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Amy Peng November 13, 2023

Investigating the Impact of Soat1 Knock-Out on Visceral Adipose Tissue Regulatory T-Cell Accumulation in TCR Transgenic Mice.

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Abstract

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Visceral adipose tissue (VAT) regulatory T cells (Tregs) are a subset of $FOXP3^+$, $CD4^+$ T cells localized in the visceral adipose tissue. These cells help maintain tissue homeostasis within the VAT and regulate organismal metabolism. Our research attempts to shed light on how modulating membrane cholesterol levels would influence the accumulation of VAT Tregs, which play important roles in limiting obesity-associated inflammation and metabolic syndrome. We attempt to answer this question by knocking out the *Soat1* gene, which catalyzes the formation of cholesterol-esters from free cholesterol, in TCR transgenic Tregs and performing a competitive transfer assay into recipient mice to measure the *Soat1* KO Treg accumulation in the visceral adipose tissue. We hypothesize that knocking out *Soat1* will increase Treg accumulation in the visceral adipose tissue due to higher free cholesterol levels in the cell membrane, which will result in more T-cell receptor clustering and signaling. However, our results showed no significant accumulation of *Soat1* KO donor Tregs within the VAT and suggests that *Soat1* function of sequestering free cholesterol into storage as cholesterol esters may not be substantial enough by itself to alter the lipid raft composition of these cells.

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Introduction

During a time where obesity prevalence among adults within the United States remains high, the need for treatment and prevention is more crucial than ever. Additionally, obesity is linked to other comorbidities such as insulin resistance, diabetes mellitus, and hypertension (Pi-Sunyer, 1999). One endocrine organ and component of body fat that helps regulate our metabolism is the visceral adipose tissue (VAT), which has been shown to result in metabolic abnormalities and obesity when inflamed or at high depositions (Shuster et al., 2012).

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that have a specialized function in suppressing inflammation, which maintain homeostasis and self-tolerance. Many studies have analyzed the phenotypes and function of Tregs residing in lymphoid organs, and only recently have people started to shift their focus on studying Tregs in non-lymphoid organs which play a specialized role in maintaining tissue homeostasis (Panduro et al., 2016). Unlike FOXP3, a transcription factor expressed ubiquitously in all Tregs, non-lymphoid tissue residing Tregs are also endowed with tissue-specific transcription factors which are crucial to their survival and function in these local environments (Josefowicz, 2013). Tregs from different tissues are transcriptionally distinct from each other with different T-cell receptor repertoires, and new research is focusing on targeting these cells in a tissue-specific manner (Zhou et al., 2015). Specifically, targeting Tregs within the VAT has garnered much interest since studies have shown that these cells have a specialized function in suppressing inflammation and promoting metabolic health, where a reduction in VAT Tregs lead to worsened inflammation and metabolic abnormalities (Muñoz-Rojas et al., 2021). Thus, designing treatments, such as

through gene therapies, may be impactful to help promote the normal functioning of VAT Treg cells to decrease the risk of developing metabolic disorders.

To explore this phenomena, previous research compared variations in gene expressions in VAT Tregs of obesity-induced mice through high-fat diet feeding (HFD) to control mice fed on a normal chow diet (NCD) using RNA-sequencing. The results from this study showed that VAT Tregs were significantly reduced during obesity, and that genes involved in the regulation of the cholesterol metabolism pathway were highly expressed in VAT Tregs from NCD-fed mice but showed a decrease in expression following high-fat diet feeding (Li et al., 2021). To investigate the role of cholesterol homeostasis in VAT Treg survival, our lab generated Treg specific knock-out mice of *Srebf2*--gene encoding for a transcription factor that regulates cholesterol biosynthesis and reuptake--and found that knocking out *Srebf2* lead to a significant decrease in VAT Treg accumulation (Elkins et al., under review). Normally, the demand for cholesterol increases drastically during T cell activation and blastogenesis, and T cells must rewire their transcriptional programs to account for this such as through the upregulation of *Srebf2*. However, another pathway that modulates intracellular cholesterol homeostasis, apart from regulating its synthesis, is the conversion of free cholesterol into cholesterol esters as a form of storage. For effective activation, Treg cells must be able to communicate with antigen-presenting cells (APCs) and other target cells through the formation of the immunological synapse: a stable interface between immune cells organized by the interaction of immunoreceptors and adhesion molecules at the cell surface. Dynamic assemblies of lipids and proteins within the cellular membrane, called lipid rafts, facilitate this reorganization of membrane proteins at the cell surface to form the immunological synapse. Free cholesterol is

known to be a key component of membrane lipids and contributes to membrane fluidity and spatial control of receptors. Thus, regulating the esterification of free cholesterol could liter to how free cholesterol level within the plasma membrane influence VAT Treg activation and proliferation. Studies show that the inhibition of cholesterol esterification increased cholesterol level in the plasma membrane of $CD8^+$ T cells, resulting in increased proliferation of $CD8^+$ T cells due to changes in the lipid rafts which in turn enhanced T-cell receptor (TCR) clustering and signaling (Yang et al., 2016). The importance of cholesterol within the lipid rafts of $CD4^+$ T cells is also supported by experiments extracting a portion of membrane cholesterol using β-cyclodextrin, resulting in a significant decrease of $CD4^+$ T cell clustering (Nguyen et al., 2005). This research illustrates the importance of cholesterol within lipid rafts, which are required for T-cell receptor clustering (TCR) and the formation of the immunological synapse. Yet, despite all these findings, knowledge on the role of cholesterol homeostasis in the accumulation of VAT tissue specific Treg cells is very limited. We seek to determine whether modulating cholesterol composition within the plasma membrane (PM) influences VAT Treg cell survival and hypothesize that knocking out the *Soat1* gene should upregulate PM cholesterol levels and promote VAT Treg accumulation. Our research utilized a VAT-Treg T cell receptor-transgenic mouse model, where adoptive transfer of Tregs from these mice into recipient mice leads to preferential accumulation of these cells in the VAT of the recipient mice. CRISPR-Cas9 was used to knock-out the *Soat1* gene encoding for the enzyme ACAT1, which functions in converting cholesterol into cholesterol esters. We then performed a competitive transfer assay by transferring *Soat1* knock-out Tregs into recipient mice and measured their accumulation within the spleen and VAT.

Materials and Methods

Designing sgRNAs for Soat1 Knock-Out

In order to knock out the *Soat1* gene, we used a previously published whole-genome library (Doench et al, 2016) to design four single guide RNAs (sgRNAs) that targeted the *Soat1* DNA sequence. The forward and reverse sgRNA oligonucleotide sequences which we designed are shown below (Table 1).

Table 1. Forward and reverse oligonucleotide sequences for the designed *Soat1* targeting sgRNAs.

Soat1 targeting sgRNAs	Forward sgRNA oligonucleotide	Reverse sgRNA oligonucleotide
sgRNA1	caccgCCCACCATTGTCCAGCGATG	aaacCATCGCTGGACAATGGTGGGc
sgRNA2	caccgTGTGGTAAATTGTTCTGATG	aaacCATCAGAACAATTTACCACAc
sgRNA3	caacgCAGTATCAGAATGAACCGGG	aaacCCCGGTTCATTCTGATACTGc
sgRNA4	caacgACGAGTACTAAATGCAGCCA	aaacTGGCTGCATTTAGTACTCGTc

Ligation of SOAT1 into MSCV Retroviral Plasmid, Expansion, and Isolation

We phosphorylated and annealed the forward and reverse sgRNA's for *Soat1* together then ligated them into a Bbsl-digested MSCV plasmid with a GFP reporter and antibiotic resistance gene. We also ligated a control sgRNA, which does not target any gene, into a BbsI-digested MSCV plasmid with an RFP reporter and antibiotic resistance gene. We then transformed the product into E. coli using heat shock before incubating the bacteria. After incubation, we spun down the bacteria and smeared them onto ampicillin antibiotic LB-agar plates before picking a colony to culture in LB broth containing 1X ampicillin. After culturing, the plasmids were isolated from the single colony using the ZymoPURE plasmid miniprep kit and sent for

sequencing to check that the sgRNA was successfully ligated into the plasmid. Once confirmed that the sgRNA was within the plasmid, we extracted more plasmid using the ZymoPURE midiprep kit.

Transfection into PlatE Cells and Production of MSCV virus

We passaged and grew PlatE cells in culture before transfecting them with a viral packaging plasmid along with the MSCV plasmids either containing the control or *Soat1* sgRNA and a viral packaging plasmid. The PlatE cells (adhered to the bottom of the plate) took up both plasmids and produced the MSCV retrovirus. We then collected the filtered supernatant media containing the retrovirus encapsulating the plasmid with control or *Soat1* sgRNA.

Guide RNA Efficiency Check

We harvested the spleen and lymph nodes of Thy1.1+ Cas9 mice and isolated their regulatory T-cells using flow cytometry. Then we infected the Tregs with the collected MSCV retrovirus containing the *Soat1* sgRNA before extracting their genomic DNA. We then performed PCR on the gDNA using *Soat1* target primers (to obtain DNA fragment of interest) and extracted the PCR product using QIAquick gel extraction kit. The PCR product was cloned into Topo vectors and transformed into E coli to replicate. We picked eight colonies, and their plasmids were isolated using the ZymoPURE plasmid miniprep kit and sent for sequencing to analyze how much of the targeted *Soat1* DNA fragment of interest had been successfully mutated out of the eight colonies to estimate the *Soat1* sgRNA efficiency. We used the sgRNA with the highest *Soat1* knock-out efficiency for the rest of the experiment.

Injection of Infected Tregs into Mice and Analysis

Studying VAT Tregs has been a challenge in the past due to its low quantity in mice (with around 5,000 cells/mice). To combat this, labs have engineered a transgenic mouse line: vTreg53 TCR-tg mice. These mice carry a pre-rearranged *Tcra* and *Tcrb* genes, which are responsible for encoding the alpha and beta chain of the TCR, respectively, of an expanded VAT-Treg clone. The Tregs from these mice express a TCR comprised of Vα2 and Vβ4 variable regions, which recognizes an antigen within the VAT and promotes clonal expansion, resulting in an elevated population of VAT Tregs (Li et al., 2018). Researchers have also devised a vaccination protocol of peptide agonists, recognized by the vTreg53 TCR, to expand these cells and found that the peptide agonist FAT1562 induced robust activation and proliferation of vTreg53 TCR-tg Tregs, as well as FAT7 which induced a more moderate response (Fernandes et al., 2020).

These two peptide agonists were also used in our experiment, where we first harvested the spleen and lymph nodes of donor CD45.1- Foxp3-thy1.1 Cas9 TCR transgenic mice and isolated their Tregs using flow cytometry. We then infected the isolated Tregs with the control and *Soat1* sgRNA MSCV viruses then combined them into a 1:1 ratio of control and *Soat1* sgRNA expressing Tregs solution. Finally, we injected the solution retro-orbitally into recipient CD45.1+ Cas9 mice. Three of the mice were put on a normal chow diet for 12 weeks before harvesting their spleen and VAT. Four mice were injected subcutaneously with FAT1562 emulsified in complete Freund's adjuvant (CFA/FAT1562) immediately after injecting the donor Tregs, before being boosted a week later with FAT1562 emulsified in incomplete Freund's adjuvant

(IFA/FAT1562). We then waited one more week before harvesting the VAT and spleen from this cohort. Another five mice were injected subcutaneously with FAT7 emulsified in CFA immediately after injection of the donor Tregs before being boosted with IFA/FAT7 a week after. Once again, we waited an extra week before harvesting this cohort's spleen and VAT. The accumulation of the donor control and *Soat1* sgRNA expressing Tregs in these tissues between each cohort of mice were analyzed using flow cytometry.

Results

Flow Cytometry Gating Strategy

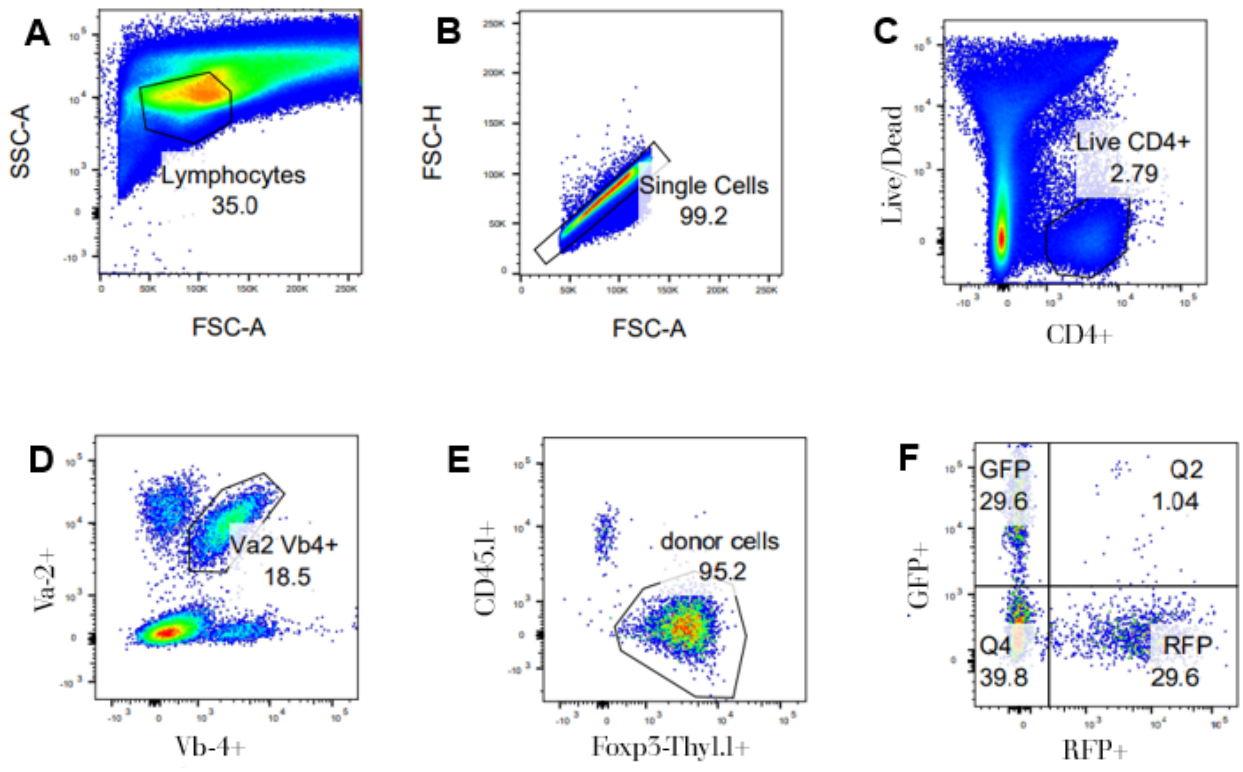

Figure 1. Gating of successive subpopulation of cells in flow cytometry for analysis after fluorescent antibody staining. (A) Flow cytometry gating strategy for isolating lymphocytes; (B)

Flow cytometry gating strategy for isolating single cells; (C) Flow cytometry gating strategy for isolating live CD4+ cells; (D) Flow cytometry gating strategy for isolating vα-2+ and vβ-4+ cells; (E) Flow cytometry gating strategy for isolating CD45.1-, Foxp3-Thy1.1+ cells; (F) Flow cytometry gating strategy for isolating GFP+, RFP- and GFP-, RFP+ cells.

We used flow cytometry to analyze the accumulation of GFP-expressing *Soat1* knock-out Tregs and RFP-control Tregs within the VAT and spleen of the recipient mice. After processing the tissues, the cells were stained with antibodies conjugated with fluorophores which bind to certain intracellular and surface markers to distinguish between different cell types. The figure above shows the gating strategy used to isolate these donors Tregs. We first gated for single cell lymphocytes (Figure 1A, 1B) before honing in on live $CD4^+$ cells (Figure 1C). From this population of cells, we gated on vα-2+ and vβ-4+ expressing cells present on the transgenic donor Tregs' TCR (Figure 1D). We then gated for CD45.1- (a marker present only in the recipient T cells) and Thy1.1+ (a surface marker for Tregs) cells to identify the donor Tregs (Figure 1E). From here, we analyzed the fluorescence intensity of the GFP and RFP expressing donor Tregs (Figure 1F) to quantify the accumulation of *Soat1* KO and control donor Tregs within the VAT and spleen.

Efficiency of Soat1 single guide RNA

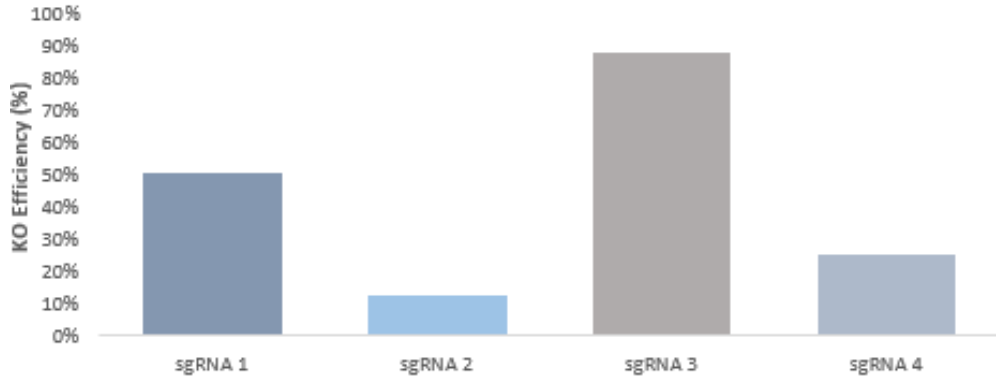

Figure 2. sgRNA percent efficiencies in knocking out *Soat1* in Thy1.1+ Cas9 expressing Tregs.

The sequencing results showed that seven out of eight (87.5% efficiency) of the selected E. coli colonies with plasmids containing genomic DNA from the infected Thy1.1+ Cas9 Tregs had mutations at the targeted *Soat1* DNA fragment of interest for sgRNA 3. The results indicate that sgRNA 3 had the highest efficiency in knocking out *Soat1* compared to the other single guide RNAs and was used to carry out the infection of CD45.1- Foxp3-thy1.1 Cas9 TCR transgenic Tregs.

Accumulation of Soat1 Knock-Out Tregs in Recipient Mice

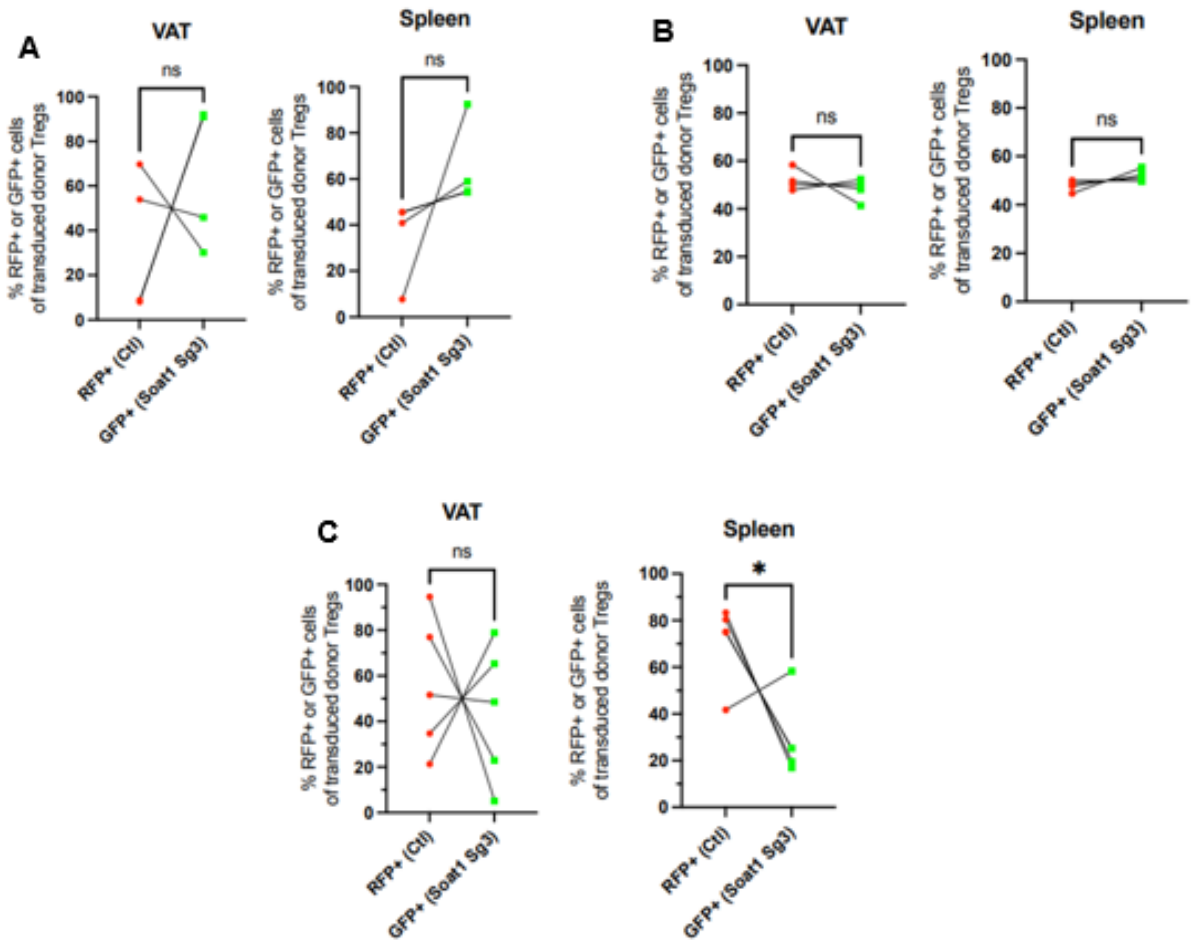

Figure 3. Percentage of control and *Soat1* KO donor Tregs accumulation within the spleen and VAT of unimmunized and immunized recipient mice. (A) Donor Tregs accumulation within the spleen and VAT of recipient NCD mice after 12 weeks; (B) Donor Tregs accumulation within the spleen and VAT of recipient NCD mice immunized with FAT1562 after 2 weeks; (C) Donor Tregs accumulation within the spleen and VAT of recipient NCD mice immunized with FAT7 after 2 weeks.

For all cohorts of mice (immunized and unimmunized), there was no difference in the accumulation of *Soat1* KO or control Tregs in the VAT (Figure 3A, $t(4)= 1.4603$, $p= 0.2180$; 3B,

t(4)= -1.2098, p= 0.2930; 3C, t(6)= -0.5647, p= 0.5927). This trend is also seen across each group's spleen samples except for the mice immunized with FAT7, where the spleens of FAT7 immunized mice show a difference in the accumulation of KO and control donor Tregs (Figure 3C, t(6)= -2.9506, p= 0.0256).

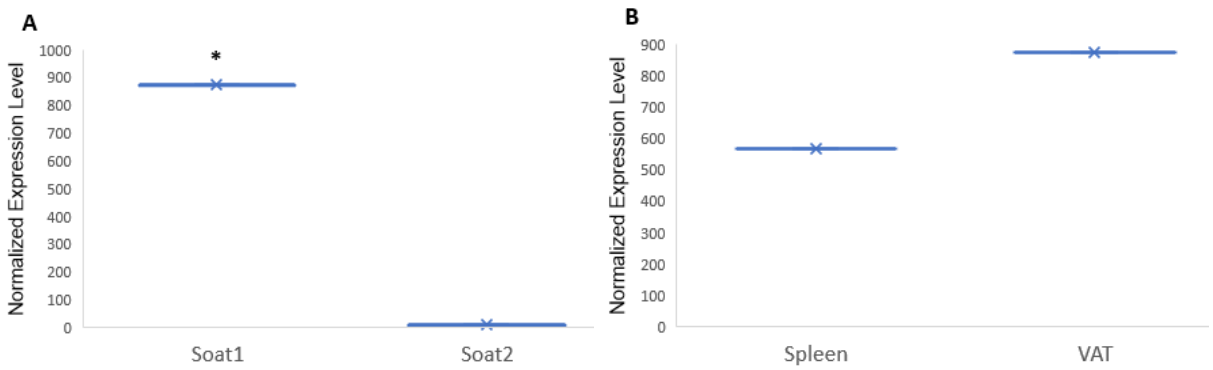

Figure 4. (A) Average *Soat1* and *Soat2* normalized expression levels in VAT Tregs of vTreg53 TCR-tg mice fed on a NCD for 16 weeks (n=2, biological replicates); (B) Average *Soat1* normalized expression level in spleen and VAT Tregs of vTreg53 TCR-tg mice fed on a NCD for 16 weeks (n=2, biological replicates).

Discussion

The results refute our hypothesis that knocking out *Soat1* would correlate with an increase in VAT Treg accumulation within the recipient mice. This seems to indicate that the pathway involved in sequestering free cholesterol into storage as cholesterol esters does not improve VAT Treg signaling and proliferation. Therefore, rescuing VAT Tregs to help promote our metabolic health by decreasing inflammation within the VAT via regulation of this pathway may not be viable. The difference in percent accumulation of *Soat1* KO donor Tregs to non-KO donor Tregs within the spleen and VAT of all the recipient mice appear to be insignificant except for

the donor Tregs that accumulated within the spleen of FAT7 immunized mice (Figure 3C, t(6)= -2.9506, p= 0.0256). However, the validity of this significance is questionable due to the small sample size of donor Tregs populating the spleen in comparison to the abundant donor Tregs that accumulated in the VAT.

One potential explanation for this trend is that knocking-out *Soat1* leads to an excessive accumulation of free cholesterol. Studies have found that an abnormally high free cholesterol to phospholipid ratio within the lipid rafts can inhibit integral membrane proteins whose function may be blocked or altered under conditions of high membrane rigidity (Tabas, 2002). Furthermore, ACAT plays an important role in maintaining intracellular cholesterol homeostasis by preventing an excessive buildup of free cholesterol which could have a cytotoxic effect by physically disrupting the integrity of intracellular structures through the formation of cholesterol crystals or by inducing ER stress mediated apoptosis (Sozen & Ozer, 2017). Our lab has also previously quantified *Soat1* transcript levels in vTreg53 TCR-tg mice using bulk RNA-sequencing and performed a paired t-test to determine if there was any difference in the expression levels of *Soat1* in either VAT or spleen Tregs. Although there does not seem to be a significant difference in *Soat1* expression between VAT Tregs and spleen Tregs (Figure 4B, t(1)=6.7740, p=0.0933), there still appears to be an approximate 50% increase in the average VAT Treg expression of *Soat1* in comparison to spleen Tregs (although more biological replicates are needed to confirm this). Additionally, we know that VAT Tregs have higher cholesterol levels and cholesterol uptake compared to lymphoid Tregs at steady state (Elkins, et al., 2023), indicating that *Soat1* may be more essential for the survival of VAT Tregs, where knocking out *Soat1* could lead to cytotoxic levels of free cholesterol within these cells. The

combined effects on the inhibition of surface proteins and cytotoxicity of excessive free cholesterol could explain why there wasn't an increase in VAT Treg accumulation if the hypothesized increase in TCR signaling and proliferation is counteracted by cell damage.

Another explanation is that ACAT is present in two isoforms, ACAT1 and ACAT2 (encoded by *Soat1* and *Soat2* respectively), where both are involved in the conversion of free cholesterol to cholesterol esters (Seo, et al., 2001). There could still be a possibility that *Soat2* expression was enough to counteract the effects of *Soat1* KO by continuing to express ACAT2 and sequester free cholesterol into cholesterol esters. However, bulk RNA-sequencing data in our lab shows that *Soat1* is more expressed in VAT Tregs of vTreg53 TCR-tg mice than *Soat2* through a paired t-test (Figure 4A, $t(1)=17.1231$, $p=0.0371$). Although ACAT2 is expressed in low amounts, it may still be productive to analyze its impact on free cholesterol esterification within these cells.

Moreover, new literature is citing the idea that distinct pools of cholesterol exist within the plasma membrane, where different pools could either be localized in microdomains or lipid rafts, involved in cholesterol metabolism and homeostasis, or essential for maintaining membrane integrity (Kennewick et al., 2023). We are unaware of which cholesterol pool ACAT1 acts on, and more specific analysis may be necessary to determine how *Soat1* KO impacts cholesterol pools within the lipid rafts.

In conclusion, although previous literatures have shown that upregulating plasma membrane (PM) cholesterol levels promotes T cell signaling and proliferation, modulating PM cholesterol levels via the ACAT1 pathway does not appear to influence VAT Treg accumulation. However, future directions branching from this experiment could be done by performing a *Soat2* KO or a

double knock-out experiment of *Soat1* and *Soat2* to determine if either isoforms of ACAT (or their combined effects) significantly impact VAT Treg accumulation. Other possible experiments could also focus on targeting specific cholesterol pools, such as the sphingolipid-associated cholesterol pools found within the lipid rafts, that have been shown to play an important role in TCR signaling.

References

Deiuliis, J., Shah, Z., Shah, N., Needleman, B., Mikami, D., Narula, V., Perry, K., Hazey, J., Kampfrath, T., Kollengode, M., Sun, Q., Satoskar, A. R., Lumeng, C., Moffatt-Bruce, S.,& Rajagopalan, S. (2011). Visceral Adipose Inflammation in Obesity Is Associated with Critical Alterations in Tregulatory Cell Numbers. PLoS ONE, 6(1), e16376. https://doi.org/10.1317/jounral.pone.0016376

Elkins, C. A., Pulavendran Sivasami, Bae, J., & Li, C. (2023). Cellular cholesterol homeostasis supports visceral adipose tissue (VAT) regulatory T cell (Treg) accumulation and promotes metabolic health. *Journal of Immunology*, *210* (1_Supplement), 76.16–76.16. https://doi.org/10.4049/jimmunol.210.supp.76.16

Fernandes, R. A., Li, C., Wang, G., Yang, X., Savvides, C. S., Glassman, C. R., Dong, S., Luxenberg, E., Sibener, L. V., Birnbaum, M. E., Benoist, C., Mathis, D., & K. Christopher García. (2020). Discovery of surrogate agonists for visceral fat Treg cells that modulate metabolic indices in vivo. *ELife*, *9*. https://doi.org/10.7554/elife.58463

Josefowicz, S. Z. (2013). Regulators of chromatin state and transcription in CD4 T-cell polarization. *Immunology*, *139*(3), 299–308. https://doi.org/10.1111/imm.12115

Kennewick KT, Bensinger SJ. (2023). Decoding the crosstalk between mevalonate metabolism and T cell function. *Immunol Rev*. 2023;317:71-94. doi:10.1111/ imr.13200

Li, C., DiSpirito, J. R., Zemmour, D., Spallanzani, R. G., Kuswanto, W., Benoist, C., & Mathis, D. (2018). TCR Transgenic Mice Reveal Stepwise, Multi-site Acquisition of the Distinctive Fat-Treg Phenotype. *Cell*, *174*(2), 285-299.e12. https://doi.org/10.1016/j.cell.2018.05.004

Li, C., Wang, G., Pulavendran Sivasami, Ramirez, R. A., Zhang, Y., Benoist, C., & Mathis, D. (2021). Interferon-α-producing plasmacytoid dendritic cells drive the loss of adipose tissue regulatory T cells during obesity. *Cell Metabolism*, *33*(8), 1610-1623.e5. https://doi.org/10.1016/j.cmet.2021.06.007

Muñoz-Rojas, A.R., Mathis, D. (2021). Tissue regulatory T cells: regulatory chameleons. *Nat Rev Immunol* 21, 597–611. https://doi.org/10.1038/s41577-021-00519-w

Nguyen, D. H., Giri, B., Collins, G., & Taub, D. D. (2005). Dynamic reorganization of chemokine receptors, cholesterol, lipid rafts, and adhesion molecules to sites of CD4 engagement. *Experimental Cell Research*, *304*(2), 559–569. https://doi.org/10.1016/j.yexcr.2004.11.022

Panduro, M., Benoist, C., & Mathis, D. (2016). Tissue Tregs. *Annual Review of Immunology*, *34*(1), 609–633. https://doi.org/10.1146/annurev-immunol-032712-095948

Pi-Sunyer FX. (1999). Comorbidities of overweight and obesity: current evidence and research issues. *Med Sci Sports Exerc*. 31(11 Suppl):S602-608. doi: 10.1097/00005768-199911001-00019.

Seo, T., Oelkers, P. M., Giattina, M. R., Worgall, T. S., Sturley, S. L., & Deckelbaum, R. J. (2001). Differential Modulation of ACAT1 and ACAT2 Transcription and Activity by Long Chain Free Fatty Acids in Cultured Cells. *Biochemistry*, *40*(15), 4756–4762. https://doi.org/10.1021/bi0022947

Shuster, A., Patlas, M., Pinthus, J. H., & Mourtzakis, M. (2012). The clinical importance of visceral adiposity: a critical review of methods for visceral adipose tissue analysis. *The British journal of radiology*, *85*(1009), 1–10. https://doi.org/10.1259/bjr/38447238

Sozen, E., & Ozer, N. K. (2017). Impact of high cholesterol and endoplasmic reticulum stress on metabolic diseases: An updated mini-review. *Redox Biology*, *12*, 456–461. https://doi.org/10.1016/j.redox.2017.02.025

Tabas, I. (2002). Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *Journal of Clinical Investigation*, *110*(7), 905–911. https://doi.org/10.1172/jci16452

Yang, W., Bai, Y., Xiong, Y., Zhang, J., Chen, S., Zheng, X., Meng, X., Li, L., Wang, J., Xu, C., Yan, C., Wang, L., Chang, C. C. Y., Chang, T. Y., Zhang, T., Zhou, P., Song, B. L., Liu, W., Sun, S. C., . . . Xu, C. (2016). Potentiating the antitumour response of CD8+ T cells by modulating

cholesterol metabolism. *Nature*, *531*(7596), 651–655. https://doi.org/10.1038/nature17412

Zhou X, Tang J, Cao H, Fan H, Li B. (2015). Tissue resident regulatory T cells: novel therapeutic targets for human disease. *Cell Mol Immunol*, 12(5),543-52. doi: 10.1038/cmi.2015.23.