Metabolic reprogramming upon CD8 T cell activation

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1. Introduction

Living organisms have developed complex metabolic networks in order to generate the biomolecules and energy required for survival and proliferation. These metabolic networks consist of a huge numbers of different metabolic enzymes catalyzing distinct chemical reactions. In principle, these reactions can be divided into two groups: anabolic reactions, which are endergonic (energy absorbing) processes to synthesize macromolecules, and catabolic reactions, which breakdown macromolecular substrates in order to generate energy and thereby also generate new products/substrates (Figure 1.1). Both networks consist of a huge variety of different enzymatic reactions forming characteristic intermediates, which can serve as new substrates for further catabolic or anabolic reactions. According to the cell's needs, the pathways for building up and breaking down cellular components can be turned on and off or sped up/slowed down. Therefore it is crucial for the cell to develop regulatory mechanisms to monitor and balance the different metabolic pathways.

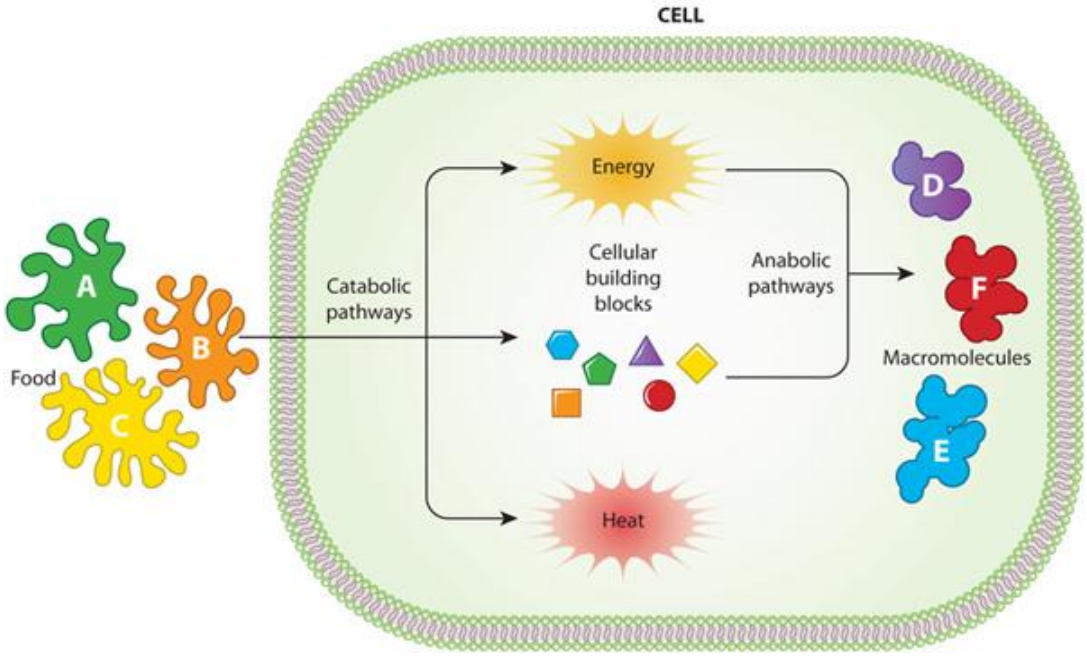

Figure 1.1. Schematic overview on cell metabolism (Nature Education 2010)

1.2. Cell catabolism – delivering the energy for life

Living systems rely on a constant supply of free energy to maintain 3 major processes, mechanical work like cellular movements or muscle contractions, active transport of molecules and ions, and synthesis of macromolecules and other biomolecules. The major energy carriers providing the required vigor are nucleotide triphosphates (NTPs), of which adenosine triphosphate (ATP) is perhaps the most famous. ATP consists of an adenosine and three phosphate groups containing two energy rich phosphoanhydride bonds. The free energy resulting from the hydrolysis of these bonds (Figure 1.2.) drives thermodynamic unfavorably enzymatic reactions.

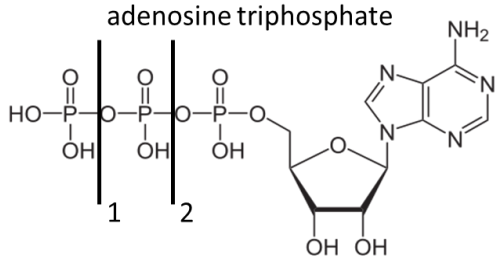

Figure 1.2. ATP structure and free energy resulting from hydrolysis of phosphoanhydride bonds

Since ATP is an energy carrier, which serves not as an energy store, there is a very high turnover of ATP, ADP, and AMP molecules. Cells are able to sense their current energy status by determining the ration between ATP and AMP through highly specific cellular energy sensors like adenosine monophosphate-activated protein kinase (AMPK) and subsequently modulate metabolic pathways to produce or consume ATP (Hardie et al. 1999).

Another role of ATP is in signal transduction pathways. Kinases use ATP as a phosphate donor to phosphorylate proteins, which commonly leads to protein activation driving signal transduction processes. Finally ATP can also be used as donor for AMP during DNA-synthesis. There are several ATP analogs like guanosine triphosphate (GTP), uridine triphosphate (UTP) and cytidine triphosphate (CTP), which are mainly used for DNA/RNA synthesis, but there are also metabolic processes which specifically utilize these triphosphates, e.g. glycogen syntheses (UTP), pyrimidine biosynthesis (CTP), and microtubule organization (GTP).

The main energy source for living cells is provided by the consumption of glucose. Glucose is metabolized through glycolysis and the subsequent tricarboxylic acid (TCA) cycle. During glycolysis a series of ten enzymatic reactions convert glucose to pyruvate (Figure 1.3.).

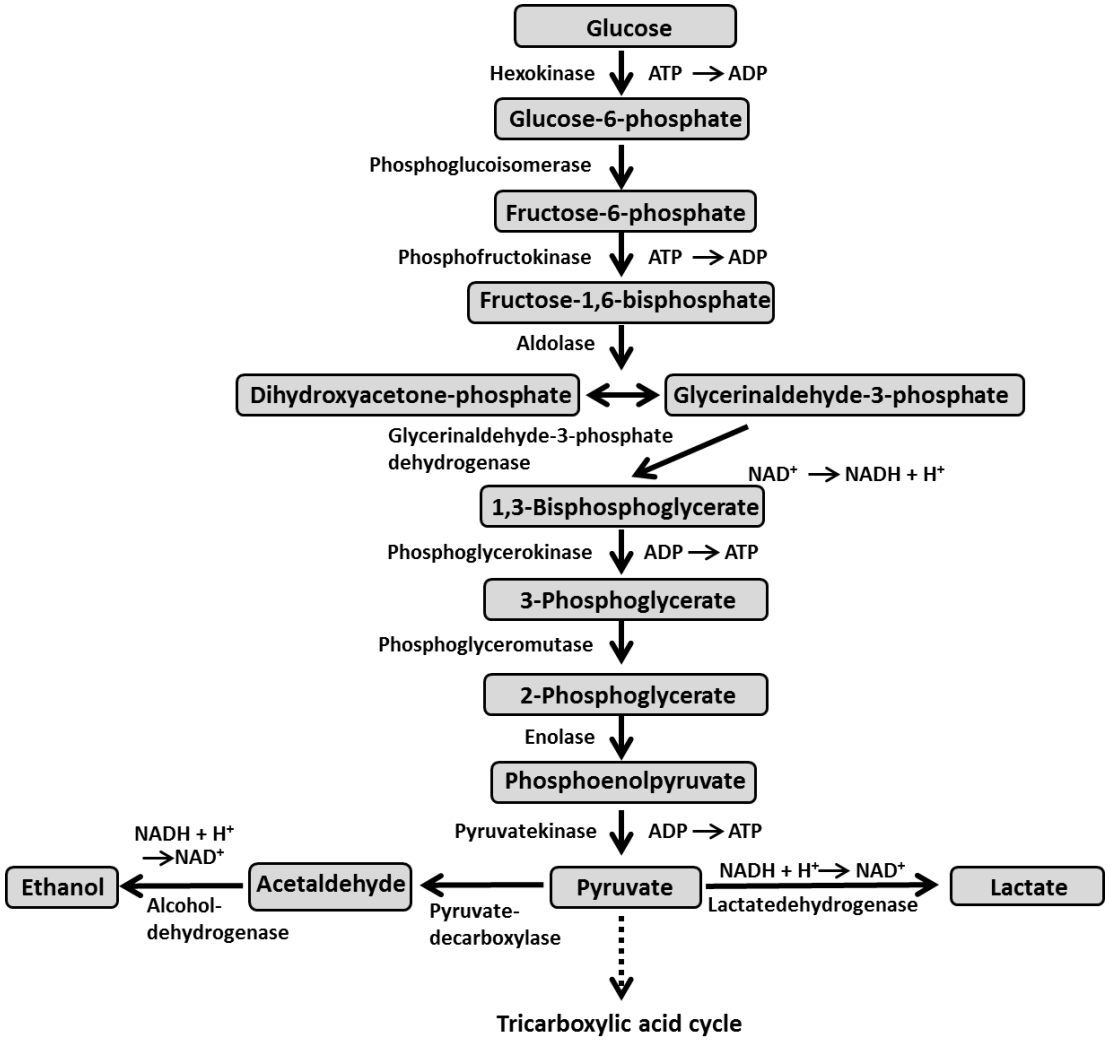

Figure 1.3. Overview glycolytic pathway from glucose to pyruvate and subsequent fermentation reactions to lactate or ethanol

The first key reaction is the phosphorylation of glucose by hexokinase thereby forming glucose-6-phosphate. This is followed by an isomerization by glucose phosphate-isomerase to fructose-6-phosphate. Further phosphorylation of fructose-6-phosphate by phosphofructokinase, consuming one ATP, leads to the formation of fructose-1,6-bisphosphate. The reaction of phosphofructokinase is a key step in regulating this process. Subsequent action of the enzyme aldolase leads to a cleavage of fructose-1,6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate. Since these two metabolites are isomers, triose phosphate-isomerase can convert dihydroxyacetone-phosphate to glyceraldehyde-3-phosphate. Two molecules of glyceraldehyde-3-phosphate are then metabolized to 1,3–bisphosphoglycerate by the enzyme glyceraldehyde-3-phosphate dehydrogenase, which reduces nicotinamide adenine dinucleotide (NAD+) to NADH.. This is followed by the conversion of 1,3–bisphosphoglycerate to 3-phosphoglycerate catalyzed by phosphoglycerate kinase, which leads to the generation of ATP. The subsequent reactions from 3-phosphoglycerate to 2-phosphoglycerate by phosphoglycerate-

mutase, followed by enolase which generates phosphoenolpyruvate leads to the last important reaction of glycolysis the formation of pyruvate by pyruvate kinase, which again leads to production of ATP. In summary, the consumption of one molecule of glucose and one NAD^+ by the glycolytic pathway leads to the net production of 2 molecules of pyruvate, 2 ATP, and one NADH.

Pyruvate can be further utilized for ATP generation by entering the tricarboxylic acid (TCA) cycle. Although this marks the main metabolic pathway in energy metabolism, two other pathways exist for metabolizing pyruvate. Under hypoxic conditions, yeast (and other microorganisms) can convert pyruvate to ethanol (Figure 1.3.). The second possibility is the generation of lactate through the activity of lactate dehydrogenase (Figure 1.3.). While this activity is also found in microorganisms, the best example is muscle cells after intensive mechanical work. Both reactions occur in the absence of sufficient oxygen and do not produce ATP, however they are needed for the regeneration of NADH (produced during glycolysis) to NAD^+ in order to maintain a high rate of glycolysis. Since all reactions of glycolysis are carried out in the cell cytoplasm, the reaction from pyruvate to acetyl-coenzyme A by the pyruvate dehydrogenase is a key step for entering the mitochondria, where the reactions of the TCA cycle occur (Figure 1.4.). Upon entering the mitochondria, acetyl-coenzyme A (acetyl coA) enters a condensing reaction with oxaloacetate to forms citrate, catalyzed by citrate synthase. Afterwards, citrate isomerizes to isocitrate. This is followed by oxidative decarboxylation by isocitrate dehydrogenase leading to the formation of α-ketoglutarate and the oxidation of NAD^+ to NADH. A second oxidative decarboxylation is characterized by the formation of succinyl coA and again the formation of NADH. Succinyl coA is than further metabolized to succinate, which leads to the formation of GTP. In the following 3 reactions, succinate is then converted to fumarate and subsequently malate, which in turn regenerates oxaloacetate enabling it to bind new acetyl-coA again. During the TCA cycle, the generation of 3 molecules of oxidized NADH per one molecule of acetyl-coA is essential for the following energy producing steps.

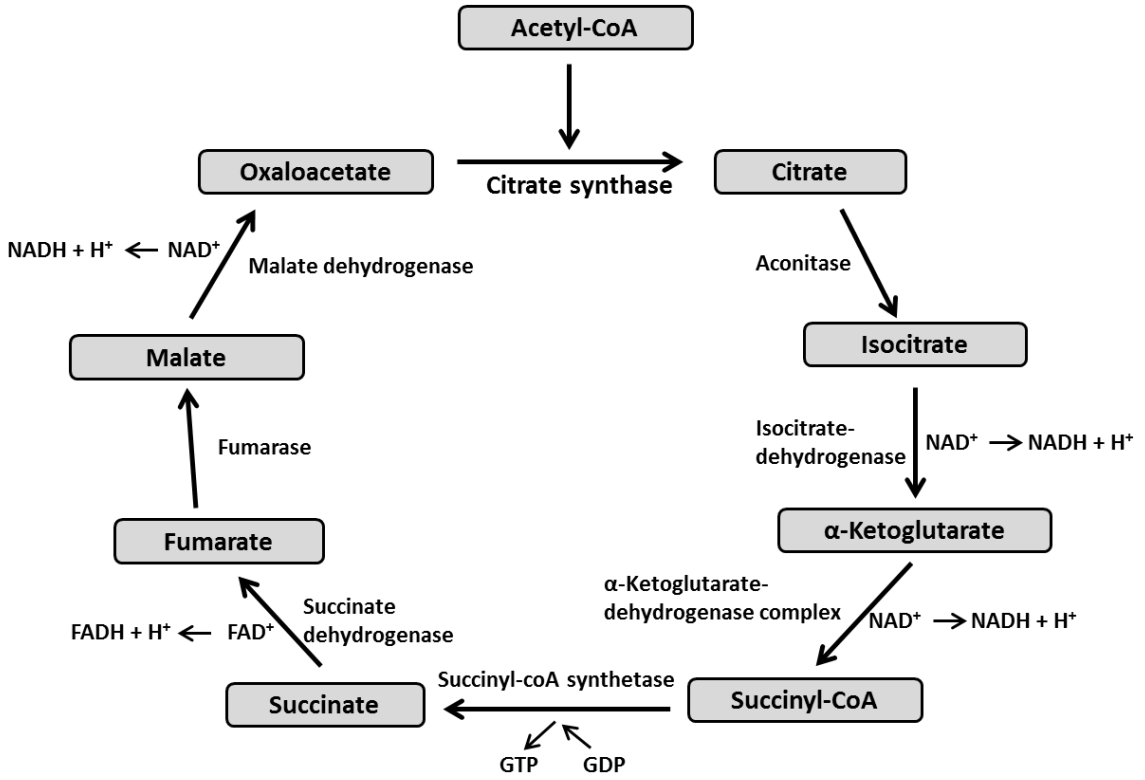

Figure 1.4. schematic overview over TCA-cycle reactions

Through the electron transport chain during the oxidative phosphorylation the reduction of NADH to NAD^+ leads to a formation of proton gradient at the mitochondrial membrane, which drives ATP synthesis by the enzyme ATP-synthase. In total, during the TCA-cycle and subsequent oxidative phosphorylation, 28 molecules of ATP are generated, making this the main energy producing process in the cell.

1.3 Metabolism in cancer

The pathways of glycolysis and the TCA-cycle are highly conserved throughout living organisms. Although the energy delivered to maintain cellular homeostasis is predominantly produced by the TCA-cycle, the process of completely breaking down glucose is rather slow. In proliferating and rapidly growing cells the demand for energy and nutrients is high, which requires changes in the metabolic signature. One example of abnormal cell proliferation is cancer. Cancer metabolism has been extensively studied in the last decades beginning with the fundamental observation made by Otto Warburg in the 1920s. He showed that in the presence of oxygen, cancer cells favored the conversion of glucose via glycolysis and subsequent reaction from pyruvate to lactate instead of further metabolizing it through the TCA-cycle and oxidative phosphorylation

(WARBURG 1956). This feature, termed the "Warburg effect", has been described for many types of tumors and is now considered a hallmark of cancer. One explanation for this altered metabolism is the selective advantage for survival and proliferation in the tumor microenvironment. During tumor progression, the oxygen levels within the tumor microenvironment vary spatially and temporally selecting for cells that constitutively upregulate glycolysis. This is characterized by the increased expression of glycolytic enzymes, glucose transporters, and inhibitors of mitochondrial metabolism (Yamamoto et al. 1990; Mathupala et al. 1997; Rivenzon-Segal et al. 2003). One mechanism to upregulate glycolysis is the stabilization of hypoxia-inducible transcription factor (HIF). HIF initiates a transcriptional program providing the basics of upregulated glycolysis under hypoxic stress (Cramer et al. 2003). Since the stabilization of HIF is coupled to oxygen levels, it is not the only factor responsible for altered metabolism. Also the increased expression and/or stabilization of certain oncogenes have been described to play a role in the upregulation of glycolysis. Mutations in the proto-oncogene Ras (Flotho et al. 1999; Stirewalt et al. 2001), AKT (Zeng et al. 2006) and PI3kinase (Jücker et al. 2002) which play an important role in insulin signaling as well as the transcription factor Myc (Dang et al. 2009; Gao et al. 2009) have all been shown to be responsible for the expression of metabolism related genes.

Often mutations of regulatory proteins lead to tumor progression, characterized by altered metabolism. These characteristic metabolic signatures are not unique to tumor cells, but in fact are features of highly proliferating cells found in higher organism. One example of such cells are T lymphocytes in the immune system. Upon recognition of their antigen, a small number of cells expand within hours to form an effector population. This strong proliferation shares similarities in its metabolic profile to cancer cells. In addition, when T lymphocytes are recruited to a site of infection to fight pathogens or to restrict tumor progression they encounter a similar microenvironment like cancer cells. Thus, by adjusting their metabolic features T lymphocytes can maintain their protective function within the immune system.

1.4. The immune system

The main task for the immune system is to prevent and/or eliminate pathogens causing a wide variety of diseases. Therefore the innate and the adaptive immune system are the pillars of the immune response. If pathogens overcome the physical barriers and enter the organism, the innate immune system provides a fast, non-specific response. If the mechanisms of the innate immune response fail to clear a pathogen, then an adaptive immune response develops. Adaptive

immunity acts by specific recognition and selective elimination of the target. Adaptive immunity is triggered when foreign antigens enter into secondary lymphoid organs by diffusion or are presented by specific antigen presenting cells (APCs). There are two types of lymphocytes characteristic for adaptive immune response. B lymphocytes regulate humoral immunity by secreting immunoglobulins, which eliminate pathogens by neutralization, agglutination, phagocytosis, and/or complement activation. B lymphocytes development in the bone marrow from pluripotent stem cells via a series of different progenitor stages which in the end leads to the clonal expansion of the mature B cells upon the binding of foreign antigen to the B cell receptor (BCR). A second type of lymphocyte, T lymphocytes, is required for cell mediated immunity. Upon triggering the specific T cell receptor (TCR), T cells proliferate and differentiate into different effector cells. Cytotoxic T cells kill infected or damaged cells, whereas Helper T cells coordinate the immune response by secreting specific cytokines. T cells develop in the thymus, where they undergo two selection processes. The first process, positive selection, ensures that only T cells with receptors that can recognize self-MHC molecules survive. The second process, negative selection, eliminates T cells that strongly recognize self-antigens on MHC molecules, thereby ensuring that only those cells able to detect foreign peptides are allowed to exit the thymus. In addition, there exist populations of regulatory lymphocytes, called native or inducible regulatory T cells (nTregs or iTregs), which control the amount of differentiated cells and inhibit further proliferation once the pathogen is cleared. A small amount of the differentiated cells remain as long lived memory cells to ensure protection should the same pathogen be encountered again.

1.4.1. T cell development and activation

T cells play a central role in the immune system and are important for cell mediated immunity. Initiation of T cell receptor signaling is a critical step for the activation of T cells and therefore requires tight control. The T cell receptor is formed during thymocyte development. These selection processes ensure that only receptors with a weak affinity for self MHC exit into the periphery. Naïve T cells can be distinguished by their expression of the surface molecules CD4 and CD8. CD4 T cells, also called helper T cells are restricted to MHC-class II recognition whereas cytotoxic CD8 T cells only recognize MHC-class I molecules. They are constantly recirculating between peripheral lymphoid organs and the bloodstream until they encounter their specific antigen. These antigens are expressed on antigen presenting cells (APCs) like B cells, dendritic cells, and macrophages as peptide-MHC class I or II complexes. Upon recognition of

antigen by the T cell receptor (TCR) an adaptive immune response is initiated (von Andrian et al. 2000; Mescher et al. 2006). The TCR consists of 2 transmembrane proteins termed the α and β chain. The extracellular domains of these two chains contain variable regions, which are capable of binding the peptide-MHC complex. Since the variable chains are formed during thymocyte development by somatic recombination every T cell harbors a different TCR with unique specificity. However, the α and β chains lack cytoplasmic domains and thus are not able to transmit signals into the cell. Signals via the TCR are transmitted via the noncovalently associated CD3 subunits, namely one heterodimer consisting of γ and ε and another of δ and ε chains, as well as a homodimer of ζ chains. These subunits possess intracellular tails containing specific signaling motifs called immunoreceptor tyrosine-based activation motifs (ITAMs). Upon triggering of the TCR these ITAMs become phosphorylated by members of the Src family of protein tyrosine kinases, namely LCK and Fyn.

Phosphorylated ITAMs provide docking sites for the tandem SH2 domains of the Syk family kinase ZAP70, which is thereby recruited to the plasma membrane and itself becomes activated via phosphorylation by Lck (Chan et al. 1992; Chan et al. 1995). One important target of ZAP70 is the transmembrane adaptor called the linker for activation of T cells (LAT). Phosphorylated LAT serves as a scaffold to recruit further downstream molecules, which activate distinct signaling pathways leading to the activation of transcription factors, such as NFAT, NF-κB and AP-1. These transcription factors are required for initiating cytokine production, T cell proliferation, and differentiation.

Translocation of the transcription factor NFAT into the nucleus is Ca^{2+} dependent. Upon recruitment and activation of PLCγ1 in the LAT complex results in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) producing two second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to the IP3 receptor on the endoplasmic reticulum inducing a transient increase in free intracellular Ca^{2+}, which binds to calmodulin which in turn activates calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase. Activated calcineurin dephosphorylates the transcription factor NFAT, thereby allowing its translocation to the nucleus (Garcia-Cozar et al. 1998).

The transcription factor AP-1, a heterodimer consisting of the transcription factors Fos and Jun, is activated via the MAP kinase pathways. The transcription factor Fos is activated by the Ras-Raf-MEK-ERK pathway which can be triggered by the recruitment of Grb2/SoS to LAT or via the activation of RasGRP by DAG (Genot et al. 2000; Jones et al. 2002). Furthermore upon TCR triggering SLP76 is recruited to phosphorylated LAT and leads to phosphorylation of Vav1, which in a series of events leads to the activation of Jnk. Phosphorylated JNK is then required for activation of Jun (Saez-Rodriguez et al. 2007).

Activation of the transcription factor NF-κB is dependent on the activation of protein kinase C (PKC) via DAG. This leads to the formation of the Carma1-BCL10-MALT1 (CBM) complex, which in turn activates the IKK complex leading to the degradation of inhibitory protein IκBα. Removal of this inhibitor allows the active transcription factor NF-κB to translocate into the nucleus (Ruefli-Brasse et al. 2003; Egawa et al. 2003).

A variety of stimuli, including soluble or immobilized antibodies (Abs) that recognize the T cell receptor (TCR), peptide-loaded APCs, or MHC-I tetramers carrying high- or low-affinity peptides, have been used to study T cell activation. It was previously shown that different stimuli used to activate the TCR can lead to either a strong/transient activation of signaling molecules or to weak/sustained signal. The former lead to apoptosis, while the latter induces proliferation (Wang et al. 2008). Interesting this was true for both thymocytes (Daniels et al. 2006) and peripheral T cells (Wang et al. 2008). However, it is poorly understood how triggering of the same receptor with ligands of different affinity can induce these different outcomes. Though, it is known that thymocytes which cannot fulfill their energy demands undergo apoptosis (Daniels et al. 2006). Therefore we hypothesized that changes in the metabolic profiles of activated T cells might contribute to cell fate specification.

1.5. Metabolic signatures of the immune system

A functional immune response requires rapid cell growth, proliferation, and the production of effector proteins. In the presence of its specific antigen, T lymphocytes must rapidly shift from a resting state to an activated one in order to accomplish these tasks. The activation of T cells is accompanied by a huge demand for ATP; the major energy source in cell metabolism. The main pathways for generating ATP are glycolysis and the TCA cycle followed by oxidative phosphorylation. Resting T cells require relatively low amounts of energy for housekeeping functions, i.e. homeostasis. Most of this energy is produced by oxidative phosphorylation (OXPHOS) through the degradation of glucose, fatty acids, and glutamine (Roos,Loos 1973). Upon activation, cellular programs direct T cells towards proliferation, differentiation, and cytokine production. Especially during rapid proliferation where T cells expand exponentially they must double their intracellular content, i.e. lipids, proteins and nucleic acids. The subsequent increasing need for energy and metabolic precursors was shown to be accomplished by a strong upregulation of glycolysis (Greiner et al. 1994). Although the generation of ATP by glycolysis is inefficient when compared to OXPHOS, upregulating glycolysis has the advantage of being a fast process and was shown to protect cells from apoptosis (Perl et al. 2002). Since upregulating

glycolysis without a corresponding increase in OXPHOS would lead to an accumulation of the end product pyruvate, it was shown that the excess pyruvate generated is converted to lactate by lactate dehydrogenase (Brand et al. 2005). This step is essential to regenerate the reducing equivalent NADH, which is needed to maintain the high glycolytic turnover. It was shown for muscle cells that high intracellular concentrations of lactate are secreted by an active cotransport via monocarboxylic transporters together with an H^+ which in turn leads to extracellular acidification. To prevent lactic acidosis the extracellular lactate is then transported via the bloodstream to the liver where during gluconeogenesis lactate is metabolized back to glucose (Juel 1997).

These observations lead to the conclusion that glucose is the major energy source of activated lymphocytes. This was also confirmed by showing that removing glucose in activated T cells leads to an inhibition of T cell proliferation and cytokine production (Greiner et al. 1994; MacDonald et al. 1979). In addition, also other metabolic pathways have been shown to play a role in T cell metabolism, e.g. increased glutamine consumption was shown to be essential for T cell function (MacDonald et al. 1979; Brand et al. 1984). The degradation of glutamine is an energy-producing pathway by entering the TCA cycle, which in the end leads to the conversion from malate to pyruvate. This pyruvate together with an upregulated glycolysis can foster the generation of lactate. Glutamine is also needed to refill intermediates of the TCA cycle, which are also used for biosynthetic processes that are essential for maintaining T cell proliferation, i.e. providing a nitrogen source for non-essential amino acids and nucleotides. Beside the generation of ATP, T cells also require NADPH to support lipid and nucleotide biosynthesis. NADPH is generated in two different processes, the pentose-phosphate-pathway dependent on glucose-6-phosphate and the last step of glutamine degradation – the conversion from malate to pyruvate. This suggests that glucose and glutamine are the major nutrients required for T cell proliferation.

1.5.1. Protein Kinase B/AKT

The most prominent pathway responsible for upregulating glycolysis is the PI3K/AKT pathway. In T cells, coligation of the TCR and the costimulatory molecule CD28 leads to direct phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) by phosphoinositide 3-kinase (PI3K), which in turn leads to increased levels of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) within the membrane. AKT translocates to the plasma membrane since it binds to PIP_3 via its PH-domain, where it can be phosphorylated by PDK1 on Thr308. For full activation, AKT requires an additional phosphorylation on Ser473 by mTOR complex 2. It was shown for T cells

that sustained activation of AKT upregulates the glucose transporter 1 (GLUT1) and increases the activity of the rate-limiting glycolytic enzyme hexokinase (Frauwirth et al. 2004). Together with the observations that AKT regulates the activity of hexokinase and phosphofructokinase the two rate limiting enzymes of the glycolysis, it can be hypothesized that AKT is responsible for regulating enzymes of glycolysis and lactate production, which leads to an increased glycolytic rate. The observations on the activating role of AKT were mostly performed in primary human T cells stimulated with CD3 and CD28, which fully activate AKT. This led to upregulation of GLUT1 and glucose uptake, which could be inhibited by addition of CTLA4 (Frauwirth et al. 2004; Parry et al. 2005).

1.5.2. Mammalian Target of Rapamycin - mTOR

In T cells, mTOR exists as one gene, but forms two distinct protein complexes: mTOR complex (mTORC) 1 and mTORC2, which differ in their inputs and substrates (Hara et al. 2002; Kim et al. 2002; Sarbassov et al. 2004). The mTORC1 complex consists of the regulatory-associated protein of mTOR (Raptor), mLST8, PRAS40, and DEPTOR. The proteins mLST8 and DEPTOR are also found in the mTORC2 complex, with the addition of RICTOR, mSIN1 proteins, and PROTOR (Kim et al. 2002). Upstream of the mTORC1 complex is the small activating GTPase Ras homolog enriched in brain (Rheb), the function of which is regulated by the GAP activity of tuberous sclerosis complex 1 (TSC-1) and TSC-2 (Zhang et al. 2003). Phosphorylation of TSC-1/2 by AKT leads to inhibition of the GAP activity and subsequent inactivation of Rheb (Tee et al. 2003).

Additionally, AKT-mediated inhibition of PRAS40 has been shown the promote mTORC1 activity independently of TSC-1/2 (Wang et al. 2007). mTORC1 is known to play a critical role in regulating mRNA translation, glucose and lipid metabolism, mitochondrial biosynthesis, and autophagy (Düvel et al. 2010). Recent studies have shown that mTORC2 is strongly and specifically activated following association with ribosomes, whereas its kinase activity is inhibited by endoplasmic reticulum stress and the glycogen synthetase kinase-3b (GSK-3b) (Chen et al. 2011). Downstream targets of mTORC2 include AKT, serum and glucocorticoid-inducible kinase 1, and protein kinase C (Guertin et al. 2006). Therefore it is known that AKT acts upstream as a regulator of mTORC1 activity, which is dependent on the phosphorylation of AKT^{T308} by the PI3K/PDK1 pathway. Additionally AKT is also a downstream target of mTORC2, which phosphorylates AKT^{S473} (Sarbassov et al. 2005; Guertin et al. 2006).

1.5.3. AMPK

Another important regulator of cellular metabolism is adenosine-monophosphate kinase (AMPK), which promotes ATP conservation and production through the activation of glycolysis, fatty acid oxidation, and the inhibition of ATP-consuming pathways, such as protein synthesis, fatty acid synthesis, gluconeogenesis, and glycogen synthesis. AMPK can be activated by an increase in the AMP:ATP ratio followed by phosphorylation through LKB1 (a serine/threonine kinase)(Hardie et al. 1999; Woods et al. 2003; Cao et al. 2010) . In addition it has been shown that Ca^{2+}-calmodulin-dependent kinase kinase 2 (CAMKK2) can activate AMPK independent of AMP levels (Hurley et al. 2005; Tamás et al. 2006). This suggests that LKB1/AMPK antagonize the PI3K/AKT/mTOR pathway, which promotes anabolism. This could be confirmed by the observations that AMPK activation was shown to be transient upon T cell stimulation (Tamás et al. 2006). Additionally, AMPK was shown to be required for memory T cell differentiation. Addition of the drug metformin caused an sustained activation of AMPK and subsequently led to increased numbers of memory T cells (Rolf et al. 2013). Recent studies showing that LKB1/AMPK influences assymetric cell division in *D. melanogaster* (Mirouse et al. 2007; Lee et al. 2007), suggest that there could be a role for AMPK in the assymetric division T cells. Since sustained activation of AKT is needed for effector T cell differentiation and AMPK activation appears to be only transient under these conditions, one could hypothesize that the contact of a T cell to an APC could also lead to a polarized distribution of metabolites.

1.5.4. Transcription factors

Recently several studies have further analyzed the connection between the major metabolic regulators and metabolism. It was investigated whether transcription factors like Hif1α and MYC play an important role in expression of metabolic enzymes. Hif1α is a transcription factor that regulates the expression of genes that encode for glycolytic enzymes as well as downregulates mitochondrial oxygen consumption by blocking the entrance of pyruvate into the TCA cycle (Firth et al. 1994; Semenza et al. 1996). Hif1α is constitutely active, but under normoxic conditions, it is rapidly degraded. Under low oxygen conditions the degradation of Hif1α is inhibited and it translocates to the nucleus where it upregulates glycolytic genes and drives the cell towards aerobic glycolysis. Another factor investigated was the proto-oncogenic transcription factor Myc, which was shown to be induced upon T cell stimulation (Douglas et al. 2001). The deletion of Myc lead to an impaired upregulation of glycolysis and glutaminolysis, and a

decreased activation of targets downstream of mTOR. These observations led to the conclusion that Myc is probably the major transcription factor regulating T cell metabolism upon activation.

1.6. Aim of the project

It was previously shown that triggering of the T cell receptor with stimuli of different affinities lead to different outcomes. Activation of CD8 T cell with soluble CD3 and CD8 antibodies lead to the strong phosphorylation of downstream molecules like ZAP70, LAT, and ERK (Wang et al. 2008). However this activation was transient and the T cells failed to induce proliferation, but rather undergo apoptosis within 24h. On the contrary, stimulation with MHC-I tetramers presenting the OVA peptide lead to a weak, but sustained activation of signaling molecules. Here, the T cells were able to survive and proliferate.

Based upon these results, I generated the hypothesis that different stimuli could induce different metabolic signatures, which in turn could explain the different phenotypes/functional outcomes. To test this hypothesis, I analyzed and compared specific metabolic features upon TCR triggering.

A second goal was to further analyze the metabolic parameters upon TCR triggering to provide a more detailed overview of how metabolism is regulated in T cells. Therefore, I analyzed the early changes in metabolism which enable T cells to switch from a resting state to an activated and proliferative phenotype. Furthermore I assessed regulatory parameters to elucidate the role of signaling proteins like AKT, mTOR and CTLA4 in upregulation of glucose metabolism and subsequent down modulation of these energy demanding processes.

2. Materials and Methods

2.1. Antibodies used in this study

Antibody	Species and clone	Application	Source
actin	mouse monoclonal IgG1(AC-15)	WB - 1:10,000	Sigma-Aldrich
anti-mouse-HRP	goat	WB - 1:10,000	Dianova
anti-rabbit-HRP	goat	WB - 1:10,000	Dianova
AMPKα	mouse monoclonal (F6)	WB - 1:1000	cell signaling technologies
AMPK –pT172	rabbit polyclonal	WB - 1:1000	cell signaling technologies
CTLA-4-PE (CD152)	mouse monoclonal (UC10-4F10-11)	FACS – 1:100	BD
CD3ε-biotin	mouse monoclonal (145-2C11)	stimulation	BD
CD8α	mouse monoclonal (53-67)	stimulation	BD
Erk1/2 – p Thr202/pTyr204	rabbit monoclonal (D13.14.4E)	WB – 1:1000	cell signaling technologies
GLUT1	rabbit monoclonal (EPR3915)	FACS - 1:100	Epitomics
Hexokinase	rabbit monoclonal (C35C4)	WB - 1:1000	cell signaling technologies
HIF1α-hydroxy- P564	rabbit monoclonal (D43B5)	WB - 1:1000	cell signaling technologies
JNK pThr183/pTyr185	rabbit polyclonal	WB- 1:1000	cell signaling technologies

LDHA	rabbit polyclonal	WB - 1:1000	cell signaling technologies
Phosphofructokinase (PFK1)	mouse monoclonal (E9)	WB - 1:1000	santa cruz biotechnologies
p70-S6K – pT389	mouse monoclonal (1A5)	WB - 1:1000	cell signaling technologies
Stat5-pY694	rabbit monoclonal (C11C5)	WB - 1:1000	cell signaling technologies
Stat5-pY694	mouse monoclonal (C47)	FACS – 1:100	BD

2.2. General reagents for cell culture

RPMI 1640 medium with NaHCO3 and stable glutamine	Biochrom AG
PBS without Ca2+Mg2+	Biochrom AG
FCS	PAN Biotech GmbH
CiproBay 200	Bayer
Trypan blue	Sigma

2.3. Inhibitors used in this study

Inhibitor	**concentration used**	**Source**
AKT V	1μM	Calbiochem
AKT VIII/AKTi	2μM	Calbiochem
AKT XII	5μM	Calbiochem
Oxamate	50mM	Sigma Aldrich
Rapamycin	2mM	Sigma Aldrich
Rotenone	100nM	Sigma Aldrich

All inhibitors were tested for induction of necrosis/apoptosis and specific function for various concentrations. The concentrations depicted are the ones used in the subsequent experiments.

2.4 Analysis of human T cells

2.4.1. Isolation and purification of human T cells

Reagents and instruments:

Ficoll	Biochrom AG
Heparin	Biochrom AG
Pan T cell isolation kit II	Miltenyi Biotec
AutoMACS	Miltenyi Biotec

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation of heparinized blood collected from healthy volunteers. A ring containing PBMCs formed during the gradient centrifugation. PBMCs were carefully aspirated and washed 3x with RPMI 1640 medium. The cells were rested in RPMI 1640 medium supplemented with 10% FCS for 2 hours in an incubator. T cells were further purified by non-T cell depletion on the AutoMACS machine using the Pan T cell isolation kit II. T cell populations of greater than 95% purity were obtained, as determined by flow cytometry analysis.

2.4.2. Stimulation of human T cells

For microbead stimulation, SuperAvidin™-coated polystyrene microspheres (Ø ~ 10 µm, Bangs Laboratories) were coated with biotinylated CD3 alone or in combination with CD28 and CD4 mAbs as indicated (10 µg/ml each) for 30 min at 37 °C in PBS. Antibody-coated microbeads were washed twice with PBS, resuspended in RPMI 1640 and incubated with T cells in a 1:1 ratio.

2.5. Animal experimentation

2.5.1. Mice condition and handling

Mice were kept in the central animal facility at the Otto-von-Guericke-University of Magdeburg and maintained in pathogen-free conditions. All experiments involving mice were performed according to the guidelines of the State of Sachsen-Anhalt,Germany.

2.5.2 Genomic DNA isolation from mouse tails

Genomic DNA was purified from a 1 cm piece of mouse tail, provided from the animal facility and placed into a 1.5 ml eppendorf tube. The mouse tail was predigested by incubation with 600 µl Tail lysis buffer and 6 µl proteinase K solution at 56°C for 3-5 h under constant shaking. After a centrifugation for 10 min at 10000 rpm the supernatant was added to a new tube containing 600µl isopropanol. Next the sample was centrifuged again for 10min at 12000 rpm and subsequently washed with 600 µl ice-cold ethanol, centrifuged again for 5 min at 12000 rpm and dried on a paper towel for 30min. The dried DNA was than resuspended in 150µl water and stored at 4°C.

2.5.3. Genotyping of OT-I-transgenic mice strain by PCR

Genotyping of the OT-I transgenic mice was performed according to the PCR protocol established by Jackson Laboratories.

Primers used for genotyping (Metabion):

Primer 1 (internal control F): 5'- CAA ATG TTG CTT GTC TGG TG -3'

Primer 2 (internal control R): 5'- GTC AGT CGA GTG CAC AGT TT-3'

Primer 3 (OT1-TCR F): 5'- AAG GTG GAG AGA GAC AAA GGA TTC-3'

Primer 4 (OT1-TCR R): 5'- TTG AGA GCT GTC TCC-3'

The following reaction mix was pipetted into the PCR tube according to the manufacturer's protocol:

PCR reaction mix	**PCR program**
Primer 1 0.25 µl (1 µM)	Hot start 94°C, 3 min
Primer 2 0.25 µl (1 µM)	Denaturation 94°C, 30 sec
Primer 3 0.125 µl (0.5 µM)	Hybridization 52°C, 30 sec 38 cycles
Primer 4 0.125 µl (0.5 µM)	Elongation 72°C, 30 sec
Taq polymerase 0.5 µl (2.5 U)	Final elongation 72°C, 2 min
5 x enhancing buffer 5 µl	

10 x reaction buffer 2.5 µl

dNTP (mix) 0.25 µl (200 µM)

dH_2O up to 23.5 µl

To each reaction 1.5 µl DNA was added

2.5.4. Gel electrophoresis of nucleic acids

1 x TAE buffer:	10 mM Tris
	0.1142% acetic acid
	1 mM EDTA pH 8.0
Agarose gel 2%:	2 g agarose
	100 ml 1 x TAE buffer
	10 µl ethidium bromide (10 mg/ml)
6 x loading buffer:	30% glycerol
	20 mM Tris (pH 7.6)
	2 mM EDTA
	0.02% bromphenolblue
	0.02% xylenxylanol
Marker:	GeneRuler 50 bp DNA Ladder

The reaction was analyzed by mixing the PCR products with 6 x loading buffer and loaded on a 2% agarose gel supplemented with ethidium bromide. Electrophoresis was carried out with a Bio-Rad Mini DNA system in TAE-buffer at 100 V for 30 min. DNA fragments were visualized by UV light observing a band at ~200bp referring to the internal TCR control and at ~350bp referring to the transgene.

2.5.5. Purification of CD8+ OT-I T cells from mouse spleen

Pan T cell isolation kit mouse	Miltenyi Biotec
AutoMACS	Miltenyi Biotec

Mice were sacrificed and the spleens were removed. To disrupt the organs and to release the cells, the spleen was mashed through a 40 µm diameter pore-size strainer with a syringe plunger at room temperature. Cells were collected in a 15 ml tube and washed by adding ice-cold PBS to a final volume of 15 ml and centrifuged for 10 min, 370 g, at 4°C. The pellet was resuspended in 10 ml ice-cold PBS and the cell number was determined.

Since OT-I TCR tg mice contain only CD8+ T cells, a Pan T cell isolation kit was used for purification. The single cell suspension was centrifuged at 370 g for 10 min. The cell pellet was resuspended in ice-cold PBS at a concentration of 10 x 10^6 cells/45 µl. Staining was performed with 5 µl Biotin-Antibody Cocktail (cocktail of biotin-conjugated monoclonal antibodies against CD11b (Mac-1), CD45R (B220), DX5 and Ter-119) and incubated for 10 min on ice. Subsequently, the cell suspension was mixed together with 40 µl of ice-cold PBS per 10 x 10^6 cells and 10 µl of anti-Biotin MicroBeads. The mixture was incubated for 15min on ice. Afterwards the cells were washed once with 10 ml of ice-cold PBS and centrifuged at 370 g for 10 min. The supernatant was removed and the cell pellet was resuspended in PBS at the concentration of 1 x 10^8cells/ml. The magnetic separation was performed by using the Auto-MACS separator. The purity of isolated cells was determined by flow cytometry.

Since CTLA4$^{-/-}$ mice provided by the group of Prof. Brunner-Weinzierl were also crossed onto the OT-I transgenic background, the purification procedure was the same as described above.

2.5.6. OT-I T cell stimulation in vitro

2.5.6.1 Stimulation with OT-I tetramers

Mouse medium:	RPMI-1640
	10% FBS
	1% antibiotics (penicillin and streptomycin)
	1.75 µl 2-mercaptoethanol (in 500 ml RPMI)

Prior to stimulation of T cells with OT-I tetramers, 0.75 µg Strep-Tactin was incubated with recombinant monomeric biotinylated-MHC-I (1 µg) in a final volume of 50 µl with PBS or mouse medium at 4°C for 45 min according to the manufacturer's instructions. Recombinant monomeric biotinylated H-2K^b molecules presenting the ovalbumin SIINFEKL peptide (OVA) or presenting the SIIQFEHL peptide (Q4H7) for the OT-I TCR were used in this study. 2 x 10^6 purified T cells were resuspended in 50 µl PBS or mouse medium. Subsequently, the cell solution was mixed with the OT-I tetramers (pMHC-strep-tactin) at 37°C for the indicated time points. Stimulation was stopped by adding 1 ml ice-cold PBS. The procedure was continued according to the specific purpose.

2.5.6.2. stimulation with CD3/CD8 mAbs

2 x 10^6 purified T cells were resuspended in 100 µl of PBS or mouse medium. Before stimulation, the cells were pre-incubated with biotinylated CD3ε mAb and biotinylated CD8 mAb (10 µg/ml) at 37°C for 1 min. Stimulation started when cells were cross-linked with 50 µg/ml streptavidin. 1 ml of ice-cold PBS was added to stop the reaction. The procedure was continued according to the specific purpose.

2.6. Surface staining and FACS (Fluorescence Activated Cell Sorting) analysis

2.6.1. Extracellular staining

1 x 10^6 cells were centrifuged for 5 min, 400 g, at 4°C. The pellet was resuspended in 100 µl of the indicated antibody solution. After 30 min of incubation in the dark at 4°C, cells were washed with PBS. The cell pellet was resuspended in 300 µl PBS and measured by the FACS Diva machine. Data were analyzed using the FlowJo software.

2.6.2. Proliferation assay

CellTrace™ CFSE Cell Proliferation Kit	Life technologies

1 x 10^6/ml purified OT-I T cells were incubated with 5µM CFSE for 20min at 37°C in the dark. After 2 subsequent washing steps with PBS the cells were resuspended in mouse medium. After labeling the cells were cultured in mouse medium using U-bottomed 96-well plates at a concentration of 1.5 x 10^4cells/well. Cells were stimulated with OT-I tetramers (1 µg) alone or in the presence of the inhibitors oxamate or rotenone for 72 h at 37°C. Cells were then analyzed for proliferation by flow cytometry.

2.6.3. Apoptosis assay

Annexin V–FITC	Biolegend
Propidium Iodide	Biolegend
Cell staining buffer	Biolegend

Cell apoptosis was analyzed by the ability of Annexin V to bind to exposed phosphatidylserine residues at the outer leaflet of the plasma membrane in combination with the application of Propidium Iodide (PI). Purified T cells were resuspended in mouse medium at the concentration of 1 x 10^6cells/ml in a 48-well cell culture plate. Cells were left untreated or treated with OT-I-tetramers (1 µg) alone or in presence of the inhibitors oxamate and rotenone at 37°C for 24h, respectively. Cells were harvested and washed with PBS. The cell pellet was resuspended in 195 µl of cell staining buffer. The staining was performed by mixing the cell solution with 5 µl of

Annexin V-FITC and incubating for 10 min at RT in the dark. Afterwards 10 µl PI were added. Samples were analyzed by flow cytometry within one hour.

2.6.4. Glucose uptake

Glucose Uptake Cell-Based Assay Kit	Cayman Chemical
Annexin V-pacific blue	Biolegend
Propidium Iodide	Biolegend

OT-I T cells were stimulated for 24h with OT-1 tetramers and then analyzed with glucose uptake cell-based assay kit (Cayman Chemical Company). Cells were harvested and starved for 15min in PBS at 37C°. Afterwards fluorescent labelled glucose (150µg/ml, 2-NBDG) was added to the cells for 15min. After subsequent washing, the cells were additionally stained as described before (2.5.2.) with Annexin V and PI to exclude apoptotic cells and analyzed via FACS immediately.

2.7. Metabolic analysis

2.7.1. Extraction of intracellular metabolites

Methanol	Roth
Chloroform	Roth
Tricine	Roth

CD8+ T cells were cultured in mouse medium in 48-well plates at a concentration of 4 x 10^6 cells/well. Cells were left unstimulated or stimulated with either CD3/CD8 mAbs or OT-I tetramers at 37°C. Cells were harvested after 2h, 4h, 6h and 8h. The cells were immediately resuspended in 600µl ice cold Methanol/Chloroform (2:1). Afterwards additional 500µL ice cold Methanol/Chloroform (2:1) were added to the samples. In a first extraction step 800µL Methanol/3,8mM Tricine (9:10) is added and leads to the separation of the aqueous and the organic phase. After vigorous vortexing the sample is centrifuged for 5min at 16000g at 0°C. Afterwards the aqueous phase is transferred to a new tube. Then again 800µL Methanol/3,8mM Tricine (9:10) is added in a second extraction step to the remaining organic phase, incubated for 5min at 4°C and subseuqently centrifuged for 5min at 16000g at 0°C. After centrifugation the

aqueous phase is pooled together with the one from the first extraction step. Afterwards the samples are boiled at 90°C for 4 min followed by a 10min centrifugation step at 16000g and 0°C. The liquid supernatant is then transferred to a new tube and dried using a SpeedVAC for 24h and can be stored at -80°C. Prior the analysis the pellets are resuspended in 600µl bidest H_2O followed by a centrifugation for 10min at 16000g at 0°C and the supernatant is transferred to a new tube and a sample of 120µL was measured with MS-coupled anion-exchange chromatography (Ritter et al. 2008) in cooperation with the group of Prof. Reichl at the Max Planck Institute for Dynamics of Complex Technisl Systems, Magdeburg.

2.7.2. Measurement of intracellular ATP

ATPlite™ Luminescence Assay System	Perkin Elmer
Luminometer	Berthold

1 x 10^6 purified CD8+ T cells were either left untreated or stimulated with CD3/CD8 mAbs or OT-I tetramers for 3´,15´,30´,60´, 120´ and 240´. After stopping the stimulation with ice-cold PBS the sample were centrifuged for 2min at 6000 rpm 4°C. The pellets were resuspended in 100µL PBS and 50µL mammalian cell lysis solution added and placed on an orbital shaker with 700rpm for 5min. Next 50µL substrate solution containing Luciferase and Luciferin was added to the samples and again placed on an orbital shaker for 5min at 700rpm. Afterwards the samples were placed in the dark at room temperature for 10min. Then the emitted light from the luciferase reaction was assessed by a Luminometer. The concentrations were calculated by a ATP standard curve added to the measurement.

2.7.3 Lactate

Lactate Colorimetric Assay Kit II	Bio Cat
Microplate reader	Tecan

1 x 10^6 purified CD8+ T cells were either left untreated or stimulated with CD3/CD8 mAbs or OT-I tetramers. After stimulation and subsequent washing with PBS, cells were lysed by adding 50µL bidestilled H_2O. Afterwards 50µL reaction mix from the kit containing assay buffer (48µL), substrate mix (2µL) and enzyme mix (2µL) was added to the sample and incubated for 30min at room temperature. Finally the samples including an appropriate standard curve were measured at a microplate reader at 450nm.

2.8. Immunoblotting

2.8.1. Cell lysis

Lysis buffer:	1% LM
	1% NP-40
	1 mM Na-monovanadate
	1 mM PMSF
	50 mM Tris-HCl (pH 7.4)
	10 mM NaF
	10 mM EDTA
	0.16 M NaCl

After stimulation, $2x10^6$ cells were washed once with ice-cold PBS. Cells were resuspended in 40 µl lysis buffer and incubated for 20 min on ice. Samples were then centrifuged for 10 min at 16,000 g, 4°C and the supernatant was transferred into a new eppendorf tube.

2.8.2. Protein concentration measurement

BSA standard (0-100 µg/ml)	Sigma
Roti-Nanoquant	Roth

Protein concentrations were determined by using the Bradford protein assay. According to the manufacturer's protocol, a working solution was prepared by diluting the 5x Roti-Nanoquant to 1x with dH2O. Samples were pre-diluted with dH2O. 50 µl of the prediluted samples were then transferred to a 96-well plate and incubated with 200 µl of 1x Roti-Nanoquant. Absorption was measured using an ELISA reader at 570 nm. BSA (0-100 µg/ml) was used as standard and the protein concentration was calculated on the basis of the derived standard curve.

2.8.3. SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

In SDS-PAGE, the migration of proteins is related to their molecular weight.

10% and 12% SDS-PAGE separating gel:	4.2 ml (10%) or 3.44 ml (12%) dH2O
	2.46 ml 1.5 M Tris-HCl, pH 8.8
	3.2 ml (10%) or 3.94 ml (12%) 30% acrylamide/BIS
	0.1 ml 10% SDS
	0.05 ml 10% APS
	5 µl TEMED
SDS-PAGE stacking gel:	2.4 ml dH2O
	0.5 ml 30% acrylamide/BIS
	1 ml 0.5 M Tris-HCl pH 6.8
	0.04 ml 10% SDS
	0.04 ml 10% APS
	0.004 ml TEMED
SDS-PAGE running buffer (1x):	25 mM Tris,
	250 mM glycine,
	0.1% SDS
5x reducing loading buffer :	50% glycerol
	330 mM Tris, pH 6.8
	10% SDS
	0.01% bromphenolblue
	10% 2-mercaptoethanol
Marker:	pageruler prestained protein ladder

Cell lysates and 5x sample buffer were mixed and boiled at 95°C for 8 min. The samples were then loaded and resolved on a 10% or 12% SDS-PAGE gel. For each lane, 40 µg of total protein were loaded. Electrophoresis was conducted with Bio-Rad Protein system. Gels were run at 120 V for 90 min.

2.8.4. Western blotting analysis and immunoblotting

Protein transfer buffer (1x):	39 mM glycine 48 mM Tris 0.037% SDS 20% methanol
	TBS 0.01M Tris 0.15M NaCl
Blocking buffer:	5% milk powder in TBS
Washing buffer:	0.02% Tween 20 in TBS
Ponceau S solution:	0.1% Ponceau S in 5% acetic acid

The proteins seperated by gel electrophoresis were then transferred to a nitrocellulose membrane, where they were detected using antibodies specific to the target proteins. In some experiments, equal loading was controlled by incubation of the membrane with Ponceau S solution for 2 min. The staining was removed by washing with TBS. Blocking of the membrane was performed using blocking buffer for 30 min to prevent unspecific binding to the membrane. Subsequently, membranes were probed with a primary antibody overnight 4°C. After washing 3 times with washing buffer, the membrane was incubated with the appropriate peroxidase-conjugated secondary antibody for 1 h at RT. The membrane was further washed 3 times with washing buffer and the bound antibodies were then visualized using an ECL (Enhanced Chemiluminescence) detection system according to the manufacturer's instructions.

3. Results

3.1. Peptide-stimulation induces metabolic reprogramming in T cells

Stimulation of T cells leads to a change from a quiescent resting state into an activated state, which is characterized by an extensive cell growth, proliferation, and the production of effector proteins, such as cytokines. In the resting state, T lymphocytes maintain their basal energy demands primarily through a mixed usage of glucose and glutamine (Brand 1985; Frauwirth,Thompson 2004). However, to meet the increased energy demands following activation, glucose metabolism increases as a source of energy and providing precursor molecules for cellular biosynthesis (Frauwirth,Thompson 2004). It was previously shown that different stimuli lead to either proliferation or apoptosis of thymocytes (Daniels et al. 2006) and mature T cells (Wang et al. 2008). However, it is poorly understood how triggering of the same receptor with ligands of different affinity can induce such different outcomes.

Since it is known that thymocytes which cannot fulfill their energy demands undergo apoptosis we hypothesized that changes in the metabolic profiles in activated T cells might contribute to cell fate specification. Therefore we stimulated purified OT-I T cells either with soluble biotinylated CD3 and CD8 monoclonal antibodies or incubated them with biotinylated H-2K^{b} molecules loaded with the SIINFEKL peptide derived from ovalbumin (OVA) (Wang et al. 2008). Stimulated T cells were then cross-linked and further assessed for different metabolic parameters.

3.1.1. Changes in intracellular ATP concentrations following T cell stimulation

To determine metabolic changes upon stimulation using the two stimuli described above, we first assessed whether the concentration of ATP changes by measuring the level of intracellular ATP. In Figure 3.1. it is shown that within 4 hours of stimulation with soluble antibodies there is a strong decrease in intracellular ATP concentrations. In contrast to this, physiological stimulation lead to a mild decrease in ATP within the first 30min following stimulation, but remained constant for the rest of the time.

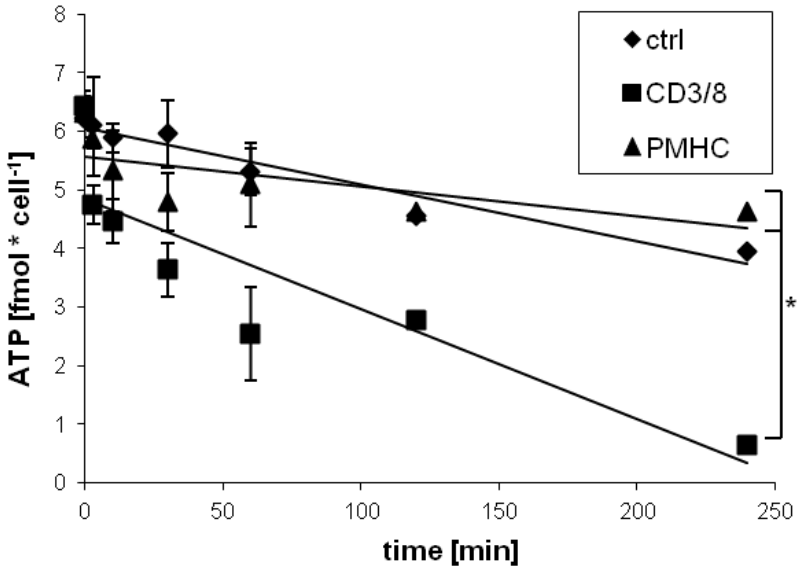

Figure 3.1. Soluble antibody stimulation leads to reduced intracellular ATP concentrations. Purified CD8+ T cells were treated with either soluble CD3/CD8 mAbs or OT-I tetramers (PMHC) for the indicated time periods. Cellular ATP production was analyzed using the ATP-Assay Kit. n=6.

These data indicate that stimulation with soluble antibodies leads to a strong decrease in ATP, which could promote apoptosis by failing to upregulate cellular metabolic responses.

3.1.2. Analyzing intermediates of glucose metabolism following T cell stimulation

It has been shown before in several studies (Frauwirth et al. 2002; Frauwirth,Thompson 2004) that stimulation of CD4+ T cells leads to increased glycolytic rates to maintain the metabolic demands of proliferating cells. Therefore we analyzed our OT-I T cells for metabolic intermediates of glycolysis to see whether this upregulation can be observed also in CD8+ T cells and furthermore compare the changes for the two different stimuli. To measure 20 different intermediates of glycolysis and TCA cycle, the lysates of stimulated T cells were separated by high-performance anion exchange chromatography and further identified by mass spectroscopy (MS)(Ritter et al. 2008).

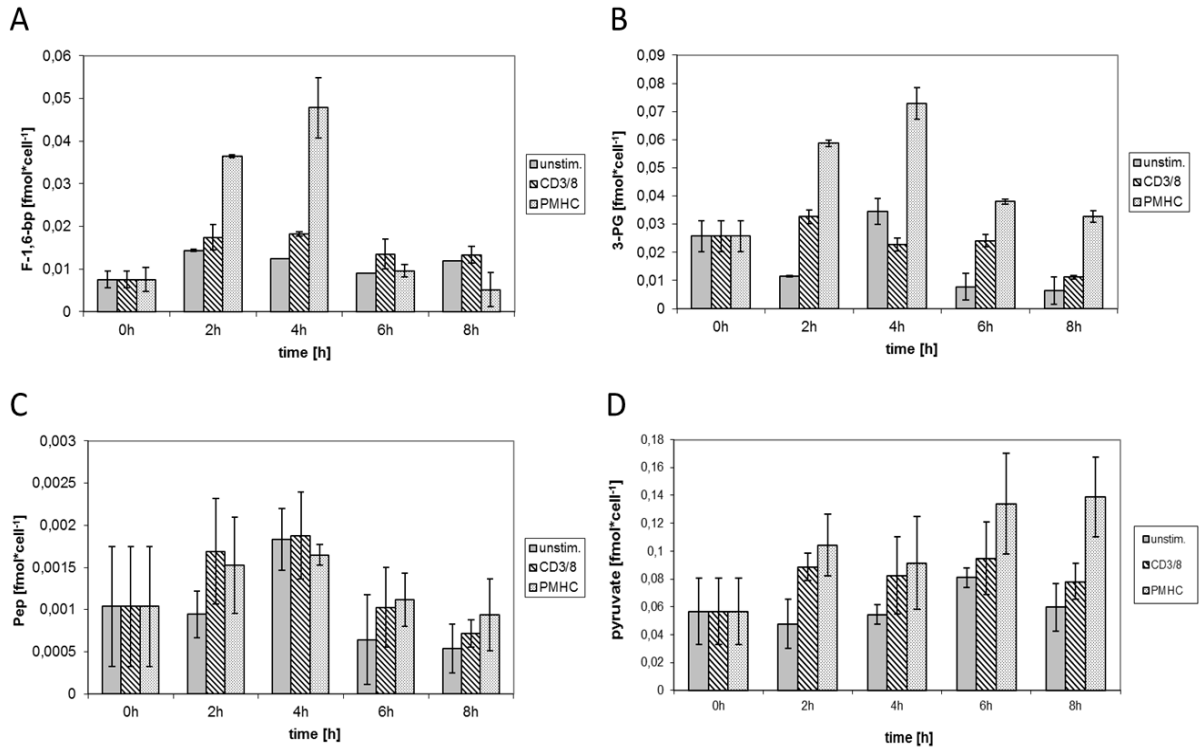

Figure 3.2. Stimulation with OT-I tetramers leads to upregulation of glycolytic metabolites. Purified CD8+ T cells were treated with either soluble CD3/CD8 mAbs or OT-I tetramers (PMHC) for the indicated time periods. Concentrations of glycolytic metabolites Fructose-1.6-bisphosphate (A), 3-phosphoglycerate (B), Phosphoenolpyruvate (C) and Pyruvate (D) where measured by MS coupled high-performance anion-exchange chromatography; n=5.

For the intermediates of glycolysis we observed a strong increase of fructose-1.6-bisphosphate (F-1,6-bp) and 3-phosphoglycerate (3-PG) 2-4 h upon tetramer stimulation, which could not be observed upon antibody stimulation (Figure 3.2). Furthermore the concentration of phosphoenolpyruvate (Pep) was not significantly increased upon both stimuli. However, pyruvate the "end product" of glycolysis shows a different kinetic, in the first hours following tetramer stimulation there was no significant increase, but at later time points we could observe an increase in pyruvate concentrations. In comparison, antibody stimulation had no impact on pyruvate concentrations.

In Figure 3.3, we analyzed further metabolites of the TCA cycle. Here, we observed a trend towards increased ketoglutarate, malate, and fumarate upon tetramer stimulation. However this increase was not statistically significant. In contrast to tetramer stimulation, changes in glycolysis and the TCA cycle were not observed after antibody stimulation.

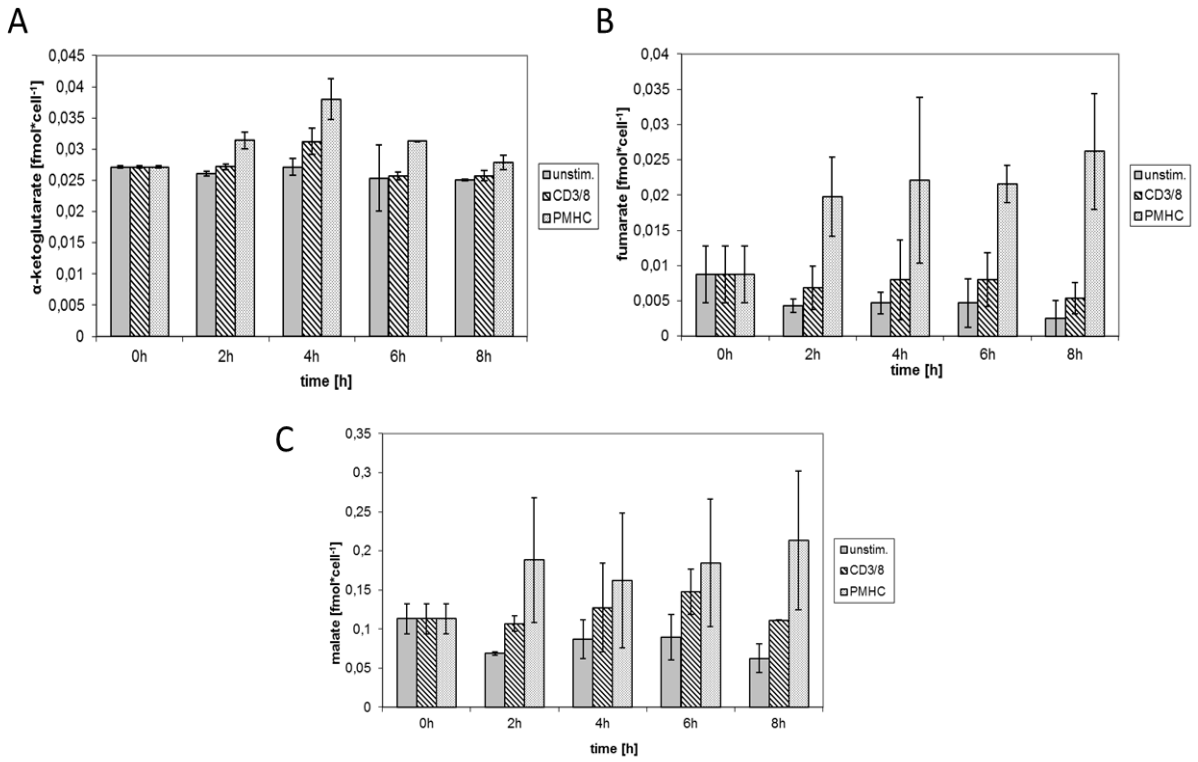

Figure 3.3. TCA cycle metabolites showed no significant changes upon stimulation. Purified CD8+ T cells were treated with either soluble CD3/CD8 mAbs or OT-I tetramers (PMHC) for the indicated time periods. Concentrations of intermediates of citric acid cycle α-ketoglutarate (A), fumarate (B) and malate (C) where measured by MS coupled high-performance anion-exchange chromatography; n=5.

Furthermore we assessed also ATP by metabolite analysis to confirm and expand the observed data for intracellular ATP concentrations and get a more detailed view about cellular energy homeostasis by additionally measuring ADP and AMP (Figure 3.4.A). Decreasing ATP concentrations measured upon soluble antibody stimulation were correlating to the experiment shown in Figure 3.1 and this trend was observed for up to 8 hours. In contrast, tetramer stimulation showed no decrease in ATP during the first 2 hours, afterwards it also decreases for up to 8 hours after stimulation. These observed effects are reflected by the inverse concentration behavior of ADP and AMP concentrations (Figure 3.4.B and C). Upon soluble antibody treatment these measurements revealed that the initial decrease in ATP lead to a strong increase in AMP, whereas ADP remained unaffected. Tetramer stimulation showed a relative stable ADP and AMP concentrations within 4 hours of the experiment. After 6h and 8h the concentrations for ADP dramatically increased.

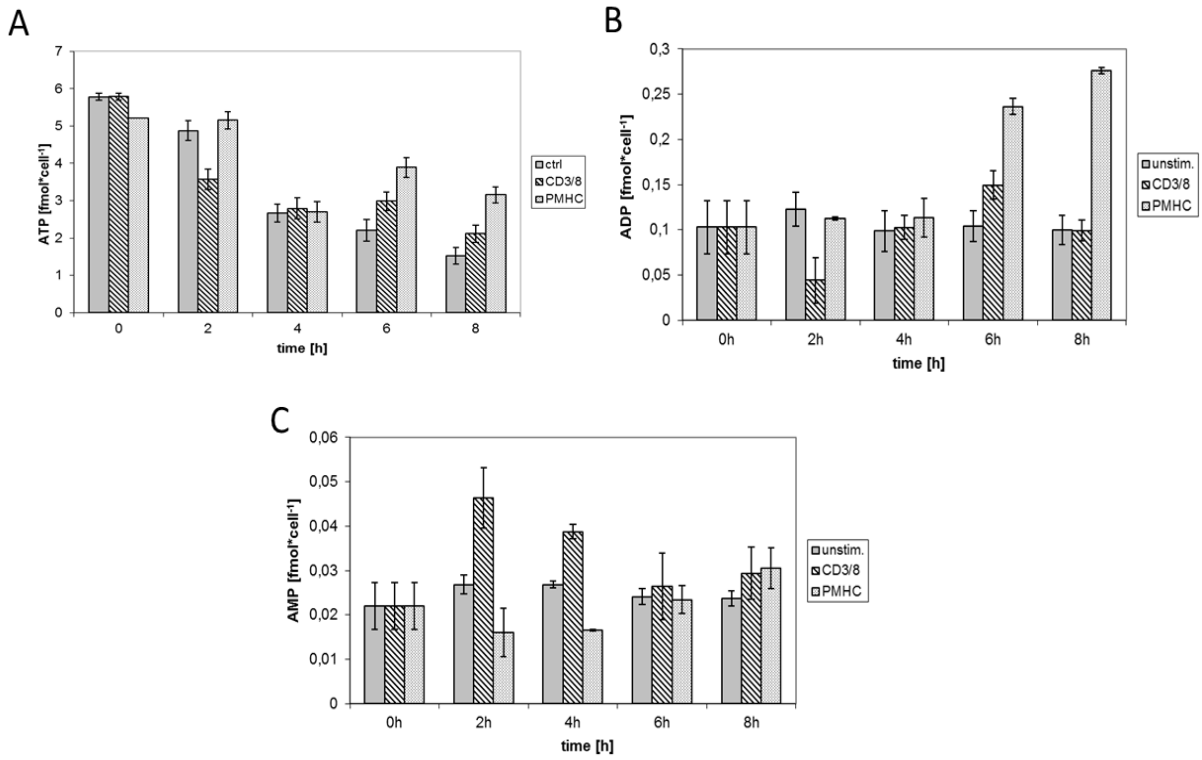

Figure 3.4. Changes in ATP with the different stimuli. Purified CD8+ T cells were treated with either soluble CD3/CD8 mAbs or OT-I tetramers (PMHC) for the indicated time periods. Concentrations of ATP (A), ADP (B) and AMP (C) where measured by MS coupled high-performance anion-exchange chromatography; n=5.

Further investigation of GTP, GDP, and GMP revealed that the effect of both stimuli did not alter significantly the changes in their concentrations. For GTP could observe a decrease after 6h to 8h (Figure3.5.A). Furthermore GDP concentrations increased at these time points (Figure 3.5.B). GMP showed a small peak after 4 hours followed by a rapid decrease in intracellular concentrations Figure 3.5.C).

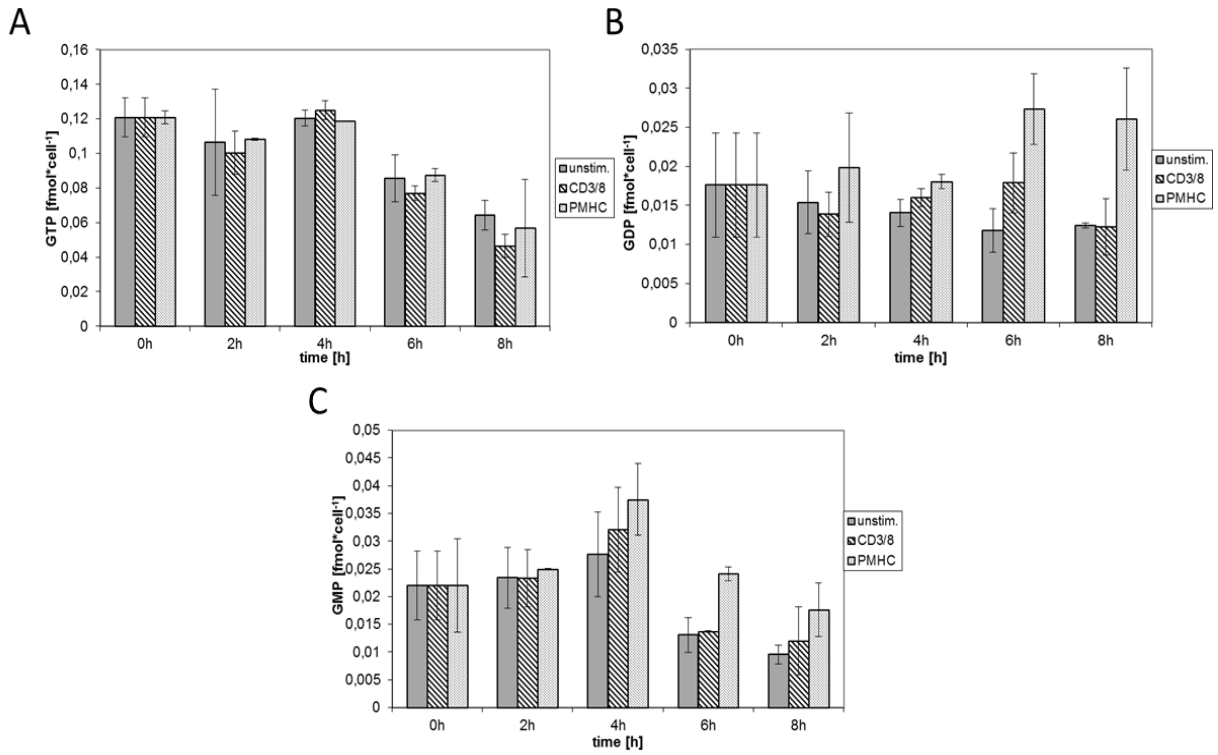

Figure 3.5. Changes in GTP concentration. Purified CD8+ T cells were treated with either soluble CD3/CD8 mAbs or OT-I tetramers (PMHC) for the indicated time periods. Concentrations of GTP (A), GDP (B) and GMP (C) where measured by MS coupled high-performance anion-exchange chromatography; n=5.

3.1.3. Elevated glycolysis leads to increased lactate production upon tetramer stimulation

The observed increase in glycolysis without significant changes in the TCA cycle lead us to hypothesize that pyruvate produced by glycolysis is converted into lactate (i.e. aerobic glycolysis). It was also shown previously that human CD4+ T cells produce lactate following CD3/CD28 stimulation. To test our hypothesis, we directly measured lactate production (Figure 3.6.).

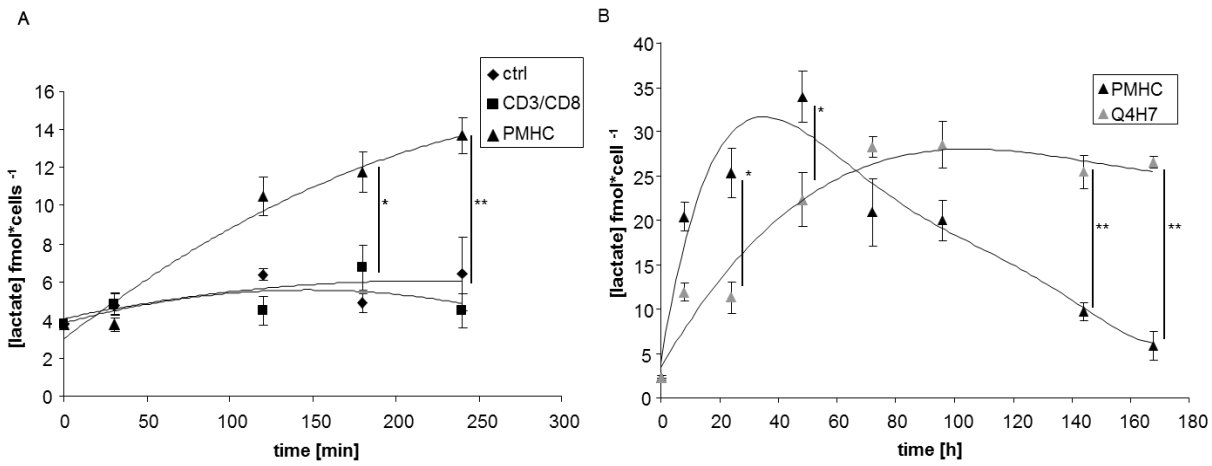

Figure 3.6. Tetramer stimulation leads to lactate production which peaks at 48h and decreased again. Purified CD8+ T cells were treated with either soluble CD3/CD8 mAbs or OT-I tetramers (PMHC) for the short term stimulation up to 4h (A) and OT-I tetramers or Q4H7 tetramers for the long term stimulation (B) lactate concentrations were assessed at indicated time points; n=7.

We observed a 3-fold increase in lactate production within the first 4 hours following tetramer stimulation, whereas there was no lactate production after antibody stimulation. When we analyzed later timepoints, we found that lactate production occurs at a high rate during the first 48 hours following stimulation. At later timepoints we observed a decrease in lactate production. Since T cells die within 24h upon antibody stimulation, it was not feasible to measure for longer stimulations. Therefore we used the low affinity peptide Q4H7 as a "negative" control, as it was shown previously that Q4H7 is a low affinity peptide which induces survival, but not T cell proliferation (Mallaun et al. 2008).

3.1.4. Metabolic inhibition leads to abrogated T cell proliferation

In the next step we examined the role of lactate production for T cell proliferation. The addition of oxamate, a lactate dehydrogenase inhibitor, lead to decreased proliferation and decreased intracellular lactate concentrations (Figure 3.7 A and B). Nevertheless, inhibiting the ATP production with rotenone lead to a complete block in proliferation, but did not effect lactate production. This inhibition is rather indirect because rotenone blocks complex I of the electron transport chain, disrupting the proton gradient at the mitochondrial membrane, which in the end abolishes the ATP synthase reaction.

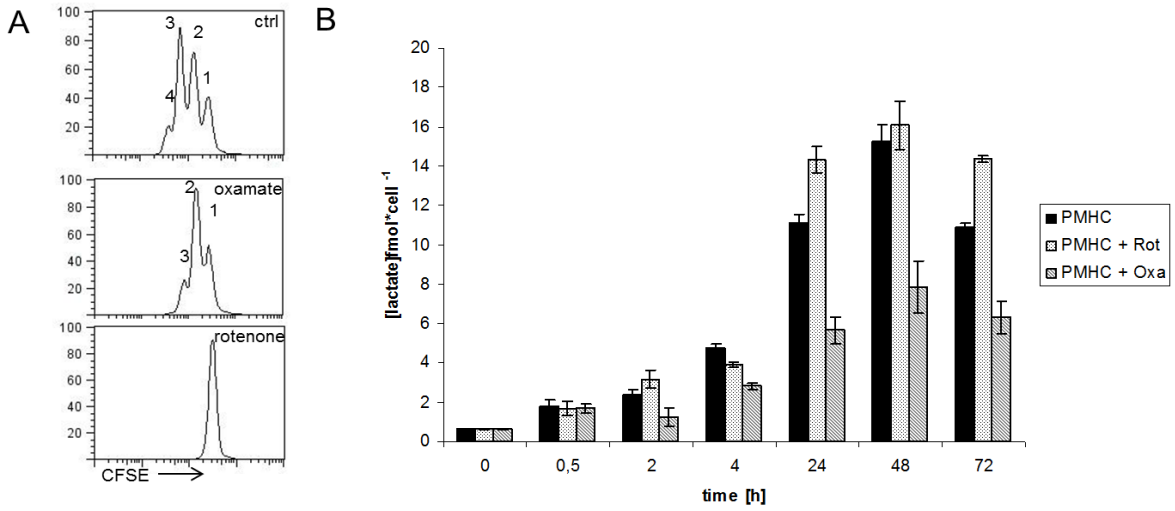

Figure 3.7. Inhibition of lactate production leads to reduced T cell proliferation. Purified CD8+ T cells were stimulated for 72h with OT-I tetramers in presence of the inhibitors rotenone and oxamate, proliferation was assessed by CFSE staining (A) and lactate production was measured at indicated time points.

To check that these inhibitory effects are not due to toxic effects of the inhibitors, we examined the induction of apoptosis and necrosis versus survival of the T cells by Annexin V/PI staining upon tetramer stimulation in presence or absence of the inhibitor. The observed data suggests that the inhibitor concentrations used had only a mild effect on T cell survival (Figure 3.8.). Rotenone lead to an increase in apoptotic cells from 22% to 26% whereas oxamate treatment increases the amount of apoptotic cells from 22% to 30%. Therefore we concluded that these findings were due to the proposed effect of the inhibitors and not due to high toxicity.

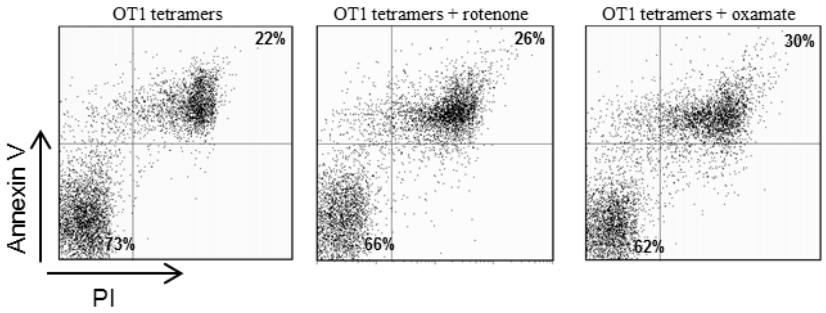

Figure 3.8. No severe toxic effects were observed upon application of the inhibitors. Purified CD8+ T cells were stimulated for 24h with OT-I tetramers in presence of the inhibitors rotenone and oxamate, apoptosis was assessed by Annexin V/PI staining

3.2. Regulation of T cell metabolic reprogramming following tetramer stimulation

In order to shed more light onto the metabolic changes occuring during T cell proliferation, we investigated the activation of known metabolic regulators like AMPK and the PI3K/AKT/mTOR pathway. We were particularl interested to further examine the regulation of metabolic reprogramming in T cells. Since only tetramer stimulation shows changes in metabolic parameters leading to T cell proliferation, we therfore focussed our work on the events following tetramer stimulation.

3.2.1. AMPK

First we analyzed the activation of the metabolic regulator AMPK, which becomes phosphorylated by LKB1 if the AMP:ATP ratio is increased. Here we show that AMPK is activated immediately by both stimuli (Figure 3.9). At later time points, antibody-stimulated T cells continue to show a sustained activation of AMPK, whereas tetramer-stimulated cells do not.

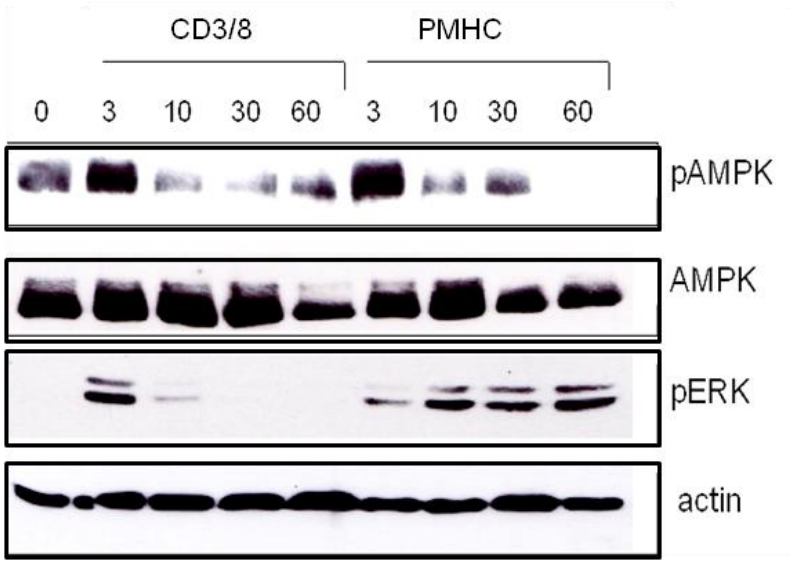

Figure 3.9. Soluble antibody stimulation leads to sustained AMPK activation. Purified CD8+ T cells were stimulated for indicated timepoints with either CD3/CD8 soluble antibodies or OT-I tetramers, whole cell lysates were immunoblotted with p-AMPK, total AMPK and p-ERK antibody, ß-actin served as loading control.

3.2.1.1. AKT and mTOR

Since it is known that the PI3K/AKT/mTOR pathway is required for metabolic regulation of cellular metabolism, we investigated the contribution of AKT and mTOR. Therefore we inhibited mTOR by using rapamycin. Rapamycin binds to the protein FK-binding protein 12 (FKbp12), the resulting FKbp12-rapamycin complex is then binding to the TSC1 protein of the mTOR complex and therefore inhibiting it´s activity (Loewith et al. 2002). Inhibition of AKT was mainly done using the AKT inibitor, AKT VIII which binds to the ph-domain of AKT and therefore inhibits the recruitment of AKT to the cell membrane. Inhibition of mTOR with rapamycin showed no effect on lactate production, while the inhibition of AKT completely abrogated lactate production (Figure 3.10.). The phosphorylation of S6K, which is downstream of AKT and mTOR was used as a positive control to ensure inhibitor function. In Figure 3.11. the observed phosphorylation of S6K was strongly reduced in presence of both inhibitors.

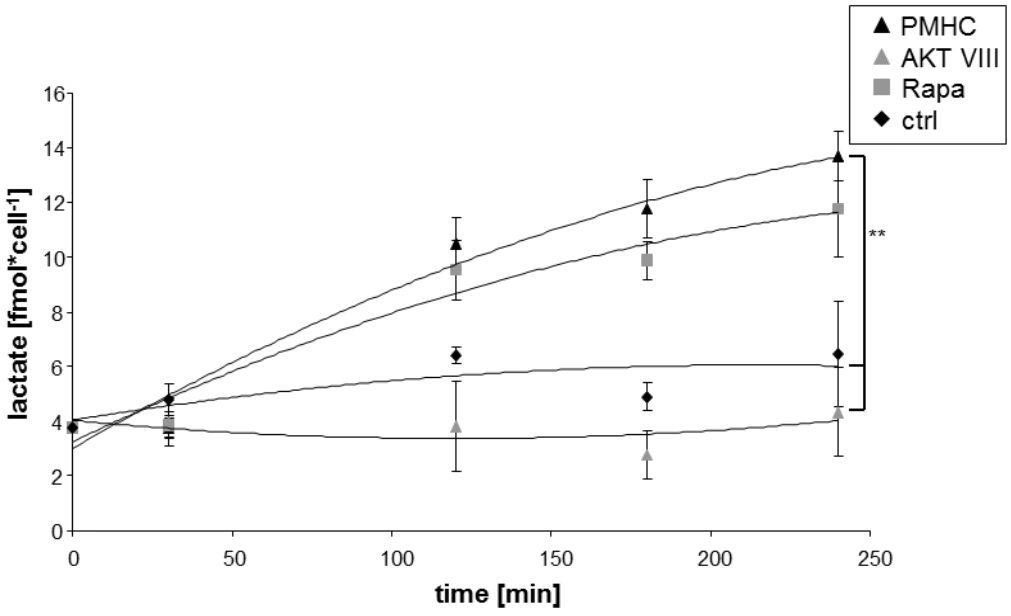

Figure 3.10. Inhibition of AKT leads to reduced lactate production. Purified CD8+ T cells were left untreated or stimulated for indicated timepoints with OT-I tetramers in the presence or absence of the inhibitors AKTVIII and Rapamycin (RAPA), cells were analyzed for intracellular lactate concentrations.

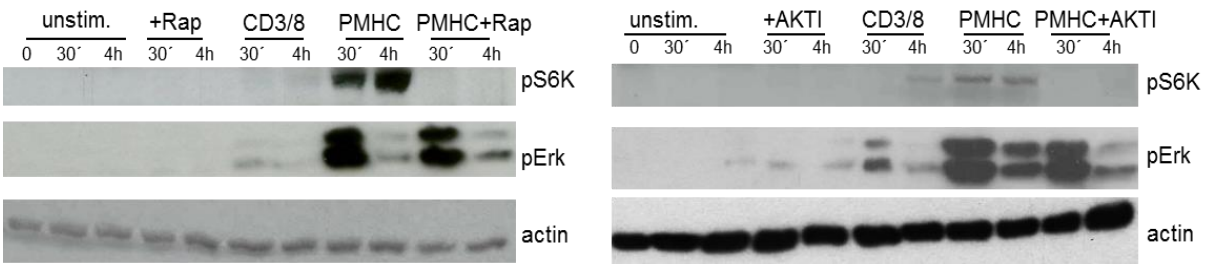

Figure 3.11. Succesful inhibition of AKT and mTOR leads to abrogated phosphorylation of S6K. Purified CD8+ T cells were left untreated or stimulated for indicated timepoints with OT-I tetramers in the presence of rapamycin (A) or AKTVIII (B), whole cell lysates were immunoblotted with p-S6K and p-ERK antibody, ß-actin served as loading control.

3.2.1.2. Regulation of metabolic enzymes by activation of AKT

Additionaly, it is shown for the first time that activation of AKT upregulates the expression of LDH, the enzyme which converts pyruvate to lactate. In Figure 3.12. this observation was confirmed using 3 specific functionally distinct AKT inhibitors, which lead to a marked reduction in LDH expression. This was accompanied by a strong and significant reduction in lactate production after 4h and 24h following stimulation in the presence of AKT inibitors.

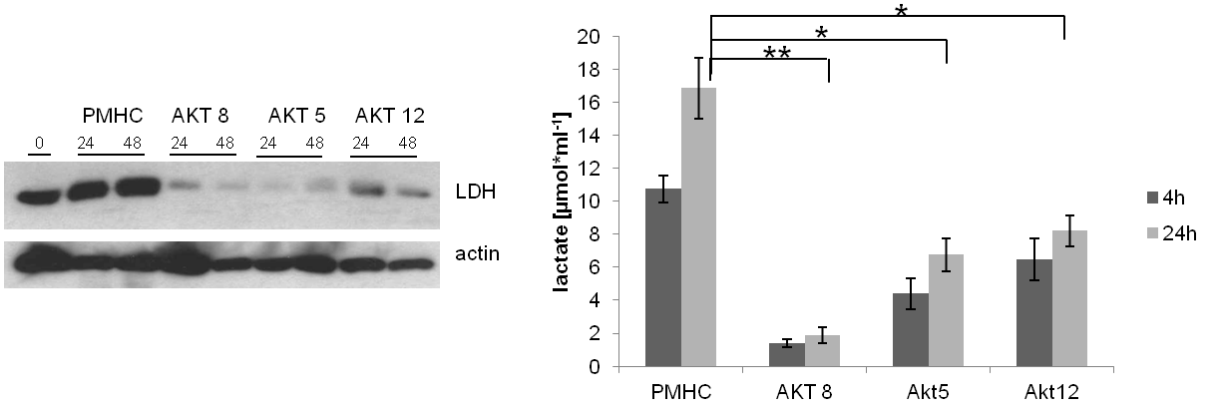

Figure 3.12. Inhibition of AKT with three different inhibitors leads to reduced upregulation of LDH. Purified CD8+ T cells were stimulated for indicated timepoints with OT-I tetramers in the presence of 3 specific AKT inhibitors, whole cell lysates were immunoblotted with total LDH and ß-actin as loading control (A) or cells were analyzed for intracellular lactate concentrations; n=3.

Furthermore we assessed the expression of hexokinase and phosphofructokinase (Figure 3.13.). Both enzymes catalyze reactions of the glycolysis and play a key role in enzymatically regulating this pathway. There was only mild increase in hexokinase expression observed upon 24 and 48h following stimulation; however phosphofructokinase showed a marked upregulation after 24h and 48h. Both expression patterns were not influenced by inhibition of AKT.

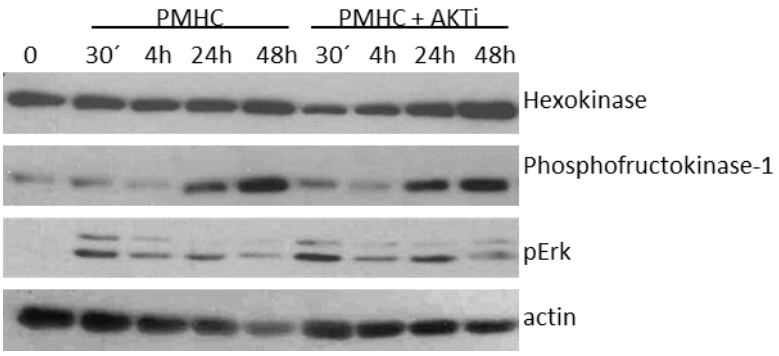

Figure 3.13. Inhibition of AKT has no effect on upregulation of hexokinase and phosphofructokinase. Purified CD8+ T cells were stimulated for indicated time points with OT-I tetramers in the presence of specific AKT inhibitor, whole cell lysates were immunoblotted with total hexokinase, total pfk1 and pERK, ß-actin served as loading control.

3.2.1.3. Hif1α is not a downstream target of AKT

Since mTOR is a downstream target of AKT and AKT-inhibition showed a strong impact on lactate metabolism in T cells, whereas mTOR did not, we searched for a direct target of AKT bypassing mTOR. Hif1α is a transcription factor which is known to regulate cellular metabolism (Cramer et al. 2003). Although it was previously described that Hif1α is mainly activated under hypoxic conditions (Shi et al. 2011), we hypothesized that AKT regulating Hif1α also under normoxic conditions. In Figure 3.14 Hif1α stabilization following T cell stimulation was examined. There is no difference observed between soluble stimulation and tetramer stimulation in the presence or absence of the AKT inhibitor.

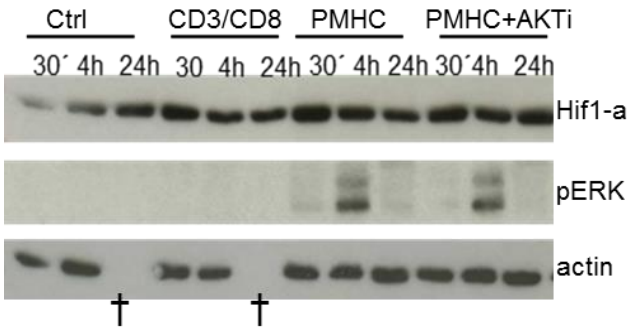

Figure 3.14. No stabilization of Hif1α was observed upon T cell stimulation under normoxic conditions. Purified CD8+ T cells were stimulated for indicated time points with soluble antibodies or OT-I tetramers in the presence of specific AKT inhibitor, whole cell lysates were immunoblotted with total Hif1α and pERK, ß-actin served as loading control.

3.2.1.4. Impact of AKT activation on expression of glucose transporter 1 (GLUT1) and glucose uptake

It was described previously for human CD4+ T cells that stimulation leads to an upregulation of glucose transporter 1 (GLUT1). This observation could be confirmed by flow cytometry measurements of GLUT1-expression (Figure 3.15.). Additionally, no significant impact of AKT inhibition on GLUT1 expression was observed.

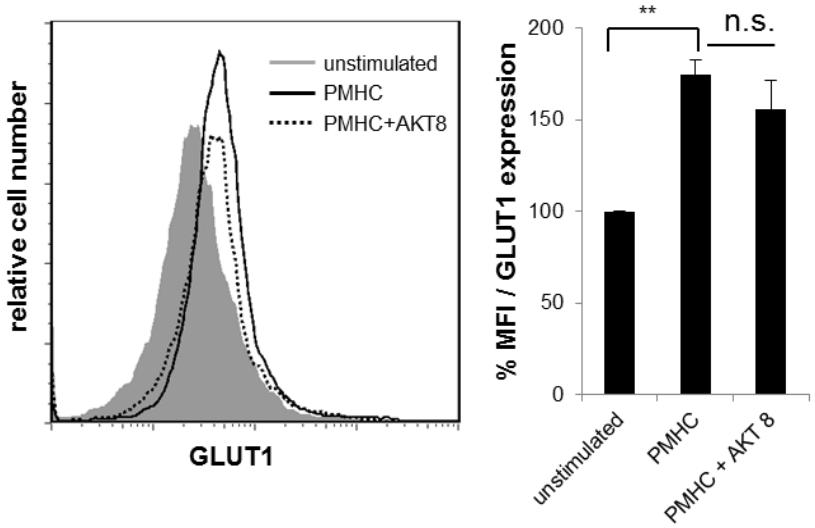

Figure 3.15. Upregulation of GLUT1 is not affected by AKT inhibition. Purified CD8+ T cells were stimulated for indicated time points with OT-I tetramers in the presence of specific AKT inhibitor, cells were analyzed by flow cytometry for GLUT1 expression.

Furthermore we analyzed the subsequent uptake of glucose by activated T cells. Here we observed an increased glucose uptake upon tetramer stimulation (Figure 3.16.), which reflects the upregulation of GLUT1 described above. This correlation was also observed in presence of the inhibitor application, which had no influence on glucose uptake.

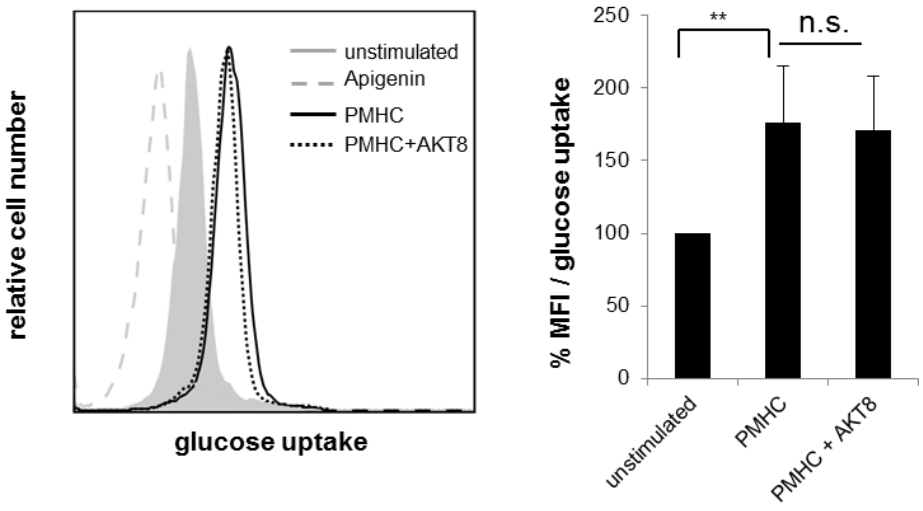

Figure 3.16. Upregulated glucose uptake upon tetramer stimulation is not altered by AKT inhibition. Purified CD8+ T cells were stimulated for 24h with OT-I tetramers in the presence or absence of specific AKT inhibitor or glucose transport inhibitor apigenin as negative control, cells were analyzed by flow cytometry for glucose uptake.

3.2.2. Switching off lactate production

In Figure 3.6B the lactate concentrations increased for 48 hours and then rapidly decreased. Therefore we hypothesized that in addition to a metabolic switch that turns on lactate production; there must also be a switch for shutting off this process.

3.2.2.1. No feedback inhibition of lactate

In our experimental setup, the activated T cells secrete lactate into the culture medium. Since *in vivo* this produced lactate is transported away, we hypothesized that the accumulating lactate might lead to feedback inhibition of its production. Feedback inhibition is a common mechanism to regulate metabolic processes and it is characterized by enzyme inhibition mediated by its product. To test this hypothesis, we added different concentrations of lactate to the stimulation reactions and assessed the intracellular lactate concentration after 4h, 24h, and 48h.

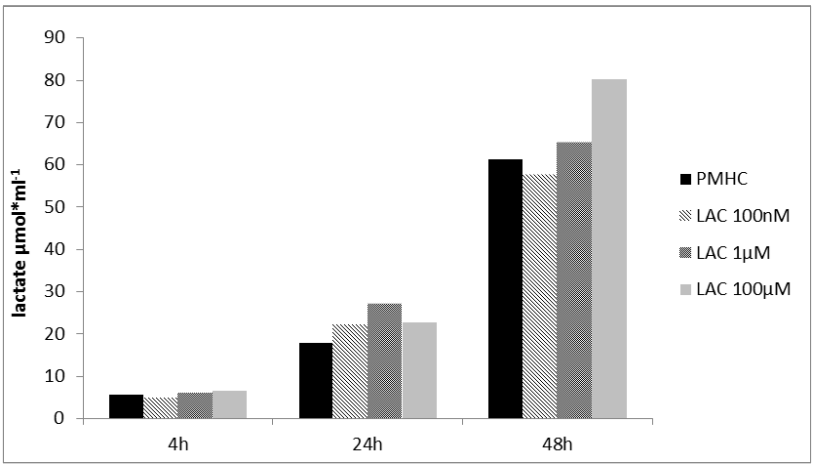

Figure 3.17. No feedback inhibition of lactate on lactate production. Purified CD8+ T cells were stimulated for 24h with OT-I tetramers in the presence or absence of different lactate concentrations, cells were analyzed for intracellular lactate concentrations, n=2.

The results shown in Figure 3.17. lead to the conclusion that there is no impact of extracellular lactate concentrations on intracellular lactate production up to a concentration of 100µM.

3.2.2.2. IL-2 is required for maintaining upregulated glycolysis

IL-2 is an autocrine growth factor which drives proliferation at later stages of T cell activation. Therefore, we tested the hypothesis that IL-2 might be involved in switching off lactate production. To test this, we applied a neutralizing IL-2 antibody to our cultures in order to prevent the binding of IL-2 to its receptor. To confirm that our neutralizing IL-2 antibody was effective and not toxic, we analyzed Stat5 phosphorylation 24h after stimulation (Figure 3.18.A) as well as apoptosis (Figure 3.18.B).

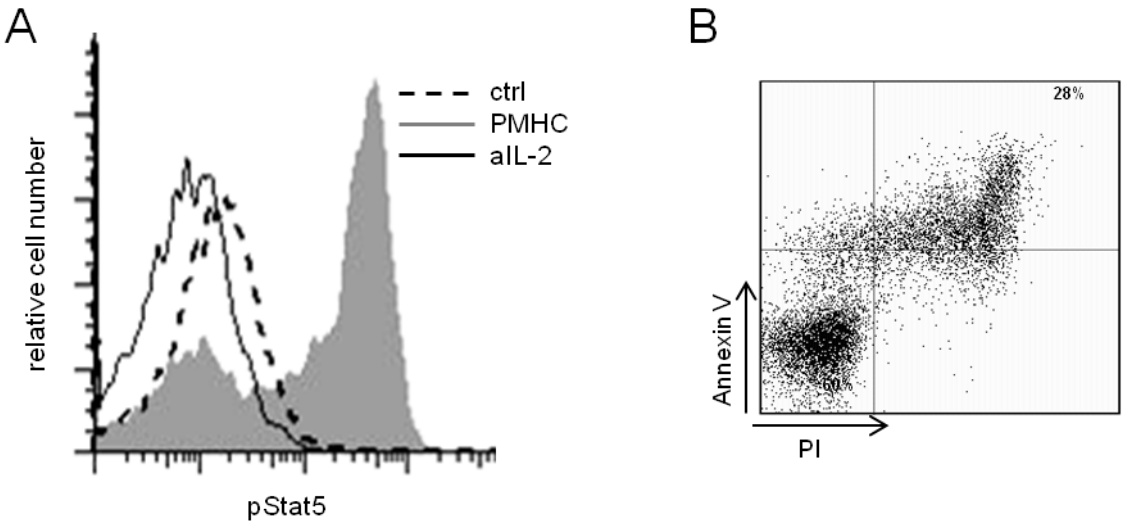

Figure 3.18 Addition of IL-2 neutralizing antibody leads to reduced pSTAT5 phosphorylation and showed no severe toxic effects. Purified CD8+ T cells were stimulated for 24h with OT-I tetramers in the presence or absence of aIL-2 antibody, cells were analyzed for pSTAT5 by intracellular flow cytometry staining (A) and apoptosis induction by AnnexinV/PI staining (B), n=2

Surpisingly, when the neutralizing IL-2 antibody was added to the culture, we observed a decreased lactate production (Figure 3.19.) after 24h stimulation which decreased further after 48h and 72h. Moreover, the addition of exogenous IL-2 to the stimulated cells fostered the production of lactate, leading to higher concentrations of lactate compared to normal tetramer stimualtion. This led us to the conclusion that IL-2 is needed to maintain lactate production and does not act as the 'OFF switch for lactate production.

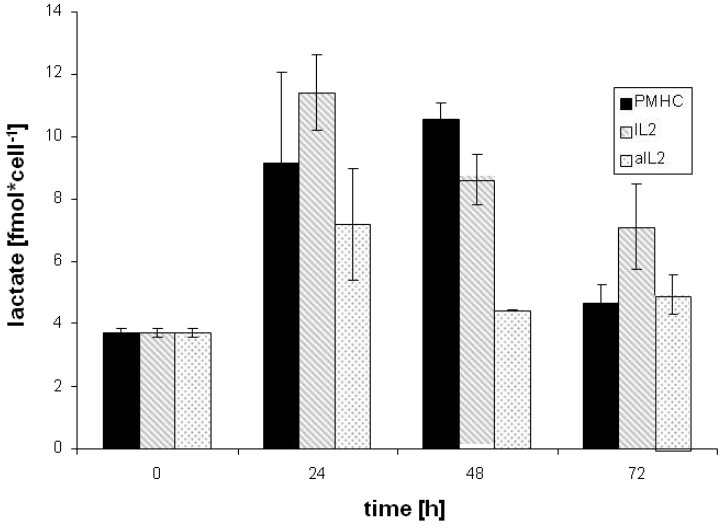

Figure 3.19 IL-2 is required to maintain lactate production. Purified CD8+ T cells were stimulated for 24h with OT-I tetramers in the presence of exogenous IL-2 or aIL-2 antibody, cells were analyzed for intracellular lactate concentrations, n=3.

3.2.2.3. Upregulation CTLA4 inhibits IL-2 production and subsequently shuts off the production of lactate

Since CTLA4 is known to be a negative regulator of T cell proliferation, we investigated the impact on CTLA4 on T cell metabolism. Therefore we analyzed the lactate production CTLA4-deficient OT-I T cells and compared them to wild-type OT-I T cells. Surprisingly the CTLA4$^{-/-}$ T cells showed a different kinetic of lactate production compared to normal OT-I T cells. After 48h of stimulation the produced lactate did not decrease, but rather continued to increase (Figure 3.20).

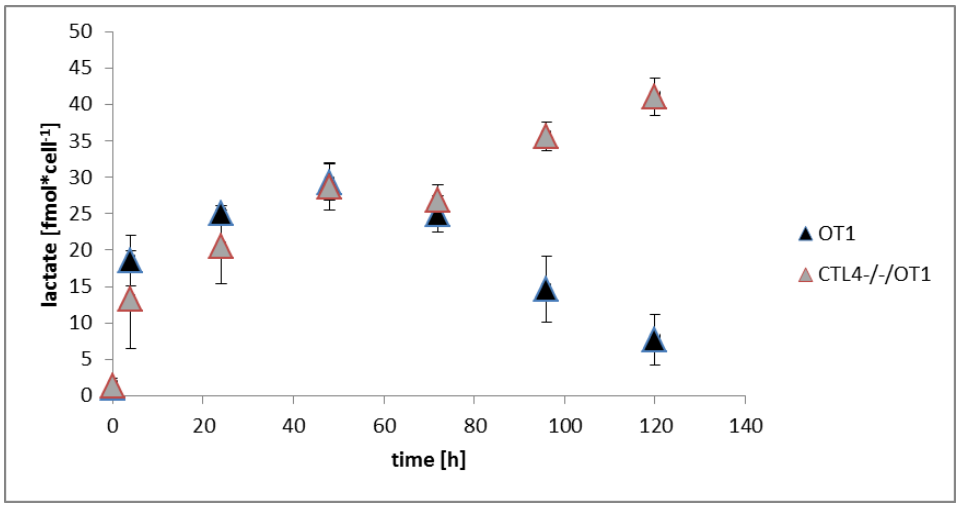

Figure 3.20. CTLA4$^{-/-}$-OT-I T cells showed no decrease in lactate production. Purified CD8+ T cells from CTLA$^{-/-}$ or CTL4$^{+/+}$ mice were stimulated with OT-I tetramers for indicated time points, cells were analyzed for intracellular lactate concentrations, n=3.

The further analysis of IL-2 production in CTLA4$^{-/-}$ T cells revealed that also IL-2 production is not decreasing after 48h of stimulation as was observed for wild-type OT-I T cells (Figure 3.21). Taken together, the similar behavior of IL-2 and lactate production combined with the observations made by adding exogenous IL-2 or neutralizing IL-2 (i.e. aIL-2 antibodies) lead us to the conclusion that the upregulation of CTLA4 leads to a decrease in IL-2 production, which subsequently results in a decreased lactate production.

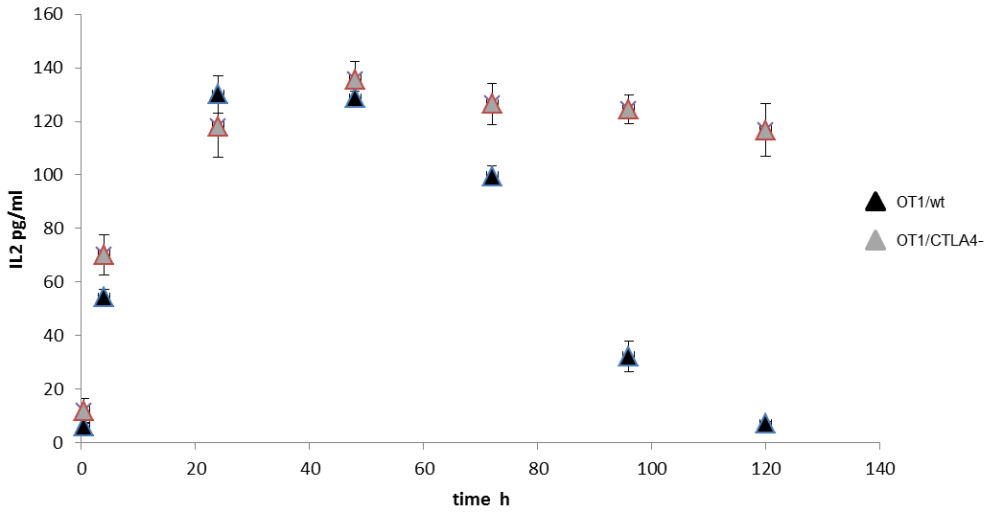

Figure 3.21. Upregulation of CTLA4 leads to decrease of IL-2 production. Purified CD8+ T cells from CTLA$^{-/-}$ or CTL4$^{+/+}$ OT-I mice were stimulated with OT-I tetramers for indicated time points, cells were analyzed for intracellular IL-2 concentrations by ELISA, n=3.

3.3. Comparing stimulation conditions for mouse and human T cells

It was previously shown that primary human T cells respond to the stimulation with CD3/CD28 coupled beads by upregulating both glycolysis and lactate production (Frauwirth et al. 2002). We confirmed this observation by stimulating primary human T cells with beads coupled with CD3 alone or in combination with CD28 (Figure 3.22.A). We found that there is a strong upregulation of lactate production upon CD3/CD28 stimulation, whereas the increase in lactate production upon CD3 stimulation alone appeared to be much weaker. When we applied these stimulation conditions to CD8+ OT-I T cells (Figure 3.22.B) both bead coupled antibodies as well as tetramers showed the same induction of lactate production. While the addition of co-stimulatory CD28 to the CD3 stimulation in human T cells leads to a significant increase in lactate production, there was only a minor increase observed in mouse T cells.

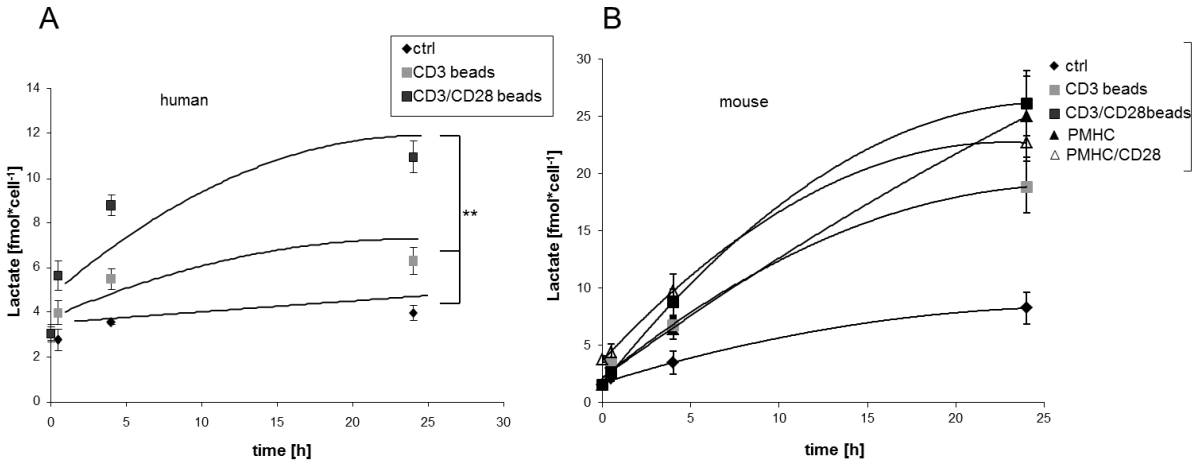

Figure 3.21 Purified human CD8+ T cells were stimulated CD3/CD28 bead coupled Abs (A) or mouse CD8+ T cells stimulated with OT-I tetramers (B) were analyzed for lactate production, n=3.

To control whether CD28 stimulation was successful, we analyzed JNK phosphorylation as we had previously shown that CD28 stimulation alone induces the activation of JNK (REF) (Figure 3.22.). From this we conclude that the addition of CD28 to the tetramer stimulation had no impact on lactate production.

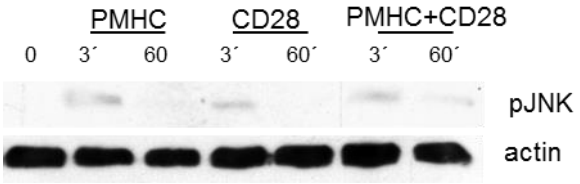

Figure 3.22. Comparable JNK phosphorylation upon stimulation with OT-I tetramers and CD28. Purified CD8+ T cells were stimulated for 3´ and 60´ with OT-I tetramers, CD28 and both together, whole cell lysates were immunoblotted with pJNK, ß-actin served as loading control, n=2.

In summary, we found that tetramer induced activation result in an increase in key glycolytic metabolites, whereas the TCA cycle remains unaffected. The upregulation of glycolysis led to a strong lactate production, which is dependent upon AKT/PKB, but not mTOR. The increased lactate production results from the upregulation of lactate dehydrogenase, which we found to be dependent on IL-2 and to be required for proliferation. Additionally, we observed an upregulation of Glucose-transporter 1 (GLUT1) as well as glucose uptake upon stimulation, which were not influenced by AKT inhibition. Furthermore knocking out CTL4, a negative regulator of T cell proliferation, led to a sustained lactate production, which correlates with a sustained IL-2 production. Thus we conclude that the upregulation of CTL4 in the later stage of T cell activation shuts off lactate production by down regulating the production of IL-2.

4. Discussion

4.1. Effect of different stimuli on T cell metabolism

Triggering the T cell receptor by pathogens *in vivo* leads to an activation of a specific network of signaling cascades. These signaling events are crucial for proliferation and differentiation leading to a functional immune response. To elucidate the complex intracellular networks soluble polyclonal stimuli such as agonistic antibodies against the TCR/CD3 complex (i.e UCHT1, OKT3), the co-receptors and/or the co-stimulatory molecules (i.e. CD28) were used for many years. However recent studies have shown that these *in vitro* stimuli are not able to mimic physiological conditions leading to a productive T cell response (Daniels et al. 2006; Wang et al. 2008; Arndt et al. 2013). It was described previously for OT-I mouse T cells that stimulation with soluble antibodies leads to strong phosphorylation of the T cell signaling network (i.e. ZAP70, AKT and ERK) which results in apoptosis (Brand et al. 1984; Wang et al. 2008). On the contrary more physiological stimuli like OT-I tetramers for mouse T cells or antibodies coupled to microbeads for human T cells showed sustained phoshorylation of signaling proteins that leads to proliferation and differentiation of T cells reflecting the *in vivo* stimulations. The reason why these stimuli have different outcomes on the T cell fate still remains unclear. Our hypothesis to explain these opposite cellular responses was that the depletion of the intracellular ATP stores following antibody stimulation could be responsible for the induction of apoptosis. It was previously shown that intracellular ATP levels can be used as a marker for cell viability in tumor cell lines meaning that if intracellular ATP concentrations fall below a certain limit, apoptosis is induced (Garland 1997; Izyumov et al. 2004). On the other hand, several studies showed that apoptotic processes require ATP to transfer apoptotic signals into the nucleus as well as for chromatin condensation and nuclear fragmentation (Tsujimoto 1997; Yasuhara et al. 1997; Kass et al. 1996). We observed a rapid consumption of ATP upon antibody stimulation, whereas ATP levels remain constant after tetramer stimulation. The question is whether the drop in ATP levels are the cause or a consequence of the resulting apoptosis. First consider that the changes in ATP levels were observed already within the first hour of stimulation. From this one is led to assume that the dramatic phosphorylation which occurs upon antibody stimulation leads to a high consumption of ATP (Leist et al. 1997). This in turn results in the induction of apoptosis, which further reinforces the reduction in ATP levels. Therefore these results suggest that following

tetramer stimulation, T cells switch on metabolic programs to generate ATP in order to counterbalance their ATP consumption.

Therefore, we next investigated the activation of AMPK, as this is the major energy sensor in cells and its activation is closely linked to intracellular ATP levels. We observed an initial activation of AMPK upon both tetramer and antibody stimulation. This initial activation was previously reported to be induced by the activation of CAMKK2 upon TCR triggering (Tamás et al. 2006). Since AMPK is further activated by a high AMP:ATP ratio, the decreased ATP levels observed upon antibody stimulation forces AMPK to remain active. In contrast, upon tetramer stimulation, T cells maintain high levels of ATP, thus inactivating AMPK. This led us to the conclusion that tetramer stimulation induces additional changes in the metabolic profiles, since the initial activation of AMPK is not sufficient to shift T cell metabolism towards glycolysis, which is needed to maintain proliferation.

4.2. Metabolic changes upon tetramer stimulation

Several previous studies analysed the upregulation of glycolysis by monitoring glucose consumption from the media, direct glucose uptake by the cell, or the upregulation of GLUT1 (Frauwirth et al. 2002; Macintyre et al. 2011; Bauer et al. 2004). We used a new method to analyze 25 metabolites and nucleotides from both glycolysis and the TCA cycle in order to further investigate the metabolic shift. In our experiments with tetramer stimulation, we clearly observed a significant increase in glycolytic metabolites compared to either the antibody stimulated or unstimulated cells. Taking a closer look at the glycolytic reactions, it is thought that there exist three rate-limiting steps at positions in the pathway where large free energy differences arise: hexokinase, phosphofructokinase, and pyruvate kinase (Rose,Warms 1966; Rapoport et al. 1976; Hue,Rider 1987). Surprisingly no increase in the concentrations of substrates for these reactions were found in our analysis. On the contrary, increased levels of fructose-1,6-bisphosphate and 3-phosphoglycerate, the respective substrates for the phosphofructokinase and glycerinaldehyde-3-phosphatedehydrogenase reactions where observed. In the recent study by Shestov et al. GAPDH was identified as a limiting step by computational modeling of glycolysis and an integrated metabolic control analysis (Shestov et al. 2014). They propose that GAPDH is the bottleneck in this pathway due to its unique placement where it can be regulated by ATP, NAD+, and the levels of glucose-derived intermediates that affects the thermodynamics of glycolysis. Furthermore in this study the authors explained that high concentrations of fructose-

1,6-bisphosphate are allosterically activating pyruvate kinase causing a depletion in the metabolites downstream of the GAPDH. This amplified the role of GAPDH as the most rate-determining step in the pathway since increased activity through GAPDH will then serve to create a balance along the pathway. This observations might help in understanding the results of our metabolite analysis in T cells, as they confirm that the overall glycolytic rate is increased. In addition after tetramer stimulation, T cells produce high amounts of lactate, which is an indication for increased aerobic glycolysis. The fact that lactate production is essential for T cell proliferation was confirmed by the observation that the addition of oxamate, a lactate dehydrogenase inhibitor, leads to decreased proliferation. We also observed small changes in the metabolites of the TCA cycle, however these were not statistically significant. Nevertheless, T cells still require the TCA cycle to maintain proliferation, as shown by inhibiting the ATP production with rotenone. This inhibition is rather indirect because rotenone blocks complex I of the electron transport chain, disrupting the proton gradient at the mitochondrial membrane, which in the end abolishes the ATP synthase reaction. In a recent study comparing metablic features of T cell subsets, the authors observed striking differences between CD8+ T cells, which showed more glycolytic metabolism, compare to CD4+ T cells, which show higher rates of mitochondrial oxidative metabolism and a greater maximal respiratory capacity (Cao et al. 2014). Nevertheless activation and proliferation of both cell types were similar sensitive to the addition of rotenone. This supports the idea that the TCA cycle is not only required for the generation of ATP, but is also essential to deliver substrates for biosynthetic processes like the generation of nucleotides, which are neede for proliferation. Furthermore it has been shown that during oxidative phosphorylation, reactive oxygen species are generated, which played a critical role in T cell activation (Sena,Chandel 2012). In summary T cells require an increased in both pathways, glycolysis and TCA cycle, to support rapid proliferation and inflammatory function.

4.3. Shutting off increased glycolysis and lactate production.

Surprisingly we observed that after 48 hours of activation, lactate production decreases. This observation led us to hypothesize that there is a switch in T cell metabolism to shut down aerobic glycolysis. IL-2 is the autocrine growth factor responsible for the switch from antigen-driven proliferation to cytokine-driven proliferation. Since IL-2 production occurs on a similar time scale, we tested the hypothesis that IL-2 might be involved in switching off lactate production. When we tested this hypothesis we found that IL-2 is required to maintain lactate production.

This observation contradicts a previous study were the authors showed that the removal of IL-2 within the first 20h of stimulation had no effect on lactate production in primary human CD4 T cells (Frauwirth et al. 2002). Under normal conditions, it is known for humans that the lactate produced is pumped out of the cell and transported by the bloodstream to organs like the liver where lactate recycling takes place. Since our *in vitro* assays lead to an accumulation of extracellular lactate we tested whether feedback inhibition, a common process for regulating enzymatic activities, can be observed. Adding up to 100µM exogenous lactate to the cells, which is about the amount of lactate we measured extracellularly *in vitro*, had no impact on the intracellular lactate production, therefore we could exclude feedback inhibition as a reason for the decreased lactate production after 48h in our experimental system. Although we can not exclude feedback inhibition *in vivo* as concentrations in the bloodstream can reach 5mM during high muscular activity. Since it is known that IL-2 induces negative regulators of T cell activation like CTLA4, which downmodulate T cell responses to prevent an overreaction of the immune system (Frauwirth et al. 2002; Parry et al. 2005), we next assessed lactate production in OT-I T cells from CTLA4$^{-/-}$ mice upon stimulation. Interestingly the decrease in lactate production could not be observed in these cells indicating that CTLA4 plays a critical role in downmodulating T cell metabolism. Furthermore this activity could be correlated to the IL-2 production in these cells. Under normal conditions the expression of IL-2 ultimately leads to the upregulation of CTLA4 (Alegre et al. 1996; Wang et al. 2001). This in turn leads to a downregulation of IL-2, which would explain the observed decrease in lactate production (Carreno et al. 2000). In a recent study, it was shown that the upregulation of BCL-6 represses genes encoding molecules involved in aerobic glycolysis that are upregulated during the effector phase of the immune response (Oestreich et al. 2014). A connection between the expression of CTLA4 and BCL-6 has been discussed recently in the generation of follicular helper T cells (Wang et al. 2015), but if it is a common feature in downmodulation T cell metabolism remains unclear and has to be addressed in further experiments. Furthermore it has been shown that a blockade of CTLA4 and PD-1 leads to an increased tumor rejection in the B16 mouse melanoma model by increased infiltration of T cells into the tumor and decreased generation of regulatory T cells (Curran et al. 2010).

In summary we showed a strong correlation of IL-2 and CTLA4 for mediating the switching of upregulated T cell metabolism. However it has to be further investigated if the downregulation of lactate production is a direct effect of CTLA4 upregulation and what might be the molecular mechaism behind this observation.

4.4. Regulation of T cell metabolism

Since it is known that the PI3K/AKT/mTOR pathway is required for metabolism, we analyzed the contribution of AKT and mTOR, as both are nvolved in signaling processes known to regulate cellular metabolism (ANDRES et al. 1955; Rathmell et al. 2003). Inhibition of mTOR with rapamycin showed no effect on lactate production, while the inhibition of AKT completely abrogated lactate production. Additionally, we show for the first time that activation of AKT upregulates the expression of lactate-dehydrogenase, the enzyme which converts pyruvate to lactate. This observation was confirmed using functionally distinct AKT inhibitors, all of which abrogated the expression of LDH.

Moreover, we observed an increase in GLUT1 expression and glucose uptake upon tetramer stimulation, which was also been previously described in primary human T cells (Rathmell et al. 2003; Frauwirth et al. 2002). In contrast to these previous studies, the upregulation of GLUT1 and glucose uptake in OT-I CD8+ T cells was not AKT-dependent. These contradictory results were also described in a recent study by Macintyre and coworkers (Macintyre et al. 2011), who showed that inhibition of AKT had no effect on glucose uptake after stimulation of P14 TCR tg T cells with gp33 peptide. Thus, we confirm the results of Macintyre showing that AKT has no impact on the uptake of glucose. However, we additionally show that AKT is required for the upregulation of lactate dehydrogenase expression. Therefore, we suggest that GLUT1 upregulation and glucose uptake are regulated in an AKT-independent manner, whereas lactate production strongly requires the activation of AKT. It was shown that phosphoinositol-dependent protein kinase 1 (PDK1), an upstream activator of AKT, is responsible for the upregulation of glucose uptake independent of the PI3K/AKT pathway (Macintyre et al. 2011). Since the role of PDK1 was assessed in T cell blasts in the presence of high IL-2 concentrations, there is a strong temporal separation from our system. We clearly observe the upregulation of LDH in parallel to the activation of AKT within 48h upon stimulation. This leads to the hypothesis that AKT activation upon stimulation induces an upregulation of LDH, whereas IL-2 production induces an AKT independent upregulation of glucose uptake via PDK1.

Surprisingly the inhibition of mTOR by rapamycin had no effect on lactate production, since it was described that mTOR is one of the crucial players in nutrient sensing in mammalian cells. Previous studies revealed an important role of mTOR in the regulation of differentiation into CD4+ T cell subsets like Th1, Th2 and Th17 cells (Michalek et al. 2011; Shi et al. 2011). Furthermore we observed no activation of HIF1α in our study which was proposed to be a link

between mTOR and upregulation of glycolytic enzymes (Shi et al. 2011). Therefore we could conclude that mTOR activation is not required for upregulation of glycolysis in CD8+ T cells. Since it was also shown that inhibition of mTOR drives memory T cell formation (Pearce et al. 2009), the addition of rapamycin is a powerful target in modulating CD4+ T cell related autoimmune diseases like multiples sclerosis (Esposito et al. 2010) with minimal effect on CD8+ T cell responses.

4.5. CD28 costimulation is dispensable for OT-I T cells stimulated with tetramers

A change in metabolism and the production of lactate has also been described for primary human CD4+ T cells (Salmond et al. 2009). We were able to confirm these previous results showing that CD3 alone induced only a weak production of lactate, whereas the addition of co-stimulation via CD28 led to a full activation of lactate production in human T cells. When we applied these conditions to our CD8+ mouse model, we see a insignificant difference between the stimulation with CD3 alone and the co-stimulation with CD28. Additionally, we show that tetramer stimulation leads to a level of lactate production similar to that after stimulation with antibodies coupled to beads. This supports the hypothesis made by Wang *et al.* that stimulation of CD8+ T cells does not require a co-stimulatory signal in contrast to CD4+ T cells (Wang et al. 2000). Similar effects were shown in a study were the blockade of the CD28 pathway hat no effect on allograft rejection mediated by CD8+ T cells (Newell et al. 1999).

4.6. Modulating metabolism

We show that CD8+ T cells undergo rapid metabolic changes following activation in order to maintain their energetic needs for proliferation and differentiation leading to a functional immune response. As mentioned before CD4+ T cells were shown to perform in a similar way upon activation. However other T cell subsets like regulatory or memory T cells require different metabolic programs according to their function. It was shown for these subsets that their metabolism mostly relying on an increased TCA cycle (Pearce et al. 2009; Delgoffe et al. 2009). The induction of fatty acid oxidation feeding into the TCA cycle leads to an enhanced generation of memory T cells. It was shown that inhibition of mTOR substantially increased the generation

of memory T cells during vaccination and virus infection. In our work we identified several key molecules to modulate T cell metabolism. Inhibition of AKT or an upregulation of CTLA4 could be possible targets for preventing the overreaction and exhaustion of T cells as seen in chronic viral infections, like HIV or HCV. Until now some metabolic inhibitors have been shown to suppress T cell responses in EAE, asthma, and graft versus host disease (Dang et al. 2011; Shi et al. 2011; Ostroukhova et al. 2012; Gatza et al. 2011). Furthermore a recent study revealed an altered metabolism in CD4+ T cells from a systemic lupus erythematosus (SLE) mouse model (Yin et al. 2015). These T cells showed besides upregulation of glycolysis and elevated mitochondrial oxidative metabolism. The addition of 2-deoxyglucose to downregulate glycolytic levels together with metformin, which downmodulates the TCA-cycle, could reverse disease biomarkers. A key goal of future work will be to determine how the activation environment *in vivo* alters metabolic reprogramming in specific disease states. Recently a new molecule was identified called lymphocyte expansion molecule (LEM, Okoye et al. 2015), which was shown to promote antigen specific CD8+ T cell expansion, effector function, and memory cell generation. LEM was observed to regulate the protein complexes of the oxidative phosphorylation pathway in the inner membrane of the mitochondria thereby upregulating the generation of reactive oxygen species which play a crucial role in T cell proliferation. Nevertheless the whole mechanism how this modulation effects T cell beahviour remains unclear. This leads to the conclusion that changes in T cell metabolism can alter T cell expansion and differentiation making metabolic regulation a powerful target for treatment of a large variety of diseases like immune-related diseases characterized by hyperactive T cells.

5. References

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6. Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular-signal-regulated kinase
GDP	guanosine-5'-diphosphate
GLUTI	glucose transporter 1
GMP	guanosine-5'-monophosphate
GTP	guanosine-5'-triphosphate
HIF1α	Hypoxia-inducible factor 1-alpha
IL	interleukin
LDH	lactate dehydrogenase
LKB1	liver kinase B1
MHC	major histocompatibility complex
mTOR	mammalian/mechanistic target of rapamycin
NADH	nicotinamide adenine dinucleotide

NTP	nucleoside triphosphate
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PDK1	3-phosphoinositide dependent protein kinase-1
TCA-cycle	tricarboxylic acid cycle
TCR	T cell receptor

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8. Curriculum vitae

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Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zu dem Thema:

„Metabolic reprogramming upon CD8 T cell activation“

selbständig verfasst habe, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr.rer.nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg den 13.07.2015

Clemens Cammann