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Assessing the effects of lactate on the immune responsiveness of T cells *in vitro*

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T cells play a key role in protecting the body from cancer. While T cells are normally effective in destroying abnormal cells, there are times when malignancies grow seemingly unchecked by the immune system. Chimeric antigen receptor (CAR)-T cell therapy implements T cells that have been genetically engineered to recognize and kill cancer cells. While CAR-T cell therapy has demonstrated efficacy in treating hematological malignancies, it has shown less promise in treating solid tumors. A possible explanation for the decreased efficacy of T cells and T cell-based immunotherapies in destroying solid tumors may lie in a phenomenon that scientists have long been aware of. The Warburg effect, first described in 1924, is the observation that cancer cells tend to consume significantly more glucose than other cells due to their shift from aerobic respiration to fermentation, resulting in the accumulation of the waste product lactate in the tumor microenvironment. This research investigated the effects of lactate on the immune responsiveness of T cells, using Jurkat E6.1 cells and human primary T cells as models. The effects of lactate on viability, CD3 surface expression, and proliferation were assessed via flow cytometry. While lactate concentration and exposure time had no significant effects on T cell viability, marked decreases in CD3 surface expression and proliferation were observed following exposure to lactate. These findings may shed light on how solid tumors evade immune detection and immunotherapies and have implications for future approaches to treating solid tumors.

Cancer is a devastating disease characterized by uncontrolled proliferation of abnormal cells. Approximately two in every five individuals will be diagnosed with cancer at some point in their lifetime,[1] and cancer accounts for nearly one in every six deaths,[2] making it a leading cause of morbidity and mortality worldwide. Consequently, understanding how cancer takes a foothold in its victims and how it can be prevented or treated is an important area of research.

Recently, the interaction between cancer and the immune system has been a hot area of study. T cells play a key role in protecting the body from cancer.[3] T cells are equipped with T cell receptors (TCRs) that interact with peptide antigens displayed by major histocompatibility complexes (MHCs) on most of the body's cells, including cancer cells. Recognition of the peptide antigen by the TCR triggers a multistep signaling cascade known as TCR signaling.[4] In cytotoxic ($CD8^+$) T cells, TCR signaling leads to the direct killing of cancer cells by inducing apoptosis.[3] In helper (CD4+) T cells, TCR signaling leads to a variety of effector functions, including directly killing cancer cells, activating and maintaining the anti-tumor responses of CD8+ T cells, and driving B cells to produce antibodies against tumor antigens.[5] While T cells are normally effective in destroying abnormal cells, there are times when malignancies grow seemingly unchecked by the immune system. The latest advances in cancer therapeutics have been targeted at enhancing or assisting the body's immune response against cancer. For example, chimeric antigen receptor (CAR)-T cell therapy implements a patient's own T cells that have been genetically engineered to recognize and kill cancer cells.[6] While CAR-T cell therapy has demonstrated efficacy in treating hematological malignancies,[3] it has shown less promise in treating solid tumors.[7] The current understanding of how solid tumors evade the immune system and immunotherapies is incomplete at best.

A possible explanation for the decreased efficacy of T cells and T cell-based immunotherapies in destroying solid tumors may lie in a phenomenon that scientists have long been aware of. The Warburg effect, named after the physiologist who first described it in 1924, is the observation that cancer cells tend to consume significantly more glucose than other cells due to their shift from aerobic respiration to fermentation.8 In some solid tumors, the shift to fermentation may be due to hypoxia when the tumor grows beyond the diffusion limit of the local blood supply,[9] but cancer cells still prefer fermentation over aerobic respiration even under normoxic conditions, a phenomenon known as aerobic glycolysis.[10] Considering that glycolysis alone is a less efficient means of energy production compared to complete glucose oxidation, this phenomenon has long puzzled scientists. A recent study discovered that increased flux through the glycolytic pathway drives the regeneration of NAD^+, a cofactor required for catabolic and anabolic reactions that enable rapid cell proliferation.[11] Shifting to fermentation may be advantageous for surviving in hypoxic

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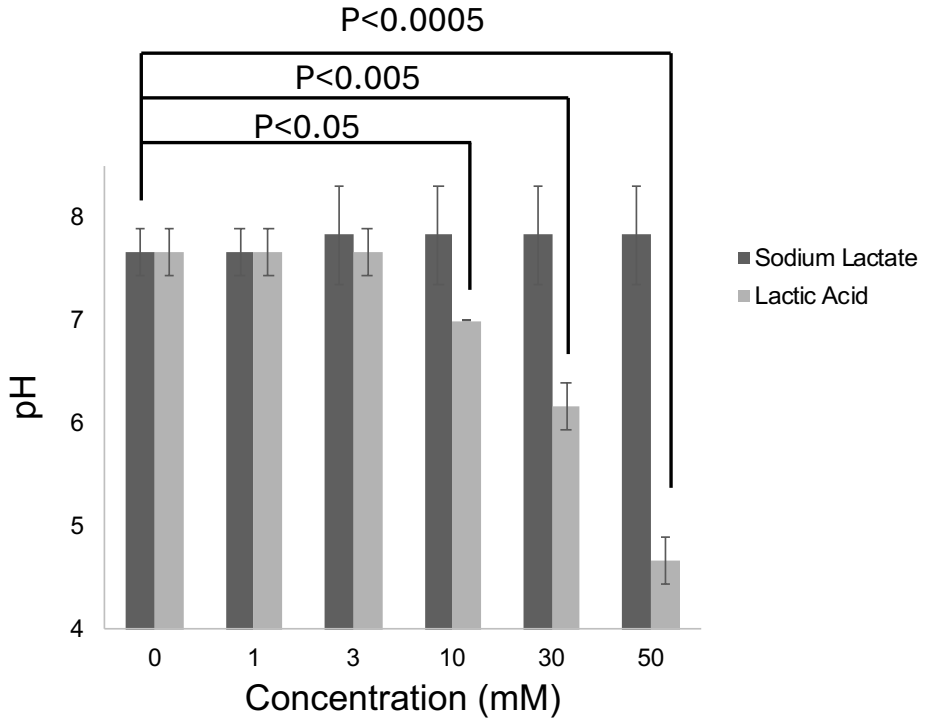

Figure 1 | Addition of sodium lactate to complete cell culture media does not change pH.
The pH of complete cell culture media containing different concentrations of lactic acid or sodium lactate was measured using universal pH indicator paper. Data from three independent experiments is displayed. Statistical significance was determined via two-tailed, homoscedastic t-tests.

environments and proliferating rapidly, but it could also have a role in immune evasion. Cancer cells performing fermentation produce large amounts of lactic acid, which quickly dissociates into lactate and H^+, acidifying the intracellular space. To combat the harmful effects of this acidification, cancer cells efflux lactate and H^+ into the tumor microenvironment (TME) through monocarboxylate transporters.[10,12] Lactate concentrations as high as 40 mM and pH levels as low as 5.6 have been reported in the TME.13 Our lab previously reported that exposure of Jurkat E6.1 cells, an immortalized $CD4^+$ T cell line, to acidic conditions potentially led to decreased TCR signaling upon stimulation of CD3.[14] However, the immunosuppressive effects of cancer cells' shift to fermentation may extend beyond acidifying the TME, as tumor-derived lactate has been demonstrated to impact the phenotypes of dendritic cells[15] and NK cells,[16] and the effects of lactate on other immune cells have yet to be investigated. Interestingly, aerobic glycolysis is not unique to cancer cells, but has also been observed in other rapidly proliferating cells, such as T cells and fibroblasts.[17,18] While it is plausible that high concentrations of lactate may inhibit the continued proliferation of cells performing aerobic glycolysis via negative feedback mechanisms, the opposite has been observed in some cases. For example, lactate has been demonstrated to stimulate the proliferation of fibroblasts in vitro[19] and promote protein synthesis in wound healing.[20]

In this study, we sought to investigate the effects of lactate on the immune responsiveness of T cells in the context of the TME—without changes in pH—to determine whether lactate itself has immunomodulatory properties. This was done by assessing viability, CD3 surface expression, and T cell proliferation in primary and immortalized T cells following exposure to lactate.

Materials & Methods

Preparation of 100x Lactic Acid and Lactate Stock Solutions

A 5 M lactic acid stock solution was prepared by dissolving the appropriate mass of sodium lactate (Thermo Scientific Chemicals, Cat. No. L14500.06) in 5 M HCl (Fisher Scientific, Cat. No. A144-212). A 5 M sodium lactate stock solution was prepared in water. The 5 M stock solutions were passed through a 0.2 micron filter (PALL Gelman Laboratory, Cat. No. 4192) and diluted with sterile-filtered diH_2O to concentrations of 100 mM, 300 mM, 1 M, and 3 M to make 100x stock solutions for the following working concentrations: 1 mM, 3 mM, 10 mM, 30 mM, and 50 mM. DiH_2O was used as a vehicle control. All stock solutions were stored at 4°C.

Jurkat E6.1 Cell Culture

The Jurkat E6.1 cells were kindly donated by Dr. Jon Houtman and Dr. Gary Weisman (previously purchased from ATCC: TIB-152TM). Jurkat E6.1 cells were grown at 37°C in 5% CO_2 using complete RPMI-1640 Medium (Sigma-Aldrich, Cat. No. R8758) supplemented with 10% FBS (Gibco, Cat. No. A52567-01). Cultures were maintained between 1x105 and 1x106 cells/mL at passage numbers below 20.

Lactic Acid, Lactate, and Media pH Assay

1 mL of complete RPMI-1640 Medium supplemented with 10% FBS was aliquoted into microcentrifuge tubes and spiked with 10 µL of the appropriate 100x lactic acid or sodium lactate stock solution. The pH of the media was measured using universal pH indicator paper (Cytiva, Cat. No. 2613-991). Data from three independent experiments were averaged. Two-tailed, homoscedastic t-tests were used to determine statistical significance with a threshold of $p \leq 0.05$.

Timepoint Viability and CD3 Surface Expression Assay

Jurkat E6.1 cells were plated (2.5x105 cells/well) 30 hours prior to analysis and incubated at 37°C in 5% CO_2. Samples were treated with sodium lactate (at working concentrations of 0, 1, 3, 10, 30, and 50 mM) 30 hours, 1 hour, 30 minutes, and 15 minutes prior to analysis. Samples were then harvested, washed, and resuspended in 1 mL DPBS (2.7 mM KCl [Fisher Scientific, Cat. No. BP366-500], 1.5 mM KH_2PO_4 [Fisher Scientific, Cat. No. BP362-1], 136.9 mM NaCl [Fisher Science Education, Cat. No. S25541A], 8.9 mM $Na_2HPO_4 \cdot 7H2O$ [EMD Millipore Corp Cat. No. 56747]). Unstained samples were analyzed using the Attune NxT Flow Cytometer.

Samples were then stained with one drop of SYTOX AADvanced Ready Flow Reagent (Invitrogen, Cat. No. R37173) and 5 μL of FITC-labelled anti-CD3 (Invitrogen, Cat. No. 11-0037-42), incubated on ice in the dark for 40 min, then analyzed using the Attune NxT Flow Cytometer. A SSC-A vs. FSC-A density plot of all events was used to visualize populations and gate the Jurkat E6.1 cells. A histogram plot of the Jurkat E6.1 cells was used to visualize fluorescence intensity detected through the BL3 channel (used to detect SYTOX) to gate live and dead cells. Counts of live and dead cells were used to calculate percent viability. A histogram plot of the live Jurkat E6.1 cells was used to visualize fluorescence intensity detected through the BL1 channel (used to detect FITC) to assess relative surface expression of CD3. X mean values of stained cells were normalized to X mean values of unstained controls and then normalized to the appropriate vehicle control for each time exposure. Data from three independent experiments were averaged. Two-tailed, homoscedastic t-tests were used to determine statistical significance with a threshold of $p \leq 0.05$.

Primary Leukocyte Isolation

Whole blood was collected from a healthy adult male human volunteer via venipuncture into BD Vacutainer EDTA tubes (Becton Dickson and Company, Cat. No. 366643). The research protocol of this study was approved by the Institutional Review Board of The University of Kansas (STUDY00150912). The volunteer who donated blood for this study gave informed consent for participation. The whole blood was diluted two-fold with DPBS, layered on top of Histopaque-1077 (Sigma-Aldrich, Cat. No. 10771-100ML), and centrifuged at 400 x g for 30 min. The buffy coat (containing leukocytes) was transferred to a clean tube, washed twice, and resuspended in DPBS. Leukocytes were used immediately after isolation.

T Cell Proliferation Assay

Freshly isolated primary leukocytes were resuspended in CFSE staining solution (5 uM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester [Sigma-Aldrich, Cat. No. 21888-25MG-F], 0.1% DMSO [Fisher Chemical, Cat. No. D128-4] in DPBS) and incubated for 10 min in a water bath set to 37°C. An equal volume of complete RPMI-1640 media with 10% FBS was added to the staining solution and incubated for 5 min to quench the staining. The stained cells were resuspended in complete RPMI-1640 media supplemented with 10% FBS and plated (500,000 cells/well). Samples were treated with sodium lactate (at working concentrations of 0, 3, 10, and 30 mM) and 60 ng/mL human IL-2 (Miltenyi Biotec, Cat. No. 130-097-743). Samples were split into stimulated and unstimulated groups. Stimulated groups were treated with 25 μL/mL of ImmunoCultTM Human CD3/CD28 T Cell Activator (STEMCELL Technologies, Cat. No. 10971). Samples were incubated at 37°C in 5% CO2 for 4 days. Samples were then harvested, washed, and resuspended in 1 mL DPBS. Samples were stained with 1 μL reconstituted LIVE/DEAD Fixable Red Dead Cell Stain (Invitrogen, Cat. No. S10274) and 5 μL PE-Cy5.5-labelled anti-CD8 (Invitrogen, Cat. No. 12-0088-42), incubated on ice for 30 min, and analyzed using the Attune NxT Flow Cytometer. A histogram plot of all events was used to visualize fluorescence intensity detected through the BL2 channel (used to detect LIVE/DEAD Fixable Red Dead Cell Stain) to gate live and dead cells. A density plot of BL3 (used to detect PE-Cy5.5) vs. BL1 (used to detect CFSE) was used to visualize proliferated and non-proliferated $CD8^+$ T cells. A histogram plot of $CD8^+$ T cells was used to visualize fluorescence intensity of CFSE to gate proliferated and non-proliferated $CD8^+$ T cells. Counts of proliferated and non-proliferated $CD8^+$ T cells were used to calculate the percentage of proliferated cells. Data from three replicates were averaged. Two-tailed, homoscedastic t-tests were used to determine statistical significance with a threshold of $p \leq 0.05$.

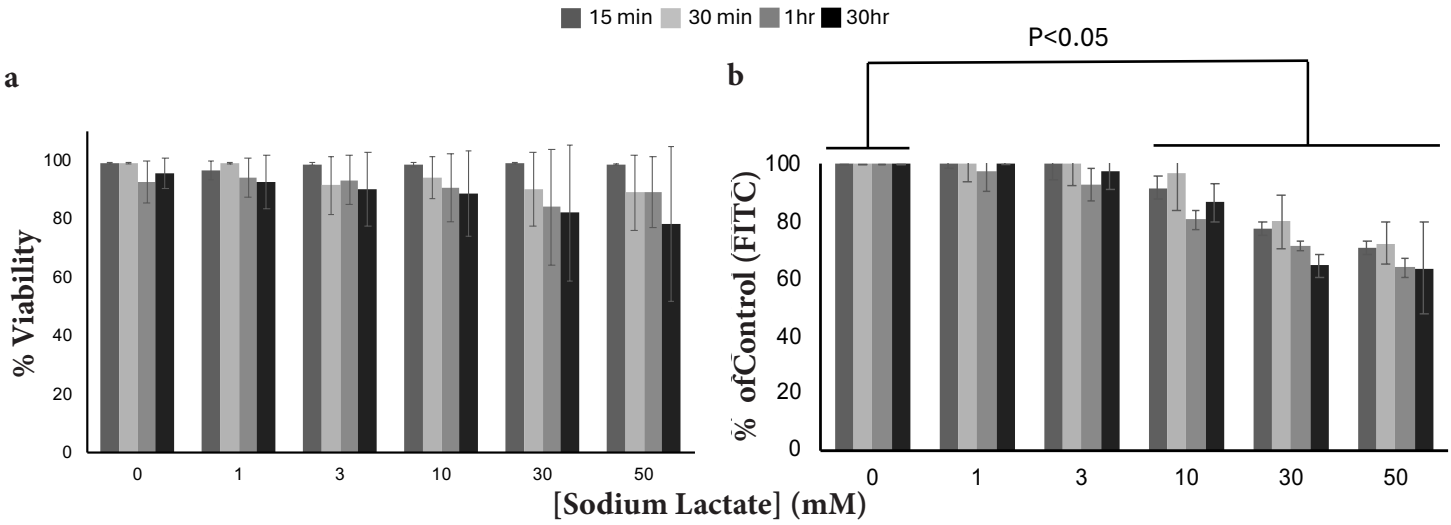

Figure 2 | Exposure to lactate decreases CD3 surface expression, but not viability, of Jurkat E6.1 cells. Jurkat E6.1 cells incubated with different concentrations of lactate for variable exposure times were analyzed via flow cytometry. **(a)** Cell viability was determined via staining with SYTOX AADvanced Ready Flow Reagent. **(b)** CD3 surface expression of live cells was determined via staining with FITC-labelled anti-CD3. Data from three independent experiments is displayed. Statistical significance was determined via two-tailed, homoscedastic t-tests. (Note that the CD3 surface expression in Jurkat E6.1 cells exposed to 10 mM lactate for 30 minutes did not differ significantly from the vehicle control.)

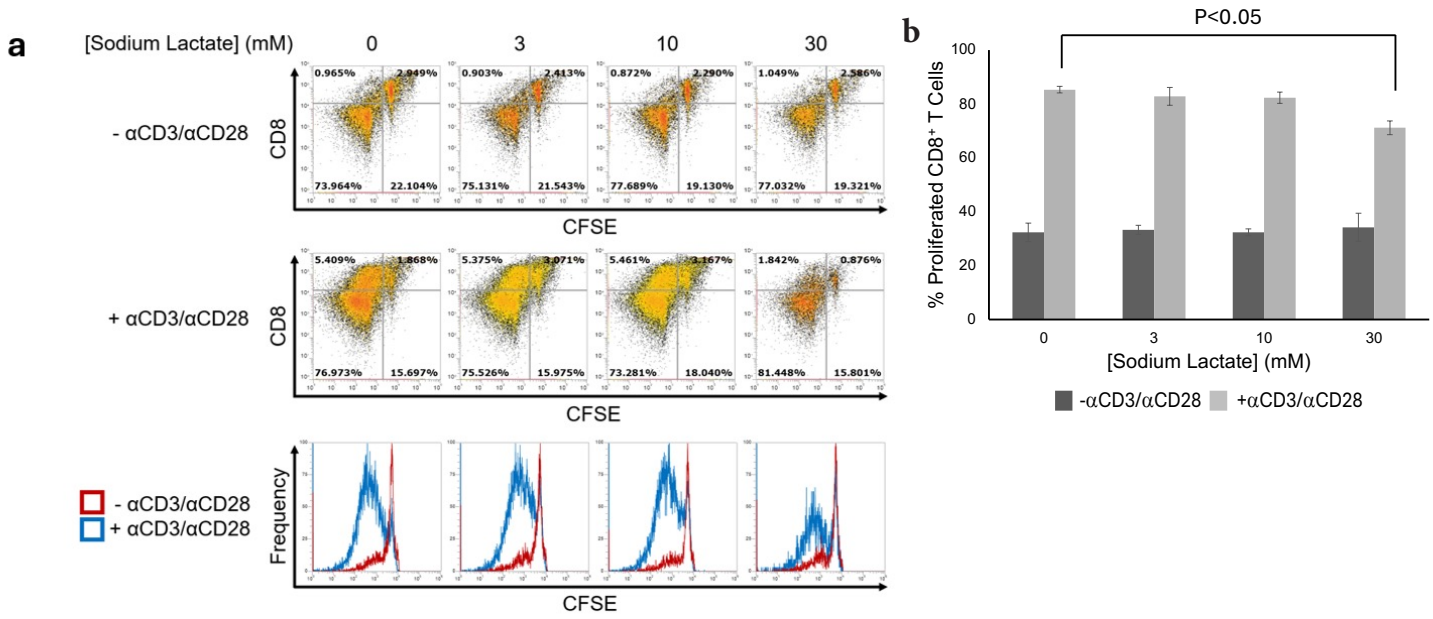

Figure 3 | Exposure to lactate impairs the proliferation of primary CD8+ T cells.
Proliferation of primary leukocytes activated via CD3/CD28 costimulation and incubated with different concentrations of lactate was analyzed via flow cytometry. (a) Top panel displays density plots of CD8 vs. CFSE for live leukocytes that were left unstimulated. Middle panel displays density plots of CD8 vs. CFSE for live leukocytes that were activated via CD3/CD28 costimulation. Bottom plot displays overlays of histogram plots of CFSE for live $CD8^+$ T cells activated via CD3/CD28 costimulation (blue) and unstimulated controls (red). (b) Data from the bottom panel of Figure 3a was replotted as a bar graph to display numerical differences in proliferation among the four treatment groups. Data from three replicates is displayed. Statistical significance was determined via two-tailed, homoscedastic t-tests.

Results

Addition of sodium lactate to complete cell culture media does not change pH

The goal of the present study was to assess the effects of lactate on the immune responsiveness of T cells independent of changes in the pH of the tumor microenvironment. To verify that the addition of sodium lactate to complete cell culture media does not change the pH of the media, we measured the pH of complete cell culture media containing the following concentrations of lactate: 0, 1, 3, 10, 30, and 50 mM. For sake of comparison, we also measured the pH of complete cell culture media containing 0, 1, 3, 10, 30, and 50 mM lactic acid. While lactic acid acidified the cell culture media at concentrations of 10 mM and above, sodium lactate did not significantly affect the pH of the cell culture media at all concentrations tested (**Figure 1**).

Exposure to lactate decreases CD3 surface expression, but not viability, of Jurkat E6.1 cells

Having established that lactate does not affect the pH of cell culture media, we next wanted to assess whether exposure to lactate decreases T cell viability and CD3 surface expression, using Jurkat E6.1 cells (an immortalized CD4+ T cell line) as a model. CD3 is an important part of the TCR complex and is vital for TCR signaling.4 CD3 surface expression in Jurkat E6.1 cells was previously verified by our lab.14 However, Jurkat E6.1 cells have been known to down-regulate CD3 surface expression over time in culture (Jon Houtman, personal communication), so we assessed CD3 surface expression of our Jurkat E6.1 cell cultures periodically via flow cytometry to ensure strong expression (data not shown). To model exposure of T cells to lactate in a TME, we exposed Jurkat E6.1 cells to complete cell culture media containing different concentrations of lactate. 1 and 3 mM lactate simulated normal physiological concentrations of lactate; 10, 30, and 50 mM lactate simulated lactate concentrations that might be found in a TME.13 0 mM lactate was used as a vehicle control. We incubated the Jurkat E6.1 cells with the lactate for four different lengths of exposure—15 minutes, 30 minutes, 1 hour, and 30 hours—to simulate infiltration of circulating and tissue-resident T cells in the TME.21 Following incubation, the cells were collected and analyzed via flow cytometry. Jurkat E6.1 cell viability remained high (>80% viable) following exposure to all lactate concentrations and all exposure times tested (**Figure 2a**). However, there was a significant decrease in CD3 surface expression in Jurkat E6.1 cells exposed to lactate concentrations of 10 mM and above (Figure 2b). This decrease was observable following 15-minute exposure times but was more pronounced following longer exposures. However, CD3 surface expression in Jurkat E6.1 cells exposed to 10 mM lactate for 30 minutes did not differ significantly from the vehicle control, possibly due to variability among repeats.

Exposure to lactate impairs the proliferation of primary CD8+ T cells

Considering the vital role of CD3 in TCR signaling, we wanted to investigate the implications that the lactate-induced decrease in CD3 surface expression had for T cell proliferation, a downstream effect of TCR signaling.3 Jurkat E6.1 cells already proliferate rapidly without stimulation, so primary T cells were used for this experiment. Leukocytes were isolated from whole blood collected from a healthy adult male human volunteer. Normal counts of peripheral blood mononuclear cells (PBMCs) in the volunteer's blood were veri-

fied via flow cytometry (data not shown). T cells were determined to strongly express CD3 and either CD4 or CD8 (data not shown). To model the stimulation of T cells in a TME, we activated primary T cells via CD3/CD28 costimulation in media containing different concentrations of lactate. CFSE was used to track cell proliferation. Following a four-day incubation, the cells were collected, and viability and proliferation of CD8+ T cells were analyzed via flow cytometry. Similar to the Jurkat E6.1 cells, the primary cells displayed no significant changes in viability across all lactate concentrations tested (>90% viable, data not shown). CD8+ T cells stimulated in media with lactate concentrations of 10 mM and below proliferated normally, but CD8+ T cells stimulated in media with 30 mM lactate demonstrated impaired proliferation (**Figures 3a and 3b**).

Discussion

T cells play a vital role in the body's defense against abnormal cells, including cancer cells.[3] T-cell-based immunotherapies—such as CAR-T cell therapy—have been explored as treatments for cancer, when the body's natural immune response is ineffective in eradicating malignant cells. While immunotherapies like CAR-T cell therapy have demonstrated success in treating hematological malignancies,[3] they have shown less promise in treating solid tumors,[7] necessitating research into how solid tumors evade the immune system and immunotherapies. The tumor microenvironments found in and around solid tumors have commonly been reported to be acidic and to contain high concentrations of the metabolic byproduct lactate, due to the Warburg effect.[13] Considering previous findings that exposure to acidic environments potentially impacted the immune responsiveness of T cells,[14] and that tumor-derived lactate impacted the phenotypes of immune cells like natural killer cells and dendritic cells,[15,16] recent evidence points to the Warburg effect as a possible explanation for the ability of solid tumors to evade the immune system and immunotherapies. In the present study, we assessed whether lactate could impact the immune responsiveness of T cells independent of a change in pH by exposing primary and immortalized T cells to different concentrations of lactate simulating both normal physiological conditions and tumor microenvironments. We first verified that the addition of lactate to complete cell culture media does not change the pH of the media. We then assessed the effects of lactate on the viability and CD3 surface expression of T cells, using Jurkat E6.1 cells as a model. While lactate had no significant effect on Jurkat E6.1 cell viability, high concentrations of lactate significantly decreased CD3 surface expression following both short (15 minutes) and long (30 hours) exposures. We investigated the implications of this finding on T cell proliferation, using primary $CD8^+$ T cells as a model. We found that, following CD3/CD28 costimulation and a four-day incubation, primary $CD8^+$ T cells treated with 10 mM lactate or less proliferated normally, whereas cells treated with 30 mM lactate proliferated significantly less

Future Directions

This study suggests that lactate—independent of a change in pH—could impact CD3 surface expression and proliferation of T cells. This corroborates the findings of other studies regarding the immunosuppressive effects of tumor-derived lactate. However, it would be interesting to further characterize the effects of lactate on the immune responsiveness of T cells—namely, to assess the effects of lactate on TCR signaling itself and downstream effects of TCR signaling, such as cytotoxicity and cytokine production. Additionally, while the mechanism by which cancer cells produce lactic acid and efflux it into the tumor microenvironment is well understood, the mechanisms by which lactate impacts immune responsiveness have not been characterized. Tracing these mechanisms may uncover potential targets for therapeutics that could be used to improve the body's natural immune response or increase the efficacy of immunotherapies against solid tumors.

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Author's Biography

Kaitlyn Sy is a senior in the Biotechnology program at the University of Kansas Edwards Campus. After completing her undergraduate degree, she will pursue her M.D. at the University of Kansas School of Medicine. She is devoted to improving outcomes for cancer patients through research, clinical practice, and community service.

Author's Contributions

K.A.S. contributed to the experimental work, design, and writing of this work; R.L.L. contributed to the design and editing of this work; J.F.T. contributed to the design and editing of this work.

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