ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24AUG1279

Citrate Synthase is Required for the Initiation of PTEN-Deficient T-Cell Acute Lymphoblastic Leukemogenesis, but not PTEN-Deficient Myeloproliferative Neoplasms

[1]Rohan Singhal
[1]Children's Research Institute, UT Southwestern Medical Center
Dallas, Texas, 75390, USA

Abstract:- The "Warburg Effect" marks the metabolic tendencies of cancer cells to primarily utilize aerobic glycolysis over oxidative phosphorylation. However, a comprehensive understanding of the interplay between altered tricarboxylic acid (TCA) cycle activity and mitochondrial dysfunction in the initiation and progression of cancer remains elusive. Particularly in T-cell acute lymphoblastic leukemia (T-ALL), an aggressive hematologic malignancy derived from immature T-cell progenitors, the role of aerobic glycolysis in leukemogenesis warrants further investigation. The PTEN gene is one of the most frequently mutated/functionally-inactivated onco-suppressors in various cancers, and PTEN mutations account for 16% percent of all T-ALL cases. In this research, I wanted to investigate if changes to aerobic respiration would initiate the leukemogenesis of PTEN-deficient T-ALL and contradict the Warburg effect, through the removal of the citrate synthase gene, a catalytic metabolite of the TCA cycle.

Mx1-Cre conditional knockout of citrate synthase (CS), a catalytic metabolite of the TCA cycle, and PTEN, an oncosuppressor often linked to T-ALL, were employed to evaluate T-ALL in wild-type, single knockout (PTEN -/-) and double knockout (CS -/-; PTEN -/-) mouse models. T-ALL-related lymphocyte concentrations were analyzed through flow cytometry of thymus, bone marrow, blood, and spleen cell samples bound to respective antibody cocktails, followed by tissue composition analysis and supplemental western blotting. Aberrant levels of T-ALL-associated lymphocytes amongst solely PTEN-deficient samples, yet a return to normalcy with CS deficiency, indicates the requirement of citrate synthase for PTEN-deficient T-ALL progression. Individual t-tests on CD3+CD4+CD8+ cells, amongst other T-ALL-associated lymphocytes, confirm significance in reduced leukemogenesis (p-value < 0.01). However, analysis of PTEN;CS-deficient myeloid cells indicates insufficient blockade of PTEN-deficient myeloproliferative neoplasms, presumably from impaired RBC maintenance post-CS deletion. This research demonstrates that the sequential context achieved via citrate synthase deletion undermines leukemogenesis and contrasts pre-existing ideals about the metabolic tendencies of cancer cells, therefore implying the dual reliance of cancer proliferation on both aerobic and anaerobic metabolic processes.

Keywords:- *Warburg Effect, Tricarboxylic Acid Cycle, T-ALL, Citrate Synthase, PTEN-Deficiency, Flow Cytometry, Leukemogenesis, Aerobic, Anaerobic, Metabolic Processes.*

I. INTRODUCTION

The "Warburg Effect" describes a fundamental shift in cancer cell metabolism, wherein cells predominantly engage in aerobic glycolysis rather than oxidative phosphorylation, even in the presence of ample oxygen. This metabolic reprogramming has been widely observed in various malignancies and underscores the metabolic plasticity of cancer cells. Despite extensive research into the Warburg Effect, the intricate dynamics between aerobic glycolysis and the tricarboxylic acid (TCA) cycle in cancer initiation and progression remain poorly understood.

T-cell acute lymphoblastic leukemia (T-ALL) represents a particularly aggressive form of leukemia originating from immature T-cell progenitors. The disease is characterized by its rapid progression and poor prognosis, often associated with genetic mutations in critical oncogenes and tumor suppressors. Among these, the PTEN gene stands out as a frequently mutated or functionally inactivated tumor suppressor, with mutations present in approximately 16% of T-ALL cases. PTEN loss disrupts cellular signaling pathways that normally restrain cell proliferation and survival, thereby contributing to leukemogenesis.

Given the pivotal role of PTEN in T-ALL, it is crucial to explore how alterations in metabolic pathways, such as those governing aerobic respiration, influence the disease. The TCA cycle, a central component of aerobic metabolism, has often been overshadowed by the emphasis on glycolysis in cancer research. To address this gap, our study focuses on the role of citrate synthase (CS), an enzyme essential for the TCA cycle, in PTEN-deficient T-ALL. By employing Mx1-Cre conditional knockout models to selectively delete CS

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24AUG1279

and PTEN, we aimed to elucidate how perturbations in aerobic metabolism affect T-ALL progression.

This investigation is driven by the hypothesis that disrupting citrate synthase will impact the leukemogenic potential of PTEN-deficient cells, potentially challenging the established metabolic paradigms associated with the Warburg Effect. Past research deleting pyruvate dehydrogenase, a precursor to the TCA cycle, showed obstruction to the initiation of PTEN-deficient T-ALL. This followed the Warburg Effect as changes made to aerobic glycolysis led to changes in cancer progression. Now, through a comprehensive analysis of T-ALL-related lymphocytes and various tissue samples, we seek to uncover the metabolic dependencies of PTEN-deficient leukemogenesis and highlight the importance of aerobic processes in cancer biology. Findings may reveal new insights into the metabolic vulnerabilities of T-ALL and offer potential avenues for therapeutic intervention.

II. METHODS

➢ *Mx1-Cre Conditional Knockout*

We used this genetic tool to delete PTEN and Citrate synthase in the respective mice by inducing the expression of the Cre recombinase enzyme under the control of the Mx1 promoter. The mice were engineered to express either Mx1+/+ (Wild Type) or Mx1 Cre/+ (knockout). All mice were injected with five intraperitoneal injections of 60 µg polyinosinic:polycytidylic acid (pIpC) every other day at 7 weeks of age to induce deletion and were analyzed at 10-12 weeks of age.

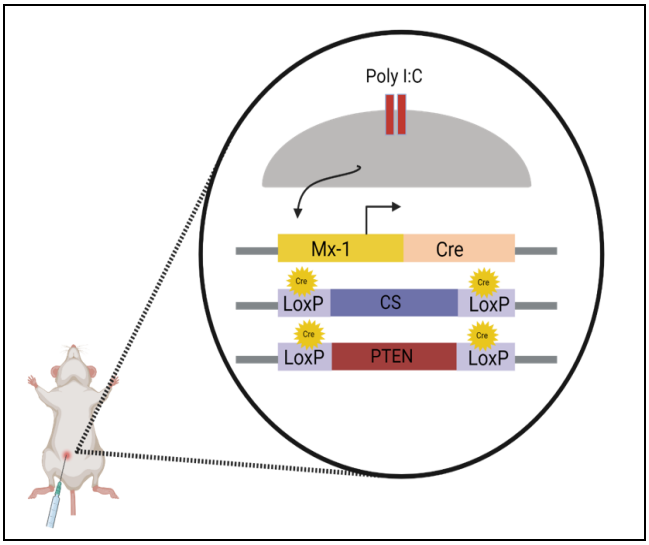

Fig 1 Expanded Molecular Diagram of Mx1-Cre mediated excision of the citrate synthase and PTEN gene to generate a conditional KO mouse model. *Cre*-mediated excision on conditional knockout mice was used due to its superiority as a mechanism in studying malignant hematopoiesis.

➢ *Cell Isolation and Tissue Composition Analysis*

Three sets of mice were used for this experiment: wild-type, single knockout (PTEN -/-), and double knockout (CS -/-; PTEN -/-) mouse models. T-cell Acute lymphoblastic leukemia develops in the bone marrow and matures in the thymus before spreading to the bloodstream and other tissue such as the spleen. In order to examine cancer prevalence in each of these mice, we dissected all mice for: femur (bone marrow), thymus, spleen, and blood samples. After taking the weight of the spleens and thymuses, a mortar and pestle were used to isolate cells from the femur, and glass slides were used to isolate cells from the spleens and thymuses. Hank's balanced salt solution aided in this process and a centrifuge was used to resuspend cells. Then, cell counts were taken using a sample processor for each tissue as well as a red blood cell count from the blood samples.

➢ *Flow Cytometry Analysis*

Once isolated, cell samples were bound to fluorescent antibody marker cocktail solutions that would distinguish between lymphocytes associated with T-ALL and reflect different light colors in a flow cytometry scan. We used this process to identify and quantify specific T-cell populations (CD3+, CD4+, CD8+) and create individual plots per sample. FlowJo quantitative population comparison gating was used to differentiate between particular cell types, and Microsoft Excel was used for significance testing with T-tests between cell populations of wild type, PTEN knockout, and PTEN/CS knockout.

➢ *Western Blotting*

In order to ensure knockout of citrate synthase was successful, supplemental western blotting was conducted using vinculin-HRP as a protein control. With remains from the previous cell samples, trichloroacetic acid and acetone were used for the precipitation of proteins, 112.5 µL solubilization buffer was used for protein resuspension, and 37.5 µL Laemmli buffer was used for SDS gel electrophoresis protein isolation. Protein bands were detected using enhanced chemiluminescence (ECL) and visualized on X-ray film, with band intensities matched to the expected molecular weights of both citrate synthase and vinculin to analyze knockout.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24AUG1279

III. RESULTS AND DISCUSSION

The presence of protein bands in all five vinculin-HRP samples but in only the wild-type samples for Citrate Synthase confirms the successful procedure of mx1-cre conditional knockout (Fig. 2).

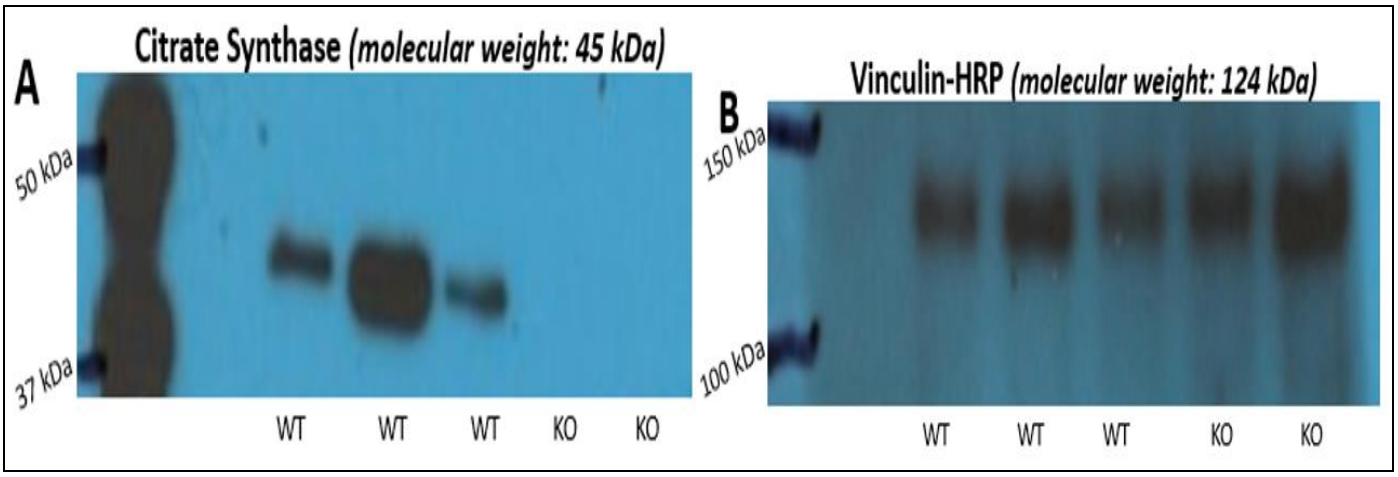

Fig 2 Western Blots testing for (A) citrate synthase deletion and (B) the loading control Vinculin-HRP; Because the citrate synthase knockout samples do not show protein bands, the absence of the CS protein is assumed.

Once the cell isolation and tissue composition analysis were completed, we were able to obtain a general idea of where cancer was present prior to our cytometry analysis. Larger thymus weights in the PTEN-deficient samples than the wild type and PTEN;CS-deficient samples signify that tumorigenesis may have occurred in the PTEN-deficient samples but not the rest (Fig. 3). In the spleen, however, tumorigenesis was presumed to have occurred in both PTEN-deficient and PTEN;CS-deficient samples (Fig. 4).

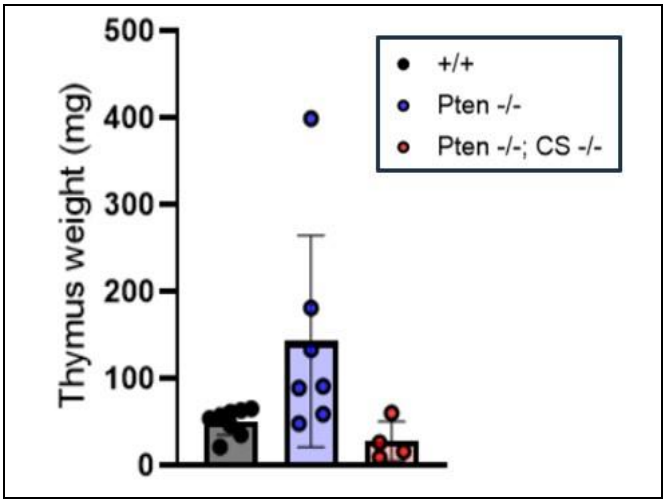

Fig 3 Weight of thymus between wild-type, PTEN-deficient, and PTEN; CS-deficient samples.

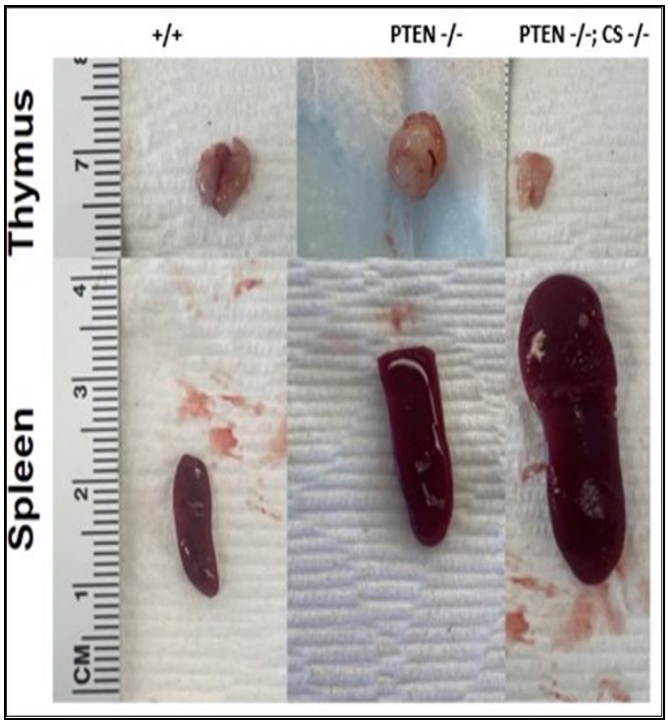

Fig 4 Representative thymus and spleen images among wild-type, PTEN-deficient, and PTEN; CS-deficient samples. Large thymus in PTEN -/- implies T-ALL & large spleen in PTEN -/-; CS -/- implies myeloid neoplasm

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24AUG1279

Based on the results shown in Fig. 3 & 4, we had an initial idea of where cancer may have been present. After binding the antibodies to our isolated cell samples from the tissues of interest, we conducted our flow cytometry analysis. Lighter colors indicate high cell concentration. Our results indicated that concentrations varied between the wild-type samples and PTEN-deficient samples, with immense concentrations of triple positive t-cells indicating T-ALL cancer in the PTEN-deficient samples. However, in the plots where citrate synthase was also deficient, cell concentrations reverted to a more wild-type-like arrangement, indicating that T-ALL was not able to develop. This was most evident in the bone marrow and thymus analysis (Fig. 5 & 6). In the thymus analysis of double positive t-cells specifically, the site of t-cell maturation, both the wild-type and double knockout samples are all CD3 negative but the PTEN-deficient samples are CD3 positive. While T-ALL development was obstructed with the deletion of citrate synthase, however, a close examination of myeloid cell concentrations depicted cancer present there, more specifically myeloproliferative neoplasms. This may have been the root of the tumorigenesis occurring in the spleens. In addition, there may be stunted normal t-cell development occurring.

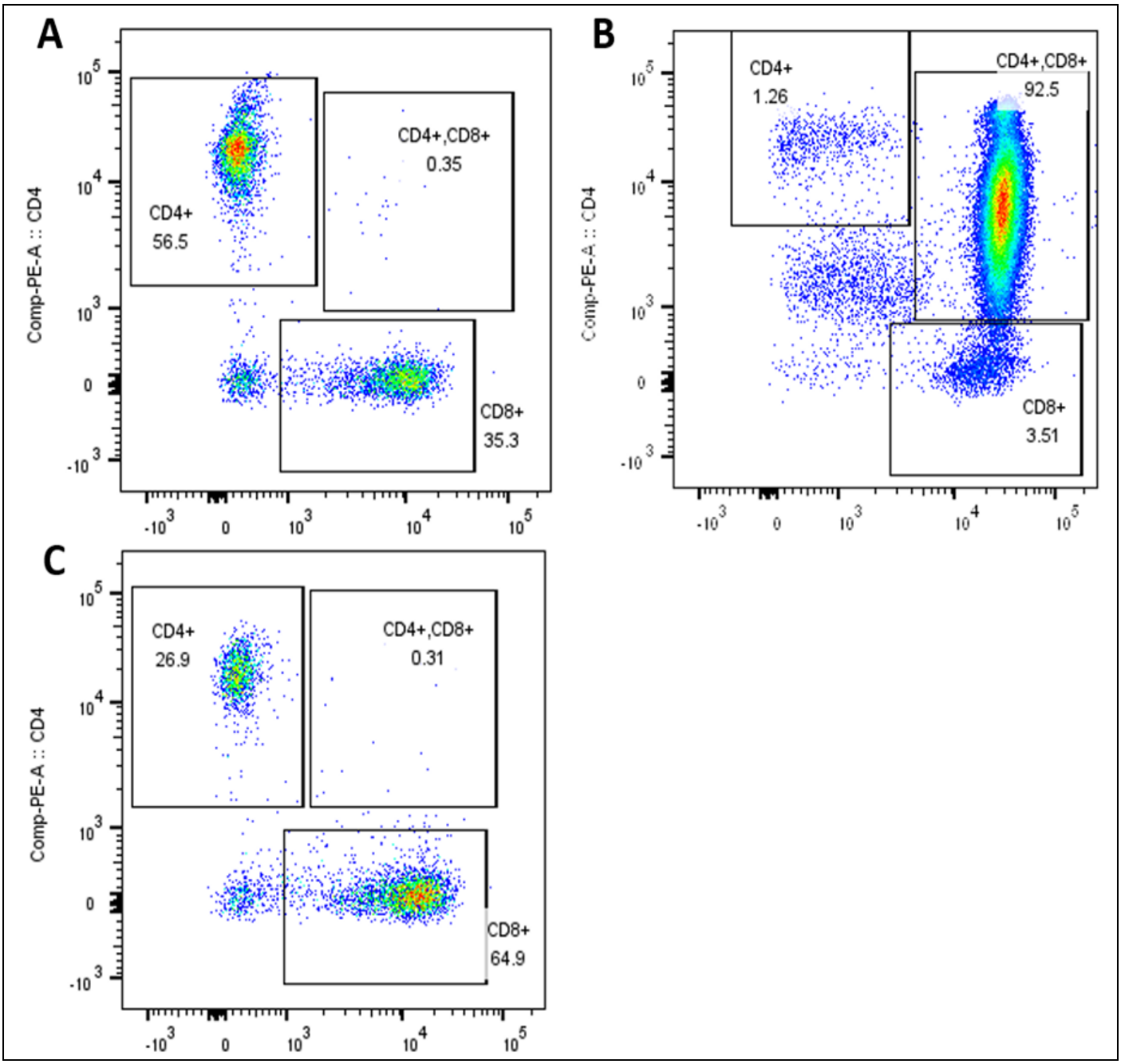

Fig 5 [Bone Marrow]. Comparison of the flow cytometry plots showing CD3+ cell concentrations in the bone marrow detected by the fluorescent CD8 and CD4 antibodies between (A) wild type, (B) PTEN-deficient, and (C) PTEN; CS-deficient samples. A high concentration of CD4+;CD8+ cells is indicative of T-cell acute lymphoblastic leukemia. Because the PTEN- plot portrays a much higher concentration of such cells compared to wild type and PTEN-/CS- cells, it can be inferred that leukemia is reduced in the citrate synthase deficient cells and similar to a WT model.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24AUG1279

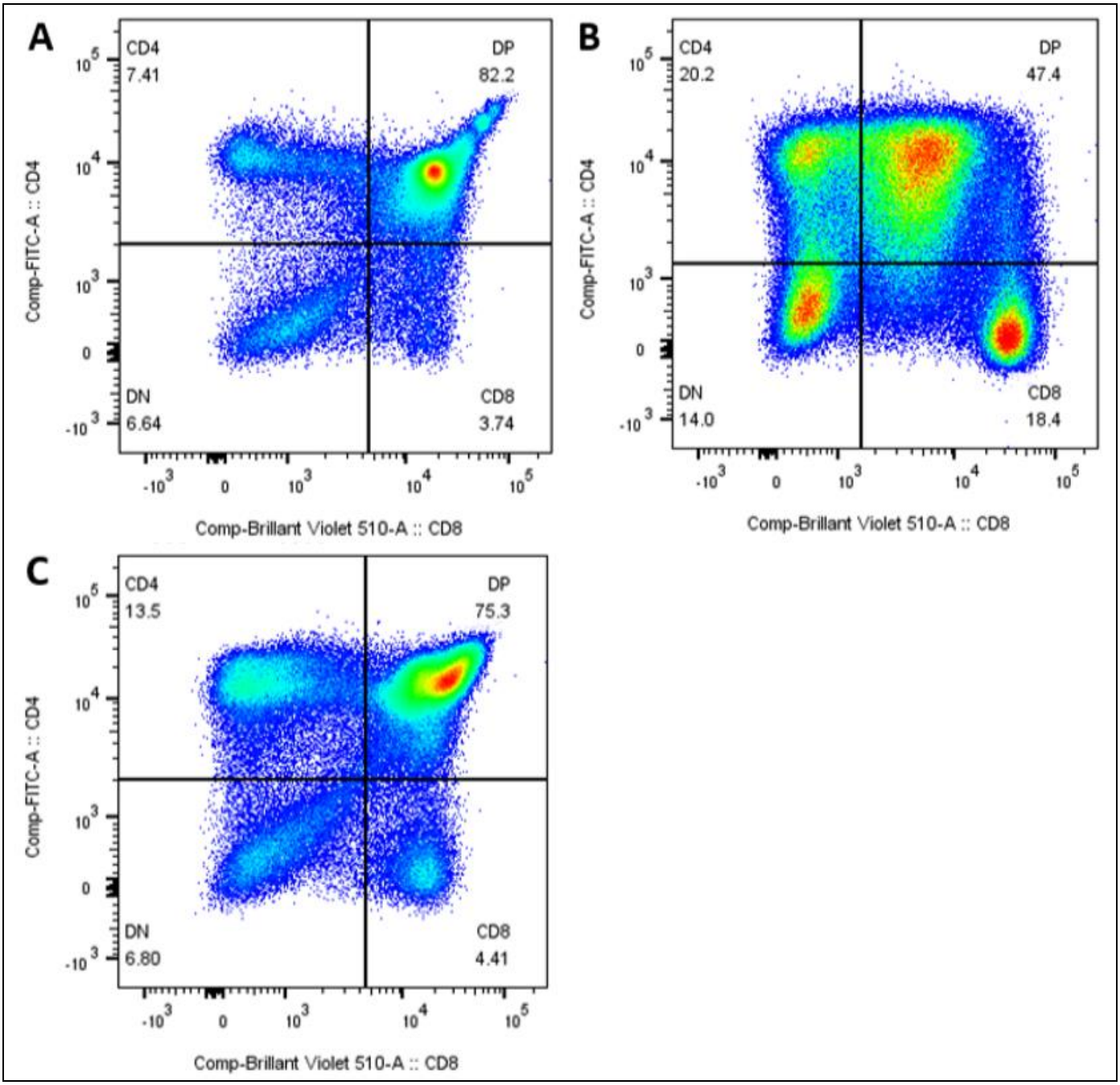

Fig 6 [Thymus]. Comparison of the flow cytometry plots showing Lineage negative cell concentrations in thymus detected by the corresponding fluorescent CD4 and CD8 antibodies between (A) wild type, (B) PTEN-deficient, and (C) PTEN; CS-deficient samples. These plots display distinctions between concentrations of CD4 cells, double negative cells, CD8 cells, and double positive cells. The thymus, site of T-ALL development, should not have any high concentrations of these four cells besides double positive. Because the PTEN -/- plot portrays a much higher concentration of all four cells compared to wild type and PTEN-/CS- cells, it can be inferred that leukemia is reduced in the CS-deficient thymus samples and evidently present in PTEN-deficient thymus samples.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24AUG1279

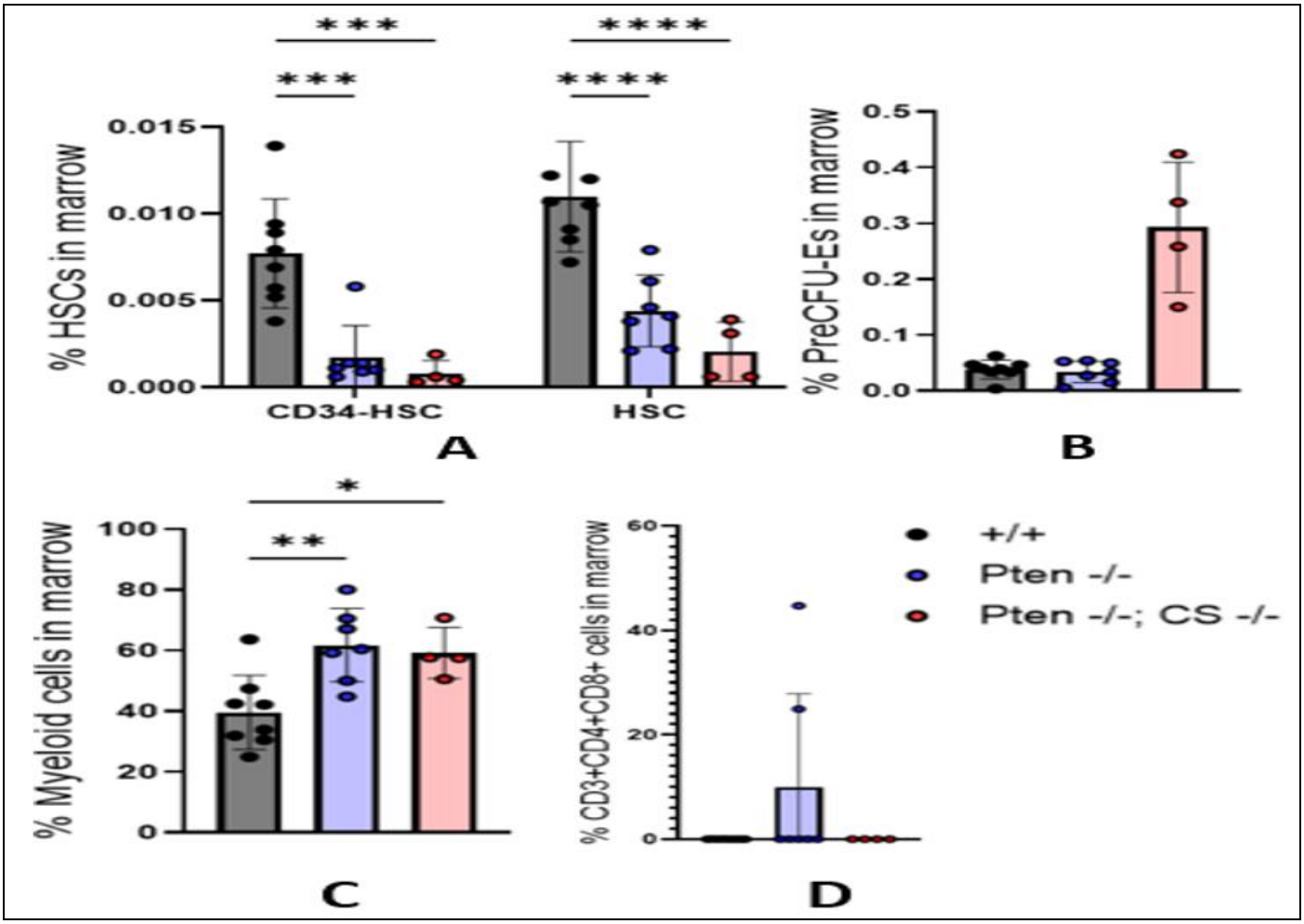

Fig 7 Comparison graphs of (A) the percentage of hematopoietic stem cells (CD34-HSC included), (B) the percentage of Pre-colony-forming unit-erythroid cells, (C) the percentage of myeloid cells, and (D) the percentage of CD3+; CD4+; CD8+ cells detected by their respective fluorescent antibodies within the bone marrow between WT, PTEN-deficient, and PTEN; CS-deficient samples. CD3+; CD4+; CD8+ cell concentrations are indicative of T-ALL, and in graph (D) it is observed that the PTEN-/CS- samples have concentrations similar to the WT model and much lower than the PTEN-deficient cells, consequently signifying absence of leukemia with citrate synthase deficiency. However, the other graphs show irregular HSC & myeloid cell concentrations for PTEN-/CS- samples signifying that although T-ALL has been obstructed, myeloproliferative neoplasms are still able to form.

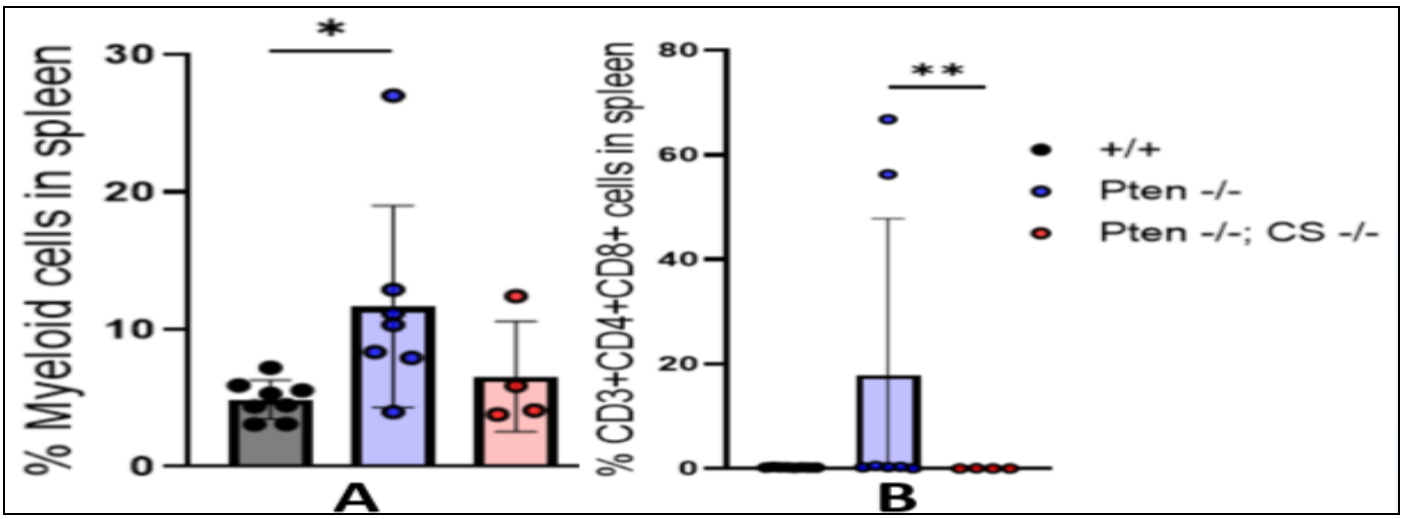

Fig 8 Comparison graphs of (A) the percentage of Myeloid cells in the spleen detected by the Mac-1 fluorescent antibody and (B) the percentage of CD3+, CD4+, and CD8+ cells in the spleen detected by the corresponding fluorescent antibodies between wild type, PTEN-deficient, and PTEN; CS-deficient samples. Cell concentration levels are indicative of reduced cancer in double KO compared to single KO.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24AUG1279

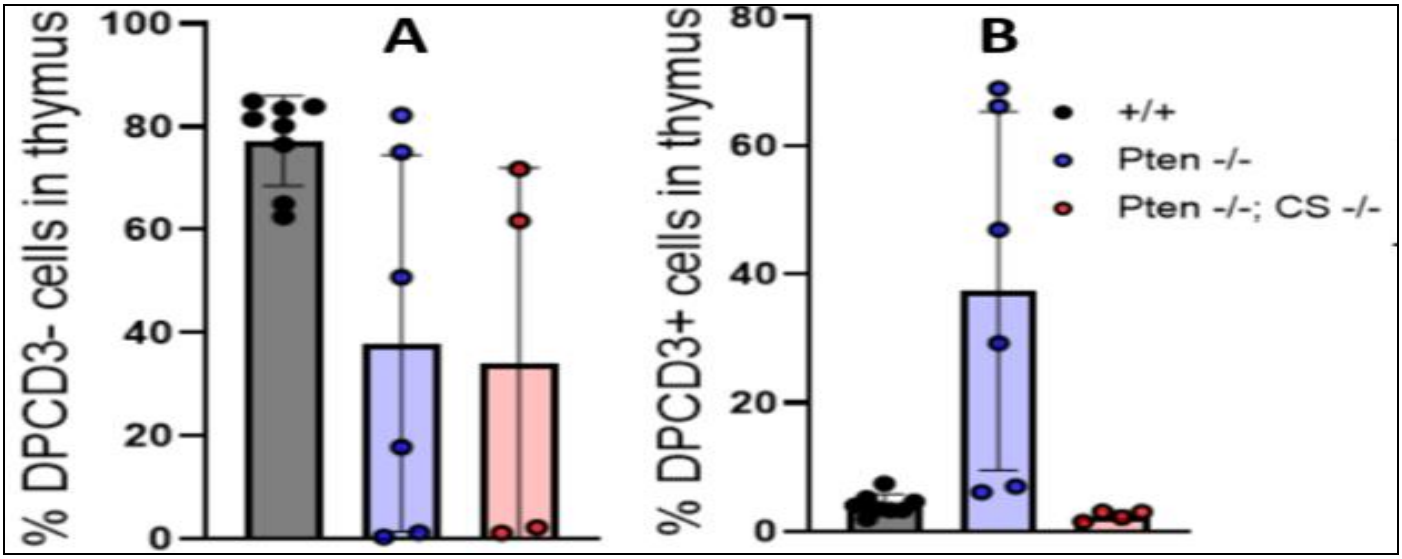

Fig 9 Comparison graphs of (A) the percentage Double positive CD3- cells in the thymus detected by the corresponding fluorescent antibody and (B) the percentage of double-positive CD3+ cells in the thymus detected by the corresponding fluorescent antibody between wild type, PTEN-deficient, and PTEN; CS-deficient samples. Cell levels in DPCD3+ indicate normalcy among WT and PTEN-/CS- and irregularity in PTEN-deficient samples.

IV. CONCLUSIONS

In this project, I investigated whether altering aerobic respiration by deleting the citrate synthase gene in PTEN-deficient T-cell acute lymphoblastic leukemia (T-ALL) might contradict the Warburg Effect and contribute to leukemogenesis. Using flow cytometry, screening of lymphocyte concentrations was done by examining tissues where T-ALL thrives most, across wild-type, PTEN-deficient, and PTEN; CS-deficient mice. The deletion of citrate synthase in a PTEN-deficient T-ALL setting results in decreased levels of associated CD3, CD4, and CD8 T-cells in the thymus in comparison to an only PTEN-deficient T-ALL model with inflated concentrations of DPCD3+ cells. The flow cytometry analysis shows little difference with myeloid cell concentrations amongst PTEN- and PTEN-/CS- cells, implying myeloproliferative neoplasms can still occur despite citrate synthase absence. With the deletion of citrate synthase obstructing the full potential of the TCA pathway and glucose metabolism, cancer development is less likely to initiate. In conclusion, the deletion of citrate synthase impedes the ability of PTEN-deficient T-cell acute lymphoblastic leukemia to form, but not for PTEN-deficient myeloproliferative neoplasms. As a next step, it will be helpful to further investigate what other metabolites are now fueling the initiation and progression of myeloproliferative neoplasms if glucose is no longer the main contributor to cancer metabolism with the removal of citrate synthase.

ACKNOWLEDGEMENT

The author wishes to acknowledge Science teachers and mentors who helped him with this project.

REFERENCES

[1]. Martelli AM, Paganelli F, Fazio A, Bazzichetto C, Conciatori F, McCubrey JA. The Key Roles of PTEN in T-Cell Acute Lymphoblastic Leukemia Development, Progression, and Therapeutic Response. Cancers (Basel). 2019 May 6;11(5):629. doi: 10.3390/cancers11050629. PMID: 31064074; PMCID: PMC6562458.

[2]. Jun S, Mahesula S, Mathews TP, Martin-Sandoval MS, Zhao Z, Piskounova E, Agathocleous M. The requirement for pyruvate dehydrogenase in leukemogenesis depends on cell lineage. Cell Metab. 2021 Sep 7;33(9):1777-1792.e8. doi: 10.1016/j.cmet.2021.07.016. Epub 2021 Aug 9. PMID: 34375613.

[3]. Tesio M, Trinquand A, Ballerini P, Hypolite G, Lhermitte L, Petit A, Ifrah N, Baruchel A, Dombret H, Macintyre E, Asnafi V. Age-related clinical and biological features of PTEN abnormalities in T-cell acute lymphoblastic leukaemia. Leukemia. 2017 Dec;31(12):2594-2600. doi: 10.1038/leu.2017.157. Epub 2017 May 25. PMID: 28539671

[4]. Icard P, Coquerel A, Wu Z, Gligorov J, Fuks D, Fournel L, Lincet H, Simula L. Understanding the Central Role of Citrate in the Metabolism of Cancer Cells and Tumors: An Update. Int J Mol Sci. 2021 Jun 19;22(12):6587. doi: 10.3390/ijms22126587. PMID: 34205414; PMCID: PMC8235534.

[5]. Starkova J, Hermanova I, Hlozkova K, Hararova A, Trka J. Altered Metabolism of Leukemic Cells: New Therapeutic Opportunity. Int Rev Cell Mol Biol. 2018;336:93-147. doi: 10.1016/bs.ircmb.2017.07.012. Epub 2017 Sep 18. PMID: 29413894.