Reprogramming of Steroid Metabolism in Natural Killer Cell Activation via Lipids

Engineering Honors Program

Capstone Project Final Report

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal and highly treatment-resistant type of pancreatic cancer. It accounts for 90% of all cases of pancreatic cancer[1] and has a five year survival rate of only 6%[2]. PDAC affects the cells that line the ducts of the pancreas, or pancreatic ductal cells. Ductal cells form the epithelial lining of the ducts that deliver digestive enzymes to the small intestine.[3] Once cancer begins to affect the function of these cells, the patient typically experiences weight loss, abdominal pain, and jaundice.[1] Although classic treatments such as radiation, chemotherapy, and surgery have largely been ineffective at lowering the mortality rate associated with PDAC, our body has innate features that have the potential to help fight this disease.

Natural Killer (NK) cells are a vital part of the innate immune system. They are a type of immune cell coming from the lymphocyte family, which also includes B-cells and T-cells. Originating in the bone marrow, NK cells are present all over the body, in the lymph nodes, spleen, tonsils, and thymus.[4] Once they have matured, they are released into the bloodstream to begin surveillance for harmful cells. NK cells scan cells in the body for markers that indicate if they are healthy or diseased. Specifically, NK cells focus on cells that were once healthy, but now could cause harm to the body, namely cells infected by a virus or cancer cells.[4] This role makes NK cells pivotal in the body's fight against cancer.

In this project, we have focused on gaining an increased understanding of NK cell metabolism. According to the National Cancer Institute, cellular metabolism is the "sum of all chemical changes that take place in a cell through which energy and basic components are provided for essential processes".[5] Essentially, we want to have a deeper understanding of the metabolic processes that contribute to the ability of NK cells to fight and kill cancer.

The NK cell metabolic processes that we zeroed in on are steroid and lipid metabolism. It is known that NK cells use a reprogrammed metabolism based on a gene called sterol regulatory element-binding protein 2 (SREBP2), which is a master regulator of sterol and fatty acid synthesis. Sterols are a subgroup of steroids, so both are metabolized in a similar way—via the mevalonate pathway.

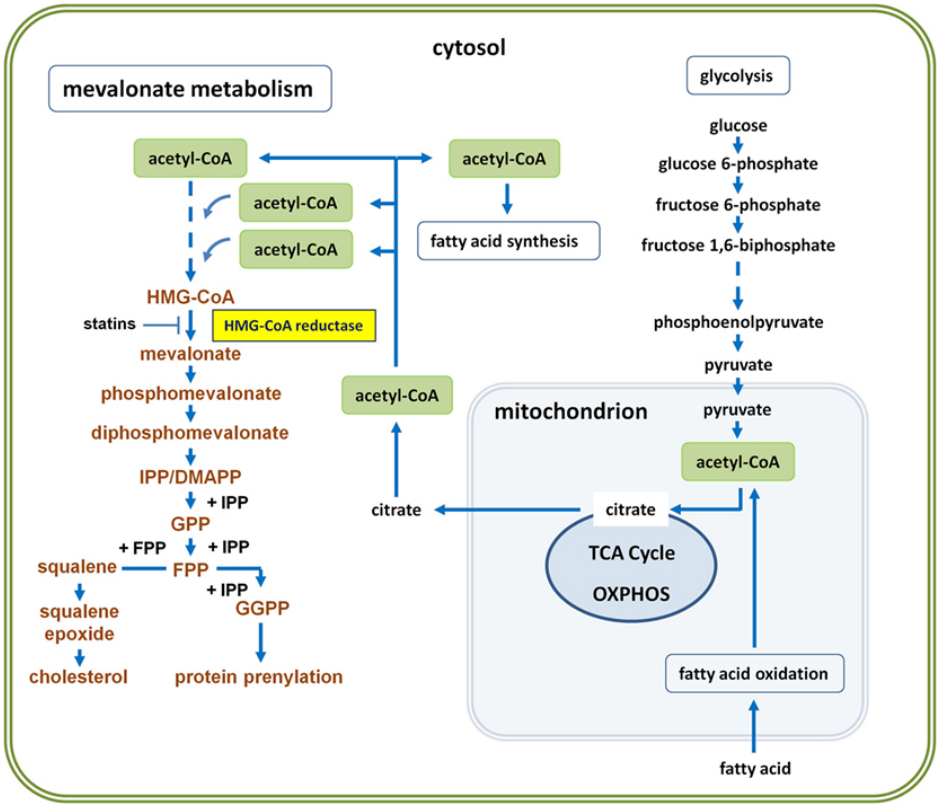

Figure 1: Diagram showing the connection between glycolysis, the citric acid cycle, and the mevalonate pathway.[6] *Reprinted from "*Mevalonate Metabolism in Immunology*" by G. Gruenbacher and M. Thurnher, 2017, Frontiers in Immunology*

In the schematic of the mevalonate pathway in Figure 1, it is shown that the building block of the pathway is acetyl-CoA, a metabolite that is derived from glucose in the citric acid cycle. Additionally, the mevalonate pathway is involved in protein prenylation, which is the addition of specific carbon chains to proteins.[7] Prenylation is a critical step for proteins to target

and bind to the membrane of the cell, which is vital to NK cell function.[8] Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are important molecules for successful protein prenylation. Another important note is that the addition of statins inhibits HMG-CoA reductase, which therefore inhibits the function of the whole mevalonate pathway and subsequent protein prenylation. In modern medicine, statins are typically used to lower cholesterol levels, as cholesterol is synthesized via the mevalonate pathway.

Another important feature of NK cells that is related to these metabolic pathways is the peroxisome. Peroxisomes are small, membrane-bound organelles that are present in virtually all eukaryotic cells. They are involved in oxidative processes using molecular oxygen.[9] Specifically, they have been found to play a vital role in the oxidative degradation of unsaturated fatty acids, which may relate to sterol metabolism.

Overall, through the course of this project, we wanted to gain an understanding of the connection between glucose, the citric acid cycle, the mevalonate pathway, statins, lipid synthesis, and protein prenylation. We investigated how these different pathways connect and how the addition of various treatments affected their function.

Objectives

In this project, we wanted to research three different points:

1. Determine the metabolic requirements of NK cells for mevalonate pathway
2. Understand how protein prenylation regulates NK cell activity
3. Investigate how peroxisomes, which metabolize unsaturated fatty acids, relate to cholesterol metabolism

Methods

All of the results collected over the course of this project were found using standard wet lab equipment and protocols listed below. The corresponding figure containing the data collected using the technique is referenced.

- Gas Chromatography-Mass Spectrometry (GC-MS) (Figure 2)
    - Purpose: to identify and measure trace amounts of a specific compound in a sample
- Cytotoxicity Assay (Figure 3)
    - Purpose: to measure the ability of various cytotoxic component to harm or kill cells
- Fluorescence Activated Cell Sorting (FACS) (Figure 4)
    - Purpose: to analyze characteristics of single cells in solution
- Western Blot (Figure 5)
    - Purpose: to detect a specific protein in a sample
- Quantitative Polymerase Chain Reaction (qPCR) (Figure 6)
    - Purpose: to measure and quantify the amplification of target genes in a sample

Each of these methods were performed using standard protocols.

Results

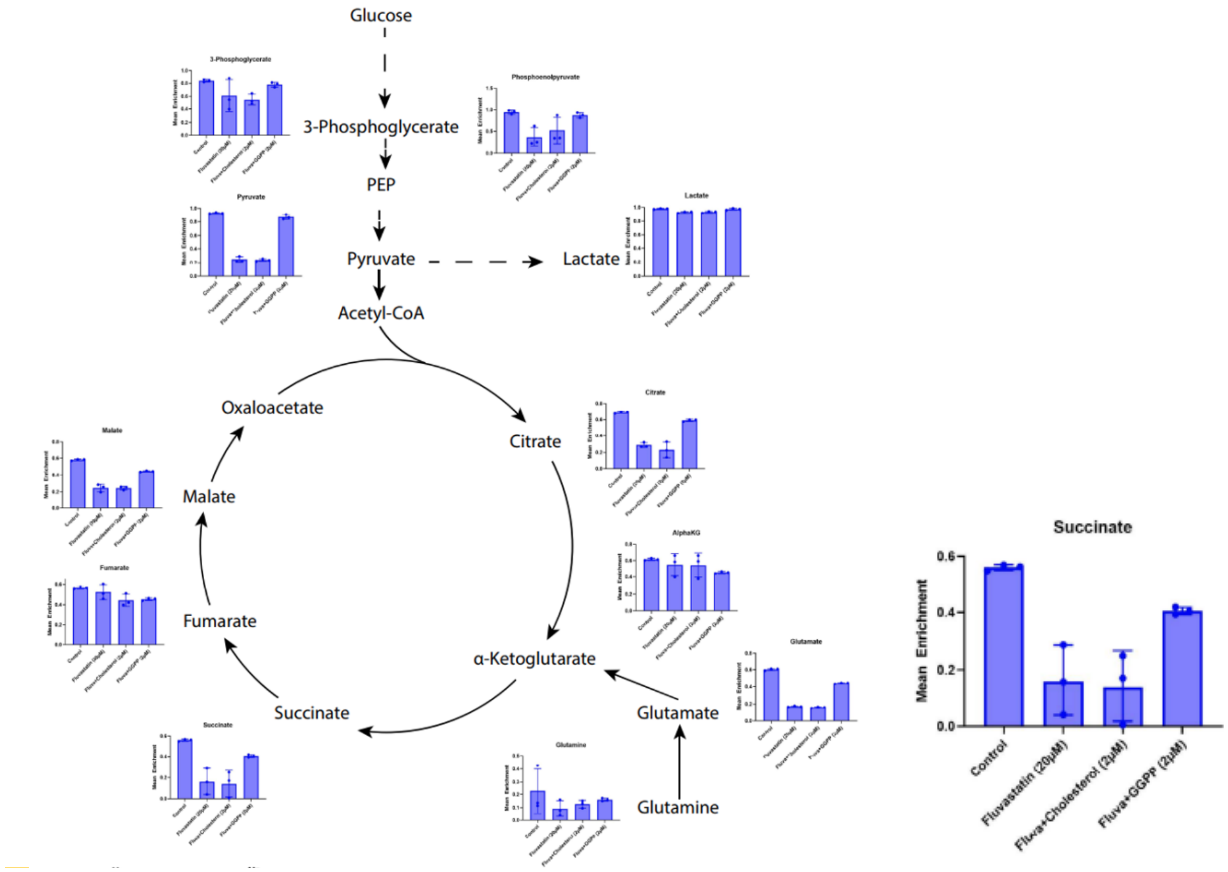

Figure 2: Glucose metabolism is altered by statin treatment in NK cells.

Figure 2 shows GC-MS data that formed the foundation of this project. The figure shows a schematic of the citric acid cycle with mean enrichment glucose tracing data from each corresponding metabolite. Although it is difficult to see the treatments and axes labels, a zoomed-in version of the tracing data for succinate is shown to the right. We used four different treatments on NK cells in this experiment: control, Fluvastatin (20 μM), Fluvastatin + Cholesterol (2 μM), and Fluvastatin + GGPP (2 μM), from left to right on the graph. Fluvastatin is a common kind of statin, which inhibits mevalonate pathway function.

Essentially, these results show that when the NK cells are treated with fluvastatin, glucose incorporation into glycolytic and citric acid cycles is down. Additionally, glucose metabolism is altered by statin treatment in NK cells. In many of the results, glucose mean

enrichment is rescued in the treatment with GGPP, a substrate for protein prenylation. This shows that protein prenylation is an important factor in NK cell function.

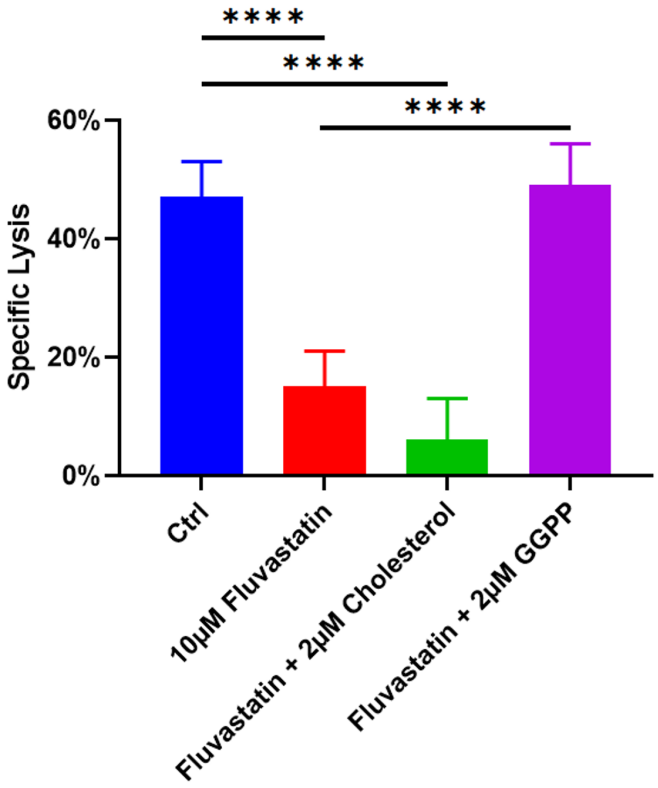

Figure 3: NK cells are dependent upon prenylation substrates for cytotoxicity.

Figure 3 shows data from a cytotoxicity assay, showing the specific lysis percentage of NK cells. Specific lysis is a measure of how well NK cells are monitoring and killing harmful cells. Cytotoxicity is a measure of how harmful an NK cell is to the invading cells. In this experiment, we found that treatment with fluvastatin significantly downregulated NK cell cytotoxicity. However, there was a significant recovery of cytotoxicity when the treatment with GGPP was applied. From this data, we can conclude that the cytotoxicity of NK cells is dependent on prenylation substrates.

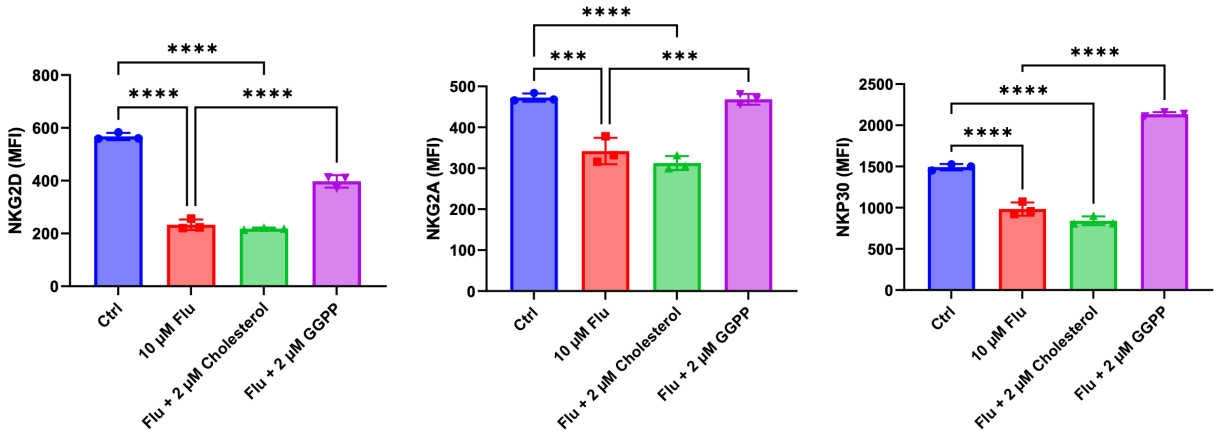

Figure 4: NK cell surface markers are downregulated by statin treatment and rescued by prenylation substrates.

Figure 4 shows data from a flow cytometry (FACS) experiment investigating the mean fluorescence intensity of the surface markers of NK cells. NK cells express a variety of activation markers on their surface. These markers help the NK cell to recognize and attack harmful cells. Surface markers are absolutely essential for an NK cell to kill a cancer cell. NKG2D is an activating receptor on the NK cell surface, NKG2A is an inhibitory receptor, and NKP30 is a cytotoxicity triggering receptor. All are important in the NK immune response.

When investigating how the expression of the surface markers changes under the same treatment conditions, we found that surface markers are downregulated by the statin treatment, but that there is significant rescue by the prenylation substrate. Again, we have identified a dependency on prenylation substrates, and we decided to investigate this further.

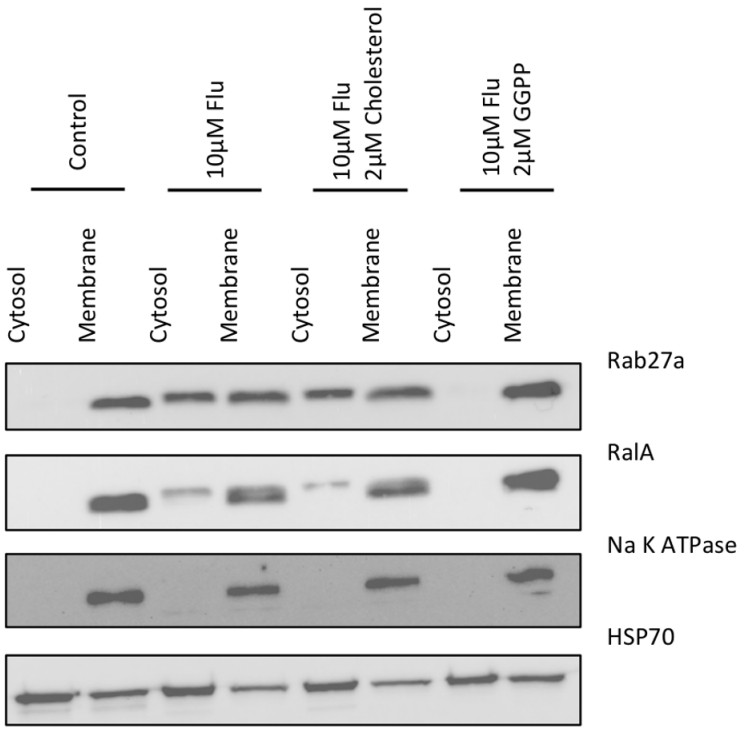

Figure 5: Statin treatment displaces vital trafficking proteins, affecting NK cell function.

Figure 5 shows the results from a Western blot experiment in which we measured the presence of different trafficking proteins in the cytosol and the membrane of NK cells under the same four treatments. As previously mentioned, protein prenylation is a critical step for proteins to bind to the cell membrane, which is vital to a cell's function. In this experiment, Na K ATPase and HSP20 are loading controls, while RalA and Rab27a are NK trafficking proteins. In the control sample, there are no trafficking proteins present in the cytosol, they are all attached to the membrane, meaning they are properly prenylated. Once the treatment with fluvastatin is applied, proteins begin to show up in the cytosol, meaning they did not get properly prenylated and are floating detached in the cell. However, when GGPP is added, the proteins are once again only found in the membrane. This experiment proved that the statin treatment disrupts protein prenylation and displaces vital proteins.

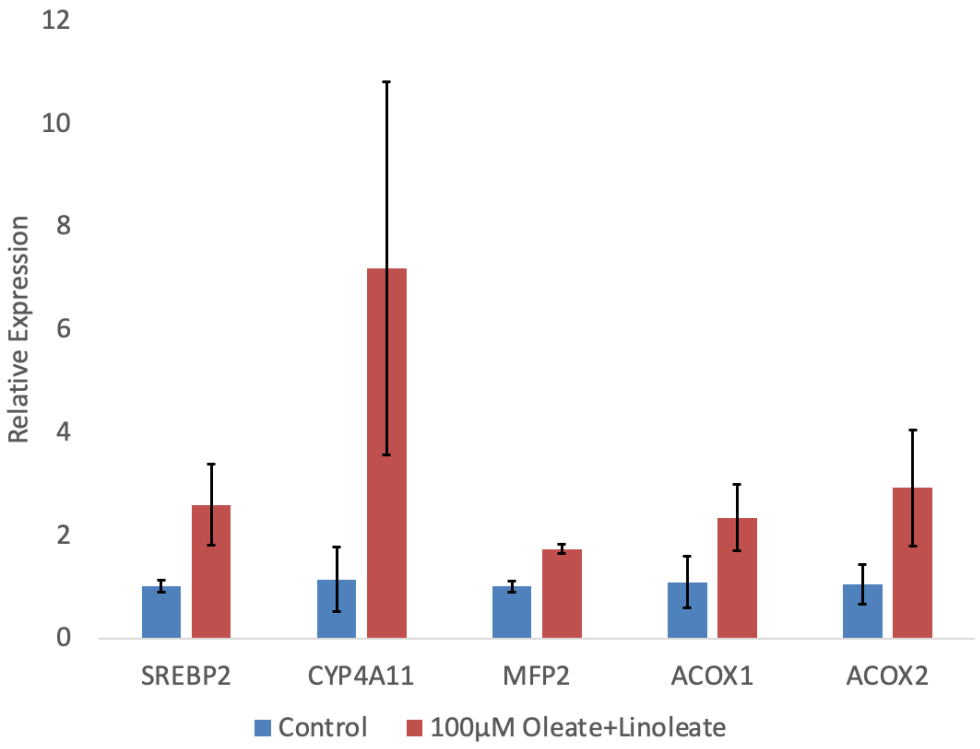

Figure 6: Lipids can regulate peroxisome and cholesterol metabolism genes.

Figure 6 shows results from a qPCR experiment measuring the relative expression of multiple genes found in cholesterol metabolism processes and the peroxisome of NK cells. We used different treatments in this experiment because we wanted to investigate the relationship between lipid metabolism and the peroxisomal function. With this experiment, we wanted to connect our previous results back to cancer and the tumor microenvironment (TME). Lipids, which are present in the TME, may affect pathways responsible for NK cell protein prenylation. Additionally, lipid metabolism changes in the TME.

We decided to measure SREBP2 because it is a master regulator for protein prenylation. CYP4A11 is a target of PPARα, a lipid sensor. MFP2 is involved in peroxisomal fatty acid oxidation. ACOX1 and ACOX2 are peroxisomal acetyl-CoA oxidases. Each gene showed increased expression when treated with lipids, showing that lipids somehow regulate peroxisome

and cholesterol metabolism genes. These results gave us a good target for future work: finding a connection between lipid metabolism and cholesterol metabolism in the TME via the peroxisome.

Discussion and Conclusion

In conclusion, we believe that we have two novel findings. First, protein prenylation is vital for NK cell function. If prenylation is disrupted, the cytotoxicity and ability of the cell to recognize cancer decreases. Second, NK cell function is determined by lipid metabolism. Somehow, lipids play a role in how the NK cell functions, which is important because lipids are present in the tumor microenvironment. Additionally, it appears that treatment with GGPP is a promising method to help keep NK cell activity robust in the tumor microenvironment. In conclusion, we were able to sufficiently complete the first two objectives of the project.

As mentioned above, our future goal is to investigate how peroxisomes connect NK lipid metabolism to cholesterol metabolism in the tumor microenvironment. This objective will help tie all of our previous findings back to our original goal of fighting PDAC. This project has a very promising future and the capacity to have an incredible impact in the world of cancer treatment.

Overall, I think we found some exciting and interesting results that are very promising for the future of the project. I am proud that I was able to learn so much about cancer, NK cells, and general lab techniques. I think this project helped build my communication, critical thinking, and time management skills, all of which will serve me well in my future career.

Acknowledgements

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References

1. Sarantis, P., Koustas, E., Papadimitropoulou, A., Papavassiliou, A. G., & Karamouzis, M. V. (2020). Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy. *World Journal of Gastrointestinal Oncology*, *12*(2), 173–181. https://doi.org/10.4251/wjgo.v12.i2.173
2. Noda, Y., Tomita, H., Ishihara, T., Tsuboi, Y., Kawai, N., Kawaguchi, M., Kaga, T., Hyodo, F., Hara, A., Kambadakone, A. R., & Matsuo, M. (2022). Prediction of overall survival in patients with pancreatic ductal adenocarcinoma: histogram analysis of ADC value and correlation with pathological intratumoral necrosis. *BMC Medical Imaging*, *22*(1). https://doi.org/10.1186/s12880-022-00751-3
3. Grapin-Botton, A. (2005). Ductal cells of the pancreas. *The International Journal of Biochemistry & Cell Biology*, *37*(3), 504–510. https://doi.org/10.1016/j.biocel.2004.07.010
4. *What Are Natural Killer Cells (NK Cells)?* (n.d.). Cleveland Clinic. Retrieved April 24, 2023, from https://my.clevelandclinic.org/health/body/24898-natural-killer-cells
5. National Cancer Institute. (2011, February 2). *https://www.cancer.gov/publications/dictionaries/cancer-terms/def/cellular-metabolism*. Www.cancer.gov. https://www.cancer.gov/publications/dictionaries/cancer-terms/def/cellular-metabolism
6. Georg Gruenbacher, & Thurnher, M. (2017). Mevalonate Metabolism in Immuno-Oncology. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.01714

7. *Prenylation - an overview | ScienceDirect Topics*. (n.d.). Www.sciencedirect.com. Retrieved April 24, 2023, from https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/prenylation#:~:text=Prenylation%20(or%20isoprenylation%2Flipidation)
8. Palsuledesai, C. C., & Distefano, M. D. (2015). Protein Prenylation: Enzymes, Therapeutics, and Biotechnology Applications. *ACS Chemical Biology*, *10*(1), 51–62. https://doi.org/10.1021/cb500791f
9. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Peroxisomes. *Molecular Biology of the Cell. 4th Edition*. https://www.ncbi.nlm.nih.gov/books/NBK26858/#:~:text=Peroxisomes%20are%20specialized%20for%20carrying