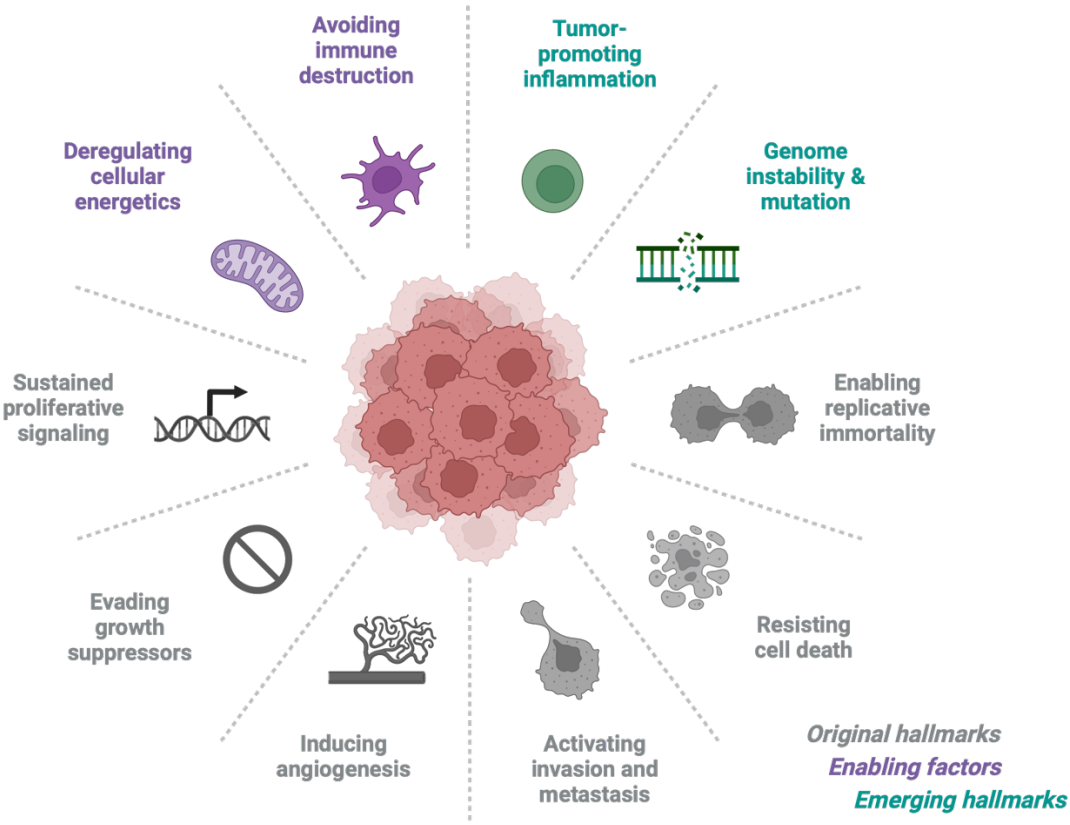


Figure 1. Hallmarks of cancer. Cancer is characterized by several biological traits that promote the development of malignancies. These fundamental features comprise continuous signaling for cell growth, evasion of mechanisms that inhibit growth, initiation of blood vessel formation (angiogenesis), facilitation of invasion and spread to distant sites (metastasis), resistance to programmed cell death, and unlimited proliferative potential. These attributes are further influenced by factors like disruption of cellular energy regulation and evasion of the immune system, which actively support and reinforce them. Moreover, emerging factors such as tumor-associated inflammation and genomic instability contribute to the ongoing evolution and advancement of cancer (5). Adapted from "Hallmarks of Cancer (Circle Layout)", by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

1.1.2 Metabolism of cancer cells

Cellular functions rely on a constant supply of energy. Glucose stands as the predominant energy source for most cells and plays a central role in both cell proliferation and survival. In non-malignant cells, the primary metabolic pathway for glucose involves oxidative phosphorylation (OXPHOS) (7). As already mentioned, tumor cells adapt their metabolism in different ways compared to healthy cells. To fulfill their heightened metabolic demands, cells nutrient uptake is increased, and furthermore, synthesis of certain metabolites is elevated. Unlike non-malignant cells, tumor cells show

modification in different pathways, such as glucose metabolism and glutaminolysis, including the tricarboxylic acid (TCA) cycle (8). Tumor cells significantly increase their glucose consumption and preferentially degrade glucose into lactate, even in the presence of ambient oxygen (Warburg 1923). This metabolic phenotype is known as the Warburg effect as it was first described by Otto Warburg in the 1920s, and it remains a topic of active research (9).

In addition, transporters related to glucose metabolism exhibit increased expression in tumor cells. This increase in insulin-independent glucose transporters (GLUT) serves to maintain higher intracellular glucose concentrations (10). Furthermore, this effect is clinically used to detect tumor cells by Positron emission tomography (PET)-based imaging, visualizing the uptake of a radioactive fluorine-labeled glucose, 18F-fluorodeoxyglucose (18F-FDG) (11). The effect was initially attributed to mutations and defects in mitochondrial enzymes. However, this holds true only for a subset of tumors (11). An advantage of high glycolytic rates is the availability of precursors for various anabolic pathways and fast energy provision(12)(13). The increased glycolysis leads to the accumulation of lactate. Lactate export occurs through the monocarboxylate transporters (MCTs) as a co-transport with a H^+ proton. As a consequence of this process, the pH value in the tumor microenvironment (TME) decreases (14). The upregulation of MCTs is also observed across a diverse range of tumors (15). In addition to glucose, rapidly proliferating cells such as tumor cells rely on lipids and glutamine as a source of energy, carbon, and nitrogen (16).

1.1.3 Glutamine's role in tumor growth and survival

Glutamine is the most abundant amino acid circulating in the bloodstream, constituting 20% of the total free amino acids in blood and is classified as a nonessential amino acid (produced by the human body) (17). Nine amino acids, including Phenylalanine, Valine, Tryptophan, Threonine, Isoleucine, Methionine, Histidine, Leucine, and Lysine are classified as essential amino acids as humans cannot produce them (18). On the other hand, twelve amino acids, Asparagine, Aspartic Acid, Cysteine, Glutamic acid, Glutamine, Glycine, Proline, Serine, Tyrosine, Selenocysteine, and Pyrrolysine, are considered non-essential as they can be synthesized by humans endogenously (19). However, glutamine is regarded as a conditionally essential amino acid, particularly

under metabolic stressed conditions, such as rapid cell proliferation and infections (20). Interestingly, glutamine contributes to almost all biosynthetic pathways in proliferating cells including cancer cells (21). Proliferating cells enhance glutaminolysis to sustain the biosynthesis of many essential biomolecules (16). Isotopic analysis revealed that approximately half of the non-essential amino acids required by tumor cells for protein synthesis are derived from metabolized glutamine (2). Furthermore, glutamine delivers precursors for nucleic acid synthesis.

To enable mitochondrial glutaminolysis, cytosolic glutamine necessitates passage across the inner mitochondrial membrane facilitated by the SLC1A5 transporter - a transporter specific to mitochondrial glutamine (22). Glutamine is degraded into glutamate and ammonium by the mitochondrial glutaminases (GLS). GLS exhibits at least three isoforms, namely GLS1, GLS2, and GAC, all of which have been identified in mitochondrial compartments (23). Subsequently, mitochondrial glutamate is converted to α-ketoglutarate, which enters the TCA cycle supporting the oxidative phosphorylation (OXPHOS) pathway to generate NADH and $FADH_2$, substrates for oxidative ATP production (24) (25) (**Figure 2.**). The conversion into alpha-ketoglutarate (α-KG) is catalyzed either by glutamate dehydrogenase 1 (GLUD1 or GDH1) or various mitochondrial aminotransferases (26) (27). Besides protein synthesis, glutamine is needed for the generation of glutathione (28), a key player in the defense mechanisms against reactive oxygen species (ROS). The importance of glutamine is underlined by the fact that some cancer cells undergo rapid cell death under glutamine deprivation (29). Glutamine can be acquired by cells either from the blood circulation, by breaking down macromolecules or by the de novo synthesis catalyzed through the enzyme glutamine synthetase (GS). GS utilizes adenosine triphosphate (ATP) to convert glutamate and ammonia into glutamine. The expression of GS is notably high in several tissues, including liver, kidney, skeletal muscle, and brain (30). With regard to malignant tissue, the expression level and enzyme activity of the GS varies between cancer types (31). For instance, GS is highly expressed in lung tumors (32), heterogeneously expressed in ovarian cancer (OVC) (33), and scarcely expressed in the basal type of breast cancer (34). Glutamine uptake takes place via various Na^+ dependent transporters, including the solute carrier (SLC) transporter members SLC1A5 (also known as ASCT2), SLC6A19 (B^0AT1), SLC6A14 ($ATB^{0,+}$), and SLC38A1.2 (SNAT1.2) (35) (36). Within the category of Na^+ -independent transporters, SLC7A5 (LAT1) and SLC7A8 (LAT2)

are the most thoroughly understood. Several cancers exhibit elevated expression levels of ASCT2 and LAT1 transporters (37). Due to the high glutamine consumption, the microenvironment of tumor cells (TME) often shows low levels of glutamine (38). The elevated expression of GS facilitates the provision of glutamine to cancer cells even in conditions of low glutamine in the environment surrounding the tumor (31). Variations in the nutritional composition and metabolic byproducts within TME have the potential to impact the activity of immune cells. This results in a dynamic interplay for crucial nutrients between immune cells and tumor cells within the TME (39).

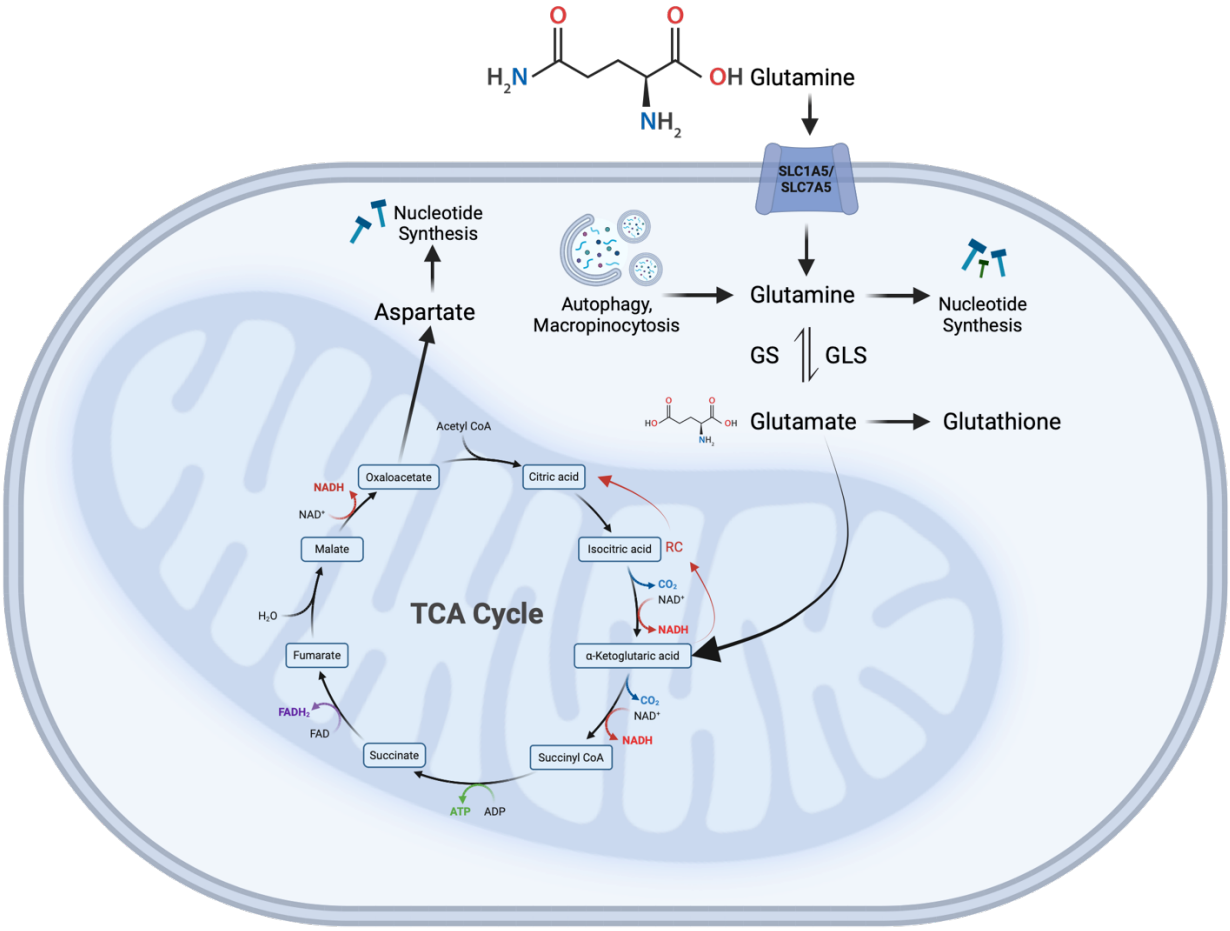


Figure 2. Glutamine metabolism in cancer cells. Glutamine can be taken up, derived from macropinocytosis or is intrinsically synthesized from glutamate via glutamine synthetase (GS). It can subsequently serve as building blocks for synthesizing hexosamine, nucleotides, and asparagine within the cytoplasm. Glutamine converted to glutamate by glutaminase (GLS), is further metabolized to α-ketoglutarate (α-KG) and enters the tricarboxylic acid (TCA) cycle. Glutamate is also used in the synthesis of the antioxidant compound glutathione. Intermediates within the mitochondrial tricarboxylic acid (TCA) cycle, including oxaloacetate (OAA) and citrate, play a crucial role in producing cytosolic aspartate for nucleotide synthesis and acetyl-CoA for lipid synthesis (13) (31). Adapted from "Cancer Metabolism in Nutrient-Deprived Conditions", by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

1.2 The human immune system

The human immune system is an interactive network of cells, tissues, organs, humoral factors, and cytokines that work together to protect the body from foreign invaders, such as bacteria and viruses. The immune system can be divided into two main branches: the innate immune system and the adaptive immune system (40).

1.2.1 The innate immune system

The innate immune system is the first line of defense against foreign invaders. It includes physical, chemical, and microbiological barriers, as well as neutrophils, monocytes, macrophages, complement system, cytokines, and acute phase proteins (41). The cellular components of the innate immunity can immediately recognize and attack a wide range of pathogens, but they do not have the ability to specifically remember them. Granulocytes, encompassing neutrophils, eosinophils, and basophils, release vesicles that carry substances like microbicidal lysozymes and peptides. Furthermore, natural killer cells employ various mechanisms to directly eliminate target cells (40) (42). Macrophages and dendritic cells are central for this part of our immunity. They take up antigens, process them and finally present them to the cells of the adaptive immune system via major histocompatibility complex I or II (MHC I and MHC II) (43). Thus, they represent an important link as so-called antigen-presenting-cells (APCs) between the innate and adaptive immune system. Another important functions of macrophages are the phagocytosis of bacteria, removal of apoptotic cells and debris from normal cell turnover in the human body (44).

1.2.2 The adaptive immune system

The acquired immunity is a hallmark of highly evolved organisms and second line of defense against foreign invaders. It consists of immune cells that can specifically identify a particular antigen from a pathogen thereby attacking the pathogen. The two main types of cells of the adaptive immune system are T lymphocytes and B lymphocytes. These cell populations develop from progenitor cells in the bone marrow (45). T cells

can directly attack and kill infected or cancerous cells, while B cells differentiate into plasma cells and produce immunoglobulins, which neutralize pathogens and opsonize target cells, triggering their destruction by other cells (45). The two main subsets of T cells are CD8-positive ($CD8^+$) and CD4-positive ($CD4^+$) T cells. As $CD8^+$ T cells have the capability to lyse target cells, they are also named cytotoxic T lymphocytes (CTLs). $CD4^+$ T cells mainly produce different effector cytokines (43). However, cytolytic activity of $CD4^+$ cells has been widely shown, making them attractive for cellular therapy approaches (46).

1.2.3 Activation and differentiation of T cells

The activation and differentiation of T cells is a complex process that involves several steps. First, T cells must encounter an antigen, which is a peptide that is unique to a pathogen, virus-infected cell, or cancer cell. The antigen is typically presented by an antigen-presenting cell (APC) that displays antigen fragments on its surface. These take up the antigen, break it down, process and present it as a peptide on major histocompatibility complex (MHC) I for $CD8^+$ and MHC II for $CD4^+$ cells (47).

MHC I molecules are found on the surface of all cells with a nucleus and expressed by platelets and play a crucial role in presenting intracellular peptides to cytotoxic $CD8^+$ T cells (48) (49). This function enables the immune system to distinguish between normal, healthy cells and those that may be infected with viruses or undergoing malignant transformation. This distinction is notable because $CD8^+$ T cells primarily engage with MHC I and are thereby able to attack and eliminate cells presenting abnormal peptides (40). $CD8^+$ T cells differentiate into cytotoxic T cells that can directly kill infected or cancer cells (45). Adequate co-stimulation is also essential, necessitating either the involvement of dendritic cells (DCs) or the presence of cytokines produced by T helper cells (50).

Conversely, MHC II molecules are specifically expressed on antigen-presenting cells (APCs), including macrophages, dendritic cells and B cells (45). In contrast to $CD8^+$ cells, $CD4^+$ T cells, which interact with MHC II, play a central role in coordinating immune responses by supporting other immune cells (51). $CD4^+$ T cells differentiate into

different subtypes, such as T helper 1, T helper 2, T helper 17 and regulatory T (T_{regs}) cells (47). Furthermore, $CD4^+$ subsets are defined by differential expression of surface molecules and endogenous production of cytokines (52).

Once the T cell recognizes the antigen, the T cell antigen receptor (TCR) binds to the specific peptide loaded on MHC molecules. For full activation, the T cell must integrate further signals. The co-receptor CD28 on T cells receives a co-stimulatory signal in response to binding to B7 (CD80/CD86) expressed by APCs (50). Moreover, cytokines such as interleukin 2 (IL-2), mainly produced by $CD4^+$ T cells, enhance their activation. T cells differentiate into different subtypes based on the type of cytokines and signals they receive. (**Figure 3.**) (50).

T cell activation depends on elevated metabolic activity (54). Upon activation, human T cells increase their cell size. The process called on-blast formation is accompanied by the expression of activation related surface markers such as CD25 and secretion of cytokines (55). This period is followed by a massive proliferative activity, the so-called clonal expansion phase. This process is necessary for the immune system to generate many T cells that can recognize and attack the specific antigen (40). It's important to note that the activation and differentiation of T cells is tightly regulated by a variety of signaling molecules and transcription factors, to ensure that the immune response is appropriate for the specific antigen and that the immune response is not overactive or self-destructive (56).

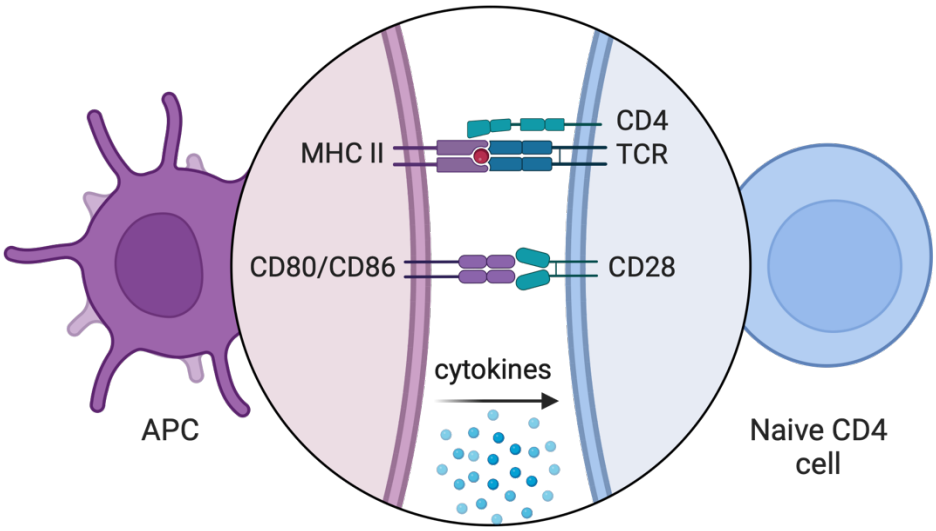


Figure 3. T cell activation and differentiation. The activation of $CD4^+$ T cells is shown in the chart: Signal 1 (binding of the T-cell receptor (TCR) to the peptide-MHCII complex on the antigen-presenting cell (APC) surface) and signal 2 (binding of the T-cell co-receptor CD28 to CD80/CD86 on the APC surface) are required for T-cell activation. The binding of polarizing cytokines to their respective receptor on the T cell surface represents signal 3. Different combinations of these cytokines influence T cell differentiation into distinct effector T cell subtypes that produce signature cytokines. Idea of the chart from British society of immunology, T cell activation, Original author - Mary Cavanagh, Imperial College London, UK, Updated by - Emily Gwyer Findlay, University of Edinburgh, UK (53). Adapted from "T Cell Activation and Differentiation (Layout)", by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

1.2.4 Function of cytotoxic lymphocytes

The CTLs target virus-infected or dysfunctional cells and trigger cell death through different mechanisms: Proinflammatory cytokines such as IFN-γ and TFN are released (57). These signaling molecules contribute to the inflammatory response by activating immune cells and promoting inflammation at the site of infection. Moreover, there is an interaction involving the Fas ligand (FasL) expressed by cytotoxic T lymphocytes (CTLs) and the Fas receptor (CD95) expressed by numerous cell types (58). This interaction triggers a signaling cascade that finally leads to apoptosis of the target cell. Furthermore, CTLs can secret cytolytic granules that contain perforin and granzymes (serin proteases) (59). Perforin creates pores in the target cell's membrane, enabling the entry of proteases, including granzyme B. Once inside, granzyme B initiates the apoptotic process, leading to the death of the target cell. (60) (61).

Importantly, an immunological memory is established by the development of so-called memory cells after initial activation of cells. Upon renewed contact with the specific antigen, those memory cells get immediately activated, accelerating the immune response (50). In addition to memory B cells, which swiftly generate highly specific antibodies upon re-infection, the pivotal role in the formation of immunological memory is carried out by memory T cells (T_{mem}), originating from effector T cells (T_{eff}) (62). Taken together, it is a dynamic system that is constantly adapting to new threats and can also learn to recognize and remember pathogens it has encountered before. T cells are also particularly important because they are responsible for the tumor surveillance as they can attack a tumor directly (43) (40).

1.2.5 Metabolism of T cells

The metabolism of T cells is closely linked to their activation and function. T cells, like all cells, require energy to carry out their various functions. They obtain this energy through a variety of metabolic pathways. Naive T cells are small and quiescent cells requiring relatively low levels of glucose, amino acids, and fatty acids to meet their fundamental energy and biosynthetic needs (43). Encountering an antigen triggers T cell activation and differentiation, reducing lipid oxidation and instead relying on a high supply of glucose and amino acids to support their proliferation and effector functions such as cytotoxicity and cytokine production (63). Previous studies conducted on both human and murine T cells have demonstrated that impairing glycolysis or limiting glucose availability as a nutrient results in a notable reduction in T cell proliferation (64). Studies on the relevance of glycolytic activity for cytokine production yielded conflicting results regarding murine and human T cells. In murine T cells, effector cytokine production appears to depend on glycolysis (64). Conversely, in human T cells, experiments with glucose transporter GLUT-1 deletion or glucose restriction did not impact cytokine production (65). Human $CD4^+$ and $CD8^+$ T cells maintained effector functions even when glycolytic activity was limited, compensating through increased mitochondrial respiration (66).

Aerobic glycolysis can be regarded as a metabolic hallmark of activated T cells (67) (68). This metabolic switch allows T cells to meet the increased energy demands of

their expanded population, and to produce the precursors needed for the biosynthesis of lipids and nucleic acids. Additionally, T cells also require amino acids, particularly glutamine, to support their growth and function. Glutamine is a conditionally essential amino acid in rapidly proliferating cells, and glutaminolysis is a crucial source of energy for T cells (69).

1.2.6 The role of glutamine in T cell function

Glutamine is a potential key amino acid that plays an important role for growth, proliferation, and cytokine production of T cells. In particular, the activation of lymphocytes is a highly energetic event. T cells are highly sensitive to glutamine levels and it is assumed that no metabolic precursor (such as proline or asparagine) or product (such as glutamate) could replace glutamine so far (3) (70).

Notably, glutamine is extensively degraded in T cells through the glutaminolysis pathway, producing mainly glutamate and to a lesser extent aspartate, alanine, lactate, pyruvate, and CO_2 (71) (72) (73). Glutamine is also required for the biosynthesis of other amino acids, such as asparagine and arginine, which are important for T cell function. Furthermore, T cells require a high level of glutamine to produce antioxidants, such as glutathione, which protect the cells from oxidative stress (74).

Additionally, research has shown that human and murine T cells hinge on glutamine for sustaining their ability to produce cytokines such as IFNγ and IL-2, which are essential for their function (3) (75). Moreover, the levels of extracellular glutamine appear to regulate the proliferation of T lymphocytes, the rate of IL-2 receptor expression (71). Further, T cells use glutamine to support the biosynthesis of the amino acids serine and glycine, which are needed for the production of cytokines (76).
In summary, glutamine is essential for both human and murine T cell activation, proliferation, and cytokine production.

1.2.7 T cells in the tumor microenvironment

Moreover, it is now known that the tumor microenvironment affects immune cell function (77). Tumor cells orchestrate a microenvironment hostile to lymphocytes. T cells entering the tumor microenvironment face significant metabolic challenges to establish and sustain an anti-tumor response (78). The acidification of the tumorous environment hinders T cell proliferation and cytokine production (55) (79). It has also been shown that elevated glucose metabolism is associated with diminished T cell infiltration into the tumor environment (80). Tumor patients have a poor prognosis if the tumors are infiltrated by a reduced number of T cells (81).

Particular emphasis must be devoted to the tumor microenvironment, where glutamine is scarce (38). This is where immune cells encounter the tumor. To fight the cancerous cells, T cells must be activated, proliferate, and produce cytokines, processes contingent on the availability of exogenous glutamine (71). Moreover, Glucose deprivation also leads to delayed on-blast formation and severely impaired proliferation of human $CD8^+$ cells. The production of a number of cytokines by human $CD4^+$ T cells is hardly affected by glucose deprivation (82) (65).

The Warburg effect in tumor cells can limit the availability of glucose in the tumor microenvironment and promote competition for nutrients and suppress the proliferation of $CD8^+$ T cells (83)(84) (85). For a long time, the ability of the immune system to fight cancer was questioned, however, with the successful development of immunotherapeutic therapies such strategies have become the focus of research (86). The immune system is capable of recognizing and destroying tumor cells, a process called cancer immunosurveillance, which acts as an important protection against cancer (87).

2. Research objectives

The objective of this research work was to investigate the role of glutamine for leukemic T cells in comparison to primary human T lymphocytes. We studied proliferation and survival of two lymphoblastic leukemia cell lines in comparison to primary $CD8^+$ T cells upon glutamine deprivation and determined the effects on primary T cells activation and proliferation.

In this regard, we analyzed the expression of the glutamine synthetase (GS) across the different cell lines. Furthermore, we investigated whether glutamine could be compensated for by supplementing glutamate in leukemia and T cell cultures. We evaluated the possible success by means of the FLUX analysis.

3. Material and methods

3.1 Material

3.1.1 Equipment

CASY cell counter	OMNI Life Science, Bremen, GER
EMax Precision microplate reader	Molecular Devices, Sunnyvale, USA
FACS Calibur	BD Bioscience, Heidelberg, GER
Fusion Pulse 6	Vilber Lourmat, Eberhardzell, GER
Incubator (BBD 6220)	Heraeus, Osterode, GER
Infrared vortex vacuum evaporator	CombiDancer, Hettich AG, Baech, SUI
Microscopes	Zeiss, Jena, GER
Multifuge 3S-R	Thermo Scientific, Waltham, USA
Multifuge 3.0R	Thermo Scientific, Waltham, USA
Pipetboy	Integra Biosciences, Fernwald, GER
Pipettes	Eppendorf, Hamburg, GER
QuadroMACS® separator	Miltenyi Biotec, Bergisch Gladbach, GER
Quickgel electrophoresis system	Helena Biosciences, Gateshead, UK
SenorDish-Reader	PreSens, Regensburg, GER
Sepatech Megafuge 1.0	Heraeus, Osterode, GER
Sepatech Megafuge 3.0	Heraeus, Osterode, GER
Thermo VarioSkan	Thermo Scientific, Waltham, USA
Vortex Genie 2	Scientific Industries, Bohemia, USA
Western Blot chamber	Biometra, Göttingen, GER

3.1.2 Consumables

Cryo tubes	Corning, Corning, NY, USA
LS columns	Miltenyi Biotec, Bergisch Gladbach, GER
Microtiter plates (6, 12, 24, 96 wells)	Costar, Cambridge, MA, USA
Pipette tips	Eppendorf, Hamburg, GER

Polystyrene Round-Bottom Tubes 5 ml	Corning Science, Tamaulipas, MEX
Scalpels, disposable	Feather, Osaka, JP
Serological Pipettes, 1/2/5/25 ml	Nerbe plus, Winsen, GER
Tubes (5 ml, 15 ml, 50 ml, 225 ml)	Eppendorf, Hamburg, GER
96 well plates U-/flat-bottom	Costar, Corning, NY, USA

3.1.3 Medium, buffers, solutions

AB-serum	Bavarian red cross, GER
Acrylamide/Bisacrylamide (30 %)	Carl Roth, Karlsruhe, Germany
ε-Amino-n-capronic acid	Sigma-Aldrich, St. Louis, MO, USA
Annexin binding buffer (10x)	BD, Franklin Lakes, NJ, USA
Aqua Ecotainer	B. Braun, Melsungen, GER
β-mercaptoethanol	Gibco/Life Technologies, Carlsbad, CA, USA
CASY Buffer	Beckman Coulter, USA
Dimethylsulfoxide (DMSO)	Sigma- Aldrich, St. Louis, MO, USA
FACSclean	BD, Franklin Lakes, NJ, USA
FACSflow	BD, Franklin Lakes, NJ, USA
FACSrinse	BD, Franklin Lakes, NJ, USA
Fetal calf serum (FCS)	Sigma- Aldrich, St. Louis, MO, USA
Glycerin	Merck, Darmstadt, Germany
Glycine	Carl Roth, Karlsruhe, Germany
H2Obidest	Braun, Kronberg im Taunus, Germany
L-glutamine	Thermo Fisher Scientific, Waltham, USA
Lymphocyte separation solution (Ficoll)	PAA, Linz, AUT
2-Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA
Methanol	Merck, Darmstadt, Germany
Non-essential amino acids	Gibco/Life Technologies, Carlsbad, CA, USA
Penicillin/Streptomycin	Thermo Fisher Scientific, Waltham, USA
Phosphate buffered saline (PBS)	Sigma- Aldrich, St. Louis, MO, USA
RIPA	Sigma- Aldrich, St. Louis, MO, USA
RPMI 1640	PAN Biotech, Aidenbach, GER

Sodium pyruvate	Gibco/Life Technologies, Carlsbad, CA, USA
Tween 20	Sigma- Aldrich, St. Louis, MO, USA

T-cell medium	ml
AB-Serum, human	50
β-mercaptoethanol	0.5
L-glutamine	5 (2 mM)
Nonessential amino acids	5
Penicillin/Streptomycin	2.5
RPMI 1640	500
Sodium pyruvate	5

Tumor cell medium	ml
RPMI 1640	500
L-glutamine	5
FCS	50 (10 %)

FACS staining buffer	ml
FCS	10 ml (2 %)
PBS	500 ml

Freezing medium	%
FCS	80
DMSO	20

MACS buffer	ml
EDTA	5 (2 mM)
FCS	5 (1 %)
PBS	500

3.1.4 Kits, reagents, and chemicals

Ammoniumchlorid	Sigma-Aldrich, St. Louis, MO, USA
CD4 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach
CD8 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach
Chemiluminescence	ECL, Amersham Bioscience, UK
Dynabeads™ T-Activator CD3/CD28	Gibco/Life Technologies, Carlsbad, USA
Glutamate	Sigma-Aldrich, St. Louis, MO, USA
[13C]glutamate	Cambridge Isotope Labs
Goat Anti-Rabbit Immunoglobulins/HRP	DakoCytomation
Human IFN-γ DuoSet ELISA	R&D Systems, Minneapolis, MN, USA
IL-2	PeproTech, Hamburg, GER
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich, St. Louis, MO, USA
7AAD, 20 µl, 559925	BD Biosciences, Franklin Lakes, NJ, USA

3.1.5 Antibodies

Antibodies for western blotting

Specificity	Source	Species cross	Weight	Dillution	Manufacturer
α -GLUL	Rabbit	Mouse	42 kDa	1:5000	Abcam
α-Tubulin	Mouse	Mouse	50 kDa	1:2000	Sigma-Aldrich

Anti-human antibodies for flow cytometry

Specificity	Conjugation	Clone	Company	Material number
Annexin V	FITC		BD	556419
CD4	PE	RPA-T4	BD	561844
CD8	PE-Cy7	SK1	BioLegend	344711
CD25	PE-Cy7	M-A251	BD	557741
α-CD69	APC	FN50	BD	555533

CD137	PE	4B4	eBioscience™	12-1379

3.1.6 Software

BioRender	BioRender, Toronto, Ontario, Canada
Excel 2021	Microsoft, Redmond, Washington, USA
FACSDiva	BD, Heidelberg, GER
FlowJo v10	FlowJo, LLC, Ashland, OR, USA
Fusion Pulse 6	Vilber Lourmat
GraphPad Prism 9.5.0 (macOS)	GraphPad Software, La Jolla, CA, USA
SenSorDish-Reader Software	PreSens, Regensburg, GER
Word 2021	Microsoft, Redmond, Washington, USA
Zotero 6.0.20 (macOS)	Center for History and New Media, George Mason University, USA

3.2 Methods

Cells were handled under a laminar air flow cabinet using sterile consumables and kept at 37 °C, 5 % CO2, 95 % relative humidity. Unless otherwise indicated, centrifugation was performed at 300 g, 4 °C for 7 minutes.

3.2.1 Tumor cell cultivation

As a model system we selected the T-ALL CCRF-CEM-C7H2 and Jurkat-FHCRC cell line. In the following, these cells will be abbreviated only to C7H2 and Jurkat cells. The C7H2 cell line was originally isolated by M. R. Norman and E. B. Thompson for in-vitro analysis (88) while Jurkat cells were derived from a 15-year old boy with acute lymphoblastic leukemia (ALL) (89). Cells were grown in a tumor cell culture medium (sec. 3.1.3) with an initial concentration of 300.000 cells per ml in a total volume of 20 ml. For the experiments, the cells were either cultivated with glutamine, without glutamine, or cultured without glutamine but with glutamate and ammonium, and split every 48h or 72h.

3.2.2 T cell isolation, stimulation and cultivation

Human peripheral blood mononuclear cells (MNCs) were isolated from the LRS cone of healthy blood donors. The separation of MNCs from erythrocytes and granulocytes was achieved through Ficoll density gradient centrifugation (30 minutes at 700g, without a break). The interface containing MNCs was harvested and washed twice with PBS. $CD4^+$ and $CD8^+$ T cells were then isolated using the MACS magnetic separation technique (Miltenyi Biotech). PBMCs were diluted in MACS buffer and incubated with magnetic anti-CD4 or anti-CD8 MicroBeads at 4 °C for 15 minutes. After incubation, cells were washed with MACS buffer, centrifuged, and resuspended.
The LS column (Miltenyi Biotech) was positioned in the magnetic field of a MACS separator. The cell suspension was applied to LS columns and magnetically separated. Following separation, purity of cells was determined by anti-CD4 and anti-CD8 staining and analyzed by flow cytometry. A purity of more than 98 % was reached. Subsequently, $0.1*10^6$ cells were seeded in a 96 well U-bottom plate in 220 µl T cell medium supplemented with IL-2 (25 IU/ml, Peprotech) together with anti-CD3/CD28 Dynabeads (Gibco/Life Technologies) in a bead to cell ratio of 1:1.

3.2.3 Cell counting, viability and size monitoring

Measurements of proliferation, viability and cell size were conducted using the CASY system (Casy® Modell TT, OLS Omni Life Science). The CASY cell counter employs an electric field to distinguish between living and dead cells. Furthermore, the system can analyze the diameter of cells. For each measurement, 50 µl of the cell suspension was combined with 10 ml CASY Buffer (Beckman Coulter).

3.2.4 Flow cytometry

Flow cytometry serves as a fluorescence-based method for detection and analysis of cells and their characteristics. The size of the cells is determined by measuring forward scattered light, while the granularity is assessed by measuring the side scattered light.

Surface markers are stained using fluorescence labeled antibodies. In this thesis the FACS Calibur (BD) was employed. The results were analyzed with the Flowjo software. BD compbeads or cells were used to determine compensation values. To assess cell autofluorescence, unstained cells were used.

Viability was analyzed using Annexin V and 7-amino-actinomycin D (7-AAD). Annexin V binds specifically to phosphatidylserine, which is exposed on the surface of early and late apoptotic cells (90). 7-AAD penetrates cells that have lost their membrane integrity and binds DNA (91). Cells were washed twice with 1 ml PBS. Then, cells were re-suspended in 400 µl 1x Annexin binding buffer diluted 1:10, stained with 5 µl Annexin V-FITC and 10 µl 7-AAD and incubated for 20 minutes in the dark. Measurements were performed with FACS Calibur. Double negative cells (Annexin V/7-AAD -/-) were defined as viable cells.

Expression of surface markers (T cells) was assessed by antibody staining. Cells were separated from the magnetic beads, centrifuged (1600 rpm, 4 min, 4°C), supernatant was discarded, and cells were washed two times with 1 ml FACS buffer. Afterwards, samples were incubated with 5 µl anti-CD4, 10 µl anti-CD8, 5 µl anti-CD25, 20 µl anti-CD95 or 5 µl anti-CD137. After an incubation period in the dark (20 min, 4°C). Cells were washed again two times with 1 ml FACS washing buffer and finally re-suspended in 400 µl FACS buffer and measured.

3.2.5 Western blot analysis

Samples were lysed in RIPA (Sigma-Aldrich), separated by 12 % SDS-PAGE and transferred to PVDF membranes, blocked with 5 % milk (Sucofin) in TBS buffer with 0.1 % Tween for one hour, and incubated with primary antibodies overnight (α-GLUL, rabbit-anti-human/mouse, Abcam, and α-Tubulin, rabbit-anti-human/mouse, Sigma-Aldrich). Next, a secondary antibody (goat-anti-rabbit, HRP, Dako Cytomation) was used. Detection was performed by chemiluminescence (ECL, Amersham Bioscience) and analyzed using the chemiluminescence system Fusion Pulse 6 (Vilber Lourmat).

3.2.6 Measurements of cellular respiration

The PreSens technology (PreSens Precision Sensing GmbH) enables the non-invasive measurement of cellular respiration in real time under cell culture conditions for several days. The concentration of oxygen is measured in a 24 well plate with a small 24-channel SDR SensorDish® Reader. O_2 sensors are integrated at the bottom of each well. To measure oxygen consumption in C7H2 and Jurkat cell lines, 0.5 x 10^6 cells were cultivated.

3.2.7 Determinations of cytokines

The concentration of IFNɣ in culture supernatants was measured using ELISA (enzyme linked immunosorbent assay). The principle of this method is a multistep reaction, with initial binding of a cytokine specific capture antibody to a microplate. In the next step, standard or samples are added, followed by a detection antibody. Subsequently, this antibody binds Streptavidin-Horseradish peroxidase (HRP), which then converts the finally added substrate tetramethylbenzidine (TMB) to a yellow dye with intensity depending on the substrate concentration. The absorbance is measured at 450 nm. Using a standard curve allows absolute quantification of cytokine concentrations. The measurements were performed according to the manufacturer´s protocol.

3.2.8 Tracing analysis

To perform the [^{13}C]-glutamate tracing in cell culture, $CD8^+$, $CD4^+$, C7H2 and Jurkat cells were cultured for 24 hours with 2 mM [^{13}C]-glutamate (Cambridge Isotope Labs) in the absence of glutamine. After the removal of the supernatant, cells were washed twice with PBS and immediately frozen in liquid nitrogen. Supernatants and cell pellets were stored at -80°C until further analysis. Detection and analysis were performed by Dr. Katja Dettmer-Wilde (Institute of Functional Genomics, University of Regensburg, Regensburg, Germany). The data are presented as mean ^{13}C enrichment.

3.2.9 Statistics

Statistics were performed by use of the software “GraphPad Prism 9.5.0” and graphs show means with standard error of the mean (SEM). When examining the characteristics of two groups, the statistical significance was determined using the Wilcoxon or Mann-Whitney test. However, when investigating the effects of a specific treatment on variables, the analysis was performed using two-way ANOVA (Analysis of Variance) followed by the post-hoc Bonferroni’s multiple comparisons test or ANOVA and post-hoc by Tukey’s multiple comparisons test. P values of < 0.05 were considered as statistically significant (*), < 0.01 as being very significant (**) and < 0.001 as highly significant (***).

4. Results

4.1 Impact of glutamine restriction on leukemic T cell lines

Leukemia cells exert a profound impact on both blood and bone marrow. One of the distinctive hallmarks of leukemia cells is their heightened demand for nutrients. First, we aimed to investigate the effects induced by glutamine restriction on human leukemic cells, as glutamine can be important for their growth and survival (92) (93).
As our model system, we selected the T-ALL CCRF-CEM-C7H2 (C7H2) and Jurkat-FHCRC cell lines. Both cell lines were cultured in the presence or absence of glutamine (2mM). Our investigation focused on tracking alterations in cell proliferation, viability, morphology, and metabolism to elucidate the impact of glutamine deprivation on these cells.

4.1.1 Impact on proliferation, viability and morphology

Cell proliferation refers to the process by which cells divide and increase in number. We investigated the impact on cell proliferation in a time dependent manner. Initially, 300.000 cells per ml in a total volume of 20ml were sown and cultured. Both C7H2 cells and Jurkat cells were cultured either with 2mM glutamine (+Gln), or without glutamine (-Gln). After 48h in culture, cell number, viability and cell size were determined using the CASY System.
Glutamine depletion resulted in a reduction in proliferation for both cell lines. In general, C7H2 cells showed a stronger proliferation activity than Jurkat cells in the presence of glutamine. Glutamine deficiency induced a decline in proliferation of C7H2 cells after 48h by 53 % (n=5) while Jurkat cells were less affected, and the reduction only amounted to 33% (n=5) (**Figure 4A**).

Reduced cell numbers may stem from decreased proliferation. The absence of glutamine exerted only a minor impact on cell viability determined using the CASY System, during this brief period (**Figure 4B**).

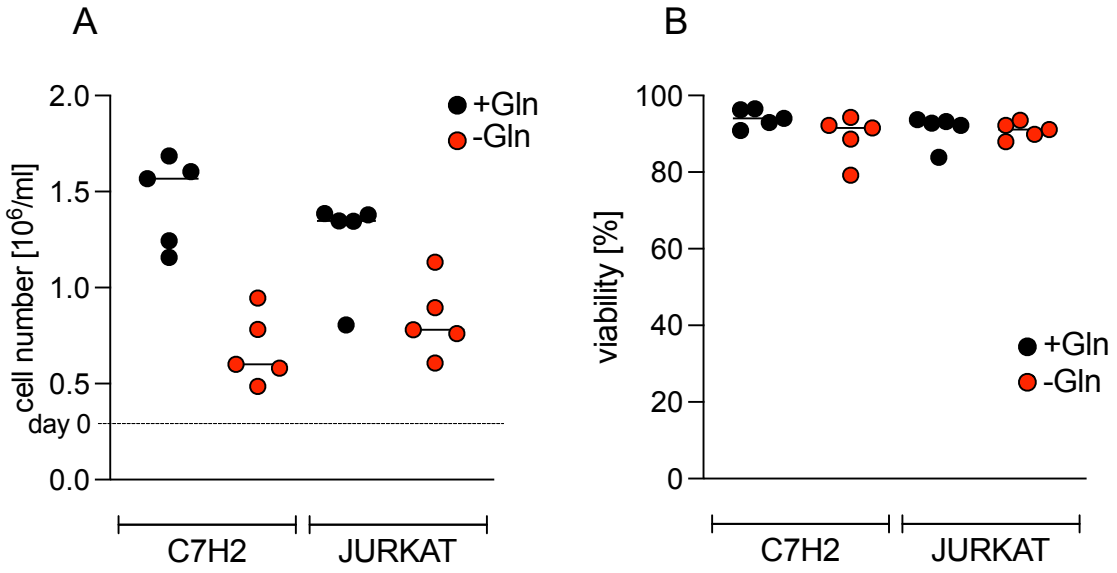


Figure 4. Impact of glutamine (gln) restriction on proliferation and viability of tumor cells.
Cell number [**A**] and viability [**B**] were determined after 48h using the CASY Cell Counter System. C7H2 and Jurkat cells were seeded at 300.00 cells/ml and incubated in the presence (+Gln) or in the absence of 2mM glutamine (-Gln) for 48h. Single data points are shown. Horizontal lines indicate the median. **A, B**: C7H2 and Jurkat n=5. Statistical significance was determined by Wilcoxon test (*p < 0.05, **p < 0.01, ***p < 0.001).

We further investigated cell viability in C7H2 and Jurkat cells upon glutamine deprivation by applying classical apoptosis staining. Annexin V and 7-AAD were used for the discrimination of dead cells by flow cytometry analysis.

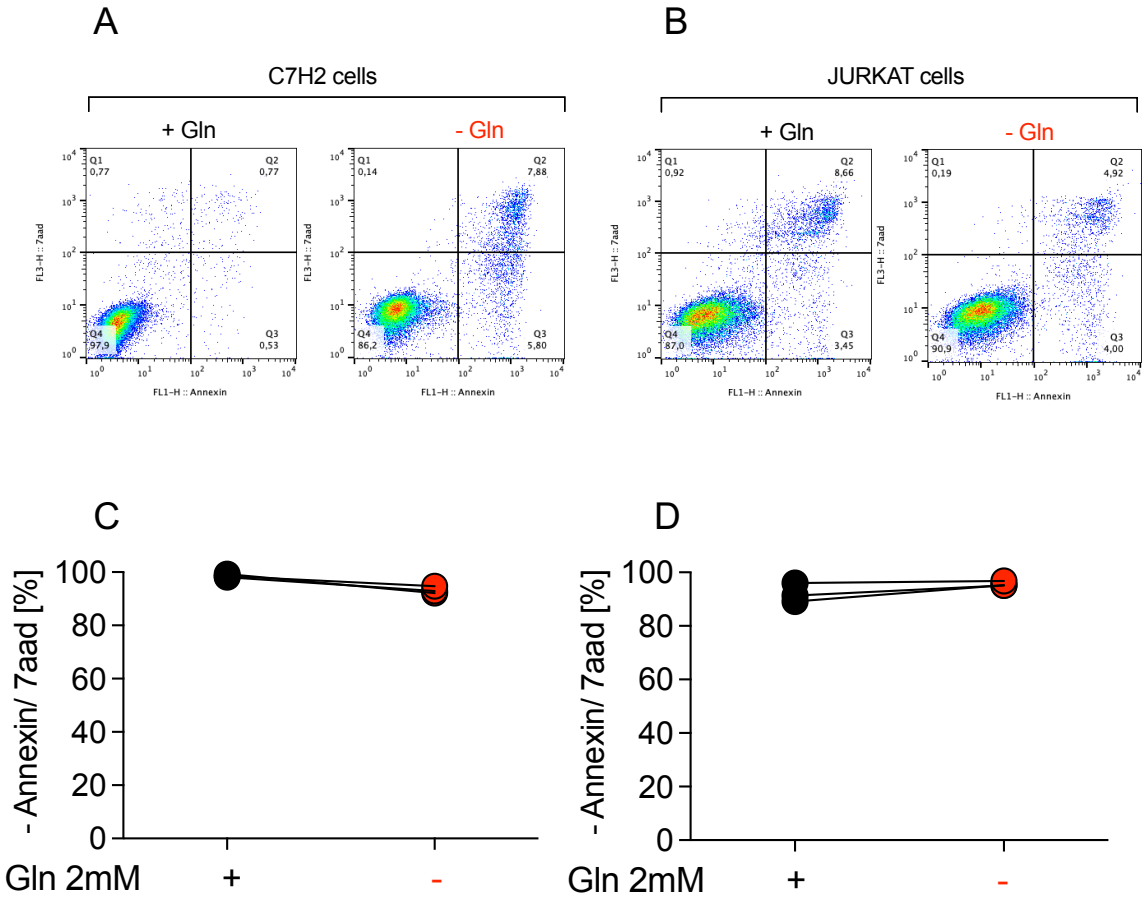


Figure 5. Impact of glutamine restriction on viability.
C7H2 [**A**] and Jurkat cells [**B**] were seeded at a density of 300.00 cells/ml and incubated in the presence (+Gln) or in the absence of 2mM glutamine (-Gln) for 48h. Viability was determined after 48h by flow cytometry with Annexin V and 7-AAD staining (**C**: C7H2, **D**: JURKAT cells). Single data points are shown. C7H2 and Jurkat n=3. Statistical significance was calculated by Mann-Whitney test (*p < 0.05, **p < 0.01, ***p < 0.001).

We noted a slight, but statistically not significant change in the expression of these apoptosis-related surface markers in both C7H2 (**Figure 5A**) and Jurkat (**Figure 5B**) cell lines under conditions of glutamine deprivation.

Concerning cell size, we observed no significant differences in both cell lines (**Figure 6A**). In addition, we documented the morphology of both cell lines through light microscopy. Jurkat cells appeared as cell clusters after 48h of glutamine deprivation, a phenomenon not observed in C7H2 cells. Furthermore, the cells of both cell lines appeared fewer in number and less closely spaced (**Figure 6B**).

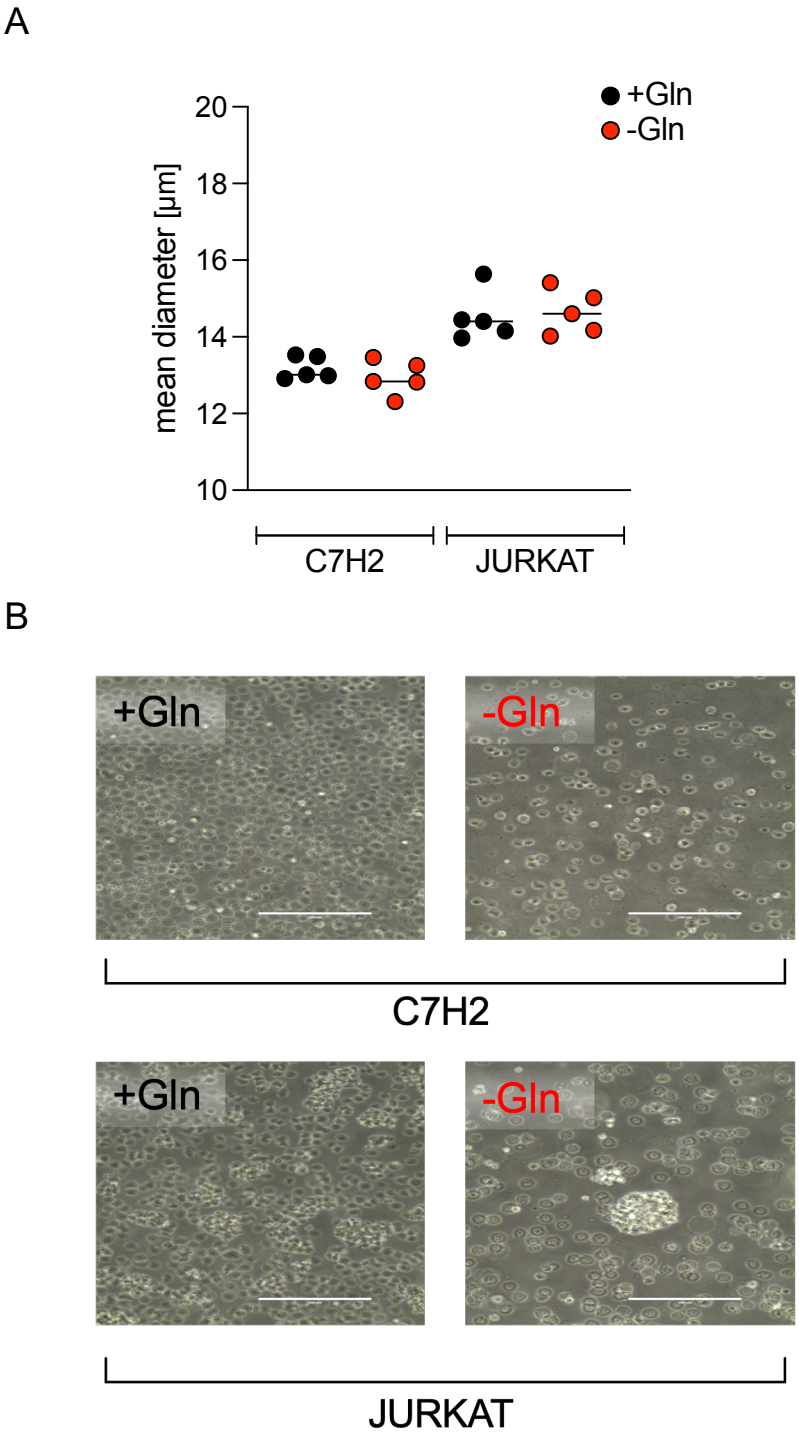


Figure 6. Impact of glutamine restriction on diameter and morphologie of tumor cells.
Mean diameter [**A**] were determined after 48h using the CASY Cell Counter System. C7H2 and Jurkat cells were seeded at 300.00 cells/ml and incubated in the presence (+Gln) or in the absence of 2mM glutamine (-Gln) for 48h. Micrographs (**B**) show the documentation of morphology of tumor cells under light microscopy. One representative picture for each condition is shown. Single data points are shown. Horizontal lines indicate the median. **A**: C7H2 and Jurkat n=5. Statistical significance was determined by Wilcoxon test (*p < 0.05, **p < 0.01, ***p < 0.001).

Taken together, short term glutamine deprivation exerted a significant impact on the proliferation of both cell lines, while survival and morphology were hardly affected. Notably, glutamine deprivation had a stronger impact on C7H2 cells than on Jurkat cells.

4.1.2 Long-term impact of glutamine deprivation

To provide a more comprehensive understanding of the effects of glutamine restriction on leukemic cells we investigated the impact of glutamine depletion in a long-term trial. Initially, we expected to see a decrease in the rate of cell division and an arrest in the cell cycle because of glutamine restriction in both cell types. This would have led to a decrease in the number of cells over time. Both cell lines were counted every 48h or 72h, reseed at 300.00 cell/mL in culture either with (+Gln) or without 2mM Glutamine (-Gln) and the theoretical yield was calculated.
In the presence of glutamine C7H2 proliferated exponentially while in glutamine deprived conditions cells hardly proliferated and finally showed no detectable proliferation at day 11 (**Figure 7A**). This indicates that the cells had attained a critical threshold in the cell cycle that made them vulnerable to the treatment. In the absence of glutamine, Jurkat cells showed a decreased rate of proliferation compared to the control group, but proliferation was only reduced, not blocked (**Figure 7B**).

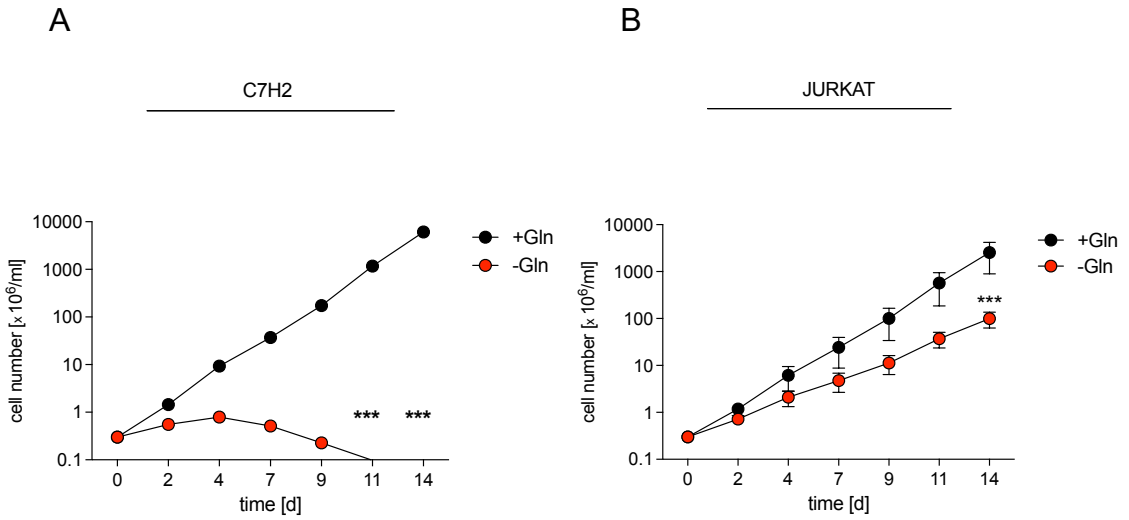


Figure 7. Long-term impact of nutrient restriction on proliferation of tumor cells. Theoretical cell yield was calculated. C7H2 **[A]** and Jurkat **[B]** cells were determined using the CASY Cell Counter System and reseed at 300.000 cell per mL every 48h or 72h. C7H2 and Jurkat cells were treated with (+Gln) and without glutamine 2mM (-Gln). Measurements every 48h or 72h. **A**, **B**: Mean+SEM, n=3. Significance was determined by two-way ANOVA and post-hoc Bonferroni's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001).

Moreover, we examined viability and cell size in both cell lines over time. The absence of glutamine exerted a significant impact, leading to a reduction in cell viability (**Figure 8A**) and diameter (**Figure 8B**) of C7H2 cells. Conversely, these parameters in Jurkat cells were not significantly altered by glutamine restriction. Within 14 days the viability of Jurkat cells remained constant (**Figure 8C**) between 87 and 100 % (n=3), both in the presence of glutamine and under glutamine-deprived conditions. In contrast, by day 9, the viability of C7H2 cells dropped below 50% (n=3) under glutamine restriction. The diameter of Jurkat cells was hardly affected by glutamine restriction over time (**Figure 8D**). These findings underscore a stronger dependence of C7H2 cells on exogenous glutamine compared to Jurkat cells.

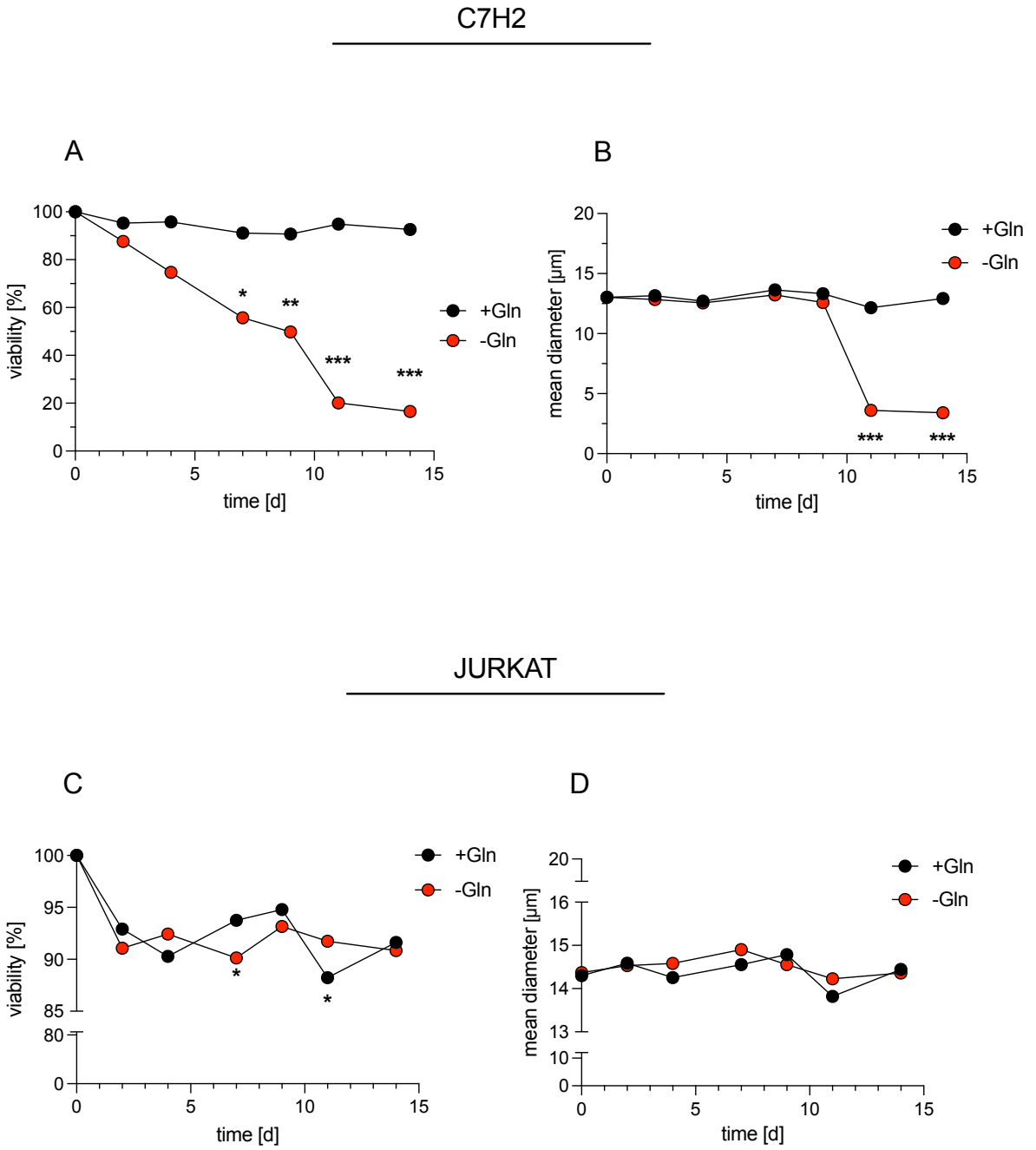


Figure 8. Impact of nutrient restriction on viability and diameter of tumor cells. Viability [**A, C**] and mean diameter [**B, D**] of both cell lines were determined using the CASY Cell Counter System and reseed at 300.000 cell per mL every 48h or 72h. C7H2 and Jurkat cells were treated with (+Gln) and without glutamine 2mM (-Gln). Measurements every 48h or 72h.Mean shown, n=3. Significance was determined by two-way ANOVA and post-hoc Bonferroni's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001).

To gain insights in the underlying mechanisms, we examined the impact of glutamine deprivation on mitochondrial respiration in both cell lines.

4.1.3 Effects on cellular respiration

As glutamine can serve as an important precursor for metabolites of the TCA cycle, providing the substrates for oxidative phosphorylation, we investigated the effects of glutamine restriction on cellular respiration. To monitor cellular respiration under cell culture conditions, we employed the PreSens technology. Initially, 500.000 cells per ml were seeded.

The control group of both cell lines exhibited a higher oxygen consumption rate compared to glutamine deprived cells, indicating the importance of glutamine uptake for cellular respiration. However, glutamine restriction resulted in a stronger decrease in mitochondrial activity in C7H2 cells, suggesting a dependency on exogenous glutamine supply (**Figure 9A**). Jurkat cells were only slightly affected by glutamine deprivation, suggesting that mitochondrial activity in Jurkat cells is not dependent on exogenous glutamine (**Figure 9B**). Notably, cellular respiration of C7H2 cells is particularly susceptible to glutamine restriction.

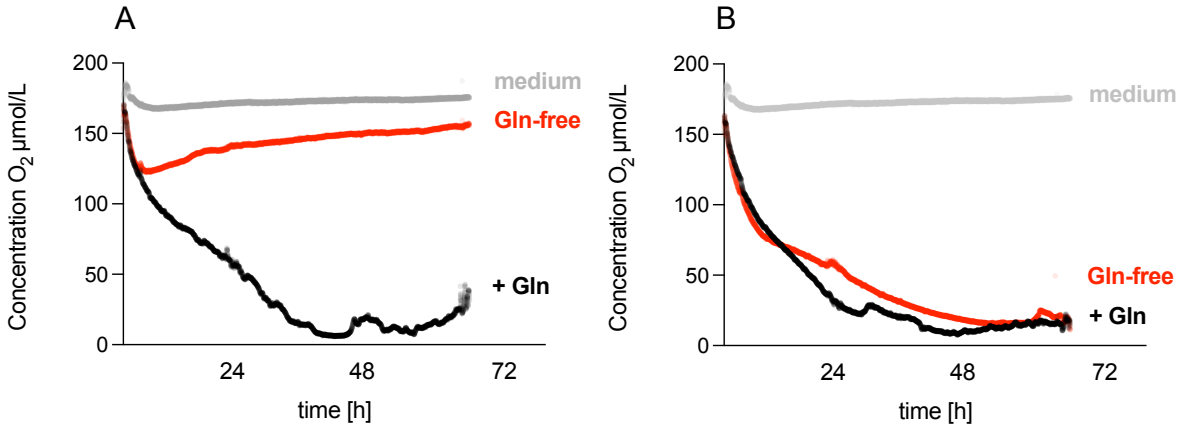


Figure 9. Mitochondrial respiration of tumor cell lines.
Oxygen consumption of C7H2 [**A**] and Jurkat [**B**] cells either with (+Gln) or without glutamine (2mM) (Gln-free) treatment were monitored for 72h non-invasively using the PreSens technology. Initially 0,4 * 10^6 cells/mM seeded (C7H2 and Jurkat, one representative experiment is shown).

Preserved respiratory activity might at least partially explain the proliferative and survival advantage of Jurkat cells under glutamine deprived conditions. The capability to

endogenously synthesize glutamine might be another key to protect cells from glutamine restriction.

4.2 Glutamine Synthetase in leukemic T cell lines

Glutamine synthetase (GS) catalyzes the conversion of glutamate and ammonia to glutamine. It is detected in many types of cells, including tumor cells (31). Glutamine synthetase plays a key role in maintaining high levels of glutamine needed for cell growth and proliferation. The lack of GS is largely responsible for exogenous glutamine addiction of various tumor cells (94). Analyzing the expression of glutamine synthetase in cancer cells under conditions of glutamine deprivation might provide important information about the role of this enzyme in tumor growth and survival. To examine the observed differences in glutamine dependency, we quantified the protein expression of glutamine synthetase in both cell types. In addition, we performed ^{13}C glutamate tracing analysis to determine the enzyme activity of GS.

4.2.1 Immunoblot analysis of GS expression

Both cell lines were cultured in the presence of glutamine (+Gln) and in the absence of glutamine (-Gln) for a period of 48h. We assessed the protein expression of glutamine synthetase (GLUL). Intriguingly, both C7H2 and Jurkat cells grown in a glutamine-rich medium exhibited a low GS expression, however, upregulated GS expression under glutamine deficiency (**Figure 10).**

As GS expression increased upon cultivation in the absence of glutamine in both cell lines, this upregulation alone cannot entirely account for the observed survival advantage of Jurkat cells in glutamine-deprived conditions.

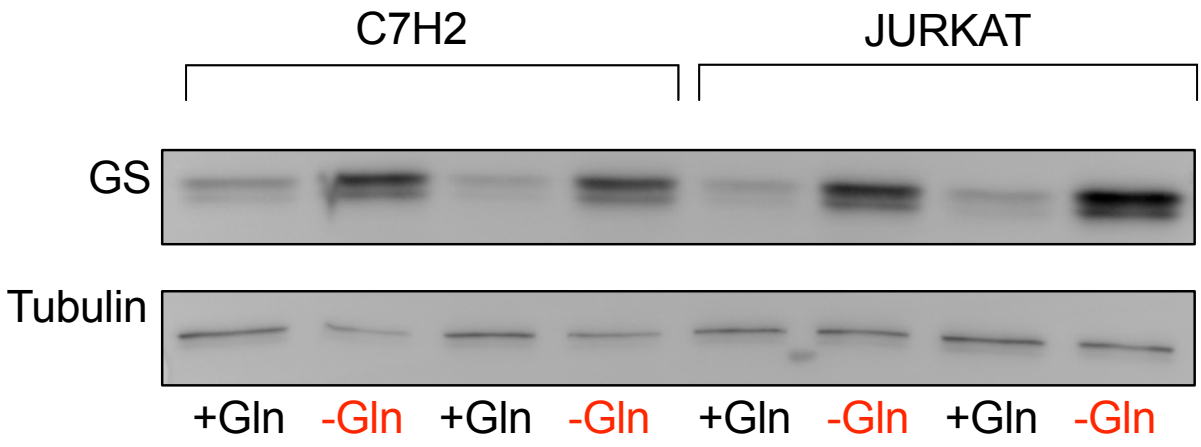


Figure 10. Immunoblot analyses of Glutamine synthetase.
C7H2 and Jurkat cells treated with (+) and without (-) gln in medium after 48 hours are shown. Tubulin was used as loading control. GLUL is overexpressed under gln deprived conditions. Two representative experiments are shown.

Nevertheless, as we cannot exclude differences in enzymatic activity, we performed metabolite tracing experiments.

4.2.2 [^{13}C]glutamate tracing analyses

C7H2 and Jurkat cells were cultivated with [^{13}C]glutamate (2mM) for 24h. Intracellular and extracellular levels of [^{13}C]glutamate and [^{13}C]glutamine were determined in cell lysates and culture supernatants by mass spectrometry. In both models system [^{13}C]glutamate supplementation resulted in intracellular [^{13}C]glutamate enrichment, showing glutamate uptake (**Figure 11A**). Furthermore, labeled glutamine was detected in both cell lines and the %enrichment was comparable, although quite low. Surprisingly, [^{13}C]glutamine was also detected in culture supernatants, indicating glutamine export (**Figure 11B**).
Taken together, both cell lines increased GS expression and glutamine synthesis from glutamate in the absence of glutamine.

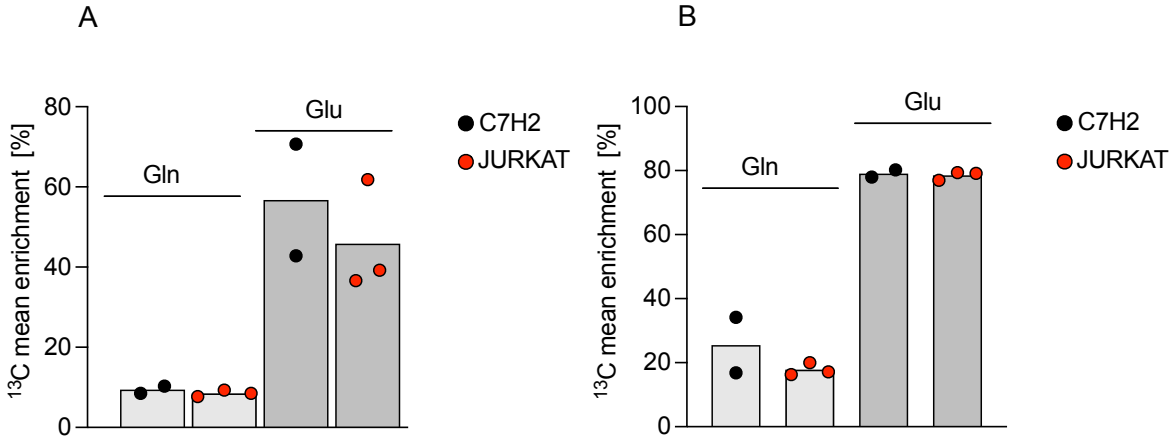


Figure 11. [^{13}C]Glutamate Tracing Analyses.
Cells were cultured with 2 mM [^{13}C]glutamate (Cambridge Isotope Labs) in the absence of glutamine for 24h. Measurements of intracellular and extracellular [^{13}C]glutamine (Gln) and [^{13}C]glutamate (Glu) by mass spectrometry. **A**: Cell pellet, **B**: Supernatant. (Mean, C7H2 n=2, Jurkat n=3). Horizontal lines show the median.

4.3 Rescue by glutamate supplementation

4.3.1 Impact on proliferation, viability and morphology

Since both leukemic cell lines exhibited GS activity, we investigated, whether glutamate supplementation could partially revert the negative impact of glutamine deprivation, with a special focus on C7H2 cells. Both cell lines were incubated for 72h either with (**I**) glutamine (2mM), (**II**) without glutamine and glutamate or (**III**) with glutamate (2mM) and without glutamine. Initial seeding involved 500.000 cells per ml.

In line with earlier observations, glutamine depletion induced a notable decrease in proliferation, particularly evident in C7H2 cells and to a lesser extent in Jurkat cells (**Figure 12A**). Glutamate supplementation mitigated the reduction in proliferation in both model systems. Moreover, glutamate supplementation could revert the slight effect on cell viability in C7H2 cells observed after 48h of glutamine restriction either determined with the CASY system **(Figure 12B**) or by Annexin V and 7-AAD staining (**Figure 12C-D**)

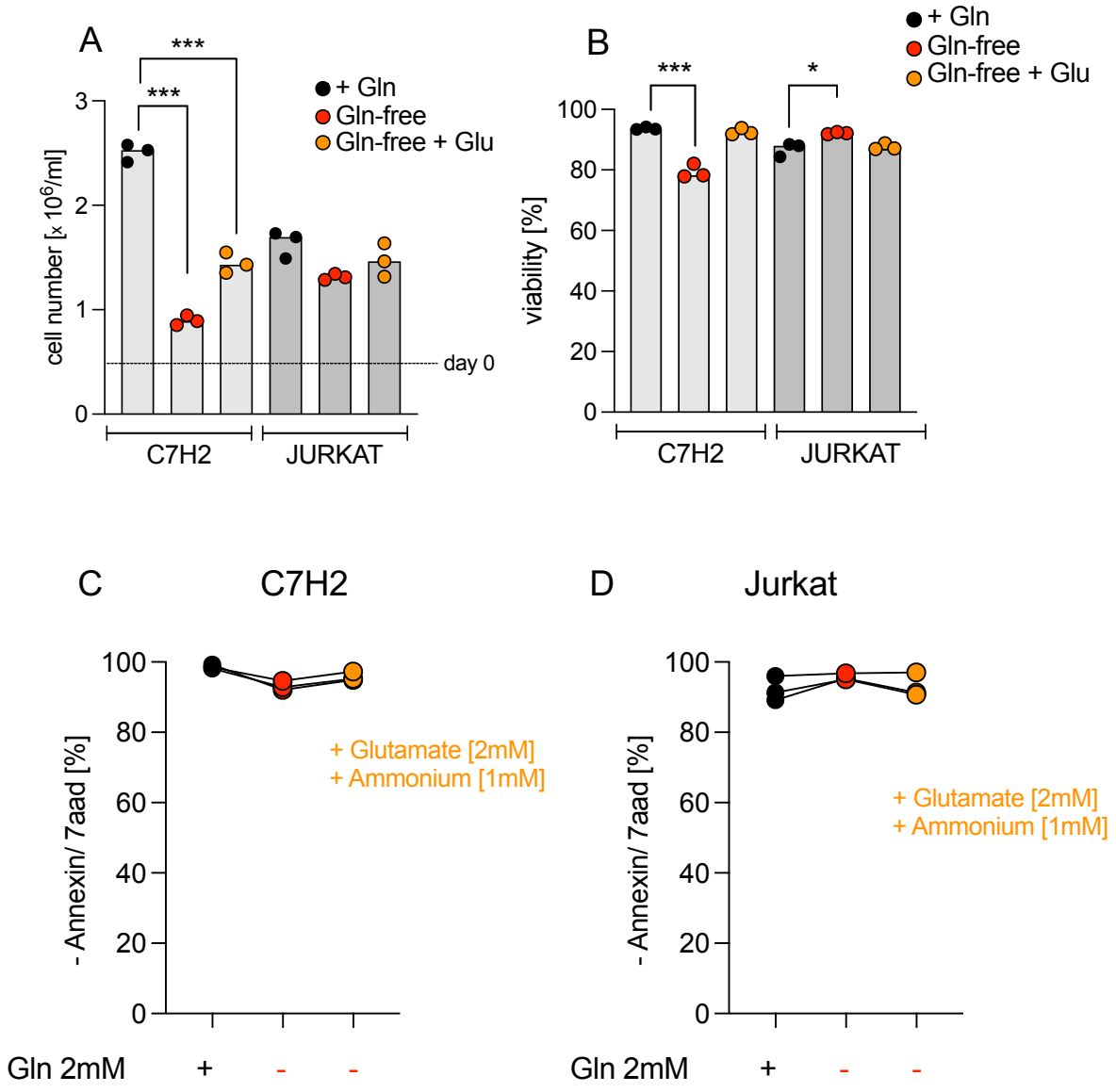
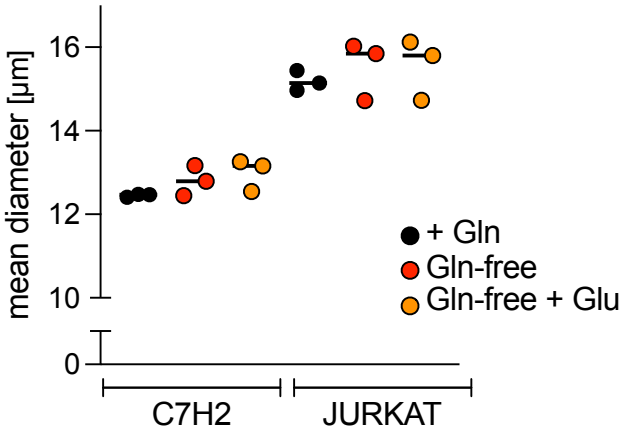


Figure 12. Impact of low-dose glutamate supplementation on proliferation and viability.
Cell number [**A**] and viability [**B**] were determined after 72h using the CASY Cell Counter System and cells were initially seeded at 500.00 cells/ml. C7H2 and JURKAT cells were incubated in the presence of glutamine (+Gln), absence of glutamine and glutamate (-Gln, -Glu) or without gln but glu (Gln: 2mM, Glu: 2mM). Viability was determined by flow cytometry with Annexin V and 7-AAD staining after 48 hours of treatment (**C, D**). n=3. Single data points are shown. Horizontal lines indicate the median. Cell number n=3, viability n=3. 12 A, B: Statistical significance was calculated with 2way ANOVA and post-hoc by Bonferroni's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). 12C, D: Statistical significance was calculated by Mann-Whitney test (*p < 0.05, **p < 0.01, ***p < 0.001).

There were no significant differences in cell size in both cell lines under the different treatments (**Figure 13A**). In addition, we documented the morphology of both cell lines. C7H2 cell density was increased by glutamate, however, not to the level of cultures grown with glutamine. As expected, there was hardly any difference in Jurkat cells between the groups exposed to glutamate and those without treatment. Both groups

showed cluster-like cell formations, which were clearly different from those in the group that underwent glutamine supplementation. (**Figure 13B**).

A



B

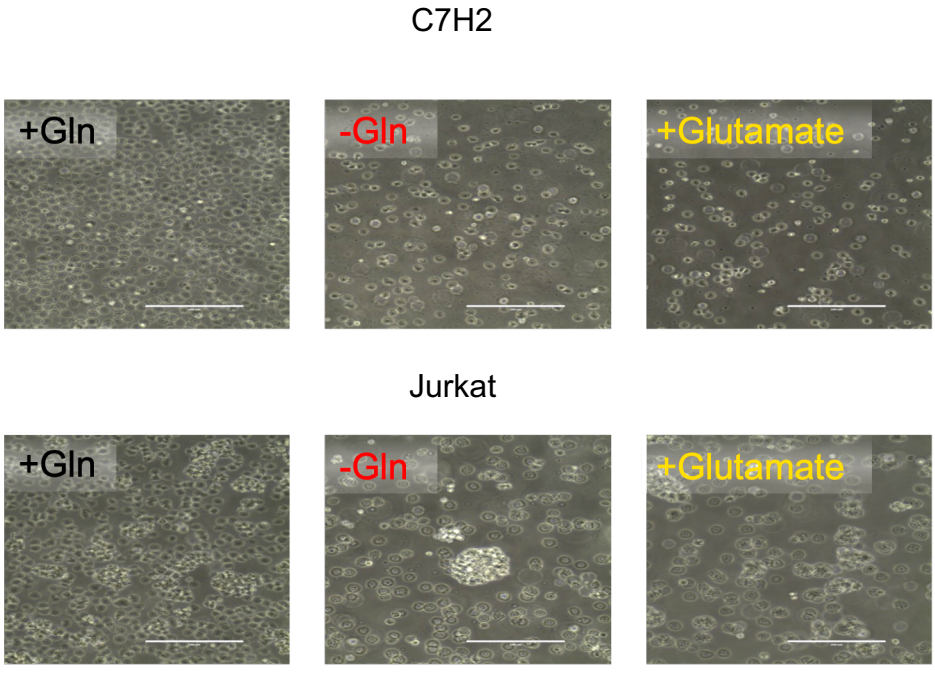


Figure 13. Impact of low-dose glutamate supplementation on diameter and morphology of tumor cells.
Diameter [**A**] was determined after 72h using the CASY Cell Counter System and cells were initially seeded at 500.00 cells/ml. C7H2 and JURKAT cells were incubated in the presence of glutamine (+Gln), absence of glutamine and glutamate (-Gln, -Glu) or without gln but glu (Gln: 2mM, Glu: 2mM). Micrographs (**B**) show the documentation of the morphology of tumor cells. One representative picture for each condition is shown. Single data points are shown. Horizontal lines indicate the median. Diameter n=3. Statistical significance was calculated with 2way ANOVA and post-hoc by Bonferronis multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

The addition of modest amounts of glutamate proved effective in boosting proliferation in C7H2 cells after 72 hours of glutamine deprivation. This suggests that, in the presence of glutamate, these cells have the capability to compensate for glutamine restriction. While Jurkat cells were generally less impacted by glutamine deprivation, the introduction of glutamate also exhibited a positive influence on both proliferation and viability in this cell line.

4.3.2 Impact on cellular respiration

As glutamate could partially rescue proliferation of C7H2 cells, the impact of glutamate on cellular respiration was determined. As already shown, glutamine restriction led to a strong decrease in mitochondrial activity only in C7H2 cells, which was mitigated by glutamate supplementation (**Figure 14A**). Jurkat cells did not show an improved respiration by substitution of glutamate (**Figure 14B**).

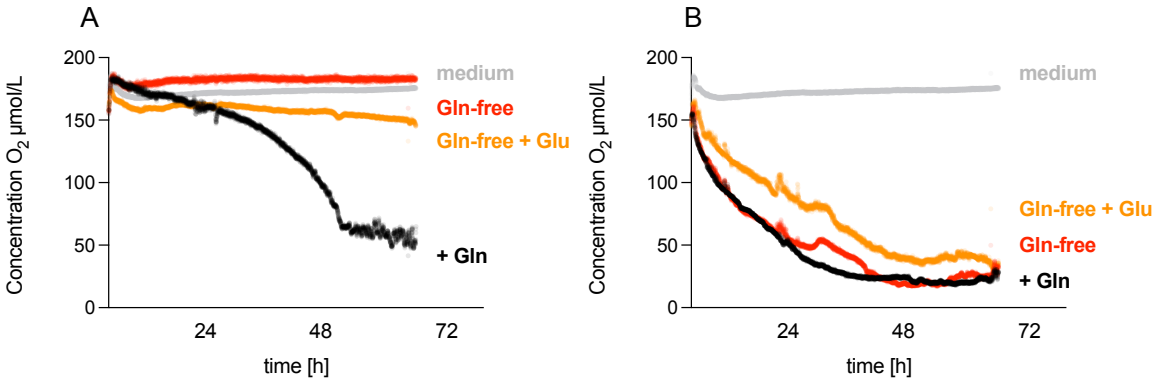


Figure 14. Mitochondrial respiration of tumor cell lines. Oxygen consumption of C7H2 [**A**] and Jurkat [B] cells in the presence (+Gln), absence of glutamine and glutamate (Gln-free) or with glutamate 2mM (Gln-free + Glu) treatment were monitored for 72h non-invasively using the PreSens technology. Initial 0,5 * 10^6 cells/mL seeded (C7H2 and Jurkat, one representative experiment is shown).

4.3.3 Impact of high dose glutamate supplementation

Considering that the uptake of glutamate might be concentration dependent, thus 2mM glutamate supplementation might not be sufficient, we investigated whether a high concentration of glutamate (10mM) could compensate for glutamine restriction. Indeed, increasing glutamate concentrations to 10 mM rescued the impact of glutamine deprivation on the proliferation of C7H2 cells. More precisely, 10mM glutamate induced an 89.5% (n=6) increase in proliferation compared to the group treated without glutamine or glutamate. (**Figure 15A**). Remarkably, Jurkat cells also exhibited increased proliferation in the presence of 10mM glutamate (**Figure 15D**).

Furthermore, high concentrations of glutamate had no discernible effect on cell viability or cell size in both cell lines (**Figure 15B+E**). The diameter of both cell lines showed hardly any change across all groups (**Figure 15C+F**). From these results, we concluded that glutamate compensates for a glutamine deficit in both cell lines.

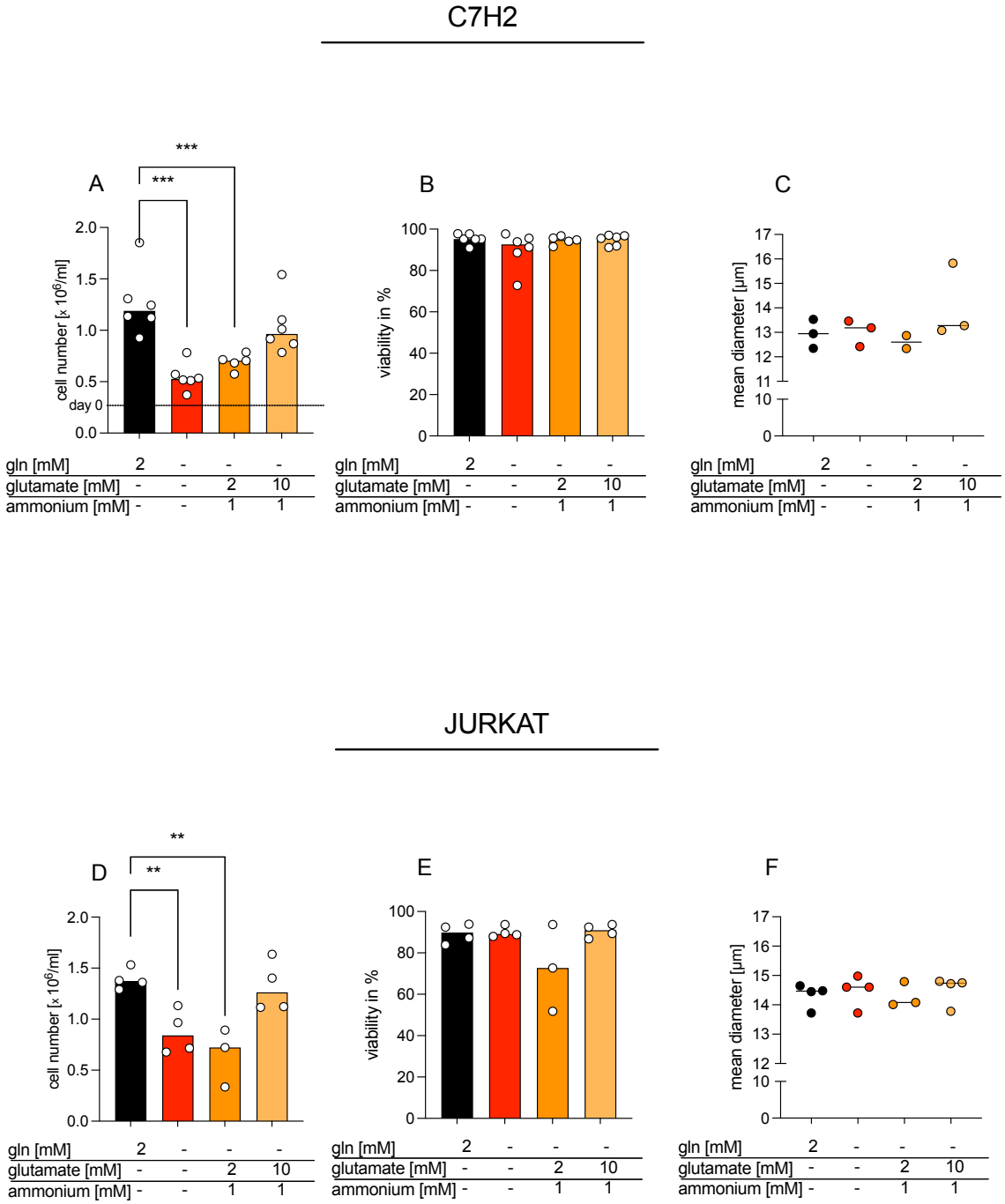


Figure 15. Impact of glutamate on proliferation, viability and diameter of tumor cells. Cell number (**A,D**), viability (**B,E**) and diameter (**C,F**) of both C7H2 and Jurkat cells were determined every 48h or 72h by CASY system. Tumor cells were incubated in the presence of 2mM glutamine (gln) or glutamine-free medium, or in glutamine-free medium supplemented with 2 - 20 mM glutamate (glu) and 1mM ammonium (am). n ≥ 3 (C7H2 2mM glu, 1mM am n=2), single data points are shown (**P value** 0.05>*>0.01>**> 0.001>***; treatment induced changes were analyzed with 2way ANOVA and post-hoc by Bonferroni's multiple comparisons test)

4.4 Impact of glutamate supplementation rescue on human T cells

The activation of T cells necessitates the presence of glutamine, and its removal results not only in the inhibition of their proliferation, but also in defects in effector functions such as TNF or IFNγ secretion (75) (3, data from the working group, PhD thesis C. Bruss). As glutamate demonstrated the ability to partially compensate for glutamine in C7H2 cells, we investigated whether glutamate could also replace glutamine in primary activated human T cells.

4.4.1 [^{13}C] glutamate tracing analyses

[^{13}C] glutamate metabolite tracing analyses were performed to investigate GS activity in CD3/CD28 activated CD8$^+$ and CD4$^+$ T cells. To ensure comparability with the results obtained for the leukemic cell lines, 2mM [^{13}C] glutamate was applied for 24h. Intracellular and extracellular levels of [^{13}C]glutamate and [^{13}C]glutamine were determined in cell lysates and supernatants, respectively. We observed no detectable amounts of [^{13}C]glutamine in CD4$^+$ and CD8$^+$ cells (**Figure 16A**) and only traces of [^{13}C]glutamine concentrations in supernatants (**Figure 16B**).

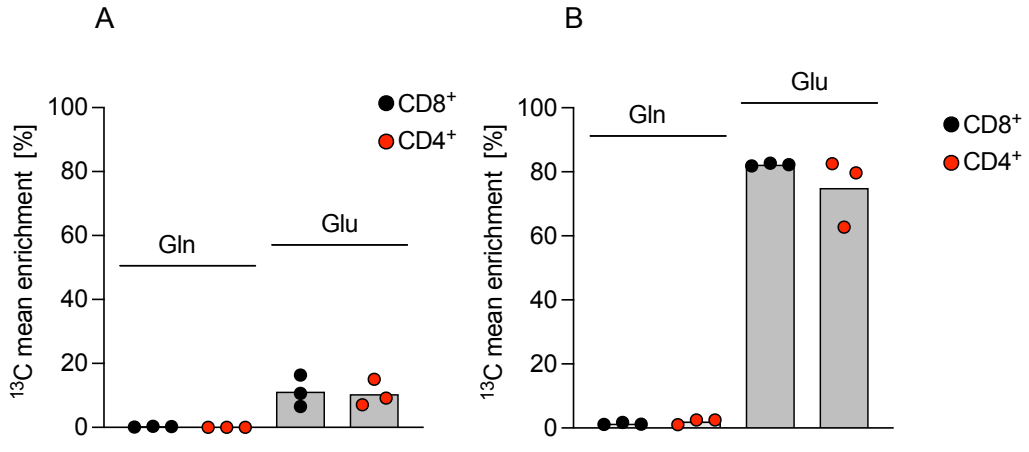


Figure 16. [^{13}C]Glutamate Tracing Analyses.
Cells were cultured with 2 mM [^{13}C]glutamate (Cambridge Isotope Labs) in the absence of glutamine for 24h. Measurements of intracellular and extracellular [^{13}C]glutamine (Gln) and [^{13}C]glutamate (Glu) by mass spectrometry. **A**: Cell pellet, **B**: Supernatant. (Mean, n=3). Horizontal lines show the median.

4.4.2 Impact on proliferation and morphology

While GS activity was not detectable in human primary T cell populations, we cannot exclude a positive impact of high extracellular concentrations of glutamate. Considering the low intracellular [^{13}C]glutamate enrichment, we hypothesized that glutamate uptake in T cells is limited and aimed to investigate the impact of higher concentrations.

Investigating forward and side scatter as indicators for cell size and granularity, cells cultivated with high doses of glutamate showed an increase in both parameters compared to glutamine restricted T cells (**Figure 17**).

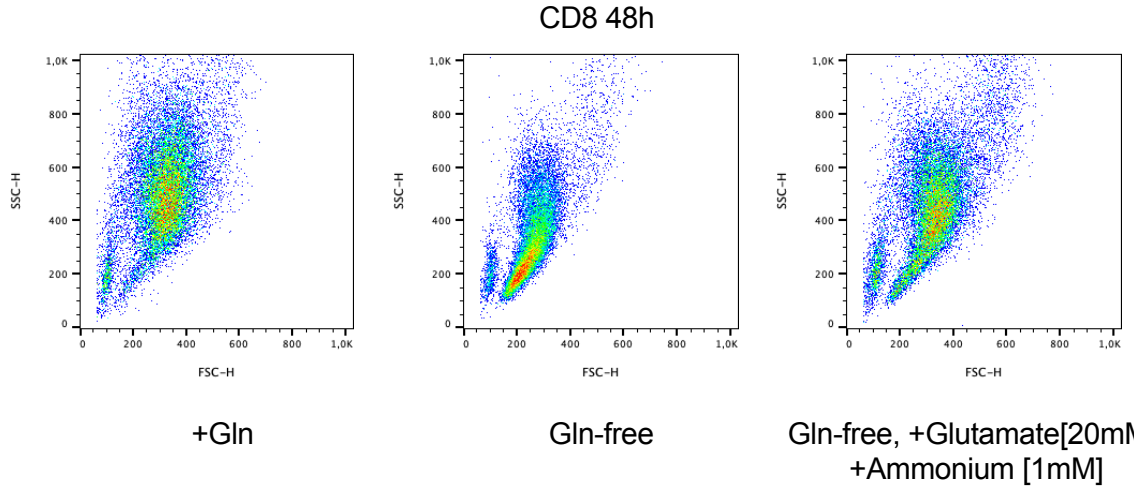


Figure 17. Impact of glutamate supplementation on cell size and granularity of stimulated human CD8$^+$ T lymphocytes cultivated in glutamine-free medium.
Flow cytometry measurement cell size and granularity after 48h in culture. T cells were incubated in the presence of 2mM glutamine (gln) or glutamine-free medium, or in glutamine-free medium supplemented with 20 mM glutamate (glu) and 1mM ammonium (am). One representative experiment is shown.

An essential effector cytokine of T cells is IFN-γ, a signaling molecule crucial for coordinating and amplifying the immune response, primarily produced by activated T cells (95). We used ELISA (Enzyme-Linked Immunosorbent Assay) technique to measure the concentration of IFN-γ in cell supernatants. As already known, the activation of T cells is directly proportional to the levels IFN-γ produced (96). T cells were either cultivated (**I**) with glutamine (2mM), (**II**) in the absence of glutamine (Gln-free and Glut-

free) and (**III**) with different amounts of glutamate (2 - 20mM) and ammonium to replace glutamine (1mM). Glutamate (20mM) and ammonium (1mM) could partially revert the negative impact on IFN-γ secretion in a concentration dependent manner. 20mM glutamate supplementation improved IFN secretion to 46 % of control level (**Figure 18A**). Accordingly, cell diameter (**Figure 18B**) and the number of cells increased, (**Figure 18C**) after 48h in culture.

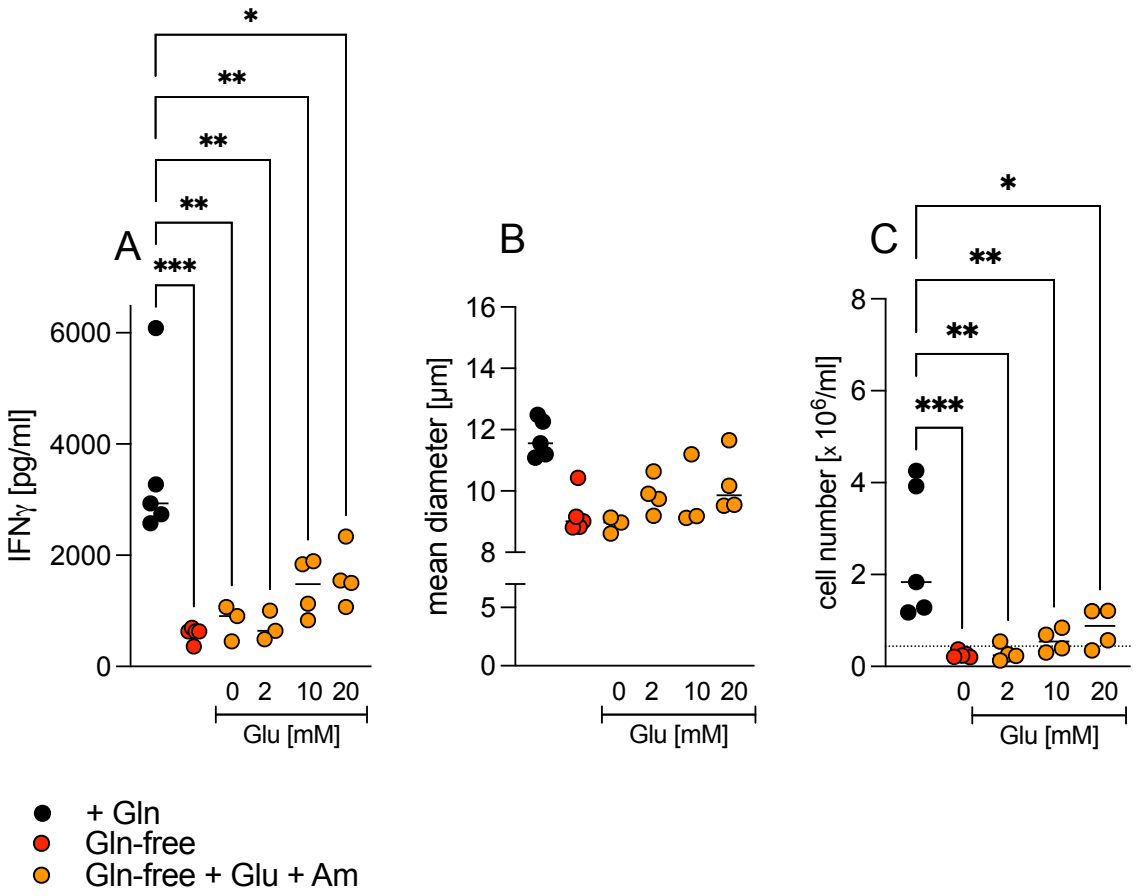


Figure 18. Impact of glutamate supplementation on IFN-γ levels, proliferation and diameter of stimulated human CD8+ T lymphocytes cultivated in glutamine-free medium. IFN-γ levels were determined in culture supernatants after 48 hours by ELISA (**A**). Diameter after 48h (**B**) and cell number on day 6 (**C**), were determined by CASY system. T cells were incubated in the presence of 2mM glutamine (gln) or glutamine-free medium, or in glutamine-free medium supplemented with 0 - 20 mM glutamate (glu) and 1mM ammonium (am). n ≥ 3, single data points are shown (**P value** 0.05>*>0.01>**>0.001>***; treatment induced changes were analyzed with ANOVA and post-hoc by Tukey's multiple comparisons test)

In a next step, we examined T cells cultures for 6 days and determined their proliferation, diameter, and viability. T cells were either cultivated (**I**) with glutamine (2mM), (**II**) in the absence of glutamine (Gln-free and Glut-free) and (**III**) with glutamate (20mM)

and ammonium (1mM) to replace glutamine and the parameters were analyzed in a time dependent manner. The addition of glutamate exerted a positive effect on cell growth but could not completely revert the effect of glutamine deprivation (**Figure 19A**). The reduced cell viability and proliferation could not be rescued by glutamate addition (**Figure 19B and C**).

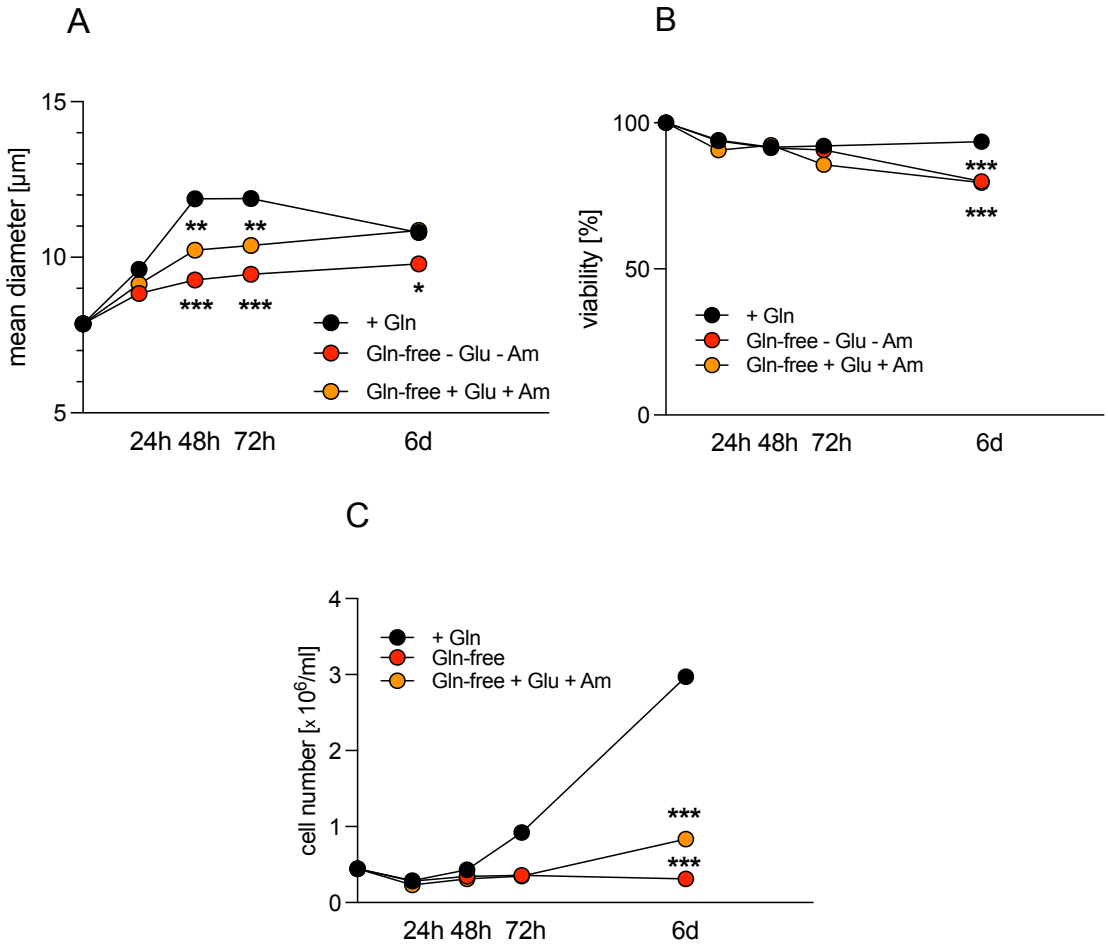


Figure 19. High concentration of glutamate cannot compensate for glutamine restriction
Flow cytometry analysis (**A**). Cell number (**B**), viability (**C**) and diameter (**D**) were determined by CASY system. T cells were incubated in the presence of 2mM glutamine (gln) or glutamine-free medium, or in glutamine-free medium supplemented with 20 mM glutamate (glu) and 1mM ammonium (am). Proliferation was measured after 24h, 48h, 72h and 6d of stimulation. n=4, mean is shown. (**P value** 0.05>*>0.01>**>0.001>***; treatment induced changes were analyzed with ANOVA and post-hoc by Bonferroni's multiple comparisons test)

These results indicate that a high dose of glutamate is partially able to compensate for glutamine in $CD8^+$ T cells in the first 48h as they show an improved on-blast formation, cytokine secretion and slightly improved proliferative activity. However, the viability of cells was affected, and this could not be rescued by glutamate supplementation.
Taken together, glutamate addition cannot fully compensate for glutamine even at very high concentrations. In our tracing experiment we did not observe glutamine synthesis from glutamate, indicating that T cells lack GS and thereby depend on exogenous glutamine provision. Further investigations are required in this regard.

5. Discussion

5.1 Glutamine addiction of C7H2, Jurkat and primary T cells: A Comparison

Cancer metabolism is a long-standing area of research in cancer biology that predates the discovery of oncogenes, “oncometabolites” and tumor suppressors by about 50 years. The field is based on the principle that metabolic activities are altered in cancer cells compared to normal cells and that these alterations support the acquisition and maintenance of their malignant properties (13). Since the discovery of the Warburg effect, glucose has been considered a central molecule in tumor metabolism research. In recent decades, scientists have also increasingly focused on the effects of amino acid metabolism in the tumor microenvironment, particularly the degradation of glutamine, one of the most thoroughly studied amino acids (2). Shifting energy generation by oxidative metabolism to glycolysis is perfectly suited to meet metabolic demands. To allow a continuous TCA flux glutamine oxidation increases in a process of anapleurosis that can provide α-ketoglutarate for the TCA cycle and metabolic intermediates for the biosynthesis of a variety of macromolecules (97). Isotope tracing analysis revealed that about half of the non-essential amino acids needed by tumor cells for protein synthesis derive from glutamine (2).

However, cancer cells are capable to switch their metabolism to adapt to low glutamine levels in order to survive and proliferate (98). We aimed to provide a more comprehensive understanding of the importance of glutamine metabolism for both leukemic and primary human T cells. Immune cells, including T cells share similarities with the metabolism of tumor cells. T cells show elevated glucose metabolism upon stimulation. Furthermore, glutamine plays a critical role in immune responses by regulating the activation and proliferation of T lymphocytes, B lymphocytes, natural killer cells and macrophages, as well as the production of antibodies and cytokines (76). T cells are critically affected by glutamine restriction (20). Glutamine deprivation strongly affected the proliferation of leukemic and primary $CD8^+$ T cells. However, viability was only affected of C7H2 cells. The differential sensitivity to glutamine restriction might be partially explained by the fact, that respiration of Jurkat cells was not glutamine dependent, whereas the respiration of C7H2 cells and $CD8^+$ T cells was significantly impaired by

glutamine depletion. Jurkat cells might be adapted to glutamine restriction by activating different metabolic pathways. Their respiration may be fueled by other substrates, glutamine independently, as fatty acid degradation or glucose metabolism. Moreover, Jurkat cells may efficiently utilize available resources. Furthermore, expression of the glutamine synthetase, encoded by the GLUL gene, enables cells to synthesize their own glutamine, rendering them less sensitive to glutamine restriction (99). The level of GLUL expression is tissue, cancer cell type dependent and has an impact on prognosis (34). Increased GLUL expression has been associated with poor prognosis in glioblastoma and hepatocellular carcinoma (100). In addition, basal-type breast cancer cells have been reported to have low GLUL expression and to be more glutamine-addicted compared with luminal-type breast cancer cells (34). Moreover, the study by Chen et al. found that patients with high levels of genes related to glutamine metabolism have a poor prognosis (101).

Thus, we investigated GLUL expression and activity in leukemic cells and primary T cells. We found that both leukemic cell lines increased the expression of GLUL under glutamine deprivation. GLUL expression solely did not explain the survival advantage of Jurkat cells under glutamine deprived conditions. Therefore, we examined GS activity by determining the conversion of ^{13}C-labeled glutamate into glutamine. Our tracing experiments revealed that both Jurkat and C7H2 cells can metabolize glutamate to glutamine and export glutamine under glutamine restriction, whereas primary T cells were not able to do the same.

Since glutamine synthetase requires glutamate as a reactant, we hypothesized that glutamate supplementation could lead to increased glutamine synthesis. As glutamate transport might be limiting, we tested 2mM glutamate doses and then higher doses of glutamate. Of course, we know that a dosage of 20mM is beyond the physiological range, although tumors display elevated glutamate levels (102). A crucial transporter in this context is the amino acid exchanger, alternatively recognized as the cystine-glutamate transporter (xCT/SLC7A11). Its significance lies in its pivotal role in sustaining redox homeostasis by swapping extracellular cystine with intracellular glutamate, thus upholding the intracellular redox equilibrium (103). The Excitatory Amino Acid Transporters (EAATs) play a crucial role in the uptake of glutamate (104). Elevated

expression of EAATs in non-neuronal cancer is associated with heightened cellular proliferation and enhanced viability (105). This phenomenon contributes to tumor advancement through the elevation of glutamate levels (105). Already the addition of low doses of glutamate enhanced the proliferation of C7H2 cells in the absence of glutamine. Given the already robust survival of Jurkat cells in the absence of glutamine, the supplementation of glutamate did not result in any noteworthy enhancement of cell survival. Higher doses of glutamate showed a further improvement in survival. Higher doses of glutamates could lead to an improvement in GS activity and thus to increased glutamine production. The presence of glutamate induces the expression of glutamine synthetase (GS) in both C7H2 and Jurkat cells. The difference may be due to the fact that Jurkat cells have increased intracellular glutamate levels, whereas C7H2 cells derive a relatively greater advantage from the uptake of extracellular glutamate. As an extracellular ligand, glutamate can initiate signaling through diverse glutamate receptors, thereby activating pathways associated with cellular proliferation (106). However, it is also possible that the high doses of glutamate fulfil compensatory functions. Glutamate can be directly converted to α-KG by GLUD, alanine or aspartate transaminase (TAs) and introduced into the TCA cycle (2). Within breast cancer, the upsurge in cellular proliferation corresponds to heightened uptake of glutamate and aspartate, serving as substrates for the tricarboxylic acid (TCA) cycle, consequently intensifying metabolic activity. The elevation of glutamate and aspartate levels in breast cancer is facilitated by the actions of EAAT2 (105).

Although T cells do not express GLUL, glutamate might support human CD8+ T cell function by other mechanisms. Human $CD4^+$ T cells are able to import glutamate quite efficiently (107). However, glutamate transporters and receptors in $CD8^+$ T cells are less investigated. Low doses of glutamate could not improve $CD8^+$ T cell function in the absence of glutamine. Nevertheless, higher doses of glutamate increased proliferation and cytokine secretion, although not to control level in $CD8^+$ T cells. Glutamate might be introduced into the TCA cycle via α-KG and fuel the TCA thus improves T cell proliferation and function. A less likely possibility would be that high glutamate concentrations lead to the expression of GLUL in T cells. Further studies of T cells treated with high glutamate concentrations would be required to confirm this.

Previous work showed that the lack of glutamine blocked the growth, proliferation, and production of cytokines such as IFN-γ and IL-2 in murine T cells (3) (75). Furthermore, this effect could not be reversed by replacing biosynthetic precursors (proline or asparagine) or products (glutamate) of glutamine (3) (70) (108). Preliminary work showed that activation of a naive T cell, which is also a high-energy event, requires a substantial increase in nutrient metabolism, including glutamine. Deficiency of glutamine was found to block T cell proliferation and cytokine production, which could not be remedied even by the supply of biosynthetic glutamine precursors (3).

In our study we have also shown that human $CD8^+$ T cells are neither activated nor proliferate in the absence of glutamine. They also did not proliferate and hardly produced any cytokines in the absence of glutamine. Christina Bruß has demonstrated in her PhD thesis that $CD8^+$ T cells are reduced in their metabolic activity under glutamine withdrawal. She also found that $CD8^+$ T cells do not express the glutamine synthetase (75). We added the ^{13}C-Glutamate (2mM) tracer analyses in this context, which also confirmed that T cells are not able to convert glutamate into glutamine.

Since glutamine is essential for T cells in their activation phase and for their growth (76), one possibility would be to let the cells produce glutamine themselves in in a glutamine-deprived environment. Since T cells do not express GS, it could be integrated into T cells by means of a vector (75). This could be done on a temporary level or on a permanent basis. Further experiments in this regard are required.

Moreover, understanding the precise role and requirement of glutamine in tumor cells and T cells is critical for the development of effective cancer therapies. This could potentially lead to strategies that selectively target glutamine metabolism in tumor cells without affecting T cell function or strategies that increase T cell resistance to glutamine deficiency.

5.2 Outlook: The micro milieu of glutamine-dependent tumors

Previous studies have shown that T cells, $CD8^+$ T cells in particular, are especially important in the fight against cancer (109). There is evidence that the metabolic characteristics of the tumor influence the metabolism of the T cells (76). In addition to tumor cells, T cells also need high levels of amino acids for their activation, differentiation and to fight the tumor. Deficiencies of certain amino acids impair the function of the immune system (76). In the tumor micromilieu, nutrient competition, e.g. for glucose or glutamine, can therefore occur between tumor cells and immune cells (39). Pan et colleagues found that the core region of solid tumors is deficient in glutamine compared to other amino acids. In some cases, glutamine could no longer be detected in the tumor environment (38). Consequently, the limited availability of glutamine in the tumor's microenvironment could potentially contribute to tumor immune escape.

Christina Bruß showed in her PhD thesis that, contrary to murine studies, glutamine and not glucose should be considered as a key limiting factor in the tumor microenvironment (75). Remarkably, even glutamine levels at the lower limit of serum concentration have a significant impact on the proliferation of $CD8^+$ T cells. Since $CD8^+$ T cells cannot express GLUL glutamine deficit leads to metabolic stress. These results underscore the importance of maintaining optimal glutamine levels in the tumor microenvironment to support robust $CD8^+$ T cell proliferation and consequently enhance the antitumor immune response (75). Apart from tumor cells, GS is also expressed by macrophages. The Mazzone research team noted elevated levels of GS protein expression in quiescent human macrophages (110). Additionally, Robert Schoeppe and Nathalie Babl demonstrated heightened constitutive GS expression in macrophages (111). Within the tumor microenvironment (TME), these cells can serve as a significant supplier of glutamine to support T cells (111).

The interplay between glutamine, tumor cells and T cells is complex and an active area of research. On the one hand, the high glutamine requirement of tumor cells may lead to glutamine deficiency in the tumor microenvironment, which may impair T cell function and contribute to tumor evasion of the immune system (38). In tumors with elevated GLUL expression, this represents a potential therapeutical target. One such

approach was demonstrated by Pillai et al. using the combination of DRP-104 with checkpoint inhibition to suppress tumorigenic metabolism in lung cancer and enhance anti-tumor T-cell responses (112). Jonathan D. Powell disrupted the immunosuppressive tumor microenvironment by employing a glutamine antagonist. His research illustrated that inhibiting glutamine metabolism in mice with tumors hampers both oxidative and glycolytic metabolism in cancer cells, resulting in reduced hypoxia, acidosis, and nutrient scarcity (113). On the other hand, strategies to inhibit glutamine metabolism in tumor cells could potentially affect T cells and especially myeloid-derived suppressor cells (MDSCs) as well (74). Myeloid cells, so-called tumor-associated macrophages, often infiltrate the tumor tissue as well (114). Clear cell renal carcinoma tumor cells trigger the secretion of IL-23 by macrophages infiltrating the tumor site through the localized depletion of extracellular glutamine. This IL-23 secretion has the potential to stimulate tumoricidal Th17 responses (115). Nevertheless, heightened GS expression in myeloid cells during starvation conditions may facilitate the secretion of glutamine for consumption by neighboring cells (111). Further investigations are necessary.

6. Conclusion

As glutamine levels are often low in the cancer environment (116), glutamine can be the limiting factor in the survival of both tumor cells and immune cells. Since different tumor cells can synthesize glutamine but not T cells due to the lack of GS expression (75), cancer cells have an advantage: they can bypass the immune system. We have found that glutamine deprivation restricts C7H2 cells in their growth more than Jurkat cells. This indicates that distinct identities of tumor cells exhibit varying levels of reliance on glutamine.

Glutamate in small doses managed to compensate for glutamine withdrawal to a small extent. These were mainly exhibited by C7H2 cells through enhanced proliferation and increased cellular respiration. Higher doses showed an even more pronounced effect in this cell line. There is a possibility that compensatory mechanisms involving alternative nutrients could offset the deficit in glutamine.

Glutamine plays a crucial role in the metabolic needs of T cells, as they exhibit a pronounced dependency on it. T cells lack the ability to independently produce glutamine, relying on the uptake of exogenous glutamine and its supply by cells that produce and secrete glutamine extracellularly. High dose of glutamate is partially able to compensate for glutamine in $CD8^+$ T cells in the first 48h as they show an improved on-blast formation, cytokine secretion and slightly improved proliferative activity.

Regarding ongoing research targeting glutaminolysis as a therapeutic strategy, the role of glutamine metabolism with a special focus on the glutamine synthetase needs further investigation. Such a strategy might be effective in tumor cells expressing glutamine synthetase but could have adverse effects on the anti-tumor response of T cells and myeloid cells. Special attention should be paid to the micromilieu of the tumor.

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8. Appendix

8.1 German abstract

Die Glutamin-Synthetase in lymphatischen Leukämiezellen und nicht-malignen T-Lymphozyten

T-Zellen zeigen während ihrer Aktivierungsphase ähnliche metabolische Eigenschaften wie Tumorzellen. Schnell wachsende und sich rasch teilende Zellen müssen ihren Stoffwechsel anpassen, wobei Glutamin als teilweise essenzielle Aminosäure, insbesondere für Zellen mit hohem Energiebedarf, eine bedeutende Rolle spielt.

Zunächst untersuchten wir die Auswirkungen des Glutamin Entzugs auf die Proliferation, das Überleben und den Stoffwechsel von Tumorzellen. Als Modellzellen wählten wir die beiden Zelllinien T-ALL CCRF-CEM-C7H2 (C7H2) und Jurkat-FHCRC. Es wurde festgestellt, dass beide Zelllinien unter Glutamin Entzug schlechter proliferierten, wobei sich der Entzug bei den C7H2-Zellen stärker auswirkte. Die Jurkat-Zellen wuchsen zwar langsamer unter Glutamin Entzug, setzten jedoch ihr Wachstum und ihre Teilung fort.

Um Einblicke in die zugrundeliegenden Mechanismen zu gewinnen, analysierten wir die Auswirkungen des Glutamin Mangels auf die mitochondriale Atmung in beiden Zelllinien. Dabei wurde festgestellt, dass die zelluläre Atmung in den C7H2-Zellen deutlich reduziert war, während die Atmung in den Jurkat-Zellen nur gering beeinträchtigt war.

Im nächsten Schritt untersuchten wir die Fähigkeit zur endogenen Synthese von Glutamin und stellten fest, dass sowohl C7H2- als auch Jurkat-Zellen unter Glutamin Entzug die Glutamin-Synthetase exprimierten. Um Unterschiede in der enzymatischen Aktivität auszuschließen, führten wir Experimente zur Rückverfolgung von Metaboliten durch. Dabei wiesen wir die Aktivität der Glutamin-Synthetase in beiden Zelllinien unter Glutamin Entzug in der Tracing-Analyse mittels markiertem ^{13}C-Glutamat nach.

Schließlich überprüften wir, ob die Zugabe von Glutamat den Glutamin Entzug ausgleichen konnte. Dabei stellten wir fest, dass hohe Dosen von Glutamat das Überleben der C7H2-Zellen deutlich verbesserte.

Im zweiten Teil der Arbeit untersuchten wir, wie sich der Glutamin Entzug bei humanen $CD8^+$ T-Zellen auswirkte. Es ist bekannt, dass diese T-Zellen unter Glutamin Entzug nicht proliferieren, nicht aktiviert werden und ihre Zytokin Produktion eingeschränkt ist. Hochkonzentriertes Glutamat in der Zellkultur konnte zumindest teilweise den Glutamin Entzug bei T-Zellen ausgleichen, was sich in einer leicht gesteigerten Proliferation und Zytokin Produktion im Vergleich zur Gruppe, bei der kein Glutamat oder Glutamin hinzugegeben wurde, zeigte.

Weitere Untersuchungen sind notwendig, insbesondere mit Blick auf das Mikromilieu des Tumors, wo Immunzellen auf den Tumor treffen. Da bestimmte Aminosäuren, wie beispielsweise Glutamin, in der Tumorumgebung in sehr geringen Konzentrationen vorkommen, könnten Tumorzellen dort einen möglichen Überlebensvorteil haben. In zukünftigen Untersuchungen sollte das Mikromilieu von Tumoren weiterhin eine wichtige Rolle spielen.

8.2 Abbreviations

Acetyl-CoA	Acetyl coenzyme A
ALL	Acute lymphatic leukemia
Am	Ammonium
ANOVA	Analysis of variance
APC	Allophycocyanin
APCs	Antigen presenting cells
ATP	Adenosin Triphosphate
α-KG	α-Ketoglutarate
7-AAD	7-Aminoactinomycin D
CD	Cluster of differentation
CO_2	Carbon Dioxide
CTLs	Cytotoxic T lymphocytes
C7H2	T-ALL CCRF-CEM-C7H2
DC	Dendritic cell
DMSO	Dimethylsulfoxid
EDTA	Ethylendiamintetraacetat
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FCS	Fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GLUD	Glutamate Dehydrogenase
Glu	Glutamate
Gln	Glutamine
GLUL/ GS	Glutamine Synthetase
GLUT	Glucose transporter
GLS	Glutaminase
IFNɣ	Interferon ɣ
IL	Interleukin
Jurkat	Jurkat-FHCRC-FADD-/-

MACS	Magnetic cell separation
MHC	Major histocompatibility complex
Ml	Mililiters
mRNA	Messenger RNA
µm	Mikrometers
N	Number of samples
NAD^+/H	Nicotinamide adenine dinucleotide
$NADP^+$/H	Nicotinamide adenine dinucleotide phosphate
Nk cells	Natural killer cells
Nm	Nanometer
O_2	Oxygen
OAA	Oxaloacetate
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PET-CT	Positron Emission Tomographie – CT
Rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
SLC	Solute-Carrier-Transporter
SSC	Sideward scatter
T-ALL	T cell acute lymphoblastic leukemia
TAM	Tumor associated macrophages
TCA	Tricarboxylic acid cycle
T_{eff}	Effector T cells
TH	T helper cells
TME	Tumor microenvironment
T_{mem}	T memory cells
T_{regs}	Regulatory T cells
W/o	Without

8.3 Publication

Schoeppe R, Babl N, Decking SM, Schönhammer G, **Siegmund A**, Bruss C, Dettmer K, Oefner PJ, Frick L, Weigert A, Jantsch J, Herr W, Rehli M, Renner K, Kreutz M. Glutamine synthetase expression rescues human dendritic cell survival in a glutamine-deprived environment. Front Oncol. 2023 Jan 19;13:1120194. doi: 10.3389/fonc.2023.1120194. PMID: 36741028; PMCID: PMC9894315.

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8.5 Curriculum vitae

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8.6 Declaration

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für die Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch Ausland in gleicher oder in ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, Juli 2024