In human alloreactive CD4+ T-cells, dichloroacetate inhibits aerobic glycolysis, induces apoptosis and favors differentiation towards the regulatory T-cell subset instead of effector T-cell subsets

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Abstract. Although kidney transplantation is the best therapy for end-stage renal disease, rejection remains a concern, and currently available immunosuppressive agents contribute to morbidity and mortality. Thus, novel immunosuppressive drugs are required. Dichloroacetate (DCA) is already used in the treatment of congenital lactic acidosis and characterized by limited toxicity. As DCA inhibits aerobic glycolysis, which is a prerequisite for CD4+ T-cell proliferation and differentiation into effector T-cells, its possible immunosuppressive role in mixed lymphocyte reaction (MLR), a model of alloreactivity, was investigated. Glucose and lactate concentrations were measured in the supernatants, and cell proliferation was assessed immunoenzymatically. CD4+ T-cells were then isolated from the MLRs and the expression of cleaved caspase-3, various enzymes involved in glycolysis, and the signature transcription factors of CD4+ T-cell subsets were evaluated by western blotting. In MLRs, DCA decreased glucose consumption and aerobic glycolysis, while it exerted a negligible effect on cell proliferation. In CD4+ T-cells, DCA induced apoptosis, and decreased the expression of glucose trasporter-1, hexokinase II, lactate dehydrogenase-A and phosphorylated pyruvate dehydrogenase, while it increased total pyruvate dehydrogenase. In addition, DCA increased the expression of transcription factor forkhead box P3, whereas it decreased the expression of T-box transcription factor TBX21, trans-acting T-cell-specific transcription factor GATA-3 and retinoic acid receptor related orphan receptor-γt. In conclusion, in alloreactive CD4+ T-cells, DCA inhibits aerobic glycolysis, induces apoptosis and favors differentiation towards the regulatory T-cell subset. These characteristics render it a promising immunosuppressive agent in the field of transplantation.

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Introduction

Kidney transplantation is the best therapeutic option for patients suffering from end-stage renal disease (1,2). However, following transplantation these patients are on a life-long immunosuppressive regiment consisting of a combination of corticosteroids, calcineurin inhibitors, mammalian target of rapamycin inhibitors, antimetabolites and, more recently, co-stimulation blockers. However, the agents that are currently used are associated with certain toxicities resulting in increased morbidity and mortality, for example cardiovascular toxicity, infection and malignancy. In addition, calcineurin and mammalian target of rapamycin inhibitors are associated with graft injury (3). Thus, there is a requirement for novel immunosuppressive agents that are able to diminish the immune alloreactive response.

Substances able to interfere with T-cell metabolism are candidates for immunosupressive agent. During T-cell activation, rapidly proliferating T-cells reprogram their metabolic pathways from pyruvate oxidation via the Krebs' cycle to the glycolytic, pentose-phosphate, and glutaminolytic pathways in order to fulfill the bioenergetic and biosynthetic demands of proliferation (4). It has also been confirmed that apart from clonal expansion, aerobic glycolysis is a prerequisite for T-cell differentiation into effector cell lineages (5). For instance, CD4+ effector T-cells (Teff) express high levels of the glucose transporter GLUT1 and are reliant on glucose metabolism, whereas regulatory T-cells (Tregs) express low levels of GLUT1 and are reliant on lipid oxidation (6). Notably, the key immunomodulatory enzyme indoleamine 2,3-dioxyganase exerts its immunosuppressive effects at least in part by affecting glucose metabolism in T-cells (7,8).

Dichloroacetate (DCA) activates pyruvate dehydrogenase (PDH) through inhibition of the mitochondrial enzyme pyruvate dehydrogenase kinase (PDK). Since PDH converts pyruvate to acetyl-CoA, DCA upregulates the influx of pyruvate into the mitochondria, increasing the ratio of glucose oxidation to

glycolysis (9). It is expected that by inhibiting aerobic glycolysis, DCA may suppress T-cell activation. In support of the above hypothesis, DCA was shown to alleviate the development of collagen-induced arthritis, a T-cell-mediated disease, in an animal study (10). Additionally, in $CD4^+$ T-cells derived from healthy individuals and stimulated by anti-CD3/CD28, DCA inhibited proliferation, increased interleukin (IL)-10 production and decreased IL-17 production, albeit at the high concentration of 20 mM. However, Treg cell differentiation was initially observed at a DCA concentration of 2 mM (11).

Compared with other potential immunosuppressive agents, an advantage of DCA is that it has already been used for the treatment of patients suffering from congenital lactic acidosis. Long-term administration of DCA in these patients has an acceptable toxicity, with the main side effect being reversible peripheral neuropathy, while no increased incidence of cardiovascular disease, infection or malignancy has yet been reported (9,12,13). Notably, regarding malignancy, DCA is currently under investigation as a treatment for cancer, since numerous types of cancer rely on aerobic glycolysis (14). This safety profile of DCA suggests that it is a good candidate for clinical trials in the field of transplantation, where the existence of potent and effective immunosuppressive agents with well-characterized toxicities renders the introduction of novel substances with unknown toxicity a challenge.

In this study, the effect of DCA on GLUT1 and certain enzymes involved in glycolysis, as well as on the expression of the signature transcription factors of the Teff Th1, Th2 and Th17 subsets and of the Treg cells, was evaluated. The two-way mixed lymphocyte reaction (MLR) was used as a model of alloreactivity (15). A DCA concentration of 1 mM was selected as it is close to the serum concentration achieved in patients with congenital lactic acidosis (9,13), and it has been shown not to be toxic for human peripheral blood mononuclear cells (PBMCs) (16).

Materials and methods

Subjects. Blood samples were collected from 5 non-related healthy volunteers (3 males and 2 females; age, 33-42 years). Healthy volunteers were individuals from the Nephrology Department of the Medical School, University of Thessaly (Larissa, Greece). A state of health was confirmed by checking medical records and conducting physical examinations and laboratory tests to perform a routine checkup by an experienced physician. The health state was confirmed by medical records, physical examination and usual laboratory tests. Informed consent was obtained from each individual enrolled into the study and the ethics committee of the Medical School of the University of Thessaly approved the study protocol.

Two-way MLR and $CD4^+$ T-cell isolation. PBMCs were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation (Histopaque 1077; Sigma-Aldrich, St. Louis, MO, USA) and counted by optical microscopy on a Neubauer plaque. Cell viability was assessed by a Trypan blue assay (Sigma-Aldrich).

For measurement of glucose consumption and lactate production, eight MLRs were performed in 12-well plates incubated for 7 days. The MLR cell-based assay is characterized as an *ex vivo* cellular immune assay that occurs between two allogenic lymphocyte populations of the same species, yet genetically distinct. In the present study, isolated PBMCs from the blood of each volunteer was paired with the PBMCs from another volunteer, generating a MLR pair. The number of PBMCs for each member of the MLR pair was $5x10^5$, summing up to $1x10^6$ PBMCs in each well. After 7 days of incubation the supernatant from each MLR was collected.

For assessment of various proteins in alloreactive $CD4^+$ T-cells, eight MLRs were performed in the same conditions as above and at the end of the 7 day period, $CD4^+$ T-cells were isolated by negative selection using the $CD4^+$ T Cell Isolation kit, Human (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). More specifically, all non-$CD4^+$ PBMCs are labeled using a cocktail of biotin-conjugated antibodies. The cocktail contains antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a that bind to the non-$CD4^+$ cell populations, whereas negative selection of highly pure $CD4^+$ T-cells is achieved by depletion of magnetically labeled cells.

For assessment of cell proliferation, eight MLRs were performed in 96-well plates for 7 days. The number of PBMCs from each member of the MLR couple was $5x10^4$, adding up to $1x10^5$ PBMCs in total in each well.

Cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Rockford, IL, USA) with L-glutamine and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and antibiotic-antimycotic solution (Sigma-Aldrich), at 37˚C in a humidified atmosphere containing 5% CO_2.

Assessment of glucose consumption and lactate production in two-way MLR. Glucose uptake was assessed by measuring the decrease of glucose concentration in the supernatants, calculated as the glucose concentration in RPMI-1640 supplemented with 10% FBS at the start of the experiment, where the maximum glucose concentration is expected, minus the final glucose concentration in the supernatants at the end of the experimental procedure. Glucose measurements were obtained using the Element Blood glucose monitor along with the test strips (Element, Infopia, Titusville, FL, USA).

Aerobic glycolysis was assessed by measuring the concentration of lactate, the end product of this pathway. Lactate was measured by loading 100 μl of each supernatant sample on to the prove of a Blood Gas Analyzer (Czito Medical, Moscow, Russia), according to the manufacturer's details.

These experiments were performed in triplicate and the results refer to the mean of the three measurements.

Cell proliferation in two-way MLR. Cell proliferation was assessed via a Cell Proliferation ELISA (Roche Diagnostics, Indianapolis, IN, USA) using bromodeoxyuridine (BrdU) labeling and immunoenzymatic detection according to the manufacturer's protocol. An ELISA reader (Microplate Reader PR2100; Sanofi Diagnostics Pasteur Inc., Redmond, WA, USA) was used to determine the optical densities (ODs) of the immunoenzymatic reaction. Proliferation index of DCA-treated MLRs was calculated by dividing the OD derived from these MLRs to the ODs derived from the untreated

MLRs. Eight MLRs were performed. These experiments were performed in triplicate and the results refer to the mean of the three measurements.

Assessment of certain protein levels in alloreactive CD4+ T-cells. Levels of GLUT1, the enzymes of glycolysis hexokinase II (HKII), lactate dehydrogenase A (LDH-A), phosphorylated at serine 293 PDH, total PDH, along with signature transcription factors T box transcription factor TBX21 (T-bet), trans-acting T-cell specific transcription factor GATA 3 (GATA-3), retinoic acid receptor related orphan receptor-γt (RORγt) and forkhead box P3 (FoxP3) of Th1, Th2, Th17 and Treg, respectively, and the apoptotic marker cleaved caspase 3, were assessed in MLR $CD4^+$ T-cells.

Isolated $CD4^+$ T-cells were counted via optical microscopy using an optical microscope (Axiovert 40C; Carl Zeiss AG, Oberkochen, Germany) and a Neubauer chamber (Paul Marienfeld GmbH & Co., KG., Lauda-Königshofen, Germany). Cell viability was assessed by trypan blue staining (Sigma-Aldrich) Equal numbers of T-cells ($5x10^5$ cells) from each MLR were lysed using the T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride and ethylenediaminetetraacetic acid and phosphatase inhibitors against acid and alkaline phosphatases, in addition to serine/threonine (PP1, PP2A, and PP2B) and tyrosine protein phosphatases (Sigma-Aldrich and Roche Diagnostics, respectively). Protein was quantified via a Bradford assay (Sigma-Aldrich) and western blotting was performed. Equal quantities of protein extracts (10 μg) from each sample were loaded for electrophoresis in 4-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Thermo Fisher Scientific, Inc.). Subsequently proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Inc.). Blots were incubated with primary antibody for 16 h, followed by anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min. Benchmark pre-stained protein ladder (Thermo Fisher Scientific Inc.) was used as a marker. Bands were visualized by enhanced chemiluminescent detection using the LumiSensor Plus Chemiluminescent HRP Substrate kit (GenScript, Piscataway, NJ, USA) and analysis was performed using the Image J software (version 1.49; National Institute of Health, Bethesda, MD, USA). In case of reprobing PVDF blots, the previous primary and secondary antibodies were safely removed via the use of Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. The PVDF blot was then reused and western blotting resumed as previously described, using a different primary antibody.

The primary antibodies used in western blotting were specific for rabbit polyclonal GLUT1 (1:200; #sc-7903; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit monoclonal HKII (1:1,000; #2867; Cell Signaling Technology, Inc.), rabbit monoclonal LDH-A (1:1,000; #2012; Cell Signaling Technology, Inc.), rabbit monoclonal PDH (1:1,000; #2784; Cell Signaling Technology Inc.), rabbit polyclonal phosphorylated (and inactivated) at serine 293 PDH (p-PDH) (1:100; #orb6670; Biorbyt, Cambridge, UK), rabbit monoclonal c-Myc (1:500; #5605; Cell Signaling Technology, Inc.), rabbit monoclonal FoxP3 (1:500; #5605; Cell Signaling Technology, Inc.), rabbit polyclonal RORγt (1:100; #orb6888; Biorbyt), rabbit monoclonal T-bet (1:500; #5852; Cell Signaling Technology, Inc.), rabbit monoclonal GATA-3 (1:500; #5852; Cell Signaling Technology, Inc.), rabbit monoclonal cleaved caspase-3 (1:500; #9664; Cell Signaling Technology, Inc.) and rabbit monoclonal β-actin (1:2,500; #4967; Cell Signaling Technology, Inc.).

Statistical analysis. SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. Regarding glucose consumption and lactate production, a paired-samples t-test was used for comparison of means. Regarding all the other evaluated variables, the effect of DCA was estimated after normalization to the values obtained from untreated cells. The ratios of the results obtained from DCA-treated cells to untreated cells were estimated first. Then the normality of each variable was evaluated and confirmed with the Kolmogorov-Smirnoff test. Finally, a one sample t-test was performed comparing the values of each case to the test value of one.

Results are expressed as the mean ± standard deviation and $P<0.05$ was considered to indicate a statistically significant difference. Standard error of the mean was also calculated.

Results

DCA decreases glucose uptake and aerobic glycolysis in MLRs. DCA decreased glucose uptake. In MLRs, the glucose consumption was 159.75±27.77 mg/dl. Treatment with DCA decreased glucose consumption significantly to 88.50±33.35 mg/dl ($P<0.001$; Fig. 1A).

DCA inhibited aerobic glycolysis as determined by the quantity of the end-product lactate. In the supernatants of the MLRs lactate concentration was 11.15±2.96 mmol/l. Treatment with DCA decreased lactate concentration significantly to 5.90±0.53 mmol/l ($P<0.001$; Fig. 1B).

In MLRs, DCA exerts a negligible effect on cell proliferation. In MLRs, DCA increased cell proliferation by a factor of 1.10±0.04 (Fig. 1C). Although statistically significant ($P<0.001$), the 10% increase in cell proliferation could be considered as negligible.

DCA induces apoptosis in alloreactive CD4+ T-cells. Apoptosis of the $CD4^+$ T-cells was assessed by the level of the cleaved (and activated) caspase-3, which is the central caspase in the execution phase of cell apoptosis where all extrinsic and intrinsic apoptotic pathways converge (17); consequently, its expression level may be used as a marker of apoptosis. DCA was observed to induce apoptosis in alloreactive $CD4^+$ T-cells, which was demonstrated by the increase in the cleaved caspase-3 level by a factor of 1.85±0.70 ($P=0.011$; Fig. 2A and B).

Effect of DCA on the levels of certain proteins in alloreactive CD4+ T-cells. In the cellular context of isolated $CD4^+$ T-cells from MLRs, DCA decreased the expression of GLUT1 by a factor of 0.55±0.18 ($P<0.001$), HKII by a factor of 0.66±0.07 ($P<0.001$) and LDH-A by a factor of 0.64±0.15 ($P<0.01$) (Fig. 3A and B).

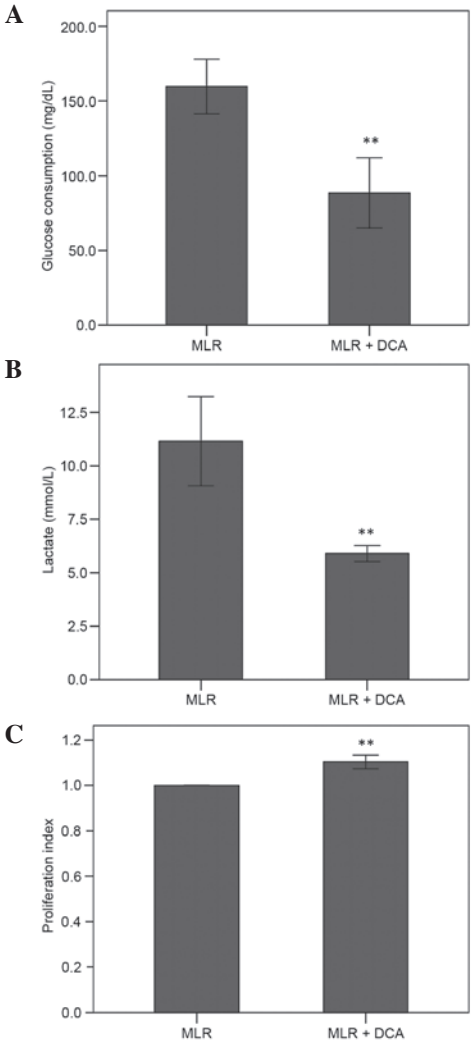

Figure 1. Effect of 1 mM DCA on glucose consumption, lactate production and cell proliferation in MLR lymphocytes. Eight independent experiments were performed. Each one was performed in triplicates and the results refer to the mean of the three measurements. (A) DCA significantly decreased glucose consumption as assessed by subtracting its concentration in the supernatant from the glucose concentration of the culture medium. (B) DCA suppressed aerobic glycolysis as determined by the level of its end-product lactate in the supernatants. (C) DCA exerted a negligible effect on cell proliferation since it induced an increase of only 10% in the proliferation index. Error bars correspond to 2 standard errors. In case of proliferation index, for reader's convenience a bar that corresponds to the control group is depicted. **P<0.001. MLR, mixed lymphocyte reaction; DCA, dichloroacetate.

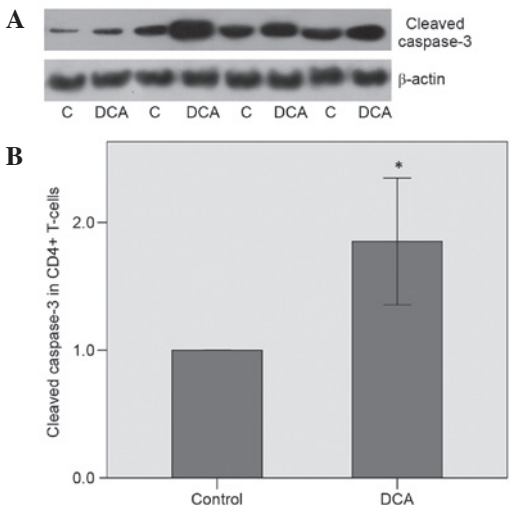

Figure 2. Effect of 1 mM DCA on cleaved caspase-3 level in CD4$^+$ T-cells isolated from the MLR. CD4$^+$ T-cells were isolated from the MLRs and cleaved caspase-3 was assessed by western blot analysis. (A) Eight experiments were performed and four of them are depicted. (B) Quantification of the results demonstrated that DCA increased the level of activated cleaved caspase-3 indicating that it induces apoptosis in alloreactive CD4$^+$ T-cells. Error bars correspond to 2 standard errors. For reader's convenience a bar that corresponds to the control group is depicted. *P<0.05. MLR, mixed lymphocyte reaction; DCA, dichloroacetate.

Conversely, DCA increased the level of PDH by a factor of 2.20±0.61 (P=0.001). Concurrently, DCA decreased the level of the phosphorylated and inactivated p-PDH by a factor of 0.49±0.08 (P<0.001) (Fig. 3).

Notably, DCA decreased the expression of c-Myc, a transcription factor that controls the expression of a number of the above proteins in T-cells (18), by a factor of 0.63±0.11 (P<0.001; Fig. 3). Thus, DCA was observed to alter the protein levels in a manner that favors decreased glucose consumption and aerobic glycolysis and increased entry of pyruvate into the Krebs' cycle.

Effect of DCA on alloreactive CD4$^+$ T-cells expression of certain transcription factors. FoxP3, RORγt, T-bet and GATA-3 are the signature transcription factors for the Treg, Th17, Th1 and Th2 cells, respectively (19). It is likely that in isolated CD4$^+$ T-cells from the MLRs, DCA favored the differentiation into the Treg subset since it increased FoxP3 expression by a factor of 2.32±0.98 (P=0.007) (Fig. 4). Conversely, it appears that DCA inhibited CD4$^+$ T-cell differentiation into the Teff subsets Th17, Th1 and Th2 since it decreased the expression of RORγt by a factor of 0.54±0.11 (P<0.001), T-bet by a factor of 0.55±0.28 (P=0.003) and GATA-3 by a factor of 0.35±0.18 (P<0.001) (Fig. 4).

Discussion

Although kidney transplantation increases survival and improves quality of life in patients suffering from end-stage renal disease (1,2), rejection remains a concern, and currently available immunosuppressive agents contribute to morbidity and mortality in these patients (3). In this study, the effect of DCA was evaluated in MLRs, and it was confirmed that DCA alters the level of certain enzymes of the glycolytic pathway in a manner that inhibits aerobic glycolysis, a prerequisite for effective differentiation of CD4$^+$ T-cells into Teffs (4-6). Then, it was confirmed that DCA decreases the expression of the signature transcription factors T-bet, GATA-3 and RORγt of the Teff subsets Th1, Th2 and Th17, respectively, while it increases the expression of the Treg transcription factor FoxP3 (19).

In MLRs, DCA decreased glucose consumption, which normally increases following T-cell activation, and lactate production, which is the end-product of aerobic glycolysis, and also increases during T-cell activation (4,5). Although statistically significant, the very slight, equal to 10%, increase in cell proliferation induced by DCA could be considered as negligible. This is possibly the result of the low DCA concentration used in the present study in order to simulate the concentration used clinically for the treatment of congenital

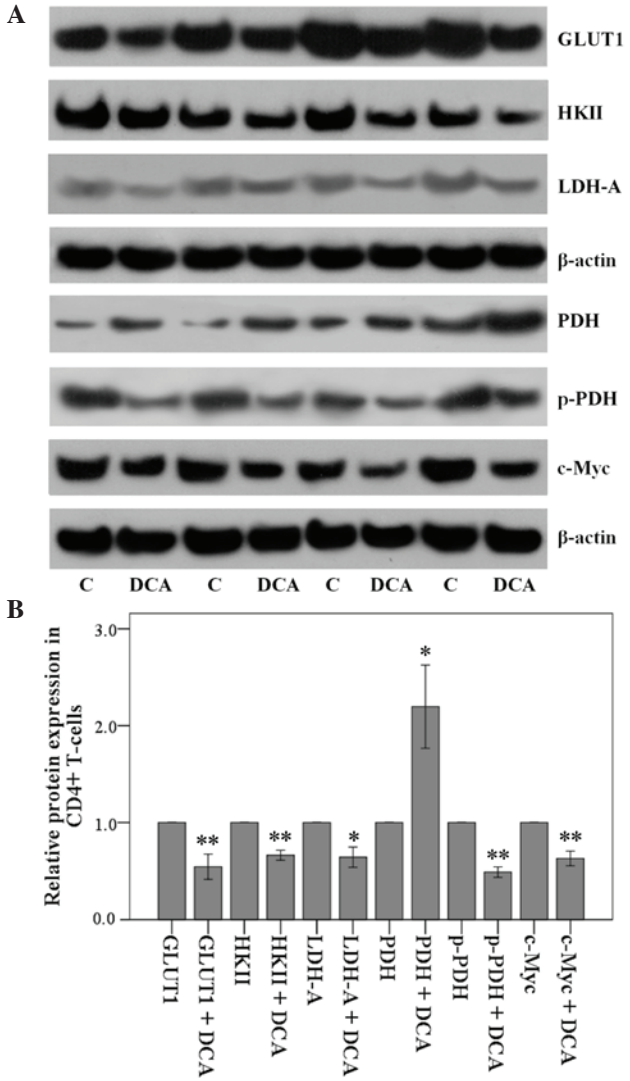

Figure 3. Effect of 1 mM DCA on certain enzymes involved in glycolysis in isolated CD4+ T-cells. Then, CD4+ T-cells were isolated from the MLRs and GLUT1, HKII, LDH-A, PDH, p-PDH and c-Myc were assessed by western blot analysis. (A) Eight experiments were performed and four of them are depicted. (B) quantification of the levels demonstrated that DCA decreased the levels of GLUT1, HKII, LDH-A, p-PDH and c-Myc, while it increased PDH. These DCA-induced alterations favor a decrease in glucose consumption and aerobic glycolysis, and diversion of glucose metabolism towards Krebs' cycle in alloreactive CD4+ T-cells. Error bars correspond to 2 standard errors. For reader's convenience bars that correspond to the control groups are depicted. *P<0.05; **P<0.001. GLUT1, glucose transporter-1; HKII, hexokinase II; LDH-A, lactate dehydrogenase-A; PDH, pyruvate dehydrogenase; p-PDH, phosphorylated PDH; MLR, mixed lymphocyte reaction; DCA, dichloroacetate.

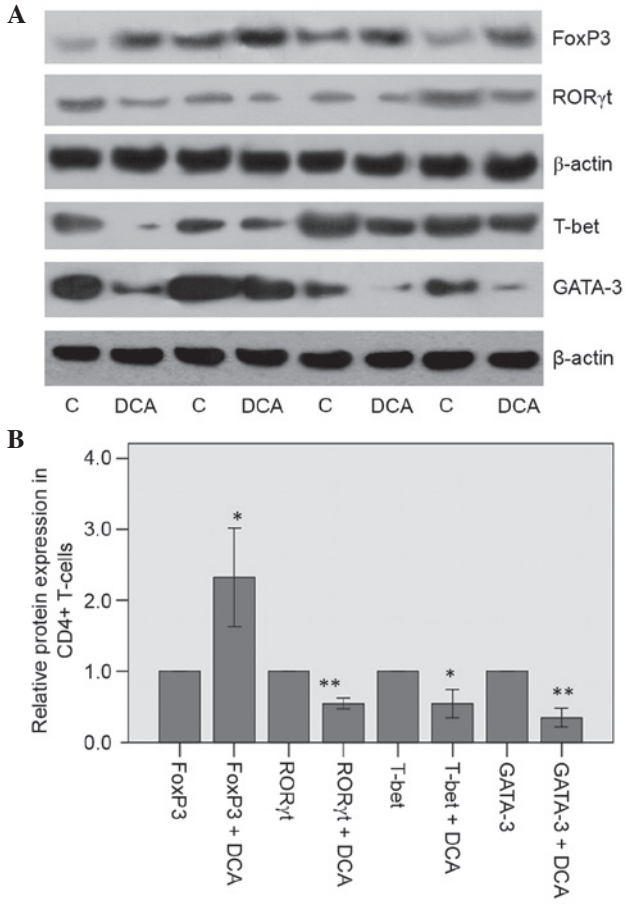

Figure 4. Effect of 1 mM DCA on the expression of signature transcription factors of CD4+ T-cell subsets from MLR. CD4+ T-cells were isolated from the MLRs and the signature transcription factors of CD4+ T-cell subsets were assessed by western blot analysis. (A) Eight experiments were performed and four of them are depicted. (B) Quantification demonstrated that DCA increased FoxP3 expression, whereas it decreased the expression of RORγt, T-bet and GATA-3. These DCA-induced alterations indicate that DCA favors CD4+ T-cell differentiation towards the regulatory T-cell subset instead of Th17, Th1 and Th2 subsets. Error bars correspond to 2 standard errors. For reader's convenience bars that correspond to the control groups are depicted. *P<0.05; **P<0.001. MLR, mixed lymphocyte reaction; DCA, dichloroacetate; FoxP3, forkhead box P3; RORγT, retinoic acid receptor related orphan receptor-γt; T-bet, T-box transcription factor TBX21; GATA-3, trans-acting T-cell-specific transcription factor GATA-3.

lactic acidosis (9,13). In another model of CD4+ T-cell activation, with anti-CD3/CD28 antibodies, DCA inhibited cell proliferation, albeit at the suprapharmacological concentration of 20 mM (11).

Then, it was evaluated whether DCA induces apoptosis in CD4+ T-cells isolated from MLRs. For this reason, the level of activated cleaved caspase-3, in which all the apoptotic pathways converge (17), was assessed. DCA increased the level of cleaved caspase-3 significantly, and consequently it is likely that it induces apoptosis in alloreactive T-cells. In cancer cell lines, DCA induces apoptosis by altering mitochondrial membrane permeability (20). Regardless of the responsible mechanism, DCA-induced apoptosis of alloreactive CD4+ T-cells could be considered to be immunosuppressive.

In order to evaluate the mechanisms involved in DCA-induced inhibition of glucose consumption and aerobic glycolysis, the levels of GLUT1, HKII, LDH-A, PDH and p-PDH were examined. In CD4+ T-cells from DCA-treated MLRs, the expression levels of the glucose transporter GLUT1 and of the first enzyme of the glycolytic pathway, HKII, decreased significantly. Thus, decreased glucose consumption in DCA-treated MLRs could be attributed to the decreased glucose influx into the cell and the deceleration of glycolysis. Notably, GLUT1 is selectively essential for CD4+ T-cell activation and effector function (21), and inhibition of HKII by 2-deoxy-D-glucose suppresses effective CD4+ T-cell activation (11).

In isolated CD4+ T-cells from the MLRs, DCA was found to decrease LDH-A, which converts pyruvate to lactate, and

to increase PDH, which irreversibly converts pyruvate to acetyl-CoA and controls its entry into the Krebs' cycle. In parallel, DCA decreased the level of the phosphorylated and inactivated p-PDH. Altogether, these DCA-induced alterations favor the diversion of glucose metabolism from aerobic glycolysis to the Krebs' cycle, contrary to what is required for $CD4^+$ T-cell proliferation and differentiation into Teffs (4,5).

The DCA-induced decrease in p-PDH is expected since DCA is a PDK inhibitor (9). Regarding the DCA-induced increase of PDH level, a study in other cell types have shown that DCA enhances PDH activity by increasing its level through inhibiting its turnover (22). However, the mechanism accounting for stabilization of PDH by DCA remains unknown (22). The transcription factor c-Myc controls metabolic reprogramming upon T-cell activation, increasing among others the expression of GLUT1, HKII and LDH-A (18). In order to assess how DCA exerts its effect on the expression of the above proteins, the c-Myc level was assessed in $CD4^+$ T-cells isolated from MLRs. Indeed, treatment of MLRs with DCA significantly decreased c-Myc expression in $CD4^+$ T-cells. This may be the underlying reason behind the decreased expression of GLUT1, HKII and LDH-A due to DCA treatment. However, the mechanisms involved in DCA-induced c-Myc downregulation remain to be elucidated.

During $CD4^+$ T-cell activation, cytokines in the T-cell microenvironment govern its differentiation into the various subsets. IL-12, IL-4 and IL-6 activate signal transducer and activator of transcription 4 (STAT4), STAT6 and STAT3 leading to the expression of the transcription factors T-bet, GATA-3 and RORγt, and ultimately to $CD4^+$ T-cell differentiation into the Teff subsets Th1, Th2 and Th17, respectively. Transforming growth factor-β (TGF-β), through SMAD2-SMAD4 induces FoxP3 expression and differentiation of $CD4^+$ T-cells into Tregs (19). $CD4^+$ effector T-cells express high levels of the glucose transporter GLUT1 and are reliant on glucose metabolism, whereas Tregs express low levels of GLUT1 and are reliant on lipid oxidation (6).

Considering that DCA decreases the level of GLUT1 and inhibits aerobic glycolysis, its effect on the signature transcription factors of Th1, Th2, Th17 and Treg subsets was evaluated. In DCA-treated MLRs $CD4^+$ T-cells, the levels of T-bet, GATA-3 and RORγt were significantly decreased indicating that DCA inhibits $CD4^+$ differentiation into the Teff subsets Th1, Th2 and Th17, respectively. Conversely, DCA treatment augmented the expression of FoxP3 indicating that it favors $CD4^+$ differentiation into Tregs. This feature of DCA to inhibit the differentiation of alloreactive $CD4^+$ T-cells into Teff subsets and concurrently to induce their differentiation into Tregs, renders DCA a promising immunosuppressant agent in the field of transplantation. Tregs may be significant in the suppression of graft rejection by Teffs, with the prospect of developing suitable treatments that will result in kidney allograft tolerance (23,24).

In conclusion, in alloreactive $CD4^+$ T-cells, DCA inhibits aerobic glycolysis, induces apoptosis and favors differentiation towards the Treg subset. Considering that DCA has already been used for a number of years in the treatment of congenital lactate acidosis with limited toxicity, the effects shown in this study suggest it may be a promising immunosuppressive agent in the field of transplantation.

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