Impact of drugs targeting tumor metabolism on CD8 T cell effector function

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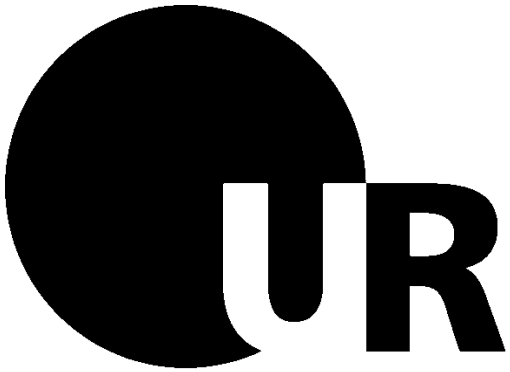

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To my parents

Meinen Eltern

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2-DG	2-deoxyglucose
2-NBDG	(2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose
7AAD	7-amino-actinomycin D
acivicin	((2S)-amino-(5S)-3-chloro-4,5-dihydro-1,2-oxazol-5-yl ethanoic acid)
ACT	adoptive cell transfer
AKT	v-akt murine thymoma viral oncogene homolog 1
AMPK	5' AMP-activated protein kinase
ANOVA	analysis of variance
APC	allophycocyanin
APC	antigen presenting cell
APS	ammonium persulfate
ARG	arginase
ATP	adenosine triphosphate
BPTES	(bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide)
BSA	bovine serum albumine
BV	brilliant violet
CAR	chimeric antigen receptor
CCL4	CC chemokine ligand 4
CCR7	C-C chemokine receptor
CD	cluster of differentiation
CM	central memory
CO_2	carbon dioxide
COX	cyclooxygenase
CTL	cytotoxic T cell
CTLA-4	cytotoxic T lymphocyte associated protein 4
ctrl	control
Cy	cyanine
DC	dendritic cell
ddH_2O	double-destilled water
dic	diclofenac
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid

DON	6-diazo-5-oxo-L-norleucine
ECAR	extracellular acidification rate
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetate
EFF	effector T cell
ELISA	enzyme-linked immunosorbent assays
EM	effector memory
EMRA	effector memory cell expressing CD45RA
ERK	extracellular signal-regulated kinase
FASL	Fas ligand
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fox	forkhead box
FSC	forward scatter
GAPDH	glycerinaldehyde 3-phosphate dehydrogenase
GCL	glutamate cysteine ligase
GDH	glutamate dehydrogenase
glc	glucose
gln	glutamine
GLS	glutaminase
GLUL	glutamine synthetase
GLUT	glucose transporter
GM-CSF	granulocyte macrophage colony-stimulating factor
GOT	glutamic-oxaloacetic transaminase
GSH	glutathione
H_2O_2	hydrogen peroxide
HBSS	Hank’s balanced salt solution
HCl	hydrochloric acid
HIF1α	hypoxia-inducible factor 1-alpha
HK	hexokinase
HRP	horse raddish peroxidase
IC_{50}	half maximal inhibitory concentration
IDO	indolamine 2,3-dioxygenase

KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
IFNγ	interferon γ
IL	interleukin
JAK-STAT	janus kinase-signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
K_i	inhibitory constant
KLRG1	killer cell lectin-like receptor
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
lum	lumiracoxib
MCT	monocarboxylate transporter
mDC	mature dendritic cell
MDSC	myeloid-derived suppressor cell
met	metformin
MFI	mean of fluorescence
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MNC	mononuclear cell
MTA	methylthioadenosine
mtATPi	oligomycin
mTOR	mechanistic target of rapamycin
mTORC1	mechanistic target of rapamycin complex 1
NAD	nicotinamide adenine dinucleotide
NFAT	nuclear factor of activated T cells
NF-kB	nuclear factor κB
NK	natural killer cell
NKT	natural killer T cell
no	number
NSAID	nonsteroidal anti-inflammatory drug
NV	naïve
O_2	oxygen
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
p38MAPK	p38 mitogen-activated protein kinase

PB	pacific blue
PBS	phosphate buffered saline
PD	programmed cell death protein
Pe	phycoerythrin
PGE	prostaglandine
PMA	phorbol-12-myristat-13-acetate
ROS	reactive oxygen species
rpm	rounds per minute
SCM	stem cell memory T cell
SEM	standard error of the mean
SSC	sideward scatter
TCA	tricarboxlic acid cycle
TCR	T cell receptor
TGF	tumor growth factor
Th	T helper cell
TIL	tumor infiltrating lymphocyte
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell
w/o	without

1. Introduction

1.1. The human immune system

The human immune system is classified into two branches: the innate (non-specific) and the adaptive (specific) immune system. Innate immunity (also called natural or native immunity) provides the early line of defense against microbes. In addition to physical barriers and the complement system (Sarma and Ward 2011) different immune cell populations are important components of the innate immune response as phagocytic cells (neutrophils, macrophages), dendritic cells (DC) and natural (NK) cells.

The immune cell populations can be categorized into either the myeloid or the lymphoid lineage. Besides macrophages, monocytes, DCs and granulocytes belong to the myeloid cells. Granulocytes consist of eosinophils, basophils and neutrophils, which secret vesicles, containing enzymes such as lysozymes and other molecules lethal to pathogens. Macrophages and DCs take up antigens, process them and present them to immune cells from the adaptive immune system. These so called professional antigen presenting cells (APCs) serve as an important link between the innate and the adaptive immune system.

Unlike the innate immune system, the adaptive immune system is highly specific to a particular pathogen and develops during a person's lifetime. Moreover, immunological memory is created after an initial response to a specific pathogen and leads to an enhanced and faster immune response in case of a reinfection. The adaptive immune system consists of lymphocytes, B and T cells, and their secreted products such as antibodies or cytokines. These two cell types as well as NK cells belong to the lymphoid lineage.

T cells and NK cells play a major role in the defense against virally infected cells. Both secrete cytokines and chemokines driving and modulating the immune response (Caligiuri 2008). In the context of the anti-tumor immune response T cells are of special importance as they are able to directly kill tumor cells beside cytokine secretion.

1.2. T cell differentiation and maturation

T cells play a central role in cell mediated immune responses. Derived from hematopoietic stem cells in the bone marrow, progenitor T cells migrate to the thymus for maturation. Within the thymus developing T cells, referred to as thymocytes, undergo several selection processes.

By positive selection the cells acquire the capability to recognize foreign antigens. A negative selection process ensures T cells to maintain self-tolerance by eliminating progenitors with receptors binding to self-antigens. After maturation T cells express the T cell receptor (TCR) and either the cluster of differentiation (CD) 4 or CD8 co-receptor on the cell surface. The TCR binds to peptides presented by major histocompatibility complex (MHC) molecules (Moss et al. 1992). MHCs interacting with T cell receptors are divided into two major forms, class I (MHC I) and class II (MHC II) molecules.

Class II molecules are expressed by professional APCs (Doyle and Strominger 1987). APCs ingest exogenous antigens in the periphery, process them into peptides and present them as epitopes via MHC II molecules on their surface. Suchlike activated APCs migrate to lymphatic organs, where the epitopes are recognized by specific CD4 T cells. Within the CD4 T cell compartment, four major populations can be distinguished: The T helper (Th) cell lineages Th1, Th2, Th17 and the regulatory T (Treg) cells (Zhu and Paul 2008). Helper T cells secrete cytokines and chemokines that activate and/or recruit other immune cell types. Th1 and Th2 cells are known as effector CD4 T cells. Th1 cells are triggered by interleukin (IL)-12 and secrete predominantly the cytokines IL-2 and interferon (IFN) γ (Zhu and Paul 2008). IL-2 stimulates survival, proliferation and differentiation of activated T cells. IFNγ, discussed more into detail in chapter 1.3.2, is critical for macrophage activation and upregulation of MHC II molecules. Th2 cells are stimulated by IL-2 and IL-4 and are characterized by a secretion of a broad range of cytokines as, among others, IL-4, IL-5 and IL-10 (Zhu and Paul 2008). IL-4 is a stimulatory factor for Th2 differentiation and serves as a positive feedback amplifier. Moreover it promotes, similar to IL-5, proliferation of B and T cells. IL-10 is an anti-inflammatory, immunosuppressive cytokine, as it downregulates MHC II expression for instance. Th17 cell differentiation is induced by tumor growth factor (TGF)-ß and IL-6, IL-21 and IL-23. This pro-inflammatory population produces IL-17 and IL-21 (Zhu and Paul 2008). IL-17 promotes secretion of chemokines recruiting other immune cells to sites of inflammation. IL-21 acts as a stimulatory factor in a positive feedback loop. Tregs characterized by expression of CD25 and forkhead box protein 3 (FoxP3) are important immunosuppressive cells releasing IL-10 and TGF-β (Zhu and Paul 2008). Within this group naturally occurring, generated in the thymus or at peripheral sites, and in cell culture TGF-ß induced Tregs can be distinguished (Shevach and Thornton 2014). TGF-ß is essential for the differentiation of naïve CD4 T cells to Tregs and maintains homeostasis of this population.

MHC I molecules interacting with CD8 as a co-receptor (Salter et al. 1990) are expressed by platelets and nearly all nucleated cells. Molecules presented by MHC class I are cytosolic proteins degraded in the proteasome and transported to the cell surface. Foreign or mutated

peptides are presented by MHC I molecules to other cells. Therefore CD8 T cells have an important role in the anti-tumor immunity by recognizing mutated cells (Hadrup et al. 2013). Due to this fact, this work is focused on CD8 T cells which will be further highlighted in the following chapters.

1.3. Human CD8 T cells

1.3.1. Differentiation of human CD8 T cells

Human CD8 T cells can be grouped into various subsets based on their antigen experience, function and molecular phenotype (Figure 1). Major subsets circulating in the blood are classified into naïve (NV) T cells and antigen-experienced populations such as central memory (CM) and effector memory (EM) T cells. These subsets are distinguished on the basis of differential expression of surface molecules, including splice variants of CD45, the lymph-node homing receptors CCR7 (C-C chemokine receptor 7) and CD62L (L-selectin) (Sallusto et al. 1999).

CD45, a protein tyrosine phosphatase exclusively expressed on cells of the hematopoietic lineage, is the most abundant leukocyte cell surface marker and plays a key role in TCR signal transduction (Tonks et al. 1988; Trowbridge and Thomas 1994). Different isoforms of CD45 are generated by alternative splicing and are expressed in cell type specific patterns on functional subsets of lymphocytes. CD45RA is found on naïve T cells as well as on a group of effector cells (EMRA T cells). After binding to the respective antigen, central and effector memory T cells lose expression of CD45RA and gain expression of CD45RO.

CCR7 and CD62L are lymphocyte homing receptors expressed on naïve and CM cells in order to track them to secondary lymphoid organs (Picker et al. 1993). In line, lack of those receptors on effector memory and effector T cells triggers migration of these subsets in peripheral regions of the body where they are recruited by chemokines into inflammatory sites. However, all three T cell subsets are present in peripheral and lymphoid tissues.

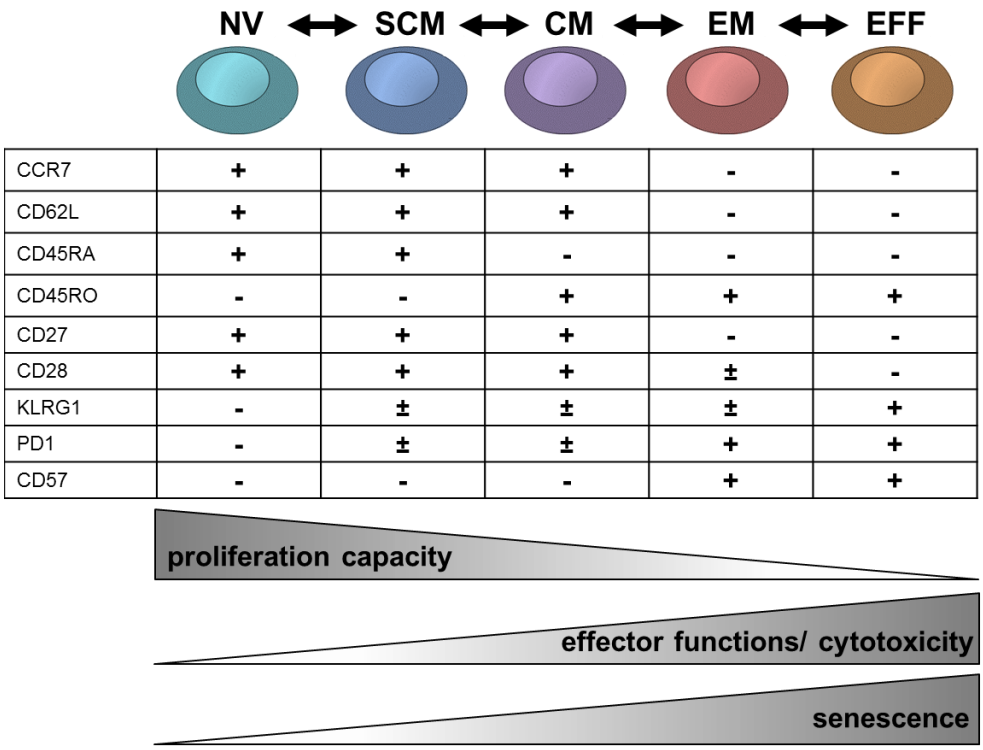

Figure 1: Model of the differentiation process of CD8 T cell subsets in humans and their relationship with functional attributes. According to Appay et al. 2008, Larbi and Tamas 2014 and Mahnke et al. 2013. CD8 subsets of circulating CD8 T cells are mainly defined according to differential expression of CCR7, CD62L, CD45RO and CD45RA. These markers change when T cells differentiate from one subset to another. Upon T cell differentiation CD8 T cells downregulate the expression of CD27 (IL-7 receptor) and CD28 (co-stimulatory receptor) and upregulate the expression of KLRG1 (killer cell lectin-like receptor-1), PD1 (programmed cell death protein 1) and CD57 (function unknown, marker for senescence). Moreover CD8 T cells lose their proliferation capacity, but acquire specific effector functions. NV: naïve T cells; SCM: stem cell memory T cells; CM: central memory T cells; EM: effector memory T cells; EFF: effector T cells.

The lineage relationship and specific phenotype of CD8 T cell subsets is still a subject of controversy in the field (Mahnke et al. 2013). After antigen exposure in the lymph nodes naïve T cells undergo proliferative expansion, differentiate into antigen experienced T cell subsets and end up as terminally differentiated effector T cells (Figure 1). CM T cells are long-lived memory cells capable to differentiate into shorter-lived EM upon antigen stimulation (Sallusto et al. 2004). Other studies propose the other way around, claiming that EM convert to CM (Wherry et al. 2003). The EM and EFF subsets seem to display a more exhausted phenotype indicated by reduced proliferative capacity and the expression of proposed senescence/ exhaustion markers such as CD57, killer cell lectin-like receptor-1 (KLRG1) and programmed cell death protein 1

(PD1) (Larbi and Tamas 2014). Moreover, the loss of the co-receptors CD27 and/ or CD28 is regarded as a characteristic for exhausted T cells.

Lately, a population of memory T cells has been defined, which has enhanced stem cell-like qualities compared to conventional CM T cells and an enhanced capacity for self-renewal. This cell type was designated memory stem T cells (SCM) (Gattinoni et al. 2011).

While CD4 Tregs are an accepted population, studies on human CD8 Tregs are rather sparse (Kapp and Bucy 2008). Patients with a leishmanial infection showed CD8 with an immunosuppressing phenotype (Holaday et al. 1993). A minor population of FoxP3 expressing CD8 T cells was detected in rheumatoid arthritis displaying superior regulatory abilities and inhibiting Th17 response (Ellis et al. 2014). A CD8, LAG-3, CD25, FoxP3, CCL4 expressing Treg subset may play a role in immunoregulation in humans through the secretion of CC chemokine ligand 4 (CCL4), which can inhibit T cell activation (Joosten et al. 2007).

1.3.2. CD8 T cells as key players in the immune response

CD8 T cells, also named cytotoxic T lymphocytes (CTL), are very important for the immune defense against intracellular pathogens such as viruses as well as for tumor immune surveillance. For a proper T cell stimulation various signals are required including antigen recognition by the TCR and secondary activating stimuli. The TCR complex is composed of two highly variable chains, the α- and the β-chain interacting with the antigens, the CD3 molecule and two ζ-chains mediating signal transduction. The CD8 co-receptor also binds to MHC I leading to a stabilization of the whole complex. Another required co-receptor, CD28, receives a co-stimulatory signal by binding to B7-1 (CD80) and B7-2 (CD86) on APCs. Besides cell to cell interaction leading to stimulation, cytokines support and enhance T cell stimulation and activation. The most important cytokine is IL-2. IL-2 is produced mainly by CD4 T cells and acts in an auto- and paracrine fashion.

After activation CD8 T cells secrete cytokines, primarily IFNγ and tumor necrosis factor (TNF), which have anti-tumor, immunoregulatory and anti-viral effects. IFNγ activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway in macrophages (Schroder et al. 2004). The JAK-STAT pathway targets gene promoters in the nucleus triggering anti-viral and adaptive immune responses (Aaronson and Horvath 2002). One well described effect of IFNγ is the inhibition of cell growth, even of tumor cells (Jonasch and Haluska 2001). This effect could be linked to the capability of IFNγ to upregulate the expression of MHC class I and II molecules (Propper et al. 2003; Schroder et al. 2004), which could result in a better

recognition by other immune cells. Moreover IFNγ orchestrates the recruitment of specific immune cells to sites of inflammation by upregulation of adhesion molecules and chemokines (Boehm et al. 1997). Besides its impact on cells of the adaptive immune system, IFNγ also triggers the innate immune response. Macrophages activated by IFNγ display increased pinocytosis and phagocytosis as well as enhanced microbial killing ability (Schroder et al. 2004).TNF triggers five different types of signals including nuclear factor κB (NF-κB) activation, extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK) (Aggarwal et al. 2012). TNF binds also to the TNF-related apoptosis-inducing ligand (TRAIL). This pathway induces apoptosis in tumor cells but not in normal cells (Wiley et al. 1995).

Moreover, CD8 T cells are capable to directly lyse cells by release of cytotoxic proteins (Peters 1991). The pore-forming protein perforin is released to facilitate granzyme influx into the target cell. Granzymes are serine proteases and cause cell death in any type of target cell. Granzyme B is the most powerful pro-apoptotic member and induces apoptosis by both caspase mediated and caspase independent pathways (Trapani 2001).

Another important mechanism how CD8 T cells kill target cells, is the binding of Fas (CD95) on their targets by the Fas ligand (FasL, CD95L) (Waring and Müllbacher 1999). This binding induces cell death mediated by caspase activation (Waring and Müllbacher 1999).

Due to their specificity and cytotoxic capability CD8 T cells comprise a powerful branch of the adaptive immune response. Regarding the ‘tumor-specific adaptive immune response’ tumor infiltrating CD8 cells have been shown to be of major importance in different types of solid tumors, including renal, ovarian, colorectal and lung cancer (Nakano et al. 2001; Sato et al. 2005; Galon et al. 2006; Kawai et al. 2008). A meta-analysis of expression signatures of 18.000 human tumors has confirmed that infiltration and activation of CD8 T cells is linked to improved patient outcome in different cancer types (Gentles et al. 2015). However, they also frequently fail to induce a sufficient anti-tumor immune response. Despite the lack of antigens and the expression of inhibitory surface markers, so called checkpoint molecules as cytotoxic T lymphocyte associated protein 4 (CTLA-4) or PD-1, which will be more elucidated later on, one reason might be their high metabolic demands upon activation and interplay with the specific metabolic tumor microenvironment.

1.3.3. Metabolism of T cells – a link between metabolism and effector functions?

In the resting state T lymphocytes cover their basal energy demand primarily through mitochondrial oxidation of glucose and glutamine (Bental and Deutsch 1993; Jones and Thompson 2007). Stimulation of T cells leads to a change from a resting towards an activated state. T cell activation requires metabolic reprogramming supporting their highly proliferative and biosynthetic phenotype (Frauwirth et al. 2002). During T cell activation and differentiation key metabolic processes such as glycolysis, mitochondrial respiration and fatty acid oxidation are recognized as crucial pathways (MacIver et al. 2013).

The metabolic reprogramming upon T cell activation is regulated on both the transcriptional and posttranscriptional level. Several studies have implicated the involvement of different signaling cascades such as 5' AMP-activated protein kinase (AMPK), AKT and mechanistic target of rapamycin (mTOR) in regulating T cell metabolism (Frauwirth et al. 2002; Pearce et al. 2009; Carr et al. 2010; Xu et al. 2012; Finlay and Cantrell 2011). Notably, those pathways are also known to be upregulated in cancer cells. Moreover, Wang and colleagues were able to show that metabolic genes involved in glucose and glutamine metabolism as well as mitochondrial biogenesis are regulated by the oncogene c-myc in murine T cells (Wang et al. 2011). Deletion of myc significantly impaired glycolytic flux and inhibited the upregulation of glutamine oxidation in active T cells. Myc, but not hypoxia-inducible factor 1-alpha (HIF1α) was required for activation-induced T cell metabolic reprogramming.

Pathways that control T cell function and cellular metabolism are linked and changes in cell metabolism can suppress or even enhance lymphocyte function (MacIver et al. 2013). As most of the publications are focused on murine T cells, it is of major importance to elucidate the role of metabolic pathways for CD8 T cell activation and function.

1.3.3.1. Glucose metabolism is strongly increased in activated T cells

Highly proliferating cells show an accelerated glucose metabolism, degrading most of the glucose into lactate, named aerobic glycolysis, first described in tumor cells by Otto Warburg (Warburg 1956). In contrast, non-proliferating cells metabolize glucose to pyruvate via glycolysis in the cytoplasm, which is subsequently oxidized to carbon dioxide (CO_2) by oxidative phosphorylation (OXPHOS) in the mitochondria. A yield of 36 mol adenosine triphosphate (ATP) is gained by 1 mol of glucose by OXPHOS, whereas only 2 mol ATP are produced by glycolysis. Nevertheless, the accelerated glycolytic flux rate and the rapid turnover of glucose allow cells to

produce sufficient amounts of ATP and at the same time to use the intermediates of the tricarboxylic acid cycle (TCA) for the biosynthesis of molecules (Fox et al. 2005; Vander Heiden et al. 2009). Already during the 1960's it has been shown that proliferating lymphocytes show an increased glucose metabolism (Cooper et al. 1963) and this phenomenon has regained attention during the last years.

After stimulation the expression of glycolysis associated genes is upregulated in human T cell populations. The expression of glycolytic enzymes and transporters such as the lactate dehydrogenase A (LDHA), the hexokinases (HK), the glucose transporter 1 (GLUT1) or the monocarboxylate transporter 1 (MCT1), responsible for lactate secretion is increased (Frauwirth et al. 2002; Macintyre et al. 2014; Renner et al. 2015). Accordingly, T cells show enhanced glucose uptake and lactate secretion in the course of stimulation. The increase in glucose metabolism has been reported in activated murine and human T cells. However, its impact on T cell function is still controversially discussed.

A strong link between glycolysis and effector functions as IFNγ production has been investigated in the murine system (Jacobs et al. 2008; Cham and Gajewski 2005; Macintyre et al. 2014). It has been reported that glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) interferes with the translation of IFNγ in murine Th1 cells (Chang et al. 2013). Murine CD4 T cells lacking LDHA, an enzyme converting pyruvate to lactate, secret less IFNγ indicating that glycolysis promotes T cell effector functions (Peng et al. 2016). Therefore glycolytic enzymes could function as metabolic checkpoints by linking T cell effector functions to glucose availability. However, another study showed that mitochondrial respiration is able to compensate for the lack of glucose to maintain IFNγ production in murine T cells (Salerno et al. 2016). Although cytokine production might require a glycolytic switch (Chang et al. 2013), glucose availability does not alter the rate of IFNγ production in murine T cells upon activation (Salerno et al. 2016). Moreover, other effector functions such as IL-2 production or cytolytic activity appear not to depend on glucose in the murine system (Cham and Gajewski 2005; Sukumar et al. 2013).

In human T cells IFNγ, TNF and IL-2 secretion is if at all moderately affected by low glucose levels (Renner et al. 2015). Cytokine secretion seems to be preserved even under very low glucose conditions (0.4 mM) (Renner et al. 2015) suggesting minor importance of glycolysis for T cell function. In another study glucose deprivation caused only tentatively reduced secretions of IL-2, TNF, IFNγ and IL-4 in human CD4 T cells (Tripmacher et al. 2008). Nevertheless, rapid IFNγ production was shown to be linked to increased glycolytic flux in human memory T cells (Gubser et al. 2013). However, the link between metabolism and human T cell effector functions is still a matter of debate.

Regarding proliferation glucose is required for murine and human T cells. Under glucose deprivation T cell expansion is strongly impaired (Chang et al. 2013, Renner et al. 2015, Tripmacher et al. 2008). Glut1 deficient CD4 and CD8 T cells have an impaired capacity to proliferate *in vitro* and *in vivo* (Macintyre et al. 2014). Nevertheless, LDHA deficiency in murine CD4 T cells had no impact on proliferation (Peng et al. 2016). These results suggest the major importance of glucose but not glucose fermentation for proliferation in the murine system. Interestingly, Glut1 deletion seemed to interfere also with CD4 T cell differentiation into Th1, Th2 and Th17 lineage, but differentiation into CD8 CTLs and CD4 Tregs were not affected (Macintyre et al. 2014).

1.3.3.2. Amino acids play an essential role in T cell activation and metabolism

Beside glucose, amino acids are regarded as key nutrients for activated T cells. It has been demonstrated that amino acids rather than glucose account for the majority of cell mass in proliferating mammalian cells, providing carbon and nitrogen to proliferating cells (Hosios et al. 2016). Amino acids fuel metabolic processes such as the TCA cycle (Newsholme et al. 2003b). Moreover amino acids are used as biosynthetic precursors for protein and nucleic acid biosynthesis. Antigen signaling through the TCR increases the uptake of several amino acids, such as phenylalanine, leucine, arginine, serine, glutamate and glutamine into T cells (Carr et al. 2010; Sinclair et al. 2013; Geiger et al. 2016). Detailed studies on the importance of amino acids for T cell function have been performed lately.

Recent work reveals that serine metabolism is enhanced in activated T cells. Serine is required for optimal T cell expansion by fueling the one-carbon metabolism and nucleotide biosynthesis (Ma et al. 2017). In addition, enzymes required for the transport or the conversion of arginine into downstream metabolites like ornithine or proline are upregulated in activated T cells. In the absence of arginine, T cells showed a reduced capacity to proliferate, impaired activity and decreased cytokine secretion (Choi et al. 2009). T cells with increased intracellular levels of arginine shift their metabolism from glycolysis to OXPHOS and display enhanced survival as well as anti-tumor activity, which is linked to the generation of CM T cells (Geiger et al. 2016).

Nevertheless, glutaminolysis plays a crucial role in T cell activation. Glutamine is with a concentration range between 550–750 µM the most abundant amino acid in the peripheral blood (Gleeson 2008). The degradation of glutamine provides precursors for the synthesis of other amino acids, proteins or nucleotides for instance (Dang 2010). When glutamine is oxidized into

glutamate, glutamate may be metabolized to produce other amino acids or may be used as anaplerotic substrate to replenish TCA cycle (Newsholme et al. 2003a; Newsholme et al. 2003b). Glutamine is converted by the glutaminase (GLS) to glutamate and two isoforms can be distinguished: the kidney- and the liver-type glutaminase. Besides glutaminase, T cell activation coordinately enhances expression of enzymes associated with glutaminolysis as the glutamic-oxaloacetic transaminase (GOT) or the glutamate dehydrogenase (GDH) (Carr et al. 2010; Sinclair et al. 2013). Subsequently, glutamate can be transformed into α-ketoglutarate by aminotransferases, transferring nitrogen to pyruvate or oxaloacetate to form aspartate or alanine, respectively. In turn α-ketoglutarate enters the TCA cycle to gain energy. Moreover, glutamate is one of the three building blocks of the antioxidant glutathione (GSH) which is comprised of three amino acids: cysteine, glutamate and glycine. GSH scavenges reactive oxygen species (ROS) and prevents thereby cellular damage. Specifically in T cells, it was shown that GSH is essential for the accelerated energy metabolism required for T cell effector functions (Mak et al. 2017). Moreover glutamine is essential for nucleotide synthesis as it acts as nitrogen donor in purine as well as in pyrimidine synthesis (Cory and Cory 2006).

Glutamine depletion blocked murine T cell growth, proliferation and cytokine production such as IFNγ and IL-2. Interestingly, this effect could not be rescued by substitution of biosynthetic precursors (proline or asparagine) or products (glutamate) of glutamine (Carr et al. 2010). Glutamine deprivation in CD4 T cells resulted in reduced mechanistic target of rapamycin complex 1 (mTORC1) activity and led to a differentiation toward a Treg cell phenotype (Klysz et al. 2015). Metzler and colleagues demonstrated that the differentiation into Tregs induced by glutamine restriction is caused by an impaired glutamine dependent nucleotide synthesis (Metzler et al. 2016). Another study assigned Treg formation under glutamine restriction to an inhibited conversion of glutamate into α-ketoglutarate and thereby preventing the production of 2-hydroxyglutarate. The formation of 2-hydroxyglutarate was essential to reduce the methylation of the *Foxp3* gene locus (Xu et al. 2017).

Taken together, glutamine is involved in a variety of pathways, summarized in Figure 2. Although glutamine is generally not regarded as an essential amino acid, T cells seem to lack the ability to compensate for glutamine deprivation, even when other pathway intermediates are provided. However, it is still not clear which pathway glutamine is involved in and which one is crucial for T cell activation as providing different intermediates only partially rescues T cell activation.

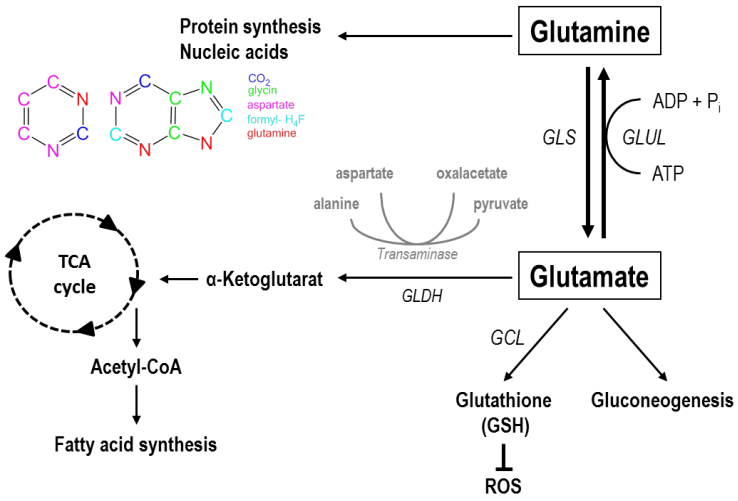

Figure 2: Pathways linked to glutamine metabolism. Glutamate is produced from glutamine through glutaminase (GLS). The reverse reaction is catalyzed by the glutamine synthetase (GLUL) producing glutamine from glutamate. Glutamate can be used as an amino group donor for the transamination of other amino acids as aspartate or alanine and is converted to α-ketoglutarate via glutamate dehydrogenase (GLDH) which is a TCA intermediate. Glutamate is one of the three building blocks for the antioxidant glutathione which is synthesized by the glutamate cysteine ligase (GCL) and protects cells from reactive oxygen species (ROS).

1.3.3.3. Mitochondrial respiration in T cells

During the last years the importance of glucose and amino acids for T cell function has been studied intensively. Only recently mitochondrial involvement in T cell activation and function regained attention.

Oxidative metabolism was shown to be important for T cell response in the murine and the human system (Sena et al. 2013; Okoye et al. 2015; Desdín-Micó et al. 2017). For murine T cells mitochondrial mass increases during the first hours of activation (Baixauli et al. 2015; Tan et al. 2017) and Fischer and colleagues described the critical role of mitochondrial biogenesis for cytokine production in human CD8 T cells (Fischer et al. 2018). Moreover, genetic approaches by deletion of the mitochondrial transcription factor A (Tfam) (Baixauli et al. 2015) or the apoptosis inducing factor (AIF) (Milasta et al. 2016), both leading to defective mitochondrial respiration, caused a strong decrease in proliferation. Interestingly, Tfam deficient CD4 T cells showed increased numbers of IFNγ cells (Baixauli et al. 2015). Balmer and Hess have shown that dysfunction of mitochondrial respiration correlates with exhaustion in murine and human CD8 T cells (Balmer and Hess 2016). T cells which lost mitochondrial mass within the tumor microenvironment were characterized by a very low cytokine production in the murine and the human system (Scharping et al. 2016; Siska et al. 2017).

Recently, Buck and colleagues reported that remodeling of mitochondria ultrastructure is critical for modulation of T cell differentiation into various subsets and moreover has an impact on anti-tumor immune response during adoptive cell transfer (ACT) (Buck et al. 2016). These findings suggest that activated T cells require not only glycolysis but also mitochondrial respiration for immune response. Therefore, the role of mitochondrial respiration has to be considered in light of the development of mitochondrial inhibitors for cancer therapy and needs further analysis.

1.3.3.4. Subset specific differences in the metabolic phenotype of T cells

Most of the studies on the link between T cell metabolism and function have been performed in bulk T cell cultures. Some publications indicate that the metabolic profile and the metabolic demands differ between T cell subsets. It has been proposed that murine CD8 memory T cells rely on lipid oxidation (Pearce et al. 2009; O'Sullivan et al. 2014). However, this finding is discussed controversially as in this study very high concentrations of etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase-1 (CPT-1A), were applied (O'Sullivan et al. 2014), probably leading to unspecific side effects. In contrast, a complete CPT-1A knockout in CD8 T cells is dispensable for the development of CD8 T cell memory and protective immunity (Raud et al. 2018) . Nevertheless, memory differentiation involves oxidative phosphorylation and changes in mitochondrial structure, as over-expression of a protein promoting mitochondrial membrane fusion fosters the generation of memory T cells *in vivo* (Buck et al. 2016). Moreover, inhibiting glycolytic metabolism by 2-deoxyglucose (2-DG) enhances memory generation in murine CD8 T cells (Sukumar et al. 2013). The metabolic profile of activated T cell subsets has been less investigated. Ecker and colleagues recently reported that IFNγ production is glucose sensitive in naïve and CM CD4 T cells (Ecker et al. 2018). Similar data have been shown for CD4 EM T cells (Dimeloe et al. 2016). In line, Gubser and colleagues showed that the accelerated effector functions of human CD8 memory T cells but not of naïve T cells is glycolysis dependent (Gubser et al. 2013). Nevertheless, it has to be taken into consideration, that those analyses have been performed in the absence of serum, which is regarded as a crucial factor for T cell activation.

Taken together T cells have a high metabolic demand. Therefore, the metabolic conditions in the tumor microenvironment could contribute to diminished anti-tumor immune responses probably in a subset dependent manner.

1.4. Impact of tumor metabolism on tumor infiltrating immune cells

Tumor cells are characterized by an altered and highly elevated metabolism in comparison to normal cells. Tumor cells reprogram and accelerate pathways including nutrient up-take and degradation in order to meet their bioenergetic, biosynthetic and redox demands. The resulting metabolic tumor microenvironment actively influences the surrounding tissue and the behavior of surrounding cells. Low nutrient levels and the accumulation of metabolites in the tumor microenvironment, produced and released by tumor cells themselves or stromal cells inside the tumor, mediate tumor promotion, invasion and metastasis (Hanahan and Coussens 2012).

The tumor microenvironment is hostile to T cells in many perspectives and many different mechanisms contribute to the failure of T cells to eradicate tumor cells. Nutrient supply is limited due to the highly elevated metabolic activity of tumor cells but also other stroma cells in the tumor microenvironment. As T cells undergo metabolic reprogramming upon stimulation to support their cell growth and effector functions, the need for nutrients is likewise elevated. This leads to a competition for nutrients between tumor cells and stroma cells including tumor infiltrating lymphocytes (TILs).

The classical example of a reprogrammed metabolic pathway in cancer is the accelerated aerobic glycolytic activity even in the presence of oxygen, the already mentioned Warburg effect (Warburg et al. 1927; Warburg 1956). As glucose is rapidly taken up by proliferating tumor cells, TILs are exposed to lowered extracellular glucose concentrations. Glucose consumption by tumor cells might metabolically restrict T cells by leading to decreased mTOR activity, glycolytic capacity and reduced effector functions (Chang et al. 2015). Ho and colleagues identified a mechanism by which glucose metabolism directly controls effector functions. Due to lowered phosphoenolpyruvate concentrations by glucose deprivation, calcium and nuclear factor of activated T cells (NFAT) signaling was suppressed, resulting in diminished anti-tumor effector functions. Nevertheless, glucose seems not to be the main restricting substrate for T cells within tumors, since in most human tumor entities an average glucose concentrations above 1 mM has been measured (Walenta et al. 2017), which has been shown to be sufficient for T cell function (Renner et al. 2015).

Besides glucose, amino acids are crucial for tumor growth (Mayers and Vander Heiden 2015; Altman et al. 2016). Many oncogenic mutations are associated with the glutamine metabolism (Altman et al. 2016). Glutamine metabolism was shown to be linked to mTORC1, and the oncogenes KRAS, MYC and p53 (Choi and Park 2018).

A variety of amino acids as glutamine, arginine, tryptophan or other amino acids are highly consumed by various tumor cell types as they are essential nutrients for tumor growth. Accordingly, Pan and colleagues showed that glutamine concentrations were consistently and significantly lower in tumor core regions (less than 100 µM) compared to the tumor periphery (about 400 µM) (Pan et al. 2016). Moreover, higher gene expression of enzymes involved in glutaminolysis are correlated with poor survival as shown in patients with ovarian cancer (Yang et al. 2014). Others demonstrated that glutamine is an important metabolite to drive tumor growth and metastasis (Zhang et al. 2014). In addition, asparagine and arginine levels correlate with tumor growth (Patil et al. 2016; Knott et al. 2018) and lowered levels of arginine are found in the tumor microenvironment (Rodriguez et al. 2009). Arginine is used by tumor cells but also other immune cells as T cells or myeloid-derived suppressor cells (MDSCs) (Renner et al. 2017). In addition, degradation of arginine by arginase secreted by MDSCs inside the tumor results in an impaired T cell responsiveness (Bronte and Zanovello 2005; Fletcher et al. 2015).

As amino acid metabolism is also important for activated T cells and other immune cells such as macrophages found in the tumor stroma, amino acid concentrations might become limiting in the tumor microenvironment (Renner et al. 2017).

Despite nutrient restriction, the secretion of metabolites formed in the course of glycolysis or amino acid degradation contributes to an immunosuppressive microenvironment. Highly glycolytic tumor cells need to efficiently export lactate to maintain glucose flux. The lactate export by the MCTs is proton-linked (Halestrap and Wilson 2012), which results in lactate accumulation and concomitantly a decrease in the extracellular pH inside the tumor. It was shown that tumor derived lactic acid suppresses proliferation and cytokine production of human cytotoxic T cells *in vitro* and *in vivo*, hence is regarded as an immunosuppressive metabolite (Fischer et al. 2007; Calcinotto et al. 2012; Mendler et al. 2012). In line, Cascone and colleagues demonstrated that increased tumor glycolysis is associated with a lower therapeutic response to adoptive T cell transfer due to suppression of anti-tumor immunity (Cascone et al. 2018). Moreover, tryptophan degradation results in the production and secretion of kynurenine regarded as an immunosuppressive metabolite (Frumento et al. 2002; Uyttenhove et al. 2003; Fallarino et al. 2006). Additionally, the adenosine metabolism is regarded as a possible pathway harming immune cell response in the tumor microenvironment. An upregulated expression of an ecto-5′-nucleotidase (CD73) was shown to result in adenosine production, affecting T and NK cells (Huang et al. 1997; Häusler et al. 2011). The accumulation of methylthioadenosine (MTA) caused by the loss of methylthioadenosine phosphorylases impairs T cell function (Henrich et al.

2016). Other waste products as prostaglandin E2 (PGE2) are also known as immunosuppressive metabolite (Kalinski 2012).

Taken together, the metabolic microenvironment in tumors has a strong impact on a T cell mediated anti-tumor immune response. On the one hand a competition for nutrients is observed, on the other hand the secretion of immunosuppressive metabolites impairs T cell function (Figure 3).

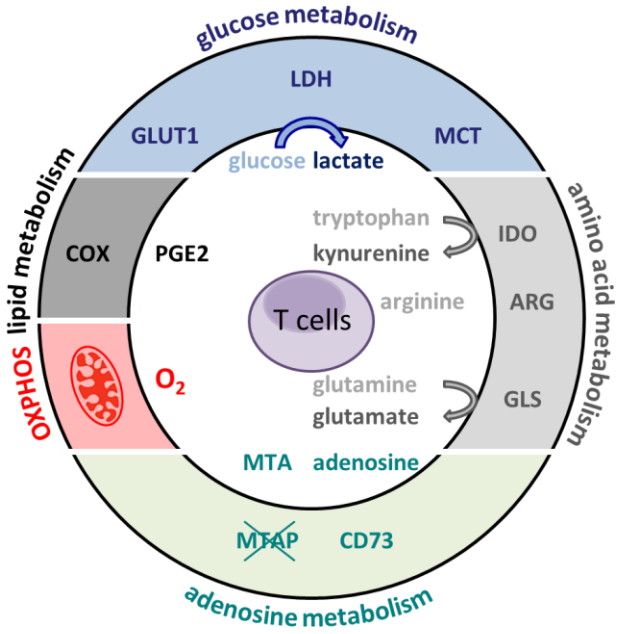

Figure 3: Metabolic hallmarks of tumor cells and the interplay between tumor cells and immune cells, according to Renner et al. 2017. Tumor cells exhibit high expression of glucose transporters (GLUT), lactate dehydrogenase (LDH), cyclooxygenase (COX), arginase (ARG), indolamine 2,3-dioxygenase (IDO), glutaminase (GLS), and oxidative phosphorylation (OXPHOS). As a consequence, glucose and the amino acids arginine, tryptophan, and glutamine are depleted from the tumor microenvironment and nutrient restriction contributes to an anergic state of anti-tumoral cytotoxic T cells. In addition, accelerated glycolysis by tumor cells results in lactate production and secretion *via* monocarboxylate-transporters (MCT). Lactate and other secreted metabolites, such as glutamate, prostaglandins (PGE2), and kynurenines, affect immune cells. Overexpression of the ecto-5′-nucleotidase (CD73) leads to adenosine formation; loss of methylthioadenosine phosphorylase (MTAP) results in methylthioadenosine (MTA) accumulation in the tumor environment.

1.5. State of the art: Targeting the tumor metabolism as an emerging strategy in cancer therapy

Targeting and thereby restricting tumor metabolism could be an interesting strategy in different perspectives; (i) metabolic restriction affects proliferation and viability of tumor cells and (ii) blocking metabolic pathways reduces the secretion of immunosuppressive metabolites. During the past decades targeting cancer metabolism has emerged as a promising strategy and selective drugs, targeting glucose metabolism, mitochondrial activity or glutaminolysis have been developed.

As tumor cells and immune cells show overlaps in their metabolic profile it will be a major challenge to develop protocols affecting tumor cell metabolism but preserving immune cell function. This is of special importance, since an effective anti-tumor immune response is known

to positively affect patient outcome (Galon et al. 2006). Therefore it is essential to understand this link and the metabolic demand in T cells which is required for a successful immune response.

A huge variety of drugs has been developed during the last years targeting different metabolic pathways. While initial studies have mainly focused on glucose metabolism, it is now clear that inhibiting mitochondrial function is another emerging approach (Tennant et al. 2010). Besides glycolysis and OXPHOS, amino acid metabolism has also been considered as a target of cancer therapy (Altman et al. 2016).

1.5.1. Targeting glucose metabolism as a promising concept for anti-tumor therapy

Glucose metabolism can be targeted at different levels. As glucose enters cells via specific transporters several inhibitors for glucose transporters have been developed and clinical trials are ongoing to test toxicity and efficacy (Cao et al. 2007; Zhan et al. 2011). An alternative strategy for inhibiting glucose metabolism relies on the inhibition of glycolytic enzymes. Compounds such as the glucose analog 2-DG or 3-bromopyruvate (HK), lonidamine (pyruvate kinase M2 and hexokinase), gossypol (LDH), dichloroacetate (pyruvate dehydrogenase kinase) are able to inhibit glucose catabolism and are currently tested in clinical trials (Yu et al. 2001; Di Cosimo et al. 2003; Maschek et al. 2004; Murray et al. 2005; Michelakis et al. 2008; Ovens et al. 2010b; Pedersen 2012).

Targeting lactate metabolism is another attractive strategy for cancer therapeutics (Doherty and Cleveland 2013). Lactic acid produced by tumor cells has emerged as a critical factor of cancer development, metastasis as well as patient survival probability (Walenta et al. 2000; Gatenby and Gillies 2004). Blocking lactate secretion affects the tumor cells itself and at the same time accumulation of the immunosuppressive metabolite lactic acid is reduced. The main transporters involved in lactate secretion are MCTs. MCTs are plasma membrane transporters carrying monocarboxylates, such as lactate, pyruvate or ketone bodies, across biological membranes (Halestrap and Wilson 2012). MCTs are encoded by the solute carrier family 16 genes (*SLC16*). Within this family 14 members have been identified so far in mammals (Halestrap and Price 1999; Juel and Halestrap 1999; Halestrap and Wilson 2012). However, only the MCTs 1 to 4 have been demonstrated to facilitate monocarboxylate transport up to now. While MCT1, 3 and 4 use the basignin protein (CD147) as ancillary protein, MCT2 uses embidigin (Halestrap and Wilson 2012).

MCT2 (*SLC16A7*) and MCT3 (*SLC16A8*) seem to play a less significant role in human tissue and have a more restricted expression pattern (Halestrap and Wilson 2012). MCT1 (SLC16A1) is expressed almost ubiquitously in all tissues such as the kidney, the liver, the heart and the skeletal muscle (Halestrap and Price 1999). MCT1 has a high lactate affinity with a K_m value of 3.5 mM and is responsible for lactate entry into or efflux out of cells depending on the metabolic state (Halestrap 2012). High expression levels of this transporter in tumors are associated with a poor outcome (Kong et al. 2016). MCT1 expression was shown to be highly elevated in breast, colorectal, gastric and cervical cancer (Pinheiro et al. 2008; Pinheiro et al. 2010; de Oliveira, Talvane Torres Antônio et al. 2012). However, a single MCT1 knockdown in human colon adenocarcinoma cells neither impaired glycolysis nor the rate of tumor growth (Le Floch et al. 2011). In line, in breast cancer cells MCT1 inhibition did not consistently alter lactate transport. However, reduced pyruvate export has been observed suggesting MCT1 to also promote pyruvate export (Hong et al. 2016).

In highly glycolytic cells such as tumor cells lactate transport is not only mediated by MCT1 but also by MCT4 (*SLC16A3*) (Halestrap and Wilson 2012). MCT4 expression is a direct downstream target of the transcription factor HIF-1α and is upregulated in response to hypoxia (Ullah et al. 2006; Perez de Heredia et al. 2010). Due to its low affinity for lactate (K_m value: 22 to 28 mM) MCT4 is in particular adapted to the release of lactate in a microenvironment already containing high amounts of lactate (Dimmer et al. 2000). MCT4 plays a key role in tumorigenesis. Knockdown experiments have shown that MCT4 is not essential for survival but required for migration and invasion of tumor cells (Gallagher et al. 2007; Izumi et al. 2011; Kong et al. 2016). Elevated levels of MCT4 have been detected in colorectal and prostate cancer (Pinheiro et al. 2008; Pértega-Gomes et al. 2011).

The elevated levels of MCT1 and MCT4 in various tumor entities provide a promising target structure. Several potent MCT1/2 inhibitors have already been developed (Murray et al. 2005; Ovens et al. 2010a; Doherty et al. 2014) and some are currently tested in clinical trials (Doherty and Cleveland 2013). However, these compounds are inactive in the presence of MCT4. Hence, the development of potent pan-MCT inhibitors or inhibitors selectively targeting MCT4 is needed. Recently, Sasaki and colleagues demonstrated that diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), is capable to block lactate transport by MCT4 (Sasaki et al. 2016). Diclofenac is able to reduce glucose uptake and lactate secretion in tumor cells. In *in vivo* experiments diclofenac significantly impaired tumor growth (Chirasani et al. 2013; Gottfried et al. 2013). New data of our laboratory demonstrate that diclofenac is capable to directly block MCT1 and MCT4

(Renner et al. under revision). Therefore diclofenac might be a candidate for a pan-MCT inhibitor used as an anti-cancer drug.

1.5.2. Targeting mitochondrial respiration in the context of tumor therapy

Besides glycolytic activity, mitochondrial respiration displays a possible target in tumor cells as well. The clinically applied multikinase inhibitor sorafenib interferes with mitochondrial function and reduces mitochondrial respiration (Tesori et al. 2015). Additionally, sorafenib was shown to decrease Treg frequency in metastatic renal cell carcinoma patients (Busse et al. 2011).

The anti-diabetic drug metformin, a biguanide, is also able to block mitochondrial respiration (Owen et al. 2000). Type 2 diabetes patients treated with metformin are known to be at lower risk for developing cancer (Dowling et al. 2007; Libby et al. 2009). Moreover, metformin was proposed as adjuvant therapy for glioma, as preliminary results reported a survival benefit of patients with grade III glioma using metformin (Seliger et al. 2019). As metformin is a frequently applied and well tolerated drug, it might be applied for tumor therapy. However, it has to be taken into consideration that metformin as well as sorafenib induce compensatory glycolytic activity (Tesori et al. 2015; Renner et al. 2018), which could in turn contribute to immune escape. Thus a combination of a glycolytic inhibitor with those drugs might be a more promising strategy (Gerthofer et al. 2018; Renner et al. 2018).

1.5.3. Targeting amino acid metabolism for tumor therapy

As tumor cells are characterized by an elevated amino acid metabolism, these pathways are also regarded as a possible target for tumor therapy and various drugs have been developed. The application of an enzyme degrading arginine resulting in arginine deprivation showed promising results in patients with leukemia (Miraki-Moud et al. 2015). Moreover, the inhibitor CB-1158 blocked arginase released by MDSCs within the tumor microenvironment, diminished tumor growth and was able to reduce suppressive capacity of these cells (Steggerda et al. 2017).

Asparaginase, the enzyme catalyzing the degradation of asparagine, is used to treat acute lymphoblastic leukemia and lymphomas for decades (Avramis 2012). Asparagine displays an important metabolite for tumor cells especially under glutamine restricted conditions (Zhang et

al. 2014). Due to this fact, the treatment with asparaginase in combination with drugs targeting glutamine metabolism could maximize the therapeutic efficacy.

Phenylacetate reduces the biologic availability of glutamine in the blood stream and is able to inhibit the proliferation of tumor cells (Samid et al. 1993). Most compounds targeting glutamine metabolism are still in the preclinical stage (Altman et al. 2016). Allosteric inhibitors of GLS, such as CB-839 or BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide), have shown promising results in preclinical models of triple-negative breast cancer and human B cell lymphoma (Shukla et al. 2012; Gross et al. 2014). CB-839 is currently under investigations in clinical trials for solid tumors, hematological tumors as well as leukemia (Clem et al. 2016). Other compounds targeting glutamine dependent pathways as acivicin ((2S)-amino-(5S)-3-chloro-4,5-dihydro-1,2-oxazol-5-yl ethanoic acid) or DON (6-diazo-5-oxo-L-norleucine) failed to get approval due to severe toxicity (Adolphson et al. 1986; Rais et al. 2016).

1.5.4. Application of anti-metabolic drugs in immunotherapy: opportunities and challenges

Tumor cells have developed several strategies to escape and suppress the immune system (Figure 3) summarized as the hallmarks of cancer (Hanahan and Weinberg 2011), including the deregulated energy metabolism and immune escape, which seem to be closely related. As the metabolic tumor microenvironment limits endogenous anti-tumor immune responses it is likely to limit the efficacy of newly developed immunotherapeutic approaches. During the last decades immunotherapy has become an important approach in treating cancer (Callahan et al. 2016). Currently investigated immunotherapies include antibodies against immune checkpoints, such as PD-1 or CTLA-4, displaying receptors with a regulatory function. CD8 T cells in tumors express high levels of PD-1 associated with an exhausted phenotype and impaired effector functions (Ahmadzadeh et al. 2009). A therapy combining nivolumab and ipilimumab, monoclonal antibodies targeting PD-1 or CTLA-4 on T cells, has shown promising response rates in metastatic melanoma (Postow et al. 2015), but in the meantime also in other tumor entities.

ACT is another promising type of immunotherapy. After isolation, genetic engineering and *in vitro* expansion of patient derived T cells, anti-tumor T cells are applied to cancer patients in order to recognize, target and destroy tumor cells. ACT of antigen-specific T cells was shown to mediate regression of established tumors (Yee et al. 2002). Moreover, ACT of chimeric antigen receptor (CAR) T cells is another promising immunotherapeutic approach. In this therapy T cells

are genetically engineered *in vitro* to potently recognize and attack tumors (Riet and Abken 2015). CAR T cells specific for CD19 (CTL019) redirecting toward leukemic cells were able to induce remission in several patients (Kalos et al. 2011; Kochenderfer et al. 2013).

Tumor infiltrating immune cells as CD8 T or NK cells can be harmed in the tumor microenvironment by nutrient restriction or immunosuppressive metabolites (Renner et al. 2017). A combination of immunotherapy with anti-metabolic agents could be a promising strategy to enhance responses to immunotherapy. However, such treatment strategies could also restrict T cell function, which needs to be considered.

1.6. Hypothesis and objectives

The metabolic profile and the link to effector functions of human CD8 T cells in particular of subsets have been insufficiently analyzed. In light of the metabolic microenvironment in solid tumors it is of crucial importance to investigate which T cell subpopulation is the most robust in terms of nutrient restriction. These subset specific analyses would allow selecting the most promising subpopulation for adoptive T cell transfer. As activated T cells and tumor cells show an overlap in their metabolic profile the application of metabolic inhibitors could adversely affect CD8 T cell function and limit their anti-tumor immune response. Therefore, the interaction between metabolically active drugs and CD8 T cell function has to be elucidated.

The aim of this project was to characterize the metabolic activity and the nutrient dependency of effector functions in human CD8 T cell subsets. Moreover, effects of clinically relevant anti-metabolic drugs, inhibiting glycolysis, glutaminolysis or cellular respiration were investigated to avoid adverse effects on T cell function when combining immunotherapeutic approaches with anti-metabolic drugs.

2. Material and methods

2.1. Materials

2.1.1. Equipment

Aria cell sorter	BD Bioscience, Heidelberg, Germany
Autoclave	Technomara, Fernwald, Germany
Balance LP1200S	Sartorius, Göttingen, Germany
Biofuge fresco	Heraeus, Osterode, Germany
CASY Cell Counter	Innovatis/Roche, Basel, Switzerland
Dimension Vista	Siemens, Munich, Germany
EVOS Cell Imaging System	OLS-Omni Life Science, Bremen, Germany
FACS Calibur flow cytometer	BD Biosciences, Franklin Lakes, NJ, USA
Forceps	Aesculap, Tuttlingen, Germany
Fusion Pulse 6	Vilber Lourmat, Collégien, France
Hemocytometer	Marienfeld, Lauda-Königshofen, Germany
Incubators	Heraeus, Hanau, Germany
Laminar Flow Cabinet Herasafe	Thermo Scientific, Waltham, MA, USA
LSR II flow cytometer	Becton Dickinson, Heidelberg, Germany
LSR Fortessa	Becton Dickinson, Heidelberg, Germany
MACSiMAG separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Microscopes	Zeiss, Jena, Germany
Multifuge 3S-R	Heraeus, Osterode, Germany
Multifuge 3.0R	Thermo Scientific, Waltham, MA, USA
Pipette (repetitive) HandyStep	Brand GmbH & CO KG, Wertheim, Germany
pH meter	Knick, Berlin, Germany
Picofuge	Heraeus, Osterode, Germany
Pipetboy	Integra Biosciences, Fernwald, Germany
Pipettes	Eppendorf, Hamburg, Germany or Gilson, Milddleton, WI, USA
Precision microplate reader	Molecular Devices, Sunnyvale (CA, USA)
QuadroMACS^{TM} separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Seahorse XFe96 Analyzer	Agilent, Santa Clara, CA, USA
SenSorDish-Reader	PreSens, Regensburg, Germany
Sepatech Megafuge 1.0	Heraeus, Osterode, Germany
Sepatech Megafuge 3.0	Heraeus, Osterode, Germany
Sonorex Ultrasonic Bath	Branson, Danbury, CT, USA

Thermo VarioSkan	Thermo Scientific, Waltham, MA, USA
Thermomixer	Eppendorf, Hamburg, Germany
Vortexer	Scientific Industries Ink., Bohemia, NY, USA
Water purification system	Millipore, Eschborn, Germany
Waterbath	Julabo, Seelstadt, Germany

2.1.2. Consumables

Cell culture dishes	BD, Franklin Lakes, NJ, USA or Eppendorf, Hamburg, Germany
Cell culture dishes (U-bottom)	Falcon, Heidelberg, Germany
Cell culture flasks	Costar, Cambridge, MA, USA or Eppendorf, Hamburg, Germany
Cell strainer (70 µm, 100 µm)	Falcon, Heidelberg, Germany
Combitips for Eppendorf multipette	Eppendorf, Hamburg, Germany
Cryo tubes	Corning, Corning, NY, USA
Hyperfilm ECL	Amersham/GE Healthcare, Chalfont St Giles, UK
Immobilon-P PVDF membrane	Merck Millipore, Billerica, MA, USA
LS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Micro test tubes (1.5 ml, 2.0 ml)	Eppendorf, Hamburg, Germany
Micropore filters	Sartorius, Göttingen, Germany
Microtiter plates (6, 12, 24, 96 wells)	Costar, Cambridge, MA, USA
Petri dishes	Falcon, Heidelberg, Germany
Pipette tips	Eppendorf, Hamburg, Germany
Plastic pipettes	Costar, Cambridge, MA, USA or Nerbe plus, GmbH Winsen/Luhe, Germany
Polystyrene test tubes	Falcon, Heidelberg, Germany
Scalpels, disposable	Feather, Osaka, Japan
Syringe Filters, sterile	Sartorius, Göttingen, Germany
Syringes and needles	BD, Franklin Lakes, NJ, USA
Tubes (5 ml, 15 ml, 50 ml, 225 ml)	Falcon, Heidelberg, Germany

2.1.3. Media, buffers and solutions

AB serum, human	Blutspendedienst des BRK, Munich, Germany
Acrylamide	Carl Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	Merck Millipore, Billerica, MA, USA
ß-mercaptoethanol	Gibco/Life Technologies, Carlsbad, CA, USA
Biocoll Separating Solution	Biochrom/Merck Millipore, Billerica, MA, US
Bovine serum albumine (BSA)	Sigma-Aldrich, St. Louis, MO, USA
Dimethylsulfoxide (DMSO)	Honeywell Riedel-de Haen /Sigma-Aldrich, St. Louis, MO, USA
Ethanol	Carl Roth, Karlsruhe, Germany
FACS clean	BD Biosciences, Franklin Lakes, NJ, USA
FACS flow	BD Biosciences, Franklin Lakes, NJ, USA
FACS rinse	BD Biosciences, Franklin Lakes, NJ, USA
Fetal calf serum (FCS)	PAA/GE Healthcare, Chalfont St Giles, UK or Gibco/Life Technologies, Carlsbad, CA, USA or Merck Millipore, Billerica, MA, USA, heat-inactivated by 56 °C, 30 minutes
Glycerin	Merck Millipore, Billerica, MA, USA
Glycine	Merck, Darmstadt, Germany
H_2O_2	Merck, Darmstadt, Germany
ddH_2O	B. Braun Melsungen, Melsungen, Germany
Hanks' Balanced Salt solution	Sigma-Aldrich, St. Louis, MO, USA
Isoton II	Beckman Coulter, krefeld, Germany
Isopropanol	B. Braun Melsungen, Melsungen, Germany
L-Alanyl-*L*-Glutamine	PAN Biotech, Aidenbach, Germany
MEM Non-Essential Amino Acid Solution	Gibco/Life Technologies, Carlsbad, CA, USA
MEM Sodium Pyruvate	Gibco/Life Technologies, Carlsbad, CA, USA
MEM Vitamin Solution	Gibco/Life Technologies, Carlsbad, CA, USA
PBS	Sigma-Aldrich, St. Louis, MO, USA or Gibco/Life Technologies, Carlsbad, CA, USA
RIPA buffer	Sigma-Aldrich, St. Louis, MO, USA
RPMI 1640	Gibco/Life Technologies, Carlsbad, CA, USA
RPMI 1640 w/o glucose	Sigma-Aldrich, St. Louis, MO, USA
TEMED	Sigma-Aldrich, St. Louis, MO, USA
Tris	USB/Affymetrix, Santa Clara, CA, USA
Triton X100	Sigma-Aldrich, St. Louis, MO, USA
Tween 20	Sigma-Aldrich, St. Louis, MO, USA
Typsin-EDTA	PAN Biotech, Aidenbach, Germany

2.1.3.1. Medium for cultivation of human T cells

Instable L-glutamine	5 ml (2 mM)
Non-essential amino acids	5 ml (0.1 mM each)
Sodium pyruvate	5 ml (1 mM)
Vitamins	2 ml
β-mercaptoethanol	0.5 ml (0.05 mM)
Penicillin/Streptomycin	2.5 ml (50 IU/ml)
AB serum, human	50 ml (10 %)
RPMI 1640	ad 500 ml

The medium was filtered through a 0.22 µm filter by the SteriCup® quick release filter system (Merck) and stored at 4 °C. Before utilization, T cell medium was supplemented with IL-2 (end concentration of 25 IU/ml, PeproTech).

2.1.3.2. Medium for cultivation of human DCs

Instable L-glutamine	5 ml (2 mM)
Penicillin/Streptomycin	2.5 ml (50 IU/ml)
FCS	50 ml (10 %)
RPMI 1640	ad 500 ml

The medium was stored at 4 °C. Before utilization, medium was supplemented with IL-4 and GM-CSF (end concentration of 144 IU/ml and 280 IU/ml, PeproTech).

2.1.3.3. Medium for cultivation of murine T cells

Instable L-glutamine	5 ml (2 mM)
Nonessential amino acids	5 ml (0.1 mM each)
Sodium pyruvate	5 ml (1 mM)
β-mercaptoethanol	0.5 ml (0.05 mM)
Penicillin/Streptomycin	2.5 ml (50 IU/ml)
FCS	50 ml (10 %)
RPMI 1640	ad 500 ml

The medium was stored at 4 °C. Before utilization, T cell medium was supplemented with IL-2 (end concentration of 10 IU/ml, PeproTech).

2.1.3.4. Freezing medium

FCS	80 %
DMSO	20 %

2.1.3.5. FACS staining buffer

FCS	10 ml (2 %)
PBS	ad 500 ml

2.1.3.6. MACS buffer

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

2.1.3.7. EDTA (200 mM)

EDTA	2.924 g
ddH_2O	ad 50 ml

The pH was adjusted to 8.0, the solution was filtered through a 0.20 µm filter to sterilize and stored at room temperature.

2.1.3.8. ACK lysis buffer (6x)

NH_4Cl (ammonium chloride)	49.64 g
$KHCO_3$ (potassium bicarbonate)	6.00 g
EDTA disodium, dihydrate	0.222 g
ddH_2O	ad 1000 ml

The pH was adjusted to 7.4, the buffer was filtered through a 0.22 µm filter by the SteriCup® quick release filter system (Merck) and stored at 4 °C. Before utilization, buffer was diluted 1:6 with ddH_2O.

2.1.4. Kits, reagents and chemicals

Acivicin in medium	Santa Cruz Biotechnology, Dallas, TX, USA
Amersham ECL Prime Western Blotting Detection Reagent	Amersham/GE Healthcare, Chalfont St Giles, UK
Ammonium chloride (NH_4Cl)	Merck, Darmstadt, Germany
Annexin binding buffer	BD Biosciences, Franklin Lakes, NJ, USA
Arginine	Sigma-Aldrich, St. Louis, MO, USA
Asparagine	Sigma-Aldrich, St. Louis, MO, USA
Aspartate	Sigma-Aldrich, St. Louis, MO, USA
BD Comp Beads	BD Biosciences, Franklin Lakes, NJ, USA
Protein transport inhibitor (monensin)	BD Biosciences, Franklin Lakes, NJ, USA
Bio-Rad DC protein assay	Bio-Rad, Munich, Germany
BPTES in medium	Sigma-Aldrich, St. Louis, MO, USA
Trypan blue	Sigma-Aldrich, St. Louis, MO, USA
CB-839 in medium	Selleckchem, Munich, Germany
CD4 (L3T4) MicroBeads, mouse	Miltenyi Biotec, Bergisch Gladbach, Germany
CD4 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
CD8a T cell Isolation Kit, mouse	Miltenyi Biotec, Bergisch Gladbach, Germany
CD8 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
Diclofenac sodium salt in medium	Fagron, Rotterdam, The Netherlands
Dimethyl α-ketoglutarate	Sigma-Aldrich, St. Louis, MO, USA
DON in medium	Sigma-Aldrich, St. Louis, MO, USA
DuoSet ELISA human IFNγ	R&D Systems, Minneapolis, MN, USA
DuoSet ELISA human TNF	R&D Systems, Minneapolis, MN, USA
DuoSet ELISA mouse IFNγ	R&D Systems, Minneapolis, MN, USA
DuoSet ELISA mouse TNF	R&D Systems, Minneapolis, MN, USA
Dynabeads™ T-Activator CD3/CD28	Gibco/Life Technologies, Carlsbad, CA, USA
EDTA (ethylenediaminetetraacetic acid)	Sigma-Aldrich, St. Louis, MO, USA
Glucose (HK) Assay Kit	Sigma-Aldrich, St. Louis, MO, USA
Glutamic acid monosodium salt hydrate	Sigma-Aldrich, St. Louis, MO, USA
Glutathione (GSH)	Sigma-Aldrich, St. Louis, MO, USA
FcR-Blocking reagent mouse	Miltenyi Biotec, Bergisch Gladbach, Germany
FoxP3 transciption factor staining buffer set	eBioscience, San Diego, CA, USA
GM-CSF	PeprochTech, Hamburg, Germany
Hypoxanthine-Thymidine Media Supplement (50x)	Sigma-Aldrich, St. Louis, MO, USA

Ionomycin	Enzo Life Sciences, Farmingdale, NY, USA
α-ketoglutaric acid sodium salt	Sigma-Aldrich, St. Louis, MO, USA
L-lactic acid ($C_3H_6O_3$)	Sigma-Aldrich, St. Louis, MO, USA
Lipopolysaccharide (LPS)	Enzo Life Sciences, Farmingdale, NY, USA
Luminol	Sigma-Aldrich, St. Louis, MO, USA
Lumiracoxib in DMSO	Selleckchem, Munich, Germany
Metformin hydrochlorid in medium	Sigma-Aldrich, St. Louis, MO, USA
Oligomycin in ethanol	Sigma-Aldrich, St. Louis, MO, USA
Potassium hydrogen carbonate ($KHCO_3$)	Fluka, Buchs, Switzerland
IL-2	PeprochTech, Hamburg, Germany
IL-4	PeprochTech, Hamburg, Germany
IL-7	PeprochTech, Hamburg, Germany
IL-15	PeprochTech, Hamburg, Germany
Phorbol-12-myristat-13-acetat (PMA)	Calbiochem, San Diego, CA, USA
SDS	Sigma-Aldrich, St. Louis, MO, USA
Sodium bicarbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium *L*-lactate ($C_3H_5NaO_3$)	Sigma-Aldrich, St. Louis, MO, USA
SR13800 (10 mM in DMSO)	kindly provided by Prof. Dr. JL Cleveland
UDP-N-acetylglucosamine	Sigma-Aldrich, St. Louis, MO, USA

2.1.5. Antibodies

2.1.5.1. Antibodies for western blotting

Table 1: Antibodies for western blotting

Specificity	Source	Species cross activity	Molecular weight	Dilution	Manufacturer
α -Actin	Rabbit	Human, animal	42 kDa	1:2000	Sigma-Aldrich
α -MCT4	Rabbit	Mouse, rat, human	43 kDa	1:1000	Santa Cruz Biotechnologies
α -GLUL	Rabbit	Mouse, rat, human	42 kDa	1:5000	Abcam

2.1.5.2. Antibodies for flow cytometry

2.1.5.2.1. Anti-human antibodies

Table 2: Anti-human antibodies for flow cytometry

Specificity	Function	Conjugation	clone	company
α-CCR7*	lymph node homing receptor	FITC	150503 REA	BD Biosciences, Miltenyi Biotech
α-CD62L*	lymph node homing receptor	APC	DREG-56 145-15	BD Biosciences, Miltenyi Biotech
α-CD45RA*	activity related marker, differentiation between naïve and memory T cells	Pe-Cy7	L48 REA	BioLegend, Miltenyi Biotech
α-CD45RO*	activity related marker, differentiation between naïve and memory T cells	Pe	UCHK1 REA	BD Biosciences, Miltenyi Biotech
α-CD3*	T cell marker	APC-H7	SK7 REA	BD Biosciences, Miltenyi Biotech
α-CD8*	part of the T cell co-receptor	Pacific Blue	RPA-T8 REA	BD Biosciences, Miltenyi Biotech
α-CD8	part of the T cell co-receptor	Pe-Cy7	SK1	BioLegend
α-granzyme B	effector molecule	FITC	GB11	BD Biosciences
α-perforin	effector molecule	APC	dG9	BioLegend
α-CD25	activity related marker for T cells	Pe-Cy7	M-A251	BD Biosciences
α-CD69	activity related marker for T cells	APC	FN50	BD Biosciences
α-CD137	activity related marker for T cells	Pe	4B4	ThermoFisher
α-phospho-mTOR	activity of mTOR	Pe	MRRBY	eBioscience™ ThermoFisher

* antibodies used for fluorescence activated cell sorting

2.1.5.2.2. Anti-mouse antibodies

Table 3: Anti-mouse antibodies for flow cytometry

Specificity	Function	Conjugation	clone	company
α-CD62L	lymph node homing receptor	BV421	Mel-14	BD Biosciences
α-CD69	activity related marker for T cells	FITC	H1.2F3	BD Biosciences
α-CD11b	myeloid marker	APC-Cy7	M1/70	BD Biosciences
α-CD8a	part of the T cell co-receptor	APC-Cy7	53-6.7	BioLegend
α-CD44	marker for memory T cells	Pe	IM7	BioLegend
α-CD137	activity related marker for T cells	APC	17B5	BioLegend
α-CD19	B cell marker	Pe	6D5	BioLegend
α-CD3e	T cell marker	FITC	145-2C11	ThermoFisher
α-CD4	part of the T cell co-receptor	APC	RM4-5	ThermoFisher
α-CD25	activity related marker for T cells	Pe-Cy7	PC61.5	ThermoFisher
α-NK1.1	NK cell marker	APC	PK136	ThermoFisher

2.1.5.2.3. Other antibodies and dyes

Table 4: Other antibodies and dyes for flow cytometry

Specificity	Function	Conjugation	clone	company
MitoTracker Green	mitochondrial content			ThermoFisher
2-NBDG	glucose analogue			ThermoFisher
7AAD	viability			BD Biosciences
Annexin V	viability	FITC		BD Biosciences
α-CD3	stimulation of murine T cells	-	145-2C11	BD Biosciences
α-CD28	stimulation of murine T cells	-	37.51	BD Biosciences

2.1.6. Databases and software

The following software tools were used to design experiments and process data.

CellQuest Pro	BD, Heidelberg, Germany
Citavi 5.7.1.0	Swiss Academic Software gmbH, Wädenswil, Switzerland
FACSDiva	BD, Heidelberg, Germany
FlowJo v9.9.6 or v10.4.1	FlowJo, LLC, Ashland, OR, USA

GraphPad Prism 8.0.0	GraphPad Software, La Jolla, CA, USA
Microsoft Excel 2003/2007/2010	Microsoft Deutschland GmbH
SenSorDish-Reader Software	PreSens, Regensburg, Germany

2.2. Methods

2.2.1. Cell culture methods for human immune cells

All cells were handled under a laminar flow cabinet with sterile consumables. If not otherwise noted, cells for cell cultivation were centrifuged at 1300 rpm for 7 minutes at 4 °C. In case of cell staining for flow cytometry, cells were centrifuged at 1600 rpm for 4 minutes at 4 °C.

2.2.1.1. Cell counting and cell size monitoring using the Casy system

The measurement of proliferation and cell size was performed on the Casy system (Casy® Modell TT, OLS Omni Life Science). These two parameters are indicators for T cell function. The Casy system uses an electric field to distinguish between living and dead cells. Besides cell number and viability, the Casy cell counter can analyze the cell diameter. 50 µl of the cell suspension were mixed with 10 ml IsoTon® II diluent (Beckman Coulter). A significant statistical certainty was obtained with a triple measurement.

2.2.1.2. Freezing and thawing of cells

In case of monocytes, cells were mixed with freezing medium (FCS with 20 % DMSO) in cryo tubes in a ratio of 1:2. The cells were slow-frozen in cryo freezing containers at -80 °C.

Frozen cells were thawed at room temperature and immediately washed with medium. After centrifugation cells were resuspended in fresh medium.

2.2.1.3. Isolation of human immune cells

2.2.1.3.1. Isolation of human mononuclear cells by density gradient centrifugation

Mononuclear cells (MNCs) were isolated from the blood of healthy donors. A process called apheresis provided the blood cells. A volunteer donor undergoes a plateletpheresis or a leukapheresis which are normally used to harvest platelets or leukocytes, respectively. The addition of citrate prevents blood coagulation.

MNCs were separated from other cells, as erythrocytes and granulocytes, with a Ficoll density gradient centrifugation. In case of a plateletpheresis about 8 ml blood were mixed with 42 ml PBS, in case of a leukapheresis about 70 ml with PBS to an end volume of 400 ml. The Ficoll solution (15 ml) was carefully covered with a layer of diluted blood (25 or 30 ml) in a centrifuge vial, without mixing the phases. After a centrifugation for 30 minutes with 1801 rpm at room temperature, the interphase of the MNCs was harvested and washed three times with PBS. The cell number was determined and the cells were used either for elutriation or for T cell isolation.

2.2.1.3.2. Separation of monocytes by counterflow centrifugation elutriation

The elutriation enables the separation of different cell populations based on their cell size. The principle of separation is the balance between centrifugal force and the counter flow drag force. It allows the enrichment of B cells (fraction I, with a diameter of 7 µm), T cells (fraction II, 8 to 9 µm) and monocytes (fraction III, 10 µm).

After the elutriation chamber was attached into the elutriator's rotor, the complete elutriation system was sterilized with 6 % H_2O_2 and washed with PBS. The settings of the peristaltic pump were calibrated with Hanks buffer (Sigma-Aldrich) to determine the required flow rate.

After harvesting the MNCs by density gradient centrifugation, cells were injected into the elutriation chamber with a flow rate of 52 ml/min. Fractions were collected using different pump flow rates (52–111 ml/min) using a fixed rotor speed of 2500 rpm at 4 °C.

Based on their size, monocytes were washed out in the last fraction, achieving more than 85 % purity. The cells were centrifuged and resuspended in medium. After cell counting, 10×10^6 cells were frozen. The surface markers CD3, CD14 and CD20 were analyzed by flow cytometry after each elutriation. Supernatants of overnight cultures (RPMI with 2 % AB serum, 2 mM glutamine and Penicillin/Streptomycin) were used to confirm the unstimulated state of the monocytes by an IL-6 elisa.

2.2.1.3.3. Maturation of monocytes to DCs

DCs for T cell stimulation were generated by incubating monocytes in medium with FCS (10 %), 224 IU/ml GM-CSF and 144 IU/ml IL-4 (both PeproTech) for four days. After two days, the DCs were matured with 100 ng/ml lipopolysaccharide (LPS, Enzo Life Sciences GmbH). After four days, matured DCs (mDCs) were harvested and were used to stimulate T cells (2.2.1.4).

2.2.1.3.4. Isolation of human CD8 T cells

CD8 T cell isolation was based on the MACS separation technique from Miltenyi Biotech. T cells are magnetically labeled with MicroBeads which are linked to a specific antibody. Magnetically labeled cells retain within a column, while unlabeled cells run through.

After determination of the cell number, MNCs were centrifuged, the supernatant was aspired and the cell pellet was resuspended in MACS buffer (40 µl per 10 x 10^6 cells). After addition of 10 µl of MicroBeads cells were incubated at 4 °C for 15 minutes. Unlabeled MicroBeads were removed with a washing step and cell pellet was resuspended in 500 µl MACS buffer. The LS column (Miltenyi Biotech) was placed in the magnetic field of a MACS separator and the column was rinsed with 3 ml MACS buffer. Cell suspension was applied onto the column. Cells labeled with MicroBeads are retained within the column, while unlabeled cells pass through. Washing steps were performed three times with 3 ml MACS buffer. The column was removed from the separator and the magnetically labeled cells were flushed out with 5 ml MACS buffer in a collection tube by firmly pushing the plunger into the column. Cells were centrifuged, the supernatant was aspired, cell pellet was resuspended in T cell medium and cell number was determined. About 12 % of the MNCs were CD8 positive.

After isolation overall purity of the CD8 T cells was checked by flow cytometry and reached always more than 98 %. The cells were stored overnight at 37 ˚C with a cell number of 10 x 10^6 cells per ml. The inactive state was checked and maintained after overnight storage (Figure 4).

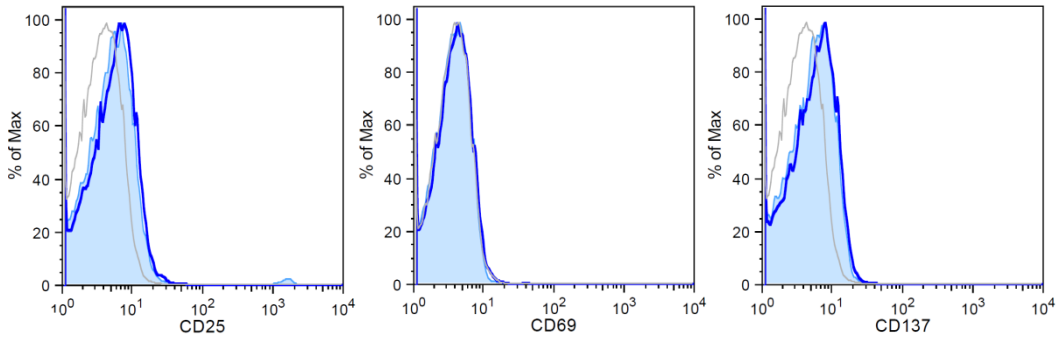

Figure 4: Analysis of the inactive state of CD8 T cells after overnight storage. CD8 T cells were isolated from MNCs of healthy donors and were stained for CD25, CD69 and CD137 before (light blue) and after overnight storage (dark blue) at 37 °C with a cell concentration of 10 x 10^6 cells per ml in RPMI with all supplements. Unstained cells were used to determine autofluorescence (grey line).

2.2.1.4. T cell stimulation, cultivation and experimental setup for human T cells

After the overnight storage the cells were harvested. To confirm the unstimulated state of the T cells, supernatants were served to measure cytokines to a later time point. The purity, the subset distribution and the activation state were checked by flow cytometry.

T cells were cultured in T cell medium with IL-2 (end concentration of 25 IU/ml, PeproTech) in a humidified atmosphere (5 % CO_2) at 37 °C in a Heraeus incubator.

CD8 T cell subsets were cultured with additional IL-7 and IL-15 (both end concentration of 5 ng/ml, PeproTech). Cells were stimulated with anti-CD3/CD28 dynabeads obtaining a bead-to-cell ratio of 1:1. 0.1 x 10^6 T cells were seeded in a 96 well U-bottom plate in 220 µl medium resulting in a cell concentration of 0.444 x 10^6 cells per ml.

Cells and supernatants were harvested after 24, 48, 72 hours and 6 days. Cells were counted to monitor proliferation and cell size. The expression of the activation related surface markers or the viability were analyzed by flow cytometry (2.2.4). Supernatants were frozen at -20 °C to determine cytokine production, lactate accumulation and glucose consumption to a later time point. To prevent nutrient restriction and acidification the cells were diluted after 72 hours, by removing 100 µl cell suspension and adding fresh medium containing IL-2.

Alternatively T cells were stimulated in a mixed leukocyte reaction (MLR) with 0.01 x 10^6 allogeneic mDCs in a 96 well U-bottom plate in 220 µl medium resulting in a cell concentration of 0.444 x 10^6 cells per ml.

In order to activate expanded T cells to a maximum, T cells were stimulated with phorbol-12-myristate-13-acetate (PMA, 0.018 µg/ml) and ionomycin (0.89 µM) for 4 hours. Supernatants were frozen at -20 °C to determine cytokine production to a later time point.

2.2.1.5. Cultivation under metabolic restriction

Glucose and glutamine metabolism was analyzed in glucose (Sigma-Aldrich) or glutamine free RPMI 1640 (Gibco/ Thermofisher). The addition of the human AB serum (10 %) resulted in a final concentration of 0.4 mM in comparison to 10.4 mM glucose or 0.05 to 0.075 mM in comparison to 2.05 to 2.075 mM glutamine in standard RPMI. The required glutamine concentration for the entire stimulation was analyzed by addition of different glutamine concentrations.

Respiration was analyzed with the inhibition of the mitochondrial ATP production by the complex V inhibitor oligomycin (final concentration 5 µM).

2.2.2. Cell culture methods for murine immune cells

2.2.2.1. Mice

To analyze MCT4 deficiency in immune cells, a $MCT4^{-/-}$ mouse model with a C57/BL6 background was used, kindly provided by Prof. Dr. John L. Cleveland (Moffitt Cancer Center & Research Institute, Tampa, FL, USA). All experiments used age- and sex-matched mice.

2.2.2.2. Isolation of murine immune cells

2.2.2.2.1. Tissue preparation of the spleen for the isolation of splenocytes

Spleens of wildtyp and $MCT4^{-/-}$ mice were collected in RPMI. A single-cell suspension was processed by scratching out cells with curved forceps in 7 ml medium with 2 mM glutamine and 50 IU/ml Penicillin/Streptomycin in a petri dish. Cells were filtered through a 100 µm cell strainer and were centrifuged. To remove erythrocytes 2 ml ACK lysis buffer (2.1.3.8) was added. After incubation for 3 minutes at room temperature, the reaction was stopped by adding 10 ml medium. After a centrifugation step the cells were resuspended in 1 ml medium and were filtered through a 100 µm cell strainer. The cell strainer was washed with 19 ml medium. The

splenocytes were counted and stored for at least 2 hours in a humidified atmosphere (5 % CO_2 at 37 °C).

To analyze the immune cell composition in the spleen 1.0 x 10^6 cells were analyzed by flow cytometry (2.1.5.2.2). To analyze the impact of MCT deficiency on T cell function, splenocytes were used to isolate CD4 and CD8 T cells (2.2.2.2.2).

2.2.2.2.2. Isolation of murine CD4 and CD8 T cells

Similar to the human T cell isolation, the murine CD4 and CD8 T cells were isolated with the MACS technology from Miltenyi Biotech. CD4 T cells were isolated by positive selection (130-049-201). The effluent after the CD4 isolation with the unlabeled remaining splenocytes was used to isolate the CD8 T cells. CD8 T cells were separated untouched by depletion (130-104-075).

The procedure for the positive selection of the murine CD4 T cells was similar to the isolation of human T cells. In short, after the centrifugation step, the cell pellet was resuspended in MACS buffer (2.1.3.6, 10 µl per 10 x 10^6 cells). The splenocytes were incubated at 4 °C for 15 minutes with MicroBeads, were washed to remove unbound beads and resuspended in 500 µl MACS buffer. The cell suspension was applied onto the prepared column. After three washing steps, CD4 T cells were flushed out, centrifuged and resuspended in medium.

CD8 T cells were isolated from the effluent after CD4 T cell isolation. After the determination of cell number, splenocytes were centrifuged, the supernatant was aspired and the cell pellet was resuspended in MACS buffer (40 µl per 10 x 10^6 cells). After addition of 10 µl of the biotin-antibody cocktail per 10 x 10^6 cells, cells were incubated at 4 °C for 5 minutes. After adding 30 µl MACS buffer and 20 µl of anti-biotin MicroBeads per 10 x 10^6 cells, cells were incubated at 4 °C for 10 minutes. Cells were applied onto the prepared column and the column was washed once with 3 ml MACS buffer. The flow through was collected containing unlabeled cells, representing the enriched CD8 T cells. CD8 T cells were centrifuged and resuspended in medium.

After isolation the overall purity of the CD4 and CD8 T cells was checked by flow cytometry. In contrast to the human T cells, the murine T cells were directly stimulated (2.2.2.2.3).

2.2.2.2.3. Stimulation, cultivation and experimental setup for murine T cells

After isolation (2.2.2.2.2), murine T cells were cultured in RPMI medium with IL-2 (end concentration of 10 IU/ml, PeproTech) in a humidified atmosphere (5 % CO_2) at 37 °C.

Cells were stimulated with anti-CD3e antibody (purified NA/LE hamster anti-mouse CD3e, clone 145-2C11, BD Biosciences, 553057, 5 µg/ml) plus anti-CD28 antibody (purified NA/LE hamster anti-mouse CD28, clone 37.51, BD Biosciences, 553294, 1 µg/ml). 96 well flat-bottom plates were coated with anti-CD3e antibody for at least two hours at 37 °C. 0.2 x 10^6 T cells were seeded in 200 µl medium resulting in a cell concentration of 1.0 x 10^6 cells per ml.

Cells and supernatants were harvested after 24 and 48 hours. Cells were counted to monitor proliferation. The expression of activation related surface markers or the viability were analyzed by flow cytometry. Supernatants were frozen at -20 °C to determine cytokine production, lactate accumulation and glucose consumption to a later time point.

2.2.3. Western blot analysis

Samples were lysed in RIPA (Sigma-Aldrich) in case of human T cells or in lysis buffer (50 mM HEPES with pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1 % Tween 20, 1 mM PMSF, 1 mM NaF, 10 mM ß-glycerophosphate, 1 mM Na orthovanadate) in case of murine T cells, separated by 10 % 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-MCT4, α-GLUL, α-β-Actin, 2.1.5.1). Detection was performed by chemiluminescence (ECL, Amersham Bioscience) and analyzed using the chemiluminescence system Fusion Pulse 6 (Vilber Lourmat).

2.2.4. Flow cytometry

Flow cytometry is a method for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell population. It allows simultaneous multi-parameter analysis of single cells. Surface marker and intracellular molecules are stained with fluorescence labeled antibodies. Next to the granularity by the sideward scatter (SSC) and the size by the forward scatter (FSC), cells can be analyzed regarding their fluorescence signal. For this thesis a BD Aria, a BD FACS Calibur, a BD LSR Fortessa and a BD LSR II were used.

In this study different markers were used for the classification and characterization of T cells (Table 2, Table 3, Table 4). Activity related surface markers as CD25 or CD69 or effector molecules as granzyme B and perforin allowed the analysis of T cell function. The results were analyzed with the Flowjo software.

BD compbeads or cells were used to determine compensation values, according to manufacturer's instructions. To determine cell autofluorescence, unstained cells were used.

2.2.4.1. Staining of extracellular surface marker

Cells were harvested in FACS tubes and washed with 1 ml of FACS wash buffer (2.1.3.5). After addition of the antibodies, cells were incubated at 4 °C for 20 minutes in the dark. The optimal antibody concentration was determined by titration for every antibody. After a washing step with 1 ml FACS wash buffer, cells were resuspended in 200-300 µl of FACS wash buffer and immediately analyzed by flow cytometry.

2.2.4.2. Antibody staining of intracellular molecules for flow cytometry

For staining of intracellular antigens as perforin or granzyme B, the intracellular staining kit from eBioscience/Thermofisher was used, following manufacturer's protocol. In short, T cells were incubated in the presence of monensin for three hours, harvested and permeabilized for 15 minutes at 4 °C. After T cells were washed twice with permeabilization buffer, T cells were stained with respective intracellular markers and incubated for 20 minutes at 4 °C in the dark. After a washing step with 1 ml FACS wash buffer, cells were resuspended in 200-300 µl of FACS wash buffer and immediately analyzed by flow cytometry.

2.2.4.3. Cell viability measurement by 7AAD and Annexin V staining

To analyze the viability of cells, 7AAD (7-amino-actinomycin D) and Annexin V were used. Cells with damaged plasma membranes are unable to prevent 7AAD from entering the cell. Once inside the cell, 7AAD binds to intracellular DNA. After initiating apoptosis, cells translocate the membrane phosphatidylserine from the inner face of the plasma membrane to the cell surface. Phosphatidylserine can be easily detected by staining with a fluorescent conjugate of

Annexin V. With this staining procedure viable (double negative), apoptotic (7AAD negative, Annexin V positive) and dead cells (double positive) can be distinguished.

The cells were harvested in FACS tubes and washed with 1 ml of PBS. The staining solution was prepared as follows: 360 µl ddH_2O, 40 µl Annexin V binding buffer, 10 µl 7AAD and 5 µl Annexin V. After addition of the staining solution the cells were incubated at room temperature for 20 minutes in the dark. The cells were immediately analyzed by flow cytometry.

2.2.4.4. Analysis of mitochondrial content by MitoTracker Green staining

In order to analyze mitochondrial content in murine T cells, cells were stained with MitoTracker Green (ThermoFisher). Unstimulated T cells were incubated with 10 nM MitoTracker Green for two hours in 1 ml RPMI medium without FCS in a humidified atmosphere (5 % CO_2) at 37 °C. After a washing step with 1 ml FACS wash buffer, cells were resuspended in 200-300 µl of FACS wash buffer and immediately analyzed by flow cytometry.

2.2.4.5. Determination of glucose uptake with 2-NBDG by flow cytometry

The fluorescent glucose analog 2-NBDG (2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose, ThermoFisher) is a tracer used for monitoring glucose uptake into living cells. T cells stimulated for 2 hours were washed with PBS and incubated in w/o glucose medium in a humidified atmosphere (5 % CO_2) at 37 °C. After one hour 2-NBDG was added with an effective concentration of 50 µM. After 45 minutes of incubation in a humidified atmosphere (5 % CO_2) at 37 °C, cells were washed twice with PBS and analyzed by flow cytometry.

2.2.4.6. Fluorescence activated cell sorting

In case of fluorescence activated cell sorting, T cells were harvested after overnight storage and washed with sterile FACS wash buffer. CD8 T cells were stained with six antibodies to distinguish between naïve ($CD3^+$, $CD8^+$, $CCR7^+$, $CD62L^+$, $CD45RA^+$, $CD45RO^-$), CM ($CD3^+$, $CD8^+$, $CCR7^+$, $CD62L^+$, $CD45RA^-$, $CD45RO^+$) and EM ($CD3^+$, $CD8^+$, $CCR7^-$, $CD62L^-$, $CD45RA^-$, $CD45RO^+$) subsets (Table 5).

Table 5: Antibodies used for fluorescence activated cell sorting with used volume for 10 x 10^6 cells

Specificity	Conjugation	Volume [µl]
CD3	APC-Cy7	20
CD8	PB	30
CCR7 (CD197)	FITC	20
CD62L	APC	60
CD45RO	Pe	90
CD45RA	Pe-Cy7	15

Cells were incubated with the antibodies at 4 °C for 20 minutes in the dark. After a washing step the cells were resuspended in FACS wash buffer to get a concentration of about 20-30 x 10^6 cells per ml. The cell suspension was filtered through a 35 µm nylon mesh to prevent a blockage and fluorescence activated cell sorting was performed immediately at a BD FACS Aria. In order to confirm separation of subpopulations, a reanalysis of all subsets was performed after fluorescence activated cell sorting (Figure 5).

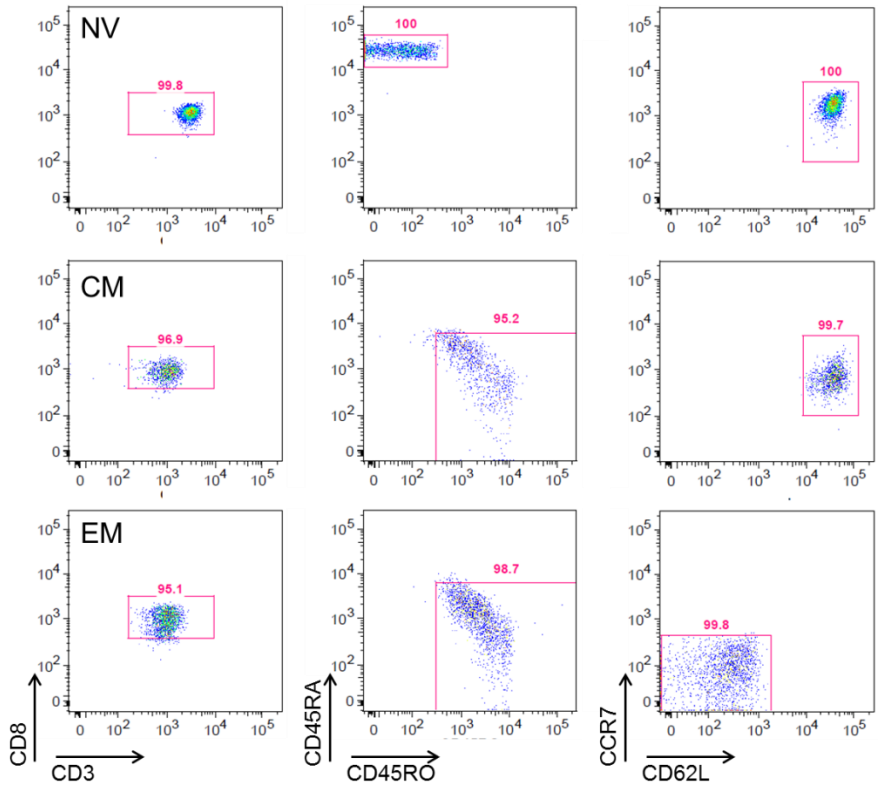

Figure 5: Purity of CD8 T cell subsets was confirmed by reanalysis after fluorescence activated cell sorting. Naïve ($CD3^+$, $CD8^+$, $CCR7^+$, $CD62L^+$, $CD45RA^+$, $CD45RO^-$), CM ($CD3^+$, $CD8^+$, $CCR7^+$, $CD62L^+$, $CD45RA^-$, $CD45RO^+$) and EM ($CD3^+$, $CD8^+$, $CCR7^-$, $CD62L^-$, $CD45RA^-$, $CD45RO^+$) subsets were separated by staining of indicated markers.

2.2.5. Methods for metabolic analysis

2.2.5.1. Determination of glucose consumption

To analyze glucose consumption, glucose concentration in supernatants was determined enzymatically after a stimulation of 24, 48 and 72 hours with the Glucose (HK) Assay Kit (Sigma-Aldrich). The glucose is phosphorylated to glucose-6-phosphate which is oxidized in the presence of nicotinamide adenine dinucleotide (NAD). During this reaction, an equimolar amount of NAD is reduced to NADH which absorbs light at a wavelength of 340 nm. The NADH increase in absorbance is directly proportional to the glucose concentration.

The measurement was performed according to the manufacturer's protocol at a Thermo VarioSkan photometer (Thermo Scientific). The glucose concentration of each sample was calculated by using a prepared standard curve.

2.2.5.2. Determination of lactate accumulation

Lactate concentration was determined enzymatically using a Dimension Vista (Siemens) and specific reagents (Roche).

2.2.5.3. Measurement of the cellular oxygen consumption with the PreSens technology

The PreSens technology (PreSens Precision Sensing GmbH) enables the measurement of respiration using viable, non-fixed cells, 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 human T cells, 0.8 x 10^6 cells were prepared with α-CD3/CD28 Microbeads in a cell to bead ratio of 1:1. The cell-bead mix was seeded in a volume of 1 ml per well. With each experiment wells were generated with medium and unstimulated T cells, respectively.

2.2.5.4. Measurement of the cellular oxygen consumption and extracellular acidification rate with the Seahorse technology

Extracellular acidification rate (ECAR, mpH/min) and oxygen consumption rate (OCR, pmol/min) of murine T cells were analyzed by a Seahorse XFe96 Analyzer (Agilent Technologies). ECAR and OCR were measured in non-buffered XF base medium (Agilent Technologies) supplemented with 2 % FCS. CD4 and CD8 T cells were immobilized onto Seahorse cell plates (0.2 x 10^6 cells per well) coated with 50 µg/ml poly-D-lysine (Sigma-Aldrich).

2.2.6. Determination of cytokines by ELISA

Cytokine secretion was determined in culture supernatants by commercially available sandwich enzyme-linked immunosorbent assays (ELISA; R&D Systems; human: DY285 for IFNγ and DY210 for TNF; murin: DY485 for IFNγ and DY410 for TNF) according to the manufacturer's protocol. In short a 96 well plate was coated with a capture antibody overnight. The plate was blocked with blocking buffer (1 % BSA) for at least one hour. The samples and the standard were incubated for two hours at room temperature. After the incubation with the detection antibody for two hours at room temperature, the plate was incubated with Streptavidin-HRP working dilution for 20 minutes. After incubation with the substrate solution for another 20 minutes the reaction was stopped with stop solution. The optical density of each well was immediately determined at 450 nm at a Precision microplate reader using a wavelength correction set to 540 nm.

3. Results

3.1. Characterization of human CD8 T cells

Tumor cells are characterized by a strongly elevated glucose and glutamine metabolism which results in reduced availability of those nutrients in the tumor microenvironment (Chang et al. 2015; Pan et al. 2016). After stimulation, primary T cells display a similar metabolic phenotype compared to tumor cells and show an accelerated glucose metabolism and glutamine dependency. A direct link between effector functions and metabolic activity has been described. Therefore, reduced nutrient levels in the tumor microenvironment could contribute to the impaired anti-tumor immune response of T cells. However, conflicting results on metabolic requirements for T cell effector functions are published (Cham and Gajewski 2005; Chang et al. 2013; Sukumar et al. 2013; Renner et al. 2015) which might result from species related differences but could also be ascribed to subset specificity. Whether CD8 T cell subsets show differences in their metabolic phenotype, this could make them more or less sensitive to the metabolic tumor microenvironment, which ultimately contribute to therapy outcome during ACT. Therefore, in a first step the metabolic characteristics of human CD8 T cell subpopulations were analyzed in relation to their function.

3.1.1. Distribution and functional characterization of CD8 T cell subsets

CD8 T cells were isolated from MNCs of healthy donors and a purity of more than 98 % was achieved (data not shown). Cells were stored overnight at a high density in culture medium. The unstimulated state of the T cells was confirmed by the determination of cytokines in the supernatant and by staining of activation related surface markers (CD25, CD69 and CD137) by flow cytometry. Purity, subset distribution and the quiescent state were maintained after overnight storage (Figure 4).

In 58 healthy donors aged from 20 to 63 years, distinct differences were evidenced between circulating CD8 T cell populations. CD8 T cells are conventionally divided into three main subsets: Naïve and memory T cells, the latter are further subdivided in central (CM) and effector memory (EM) T cells.

These subsets can be distinguished by a specific expression pattern of the surface markers CD45RA, CD45RO, CD62L and CCR7 (Figure 6). Naïve T cells are characterized by expression of CCR7, CD62L and CD45RA whereas CD45RO is not expressed. Memory subsets are

CD45RO positive and CD45RA negative. Regarding the two homing receptors CCR7 and CD62L, the CMs are double positive, the EMs double negative.

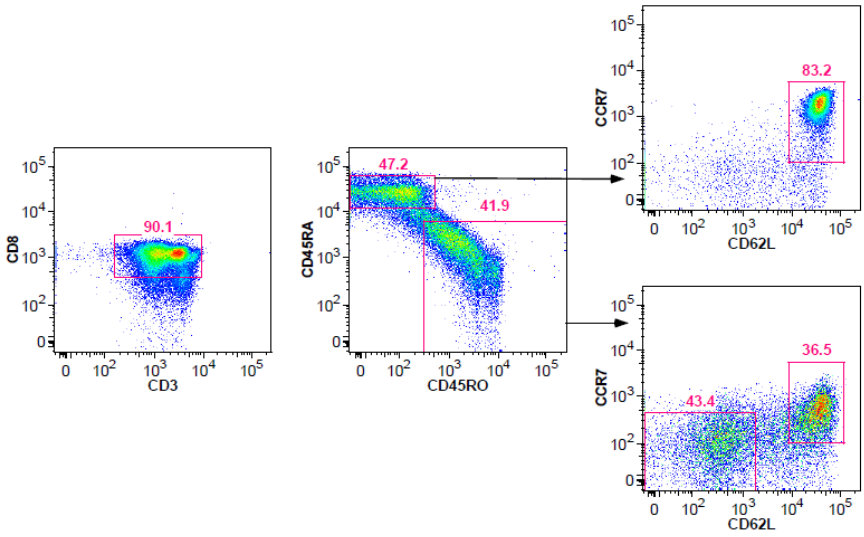

Figure 6: Staining of surface markers associated with differentiation state of CD8 T cell subsets. CD8 T cells were isolated by magnetic bead separation, stored overnight, stained for surface markers and analyzed by flow cytometry. In case of fluorescence activated cell sorting, cells were stained for CD3 and CD8 together with the differentiation markers to guarantee highest possible purity. Naïve: $CD45RA^+$ $CD45RO^-$ $CCR7^+$ $CD62L^+$, CM: $CD45RA^-$ $CD45RO^+$ $CCR7^+$ $CD62L^+$, EM: $CD45RA^-$ $CD45RO^+$ $CCR7^-$ $CD62L^-$. Data from one representative donor are shown.

On average about 36.6 % of CD8 T cells were assigned to the naïve, about 18.9 % to the EM and 7.2 % to the CM T cell subset (Figure 7 A). The remaining cells were not clearly assignable. The percentage of naïve CD8 T cells decreased whereas CD8 memories accumulated linearly with age, while CM number increased only slightly (Figure 7 B).

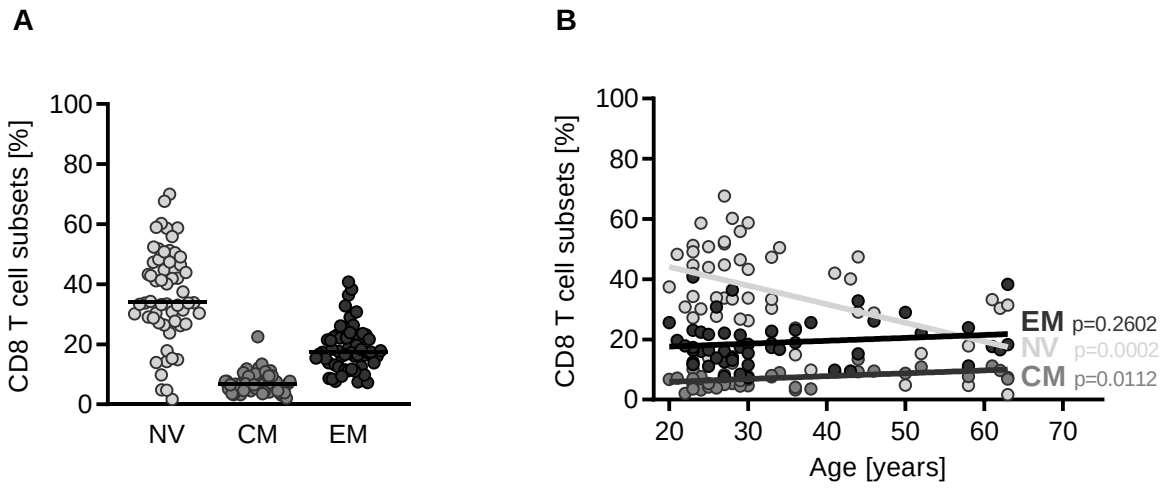

Figure 7: Distribution of naïve, CM and EM subsets in bulk CD8 T cells. CD8 T cells were isolated from healthy donors and were stained for CD45RA, CD45RO, CCR7 and CD62L to differentiate between naïve (light grey), CM (grey) and EM cells (black). (A) Percentage for naïve, CM and EM T cell subsets in total T cells. Horizontal lines indicate the median (n=58). (B) Frequency distribution of naïve, CM and EM subsets in bulk CD8 T cells correlated with age. The linear regression is shown. The corresponding p values are shown in the graphs for each regression line, determined by Pearson's r (n=52).

IFNγ levels were determined in supernatants after 48 hours of α-CD3/CD28 stimulation in bulk T cells by ELISA. The more T cells were assigned to the EM subset in total CD8 T cells the more IFNγ was produced by trend (Figure 8).

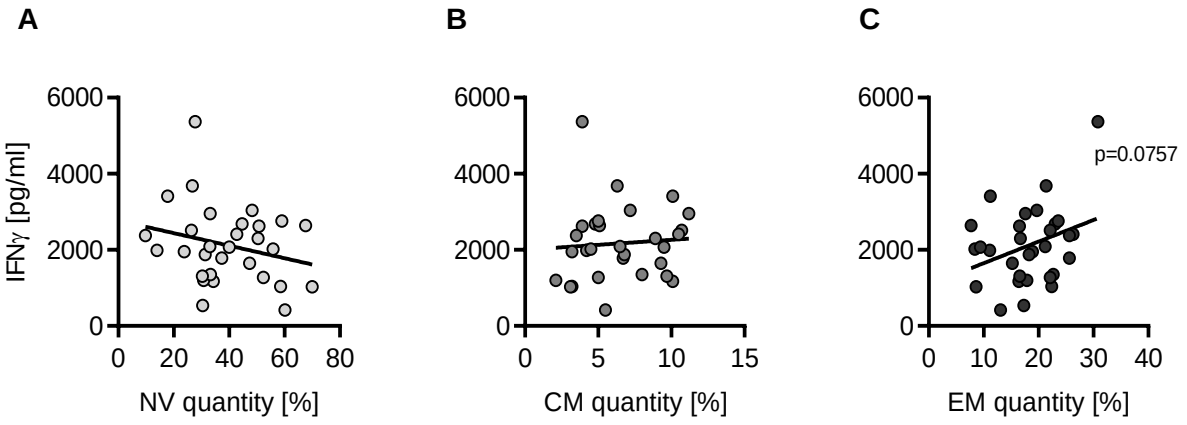

Figure 8: Relation between IFNγ secretion and T cell subset distribution in bulk CD8 T cells. CD8 T cells were isolated from healthy donors and were stained for CD45RA, CD45RO, CCR7 and CD62L to distinguish between naïve (light grey), CM (grey) and EM cells (black). CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. IFNγ levels were determined in culture supernatants after 48 hours by ELISA. The linear regression for naïve, CM and EM are shown. The corresponding p values are shown in the graphs for each regression line, determined by Pearson's r (n=30).

To get the highest possible purity, CD8 T cell subsets were separated by fluorescence activated cell sorting. After gating on CD3 and CD8 double positive T cells, the subsets were sorted according to their expression profile of CD45RA, CD45RO, CD62L and CCR7 (Figure 6). The separation of the subpopulations was confirmed by reanalysis of all subsets after sorting (Figure 5).

In line with the observed differences in the capability of bulk CD8 T cells to secrete cytokines depending on subset composition, the capability to produce IFNγ was different in the sorted subsets (Figure 9, Table 6). In contrast to naïve T cells, memory subpopulations were characterized by a high production of IFNγ (Figure 9 A) as well as TNF (Figure 9 B). CM T cells showed superior capacity to produce IFNγ over EM T cells after 48 hours. According to the literature, cytokine levels produced by memory T cells peaked already after 24 hours upon stimulation and did not markedly increased after 48 hours, whereas in naïve T cells cytokine production was further increased after 48 hours.

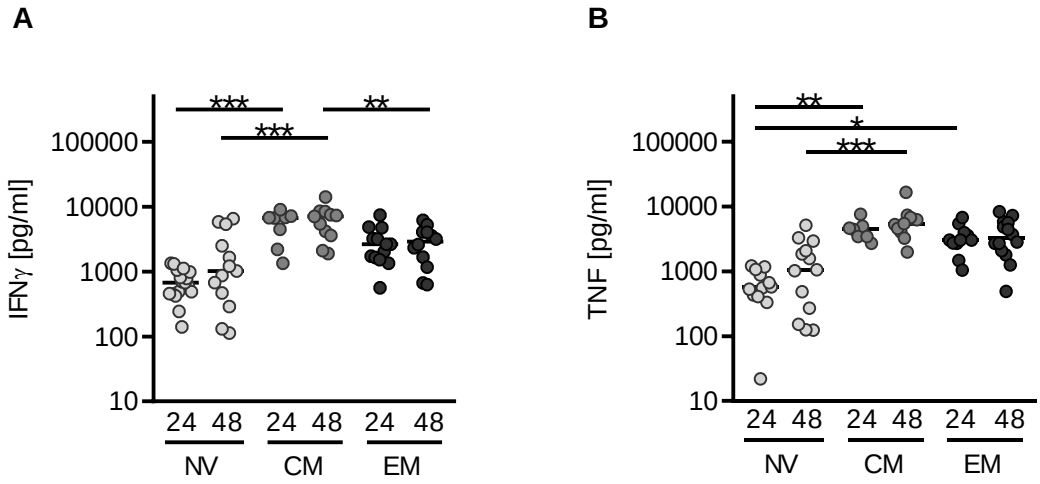

Figure 9: Cytokine secretion of CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. (A) IFNγ and (B) TNF levels were determined in culture supernatants after 24 and 48 hours by ELISA; horizontal lines indicate the median from independent donors (n≥7). Significance was determined by one-way ANOVA and post hoc Dunnett's multiple comparisons test.

Table 6: Average IFNγ and TNF production of CD8 T cell subsets.

	IFNγ [pg/ml]		TNF [pg/ml]	
	24 hours	**48 hours**	**24 hours**	**48 hours**
NV	677	1018	566	1051
CM	6717	7080	4428	5412
EM	2628	2899	3036	3263

CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. IFNγ and TNF levels were determined in culture supernatants after 24 and 48 hours by ELISA; median from data from Figure 9 are shown. For results for statistical significance see Figure 9.

As a first read out, proliferation was monitored as an indicator for the quality of T cell culture with the CASY system. After 24 hours a slight proliferation was already observed in naïve T cells, in line with a rather low cytokine production, whereas CM and EM, producing high cytokine levels within the first 48 hours, started to proliferate beyond 48 hours (Figure 10). However, proliferative capacity was similar in naïve, CM and EM T cells.

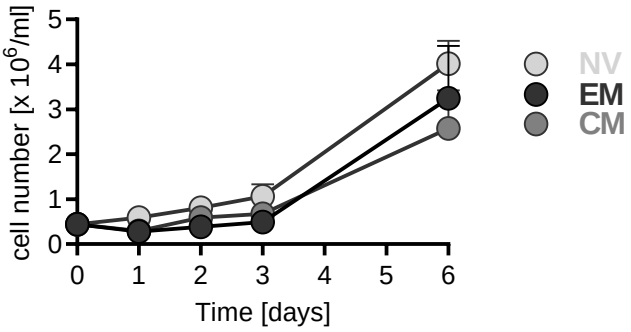

Figure 10: Proliferation of CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Cell number was determined after 24, 48, 72 hours and 6 days of stimulation by the CASY system. Data are shown as mean±SEM (n=5 for naïve, n=3 for CM, n=4 for EM).

3.1.2. Metabolic characteristics of CD8 T cell subsets

Due to the fact that CD8 T cell subsets showed differences regarding their cytokine profile and their proliferative activity, the metabolic phenotype of the sorted CD8 subsets was investigated. Therefore glucose metabolism and respiratory activity were analyzed in the course of stimulation.

Glucose consumption and lactate production were determined as parameters for glycolytic activity of stimulated CD8 T cells (Figure 11). Lactate production was only slightly detectable in the first 24 hours of activation in all subsets (data not shown) but was increased beyond 24 hours and accelerated after 48 hours concomitant with proliferation in all three subsets (Figure 10). Although there was a slight difference regarding proliferation kinetics in naïve T cells compared to memory T cells, glycolytic activity was similar in all three subsets.

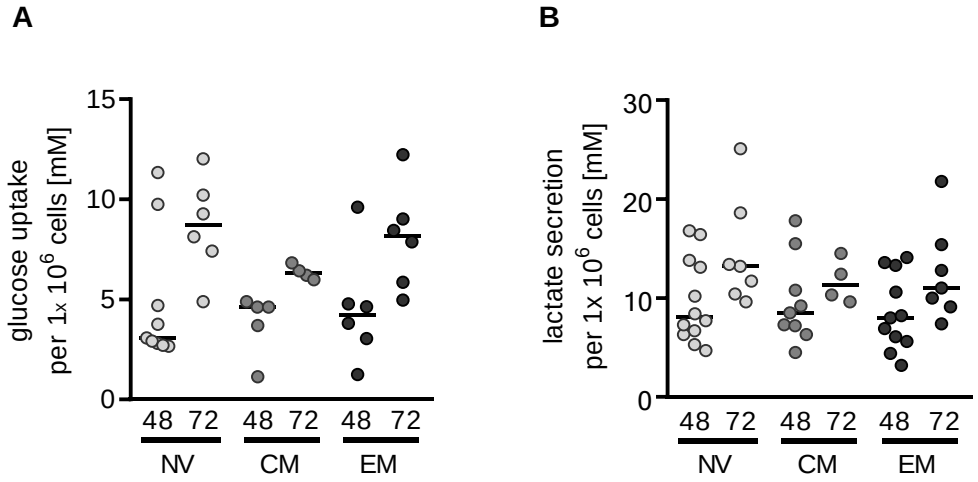

Figure 11: Glycolytic activity of CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Glucose and lactate concentrations were determined in cell culture supernatants after 48 and 72 hours and were normalized to cell number. Glucose concentration was determined by the Glucose (HK) Assay Kit, lactate secretion by enzymatic assays in culture supernatants. Horizontal lines indicate the median from independent donors (n≥4). Significance was determined by one-way ANOVA and post hoc Dunnett's multiple comparisons test.

To analyze cellular respiration the PreSens technology was applied. Respiration was immediately elevated upon stimulation in all three subsets; however naïve T cells showed a higher mitochondrial respiratory activity compared to the memory subsets (Figure 12).

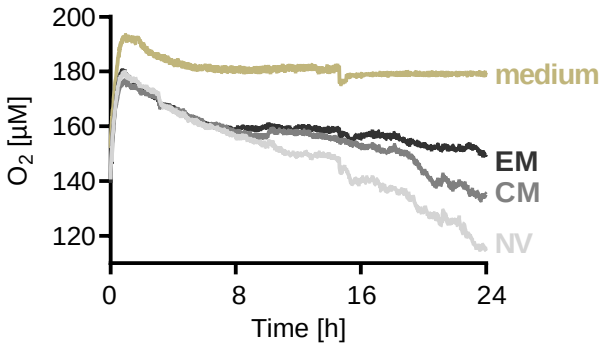

Figure 12: Mitochondrial respiration of CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Cellular respiration was measured with the PreSens technology in bulk CD8 T cells or CD8 T cell subsets at a concentration of 0.8 x 10^6 cells per ml under cell culture conditions (n=6 for naïve and EM, n=5 for CM).

CD8 T cell subsets showed some differences in metabolic activity upon stimulation. Although all three subsets had a similar glycolysis rate, naïve showed a higher oxygen consumption rate in comparison to CM and EM cells, suggesting that mitochondrial activity is of special importance for the naïve subpopulation.

3.2. Nutrient restriction in CD8 T cells - Characterization of CD8 T cell subsets under nutrient restriction

Within a tumor competition for nutrients between tumor cells and immune cells might contribute to the impaired anti-tumor immune response. To investigate a possible impact of nutrient restriction on the function of CD8 T cell subpopulations, the metabolic requirements for effector functions were characterized in all three subsets. The relevance of different metabolic pathways for T cell function was determined by stimulating T cells under nutrient restricted conditions.

To mimic nutrient restriction T cell function was analyzed under glucose (w/o glc) or glutamine (w/o gln) deprived conditions and mitochondrial inhibition with the ATP synthase inhibitor oligomycin (mtATPi). The effect of glucose deprivation was analyzed in glucose free RPMI 1640 supplemented with 10 % AB serum, which resulted in a final concentration of 0.4 mM glucose. The effect of glutamine deprivation was analyzed in glutamine free RPMI 1640 supplemented with 10 % AB serum which leads to a glutamine amount of 0.05 to 0.075 mM.

Viability of T cells was not affected under nutrient restricted conditions after 72 hours (Figure 13). After 6 days viability was still not affected in CM T cells, whereas naïve and EM T cells showed a reduction in viability under glutamine deprivation by trend (naïve: 93.7 % in control cells to 84.7 % under glutamine deprivation, CM: 92.7 % to 87.0 %, EM: 86.7 % to 67.9 %). Lactate production was analyzed as a parameter for glycolytic activity.

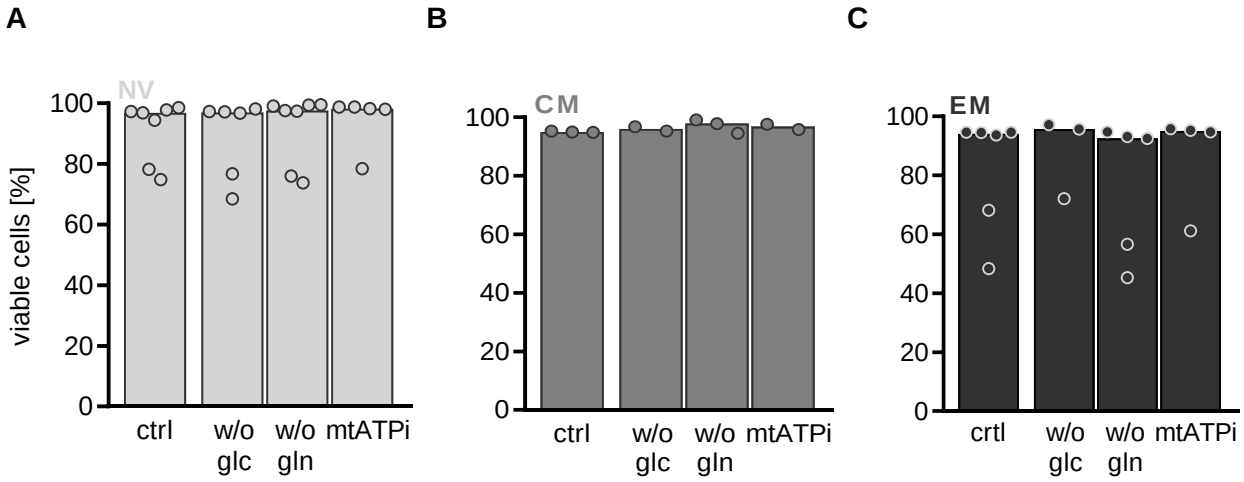

Figure 13: Impact of nutrient restriction on viability of CD8 T cell subsets. T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. As medium for control (ctrl) cells RPMI medium supplemented with 2 mM glutamine and 10 % AB serum (total of 10.4 mM glucose) was used. Glucose restricted conditions (w/o glc) were defined as glucose free medium supplemented with 10 % AB serum resulting in a total of 0.4 mM glucose. For glutamine restricted conditions (w/o gln) a glutamine free RPMI medium was used supplemented with 10 % AB serum resulting in a total of 0.05 to 0.075 mM glutamine. Mitochondrial ATP production was blocked by oligomycin (mtATPi) treatment (5 µM). (A, B, C) After 72 hours of stimulation viability was determined with the CASY system. Data are shown as median (n≥3, with exception of the CMs under glucose deprivation: n=2). Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test.

In contrast to the glycolytic activity, mitochondrial activity was similar to controls in all three subsets under glucose free conditions even initially elevated in EM T cells (Figure 14). Surprisingly, mitochondrial inhibition significantly reduced lactate production in the naïve subset but had no impact on CM and EM T cells. Again, the reduced lactate secretion under mitochondrial inhibition in naïve cells could be either the result of a delayed activation or pointing towards a direct contribution of glutamine degradation to lactate production via the citric acid cycle. To answer this question flux experiments need to be performed.

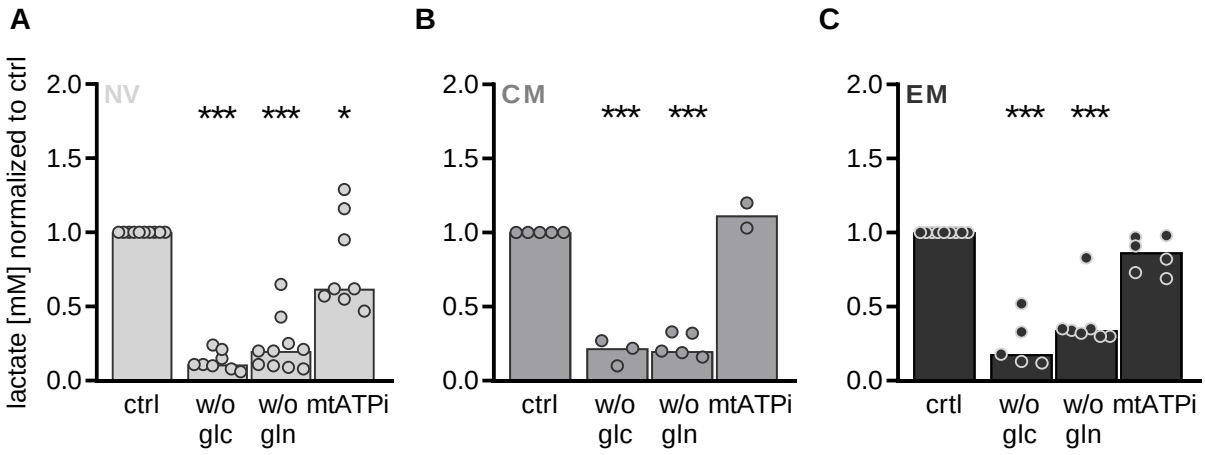

Figure 14: Impact of nutrient restriction on glycolysis in human CD8 T cell subsets. T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. For detailed cultivation conditions see Figure 13. Glycolysis was investigated by measuring lactate accumulation in supernatants after 48 hours. Data are shown as median normalized to control (n≥3, with exception of the CMs treated with mtATPi: n=2). Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, ***p<0.001).

Glutamine deprivation did not affect respiration in the early phase of activation, but led to a strong decrease in mitochondrial activity in all the subsets beyond 8 hours, comparable to mitochondrial inhibition with oligomycin (Figure 15).

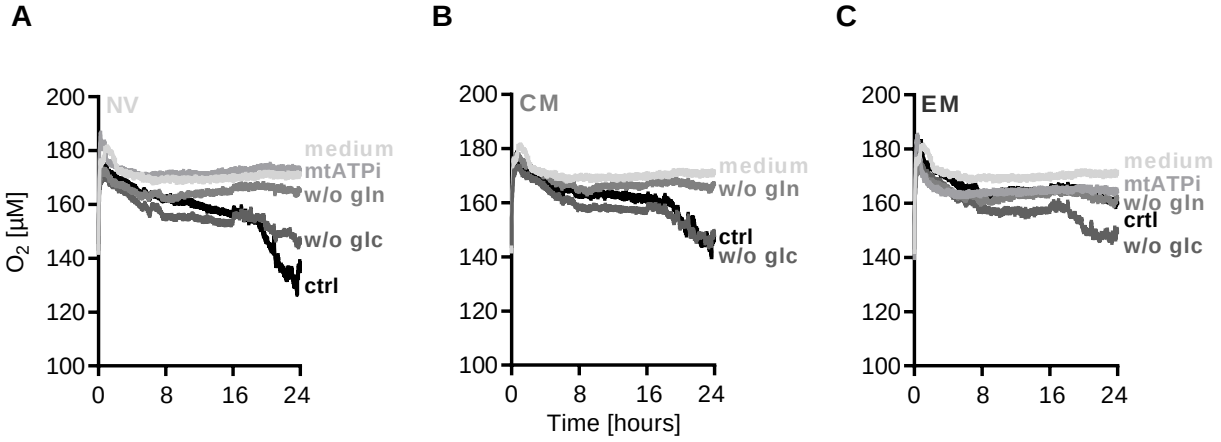

Figure 15: Impact of nutrient restriction on mitochondrial respiration of CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. For detailed cultivation conditions see Figure 13. Cellular respiration was measured with the PreSens technology in freshly stimulated CD8 T cell subsets at a concentration of 0.8 x 10^6 cells per ml under cell culture conditions. Data from one representative donor are shown.

The results obtained on glycolytic activity and respiration under glutamine deprivation denoted a deficient activation. Upon stimulation, human T cells go through an initial growth phase - the so called “on-blast” formation. Glutamine restriction had a strong effect on the on-blast formation indicating an impaired activation. In contrast, increase in cell size was only slightly affected by glucose deprivation in naïve T cells and preserved in the memory subsets. Inhibiting mitochondrial function affected cell size in naïve, slightly in CM but not in EM cells (Figure 16).

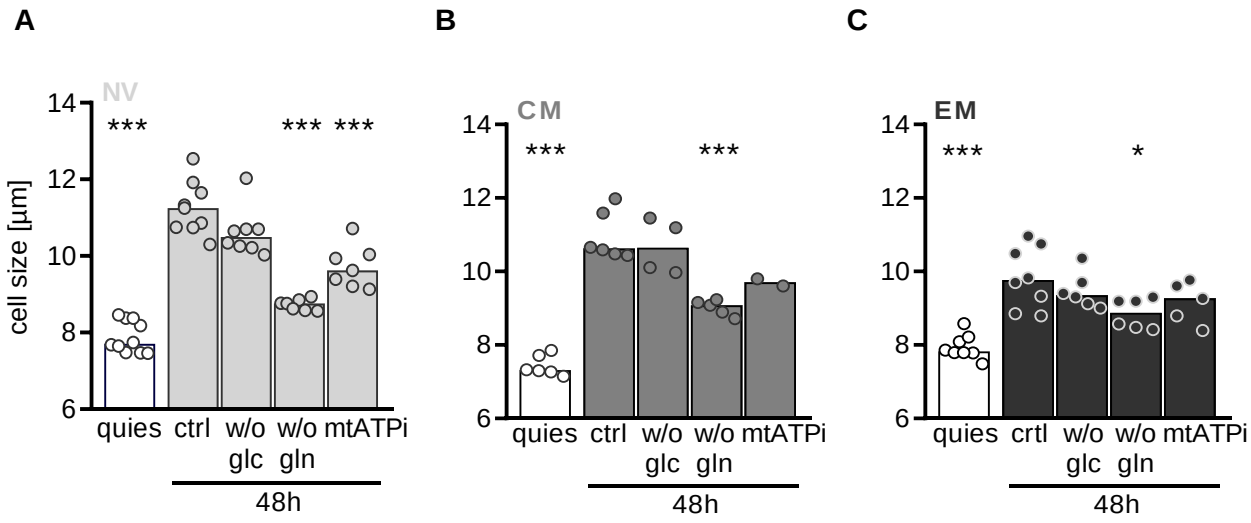

Figure 16: Impact of nutrient restriction on increase in cell size in CD8 T cell subsets. Quiescent CD8 T cell populations (quies) were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. For detailed cultivation conditions see Figure 13. Cell diameter was determined after 48 hours of stimulation under various conditions by the CASY system. Data are shown as median (n≥3, with exception of the CMs treated with mtATPi: n=2). Asterisks show significant differences between stimulated control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett’s multiple comparisons test (*p<0.05, ***p<0.001).

Cytokine production precedes proliferation and takes place immediately after stimulation. Although, we detected no significant effect of glucose deprivation and only partially of mitochondrial inhibition on IFNγ secretion in CD8 T cells subsets, donor dependent effects were observed (Figure 17 A, B, C).

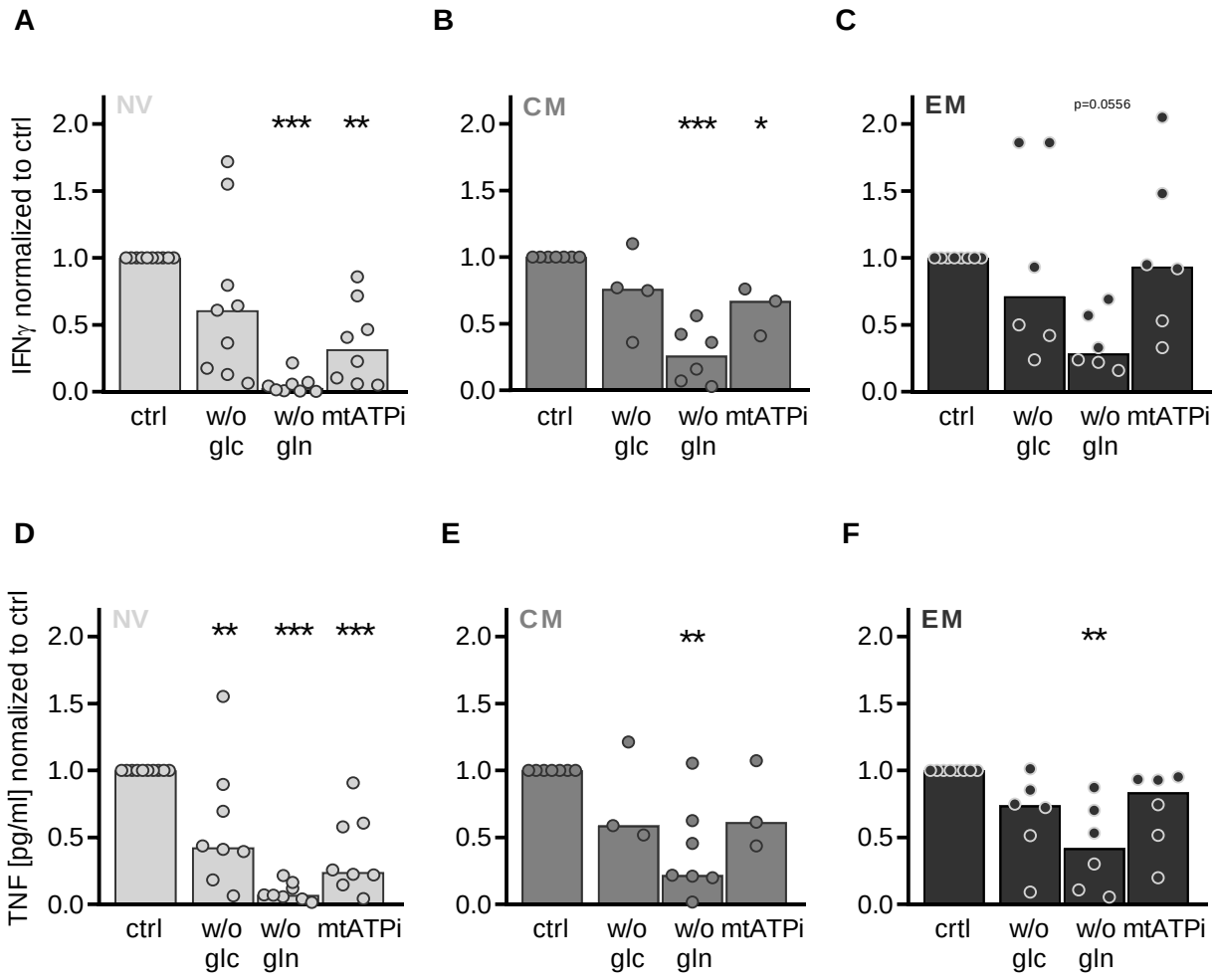

Figure 17: Impact of nutrient restriction on cytokine production of CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. For details on culture conditions see Figure 13. (A, B, C) IFNγ or (D, E, F) TNF levels were determined in culture supernatants after 48 hours by ELISA. Data are shown as median normalized to control levels (n≥3). Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

In particular for the naïve and the EM T cells the variation between donors was pretty high. Depending on the donor, cytokine production seemed to be induced or strongly reduced under glucose deprivation. Similar results were obtained for oligomycin treatment, an effect we could not delineate analyzing bulk CD8 T cells (Renner et al. 2015).

In contrast, glutamine deprivation led to a strongly reduced cytokine production in all three subsets. The impact of glutamine deprivation in the naïve T cells was distinct, since only 5 % of the control IFNγ level was preserved. In the memory T cell subsets IFNγ production was

maintained to 27 % in the CM and to 37 % in the EM subset. Similar results were obtained for TNF production (Figure 17 D, E, F). Interestingly, CM and EM T cells from the same donor were glutamine dependent to a similar extent.

In contrast to cytokine secretion proliferation was strongly decreased under glucose restricted conditions and a reduction of 50 % was observed by mitochondrial inhibition in all three subsets. However, especially in EM T cells the effect of oligomycin on proliferation was donor specific. Again, glutamine deprivation exerted a strong inhibitory effect in all three subsets (Figure 18).

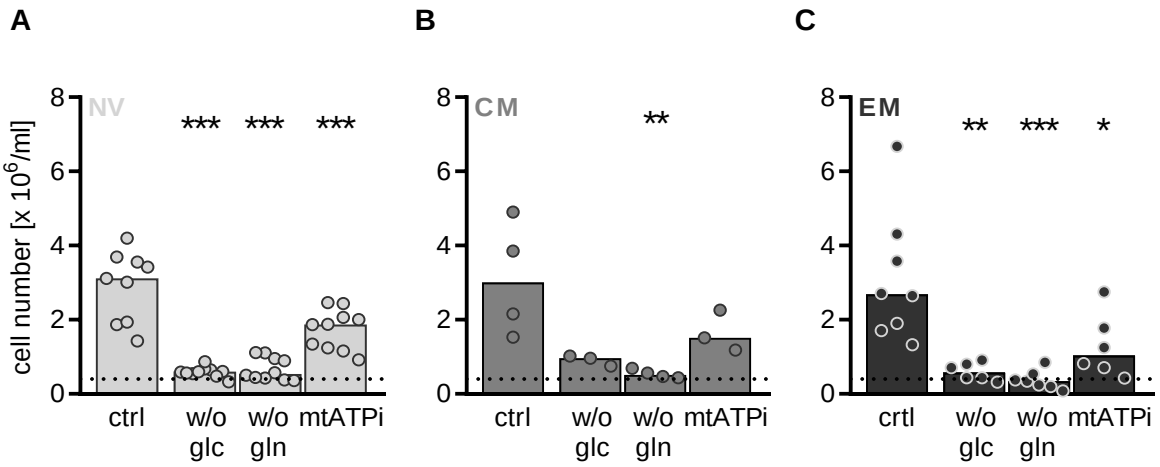

Figure 18: Impact of nutrient restriction on proliferation of CD8 T cell subsets. T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. For detailed cultivation conditions see Figure 13. (A, B, C) Cell number after 6 days of stimulation was determined with the CASY system. Data are shown as median (n≥3). Dotted line indicates starting cell number. Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

Taken together CD8 T cell subsets were affected to different extents by nutrient restriction. Although a donor specific variation was observed, naïve T cells were quite sensitive to mitochondrial inhibition in line with their higher respiration rate. Despite proliferation, CM and EM T cells seemed to be only slightly affected by glucose deprivation and mitochondrial inhibition in general, although donor specific effects have to be taken into consideration. Glutamine restriction distinctly reduced T cell function in all three subsets which indicates a strong dependency on glutamine metabolism.

3.3. The role of glutamine metabolism in human CD8 T cells

In contrast to glucose deprivation or mitochondrial inhibition, glutamine deprivation had a strong impact on T cell function in all CD8 T cell subsets. Although the glutamine dependency in T cells has been shown before, it is still a subject of controversy in the field, which pathway is glutamine crucial for. Moreover, there is nothing known about the limiting glutamine concentration still allowing functional T cell response. Therefore, in the following part glutamine titrations were performed and CD8 T cell function analyzed. Moreover, glutamine derived metabolites were supplemented in order to elucidate those glutamine dependent pathways essential for CD8 T cell activation. As all three subsets showed a strong glutamine dependency, the following set of experiments was performed in bulk CD8 T cell cultures.

3.3.1. Glutamine is essential for CD8 T cells, but low concentrations are sufficient to maintain effector functions

To gain more insight into the link between glutamine metabolism and T cell function, bulk CD8 T cells were cultured in the presence of various glutamine concentrations. Applied glutamine concentrations were chosen in light of blood levels of about 0.5 mM and with regard to levels as low as 0.1 mM which have been detected in tumors (Pan et al. 2016). Since glutamine is an important precursor molecule for a variety of metabolic pathways, mitochondrial respiration and lactate production were analyzed as parameters for metabolic activity of stimulated CD8 T cells (Figure 19).

Surprisingly, respiration was higher beyond 12 hours administering the physiologic glutamine concentration of 0.5 mM and 0.25 mM compared to the standard culture concentration (2 mM). As observed in the single subsets, there was no difference in cell respiration between control and glutamine deprived T cells within the first 12 hours, indicating that the TCA is not restricted (Figure 19 A). However, beyond 12 hours, respiration was reduced under glutamine concentrations lower than 0.25 mM. Similar results were obtained for lactate secretion determined in supernatants after 48 hours of stimulation, which was significantly reduced at concentrations lower than 0.25 mM glutamine.

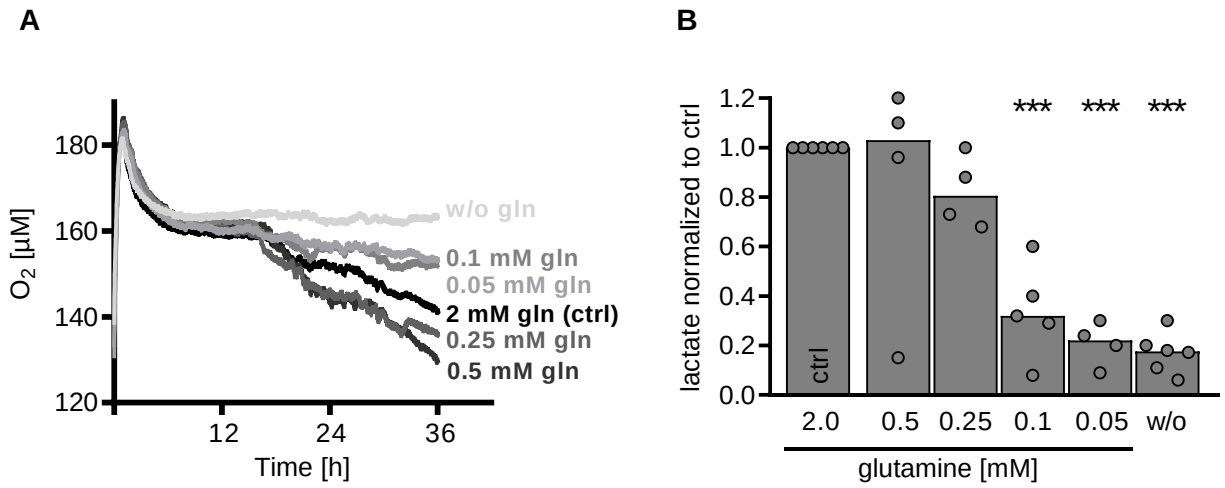

Figure 19: Impact of glutamine concentration on metabolic activity in human bulk CD8 T cells. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. (A) Cellular respiration was measured with the PreSens technology in freshly stimulated CD8 T cells under indicated glutamine concentration at a concentration of 0.8 x 10^6 cells per ml under cell culture conditions (n= 4 for control and w/o glutamine, n=3 for 0.5, 0.25, 0.1, 0.05 mM glutamine. (B) Glycolysis was investigated by measuring lactate accumulation in supernatants after 48 hours. Data are shown as median normalized to control levels (n≥4). Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (***p<0.001).

Glutamine restriction caused a decrease in the metabolic activity of CD8 T cells in a concentration dependent manner (Figure 19). The limiting concentration affecting T cell function in terms of the expression of CD25 expression, on-blast formation, cytokine secretion and proliferation were investigated (Figure 20). It has to be mentioned that glutamine concentrations might be slightly higher as indicated due to the additional glutamine level added with the serum (0.05 to 0.075 mM).

The increase in cell size and proliferation were reduced when glutamine concentrations were as low as 0.25 mM glutamine, half the level normally detected in blood. A concentration of 0.1 mM was limiting for CD25 expression and the secretion of IFNγ (Figure 20 E) and TNF (Figure 20 F). However, cytokine secretion was not completely inhibited. Under glutamine deprivation IFNγ production was maintained to 29.0 %, TNF production to 32.4 % of the levels observed in control CD8 T cells.

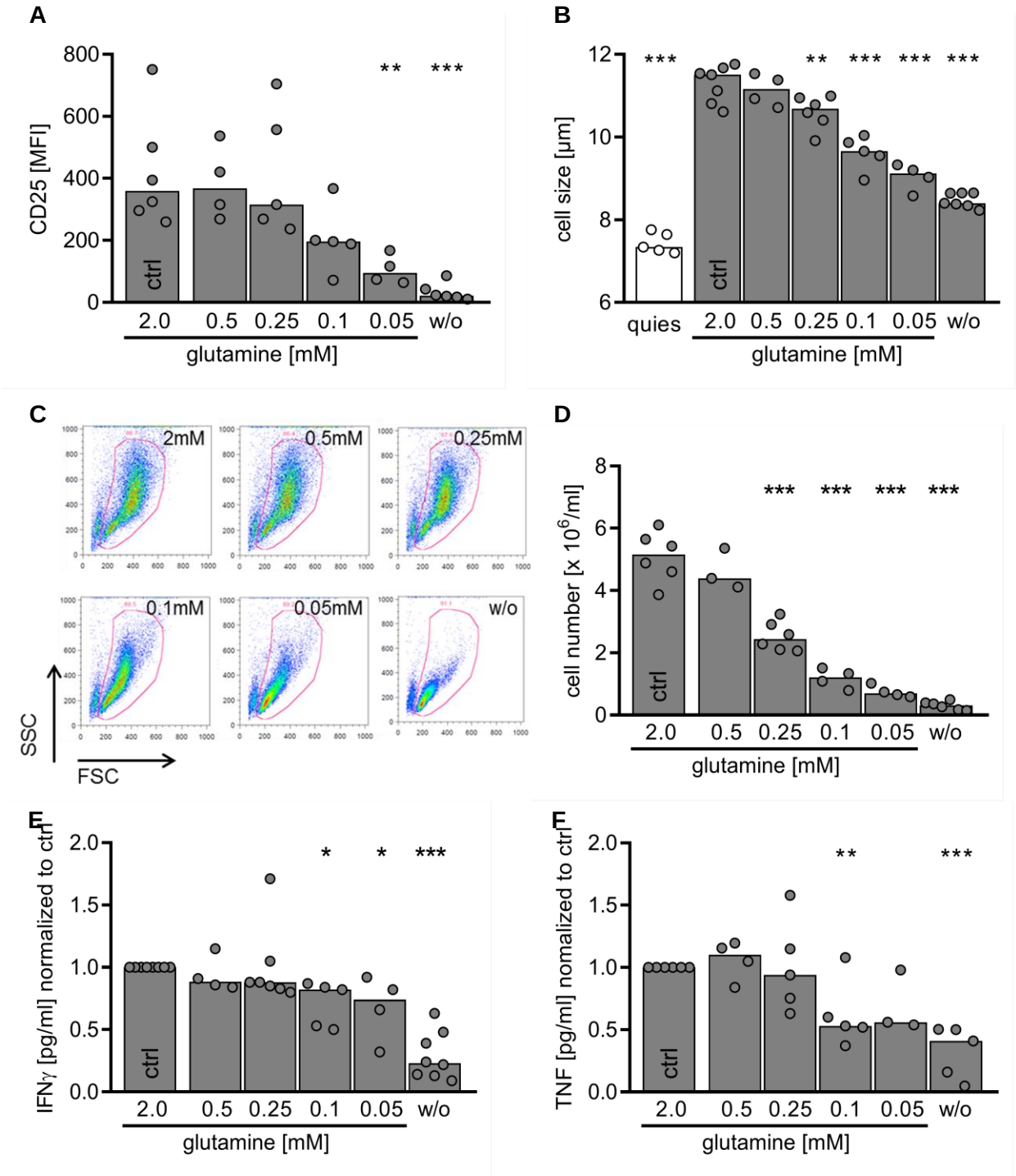

Figure 20: Impact of glutamine concentration on T cell function in human bulk CD8 T cells. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. (A) The surface expression of the activation related marker CD25 was determined after 48 hours by antibody staining and subsequent analysis by flow cytometry, shown is the median fluorescence intensity (MFI). (B) Cell size and (C) granularity were analyzed by flow cytometry with forward and side scatter plots. (C) One representative donor is shown. (B, D) Cell diameter after 48 hours and proliferation after 6 days were determined in T cells by the CASY system. (E) IFNγ and (F) TNF levels were determined in culture supernatants after 48 hours by ELISA. (A, B, D, E, F) Data are shown as median and (E, F) are normalized to control (each n≥3). Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

To analyze whether glutamine is only important for the on-set of T cell activation or also required later on, T cells were activated in the presence or absence of 2 mM glutamine for the first 24 hours and subsequently deprived for glutamine. For comparison all groups were washed after 24 hours and new medium with or without glutamine depending on the treatment group was added. When glutamine was deprived after 24 hours, CD8 T cell function was again strongly affected (Figure 21).

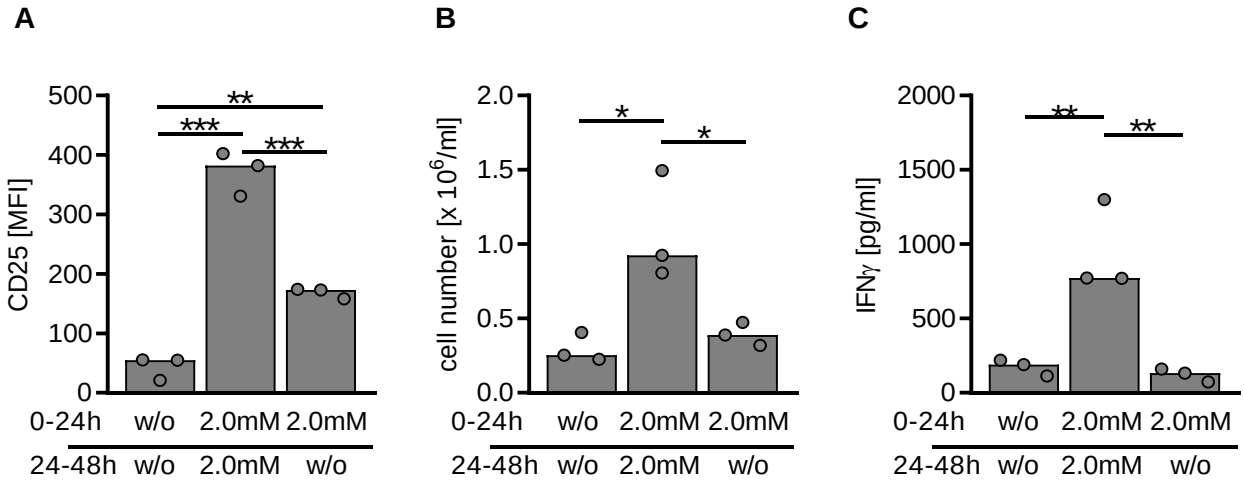

Figure 21: Glutamine is required in the whole course of stimulation. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. T cells were activated in the absence or presence of 2 mM glutamine for 48 hours in comparison to T cells stimulated in the presence of glutamine (2.0 mM) for the first 24 hours, and subsequently cultured in the absence of glutamine. For comparison all groups were washed after 24 hours and new medium was added. (A) CD25 expression was determined after 48 hours by antibody staining and subsequent analysis by flow cytometry, shown is the median fluorescence intensity (MFI). (B) Proliferation was determined after 72 hours in T cells by the CASY system. (C) IFNγ levels were determined in culture supernatants after 48 hours by ELISA. (A, B, C) Data are shown as median (n=3). Asterisks show significant differences between treatments. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01,***p<0.001).

CD25 expression was significantly elevated in comparison to a complete glutamine deprivation but still reduced by more than 50 % in comparison to control level (Figure 21 A). Similar results were obtained for proliferation (Figure 21 B). IFNγ secretion was strongly reduced after glutamine removal similar to the amount of a complete deprivation (Figure 21 C). Those data show, that glutamine is constantly required even when T cells are pre-activated.

mTOR is a master regulator of different cellular processes including cell growth, proliferation or survival for instance. Moreover mTOR was shown to regulate T cell differentiation and

function as well as metabolic activity (Waickman and Powell 2012). Although glutamine does not directly affect mTORC1 activity in the murine system, transport processes in which glutamine is involved induce mTORC1 (Cohen and Hall 2009). To investigate whether glutamine is required for mTOR activity for the early activation in human T cells, mTOR activation was analyzed by flow cytometry. In the presence of glutamine mTOR activation was strongly increased after 24 hours and remained high (Figure 22 A). Under glutamine deprivation mTOR upregulation was blocked. mTOR activation was reduced when glutamine was removed after 24 hours of stimulation in the presence of glutamine (Figure 22 B), indicating that glutamine presence is an absolute requirement for mTOR activity.

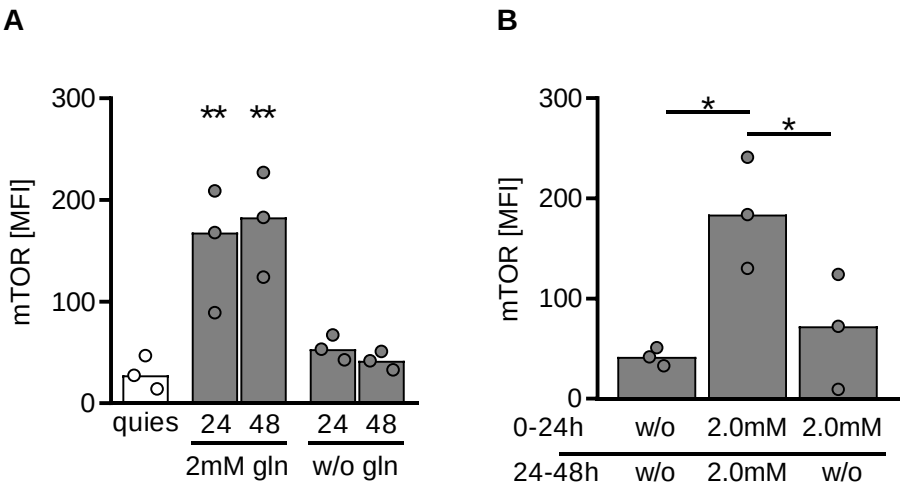

Figure 22: Impact of glutamine deprivation on mTOR activity in human bulk CD8 T cells. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. (A) mTOR expression was analyzed after 24 and 48 hours in the presence or absence of glutamine. (B) T cells were activated in the presence or absence of 2 mM glutamine for 48 hours in comparison to T cells stimulated in the presence of glutamine (2.0 mM) for the first 24 hours, and subsequently cultured in the absence of glutamine. For comparison all groups were washed after 24 hours and new medium was added. mTOR expression was determined by antibody staining and subsequent analysis by flow cytometry, shown is the median fluorescence intensity (MFI). Data are shown as median (n=3). Asterisks show significant differences between (A) quiescent and stimulated cells and (B) between treatments. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01).

T cells cultured in glutamine concentrations lower than 0.25 mM remained in a quiescent state, maybe cohesive to blocked mTOR activation. Reduced proliferation, CD25 expression and in particular on-blast formation indicated insufficient T cell activation at glutamine concentrations lower than blood levels. Regarding cytokine secretion glutamine deprivation led to different results. IFNγ secretion was reduced at concentrations lower than 0.1 mM, but strongly inhibited

under glutamine deprived conditions. In contrast the impact of glutamine reductions lower than 0.1 mM on TNF production was similar as the impact of a glutamine deprivation.

In order to elucidate for which pathway glutamine is essential, experiments were performed to rescue T cell activation by providing those metabolites glutamine is a precursor for.

3.3.2. Biosynthetic precursors and other amino acids are not able to substitute for glutamine

T cells were highly sensitive to glutamine deprivation. To define the crucial pathway requiring glutamine, T cells were analyzed in the absence of glutamine but in the presence of various metabolites which glutamine is the precursor for. Glutamine can be degraded to glutamate and subsequently to α-ketoglutarate, an important intermediate of the TCA cycle, delivering substrates for oxidative phosphorylation. Moreover, glutamate, together with cysteine and glycine, is a component of GSH which acts as an antioxidant and prevents oxidative stress. Glutamine also serves as an amino group donor for nucleotide synthesis (Figure 2). Therefore, T cells were stimulated in the absence of glutamine but providing GSH (2 mM), glutamate (1 mM), cell permeable dimethyl-α-ketoglutarate (1 mM) as well as the purine and pyrimidine derivates hypoxanthine and thymidine (100 and 16 µM). The addition of hypoxanthine and thymidine has been shown to partially rescue glutamine deprivation in the murine system (Wang et al. 2011). Single supplementation of these metabolites was not sufficient to replace glutamine (data not shown). Even the addition of all metabolites simultaneously did not rescue T cell activation (subst., Figure 23). Low CD25 expression as well as blocked increase in cell size clearly indicated an inactive state of T cells stimulated in the presence of all above mentioned metabolites (Figure 23 A, B). Moreover, CD8 T cells were not capable to produce IFNγ (Figure 23 C) or to proliferate (data not shown). These results indicate that glutamine is primarily essential for T cell activation.

Glutamine can also fuel protein glycosylation contributing to the formation of UDP-N-acetyl-glucosamine (Swamy et al. 2016). However, the addition of UDP-N-acetyl-glucosamine was not able to rescue T cell function under glutamine deprived conditions (data not shown).

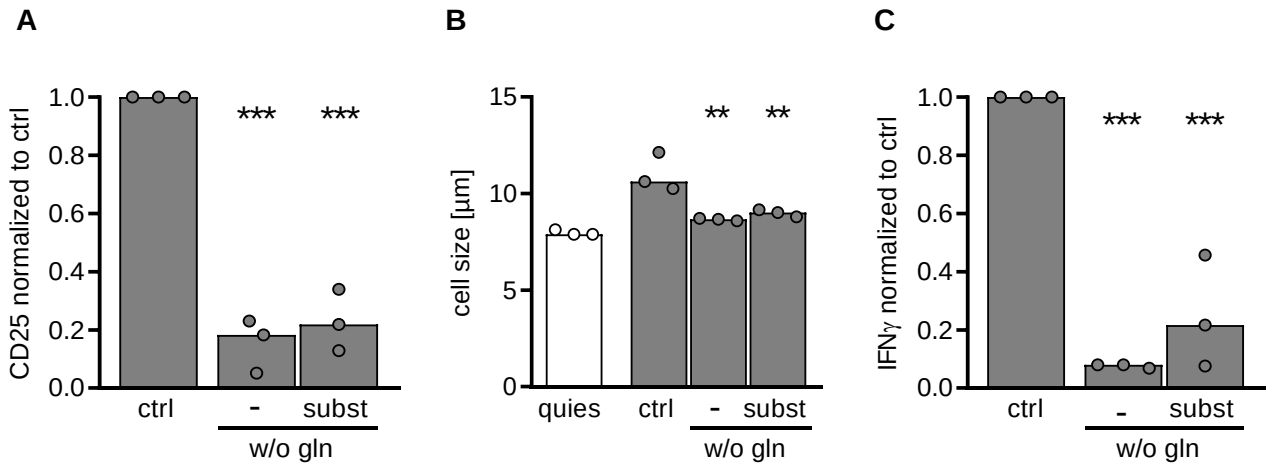

Figure 23: Exogenous addition of metabolites directly linked to glutamine metabolism is not able to substitute for glutamine. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1 under indicated conditions: w/o gln - in absence of glutamine, subst – T cells were treated with GSH (2 mM), glutamate (1 mM), α-ketoglutarate (1 mM), hypoxanthine (100 µM) and thymidine (16 µM). (A) Expression of CD25 was determined after 48 hours by antibody staining and subsequent analysis by flow cytometry, shown is the median fluorescence intensity (MFI). Data are normalized to control. (B) Cell diameter of unstimulated (quies) and stimulated T cells was determined after 48 hours by the CASY system. (C) IFNγ levels were determined in culture supernatants after 48 hours by ELISA. (A, B, C) Data are shown as median and (A, C) normalized to control (n=3). Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (**p<0.01, ***p<0.001).

Other potential amino group donors are arginine and asparagine. Both amino acids administered at a concentration of 2 mM were not able to compensate for glutamine deprivation (data not shown). Additionally, according to a study with murine T cells (Carr et al. 2010), the amino acids aspartate and low levels of glutamate (2 mM) alone were tested, which were also not able to replace glutamine (data not shown). However, we have to admit, that the uptake of those metabolites might be limited and thus the supplementation is not efficient. To test this notion, higher concentrations of glutamate were tested. Indeed, first results indicate that a high concentration of glutamate (20 mM) is partially able to compensate for glutamine. Although the applied concentration of 20 mM glutamate seems not to be reachable under physiological conditions, this is the first time showing that T cell function is not completely blocked in the absence of glutamine. However, this warrants further investigations.

Taken together, these results indicate that glutamine itself is essential for T cell activation and is hardly replaceable by its degradation products.

In parallel with this project, myeloid cells were cultured under glutamine deprived conditions (performed by R. Schöppe in line with a medical doctoral thesis). Monocytes and macrophages were unaffected by glutamine deprivation (data not shown), indicating that myeloid cells are able to substitute for glutamine. A possible explanation why macrophages are not affected by glutamine deprivation might be their high expression of glutamine synthetase (GLUL), an enzyme converting glutamate and ammonia to glutamine (Figure 2). In contrast to macrophages, neither bulk, nor naïve or EM CD8 T cells did express this enzyme, even after a longer period of stimulation (Figure 24). Furthermore, the expression of the enzyme could not be elevated by glutamine deprivation as observed in myeloid cells (data not shown). These data indicate that glutamine sensitivity of CD8 T cells might be attributed to the lack of GLUL and thereby the inability to synthesize glutamine.

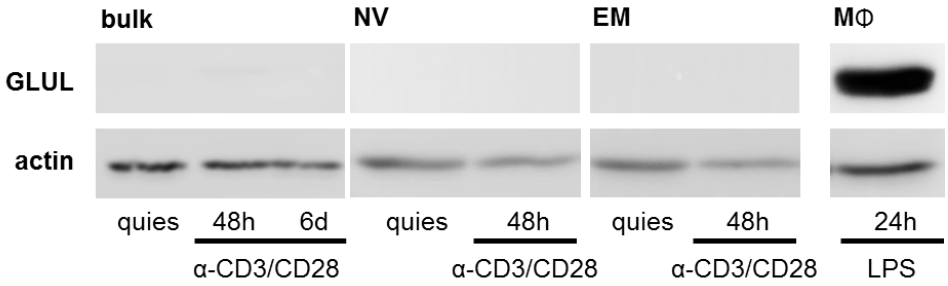

Figure 24: GLUL is expressed in myeloid cells, but not in CD8 T cells. T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Immunoblot analyses of GLUL in quiescent and stimulated CD8 T cells after 48 hours are shown. Macrophages (MΦ) were used as positive control. Actin was used as loading control. One representative experiment is shown.

Therefore, artificial overexpression of GLUL might be a strategy to support T cell function in a tumor microenvironment characterized by low glutamine concentrations. This approach is currently under investigation.

3.4. Anti-metabolic targeting to restore immune cell effector functions of human CD8 T cells in the tumor microenvironment

Tumor cells are addicted to glutamine, suggesting glutamine metabolism as a possible therapeutic target. Compounds targeting glutamine metabolism have shown promise in preclinical trials (Altman et al. 2016). However, glutamine is also an essential amino acid during T cell stimulation. The supplementation of glutamine-derived metabolites was not able to

substitute for glutamine. Due to these facts, the application of glutamine analogs or drugs blocking glutamine metabolism as currently investigated in the context of cancer therapy could lead to a strong block in the T cell mediated anti-tumor immune response. Therefore, in the next chapter the impact of such drugs was analyzed.

Besides nutrient restriction within the tumor microenvironment immune cells have to deal with the accumulation of waste metabolites released by tumor cells. The highly glycolytic phenotype of tumor cells leads not only to reduced glucose levels, but also to an accumulation of lactic acid. Lactic acid strongly suppresses the function and viability of T cells (Fischer et al. 2007). In line, we demonstrated that reduction of tumor derived lactic acid by down-regulation of LDHA improved T and NK cell function and thereby immunosurveillance and tumor growth control (Brand et al. 2016). Since lactic acid is known as immunosuppressive metabolite, the reduction of the lactic acid secretion might restore tumor-directed immune response. Therefore, the application of anti-glycolytic drugs could be a promising strategy to strengthen the endogenous anti-tumor immune response, but also the response to immunotherapeutic approaches. As in this study in some donors an impact of glucose deprivation on cytokine secretion of CD8 T cells was observed, especially in the naïve subset, adverse effects of such drugs need to be carefully investigated.

3.4.1. Pharmacologic blockade of glutamine metabolism in human CD8 T cells

In light of our data on the importance of glutamine for T cell activation, the application of drugs inhibiting glutamine metabolism for the treatment of cancer might be a double edged sword. To investigate the impact of such inhibitors, CD8 T cells were treated with the two glutamine analogues acivicin and DON in comparison to two inhibitors specifically blocking glutaminase. As acivicin and DON are analogs of glutamine they inhibit all glutamine utilizing enzymes. Due to their structural similarity to glutamine those two substances enter the catalytic center of glutamine utilizing enzymes and inhibit by covalent binding, therefore those two drugs can be regarded as pan-inhibitors. For DON an IC_{50} value of 235 µM was proposed in fibroblasts (Crosby et al. 2015), but it was shown to have a strong inhibitory effect already at a concentration of 5 µM on glutaminase activity in rat CD4 T cells (Lee et al. 2015). For acivicin an inactivation of the glutamine dependent aldehyde dehydrogenase was shown at a concentration of 5.4 µM, which inhibited growth of human hepatocyte carcinoma cell line. In this study 0.2 and 0.4 µM for acivicin and 5 and 10 µM for DON were supplemented. In comparison to the two pan-inhibitors, CD8 T cells were treated with two inhibitors specifically targeting the transformation of

glutamine to glutamate. BPTES and CB-839 were used as specific glutaminase inhibitors (Gross et al. 2014). BPTES as well as CB-839 were shown to have an impact on cell growth and viability in various tumor cell lines *in vitro* and *in vivo* (Gross et al. 2014; Xiang et al. 2015; Le et al. 2012; Nagana Gowda et al. 2018). An IC_{50} value of 3.3 µM was shown for BPTES in P493 human lymphoma B cells (Shukla et al. 2012) and between 25 and 35 µM for CD4 T cells. IC_{50} values for glutaminase inhibition by CB-839 were shown to be lower than 50 nM (Gross et al. 2014). To ensure an inhibitory effect of both substances, higher concentrations (BPTES: 25 and 50 µM, CB-839: 0.1 and 1.0 µM) were applied.

Similar to the results obtained for glutamine deprivation, DON and acivicin reduced the expression of activation related surface markers as well as blocked on-blast formation, indicating that T cell activation is inhibited (Table 7).

Table 7: Impact of pan-inhibition of glutamine metabolism in comparison to glutaminase inhibition on T cell function.

	ctrl	acivicin [µM]		DON [µM]		CB-839 [µM]		BPTES [µM]	
		0.2	0.4	5	10	0.1	1.0	25	50
diameter [µm]	11.3 ±0.2	9.1	9.2	9.8** ±0.1	9.3*** ±0.0	11.3 ±0.5	11.2 ±0.5	10.1* ±0.2	10.2* ±0.1
CD25 [MFI]	299 ±14	58.9	40.2	325 ±72	141 ±48	338 ±81	323 ±77	294 ±114	277 ±125
IFNγ [pg/ml]	1945 ±262	607	486	866* ±231	593** ±108	1827 ±153	2333 ±174	1127 ±283	1166 ±317
TNF [pg/ml]	2354 ±407	637	659	1252 ±189	1066 ±266	2130 ±502	2124 ±367	2576 ±100	3464 ±466
viability 72h [%]	76 ±4	93	93	62 ±10	62 ±12	79 ±3	80 ±5	66 ±5	65 ±5
viability 6d [%]	71 ±6	74	75	43 ±14	39 ±15	76 ±7	79 ±6	68 ±6	66 ±10
cell no [x 10^6/ml]	4.0 ±0	0.4	0.4	0.6*** ±0.1	0.4*** ±0.1	4.1 ±0.5	4.1 ±0.5	2.9 ±0.8	3.3 ±0.5

CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Inhibitors were applied at the following concentrations: 0.1 or 1.0 µM CB-839, 25 or 50 µM BPTES, 10 or 25 µM DON and 0.2 or 0.4 µM acivicin. The surface expression of the activation related surface marker CD25 was determined after 48 hours by antibody staining and subsequent analysis by flow cytometry, shown is the median fluorescence intensity (MFI). Cell diameter after 48 hours and proliferation after 72 hours and 6 days were determined by the CASY system. Cytokine levels were determined in culture supernatants after 48 hours by ELISA. Data are shown as mean±SEM. Asterisks show significant differences between control and treatment (n=2 for acivicin, n=3 for DON, CB-839 and BPTES). Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

Moreover, both inhibitors induced proliferation arrest, reduced or even blocked cellular respiration (Figure 25 A, B) and decreased cytokine secretion by more than 50 %. Viability was not affected within the first 72 hours. In contrast to acivicin, DON induced cell death after 72 hours by trend and an even stronger effect was observed after 6 days. Taken together, the application of the two pan-inhibitors resembled the phenotype observed under glutamine deprived conditions. As glutamine deprivation did not affect viability of T cells, these results point towards an unspecific effect of DON.

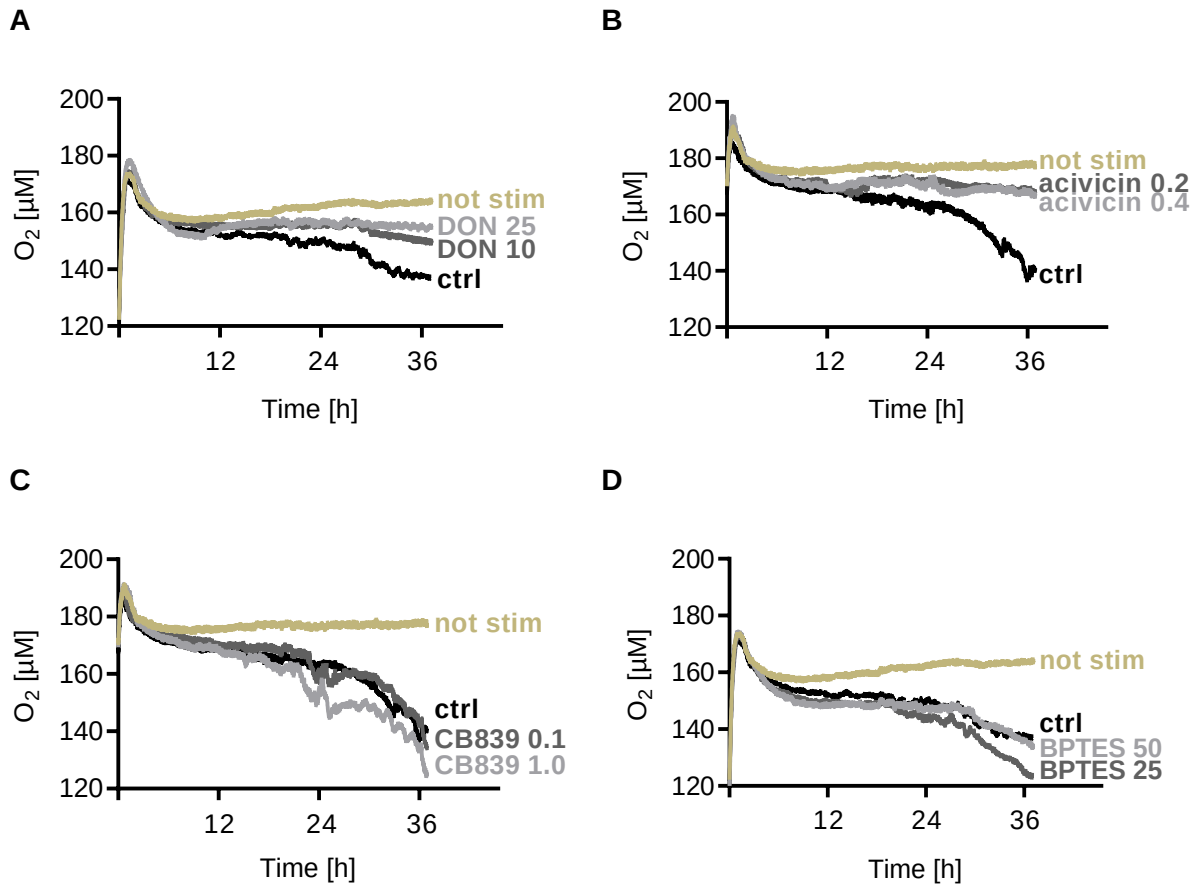

Figure 25: Impact of pan-inhibition on glutamine metabolism in comparison to specific glutaminase inhibition on respiration. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Cellular respiration was measured with the PreSens technology in freshly stimulated cells at a concentration of 0.8 x 10^6 cells per ml under cell culture conditions (n=2). Inhibitors were applied at the following concentrations: (A) 5 and 10 µM DON or (B) 0.2 and 0.4 µM acivicin and (C) 0.1 and 1.0 µM CB-839 or (D) 25 and 50 µM BPTES.

In contrast to the pan-inhibition, the application of the glutaminase inhibitors had no impact on CD8 T cell function (Table 7). Neither BPTES nor CB-839 reduced the expression of the activation related surface marker CD25 or affected cytokine production in CD8 T cells (Table 7).

Moreover, the application of 50 µM BPTES led to a slightly increased TNF secretion. Viability, proliferation and activation state of CD8 T cells were not affected by glutaminase inhibition. Contrary to expectations, not even cellular respiration was reduced (Figure 25 C, D).

Taken together, the application of glutamine analogs has a strong negative impact on T cell function. However, glutaminase inhibitors did not affect T cells. Either glutaminase is not essential for a T cell response or the inhibitors do not enter the cells. As high concentrations of glutamate partially rescued T cell activation, an involvement of glutaminase in T cell activation cannot be excluded. Taken together, BPTES and CB-839 inhibitors might display two promising drugs in the context of cancer therapy.

3.4.2. Pharmacological blockade of glucose metabolism by MCT inhibitors - Impact of NSAIDs on the function of human CD8 T cell subsets in the early phase of activation

Previously, the non-steroidal anti-inflammatory drug (NSAID) diclofenac was shown to be capable to block lactate secretion in a variety of tumor cell lines and is capable to reduce *in vivo* tumor growth of B16 mouse melanoma cells (Gottfried et al. 2013, Renner et al. under revision). Further analysis revealed that diclofenac and lumiracoxib, which shows a very high structural similarity, directly block MCT1 and MCT4 (Renner et al. under revision). Diclofenac has K_i of 1.45 µM for MCT1 and 0.14 µM for MCT4 (Renner et al. under revision). In line, lumiracoxib displaying a K_i value of 4.15 µM for MCT1 and 1.12 µM for MCT4 blocks lactate secretion in different melanoma cell lines (Brummer et al. 2019). Therefore, both NSAIDs might be promising drugs in the context of ACT.

In order to elucidate whether there are subset specific effects, the impact of diclofenac and lumiracoxib was analyzed in CD8 T cell subsets. These analyses allowed the determination of the most promising subset for immunotherapy in combination with anti-metabolic drugs.

While quiescent human CD8 T cells express very low levels of MCT1 and MCT4, both transporters are upregulated upon stimulation (Renner et al. under revision). As the first stimulation represents a primary immune response, the following experiments were performed to identify the impact of MCT inhibition on the efficacy of primary activation. Within the first 48 hours of stimulation, diclofenac as well as lumiracoxib inhibited glycolysis. Both substances

reduced glucose consumption and significantly lactic acid secretion in naïve and EM CD8 T cells (Figure 26).

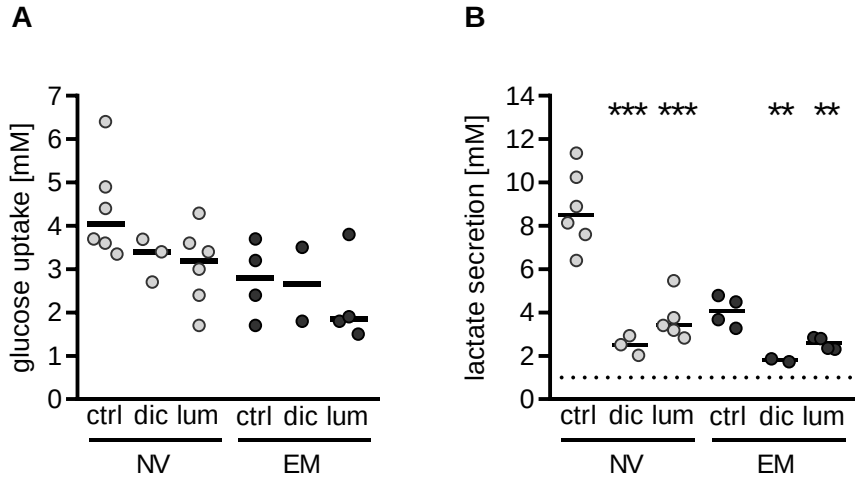

Figure 26: Impact of NSAIDs on glycolytic activity of CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Each symbol represents an individual donor. Horizontal lines indicate the median. Inhibitors were applied at the following concentrations: 0.2 mM lumiracoxib (lum) and 0.2 mM diclofenac (dic). (A) Glucose and (B) lactic acid levels were measured enzymatically in culture supernatants after 48 hours of stimulation (n≥2). Dotted line in (B) indicates lactate level in culture medium. Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

In contrast to glycolytic activity, on-blast formation was not affected in naïve or EM T cells (Figure 27 A). Long-term diclofenac treatment led to a decreased proliferation in naïve T cells, and induced a proliferation arrest in EM T cells (Figure 27 B). Surprisingly, although lumiracoxib reduced lactate secretion in naïve T cells, the proliferation was not affected. In the EM subset, the negative impact of lumiracoxib on proliferation was distinct, but not significant. These results might be explained with the lower K_i values of diclofenac for both MCTs, also reflected in a stronger impact on lactate secretion (Figure 26 B). Regarding cytokine production, NSAID treatment had no significant impact on IFNγ and TNF secretion. Nevertheless, naïve T cells produced less IFNγ in the presence of lumiracoxib by trend.

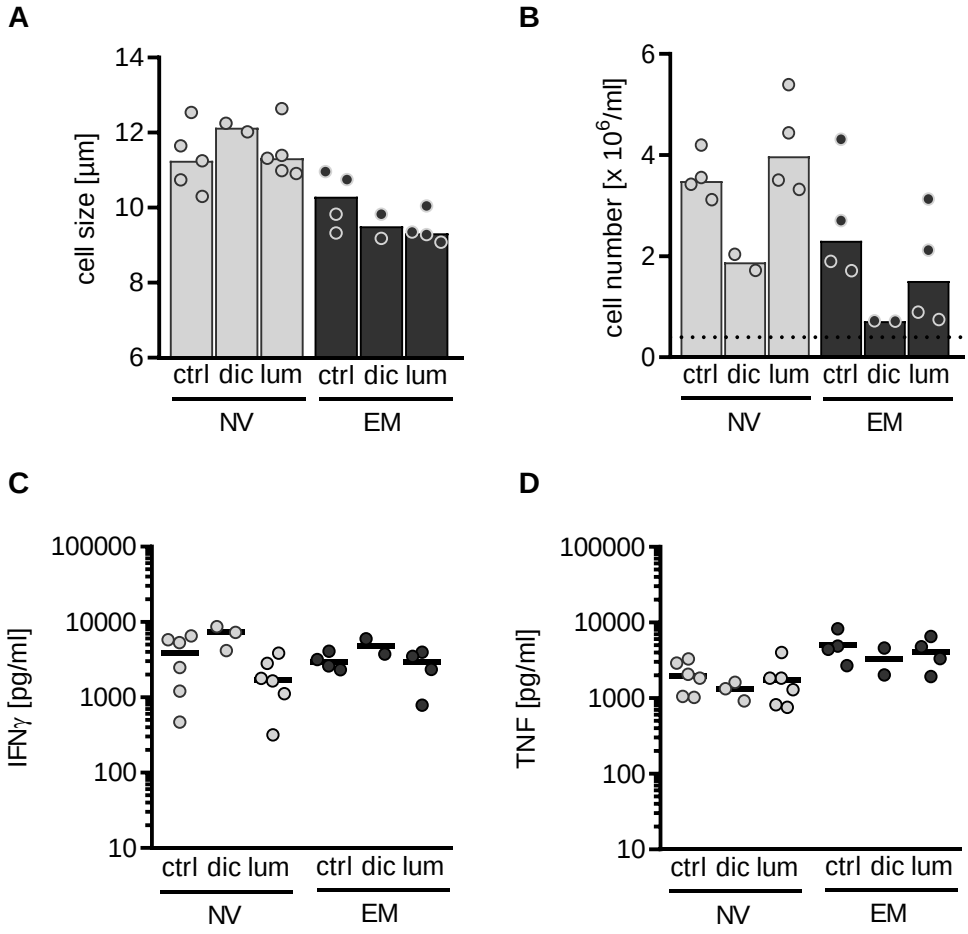

Figure 27: Impact of NSAIDs on T cell function in CD8 T cell subsets. CD8 T cells were stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Inhibitors were applied at the following concentrations: 0.2 mM diclofenac (dic) and 0.2 mM lumiracoxib (lum). (A) Cell diameter were determined after 48 hours and (B) proliferation after 6 days by the CASY system (n=2 for diclofenac, n≥4 for lumiracoxib). (C) IFNγ and (D) TNF levels were determined in culture supernatants after 48 hours by ELISA. Each symbol represents an individual donor. Horizontal lines indicate the median. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test.

Taken together, the NSAIDs diclofenac and lumiracoxib exerted a significant impact on glucose metabolism in both subsets. However, cytokine secretion was preserved and CD8 T cells still proliferated at least to some extent in the course of primary stimulation.

Both NSAIDs did not completely block lactate secretion most likely due to a remaining activity of MCT1 and MCT4. Thus, the impact of a complete blockade of either transporter was investigated in the next set of experiments.

3.5. Impact of MCT4 deficiency on T cell function

Quiescent human T cells show very low levels of MCT1 and MCT4, however, expression of both MCTs is strongly increased after stimulation (Renner et al. under revision). The role of MCT1 has been investigated in the murine system applying a specific MCT1/2 inhibitor. Blocking lactate transport by this inhibitor reduced lactate secretion and in line proliferation of human T cells (Murray et al. 2005). Data of our group show a minor and transient effect of a MCT1/2 inhibition in human T cells due to an increased MCT4 expression in the course of activation. The impact of a MCT4 blockade alone or even a complete blockade of MCT1 and MCT4 was not elucidated so far. Unfortunately, there is no MCT4 inhibitor commercially available. Nevertheless a MCT4 knock out mouse model is available (kindly provided by Prof. John L. Cleveland) and combining the MCT1/2 inhibitor to T cells from these knockout mice mimics a complete blockade of both transporters. As there is little known about the role of MCT4 for immune cell development and function, basic characteristics of these mice with special focus on T cells were investigated.

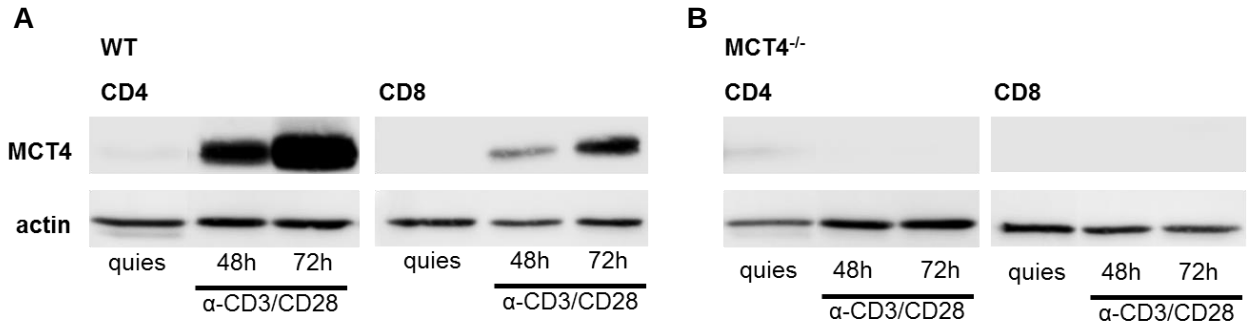

Figure 28: Immunoblot analysis of MCT4 expression in murine CD4 and CD8 T cells. T cells were stimulated with plate-bound α-CD3 (5 µg/ml) and soluble α-CD28 (1 µg/ml). Expression level of MCT4 from (A) wildtyp (WT) and (B) MCT4 knockout mice ($MCT4^{-/-}$) in quiescent (quies) and stimulated CD4 and CD8 T cells after 48 and 72 hours is shown. Actin was used as loading control. One representative experiment is shown.

In a first step, the MCT4 expression in WT and MCT4 deficient T cells was examined to confirm the genetic knock out. Protein levels were quantified in quiescent and activated murine T cells (Figure 28). In quiescent WT CD4 as well as in CD8 T cells MCT4 was not expressed but expression increased upon stimulation (Figure 28 A). As expected, MCT4 expression was not detectable in T cells isolated from spleens from $MCT4^{-/-}$ mice, even after 72 hours of stimulation (Figure 28 B).

3.5.1. Immune cell composition in spleens from wildtyp and MCT4 deficient mice

The MCT4$^{-/-}$ mice developed phenotypically normal. To analyze the immune cell composition splenocytes were isolated from healthy mice and were analyzed by flow cytometry (see Figure 29 A, C for gating strategies). The percentage of B cells (CD19^{+}), myeloid cells (CD11b^{+}), NK (CD3ε^{-}, NK1.1^{+}) and NKT (CD3ε^{+}, NK1.1^{+}) cells relative to levels of total living cells was determined. The level of B cells was significantly lowered and the percentage of myeloid cells by trend in MCT4$^{-/-}$ mice. While numbers of B cells and myeloid cells were reduced, the NK and NKT cell compartment showed a higher frequency in spleens of MCT4$^{-/-}$ mice (Figure 29 B). Moreover, a greater proportion of T cells (CD3ε^{+}) was detected in MCT4$^{-/-}$ mice among entire living cell, reflected in increased percentage of CD4 (CD3ε^{+}, CD4^{+}) and CD8 (CD3ε^{+}, CD8α^{+}) T cells. However, the CD4 to CD8 ratio was not altered by MCT4 deficiency (Figure 29 E).

Taken together, the immune cell composition in splenocytes of MCT4$^{-/-}$ mice was altered in some aspects; however, the observed differences were not strongly pronounced.

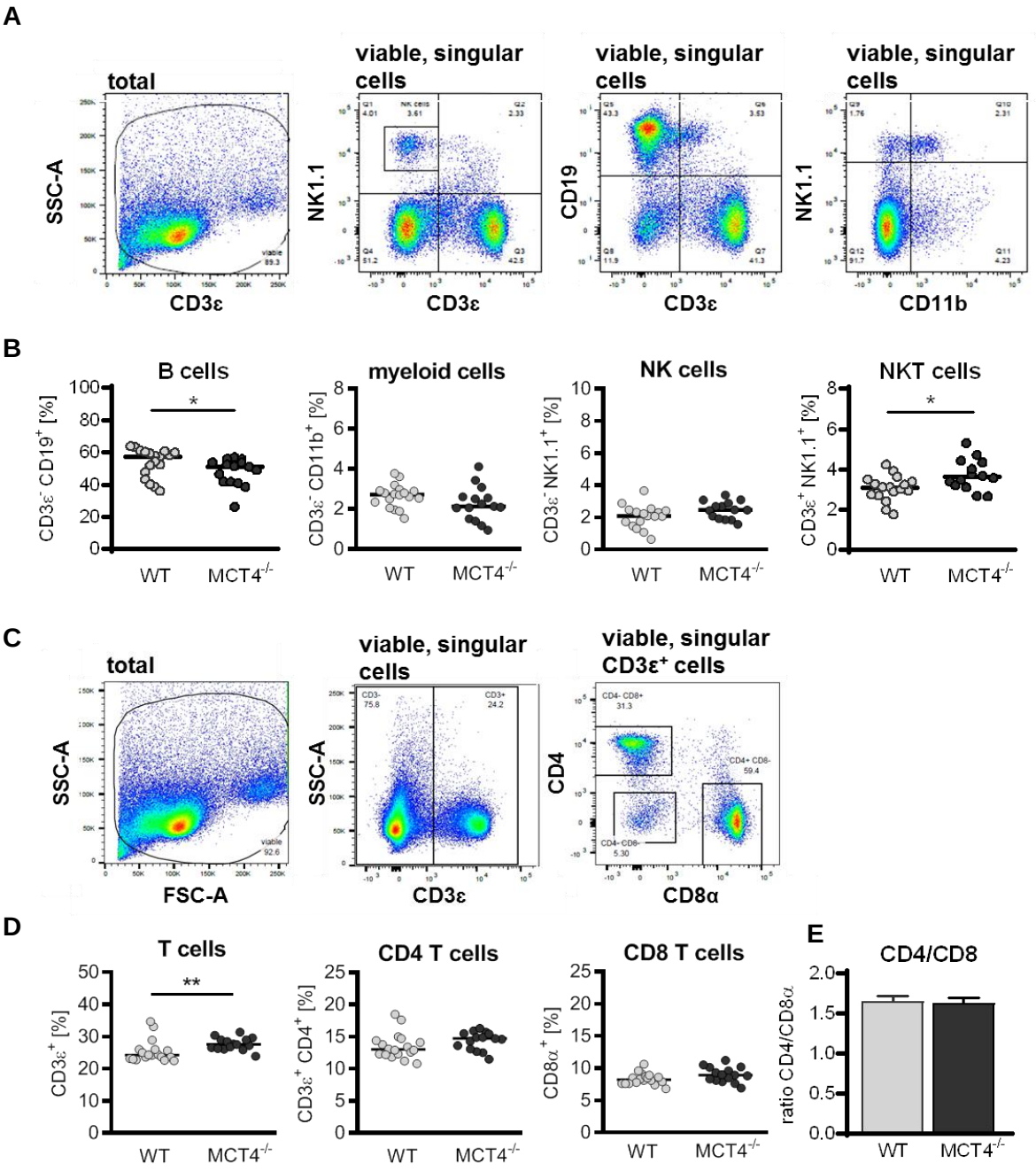

Figure 29: MCT4 deficiency does moderately alter immune cell composition in spleens. (A) Gating strategy for flow cytometry analysis of living singular cells (FSC-W^{low}), B cells (CD3ε^{-}, CD19^{+}), myeloid cells (CD3ε^{-}, CD11b^{+}), NK cells (CD3ε^{-}, NK1.1^{+}), and NKT cells (CD3ε^{+}, NK1.1^{+}) in spleens from WT and MCT4$^{-/-}$ mice. One representative WT spleen is shown. (B) Percentage of immune cell populations within living cells: B cells, myeloid cells, NK and NKT cells isolated from spleens from WT (light grey) and MCT4$^{-/-}$ (dark grey) mice. Each symbol represents cells from an individual mouse; horizontal lines indicate the median. (C) Gating strategy for flow cytometry analysis of living singular cells (FSC-W^{low}): T cells (CD3ε^{+}), CD4 T cells (CD3ε^{+}, CD4^{+}) and CD8 T cells (CD3ε^{+}, CD8α^{+}) in spleens from WT and MCT4$^{-/-}$ mice. One representative WT spleen is shown. (D) Percentage of immune cell populations within living cells: T cells, CD4 and CD8 T cells. Each symbol represents an individual mouse; horizontal lines indicate the median. (E) CD4/CD8 ratio in spleens from WT and MCT4$^{-/-}$ mice. Asterisks show significant differences. Significance was determined by Mann-Whitney test (*p<0.05, **p<0.01).

3.5.2. Metabolic and functional characterization of murine MCT4$^{-/-}$ CD4 and CD8 T cells

Next, CD4 and CD8 T cells were isolated from spleens of healthy WT and MCT4$^{-/-}$ mice and stimulated with α-CD3 and α-CD28 antibodies. Glucose metabolism, respiration as well as T cell function were investigated.

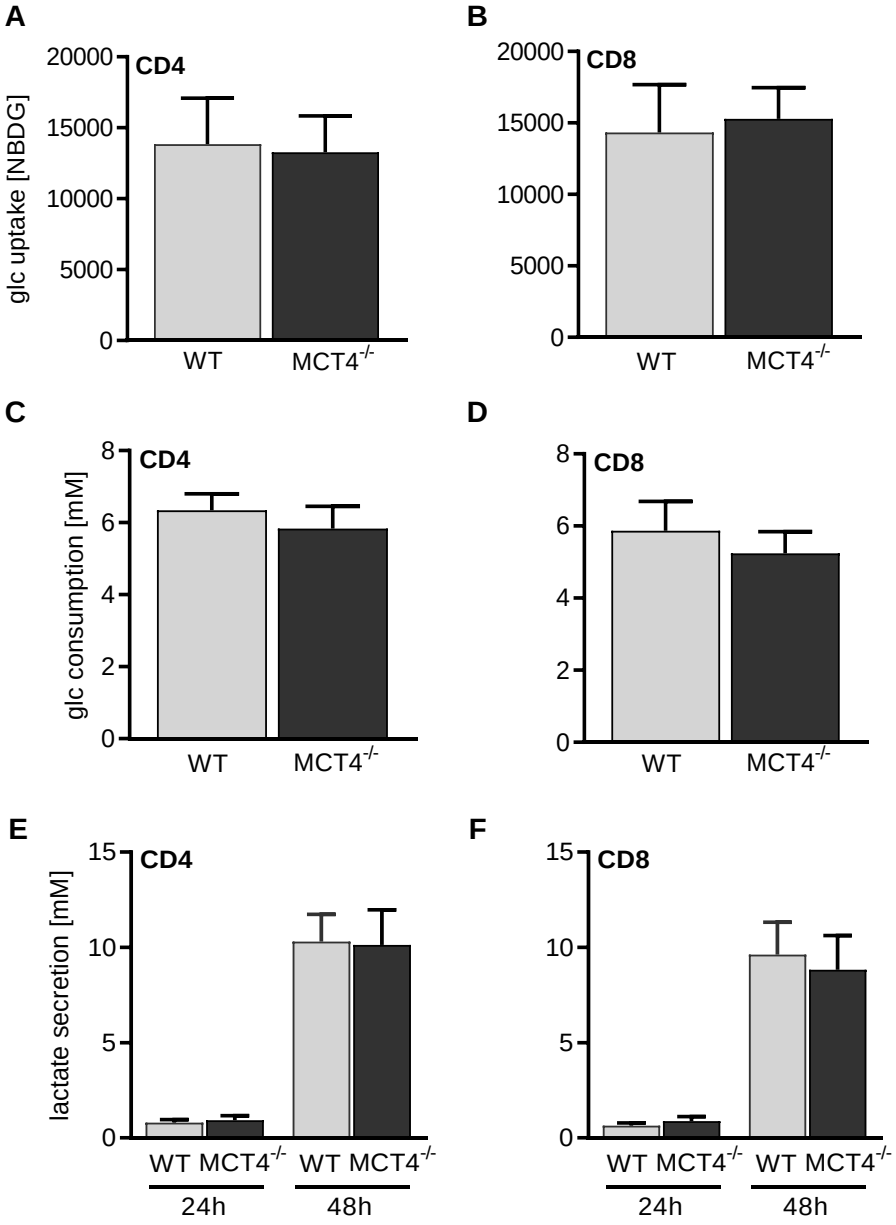

Figure 30: MCT4 deficiency has no impact on glycolytic activity in CD4 and CD8 T cells. T cells were stimulated with plate-bound α-CD3 (5 µg/ml) and soluble α-CD28 (1 µg/ml). (A) CD4 and (B) CD8 T cells were stimulated for 2 hours and incubated with 2-NBDG, followed by subsequent analysis by flow cytometry (n=3, mean+SEM). (C, D) Glucose and (E, F) lactate concentration were measured enzymatically in culture supernatants of (C, E) CD4 and (D, F) CD8 T cells after 48 hours of stimulation (n≥8, mean+SEM). Significance was determined by Mann-Whitney test.

Early glycolytic activity was investigated by measuring the incorporation of the fluorescent glucose analogue 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) by flow cytometry. Neither in CD4 nor in CD8 T cells, MCT4 deficiency showed an effect on glucose uptake immediately after stimulation (Figure 30 A, B). To quantify the long-term effect of MCT4 deficiency on glucose metabolism, glucose and lactate concentrations were measured in the supernatants after 48 hours of stimulation. Interestingly, although the MCT4 level is strongly increased in WT cells after 48 hours (Figure 28 A), there was no difference in glucose uptake (Figure 30 C, D) or lactate secretion (Figure 30 E, F) observed in WT and MCT4$^{-/-}$ T cells.

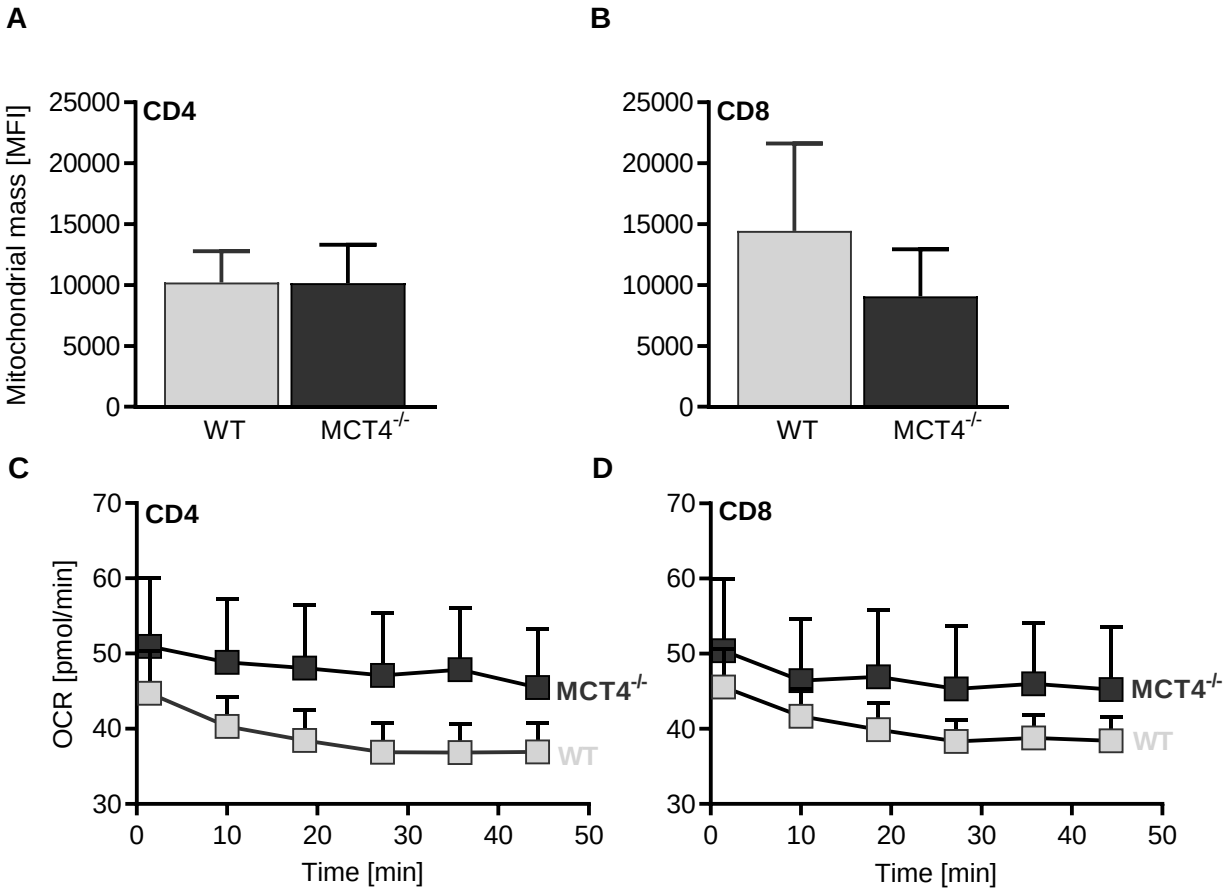

Figure 31: MCT4 deficiency does not affect mitochondrial mass, but has an impact on respiration. Mitochondrial mass of (A) CD4 and (B) CD8 T cells was determined by MitoTracker Green staining and subsequent analysis by flow cytometry (n=6, mean+SEM). Oxygen consumption rate was measured in (C) CD4 and (D) CD8 T cells stimulated with plate-bound α-CD3 (5 µg/ml) and soluble α-CD28 (1 µg/ml) for 2 hours, using the Seahorse Technology, in the presence of 2 % serum (n=3, mean+SEM). Significance was determined by Mann-Whitney test.

Since MCT4 deficiency could impact other pathways than glycolysis, we examined mitochondrial mass and activity. Therefore, mitochondrial mass was analyzed by MitoTracker Green staining and respiration using the Seahorse technology in the presence of 2 % serum (Figure 31). Although WT and MCT4$^{-/-}$ T cells showed a similar mitochondrial content (Figure 31 A, B), MCT4$^{-/-}$ T cells were characterized by a tentatively higher oxygen consumption rate (OCR) than WT cells (Figure 31 C, D).

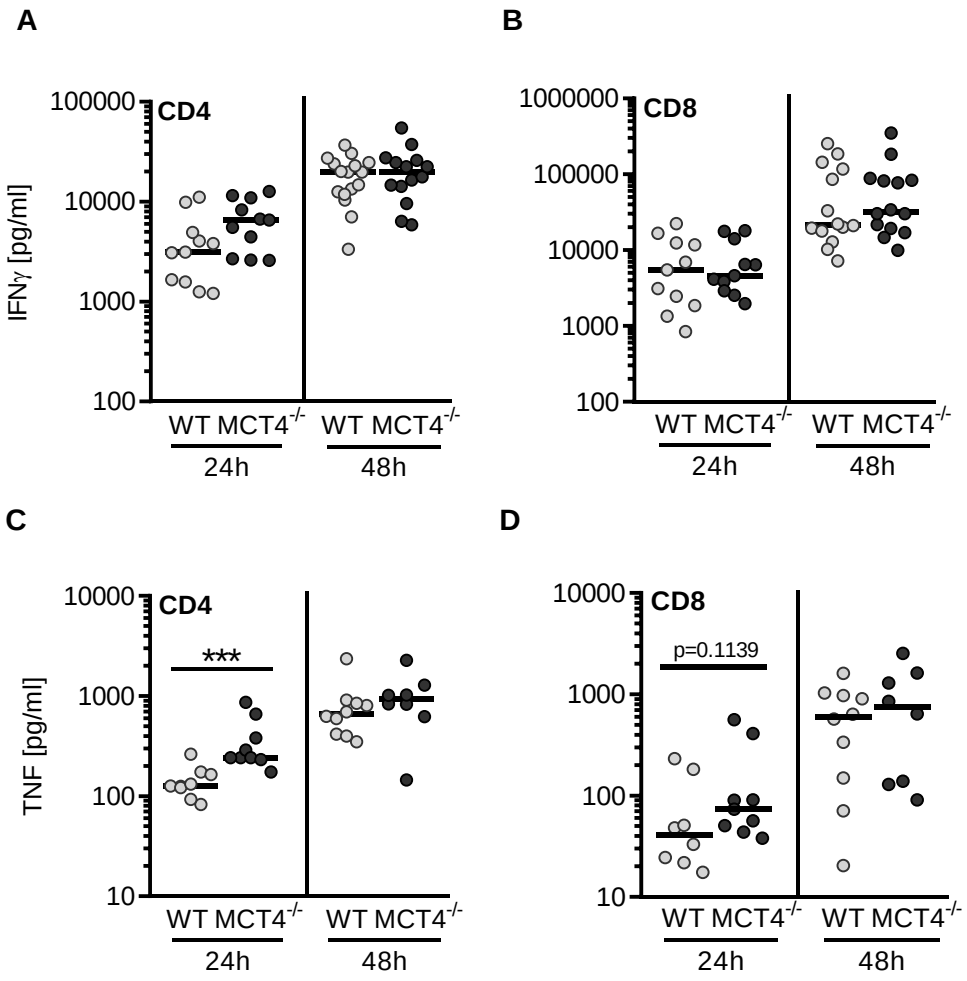

Figure 32: MCT4 deficiency has no impact on cytokine production in CD4 and CD8 T cells. T cells were stimulated with plate-bound α-CD3 (5 µg/ml) and soluble α-CD28 (1 µg/ml). (A, B) IFNγ (n≥11) and (C, D) TNF levels (n≥8) were determined in culture supernatants from (A, C) CD4 and (B, D) CD8 T cells after 24 and 48 hours by ELISA; horizontal lines indicate the median. Asterisks show significant differences. Significance was determined by Mann-Whitney test (***p<0.001).

To investigate whether MCT4 deficiency has a direct impact on effector functions, IFNγ and TNF secretion was measured in supernatants after 24 and 48 hours by ELISA (Figure 32). CD4 as well as CD8 T cells produced already high levels of IFNγ and TNF in the first 24 hours, but concentration in the supernatants was still increased after 48 hours. MCT4 deficiency did not impair cytokine secretion, rather accelerated IFNγ and TNF production at least in CD4 T cells.

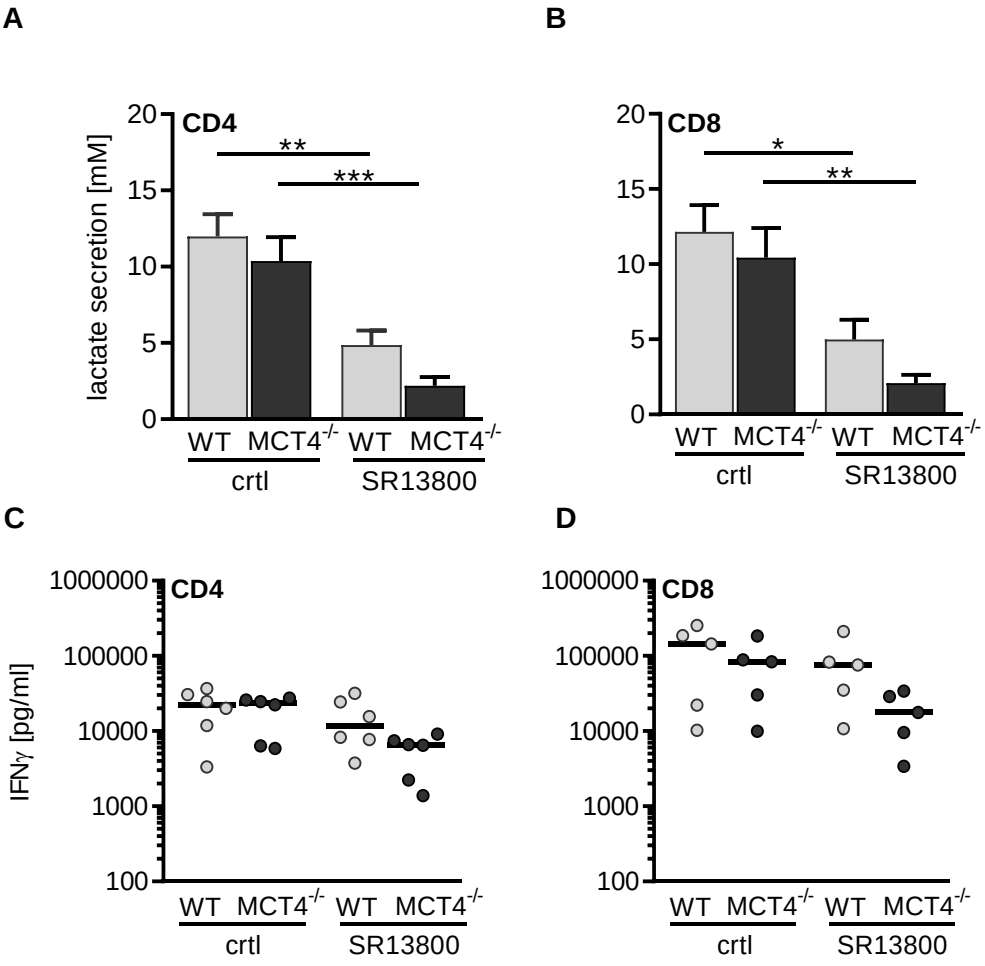

Figure 33: MCT4 deficiency only moderately affects T cell function in CD4 and CD8 T cells, even in combination with MCT1/2 inhibition. T cells were stimulated with plate-bound α-CD3 (5 µg/ml) and soluble α-CD28 (1 µg/ml). SR13800 was applied at a concentration of 1 µM. Lactate levels were measured enzymatically in 48 hour culture supernatants of (A) CD4 and (B) CD8 T cells (n=6, mean+SEM). IFNγ levels were determined after 48 hours in supernatants of stimulated (C) CD4 and (D) CD8 T cells by ELISA (n≥5, median). Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

In comparison to WT T cells, MCT4$^{-/-}$ T cells showed a similar glycolytic activity and preserved effector functions when stimulated *in vitro*. These findings suggest that MCT4 is not essential at least during primary stimulation to maintain effector functions and metabolic steady state conditions in murine T cells. However, whether there is a negative effect *in vivo* needs to be investigated.

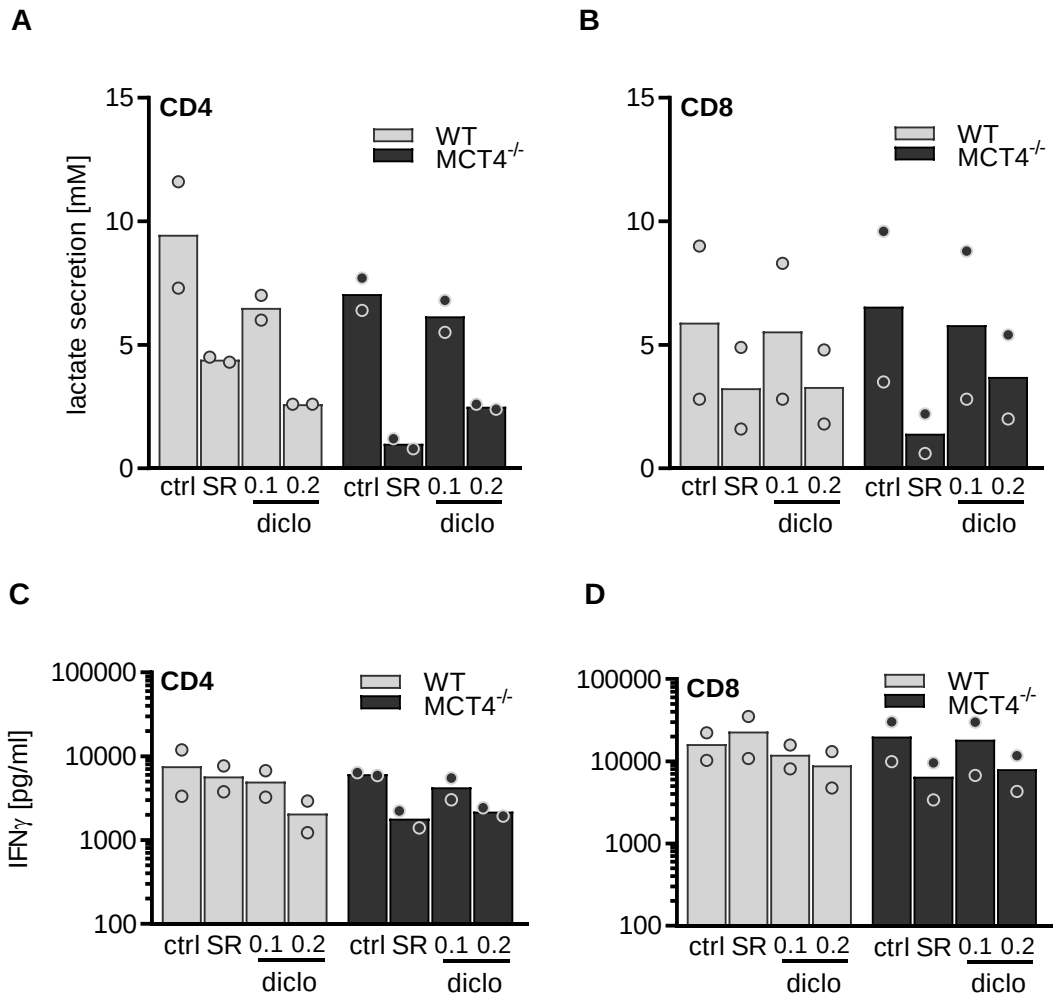

Figure 34: Diclofenac shows a comparable effect on lactate production and IFNγ secretion as a combined blockade of MCT1, MCT2 and MCT4 in CD4 and CD8 T cells. T cells were stimulated with plate-bound α-CD3 (5 µg/ml) and soluble α-CD28 (1 µg/ml). Inhibitors were applied at the following concentrations: 1 µM SR13800 (SR), 0.1 mM and 0.2 mM diclofenac (diclo). Lactate levels were measured enzymatically in 48 hour culture supernatants of (A) CD4 and (B) CD8 T cells. IFNγ levels were determined in supernatants after 48 hours of stimulated (C) CD4 and (D) CD8 T cells by ELISA (n=2, median).

As tumor cells are characterized by the expression of several MCT transporters, anti-metabolic therapy could be optimized using pan-MCT inhibitors, blocking MCT1, MCT2 and MCT4. To mimic a pan-MCT inhibition, $MCT4^{-/-}$ T cells were analyzed in the presence of the MCT1/2 inhibitor SR13800, kindly provided by Prof. John L. Cleveland. WT as well as $MCT4^{-/-}$ T cells showed a strong reduction in lactate release in the presence of 1.0 µM SR13800 after 48 hours of stimulation (Figure 33 A, B).

In the presence of the MCT1/2 inhibitor SR13800 lactate secretion was reduced to about 40 % of control cells of WT CD4 and CD8 T cells. In $MCT4^{-/-}$ CD4 and CD8 T cells, SR13800 application led to a reduction in lactate secretion to about 15 % of control cells. Although both transporters were blocked and lactate release was strongly diminished, $MCT4^{-/-}$ T cells still produced high amounts of IFNγ, although a decrease was detected (Figure 33 C, D).

Finally, the impact of diclofenac targeting MCT1 and MCT4 was analyzed in comparison to the treatment with SR13800 targeting MCT1 and MCT2 in $MCT4^{-/-}$ T cells (Figure 34). High concentrations of diclofenac reduced lactate secretion comparably in WT and $MCT4^{-/-}$ cells. However the impact was slightly lower compared to a complete MCT1 and MCT4 blockade as achieved by the application of the MCT1/2 inhibitor to $MCT4^{-/-}$ T cells. IFNγ secretion was comparably affected by 0.2 mM diclofenac in comparison to a pan-MCT blockade. These data show on the one hand that a pan-MCT inhibition still allows the secretion of important effector cytokines such as IFNγ and on the other hand that diclofenac exerts similar effects as a specific MCT inhibition.

3.6. Sorting through CD8 subsets: Which T cell subset is appropriate for adoptive immunotherapy in combination with anti-metabolic therapy?

In freshly isolated and activated T cells an inhibition of MCTs showed only minor effects, probably due to their low expression. For ACT therapy, an *ex vivo* expansion of modified antigen specific T cells is required, which might also alter the sensitivity of T cells to metabolic inhibition.

3.6.1. Optimizing the expansion protocol of CD8 T cell subsets for adoptive T cell transfer

In a first step, different stimulation/ expansion protocols were evaluated. One objective was to preserve the expression of the homing receptors CCR7 and CD62L in naïve and CM T cells. T cell subpopulations were stimulated either with α-CD3/CD28 beads or in a mixed lymphocyte

reaction (MLR) with allogeneic mDCs in the presence of IL-2 and expanded for two weeks. A MLR represents a more physiological stimulus, compared to activation with α-CD3/CD28 beads. Different interleukin cocktails combining IL-7, IL-15 and IL-21 were tested and viability, overall yield in cell number and capacity to produce important effector cytokines as IFNγ, granzyme B or perforin and the expression of the two homing receptors were examined after two weeks of expansion.

The yield in cell number is an important parameter for choosing the stimulation protocol for an *ex vivo* expansion. The yield after 13 days of expansion was calculated (Figure 35 A, B, C). Surprisingly, in all three subsets the yield was four times higher by stimulation with mDCs compared to stimulation with α-CD3/CD28 beads. The supplementation of IL-7 and IL-15 additionally to IL-2 had no further promoting effect on proliferation of CD8 T cell subsets. Viability was high and comparable between stimulation with α-CD3/CD28 beads or mDCs and additional supplementation of IL-7 and IL-15 had only a slightly positive effect (Figure 35 D, E). The addition of IL-21 to the interleukin cocktail of IL-2, IL-7 and IL-15 did not improve the outcome (data not shown).

CCR7 expression was stable when stimulated with α-CD3/CD28 beads in naïve T cells, but decreased upon cultivation with mDCs (Figure 36). CD62L expression was comparably reduced under both stimulation protocols (Figure 37). The addition of IL-7 or IL-15 did not show a clear positive effect on the preservation of CCR7 or CD62L in the long-term. Particularly, CM T cells lost their CCR7 receptor independent of the stimulation protocol (Figure 36).

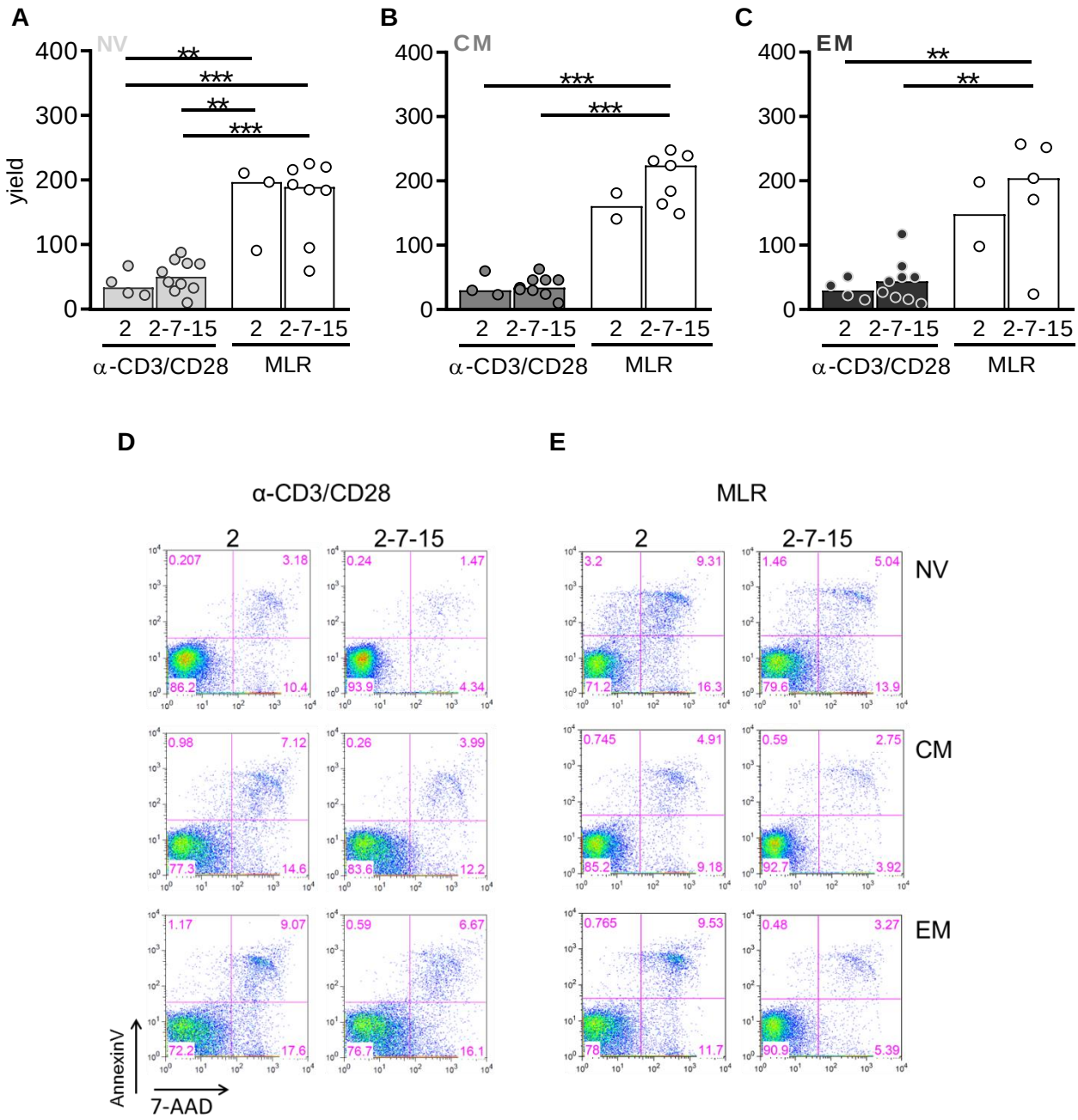

Figure 35: Impact of various stimulation methods on proliferation and viability of CD8 T cell subsets. CD8 T cells were stimulated either with α-CD3/CD28 beads at a cell to bead ratio of 1:1 or in a MLR with mDCs in the presence of IL-2 alone (25 IU/ml) or the interleukin cocktail IL-2 (25 IU/ml), IL-7 (5 ng/ml) and IL-15 (5 ng/ml) for 13 days. Proliferation of (A) naïve, (B) CM and (C) EM CD8 T cells was determined with the CASY system (n≥3, with the exception of CM and EM stimulated in a MLR in the presence of single IL-2: n=2, median). Asterisks show significant differences between indicated conditions. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (**p<0.01, ***p<0.001). (D, E) Viability was measured by Annexin V/ 7-AAD antibody staining and subsequent analysis by flow cytometry. Data from one representative donor are shown.

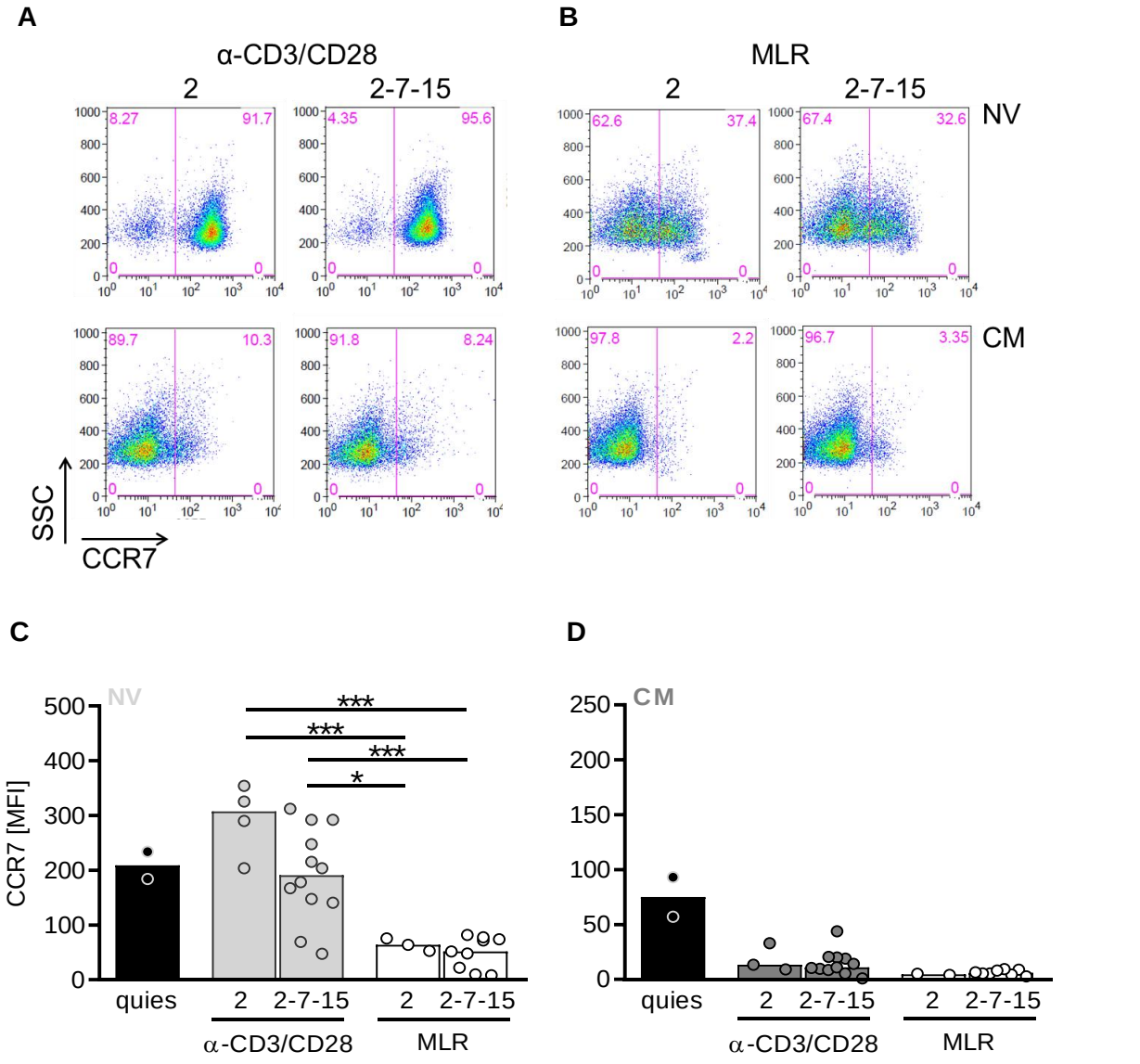

Figure 36: Impact of different stimulation methods on the expression of CCR7 in CD8 T cell subsets. CD8 T cells were stimulated either with α-CD3/CD28 beads at a cell to bead ratio of 1:1 or in a MLR with mDCs in the presence of single IL-2 (25 IU/ml) or the interleukin cocktail IL-2 (25 IU/ml), IL-7 (5 ng/ml) and IL-15 (5 ng/ml). Expression of CCR7 in (A, B, C) naïve and (A, B, D) CM CD8 T cells was measured in quiescent (quies) state and after 13 days of expansion by antibody staining and subsequent analysis by flow cytometry (n≥3, with the exception of CM stimulated in a MLR in the presence of single IL-2: n=2, median). (A, B) One representative donor is shown. Asterisks show significant differences between indicated conditions. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, ***p<0.001).

Regarding CD62L expression, a strong reduction was observed (Figure 37), but nearly one-third was preserved with both stimulation methods. Comparable to the results in naïve cells, the

addition of the different interleukins did not preserve the CCR7 or CD62L expression profile in CM cells during expansion.

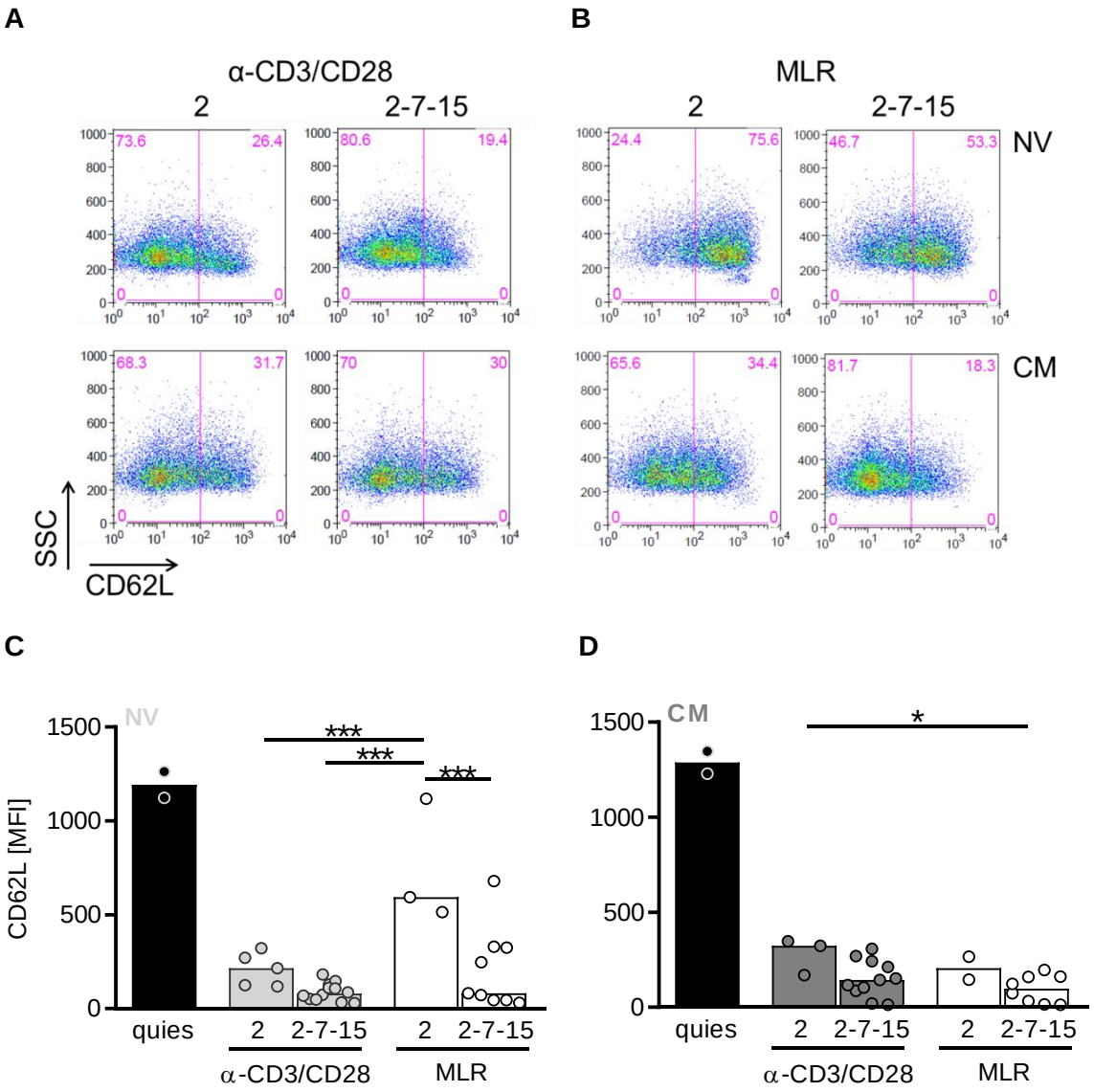

Figure 37: Impact of different stimulation methods on the expression of CD62L in CD8 T cell subsets. CD8 T cells were stimulated either with α-CD3/CD28 beads at a cell to bead ratio of 1:1 or in a MLR with mDCs in the presence of single IL-2 (25 IU/ml) or the interleukin cocktail IL-2 (25 IU/ml), IL-7 (5 ng/ml) and IL-15 (5 ng/ml). Expression of CD62L in (A, B, C) naïve and (A, B, D) CM CD8 T cells was measured in quiescent (quies) state and after 13 days of expansion by antibody staining and subsequent analysis by flow cytometry (n≥3, with the exception of CM stimulated in a MLR in the presence of single IL-2: n=2, median). (A, B) One representative donor is shown. Asterisks show significant differences between indicated conditions. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, ***p<0.001).

As we observed a slightly better cell viability in the presence of the interleukin cocktail IL-2, IL-7 and IL-15 and as in the literature this combination is recommended (Geginat et al. 2003; Cha et al. 2010; Gargett and Brown 2015), the interleukin cocktail IL-2 in combination with IL-7 and IL-15 was used in further experiments. Next the capability to secret IFNγ and to express perforin and granzyme B was analyzed after expansion with α-CD3/CD28 beads or mDCs. T cells were stimulated with PMA/ ionomycin in order to activate T cells to a maximum (Figure 38).

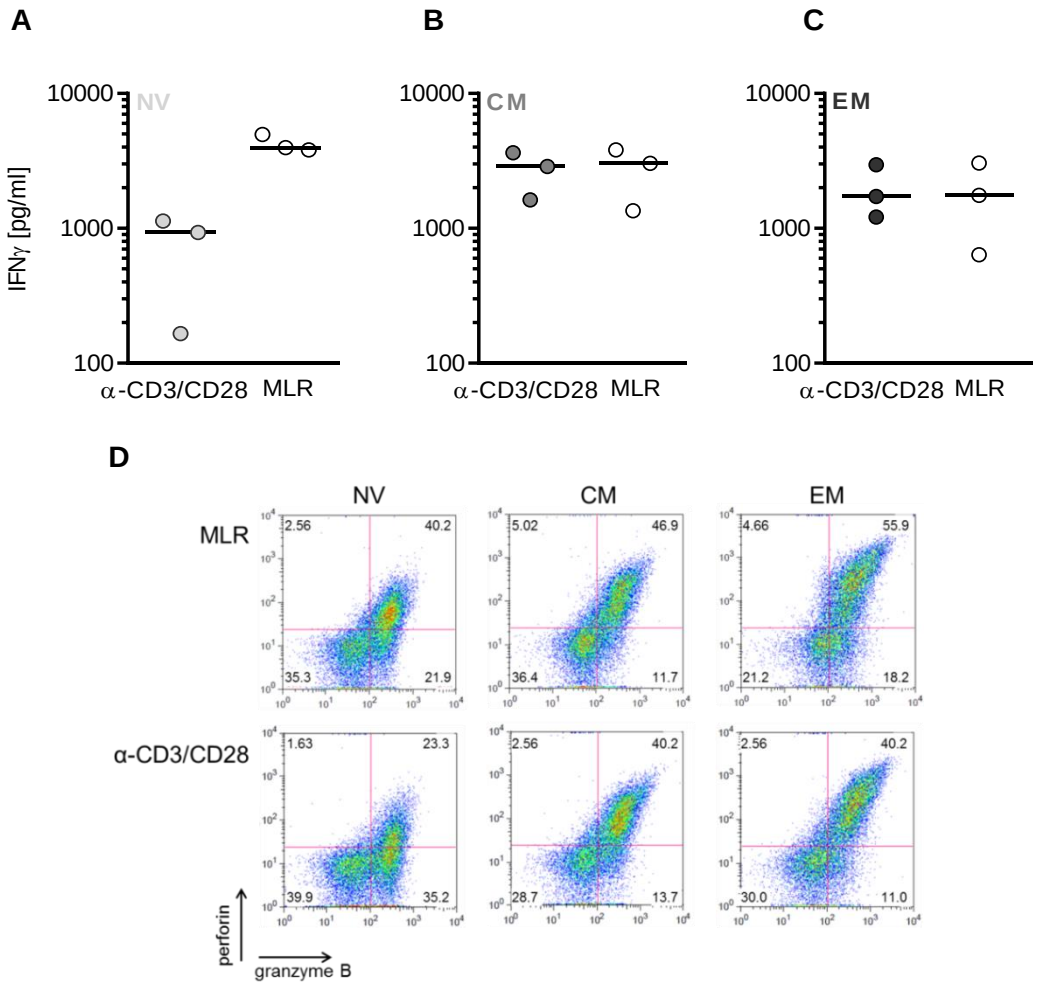

Figure 38: Impact of various stimulation methods on effector functions of CD8 T cell subsets. CD8 T cells were expanded either with α-CD3/CD28 beads at a cell to bead ratio of 1:1 or in a MLR with mDCs in the presence of the interleukin cocktail IL-2 (25 IU/ml), IL-7 (5 ng/ml) and IL-15 (5 ng/ml). IFNγ secretion of expanded (A) naïve, (B) CM and (C) EM CD8 T cells was determined in culture supernatants after 4 hours stimulation with PMA/ionomycin by ELISA. Asterisks show significant differences between indicated conditions (n=3). Horizontal lines indicate the median**.** Significance was determined by Mann-Whitney test. (D) Production of perforin and granzyme B was measured by intracellular antibody staining and subsequent analysis by flow cytometry. Data from one representative donor out of two are shown.

Naïve T cells expanded with mDCs were more potent in producing effector molecules like IFNγ or perforin and granzyme B. CM and EM T cells secreted high amounts of IFNγ as well as perforin and granzyme B, independently which stimulation method was used.

The stimulation with mDCs seemed to be the most promising strategy regarding yield and capability to produce important effector molecules, in particular for naïve T cells. However, stimulation with α-CD3/CD28 beads seemed to be favorable regarding CCR7 expression, particularly in the naïve subset. Due to results obtained in this thesis, expanding T cells in a MLR in combination with the interleukin cocktail IL-2, IL-7 and IL-15 seemed to be favorable and was used in further experiments.

3.6.2. Impact of metabolic targeting on T cell function in expanded human CD8 T cell subsets

The impact of metabolic inhibition on T cell function was analyzed in CD8 T cell subsets (Table 8). After expansion with mDCs, T cells were restimulated with anti-CD3/CD28 to foster the production of effector cytokines. Besides both NSAIDs diclofenac (0.2 mM) and lumiracoxib (0.2 mM), T cell function was examined in the presence of metformin (1.0 and 10.0 mM), the glutaminase inhibitor CB-839 (0.1 and 1.0 µM) and the glutamine analogue DON (5.0 and 10.0 µM). Metformin is an anti-diabetic drug, inhibiting complex I of the respiratory chain and was shown to reduce tumor growth (Owen et al. 2000; Dowling et al. 2007).

Comparable to the results obtained in primary activated CD8 T cells subsets, diclofenac and lumiracoxib had only a slight impact on T cell function in expanded T cell subsets (Table 8). Neither the activation state analyzed by CD25 expression and on-blast formation nor cytokine secretion were affected. However, in long-term both NSAIDs reduced proliferation after 6 days. While proliferation in naïve T cells was not and in CM T cells was only slightly affected, even a proliferation arrest in the EM subset was detected.

For metformin similar results as for NSAIDs were achieved. Activation status as well as cytokine production were not reduced. However, long-term treatment with metformin reduced proliferation after 6 days in all three subsets significantly applying a rather high concentration of metformin. The impact was not as strong applying 1 mM metformin, which rather reflects a concentration achievable under physiologic conditions.

Table 8: Impact of metabolic inhibition on T cell function in CD8 T cell subsets after expansion.

		ctrl	dic [mM]	lum [mM]	met [mM]		DON [µM]		CB-839 [µM]	
			0.2	0.2	1.0	10.0	5.0	10.0	0.1	1.0
cell number [x 10^6/ml]	NV	1.2 ±0.2	0.8 ±0.1	1.0 ±0.0	1.1 ±0.0	0.6* ±0.0	1.1 ±0.1	0.8 ±0.2	1.6 ±0.2	1.5 ±0.2
	CM	2.7 ±0.7	1.0 ±0.2	1.9 ±0.7	1.4 ±0.3	0.7* ±0.0	1.7 ±0.5	1.0 ±0.5	2.7 ±0.6	2.6 ±0.5
	EM	1.8 ±0.2	0.4*** ±0.0	0.6*** ±0.0	0.9** ±0.1	0.4*** ±0.0	1.2 ±0.3	0.7** ±0.3	1.8 ±0.2	1.7 ±0.1
cell diameter [µm]	NV	9.5 ±0.2	9.9 ±0.1	9.7 ±0.1	9.6 ±0.0	9.3 ±0.1	9.4 ±0.0	9.2 ±0.1	9.6 ±0.0	8.5 ±1.0
	CM	10.1 ±0.1	10.0 ±0.1	10.0 ±0.1	9.8 ±0.1	9.6 ±0.1	9.8 ±0.0	9.5* ±0.1	9.9 ±0.2	9.9 ±0.1
	EM	9.0 ±0.0	9.0 ±0.1	9.0 ±0.1	9.0 ±0.1	9.0 ±0.1	8.9 ±0.1	8.9 ±0.0	8.8 ±0.0	8.9 ±0.1
CD25 [MFI]	NV	561 ±73	678 ±170	788 ±38	583 ±100	428 ±7	571 ±58	585 ±93	587 ±18	497 ±50
	CM	669 ±27	763 ±15	777 ±24	650 ±20	625 ±66	794 ±27	609 ±58	673 ±25	665 ±66
	EM	653 ±54	614 ±114	688 ±73	579 ±81	393 ±166	622 ±108	499 ±53	672 ±96	585 ±55
IFNγ [pg/ml]	NV	3640 ±349	4342 ±975	4264 ±462	4359 ±316	6465 ±368	4737 ±1112	3653 ±527	4695 ±859	3984 ±631
	CM	6471 ±822	7737 ±765	6454 ±453	7091 ±910	7795 ±1037	6215 ±1623	6018 ±1348	5642 ±1468	5546 ±1112
	EM	6736 ±1000	7659 ±436	6247 ±751	5750 ±443	7274 ±263	5485 ±332	5570 ±311	6115 ±76	5128 ±291
TNF [pg/ml]	NV	2671 ±479	2149 ±146	2741 ±675	2408 ±554	2653 ±629	3089 ±1156	2224 ±636	2641 ±659	2472 ±363
	CM	5548 ±2096	6326 ±2761	5120 ±1598	5647 ±1838	5239 ±1738	4215 ±1397	4903 ±1442	5151 ±1611	4865 ±1114
	EM	5325 ±2072	5395 ±2120	5527 ±1773	4569 ±1524	4508 ±1571	3472 ±266	3755 ±373	4403 ±487	3931 ±446

CD8 T cells were expanded with mDCs for 13 days and afterwards stimulated with α-CD3/CD28 beads at a cell to bead ratio of 1:1. Inhibitors were applied at the following concentrations: 0.2 mM diclofenac (dic), 0.2 mM lumiracoxib (lum), 1.0 and 10.0 mM metformin (met), 5.0 and 10.0 µM DON as well as 0.1 and 1.0 µM CB-839. After 48 hours cell diameter and after 6 days cell number were determined by the CASY system. Expression of the activation related surface marker CD25 was measured after 48 hours by antibody staining and subsequent analysis by flow cytometry. IFNγ and TNF levels were determined in culture supernatants after 48 hours by ELISA. Data are shown as mean±SEM (n=3). Asterisks show significant differences between control and treatment. Significance was determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001).

Targeting glutamine metabolism, neither DON nor CB-839 had a strong impact on T cell function. Preserved effector functions in the presence of DON is in sharp contrast to results obtained on primary stimulated T cells. There are two possible explanations, either glutamine metabolism is not required in expanded T cells or they become resistant to the inhibitor. To test whether glutamine metabolism is essential also for expanded T cells, CD8 T cells subsets were deprived for glutamine after expansion and restimulated. Glutamine deprivation still strongly reduced IFNγ secretion, in particular in naïve and CM T cells (naïve: 3041 in control T cells to 710 pg/ml under glutamine deprivation, CM: 8086 to 2322 pg/ml, EM: 6708 to 4399 pg/ml), comparable to freshly isolated T cells. Since this experiment was performed only once, these results warrant further investigation. Nevertheless, 10 µM DON reduced on-blast formation in CM after 48 hours and proliferation of EM T cells after six days.

Taken together, anti-metabolic drugs did not impair T cell effector functions in all three subsets after expansion; however, an effect on proliferation was detected. Based on our findings, substances targeting tumor metabolism should be feasible in combination with ACT in all three subsets.

4. Discussion

4.1. Nutrient competition in the tumor microenvironment - does nutrient restriction blunt T cell function?

Accelerated metabolic activity is not only a well-characterized property of tumor cells but also observed in several types of immune cells upon stimulation, including T cells. Both tumor cells and T cells have a high demand for nutrients as glucose, amino acids and fatty acids (Vander Heiden et al. 2009; Lunt and Vander Heiden 2011; Bantug et al. 2018). Thus similar metabolic pathways are activated in tumor cells and T cells. This overlap in the metabolic profile results in nutrient competition in the tumor microenvironment (Uyttenhove et al. 2003; Mellor and Munn 2004; Rodriguez et al. 2009; Srivastava et al. 2010; Munn and Mellor 2013; Chang et al. 2015). Furthermore, the high metabolic activity of tumor cells can lead to nutrient restricted conditions and has thereby a strong impact on immune cell function as several studies demonstrated a direct link between metabolism and T cell fate and function (Pearce et al. 2013; O'Sullivan and Pearce 2015; Patsoukis et al. 2016). In line, Irving and colleagues proposed that metabolic competition is underestimated in the context of immunotherapeutic approaches (Irving et al. 2017).

While in quiescent T cells mitochondrial respiration is regarded as the main energy providing pathway, activated T cells rely on glycolysis, mitochondrial respiration and fatty acid oxidation to cover their metabolic requirements (Bental and Deutsch 1993; Jones and Thompson 2007; Michalek and Rathmell 2010; Pearce 2010; van der Windt and Pearce 2012; MacIver et al. 2013). It is still a matter of debate, which metabolic pathways are crucial for the different aspects of T cell function and whether there are subset specific differences. As CD8 T cells are important for the anti-tumor immune response, the impact of the metabolic tumor microenvironment on their function is of special interest.

4.1.1. Functional and metabolic characteristics of human CD8 T cell subsets

4.1.1.1. Glucose metabolism and T cell function

The link between glucose metabolism and T cell function has been investigated, especially in the murine system. Several publications show that glycolysis is crucial for effector functions and

proliferation of murine T cells (Cham and Gajewski 2005; Chang et al. 2013; Ho et al. 2015; Peng et al. 2016; Siska and Rathmell 2016).

Glucose restriction as well as administration of the non-metabolizable glucose analogue 2-DG, mimicking glucose deprivation by blocking the first step of glycolysis, suppresses IFNγ, but not IL-2 production in murine CD8 T cells (Cham and Gajewski 2005). These results were supported by Jacobs and colleagues who demonstrate that IFNγ production is more sensitive to glucose availability than IL-2 (Jacobs et al. 2008). Besides inhibition of IFNγ production, other effector molecules as perforin, granzymes or TNF are also affected by 2-DG (Cham et al. 2008). However, it has to be taken into consideration that 2-DG is not a specific glycolytic inhibitor as concentrations as high as 10 mM also block mitochondrial respiration (Renner et al. 2015). There are different aspects discussed, how glycolytic activity relates to cytokine secretion. It has been reported that glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme, is bound to the mRNA of IFNγ thereby blocking translation (Nagy and Rigby 1995; Chang et al. 2013). When glycolysis is engaged, GAPDH binding to cytokine mRNA is resolved and translation is initiated. Furthermore, LDHA activity mediating aerobic glycolysis and therefore relieving mitochondrial respiration provides more citrate for the generation of acetyl-coenzyme A which fosters histone acetylation and promotes IFNγ expression (Peng et al. 2016). Another study shows that glucose restriction (0.1 mM glucose) compromises nuclear translocation of NFAT1 in murine CD4 T cells, which is associated with the lack of the glycolytic metabolite phosphoenolpyruvate (Ho et al. 2015). IFNγ as well as IL-2 production is impaired, whereas TGF-β secretion is induced.

Not many studies have investigated the link between metabolism and T cell function in the human system so far. Comparable to murine T cells, human bulk T cells also upregulate their glucose metabolism upon stimulation (Bental and Deutsch 1993; Frauwirth et al. 2002). However, glucose deprivation does not affect IFNγ, TNF or IL-4 production on mRNA or protein level in human bulk CD4 and CD8 T cells (Tripmacher et al. 2008; Renner et al. 2015). A possible explanation might be that human T cells are able to compensate a lack in glucose by increased OXPHOS, denoted as metabolic plasticity (Frauwirth et al. 2002; Tripmacher et al. 2008; Renner et al. 2015).

Only two studies investigated glucose metabolism and mitochondrial function in a subset specific manner in human CD4 and CD8 T cells (Gubser et al. 2013; Dimeloe et al. 2016). Thus, in this study glycolytic activity and the impact of glucose metabolism on T cell function was investigated in CD8 subpopulations.

In a first step, naïve, CM and EM CD8 T cells were purified from healthy donors by fluorescence activated cell sorting and were characterized in terms of functional capabilities. In line with the literature, both memory populations showed a more rapid and higher cytokine production in comparison to naïve T cells (Appay et al. 2008; Mahnke et al. 2013). Especially the rare CM T cell subset was characterized by a high IFNγ and TNF secretion accordingly to other studies (Tussey et al. 2000; Hislop et al. 2001). The rapid recall ability of memory T cells might be explained by epigenome remodeling. Open chromatin marks (H3K4me1, H3K4me3, H3K27ac) at transcriptional start site of IFNγ correlate with increased expression and inducibility of IFNγ in resting memory cell s (Barski et al. 2017). Nevertheless, other reports found that memory subsets are equally efficient in acquiring effector functions (Ravkov et al. 2003; Wherry et al. 2003).

With regard to proliferation, no difference was detected between all three subsets in this study. Although less differentiated CD8 T cell subsets are shown to have a higher proliferative capacity than experienced T cells (Migliaccio et al. 2006), proliferation correlates strongly with cytokine availability, while especially memory T cell proliferation depends on the presence of IL-7 and IL-15 (Geginat et al. 2003).

To investigate the link between T cell function and glycolytic activity, T cells were analyzed under glucose deprived conditions. There is only little known about this link in human CD8 T cell subsets. Gubser and colleagues showed that human EM CD8 T cells display an enhanced extracellular acidification rate regarded as a measure for glycolytic activity in comparison to naïve T cells upon stimulation, although the expression profile of key glycolytic enzymes is similar (Gubser et al. 2013). Glycolytic activity was directly analyzed upon activation with the seahorse technology in fixed cells in the absence of serum and linked to the rapid on-set of cytokine secretion after 12 hours (Gubser et al. 2013).

In our study, glycolytic activity was almost undetectable within the first 24 hours in all three subsets determined by glucose uptake and lactate secretion in non-fixed T cells in the presence of 10 % serum. Nevertheless, cytokine secretion was immediately elevated especially in memory CD8 T cells.

To further elucidate the importance of glucose metabolism for T cell function, CD8 T cell subsets were stimulated in glucose free medium in the presence of 10 % serum (final glucose concentration: 0.4 mM). Viability and activation-associated increase in cell size were not affected by glucose restriction. On average secretion of IFNγ and TNF was only slightly reduced under glucose deprived conditions in all three subpopulations. In line, Gubser and colleagues reported that low concentrations of glucose (0.5 mM) are sufficient to drive IFNγ production in EM CD8

T cells (Gubser et al. 2013). Surprisingly, depending on the donor, cytokine production could be either induced or diminished by glucose deprivation in this study. The variation was most pronounced in the naïve subset.

The controversy on the importance of glycolysis for IFNγ secretion in the literature might be related to donor variations, species differences or different experimental protocols. A major difference in the experimental protocols is the absence of serum or the use of dialyzed serum whereas in this study complete serum was applied. Studies which showed a compromised cytokine secretion or activation in human T cells (Dziurla et al. 2010; Macintyre et al. 2014) were also performed in the presence of dialyzed serum. Recent publications suggest that serum is essential for T cell activation (Ecker et al. 2018; Medvec et al. 2018). Finally, in some studies rather high concentrations of 2-DG (Gubser et al. 2013; Cham and Gajewski 2005) were used which has unspecific side effects as blocking mitochondrial respiration (Renner et al. 2015).

As an important carbon source, glucose is shown to be indispensable for T cell proliferation in the murine (Wang et al. 2011; Chang et al. 2013) and the human system (Tripmacher et al. 2008; Renner et al. 2015). According to the findings in the literature, glucose deprivation caused a block in proliferation in CD8 T cell subsets. Surprisingly, Peng and colleagues showed that LDHA, the enzyme converting pyruvate to lactate to regenerate NAD^+ from NADH, is not crucial for proliferation of murine CD4 T cells (Peng et al. 2016). These findings suggest that not glucose fermentation but glucose metabolism is required for T cell proliferation, which is supported by the fact that murine as well as human Glut1 deficient CD4 T cells show an impaired proliferation (Macintyre et al. 2014),

Taken together, aside from a diminished proliferation, effector functions of CD8 T cell subsets were only partially affected by glucose deprivation. However, a donor specific variation was observed, mainly in naïve cells.

Besides glucose metabolism, oxidative phosphorylation is elevated upon T cell stimulation. Therefore the role of oxidative phosphorylation was analyzed in CD8 T cell subsets.

4.1.1.2. The dependency on mitochondrial respiration is CD8 T cell subset specific

Not only glucose, but also oxygen availability is limited in the tumor microenvironment (Vaupel et al. 2001). Oxidative phosphorylation is shown to be of crucial importance for T cells in the murine system (Chang et al. 2013; Sena et al. 2013; Okoye et al. 2015; Baixauli et al. 2015). An immediate increase in mitochondrial respiration is reported upon T cell activation (Chang et al. 2013; Sena et al. 2013; van der Windt et al. 2013). Blocking OXPHOS by oligomycin

simultaneously with stimulation results in a reduced proliferation, whereas T cells are able to proliferate when oligomycin is administered 24 hours postactivation, indicating that respiration is essential for the on-set of proliferation (Chang et al. 2013).

Beside a general increase in respiration upon activation, subset specific differences have been reported in murine T cells. IL-15 induced memory T cells have more mitochondrial mass and show a higher OCR than naïve T cells (van der Windt et al. 2013). In line, Buck and colleagues reported a distinct mitochondrial structure in murine effector and memory T cells (Buck et al. 2016). Activated IL-2 induced effector T cells have punctate mitochondria, while IL-15 induced memory T cells maintain fused networks (Buck et al. 2016). As mitochondrial morphology and function are closely linked, those data indicate a subset specific reliance on oxidative phosphorylation.

Mitochondrial respiration is not only important for murine but also for human CD4 and CD8 T cell populations (Renner et al. 2015; Fischer et al. 2018). However, there is little knowledge about mitochondrial respiration in stimulated human CD8 T cell subsets. Human EM CD4 T cells have more and morphologically distinct mitochondria than naïve T cells (Dimeloe et al. 2014; Dimeloe et al. 2016). Quiescent naïve and EM CD8 T cells show a similar basic respiration (Gubser et al. 2013). However, EM T cells have a higher spare respiratory capacity than naïve T cells (Gubser et al. 2013).

To compare mitochondrial activity in human CD8 T cells, naïve, CM and EM CD8 T cells were stimulated and respiration was analyzed under culture conditions in the presence of serum. In the first eight hours, naïve, CM and EM T cells respired on a similar level; however, afterwards mitochondrial respiration was higher in naïve T cells compared to both memory subsets. Notably, EM cells showed the lowest oxygen consumption of all three subsets analyzed. In line with our data, Fischer and colleagues showed that naïve CD8 T cells rapidly increase mitochondrial mass and respiration after activation which is critical for effector functions as cytokine secretion (Fischer et al. 2018).

The importance of OXPHOS was tested in CD8 T cell subsets by blocking mitochondrial ATP production with oligomycin. It was shown that oligomycin application, similar to hypoxia, causes apoptosis in CD4 naïve T cells (Dimeloe et al. 2016). Viability was not altered by oligomycin treatment in all three CD8 T cell subsets analyzed in our hands. T cell function was strongly affected in naïve T cells, but only moderately in both memory subsets. A reduced increase in cell size, indicating a diminished activation state, was observed in naïve T cells, but not in the memory subsets. The reduced activation state in naïve T cells was further supported by a lowered lactate secretion after 48 hours, although variation between donors was observed.

Inhibiting OXPHOS diminished IFNγ and TNF secretion in naïve but not in EM cells, and in CM cells IFNγ secretion was only slightly reduced. Again, a donor variance was observed.

In bulk CD8 T cells, oligomycin treatment leads to a reduced on-blast formation and proliferation, but has no significant effect on cytokine production (Renner et al. 2015). As the effect was more pronounced on naïve cells which show limited cytokine secretion, a negative effect on naïve cells can be overlooked analyzing bulk cultures.

Taken together, mitochondrial inhibition mainly affected the naïve subset in line with the most pronounced increase in respiration upon stimulation.

Besides glucose metabolism and mitochondrial respiration, T cells rely on glutamine metabolism. Therefore the role of glutamine metabolism was analyzed by glutamine titration and restriction experiments.

4.1.1.3. Glutamine is an essential substrate during T cell stimulation

Tumor cells are frequently characterized by an increased consumption of glutamine (Altman et al. 2016), which could result in limited glutamine amounts in the tumor microenvironment. However, only a limited number of publications determined glutamine concentrations in the tumor. Pan and colleagues measured glutamine levels in pancreatic tumors and detected levels as low as 100 µM in the core and 400 µM in the periphery (Pan et al. 2016).

A variety of studies have shown that glutamine is also an essential amino acid for stimulated murine and human T cells (Carr et al. 2010; Wang et al. 2011; Newsholme et al. 2003a; Newsholme et al. 2003b). Therefore, restricted glutamine concentrations in the tumor microenvironment might contribute to tumor immune escape.

In the murine system glutamine availability is crucial for cell growth, the expression of activation-related surface markers and cytokine secretion in CD4 and CD8 T cells (Carr et al. 2010; Johnson et al. 2018; Wang et al. 2011, Csibi et al. 2013). In line, glutamine was shown to be essential for the production of GSH which is required for T cell growth and IL-2, IL-17 and IFNγ secretion (Mak et al. 2017).

Beside an important role for T cell activation, glutamine metabolism is also involved in CD4 T cell differentiation. Recently Johnson and colleagues reported that glutamine metabolism promotes differentiation of CD4 T cells to a Th17 phenotype, but impedes differentiation to Th1 cells (Johnson et al. 2018). Glutamine restriction is also linked to the induction of a Treg phenotype (Klysz et al. 2015).

As in the murine system, Metzler and colleagues demonstrated that glutamine restriction drives induction of a Treg like phenotype also in human CD4 T cells (Metzler et al. 2016). Glutamine dependency of different human T cell subpopulations is less investigated. For CD4 T cells, flux analyses demonstrated that glutamine can be degraded by the TCA to produce either citrate or pyruvate, the latter can be converted into lactate. Moreover, via the conversion of citrate into acetyl-CoA, glutamine fuels fatty acid synthesis in naïve and CM T cells, but not in EM T cells (Ecker et al. 2018).

To our knowledge, data on the importance of glutamine for CD8 T cell activation and respective subsets are not available. Therefore, CD8 T cells were stimulated under various concentrations of glutamine. Glutamine concentrations supplemented were 2 mM, the typical amount used for *in vitro* cultures, 0.5 mM reflecting blood level, and 0.25 to 0.05 mM to mirror the tumor situation. The application of serum should result in an additional glutamine supplementation of 0.05 to 0.075 mM. Besides the observed decreased glutamine concentrations in the tumor, these titration experiments were of special interest as during advanced malignant diseases glutamine plasma levels can be reduced by 20-30 % (Klimberg and McClellan 1996).

Already at 0.25 mM glutamine on-blast formation and proliferation were significantly reduced; however, IFNγ and TNF secretion were maintained in bulk CD8 T cells. At 0.1 mM glutamine, which is detected in tumor cores (Pan et al. 2016), the secretion of both cytokines was strongly diminished. Interestingly, metabolic activity in terms of glycolysis and respiration was impaired at glutamine concentrations as low as 0.1 mM, but not at higher concentrations. Cell growth and proliferation were affected at a concentration of 0.25 mM, but metabolic activity was preserved, indicating that glutamine itself seems to be important for proliferation.

Experiments with CD8 T cell subsets demonstrated that all three subsets were strongly affected regarding proliferation, metabolic activity and cytokine secretion by glutamine deprivation. Nevertheless, some differences in their dependency for glutamine were detected as IFNγ and TNF secretion was not completely blocked in memory subsets as observed in naïve T cells.

In order to elucidate which glutamine dependent pathway is crucial for T cell activation, glutamate, α-ketoglutarate, GSH, aspartate, arginine, asparagine alone or a combination of glutamate, α-ketoglutarate, GSH, hypoxanthine and thymidine were supplemented. As in other studies (Carr et al. 2010), we failed to completely rescue T cell function. In contrast to a study on murine T cells, in which CD4 T cell proliferation is partly recovered by the application of α-ketoglutarate as well as hypoxanthine and thymidine (Wang et al. 2011). These mixture of

metabolites administered in the same concentration did not rescue human CD8 T cell activation and proliferation in this study. Asparagine, able to rescue proliferation under glutamine starvation in tumor cells (Pavlova et al. 2018), could not replace glutamine in CD8 T cells. Glutamine is shown to be important for the generation of GSH to scavenge ROS in murine T cells (Mak et al. 2017), but GSH administration failed to substitute for glutamine in human T cells in our hands. Either glutamine could not be replaced or the metabolites administered were not efficiently taken up. To partially account for uptake limitations the cell permeable dimethyl-α-ketoglutarate was used in this study, but was also not effective.

According to the study of Geiger and colleagues, stimulated human CD4 T cells are able to import glutamate quite efficiently (Geiger et al. 2016). However, there is only little knowledge about transporters and receptors for glutamate in human CD8 T cells. To overcome a possible limitation due to a low uptake rate, glutamate was supplemented at very high concentrations. Indeed, preliminary results denoted that glutamate as high as 20 mM improved cytokine secretion to 50 % of control levels; however, glutamate supplementation had no effect on proliferation.

Studies have previously shown that there is a link between mTOR and glutamine metabolism. In line, in this study mTOR activation was reduced in the absence of glutamine. Furthermore, glutamine withdrawal after 24 hours of stimulation resulted in a drop in phospho-mTOR, indicating that glutamine is necessary to sustain mTOR activation.

Moreover, extracellular glutamine is shown to be an obligatory signal for the mTOR pathway (Fumarola et al. 2005). Glutamine acting as an exchange molecule facilitates the uptake of other amino acids as leucine and this is linked to mTOR activation (Nicklin et al. 2009). mTOR complex 1 activation (mTORC1) promotes glutamine metabolism by activating the glutamate dehydrogenase, the enzyme for the deamination of glutamate to α-ketoglutarate (Csibi et al. 2013). Glutamine metabolism is linked to mTORC1, as α-ketoglutarate stimulates mTORC1 activation, which indicates that mTORC1 is a downstream target of glutamine (Choi and Park 2018). However, in this study addition of cell permeable α-ketoglutarate was not able to replace glutamine.

Taken together, the data presented in this thesis strongly underline that glutamine, although regarded as non-essential amino acid, seems to be crucial for CD8 T cell activation. Preliminary data suggest, that the sensitivity might be related to the observed limited expression of glutamine synthetase and thus in the inability of T cells to synthesize glutamine from glutamate. Our results implicate that rather glutamine than glucose has to be considered as limiting factor in the tumor microenvironment. Already at concentrations half the level normally detected in blood,

CD8 T cell proliferation was significantly reduced. Further experiments overexpressing glutamine synthetase will reveal whether the sensitivity of T cells to glutamine deprivation can be overcome. Such approaches might be a promising strategy in the context of ACT.

In light of the presented data, the application of anti-metabolic drugs especially inhibitors of glutamine metabolism could have adverse effects on T cell function and thereby patient outcome.

4.2. Impact of drugs targeting tumor metabolism on human CD8 T cells

4.2.1. Impact of pharmacologic blockade of glutamine metabolism in CD8 T cells

Targeting amino acid metabolism has been exploited for quite a long time. Asparaginases are a cornerstone of treatment protocols for acute lymphoblastic leukemia and lymphoma (Avramis 2012). ADI-PEG20, an arginine degrading inhibitor (Miraki-Moud et al. 2015), is already tested in clinical trials (NCT01287585). Moreover, blocking glutamine metabolism has become an attractive anti-cancer therapeutic target (Altman et al. 2016).

With regard to the importance of glutamine for T cell activation, the application of glutamine analogues or pathway inhibitors has to be regarded with caution. Due to this fact the impact of drugs targeting the glutamine metabolism were analyzed in this study.

In several clinical studies, the glutamine analogue DON demonstrated promising results in patients with hematological malignancies or solid tumors (Eagan et al. 1982; Lynch et al. 1982; Sullivan M. P. et al. 1962). Moreover, DON is shown to sensitize pancreatic cancer cells to chemotherapy *in vitro* and improved the efficacy of gemcitabine treatment (Chen et al. 2017). T cell function of freshly isolated and stimulated bulk CD8 T cells was strongly inhibited in the presence of DON and also acivicin as on-blast formation, cytokine secretion as well as proliferation were decreased. Notably, due to severe side effects especially due to neurological toxicity, acivicin failed to get approval (Adolphson et al. 1986; Olver et al. 1998). Moreover, DON was declined due to dose limiting toxicities particularly in the gastrointestinal tract (Rais et al. 2016).

In contrast to glutamine analogues, the application of two different glutaminase inhibitors had no impact on CD8 T cell function as CD25 expression, on-blast formation, proliferation as well as IFNγ and TNF secretion were not altered. These results are in line with other studies, in which glutaminase inhibition by BPTES or CB-839 does not impede cell growth and proliferation of

primary human T lymphocytes *in vitro* (Xiang et al. 2015). However, BPTES administration, leading to increased intracellular glutamine levels, reduces proliferation and cytokine secretion in human CD4 T cells (Sener et al. 2016).

Johnson and colleagues recently reported that the response to CB-839 treatment in murine CD4 T cells is subset specific (Johnson et al. 2018). Proliferation of Th17, but not of Th1 cells is reduced. Surprisingly, Th1 T cells produce more IFNγ in the presence of CB-839. Those findings are in contrast to the results of Sener and colleagues (Sener et al. 2016) and might indicate a species related difference between human and murine T cells.

Taken together glutaminase inhibition has a moderate effect on T cell function in comparison to glutamine analogues and therefore the administration might be an interesting option in the context of cancer therapy. Unfortunately, the efficacy of these inhibitors is restricted to only a few tumor entities since a variety of tumor cells can fuel their glutamine metabolism by synthetizing glutamine from glutamate by the glutamine synthetase (Tardito et al. 2015).

Surprisingly, in expanded T cells, T cell function was preserved in the presence of DON. An experiment with expanded CD8 subsets under glutamine deprived conditions indicated that T cells were still dependent on glutamine metabolism. Whether DON was not taken up any longer or T cells exported the inhibitor by multidrug resistance (MDR) pumps, as it is observed in tumor cells (Gottesman and Pastan 2015), needs further investigation. These observations are of interest especially in the context of ACT, as T cells need to be expanded before transfer. Moreover, the role of tumor metabolism during cancer therapy has gained attention in the past years. Due to this fact, inhibitors of glutamine metabolism could be used as combinatory drug, in particular with regard to immunotherapeutic approaches (Cervantes-Madrid et al. 2015).

Taken together, glutaminase inhibiting drugs did not show any impact on proliferation or T cell effector functions as IFNγ or TNF secretion in freshly stimulated CD8 T cells or expanded CD8 T cell subsets. Promising results were obtained with the glutamine analogue DON, which showed no impact on the effector functions in expanded T cell subsets.

4.2.2. Targeting glycolysis via inhibition of MCT1 and MCT4 in T cells

Beside a highly elevated demand for amino acids, tumor cells frequently show an increased glycolytic activity. As a result, lactic acid accumulates in the microenvironment of tumors displaying the Warburg phenotype. It has been shown by our group and others that lactic acid suppresses the anti-tumor immune response not only of T cells (Fischer et al. 2007; Calcinotto et al. 2012; Mendler et al. 2012) but also of other cell types as NK cells or monocytes (Dietl et al.

2010; Husain et al. 2013). The main transporters responsible for the secretion of lactate, in co-transport with a proton, are the monocarboxylate transporters (MCTs). MCT1 is ubiquitously expressed on normal cells and in line on several tumor entities, as for example colorectal, breast, gastric, cervical cancer and glioma (Pinheiro et al. 2008; Pinheiro et al. 2010; de Oliveira, Talvane Torres Antônio et al. 2012; Miranda-Goncalves et al. 2013). In addition, MCT4 is expressed on highly glycolytic cells and thus frequently detected on tumor cells as renal cell carcinoma, cervical or prostate cancer (Doherty and Cleveland 2013).

Due to their strongly elevated expression in a high proportion of tumors, MCTs represent a promising therapeutic target structure. However, blocking only one transporter is of limited efficacy. It has already been shown for a human colon adenocarcinoma cell line that cells are insensitive to MCT1 inhibition, as they are able to compensate by MCT4 (Marchiq et al. 2015). Vice versa, a MCT4 disruption only hardly affects metabolic activity in these cells. However, MCT4 disruption combined with MCT1 inhibition does not induce cell death but impairs tumor growth. Known MCT inhibitors mainly target MCT1 and MCT2 and are already tested in clinical trials (Murray et al. 2005; Ovens et al. 2010a; Ovens et al. 2010b; Doherty et al. 2014), while inhibitors for MCT4 are currently developed (Doherty and Cleveland 2013). Since various tumor entities are characterized by elevated MCT4 levels, compounds disabling MCT4 to efficiently reduce lactate secretion are needed.

Expression levels and importance of those transporters in T cells has not been systematically investigated and especially data on the human system are lacking. Murray and colleagues showed that an MCT1/2 inhibitor reduces lactate secretion in primary stimulated human T cells and reduces proliferation (Murray et al. 2005). However, they did not investigate effector functions. Moreover, in this publication an increased expression of MCT4 in the course of stimulation is briefly mentioned. Thus, the application of pan-MCT inhibitors might impede T cells.

Our group has already shown that diclofenac reduces lactate secretion and diminishes tumor growth *in vivo* (Chirasani et al. 2013; Gottfried et al. 2013). New data of Sasaki and colleagues and our group show that diclofenac directly targets MCT1 and MCT4 activity (Sasaki et al. 2016; Renner et al. under revision). Similar to diclofenac, lumiracoxib, a diclofenac derivative, is capable to target both transporter and reduces lactate secretion in human melanoma cell lines (Brummer et al. 2019). Diclofenac and lumiracoxib have a high structural similarity, as only one chloride is replaced by a fluoride molecule. Both substances are cyclooxygenase (COX) inhibitors. Diclofenac inhibits prostaglandin synthesis by inhibiting COX-1 and COX-2 (Gan 2010) and is used as an anti-inflammatory and analgesic drug, also during cancer therapy

(Mercadante 2001). Lumiracoxib is a selective COX-2 inhibitor (Tacconelli and Patrignani 2004), but was withdrawn from the market in a variety of countries due to its potential causing liver failure, however only at high concentrations. Besides blockade of lactate accumulation, both substances are developed to reduce prostaglandin E2 secretion. Prostaglandin E2 has been shown to suppresses T cell function and the response to checkpoint inhibition (Zelenay and Reis e Sousa 2016). Thus the application of diclofenac or lumiracoxib might block the secretion of two immunosuppressive metabolites, lactate and prostaglandin E2.

Diclofenac and lumiracoxib exerted similar effects on T cell function in freshly isolated and activated human CD8 T cell subsets. Application of both substances led to a reduction in glucose consumption and lactate secretion. In accordance with a stronger effect on lactate secretion, which can be explained by the higher K_i value of lumiracoxib for both transporters, proliferation was stronger reduced under diclofenac treatment after six days. Cytokine secretion was only slightly affected in naïve and preserved in EM T cells. Taken together, diclofenac as well as lumiracoxib reduced glycolytic activity and proliferation in CD8 T cell subsets. However, T cell effector functions were preserved or only slightly lowered by both substances indicating that T cells are able to tolerate MCT inhibition.

This notion was supported by our findings analyzing MCT4$^{-/-}$ T cells. As nothing is known about the importance of MCT4 for T cell activation, CD4 as well as CD8 T cells from MCT4$^{-/-}$ mice were investigated. Surprisingly, both MCT4$^{-/-}$ T cell populations showed no differences regarding glycolytic activity when stimulated with α-CD3/CD28 antibodies in comparison to WT T cells. Disruption of the MCT4 gene did not modify the transport of lactate, since both cell types secreted the same amount of lactate. These results show again that lacking or inhibition of one MCT can be compensated by another MCT transporter. Notably, MCT4 deficiency seemed to shift metabolism to respiration indicated by a higher oxygen consumption rate in MCT4$^{-/-}$ T cells. Since there was no drop in cytokine secretion caused by MCT4 deficiency, a link between MCT4 and effector functions in freshly stimulated T cells was not observed, at least in an *in vitro* setting. However, MCT4 expression might be important in expanded T cells, which needs to be clarified.

Our findings in T cells are in contrast to results published for macrophages. In knockdown experiments with siRNA specific for MCT4, murine macrophages are characterized by intracellular lactate accumulation and decreased glycolytic activity. Moreover, effector functions are reduced in MCT4$^{-/-}$ macrophages, measured by expression of inducible nitric oxide synthase (iNOS), TNF and IL-6 secretion (Tan et al. 2015). It was recently shown that oral squamous carcinoma tumors grow more aggressive in MCT4$^{-/-}$ mice than in WT mice (Bisetto et al. 2018).

The number of infiltrating macrophages analyzed by immunohistochemistry is decreased in tumors in MCT4$^{-/-}$ mice. Analyzing the circulating monocytes demonstrate significant lower levels possibly resulting in a defect in the recruitment of macrophages to the tumors.

To investigate whether complete blockade of MCT1 and MCT4 has a negative impact, a MCT1/2 inhibitor was applied to MCT4$^{-/-}$ T cells in comparison to WT T cells. A mouse with a complete knock out of both transporters is not available, as a complete MCT1 knockout has shown to be embryonically lethal (Lengacher et al. 2013; Morrison et al. 2014) and mice with a basignin knockout, the anchor protein for MCT1 and MCT4, are sterile and show various neurological abnormalities (Muramatsu and Miyauchi 2003).

T cell function of MCT4$^{-/-}$ T cells in comparison to WT T cells was analyzed in the presence of the MCT1/2 inhibitor SR13800. In addition, these results were compared to treatment with diclofenac. Supplementation of the MCT1/2 inhibitor strongly reduced lactic acid transport in both, WT and MCT4$^{-/-}$ T cell populations, but in MCT4$^{-/-}$ T cells to a stronger extent. MCT1 and MCT4 inhibition reduced cytokine secretion but did not lead to a complete blockade. WT T cells treated with diclofenac leading to a MCT1 and MCT4 inhibition showed a similar impact on IFNγ secretion as MCT4$^{-/-}$ T cells treated with the MCT1 inhibitor SR13800. In murine T cells a trend to reduced cytokine secretion was observed in the presence of diclofenac. This effect is not observed in human T cells (Renner et al. under revision). Those data suggest that there might be a difference between the murine and the human system, which should be further investigated.

To our knowledge this is the first study investigating the impact of MCT4 on T cell function. As MCT4 deficiency did not show any impact on effector functions in T cells *in vitro*, MCT4 as well as a simultaneous MCT1 blockade could display a promising strategy in cancer therapy. Due to the fact, that diclofenac and lumiracoxib inhibit MCT1 as well as MCT4, they might be promising candidates for cancer therapy. However, a therapy with MCT inhibitors alone might not be effective. In a head and neck squamous cell carcinoma (HNSCC) cell line, the double disruption of MCT1 and MCT4 does not show any effects on cell growth under normoxia, but leads to a growth inhibition under hypoxic conditions (Boasquevisque et al. 2017). These findings suggest that glycolytic inhibition in tumor cells could be compensated by alternative metabolic pathways as respiration.

Nevertheless, the reduction in lactic acid secretion supports an endogenous anti-tumor response, regarded as crucial for patient survival (Galon et al. 2006). In accordance, a MCT4 knockdown in 4T1 breast cancer cells by shRNA or the application of the MCT4 inhibitor 7acc1

lead to a strengthened cytotoxicity of NK cells, which is correlated with reduced tumor growth (Long et al. 2018).

Therefore, a combination of anti-glycolytic drugs with immunotherapeutic approaches could be extremely beneficial as recently suggested by Cascone and colleagues (Cascone et al. 2018). In case of an ACT, a sufficient *in vitro* expansion of patient derived T cells is required. To exclude effects of metabolic targeting on expanded T cells, the impact of metabolic inhibitors was finally investigated in CD8 T cell subsets after expansion.

4.2.3. Impact of anti-metabolic targeting on T cell function in expanded CD8 T cell subsets in the context of immunotherapeutic approaches

So far immunotherapeutic approaches as ACT demonstrate efficacy particularly in patients with leukemia and lymphoma. Regarding various solid tumors, the success of ACT is moderate and only in a small cohort of patients a tumor regression can be achieved (Goff et al. 2016). In contrast to hematologic malignancies, solid tumors display an immunosuppressive metabolic microenvironment (Renner et al. 2017). In line, recent publications clearly show that a high glycolytic activity impairs the endogenous anti-tumor immune response (Brand et al. 2016) which could limit the efficacy of immunotherapeutic approaches (Ascierto et al. 2016; Cascone et al. 2018).

Transferred T cells require a successful engraftment, long-term *in vivo* persistence and regeneration of effector as well as memory T cell populations. Beside culture conditions such as the duration in culture (Kalos et al. 2011) or the capability to persist *in vivo*, the outcome depends on the differentiation stage of reinfused cells (Klebanoff et al. 2012). It is still controversially discussed which T cell subsets could mediate a highly effective cancer regression in patients upon ACT. Although effector T cells are poised to rapidly execute effector functions upon activation, as evidenced by their capacity to release large amounts of cytokines and their ability to rapidly lyse targets, less differentiated T cell subsets seem to be superior in mediating antitumor immunity (Klebanoff et al. 2012). There is evidence that adoptively transferred T cells derived from CMs have a greater capacity than cells derived from EMs to persist *in vivo* (Berger et al. 2008). In a study with CD19-specific CAR-modified memory stem CD8 T cells, it was shown that these cells mediate superior and more durable antitumor responses than CD8 T cells generated with clinical protocols currently under investigation (Sabatino et al. 2016).

Taken together, ACT of less differentiated T cell subsets have consistently demonstrated superior *in vivo* expansion, persistence and antitumor capacities relative to the more experienced T cells. However, our results suggest that EM T cells should also be considered for ACT, as they were less sensitive to nutrient restriction. Moreover, in particular CD8 EM T cells are capable to infiltrate tumors (Siska et al. 2017). Furthermore, tumor infiltrating CD45RO positive cells, displaying memory T cells, are reported to be associated with a better prognosis during cancer therapy (Hadrup et al. 2013).

For ACT an *ex vivo* expansion of T cells is indispensable. Several expansion protocols were tested in order to obtain cells with a preserved CD62L and CCR7 expression and a capacity to produce important effector molecules. Stimulation in a MLR in combination with the interleukin cocktail of IL-2, IL-7 and IL-15 was favorable in comparison to a commonly used α-CD3/CD28 stimulation due to a higher proliferation rate and a higher capacity to secret IFNγ as well as perforin and granzyme B after expansion.

After expansion, the impact of several drugs targeting tumor metabolism on CD8 T cells was determined. T cell function of CD8 T cell subsets was analyzed in the presence of the MCT inhibitors diclofenac and lumiracoxib, the anti-diabetic drug metformin displaying a mitochondrial inhibitor, and finally DON and CB-839, inhibitors for glutamine metabolism.

Aside from proliferation, T cell function was not impaired by inhibition of glycolysis in all three subsets, reflecting the results obtained in bulk CD4 and CD8 T cells (Renner et al. under revision). Metformin is shown to improve T cell function in exhausted CD8 T cells derived from patients with breast or lung cancer, as they secret higher amounts of IFNγ, IL-2 and TNF (Watanabe et al. 2017). Metformin had no inhibitory effect on expanded CD8 T cell subsets; however, supplementation did not improve T cell function. The improved function in T cells isolated from patients might be an indirect effect due to a lower tumor growth and thereby a less harmful tumor microenvironment.

Taken together, glycolytic and mitochondrial inhibitors had no impact on T cell function as proliferation or cytokine secretion of expanded CD8 T cells. Strikingly, although the application of DON impaired the function of freshly isolated T cells, DON did not affect function in expanded CD8 T cell subsets. Comparable to the results obtained for primary T cells, CB-839 did not show any implication on T cell function comparable to the results on primary activated T cells.

As T cells expanded in a MLR were resistant to metabolic inhibitors after expansion, the application of specific anti-metabolic drugs might be supportive during ACT or other immunotherapeutic approaches.

4.3. Perspectives: The potential of metabolic targeting to support immunotherapy

In the last decades, immunotherapy has become an important treatment strategy for several tumor entities. However, regarding the conditions in the microenvironment of solid tumors immunotherapy may require combinatorial treatment regimens (Abken 2015; Beatty and O'Hara 2016). The combination of anti-metabolic drugs with immunotherapy is an emerging strategy in this context. A blockade of CD73, an enzyme that catabolizes the hydrolysis of extracellular adenosine monophosphate, which is known to impair T and NK cell immune response (Häusler et al. 2011), was shown to enhance PD-1 and CTLA-4 therapy (Allard et al. 2013). Specific glutaminase inhibitors do not affect T cell function. Accordingly, glutaminase inhibition has already been shown to support immunotherapy with PD-1 and PD-L1 antibodies (Gross et al. 2016).

High lactate concentrations in the tumor microenvironment dampens the efficacy of ACT (Cascone et al. 2018). In advanced renal cell carcinomas (RCC), the metabolic profile of tumors is associated with the response rate and overall survival of patients treated with PD-1 therapy (Ascierto et al. 2016).

However published data suggest that application of anti-glycolytic drugs might impair T cell function. The data in this thesis show that glucose metabolism is of minor importance for T cell effector functions. In line, the anti-glycolytic NSAIDs diclofenac and lumiracoxib did not block T cell effector functions and are therefore promising supplements during immunotherapy. Moreover, both NSAIDs are shown to reduce simultaneously extracellular lactate (Sasaki et al. 2016; Brummer et al. 2019; Renner et al. under revision) and prostaglandin E2 accumulation (Gan 2010). The latter is also known as an immunosuppressive metabolite (Kalinski 2012) and COX inhibition was shown to support checkpoint therapy (Zelenay and Reis e Sousa 2016). However, our group demonstrated that the application of diclofenac, but not aspirin effectively supports checkpoint therapy in a subcutaneous model of triple negative breast cancer *in vivo* (Renner et al. under revision). Those data argue for the application of NSAIDs inhibiting lactate and prostaglandin E2 secretion at the same time.

Beside the application of anti-metabolic drugs, a genetic approach to render T cells more competitive in nutrient acquisition or making T cells more resistant to inhibitory metabolites in the tumor microenvironment could be promising. Overexpression of PPAR-gamma co-activator 1α (Scharping et al. 2016; Bengsch et al. 2016), a master regulator of mitochondrial biogenesis (Lin et al. 2005), or phosphoenolpyruvate carboxykinase 1 (Ho et al. 2015) improve the efficacy of adoptively transferred T cells. Inhibiting cholesterol esterification by genetic ablation or

pharmacological inhibition leads to enhanced effector functions and proliferation in CD8 T cells as well as better controlling of tumor progression (Yang et al. 2016). With regard to our results, artificial overexpression of the glutamine synthetase (GLUL) might be also a strategy to boost T cell function in a tumor microenvironment characterized by low glutamine concentrations.

Finally, although plasticity remains very challenging, metabolic pathways seem to be associated with differentiation into different T cell subsets (Buck et al. 2016; Patsoukis et al. 2016; Patel and Powell 2017). Manipulation or reprogramming of metabolism with metabolic active substances to induce the development of special T cell subsets could ameliorate the current protocols for cancer immunotherapy (Zhang and Ertl 2016; Dugnani et al. 2017).

In conclusion, this study reveals that effector functions of CD8 T cell subsets are only partly compromised by drugs targeting glycolysis, glutamine metabolism or cellular respiration. Although the application of anti-metabolic drugs affects some aspects of T cell function, the highly accelerated metabolism of tumor cells has such a strong immunosuppressive effect that the application of anti-metabolic drugs might still be beneficial, especially in the context of immunotherapy.

5. Summary

Novel, promising strategies to fight cancer include immunotherapeutic approaches these days. Such strategies are regarded as breakthrough of the 21th century. The Nobel Prize of medicine was recently awarded to the discovery of ways to remove the immune system's "brakes" (checkpoints) and thereby unleashing immune cells to attack tumors. However, only in a limited cohort of patients a persisting tumor regression is achieved. Besides avoiding immune recognition by the expression of such checkpoint molecules or the lack of antigen presentation, the metabolic tumor microenvironment contributes to diminished anti-tumor immune responses. The tumor microenvironment is characterized by low levels of nutrients and at the same time the accumulation of immunosuppressive metabolites as lactic acid, both negatively affecting the anti-tumor immune response. Therefore, targeting tumor metabolism could improve the efficacy of immunotherapeutic approaches. The link between metabolism and T cell effector functions is still a matter of debate and data on the human system are rather sparse particularly for different CD8 T cell subsets. Understanding this link is a prerequisite for the development of therapies targeting tumor cells, but preserving immune cell functions.

Thus in this thesis, CD8 T cell subsets (naïve, central and effector memory T cells) were characterized in terms of their metabolic demands for different aspects of function as proliferation or cytokine secretion. Nutrient restriction experiments were performed to reveal metabolic dependencies of CD8 T cells and finally the impact of anti-metabolic drugs as proposed for cancer treatment was analyzed.

In comparison to murine T cells, human CD8 T cells seem to be less dependent on glucose metabolism, as cell growth and effector functions as cytokine secretion were only slightly compromised under glucose deprived conditions. However, proliferation was blocked in all three subsets. Interestingly, the variation between donors was pretty high especially in naïve T cells.

In contrast, glutamine was essential for CD8 T cell function as glutamine restriction led to insufficient activation of all CD8 T cell subsets. Nevertheless, effector functions as IFNγ and TNF secretion were less compromised under glutamine deprived conditions in memory subsets than in naïve T cells. Titration experiments showed that glutamine concentrations as low as 0.1 mM inhibited T cell activation and IFNγ and TNF secretion, a concentration which has been already detected in tumors. Proliferation was already significantly reduced at a glutamine concentration of 0.25 mM. These results showed that not only glucose but also glutamine is a substrate, tumor cells and immune cells have to compete for in the tumor microenvironment.

Experiments failed to find a combination of metabolites downstream of glutamine degrading pathways which can substitute for glutamine, indicating that glutamine itself is crucial for T cells. Preliminary results suggest that the lack of glutamine synthetase, an enzyme catalyzing the reaction of glutamate to glutamine, could play a role in the dependency on glutamine in T cells.

Next, the role of cellular respiration was analyzed by application of oligomycin, an ATP synthase inhibitor. T cell functions as proliferation as well as IFNγ and TNF secretion were diminished in naïve T cells but only slightly in central and effector memory T cells.

Although effector memory T cells are regarded as an exhausted subset, our findings suggest that these cells are less affected by nutrient restriction than naïve T cells. Therefore also effector memory T cells should be considered in terms of immunotherapeutic approaches as adoptive T cell transfer.

Finally, as activated T cells show an accelerated metabolism similar to tumor cells, the impact of anti-metabolic drugs was investigated in freshly stimulated CD8 T cells. With special regard to adoptive T cell transfer, the impact of clinically relevant anti-metabolic drugs was investigated in expanded CD8 T cells.

Due to the importance of glutamine for T cell activation, the application of inhibitors targeting glutamine metabolism has to be considered carefully. Due to this fact the impact of glutamine analogues (DON and acivicin) as well as glutaminase inhibitors (BPTES and CB-839) was analyzed in this study. Inhibition of glutaminase, converting glutamine to glutamate, had no impact on T cell function in primary stimulated CD8 T cells, whereas the glutamine analogs strongly impaired T cell function and metabolic activity. Surprisingly, DON did not show a strong impact on expanded CD8 subsets.

Since glucose metabolism is of major importance for tumor cells, the impact of drugs targeting glycolysis on T cells was analyzed. Inhibition of monocarboxylate transporter (MCTs) in tumor cells, to block lactic acid secretion, affects tumor cells itself but might also support the anti-tumor immune response by reducing lactate accumulation. Up to now potent MCT inhibitors have been developed only for MCT1 and MCT2. The two non-steroidal anti-inflammatory drugs (NSAIDs) diclofenac and lumiracoxib, both known cyclooxygenase (COX) inhibitors, target MCT1 and MCT4. As they are the only identified inhibitors of MCT1 and simultaneously MCT4, they might be promising substances in the context of tumor therapy. Although an inhibition of glycolytic activity was observed, cytokine secretion was maintained in human naïve and effector memory CD8 T cell subsets and also in expanded CD8 T cell subsets. Only a reduction in proliferation was observed after six days.

These results were further supported by investigating a complete MCT1 and MCT4 inhibition in murine T cells. To mimic this pan-MCT inhibition, MCT4 deficient T cells were cultured in the presence of a MCT1/2 inhibitor. Although the glycolytic activity was almost completely blocked, murine T cells were still able to secret important effector cytokines such as IFNγ; however levels were moderately reduced.

In this thesis, CD8 T cells showed subset specific differences in their metabolic activity. While glucose restriction did not harmed T cell function, glutamine deprivation had a strong impact on the T cell immune response. Therefore, targeting glucose metabolism is a promising strategy in cancer therapy, while drugs acting on the glutamine metabolism have to be considered carefully. Nevertheless, T cells were unaffected by glutaminase inhibitors. These findings suggest that anti-metabolic drugs are promising supplements in immunotherapeutic approaches as checkpoint blockade or adoptive T cell transfer therapy.

6. Zusammenfassung

Immuntherapeutische Ansätze stellen heutzutage neuartige, erfolgsversprechende Strategien zur Krebsbehandlung dar. Diese neuen Vorgehensweisen gelten als Durchbruch des 21. Jahrhunderts. So wurde kürzlich der Nobelpreis für Medizin für die Entdeckung verliehen, „die Bremsklötze“ des Immunsystems (sogenannte Checkpoints) zu lösen und dadurch Immunzellen zur Tumorbekämpfung zu befähigen. Allerdings wird eine andauernde Tumorregression durch eine solche Therapie nur in einer begrenzten Patientengruppe erreicht. Neben der Tatsache, dass Immunzellen aufgrund solcher Checkpoints und einer fehlenden Antigen-Präsentation den Tumor nur unzureichend erkennen, trägt das metabolische Tumormikromilieu zu einer verringerten anti-tumoralen Immunantwort bei. Das Tumormikromilieu ist nämlich durch einen Nährstoffmangel und gleichzeitig einer Anhäufung immunsuppressiver Metabolite gekennzeichnet, wie beispielsweise der Milchsäure, was die anti-tumorale Immunantwort abschwächt. Daher könnte die Erfolgsquote einer Immuntherapie durch das Eingreifen in den Tumormetabolismus gesteigert werden. Der Link zwischen Metabolismus und humanen T-Zell-Effektor-Funktionen ist immer noch unzureichend geklärt, wobei es an Untersuchungen zum humanen System mangelt, vor allem in Bezug auf Daten für verschiedene CD8 T-Zell-Subpopulationen. Diesen Link zu verstehen ist essentiell für die Entwicklung neuer Therapieansätze, bei denen gezielt Tumorzellen bekämpft, gleichzeitig aber T-Zell-Funktionen erhalten bleiben.

Deshalb wurden in dieser Arbeit die metabolischen Anforderungen charakterisiert, die für die Aufrechterhaltung von Funktionen, wie Proliferation oder Zytokinproduktion, in CD8 T-Zell-Subpopulationen (naive Zellen, zentrale und Effektor-Gedächtniszellen) nötig sind. Mit Hilfe von Nährstoffentzugs-Experimenten wurde die metabolische Abhängigkeit von CD8 T-Zellen untersucht und zudem der Einfluss von anti-metabolischen Substanzen analysiert, die in der Krebsbehandlung Anwendung finden könnten.

Im Vergleich zu murinen T-Zellen, schienen humane T-Zellen weniger auf den Glukosemetabolismus angewiesen zu sein, da das Zellwachstum und die Effektor-Funktionen, wie die Zytokinproduktion, nur leicht durch einen Glukoseentzug beeinträchtigt waren. Allerdings wurde die Proliferation in allen drei Subpopulationen inhibiert. Interessanterweise war vor allem in den naiven T-Zellen eine hohe Spendervarianz zu beobachten.

Im Gegensatz dazu war der Glutaminstoffwechsel essentiell für die Funktionen der CD8 T-Zellen, da ein Glutaminentzug zu einer unzureichenden Aktivierung in allen Subpopulationen

führte. Allerdings waren die Effektorfunktionen, in Form von IFNγ- und TNF-Sekretion, unter Glutaminentzug weniger stark in den Gedächtniszellen als in den naiven T-Zellen beeinflusst. Titrationsexperimente zeigten, dass Glutaminkonzentrationen von 0.1 mM die T-Zell-Aktivierung sowie die IFNγ- und TNF-Sekretion inhibierten, eine Konzentration, die schon in Tumoren nachgewiesen wurde. Die Proliferation war bereits bei einer Konzentration von 0.25 mM signifikant reduziert. Diese Ergebnisse demonstrieren, dass nicht nur Glukose sondern auch Glutamin einen Nährstoff darstellt, um den Tumorzellen und Immunzellen im Tumormikromilieu konkurrieren.

Indem eine Kombination aus verschiedenen Metaboliten supplementiert wurde, die mit dem Glutaminstoffwechsel in Verbindung gebracht werden, wurde versucht ein Ersatzmetabolit für Glutamin zu finden. Das Scheitern dieser Experimente deutet darauf hin, dass Glutamin selbst einen essentiellen Nährstoff für T-Zellen darstellt. Vorläufige Ergebnisse suggerieren, dass die Glutaminabhängigkeit von T-Zellen in der Abwesenheit der Glutamin-Synthetase begründet sein könnte, einem Enzym, das die Reaktion von Glutamat zu Glutamin katalysiert.

In einem nächsten Schritt wurde die Rolle der zellulären Atmung mit Hilfe von Oligomycin analysiert, ein Inhibitor der ATP-Synthase. T-Zell-Funktionen wie die Proliferation oder die IFNγ- und TNF-Sekretion waren stark gehemmt in naiven T-Zellen, aber nur leicht in den zentralen und den Effektor-Gedächtniszellen.

Obwohl die Effektor-Gedächtniszellen als geschwächte Subpopulation gelten, zeigen unsere Ergebnisse, dass diese Zellen weniger stark von einem Nährstoffmangel als naive T-Zellen betroffen sind. Somit sollten auch Effektor-Gedächtniszellen bei immuntherapeutischen Ansätzen, wie dem adoptiven T-Zell-Transfer, berücksichtigt werden.

Da aktivierte T-Zellen einen erhöhten Stoffwechsel aufweisen, ähnlich dem in Tumorzellen, wurde schließlich die Auswirkung von anti-metabolischen Substanzen auf frisch stimulierte CD8 T-Zellen untersucht. Im Hinblick auf einen adoptiven T-Zell-Transfer, wurde zudem der Effekt von klinisch relevanten anti-metabolischen Substanzen auf expandierte T-Zell-Subpopulationen untersucht.

Unter besonderer Berücksichtigung der Bedeutung des Glutamins für die T-Zell-Aktivierung, sollte die Applikation von Inhibitoren, die in den Glutaminstoffwechsel eingreifen, sorgfältig geprüft werden. Infolgedessen wurde in dieser Arbeit der Einfluss von Glutamin-Analoga (DON und Acivicin) sowie Glutaminase-Inhibitoren (BPTES und CB-839) untersucht. Die Inhibition der Glutaminase, die Glutamin zu Glutamat umwandelt, zeigte keinen Effekt auf die T-Zellfunktionen in CD8 T-Zellen, wohingegen die Glutamin-Analoga die T-Zell-Funktionen und die metabolische

Aktivität stark supprimierten. Überraschenderweise, zeigte DON keinen Effekt mehr auf expandierte CD8 Subpopulationen.

Da der Glukose-Metabolismus eine übergeordnete Rolle für Tumorzellen spielt, wurde der Einfluss von Substanzen untersucht, die auf die Glykolyse wirken. Die Inhibition der Monocarboxylat-Transporter (MCTs) in Tumorzellen, durch welche die Laktatsekretion blockiert wird, würde Tumorzellen direkt beeinflussen und könnte zusätzlich die anti-tumorale Immunantwort unterstützen, indem eine Laktatakkumulation verhindert wird. Bislang wurden wirksame Inhibitoren lediglich für MCT1 und MCT2 entwickelt. Die beiden nichtsteroidalen Antirheumatika (NSARs, engl. NSAIDs) Diclofenac und Lumiracoxib, beide auch als Cyclooxygenase (COX)-Inhibitoren bekannt, hemmen MCT1 und zugleich MCT4. Da diese die einzigen bekannten Inhibitoren sind, die neben MCT1 auch MCT4 blockieren, könnten diese vielversprechende Substanzen im Kontext zur Tumortherapie darstellen. Obwohl eine Reduktion der glykolytischen Aktivität festgestellt wurde, war die Zytokinproduktion sowohl in naiven als auch in Effektor-Gedächtniszellen sowie in den expandierten Subpopulationen erhalten. Lediglich eine Hemmung der Proliferation war nach sechs Tagen zu beobachten.

Diese Ergebnisse wurden zusätzlich durch eine vollständige Blockade von MCT1 und MCT4 in murinen T-Zellen bestätigt. Um eine pan-Inhibition zu imitieren, wurden MCT4 defiziente T-Zellen in Anwesenheit von einem MCT1/2 Inhibitor kultiviert. Obwohl die glykolytische Aktivität beinahe vollständig inhibiert war, waren die murinen T-Zellen immer noch in der Lage wichtige Effektorzytokine wie IFNγ zu produzieren, wobei die Werte zu einem gewissen Maß reduziert waren.

Die in dieser Arbeit analysierten CD8 T-Zellen wiesen Gruppen-spezifische Unterschiede in ihrer metabolischen Aktivität auf. Während ein Glukoseentzug die T-Zell-Funktionen nicht schädigte, rief ein Glutaminentzug einen starken Effekt auf die T-Zell-Immunantwort hervor. Deshalb stellt der Glukosemetabolismus eine aussichtsreiche Zielstruktur in der Krebstherapie dar, während Medikamente, die auf den Glutaminstoffwechsel wirken, einer sorgfältigen Prüfung bedürfen. Dennoch blieben T-Zellen von den Glutaminase-Inhibitoren unbeeinträchtigt. Diese Ergebnisse zeigen, dass anti-metabolische Substanzen eine vielversprechende Ergänzung zu immuntherapeutischen Ansätzen darstellen, wie beispielsweise einer Checkpoint Blockade oder einem adaptiven T-Zell-Transfer.

7. References

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