Effects of limiting citrate derived Acetyl-CoA production on the development of exhaustion by $CD8^+$ T cells

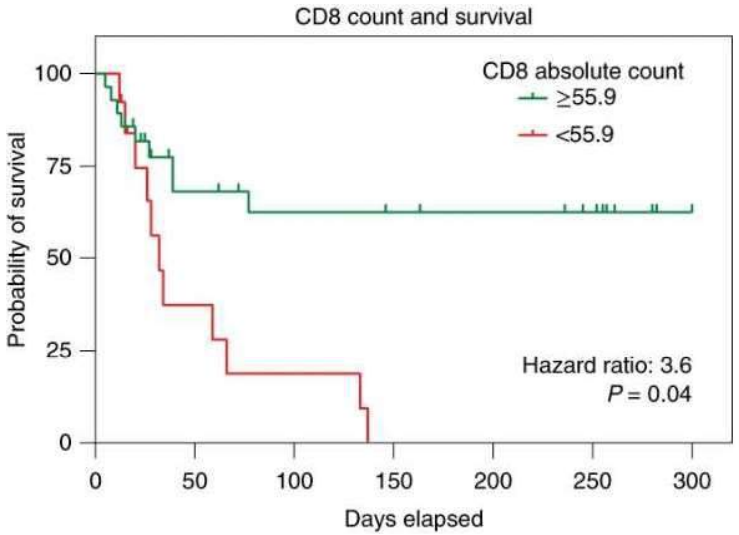
Presentation by Alessandra Azure

Washington AJAS

UPMC | HILLMAN CANCER CENTER

Healthy T cells kill cancer cells. They promote cancer patient survival.

- T cells are part of the adaptive immune system – they are specialized
  - **CD8⁺ T cells** are "killer" T cells that kill virally infected and cancerous cells
  - T cell produce **cytokines**, which are signals cells use to communicate
    - IFNγ and TNF-α are pro-inflammatory

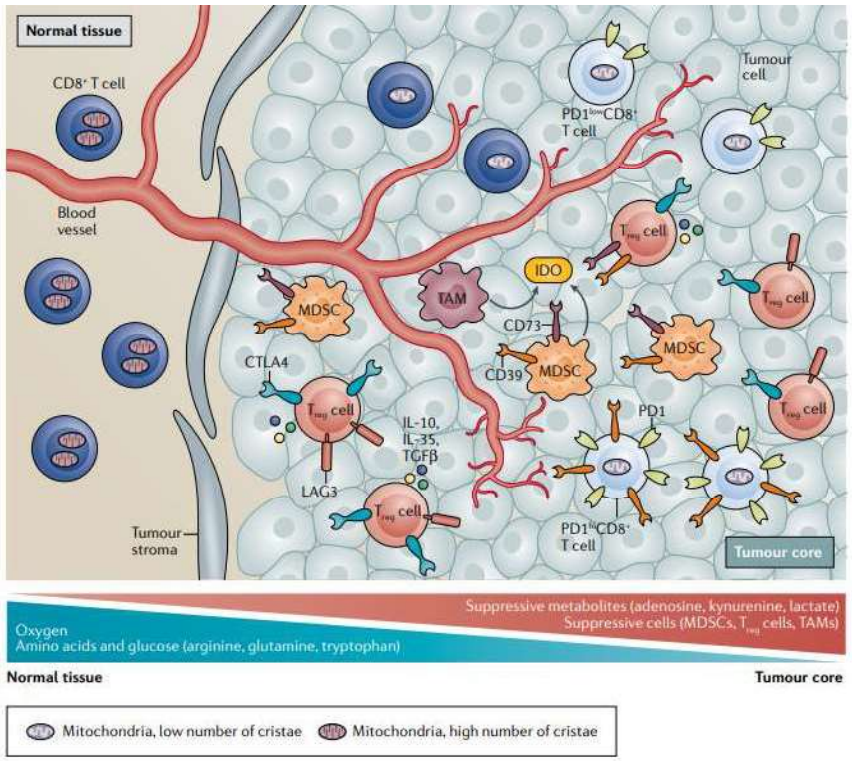


- CD8⁺ T cells can become **exhausted**
  - Exhaustion = hypofunctional state where some anti-tumor effector function is lost
  - Associated markers: PD-1, TIM-3

What *causes* T cell exhaustion?

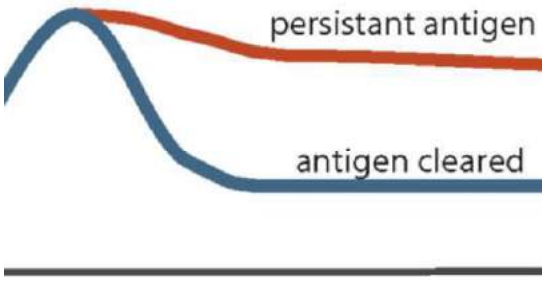
Ma, J. *et al*. Immunotherapy Cancer, 2019

Chronic activation and the suppressive TME drive T cells to exhaustion.



Conditions of the tumor microenvironment (**TME**):

- Nutrient insufficiency
- Low oxygen (hypoxia)
- Waste buildup
- Increased presence of inhibitor receptor ligands
- Immunosuppressive Treg cells

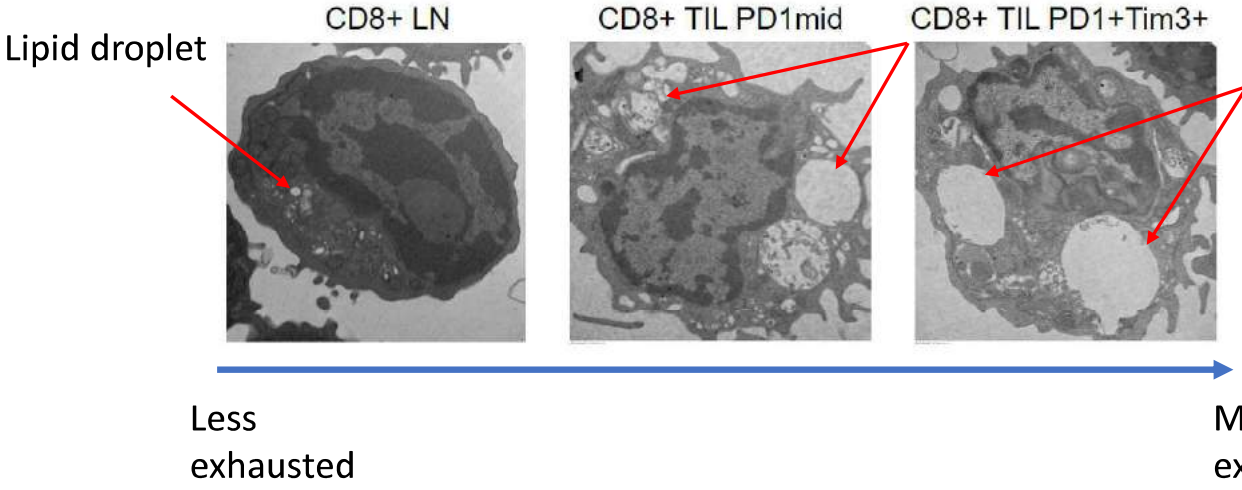


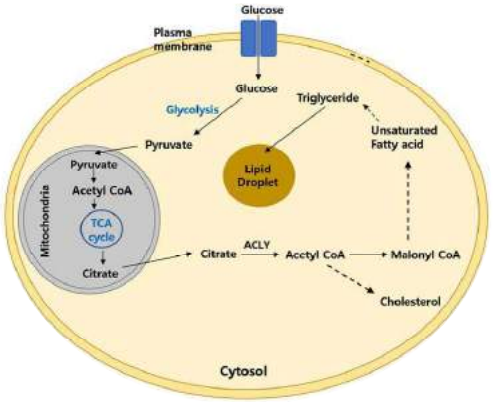
- Persistent antigen exposure and the harsh TME cause T cells to become **exhausted**

DePeaux, K., Delgoffe, G.M. Nat Rev Immunol, 2021

Exhausted T cells change the way they produce energy.

- Exhausted T cells lose full **mitochondria** function
  - Causes **metabolic insufficiency**
    - Cells shift metabolism to alternate pathways outside mitochondria – possibly *fatty acid synthesis pathway*





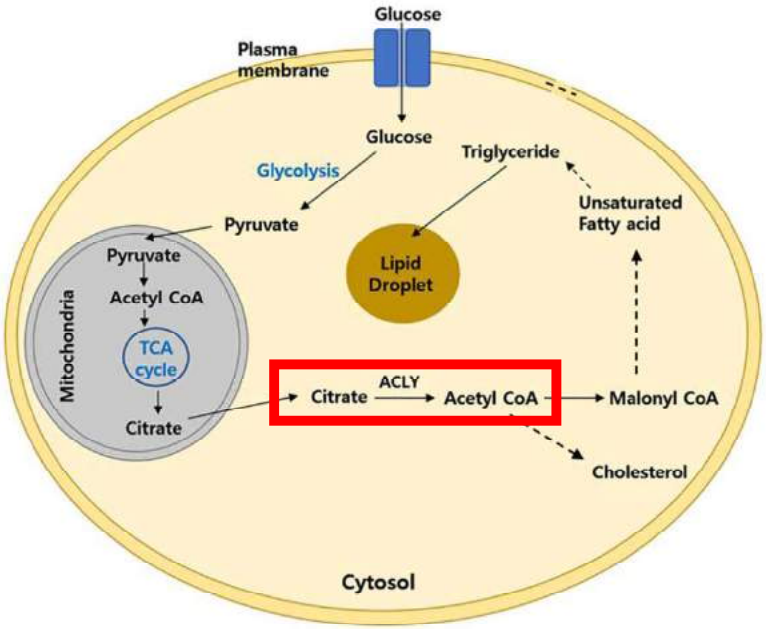
How is lipid accumulation related to the progression of T cells towards exhaustion?

- **Fatty Acids** are one type of nutrient that provides energy to T cells; **lipid droplets** store fatty acids
  - Dysregulation of the fatty acid synthesis pathway may be detrimental to T cells

Nicole Scharping

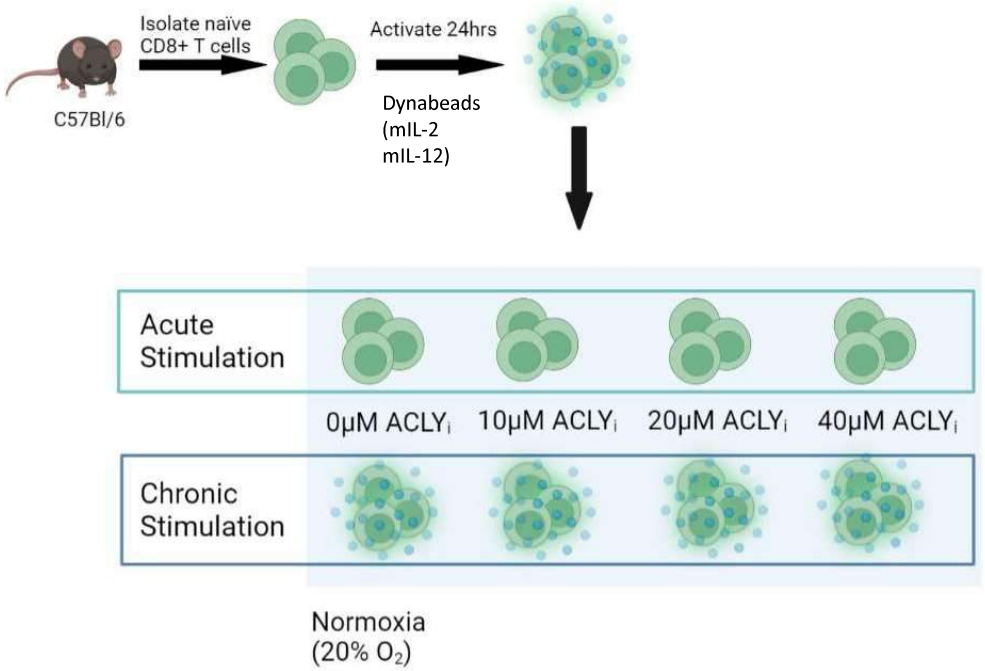
Cheon, S. *et al.* Journal of Molecular Medicine, 2021

Does inhibiting citrate derived Acetyl-CoA production impact the development of exhaustion in CD8^{+} T cells?

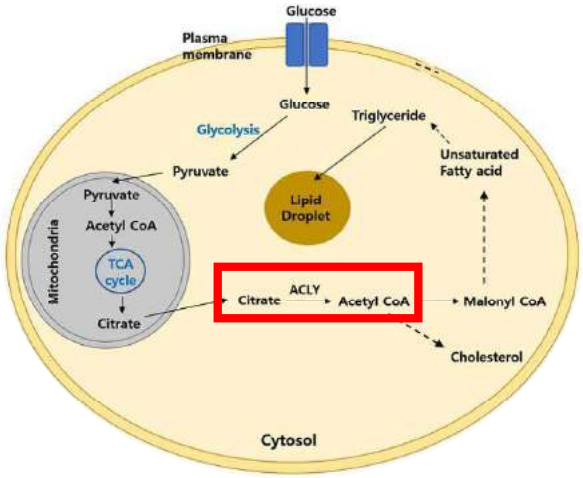


Methods: Evaluating ACLYi in vitro

Experiment Goal: Investigate the impact of inhibiting Acetyl-CoA production on the development of exhaustion by $CD8^+$ T cells.

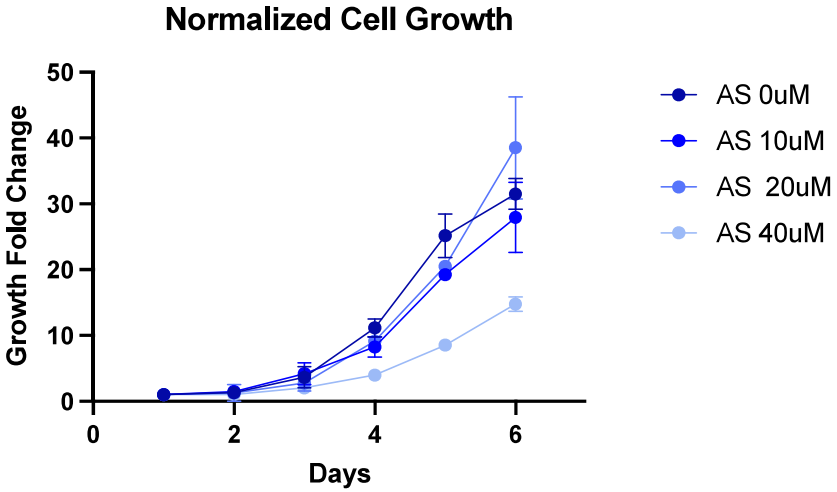


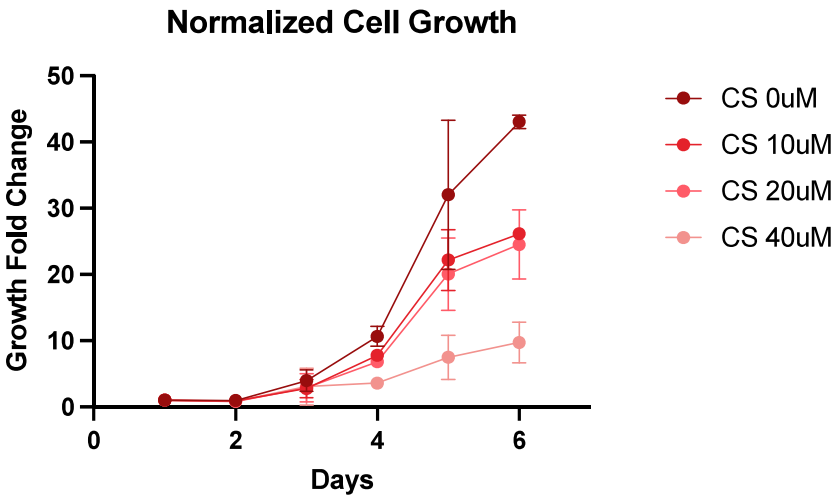
ACLYi is an inhibitor of ATP-citrate lyase (ACLY), an enzyme that catalyzes the conversion of citrate to Acetyl-CoA



- **T cell receptor (TCR)** was activated with αCD3/αCD28 dynabeads
- 6 day CS period
- ** Media contains **ACLYi** at appropriate experimental concentration ** (dosage not yet established)
- Analyzed via flow cytometry – single cell analysis of surface markers and cytokines

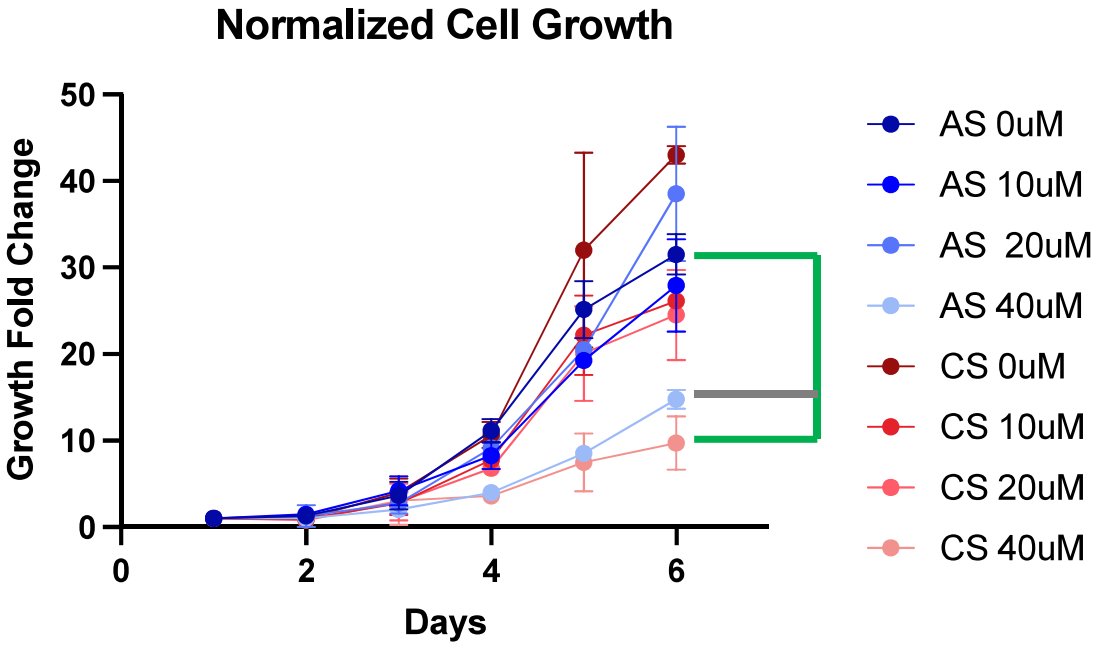
ACLYi and chronic stimulation decrease cell proliferation





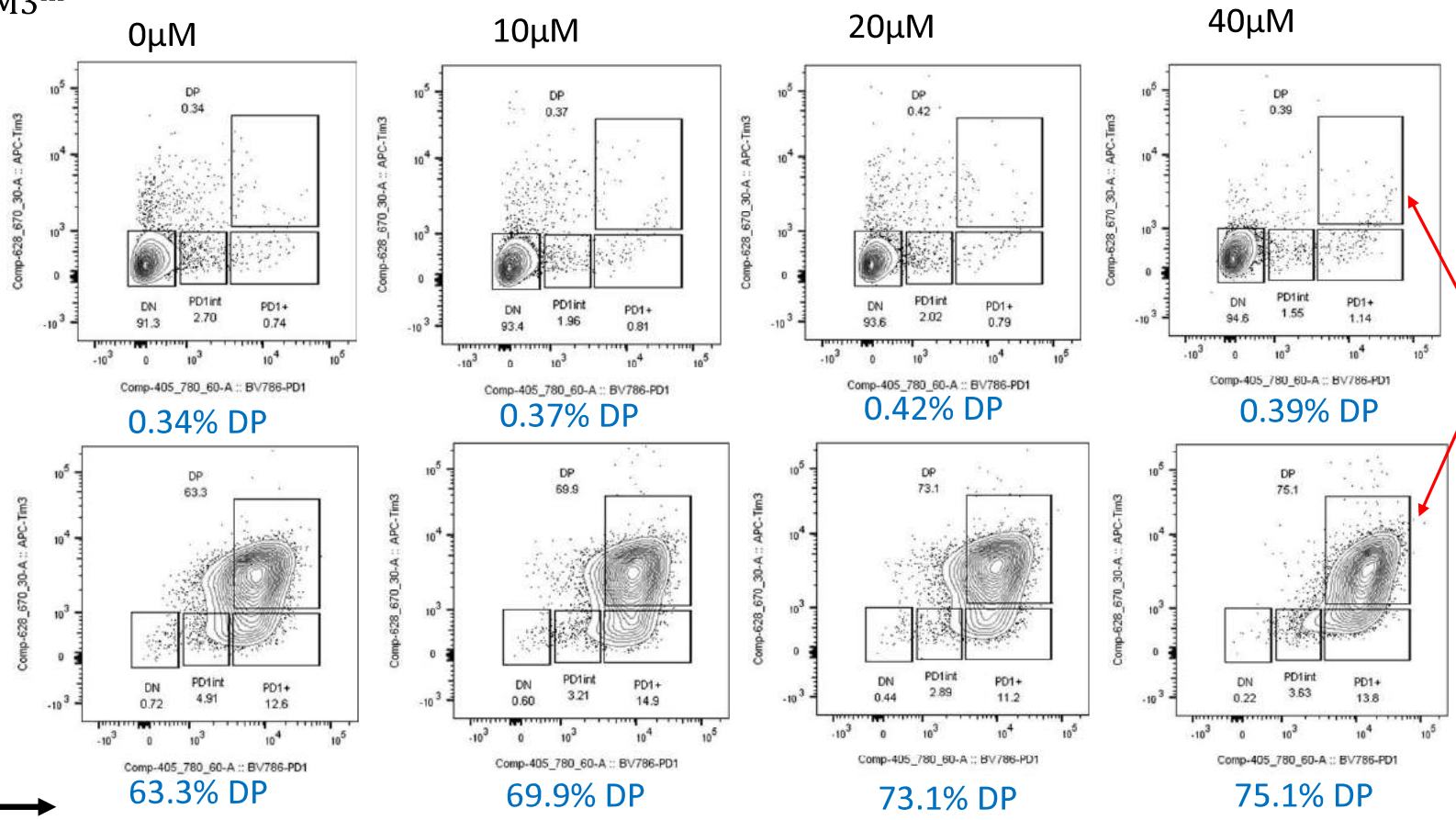
As inhibitor concentration increased, cell proliferation decreased, possibly due to *metabolic stress.*

Chronically stimulated cells proliferated less than acutely stimulated cells.



No notable change in PD-1 or TIM-3 expression was observed at any inhibitor concentration.

DP = $PD1^{hi}$ $TIM3^{hi}$



Repeat 2

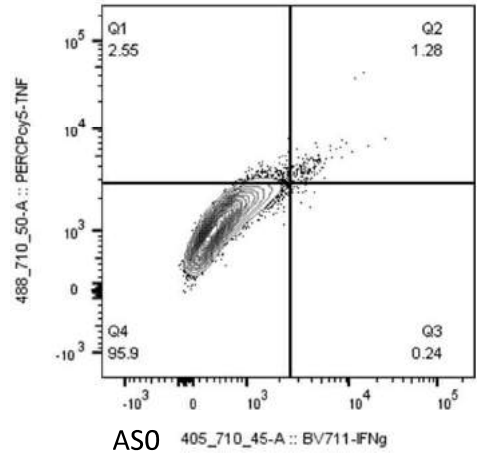
TIM-3 and PD-1 are surface markers associated with T cell exhaustion.

Cytokine production indicates the functional capacity of T cells.

- IFNγ and TNF-α are pro-inflammatory cytokines that stimulate an immune response

DN = double negative
DP = double positive

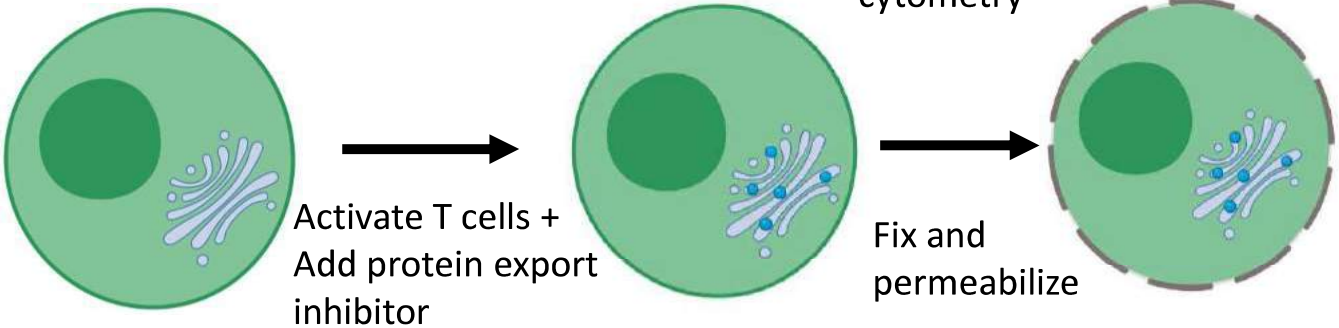
Unstimulated controls



TNF-α

IFNγ

Method: Cytokine Intracellular Staining



Activate T cells + Add protein export inhibitor

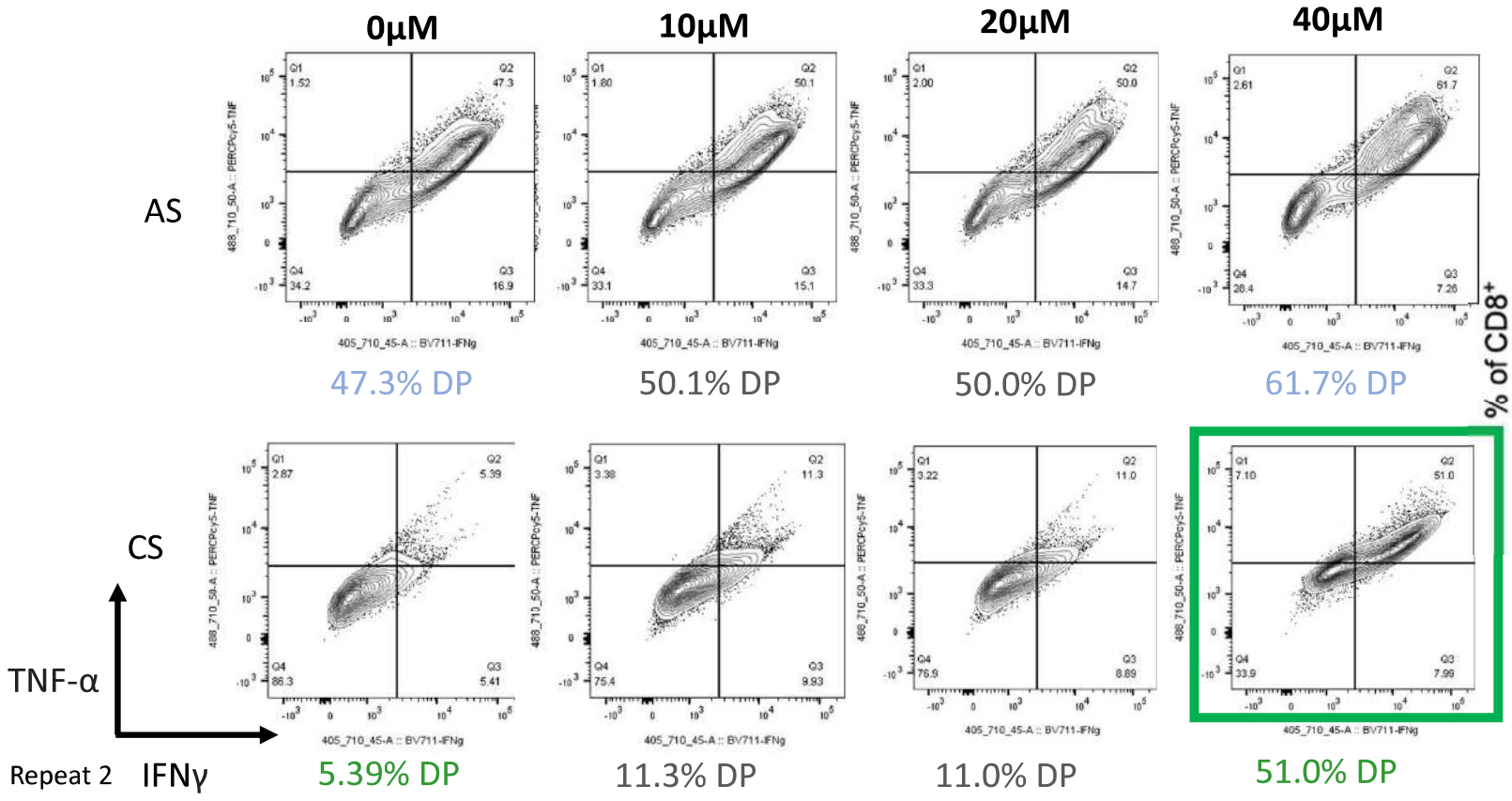
Fix and permeabilize

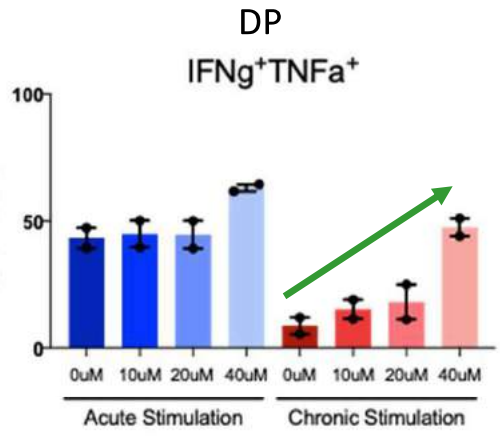
Add cytokine-specific antibodies + Analyze by flow cytometry

Observed **increase** in cytokine production in cell groups treated with 40 µM ACLYi.

Restim TCR with αCD3/αCD28

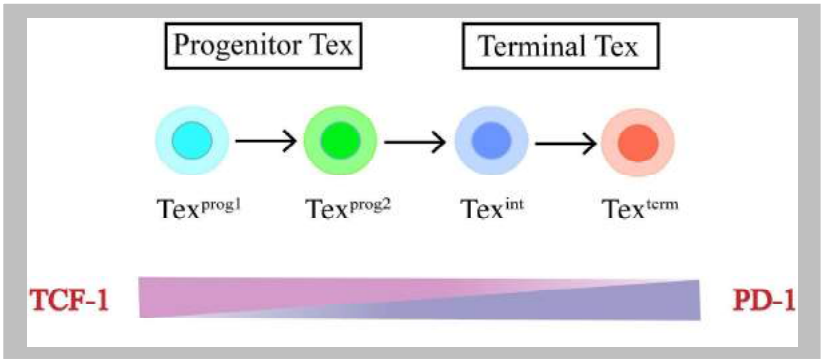
DP = IFN^{hi} TNF^{hi}





Putting it together

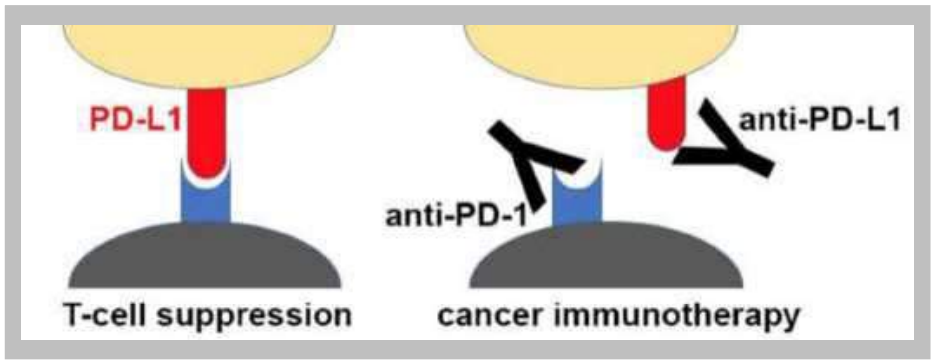
- Limiting Acetyl-CoA production may impact $CD8^+$ T cell effector function
  - Sustained cytokine signaling would promote antigen presentation and recruitment of immune cells
- Optimal ACLYi concentration likely lies around 30µM
  - Higher inhibitor concentration = less cell proliferation, more cytokine impact
- May be possible that ACLYi is delaying T cell progression to complete/ terminally differentiated exhaustion



Zhang, J. *et al*. FASEB, 2021

Future Direction

- Hypoxia repeat testing (1.5% O_2); Microscopy
- In vivo mice tumors
  - Investigate how surface marker expression interacts with cytokine production
    - Combination with anti-PD1 immunotherapy



- *Big Picture*: Make cancer patients' T cells work better for longer
  - Better way of treating cancer
  - Early clinical trial ongoing with similar inhibitor

Metabolism!

Crown Bioscience, 2021

Acknowledgements

Delgoffe Lab

- **Kellie Spahr**
- **Dr. Greg Delgoffe**

Hillman Academy

- **Dr. David Boone**
- **Steven Jones**

UPMC | HILLMAN CANCER CENTER

University of Pittsburgh

References

Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Lymphocytes and the Cellular Basis of Adaptive Immunity. Available from: https://www.ncbi.nlm.nih.gov/books/NBK26921/

American Cancer Society. “Cancer Facts & Figures 2022”. Atlanta: American Cancer Society; 2022.

Augustin RC, Delgoffe GM, Najjar YG. Characteristics of the Tumor Microenvironment That Influence Immune Cell Functions: Hypoxia, Oxidative Stress, Metabolic Alterations. Cancers (Basel). 2020 Dec 17

Blank, C.U., Haining, W.N., Held, W. et al. Defining ‘T cell exhaustion’. Nat Rev Immunol 19, 665–674 (2019). https://doi.org/10.1038/s41577-019-0221-9

Chang CH, Pearce EL. Emerging concepts of T cell metabolism as a target of immunotherapy. Nat Immunol. 2016 Apr;17(4):364-8. doi: 10.1038/ni.3415. PMID: 27002844; PMCID: PMC4990080.

Cheon, So Yeong & Cho, KyoungJoo. (2021). Lipid metabolism, inflammation, and foam cell formation in health and metabolic disorders: targeting mTORC1. Journal of Molecular Medicine. 99. 10.1007/s00109-021-02117-8.

Delgoffe GM. Filling the Tank: Keeping Antitumor T Cells Metabolically Fit for the Long Haul. Cancer Immunol Res. 2016 Dec;4(12):1001-1006. doi: 10.1158/2326-6066.CIR-16-0244. PMID: 27908931; PMCID: PMC5408882.

DePeaux, K., Delgoffe, G.M. Metabolic barriers to cancer immunotherapy. *Nat Rev Immunol* 21

References cont.

Dominguez, M., Brune, B., Namgaladze, D. Exploring the Role of ATP-Citrate Lyase in the Immune System. Frontiers in Immunology (2021). https://doi.org/10.3389/fimmu.2021.632526

Ma, J., Zheng, B., Goswami, S. *et al.* $PD1^{Hi}$ $CD8^{+}$ T cells correlate with exhausted signature and poor clinical outcome in hepatocellular carcinoma. *j. immunotherapy cancer* 7**,** 331 (2019).

"MojoSort™ Streptavidin Nanobeads Protocol - Negative Selection." *BioLegend*. https://www.biolegend.com/fr-lu/protocols/mojosort-streptavidin-nanobeads-protocol-negative-selection.

Petan T, Jarc E, Jusović M. Lipid Droplets in Cancer: Guardians of Fat in a Stressful World. Molecules. 2018 Aug 3;23(8):1941. doi: 10.3390/molecules23081941. PMID: 30081476; PMCID: PMC6222695.

Scharping, N.E., Rivadeneira, D.B., Menk, A.V. et al. Mitochondrial stress induced by continuous stimulation under hypoxia rapidly drives T cell exhaustion. Nat Immunol 22, 205–215 (2021). https://doi.org/10.1038/s41590-020-00834-9

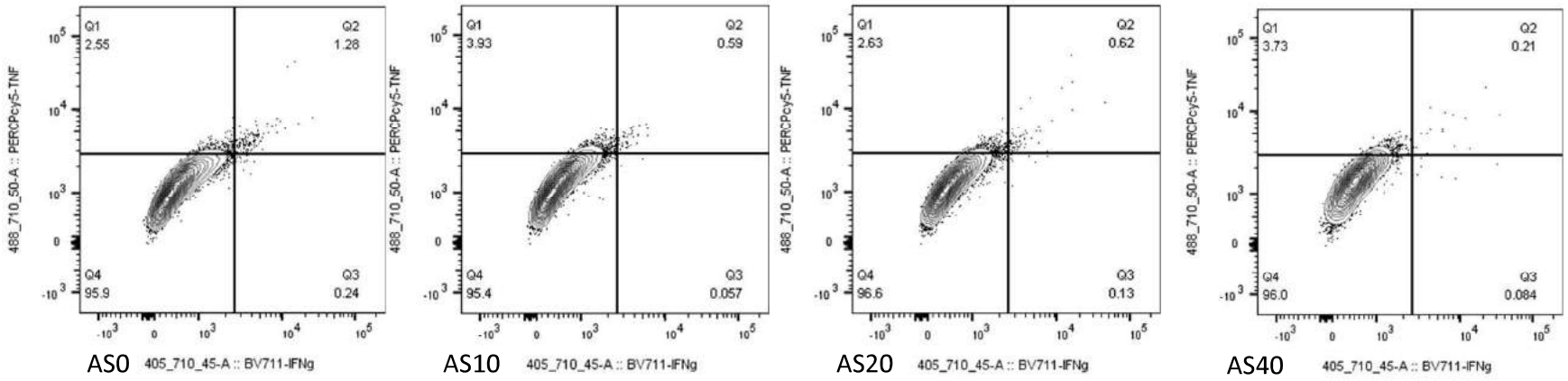
"Study of MTB-9655, an Inhibitor of ACSS2, in Patients With Advanced Solid Tumors." *ClinicalTrials.gov.* ID: NCT04990739

Sugiura A, Rathmell JC. Metabolic Barriers to T Cell Function in Tumors. J Immunol. 2018 Jan 15;200(2):400-407. doi: 10.4049/jimmunol.1701041. PMID: 29311381; PMCID: PMC5777533.

Wherry, E., Kurachi, M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol 15, 486–499 (2015). https://doi.org/10.1038/nri3862

Appendix – All Cytokine unstim controls

Unstimulated controls

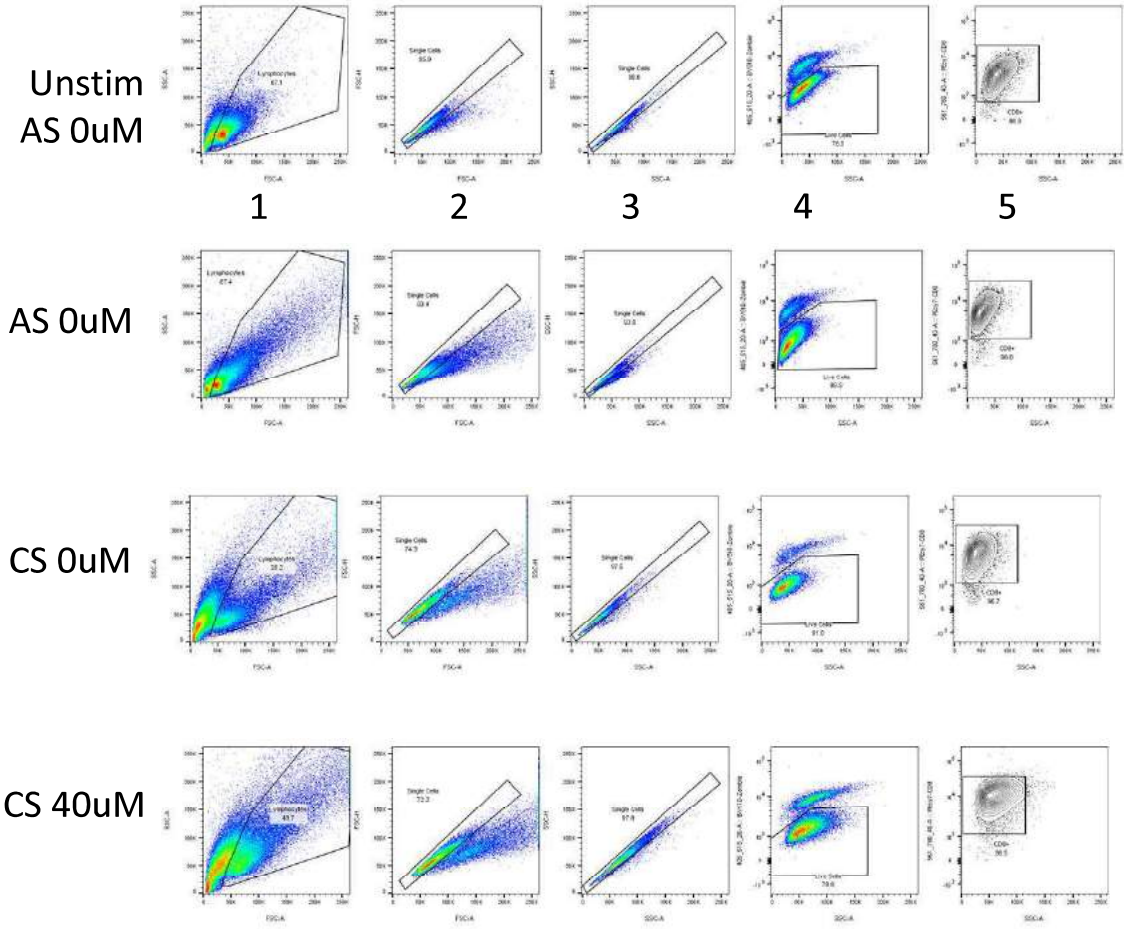


TNF-α

IFNγ

Appendix - Gating

AS = acute stimulation
CS = chronic stimulation



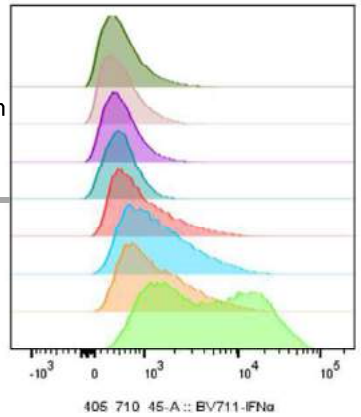
Gaiting (1) lymphocytes
(2/3) singlets
(4) live cells
(5) $CD8^+$ T cells

Appendix - Cytokines

CS; aCD3/aCD28 restim

Observed noticeable increase in cytokine production in cell group treated with 40 µM ACLYi concentration.

Repeat 1

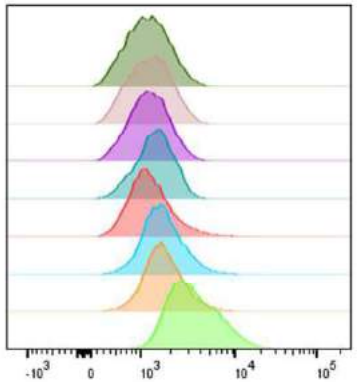


Unstim cntrls

CS0
CS10
CS20
CS40

TNF-α

Sample Name	Subset Name	Count	Geometric Mean : 405_710_45-A
Unstim ctrls_2_AS0_un_017.fcs	CD8+	30410	564
Unstim ctrls_2_AS10_un_018.fcs	CD8+	19332	534
Unstim ctrls_2_AS20_un_019.fcs	CD8+	30655	550
Unstim ctrls_2_AS40_un_020.fcs	CD8+	8375	539
CS_ACLYi_CD3 restim_2_CS0_CD3_005.fcs	CD8+	16835	963
CS_ACLYi_CD3 restim_2_CS10_CD3_006.fc	CD8+	15792	1377
CS_ACLYi_CD3 restim_2_CS20_CD3_007.fc	CD8+	16320	1326
CS_ACLYi_CD3 restim_2_CS40_CD3_008.fc	CD8+	23420	3952

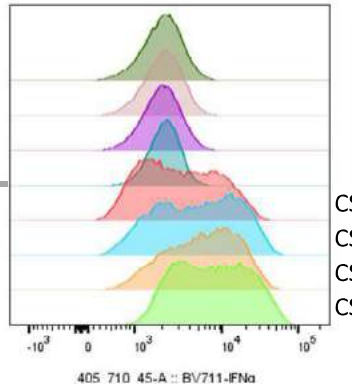


CS0
CS10
CS20
CS40

IFNγ

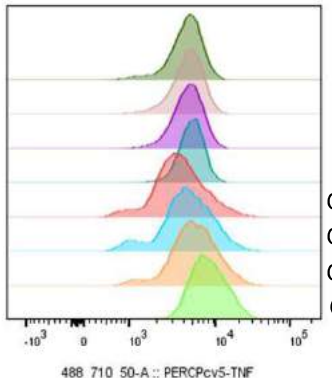
Geometric Mean : 488_710_50-A
1191
1243
1233
1383
1295
1690
1717
3379

Repeat 2



CS0
CS10
CS20
CS40

Sample Name	Subset Name	Count	Geometric Mean : 405_710_45-A
Unstim ctrls_1_AS0_un_017.fcs	CD8+	28027	1987
Unstim ctrls_1_AS10_un_018.fcs	CD8+	29374	2015
Unstim ctrls_1_AS20_un_019.fcs	CD8+	27007	1965
Unstim ctrls_1_AS40_un_020.fcs	CD8+	15591	2079
CS_ACLYi_CD3 restim_1_CS0_CD3_005.fcs	CD8+	27216	3028
CS_ACLYi_CD3 restim_1_CS10_CD3_006.fc	CD8+	19476	4806
CS_ACLYi_CD3 restim_1_CS20_CD3_007.fc	CD8+	24566	5509
CS_ACLYi_CD3 restim_1_CS40_CD3_008.fc	CD8+	32880	7209



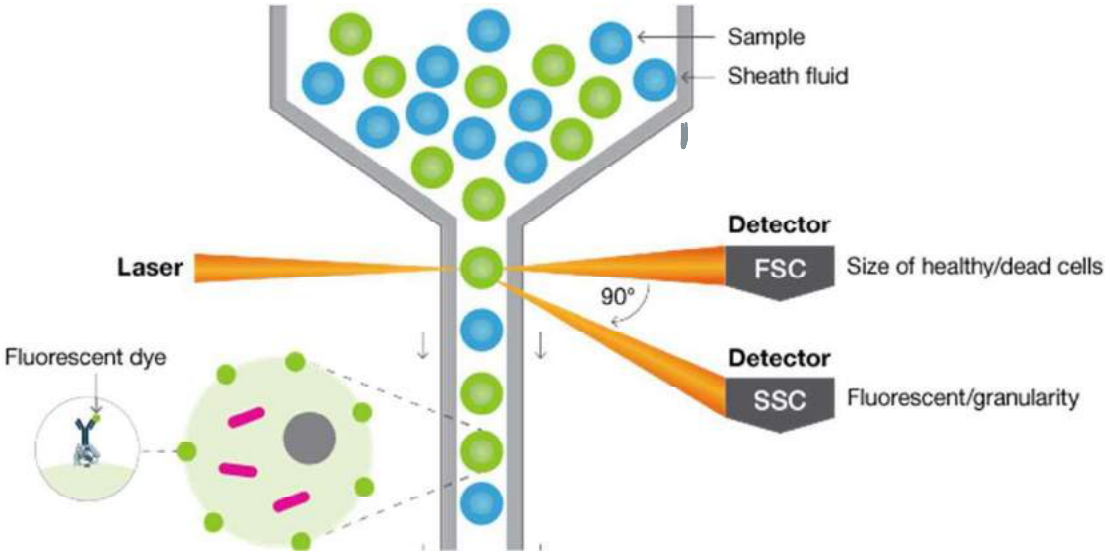
CS0
CS10
CS20
CS40

Geometric Mean : 488_710_50-A
4122
4312
4362
4839
3690
4317
5230
7886

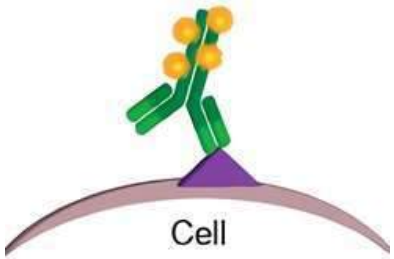
IFNγ and TNF-α are pro-inflammatory cytokines that stimulate an immune response.

Appendix – Flow Cytometry

- Cells were analyzed for surface markers, intracellular markers, and cytokine production via **flow cytometry**



A flow cytometer performs single-cell analysis of stained markers.



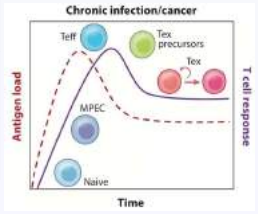
The Effects of Limiting Citrate-Derived Acetyl-CoA Synthesis on the Development of Exhaustion in CD8⁺ T Cells

Alessandra Azure[1], Kellie Spahr[2]

[1]Washington State, UPMC Hillman Academy, [2]Department of Immunology, University of Pittsburgh

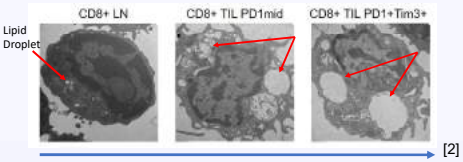
INTRODUCTION

- CD8⁺ T cells are a subset of T cells that kill cancer cells
- IFNγ and TNF-α are pro-inflammatory cytokines. They promote an anti-tumor immune response



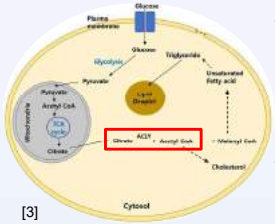
[1]

- T cells can lose their effector function when entering a hypofunctional state called '**exhaustion**'
- Driven, in part, by chronic activation due to persistent antigen exposure
- PD-1 and TIM-3 are surface markers highly expressed by exhausted T cells – serve as phenotypic indicators of exhaustion



[2]

- Exhausted T cells lose full mitochondria function, causing them to shift metabolism to alternate pathways – possibly the fatty acid synthesis (FAS) pathway
  - Observations of lipid accumulation in exhausted cells



[3]

- Acetyl-CoA is a key metabolite in the FAS pathway. ATP Citrate Lyase (ACLY) is an enzyme that catalyzes the conversion of citrate to acetyl-CoA in the cytosol.
- By inhibiting ACLY, citrate-derived acetyl-CoA production is therefore limited

GOALS

- **Big Picture:** Prevent T cell progression to exhaustion using a metabolism-focused approach
  - *Why it matters*: Exhausted T cells fight cancer poorly. Preventing exhaustion could improve cancer patient survival outcomes.
- Investigate: **Does inhibiting citrate-derived Acetyl-CoA production impact the development of exhaustion in CD8⁺ T cells?**
  - If so, could the observed accumulation of lipid droplets in exhausted T cells be a *driver* of exhaustion?

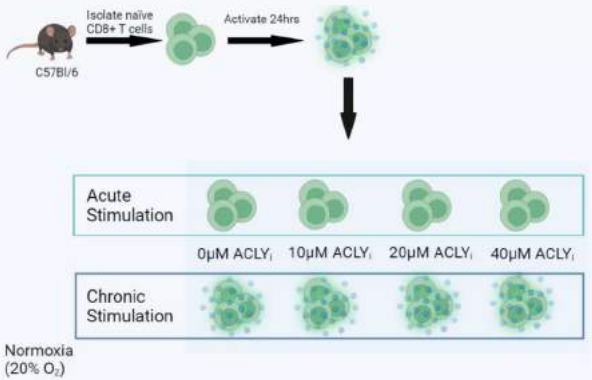
HYPOTHESIS

Limiting Acetyl-CoA production will help CD8⁺ T cells retain their anti-tumor capabilities.

- Because exhausted T cells show decreased ability to extract energy from lipids; so, preventing T cells from over-utilizing the fatty acid synthesis pathway may improve their ability to function

METHODS

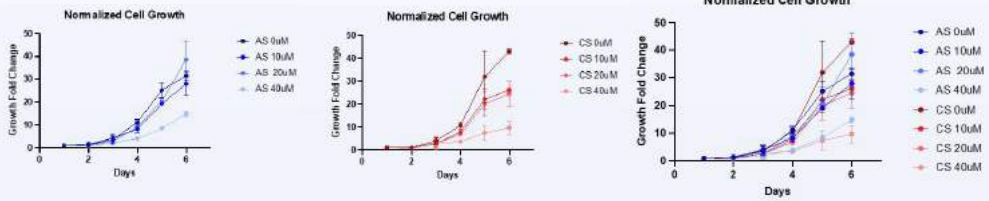
- CD8+ T cells were isolated via negative selection using MojoSort™ Streptavidin Nanobeads
- T cell receptor (TCR) was activated *in vitro* with αCD3/αCD28 Dynabeads™ (+ mulL-2)
- Acute Stimulation (AS) and Chronic Stimulation (CS) groups
  - AS = 24-hour TCR stimulation only
  - CS = 6 days continuous TCR stimulation
- Media contains ATP Citrate Lyase inhibitor (**ACLYi**)
  - BMS-303141
  - 4 concentrations used; 0μM = control
- All cells were analyzed using flow cytometry
  - Cytokine staining included un-restimulated controls; a protein export inhibitor was added to cells prior to restimulation and cytokine staining
  - N = 2 full repeats



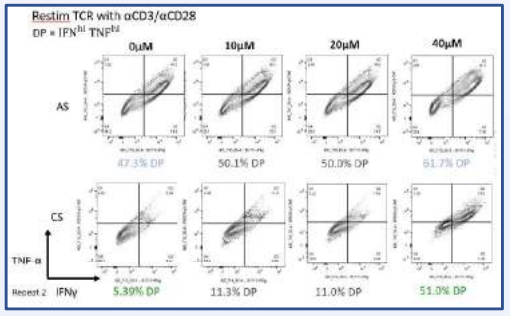
RESULTS

Cell Growth

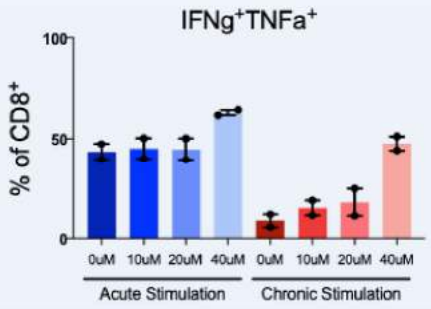
As inhibitor concentration increased, cell proliferation decreased, possibly due to metabolic stress. As expected, chronically stimulated cells proliferated less than acutely stimulated cells.



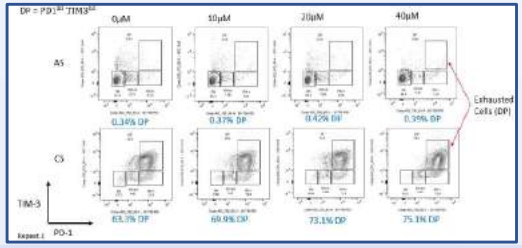
Cytokine Production of IFNγ and TNF-α



Cells treated with 40μM of ACLYi consistently produced notably more pro-inflammatory cytokines (IFNγ and TNF-α) than cells treated with lower inhibitor concentrations or no inhibitor.



Surface Markers PD-1 and TIM-3

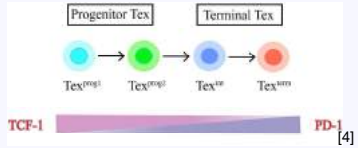


Flow cytometry analysis revealed little change in PD-1 or TIM-3 expression between inhibitor concentrations. As expected, the CS group exhibited a higher percentage of PD-1hi TIM-3hi cells than the AS groups overall.

CONCLUSIONS

- Inhibiting Acetyl-CoA synthesis does impact CD8⁺ T cell effector function
  - **Sustained cytokine signaling would promote antigen presentation and recruitment of immune cells – therefore *improving the body's immune response against cancer***
  - Little to no observed impact on phenotypic exhaustion
- Optimal ACLYi concentration likely lies around 30μM
  - Higher inhibitor concentration = less cell proliferation; more cytokine impact
- May be possible that ACLYi is delaying T cell progression to terminally differentiated exhaustion
  - Results provide evidence to the merit of investigating lipid accumulation as a driver of exhaustion

"Spectrum of exhaustion"



[4]

FUTURE DIRECTIONS

- Repeat testing under ***hypoxia*** (1.5% O_2) to mimic conditions of the tumor microenvironment
- Use microscopy to visualize changes in lipid droplet size
- Making the jump to *in vivo*:
  - Test CD8⁺ T cells treated with ACLYi in *in vivo* mouse tumors; observe rates of IFNγ and TNF-α production in TILs
  - Pair with a PD-1/PD-L1 blockade therapy; mitigate immunosuppressive effects of PD-1 binding
- *Bench to bedside transition*:
  - Current First-In-Human clinical trial use a similar, orally-available inhibitor of Acetyl-CoA production (via ACSS2) in patients with advanced solid tumors
  - Immunotherapy potential

REFERENCES

[1] "Measuring CD8+ T Cell Exhaustion," *Cofactor Genomics*, 2019 https://cofactorgenomics.com/wk-33-cd8-t-cell-exhaustion/. Accessed Mar. 22, 2023.
[2] Image credit: Nicole Scharping
[3] Cheon, S. *et al.* Journal of Molecular Medicine, 2021. Image has been edited for clarity.
[4] Zhang, J. *et al.* Federation of American Societies for Experimental Biology, 2021
- Chang CH, Pearce EL. Emerging concepts of T cell metabolism as a target of immunotherapy. Nat Immunol. 2016 Apr;17(4):364-8. doi: 10.1038/ni.3415. PMID: 27002844; PMCID: PMC4990080.
- Delgoffe GM. Filling the Tank: Keeping Antitumor T Cells Metabolically Fit for the Long Haul. Cancer Immunol Res. 2016 Dec;4(12):1001-1006. doi: 10.1158/2326-6066.CIR-16-0244. PMID: 27908931; PMCID: PMC5408882.
- DePeaux, K., Delgoffe, G.M. Metabolic barriers to cancer immunotherapy. Nat Rev Immunol 21
- Scharping, N.E., Rivadeneira, D.B., Menk, A.V. et al. Mitochondrial stress induced by continuous stimulation under hypoxia rapidly drives T cell exhaustion. Nat Immunol 22, 205–215 (2021). https://doi.org/10.1038/s41590-020-00834-9
- "Study of MTB-9655, an Inhibitor of ACSS2, in Patients With Advanced Solid Tumors," ClinicalTrials.gov. ID: NCT04990739

ACKNOWLEDGEMENTS

Many thanks to my mentor and all those who have supported me throughout this project:

Delgoffe Lab, University of Pittsburgh

Kellie Spahr
Greg Delgoffe, PhD
Dayana Rivadeneira, PhD

Hillman Academy

David Boone, PhD
Steven Jones
Tulia Bruno, PhD