STUDENT PAPER 2

Use of CRISPR/Cas9 Gene Editing Methods to Investigate the Mechanism of Trem2-Dependent Gene Expression in Macrophages

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ABSTRACT

Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) is a surface receptor expressed in macrophages during tissue injury. This receptor plays a role in driving phagocytosis and dampening inflammation. Because of this, it plays a large part in diseases such as Alzheimer's disease, liver fibrosis, and metabolic syndrome. Each of these diseases all have a population of TREM2-expressing macrophages that does not exist in healthy tissue. However, the exact pathway in which TREM2 is involved in these diseases is rather unknown. Macrophage gene expression is regulated by a variety of transcription factors such as ATF3 and TFEB. These transcription factors have been suggested to be involved in some of the disease processes mentioned above by RNA-seq or ChIP-seq experiments.

The research question we addressed was how these two transcription factors directly affect transcription in macrophages, specifically in the TREM2 pathway. CRISPR/Cas9 gene editing was used to generate loss of function alleles for each transcription factor. RNA-seq was then used to compare gene expression to define the gene-specific transcriptional roles of each factor and determine whether they play roles downstream of TREM2 signaling.

Results showed that Atf3 knockout had very few genes upregulated or downregulated in the RNA seq compared to Trem2 knockout. Tfeb, on the other hand, had 13 genes in common with Trem2 knockout that were expressed lower than the control and 10 genes in common expressed higher than the control. The Tfeb knockout had no difference in Trem2 expression between the knockout population and control, further providing evidence that Tfeb is located downstream of Trem2. Because Trem2 levels stayed consistent in the Tfeb KO, it is likely that some of the effects of Trem2 on the macrophage disease population genes are directly mediated through Tfeb.

INTRODUCTION

The immune system has a crucial role in protecting and healing the body when fending against pathogenic organisms. Specifically, macrophages are a key part of the first line response of the immune system through phagocytosis of bacteria or infected cells and by activating the inflammatory cascade. They not only provide protection against foreign entities but also assist other immune cells in the healing process. They help the body stabilize homeostasis and aid in tissue repair (Troutman et al., 2021). In addition, they have been described as regulators of tissue repair, regeneration, and fibrosis in multiple other

disease contexts (Wynn and Vannella, 2016). Because of their role in these specific functions of the immune system, they have been declared as potential therapeutic targets, which is why there has been an increased level of research interest surrounding this topic (Wynn and Vannella, 2016).

Several macrophage phenotypes are known to be found in diseased tissues; however, there is only a limited understanding of what exactly causes the diversification of macrophages to result in these diseased phenotypes (Seidman et al., 2020). Seidman et al. delved into understanding how environmental signals related to diseases modify macrophage gene expression in a nonalcoholic fatty liver disease (NASH) mouse model. They identified a substantial increase in the expression of Triggering Receptor Expressed on Myeloid Cells 2 (Trem2) in Kupffer Cells in the NASH model, a gene that is only minimally expressed in Kupffer Cells of healthy livers (Seidman et al., 2020). The NASH diet resulted in more than 800 genes in Kupffer Cells being differentially expressed, showing how environmental signals have a profound impact on macrophage gene expression (Seidman et al., 2020).

TREM2 in particular is a type of surface receptor typically expressed in a subset of macrophages during different types of tissue injury (Gratuze et al., 2018). This receptor plays a role in driving phagocytosis and lipid catabolism, and it was also found to be pushing the remodeling of immune cells on the tissue level (Jaitin et al., 2019). The TREM2 pathway seems to be involved in sensing tissue damage and restricting its spread (Deczkowska et al., 2020). TREM2 has been identified as playing a major role in cell types such as microglia in Alzheimer's disease, Kupffer cells in nonalcoholic steatohepatitis (NASH), and adipose tissue macrophages in obesity (Troutman et al., 2021). The goal of this research is to better understand the transcriptional pathways in macrophages and relate them to transcriptional changes conferred by TREM2. This may provide information to potentially target the transcriptional regulation of macrophages in human disease. Nonalcoholic steatohepatitis, obesity, atherosclerosis, and Alzheimer's disease all have a population of TREM2-expressing macrophages that do not exist in healthy tissue (Troutman et al., 2021). These populations are named disease-associated macrophages (DAM) in Alzheimer's disease, lipid-associated macrophages (LAM) in obesity, and scar-associated macrophages (SAM) in fibrosis (Troutman et al., 2021). Based on knockout experiments, TREM2 has been identified as a key regulator of these populations (Troutman et al., 2021).

However, how TREM2 modulates gene expression is unknown (Xiong et al., 2019). TREM2 binds to a large variety of ligands, including phospholipids, lipoproteins, and apoptotic cells (Deczkowska et al., 2020). Pharmacologically, TREM2 may not be the best place to target therapy because in these disease contexts there are a lot of ligands already present that TREM2 is a receptor for, so it may be better to target something downstream and activate it for less system wide effects and more specificity. Macrophage gene expression is known to be regulated by a variety of transcription factors, including ATF3, EGR2, and members of the MiT/TFE family (Troutman et al., 2021). These transcription factors have been suggested to be involved in some of the disease processes mentioned above by RNA-seq or ChIP-seq experiments (Seidmann et al., 2020, Troutman et al., 2021).

The MiT transcription factors are known to be related to inflammatory and immune responses. Specifically, they have large roles in autophagy, lysosomal biogenesis, and lipid catabolism (Irazoqui 2020). The MiT/TFE transcription family consists of MITF, TFEB, TFEC, and TFE3. Because of their role in inflammatory responses and innate immunity, it makes them strong candidates to be connected with the TREM2 functional pathway as well as possible places of attack for several diseases (Irazoqui 2020). When the NASH diet was introduced to mice, the transcription factor ATF3, from the ATF/AP-1 family, increased in expression followed by TREM2 upregulation (Troutman et al., 2021). This makes ATF3 a good candidate to test in this experiment because of its connection to the TREM2 pathway.

The research question we addressed is how these transcription factors, ATF3 and TFEB, directly affect transcription in macrophages, specifically how these transcription factors are related to the TREM2 pathway. Our Lab defined TREM2 dependent enhancers using epigenetic methods and using computational methods identified an enrichment of motifs for ATF3 and members of the MiT/TFE family of transcription factors. These previous results led to the hypothesis that ATF3 and TFEB (a member of the MiT/TFE Family) are located downstream of TREM2 signaling.

FIGURE 0:

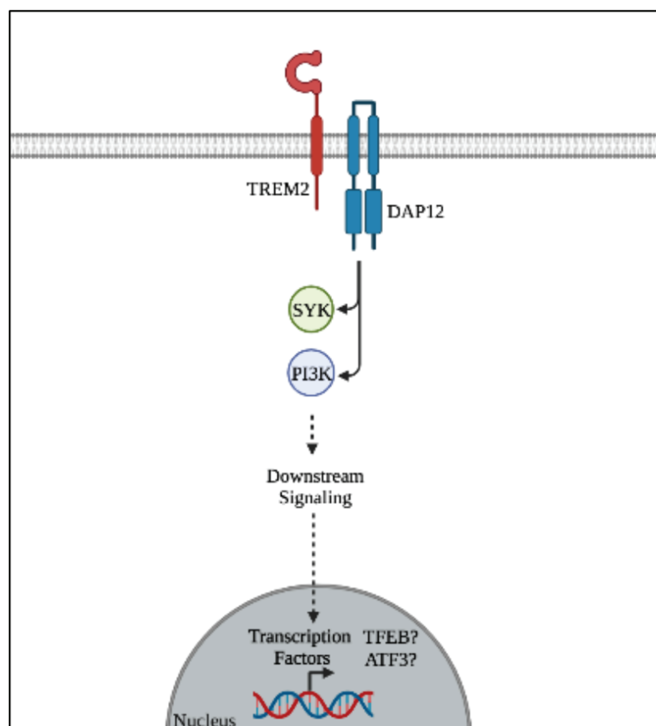


Figure 0: Known signaling pathway of TREM2. The hypothesis was that TFEB and ATF3 were potential transcription factors downstream that TREM2 uses to modulate gene expression.

Adapted from Dezykowska, Weiner, and Amit, Cell 2020.

Created with BioRender.com

Figure 0 shows the known pathway of TREM2 along with where ATf3 and TFEB potentially fit in the pathway. To test this hypothesis, we looked for gene expression overlap between CRISPR knock outs of these transcription factors and the knockout of TREM2 itself in a cultured macrophage model. This hypothesis was tested by using CRISPR/Cas9 gene editing to generate loss of function mutations for each transcription factor in a model macrophage cell line. FACS sorting was used to isolate single cells for clonal expansion, allowing the identification of cells with homozygous frameshift mutations resulting in complete loss of function. RNA-seq was then used to compare the gene expression of wild type and mutant cells to define how they affect gene expression. We then compared these generated knockout cells to the loss of TREM2 in the same cell type, a dataset already created by our laboratory. By using CRISPR, FACS sorting, and RNA-seq, we conducted a comprehensive study of how these factors exert their effect on macrophage gene expression.

Methods

First, gRNA sequences were incorporated into plasmids for cloning. CHOP CHOP (https://chopchop.cbu.uib.no) was used to determine the optimal guide RNA sequence to generate primers for cloning.

Annealing of primers for gRNA was done at 50 µM each of reverse and forward primer in 11xDuplex buffer (1.1M Potassium Acetate, 300mM Hepes), starting at 95°C for 5 min, followed by a decrease in temperature to 25°C at a rate of 5°C/min. BsmBI-digested LentiGuide-mCherry and the annealed primers were ligated by T4 DNA ligase at 16°C overnight.

The ligation mixture and Stbl2 competent cells were incubated on ice followed by a 30 second 42°C heat shock. Bacteria were cultured in SOC media and then plated on an LB agar plate with ampicillin at 37°C overnight.

Each colony grown on the plates was extracted and cultured in LB media with ampicillin overnight at 37°C while shaking at 250RPM. DNA was then extracted from the cultured colonies using the Zymo Mini Prep purification kit. DNA was sent for Sanger sequencing to confirm the

insertion of the correct gRNA sequence into the plasmid.

Once the correct sequence was confirmed, 100mL LB (Amp+) cultures of the transformed bacteria were incubated overnight in incubator (250 RPM, 37°C). Then, Macherey-Nagel Midi Prep DNA purification kit was completed to procure 5 µg of plasmid DNA.

293T cells were hawed and then cultured in DMEM10 which includes DMEM with glutamate, penicillin, streptomycin, and 10% fetal bovine serum (FBS) until 70-80% confluency was reached. The cells were trypsiniszed and counted to add ~3.5 x 106 293T cells into every 10 cm dish with DMEM10 as media.

The media was changed to DMEM supplemented as above, except with 30% FBS. 5µg of the lentiviral vector, 3.75µg of psPAX2, and 1.25µg of pVSVG along with XtremeGene HP was mixed in Opti-MEM media and then added to the 293T cells in the 10 cm plates. 24 hours and 48 hours after lentiviral vector was added, supernatant media containing the virus was collected and filtered through 0.45 µm filters. 10µg/mL fibronectin was added to a 12 well plate and placed in an incubator for 1 hour (37°C).

Cas9-expressing conditionally immortalized macrophages (ER HoxB8 cells, generated as described in Shen et al., eLife 2022) were cultured in RPMI media with glutamate, penicillin/streptomycin, 10% FBS, 20ng/mL GM-CSF, and 0.5µM β-estradiol to prevent differentiation. LentiBlast and polybrene were added to each well along with 500,000 cells in RPMI. Finally, the virus or DMEM for the negative control well was added. Transduction was carried out during centrifugation at 1000g for 90 minutes at room temperature. After 5 days, successfully transduced cells (mCherry positive) were single-cell sorted by FACS into 96-well plates.

Of the grown clones of cells, DNA was isolated through the Qiagen Nucleic Acid Extraction Kit. PCR was conducted to amplify the genomic region targeted with gRNA and gel electrophoresis was run with the PCR product. DNA was extracted from the gel, purified, and Sanger sequenced to assess for clones with homozygous frameshift mutations on both alleles.

Clones with homozygous frameshift mutations (as well as clones with control gRNA) were differentiated into macrophages. Cells were washed twice with PBS, followed by differentiation in DMEM with 17 ng/ml M-CSF for 7 days. Cells were lysed in TRIzol Reagent and RNA was isolated. The RNA was fragmented, reverse transcribed to cDNA, and then the libraries were prepared and amplified. Finally, it was sequenced on a NextSeq 500 or Hi-Seq 4000 (Illumina, San Diego, California). It was performed as done in Cobo et al. RNA-seq analysis was completed through the HOMER pipeline to better understand the gene expression of wild type and mutant cells (Heinz et al. 2010).

Results

RNA seq analysis was done through the HOMER pipeline and it was used to calculate the log2 Fold Change and the p adjusted values (Heinz et al. 2010). These two values were then used to determine which genes in the knockout cells were significantly upregulated and downregulated. For a gene to be considered significantly upregulated or downregulated, it required a 1.5 times difference in transcripts per million when comparing the control to the knockout population and a p adjusted value of less than 0.05. The red dots in Figures 1 and 3 represent significant upregulation and the blue dots represent significant downregulation.

FIGURE 1:

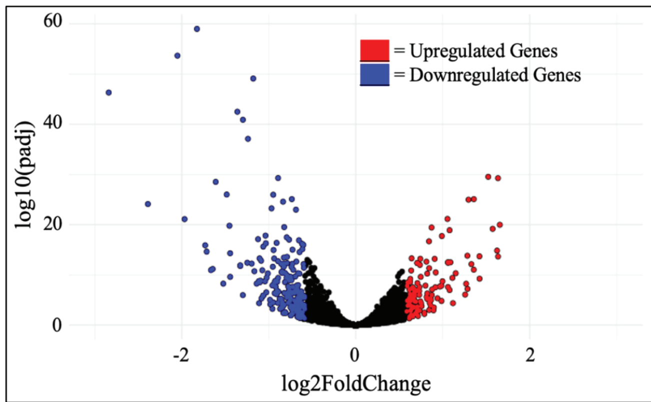
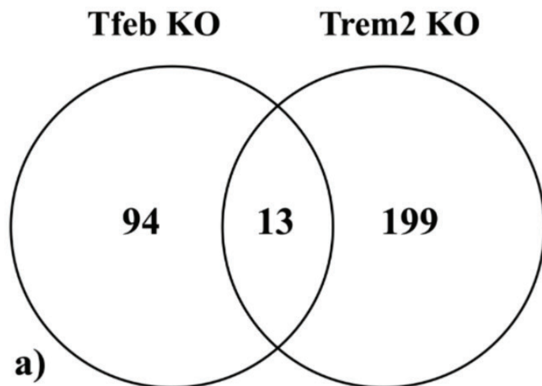


Figure 1: Volcano Plot of RNA seq results of Tfeb KO cells vs. control. Upregulation and downregulation for genes were determined by p adj < 0.5 and Fold change > 1.5 or < -1.5.

FIGURE 2:



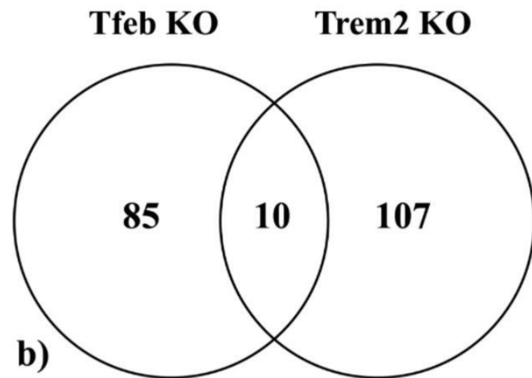


Figure 2: There are several genes regulated in common between the Tfeb KO and Trem2 KO compared to the control.

- Venn Diagram of downregulated genes
- Venn Diagram of upregulated genes

For Tfeb KO, Figure 1 shows there were several genes that were both upregulated and downregulated. Figure 2a identifies 13 genes in common that were downregulated in both populations of knockout cells compared to the control. These 13 genes were Cx3cl1, Gadd45g, Tmem132a, Zfp503, Pparg, Gngt2, Slc15a3, At3, Cd9, Tgm2, Sdc3, Plekho2, and Gnas. There were 10 genes in common that were upregulated in both populations compared to the control (Figure 2b). The 10 genes were Ccnb2, Cenpa, Ccl2, Mtus1, Ncapg2, Nt5dc2, Slc16a6, D17H6S56E-5, Egr1, and Rhob.

FIGURE 3:

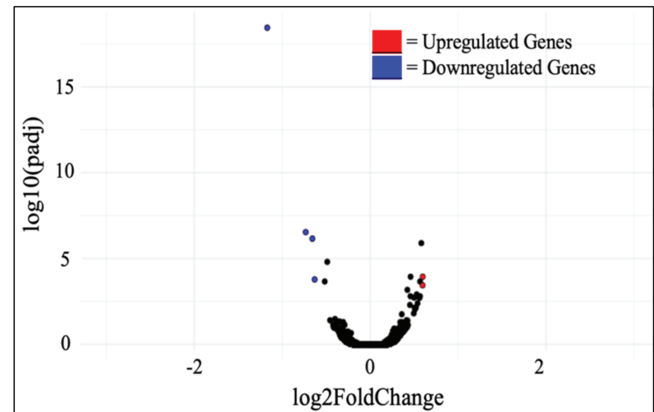


Figure 3: Volcano Plot of RNA seq results of control vs. Atf3 KO cells. Upregulation and downregulation for genes were determined by p adj < 0.5 or Fold change > 1.5 and < -1.5.

FIGURE 4:

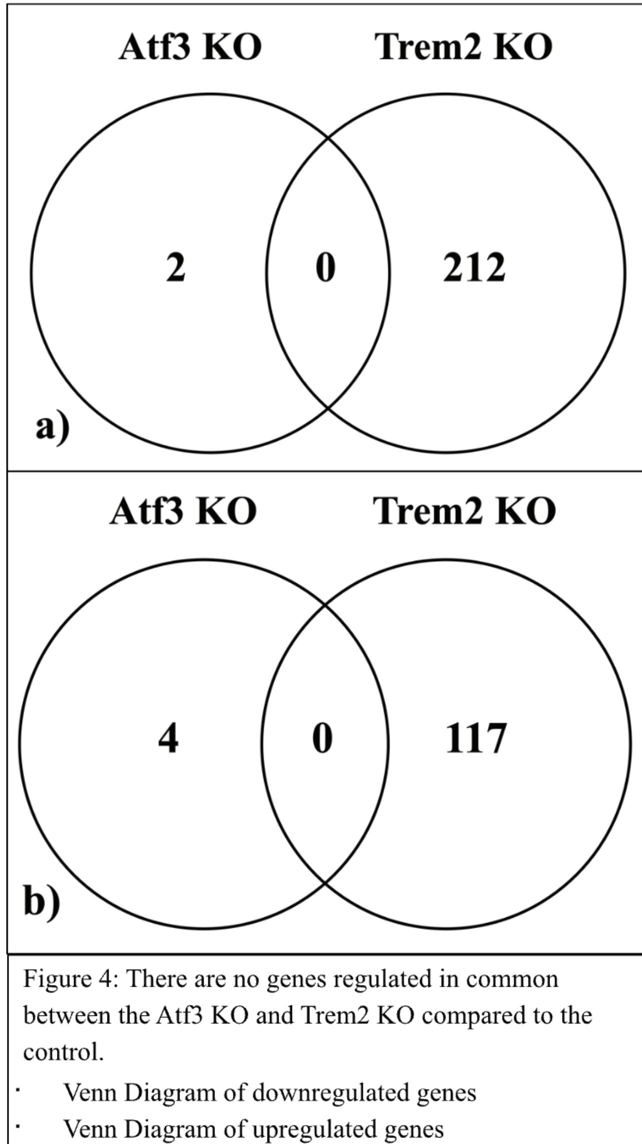


Figure 4: There are no genes regulated in common between the Atf3 KO and Trem2 KO compared to the control.

- Venn Diagram of downregulated genes
- Venn Diagram of upregulated genes

For Atf3 KO, Figure 3 has very few genes that were significantly upregulated and downregulated. The venn diagrams in Figure 4 reveals that there were zero genes upregulated in common between Atf3 KO and Trem2 KO.

FIGURE 5:

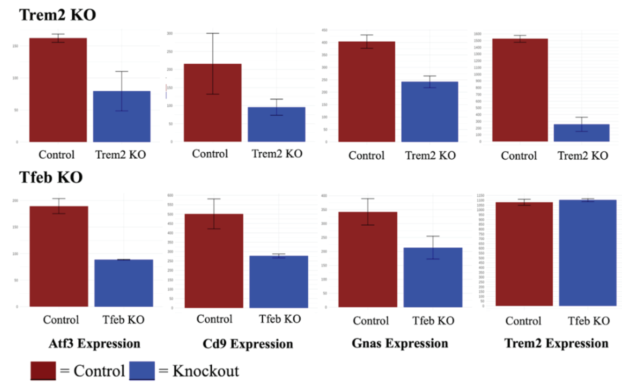


Figure 5: Bar plots comparing gene expression of select genes between the knockout population and the control population.Y-axis represents transcripts per million.

The genes chosen in Figure 5 are all signature genes of the earlier mentioned DAMs, LAMs and SAMs macrophages from the Troutman et al. literature review. The first row of plots of Figure 5 are from the Trem2 KO cells while the second row of plots is from the Tfeb KO cells.

Previous research showed there were 6 genes that were downregulated in these DAM/LAM/SAM populations as well as Trem2 KO when comparing to the control (Prohaska 2022). In Tfeb KO, Atf3, Cd9, and Gnas, 3 of the 6 genes, were found downregulated as well. For the first three sets of plots in Figure 5 from the left to the right, the gene expression profile is similar where the knockout of the gene in question resulted in a decrease in gene expression compared to the control. In the fourth set of plots, the Trem2 KO resulted in a decreased expression of Trem2 in the cells. However, the knockout of Tfeb results in no significant change in Trem2 expression when comparing the control and the Tfeb KO cells.

Discussion

The results support the potential role of Tfeb in downstream Trem2 signaling, but not Atf3. Because Trem2 levels stayed consistent in the Tfeb KO, it is likely at least some of the effects of Trem2 on the DAM, LAM, and SAM genes are directly mediated through Tfeb. It further provides evidence that Tfeb is located downstream of Trem2.

For Atf3, there was little significant upregulation and downregulation of genes. Therefore, Atf3 KO needs to be tested in different conditions such as amylin and myeloid to see if there will be a difference in the gene expression after knockout.

These KO experiments were performed in an immortalized cell line in vitro in which the Trem2 pathway is constitutively activated. Previous research has shown that the in vivo environment is very important in maintaining macrophage identity, including induction of the Trem2 pathway itself (Gosselin et al., 2017). Therefore, there may be differences in the consequences of these knockouts in an in vivo setting. The Tfeb and Atf3 KO must be performed in an in vivo setting to compare the generated data from this experiment to better understand these experiments.

In the future, further KO experiments can be conducted with other transcription factors in the MiT/TFE and ATF/AP-1 families. Glass Lab RNA seq data has found that three members of the MiT/TFE family and more than six members of the ATF/AP-1 family are expressed in HoxB8 cells. It is likely that these factors are at least partially redundant, which would result in more modest effects when a single factor is deleted. As a result, more severe phenotypes might be expected if combinations of KOs were generated. Additional downstream experiments could be performed by investigating the consequences of macrophages with knockouts of these transcription factors in specific disease models such as Alzheimer's disease, metabolic syndrome, and liver fibrosis.

All in all, the data discussed here may help uncover the Trem2 pathway so that we may potentially create novel therapies for diseases that are currently challenging to treat.

CITATIONS

Cobo, Isidoro et al. "DNA methyltransferase 3 alpha and TET methylcytosine dioxygenase 2 restrain mitochondrial DNA-mediated interferon signaling in macrophages." Immunity vol. 55,8, 2022, pp. 1386-1401.e10., doi:10.1016/j.immuni.2022.06.022

Deczkowska, Aleksandra, et al. "The Physiology, Pathology, and Potential Therapeutic Applications of the Trem2 Signaling Pathway." Cell, vol. 181, no. 6, 2020, pp. 1207–1217., https://doi.org/10.1016/j.cell.2020.05.003.

Gosselin, David et al. "An environment-dependent transcriptional network specifies human microglia identity." Science (New York, N.Y.) vol. 356,6344 (2017): eaal3222. doi:10.1126/science.aal3222

Gratuze, Maud, et al. "New Insights into the Role of Trem2 in Alzheimer's Disease." Molecular Neurodegeneration, BioMed Central, 20 Dec. 2018, https://molecularneurodegeneration.biomedcentral.com/articles/10.1186/s13024-018-0298-9#citeas.

Heinz S, Benner C, Spann N, Bertolino E et al. "Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities." Mol Cell, 28 May 2010; 38(4):576-589., 10.1016/j.molcel.2010.05.004

Irazoqui, Javier E. "Key Roles of MIT Transcription Factors in Innate Immunity and Inflammation." Trends in Immunology, vol. 41, no. 2, 2020, pp. 157–171., https://doi.org/10.1016/j.it.2019.12.003.

Jaitin, Diego Adhemar, et al. "Lipid-Associated Macrophages Control Metabolic Homeostasis in a trem2-Dependent Manner." Cell, U.S. National Library of Medicine, 27 June 2019, https://pubmed.ncbi.nlm.nih.gov/31257031/.

Seidman, Jason S., et al. "Niche-Specific Re-Programming of Epigenetic Landscapes Drives Myeloid Cell Diversity in Nash." SSRN Electronic Journal, 16 June 2020, pp. 1057–1074., https://doi.org/10.2139/ssrn.3541367.

Shen, Zeyang, et al. "Systematic Analysis of Naturally Occurring Insertions and Deletions That Alter Transcription Factor Spacing Identifies Tolerant and Sensitive Transcription Factor Pairs." ELife, ELife Sciences Publications, Ltd, 20 Jan. 2022, https://elifesciences.org/articles/70878.

Prohaska, Thomas Andreas "The Role of TREM2 in Myocardial Injury and Transcriptional Regulation of Macrophage Phenotypes." UC San Diego, 2022, ProQuest ID: Prohaska_ucsd_0033D_21481. Merritt ID: ark:/13030/m5q02dvt. Retrieved from https://escholarship.org/uc/item/7x0637d7

Troutman, Ty D., et al. "Exploiting Dynamic Enhancer Landscapes to Decode Macrophage and Microglia Phenotypes in Health and Disease." Molecular Cell, vol. 81, no. 19, 2021, pp. 3888–3903., https://doi.org/10.1016/j.molcel.2021.08.004.

Wynn, Thomas A., and Kevin M. Vannella. "Macrophages in Tissue Repair, Regeneration, and Fibrosis." Immunity, vol. 44, no. 3, 2016, pp. 450–462., https://doi.org/10.1016/j.immuni.2016.02.015.

Xiong, Xuelian, et al. "Landscape of Intercellular Crosstalk in Healthy and Nash Liver Revealed by Single-Cell Secretome Gene Analysis." Molecular Cell, Cell Press, 8 Aug. 2019, https://www.sciencedirect.com/science/article/pii/S1097276519305830.

Mohnish Alishala

Faculty Mentorship Program: 2021–22

Biography:

My name is Mohnish Alishala and I am a third-year human biology major. At UCSD, I am a head coach for StRIVE, a club where we coach adult students with disabilities to help them transition to independent living. I am also a member of Camp Kesem where we fundraise money throughout the year to create summer camps for children whose parents have cancer. My involvement on campus gives me an opportunity to make a positive impact on other people.My research interests focus on the immune system and how our body fights against illness. This ties in with my career goal of becoming a physician.

"As an aspiring physician, I was interested in pursuing biomedical research to learn more about the immune system from a different perspective."

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