AUS DEM LEHRSTUHL FÜR INNERE MEDIZIN III

PROF. DR. WOLFGANG HERR

DER FAKULTÄT FÜR MEDIZIN

DER UNIVERSITÄT REGENSBURG

Impact of an anti-metabolic therapy on leukemic and non-malignant T cells

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

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Fakultät für Medizin
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Dekan:	Prof. Dr. Dr. Torsten E. Reichert
1. Berichterstatter:	Prof. Dr. Marina Kreutz
2. Berichterstatter:	PD Dr. Stephan Schreml
Tag der mündlichen Prüfung:	21. September 2017

Für meine Eltern.

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1. Introduction

Tumor cells display and depend on increased metabolic activity to sustain proliferation. Among other pathways glycolysis is strongly elevated. Inhibition of this vital metabolic pathway is a fairly new approach and several anti-glycolytic drugs are currently under investigation in clinical trials (1, 2). However, beside tumor cells other organs and tissues also rely on glucose metabolism and the suppression of this pathway might result in adverse side effects. In particular, activated T lymphocytes show a similar metabolic phenotype compared to cancer cells. Thus, anti-glycolytic drugs might impede T cell function. As T cell infiltration and activation are important for the prognosis of tumor patients, preservation of T cell function is crucial (3). The link between glucose metabolism and T cell function has been mainly investigated in the murine system and data on the human system are rather sparse and contradictory. However, analyzing the importance of glucose metabolism and the impact of glycolytic inhibitors on human T cell function is a prerequisite for the development of treatment strategies selectively affecting tumor cells.

1.1 Tumor cells

1.1.1 Characteristics of a tumor cell

In 2000 Hanahan and Weinberg defined the main attributes of neoplastic cells – the well-known “Hallmarks of Cancer” (4).

The hallmarks include (i) a *sustained proliferation and cell growth* by increased receptor sensitivity, autocrine hormone secretion or constitutive pathway activation; (ii) *evasion of growth suppressors* by loss of tumor suppressor genes such as retinoblastoma 1 (RB1) or tumor protein p53 (TP53) or corruption of the anti-proliferative effect of the transforming growth factor β (TGFβ); (iii) *resistance to cell death* via increase of anti-apoptotic (e.g. Bcl-2) and decrease of pro-apoptotic proteins (e.g. Bax, Bak); (iv) *enabling of replicative immortality* by the up-regulation of the enzyme telomerase, which elongates replication-limiting, telomeric DNA-endings; (v) *induction of angiogenesis* to assure removal of waste products and supply with

essential nutrients and oxygen; (vi) *activation of invasion and metastasis* via down-regulation of adhesion molecules and genetic transition into migrating cells.

In 2011 two emerging hallmarks were added by the authors: *evasion of immune destruction* and *re-programming of energy metabolism* (5).

1.1.2 Tumor cell metabolism

In the 1920s Otto Warburg, a German scientist, reported increasing lactic acid concentrations in tumor cell cultures, resulting from accelerated glucose metabolism (6). This physiological process is well known from muscle cells switching from Krebs´ cycle (= tricarboxylic acid cycle; TCA cycle) derived energy production to glucose fermentation under oxygen limitation. Surprisingly, Warburg demonstrated, that tumor cells exhibited the same metabolic phenotype even in the presence of oxygen (= Warburg effect, aerobic glycolysis). Nowadays, this feature is used in diagnosis to detect tumor cells by fluorodeoxyglucose traced positron emission tomography–computed tomography (FDG-PET/CT) (7).

The highly glycolytic state is achieved by up-regulating the expression of **enzymes linked to glycolysis**. Hexokinase (HK), the first enzyme in the glycolytic pathway, and other glycolytic key enzyme such as phosphofructokinase (PFK) or lactate dehydrogenase (LDH) are elevated on transcriptional and protein level (8, 9). In a variety of tumors LDH-A is upregulated and its expression negatively correlates with patient survival (10). LDH-A degrades pyruvate to lactate thereby regenerating NAD^+ which is necessary to maintain the glycolytic flux (11).

Besides an up-regulated expression, also an altered activity, reaction rate and substrate specificity of glycolytic enzymes is observed and **tumor specific isoforms** of different enzymes are detected as a result of alternative splicing processes after transcription. For example in normal cells HK is expressed as isoform HK-I. Tumor cells, however, express the isoform HK-II, which in contrast is not negatively regulated by its product glucose-6-phosphate thus ensuring a continued glycolytic flux. Furthermore, HK-II is bound to the outer mitochondrial membrane thus (i) enabling an immediate access to mitochondrially produced ATP and (ii) preventing the apoptosis inducing release of cytochrome *c* (11, 12). Non-transformed cells

express the M1 isoform of the pyruvate kinase (PKM1). However, almost every tumor cell expresses the subtype M2 (PKM2), which has intrinsically a lower enzymatic activity and is impeded by different kinases. Consequently, glycolytic intermediates accumulate and promote the anabolic metabolism by flux into downstream pathways such as the pentose phosphate pathway. In addition PKM2 is described as a co-factor for transcription (11, 13).

Furthermore, the expression of **transporters linked to glucose metabolism** is increased in tumor cells. To ensure high intracellular glucose concentrations, the amount of insulin-independent glucose transporters (GLUT) is elevated on transcriptional level but also by increased surface trafficking of cytosolic transporters (14, 15). The transport of glucose across the lipid membrane by GLUT is energy independent. Especially GLUT1, GLUT3 and GLUT4 show a high affinity to glucose and ensure a sufficient glucose flux into cancer cells even under low-glucose conditions (11, 16). Accordingly, overexpression of GLUT1 has been related to poor prognosis in tumor bearing patients (17). The expression of monocarboxylate transporters (MCT) is also up-regulated in a variety of tumors (18). MCTs belong to the solute carrier 16 (SLC16) family (19) and transport lactate across the plasma membrane in co-transport with a proton thereby maintaining the intracellular pH and cell homeostasis (20). Notably, the MCTs bidirectionally transport substrates depending on the intra- and extracellular concentration gradients of protons and substrates as lactate (21). The most common monocarboxylate transporters in cancer cells are MCT1 and MCT4: While MCT1 is found in many tissues and transports a wider range of substrates (e.g. lactate, benzoate, butyrate), MCT4 in contrast is mainly expressed in highly glycolytic cells such as tumor cells. Accordingly, MCT4 exhibits higher Km values (MCT4: Km = 20 mM) compared to other members of the MCT family (MCT1: Km = 5 mM) enabling a sufficient export of lactate even under conditions of extensive production and preventing loss of pyruvate across the cell membrane (18, 22). Interestingly, MCT1 and MCT4 expression varies even within a tumor: while hypoxic areas preferentially express MCT4 to release lactate, normoxic regions take up and metabolize the monocarboxylate via MCT1 (23, 24). Furthermore, MCT surface expression is regulated by the matrix metalloproteinase inducer basignin (= CD147), which is required for the transport to

and insertion of MCTs into the cell membrane. Its activation in turn promotes matrix degradation, invasion and metastasis (25).

Initially, the Warburg phenotype was attributed to mitochondrial disorders, however even increased mitochondrial content and energy production have been observed in different tumors (26–28). Other reasons for the switch to glycolysis are currently discussed. Glycolysis yields only two molecules ATP from one molecule glucose compared to 36 molecules ATP gained by mitochondrial oxidative phosphorylation (OXPHOS). However, despite the lower yield of ATP, glycolysis is the most time-effective and least energy demanding process by which a cell can gain additional energy (29). When energy production is covered by glycolysis, the intermediates of the TCA cycle can be used for the generation of building blocks.

Thus accelerated glycolysis is necessary to accomplish the high energy demand of proliferating cells required for the production of biomass and to ensure DNA replication and cell division. Moreover, glycolytic intermediates are required for the generation of building blocks as shown in figure 1 (30).

The flux of glucose-6-phosphate to the pentose phosphate pathway (PPP) facilitates the synthesis of nucleotides (ATP, GTP, TTP, CTP) being essential for DNA replication and RNA transcription. Furthermore, $NADP^+$ is reduced to NADPH, which is important for the replenishment of the antioxidant glutathione and serves as a reducing equivalent for lipid biosynthesis. Glyceraldehyde-3-phophate, 3-phosphoglycerate and acetyl-CoA are the carbon sources for lipid biosynthesis. In addition, 3-phosphoglycerate is essential for amino acid biosynthesis of glycine and cysteine via serine (30).

Additionally, proliferating cells utilize the amino acid glutamine as alternative carbon- and essential nitrogen-source. Moreover, the degradation products of glutaminolysis replenish the pool of mitochondrial intermediates (= anaplerosis) and thereby - replacing glucose - maintain mitochondrial energy production. Vice versa, substrates of the TCA cycle are transported to the cytosol (= cataplerosis) and serve as precursors of the amino acids proline and arginine. Moreover, the intermediates α-ketoglutarate (αKG) and citrate are converted into acetyl-CoA by cytosolic ATP-citrate lyase (ACL) and used as the main building block of cholesterol and other lipids (figure 1).

First described in solid tumors, these alterations in metabolism are also observed in leukemia and lymphoma cells (31, 32)

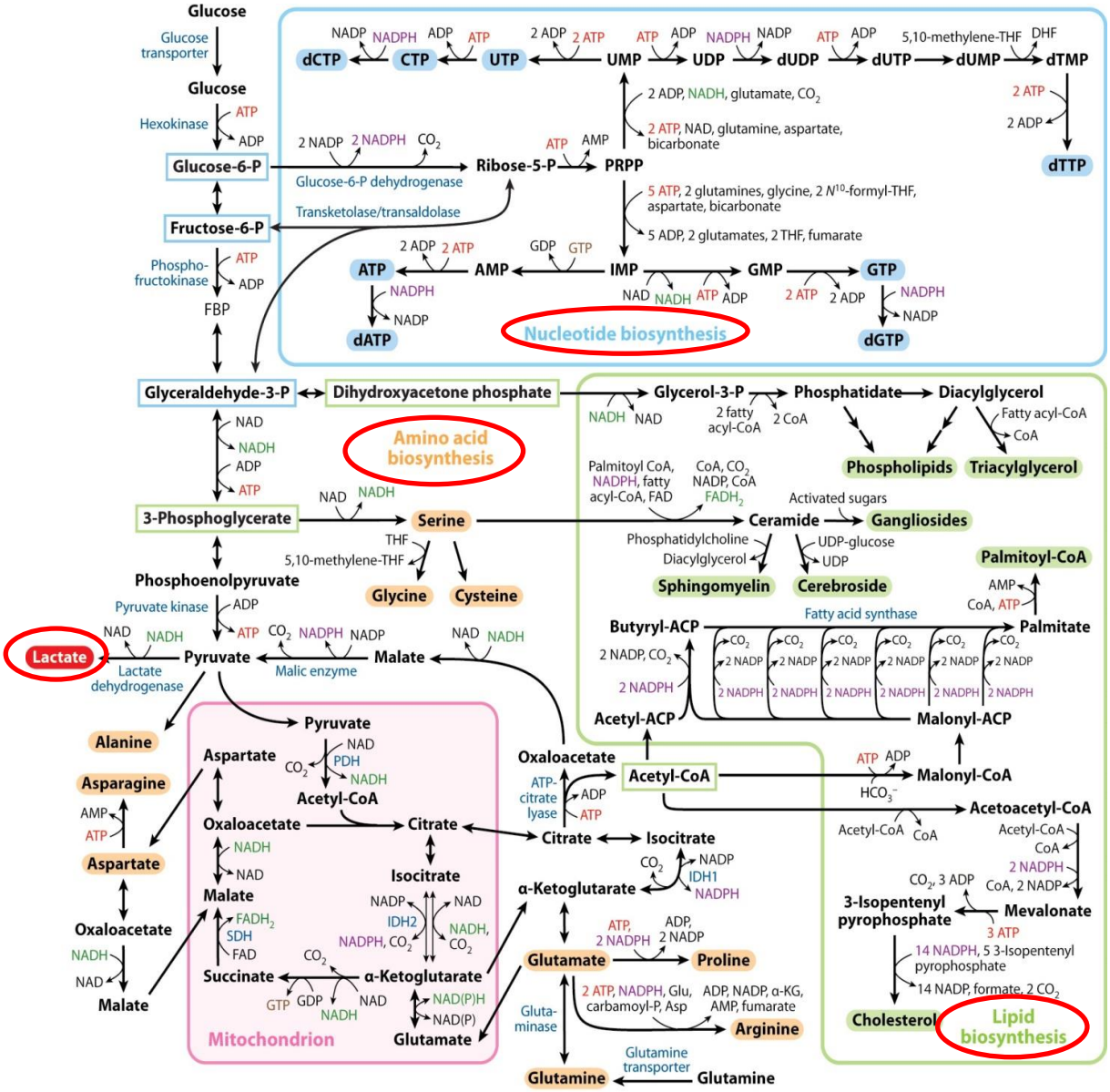

Figure 1. Glucose and glutamine metabolism of neoplastic cells
(modified after Lunt et. al, Annual review of cell and developmental biology, 2011 (30))

1.1.3 Regulation of tumor cell metabolism

As described above, the Warburg effect supports tumor growth and proliferation by providing essential building blocks and ATP. Mitogenic activation normally depends on the interaction of growth factors and their receptors. In cancer cells, however, pathways downstream of those receptors can be constitutively activated in the absence of extracellular stimuli by mutations, enhanced intrinsic activity or elimination of negative regulators. Moreover, it has been shown, that tumor suppressors and proto-oncogenes directly activate cell metabolism.

1.1.3.1 The PI3K/Akt/mTORC1 pathway

The PI3K/Akt/mTORC1 pathway is a complex signaling network and highly activated in many tumors (33). Phosphatidylinositol-3-kinase (PI3K), a lipid kinase, is activated by receptor protein tyrosine kinases (RPTK) and G protein coupled growth factor receptors like epidermal growth factor receptor (EGFR) or insulin-like growth factor receptor (IGFR). Upon activation PI3K phosphorylates phosphatidylinositol-2-phosphate (PIP2) to phosphatidylinositol-3-phosphate (PIP3). Proteins expressing pleckstrin homology domains (PH) such as 3-phosphoinositide dependent kinase 1 (PDK1) bind to PIP3 and activate the serine threonine kinase Akt by conformational change and phosphorylation. Conversely, phosphatase and tensin homolog (PTEN) dephosphorylates PIP3 to PIP2 and prevents overstimulation of Akt.

Akt is expressed in three different isoforms: Akt1, Akt2 and Akt3. All of them modulate numerous regulators of cell survival, cell cycle progression and metabolism on a transcriptional or post-translational level (34). By phosphorylation Akt increases the activity of the rate-limiting glycolytic enzyme HK (35). Moreover, the association of HK with the outer mitochondrial membrane (as described above) is promoted (36). The stimulation of PFK2 results in an accumulation of the allosteric PFK1 activator fructose-2,6-bisphosphate thus indirectly enhancing glycolysis (37). Glucose uptake is supported by trafficking of intracellularly stored GLUT1 to the plasma membrane, even though underlying mechanisms have not yet been fully understood (38). In addition, Akt phosphorylates and therewith inactivates pro-apoptotic factors such as

Bad, procaspase-9 or transcriptional factors of the death ligand Fas (39, 40). Importantly, Akt promotes the formation of the mammalian target of rapamycin complex 1 (mTORC1)(34). Besides the post-translational modulation also transcriptional effects are described: Akt and mTORC1 control the expression of almost all genes involved in glucose uptake (41) and glycolysis (42, 43) by the transcription factors HIF1α and c-Myc (see below). Akt activates the nuclear factor κB (NFκB) and the cyclic AMP response element-binding protein (CREB), both upregulating the expression of survival genes (44). Moreover, mTORC1 up-regulates the messenger RNA (mRNA) translation by the effector proteins S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E (eiF-4E)- binding protein 1 (4E-BP1) and accelerates the cellular turnover (45).

1.1.3.2 Hypoxia inducible factor 1α (HIF1α)

Different stimuli, such as Akt and mTORC1 activity, hypoxia or reactive oxygen species (ROS) inhibit the constant degradation of HIF1α by proteasomes. Dimerization with the constitutively expressed HIF1β protein leads to the activation of the HIF-complex. Upon heterodimerization the complex acts as a transcription factor on hypoxia-responsive elements (HREs) itself, but also regulates further transcription factors. Target genes upregulate glucose metabolism, cell differentiation and metastasis: activated HIF up-regulates GLUTs, glycolytic enzymes e.g. hexokinase (9) as well as LDH-A and pyruvate dehydrogenase kinase 1 (PDK1). This in turn restricts the uptake of glucose-derived pyruvate by mitochondria and decreases cellular respiration (46). As Ullah et al. show, MCT4 but not MCT1 is increased by HIF1α expression (47). Additionally, the vascular endothelial growth factor (VEGF) is an important target gene of the HIF-complex. VEGF induces angiogenesis and improves oxygen and nutrient supply, but also facilitates hematogenous metastasis of tumor cells. Furthermore, the matrix metalloproteinases 2 and 9 (MMP2, MMP9) are up-regulated and cell adhesion molecules (such as E-cadherin) down-regulated (48), which supports metastasis. There is evidence, that HIF promotes the de-differentiation of cancer cells and prevents differentiation of progenitors (49, 50).

1.1.3.3 c-Myc amplification

Myc is a transcription factor, which regulates the expression of a variety of human genes upon dimerization with the protein Myc-associated factor x (Max). Myc is the product of the c-Myc gene (cellular myelocytomatosis gene) and amplification is caused either by an activating mutation, by oncogenes inducing gene expression or by a decreased protein degradation as a result of an inefficient ubiquitination (51). Especially, the growth factor receptor-associated phosphorylation cascade Ras (rat sarcoma) – Raf (rat fibrosarcoma) – MEK (Mitogen/Extracellular signal-regulated kinase) – ERK (extracellular signal-regulated kinase) and the PI3K/Akt/mTOR pathway promote the induction of Myc expression (52). Amplified Myc results in the overexpression of glucose transporters and several glycolytic enzymes such as HK, PFK, LDH-A and PDK. Myc also initiates the alternative splicing of pyruvate kinase to PKM2 (53). Beyond that, Myc supports the upregulation of glutaminolysis by increasing the expression of amino-acid transporter 2 (ASCT2) and glutaminase (GLS), catalyzing the reaction of glutamine to glutamate (13, 52). As a consequence cells become glutamine dependent, which intriguingly leads to apoptosis in case of glutamine withdrawal in Myc overexpressing cells (54). Myc also stimulates ribosome biogenesis and increases nucleotide biosynthesis by expression of several PPP enzymes (52). Myc regulates cell cycle progression by increased transcription of cyclin dependent kinases (Cdks), cyclins or associated transcription factors like E2F and the antagonism of the cell cycle inhibitors $p21^{Cip}$ and $p27^{Kip}$ (55). Cyclins enable the transition of G_1- (gap 1 phase) to S-phase (synthesis phase) in the cell cycle and support proliferation (56).

1.1.3.4 Loss of p53 expression

p53, also known as “the guardian of the genome”, is a tumor suppressor accumulating intracellularly as a consequence of DNA damages like strand brakes. Thereupon, DNA repair systems are activated while cell cycle is arrested. Failure of DNA repair leads to increasing p53 levels and the induction of apoptosis by caspases (57). Moreover, p53 also affects cell metabolism by blocking glycolysis via the protein

TIGAR (TP53-induced glycolysis and apoptosis regulator), which impedes the enzymatic reaction of fructose-6-phosphate to fructose-2,6-bisphophate (58). On the other hand p53 enhances mitochondrial enzyme synthesis as cytochrome c oxidase 2 (SCO2) expression, which in turn increases oxidative phosphorylation (OXPHOS) (59, 60). A loss of function mutation in the p53 tumor suppressor gene promotes cell cycle and enhances glycolysis (8).

1.1.4 Inhibition of tumor glucose metabolism – a promising therapeutic strategy?

As tumor cells - in contrast to non-malignant cells – display a highly elevated glycolysis, inhibition of this metabolic pathway is an emerging therapeutic strategy. Glycolytic restriction affects tumor cells in many ways: (i) reduced energy supply resulting in increased (chemo-/radio-) therapeutic sensitivity (61–63), (ii) growth inhibition due to lack of necessary building blocks and (iii) diminished extracellular lactate accumulation. There is growing evidence that lactic acid is not only the end product of aerobic glycolysis but also represents an alternative fuel for respiring tumor cells. Tumor-associated cells, such as fibroblasts, can also metabolize lactate instead of glucose, which prevents a competition for nutrients between tumor, tumor-associated and immune cells (23, 64). Furthermore, the acidic tumor milieu alters immune cell function and the extracellular matrix and promotes invasion and metastasis (65). Numerous studies showed a correlation between pH level and drug resistance of tumor cells (20). As shown by Fischer et al., high extracellular lactic acid concentration blocks the secretion of lactate by activated human T cells. This results in an impaired T cell function thereby contributing to the immune escape of tumor cells (66, 67). Additionally, lactic acid decreases the ability of monocytes to produce the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) playing an important role in the anti-tumor immune response (68). Furthermore, Colegio et al. demonstrated, that tumor-derived lactate is taken up by tumor associated macrophages (TAMs) and leads to an increased expression of the vascular endothelial growth factor (VEGF) and arginase 1 - both tumor promoting factors (69). This implies, that lactate acts not only as an immunosuppressor, but is capable to

recruit and support pro-tumorigenic immune cells fostering tumor growth, maintenance and metastasis (70).

Interfering with the glycolytic pathway is possible on different levels: (i) on the level of signal transduction and transcription and (ii) on the protein level of enzymes and transporters of the metabolic pathway itself.

(i) Signaling pathways involved in the regulation of metabolism are already targeted by many substances resulting in an interrupted growth and proliferation or even the induction of apoptosis in tumor cells:

Growth factor receptor: The monoclonal antibody cetuximab impedes EGF-receptor mediated growth signaling via the PI3K/Akt/mTORC1 pathway, which is successfully applied in the treatment of colorectal cancer (71, 72).

PI3K/Akt: Akt inhibition via perifosine in combination with other Akt inhibitors is in the focus of new therapeutic strategies for T-ALL (73).

mTORC1: rapamycin targets mTORC1 and thereby diminishes its transcriptional effects on genes up-regulating glycolysis. Additionally, the antidiabetic drug metformin induces AMP-kinase (AMPK), which indirectly inhibits mTORC1 (74).

HIF1: hypoxia and other factors stabilize the transcription factor subunit HIF1α (as shown above). For activation necessary dimerization with the β-subunit can be blocked by the drug acriflavine (75).

(ii) Glucose metabolism can be targeted on several steps and inhibitors of glycolytic enzymes as well as of transporters are currently under investigation (figure 2). The first step in glucose metabolism is the uptake of glucose by different transporters. GLUT1, overexpressed in many tumor cells (14), is targeted by the drugs phloretin (76) and fasentin (77). Furthermore, hexokinase is impeded by various substances such as lonidamine (78), 3-bromopyruvate (79) or 2-deoxyglucose (2DG) (11, 80). 2DG, a glucose analogue, is converted into 2-deoxyglucosephosphate (2DG-P), which cannot be further metabolized. Its accumulation leads to a feed-back inhibition and a subsequent glycolytic arrest (81). 2DG application results in a release of hexokinase II (HK II) from the outer mitochondrial membrane, affecting the integrity of mitochondria and their function (63, 82). Similar effects are achieved by the inhibition of phosphofructokinase and pyruvate kinase (11). Another approach is the blockade

of pyruvate dehydrogenase kinase 1 (PDK1) by dichloroacetate (DCA), which reduces its inhibitory effect on pyruvate dehydrogenase (PDH). This results in a regained flux of pyruvate into the TCA cycle, increased OXPHOS and a drop in lactate excretion (83). Next, lactate dehydrogenase (LDH) is impeded by FX11 and oxamate, which leads to ATP depletion and a reduced chemotherapeutic drug resistance (84, 85). Additionally, the close cooperation of LDH with the transcription factor Oct-4 (Octamer binding transcription factor 4) is compromised (86). Blockade of monocarboxylate transporters MCT1 and MCT4 has anti-tumorigenic effects by blocking lactate efflux which results in an intracellular acidification and cell death (87, 88). A specific inhibitor of MCT1 (AR-C155858) was developed by AstraZeneca (89) and effectively blocks growth of transformed fibroblasts (90). Another MCT1 inhibitor (AZD3965) induces cell death of breast cancer and Burkitt lymphoma cell lines (88). Importantly, efficient inhibition of glycolysis implies simultaneous blockade of both MCT transporters (22, 90).

Recently it has been shown that diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is a potent glycolytic inhibitor in several tumor cells (melanoma and histiocytoma cells). The observed effects are independent of cyclooxygenase (COX) inhibition, as neither the unspecific COX-inhibitor aspirin nor the selective COX-2 inhibitor NS-398 showed an effect on proliferation and lactate production (91).

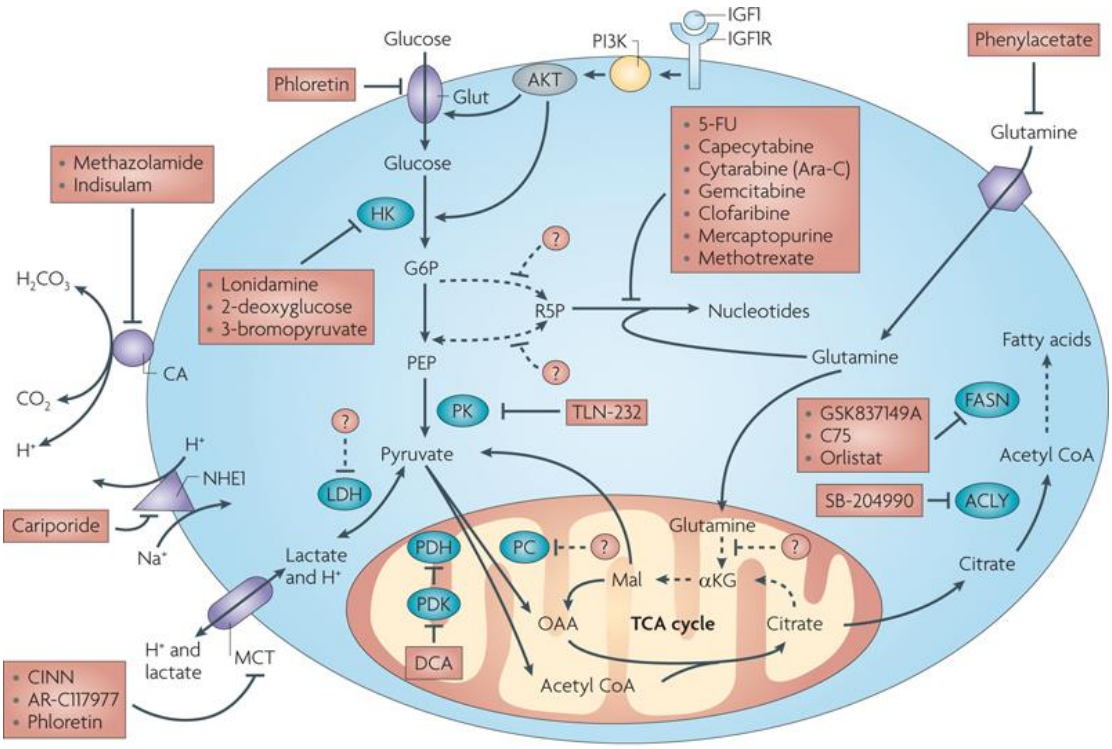

Figure 2. Targets and appropriate anti-metabolic compounds already used in treatment of tumors (by Tennant et al., Nature Reviews Cancer, 2010 (80), reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, © 2010)

At first glance, inhibition of glycolysis seems to be a selective therapeutic strategy for tumor cells. However, many of the above mentioned targets are ubiquitously expressed in non-malignant cells (e.g. GLUT and glycolytic enzymes), so that blockade of this metabolic phenotype may hit other glucose-dependent cells.

Several studies show a comparable metabolic switch in T lymphocytes upon activation (92, 93), which raises concerns of a reduced immunofunction under anti-glycolytic treatment. As T cell infiltration and activation in the tumor tissue are correlated with a better prognosis (3, 94, 95), anti-glycolytic drugs might compromise T cell function thus negatively affecting patient outcome. However, the inhibition of glycolysis results in reduced tumor cell proliferation, viability and concomitantly lowered extracellular lactic acid concentration. Consequently, the anti-tumor immune response might be improved. In summary, the overlapping metabolic profile and complex interactions between tumor and immune cells challenge the application of anti-glycolytic therapies for tumor therapy.

1.2 Activation, effector function and metabolism of human T lymphocytes

The human immune system is challenged by a variety of different pathogens. Besides the unspecific response of the innate immune system, a successful defense against infections or degenerated cells depends on the adaptive immune response. Naive cells surveil the tissue and blood using a wide variety of different and unique recognition receptors and, upon activation, undergo massive clonal expansion to ensure pathogen clearance. To do so, immune cells activate several important metabolic pathways to meet their demands.

1.2.1 Activation of T lymphocytes

To proliferate and differentiate, T cells rely on antigen recognition, co-stimulation and cytokines. Antigen recognition is the first step of activation ensuring a specific immune response. Therefore, the presentation of foreign proteins by major histocompatibility antigens (MHC) is mandatory and two different complexes, MHC I and MHC II, recruit specific subpopulation of T cells. Upon recognition of unspecific microbial patterns, dendritic cells (DCs) or macrophages in the periphery take up and process foreign extracellular antigens (for example bacteria or fungi). After degradation, antigen fragments consisting of up to 25 amino acids (AA) are presented by the major histocompatibility complex II (MHC II), which is only expressed on professional antigen presenting cells (APCs) like DCs or macrophages. The activated APCs thereupon migrate to secondary lymphoid tissues such as lymph nodes or the spleen, where circulating naïve $CD4^+$ lymphocytes (= T helper cells, T_h cells) expressing CD4-assosciated T cell receptors (TCRs) are stimulated. In contrast, $CD8^+$ T cells (= cytotoxic T cells, CTLs) are activated by the major histocompatibility complex I (MHC I) expressed by every nucleated human cell. In the course of the turnover of cellular proteins, short amino acid chains (< 10 AA) are connected with the histocompatibility complex and presented at the cell surface. Virus- or tumor-caused alterations in those patterns are detected by specific, CD8-associated TCRs and result in an activation of the CTL. Similar to $CD4^+$ T cells, activation of $CD8^+$ T cells can be also initiated by antigen presenting cells, which

requires a pathway called “cross-presentation”: for reasons, which have not yet been entirely explained, APCs are able to ingest infected cells or secreted tumor- or virus-proteins and load to MHC I instead of MHC II. Furthermore, cytokines produced by $CD4^+$ T cells also recognizing the respective pathogen (see below) are able to enhance or even initiate the differentiation of CTLs. It is assumed, that especially CTLs coping with latent viral infections or tumors eliciting a limited innate immune response depend on the interaction with both, APCs and $CD4^+$ helper cells (81).

The T cell receptor complex consists of (i) the receptor part with two highly varying, via disulfide bonds connected chains (α- and β-chain) recognizing the cell-specific, MHC-bound antigen, and (ii) the non-covalently associated CD3- and ζ-proteins, which transduce the stimulation signal. The CD4- respectively CD8-molecules are transmembrane glycoproteins of the Ig superfamily, which bind to non-polymorphic sections of the MHC and are responsible for the MHC-restriction of the particular cell (CD4 to MHC II, CD8 to MHC I). Intracellular kinases connected to these molecules enable the signal transduction by phosphorylation of CD3- and ζ-proteins (81).

Besides the TCR stimulation, further co-stimuli are necessary to fully activate T cells. These additional signals are cell-to-cell- or cytokine transmitted and depend on a prior APC stimulation. The TCR stimulation without co-stimulation results in anergy, describing a cellular hyporesponsiveness to a further T cell receptor stimulation.

Binding of CD28, expressed on the surface of T cells, by the APC ligands B7-1 and B7-2 (= CD80 and CD86) amplifies or even initiates the signal transduction especially via the activation of the PI3K/Akt pathway (96). Its importance is underlined by the fact, that knock-out of or mutations in genes encoding these proteins result in severe immune deficiency (17, 81). While about 80 % of $CD4^+$ T cells express CD28, only about 50 % of $CD8^+$ T cells express this co-stimulatory ligand (97). Also the CD40 ligand (CD40L), a tumor necrosis factor superfamily membrane protein on the helper T cell surface, binding the receptor CD40 on the APC surface leads to an increased B7-1/B7-2 expression and cytokine production thus providing a positive feedback loop. This process called licensing additionally enhances CTL differentiation (81).

Besides the membrane bound co-stimulatory molecules also humoral factors support and enhance the activation of lymphocytes. The most important cytokine for T cell stimulation is IL-2, which is early produced by innate immune cells and later

synthesized and secreted by $CD4^+$ T helper cells, acting in an auto- as well as paracrine manner. The secretion peak is reached 8-12 hours upon antigen recognition and IL-2 interacts with the type I cytokine receptors. IL-2 binding to the IL-2 receptor α (IL2-Rα, CD25) supports cell cycle progression and proliferation, survival and secretion of effector proteins such as IFNɣ (81, 92). Other cytokines playing a crucial role in T cell stimulation are IL-12, IL-15 and IL-21.

Upon ligation of the TCR and co-stimulation, the PI3K pathway is activated. The consecutive activation of the downstream kinase Akt contributes to cell survival by inhibition of members of the Bcl-2 family. Also the MAP (mitogen-activated protein) kinase cascade, involving Ras/Raf/MEK/ERK, is initiated and results in the synthesis and activation of the transcription factor activator protein 1 (AP-1). The TCR-dependent activation of the enzyme phospholipase C ɣ1 (PLCɣ1) catalyzes the reaction of the membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP2) into the soluble inositol-1,4,5-triphosphate (IP3) and diacyl glycerol (DAG). IP3 leads to an increase of cytosolic calcium and via calmodulin and calcineurin to the activation of the transcription factor nuclear factor of activated T cells (NFAT), whereas DAG recruits the protein kinase C (PKC) and therewith activates the nuclear factor κB (NFκB). These transcription factors are responsible for the expression of anti-apoptotic proteins, enhanced proliferation, increased cytokine production as well as differentiation of naive T cells into effector and memory cells (81).

Besides activating co-receptors also inhibiting counterparts (= immune checkpoints) play a crucial role in balancing the immune response avoiding both immunodeficiency and autoimmune reactions. B7-1 and B7-2, ligands on the surface of APCs, are bound by the membranous T cell molecule cytotoxic T lymphocyte antigen 4 (CTLA-4, CD154), which is also engaged upon activation and shows a considerably higher affinity to B7 compared to the CD28 receptor. Especially when B7 levels are low, this connection leads to a negative regulation of the immune response and self-tolerance. A similar effect is achieved by the connection of the programmed death ligand 1 (PD-L1) on APCs to its receptor programmed death 1 (PD-1) on T cells. These mechanisms mediate the inactivation of the immune system to prevent systemic damage by overstimulation or the development of autoimmune reactive T cell clones by MHC-presentation of autologous peptides without expression of co-stimuli (81).

1.2.2 Effector functions of activated T cells

T cells are responsible for the cell-mediated part of the adaptive immune response. $CD8^+$ T cells directly eliminate infected and transformed cells by apoptosis inducing ligands and cytotoxic proteins. In contrast, $CD4^+$ T cell subpopulations attract, control and activate innate immune cells like macrophages and neutrophils by cytokine secretion and surface molecule expression. Depending on the particular T cell subtype different leukocytes are recruited. Additionally, $CD4^+$ T cells can acquire a cytotoxic phenotype contributing to lysis of malignant or infected host cells (98, 99).

$CD8^+$ T cells (CTLs) directly attack every kind of cell harboring intracellular microbes, but also neoplastic cells. Following differentiation and activation in secondary lymphoid organs, CTLs enter infection sites and bind to target cells presenting specific virus- or tumor-altered cellular proteins by class I MHC. After binding to the MHC I, the immunologic synapse is formed by the adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 1 (LFA-1). This close connection prevents damage of healthy neighboring cells. CTLs secret perforin and granzymes, which perforate the target cell membrane and induce apoptosis. Furthermore, CTLs express the membrane protein Fas ligand (FasL, CD95), which binds the ubiquitously expressed death receptor Fas and also mediates cell death via caspase activation (81).

To avoid recognition and elimination, tumor cells engage many mechanisms. As T cell activation relies on the MHC presentation, many tumors show downregulated MHC I expression to get “invisible” to CTLs. Another strategy is the suppression of T cell activation by upregulated PD-L1 expression. Furthermore, tumor antigens can be presented on MHC II but with low B7 levels on the APCs. This combination supports the CTLA-4- instead of the CD28-binding by B7 family members also inhibiting T cell activation. Additionally, tumor produced humoral factors such as transforming growth factor β (TGFβ) prevent T cell proliferation and effector functions (81).

Activated $CD4^+$ T cells differentiate in a variety of subpopulations: T_h1, T_h2 and T_h17 cells, displaying different functions.

T_h1 cells are mainly activated by APCs secreting IL-12 upon pathogen recognition. The T_h1 subpopulation is characterized by production and secretion of IFNγ

supporting macrophage activation and the killing of phagocytosed microbes by reactive oxygen species (ROS) and lysosomal enzymes. Beyond that IFNɣ-activated macrophages secret chemokines to recruit innate immune cells and produce IL-12 to amplify the T_h1 response. An IFN-mediated antibody switch of IgG to subclasses promoting opsonization and phagocytosis of extracellular microbes supports this mechanism. Additionally, IFNɣ increases the MHC expression and MHC-mediated pathogen presentation leading to a stronger activation thereby resulting in a positive feedback loop.

T_h2 cells in contrast are primarily stimulated by allergens and chronic inflammations without innate immune cells. These cells coordinate an immune response performed by mast cells, basophils and eosinophils via secretion of IL-4, IL-5 and IL-13. Thereby, IL-4 and IL-13 regulate the antibody switch to IgE, whereas IL-5 stimulates eosinophils. Further on, especially IL-13 increases the mucosal barrier function by stimulation of mucus production.

Immune response mediated by T_h17 cells is stimulated by extracellular fungi and bacteria, which are recognized and presented via the MHC II by APCs. IL-17 produced by T_h17 cells activates neutrophils and monocytes.

$CD4^+$ T helper cells enhance the antitumor response by production of cytokines which are necessary for the differentiation of $CD8^+$ effector cells. Additionally, secretion of IFNɣ and TNFα increases MHC I expression in target cells thus supporting recognition by CTLs (81).

1.2.3 Metabolism of T lymphocytes

1.2.3.1 Metabolism of quiescent T cells

As shown in figure 3, quiescent cells cover their energy demand utilizing glucose and fatty acids to generate ATP by oxidative phosphorylation (OXPHOS) (100, 101). This metabolic phenotype is not static, but relies on different growth signals to prevent apoptosis and maintain intracellular glucose concentrations. A permanent, weak T cell receptor (TCR) signal is necessary to maintain a critical amount of GLUT1 on the surface and oxidative energy production by stimulation of mitogen-activated protein kinase (MAPK) and AMP-kinase (AMPK) pathways (92).

Besides the TCR signals also interleukin 7 (IL-7) prevents cell death via stabilizing the balance between pro- (Bim) and anti-apoptotic members (Bcl-2 and Mcl-1) of the B cell lymphoma 2 family (102) by binding to the IL-7 receptor (IL-7R). This homeostasis is a critical process to avoid both, immunodeficiency by lacking of adequate cell numbers and autoimmune diseases by uncontrolled proliferation. Additionally, also IL-7 promotes the glucose metabolism and glycolysis by expression and the surface trafficking of GLUT1 and therewith increased glucose uptake via the activation of the januskinase (Jak)/signal transducer and activator of transcription 5 (STAT5) and the PI3K/Akt pathway. In contrast to activated T cells, quiescent cells show a delayed, but at low levels constant Akt activation (103).

1.2.3.2 Metabolism and function of activated T cells

Upon activation **murine T cells** immediately increase the expression of various surface molecules as well as the secretion of cytokines, followed by a massive clonal expansion. For this reason, stimulated T cells have a high demand for biomolecules (DNA, lipids, amino acids) required for cell growth, proliferation and production of effector proteins. To meet this demand increased metabolic activity is necessary (104) and T cell metabolism shifts, as shown in figure 3 (101), from OXPHOS to glycolysis to support the highly proliferative phenotype (105). Binding of the TCR complex and co-stimulatory receptors results in increased PI3K/AKT/mTORC1, AMPK and MAP kinase activity, regulating transcription factors such as c-Myc or

NFAT (106, 107). Glycolysis, glutaminolysis and mitochondrial activity are upregulated by expression of rate-limiting enzymes and the surface trafficking of appropriate transporters (96, 108, 109). The metabolic profile of activated T cells closely resembles the profile observed in tumor cells.

Frauwirth et al. demonstrated, that CD28 co-stimulation is crucial for the enhancement of glucose metabolism in T cells by upregulation of the PI3K/Akt pathway (96). This results in increased GLUT1 and GLUT3 levels to foster glucose up-take required for the replenishment of intracellular building blocks (110) as well as elevated activity of glycolytic enzymes e.g. HK and PKM2 (101). Neither TCR/CD3 stimulation alone nor IL-2 binding to IL-2 receptors are able to induce and maintain a comparable metabolic state in T cells (96). Interestingly, the CD28 ligation alone does not induce glucose metabolism and stimulation of further, TCR-associated pathways, such as the MAP kinase cascade, is required. Especially ERK is necessary to increase the hexokinase activity (111). Furthermore, it is well-known, that some of the glycolytic enzymes are able to affect genetic transcription or stabilize transcriptional factors, respectively, representing a linkage between the T cell effector function and metabolism (112). Similar to tumor cells, glucose serves as a carbon source for nucleotides and NADPH redox equivalents via the pentose phosphate pathway as well as amino acid synthesis. Provision of these building blocks is necessary for the proliferation (111, 113) and inhibition of glycolysis by 2DG results in a significant proliferation arrest of murine T cells (107, 114, 115). Furthermore, in the murine system a strong link between the production of the effector cytokine interferon ɣ and glycolysis is reported (113, 116, 117). Glucose regulates the dissociation of the enzyme glyceraldehyde-3-phophate dehydrogenase (GAPDH) from the 3´ UTR (= three prime untranslated region) of IFNɣ, which enables the translation of IFNɣ (118). Accordingly, it is shown that 2DG inhibits the IFNɣ production (119). Cham and Gajewski demonstrated that 2DG also reduces IL-2 production in murine T cells (120). On the other hand it has been shown that glucose starvation affects whether IL-2 production by murine $CD4^+$ cells nor cytotoxic activity of murine $CD8^+$ T cells (113, 121).

Upregulated glutamine uptake replenishes the TCA cycle, supports maintenance of mitochondrial membrane potential and acts as a biomolecular precursor of lipids and amino acids (92, 104, 122–125). Inhibition of glutaminolysis is shown to impede

murine T cell growth and proliferation (126), whereas IFNɣ and IL-2 are produced in glutamine free environment. However, presence of glutamine results in a further stimulation (127, 128).

Studies analyzing the importance of mitochondrial energy production for proliferation and effector functions of activated T cells are contradictory. Mitochondrial ATP seems to be important for proliferation and memory T cell development (118). Also, IL-2 production depends on OXPHOS and reactive oxygen species (ROS) generation (129). However, the inhibition of mitochondrial respiration has no impact on the IFNɣ production in murine $CD4^+$ T cells (118), whereas the blockade in murine $CD8^+$ T cells is shown to distinctly reduce interferon secretion (130).

The link between metabolism and function of **human** T lymphocytes is much less elucidated, but numerous aspects show significant differences between the murine and the human immune system (131). For example, glucose restriction results in severely impaired proliferation but preserved IFNɣ production in human T cells, whereas murine T cells cannot maintain interferon secretion. Glycolytic inhibition by 2DG in contrast affects effector functions (132). Upon activation, increased glucose and glutamine uptake are linked to proliferation and cytokine production (96, 108, 133). However, human memory $CD8^+$ T cells rely on an early glycolytic switch to ensure a sufficient IFNɣ production (114), whereas human $CD4^+$ T cells maintain their functions even under conditions of energy restriction (134). Taken together, the link between metabolism and effector functions is still not clear.

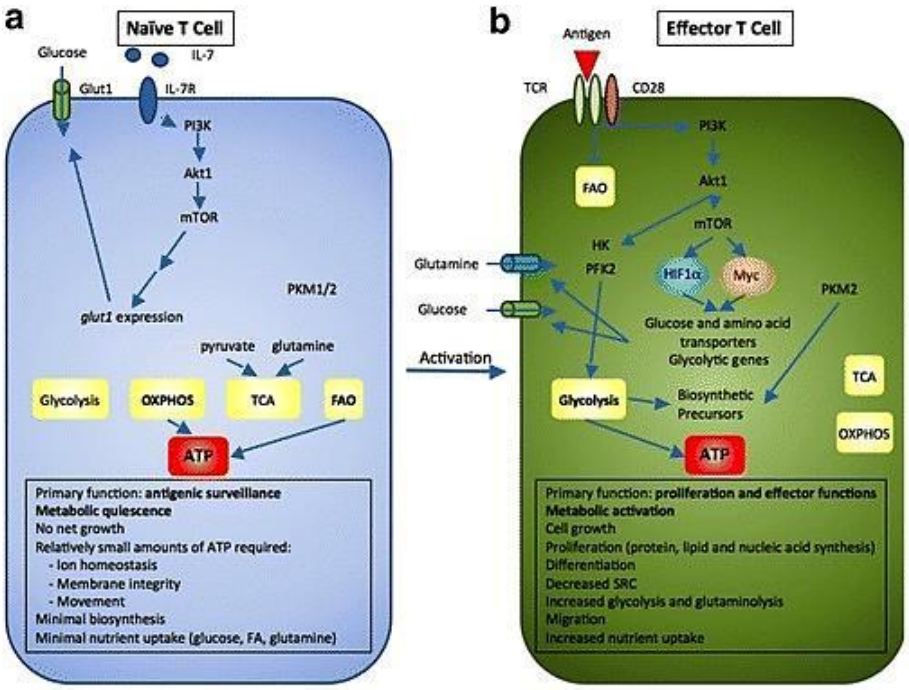

Figure 3. Metabolic re-programming during T cell activation
(a) Naïve T cells surveil secondary lymphoid tissues. Therefore necessary energy is mainly produced by OXPHOS and fatty acid oxidation (FAO). Low levels of IL-7 binding to IL-7 receptor (IL-7R) ensure adequate amounts of surface glucose transporters via PI3K/Akt/mTOR pathway. (**b)** Upon activation T cells undergo a massive clonal expansion. TCR/CD28-mediated pathways inhibit FAO and OXPHOS, while upregulated glucose transporters and glycolytic enzymes maintain high levels of important building blocks (modified after Herbel et al., Clinical and translational medicine, 2016, reprint permitted by the authors (101))

2. Research objectives

One of the main aspects of nowadays cancer research is the development of targeted therapies to reduce treatment related adverse side effects and increase therapeutic efficacy. Therefore, characteristics distinguishing tumor cells from non-malignant cells are under investigation. In this context also the altered tumor cell metabolism is under consideration. Most solid tumors but also leukemic cells are characterized by an upregulation of glucose metabolism. Its inhibition is shown to effectively impair tumor growth and viability. Moreover, anti-glycolytic treatment reduces the secretion of the immunosuppressive metabolite lactic acid. Taken together, glucose metabolism represents a promising therapeutic target.

However, it has been shown, that murine primary T cells also rely on glucose metabolism to sustain proliferation and effector functions. In line, glycolytic inhibition impairs proliferation and secretion of key cytokines such as IFNɣ. As the number and activity of tumor infiltrating T cells positively correlate with patient prognosis, anti-glycolytic treatment might severely affect patient outcome.

The aim of this study was the development of an anti-glycolytic treatment, effectively targeting tumor glucose metabolism, but preserving effector functions of human T cells. Data on the link between metabolism and human T cell function are rather sparse. In this study, therefore, glucose metabolism was analyzed in relation to function of stimulated human $CD4^+$ and $CD8^+$ T cells. In addition, the impact of two glycolytic inhibitors, 2DG and diclofenac, on proliferation and viability of malignant T lymphocytes was investigated in comparison to primary $CD4^+$ and $CD8^+$ T cells. Finally, effects of glycolytic inhibitors on effector functions such as IFNɣ production of human T cell populations were examined.

3. Material and methods

3.1 Material

3.1.1 Devices

Advia 1650	Bayer HealthCare, Tarrytown (NY, USA)
CASY cell counter	Roche Innovartis, Bielefeld
EMax Precision microplate reader	Molecular Devices, Sunnyvale (CA, USA)
FACS Calibur	BD Bioscience, Heidelberg
Incubator (BBD 6220)	Heraeus, Osterode
Multifuge 3S-R	Thermo Scientific, Waltham
Multifuge 3.0R	Thermo Scientific, Waltham
Sepatech Megafuge 1.0	Heraeus, Osterode
Sepatech Megafuge 3.0 cooling centrifuge	Heraeus, Osterode
Thermo VarioSkan	Thermo Scientific, Waltham
Vortex Genie 2	Scientific Industries, Bohemia (NY, USA)

3.1.2 Pipettes, plates, flasks, tubes

Research Plus (pipettes), 10/100/1000 µl	Eppendorf AG, Hamburg
Serological Pipettes, 1/2/5/25 ml	Nerbe plus, Winsen
Pipetboy acu	Integra Bioscience, Zizers (CH)
Polystyrene Round-Bottom Tubes 5 ml (FACS tubes)	Corning Science, Tamaulipas (Mexico)
Tube Racks 15/50 ml	Greiner Bio-One, Kremsmünster (AUT)
96 well plates U-/flat-bottom	Costar, Corning

3.1.3 Medium, buffers, solutions

Annexin binding buffer	BD, Franklin Lakes (NJ, USA)
Aqua Ecotainer	B. Braun, Melsungen
FACSclean	BD, Franklin Lakes (NJ, USA)
FACSflow	BD, Franklin Lakes (NJ, USA)
FACSrinse	BD, Franklin Lakes (NJ, USA)
Lymphocyte separation solution (Ficoll)	PAA, Linz (AUT)
Phosphate buffered saline (PBS)	Biochrome, Berlin
Dulbecco´s PBS without Ca^{2+}/Mg^{2+}	PAA

T cell medium

500 ml RPMI 1640	PAN Biotech, Aidenbach
5 ml instable L-glutamine	Biochrome, Berlin
5 ml Nonessential amino acids	Gibco
5 ml Sodium pyruvate	Gibco
0.5 ml β-mercaptoethanol	Gibco
2.5 ml Penicillin/Streptomycin (10.000U/ml)	Gibco
10 % AB-Serum, human	PAN Biotech, Aidenbach

Tumor cell (C7H2) medium

500 ml RPMI 1640	PAN Biotech, Aidenbach
2 ml instable L-glutamine	Biochrome, Berlin
10 % FCS	Gibco

DC medium

500 ml RPMI 1640	PAN Biotech, Aidenbach
1.0 % instable L-glutamine	Biochrome, Berlin
2.5 ml Penicillin/Streptomycin (10.000U/ml)	Gibco
10 % FCS (fetal calf serum)	Gibco

FACS staining buffer

PBS + 2 % AB-serum

MACS buffer

PBS + 2 mM EDTA + 1 % FCS

3.1.4 Cell separation

QuadroMACS® separator	Miltenyi Biotec, Bergisch Gladbach
LS Columns	Miltenyi Biotec, Bergisch Gladbach
CD4 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach
CD8 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach

3.1.5 Apoptosis staining

Dye	Company	Vol./test	Material number
FITC Annexin V	BD	5 µl	556419
7-AAD	BD	20 µl	559925

3.1.6 Antibodies and isotypes

Anti-human antibody	Conjugate	Company	Clone	Isotype (Mouse)	Vol./test	Material number
CD4	PE	BD	RPA-T4	IgG1, κ	5 µl	561844
CD8	PE-Cy7	BioLegend	SK1	IgG1, κ	10 µl	344711
CD25	PE-Cy7	BD	M-A251	IgG1, κ	5 µl	557741
CD95	FITC	BD	DX2	IgG1, κ	20 µl	561975
CD137	PE	eBioscience	4B4	IgG1, κ	5 µl	12-1379

Isotype (Mouse)	Conjugate	Company	Clone	Vol./test	Material number
IgG1, κ	FITC	BD	MOPC-21	20 µl	555909
	PE-Cy7	BioLegend	MOPC-21	20 µl	400126
	PE	BD	MOPC-21	20 µl	555749

3.1.7 Kits, cytokines

Dynabeads® Human T-Activator CD3/CD28	Gibco/Invitrogen
Human IFN gamma DuoSet ELISA	R&D Systems, Wiesbaden
Human IL-2 DuoSet ELISA	R&D Systems
Human IL-10 DuoSet ELISA	R&D Systems
Glucose (HK) Assay Kit	Sigma, St. Louis (USA)
IL-2	PeproTech, Hamburg

3.2 Methods

3.2.1 Tumor cell line cultivation

As representative of a malignant T-ALL cell line CEM-CCRF-C7H2 cells, first isolated by Norman and Thompson in 1977 (135), was used. These cells were cultivated in tumor cell medium at a starting concentration of 300.000 cells per ml in a total volume of 20 ml. Incubation was performed in a humidified atmosphere (5 % CO_2, 95 % air) at 37° Celsius and cells were split every second day.

3.2.2 T cell isolation, stimulation and cultivation

Human peripheral blood mononuclear cells (PBMCs) were separated from blood of healthy donors by leukapheresis via a density gradient centrifugation (2000 rpm, 25 min, room temperature) over Ficoll/Hypaqua and subsequent countercurrent centrifugation (elutriation). Cells were collected from the interphase and washed with PBS (two times 1800 rpm, 7 min, 4°C, third time 1200 rpm, 7 min, 4°C). The study was approved by the local ethical committee and all human participants gave written informed consent.

$CD4^+$ and $CD8^+$ T cells were isolated by magnetic separation. Therefore, 10^8 monocyte-depleted PBMCs were solved in 160 µl MACS buffer and incubated with 40 µl magnetic anti-CD4 or anti-CD8 MicroBeads. After incubation, cells were washed with MACS buffer, centrifuged and resuspended. The cell suspension was applied on LS columns and magnetically separated by a MACS separator. After separation, purity of populations was determined by anti-CD4 and anti-CD8 staining and analyzed by flow cytometry. Thereby a purity of more than 98 % was achieved (figure 4).

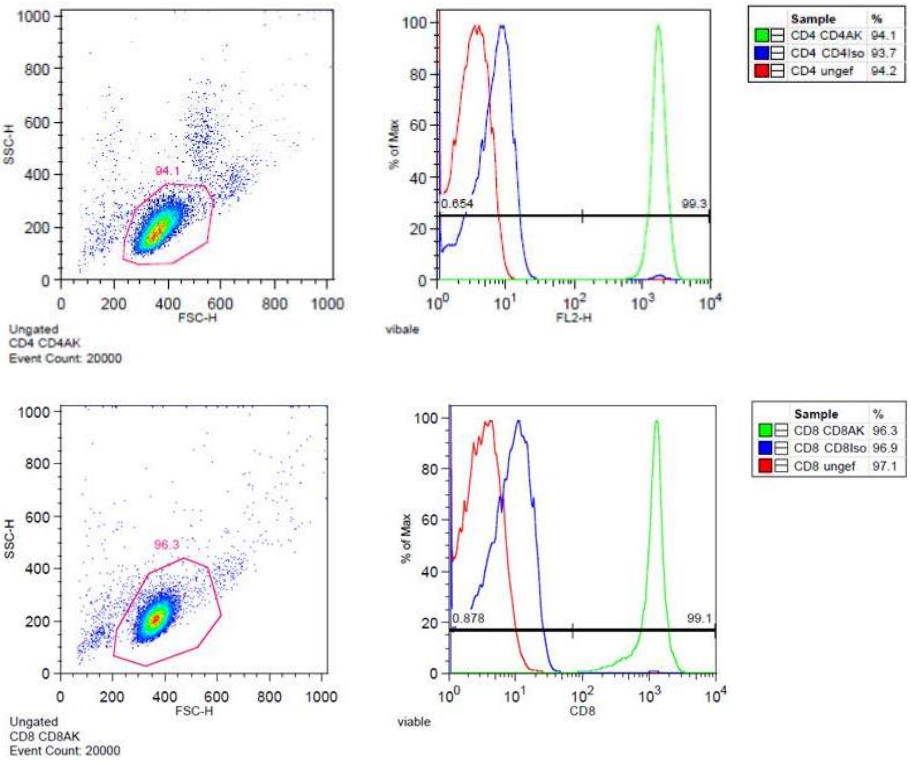

Figure 4. Purity of freshly isolated example donor $CD4^+$ and $CD8^+$ T cells
Subpopulations were stained with anti-$CD4^+$ and anti-$CD8^+$ antibodies immediately after magnetic bead separation and analyzed by flow cytometry.

Subsequently, cells were solved in T cell medium supplemented with IL-2 (100 IU/ml) and plated on 96 well plates together with anti-CD3/CD28 beads in a ratio of 1:1 (10^5 cells, 10^5 beads, total volume 225 µl). As a control T cells were plated without anti-CD3/CD28 beads under identical conditions (= quiescent T cells). Plated T cells were cultured in a humidified atmosphere (5 % CO_2, 95 % air) at 37° Celsius. In figure 5 a detailed experimental time course is presented.

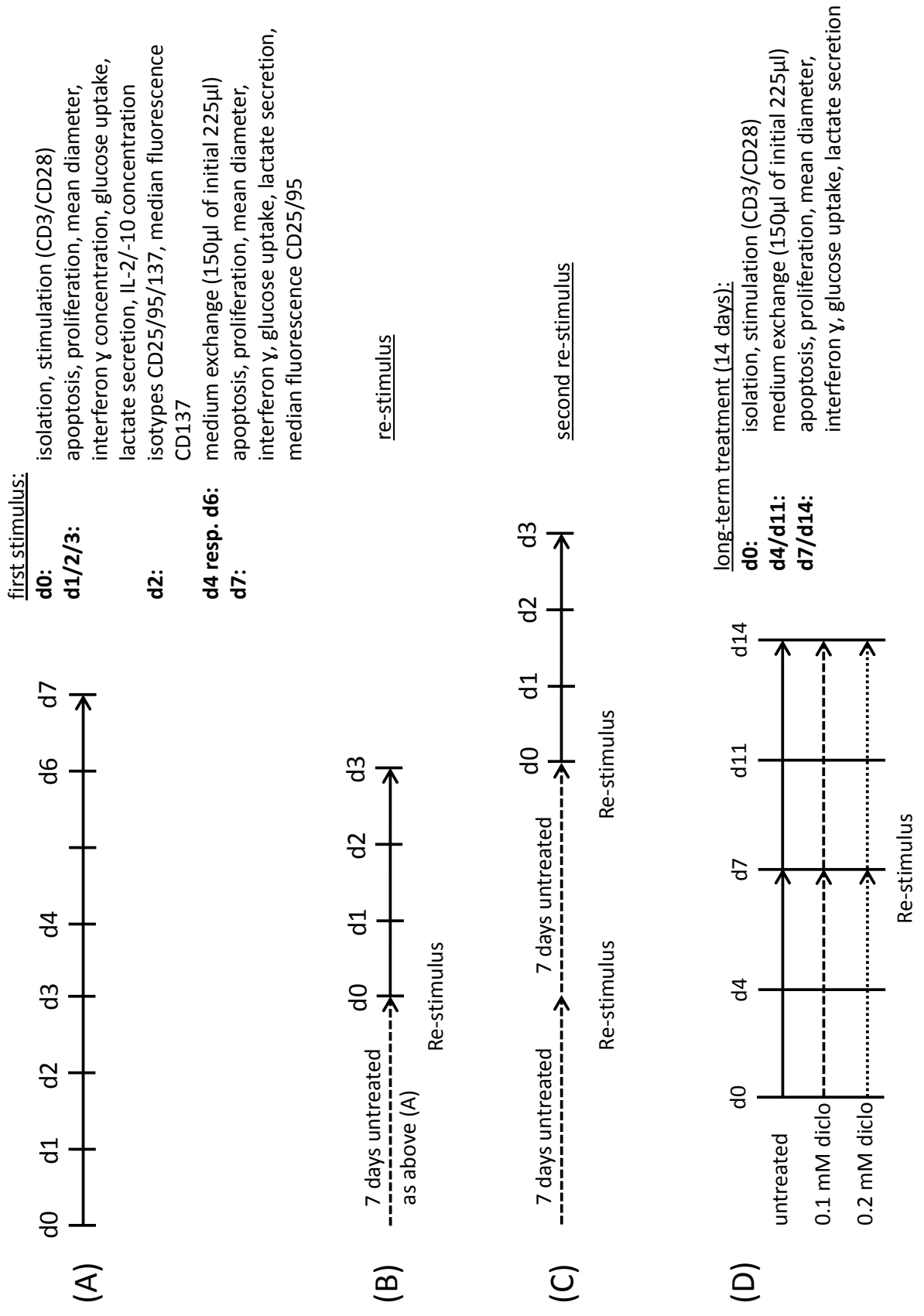

Figure 5. Schedule of T cell cultivation and time points of measurements

3.2.3 Mixed leukocyte reaction (MLR)

T cells were stimulated in a mixed leukocyte reaction (MLR), representing a more physiological stimulus. In a MLR T cells are activated by allogenic antigen presenting dendritic cells (DCs). Monocytes were differentiated to dendritic cells by stimulation of 1.5 x 10^6 cells per ml with IL-4 (144 U/ml), colony stimulating factor GM-CSF (224 U/ml) and after 4 days matured with lipopolysaccharide LPS (100 ng/ml) in DC medium for two days. Matured DCs were centrifuged (1600 rpm, 4 min, 4 °C) and re-suspended in T cell medium. DCs were plated on 96 well plates with freshly isolated T cells at a ratio of 1:10 (10.000 DCs, 100.000 T cells) in a total volume of 225 µl T cell medium supplemented with IL-2 (100 IU/ml). A medium exchange (150 µl of initial 225 µl) was performed on day four and T cells were harvested after seven days of stimulation.

3.2.4 Determination of cell number and cell size

Measurement of proliferation and cell growth was performed with the CASY system. Cells were pooled and separated from anti-CD3/CD28 beads by magnetic separation. 50 µl of the cell suspension were mixed with 10 ml of CASY buffer. Principle of the CASY technology is the electric acquisition of cells passing through a measurement pore. The resulting signal depends on the cell volume (electrical current exclusion, ECE), which leads to detection of cell size and number.

3.2.5 Measurement of glucose consumption and lactate secretion

After magnetic separation from anti-CD3/CD28 beads and pooling, cell suspension was centrifuged (1600 rpm, 4 min, 4°C) and supernatant was drawn and stored at -20 °C for determination of glucose and lactate concentrations. Glucose consumption and lactate secretion were calculated as the difference between the concentration in the standard medium and culture supernatant.

Glucose was measured by an enzymatic assay. In a first reaction, glucose was converted into glucose-6-phosphate (G6P) by the enzyme hexokinase degrading ATP. In a second step, G6P and NAD were converted to 6-phosphogluconate and NADH by glucose-6-phosphate dehydrogenase (G6PDH). NADH was measured at 340 nm by a spectrophotometer (Thermo VarioSkan plate reader). Glucose level was calculated by a linear standard curve (measurement of a serial diluted standard sample).

Lactate was also determined enzymatically by means of an ADVIA 1650 analyzer using reagents from Roche at the Department of Clinical Chemistry (University Hospital of Regensburg). Therefore, 250 µl of sample was diluted with 250 µl PBS and measured.

3.2.6 Determination of cytokines

Measurement of the cytokines IFNɣ, IL-2 and IL-10 was performed by using ELISA-kits (enzyme linked immunosorbent assay) in culture supernatants after centrifugation (1600 rpm, 4 min, 4°C). In brief, the principle of this measuring method is a multistep reaction, with initial binding of a cytokine specific capture antibody to a microplate. In the next step, standard or samples are added, followed by a detection antibody. Subsequently, this antibody binds Streptavidin-Horseradish peroxidase (HRP), which then converts the finally added blue substrate tetramethylbenzidine (TMB) to a yellow dye with intensity depending on the substrate concentration. This color change is measured by a microplate reader set to 450 nm with wavelength correction set to 540 nm. The achieved row data are converted to cytokine concentrations by using a standard curve. The measurements were performed according to the manufacturer´s protocol.

3.2.7 Flow cytometry

Cells solved in FACS buffer were aspirated and excited by a laser beam. Deflection of the laser light causes a light scatter depending on cell size (“forward scatter”) and

intracellular granularity (“sideward scatter”). Staining with fluorescent dyes conjugated to antibodies enables determination of intracellular and surface proteins. In this work, flow cytometry was used to measure viability and expression of the surface markers CD4, CD8, CD25, CD95 and CD137.

Viability was determined by FITC labeled Annexin V and 7-amino-actinomycin D (7-AAD) staining. Annexin V binds to phosphatidylserine, which is switched to the outside of the plasma membrane in apoptotic cells. 7-AAD interacts specifically with cytosine and guanine of cellular DNA, which is only possible in late apoptotic cells with a disrupted plasma membrane. Cells were harvested, pooled and separated from beads at different time points (Fig. 5). 0.25 x 10^6 cells were centrifuged (1600 rpm, 4 min, 4°C), washed two times with 1 ml PBS, supernatant was discarded and cells were stained. Subsequently, cells were re-suspended in 400 µl Annexin binding buffer diluted 1:10, stained with 5 µl Annexin V FITC and 20 µl 7-AAD and incubated for 20 minutes in the dark. Unstained and single-stained (Annexin V FITC or 7-AAD) cells were used to compensate and calibrate measurement settings. Measurements were performed with FACS Calibur, analyzation of data with the Cell Quest software. Double negative cells (Annexin V/7-AAD -/-) were defined as viable cells.

Expression of surface markers was measured by antibody staining. Antibodies applied were anti-CD4/CD8 (purity control upon isolation), anit-CD137 (early activation marker) and anti-CD25/CD95 (activation markers) at different time points (figure 5). Cells were separated from the magnetic beads, centrifuged (1600 rpm, 4 min, 4°C), supernatant was discarded and cells were washed two times with 1 ml FACS buffer. Cells were stained with 5 µl anti-CD4, 10 µl anti-CD8, 5 µl anti-CD25, 20 µl anti-CD95 or 5 µl anti-CD137. After an incubation period (20 min, 4°C), cells were washed again two times with 1 ml FACS washing buffer and finally re-suspended in 400 µl FACS buffer and measured. Quiescent, stained cells respectively isotype stained activated T cells were used as controls for unspecific antibody staining.

3.2.8 Restriction of glycolysis

To inhibit glucose metabolism, two different agents were used: the hexokinase inhibitor 2-deoxyglucose (2DG) and the non-steroidal anti-inflammatory drug (NSAID) diclofenac (diclo). 2DG was solved in standard medium to a stock concentration of 200 mmol/l (200 mM) and applied in concentrations of 1 mM, 5 mM and 10 mM. Diclofenac was solved in standard medium to a stock concentration of 8 mM and applied in concentrations of 0.1, 0.2 and 0.4 mM.

3.3 Statistics

Statistics were performed by use of the software “GraphPad Prism 5” and depicted graphs show means with standard error of the mean (SEM). When comparing two groups, significance levels were calculated by the paired and two-tailed Student´s t test. In contrast, treatment induced changes were analyzed by ANOVA and post-hoc Tukey´s multiple comparison test. P values of < 0.05 were considered as statistically significant (*), < 0.01 as being very significant (**) and < 0.001 as highly significant (***).

4. Results

4.1 Impact of glycolytic inhibition on leukemic T-ALL cells

In a first step the impact of targeting glucose metabolism on human leukemic cells was investigated. The human childhood T-ALL cell line CCRF-CEM-C7H2 (C7H2) was used as a model system for leukemia. We compared the impact of diclofenac, recently described as a glycolytic inhibitor, with 2DG, a well-established glycolytic inhibitor. Diclofenac was applied in concentrations of 0.1 mM, 0.2 mM and 0.4 mM and 2DG in concentrations of 1 mM, 5 mM and 10 mM.

4.1.1 Impact on glucose consumption and lactate production

As shown in figure 6A the untreated cultures metabolized 85 % of medium glucose within 72 hours of cultivation. 2DG and diclofenac treated cells showed a significant reduction of glucose consumption in a concentration dependent manner. Similar levels of glycolytic inhibition were obtained with applied concentrations of both inhibitors. A 2DG concentration of 1 mM decreased glucose consumption by about 30 %, application of 5 and 10 mM resulted in an almost complete inhibition (fig. 6A). 0.1 mM diclofenac diminished glucose consumption by more than 60 % and the two-fold concentration resulted in an almost complete blockade (fig. 6A).

The increased glucose metabolism resulted in a highly elevated lactate production and secretion in C7H2 cell cultures (fig. 6B). Treatment with the two different inhibitors diminished lactate secretion in a concentration dependent manner. Lactate production was reduced significantly by all treatments and especially high doses (0.2 mM diclofenac and 10 mM 2DG) achieved a reduction by about 90 %.

Glycolytic activity was compromised more effectively by 0.1 mM diclofenac compared to 1 mM 2DG treatment, whereas 0.2 mM diclofenac showed comparable effects to 5 and 10 mM 2DG.

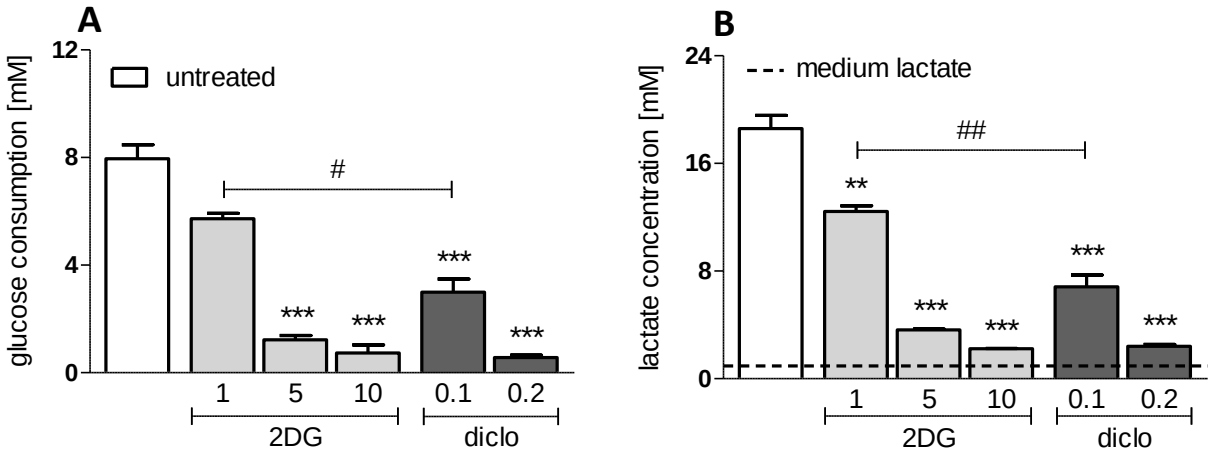

Figure 6. Impact of 2-deoxyglucose and diclofenac on glucose metabolism of C7H2 cells
C7H2 cells were incubated with different concentrations of the glycolytic inhibitors diclofenac (diclo) and 2-deoxyglucose (2DG) for 72 hours. (A) Glucose and (B) lactate concentrations were measured in the culture supernatant. **A and B** untreated and 0.1 diclo n=9, 0.2 diclo n=8, 2DG n=3 (**P value** 0.05>*>0.01>**>0.001>***, 0.05>#>0.01>##>0.001>###, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

4.1.2 Impact on proliferation and viability

Glucose metabolism is pivotal for the proliferation of cells. Therefore, cell division was analyzed under 2DG and diclofenac treatment.

Both inhibitors led to a reduction in proliferation. Low doses of 1 mM 2DG and 0.1 mM diclofenac slightly diminished cell number by about 20 %, whereas higher concentrations of both inhibitors severely and comparably suppressed cell division (fig. 7A).

Untreated controls of C7H2 cells showed an overall survival of 92 % over the entire incubation time (fig. 7B). As already mentioned, the metabolism of malignant cells is characterized by a shift to glycolysis, which allows the presumption of an important and particular role of glucose metabolism. Therefore, the impact of glycolytic inhibition on the viability of C7H2 cells was analyzed.

Interestingly, 2DG had only little impact on tumor cell viability. Even 10 mM 2DG, which is clinically not applicable, reduced cell viability only by 35 %. Despite comparable effects on glucose metabolism and proliferation, diclofenac exerted a stronger impact and reduced cell viability very effectively. Within 72 hours 0.2 mM induced a decline in cell viability by 76 %.

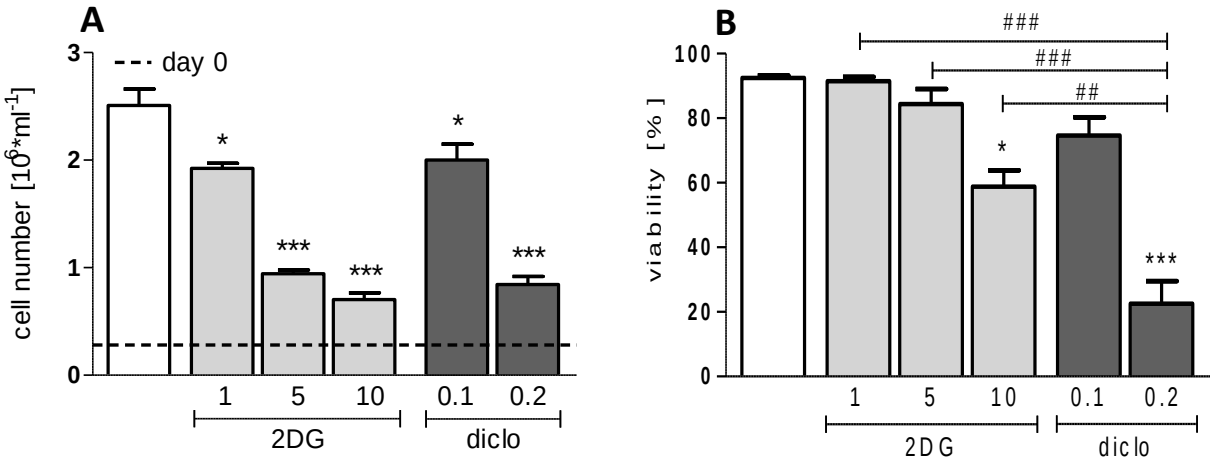

Figure 7. Impact of 2-deoxyglucose and diclofenac on cell number and viability of C7H2 cells
C7H2 cells were incubated with different concentrations of the glycolytic inhibitors diclofenac (diclo) and 2-deoxyglucose (2DG) for 72 hours. (A) Cell number was determined with the CASY system and (B) viability by flow cytometry using Annexin V and 7-AAD staining. **A** untreated and diclo n=5, 2DG n=3; **B** untreated and 0.1 diclo n=11, 0.2 diclo n=9, 2DG n=3. (**P value** 0.05>*>0.01>**>0.001>***, 0.05>#>0.01>##>0.001>###, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

Taken together, both glycolytic inhibitors diminished glucose metabolism effectively and to a comparable extent. Both inhibitors significantly reduced proliferation, however only diclofenac exerted a significant impact on cell viability, pointing towards additional effects of diclofenac.

4.2 Characterization of primary human T cells

Activated murine T cells have a similar metabolic phenotype compared to tumor cells in terms of aerobic glycolysis, thereby also reflecting the Warburg effect. Due to the fact that most of the studies were performed in the murine system so far, we characterized the kinetics of glucose metabolism in correlation to cell proliferation and growth, as well as interferon ɣ production and viability in human bulk $CD4^+$ and $CD8^+$ T cells. T cells, purified from healthy donors were stimulated with anti-CD3/CD28 beads at a cell to bead ration of 1:1. After 7 days cells were collected, diluted and restimulated (restimulation) for another week followed by a third stimulation. During each stimulation period, samples were analyzed after 24, 48, 72 hours and 7 days. In comparison, quiescent bulk T cells were analyzed.

4.2.1 Characterization of stimulated human $CD4^+$ T cells

4.2.1.1 Metabolic characterization

During the first 24 hours of stimulation glucose consumption and lactate production were almost below the limit of quantification, but increased after 24 hours and strongly accelerated beyond 48 hours (fig. 8A/B). This general pattern was also observed during restimulation however restimulated $CD4^+$ T cells showed an increased glycolytic activity as reflected by accelerated and significantly elevated lactate accumulation. Stimulated and restimulated $CD4^+$ T cells maintained a highly glycolytic phenotype up to 7 days (data not shown). A second restimulation did not result in any further changes with regard to glucose consumption and lactate secretion (n=4, data not shown).

Quiescent cells kept for 7 days under the same culture conditions were glycolytically inactive (n=3, data not shown).

Interestingly, lactate levels detected exceeded concentrations achievable when glucose is completely converted into lactate, strongly indicating that glycolysis is not the only source of lactate production.

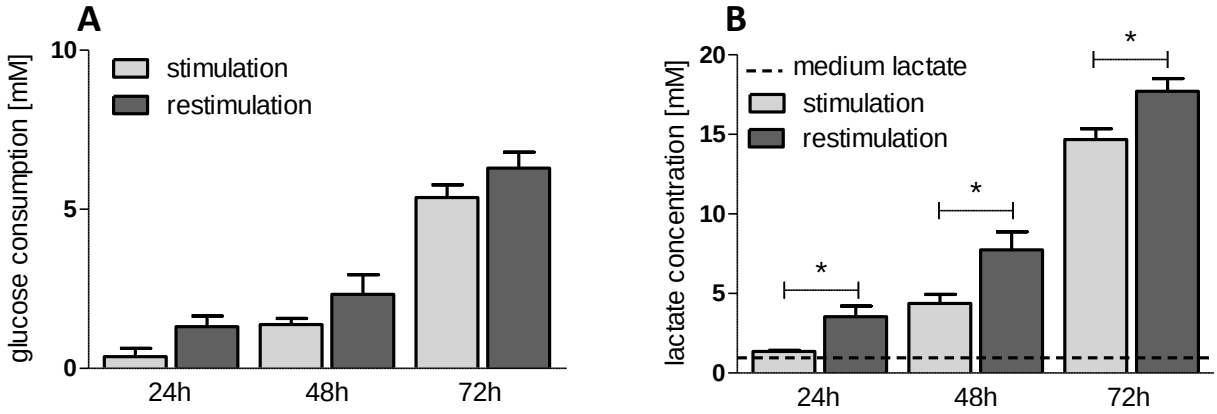

Figure 8. Metabolic characterization of human stimulated and restimulated $CD4^+$ T cells
(A) Glucose and (B) lactate levels were measured enzymatically in culture supernatants. **A** stimulated 24h n=5, 48h n=7 and 72h n=4, restim n=4, **B** stim 24h n=5, 48h n=7 and 72h n=8, restim 24h n=5, 48h and 72h n=4. (**P value** 0.05>*>0.01>**>0.001>***, differences between stimulation and restimulation were analyzed with the Student´s t-test, paired and two-tailed)

4.2.1.2 Functional characterization

No significant increase in cell number was observed in stimulated and restimulated cells during the first 48 hours of culture (fig. 9A) however a strong increase in cell size was measured (fig. 9B). The onset of proliferation was detected beyond 48 hours, concomitantly with a strongly accelerated glucose metabolism. No significant differences between stimulation and restimulation were detectable (fig. 9A). Until day seven a final cell number of $3.6 * 10^6 \pm 0.20$ cells/ml (stimulus, n=10) and $3.16 * 10^6 \pm 0.49$ cells/ml (restimulus, n=6) was achieved. After a second restimulation the proliferative capacity within the first 72 hours was reduced ($1.13*10^6 \pm 0.09$, n=4). Unstimulated $CD4^+$ cells showed a minimal proliferation resulting in $0.7 * 10^6 \pm 0.1$ cells/ml within 72 hours (n=3).

The first two days were characterized by a significant cell growth in stimulated cells (so-called “on-blast formation”). This initial increase in cell size was followed by a slight shrinkage during the proliferative phase until day 7 (data not shown). Another significant increase in cell size was detected during the first 48 hours of restimulation, however to a much lower extent compared to the first activation (fig. 9B). When stimulated a third time, cells showed a slight, but significant cell growth from 9.2 ± 0.1 µm to 10.7 ± 0.3 µm within 72 hours (n=4). Quiescent T cells did not increase their cell size (n=3, data not shown).

Stimulated T cells exhibited a constant viability of 89.7 ± 1.0 % until day 3 (n=4). After 7 days of cultivation a decline to 71.3 ± 5.4 % (n=4) was observed, which may be related to increasing lactate levels in culture supernatants, known to affect T cell viability. The loss of viability was reversible and after restimulation cells recovered and showed a high viability of 89.7 ± 0.38 % on day 3 (n=4). The same was observed during a second restimulation. As expected, unstimulated $CD4^+$ T cells showed a slight decline in viability over time from 79.5 % ± 13.7 % after 24 hours (n=3) to 71.2 % ± 17.3 % after 72 hours (n=3) .

It has been proposed in the murine system, that interferon ɣ production is glucose dependent. As shown in figure 9C stimulated cells produced high levels of interferon ɣ already in the first 24 hours of stimulation while glucose consumption was very low. Interferon ɣ levels remained high up to 72 hours, but concentration dropped to 22.5 ± 8.3 pg/ml on day seven (n=10). Restimulated cells reached higher IFNɣ concentrations compared to stimulated T cells, higher levels were maintained over time and even after 7 days significant levels were detectable (253.4 ± 175.0 pg/ml, n=5).

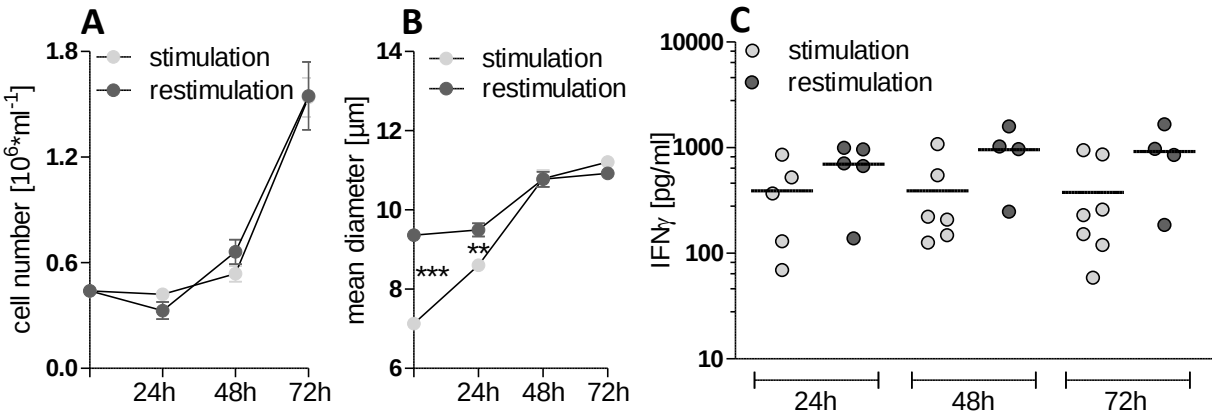

Figure 9. Functional characterization of human stimulated and restimulated $CD4^+$ T cells
(A) Cell number and (B) mean diameter were determined by CASEY system; (C) Measurement of interferon ɣ concentrations was performed by ELISA **A and B** n=4; **C** stimulation 24h n=5, 48h n=6 and 72h n=7, restimulation 24h n=5, 48h and 72h n=4; (**P value** 0.05>*>0.01>**>0.001>***, differences between stimulation and restimulation were analyzed with the Student´s t-test, paired and two-tailed)

4.2.2 Characterization of stimulated human CD8$^+$ T cells

4.2.2.1 Metabolic characterization

Glucose uptake was below the limit of detection in stimulated CD8$^+$ T cells within the first 24 hours (fig. 10A). On day 3 about 50 % of initially available glucose was taken up into the cells and the high glycolytic activity persisted over the whole stimulation period (data not shown). After restimulation, glucose uptake was significantly accelerated in the first 24 hours compared to stimulated cells, however beyond 24 hours there was no difference detectable between stimulated and restimulated CD8$^+$ T cells. During stimulation a slight but significant increase in lactate secretion was detected after 24 hours and strongly increased beyond 72 hours (fig. 10B).

During restimulation significantly elevated lactate secretion was measured only during the first 24 hours compared to stimulated CD8$^+$ T cells. A second restimulation led to a low glycolytic activity within the first 48 hours and a diminished activity beyond 48 hours compared to stimulated and restimulated CD8$^+$ T cells (data not shown). After 72 hours only 18 % of glucose was consumed and lactate levels of 9.5 mM (n=2) were detected. In comparison, stimulated and restimulated CD8$^+$ T cells were highly glycolytic beyond 48 hours of stimulation and re-stimulation.

In quiescent CD8$^+$ T cells lactate levels increased only marginally (data not shown, n=2).

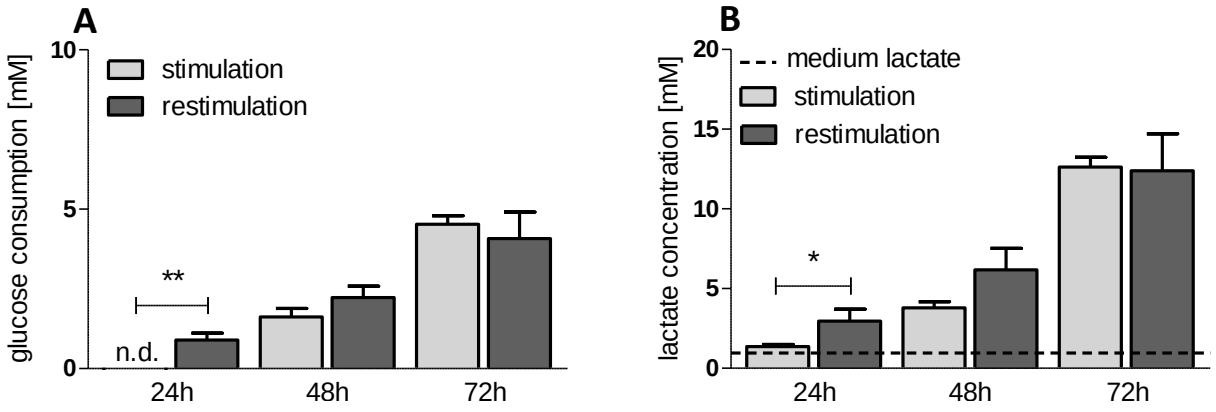

Figure 10. Metabolic characterization of human stimulated and restimulated CD8$^+$ T cells
(A) Glucose and (B) lactate levels were measured enzymatically in culture supernatants. **A** stimulation 24h and 48h n=6, 72h n=4, restimulation 24h n=5, 48h and 72h n=4, **B** stimulation 24h and 48h n=6, 72h n=4, restimulation 24h n=5, 48h and 72h n=4. (**P value** 0.05>*>0.01>**>0.001>***, differences between stimulation and restimulation were analyzed with the Student´s t-test, paired and two-tailed)

4.2.2.2 Functional characterization

As shown in figure 11A, cell number increased only slightly within the first two days of stimulation. Between day 2 and 3 cell proliferation was significantly accelerated and after 7 days a maximum cell number of 2.7 ± 0.37 * 10^6 cells/ml (stimulation, n=9) and 2.4 ± 0.77 * 10^6 cells/ml (restimulation, n=5) was achieved. During a second restimulation cells showed a lower proliferation potential and the average cell number after 72 hours amounted to 0.78 * 10^6 cells/ml (n=2), half of the cell number reached during stimulation and restimulation. Within 72 hours, quiescent T cells showed only a slight increase in cell number (n=2, data not shown).

Stimulated $CD8^+$ T cells increased their size comparable to $CD4^+$ T cells (fig. 11B), whereas restimulated cells grew significantly less within the first 72 hours of activation. Between day 3 and 7 a general shrinkage in cell size was observed and the final diameter was diminished to 9.1 ± 0.1 µm in stimulated (n=9) and 8.3 ± 0.2 µm in restimulated (n=5) $CD8^+$ T cells. Equal growth characteristics were observed in two-times restimulated $CD8^+$ T cells (data not shown). Quiescent $CD8^+$ T cells showed a negligible growth of 11 % within 72 hours (n=2).

Within the first 3 days of stimulation viability remained constant between 80 and 90 % (n=4). After a week of stimulation, a significant lower percentage of 72.6 ± 5.2 % (n=4) of viable cells was measured, correlating with increasing lactate concentrations in culture supernatants, which had been shown to affect cell viability of human T cells. Restimulation and associated dilution of cells as well as complete medium exchange raised cell viability again to 90.4 ± 1.5 % (n=4) after 72 hours. However with increasing proliferation and lactic acid production again a drop to 78.8 ± 1.4 % (n=3) was observed between day 3 and 7. Within a second restimulation $CD8^+$ T cell viability was constantly compromised and reached a maximum of 72.6 % (n=2) after 72 hours. Quiescent cells exhibited a decrease in viability within 72 hours from 91.4 (n=2) to 82.6 (n=2).

Already after 24 hours of activation interferon ɣ concentrations of about 500 pg/ml were measured (fig. 11C). Concentrations stayed roughly constant until day 3, but dropped sharply afterwards concomitant with a strongly accelerated proliferation (on day seven 44 ± 15.5 pg/ml, n=7). There was no difference detectable between stimulated and restimulated $CD8^+$ T cells. In contrast to $CD4^+$ T cells, interferon ɣ levels were not elevated and partially maintained during restimulation. A second restimulation resulted in lower concentrations of interferon ɣ with a maximum of 307.9 pg/ml on day 3 (n=2).

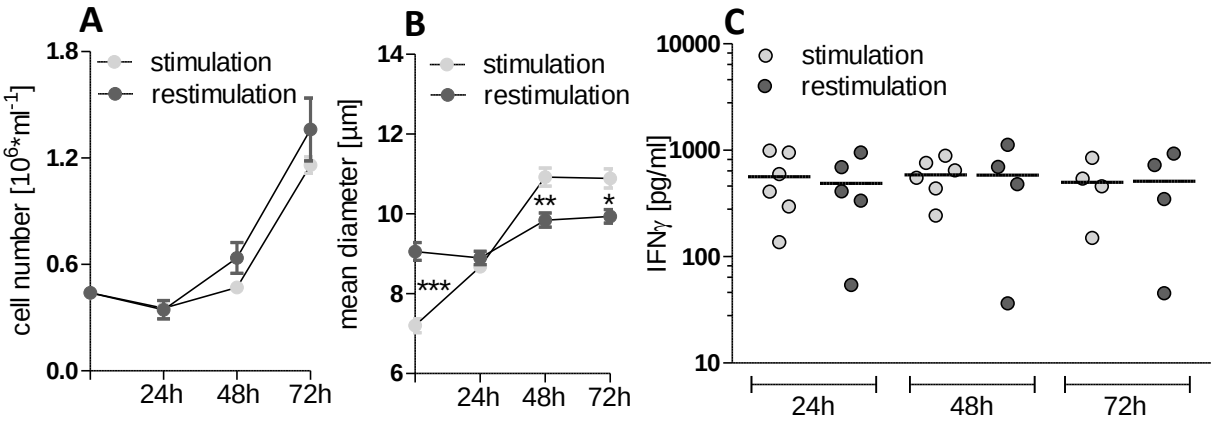

Figure 11. Functional characterization of human stimulated and restimulated $CD8^+$ T cells
(A) Cell number and (B) mean diameter were determined by CASEY system; (C) Measurement of interferon ɣ concentrations were performed by ELISA. **A and B** n=4; **C** stimulation 24h and 48h n=6 and 72h n=4, restimulation 24h n=5, 48h and 72h n=4; (**P value** 0.05>*>0.01>**>0.001>***, differences between stimulation and restimulation were analyzed with the Student´s t-test, paired and two-tailed)

4.2.3 Comparison between metabolic activity in $CD4^+$ and $CD8^+$ T cells

Both T cell subpopulations showed an increased glycolytic activity with accelerated glucose consumption and lactic acid secretion beyond 48 hours of stimulation and restimulation. Thereby, glycolytic activity was higher in $CD4^+$ compared to $CD8^+$ T cells. Early glycolytic activity was increased significantly in restimulated T cells compared to stimulated T cells. A second restimulation had only little impact on glycolytic activity of $CD4^+$ T cells, but glucose consumption and lactate secretion were reduced by an average of 25 % in $CD8^+$ T cells within 72 hours.

Proliferation strongly correlated with glucose metabolism and both subpopulations started to proliferate after a 48-hour growth period. Cell size was nearly equal in

stimulated $CD4^+$ and $CD8^+$ T cells, but while growth was maintained in restimulated $CD4^+$ cells, it was significantly diminished in the $CD8^+$ T cells beyond 48 hours. Moreover, proliferation was distinctly stronger in restimulated $CD4^+$ compared to $CD8^+$ T cells beyond 48 hours. Viability was slightly higher in $CD4^+$ compared to $CD8^+$ T cell cultures and both subtypes showed reduced viability upon 24 hours of restimulation, which was recovered until day 3 only in $CD4^+$ T cells.

Interferon ɣ secretion was constant until day 3 in both stimulated cell types, but higher in $CD8^+$ than $CD4^+$ T cells. Upon restimulation, IFNɣ secretion was strongly, but not significantly increased in $CD4^+$ T cells, whereas $CD8^+$ cells maintained the concentration level of first time stimulation. Remarkably, the second restimulation resulted in slightly diminished concentrations in both populations, with in average 2.5-fold higher levels in $CD4^+$ T cells.

4.3 Impact of anti-metabolic drugs on human T cells

As shown in the first part malignant T-ALL cells were sensitive to glycolysis inhibiting drugs with regard to glucose metabolism, proliferation and viability. The application of anti-glycolytic drugs is an emerging strategy in cancer therapy. However, as demonstrated in the second part, also human T cells have an increased glucose metabolism upon activation. Therefore the question must be asked what consequences could arise regarding functionality and efficacy of the anti-tumor immune response of T cells in the presence of anti-glycolytic drugs. Therefore, the impact of 2DG and diclofenac on human T cell function was analyzed. The experimental set-up was equal to the one applied for metabolic characterization of quiescent, stimulated and restimulated human T cells.

4.3.1 Impact on quiescent human T cells

In a first step quiescent (i.e. not stimulated) bulk $CD4^+$ and $CD8^+$ T cell cultures were treated with 0.1 and 0.2 mM diclofenac. As shown in the first part glucose metabolism and proliferation is almost undetectable in unstimulated T cells resulting in an limited impact of anti-metabolic drugs. Importantly, viability was preserved (data not shown). Because of negligible effects, experiments were performed only two-times (n = 2).

4.3.2 Impact on stimulated human T cells

As the first stimulation represented an early immune response, the following experiments were performed to identify the influence of anti-metabolic drugs on the efficacy of primary activation.

4.3.2.1 Glucose metabolism

Within the first 3 days of stimulation 2DG was a strong inhibitor of glycolysis and already 1 mM 2DG reduced glucose consumption significantly by more than 80 %, moreover 5 and 10 mM 2DG led to a complete blockade in uptake (fig. 12A/B). Interestingly, after 7 days, the impact of 1 mM 2DG was significantly reduced in both populations and glucose uptake was diminished by only 25 %, whereas 5 and 10 mM still resulted in a complete block (table 1).

Already 0.1 mM diclofenac exerted a significant effect on both populations and reduced glucose consumption by about 50 % in $CD4^+$ and 60 % in $CD8^+$ T cells cultures after 72 hours. 0.2 mM diclofenac diminished glycolytic activity by 75 % compared to untreated cells (fig. 12A/B). As observed in 2DG treated T cell populations, beyond day 3 a reduced impact on glycolytic inhibition was observed. After 7 days, glucose consumption was 80 % under 0.1 mM and 50 % under 0.2 mM diclofenac treatment in comparison to untreated cells in both T cell populations (table 1).

The reduced glucose metabolism was also reflected in a strongly diminished lactate secretion (fig. 12C/D). 1 mM 2DG reduced lactate levels in culture supernatants very effectively by about 80 % in $CD4^+$ and $CD8^+$ T cells and the application of 5 and 10 mM 2DG induced a nearly total block in lactate secretion in the first 72 hours. After 7 days the reduced impact of 1 mM 2DG on glucose consumption was also observed analyzing lactate secretion (only by about 30 % reduced lactic acid) in both populations, whereas 5 and 10 mM were still capable to block lactate secretion (table 1).

Taken together, inhibition of lactate secretion under diclofenac treatment corresponded to glucose consumption in the first 72 hours as well as after 7 days. 1 mM 2DG and 0.2 mM diclofenac exerted comparable effects on both populations, whereas 0.1 mM diclofenac had a significantly lower impact on glycolysis of T cells. 5 mM and 10 mM 2DG reduced glycolysis significantly stronger than 0.2 mM diclofenac.

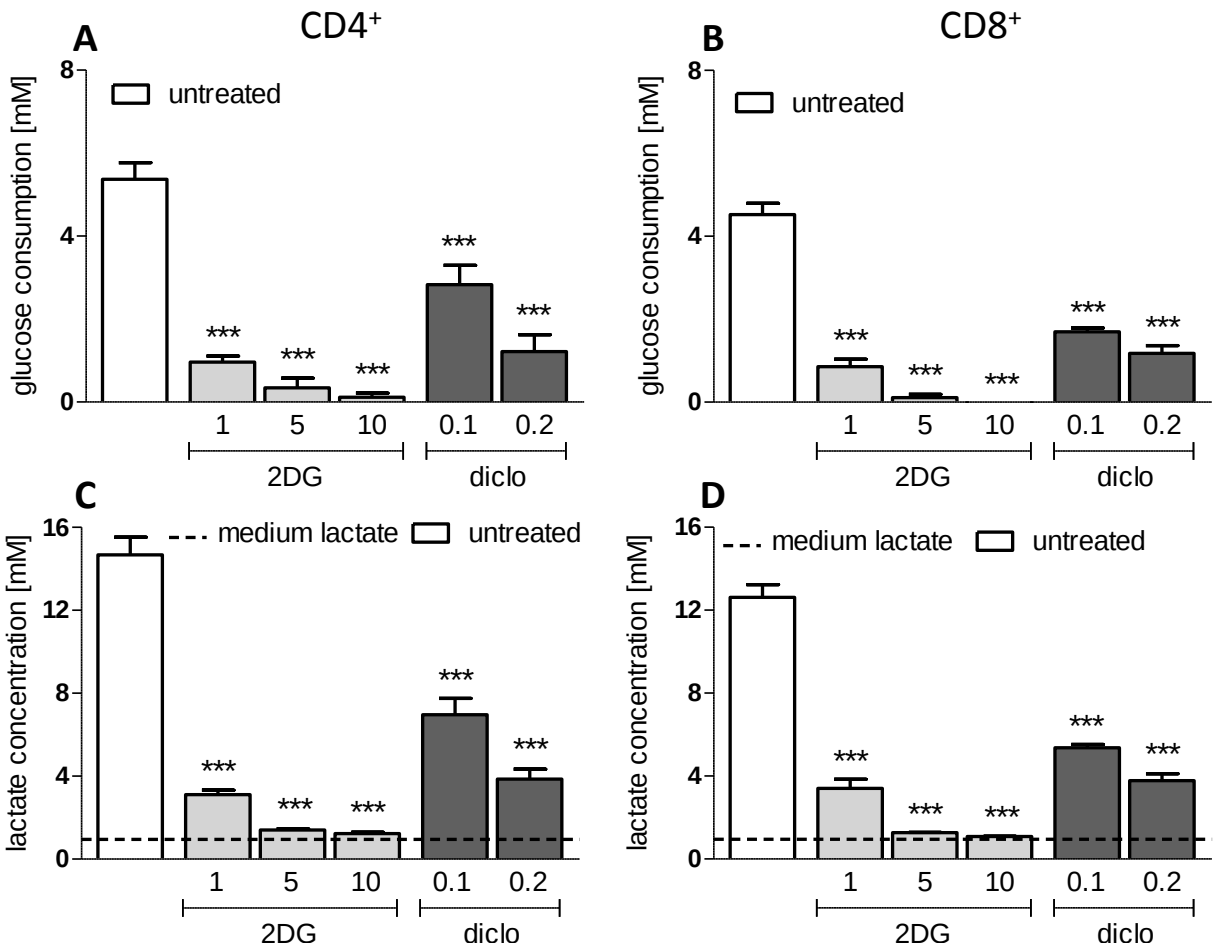

Figure 12. Impact of 2DG and diclofenac on glucose metabolism of stimulated human CD4$^+$ and CD8$^+$ T lymphocytes
(A/B) Glucose and (C/D) lactate levels are measured enzymatically in culture supernatants after 72 hours of stimulation; Untreated and 2DG n=4, diclo n=3 (**P value** 0.05>*>0.01>**>0.001>***; treatment induced changes are analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

Summarizing, 2DG and diclofenac had a significant impact on aerobic glycolysis within 72 hours, which showed a compensation concerning 1 mM 2DG and both diclofenac concentrations within 7 days. Despite an exchange of the cell medium containing the initial 2DG and diclofenac concentration on day 4, the degradation of the active agents within the stimulation period cannot be completely excluded and has to be considered.

Table 1. Impact of 2DG and diclofenac on stimulated $CD4^+$ and $CD8^+$ T cells after 7 days
Glucose and lactate levels were measured enzymatically in culture supernatants after 7 days of stimulation; mean diameter was determined by CASEY system; Measurement of interferon ɣ concentrations was performed by ELISA (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

7 days		untr.	1 mM 2DG	5 mM 2DG	10 mM 2DG	0.1 mM diclo	0.2 mM diclo
glucose consumption [mM]	$CD4^+$	7.7 ± 0.7 (n=8)	5.6 ± 1.0 (n=5)	0.2 ± 0.2*** (n=5)	-0.4 ± 0.3*** (n=4)	6.3 ± 0.7 (n=8)	3.9 ± 0.6** (n=8)
	$CD8^+$	6.5 ± 0.9 (n=7)	5.2 ± 2.6 (n=5)	-0.1 ± 0.2*** (n=5)	-0.5 ± 0.4*** (n=4)	5.0 ± 0.9 (n=7)	2.9 ± 1.2* (n=7)
lactate secretion [mM]	$CD4^+$	20.1 ± 1.3 (n=9)	13.3 ± 2.1 (n=4)	2.0 ± 0.2*** (n=4)	1.3 ± 0.1*** (n=4)	15.6 ± 1.5 (n=8)	11.3 ± 1.6*** (n=8)
	$CD8^+$	17.9 ± 1.8 (n=8)	13.6 ± 2.4 (n=5)	2.2 ± 0.2*** (n=5)	1.3 ± 0.1*** (n=4)	11.9 ± 1.8 (n=7)	9.1 ± 2.0** (n=7)
mean diameter [µm]	$CD4^+$	9.4 ± 0.1 (n=10)	9.5 ± 0.1 (n=4)	9.1 ± 0.2 (n=4)	8.6 ± 0.1** (n=4)	9.5 ± 0.1 (n=8)	9.4 ± 0.1 (n=8)
	$CD8^+$	9.1 ± 0.1 (n=9)	9.4 ± 0.1 (n=4)	9.3 ± 0.1 (n=4)	9.0 ± 0.1 (n=4)	9.1 ± 0.1 (n=7)	8.8 ± 0.1 (n=7)
viability [%]	$CD4^+$	71.3 ± 5.4 (n=4)	91.1 ± 1.6** (n=4)	83.9 ± 2.6 (n=4)	75.6 ± 2.3 (n=4)	85.8 ± 1.1 (n=3)	77.5 ± 2.9 (n=3)
	$CD8^+$	72.6 ± 5.2 (n=4)	91.9 ± 1.3* (n=4)	85.2 ± 1.5 (n=4)	76.8 ± 2.4 (n=4)	86.1 ± 2.2 (n=3)	72.1 ± 6.8 (n=3)
interferon ɣ [pg/ml]	$CD4^+$	20.1 ± 8.8 (n=9)	10.6 ± 2.6 (n=5)	3.5 ± 2.2 (n=5)	2.4 ± 2.2 (n=4)	39.4 ± 15.9 (n=8)	64.8 ± 25.2 (n=8)
	$CD8^+$	44.2 ± 13.0 (n=8)	20.3 ± 3.9 (n=5)	15.3 ± 3.1 (n=5)	12.9 ± 3.8 (n=4)	113.7 ± 60.9 (n=7)	156.1 ± 66.1 (n=7)

4.3.2.2 Cell growth, proliferation and viability

Upon stimulation “on-blast” formation of T cells took place immediately. Treatment with 2DG impaired cell growth significantly in both subtypes and application of 10 mM 2DG reduced cell size by up to 20 % (n = 4, data not shown). In contrast, neither $CD4^+$ nor $CD8^+$ T cell size was reduced by treatment with diclofenac (n = 3, data not shown). Only 10 mM 2DG exerted a persisting impact on $CD4^+$ T cells up to 7 days (table 1).

Within the first 72 hours, 2DG and diclofenac reduced proliferation in both subpopulations, but statistical significance was only reached in $CD4^+$ T cells (fig. 13A-D). In line with a reduced impact on glucose metabolism after day 3, the impact of 1 mM 2DG on proliferation was also diminished but still significant in $CD4^+$ T cells. 5 and 10 mM 2DG lowered proliferation strongly in both subpopulations reflecting a strong impact on glycolysis (fig. 13A/B). 0.1 mM diclofenac reduced cell number only significantly in $CD4^+$ T cells, whereas 0.2 mM impaired proliferation in both populations (fig. 13C/D). However, the impact of diclofenac was more pronounced in $CD4^+$ T cell cultures after 7 days (table 1).

Glycolytic inhibition with 2DG had almost no impact on T cell viability and only 10 mM 2DG affected $CD4^+$ T cells significantly. Diclofenac treatment had no effect on T cell viability, which is in contrast to the leukemic cell line (fig. 13E/F). The drop in viability at the end of a stimulation period (after 7 days) observed in control cell cultures was not detected in treated cells. This might be the result of reduced lactic acid levels in cell cultures (table 1).

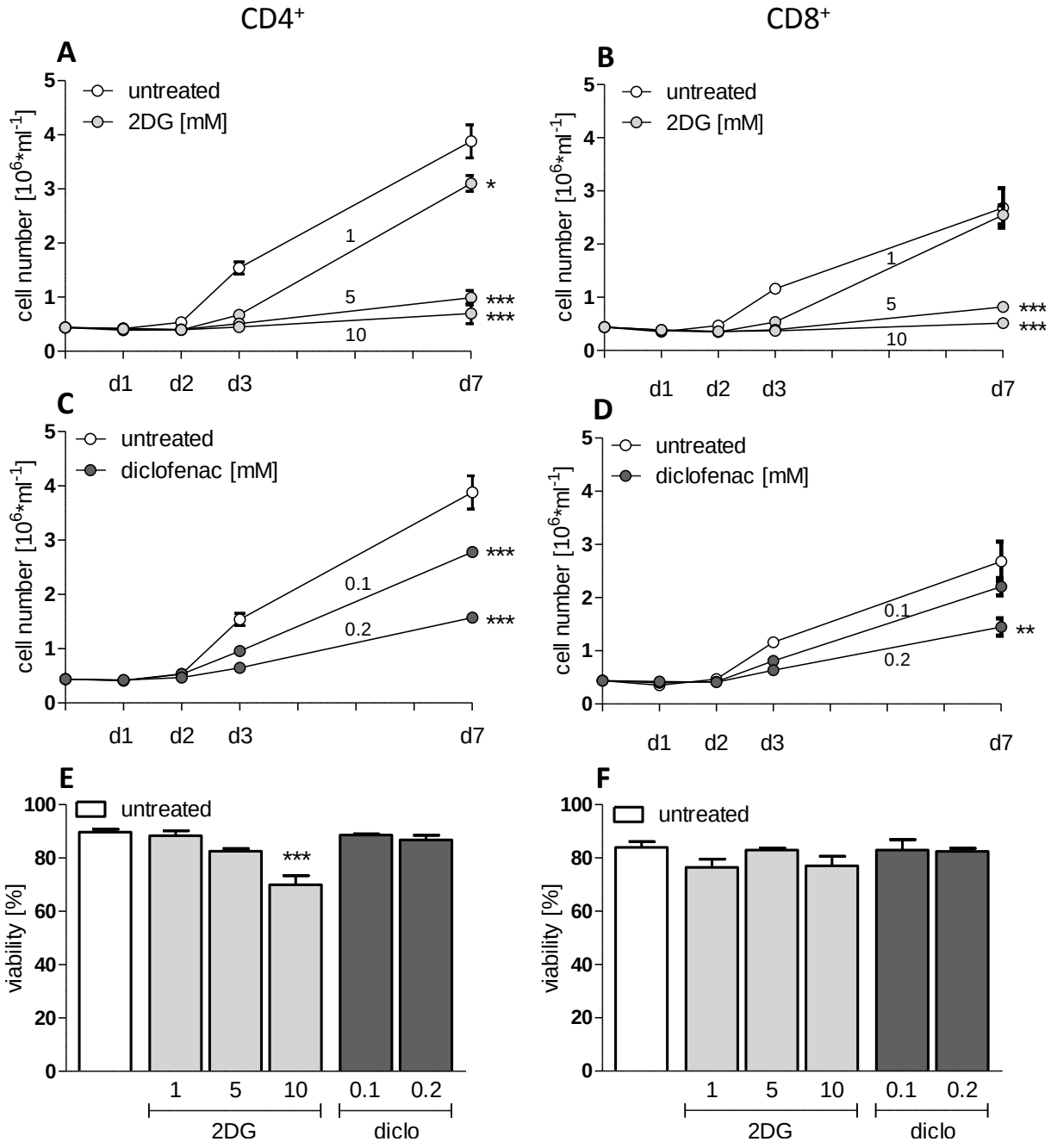

Figure 13. Impact of 2DG and diclofenac on proliferation and viability of stimulated human CD4$^+$ and CD8$^+$ T lymphocytes

(A-D) cell number was determined by CASEY system and (E-F) viability by flow cytometry with Annexin V and 7-AAD staining after 72 hours of treatment. **A** untreated and 2DG n=4, diclo n=3; **B** untreated 3d n=4 and 7d n=9, 2DG 3 and 7d n=4, diclo 3d n=3 and 7d n=7; **C and D** untreated and 2DG n=4, diclo n=3 (**P value** 0.05>*>0.01>**>0.001>***; treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

4.3.2.3 Impact on interferon ɣ and IL-2 production

When treated with glycolytic inhibitors an opposite effect on interferon ɣ secretion was observed. 2DG treatment led to decreased interferon ɣ levels in a concentration dependent manner, whereas diclofenac had no inhibiting but even more a beneficial effect on interferon ɣ secretion (fig. 14A/B).

Interleukin 2 (IL-2) stimulates the proliferation of T cells and is secreted to a much lower extent by $CD8^+$ than $CD4^+$ T lymphocytes. While $CD4^+$ T cells produced less IL-2 under 2DG treatment, 2DG had no impact on IL-2 secretion in $CD8^+$ T cells. Diclofenac exerted only a marginal effect on IL-2 secretion in T lymphocytes (fig. 14C/D).

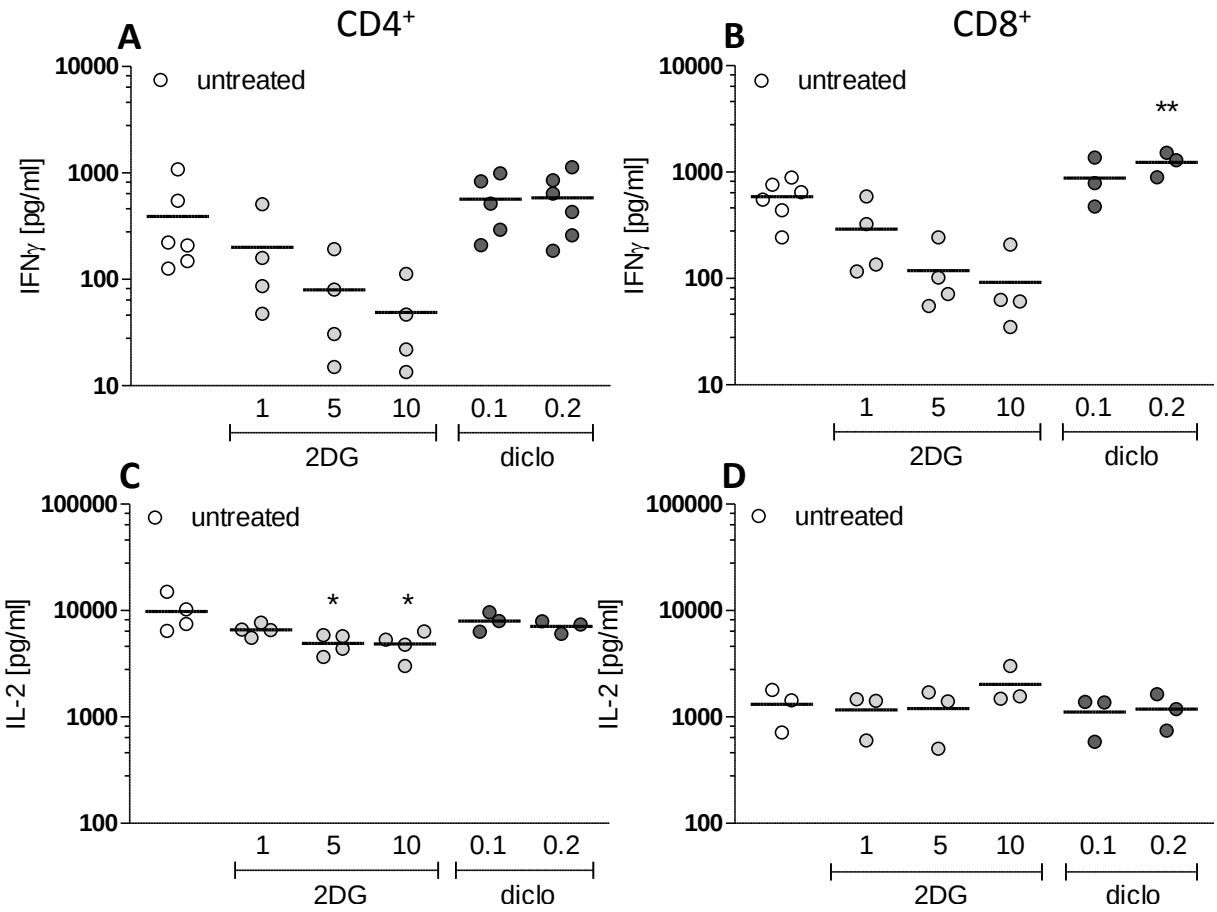

Figure 14. Impact of 2DG and diclofenac on interferon ɣ and interleukin 2 secretion of stimulated human $CD4^+$ and $CD8^+$ T lymphocytes
(A/B) Interferon ɣ and (C/D) IL-2 are measured after 48 hours of stimulation in culture supernatants by Elisa; **A** untreated n=6, 2DG n=4, 0.1 mM diclo n=5, 0.2 mM diclo n=6; **B** untreated n=6, 2DG n=4, diclo n=3; **C** untreated and 2DG n=4, diclo n=3; **D** n=3; (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes are analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

Another important cytokine mainly produced by $CD4^+$ T cells is interleukin 10 (IL-10), which has an anti-inflammatory and regulating effect on the immune response suppressing T cell activity. 0.1 mM diclofenac significantly increased IL-10 production, whereas 2DG significantly reduced IL-10 secretion (table 2).

Table 2. Impact of 2DG and diclofenac on IL-10 secretion of $CD4^+$ T cells after 48 hours
IL-10 was measured in culture supernatants after 48 hours of stimulation by ELISA; **A** untreated n=6, 2DG n=4, 0.1 mM diclo n=5, 0.2 mM diclo n=6; **B** untreated n=6, 2DG n=4, diclo n=3; **C** untreated and 2DG n=4, diclo n=3; **D** n=3; (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

IL-10 [pg/ml]; (n=3)	**untreated**	**1 mM 2DG**	**5 mM 2DG**	**10 mM 2DG**	**0.1 mM diclofenac**	**0.2 mM diclofenac**
$CD4^+$	4716 ± 103.5	1472 ± 247.6***	286.4 ± 26.0***	121.1 ± 16.1***	6590 ± 797.2*	4743 ± 360.5

To sum up, cytokine production was only affected by 2DG treatment, but not by diclofenac. As both inhibitors reduced glycolysis to a comparable extent, these results strongly indicate adverse side effects of 2DG.

4.3.2.4 Expression of the activation-related surface markers CD137, CD25 and CD95

CD137, a member of the tumor necrosis factor (TNF) receptor family, is expressed mainly on activated $CD8^+$ T cells acting as a co-stimulatory molecule. After 48 hours of stimulation $CD8^+$ T cells showed - compared to $CD4^+$ T cells (fig. 15A) - a highly increased expression (fig. 15D). Application of 2DG or 0.2 mM diclofenac slightly lowered CD137 expression in $CD8^+$ T cells, whereas its expression was not affected by both inhibitors in $CD4^+$ T cells.

As a marker of activated T cells CD25, part of the IL-2 receptor, was measured after seven days of stimulation and, in contrast to CD137, less expressed in $CD8^+$ (fig. 15E) compared to $CD4^+$ T cell cultures (fig. 15B). Both subpopulations were

negatively affected by 2DG treatment with 5 and 10 mM, whereas diclofenac had only a marginal impact.

CD95, better known as Fas receptor, is expressed by mature T cells and, when bound by the Fas ligand, induces apoptosis. Measured after seven days of stimulation CD95 was not significantly affected by both glycolytic inhibitors (fig. 15C/F).

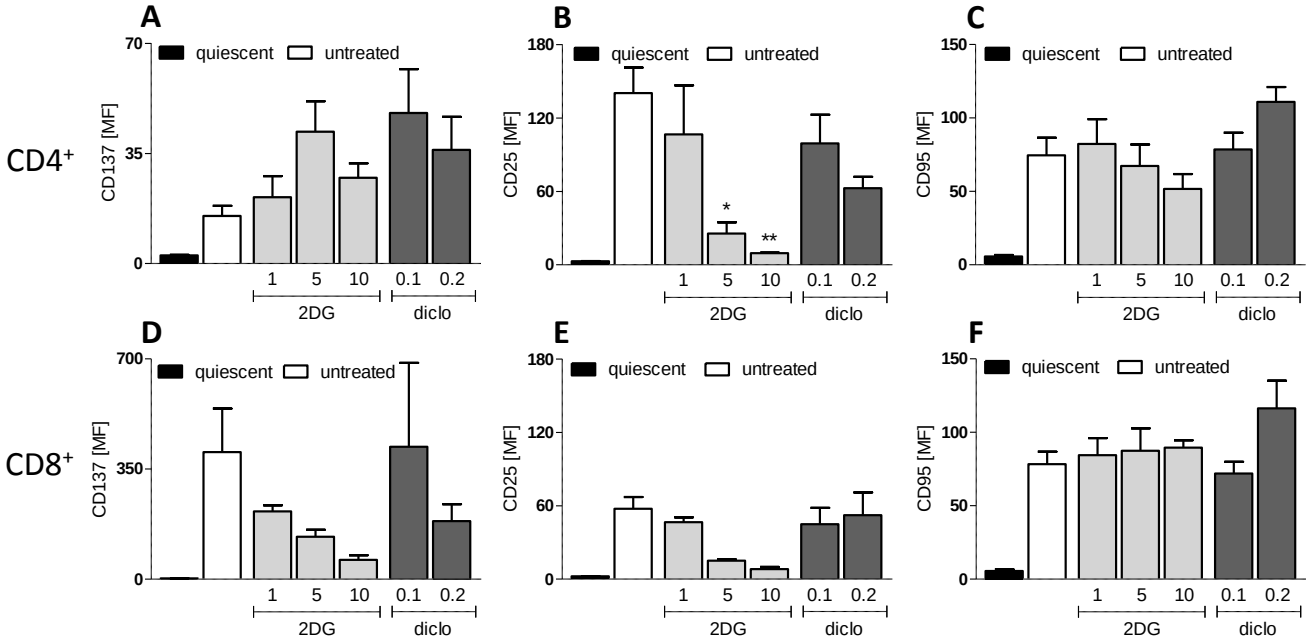

Figure 15. Impact of 2DG and diclofenac on expression of surface markers CD137, CD25 and CD95 of stimulated human $CD4^+$ and $CD8^+$ T lymphocytes
Cells were stained for flow cytometry with anti-CD137 (after 48 hours), anti-CD25 and anti-CD95 antibodies (after 7 days); bars show the median fluorescence ± SEM; **A** quiescent n=7, untreated and diclo n=6, 2DG n=3; **B and C** quiescent n=7, untreated n=4, 2DG and diclo n=3; **D** untreated n=6, 2DG n=4, diclo n=3; **D** quiescent and untreated n=6, 1mM 2DG n=2, 5 and 10 mM 2DG n=3, 0.1 mM diclo n=4, 0.2 mM diclo n=5; **E** quiescent n=7, untreated n=5, 1 mM 2DG n=4, 5 and 10 mM 2DG n=3, diclo n=4; **F** quiescent n=6, untreated n=5, 1 and 5 mM 2DG n=4, 10 mM 2DG n=3, diclo n=4; (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

In summary, both glycolytic inhibitors exerted only marginal effects on the expression of activation related surface markers.

4.3.3 Impact on restimulated human T cells

After characterizing the impact of anti-glycolytic drugs on the activation of freshly isolated $CD4^+$ and $CD8^+$ T cells, the effects of equal drug concentrations on fully stimulated immune cells were investigated. Restimulated T cells display an increased glycolytic activity thereby the metabolic profile is more comparable to tumor cells. The experimental set-up was the same as applied to stimulated T cells. Additionally the impact of 0.1 and 0.2 mM diclofenac on T cells expanded for two weeks and restimulated once again, representing a long-term cell culture, were investigated. During this third stimulation period we analyzed only the impact of diclofenac, which showed – in contrast to 2DG treatment – promising results concerning preserved effector function of T cells under treatment.

4.3.3.1 Glucose metabolism

2DG and diclofenac exerted a significant impact on glycolysis in restimulated T cells, however the inhibition was less pronounced compared to stimulated T cells. After restimulation 1 mM 2DG reduced glucose consumption by 50 % (in contrast to 80 % during stimulation) and 5 mM treated cells exhibited an uptake of 20 % of control cultures (during stimulation a complete block was observed). Only 10 mM 2DG blocked glucose consumption utterly within the first 72 hours (data not shown).

Effects of diclofenac were also less pronounced and 0.1 mM treated cells consumed 70 % (fist stimulation 50 %) of initially available medium glucose and cells treated with 0.2 mM 50 % compared to 25 % during the first stimulation in both T cell populations (data not shown).

Reduction of lactic acid secretion by both inhibitors was significant in $CD4^+$ T cells, whereas $CD8^+$ T cells again were affected only by high-dose 2DG application (fig. 16A/B).

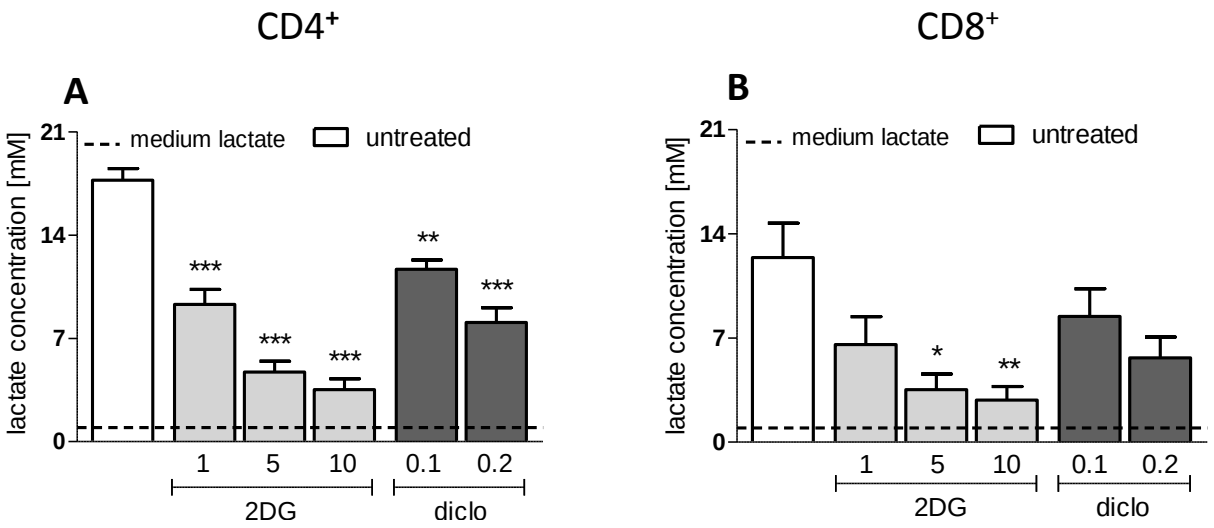

Figure 16. Impact of 2DG and diclofenac on lactate secretion of restimulated human $CD4^+$ and $CD8^+$ T lymphocytes
Lactate levels were measured enzymatically in culture supernatants after 72 hours. Untreated and 2DG n=4, diclo n=3 (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

4.3.3.2 Cell growth, proliferation and viability

The slight increase in cell size during restimulation (10-15 %) was significantly reduced only by the application of 5 mM and 10 mM 2DG in $CD4^+$ T cells. Diclofenac had only marginal effect on the diameter of both subtypes (data not shown).

Restimulated T cells showed a high proliferative capacity and 5 mM 2DG, 10 mM 2DG and 0.2 mM diclofenac reduced proliferation significantly in both populations, whereas 1 mM 2DG impeded proliferation only in $CD8^+$ T cell cultures (fig. 17A/B). This is in line with the reduced impact of glycolytic inhibition on restimulated T lymphocytes.

Restimulation had no effect on viability and untreated cultures showed a viability of 90 %. Only 10 mM 2DG reduced viability significantly (fig. 17C/D).

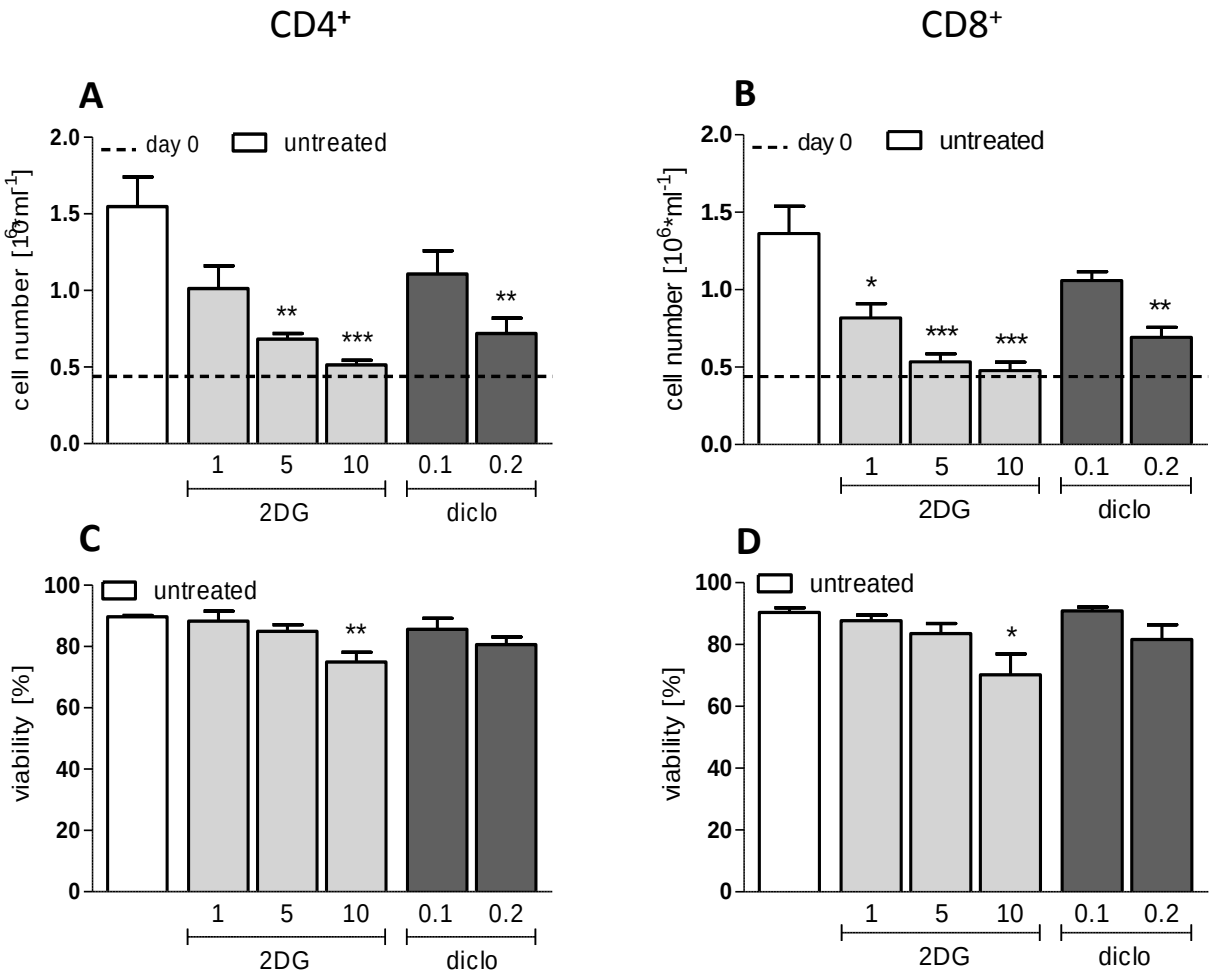

Figure 17. Impact of 2DG and diclofenac on proliferation and viability of restimulated human $CD4^+$ and $CD8^+$ T lymphocytes
(A/B) Cell number was determined by CASEY system after 72 hours; (C/D) viability was analyzed by flow cytometry with Annexin V and 7-AAD staining measurement. Untreated and 2DG n=4, diclo n=3 (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

4.3.3.3 Interferon ɣ, IL-2 and IL-10 secretion

The remarkable, distinct effects of 2DG and diclofenac were observed again in restimulated T cells. 2DG reduced interferon ɣ secretion, whereas diclofenac preserved or marginally increased secretion (fig. 18A/B).

IL-2 concentrations of $CD4^+$ T cell cultures were not significantly affected by a 2 DG treatment (data not shown, n=4). In contrast, both doses of diclofenac led to an increase in supernatant IL-2 concentration of $CD4^+$ T cells, which was even significant in the case of 0.1 mM diclofenac (data not shown, n=3). Upon 48 hours of restimulation, $CD8^+$ T cells secreted no IL-2 (data not shown).

Similar to interferon ɣ, 2DG reduced IL-10 production significantly by 60 % (1 mM), 90 % (5 mM) and 95 % (10 mM, n=3, data not shown), whereas diclofenac exerted no significant effects.

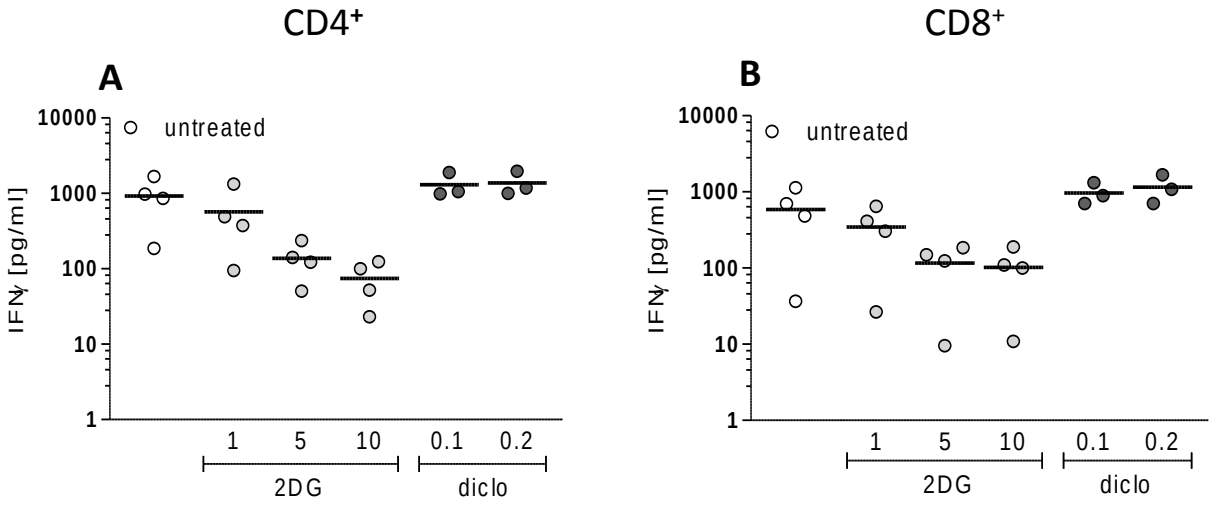

Figure 18. Impact of 2DG and diclofenac on interferon ɣ secretion of restimulated human $CD4^+$ and $CD8^+$ T lymphocytes
Analysis of interferon ɣ concentrations was performed by ELISA after 48 hours of restimulation; Untreated and 2DG n=4, diclo n=3 (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

4.3.3.4 Impact of diclofenac on two-times restimulated T cells

A second restimulation resulted in a reduced glycolytic activity. Especially $CD8^+$ T cells consumed less than 20 % of initial medium glucose resulting in reduced lactate secretion. Treatment with diclofenac had only a marginal inhibitory effect on metabolism of $CD8^+$ T cells. In contrast, $CD4^+$ T cells were glycolytically more active and the impact of diclofenac was significant (table 3).

After 72 hours mean diameters of two times restimulated cells were comparable to one time restimulated cells and diclofenac application had no observable effect (table 3).

The proliferation capability and cell number of untreated, two-times restimulated T cells was reduced and inhibition of proliferation by 0.2 mM diclofenac was significant in $CD4^+$ T cells (table 3).

Viability was again not affected by the treatment with diclofenac (table 3).

Interferon ɣ secretion was already low after restimulation and further restimulated cells only produced 70 % ($CD4^+$) and 50 % ($CD8^+$) of levels detected in one time restimulated cell supernatants displaying an exhausted phenotype. However, even in multiple stimulated T cells diclofenac exerted no significant effect on interferon ɣ secretion (table 3).

Table 3. Impact of 2DG and diclofenac on two times re-stimulated $CD4^+$ and $CD8^+$ T cells
Glucose and lactate levels were measured enzymatically in culture supernatants; cell number and mean diameter were determined by CASEY system; Measurement of interferon ɣ concentrations were performed by ELISA, viability was analyzed by flow cytometry with Annexin V and 7-AAD staining (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

72 hours		**untreated**	**0.1 mM diclo**	**0.2 mM diclo**
glucose consumption [mM]	$CD4^+$ (n=4)	4.7 ± 0.4	2.6 ± 0.3*	1.6 ± 0.7**
	$CD8^+$ (n=2)	1.7	1.0	0.7
lactate [mM]	$CD4^+$	13.5 ± 1.3	8.5 ± 0.8*	7.1 ± 0.8**
	$CD8^+$	9.5	5.8	5.3
cell number [x 10^6/ml]	$CD4^+$	1.1 ± 0.1	0.8 ± 0.1	0.6 ± 0.1**
	$CD8^+$	0.8	0.6	0.5
mean diameter [µm]	$CD4^+$	10.7 ± 0.3	10.7 ± 0.3	10.7 ± 0.3
	$CD8^+$	9.8	9.7	9.4
viability [%]	$CD4^+$	88.8 ± 1.2	81.3 ± 4.6	79.6 ± 2.8
	$CD8^+$	72.6	72.3	68.4
interferon ɣ [pg/ml]	$CD4^+$	669.9 ± 234.0	447.8 ± 235.7	567.2 ± 220.2
	$CD8^+$	307.9	268.0	283.7

4.3.4 Impact of continuous diclofenac exposure

Therapeutic benefits of medical approaches depend – among other factors – on long term toleration by the patient. Because of this the hereinafter described experiments were performed to analyze the effect of permanent anti-metabolic treatment by diclofenac on human T cells.

T cells maintained their highly glycolytic phenotype during 14 days of culture. Within the two weeks of continuous application, diclofenac reduced glucose uptake of $CD4^+$ T cells by 30 % (0.1 mM, n=5) and 60 % (0.2 mM, n=5). $CD8^+$ T cells in contrast were impaired stronger by 65 % (0.1 mM, n=3) and 80 % (0.2 mM, n=3). However, differences between both subpopulations did not reach statistical significance (data not shown). Similar observations were made with respect to lactate secretion (fig. 20A). While both untreated subpopulations produced nearly equal amounts of lactate, the reduction was more effective in $CD8^+$ T cell cultures.

Diclofenac had no impact on cell size after 14 days of continuous treatment (data not shown). Proliferation of $CD4^+$ T cells was not affected by 0.1 mM diclofenac, while $CD8^+$ cell number was reduced by 40 % (fig. 20B). The effect of 0.2 mM diclofenac was similar in both T cell populations. Furthermore, diclofenac treatment had no negative impact on viability in long-term cultures (fig. 20C).

Analyzing cytokine production revealed that even a long-term treatment with diclofenac did not affect IFNɣ levels (fig. 20D). Moreover, no impact on CD25 expression was detected and we even observed a significantly increased expression in $CD4^+$ T cells continuously treated with 0.2 mM diclofenac (data not shown, $CD4^+$ n=5, $CD8^+$ n=3). CD95 expression was increased by 50 % in $CD4^+$ and by 75 % in $CD8^+$ T cells under diclofenac treatment (data not shown, $CD4^+$ n=4, $CD8^+$ n=3). Statistical significance was not reached however a clear trend was observed.

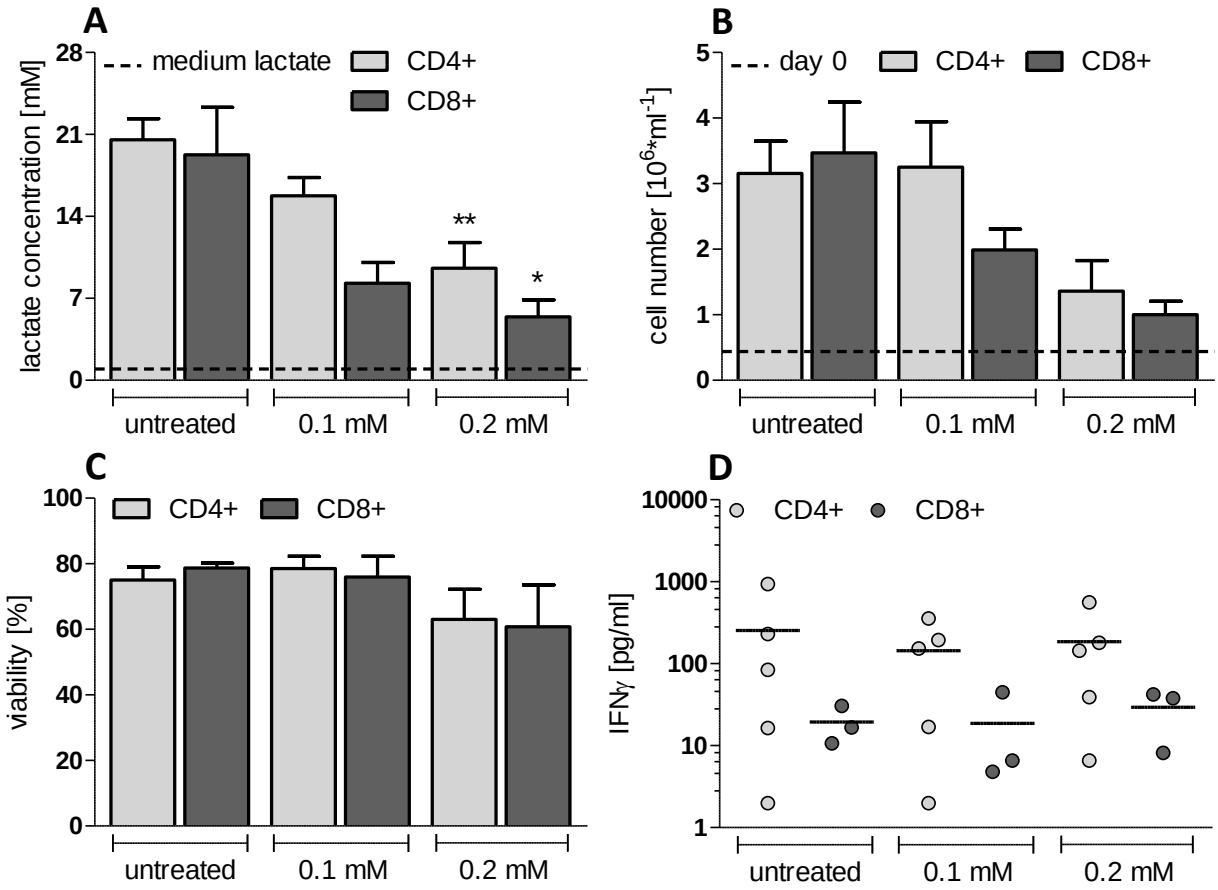

Figure 19. Impact of diclofenac on lactate concentration, proliferation, viability and interferon ɣ secretion of continuously treated human $CD4^+$ and $CD8^+$ T lymphocytes
(A) lactate levels were measured enzymatically in culture supernatants after 14 days of continuous diclofenac application; (B) cell number was determined by CASEY system; (C) viability was analyzed by flow cytometry with Annexin V and 7-AAD staining; (D) measurement of interferon ɣ concentrations was performed by ELISA **A, C and D** $CD4^+$ n=5, $CD8^+$ n=3, **B** $CD4^+$ untreated n=6, $CD4^+$ diclo n=5, $CD8^+$ n=3 (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

4.3.5 Impact on a mixed leukocyte reaction (MLR)

In all aforementioned experiments T cells were stimulated with anti-CD3/CD28 beads, which represent a strong but perhaps not physiologic activation stimulus for T cells. To apply a more physiologic stimulus, T cells were also activated with mature dendritic cells in an allogeneic setting and the impact of diclofenac and 2DG on T cell populations was investigated. In this set-up pre-matured dendritic cells (mDCs) of one donor are incubated together with $CD4^+$ lymphocytes of another donor. In this setting, however, additional effects of 2DG and diclofenac on DCs cannot be completely excluded.

Glucose and lactate concentrations measured after one week of stimulation showed two major results: (i) control cells were highly glycolytic and secreted large amounts of lactate and (ii) the anti-glycolytic treatment was effective. As a control culture lymphocytes alone were analyzed again concerning glucose consumption and lactate secretion after 7 days and showed negligible activity (table 4).

Proliferation was strongly affected by 2DG and a highly significant abatement was observed after 7 days comparable to anti-CD3/CD28 activated T cells. Also diclofenac impaired proliferation effectively, but to a less extent. While cell number was strongly diminished by 2DG and diclofenac the mean diameter only dropped slightly under 2DG treatment and diclofenac showed no impact, comparable to the results gained in anti-CD3/CD28 activated T cells (table 4).

After 7 days of co-cultivation about 90 % of untreated lymphocytes were viable. While significant reduction of viability was obtained by 5 mM 2DG treatment, diclofenac in contrast led only to a slight and not significant reduction (table 4).

CD25 expression was higher than in bead-stimulated cultures and anti-glycolytic treatments with 2DG strongly reduced the expression of CD25. Especially 5 mM 2DG diminished CD25 expression significantly to the level found in unstimulated lymphocytes (table 4).

In addition CD95 expression was reduced by 2 DG (significant by 5 mM 2DG) and in contrast diclofenac raised the median fluorescence however not significant (table 4).

The interferon ɣ secretion of 2DG treated co-cultures was almost completely inhibited, whereas diclofenac led to maintained or even increased production.

IL-2 and IL-10 were produced to a less extent compared to anti-CD3/CD28 activated T cells. Neither 2DG nor diclofenac exert significant impact on IL-2 and IL-10 secretion (table 4).

Table 4. Impact of 2DG and diclofenac on $CD4^+$ lymphocytes activated in a mixed leukocyte reaction (MLR) Glucose and lactate levels were measured in culture supernatants after 7 days of allogenic activation via MLR; Cell number and mean diameter were determined by CASEY system; Measurement of interferon ɣ/IL-2/IL-10 concentrations were performed by ELISA; Viability was analyzed by Annexin V and 7-AAD staining and surface markers by anti-CD25/-CD95 staining for flow cytometry (**P value** 0.05>*>0.01>**>0.001>***, treatment induced changes were analyzed with ANOVA and post-hoc by Tukey´s multiple comparison test)

after 7 days	**untreated**	**1 mM 2DG**	**5 mM 2DG**	**0.1 mM diclo**	**0.2 mM diclo**	**only $CD4^+$**
glucose consumption [mM] (n=4)	8.4 ± 0.1	0.5 ± 0.3***	-0.8 ± 0.3***	4.5 ± 0.8**	1.6 ± 1.1***	-2.1 ± 0.6 (n=3)
lactate secretion [mM] (n=4)	22.2 ± 0.8	3.0 ± 0.9***	1.3 ± 0.0***	12.0 ± 1.3***	6.1 ± 1.0***	1.1 ± 0.1 (n=3)
proliferation [10^6/ml] (n=4)	2.9 ± 0.2	0.7 ± 0.1***	0.5 ± 0.1***	1.7 ± 0.2***	1.1 ± 0.1***	0.4 ± 0.2 (n=2)
mean diameter [µm] (n=4)	10.3 ± 0.3	9.2 ± 0.4	8.3 ± 0.1**	10.3 ± 0.3	10.0 ± 0.3	7.5 ± 0.2 (n=2)
viability [%] (n=4)	89.6 ± 0.8	76.5 ± 1.9	56.1 ± 9.0***	87.7 ± 1.6	82.5 ± 2.3	87.0 ± 4.0 (n=2)
CD25 [median fluorescence] (n=4)	263.0 ± 72.9	73.3 ± 44.3	4.0 ± 0.4*	251.7 ± 66.1	193.1 ± 23.2	3.0 ± 0.1 (n=7)
CD95 [median fluorescence] (n=4)	72.1 ± 6.8	60.6 ± 13.3	8.0 ± 2.4***	80.6 ± 4.7	87.5 ± 8.3	5.7 ± 1.0 (n=7)

IFNɣ [pg/ml] (n=4)	83.9 ± 9.7	5.1 ± 3.4	n.d.	95.5 ± 24.3	85.4 ± 41.2	n.d. (n=1)
IL-2 [pg/ml]	605.0 ± 196.7 (n=4)	543.6 ± 243.8 (n=3)	423.7 ± 350.3 (n=2)	474.4 ± 193.0 (n=3)	516.3 ± 234.0 (n=3)	100.9 ± 87.9 (n=3)
IL-10 [pg/ml] (n=4)	10.1 ± 4.3	3.5 ± 3.1	8.3 ± 4.9	12.9 ± 6.2	14.4 ± 5.9	n.d. (n=1)

5. Discussion

5.1 Metabolic features of malignant and primary human T cells

Upregulated glycolysis despite a sufficient oxygen supply (= Warburg effect) is a metabolic feature of malignant cells, which is well known for many years and found in both, solid tumors and leukemia (6, 31). Tumor cells degrade glucose mainly to lactate, which is secreted in co-transport with a proton, resulting in lactate accumulation and concomitant acidification, referred as lactic acid, in the microenvironment of solid tumors. This glycolytic phenotype is shown to correlate directly with a poor prognosis. Patients suffering from hepatocellular carcinoma with a high GLUT1 expression reveal a significantly reduced survival rate compared to carcinomas with a low GLUT1 expression (136). Similar results are found with regard to the expression of lactate dehydrogenase (LDH) in melanoma patients (137) as well as for MCT1 expression in patients with bladder carcinoma (138) respectively MCT4 expression in oral squamous cell carcinoma (139).

In line, high extracellular lactate levels have a negative impact on patient prognosis, shown for cervix carcinoma by Walenta et al. (140). Several reasons are responsible for its pro-tumorigenic effects. Lactate exposure enhances mobility of tumor cells by promoting metastasis and cell spread (140–143). Moreover, lactic acid has profound effects on immune cell function. Dietl et al. demonstrated, that extracellular lactic acid reduces tumor necrosis factor α (TNF-α) secretion of monocytes thereby compromising the immune function (68). Furthermore, tumor derived lactate acts as a recruiting signal to tissue macrophages, polarizes a M2 phenotype (so called tumor-associated macrophages, TAMs) and induces the expression of vascular endothelial growth factor (VEGF) and arginase 1 (Arg1) shown by Colegio et al. Resulting neovascularization and nutrient provision promotes tumor growth (69). In addition, cytotoxicity and cytokine secretion of T cells is impeded in a lactic acid-rich milieu most likely due to intracellular accumulation and disturbed lactate efflux (66). Recently, Brand et al. proved a direct link between tumor-derived lactic acid and the inhibition of tumor immunosurveillance by T and NK cells in vivo (67).

Given that glucose consumption promotes tumor proliferation while increasing lactate levels impede the anti-tumor immune response, inhibition of tumor glycolysis is a

promising therapeutic approach. Several anti-glycolytic substances are currently under investigation and clinical trials have been initiated (144). Furthermore, synergistic effects of anti-angiogenic antibodies or conventional chemotherapeutic drugs in combination with anti-glycolytic substrates have already been proven. After a short period of initial regression, breast cancer cells for instance resume their growth under the treatment with sunitinib, a multi-targeted inhibitor of the receptor tyrosine kinase, due to metabolic reprogramming towards the anaerobic glycolysis. The combinatorial treatment by sunitinib with glycolytic inhibitors or knock-out of MCT4 prevents the recurrence of the tumor (145). A similar effect is shown for the hexokinase inhibitor 2DG which sensitizes the acute lymphoblastic leukemia cells to the treatment with prednisolone (146) and re-sensitizes glucocorticoid resistant cells to dexamethasone (63).

However, anti-glycolytic treatment might impede T cell function, which is considered as important for the anti-tumor immune response and patient survival. Numerous studies point out, that the activation of murine bulk T lymphocytes results in an upregulated glycolysis, which provides biomass and energy and is inevitable for proliferation and effector function such as IFNγ secretion (122, 147–149). IFNγ is of special importance for the anti-tumor immunity as it exerts several immunosupportive effects. The upregulation of MHC I expression on tumor cells resulting in a stronger immunogenicity and increase in sensitivity to cytotoxic T cells is described (81). Furthermore, IFNγ activates macrophages of the M1 phenotype, which are capable of killing tumor cells. The key role of interferon is underlined by the fact that the deficiency of this cytokine or appropriate receptors leads to increased tumor incidence (81). In murine T cells, IFNγ translation is reported to strongly depend on glucose supply, whereas IL-2 secretion is not affected by impaired glycolysis (113, 118, 120, 150). Accordingly, the inhibition of glycolysis or glucose starvation leads to a restricted effector function of murine T cells (120). Considering those consequences of an anti-glycolytic therapy, it is surprising, that only little is known about the link between metabolism, cell cycle progression and effector functions in human T cells (3, 94, 95). Therefore, we analyzed the glucose metabolism in stimulated human $CD4^+$ and $CD8^+$ T cells in relation to effector functions.

Upon stimulation T cells grow and produce cytokines and after a 48 hour period of cell growth ("on-blast" formation), stimulated and restimulated human $CD4^+$ and $CD8^+$ T cells start to proliferate. During the first 24 hours of stimulation glucose metabolism is only marginally elevated in both populations, beyond 24 hours glycolysis is increased and a highly glycolytic state is achieved beyond 48 hours. This general pattern is observed in stimulated and restimulated $CD4^+$ and $CD8^+$ T cells, although glycolytic activity is higher in restimulated T cells. Generally, $CD4^+$ lymphocytes slightly outperform $CD8^+$ lymphocytes in terms of proliferation and glucose metabolism. Interferon ɣ is secreted by T cells immediately upon activation (quiescent cells do not secret any IFNɣ) thus independently of glucose consumption. Despite continuous stimulation and persistent glucose uptake, measurable IFNɣ concentrations drop sharply beyond 48 hours. Thus a direct link between glycolysis and IFNɣ secretion in human T lymphocytes seems unlikely, which would be a major difference between human and murine T cells.

Taken together, our results show an upregulated glycolysis in proliferating human T cells, which is similar to tumor cells. Remarkably, important effector functions seem to be decoupled of glucose supply and consumption. Therefore, glycolytic inhibition should affect T cell proliferation, but not effector functions.

5.2. Impact of an antiglycolytic treatment on leukemic versus primary T cells

We examined the impact of 2DG and diclofenac on a human leukemic T-ALL cell line in comparison to primary human $CD4^+$ and $CD8^+$ T cells.

The anti-metabolite **2-deoxyglucose** is enzymatically phosphorylated to 2-deoxyglucose-6-phosphate, which cannot be further metabolized and induces a feedback inhibition on glucose metabolism. Administration of 2DG can result in adverse side effects, e.g. dizziness, fatigue, confusion, anorexia and QT prolongation, depending on the administered concentration. However, Raez et al. did not find any severe adverse effects in ten solid tumor bearing patients, treated with 2DG concentrations of up to 45 mg/kg (151). This corresponds to a serum concentration of 4.4 mM, based on an average patient with a bodyweight of 80 kg, 5

liters of blood volume and an assumed oral bioavailability of 100 %. In line, we performed our experiments in a range of 1 to 10 mM 2DG.

In 2013 our group showed that **diclofenac**, a non-steroidal anti-inflammatory drug (NSAID), exerts an inhibitory effect on lactate secretion and proliferation of several different tumor cell lines in vitro and reduces growth of murine B16 melanoma cells in vivo (91). Additionally diclofenac inhibits lactate formation as shown in a murine glioma model (152). This effect is due to blocked lactate transport by MCT1 and MCT4 resulting in reduced extracellular lactic acid concentrations and intracellular accumulation, which impedes glycolysis. As demonstrated by Holger Becker (TU Kaiserslautern, unpublished) already low concentrations of diclofenac significantly reduce the activity of MCT1 (Ki 1.45 ± 0.04 µM) and MCT4 (Ki 0.14 ± 0.01 µM). In contrast to 2DG, adverse drug reactions of diclofenac are rare and well-known (12 % of treated patients) (153). The most common side effects include a disturbed gastrointestinal system (abdominal pain, nausea, peptic ulceration), skin appearances (rash, urticarial, dermatitis), dizziness as well as renal (oliguria, proteinuria) and cardio-vascular symptoms (edema, hypertension) (154).

The human childhood T-ALL cell line CCRF-CEM-C7H2 has a highly glycolytic phenotype. The available medium glucose is almost entirely taken up within 72 hours correlating with a strong increase in cell number. Application of 2DG or diclofenac reduces glycolysis and proliferation significantly and to a comparable extent by using 5 mM 2DG or 0.2 mM diclofenac. These findings underline the importance of glycolysis-derived biomass for tumor expansion also in leukemic cells.

In $CD4^+$ and $CD8^+$ T cell cultures 2DG also exerts a significant effect on glucose consumption and lactate secretion. Even the administration of the lowest dose of 1 mM 2DG results in a nearly total glycolytic blockade within the first 72 hours. The anti-glycolytic effect of 1 mM 2DG on the glycolysis of $CD4^+$ T cells is significantly stronger than the effect of 0.1 mM diclofenac and therewith represents a considerable difference to the impact on C7H2 tumor cells. This tendency can also be found in $CD8^+$ T lymphocytes. However, cells that have been treated and restimulated for seven days seem to be less affected by 2DG treatment, which suggests a possible development of a compensatory mechanism. Although medium

exchange after 4 days should ensure constant concentrations, degradation of the drug cannot be completely excluded. Surprisingly, the impact of 2DG on T cell activation is despite a comparable glycolytic inhibition much stronger compared to diclofenac. On-blast formation is significantly affected by 2DG, whereas diclofenac has only slight effects on cell growth of both T cell populations. 2DG blocks proliferation almost entirely within the first 72 hours, whereas diclofenac treated cells are less affected. In line with our results on glucose deprivation (132), high dose 2DG almost completely block proliferation, whereas 0.1 mM diclofenac treated cells show an increase in cell number by about 50 %. Moreover, the activation induced expression of CD25 is significantly repressed by 2DG, but not by diclofenac treatment.

Taken together, both substances effectively reduce the glucose metabolism of leukemic and primary human T cells. However, diclofenac has a more pronounced negative impact on tumor cells, but is better tolerated by primary human T cells and preserves proliferation and activation.

Both glycolytic inhibitors reduce the viability of C7H2 cells, but a significant stronger effect of diclofenac was observed. A conceivable reason is the cytotoxic intracellular lactic acid accumulation by inhibition of monocarboxylate transporters. As shown by Barry and Eastman, intracellular acidification results in the activation of deoxyribonuclease II which leads to apoptosis and cell death (155). Nevertheless, further direct apoptosis inducing effects of diclofenac cannot be excluded. T cells treated with high doses diclofenac show, despite a similar impact on glycolysis, a totally preserved survival rate. In contrast, the application of 10 mM 2DG diminishes viability significantly and to a comparable extent in malignant and non-malignant T cells. Interestingly, beyond 72 hours of stimulation, viability of T cells is improved by the application of both anti-glycolytic agents, which may be due to the reduced extracellular, cytotoxic lactate levels compared to untreated cell cultures. As shown by Fischer et al. (66), lactic acid exerts a strong negative impact on T cell viability and effector functions in a concentration dependent manner. Accordingly, the therapeutic application of glycolytic inhibitor diclofenac directly reduces the viability of tumor cells while T cell viability is preserved or even increased by concomitantly reduced lactic acid secretion.

Along with the murine data, IFNɣ secretion of 2DG treated human T cells is reduced. Furthermore, the production of the cytokines IL-2 and IL-10 is compromised by the 2DG treatment in $CD4^+$ T cells, whereas the IL-2 secretion of $CD8^+$ T cells is preserved. While glucose metabolism is impeded effectively by diclofenac, IFNɣ production is utterly preserved and stimulated $CD8^+$ T cells treated with 0.2 mM even show significantly higher IFNɣ levels. Furthermore, IL-2 secretion is only marginally affected by diclofenac and not altered concentrations of IL-10 are found in $CD4^+$ lymphocytes. On the basis of these results it seems irritating, that 2DG and diclofenac display such strong differences concerning the impact on T cell effector function despite comparable effects on glycolysis. Accordingly, a direct link between glucose metabolism and IFNɣ secretion in human T cells is not likely. This is supported by the fact, that also glucose starvation has no impact on cytokine secretion (132). Furthermore, oligomycin, an irreversible inhibitor of the mitochondrial ATP-synthase, has no effect on the IFNɣ production in human T cells as well (132). In further analysis we could show, that 2DG not only inhibits glycolysis but also blocks respiration. Although T cells showed some metabolic flexibility as glucose deprivation can be compensated by increased respiration, blocking of both pathways is deleterious for T cell function (132).

As shown above, 2DG application compromises the early on-blast formation, proliferation and CD25 expression, which reflects an unstimulated, quiescent state of the treated cells. These findings support two hypotheses:

(i) T cell activation depends at least on one energy and biomass delivering pathway

(ii) Respiration and anaerobic glycolysis seem to be interchangeable and therewith compensatory

The physiological activation of an adaptive immune response involves a multicellular process and in addition antigen-presenting cells are possible targets of an anti-glycolytic therapy. To assure physiological relevance, we also examined the consequences of the 2DG and the diclofenac treatment on an allogenic mixed leukocyte reaction (MLR) of $CD4^+$ T cells with dendritic cells (DCs). After 7 days of stimulation, the data acquired in anti-CD3/CD28 stimulated cells are nearly congruent

with MLR results. Nevertheless, additional impacts of 2DG and diclofenac on the maturation of dendritic cells cannot be excluded by our experiments.

Due to the constant lactate secretion by tumor cells, a long-term application of diclofenac without compromising the patients´ immune system is mandatory to ensure sustainable therapeutic success. Therefore, we analyzed the impact of an uninterrupted diclofenac treatment on stimulated T cells. Even after 14 days of treatment diclofenac has no impact on the IFNɣ secretion, while a persisting, but significant effect on glucose metabolism is observed.

Our results contrast murine data and several explanations could be responsible for those differences between human and murine cells:

Murine IFNɣ secretion strongly depends on glucose metabolism, whereas low glucose conditions preserve IFNɣ production in humans (113, 119, 132). Furthermore, Datta et al. demonstrated, that the blockade of the mTOR pathway impedes T cell motility in addition to the expression of migration-related surface markers in the murine, but not in the human immune system (115).Thus it is likely, that human and murine cells differ in their immune cell metabolism more than expected.

In addition, experimental conditions have to be considered. The experiments showing the link between glucose metabolism and IFNɣ production in the murine system are performed in medium without serum or applying dialyzed serum. In contrast, human T cells are cultivated in non-dialyzed, AB- or fetal calf serum (FCS) containing medium. To exclude impacts of the different experimental set-ups, further experiments are necessary.

Based on our results it seems possible to apply anti-glycolytic drugs reducing lactate secretion by tumor cells while preserving immune cell effector functions, however at the expense of reduced T cell proliferation.

5.3 Outlook: Glycolytic inhibition complements immunotherapeutic approaches

Immunotherapeutic approaches are one of the most promising and paradigm-shifting strategies against tumor burden. The application of so called checkpoint inhibitors results in the release of T cell anergy and leads to the destruction of tumor cells. Promising results are already achieved in metastatic melanoma (156), advanced squamous non-small cell lung cancer (NSCLC) (157) and several others (158, 159). The checkpoints CTLA-4 (cytotoxic T lymphocyte antigen 4) and PD-1 (programmed death 1) are receptors on the cell surface of T cells and negatively regulate their activation and proliferation upon binding by appropriate ligands (81). Those ligands, such as the B7 family and PD-ligand 1 and 2 (PD-L1/-L2), are not only upregulated in the course of inflammation to avoid a permanent and unrestricted stimulation (81), but are also expressed by tumor cells to suppress the response of invading T cells (160). Therefore, the blockade of those co-inhibitory receptors by antibodies like ipilimumab (Anti-CTLA-4) and nivolumab (Anti-PD-1) preserves and enhances the anti-tumor immune response. It turns out that especially combinations of CLTA-4- and PD-1-blockades are effective (81). However, after an initial strong response to checkpoint inhibition many patients develop treatment resistance (161). Furthermore, checkpoint inhibition results in strong and unrestricted immune response thus severe immune-related adverse effects (irAEs) have been observed. These include most importantly endocrinopathies (like hypophysitis or hypothyroidism), pneumonitis, colitis, skin appearances (like pruritus and vitiligo) as well as an increase of hepatic enzymes (162). Hence, the development of combinatorial treatment schemes lowering necessary concentrations of checkpoint inhibitors thus reducing possible irAEs and avoiding resistance is of major interest.

The success of checkpoint inhibition seems to be connected with tumor glucose metabolism as a high concentration of serum lactate dehydrogenase A (LDH-A) correlates with a poor outcome upon PD-1 and CTLA-4 blockade (163, 164). Beside lactate accumulation also secretion of PGE_2, which is detected in a variety of tumors and assumed to be a primary driver of carcinogenesis (165–168), limits immune cell function. In line, Zelenay et al. demonstrated that the non-selective COX-inhibitor Aspirin enhances the efficacy of immunotherapy by anti-PD-1 antibodies in $BRAF^{V600E}$ mutated melanoma cells. Interestingly, selective COX2-inhibitors (so-

called coxibes) show a synergism with anti-PD1 treatment as well, albeit to a lesser extent. (169).

Based on our data, we hypothesize, that diclofenac, an unselective COX1 and COX2 inhibitor might be much more efficient in combination with checkpoint inhibitors, as it combines the benefits of reduced PGE_2- and lactate secretion. Necessary concentrations are only marginally higher than achieved with commonly used daily administration of 100 mg per os (152). Additionally, diclofenac is bound to 99 % to serum albumin (153). To liberate diclofenac from albumin and therewith achieve higher effective concentrations a simultaneous administration of agents with stronger plasma protein binding could be discussed.

Taken together, anti-glycolytic drugs are promising supplements in immunotherapeutic approaches. Especially diclofenac lends itself to the combination with checkpoint inhibitors and offers the following advantages over the application of comparable glycolytic inhibitors such as 2DG:

- extensive therapeutic experiences and a manageable spectrum of adverse effects
- a significantly reduced tumor cell viability, but sustained immune cell viability
- preserved immune cell activation and effector functions while effectively reduced tumor lactate secretion

6. Conclusion

Human T-ALL cells as solid tumor cells exhibit a metabolic phenotype characterized by an upregulated glucose uptake and lactate secretion despite sufficient oxygen supply (= Warburg effect). Stimulated and re-stimulated human T lymphocytes show a similar metabolic shift towards aerobic glycolysis. However, glucose metabolism is not elevated during the initial phase of stimulation when cell growth and cytokine production takes place. Thus, IFNɣ secretion seems to be decoupled from glucose consumption. Comparing the impact of glycolytic inhibition on primary and malignant lymphocytes reveals that glucose uptake and lactate secretion are reduced in malignant and non-malignant T cells. The glycolytic inhibitors 2-deoxyglucose and diclofenac effectively reduce proliferation in malignant and non-malignant lymphocytes. However, despite reduced glycolysis diclofenac treatment preserves effector functions as the secretion of IFNɣ. In contrast, the application of 2DG simultaneously impairs glycolysis and cellular respiration and thereby compromises T cell activation, proliferation and effector functions.

This study demonstrates that the application of the anti-glycolytic drug diclofenac effectively impairs tumor glucose metabolism and viability, while T cell effector functions are totally preserved. Especially immune therapies (e.g. checkpoint inhibition) could benefit from these insights, as tumor derived lactic acid is a strong immunosuppressive metabolite fostering immune escape.

7. References

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8. Appendix

8.1 German abstract

Auswirkungen einer anti-metabolischen Therapie auf leukämische und nicht-maligne T-Lymphozyten

Solide und leukämische Tumorzellen weisen einen bemerkenswerten Stoffwechsel auf: Trotz suffizienter Sauerstoffzufuhr zeigt sich eine gesteigerte Glykolyse mit erhöhtem Glukoseverbrauch und vermehrter Laktatsekretion (= aerobe Glykolyse). Diese Eigenschaft wird als Warburg-Effekt bezeichnet und ist weniger Ausdruck einer metabolischen Degeneration, sondern stellt vielmehr einen Wachstumsvorteil für Tumorzellen dar. Die in der Glykolyse anfallend Zwischenprodukte sind Bausteine für Zellwachstum und Proliferation. Darüber hinaus übt Laktat, das glykolytische Endprodukt, einen immunsuppressiven Effekt aus und schränkt dadurch die Prognose von Tumorpatienten ein. Die Hemmung dieses charakteristischen Stoffwechsels mittels antiglykolytischer Substanzen tritt zunehmend in den Fokus der Forschung. Zu diesem Zwecke untersuchten wir die Auswirkungen von 2-Deoxyglucose (2DG) und Diclofenac auf kindliche T-ALL Zellen. Beide Wirkstoffe hemmten die Glykolyse und Proliferation signifikant, jedoch wurde eine effektive Reduktion der Viabilität um bis zu 75 % nur unter Anwendung von Diclofenac beobachtet.

Murine Daten deuten darauf hin, dass T-Lymphozyten bei Aktivierung ihren Stoffwechsel ebenfalls zu Gunsten der aeroben Glykolyse umstellen. Da nur wenige Daten bezüglich humaner T-Zellen vorliegen, analysierten wir in einem zweiten Schritt die Interaktion von Stoffwechsel und Effektorfunktionen in ein- und zweifach stimulierten $CD4^+$ und $CD8^+$ Spenderlymphozyten. Unmittelbar nach Aktivierung mittels anti-CD3/-CD28 Antikörpern war eine Sekretion von Interferon ɣ zu verzeichnen, wohingegen die Steigerung der Glykolyse und der Proliferation erst einem 48-stündigen Intervall des Zellwachstums („on-blast formation“) folgte.

In vorausgehenden Experimenten wurde gezeigt, dass die Hemmung der Glykolyse zu einer erheblichen Störung der Effektorfunktionen und insbesondere der IFNɣ-Sekretion durch murine T-Zellen führt. Diese Tatsache stellt eine große

Herausforderung für die klinische Anwendbarkeit von Glykolyseinhibitoren dar, zumal die Infiltration von Tumorgewebe durch funktionstüchtige T-Zellen grundlegend prognosebestimmend ist. In unseren Experimenten konnten wir zeigen, dass die Anwendung hoher Dosen von 2DG die Glykolyse und Proliferation nahezu komplett blockiert und die Sekretion wichtiger Effektorzytokine (IFNɣ, IL-2, IL-10) teils deutlich beeinträchtigt. In einigen experimentellen Konstellationen war sogar die Viabilität der humanen T-Zellen signifikant reduziert. Unter Berücksichtigung der ebenfalls vermindert exprimierten Reifungsmarker (CD137, CD25, CD95) muss von einer Beeinträchtigung der Aktivierbarkeit von humanen T-Zellen unter Anwendung von 2DG ausgegangen werden.

Im Gegensatz dazu konnte Diclofenac die Zytokinproduktion trotz vergleichbarer Auswirkungen auf die Glykolyse vollständig erhalten oder sogar steigern. Diese Ergebnisse widerlegen eine direkte Verknüpfung von Glukosestoffwechsel und sekretorischer Effektorfunktion humaner T-Lymphozyten. Bemerkenswert ist, dass Diclofenac keinen negativen Effekt auf die Viabilität der behandelten Zellen ausübte. Um die physiologische Relevanz unserer Ergebnisse zu bestätigen, analysierten wir anschließend die Auswirkungen beider Substanzen auf eine allogene Lymphozytenreaktion (= mixed leukocyte reaction, MLR). Die dabei erzielten Resultate konnten die Ergebnisse der anti-CD3/-CD28 stimulierten T-Zellen reproduzieren, wenn auch weitere Einflüsse von 2DG und Diclofenac auf die Antigen-präsentierenden Zellen hierbei nicht ausgeschlossen werden konnten. Der Erfolg einer medizinischen Therapie bemisst sich unter anderem an der Langzeitverträglichkeit und unsere Ergebnisse demonstrierten eine persistierende Effektorfunktion und Viabilität sogar unter eine kontinuierlichen, 14-tägigen Applikation von Diclofenac.

Auf Grund dieser Erkenntnisse erscheint eine Kombinationstherapie von Glykolyseinhibitoren mit sogenannten Checkpoint-Inhibitoren vielversprechend. Checkpoint-Inhibitoren lösen eine gesteigerte, wenn auch ungerichtete Immunantwort von körpereignen Immunzellen gegen die Fremdantigen-präsentierenden Tumorzellen aus. Diese Antwort wird jedoch durch vermehrt ins Tumormilieu sezernierte Milchsäure reduziert bzw. im Verlauf sogar vollständig unterdrückt, was einer Resistenzentwicklung gleich kommt. Eine Inhibition der

Laktatproduktion in Tumorzellen könnte diesen tumoreigenen Schutz vor einer zielgerichteten Immunabwehr aufheben.

Weitere Experimente sind notwendig um die Interaktion von Metabolismus und Effektorfunktion humaner T-Zellen vollständig zu verstehen und die vielversprechenden antimetabolischen Therapien zur optimieren.

8.2 Abbreviations

- 2DG — 2-deoxygluse
- 3´ UTR — three prime untranslated region
- 4E-BP1 — eukaryotic initiation factor 4E binding protein 1
- 7-AAD — 7-aminoactinomycin
- AA — amino acid
- acetyl-CoA — acetyl coenzyme A
- ACL — ATP-citrate lyase
- Akt — protein kinase B
- AMPK — adenosine monophosphate activated protein kinase
- ANOVA — analysis of variance
- AP-1 — activation protein 1
- APC — antigen presenting cell
- arg1 — arginase 1
- ASCT2 — amino-acid transporter 2
- ATP — adenosine triphosphate
- Bad — Bcl-2-associated death promoter
- Bak — Bcl-2 homologous antagonist killer
- Bax — Bcl-2-associated X protein
- Bcl-2 — B cell lymphoma 2
- bHLH — basic helix-loop-helix
- Bim — Bcl-2-like protein 11
- CD — cluster of differentiation
- Cdk — cyclin dependent kinase
- CO_2 — carbon dioxide
- COX — cyclooxygenase
- CREB — cyclic AMP response element-binding protein
- CTL — cytotoxic T cell
- CTLA-4 — cytotoxic T lymphocyte antigen 4
- CTP — cytidine triphosphate
- DAG — diacyl glycerol
- DC — dendritic cell
- DCA — dichloroacetate
- diclo — diclofenac
- DNA — deoxyribonucleic acid
- E-cadherin — epithelial cadherin
- EGF — epidermal growth factor
- EGFR — epidermal growth factor receptor
- ELISA — enzyme-linked immunosorbent assay
- ERK — extracellular-signal-regulated kinase
- et al. — et altera
- FACS — fluorescence-activated cell sorting
- FCS — fetal calf serum
- FasL — Fas ligand
- fig. — figure

- FITC — fluorescein isothiocyanate
- G_1-phase — gap 1 phase
- G6P — glucose-6-phosphate
- G6PDH — glucose-6-phosphate dehydrogenase
- GAPDH — glyceraldehyde-3-phosphate dehydrogenase
- GATA3 — trans-acting T-cell-specific transcription factor
- GKS-3 — glycogen synthase kinase 3
- GLS — glutaminase
- GLUT — glucose transporter
- GM-CSF — granulocyte macrophage colony-stimulating factor
- GTP — guanosine triphosphate
- HIF1α — Hypoxia inducible factor 1α
- HK — hexokinase
- HRE — hypoxia-responsive elements
- HRP — Streptavidin-Horseradish peroxidase
- ICAM-1 — intercellular adhesion molecule 1
- i.e. — id est
- IFNɣ — interferon ɣ
- IGFR — insulin-like growth factor receptor
- IL — interleukin
- iNOS — inducible nitric oxide synthase
- IP3 — inositol-1,4,5-triphosphate
- irAE — immune-related adverse effect
- IRF4 — interferon regulatory factor 4
- IU — international unit
- Jak — januskinase
- LDH — lactate dehydrogenase
- LFA-1 — lymphocyte function-associated antigen 1
- LPS — lipopolysaccharide
- MACS — magnetic cell separation
- MAPK — mitogen-activated protein kinase
- Max — Myc-associated factor x
- Mcl-1 — induced myeloid leukemia cell differentiation protein
- MCT — monocarboxylate transporter
- Mdm2 — mouse double minute 2 homolog
- MEK — Mitogen/Extracellular signal-regulated kinase
- MHC — major histocompatibility complex
- MLR — mixed leukocyte reaction
- mM — millimolar = millimole per liter
- MMP — matrix metalloproteinase
- mRNA — messenger RNA
- mTORC1 — mammalian target of rapamycin complex 1
- Myc gene — myelocytomatosis gene
- n — number (of samples)
- NAD^+/H — nicotinamide adenine dinucleotide
- $NADP^+$/H — nicotinamide adenine dinucleotide phosphate
- NFAT — nuclear factor of activated T cells

- NFκB — nuclear factor κB
- NK cells — natural killer cells
- nm — nanometer
- NO — nitric oxide
- NSAID — non-steroidal anti-inflammatory drug
- NSCLC — non-small cell lung cancer
- Oct-4 — octamer binding transcription factor 4
- OXPHOS — oxidative phosphorylation
- $p21^{Cip}$ — cyclin-dependent kinase inhibitor 1
- p53 — protein 53
- PBMC — peripheral blood mononuclear cell
- PBS — phosphate buffered saline
- PD-1 — programmed death 1
- PDH — pyruvate dehydrogenase
- PDK1 — 3-phosphoinositide dependent kinase 1
- PD-L1 — programmed death ligand 1
- PE — phycoerythrin
- PET-CT — Positron emission tomography – CT
- PFK — phosphofructokinase
- pg — picogram
- PGE_2 — prostaglandin E_2
- PH — pleckstrin homology domain
- pH — pondus hydrogenii
- PI3K — phosphatidylinositol-3-kinase
- PIP2 — phosphatidylinositol-2-phosphate
- PIP3 — phosphatidylinositol-3-phosphate
- PK — pyruvate kinase
- PKC — protein kinase C
- PLCγ1 — phospholipase C γ1
- PPP — pentose phosphate pathway
- Raf — rat fibrosarcoma
- Ras — rat sarcoma
- RB — retinoblastoma
- RORγt — RAR-related orphan receptor γt
- ROS — reactive oxygen species
- rpm — rounds per minute
- RPTK — receptor protein tyrosine kinases
- S6K1 — S6 kinase 1
- SEM — standard error of the mean
- SCO2 — cytochrome c oxidase 2
- SKP2 — S-phase kinase-associated protein 2
- S-phase — synthesis phase
- STAT — signal transducer and activator of transcription
- T-ALL — T cell acute lymphoblastic leukemia
- TAM — tumor associated macrophages
- TCA cycle — tricarboxylic acid cycle
- TCR — T cell receptor

- TGFβ — transforming growth factor β
- T_h cell — T helper cell
- TIGAR — TP53-induced glycolysis and apoptosis regulator
- TMB — tetramethylbenzidine
- TNFα — tumor necrosis factor α
- TP53 — tumor protein p53
- TSC 1/2 — tuberous sclerosis complex 1/2
- TTP — thymidine triphosphate
- VEGF — vascular endothelial growth factor
- αKG — α ketoglutarate

8.3 Publication

Renner K, Geiselhöringer A, **Fante M**, Bruss C, Färber S, Schönhammer G et al. Metabolic plasticity of human T cells: Preserved cytokine production under glucose deprivation or mitochondrial restriction, but 2-deoxy-glucose affects effector functions. European journal of immunology 2015; 45(9):2504–16.

8.4 Acknowledgement

Der größte Dank gebührt meiner Betreuerin, **Dr. Kathrin Renner-Sattler**, für die Überlassung des Themas und die umfassende Unterstützung, ohne die die erfolgreiche Durchführung der Arbeit nicht möglich gewesen wäre. Die zahlreichen Diskussionen in freundschaftlicher Atmosphäre, die Erfolge, aber auch Rückschläge haben mich nicht nur für dieses Thema, sondern auch für die Grundlagenforschung und Laborarbeit im Allgemeinen nachhaltig begeistert.

Darüber hinaus bedanke ich mich herzlichst bei meiner Doktormutter, **Prof. Dr. Marina Kreutz**, für die Bereitstellung der Forschungseinrichtung und die kritische, aber immer wohlgemeinte Prüfung von Experimenten und Ergebnissen. Erst durch diesen Diskurs ist mir die Komplexität meines Themas bewusst geworden.

Bei den Direktoren **Prof. Dr. Wolfgang Herr** und **Prof. Dr. Reinhard Andreesen** bedanke ich mich für die Möglichkeit, meine Arbeit am Lehrstuhl für Hämatologie und internistische Onkologie anzufertigen. Die Freude an der hämatologischen Forschung hat viel dazu beigetragen, mich auch für die Facharztausbildung in der Hämatologie und Onkologie in Regensburg zu entscheiden.

Ein besonderer Dank gilt **Stephanie Färber** für die selbstlose Hilfe bei allen hier genannten Experimenten, die zuverlässige Zusammenarbeit und die tolle Zeit im Labor. Ebenso ergeht ein großer Dank an die vielen anderen, immer hilfsbereiten medizinisch-technischen Assistentinnen.

Ich möchte mich bei meiner Schwester **Katharina Richter** für die sprachlichen und grammatikalischen Korrekturen in dieser Arbeit bedanken, die sie trotz fachlicher und räumlicher Distanz mit größter Sorgfalt für mich angefertigt hat.

Großer Dank geht an **Ulrike Wassermann**, die - wann immer notwendig - für Motivation und Aufmunterung gesorgt und mir und meiner Arbeit stets volles Verständnis entgegen gebracht hat.

Die Doktorarbeit ist der aktuellste von zahlreichen Zwischenschritten meiner bisherigen Ausbildung. Jeden dieser Schritte haben meine Eltern, **Petra und Bernhard Fante**, bedingungslos unterstützt. Deswegen widme ich diese Arbeit und alle damit verknüpften Anstrengungen und Mühen meinen Eltern.

8.5 Declaration

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

Regensburg, Juni 2017