Metabolic plasticity of human T cells: Preserved cytokine production under glucose deprivation or mitochondrial restriction, but 2-deoxy-glucose affects effector functions

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The strong link between T-cell metabolism and effector functions is well characterized in the murine system but hardly investigated in human T cells. Therefore, we analyzed glycolytic and mitochondrial activity in correlation to function in activated human CD4 and CD8 T cells. Glycolysis was barely detectable upon stimulation but accelerated beyond 24 h, whereas mitochondrial activity was elevated immediately in both T-cell populations. Glucose deprivation or mitochondrial restriction reduced proliferation, had only a transient impact on “on-blast formation” and no impact on viability, IFN-γ, IL-2, IL-4, and IL-10 production, whereas TNF was reduced. Similar results were obtained in bulk T cells and T-cell subsets. Elevated respiration under glucose restriction demonstrated metabolic flexibility. Administration of the glycolytic inhibitor 2-deoxy-glucose suppressed both glycolysis and respiration and exerted a strong impact on cytokine production that persisted for IFN-γ after removal of 2-deoxy-glucose. Taken together, glycolytic or mitochondrial restriction alone compromised proliferation of human T cells, but barely affected their effector functions. In contrast, effector functions were severely affected by 2-deoxy-glucose treatment.

Keywords: 2-deoxy-glucose · ATP · Cytokines · Glucose deprivation · Human CD4 T cells · Human CD8 T cells · Metabolism · Mitochondrial inhibition

Introduction

The transition from quiescent to effector lymphocytes increases the metabolic demands of T cells, as growth, proliferation and effector functions depend on ATP and biomolecule synthesis [1–4]. Numerous studies have investigated metabolic alterations of murine T cells upon stimulation. Murine T-cell activation leads to increased glucose metabolism that drives proliferation and effector functions [5–8]. Most studies agree, that proliferation depends on glucose and glutamine uptake and a strong link between glycolytic activity and IFN-γ production has been reported [7, 9, 10]. Pearce and colleagues have demonstrated
that glucose is especially important for the initiation of IFN-γ translation. Glucose triggers the dissociation of glyceroldehyde-3-phosphate dehydrogenase (GAPDH) from the 3' UTR of IFN-γ mRNA thereby allowing cytokine production [11]. In contrast, production of other cytokines such as IL-2 appears not to depend on glucose [6, 7]. Beside similarities, as activation of glucose metabolism upon stimulation, murine T-cell subpopulations adopt distinct metabolic pathways to different extents. CD4 T cells remain more oxidative and CD8 T cells more glycolytic, thereby exhibiting a higher proliferative capacity [12].

Many studies have used the glycolytic inhibitor 2-deoxyglucose (2-DG) to prove the importance of glucose metabolism for T-cell function. However, glucose deprivation and 2-DG treatment do not always result in the same effects. Cham and Gajewsky showed that IL-2 production was not affected by glucose deprivation but reduced by 2-DG treatment [6]. Further, MacDonald and colleagues suggested that 2-DG altered cytolysis and conjugate formation of murine T cells with target cells independent of energy depletion [13]. Interestingly, a direct inhibitory effect of 2-DG on cell respiration has been shown in adipocytes [14]. This is of special importance considering that mitochondrial oxidative phosphorylation (OXPHOS) and reactive oxygen species production are involved in proliferation and IL-2 production, respectively [11, 15].

Data in the human system are rather sparse. Elevated glucose and glutamine uptake has been observed upon activation of human lymphocytes and linked to proliferation and cytokine production [16–18]. However, glycolytic or mitochondrial activity was dispensable during the early phase of PMA/ionomycin stimulation of human CD4 T cells [19]. Nevertheless, an early glycolytic switch has been related to an accelerated production of IFN-γ in human memory CD8 T cells [20].

A detailed understanding of the relationship between metabolism and human T-cell function is mandatory also in light of newly developed protocols for cancer therapy targeting glycolytic or mitochondrial activity. Such protocols might disproportionately affect the adaptive immune system [21] and thereby the anti-tumor immune response, which is regarded as important for patient outcome [22].

In this study, we characterized and directly compared the activation-induced metabolic reprogramming of isolated stimulated and restimulated human CD4 and CD8 T cells and investigated the impact of metabolic restriction. As our key finding, we demonstrate that glucose metabolism and mitochondrial function are important for CD4 and CD8 T-cell proliferation, but of minor importance for cytokine production and viability. Administration of 2-DG, in contrast, severely compromised glycolytic and mitochondrial activity and cytokine production in CD4 and CD8 bulk populations and related subsets. Taken together, we show that important effector functions of T cells are barely affected by inhibiting separately glucose or mitochondrial activity, however, they are severely compromised by 2-DG.

Results

Glucose metabolism in human CD4 and CD8 T cells

CD4 and CD8 T cells were stimulated for 6 days (denominated stimulated). After 6 days cells were collected, diluted and stimulated again (denominated restimulated). The restimulation procedure was thought to reflect the in vivo situation, where T cells that evaded from the lymph node are restimulated at the site of inflammation. We analyzed the mRNA expression kinetics of important proteins involved in glycolysis. In both T-cell populations, mRNA levels of glycolytic enzymes and transporters were increased upon stimulation albeit the initial kinetics were different (Fig. 1A and B). In CD4 T cells, mRNA expression of lactate dehydrogenase A (LDHA), hexokinase (HK) I and II and monocarboxylate transporter 1 (MCT1) was strongly elevated after the first 6 h, whereas in CD8 T cells expression was moderately increased after 6 h but highly elevated after 48 h (Fig. 1A and B). Glucose transporter 1 (GLUT1) expression remained increased beyond 6 h in both T-cell populations (Fig. 1A and B). High levels of mRNA expression were maintained during restimulation (data not shown).

Glucose metabolism was investigated by measuring glucose uptake and lactate secretion. Glucose metabolism was almost not detected in quiescent T cells cultivated in medium supplemented with the same concentration of IL-2 as activated T cells (Supporting Information Fig. 1). Activated T-cell populations showed limited glycolytic activity as shown by the secretion of low levels of [2,3-13C]lactate converted from labeled glucose (Supporting Information Fig. 1). Increased glycolytic activity was found beyond 24 h, especially in CD4 T cells (Fig. 1C–F). To further investigate glucose metabolism, we determined extracellular acidification rates (ECAR) by T cells activated for 24, 48 and 72 h. In line with the observed elevated lactate levels, the glycolytic activity was also reflected by an increased ECAR in the course of activation (Fig. 1G and H). CD4 T cells stimulated for 24 h were significantly more active compared to CD8 T cells (40 ± 4 versus 26 ± 3**, p < 0.001, respectively).

It has been reported that memory T cells have an increased glucose metabolism in the early phase of stimulation. However, we could not find any correlation between lactate secretion and the percentage of CD95-positive T cells in our bulk population, which are regarded as memory T cells.

T cells maintained a high glycolytic activity during the whole stimulation period of 6 days (data not shown). Restimulated T cells, displayed a significantly higher initial glucose metabolism in both T-cell populations (Fig. 1C–F). Again, glucose metabolism showed a higher level in restimulated CD4 T cells.

Mitochondrial activity in human CD4 and CD8 T cells

We analyzed mitochondrial function in the course of human CD4 and CD8 T-cell activation. Here we determined the expression
Figure 1. Kinetics of glucose metabolism in human CD4 and CD8 T cells. Human T cells were activated with anti-CD3/CD28 beads at a cell-to-bead ratio of 1:1. Samples were harvested at indicated time points. (A, B) The mRNA expression of glycolytic enzymes and glucose and lactate transporters was determined by RT-qPCR and normalized to 18S mRNA. Each symbol shows a different transcript, data are shown as mean ± SEM, n = 3 different donors. (C, D) Glucose consumption ([glucose] = [medium glucose] – [glucose in culture supernatant]) was determined upon activation as well as (E, F) lactate secretion by enzymatic assays in culture supernatants. Dotted black line shows medium lactate concentration due to serum addition. Data are shown as mean ± SEM, n = 10 different donors. (G, H) ECAR was determined with Seahorse XFp bioanalyzer in T-cell populations activated for indicated time points. Shown is the mean of n = 3 different donors ± SEM, each donor was analyzed in triplicates. *p < 0.05, **p < 0.01, ***p < 0.001 (A, B Student’s t-test, paired, two-tailed; C-H ANOVA and post-hoc by Bonferroni’s Multiple Comparison Test).

of two enzymes closely linked to mitochondrial activity, pyruvate dehydrogenase, recently linked to murine CD4 T-cell differentiation [23], and the citric acid cycle enzyme malate dehydrogenase. Respiration was analyzed applying the PreSens technology, allowing long-term monitoring of cellular respiration under cell culture conditions in nonfixed cells. In contrast to the significant increases observed for glycolytic enzymes, expression of PDH and MDH expression was not changed significantly (Fig. 2A and B). Cellular respiration was immediately elevated upon stimulation and remained high in contrast to the delayed onset of glycolysis.
Figure 2. Mitochondrial activity in human CD4 and CD8 T cells. T-cell populations were stimulated with anti-CD3/CD28 beads at a cell-to-bead ratio of 1:1. Expression of PDH and MDH mRNA was determined by RT-qPCR and normalized to 18S mRNA in (A) CD4 and (B) CD8 T cells. Each symbol shows a different transcript, data are shown as mean ± SEM, n = 3 different donors. (C, D) Cellular oxygen consumption was determined with the PreSens technology. Shown is the mean of oxygen consumption of n = 5 different donors for each condition. (E) Mitochondrial basic activity (ROUTINE) and capacity (ETS, determined by uncoupling with FCCP) were determined by high-resolution respirometry in restimulated T cells. O2 consumption related to ATP production (O2_ATP) was calculated as the difference between ROUTINE and LEAK respiration. Bars show the mean ± SEM, n = 5 different donors. **p < 0.01, ***p < 0.001 (Student’s t-test, paired, two-tailed).

(Fig. 2C and D). The same pattern was observed in restimulated T cells; however, respiration showed significantly lower values in CD8 T cells. Therefore, we analyzed mitochondrial respiratory function by high-resolution respirometry in restimulated T cells. CD8 T cells showed significantly decreased basic oxygen consumption (ROUTINE), capacity of the respiratory system (electron transfer system) and respiration related to ATP production (Fig. 2E). Taken together, mitochondrial function was lower in restimulated CD8 T cells. To gain insight whether reduction in mitochondrial oxygen consumption is related to a reduction in mitochondria content, activity of citrate synthase, a well-established marker for mitochondrial content was determined. No difference between CD4 and CD8 T cells was detectable (0.0092 ± 0.00075; 0.0084 ± 0.0037, respectively) suggesting differences in the amount of respiratory enzymes or in substrate flux between the two T-cell populations. In summary, beside a similar general metabolic pattern upon stimulation in CD4 and CD8 T cells, CD4 T cells were metabolically more active.

Metabolic inhibition in human CD4 and CD8 T cells

In the murine system, it has been demonstrated that T-cell function is closely related to glycolytic and mitochondrial activity. Therefore, we investigated the role of glucose metabolism and respiration for human T-cell function by applying inhibitors of glycolysis (2-DG) and respiration (oligomycin) in comparison to glucose deprivation.

Freshly isolated human CD4 and CD8 T cells were stimulated either in the presence of increasing concentrations of 2-DG and in comparison T cells were cultured in glucose-free medium. Both T-cell populations were very sensitive to 2-DG treatment, as
already 1 mM 2-DG resulted in a ~80% reduction in glucose consumption (data not shown) and a strong inhibition in lactate secretion (Fig. 3A). Concentrations of 5 and 10 mM 2-DG exerted an additional impact, resulting in a more than 95% inhibition in glycolytic activity comparable to that of glucose deprivation (Fig. 3A and B). During restimulation, 2-DG affected glucose metabolism significantly less compared to primary stimulation in CD4 T cells. Interestingly, mRNA levels of \(\text{LDHA, GLUT1 and MCT1}\) were not affected by glucose deprivation (Supporting Information Fig. 2).

To address the necessity of mitochondrial derived ATP for T-cell activation, oligomycin, an irreversible inhibitor of the mitochondrial ATP synthase, was applied. Addition of oligomycin resulted in a transient reduction in glucose consumption (data not shown) and lactate secretion in both T-cell populations during the first 72 h (Fig. 3C and D). After 5 days, the impact on lactate secretion was no longer detected (data not shown). Again, mRNA expression of \(\text{LDHA, GLUT1 and MCT1}\) was not affected by oligomycin treatment (Supporting Information Fig. 2).

To test whether oligomycin exerts a continuous impact, we applied oligomycin 24 and 48 h after activation (nominated oligo 24 and oligo 48, respectively). As evident from Figure 3C and D, delayed administration of oligomycin had either no or a smaller effect on lactate accumulation in the cell supernatant. No impact of oligomycin was observed during restimulation, indicating a particular role of OXPHOS in the transition from quiescent to effector T cells.

Impact of metabolic restriction on cell size, proliferation and viability

After establishing the parameters for metabolic restriction, we analyzed the impact of glycolytic and mitochondrial inhibition on T-cell function.

Upon primary stimulation, human CD4 and CD8 T cells increased cell size within the first 48 h, whereas T-cell proliferation started beyond 48 h (Supporting Information Fig. 3). The increase in cell size was significantly delayed by 2-DG as well as mitochondrial energy depletion, but not by glucose deprivation (Table 1). Notably, 2-DG had a more pronounced effect than glucose deprivation, although glycolytic reduction was comparable (Fig. 3A and B). Nevertheless, after 6 days, cell size was no longer affected by metabolic restriction (data not shown).

Proliferation strongly correlated with increased glucose metabolism. CD4 T cells proliferated more during the first...
Table 1. Impact of metabolic restriction on increase in cell size/"on-blast formation"

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<th>Quiescent</th>
<th>Stimulated</th>
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<tr>
<td></td>
<td>Untreated</td>
<td>1 mM 2-DG</td>
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<tr>
<td>CD4</td>
<td>7.2 ± 0.3</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td>CD8</td>
<td>7.2 ± 0.4</td>
<td>11.1 ± 0.6</td>
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Cell diameter [μM] was determined in T cells stimulated for 48 h by the CASY system. Data are shown as mean ± SD for n = 6 different donors for 2-DG treatment, n = 4 for w/o glucose, n = 5 for oligo, n = 3 for oligo 24 and oligo 48. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA and posthoc by Bonferroni’s Multiple Comparison Test).

72 h (Supporting Information Fig. 3), but after 6 days cell numbers were similar between both subsets (data not shown). Resimulated CD8 T cells showed significantly less active glycolysis than CD4 counterparts and proliferated less after 6 days (3.19 ± 1.23 × 10⁸ cells/mL and 4.385 ± 0.76 × 10⁸ cells/mL, p = 0.02, respectively). In both T-cell populations, glucose restriction and 2-DG treatment resulted in strong reduction in proliferation (Fig. 4A and B), however after 6 days, 1 mM 2-DG exerted only a slight impact (data not shown). During resimulation, T cells showed a reduced sensitivity to 2-DG as complete inhibition of proliferation was only found with 10 mM 2-DG.

Inhibition of mitochondrial ATP synthesis strongly compromised the onset of proliferation in stimulated T-cell populations (Fig. 4C and D). This initial reduction might be partially explained by the adverse effect of oligomycin on glucose metabolism. Hence, oligomycin exerted only a slight impact on glucose metabolism and reduced impact on the onset of proliferation in restimulated T cells (Fig. 4C and D). Oligomycin continued to significantly diminish proliferation to about 50% of controls during both stimulation periods (data not shown). Delayed administration of oligomycin 24 and 48 h after T-cell stimulation and restimulation, respectively, exerted no significant impact on proliferation.

Taken together, mitochondrial energy supply seems to be important for triggering the glycolytic machinery in the initial phase of stimulation but also contributes to the energy supply needed for proliferation.

Neither glycolytic nor mitochondrial restriction exerted a strong impact on cell viability. Maximum reduction of cell
viability of 20% was observed under glucose-deprived conditions and 10 mM 2-DG treatment after 6 days (Supporting Information Table 1).

**Impact of metabolic restriction on surface marker expression and cytokine secretion**

First, we measured the expression of activation-related surface molecules (Supporting Information Fig. 4A–D). Glucose deprivation and mitochondrial inhibition did not diminish CD25 (IL-2 receptor) expression; however, 2-DG exerted again a significant impact in CD4 T cells. Inhibition of mitochondrial ATP production lowered CD137 expression, yet exerted no negative impact on other surface molecules (Supporting Information Fig. 4A–D).

Next we investigated the impact of metabolic restriction on cytokine production, as particularly IFN-γ synthesis has been linked to glucose metabolism. We detected high expression of IFN-γ mRNA after 6 h of stimulation and restimulation (Fig. 5A and B). High concentrations of IFN-γ protein were measured in culture supernatants after 24 h of stimulation or restimulation, peaked at 48 h and decreased after 72 h (data not shown); hence 48 h values are shown (Fig. 5C and D).

Neither glucose deprivation nor mitochondrial inhibition blocked IFN-γ production in freshly stimulated T cells, both at the mRNA (Supporting Information Fig. 5) and protein level (Fig. 5C and D). However 2-DG significantly reduced IFN-γ production in stimulated CD4 and CD8 T-cell populations (Fig. 5C and D, respectively). Moreover, 2-DG induced a significantly persisting inhibitory effect on IFN-γ production in CD8 T cells and a clear trend was observed in CD4 T cells (Fig. 5E and F, 1 mM 2-DG).
Table 2. Impact of metabolic restriction on TNF production in the main related CD4 and CD8 T-cell subsets, naïve, effector memory (EM) and central memory (CM)

<table>
<thead>
<tr>
<th></th>
<th>untreated [pg/mL]</th>
<th>w/o glucose</th>
<th>1 mM 2-DG</th>
<th>5 mM 2-DG</th>
<th>oligo 2-DG</th>
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<tbody>
<tr>
<td>CD4</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Naïve I</td>
<td>728</td>
<td>2.3</td>
<td>0.86</td>
<td>0.91</td>
<td>1.23</td>
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<tr>
<td>Naïve II</td>
<td>242</td>
<td>1.30</td>
<td>0.78</td>
<td>0.72</td>
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<tr>
<td>EM I</td>
<td>9242</td>
<td>0.44</td>
<td>0.39</td>
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<td>0.73</td>
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<tr>
<td>EM II</td>
<td>3614</td>
<td>0.49</td>
<td>0.46</td>
<td>0.34</td>
<td>0.54</td>
</tr>
<tr>
<td>CM I</td>
<td>3532</td>
<td>2.05</td>
<td>1.00</td>
<td>0.92</td>
<td>1.06</td>
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<tr>
<td>CM II</td>
<td>2312</td>
<td>0.81</td>
<td>0.54</td>
<td>0.34</td>
<td>0.56</td>
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<tr>
<td>CD8</td>
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<tr>
<td>Naïve I</td>
<td>1576</td>
<td>0.38</td>
<td>0.07</td>
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<tr>
<td>Naïve II</td>
<td>123</td>
<td>0.44</td>
<td>0.21</td>
<td>0.34</td>
<td>0.36</td>
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<tr>
<td>EM I</td>
<td>3702</td>
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<td>0.50</td>
<td>0.35</td>
<td>0.53</td>
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<tr>
<td>EM II</td>
<td>487</td>
<td>0.73</td>
<td>0.64</td>
<td>0.59</td>
<td>0.69</td>
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</table>

Absolute values for TNF secretion of two different donors stimulated for 48 h without any treatment are shown. Impact of metabolic restriction on TNF production was calculated relative to untreated control cells.

$p = 0.053$; 5 mM 2-DG $p = 0.06$). However glucose metabolism and proliferation were completely restored when 2-DG pretreated T-cell cultures were washed and restimulated in the absence of 2-DG (Supporting Information Fig. 6A–D).

Next we determined the production of other cytokines under metabolic restriction and obtained similar results. 2-DG also diminished TNF secretion, IL-10 and IL-4 in stimulated T cells (Supporting Information Fig. 7A–D). In contrast, IL-2 production was not affected by 2-DG (Supporting Information Fig. 7E and F). Again an impact of glucose deprivation or mitochondrial inhibition was not detected on these cytokines. A strong impact of 2-DG but hardly no impact of glycolytic or mitochondrial restriction was observed also in restimulated T cells, with the only difference that IFN-γ production was 50% reduced by glucose deprivation in CD4 T cells (data not shown).

Different metabolic demands have been reported for CD4 and CD8 T-cell subsets. Therefore we measured cytokine production under the various treatments in the major CD4 and CD8 T-cell subsets of two donors. Despite differences in the total amount of cytokine production, metabolic restriction had comparable effects on cytokine production in bulk cultures and T-cell subsets (Fig. 5G and H, Supporting Information Fig. 8 and Table 2). Moreover, proliferation was comparably affected in T-cell bulk and related subsets (data not shown).

Figure 2C and D shows that mitochondrial respiration was immediately elevated upon stimulation. We analyzed cellular respiration of T cells subjected to glucose-free medium in comparison to 2-DG treatment. T cells were able to transiently increase respiration in the absence of glucose (Fig. 6A and B). However, 1 mM 2-DG blocked this compensatory mechanism (data not shown). Moreover, higher 2-DG concentrations exerted a concentration-dependent decrease in mitochondrial respiration (Fig. 6C and D). Glucose deprivation might be compensated by increased respiration, which is blocked by 2-DG. An inhibition of both pathways could result in severely compromised ATP levels thereby affecting cytokine production. Surprisingly, we detected even higher ATP

**Figure 6.** Mitochondrial respiration in glucose-free medium in comparison to 2-DG treatment. Cellular respiration was determined with the PreSens technology in freshly stimulated CD4 and CD8 T cells at a concentration of $2 \times 10^{6}$ cells/mL. (A, B) Human CD4 and CD8 T cells were cultivated in glucose-free medium (w/o glucose) or (C, D) with 2-DG in standard glucose medium. Data are shown as mean of $n = 6$ different donors for glucose deprivation, one representative experiment for 2-DG treatment.
levels in CD4 and CD8 T cells treated with 5 or 10 mM 2-DG (Table 3). Therefore we conclude, that a collapse in cellular ATP levels cannot explain the severe impact of 2-DG on cytokine production.

**Table 3. Impact of metabolic restriction on cellular ATP levels in stimulated CD4 and CD8 T cells**

<table>
<thead>
<tr>
<th></th>
<th>Quiescent</th>
<th>Stimulated</th>
<th>Untreated</th>
<th>w/o Glucose</th>
<th>1 mM 2-DG</th>
<th>5 mM 2-DG</th>
<th>10 mM 2-DG</th>
<th>oligo</th>
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<tr>
<td>CD4</td>
<td>462 ± 63</td>
<td>144 ± 43</td>
<td>111 ± 33</td>
<td>73 ± 25</td>
<td>210 ± 93</td>
<td>400 ± 167*</td>
<td>117 ± 24</td>
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<tr>
<td>CD8</td>
<td>144 ± 27</td>
<td>199 ± 76</td>
<td>66 ± 6’</td>
<td>63 ± 22’</td>
<td>175 ± 131</td>
<td>325 ± 212</td>
<td>83 ± 20</td>
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Data are shown as mean ± SD for n = 4 different donors. *p<0.01 (ANOVA and posthoc by Bonferroni’s Multiple Comparison Test).

Metabolic restriction in a mixed leukocyte reaction (MLR)

T-cell stimulation normally occurs in lymph nodes where T cells interact with antigen presenting cells and are primed for T-cell function. We aimed to confirm our results obtained in anti-CD3/CD28 stimulated T cells by applying a more physiologic stimulus. We activated freshly isolated CD4 T cells with mature dendritic cells in an allogeneic setting. We applied 1 and 5 mM 2-DG, as both concentrations led to a strong and significant reduction in glucose metabolism in anti-CD3/CD28 stimulated T cells. 2-DG and glucose deprivation inhibited glucose metabolism and we detected a reduction in glucose metabolism by oligomycin as observed in anti-CD3/CD28 stimulated T cells (Fig. 7A). In both stimulation protocols, glucose deprivation and inhibition of OXPHOS diminished proliferation, but did not reduce IFN-γ production and surface marker expression. However 2-DG reduced all these parameters (Fig. 7B–E). Viability was slightly affected by 5 mM 2-DG and glucose deprivation (Fig. 7F).

Discussion

T-cell activation leads to metabolic alterations, which have been intricately linked to T-cell function [5–9]. Upon immune stimulation, they undergo metabolic reprogramming to increase their rates of glycolysis and oxidative phosphorylation [8, 24]. Particularly elevated glucose metabolism has been related to cell growth, proliferation, viability and important effector functions such as IFN-γ production in the murine system [2, 6, 11]. Up to now only few studies investigated the metabolic requirements [17, 19, 20, 25] for human CD4 and CD8 T-cell activation and function in direct comparison and data on the long-term impact of metabolic restriction are sparse. We analyzed metabolic reprogramming upon stimulation and restimulation in human CD4 and CD8 T cells. Within the first 24 h, activation resulted in limited glycolytic activity; however, glucose metabolism was increased concomitantly with the onset of proliferation and maintained high throughout the whole stimulation period. In contrast, respiration was immediately turned on upon stimulation in both T-cell populations. Accelerated glycolytic activity was detected during restimulation as T cells were already primed for glucose metabolism. Despite this common pattern, our analysis also revealed that CD4 T cells show elevated glycolytic and mitochondrial activity and increased proliferative capacity compared to CD8 T cells. The opposite has been observed in the murine system [12].

Blocking mitochondrial ATP production delayed “on-blast” formation, the onset of glycolysis and proliferation and exerted an impact on the proliferative capacity. However the production of a variety of cytokines was not dependent on OXPHOS. Moreover, mitochondrial ATP was less important in the course of activation and during restimulation. Our data strongly indicate that oxidative phosphorylation is of special importance for the initial phase of activation. However, it has been shown in a murine model of graft versus host disease that unlike T cells activated in vitro, alloreactive T cells in vivo seem to heavily depend on OXPHOS for their ATP synthesis [26]. Furthermore, an upregulation of OXPHOS has also been demonstrated in autoimmune disease, which was normalized after glucocorticoid treatment [27].

Glucose deprivation resulted also in delayed “on-blast” formation and severely impaired proliferation. The production of a variety of cytokines was barely affected by glucose deprivation, which is in accordance to results recently published in human CD8 T cells [20], but seems to be different in the murine system. In our hands glucose deprivation resulted in a transient increase in mitochondrial respiration, indicating metabolic flexibility of human T-cell populations in the early phase of activation, which was also suggested for PMA/ionomycin stimulated human CD4 T cells and for murine CD4 T cells [11, 25]. Both glucose deprivation and 2-DG reduced glycolysis to a similar extent, however cytokine production was maintained under low glucose conditions, whereas treatment with 2-DG resulted in strongly diminished cytokine levels. This is in contrast to the findings in the murine system where 2-DG and glucose deprivation exerted similar suppressive effects [6]. Further analyses revealed that 2-DG inhibited not only glucose metabolism but also reduced mitochondrial respiration in human T cells. An effect of 2-DG on mitochondrial respiration has also been observed in rat adipocytes [14]. A likely explanation is that 2-DG releases HKII from the voltage-dependent anion channel, thereby affecting mitochondrial integrity and function [28, 29].

In line with this observation Trippacher reported that simultaneous inhibition of glucose metabolism and OXPHOS reduced the number of cytokine producing human CD4 T cells [19].
Figure 7. Impact of metabolic inhibition on T cells stimulated in an MLR. 10⁵ CD4 T cells were stimulated with 10⁴ mDCs over 7 days in the presence of 2-DG or oligomycin (oligo) in standard glucose medium or in glucose-free medium (w/o glucose). Samples were taken after 5 and 7 days. (A) Lactate secretion was measured in culture supernatant (lactate level in the culture medium is symbolized by the dotted black line), (B) cell number determined by cell counting and (C) cytokine production by ELISA. (D) Viability was determined by Annexin V/7-AAD staining. (E, F) The surface expression of CD25/CD95 was determined by antibody staining and subsequent analysis by flow cytometry, shown is the mean of the median fluorescence intensity (MFI). (A and B, D–F) Bars show the mean + SEM of n = 3 different donors for glucose deprivation, n = 4 different donors for 2-DG treatment and n = 4 different donors for oligomycin treatment. (C) Each dot represents an individual donor. *p < 0.05, **p < 0.01, ***p < 0.001 show significant differences between control and treatment (ANOVA and posthoc by Bonferroni’s Multiple Comparison Test).

Furthermore, a block in cytolytic activity of murine CD8 T cells was described when combining glycolytic and mitochondrial inhibition [30]. We also observed a blocked IFN-γ production when T cells were treated with oligomycin in glucose-free medium (unpublished data). However, severely compromised ATP levels were not detected under either treatment and 2-DG even increased ATP levels. Probably this is due to a block in ATP consumption as a result of decreased T-cell activation.

In the murine system, low concentrations of 2-DG resulted in a shift toward a more memory-like phenotype [31]. In our hands 2-DG affected cytokine production in memory and naïve CD4 and CD8 T cells, proliferation was also reduced to similar levels (unpublished data). Moreover, 2DG treatment caused a dramatic decrease in CD25 and CD95 expression. Taken together our data rather indicate that 2DG but not low glucose is blocking T-cell activation and that this is the reason for diminished T-cell effector functions. A possible impact on differentiation cannot be excluded.

Furthermore, the impact of 2-DG on IFN-γ secretion was only partially reversible, as IFN-γ secretion remained diminished even after termination of 2-DG treatment and full restoration of glucose metabolism. These results further underline that 2-DG exerts other effects beside glycolytic restriction.

The physiologic relevance of our results is supported by the fact, that data obtained in anti-CD3/CD28 stimulated T cells were fully reproduced when T cells were stimulated with mDCs in an allogeneic setting. In this setting, we cannot exclude that 2-DG exerts additional effects on DCs. However again, glycolytic restriction might not be the underlying mechanism, since we did not
observe an impact on cytokine production and surface marker expression by glucose deprivation.

Taken together, effector functions in human T-cell populations are hardly affected under conditions of restricted glycolytic or mitochondrial activity, but suppression of both pathways results in severely compromised T-cell function. This is of physiologic relevance as limited oxygen tension and nutrient availability occur also in vivo in inflamed tissues or in the tumor microenvironment. Viability of anti-CD3/CD28 stimulated T cells was maintained under complete metabolic restriction. In contrast to PMA/ionomycin stimulated human CD4 T cells which undergo apoptosis under similar metabolic conditions [25]. This might be explained by immediate and stronger glycolytic activity of PMA/ionomycin stimulated T cells (Supporting Information Fig. 1). Analysis of the relation between metabolism and human T-cell function in comparison to the murine system is of consequence for the testing in mice of anti-metabolic drugs for the treatment of autoimmune and malignant diseases [32]. The application of anti-glycolytic drugs is an emerging strategy in cancer treatment, affecting tumor cells itself and simultaneously reducing the secretion of immunosuppressive metabolites such as lactic acid [33]. Based on our findings it seems conceivable to sustain T-cell effector function when targeting either glycolysis or respiration. However caution is advised when protocols are developed to inhibit both pathways simultaneously [34, 35], hence the sequential application of metabolic inhibitors could preserve T-cell function.

Materials and methods

T-cell isolation, stimulation and cultivation

The study was approved by the local ethical committee; all human participants gave written informed consent. Human T cells and monocytes were isolated from PBMCs of healthy donors. CD4 and CD8 subpopulations were enriched by Miltenyi (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) magnetic bead separation, achieving more than 98% purity. Naive, central memory and effector memory T cells were isolated by specific surface marker staining and cell sorting with the FACS Aria (details are given in the Supporting Information Fig. 9). Stimulation of T cells was performed either with anti-CD3/CD28 beads (Gibco, Life Technologies, Darmstadt, Germany) at a cell-to-bead ratio of 1:1 or with PMA (20 nM) and ionomycin (1 μM) or in mixed lymphocyte reaction (MLR) with allogeneic mature dendritic cells. T cells were cultured in RPMI 1640 medium supplemented with 10% human AB serum, 2 mM glutamine (PAN-Biotech GmbH, Aidenbach, Germany), essential and nonessential vitamins, pyruvate, β-mercaptoethanol, penicillin and streptomycin (all Gibco Life Technologies, Darmstadt, Germany) and 100 IU/mL rHL-2 (PeproTech, Hamburg, Germany) in a humidified atmosphere (5% CO2, 95% air) at 37°C (Heraeus Incubator). Cell number and cell size were determined using the CASY system (Roche Innovartis, Bielefeld, Germany).

Metabolic restriction

Glucose metabolism was inhibited by applying 2-DG or stimulation in glucose-free RPMI 1640 supplemented with all additives listed above including 10% AB serum, which resulted in a final concentration of 0.4 mM glucose in comparison to 10.4 mM in standard RPMI. Mitochondrial ATP production was inhibited by complex V inhibitor oligomycin (final concentration 5 μM). Stability over time was controlled in preliminary experiments revealing blocked mitochondrial respiration over 7 days.

Flow cytometry

T cells were stained with anti-CD137 (eBioscience, Frankfurt am Main, Germany), anti-CD25 and anti-CD95 (both BD Pharmingen, Heidelberg, Germany), quiescent cells were stained as negative controls. Apoptosis was determined by Annexin V/7-AAD staining (BD Pharmingen, Heidelberg, Germany). Flow cytometry was performed using BD FACS Calibur instrument (Becton Dickinson, San Jose, CA, USA), data were analyzed with the FlowJo software. Gating strategies are shown in the Supporting Information Figures 10 and 11.

Determination of cytokines

Cytokine secretion was determined in culture supernatants by commercially available enzyme-linked immunosorbent assays (ELISA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol.

Determination of glycolytic and mitochondrial activity

Medium glucose concentration was measured by a commercially available kit (R-Biopharm, Darmstadt, Germany). Lactate concentration was determined enzymatically using an ADVIA 1650 instrument (Bayer, Tarrytown, NY, USA) and specific reagents (Roche, Mannheim, Germany). Extracellular acidification rate (ECAR, μM/min) was measured in nonbuffered RPMI-1640 medium (Sigma Aldrich) using an XFp extracellular flux analyzer (Seahorse Bioscience, Billerica, MA, USA). CD4 and CD8 T cells were immobilized onto Seahorse cell plates (0.3 × 106 cells per well) coated with Corning® Cell-TakTM Cell and Tissue Adhesive referring to the manufacturer’s protocol. Glycolytic activity was determined as the conversion of [1,2-13C2]glucose (10 mM) to [2,3-13C]lactate in culture supernatants (see Supporting Information Data). Respiration was determined under cell culture conditions by the PreSens technology (PreSens Precision Sensing GmbH, Regensburg, Germany) and with the oxigraph O2-k (Orboros Instruments, Innsbruck, Austria). Detailed information is given in Supporting Information Data. Citrate synthase activity was determined as described [36].

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ATP measurement

Cellular ATP levels were determined by a commercial kit (ATP determination kit, Life Technologies) according to the manufacturer's protocol.

Reverse transcription–quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from T cells using RNeasy Spin Columns (Qiagen, Hilden, Germany). Reverse transcription was performed with 500 ng RNA in a total volume of 20 μL using M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). To quantify gene expression, the Mastercyler Ep Realplex (Eppendorf, Hamburg, Germany) was used. For RT-qPCR 1 μL cDNA, 1 μL of specific primers or 0.5 μL of 18S primers (10 μM) and 5 μL Quant iT Fast SYBR Green PCR Kit (Qiagen, Hilden, Germany) in a total of 10 μL were applied. Primer sequences are listed in Supporting Information Table 1. All data were normalized to 18S RNA, which was stably expressed.

Chemicals

Unless noted otherwise, all chemicals were purchased from Sigma (Deisenhofen, Germany).

Statistical analysis

Statistics were performed using the GraphPad 5 software. Significance was determined by ANOVA and posthoc by Bonferroni's Multiple Comparison Test as well as the Students t-test paired and two-tailed.

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References

Abbreviations: 2-DG: 2-deoxy-glucose · ECAR: extracellular acidification rate · ETS: electron transfer system · GAPDH: glyceraldehyde-3-phosphate dehydrogenase · GLUT: glucose transporter · HK: hexokinase · LDHA: lactate dehydrogenase A · MCT: monocarboxylate transporter · oligo: oligomycin · ROX: residual oxygen consumption · OXPHOS: oxidative phosphorylation

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