Utilizing Single-Cell RNA-sequencing to explore Immunomodulation in Glioblastoma

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I. Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults[1]. It has a 5-year prognosis of less than 5% survival; it is incredibly resistant to the standard of care including surgical resection, chemotherapy, and radiation treatment; and it has an extremely high recurrence rate. Therefore, various efforts to improve therapeutic outcomes have been proposed and developed over the past decade. Immunotherapy, which is harnessing the body's innate defensive mechanisms to fight a disease, has emerged as an effective therapy for many forms of cancer including leukemia, cervical cancer, and bladder cancer among others due to its synergistic effects with other forms of therapy, reduced number of side effects, and overall ability to induce lower recurrence rates. It's lack of effectiveness in GBM is thought to be due to the low mutational load of the tumor, low levels of antigen shed, low immune infiltration, and local and systemic immune suppression[12].

Amongst immunotherapies, one that has gained a lot of traction in recent years is Immune Checkpoint Blockade (ICB) which has been approved to treat numerous types of cancers[8] in including Hodgkin lymphoma, liver cancer, lung cancer among many others. However, ICB has been found to only have a limited effect in treating GBM[2]. This may in part be due to the dense infiltration of pro-tumor myeloid cells in the tumor-microenvironment (TME)[3]. Targeting these stromal cell populations may potentially enable an increase in ICB treatment sensitivity. These cells have been found to engage with tumor stimuli[9] through calcium signaling which then eventually leads to pro-tumor processes further down-stream. Preliminary data in the lab identified CaMKK2 as a pro-tumor factor enabling ICB resistance as a component of the calcium signaling pathway. Further, early experiments indicated CaMKK2 inhibited the

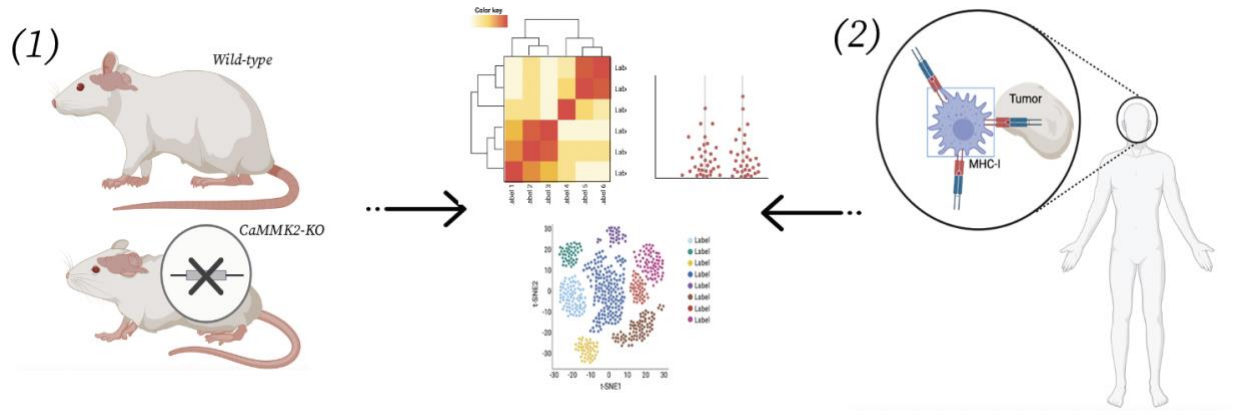
immunostimulatory capacity of Tumor Associated Macrophages (TAMs). Therefore, CaMKK2 has emerged as an increasingly relevant target for therapeutic intervention to boost ICB response.

II. Research Goals and Hypothesis

The goal of this work is to elucidate the CaMKK2-dependent immunophenotypic alterations in the glioblastoma TME, as well as the connection between antigen presentation and T cell function. *I hypothesize that CaMKK2 enhances a pro-tumor phenotype in TAMs and that a more immunostimulatory TME correlates with improved T cell response.*

III. Methods

A visual summary of the conducted experimental methods is given below, along with a more detailed description of each section of the overall project.



(1) Wildtype (WT) and CaMKK2 -/- mice were implanted with CT2a, a preclinical glioma model, and immune cells were harvested and processed for single cell RNA-sequencing (scRNA- seq) on day 14 post-implantation. A quantitative analysis workflow for this genomic data was prepared based on the Seurat6 pipeline, including processing, clustering,

validation of manual cell-type annotation, differential expression (DE), and GO Biological Process Enrichment.

(2) To better understand the relationship between antigen-presentation and TAM and T cell function we utilized a publicly available patient scRNA-seq data set[4] and stratified the patients based on MHC-I expression on their tumors.

IV. Results

i. Calcium-related Immunomodulation

Upon collecting the single-cell RNA sequencing data after processing and alignment, the data was loaded in R and processed through the Seurat package[6]. This data was then filtered and normalized as per standard single-cell protocol (*see Fig. 1*).

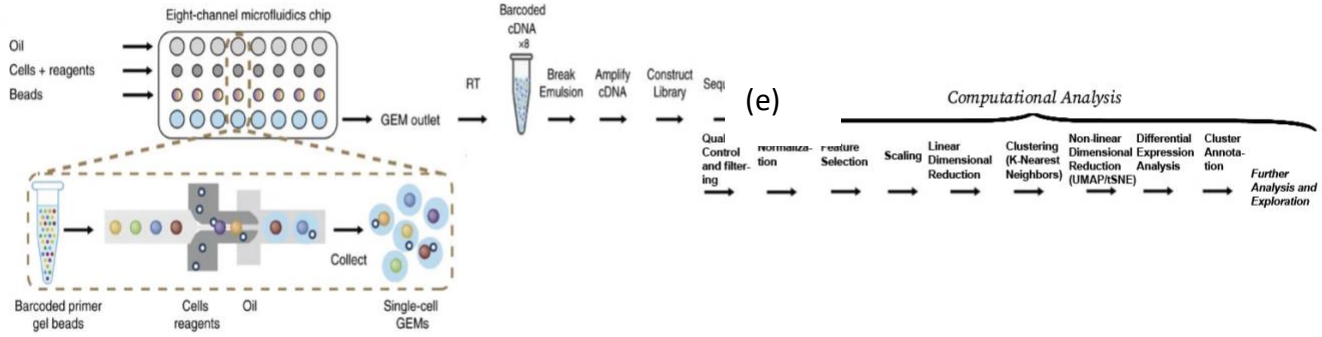


Figure 1. A visualization of the 10x Genomics single-cell sequencing platform and the subsequential Seurat computational analysis pipeline. (derived from Zheng et al, Nature Comms[5] and the Satija Lab)

In preparation for downstream analysis of gene expression, genes that are highly variant from cell-to-cell are identified to help highlight biological signals in the dataset[10,11] and for Principal Component Analysis (PCA). Then the data was scaled, linearly dimensionally reduced (PCA) (*see Fig 2b, c, d*), and clustered (*see Fig. 2d*); the majority of data variance was found to be

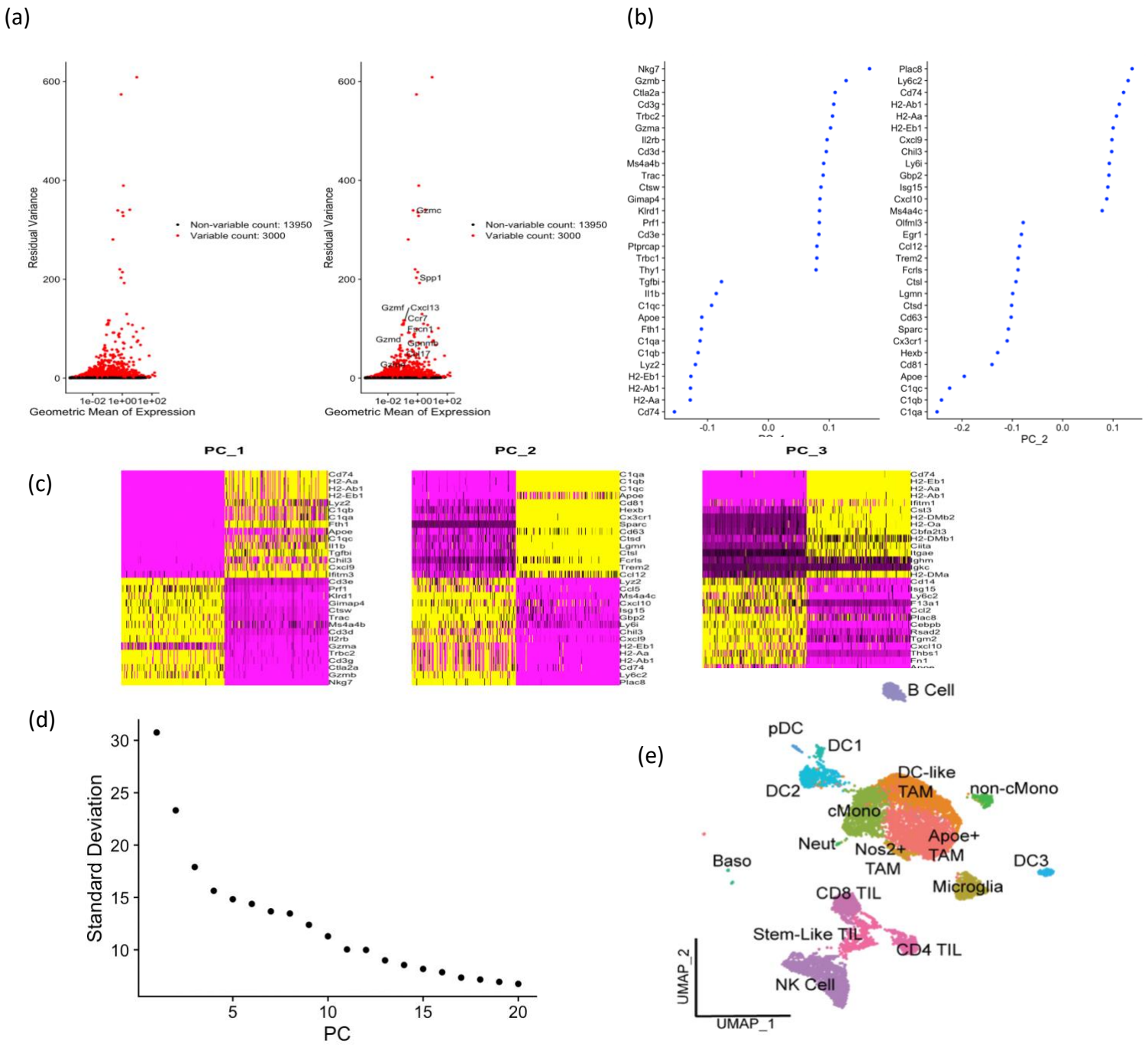
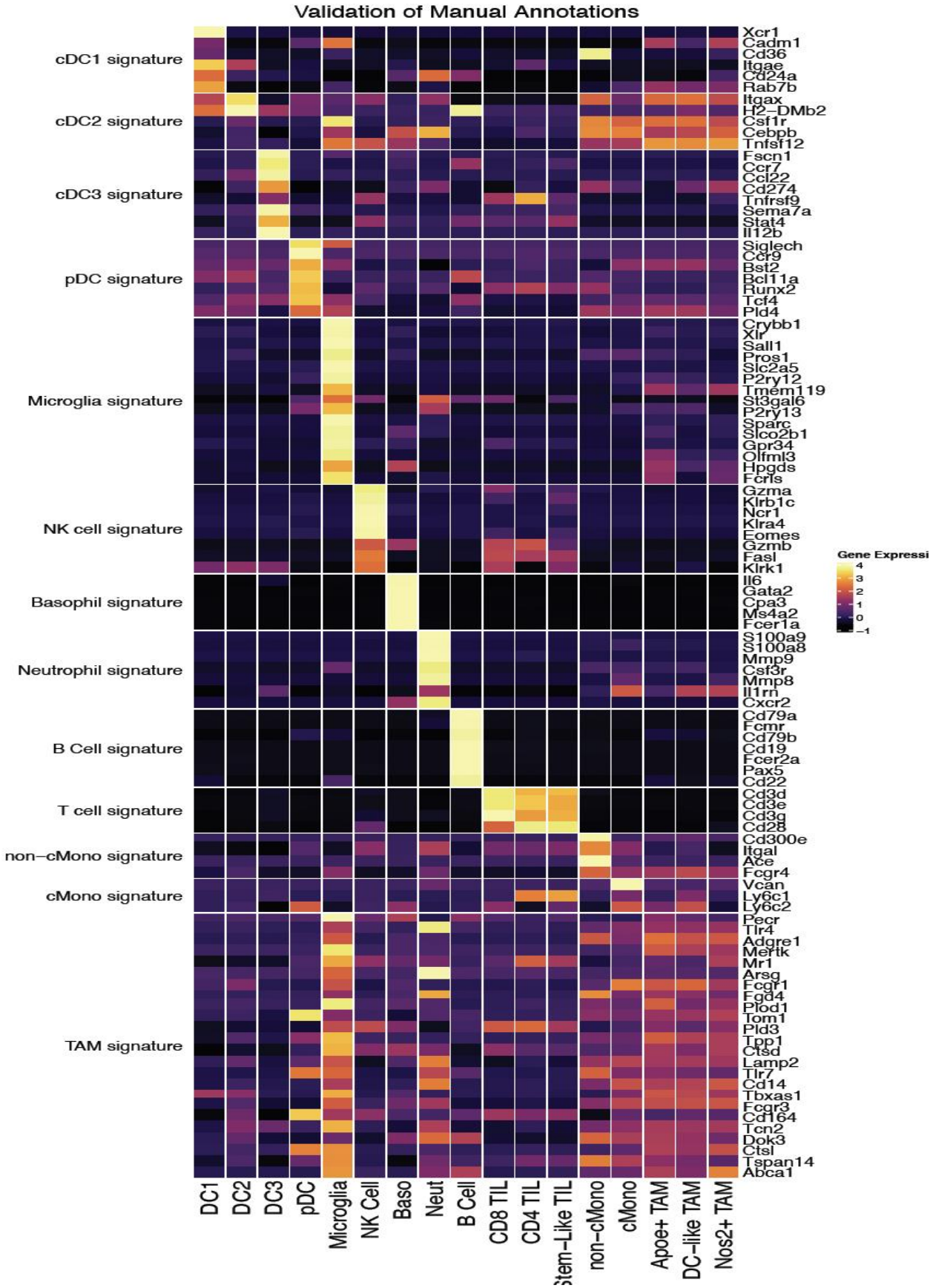


Figure 2. Standard Seurat Workflow for Processing and Exploring Data. (a) Identification of highly variable gene features (b) Visualization of Principal Components after linear dimensionality reduction (in this case, PCA) (c) Visualization of PC'S 1-3; the DimHeatPlot function is a great tool in elucidating sources of data set heterogeneity (d) An elbow plot for the processed data set; by the placement of the "elbow" we can see that this data set is mostly explained by 11 or so PC's. (e) Non-linear Uniform Manifold Approximation (UMAP) dimensional reduction plot of collected murine scRNA-seq data; clusters were manually annotated. (f) (On the following page) The validation of manual annotations using bona fide gene signatures.

(f)


Validation of Manual Annotations
cDC1 signature
cDC2 signature
cDC3 signature
pDC signature
Microglia signature
NK cell signature
Basophil signature
Neutrophil signature
B Cell signature
T cell signature
non−cMono signature
cMono signature
TAM signature
Xcr1
Cadm1
Cd36
Itgae
Cd24a
Rab7b
Itgax
H2−DMb2
Csf1r
Cebpb
Tnfsf12
Fscn1
Ccr7
Ccl22
Cd274
Tnfrsf9
Sema7a
Stat4
Il12b
Siglech
Ccr9
Bst2
Bcl11a
Runx2
Tcf4
Pld4
Crybb1
Xlr
Sall1
Pros1
Slc2a5
P2ry12
Tmem119
St3gal6
P2ry13
Sparc
Slco2b1
Gpr34
Olfml3
Hpgds
Fcrls
Gzma
Klrb1c
Ncr1
Klra4
Eomes
Gzmb
Fasl
Klrk1
Il6
Gata2
Cpa3
Ms4a2
Fcer1a
S100a9
S100a8
Mmp9
Csf3r
Mmp8
Il1rn
Cxcr2
Cd79a
Fcmr
Cd79b
Cd19
Fcer2a
Pax5
Cd22
Cd3d
Cd3e
Cd3g
Cd28
Cd300e
Itgal
Ace
Fcgr4
Vcan
Ly6c1
Ly6c2
Pecr
Tlr4
Adgre1
Mertk
Mr1
Arsg
Fcgr1
Fgd4
Plod1
Tom1
Pld3
Tpp1
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Tbxas1
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Ctsl
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DC3
pDC
Microglia
NK Cell
Baso
Neut
B Cell
CD8 TIL
CD4 TIL
Stem−Like TIL
non−cMono
cMono
Apoe+ TAM
DC−like TAM
Nos2+ TAM

explained by the top 11 PC's. Clustering was based on the creation of K-nearest neighbors (KNN) graph and then the 'drawing' of boundaries around locally similar cell "communities".

Additionally, non-linear dimensionality reduction tools were utilized to project and explore the local and global similarities of the data set (*see Fig 2d*). To label clusters, manual annotations were made based on reviewing differentially expressed genetic markers with canonically known markers. These annotations were confirmed by validation through the creation of a heat map. Bona fide expression signatures for the cell-types of interest were collected and then, utilizing the Seurat[6] and ComplexHeatMap[7] packages in R, we visually validated the collected expression signatures of the annotated clusters (*see Fig 2f*).

After this was completed, differential expression analysis was conducted on the data when stratified for genotype (CaMKK2 KO -/-, Wild-type). We investigated marker genes that exhibited large differences in expression between the two genotype identity classes. By correlating this expression data with genes known to be associated with immune programs, we found TAM populations from CaMKK2 KO mice had upregulated gene programs associated with IFN response, immunostimulation, and antigen processing, and downregulation in disease-associated microglia genes, supporting the claim that CaMKK2 induces a pro-tumor TAM phenotype (*see Fig. 3*).

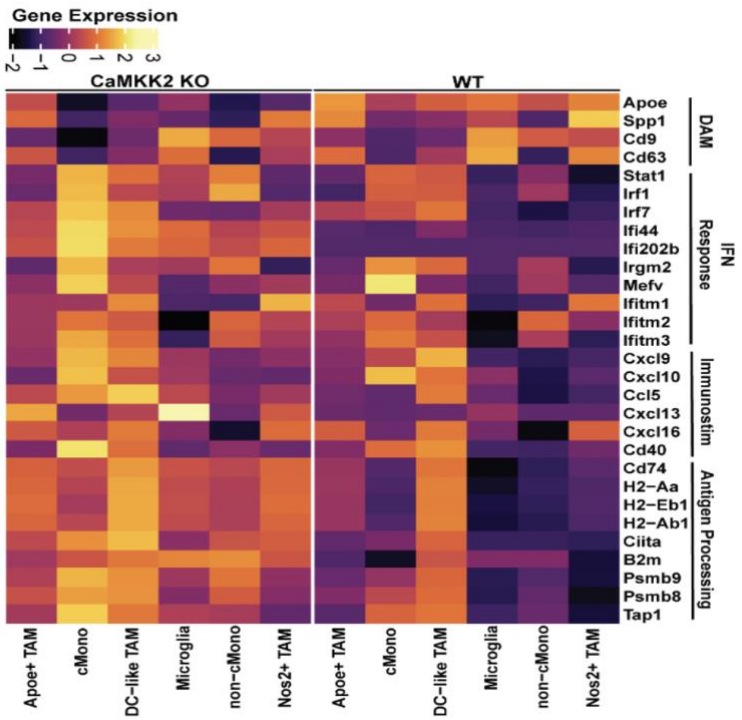

Figure 3. Differential Expression analysis between WT and CaMKK2 KO revealed modulation in immune processes. Here we have cell-type gene expression for CaMMK2 vs KO for selected immune programs.

ii. MHC-I-related Immunostimulation

The publicly available data set was processed in a similar manner to previous set. The data was stratified based on patient data and then ordered based on MHC-1 expression allowing the identification of the Top 33% and Bottom 33% patients (n=9) (see Fig. 4a). Following this differential expression analysis and GO Biological Process Enrichment were conducted on the data set (see Fig. 4b, c). We found that High tumor MHC-I expression was associated with a pro-inflammatory phenotype in Macrophages (-CD163, -CXCR4, -CCL20, ↑CCL4) and T cells (-CD69, ↑CD48), demonstrating that higher antigen presentation in the TME is associated with more anti-tumor immune phenotypes. These results were corroborated by the processes highlighted in the GO Biological Process Enrichment including

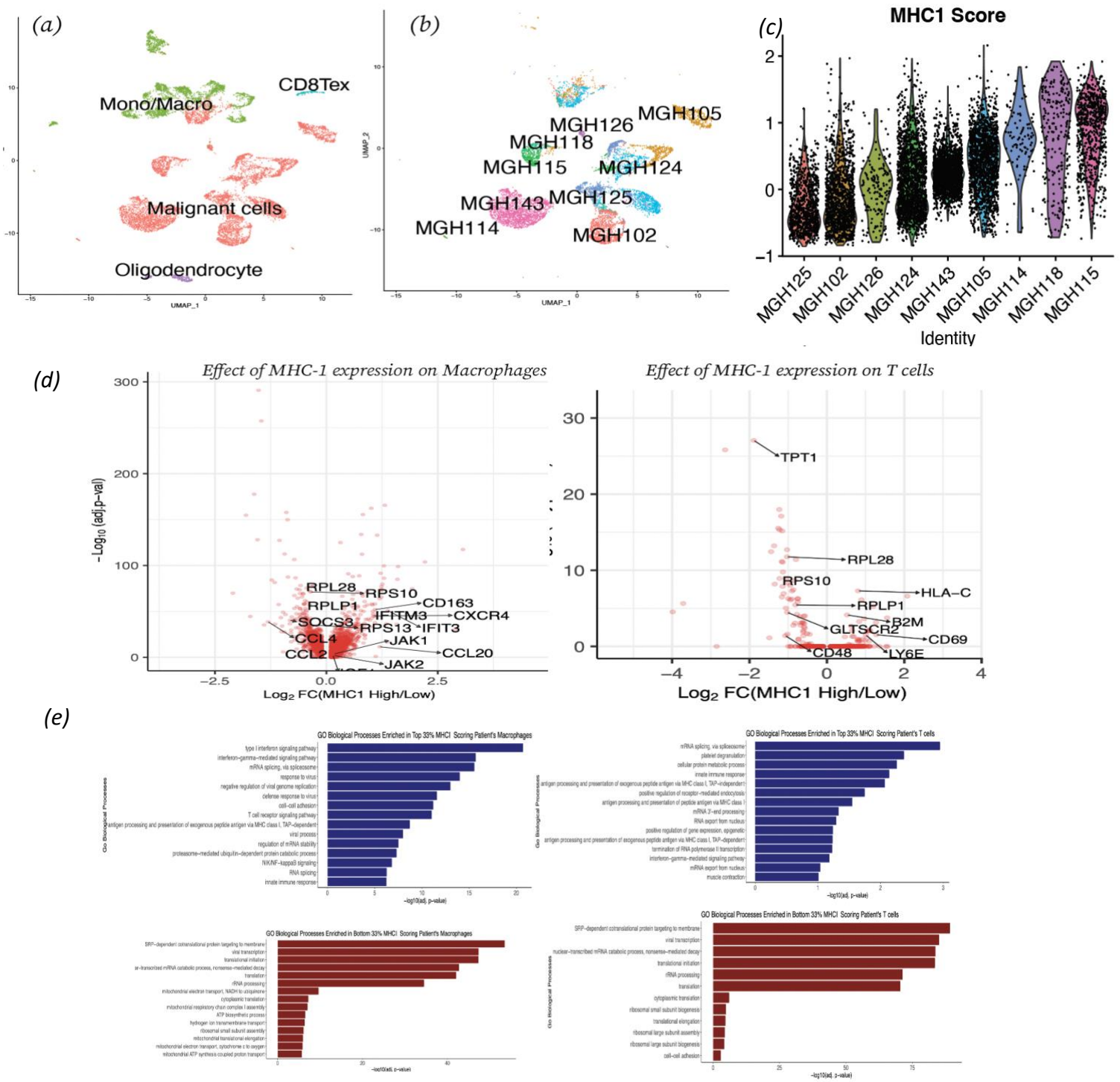

Figure 4. Exploring the connection between antigen presentation and immune cell function a) UMAP plot and pre-annotations reveals major cell types present in the immune data set. (b) Cells in UMAP projection grouped and labelled by patient ID. (c) Immune cells stratified by patient-ID and sorted by MHC-1 expression. (d) Volcano Plots for differential expression analysis between Low/High MHC-I expression for T cells and Macrophages (e) GO Biological processes analysis for Top and Bottom 33% T cells and Macrophages

V. Conclusion

(1) CaMKK2 deletion induced immune cells toward a more pro-inflammatory phenotype, including enhanced antigen-presentation, which partially explains the observed ICB responsiveness in CaMKK2 deficient mice. (2) MHC-I expression is correlated with a pro-inflammatory phenotype in TAMs and T cells implying immune dysfunction is partially driven by restricted antigen processing and presentation.

Therefore, CaMKK2 should be further investigated as a possible therapeutic target alongside ICB to lower treatment resistance by enhancing T cell response through a more immunostimulatory TAM phenotype.

VI. Acknowledgments

Thank you to Dr. John Sampson and the Sampson Lab for opening their doors to me and supporting my research endeavors; specifically, thank you to William Tomaszewski for his endless mentorship and support by sharing his data and experience with me while helping me grow as a scientist and a person; Karolina Woroniecka and Emily Lerner for providing data and collaboration.

VII. References

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