Glucose Oxidation Is Critical for CD4<sup>+</sup> T Cell Activation in a Mouse Model of Systemic Lupus Erythematosus

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Glucose Oxidation Is Critical for CD4⁺ T Cell Activation in a Mouse Model of Systemic Lupus Erythematosus

Yiming Yin,¹ Seung-Chul Choi,¹ Zhiwei Xu, Leilani Zeumer, Nathalie Kanda, Byron P. Croker, and Laurence Morel

We have previously shown that CD4⁺ T cells from B6.Sle1Sle2.Sle3 lupus mice and patients present a high cellular metabolism, and a treatment combining 2-deoxy-D-glucose, which inhibits glucose metabolism, and metformin, which inhibits oxygen consumption, normalized lupus T cell functions in vitro and reverted disease in mice. We obtained similar results with B6.Lpr mice, another model of lupus, and showed that a continuous treatment is required to maintain the beneficial effect of metabolic inhibitors. Further, we investigated the relative roles of glucose oxidation and pyruvate reduction into lactate in this process. Treatments of B6.Sle1Sle2.Sle3 mice with either 2-deoxy-D-glucose or metformin were sufficient to prevent autoimmune activation, whereas their combination was necessary to reverse the process. Treatment of B6.Sle1Sle2.Sle3 mice with dichloroacetate, an inhibitor of lactate production, failed to effectively prevent or reverse autoimmune pathology. In vitro, CD4⁺ T cell activation upregulated the expression of genes that favor oxidative phosphorylation. Blocking glucose oxidation inhibited both IFN-γ and IL-17 production, which could not be achieved by blocking pyruvate reduction. Overall, our data show that targeting glucose oxidation is required to prevent or reverse lupus development in mice, which cannot be achieved by simply targeting the pyruvate-lactate conversion. The Journal of Immunology, 2016, 196: 80–90.

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Abbreviations used in this article: ANA, anti-nuclear autoantibody; B6, C57BL/6J; DCA, dichloroacetate; 2DG, 2-deoxy-D-glucose; DN, double-negative; ECAR, extracellular acidification rate; GC, germinal center; GN, glomerulonephritis; Met, metformin; MFI, mean fluorescence intensity; MPC, mitochondrial pyruvate carrier; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; SLE, systemic lupus erythematosus; TC, B6.NZM.Sle1.Sle2.Sle3; Teff, effector T cell; Treg, regulatory T cell; TZD, thiazolidinedione.

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findings that Met decreases oxygen consumption in T cells (19). Met also normalized the excessive production of IFN-γ by CD4+ T cells from TC mice and SLE patients (19). This suggested a critical involvement of mitochondrial hyperactivity in lupus T cells. In support of this hypothesis, mitochondrial membrane hyperpolarization, an indication of high mitochondrial metabolism, was reported in T cells from SLE patients (22). In addition, increased pyruvate oxidation by CD4+ T cells has been demonstrated in mouse models of SLE (23).

In this article, we report that CD4+ T cells from B6.1pr mice, another model of spontaneous lupus, also have a high metabolism, and that Met+2DG was effective in reversing immune pathology as it did in TC mice. We also provide evidence for a critical role of mitochondrial metabolism and pyruvate oxidation in the regulation of TC CD4+ T cells. The selective inhibition of pyruvate conversion into lactate by dichloroacetate (DCA) was less effective than the inhibition of glucose metabolism by 2DG in either preventing or reverting disease in TC mice. Moreover, we showed that the in vitro activation of CD4+ T cells enhanced pyruvate oxidation in addition to lactate production. By using small-molecule inhibitors, we showed that pyruvate oxidation is important for IFN-γ production, whereas both pyruvate oxidation and its conversion into lactate are important for IL-17A production. Overall, these results provided new insights into how glucose metabolism regulates T cell function and autoimmunity, and revealed the importance of glucose oxidation in lupus development.

Materials and Methods

Mice and in vivo treatments

TC mice have been described previously (24). C57BL/6 (B6) and B6.MRL-Faslpr (B6.1pr) mice were originally purchased from The Jackson Laboratory. Only female mice were used in this study at the age indicated for each experiment. Treatment was performed with metabolic inhibitors (all from Sigma) dissolved in drinking water: Met (3 mg/ml), 2DG (5 mg/ml), DCA (2 mg/ml), or a combination of two of these drugs for the duration indicated for each study. For each treatment study, contemporaneous age-matched control mice received plain drinking water. Preventive treatments were performed in 2-mo-old mice, and reversal treatments were performed in mice at least 7 mo of age and all anti-dsDNA IgG positive for TC mice, and 4 mo of age for B6.1pr mice. Peripheral blood was collected to analyze autoantibody production; body weight and blood sugar levels were monitored weekly and biweekly, respectively. At the end of the treatment, spleens were collected for flow-cytometry and metabolic analysis of CD4+ T cells, and kidneys were evaluated for renal pathology. All experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Metabolic measurements

Splenocyte suspensions were enriched for CD4+ T cells by negative selection with magnetic beads (Miltenyi) yielding CD4+ cell population with a purity >90%. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using with a XF96 Extracellular Flux Analyzer under mitochondrial stress test conditions (Seahorse). Assay buffer was made of nonbuffered RPMI 1640 medium supplemented with 2.5 μM dextrose, 2 mM glutamine, and 1 mM sodium pyruvate (all from Sigma). Baseline ECAR and OCR values were averaged among at least four technical replicates per sample for the first three successive time intervals. In some assays, T cells were stimulated for 24 h with plate-bound anti-CD3e (2 μg/ml) and soluble anti-CD28 (1 μg/ml) in RPMI 1640 before analysis.

Flow cytometry

Single-cell suspensions were prepared from spleens using standard procedures. After RBC lysis, cells were blocked with anti-CD16/32 Ab (2.4G2) and stained in FACS staining buffer (2.5% FBS, 0.05% sodium azide in PBS). Fluorochrome-conjugated Abs used were to B220 (RA3-6B2), BCL6 (K112-91), CD4 (RAM4-5), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD95 (Jo2), CD122 (TM-b1), CD152 (UC10-4B9), CD154 (MR1), FOXP3 (FJK-16s), ICOS (15F9), IFN-γ (XMG1.2), IL-17A (TC11-18H10.1), Ly-77 (GL7), PD-1 (RMP-1-30), purchased from BD Biosciences, eBioscience, and BioLegend. Follicular T cells were stained as previously described (19) in a three-step process using purified CXCRC5 (2G8) followed by biotinylated anti-rat IgG (Jackson Immunoresearch) and PerCP5.5-labeled streptavidin in FACS staining buffer on ice. Dead cells were excluded with fixable viability dye (eFluor780; eBioscience). Data were collected on LSRFortessa (BD Biosciences) and analyzed with FlowJo (Tree Star) or FCS Express (DeNovo) software. IFN-γ and IL-17A production were analyzed in cells treated with the leukocyte activation mixture (BD Biosciences) for 5 h and the Fixation/Permeabilization kit (eBioscience).

Cell culture

Freshly isolated splenic CD4+ T cells (5 × 10^5 per well) were polarized for 3 d with plate-bound anti-CD3e (2 μg/ml) and soluble anti-CD28 (1 μg/ml) in RPMI 1640. Th1 polarization was performed by adding IL-12 (10 ng/ml) and anti–IL-4 (10 μg/ml), and Th17 polarization was performed by adding TGF-β (3 ng/ml), IL-6 (50 ng/ml), 6-Formylindolo (3,2-b) carbazole (300 nM; Enzo Life Sciences), anti–IL-4, and anti–IFN-γ (10 μg/ml each). Metabolic inhibitors (all from Sigma), Met (1 mM), 2DG (1 mM), DCA (10 mM), UK5099 (10 μM), or troglitazone (10 μM), were added to the cell cultures at the beginning of polarization. Gene expression for metabolic enzymes was measured by quantitative RT-PCR using Sybr Green incorporation as previously indicated (19).

Ab measurement

Serum anti-dsDNA and anti-chromatin IgG were measured by ELISA, and anti-nuclear autoantibodies (ANAs) were measured by immunofluorescence on Hep-2 cells as previously described (24). To normalize for interindividual variations in short-term treatments, we compared anti-dsDNA IgG levels for each mouse as percent change from the initial pretreatment value. ANA images were acquired with the same settings on an immunofluorescence microscope, and mean fluorescence intensity (MFI) was computed with ImageJ (http://imagej.nih.gov/ij/) after background subtraction.

Renal pathology

GN was scored semiquantitatively in a blinded fashion by a renal pathologist (B.P.C.) as previously described (25). In brief, the type of lesion was scored by order of increasing severity: none, mesangial matrix, mesangial cellular, and proliferative global GN; and the extent of the lesions was scored on a scale of 1 to 4. The glomerular deposition of C3 and IgG immune complexes was performed on frozen kidney sections as previously described (25). Glomerular size and the extent of C3 or IgG deposits were measured from sections averaging three to six glomeruli per sample, using Meta Morph 7.5 (BioImaging Solutions).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 6.0 software. Unless indicated, data were normally distributed, and graphs show means and SEM for each group. Results were compared with two-tailed t tests with a minimal level of significance set at p < 0.05. Bonferroni corrections were applied for multiple comparisons. Time-course results were compared by two-way ANOVA. Each in vitro experiment was performed at least twice with reproducible results.

Results

Met+2DG treatment decreased autoimmune pathology in B6.1pr mice

To address whether the combination of Met + 2DG was effective in other models of lupus, we selected B6.1pr mice, a simplified model driven by FAS deficiency and characterized by an expansion of CD4+ CD8– double-negative (DN) T cells. As TC T cells, CD4+ T cells from B6.1pr mice showed an elevated aerobic glycolysis as shown by ECAR (Fig. 1A) and OCR (Fig. 1B). Similar results were obtained after in vitro activation of CD4+ T cells from young mice with anti-CD3 and CD28 Abs (Fig. 1C, 1D), with B6.1pr CD4+ T cells maintaining a high spare respiratory capacity (Fig. 1E). The Met+2DG treatment for 7 wk of anti-dsDNA IgG2a mice significantly decreased CD4+ T cell ECAR (11.73 ± 0.91 versus 17.58 ± 1.24 μPm/min, p < 0.001) and OCR (74.19 ± 2.30 versus 103.6 ± 13.51 pmol/min, p < 0.05). The treatment also limited lymphoid expansion (Fig. 1F) and prevented serum anti-dsDNA IgG (Fig. 1G) and ANA (Fig. 1H) to increase. Renal pathology is very mild in B6.1pr mice; however, the Met+2DG treatment limited the extent of
IgG immune complexes deposited in the glomeruli (Fig. 1I) and limited glomerular expansion (Fig. 1J), a marker of lupus nephritis. The treatment did not affect the percentage of DN T cells (Fig. 1K), but it decreased the percentage of CD4+ T cells (10.98 ± 0.54 versus 15.27 ± 0.50%, p = 0.002), as well as their activation (Fig. 1L). Met+2DG did not affect the large percentage of CD44+CD62L− CD4+ effector memory T cells (TEMs), but expanded the percentage of naive CD44−CD62L+ T cells (Fig. 1M) and decreased the percentage of CXCR5+PD-1+BCL6+PD-1+PD-1+FOXP3+CD4+ follicular Th cells (Tfh) (Fig. 1N). Accordingly, the percentages of germinal center (GC) B cells and plasma cells (PCs) (O). *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 1.** Met+2DG treatment decreased autoimmune pathology in B6.lpr mice. Basal ECAR (A) and OCR (B) in CD4+ T cells from 6-mo-old mice (n = 4–10/strain). Basal activated ECAR (aECAR) (C), activated OCR (aOCR) (D), and activated spare respiratory capacity (aSRC) (E) in CD4+ T cells from 2-mo-old mice after 24 h activation with anti-CD3 and anti-CD28 Abs (n = 3/strain). (F–O) Four-month-old B6 mice were treated with Met+2DG for 7 wk (n = 5), when analysis was performed comparatively with age-matched controls (n = 4). (F) Splenocyte numbers. (G) Effect of treatment on serum anti-dsDNA IgG. (H) ANA levels measured as MFI in terminal sera. (I) Percentage of glomerular area with IgG deposits. (J) Glomerular surface area measured in arbitrary units (AU). Percentage of CD4+CD8− DN T cells (K), CD69+ (L), CD44+CD62L− Tfh (M), CD4+ T cells and percentage of CD95+GL7+GC B220+ B cells and plasma cells (PCs) (O). *p < 0.05, **p < 0.01, ***p < 0.001.

Cessation of Met+2DG treatment resulted in flares

We have shown that a 5-h in vitro treatment with Met was sufficient to reduce IFN-γ production (19), suggesting that T cells respond rapidly to metabolism alterations. It is unknown, however, whether the in vivo Met+2DG treatment reset the immune system or, to the contrary, whether a continuous treatment is required to maintain therapeutic benefits. To address this question, we performed a reversal Met+2DG treatment for 8 wk; then the treated mice were split into three cohorts: same Met+2DG treatment or plain water for 2 or 6 additional weeks, and compared with age-matched mice that were never treated. As previously reported (19), the Met+2DG treatment decreased splenomegaly (Fig. 2A), anti-dsDNA IgG (Fig. 2B), renal pathology (Fig. 2C), CD4+ T cell activation (Fig. 2D, 2E), Tfh (Fig. 2F), and GC B cell expansion (Fig. 2G). However, the therapeutic effects disappeared after the treatment was stopped for as little as 2 wk (Fig. 2), showing that CD4+ T cells required continuous exposure to Met+2DG to downregulate their activation and effector differentiation. We tested this hypothesis in vitro by comparing the continuous effect of Met for 6 d with only for the first 3 d on Th1 polarization (Fig. 2H). The removal of Met in the last 3 d resulted in a percentage of CD4+ T cells expressing IFN-γ and a level of expression that were equivalent to that of cells that were never treated with Met. These values were
significantly higher than that of cells maintained on Met for 6 d in both strains. Overall, these results indicate that continuous inhibition of T cell metabolism is required to maintain the anti-inflammatory effect.

**Monotherapy with either Met or 2DG prevented disease development**

We have shown that monotherapy with either Met or 2DG did not reverse disease in TC mice (19). To assess whether any of these drugs was able to prevent autoimmune activation and pathology to develop, we treated TC mice and B6 controls starting at 8 wk of age. Met treatment for 22 wk resulted in a greater body weight gain in TC mice (Fig. 3A), but had no effect in B6 (data not shown), suggesting that the weight gain in Met-treated mice may be associated with a decreased morbidity. Accordingly, Met reduced splenomegaly (calculated as spleen/body weight ratio to account for the difference in body weight; Fig. 3B). The production of anti-dsDNA (Fig. 3C) and anti-chromatin (Fig. 3D) IgG remained below pretreatment levels until the last 2 wk of treatment. Terminal serum ANAs were also significantly decreased by the treatment (Fig. 3E). CD4+ T cell activation (Fig. 3F, 3G) and TFH expansion (Fig. 3H) were reduced by the Met treatment, and there was a trend for a reduction of GC B cells (Fig. 3I). The severity of renal pathology was also significantly reduced with a shift to mesangial from proliferative GN (Fig. 3J). Similarly, we treated 8-wk-old TC mice with 2DG for 16 wk and observed a

**FIGURE 2.** Cessation of Met+2DG treatment resulted in flares. Eight-month-old TC mice were treated with Met+2DG for 8 wk; then mice were maintained on treatment (Met+2DG) or switched to plain water for an additional 2 or 6 wk (Met+2DG 2 and Met+2DG 6). Untreated TC and B6 mice (Ctrl) were analyzed at the same time. (A) Splenocyte numbers. (B) Changes in individual serum anti-dsDNA IgG levels in mice in which treatment ceased for 2 wk as compared with untreated mice. (C) GN score distribution: P4, proliferative GN score 4; Mc3-4, mesangial cellular scores 3 and 4 (grouped Met+2DG 2 and Met+2DG 6). Percentages of CD69+ (D), CD44+ CD62L− TEM (E), and TFH (F) CD4+ T cells. (G) Percentages of B220+ B cells with a GC phenotype. n = 4–8/group. (H) B6 and TC CD4+ T cells were polarized in vitro with Met for 3 d, then washed and split into two fractions cultured in the same Th1 conditions with (black) or without (hatched) Met for an additional 3 d. Their percentage of IFN-γ+ CD4+ T cells, as well as the level of MFI of the IFN-γ+ production, was compared with that of cells polarized for 6 d without Met (white). Representative FACS overlays are shown on the right for each strain. n = 3/strain. *p < 0.05, **p < 0.01, ***p < 0.001.
modest but significant weight gain (Fig. 4A), as well as a reduced lymphoid expansion in the spleen (Fig. 4B) of treated mice. Serum anti-dsDNA IgG remained low in treated mice and significantly lower than in control mice (Fig. 4C, 4D). Similarly, terminal serum ANAs were decreased by the treatment (Fig. 4E). Finally, CD4+ T cell activation (Fig. 4F, 4G), TFH and GC B cell expansion (Fig. 4H, 4I), and the severity of renal pathology (Fig. 4J) were all reduced by 2DG. These results showed that either Met or a global reduction of glucose metabolism was sufficient to prevent autoimmune activation and pathology in TC mice.

Inhibition of pyruvate reduction does not prevent or reverse lupus

To determine whether the reduction of pyruvate to lactate was the critical branch of glucose metabolism in TC T cells, we used DCA, an inhibitor of PDK1, which inhibits pyruvate dehydrogenase...
(PDH) and pyruvate oxidation (26). The resulting effect of DCA is to favor the oxidation of pyruvate to the expense of its conversion to lactate (27, 28). DCA globally decreased CD4+ T cell metabolism with a greater effect on glycolysis than OXPHOS (Fig. 5A, 5B), leading to a lower ECAR/OCR ratio (Fig. 5C). Eight-week-old TC mice were treated with DCA or 2DG for 16 wk and compared with age-matched controls. As expected, 2DG reduced both ECAR and OCR in the CD4+ T cells of treated mice, but no difference was observed for DCA-treated mice (Fig. 5D, 5E). This preventive DCA treatment had no significant effect on lymphoid expansion (Fig. 5F), anti-dsDNA IgG production (Fig. 5G), CD4+ T cell activation and differentiation (Fig. 5H–J), as well as GC B cell induction (Fig. 5K). Finally, DCA had no significant effect on the expression of key T cell activation markers as compared with 2DG (Fig. 5L–O).

We further evaluated the effect of DCA treatment on disease reversal. Eight-month-old TC mice were treated with DCA or Met for 2 mo and compared with age-matched controls. In older mice, the DCA treatment significantly reduced aerobic glycolysis while...
increasing OXPHOS, leading to a shift toward mitochondrial metabolism in CD4+ T cells (Fig. 6A). In these mice, DCA resulted in body weight loss (Fig. 6B) and lymphoid expansion (Fig. 6C). DCA treatment had either no effect or increased autoantibody levels (Fig. 6D–F), and had no effect on CD4+ T cell activation and differentiation (Fig. 6G–J) or GC B cell expansion (Fig. 6K). DCA also did not change the expression of surface markers on CD4+ T cells (Fig. 6L–O) and renal pathology (Fig. 6P). Finally, we compared the combination of Met+DCA with the Met+2DG treatment to assess whether the effective branch of glucose metabolism blocked by 2DG was aerobic glycolysis (Supplemental Fig. 1). The Met+DCA treatment, however, did not change CD4+ T cell metabolism (Supplemental Fig. 1A, 1B), most likely due to the opposite effects of the two drugs on glucose metabolism. There was only a significant decrease in the expansion of TEm (Supplemental Fig. 1H), but all the other biomarkers, including renal pathology, were

**FIGURE 6.** DCA treatment did not reverse disease. Eight-month-old TC mice were treated with DCA or Met for 8 wk, when analysis was performed. (A) Effect of treatment on CD4+ T basal ECAR, basal OCR, basal ECAR/OCR ratio, and mitochondrial test OCR plots compared by two-way ANOVA. (B) Body weight change during treatment. (C) Splenocytes numbers. Serum anti-dsDNA (D) and anti-chromatin (E) IgG for individual mice with initial (I) and terminal (T) values for each treatment. (F) ANA levels measured as MFI in terminal sera. Percentage of CD69+ (G), TEM (H), Tfh (I), and Treg (J) CD4+ T cells. (K) Percentages of GC B220+ B cells. Expression levels of surface markers on total CD4+ T cells: PD-1 (L), CD40L (M), CTLA-4 (N), and CD95 (O). (P) GN score distribution: Pg4, proliferative GN score 4; Mc4, mesangial cellular score 4; Mm4, mesangial matrix score 4, n = 10/group, n = 5–7/group. *p < 0.05, **p < 0.01, ***p < 0.001.
unchanged. Overall, these results showed that the inhibition of pyruvate reduction to lactate by DCA, even in combination with Met, is not effective in preventing or reverting autoimmune pathology in TC mice, suggesting that lactate production is not the critical glycolytic pathway in the CD4⁺ T cells of TC mice.

**CD4⁺ T cell activation increases pyruvate oxidation**

The results from in vivo treatments suggested that OXPHOS is an important pathway relative to pyruvate reduction to lactate for CD4⁺ T cell activation in the TC model of lupus. Consistent with this hypothesis, thiazolidinediones (TZDs) reduced T cell activation, ANA production, and renal pathology in SLE (29, 30), which has been attributed to their peroxisome proliferator-activated receptor γ agonist activity (22). However, TZDs have been recently shown to be acute MPC inhibitors, effectively shutting down pyruvate oxidation (31, 32). Interestingly, pioglitazone (a TZD) normalized IFN-γ, but not IL-17, production in T cells from lupus patients (29), suggesting a different requirement of pyruvate ox-

**FIGURE 7.** CD4⁺ T cell activation increased pyruvate oxidation. (A) B6 CD4⁺ T cells were activated with anti-CD3 and anti-CD28 (Th0) for 1 or 3 d, after which metabolic gene expression was compared with unstimulated (N) cells. For clarity, significance values of the 3-d time point were omitted when the difference for d1 was significant. (B) B6 (white bars) and TC (black bars) CD4⁺ T cells were activated (Th0) or polarized into Th1, Th17, and Treg conditions for 3 d. Metabolic gene expression was compared with Th0 values. Brackets with horizontal bases indicate significant values for both B6 and TC values. Otherwise, differences were significant for B6 values only (Pdk1, Gls2) or TC values only (Cpt1a). In addition, significant differences between strains for each subset are indicated with horizontal lines. Gene expression in unstimulated (N) cells is shown as reference. Gene expression was normalized to Ppia, then to the mean value for N B6 cells. n = 6/strain. *p < 0.05, **p < 0.01, ***p < 0.001. RQ, relative quantity.
dation in these two T cell subsets. To further test how pyruvate utilization regulates CD4+ T cells function, we compared the expression of key metabolic genes between in vitro–activated and untouched naive CD4+ B6 T cells (Fig. 7A). Anti-CD3/CD28 activation significantly upregulated \( \text{Hk2} \) expression, confirming that T cell activation increases glucose metabolism. Activated CD4+ T cells also showed a higher expression of lactate dehydrogenase A (\( \text{Ldha} \)) and lactate transporter MCT4 (\( \text{Slc16a3} \)), confirming an increased conversion of pyruvate into lactate. Importantly, we also observed a significant downregulation of \( \text{Pdk1} \) levels, indicating an increased pyruvate oxidation. Anti-CD3/CD28 activation also showed a higher expression of lactate dehydrogenase A (\( \text{Ldh} \)) and lactate transporter MCT4 (\( \text{Slc16a3} \)), confirming an increased conversion of pyruvate into lactate. Importantly, we also observed a significant downregulation of \( \text{Pdk1} \) levels, indicating an increased pyruvate oxidation. Anti-CD3/CD28 activation also strongly upregulated \( \text{Gls2} \) and \( \text{Odc} \), confirming the upregulation of glutamine metabolism in activated CD4+ T cells (15). In contrast, \( \text{Cpt1a} \) levels were downregulated, indicating a decreased fatty acid oxidation. Thus, these results showed that in vitro CD4+ T cell activation increased glutamine and glucose metabolism, with both pyruvate oxidation and reduction into lactate. To investigate whether CD4+ T cell activation depends on pyruvate oxidation under different cytokine environments, we measured the expression of the same metabolic genes after Th1, Th17, and Treg polarization in both B6 and TC CD4+ T cells (Fig. 7B). Metabolic enzyme expression was globally similar between the two strains in all three polarized subsets, except for \( \text{Slc16a3}, \text{Pdk1}, \) and \( \text{Gls2} \), which were significantly lower in polarized TC than B6 T cells. These results support a skewing toward pyruvate oxidation in TC T cells. Importantly, all activated and polarized subsets presented a significantly lower \( \text{Pdk1} \) expression, as well as a higher \( \text{Gls2} \) and \( \text{Odc} \) expression compared with naive cells (Fig. 7B). This suggested a higher involvement of both glucose and glutamine oxidation in all subsets. Therefore, increased substrate oxidation is a general feature of T cell activation in vitro, regardless of polarizing condition.

Finally, we assessed the requirement of pyruvate oxidation for IFN-\( \gamma \) and IL-17A production using in vitro polarization in the presence of pharmacological inhibitors. In addition to DCA, we used two MPC inhibitors, UK5099 and troglitazone (a TZD), that are expected to have an effect opposite to DCA, that is, blocking pyruvate oxidation versus blocking lactate production. Consistent with a previous report (26), DCA inhibited IL-17A production (Fig. 8A, 8B) and increased Foxp3 expression (data not shown) in Th17-polarized B6 T cells. The same result was obtained with TC T cells, although IL-17A production remained higher than in B6 T cells. In addition, TZD, UK5099, and Met significantly inhibited IL-17A production in these conditions (Fig. 8A, 8B). These data indicate that disruption of either lactate production or OXPHOS, including pyruvate oxidation, impairs IL-17A production. In contrast, DCA significantly enhanced the percentage of CD4+ T cells producing IFN-\( \gamma \) (Fig. 8A, 8C), as well as the amount of...

**FIGURE 8.** DCA enhanced Th1 polarization. CD4+ T cells from 2-mo-old TC and B6 mice were polarized in Th1 or Th17 conditions in the presence of DCA, TZD, UK5099 (UK), or Met for 3 d, after which intracellular IL-17A or IFN-\( \gamma \) was compared with untreated controls (Ctrl). (**A**) Representative IFN-\( \gamma \) and IL-17A production in TC CD4+ T cells, Ctrl, and treated with DCA or TZD. (**B**) Percentage of IL-17A+ CD4+ T cells. (**C**) Percentage of IFN-\( \gamma \)-producing CD4+ T cells. (**D**) Representative IFN-\( \gamma \) staining in TC CD4+ T cells treated with DCA (solid line), TZD (broken line), and Ctrl (filled histogram). The IFN-\( \gamma \) gate is shown. (**E**) MFI of the IFN-\( \gamma \)-producing population as shown in (**D**) for DCA-treated and Ctrl CD4+ T cells. (**B** and **C**) Comparisons were made to the Ctrl samples in each strain. (**E**) Statistical significance is shown for paired t tests between each treated and Ctrl sample. \( n = 6/\text{strain}. \) *\( p < 0.05, ** p < 0.01, *** p < 0.001. \)
Multiple studies have also shown that CD4+ T cell activation inhibition of MPC targets both Th1 and Th17 cells. These results have different requirements on glucose metabolism. Inhibition of also relies on lactate production. Therefore, Th1 and Th17 cells have different requirements on glucose metabolism. Inhibition of glucose reduction by DCA selectively targets Th17, whereas inhibition of MPC targets both Th1 and Th17 cells. These results also explain why DCA was not as effective as 2DG in the in vivo treatments.

Discussion

In this study, we first showed that CD4+ T cell metabolism was elevated in B6.lpr mice, another model of lupus with a different cause than the TC model, which includes a strong contribution of Th17 cells (33). Furthermore, the same Met+2DG treatment was effective in suppressing autoimmune manifestations in B6.lpr mice, although neither the effector memory nor DN T cells were affected. The treatment was very effective in reducing the number and percentage of Treg in both strains. Treg have been identified as a strong disease biomarker in human SLE (34, 35), and their functional metabolism depends on both glucose metabolism and OXPHOS (36). The effectiveness of Met+2DG in the two lupus models may be linked to the targeting of this T cell subset. Nonetheless, our results showed that T cell metabolic defects in SLE are not model-dependent and suggest that treatments with metabolic inhibitors may be beneficial in patients with a range of etiologies. We also showed that the therapeutic benefit of Met+2DG rapidly disappears when treatment is stopped. We have previously shown that a 5h in vitro treatment with Met is sufficient to inhibit IFN-γ production by CD4+ T cells (19). This and our new in vivo results demonstrated that the activation of lupus T cells is tightly controlled by their energy state, and removal of metabolic blocks quickly leads to reactivation and differentiation into effector subsets. Our results also showed that treatment with metabolic inhibitors do not reset the immune system, which has consequences for future translational studies.

Activation of CD4+ T cells increases both lactate production and OXPHOS (15, 18). A number of studies have shown that the differentiation into inflammatory effector CD4+ T cells, especially Th1 and Th17 cell subsets, relies on aerobic glycolysis (11, 37). Multiple studies have also shown that CD4+ T cell activation requires mitochondrial metabolism, specifically OXPHOS and aerobic oxygen species production (18, 38). The relative contribution of these metabolic pathways to lupus pathogenesis has both mechanistic and translational implications. Indeed, most of the studies regarding the metabolic requirements of effector CD4+ T cells have been conducted with either in vitro studies or with short-term acute autoimmune inductions such as the EAE model. Contrary to what we have reported in disease reversal studies (19), monotherapy with either 2DG or Met effectively prevented or significantly delayed disease in TC mice. This indicates that CD4+ T cells that have been chronically activated by autoantigens require the inhibition of both glucose metabolism and mitochondrial oxidation to be normalized, but this activation can be prevented by targeting either pathway. The fact that autoimmune activation and pathology were prevented by Met suggests that the conversion of pyruvate to lactate, which is not targeted by Met (19), is not critical in this process. CD4+ T cell activation requires only OXPHOS, but once activated, CD4+ T cells can use either OXPHOS or aerobic glycolysis (39). In light of these findings, our results indicate that the activation of autoimmune TC CD4+ T cells can be prevented by blocking glucose OXPHOS with either 2DG or Met. Then both 2DG and Met are required to normalize chronically activated CD4+ T cells to inhibit both aerobic glycolysis and OXPHOS. The lack of efficacy of Met+DCA treatment comparatively with Met+2DG suggests that the first 10 steps of glycolysis (i.e., conversion of glucose to pyruvate) are important for the activation of TC T cells. This is most likely due to the production of NADPH through the pentose phosphate pathway and the synthesis of amino acids and nucleotides from glycerol-dehyde 3-phosphate, two key pathways for macromolecule synthesis that are mobilized in activated T cells (40). In addition, other sources of OXPHOS that are blocked by Met, but not by 2DG, may be involved. Anaerobic glucose metabolism is critical for CD4+ T cell activation, both in vitro (15) and in vivo (41). It is therefore likely that glatiramer also contributes to the activation of lupus T cells, which should be tested in future reversal studies combining glucose and glutamine inhibitors.

Our in vitro studies have specifically compared pyruvate oxidation and reduction in CD4+ T cell activation and polarization into Th1 and Th17 subsets. After activation, CD4+ T cells up-regulated Hk2, Idh2, and Slc16a3, but downregulated Pdk1, indicating that both lactate production and pyruvate oxidation were enhanced after CD4+ T cell activation. Th1 and Th17 polarization were associated with the downregulation of Pdk1 and upregulation of Gls2 and Odc, indicating the prevalence of mitochondrial metabolism fueled by pyruvate and glucose for IFN-γ and IL-17A production. These results were confirmed with metabolic inhibitors probing pyruvate utilization, showing that both IFN-γ and IL-17A were targeted by MPC inhibitors. IL-17A is, however, different from IFN-γ in that it also requires pyruvate reduction. Overall, our results combining gene expression and metabolic inhibitors suggest that pyruvate oxidation is critical for T cell activation and inflammatory cytokine production. A previous study has found that neither ATP production nor a functioning electron transport chain was required for IFN-γ production (39), but the inhibitors were added to cells already polarized, when we have shown that Met was less effective (and therefore mitochondrial oxidation was less required) than when polarization is initiated (19). A recent proteomic study of Th1-polarized human CD4+ T cells showed a drastic upregulation of pyruvate oxidation and TCA cycle utilization (42), confirming our results showing a strong involvement of mitochondrial oxidation in IFN-γ production. Our in vitro results suggest that both glucose and glutamine are involved, but their respective contribution to the spontaneous chronic activation of lupus T cells remains to be determined.

IFN-γ is the dominant cytokine produced by CD4+ T cells in the TC mouse model of lupus (19). The enhancement of IFN-γ production by DCA is likely a major reason why treatment with this metabolic inhibitor was not effective as 2DG in this model. Other models of lupus are dominated by other types of effector T cells. Th17 cells play a dominant role in B6.lpr mice (33), and therefore DCA may be more effective, although we have shown that the combination of Met and 2DG is effective at suppressing autoimmune production and preventing the development of early renal pathology. The BXSB.Yaa model is dominated by Treg cells (43, 44) and type I IFN (45). Therefore, it will be very informative to assess the efficacy of metabolic inhibitors in these other models of SLE.

In summary, we provide evidence that glucose oxidation plays a significant role in the activation of CD4+ T cells in the TC mouse model of lupus, and the inhibition of this metabolic pathway is critical to normalize their function and the associated autoimmune pathology. This is similar to the graft-versus-host disease model, in which T cells chronically activated by autoantigens increased both aerobic glycolysis and OXPHOS, but an ATP synthase inhibitor was sufficient to block graft-versus-host disease (46). We propose
that mitochondrial oxidation of glucose, and potentially glutamine, is critical to sustain T cells chronically activated by autoreactive T cells.

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Disclosures

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