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MicroRNA-124 Enhances T Cells Functions by Manipulating the Lactic Acid Metabolism of Tumor Cells

Mohammad Khakpoor-Koosheh[1] , Hosein Rostamian[1], Elham Masoumi[2], Leila Jafarzadeh[3], Keyvan Fallah-Mehrjardi[1], Mohammad Javad Tavassolifar[1], Farshid Noorbakhsh[1], Hamid Reza Mirzaei[1], Jamshid Hadjati[1], and Nima Rezaei[1,4]

[1] Department of Medical Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Immunology, School of Medicine, Ilam University of Medical Sciences, Ilam, Iran
[3] Department of Laboratory Sciences, Sirjan School of Medical Sciences, Sirjan, Iran
[4] Research Center for Immunodeficiencies, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

High production of lactic acid is a common feature of various tumors. Lactic acid is an immunosuppressive molecule with crucial roles in tumor cells' immune escape, which could largely be attributed to its negative effects on the T cells present in the tumor microenvironment (TME). Strategies that decrease the glycolysis rate of tumor cells could enhance immunosurveillance and limit tumor growth. Pyruvate kinase M2 (PKM2) is a key enzyme in the glycolysis pathway, and it plays a vital role in lactic acid buildup in the TME. MicroRNA (miR)-124 has been shown to be able to decrease tumor cell lactic acid synthesis indirectly by reducing PKM2 levels.

In this study, we first overexpressed miR-124 in the tumor cells and evaluated its effects on the PKM2 expression and lactic acid production of the tumor cells using quantitative real-time polymerase chain reaction (qRT-PCR) and spectrophotometry, respectively. Then, we cocultured miR-124–treated tumor cells with T cells to investigate the effects of miR-124 overexpression on T cell proliferation, cytokine production, and apoptosis.

Our results demonstrated that miR-124 overexpression could significantly reduce the amount of lactic acid produced by tumor cells by manipulating their glucose metabolism, which led to the augmented proliferation and IFN-γ production of T cells. Moreover, it rescued T cells from lactic acid-induced apoptosis.

Our data suggest that lactic acid is a hindering factor for T-cell–based immunotherapies; however, manipulating tumor cells' metabolism via miR-124 could be a promising way to improve antitumor responses of T cells.

Keywords: Lactic acid; Metabolism; MIRN124 microRNA, human; T-lymphocytes; Tumor microenvironment

Corresponding Authors: Nima Rezaei, MD, PhD;
Department of Medical Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 6692 9234, Fax: (+98 21) 6692 9235, E-mail: rezaei_nima@tums.ac.ir

Jamshid Hadjati, PhD;
Department of Medical Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 6405 3268, Fax: (+98 21) 6641 9536, E-mail: hajatij@tums.ac.ir

- The first and second authors have contributed equally in this study.

INTRODUCTION

Cancer immunotherapy using adoptive cellular therapy (ACT) has shown great success in treating hematological malignancies and immunogenic tumors such as renal cell carcinoma, but its efficacy in the context of solid tumors is restricted, and desirable results are yet to be achieved.[1,2]. Solid tumors are complex, and dynamic immunosuppressive microenvironments have been identified as one of the major obstacles limiting ACT efficacy.[3-5] One of the hindering factors in tumor microenvironments (TME) is lactic acid accumulation, which results from metabolic alterations in tumor cells leading to high utilization of glycolysis in hypoxic or even normoxic conditions, a phenomenon known as the Warburg Effect.[6] Tumor acidosis is now considered a hallmark of tumors, and it plays a vital role in evading immune responses, tumor progression, angiogenesis, and metastasis.[7,8] The acidic pH of TME induces apoptosis in tumor-specific T cells and causes a decrease in their cytolytic activity and cytokine secretion.[9]

Thus, targeting tumor cell glycolysis and the enzymes involved in this process could potentially restrict tumor growth and metastasis by improving the immunosurveillance and function of T cells.[10]

As the levels of lactic acid in the TME demonstrate the prevalence of glycolytic phenotypes, it can be a valuable marker for detecting the tumors more likely to respond to anti-glycolytic agents.[11] An approach for inhibiting lactate production is targeting enzymes responsible for the glycolysis process in which lactate is generated as an end-product.[11] The final rate-limiting step of glycolysis is catalyzed by pyruvate kinase (PK). PK has four isoforms: Pyruvate kinase M (PKM)1, PKM2, PKL, and PKR. The *PKM* gene can produce either the PKM1 or PKM2 isoforms by alternative splicing. Polypyrimidine tract-binding protein 1 (PTBP1) is a PKM1/PKM2 splicer and mediates the PKM isoform switch from PKM1 to PKM2.[12] PKM2 is overexpressed in many types of tumors and alters glucose metabolism from the standard respiratory chain toward lactate production in tumor cells.[13] PKM2 is vital to tumor cell metabolism, making it an excellent cancer therapy target.

MicroRNAs (miRNAs) are noncoding single-stranded RNAs, about 20 to 24 nucleotides long, that are potent posttranscriptional regulators. By altering the expression of messenger RNAs, miRNAs play crucial regulatory functions in cellular processes and are extensively involved in human diseases such as cancer.[14] The expression of miR-124 has been shown to be decreased in a number of human neoplasms, and it is suggested to be related to the occurrence, growth, and prognosis of tumors.[15] miR-124 regulates PKM isoform expression by targeting PTBP1, through which it switches the PKM gene expression from PKM2 to PKM1.[16] Therefore, as glucose is primarily metabolized by oxidative phosphorylation rather than glycolysis, we hypothesized that the overexpression of miR-124 in HCT-116 cells could lead to lower rates of glycolysis and, consequently, the level of lactate production.[16]

Here, we investigated the effects of targeting tumor metabolism using miR-124 on the activity and survival of T cells.

MATERIALS AND METHODS

Cell Culture and Media

The human colorectal cancer cell line, HCT-116, was obtained from the Iranian Biological Resource Center (IBRC). The cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA) and incubated at 37°C in 5% CO_2. Before use, FBS was heat-inactivated at 56°C for 30 minutes.

Plasmid Extraction

The lentiviral vector (pLenti-III-mico-GFP) containing hsa-miR-124-3p was purchased from Bon Yakhteh (Tehran, Iran). To extract miR-124-containing plasmids from their host bacteria (*Escherichia coli* DH5α strain), the bacteria were cultured in 20 mL of Luria–Bertani (LB) medium, supplemented with 50 µg/mL kanamycin, and incubated in a shaking incubator at 150 RPM at 37°C overnight to obtain a homogeneous solution of bacteria. The plasmids were then extracted using the FavorPrep™ plasmid DNA Extraction Mini Kit (Favorgen, Taiwan), according to the manufacturer's protocol. The concentration of plasmid was measured by Thermo Scientific NanoDrop™ 2000.

Stable Cell Line Generation

To generate HCT-116 cells that stably express miR-124 (HCT-116+miR-124 cells), HCT-116 cells were transfected with miR-124–containing plasmids using TurboFect Transfection Reagent (Thermo Fisher

Scientific, USA), according to the manufacturer's instructions. Briefly, HCT-116 cells were seeded in a 24-well plate (5×10^4 in each well) and cultured for 24 hours to reach 70% to 90% confluency. Afterward, 0.5µg of DNA was diluted in 100 µL of RPMI, and then 2 µL of TurboFect Transfection Reagent was added. Finally, to transfect HCT-116 cells, 100 µL of the transfection reagent/DNA mixture was added dropwise to each well. To select stably transfected cells, they were cultured in RPMI 1640 medium containing 10% FBS and 2 µg/mL puromycin for 4 weeks. Afterward, the green fluorescent protein (GFP) expression of cells was assessed using flow cytometry.

Lactate Measurement

HCT-116+miR-124 and control cells were seeded into a 48-well plate (2×10^5 cells/well) and incubated for 48 hours. Lactate concentration in the supernatant was measured at 24 and 48 hours using a colorimetric method with a lactate assay kit (Greiner Diagnostic GmbH, Germany).

PKM2 Expression Assessment

To evaluate the gene expression levels of *PKM2*, total RNA was extracted from miR-124 transfected and non-transfected HCT-116 cells using RNX-plus solution (Sinaclon, Iran). Thermo Scientific NanoDrop™ 2000 was used to measure RNA concentration. To remove genomic DNA, the extracted RNA was treated with DNase I (Fermentas, USA). After that, a cDNA synthesis kit (Thermo Fisher Scientific, USA) was used to synthesize cDNA. Real-time PCR was carried out using ABI StepOnePlus (Applied Biosystems, USA). All primers used in the qRT-PCR analysis are listed in the supplementary Table 1. 18S ribosomal RNA (rRNA) and beta-actin were used as internal controls. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels. PKM2 expression was normalized to the geometric mean of the housekeeping genes, 18S rRNA, and beta-actin.

PBMC Isolation and T-cell Enrichment

Peripheral blood mononuclear cells (PBMCs) were acquired from healthy donors. PBMCs were isolated by Lymphodex using density gradient centrifugation (inno-train Diagnostik GmbH, Germany). Isolated PBMCs were seeded in a 24-well plate (1.5×10^6 cells/well) and cultured in RPMI 1640 containing 10% FBS, 100 IU hIL-2 (Miltenyi Biotec, Germany), 3 µg/mL anti-CD3 antibody (Miltenyi Biotec, Germany), and 10 µg/L anti-CD28. The results are presented in Supplementary Figure 1.

T Cell Proliferation and Cytokine Assay

First, to stop HCT-116 cell proliferation, they were treated with 25 µg/mL mitomycin C (Sigma, St. Louis, MO) for 30 minutes. For measuring T-cell proliferation, 1×10^6 T cells were treated with 2.5 µM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, USA) for 10 minutes at 37°C and then 4 mL of FBS was added to quench the reaction (incubated at 37°C for 2 minutes). After washing the cells with 15 mL of PBS, they were cocultured with HCT-116 cells or HCT-116+miR-124 cells at 1:1 ratios in RPMI 1640 complete media that contained anti-CD3 (3 µg/ml) and anti-CD28 (10 µg/mL) antibodies to trigger the unspecific proliferation of T cells. After 72 h of incubation, the cells were analyzed using a flow cytometer (BD FACSCalibur Biosciences, USA). In order to detect T cells, the cells were stained with anti-CD3-APC (BioLegend, USA). For IL-2 and IFN-γ assessment, the cell-free supernatant was harvested 24 hours after the coculture of tumor cells with T cells and then kept at −70°C until the cytokine assessment with ELISA kit (R&D Systems, USA).

Apoptosis Assay

Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) Apoptosis Kit (Zist Exir Teb, Iran) was used to evaluate the apoptosis of T cells. HCT-116 and HCT-116+miR-124 cells (2×10^5 cells/well) were cocultured with T cells in 24-well plates in complete RPMI 1640 medium (10% FBS) for 48 hours at a 1:1 ratio. T cells cultured in complete RPMI 1640 medium in the absence of HCT-116 cells and lactic acid were used as the negative control. Those cultured in the presence of 30 mM lactic acid were used as a positive control for lactic acid. After 48 hours of culture, the supernatant was harvested and centrifuged at 1500 rpm for 10 minutes. Annexin V-FITC/PI staining of the cells was performed according to the manufacturer's protocol (Zist Exir Teb, Iran). In order to detect the T cells, the cells were stained with anti-CD3-APC (BioLegend, USA) and then analyzed by flow cytometer (BD FACSCalibur Biosciences, USA).

Flow Cytometric Analysis

The flow cytometric analysis was performed using FlowJo software (v7.6.1) (Tree Star Inc.). All experiments were done in triplicate and repeated three times.

Statistical Analysis

Prism 7 software was used to perform statistical analysis. The normality of the data was examined using the Kolmogorov-Smirnov test. The between-group comparisons were made using an independent t-test and analysis of variance (ANOVA) with Tukey's post hoc test. *p* values less than 0.05 were considered statistically significant.

RESULTS

miR-124 Decreased Tumor Cells' Lactic Acid Production

To change the TME in favor of T cells, we manipulated the tumor's metabolic condition by targeting its lactic acid production via miR-124. The human colorectal cell line HCT-116 was chosen for this study due to its ability to produce high amounts of lactic acid. We first transfected HCT-116 cells with the miR-124 plasmid containing the puromycin resistance gene. The transfection efficacy was checked via flow cytometry (Figure 1A). After 4 weeks of culturing transfected tumor cells in the presence of puromycin, a stably expressing miR-124 cell line was generated.

The relative expression of PKM2 was evaluated using real-time PCR in the transfected and control groups. As expected, the results indicated that PKM2 expression was remarkably lower in the miR-124 transfected group than in the untransfected group (p=0.005) (Figure 1B).

Subsequently, to confirm that miR-124 overexpression could successfully reduce lactate production, HCT-116+miR-124 cells were cultured in complete RPMI 1640 media for 48 hours. The lactic acid concentration was measured at two time points (24 and 48 hours), as we postulated that it would take some time to spot the effect of miR-124 on lactic acid production. The results showed that miR-124 overexpression in tumor cells could significantly reduce the lactic acid production of transfected HCT-116 compared to the control group (p<0.0001) (Figure 1C). miR-124 suppressive effects on lactic acid production could also be observed in the first 24 hours (p=0.03). In addition, to rule out the possibility that this decrease might be due to increased cell death in the transfected group, a viability assessment was performed using a trypan blue dye exclusion assay; nearly all cells were found to be alive in both groups (Supplementary Figure 2).

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Overexpression of miR-124 in Tumor Cells Could Enhance the Proliferative Capacity of T Cells

To evaluate how this decrease in lactic acid production of miR-124 HCT-116 cells could affect T cell proliferation, CFSE-labeled T cells were cocultured with untransfected and transfected HCT-116 cells in 1:1 ratios for 72 hours. As the T cells were not specific for HCT-116 cell antigens, anti-CD3 and anti-CD28 were added to the media to provide T cells with signals to proliferate. T cells cultured in the presence or absence of the 20 mM added lactic acid were considered positive and negative lactic acid controls, respectively.To simulate the TME condition, positive control groups were exposed to 20mM of lactic acid[18,19]. The representative flow cytometric data of T cell proliferation are shown in Figure 2A, C. Unsurprisingly, due to reduced lactic acid production in HCT-116+miR-124 cells, the suppressive effects of lactic acid on T cell proliferation were also decreased, and T cell proliferation in these cells was superior to that of cells cocultured with untransfected HCT-116 cells (p=0.001) (Figure 2D). However, miR-124 overexpression in tumor cells could not fully recover the proliferation capacity of T cells, as those T cells cultured in plain media showed better proliferation capability (p<0.0001).

Cytokine Secretion of T Cells was Improved by the Overexpression of miR-124 in Tumor Cells

To evaluate the IL-2 and IFN-γ secretion of T cells, which are crucial cytokines for T cell activity, they were cocultured with tumor cells. After 24 hours, the supernatant was collected, and the concentration of cytokines was assessed using ELISA. The results indicated that miR-124 overexpression in HCT-116 cells could significantly improve IFN-γ secretion in nearby T cells compared to the untransfected group (p<0.0001); however, no significant difference was observed regarding IL-2 secretion by T cells (p= 0.14). Similar to the proliferation results, miR-124 overexpression could not recover the cytokine secretion of T cells to the level of the T-media group (p<0.0001) (Figure 3A and B).

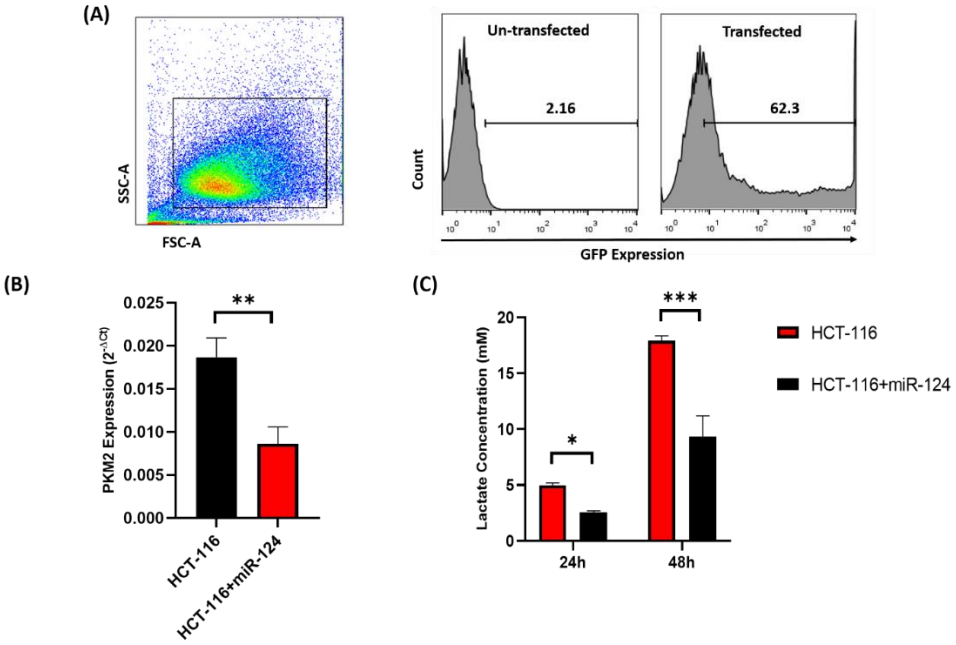

Figure 1. miR-124 decreased tumor-derived lactic acid. (A) Representative flow cytometry plots for the analysis of green fluorescent protein (GFP) expression in HCT-116 cells transfected with miR-124 plasmid at 48 hours post-transfection. HCT-116 and HCT-116+miR-124 cells were cultured in complete RPMI 1640 media (2×10^5 cells/well). (B) Gene expression of *PKM2* in HCT-116 and HCT-116+miR-124 cells. (C) The lactic acid concentration in the supernatant of the cultured cells was evaluated using a spectrophotometer after 24 and 48 hours of culture. ANOVA and Student's t-test were used for statistical analyses as appropriate. Data are presented as mean±standard deviation of three independent experiments. (*p<0.05; **p<0.01; ***p<0.001)

miR-124 Overexpression in Tumor Cells Rescues T Cells from Lactic Acid-induced Apoptosis

The influence of tumor-derived lactic acid on T cell apoptosis was investigated via Annexin V-FITC/PI double staining. Figure 4A and 4B show the representative results of the apoptosis assay performed on various groups. 30 mM of lactic acid was added to T-cell culture medium for this group to serve as a positive control for apoptosis. Flow cytometric analysis demonstrated that miR-124 overexpression in tumor cells could significantly reduce late apoptotic T cells compared to the T-HCT-116 group (p=0.001). Also, viable T cells were significantly higher in the T-HCT-116+miR-124 group compared to the T-HCT-116 group (p<0.0001). The findings of the apoptosis assay suggest that lactic acid could induce late-stage apoptosis in T cells; however, the comparison between the populations of early-apoptotic and necrotic T cells in T-HCT-116+miR-124 and T-HCT-116 groups showed no significant difference (Figure 4C).

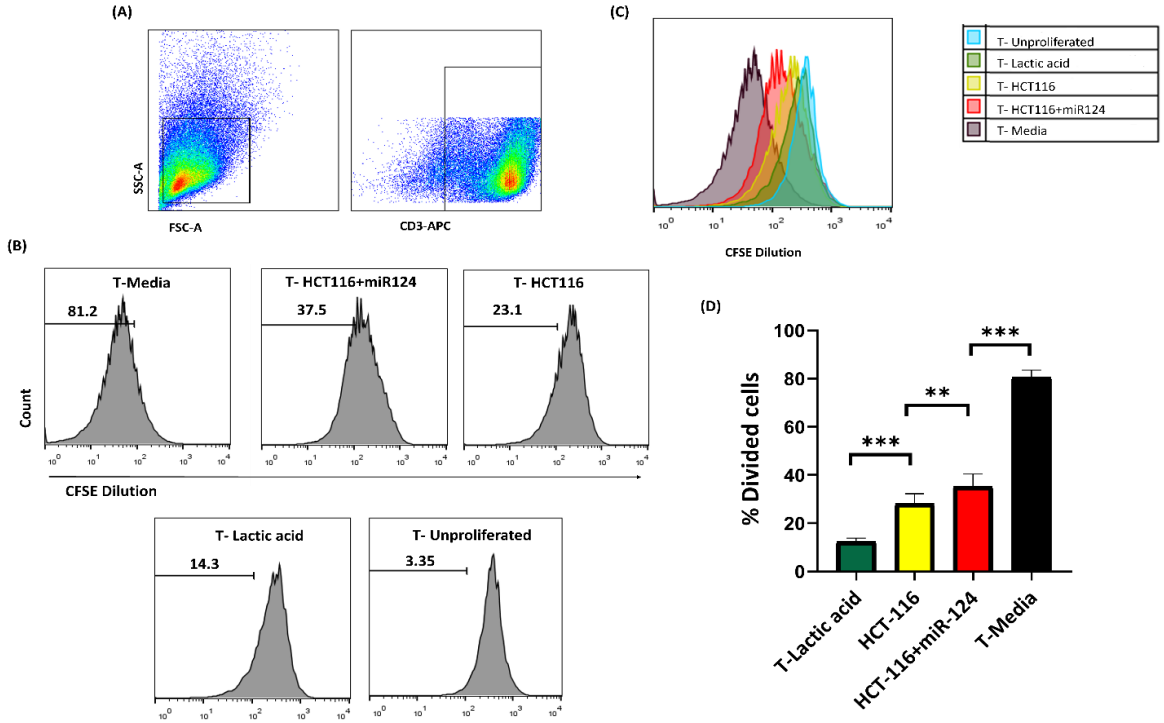

Figure 2. miR-124 improved the proliferation of T cells. Mitomycin C-treated HCT-116 and HCT-116+miR-124 cells (2×10^5 cells/well) were cocultured with CFSE-labeled T cells at a 1:1 ratio for 72 hours in the presence of anti-CD3/CD28 antibodies. In order to differentiate T cells from tumor cells, anti-CD3-APC staining was used. (A) Representative gating strategy for identification of live T cells. (B) Representative histograms indicating the percentage of T cell proliferation in the five groups. (C) Overlaid histogram plots of CFSE-labeled T cells in various groups. (D) Bar graph shows the average percentage of T cell proliferation in various groups. (E) Lactic acid concentration in the supernatant of mitomycin C-treated cells and the control group. ANOVA test was used for statistical analysis of the data. Data are presented as mean±standard deviation of three independent experiments. (p<0.01; ***p<0.001) IL: interleukin, CFSE: carboxyfluorescein succinimidyl ester.**

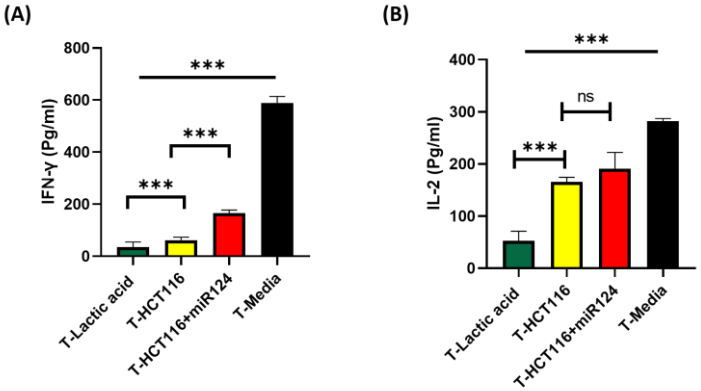

Figure 3. miR-124 boosted cytokine secretion by T cells. Mitomycin C-treated HCT-116 and HCT-116+miR-124 cells (2×10^5 cells/well) were cocultured with T cells at 1:1 ratios, in the presence of anti-CD3/CD28 antibodies. (A and B) After 24 hours, the supernatants were harvested and the concentrations of IL-2 and IFN-γ was measured using ELISA. Data are presented as mean ± standard deviation of three independent experiments. ANOVA followed by Tukey's post hoc test was used to compare group means. (*p<0.001) IL: interleukin, ns: not significant.**

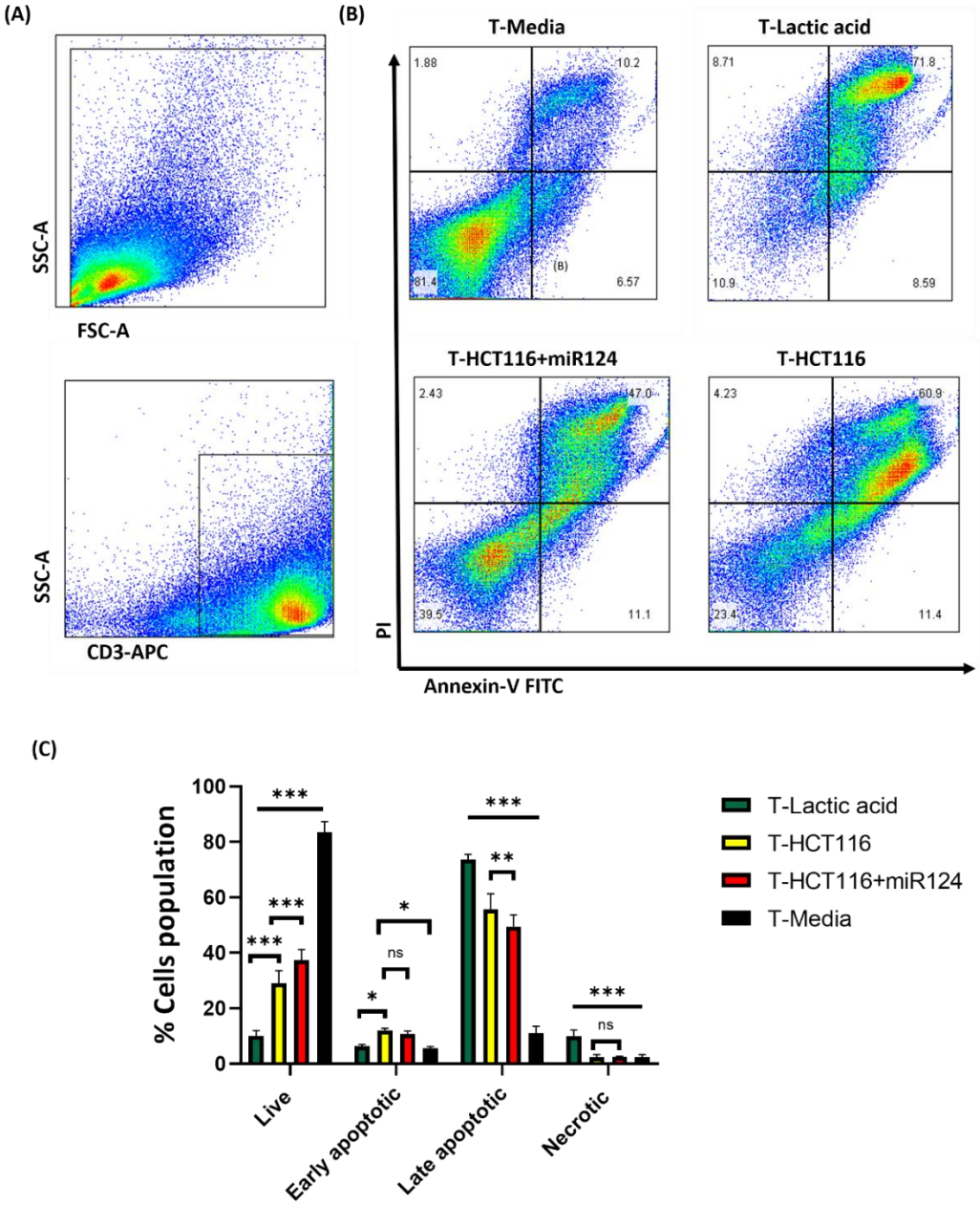

Figure 4. miR-124 enhanced T-cell viability. HCT-116 and HCT-116+miR-124 cells (2×10^5 cells/well) were cocultured with T cells at 1:1 ratios. After 48 hours, T cell apoptosis was assessed by the Annexin V-FITC/PI Apoptosis Detection Kit. In order to differentiate T cells from tumor cells, anti-CD3-APC staining was used. (A) Representative gating strategy for the identification of T cells. (B) Representative density plots showing Annexin V (X-axis) and PI (Y-axis) staining of T cells. Annexin V-positive and PI-negative populations indicate early-apoptotic cells; Annexin V/PI double-positive population indicates late apoptotic cells; Annexin V-negative and PI-positive populations indicate cell populations undergoing necrosis; and Annexin V/PI double-negative population indicates live cells. (C) The bar graph shows the average percentage of various cell populations in different conditions. Data are presented as mean±standard deviation of three independent experiments. ANOVA followed by Tukey's post hoc test was used to compare the group means (*p<0.05; **p<0.01; ***p<0.001). ns: not significant, PI: propidium iodide.

DISCUSSION

High levels of lactic acid in the TME are positively correlated with the progression of various tumors.[18,20-22] This could be due to the effects of high lactic acid concentrations on T cell function.[23] High levels of extracellular lactate can block the necessary export of intracellular lactate in T cells. Besides, T cells can be rendered dysfunctional due to the disruption of aerobic glycolysis by excessive amounts of extracellular lactate.[24,25] Therefore, developing strategies to decrease the lactic acid production of tumor cells could provide a beneficial way to overcome this challenge in cancer immunotherapy. Here, we showed that lactic acid could negatively impact T-cell function in vitro and dampen T cell proliferative ability, which is in line with previous studies. A reason for this proliferative hindrance is the blockade of glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate dehydrogenase by lactic acid, diminishing subsequent glycolytic intermediates such as 3-phosphoglycerate derivative serine, which is required for T cell proliferation.[26,27] In agreement with a previous study, we have shown that IL-2 and IFN-γ secretion by T cells is weakened by lactic acid.[24] A proposed mechanism for lower secretion of IFN-γ is the regulation of nuclear factor of activated T-cells (NFAT), a major regulator of IFN-γ transcription, by intracellular acidification.[23,27]

Targeting the glycolysis pathway of tumor cells could be a promising strategy to reduce their lactic acid secretion, which is a hindering factor in the antitumor immune responses of T cells.[28] Also, as this strategy can enhance immunosurveillance, it could be considered a great way to boost the efficacy of immunotherapy.[25,29,30]

PKM2 is a crucial rate-limiting enzyme in glycolysis and plays a vital role in the metabolism of tumor cells. PKM2 has gained much attention in recent years due to its role in tumor development and has been proposed as an attractive target for treating tumors.[31] High expression of PKM2 can lead to increased glucose consumption and lactate synthesis, promoting the proliferation and metastasis of tumor cells.[32] miR-124 can regulate PKM2 and the Warburg effect in colorectal cancer cells by targeting PTBP1. Therefore, overexpression of miR-124 in colorectal cancer cells can drastically suppress lactate production and promote oxidative phosphorylation.[16,17,33,34] Prior studies have already discussed the direct effects of miR-124 overexpression on tumor cells. Here, we intended to use miR-124, because of its antiglycolytic effects, to manipulate the TME in favor of T cells. The results demonstrated that miR-124 overexpression in tumor cells could improve T cells' proliferative ability and cytokine production.

Interestingly, although lactic acid levels were higher in the untransfected group than the lactic acid group, the proliferation of T cells cocultured with untransfected tumor cells was remarkably higher than in the lactic acid group. This could be explained by the finding that T cells could benefit from low amounts of lactic acid and can actually improve their function.[35-37] In our study, the higher proliferation of T cells in the untransfected group might be due to the initial utilization of tumor-derived lactate by T cells. However, the gradual increase in lactic acid concentrations over time makes T cells prone to apoptosis and renders them dysfunctional. Contrarily, the sudden encounter of T cells with high levels of lactic acid in the lactic acid group is detrimental and drastically decreases their proliferation. Although T cells cocultured with the miR-124–treated group showed significantly higher IFN-γ compared with the untreated group, IL-2 levels were not significantly different between the two groups. This could be due to the presence of other molecules that suppress IL-2 secretion of T cells, such as adenosine and Transforming growth factor beta (TGF-β).[38,39]

miR-124 overexpression managed to reduce T-cell apoptosis and improve their viability. In addition, other studies have used dichloroacetate and diclofenac in an attempt to enhance T-cell–mediated antitumor responses by reprogramming tumor metabolism and reducing the production of lactic acid. The results indicated that both of these drugs were able to improve the viability and function of T cells, which is in agreement with our results; besides, diclofenac also managed to improve the outcome of immune checkpoint blockade therapy when used in combination with anti-PD1 antibody.[30]

CD276 (a member of the B7/CD28 superfamily) is an immune checkpoint highly expressed in various tumor types and positively correlated with tumor progression.[40] A recent study showed that CD276 exerts its oncogenic effects through the induction of the glycolysis pathway via induction of PKM2 phosphorylation and increasing the lactic acid production in tumor cells.[41] Interestingly, knocking down CD276 in cancer cells reduced their PKM2 activation and lactate production, leading to improved function and survival of T cells, which is in line with our findings.[41]

This study demonstrated that lactic acid accumulation in the TME is a hindering factor for T cell

response against tumors. Therefore, restricting tumor lactic acid could be a beneficial way to increase the efficacy of antitumor immunotherapies. Our study revealed that miR-124 overexpression could be used as a strategy to reprogram tumor metabolism in favor of T cells. Strategies to manipulate tumor metabolism could pave the way for developing new treatments and improving the existing ones.

STATEMENT OF ETHICS

The protocols of this research were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (Ethics Committee Approval Number: IR.TUMS.MEDICINE.REC.1398.137).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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None.

REFERENCES

1. Maus MV, Grupp SA, Porter DL, June CHJB. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. Blood. 2014;123(17):2625-35.
2. Melero I, Rouzaut A, Motz GT, Coukos GJCd. T-cell and NK-cell infiltration into solid tumors: a key limiting factor for efficacious cancer immunotherapy. Cancer Discov. 2014;4(5):522-6.
3. Labani-Motlagh A, Ashja-Mahdavi M, Loskog AJFiI. The tumor microenvironment: A milieu hindering and obstructing antitumor immune responses. Front Immunol. 2020;11:940.
4. Fallah-Mehrjardi K, Mirzaei HR, Masoumi E, Jafarzadeh L, Rostamian H, Khakpoor-Koosheh M, et al. Pharmacological targeting of immune checkpoint A2aR improves function of anti-CD19 CAR T cells in vitro. Immunol Lett. 2020;223(26):44-52.
5. Rostamian H, Fallah-Mehrjardi K, Khakpoor-Koosheh M, Pawelek JM, Hadjati J, Brown CE, et al. A metabolic switch to memory CAR T cells: Implications for cancer treatment. Cancer Lett. 2021;500(14):107-18.
6. Ward PS, Thompson CBJCc. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer cell. 2012;21(3):297-308.
7. Corbet C, Feron OJNRC. Tumour acidosis: from the passenger to the driver's seat. Nat Rev Cancer. 2017;17(10):577.
8. Pérez-Tomás R, Pérez-Guillén IJC. Lactate in the Tumor Microenvironment: An Essential Molecule in Cancer Progression and Treatment. Cancers (Basel). 2020;12(11):3244.
9. Balgi AD, Diering GH, Donohue E, Lam KK, Fonseca BD, Zimmerman C, et al. Regulation of mTORC1 signaling by pH. PloS one. 2011;6(6):e21549.
10. Romero-Garcia S, Moreno-Altamirano MMB, Prado-Garcia H, Sánchez-García FJJFii. Lactate contribution to the tumor microenvironment: mechanisms, effects on immune cells and therapeutic relevance. Front Immunol. 2016;7(2):52.
11. Ganapathy-Kanniappan S, Geschwind J-FHJMc. Tumor glycolysis as a target for cancer therapy: progress and prospects. Mol Cancer. 2013;12(1):1-11.
12. Clower CV, Chatterjee D, Wang Z, Cantley LC, Vander Heiden MG, Krainer ARJPotNAoS. The alternative splicing repressors hnRNP A1/A2 and PTB influence pyruvate kinase isoform expression and cell metabolism. Proc Natl Acad Sci. 2010;107(5):1894-9.
13. Zahra K, Dey T, Mishra SP, Pandey UJFio. Pyruvate kinase M2 and cancer: the role of PKM2 in promoting tumorigenesis. Front Oncol. 2020;10:159.
14. Peng Y, Croce CMJSt, therapy t. The role of MicroRNAs in human cancer. Signal transduction and targeted therapy. 2016;1(1):1-9.
15. Jia X, Wang X, Guo X, Ji J, Lou G, Zhao J, et al. MicroRNA-124: an emerging therapeutic target in cancer. Cancer Medicine. 2019;8(12):5638-50.
16. Sun Y, Zhao X, Zhou Y, Hu YJOr. miR-124, miR-137 and miR-340 regulate colorectal cancer growth via inhibition of the Warburg effect. Oncology reports. 2012;28(4):1346-52.
17. Taniguchi K, Sugito N, Kumazaki M, Shinohara H, Yamada N, Nakagawa Y, et al. MicroRNA-124 inhibits cancer cell growth through PTB1/PKM1/PKM2 feedback cascade in colorectal cancer. Cancer Lett. 2015;363(1):17-27.
18. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfør K, Rofstad EK, et al. High lactate levels predict

likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. 2000;60(4):916-21.
19. Walenta S, Salameh A, Lyng H, Evensen JF, Mitze M, Rofstad EK, et al. Correlation of high lactate levels in head and neck tumors with incidence of metastasis. 1997;150(2):409.
20. Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW, et al. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. 2001;51(2):349-53.
21. Yokota H, Guo J, Matoba M, Higashi K, Tonami H, Nagao YJJoMRIAOJotISfMRiM. Lactate, choline, and creatine levels measured by vitro 1H-MRS as prognostic parameters in patients with non-small-cell lung cancer. 2007;25(5):992-9.
22. Qian J, Gong Z-c, Zhang Y-n, Wu H-h, Zhao J, Wang L-t, et al. Lactic acid promotes metastatic niche formation in bone metastasis of colorectal cancer. Cell Commun Signal. 2021;19(1):1-15.
23. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. Cell Metab. 2016;24(5):657-71.
24. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, et al. Inhibitory effect of tumor cell–derived lactic acid on human T cells. 2007;109(9):3812-9.
25. Pilon-Thomas S, Kodumudi KN, El-Kenawi AE, Russell S, Weber AM, Luddy K, et al. Neutralization of tumor acidity improves antitumor responses to immunotherapy. Cancer Res. 2016;76(6):1381-90.
26. Quinn III WJ, Jiao J, TeSlaa T, Stadanlick J, Wang Z, Wang L, et al. Lactate limits T cell proliferation via the NAD (H) redox state. Cell Rep. 2020;33(11):108500.
27. Lim AR, Rathmell WK, Rathmell JCJE. The tumor microenvironment as a metabolic barrier to effector T cells and immunotherapy. Elife. 2020;9:e55185.
28. Calcinotto A, Filipazzi P, Grioni M, Iero M, De Milito A, Ricupito A, et al. Modulation of microenvironment acidity reverses anergy in human and murine tumor-infiltrating T lymphocytes. Cancer Res. 2012;72(11):2746-56.
29. Cascone T, McKenzie JA, Mbofung RM, Punt S, Wang Z, Xu C, et al. Increased tumor glycolysis characterizes immune resistance to adoptive T cell therapy. Cell Metab. 2018;27(5):977-87. e4.
30. Renner K, Bruss C, Schnell A, Koehl G, Becker HM, Fante M, et al. Restricting glycolysis preserves T cell effector functions and augments checkpoint therapy. Cell Rep. 2019;29(1):135-50. e9.
31. Luo W, Semenza GLJTiE, Metabolism. Emerging roles of PKM2 in cell metabolism and cancer progression. Trends in Endocrinology & Metabolism. 2012;23(11):560-6.
32. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. 2008;452(7184):230-3.
33. Qiu Z, Guo W, Wang Q, Chen Z, Huang S, Zhao F, et al. MicroRNA-124 reduces the pentose phosphate pathway and proliferation by targeting PRPS1 and RPIA mRNAs in human colorectal cancer cells. Gastroenterology. 2015;149(6):1587-98. e11.
34. Taniguchi K, Ito Y, Sugito N, Kumazaki M, Shinohara H, Yamada N, et al. Organ-specific PTB1-associated microRNAs determine expression of pyruvate kinase isoforms. Sci Rep. 2015;5(1):1-8.
35. Rundqvist H, Veliça P, Barbieri L, Gameiro P, Cunha PP, Gojkovic M, et al. Lactate potentiates differentiation and expansion of cytotoxic T cells. 2019.
36. Wen J, Cheng S, Zhang Y, Wang R, Xu J, Ling Z, et al. Lactate anions participate in T cell cytokine production and function. Sci China Life Sci. 2021:1-11.
37. Zhang Y, Wen J, Sun B. Lactate is critical for cytokines production of T cell after TCR activation. Am Assoc Immnol. 2020;204(1 Supplement):240.3.
38. Polanczyk MJ, Walker E, Haley D, Guerrouahen BS, Akporiaye ETJJotm. Blockade of TGF-β signaling to enhance the antitumor response is accompanied by dysregulation of the functional activity of CD4+ CD25+ Foxp3+ and CD4+ CD25− Foxp3+ T cells. J Transl Med. 2019;17(1):1-12.
39. Masoumi E, Jafarzadeh L, Mirzaei HR, Alishah K, Fallah-Mehrjardi K, Rostamian H, et al. Genetic and pharmacological targeting of A2a receptor improves function of anti-mesothelin CAR T cells. 2020;39(1):1-12.
40. Kanchan RK, Perumal N, Atri P, Chirravuri Venkata R, Thapa I, Klinkebiel DL, et al. MiR-1253 exerts tumor-suppressive effects in medulloblastoma via inhibition of CDK6 and CD276 (B7-H3). Brain Pathol. 2020;30(4):732-45.
41. Yue G, Tang J, Zhang L, Niu H, Li H, Luo SJJoGO. CD276 suppresses CAR-T cell function by promoting tumor cell glycolysis in esophageal squamous cell carcinoma. J Gastrointest Oncol. 2021;12(1):38.