

Transcriptional Analysis Reveals Metabolic and Cellular Dysfunction of Tumour Infiltrating NK Cells in Glioblastoma Patients



A Thesis Presented to the University of Dublin,
Trinity College in fulfilment of the requirement for
the degree of Research Masters Master of Science
(MSc) in Immunology

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2026

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Declaration

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Abstract

Glioblastoma (GBM) is the most common primary brain tumour with a poor 5-year patient survival rate of less than 7%. The tumour microenvironment (TME) of GBM is characterised by low infiltration of immune cells and immunosuppression. Natural Killer or NK cells are potent anti-cancer immune cells which classically kill cancer cells and thus, they represent a potential source of immunotherapy for GBM. However, they poorly infiltrate into GBM tumour sites. NK cells are classified into cytokine producing CD56^{bright} NK cells and cytotoxic CD56^{dim} NK cells, with both subsets becoming dysfunctional in tumour. One potential source of immunosuppression is TGF- β which is abundant in GBM and known to inhibit NK cell function. However, further research is required to characterise its dysfunctional effects and influence on the metabolic and physiological state of NK cells in GBM. Single cell RNA sequencing (scRNA-seq) advancements have revealed dysfunctional transcription patterns of NK cells in tumour. This work analysed a scRNA-Seq NK cell dataset published by Netskar et al., 2024, to investigate the differential expression patterns of NK cells from GBM patient tumours (GBM-NK cells) with reference NK cells from peripheral blood of healthy donors (HD-PBMC-NK). Global unbiased analysis showed GBM-NK cells had distinct transcriptional patterns from HD-PBMC-NK cells and an enrichment in CD56^{bright} NK cells. Interestingly, there was an apparent overall higher gene expression level in NK cells from tumour compared to blood. Cellular stress and apoptosis related pathways were upregulated while IFN- γ signalling and other cytotoxic pathways related to CD56^{dim} NK cell function were downregulated in GBM-NK cells. We then employed a hypothesis driven approach and selected candidate gene sets for closer analysis on a gene level, revealing amongst others an upregulation of oxidative stress, cellular response to ROS and cytochrome c associated apoptosis in NK cells in the tumour. The complexity of the TME was indicated by contradictory pathway enrichment such as increased signature for both mitochondrial fission and fusion or an increased expression of oxidative phosphorylation signature while some ETC complexes were significantly downregulated. Examination of TGF- β also identified some contradictory findings; there was evidence of higher TGF- β signalling in tumour NK cells but these also had elevated IFNG and MYC gene expression that would be predicted to be lower in the presence of TGF- β signalling. Overall, this thesis highlights that GBM-NK have a stressed, metabolically dysregulated and impaired mitochondrial phenotypes, at least at the RNA level. Cross-referencing TGF- β analysis in combination with other inhibitory signalling pathways known to exist in GBM such as EGFR, adenosine signalling etc., could help illustrate how GBM-NK develop an altered transcriptional expression profile. Combined with functional validation, these data will help inform the best design of NK cell immunotherapy for GBM tumours.

Acknowledgements

Firstly, I would like to thank my supervisor, Clair Gardiner for providing me with this wonderful opportunity of research. Thanks, Clair, for your mentorship and support as you helped me develop my skills as a research scientist and for helping me transition into immunology academic research after my undergraduate in neuroscience. Especially for your patience and insight to help me simplify complex concepts in concise ways. I would also like to thank my Co-supervisor David Finlay who very generously provided grant support for my Masters which helped make this research project feasible for me. Thank you, Dave for your encouragement, insight and enthusiasm for my research. It has been such a privilege to work under the tutelage of such great scientists but also such kind people. I am grateful for the many chats I had with you both.

Furthermore, it has been such joy getting to know everyone from both labs, over the past two years working as an RA and Masters Student. From my first interview everyone was so welcoming, and friendly. I always felt I could ask anyone for help with any issues and equally important it was great to work with such nice people. I would also like to extend a heartfelt thank you to my Gardiner lab peers Amal, Benedikt (for the times you visited us), Conor, Eimear, Ethan, Jack and Gearoid. It has been the best craic getting to know you all every day on in work but also for the great time spent together on lunchbreaks (shoutout Andrea and Laura for helping me get through long work days with some great chats), coffee trips, nights out. I would especially like to thank Conor De Barra who I have worked more closely with as part of our other lab research projects. Thank you for your advice and the many chats on movies, musical gossip and general nerdism. I would also like to Barry Scott for your helping me find my footing with RNA-seq analysis and bioinformatics, your tips and advice really set me at ease as you gave me direction to teach myself new analytical skills in Python. Furthermore, I was very lucky to have gotten to know John Scanlan who has been a tremendous help and has given me some great direction. Thank you for being very generous with your time and support for my many questions. Finally, I would like to thank my family who have

encouraged me throughout this project. In particular, I would like to thank my brother David for your support as I wrote my thesis and your encouragement.

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Abbreviations

AC	: Astrocyte-like
ADCC	: Antibody-Dependent Cellular Cytotoxicity
AMP	: Adenosine Monophosphate
AMPK	: AMP-Activated Protein Kinase
ATP	: Adenosine Triphosphate
BAD	: Bcl-2-Associated Agonist of Cell Death
BBB	: Blood–Brain Barrier
BMPR	: Bone Morphogenetic Protein Receptor
CAR	: Chimeric Antigen Receptor
CEBPD	: CCAAT/Enhancer Binding Protein Delta
CELLROX	: Cellular Superoxide Indicator
COX	: Cytochrome c Oxidase
DE	: Differential Expression
DEG	: Differentially Expressed Gene
DNA	: Deoxyribonucleic Acid
DUOX	: Dual Oxidase
EGFR	: Epidermal Growth Factor Receptor
ES	: Enrichment Score
ETC	: Electron Transport Chain
FC	: Fold Change
FDR	: False Discovery Rate
FGF	: Fibroblast Growth Factor
FTL	: Ferritin Light Chain
GADD34	: Growth Arrest and DNA Damage-Inducible Protein 34
GARP	: Glycoprotein A Repetitions Predominant
GBM	: Glioblastoma
GBM-NK	: Glioblastoma tumour infiltrating NK cells
GBM-PBMC-NK	: NK cells from peripheral blood of Glioblastoma patients
GDF11	: Growth Differentiation Factor 11
GLUT3	: Glucose Transporter 3
GNLY	: Granulysin

GO : Gene Ontology
GOBP : Gene Ontology Biological Process
GSC : Glioblastoma Stem Cell
GSEA : Gene Set Enrichment Analysis
GSEAPY : Gene Set Enrichment Analysis in Python
GSVA : Gene Set Variation Analysis
GZMB : Granzyme B
HD-PBMC-NK : Healthy Donor Peripheral Blood Mononuclear Cell Natural
Killer Cells
HLA : Human Leukocyte Antigen
HMDB : Human Metabolome Database
HNSCC : Head and Neck Squamous Cell Carcinoma
HPV : Human Papillomavirus
ID2 : Inhibitor of DNA Binding 2
ID3 : Inhibitor of DNA Binding 3
IDH : Isocitrate Dehydrogenase
IFN- γ : Interferon Gamma (protein)
IFNG : Interferon Gamma (gene)
KIR : Killer-cell Immunoglobulin-like Receptor
KS : Kolmogorov–Smirnov Test
LILLIEFORS : Lilliefors Test for Normality
MAPK : Mitogen-Activated Protein Kinase
MCT: Monocarboxylate Transporter
MES : Mesenchymal-like
MFN1 : Mitofusin 1
MFN2 : Mitofusin 2
MGI : Mouse Genome Informatics
MHC : Major Histocompatibility Complex
MICA : MHC Class I Polypeptide-Related Sequence A
MICB : MHC Class I Polypeptide-Related Sequence B
Mitofission : Mitochondrial Fission
Mitofusion : Mitochondrial Fusion
MITOSOX : Red (Mitochondrial Superoxide Indicator)

MSigDB : Molecular Signatures Database

mRNA : Messenger RNA

UPR^{mt} : mitochondrial unfolded protein response

MYC: MYC Proto-Oncogene

NADPH : Nicotinamide Adenine Dinucleotide Phosphate (Reduced)

NCAM1 : Neural Cell Adhesion Molecule 1

NES : Normalized Enrichment Score

NF- κ B : Nuclear Factor kappa-light-chain-enhancer of activated B cells

NK : Natural Killer (cell)

NPC : Neural-Progenitor-like

NRF2 : Nuclear Factor Erythroid 2–Related Factor 2

OC : Oligodendrocyte-like

OPC : Oligodendrocyte Progenitor-like

ORA : Over-representation Analysis

OXPHOS : Oxidative Phosphorylation

PBMC : Peripheral Blood Mononuclear Cells

PCA : Principal Component Analysis

PCR : Polymerase Chain Reaction

PDGF : Platelet-Derived Growth Factor

PDGFRA : Platelet-Derived Growth Factor Receptor Alpha

PDL1 : Programmed Death-Ligand 1

PPA1 : Inorganic Pyrophosphatase 1

PRDX : Peroxiredoxin (family)

RNA : Ribonucleic Acid

ROS : Reactive Oxygen Species

ScANVI : single-cell Annotation using Variational Inference

scVI - single-cell Variational Inference

SCANPY : Single-Cell Analysis in Python

SCENIC : Single-Cell Regulatory Network Inference and Clustering

SCENITH : Single-Cell Energetic Metabolism by Profiling Translation Inhibition

TERT : Telomerase Reverse Transcriptase

TFRC : Transferrin Receptor

TGF : Transforming Growth Factor

TGFBR1 : Transforming Growth Factor Beta Receptor 1
TGFBR3 : Transforming Growth Factor Beta Receptor 3
TGIF1 : TGFB-Induced Factor Homeobox 1
TIGIT : T cell Immunoreceptor with Ig and ITIM Domains
TME : Tumour Microenvironment
TNF : Tumour Necrosis Factor
TNFAIP3 : TNF Alpha-Induced Protein 3
TNFSF10 : TNF Superfamily Member 10 (also TRAIL)
TP53 : Tumour Protein 53 (p53)
TRAIL : TNF-Related Apoptosis-Inducing Ligand
TXN : Thioredoxin
TXNRD1 : Thioredoxin Reductase 1
UMAP : Uniform Manifold Approximation and Projection
VEGF : Vascular Endothelial Growth Factor
WHO : World Health Organization

1. Introduction

1.1 Glioblastoma: An Overview

Glioblastoma is the most common primary brain tumour, with a poor patient prognosis: median survival rate of 12 months and 5-year survival rate of less than 7% (Bafiti et al., 2022; D.-x. Yang et al., 2019; Ye et al., 2021). Glioblastoma is a grade 4 glioma (originating from malignant glial cells – mainly astrocytes) with diagnostic criteria of wildtype isocitrate dehydrogenase (IDH) expression and presentation of at least one of the following molecular profiles: the TERT promoter mutation, Chromosome 7 gain/chromosome 10 loss (+7/-10), EGFR amplification (Park et al., 2023). Glioblastoma is a highly aggressive cancer with rapid onset and invasive proliferation – typically within cerebral hemispheres – which makes surgical removal highly difficult (Schaff & Mellinghoff, 2023). Glioblastoma cells are also highly resistant to chemotherapy treatments, and recurrence is a typical feature of oncology (Vredenburg et al., 2007). This recurrence and persistence partially stem from genetic heterogeneity of glioblastoma stem cells (GSCs) which possess self-renewal properties and the capacity to differentiate into various tumour cell subtypes (Auffinger et al., 2015). Four mutational subtypes of GSCs with variant tumorigenic and treatment response properties have been identified by single cell RNA sequencing analysis: neural-progenitor-like (NPC-like), oligodendrocyte (OC)-progenitor-like (OPC-like), astrocyte-like (AC-like), and mesenchymal-like (MES-like) (Gisina et al., 2022; Cyril Neftel et al., 2019; Patel et al., 2014; Suvà & Tirosh, 2020). Moreover, recent evidence from single cell RNA sequencing analysis have revealed these mutations are often coexpressed within different tumoural regions and can transform following treatment (Prager et al., 2020; Suvà & Tirosh, 2020). In addition to this phenotypic plasticity, treatment resistance is exacerbated by immune evasion properties of glioblastoma. Glioblastomas are tumour ‘cold’ cancer types, meaning there is a low degree of immune cell infiltration into tumour sites and an inhibited anti-cancer immune response (Bikfalvi et al., 2023). This is facilitated by its immunosuppressive tumour microenvironment (TME), consisting of myeloid derived suppressive cells and regulatory T cells, which impede immune cell activation through

secretion of inhibitory cytokines such as IL-10, and the generation of reactive oxygen species (ROS) to induce dysfunction and hinder immune cell viability (Liu et al., 2024). Furthermore, exosomes (small membrane-bound extracellular vesicles) produced by cancer cells in TME, target immune cell inhibition through immunosuppressive cytokines and metabolites – such as TGF- β and indoleamine etc (Liu et al., 2024; Poorva et al., 2025). Thus, the development of TME resistant immunotherapies (enhancing the immune system anti-tumour response) is a developing field of research (Liu et al., 2024).

1.2 Natural Killer Cells – the innate anti-tumour immune cell

A key immune cell posed for immune therapeutic treatment of glioblastoma are Natural Killer (NK) cells (Wu et al., 2025). NK cells are innate lymphocytes with cytotoxic and cytokine-producing functions which target viruses and cancer cells (Abbassi-Daloui et al., 2020; Caligiuri, 2008). NK cells carry out cytolytic functions mainly via two modes: activation of killer receptors upon recognition of non-self-tissues (contingent upon activating and inhibitory receptors engagement to trigger or repress cytotoxic response) or via antigen-dependent cellular cytotoxicity – killing antigen presenting cells marked for cytolytic destruction (Poorva et al., 2025). Thus, NK cells fulfil the role of immunological surveillance and tumour clearance (Vivier et al., 2008). NK cells circulating in peripheral blood consist of two subtypes: CD56^{bright} NK cells – function to produce cytokines such as IFN- γ to support host immune and anti-tumour responses, and CD56^{dim} NK cells – function to execute cytolytic effector function and kill target cell: secrete granules containing perforin to puncture target cell membrane and enable secreted granzymes to induce apoptosis (Vivier et al., 2008). Furthermore, NK cells induce apoptosis via death receptor signalling via tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or Fas ligand (FasL) binding to respective target cell receptors (Poorva et al., 2025). High expression of CD16 receptor in CD56^{dim} NK cells is critical to ADCC function (Yeap et al., 2016). Typically, CD56^{bright} and CD56^{dim} NK cells exist in 1:9 proportions in circulating blood (Poli et al., 2009). Additionally, CD56^{bright} and CD56^{dim} NK cell present differential underlying metabolism upon activation: CD56^{bright} function is supported preferentially through oxidative phosphorylation and CD56^{dim} NK through glycolysis (Keating et al., 2016).

Sufficient activation of NK cells and supported metabolism are critical to effect tumour clearance (Miao et al., 2024).

1.3 NK cells vs Glioblastoma

With their potent anti-tumour function, NK cells are central figures for immunotherapy development (Poorva et al., 2025). It has been discovered that NK cells cross the blood-brain barrier and infiltrate tumour sites of glioblastoma patients. Indeed, increased NK cell infiltration and elevated NK cell signature from glioblastoma patients has correlated strongly with improved patient prognosis (Kmiecik et al., 2013; Kmiecik et al., 2014; C. Li et al., 2022). Furthermore, (Yeo et al., 2022) highlight that NK in vivo cells do not infiltrate contralateral brain tissue, and glioblastoma mouse model (using patient derived tumour cell lines) analysis by (Kirschenbaum et al., 2024), showcase an increased tumour infiltration over time. Glioblastoma tumour cells possess many ligands for activating NK cell receptors. Furthermore, experimental work has shown NK cells can bind to and eliminate glioblastoma tumour and GSC cells (Breznik et al., 2022). However, one aspect of immune evasion by GSC cells in vivo, is the downregulation of NKG2D receptor ligands (e.g., MICA, MICB, ULBP) which normally trigger NK cell cytotoxicity (Zhong et al., 2022), and the upregulation of inhibitory MHC class I molecules (HLA-E etc.) which bind to NK cell-inhibitory receptors like KIR and NKG2A (Hrbac et al., 2022). However, to harness the innate cytotoxicity of NK cells against GSCs, recent research efforts have focused on the development of CAR-NK cells (chimeric antigen receptor or CAR presenting NK cells): engineered to recognise antigens specific to GSCs such as tumour promoter antigen EGFRvIII (J Han et al., 2015). These CAR-NK cell therapies have shown promise in vitro and are currently under clinical trials. Moreover, CAR-NK cells boast advantages over CAR-T cell immunotherapies, including superior safety profiles (low risk of cytokine release syndrome (CRS), neurotoxicity, and graft-versus-host disease) and immediate cytotoxic effects independent of wider immune cell responses (Poorva et al., 2025).

1.4 TME Challenge to NK cell immunotherapies

However, challenges remain which hinder CAR-NK cell efficacy in in-vivo: difficulties crossing the BBB for systemically introduced CAR-NK cells, short-

term activation compared to sustained CAR-T cell response, reduced viability and downregulated receptors resultant of immunosuppression within the TME (Poorva et al., 2025). A major hurdle for CAR-NK cell function are the metabolic challenges in the TME. Solid tumours are known to be hypoxic environments which hinder oxidative phosphorylation of immune cells (Wang et al., 2022). Furthermore, the upregulated shifts towards of glycolysis in tumour cells (Warburg Effect), conversely corresponds with glucose deprivation and downregulated glycolysis in immune cells as tumour cells sequester available glucose and nutrients (Liberti & Locasale, 2016; Wang et al., 2022). Additionally, TME is quite acidic and the generation of reactive oxygen species by MDSC cells have been shown to induce dysfunction and damage NK cells in solid tumours (Certo et al., 2021; Kotsafti et al., 2020). These conditions have been shown to downregulate NK cell infiltration in TME, particularly for CD56^{dim} NK cells, as excessive ROS levels downregulated CD16 surface expression and other immunosuppressive cytokines suppress cytotoxicity (Aboelella et al., 2021).

1.5 TGF- β inhibition of NK cell function

One immunosuppressive pathway includes TGF- β signalling (produced by MDSCs and GSCs) which is broadly described as an inhibitor of NK cell function (Close et al., 2019; Groth et al., 2019; Shaim et al., 2021). Canonical TGF- β signalling pathways involves TGF- β ligand binding TGF- β receptor 2 on NK cells (recruiting activated TGF- β receptor 2) and resultant activation of TGF- β receptor one cascades: cytoplasmic kinase receptor domain phosphorylates SMAD2 and SMAD3 to form a complex with SMAD4 which is then translocated to nucleus to modulate gene expression and thereby directing cell responses (Deng et al., 2024). TGF- β signalling has been broadly described to inhibit NK cell proliferation (CD56^{dim} NK cells especially), cytotoxicity, cytokine production and metabolism (Schuster & Kriegelstein, 2002; Slattery et al., 2021; V. Zaiatz-Bittencourt et al., 2018). TGF- β has been shown to be elevated within glioblastoma TME with upregulated protein signatures expression in tumour infiltrating NK cells isolated from patient tumour tissue samples (GBM-NK) (Close et al., 2019). Furthermore, (Shaim et al., 2021) have demonstrated the mechanism underlying GSC cell contact mediated TGF- β signalling which results in downregulated NK cell receptors, impaired cytotoxicity, downregulated

cytokine production and functions reduced tumour clearance in mouse models. Additionally, (Kirschenbaum et al., 2024) revealed TGF- β signalling in mouse models of glioblastoma can induce NK cell dysfunction within 24 hours. Thus, multiple facets of tumour oncology induce immunotherapy failure but also deregulate GBM-NK cell; causing low infiltration of NK cells in tumour. However, many aspects of TGF- β induced dysfunction characterised in other healthy and cancer tissues (Slattery et al., 2021; Viel et al., 2016), have yet to be examined and general aspects of GBM-NK physiology such as mitochondria morphology and bioenergetics remain under researched.

1.6 Single Cell RNA-seq – a tool for bioinformatic analysis of GBM-NK cells

The cellular responses of GBM-NK cells to these challenging stimuli experienced within the TME, coincide with changes in gene expression (Dean et al., 2024; Morimoto et al., 2021). Genes transcribed in response to upregulated cellular pathways and alterations in metabolic or physiological states, can be detected through transcriptomic analysis techniques (Kennedy et al., 2024; Subramanian et al., 2005). Single-cell RNA-sequencing (scRNA-seq) is one such genomic technique which enables high resolution quantitative analysis of mRNA molecules expressed in an individual identified cell on a genomic scale. This powerful tool, assessing gene expression variation on a cellular level, is highly informative for providing insight into the physiological state of cells (Hwang et al., 2018). However, scRNA-seq does not capture protein translation or pathway activity with complete accuracy (Bauernfeind & Babbitt, 2017; Li et al., 2020). Therefore, the use of transcriptional analysis has been employed to provide corroborative data for experimental analysis but also for predictive assessments of cellular biology. Indeed, transcriptomic analysis has been employed to highlight multiple aspects of complex cellular biology which are otherwise difficult to probe. ScRNA-seq is increasingly utilised to characterise functional, physiological and metabolic states of tumour infiltrating immune cells and has uncovered novel biological signatures which uniquely represent distinct cellular biology – such as differential phenotypes of immune cells in pathology compared to physiological state (Tang et al., 2023). Therefore, one

approach to scRNA-seq is the analysis of differential expression patterns between cell types and cell populations of interest. Many recent studies have begun to compare the divergent transcriptome of GBM-NK cells to reference NK cells – such as peripheral NK cells derived from the PBMCs (Peripheral Blood Mononuclear Cells isolated from blood) of healthy donors (HD-PBMC-NK cells) or patients (GBM-PBMC-NK cells) (Kirschenbaum et al., 2024; Monaco et al., 2022; Netskar et al., 2024; Schmassmann et al., 2023; Shaim et al., 2021).

1.7 Current paradigm of GBM-NK Transcriptomics

Transcriptomic analysis of glioblastoma cells has been widely examined (Ordóñez-Rubiano et al., 2025), however in more recent years RNA-seq studies have emerged for previously lacking transcriptome analysis of GBM-NK cells. However, the scope of GBM-NK differential gene analysis to HD-PBMC-NK cells is limited. To my knowledge two studies have directly compared these two populations: (Kennedy et al., 2024) compared two separate datasets for GBM-NK and HD-PBMC-NK cells and noted an upregulation of common markers for hypoxia in GBM-NK cells related to mitophagy and glycolysis: Bcl2-interacting protein 3 (*BNIP3*)—a mitochondrial protein involved in mitophagy and prolonged NK cell responses; glycolytic enzyme enolase 2 (*ENO2*) and glucose transporter GLUT3 (*SLC2A3*). Additionally, (Shaim et al., 2021) assessed publicly available datasets to uncover transcriptomic evidence supporting experimental analysis that: activating NK cell cytotoxic function markers (granzymes – GZMB etc., killer receptors NKp44 etc.) were significantly decreased in GBM-NK transcriptome while there was an upregulation of inhibitory NK receptor genes (*KIR2DL4* etc.) and TGF- β signalling markers (*SMAD4* etc.). However, to my knowledge analysis of differential expression patterns between GBM-NK and HD-PBMC-NK cells has not been assessed on a gene set level nor has gene set enrichment analysis (GSEA) been performed for this comparison. GSEA is a computational test determining if a defined gene set for a pathway showcases statistically significant, differences between two biological groups i.e., phenotypic analysis of expression (Subramanian et al., 2005). However, transcriptomic analysis of GBM-NK cells by (C. Li et al., 2022) highlighted some gene expression patterns are commonly upregulated across clinical GBM molecular subtypes: expression profiles associated with poor clinical outcomes

were identified – BID, pro-apoptotic gene; CD70, associated with T cell deficiency; UL16, ligand inactivating NKG2D killer receptor. Correlation analysis of risk gene signature score strongly correlated with NK cell immune checkpoint (inhibit immune response and induce dysregulation immune response/exhaustion) PDL1 expression. Furthermore, GBM-NK cells sourced from recurrent and newly diagnosed glioblastoma patients also share transcriptional signatures: downregulation of IFN- γ signalling and cell cycle pathways; upregulation of hypoxia (Monaco et al., 2022). This may warrant generalisation of transcriptomic findings for GBM-NK cells as common pathology wide phenotypes. Transcriptomic analysis however has also identified some heterogeneity of GBM-NK expression: NK cells in peripheral region of tumour site displayed downregulation of proliferation, stimulatory receptor and NF- κ B signalling (promotes NK cell cytokine production e.g., IFN- γ) genes (Schmassmann et al., 2023). Furthermore, scRNA-seq analysis of IL-21 stimulated NK cells with improved cytotoxicity against GSC cells in vitro, conversely highlighted the potential effects of the TME on GBM-NK cells: ROS gene signature is significantly decreased while oxidative phosphorylation and cytotoxicity gene signatures are significantly increased in IL-21 stimulated vs unstimulated NK cells – both cocultured with patient derived GSCs in vitro (Kennedy et al., 2024; Shanley et al., 2024). Thus, to date transcriptomic analysis provide partially validated evidence that GBM-NK cells showcase elevated TGF- β signalling, inhibited cytotoxicity, hypoxic phenotypes (suggesting glycolytic shift and upregulated mitophagy), with suggestive evidence for potentially upregulated signatures for apoptotic pathways, ROS production and dysregulated oxidative phosphorylation signatures compared to healthy NK cells. However, many aspects of cellular biology such as oxidative stress, ROS, mitochondrial health and morphology, and metabolism require further research and direct transcriptomic analysis to characterise the functional state of GBM-NK cells and the biological pressures they experience within the TME.

1.8 Dataset Studied and Aims of Analysis

This thesis hoped to further expand our knowledge on the unique gene expression patterns of GBM-NK cells and investigate new research questions of

interest for understanding NK cell physiology and dysfunctional stimuli within TME. For this analysis, we chose to analyse the differential gene expression patterns of GBM-NK and HD-PBMC-NK populations, compiled from datasets sourced within the pan-cancer scRNA-seq NK cell atlas published by (Netskar et al., 2024). This atlas compiled a myriad of single cell and bulk RNA-seq datasets and offered a large sample for assessment. This ready-to-use open access data resource(allowed us to evaluate differential transcriptomic readouts potentially underlying many aspects of GBM-NK phenotypes divergent from HD-PBMC-NK cells. Our analysis approach was twofold: an initial exploratory approach highlighted global transcription pattern differences and highlighted gene set pathways that were differentially enriched within GBM-NK cells; and a resultant closer analysis of select candidate gene sets of interest We grouped our candidate gene set into categories of interest based on current literature paradigms and underexplored areas:TGF- β , General Mitochondrial Health, Oxidative Phosphorylation, Mitochondrial Morphology, and Glycolysis and Gluconeogenesis differential signature expressions in GBM-NK cells. Furthermore, we hoped to gauge the influence of an upregulated TGF- β signalling expression profile on GBM-NK cells: performing correlative analysis for metabolic, mitochondrial bioenergetic and cellular stress signatures and targeted GSEA.

Thus, the chief objectives for this research were to evaluate transcriptome wide and pathway specific differential expression patterns between GBM-NK and HD-PBMC-NK cells and thereby infer the functional state of NK cells infiltrating the TME of glioblastoma. The goal was to focus particularly on cellular metabolism, mitochondrial morphology, mitochondrial bioenergetic capacity, and cellular stress programmes e.g., ROS which have been largely neglected to date. Moreover, rather than a more limited examination of individual markers, we sought to undertake a more general gene set pathway assessment which would define more deeply embedded dysregulation and the potential for novel target identification. These data will also inform future NK cell therapies e.g. CAR-NK cells how to better survive and work in the GBM TME.

2. Methodology

2.1 Dataset Background

For this thesis we analysed transcript expression of NK cells sourced from a publicly available scRNA-seq NK cell pan cancer atlas, published by (Netskar et al., 2024). This atlas dataset was ready for use upon download with all previous dataset preparations made. These included the removal of doublets, filtering out low expression profile cells with less than 1000 molecules expressed, filtering out low genes expressed by less than 10 cells, normalisation of data via log(transformation), performed batch/dataset variations: maintained cell-to-cell variation independent of donor/patient sample-to-sample variation (cells which would otherwise cluster by donor or dataset of origin were amalgamated into a homogeneous population e.g., GBM-NK or CD56^{dim} GBM-NK etc.), uniformly annotated cell populations – harmonised multiple dataset annotations with scANVI model, deconvoluted bulk RNA-seq from tumour datasets using BayesPrism, mapped RNA to reference genome and converted data into readable expression matrix, and performed quality control tests – presented in publication (Netskar et al., 2024). This atlas consisted of HD-PBMC-NK cells, six tissue resident NK cell subtypes, and tumour infiltrating NK cells sourced from seven cancer types. We filtered this atlas dataset for these select annotated groups: NK cell populations labelled within the published dataset as ‘source=PBMC’ were renamed HD-PBMC-NK and ‘source=Glioblastoma’ were renamed GBM-NK. NK cells from ‘healthy’ brain dataset sources had low tissue residency scores and were excluded from this thesis analysis (Netskar et al., 2024): metadata pertaining to low age deaths also justified this exclusion of ‘healthy’ brain resident NK cells. NK cells were mainly annotated by (Netskar et al., 2024) using CellTypist (where available cite-seq data and previous annotations were incorporated into homogenous annotation protocol (Netskar et al., 2024)) which included marker expression of the following genes: NKG7, GNLY, CST3, MALAT1, SPINK2, KLRD1, HMOX1, IGFBP4, TRBV10-2, CD3E (Domínguez Conde et al., 2022). To distinguish CD56^{bright} from CD56^{dim} NK cells, CellTypist was again employed by (Netskar et al., 2024) to demarcate FCGR3A (CD16) positive (CD56^{dim}) and negative

(CD56^{bright}) cells; marker genes included: GNLY, FCGR3A, NKG7, FCER1G, TYROBP, IGFBP7, GZMB, PTGDS, MYOM2, SPON2, SH2D1B (Domínguez Conde et al., 2022). We verified the expression of these markers in NK cells annotated as GBM-NK and HD-PBMC-NK cells and additionally for assessed differential expression of CD16 in NK cell subsets for both populations. We did so through the generation of violin plots and found GBM-NK showed an elevated expression profile for all markers relevant to cell types - **see Supplementary Figures 2, 3**. For each gene available in the analysed dataset, (Netskar et al., 2024) also computed the mean expression and frequency per population: sum of gene expression readout in each cell normalised for the total number of cells in a population; frequency/detection rate of gene (percentage of population expressing gene). There was gene expression and frequency information for a total of 14337 genes available in the dataset. The steps taken by (Netskar et al., 2024) to source datasets and annotate GBM-NK and HD-PBMC-NK populations, along with a breakdown of the number of datasets compiled per population is illustrated in Figures 1 and 2.

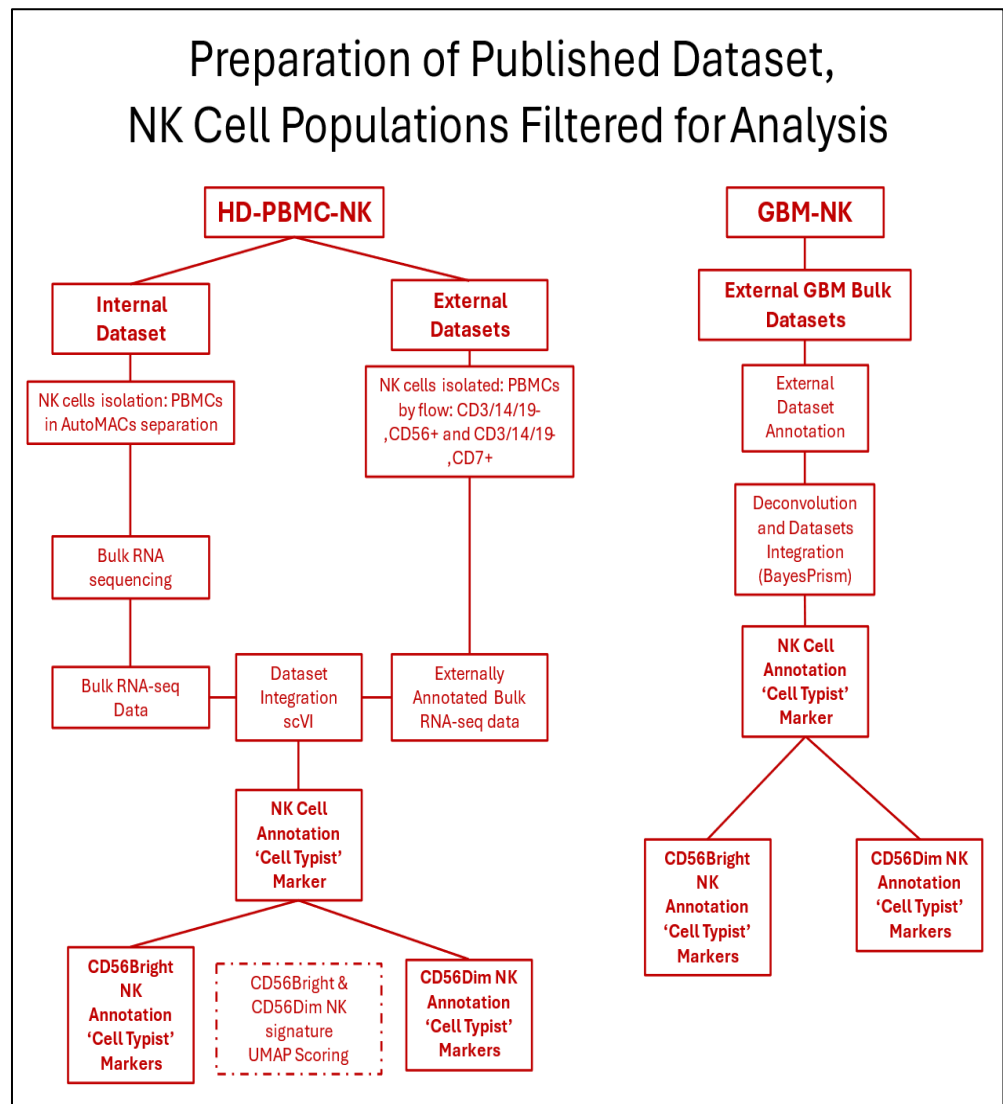


Figure 1: Preparation of Published Dataset by (Netskar et. al, 2024) concerning the NK Cell Populations Chosen for Thesis Analysis. A brief overview of the original steps taken by (Netskar et al., 2024) to prepare an annotated pan-cancer NK cell RNA-seq dataset - specifically concerning the NK cells populations filtered from this dataset resource for analysis in this thesis. Left) is a stepwise summary procedure for the collection and preparation of RNA-seq data for the HD-PBMC-NK population (NK cells sourced from the PBMCs of healthy donors): how the NK cells were sourced collected from internal and external sources and integrated into a uniform population. We relabelled this HD-PBMC-NK population from cell populations annotated as 'source=PBMC' by (Netskar et al., 2024). For external datasets we have also included the flow panels used in original datasets to annotate NK cell from bulk: either CD3/CD14/CD19 – and CD56 + or CD7+ combinations. These panels are accessible in the metadata of original datasets referenced in (Netskar et al., 2024). For internal datasets details of AutoMACs separation protocol used to isolate NK cells from PBMCs are likewise outlined in (Netskar et al., 2024). Right), is also a stepwise summary procedure for the collection and preparation of RNA-seq data for the GBM-NK population (NK cells sourced from the brain tumour tissues of glioblastoma patients) collected from external datasets. GBM-NK cells were relabelled from

original annotations by (Netskar et al., 2024) - “Source=glioblastoma”. See (Netskar et al., 2024) for a detailed breakdown of sources used to create annotated pan cancer NK cell single cell RNA-seq atlas). In addition to the input of preexisting annotations from external dataset sources, Cell Typist was used to annotate NK cells and CD56Bright and CD56Dim NK cells populations. Gene markers used by Cell Typist for NK cell annotation: NKG7, GNLY, CST3, MALAT1, SPINK2, KLRD1, HMOX1, IGFBP4, TRBV10-2, CD3E. CD56^{bright} from CD56^{dim} NK cells were differentiated by marker genes: GNLY, FCGR3A, NKG7, FCER1G, TYROBP, IGFBP7, GZMB, PTGDS, MYOM2, SPON2, SH2D1B. The dashed outlined box refers to additional steps taken by (Netskar et al., 2024) to verify Cell Typist annotation of CD56^{bright} from CD56^{dim} NK populations: assessing the distribution of these two subset populations by cell marker UMAP expression profiles – see (Netskar et al., 2024) for more details. scVI - single-cell Variational Inference, python package used for integration of different datasets. BayesPrism - computational models used to deconvolute bulk RNA-seq from tumour datasets.

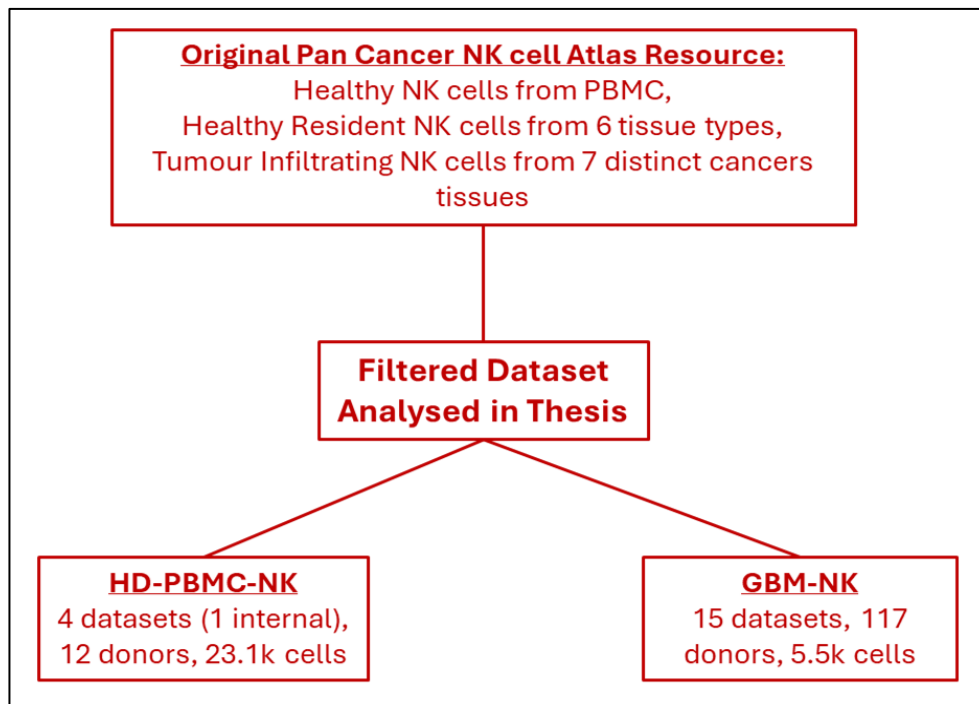


Figure 2: Summary Illustration of Selected NK cells Populations Filtered from (Netskar et al., 2024) Dataset for Analysis. Diagram includes a categorical overview of the sources (Netskar et al., 2024) used to compile the pan-cancer NK cell atlas and a more detailed breakdown of the NK cell populations analysed in this thesis: the number of datasets, donors and cells. HD-PBMC-NK – NK cells sourced from PBMCs of healthy donors; GBM-NK – NK cells sourced from the brain tumour tissues of glioblastoma patients.

2.2 General Approach

Dataset analysis was undertaken on a population level of comparison as opposed to investigating cell to cell differences. In a similar manner to the pseudobulk approach of (Netskar et al., 2024) and others (Tirosh et al., 2016; Zilionis et al., 2019) we investigated if differential gene expression was based on expression pattern differences of GBM-NK and HD-PBMC-NK cells. We justified this approach as tumour specific conditions likely contribute to chronic changes in transcriptome expression profile (Verhaak et al., 2010; Wang et al., 2017). Thus, differential analysis of both populations served to highlight common expression patterns trends unique to GBM-NK cells compared to HD-PBMC-NK cells. This approach stemmed from findings by (C. Li et al., 2022) who found gene signatures in GBM-NK cells associated with higher risk of worse patient prognoses was independent of molecular diagnoses of tumour. Additionally, GBM-NK sourced from recurrent and newly diagnosed patients share many dysfunctional transcription patterns differences to peripheral NK cell controls (Monaco et al., 2022).

Our analysis design was two-fold: an initial exploratory approach highlighted general transcriptional pattern differences in a largely unsupervised manner; followed by a subsequent closer analysis of expression patterns for gene sets of interest. Our hypothesis driven analyses of candidate gene set pathways were informed in part by literature and laboratory interests but also results from initial over-representation analysis (ORA). In contrast to a standard GSEA performed on the transcriptome-wide ranked list of DEGs, ORA involves the select input of statistically significant DEGs for enrichment analysis – highlighting which gene sets are represented at a greater than expected expression level (Das, McClain and Rai, 2020). In a similar manner to ranked GSEA, ORA provided an overview of gene set pathways that are significantly enriched or represented in GBM-NK vs HD-PBMC-NK cells and therefore acted as a triage for selection of candidate gene sets for analysis. This approach was implemented as for transcriptomic analysis undertaken by (Abbassi-Daloii et al., 2020; Monaco et al., 2022). Furthermore, exploratory analysis would provide additional context to the interpretation of cellular states of cell populations when investigating pathways in detail.

2.3 Gene set curation

Candidate gene sets chosen for analysis (TGF- β and ROS) were curated from multiple gene set subsets and gene sets sourced from different gene set library databases e.g., Elsevier Pathway Collection. The breakdown of sources utilised for each gene set is given in Results **Figure 14**. Gene sets included additional genes of interest currently under investigation by our research group or genes known from the literature to be relevant to a given pathway. For example, the genes for GARP and its subunits (transmembrane cell surface docking receptor for latent TGF- β (Stockis et al., 2009)) were added to the TGF- β gene set. In some cases, mouse gene sets (MGI source used for mitochondrial fission gene set) were added to this candidate gene set curations to investigate novelty in the human gene set analysis. Rate limiting metabolism gene sets were sourced from literature (Arnold, 2012; Zuo et al., 2021).

2.4 Gene set Analysis

Every gene available in the dataset had a given expression and frequency average for an annotated cell group. For example, mean expression for 'ABC' gene was the sum of its expression from every cell in X population of NK cells, normalised to the number of cells in X population. The frequency for 'ABC' gene was the percentage of cells in population X which had a non-zero value for expression (Netskar et al., 2024). An overview of the relative expression of candidate gene sets in GBM-NK and HD-PBMC-NK cells was undertaken through comparison of weighted averages of gene set in populations under comparison. The weighted average gene set expression per cell was calculated as the sum of mean expressions for every available gene in gene set normalised to the size of the gene set. The weighted average gene set frequency was calculated as the sum of gene frequencies for every available gene in gene set normalised to the size of the gene set. Gene set expression could be interpreted as the average expression value for which any gene in a gene set is normalised to the number of cells in a population, while gene set frequency could be interpreted as the average detection rate or percentage of cells expressing any given gene set gene in a population. The Log₂ fold change in weighted averages were calculated in Excel. This weighted average approach was as used in other

RNA-seq studies (Aibar et al., 2017; C. Li et al., 2022; C. Neftel et al., 2019; Sade-Feldman et al., 2018).

2.5 UMAP Visualisation of Dataset

Global transcriptional pattern differences in transcriptome of GBM-NK and HD-PBMC-NK were visualised in UMAPs. This was paired with comparative analysis forcing datasets into two unbiased clusters using K-means clustering algorithm. UMAPs were generated for annotated cell groups and overlap of transcriptional patterns was assessed qualitatively but also quantitatively via two matrices: Jaccard index for assessing direct overlap of cells with similar transcription patterns and a positive Jaccard Similarity Index (exact overlap occurrence divided by non-overlap occurrence), interpreted that both populations display different transcriptome states. Silhouette score to assess how strong cells cluster to populations. Silhouette scores ranged from -1: when groups cluster more closely to a different group, to 0: when cells do not strongly cluster to any group, to +1: when cells cluster very strongly to their annotated group. UMAP generation from unbiased Leiden Clustering was undertaken to visualise heterogeneity of the dataset. This follows similar approach to immune cell transcriptomic analysis in literature (Aybey et al., 2023; Won et al., 2024; C. Zhang et al., 2022).

2.6 Differential Expressed Gene Analysis

Differential gene analysis compared relative expression of every gene from GBM-NK and HD-PBMC-NK cells was performed in Python using Wilcoxon ranked test. Genes were labelled significant if Log_2 fold change in expression $> +1$ with an adjusted p-value < 0.05 (significantly increased) or Log_2 fold change < -1 with an adjusted p-value < 0.05 (significantly decreased). These thresholds of significance followed common standards used in transcriptomic studies (McDermaid et al., 2019). Data was extracted from python into Microsoft Excel files. This Python SCANPY package computation follows the same principle as limma tool differentiation, commonly used in transcriptomic studies (C. Li et al., 2022).

2.7 PCA plots and DEG analysis

PCA plots were generated from the top 2000 variable genes or variable components of differential gene analysis between two populations. For each PCA, a Wilcoxon ranked sum test was performed to uncover and plot the top 20 differentially expressed genes (DEGs) that are upregulated. The top 20 DEGs for each population comparison were plotted and ranked in descending order of z-scores obtained from Wilcoxon rank-sum test - see **Supplementary Tables 1-3** for Log₂ Fold Change and, significance values. This approach to identifying genes which contribute to the greatest variation between populations was inspired by (Chatterjee et al., 2024) and was undertaken to observe commonality of differential expression CD56^{bright} and CD56^{dim} GBM-NK vs HD-PBMC-NK cells.

2.8 Enrichr ORA

Enrichr is a widely used web-based gene set enrichment tool which was used for our ORA results of top upregulated and downregulated GBM-NK genes (Kuleshov et al., 2016; Monaco et al., 2022). (Hong et al., 2014) support this approach, finding: “separate analysis of up- and downregulated genes could identify more pathways that are really pertinent to phenotypic difference. In conclusion, analysing up- and downregulated genes separately is more powerful than analysing all of the DE genes together” From approximately 7000 significantly increased DEGs (genes with significantly increased expression in GBM-NK), the top 2050 genes (ranked by B statistic) were inputted into Enrichr. Gene set Database libraries selected are outlined in **Figure 9** and were chosen to showcase a range of enriched gene set pathways that highlighted multiple aspects of cellular physiology that may be altered in GBM-NK. Enriched gene sets were ordered by ‘combined rank’ computed by Enrichr. This combined rank was calculated as follows: log of the p-value, obtained from Fisher’s Exact Test (proportion’s test that for any given genes assumes its binomial distribution and independence of expression within any given gene set) was multiplied by Enrichr’s odd’s ratio or z-score: standard deviation of gene’s expression from predetermined expected mean rank within a given gene set (Kuleshov et al., 2016). Enriched gene sets were grouped in categories of

cellular function and tabulated. Similar gene sets enriched across libraries were highlighted in the same colour to exhibit ORA trends. Metabolite network was generated by Enrichr web tool packages: building connections between metabolite gene sets (represented by nodes) based upon the similarity of their gene content.

Enrichr allowed us to probe the enrichment of a myriad of gene sets from hundreds of gene set libraries simultaneously.

2.9 Volcano Plot for Significantly Increased and Decreased genes

Volcano plots plotted gene set genes based on their Log₂ fold change in expression and adjusted p-values from DEG analysis of GBM-NK vs HD-PBMC-NK cells. Again, Log₂ fold change increase > +1 with an adjusted p-value < 0.05 denoted significantly increased genes. Conversely, significantly decreased genes were defined as having a Log₂ fold change increase < -1 with an adjusted p-value < 0.05. This followed approach of many transcriptomic studies investigating immune cells expression profile (McCarthy et al., 2012; McDermaid et al., 2019).

2.10 Ranking significantly increased genes rankings

To help identify which genes were strongly elevated in GBM-NK cells, significantly increased genes were ranked by greatest log fold change in expression and greatest frequency respectively. This follows a similar approach to (Darmanis et al., 2017) who highlight significantly increased genes that were expressed at relevant frequency.

2.11 Targeted analysis of High TGF- β signature in GBM-NK cells

GBM-NK cells which expressed all 37 significantly increased marker genes (identified in **Fig. 19**) at significantly greater expression level (see parameters defined in 2.9) in GBM-NK than HD-PBMC-NK cells were defined as High TGF- β scoring GBM-NK cells. Whereas Low TGF- β scoring GBM-NK cells expressed none of these significant genes at levels significantly greater than HD-PBMC-NK cells. Volcano plots were then generated to illustrate the differential gene expression of functional gene sets in High vs Low TGF- β GBM-NK cells. This correlative approach was as performed by immune cell transcriptional analysis by (Kennedy et al., 2024; Monaco et al., 2022). GSEA of High vs Low TGF- β GBM-

NK cells, was performed for the entire ranked transcriptome. GSEA analysis assessed gene set enrichment from the KEGG 2016 database library and set the following criteria: gene sets tested were sized between 15-500 genes. We applied the GSEAPY (Gene Set Enrichment Analysis in Python) computational protocol for this analysis (Fang et al., 2022). GSEA results were filtered for significantly enriched gene sets by the following statistical criteria: gene sets with $NES > 1.5$ & $FDR\ q\text{-value} < 0.25$ were deemed positively enriched in High TGF- β GBM-NK cells; gene sets with $NES < -1.5$ & $FDR\ q\text{-value} < 0.25$ were deemed negatively enriched. The $FDR\ q\text{-value} < 0.25$ was a liberal significance threshold for the sake of exploratory analysis. Leading genes for enrichment were also given in the excel output file rendered from python analysis and helped interpret pathway enrichment in High TGF- β scoring GBM-NK cells. This GSEA analysis of NK cells with specifically enriched signatures was inspired by transcriptomic analysis of (Luo et al., 2025).

2.12 Statistical Analysis and Data Presentation

Data was analysed using Spyder Python software. For each level of analysis, data was deemed to be non-parametric from the following normality tests: Lilliefors and Anderson–Darling normality tests. Representative normality tests and QQ-plots illustrating non-normality are provided in **Supplementary Figure 1**. RNA-seq data is typically characterised as having non-normal distribution in (Li & Tibshirani, 2013). Differential gene expression was tested for significance using Wilcoxon rank-sum test (Mann–Whitney U test) and P-values were adjusted using Benjamini–Hochberg False Discovery Rate procedure. For Differential gene expression analysis, genes were ranked by Log₂ Fold Change in gene expression. For initial exploratory analysis, Enrichr was used to perform ORA on top increased or decreased DEGs generated from Python programmed analysis. Enrichr computed gene set enrichment tests from Fisher’s Exact Test (Kuleshov et al., 2016). DEG analysis of high vs low TGF- β signature and subsequent GSEA was all performed in Python.

All plots were generated from Python except for candidate gene set expression and frequency bar charts, which were made using GraphPad Prism 10 software. Metadata and data tables were made in Microsoft Excel. Flow chart diagrams and data summary tables were made in Microsoft PowerPoint.

2.13 Code Availability and Metadata

Python script templates used to generate and analyse Netskar et al., 2024 dataset uploaded to GitHub Repository 'Master-Sc-Code-AndrewR' and can be accessed through the following link: <https://github.com/anydrew/Master-Sc-Code-AndrewR.git>.

2.14 Assumptions

However, with these protocols we made general assumptions for the interpretation of our analysis. These including the following:

1. It was expected that the majority of GBM-NK cells were annotated correctly by (Netskar et al., 2024)
2. NK cells were corrected identified and tissues sources retained when annotated by (Netskar et al., 2024). In addition to control tests published by (Netskar et al., 2024), we performed limited diagnostic testing validating the expression of markers identifying NK cells and markers identifying CD56^{bright} and CD56^{dim} cells – see **Supplementary Figures 2, 3.**
3. We assumed that HD-PBMC-NK cells were 'healthy' and represented physiological peripheral NK cell transcription expression patterns. HD-PBMC-NK cells were taken ex-vivo and not stimulated before sequencing.
4. Differential gene/ gene set expression patterns in GBM-NK were specific to glioblastoma tumour conditions - study by (Schmassmann et al., 2023) showcases tumour specific expression patterns are observed in GBM-NK vs GBM-PBMC-NK. Additionally, (Netskar et al., 2024), calculated High tissue residency scores for GBM-NK cells indicating strong tumour infiltration for these cells.
5. GBM-NK expression patterns encapsulate a mixed population: GBM-NK cells likely under different cellular states: quiescent, activated and exhausted, depending on exposure time upon infiltration into TME and other temporal/spatial dynamics (Kirschenbaum et al., 2024; Schmassmann et al., 2023)– further phenotypic subtypes characterised by (Netskar et al., 2024)

6. Our analysis assessed GBM-NK as a global population, therefore provided a general overview of transcriptomic differences in NK cells found in the TME.
7. Similarly, a CD56^{bright} skewed effect may have influenced ORA of GBM-NK cells – outlined in limitations. However, for the purposes of our candidate approach, gene set analyses appeared broadly consistent across subsets.
8. Metabolic gene set analysis was not a direct measure of metabolism but metabolic potential. For example, an upregulation of metabolic enzymes and cellular machinery responsible for sustaining metabolism (such as mitochondrial complexes) represent a potentially altered machinery which could influence metabolic capacity of cells.
9. The scRNA-seq dataset captured both transient and persistent changes to NK cell transcription at the point of sample sequencing (Ding et al., 2022). Our data included analysis of potentially durable changes to transcription such as mitochondrial complex assembly genes and transient gene expression of Immediate-early activation genes (AP-1 subunits etc.) for example oxidative stress cytokine stimulation in response to and cell receptor activation (Dean et al., 2024; Fan et al., 2024; Lau et al., 2022). However, we did not regress out transient genes (as some papers do, (Mathew et al., 2021; O'Connor et al., 2025)) as they also provided insight – for example cell cycle gene changes (top hits from our ORA) represent proliferation states of NK cells are biological relevant results of interest.
10. Differential gene analysis of High TGF- β signature GBM-NK cells could only be interpreted as correlative and not causal evidence of TGF- β inhibitory signalling, due to presence of multiple confounded biological factors in the TME – see limitations.
 - a. Furthermore, candidate gene set analysis cannot be attributed to the influence of a single enriched pathway. For example, TGF- β signalling effects may inhibit NK cells

while another competing stimuli in the TME may influence NK cell activation and inhibition – such as hypoxia, inflammation etc (Ghosh et al., 2022; Sedgwick et al., 2020).

11. Transcriptomic analysis underlines immediate programmed cellular response to stimuli but may not correspond to direct proteomic and pathway alteration – until validated by future experimental testing (Bauernfeind & Babbitt, 2017).

3. Results

3.1 Global Overview and Analysis of Data

This dataset exhibited highly diverse expression patterns: unsupervised Leiden clustering generated over 150 unique clusters of cells (even under low resolution parameters – see methodology section 2.5), as illustrated by UMAP in **Figure 3A**. Furthermore, to validate the dataset comparisons, a UMAP was generated from the unbiased K means clustering of the dataset into two groups, (**Fig. 3B**, cluster 1 in orange and cluster 2 in blue) and broadly resembled the structure of GBM-NK and HD-PBMC-NK populations mapped to these respective dataset annotations (**Fig. 3C**). This UMAP (**Fig. 3C**) also identifies ‘overlapping’ cells from the two datasets (shown in yellow – sample box to illustrate some yellow cells). However, it can clearly be seen that GBM-NK (red) and HD-PBMC-NK (blue) annotated populations, are largely non-overlapping. Indeed, out of a total of 28,600 cells analysed (23.1k from HD-PBMC-NK and 5.5k from GBM-NK), only 91 cells overlap in having a similar transcriptional profile, just 0.3% of the total cells analysed. This Jaccard Similarity Index, supporting that both populations display different transcriptome states. Overlapping cells are shown in UMAP **Fig. 3E** as yellow dots overlaying the total dataset population in grey. These overlapping cells can be seen to be distributed throughout but perhaps preferentially associate more strongly with the HD-PBMC-NK cluster. However, quantifying direct overlap on UMAPs does not provide the full picture: UMAPs visualise complex multidimensional data onto a 2D space and do not account for depth or proximity of clusters to each other. The Silhouette score partially resolves this by signifying how strongly groups cluster.. A Silhouette score of 0.3244 for **Figure 3C** signifies that both groups positively segregate (albeit moderately) and supports the qualitative interpretation of the UMAP visualisation that, although there is some overlap, GBM-NK can be distinguished from HD-PBMC-NK cells based on their transcription patterns.

It is known that CD56^{bright} cells account for approximately 10% of circulating NK cells in a healthy donor (Caligiuri, 2008; Cooper et al., 2001). Indeed, with approximately 23k cells for HD-PBMC-NK analysis, the data shown here support

this with 7% of CD56^{bright} cells in HD-PBMC-NK. In contrast, GBM-NK cells display an enrichment of CD56^{bright} and from a total of 5.5k GBM-NK cells used in the original analysis, over 65% are classified as CD56^{bright} (**Fig. 4**). These cell groups were annotated using Cell Typist transcription markers, for which the expression profiles were validated using Violin Plots in GBM-NK and HD-PBMC-NK populations – GBM-NK even showcased stronger expression profile for general NK cell and CD56^{dim} NK cell markers (**see Supplementary Figures 2, 3**).

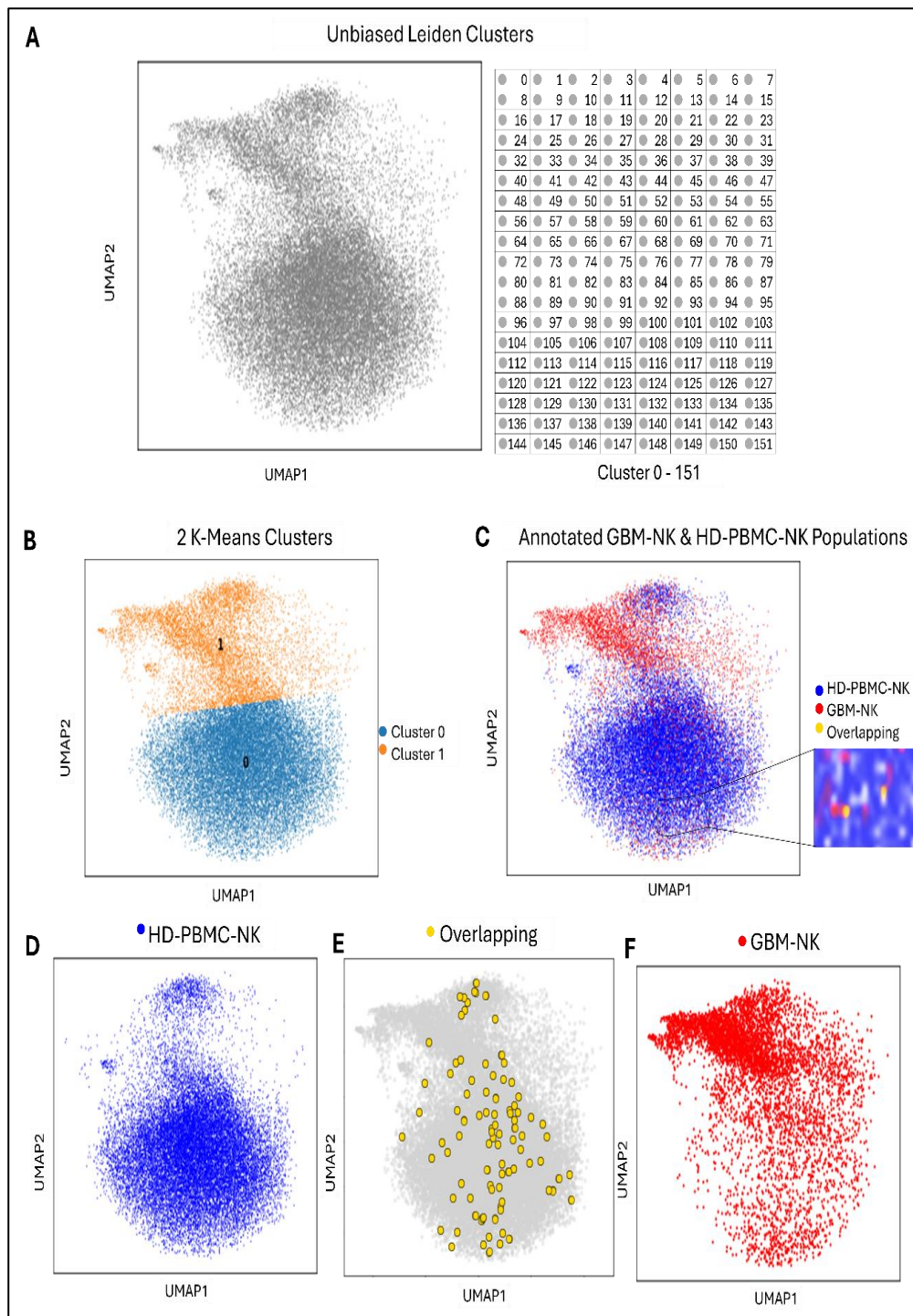


Figure 3: GBM-NK Cells are Transcriptionally Distinct from HD-PBMC-NK Cells. NK cell populations were previously annotated within the downloaded dataset (Netskar et al. 2024). Tissue sources were gathered from the metadata of original compiled dataset with NK cells annotated using CellTypist. Data filtered for NK cells in circulation of healthy donors (HD-PBMC-NK; 23,100 cells) or GBM tumour (GBM-NK; 5,500 cells) were used to generate **A)** first an unbiased UMAP denoting 151 clusters rendered by unsupervised Leiden Clustering (resolution 0.5) – legend colour code undistinguishable due to number of clusters). **B)** Unbiased UMAP of two clusters labelled by K means clustering machine learning **C)** Unified UMAP of both GBM-NK and HD-PBMC-NK populations – overlapping yellow cells observed in enhanced box beside

UMAP. Jaccard Similarity Index, 0.0032; Silhouette Score 0.3244 **D**) Individual UMAP filtered for HD-PBMC-NK population, 23.1k cells. **E**) UMAP of overlapping cells (91 cells). **F**) Individual UMAP filtered for GBM-NK population, 5.5k cells. Grey dots and numbers represent Leiden clusters **A** (denote total dataset populations GBM-NK and HD-PBMC-NK in **E** and provide background for overlain yellow overlap cells), blue dots denote HD-PBMC-NK cells (denote cluster 0 in **B**), orange dots denote cluster 1 cells (**B**), yellow dots denote overlapping cells, and red dots denote GBM-NK cells.

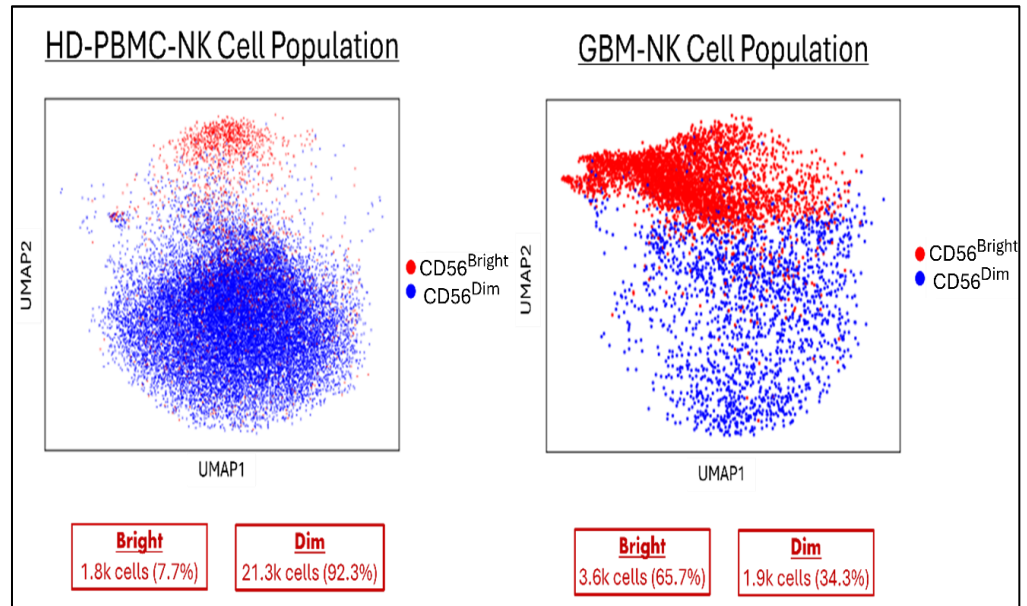


Figure 4: GBM-NK have a greater enrichment of CD56^{bright} cells compared to HD-PBMC-NK cells. Individual UMAPs generated for HD-PBMC-NK (left) and GBM-NK populations (right) with CD56^{bright} (CD56^{bright}) and CD56^{dim} cells (CD56^{dim}) annotated by red and blue dots respectively. Cell Typist was used by Netskar et al., 2024 to annotate respective CD56^{bright} and CD56^{dim} subsets based upon greater expression of *NCAM1* (encoding CD56) and *FCGR3A* (encoding CD16) respectively. Quantity (cell number per thousand/k) and percentage of CD56^{bright} and CD56^{dim} cells within total HD-PBMC-NK and GBM-NK populations marked in tables below respective graphs.

Furthermore, the generation of NK cell subset UMAPs for both cohorts, suggests that the GBM-NK population displays greater variation between transcriptional states of CD56^{bright} and CD56^{dim} cells, compared to HD-PBMC-NK population. This is indicated by less overlap of subsets for when examining individual and unified UMAPs for HD-PBMC-NK (**Fig. 5A-C**) and GBM-NK (**Fig. 5E-G**) populations. CD56^{bright} cells appear to polarise further from CD56^{dim} cells in GBM-NK compared to HD-PBMC-NK, where there is slightly more regional overlap between their respective CD56^{bright} and CD56^{dim} cells. This is supported

by quantitative assessments scoring for clustering strength of CD56^{bright} and CD56^{dim} subsets in both GBM-NK and HD-PBMC-NK: Silhouette scores – 0.4637 for GBM-NK; 0.4101 for HD-PBMC-NK cells. Although, there are more directly overlapping cells identified in HD-PBMC-NK (44 cells) vs GBM-NK (8cells) (**Fig. 5D & 3H**), relative to the total population of both cohorts direct overlap is considerably low for both: Jaccard Similarity index - 0.002 in HD-PBMC-NK; 0.0014 in GBM-NK. Furthermore, the overlapping cells identified in HD-PBMC-NK cells appear to be congregate closer to the CD56^{dim} cluster region while overlapping cells in GBM-NK are mapped to the CD56^{bright} cluster region. Thus, evidence suggests a slightly stronger divergence of CD56^{bright} and CD56^{dim} transcriptomic patterns in GBM-NK compared to HD-PBMC-NK.

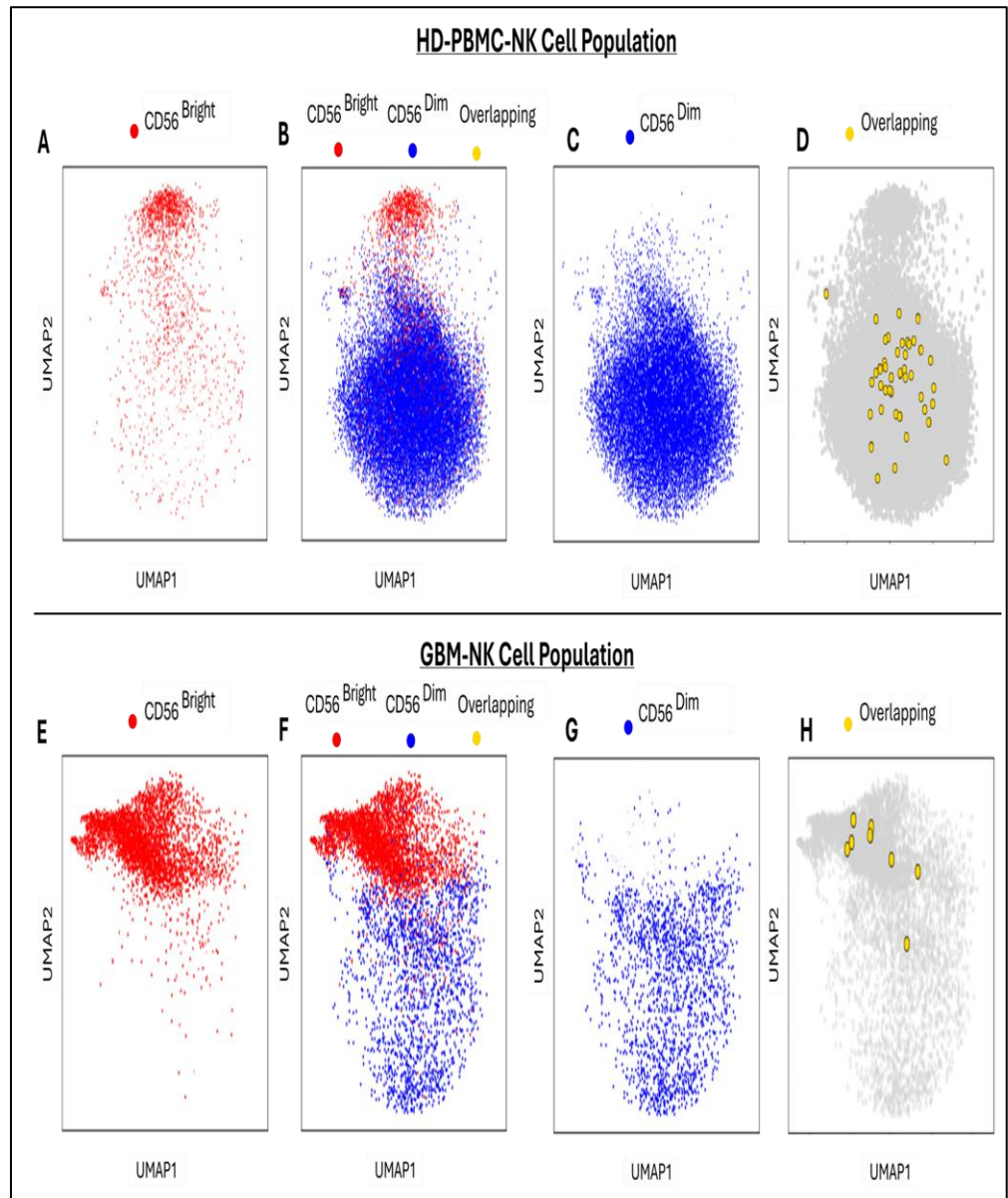


Figure 5: GBM-NK cohort have more inherent variability in NK cell subsets compared to HD-PBMC-NK cohort. Individual UMAPs generated for HD-PBMC-NK (top) and GBM-NK populations (bottom) with $CD56^{\text{bright}}$ and $CD56^{\text{dim}}$ annotated by red and blue dots respectively, along overlapping regions illustrated by yellow dots. Cell Typist was used by Netskar et al., 2024, to annotate respective $CD56^{\text{bright}}$ and $CD56^{\text{dim}}$ subsets based upon greater expression of *NCAM1* (encoding CD56) and *FCGR3A* (encoding CD16) respectively. For both HD-PBMC-NK and GBM-NK Populations: **A, E**) Individual UMAP filtered for $CD56^{\text{bright}}$ Population, 1.8k cells in HD-PBMC-NK; 3.6k cells in GBM-NK. **B, F**) Unified UMAP of both $CD56^{\text{bright}}$ and $CD56^{\text{dim}}$ subset populations. **B**) Jaccard Similarity Index, 0.002; Silhouette Score 0.4101. **H**) Jaccard Similarity Index, 0.0014; Silhouette Score 0.4637. **C, G**) Individual UMAP filtered for $CD56^{\text{dim}}$ population, 21.3k and 1.9k cells in HD-PBMC-NK and GBM-NK respectively. **C, G**) UMAP of overlapping $CD56^{\text{bright}}$ and $CD56^{\text{dim}}$ cells (44 cells in HD-PBMC-NK and 8 in GBM-NK). Blue dots denote $CD56^{\text{dim}}$ cells, yellow dots denote overlapping cells and red dots $CD56^{\text{bright}}$ cells. Grey Dots denote total dataset

populations GBM-NK and HD-PBMC-NK in **D and H**, and provide background for overlain yellow overlap cells).

In order to address if CD56^{bright} cells from tumours are transcriptionally similar to circulating counterparts, we directly compared these and found that CD56^{bright} populations in GBM-NK were transcriptionally divergent from CD56^{bright} cells in HD-PBMC-NK. Although there was some overlap, as expected from cells from the same subset type, the UMAP displaying all CD56^{bright} cells in HD-PBMC-NK (blue) and GBM-NK (red) populations clearly illustrated that CD56^{bright} cells in GBM-NK deviate from the transcriptional patterns of CD56^{bright} cells in HD-PBMC-NK (**Fig. 6A**) This is also evident when observing individual UMAPs for CD56^{bright} NK cells from both cohorts (**Fig. 6C,D**). Although it is a weaker score, CD56^{bright} cells in both cohorts positively clustered to themselves quantitatively: Silhouette score of 0.2058. Furthermore, a small number of 11 directly overlapping cells were identified in **Figure 6B** and along with a low Jaccard Index of 0.0020, support the conclusion that CD56^{bright} cells in GBM-NK can be transcriptionally distinguished from HD-PBMC-NK.

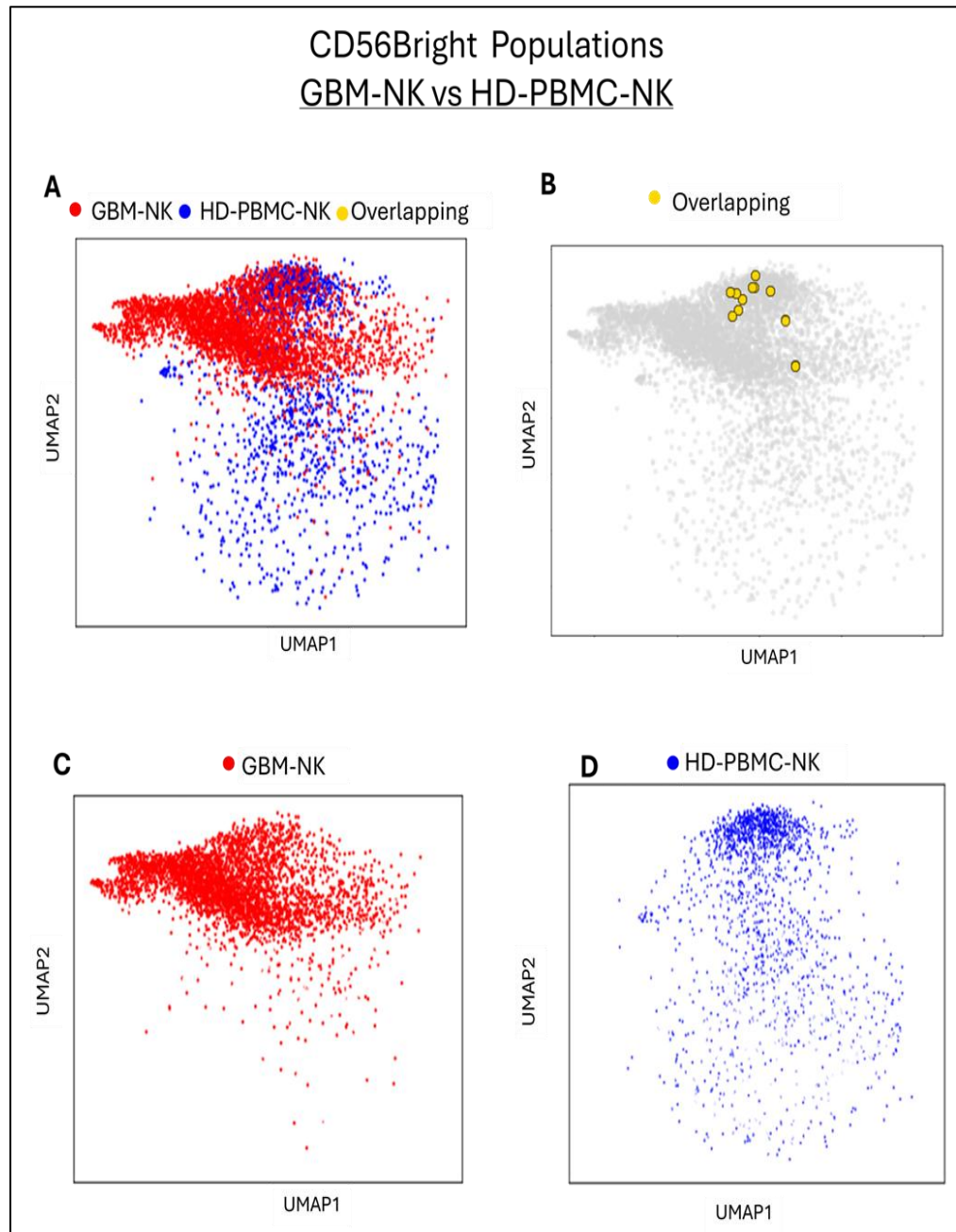


Figure 6: CD56^{Bright} NK cells in tumours are transcriptionally different to CD56^{Bright} cells in healthy donors. UMAPs generated for CD56^{Bright} cells in HD-PBMC-NK and GBM-NK populations. **A)** Unified UMAP of CD56^{Bright} cells from GBM-NK (red) and HD-PBMC-NK (blue) populations – overlapping cells signified by yellow dots. Jaccard Similarity Index 0.0020, Silhouette Score 0.2058. **B)** UMAP of overlapping cells (11 cells). **C)** Individual UMAP filtered for CD56^{Bright} Population in GBM-NK, 1.8k cells. **D)** Individual UMAP filtered for CD56^{Bright} population of HD-PBMC-NK cohort, 3.6k cells. Blue dots denote HD-PBMC-NK cells, red dots GBM-NK cells and **(B)**, yellow dots denote overlapping cells. Grey Dots denote total dataset populations GBM-NK and HD-PBMC-NK in **B** and provide background for overlain yellow overlap cells).

A similar analysis of CD56^{dim} populations showed CD56^{dim} populations in GBM-NK diverge to lesser degree from HD-PBMC-NK CD56^{dim} cells. This is observed by the largely overlapping regions of CD56^{dim} cells in HD-PBMC-NK (blue) and GBM-NK (red) cohorts in the UMAP illustrated in **Figure 7A** with directly overlapping (70) cells identified in **Figure 7B**. However, despite large overlapping regions, when studying the unified UMAP of CD56^{dim} cells with individual CD56^{dim} UMAPs for both cohorts, it is clear there are different transcriptional patterns in CD56^{dim} GBM-NK cells (**Fig. 7C,D**) Despite some evidence of closer overlap between cohorts (relatively little direct overlap - Jaccard Similarity Index 0.0031) GBM-NK cells do cluster positively to themselves albeit weakly from HD-PBMC-NK: Silhouette score of 0.1791.

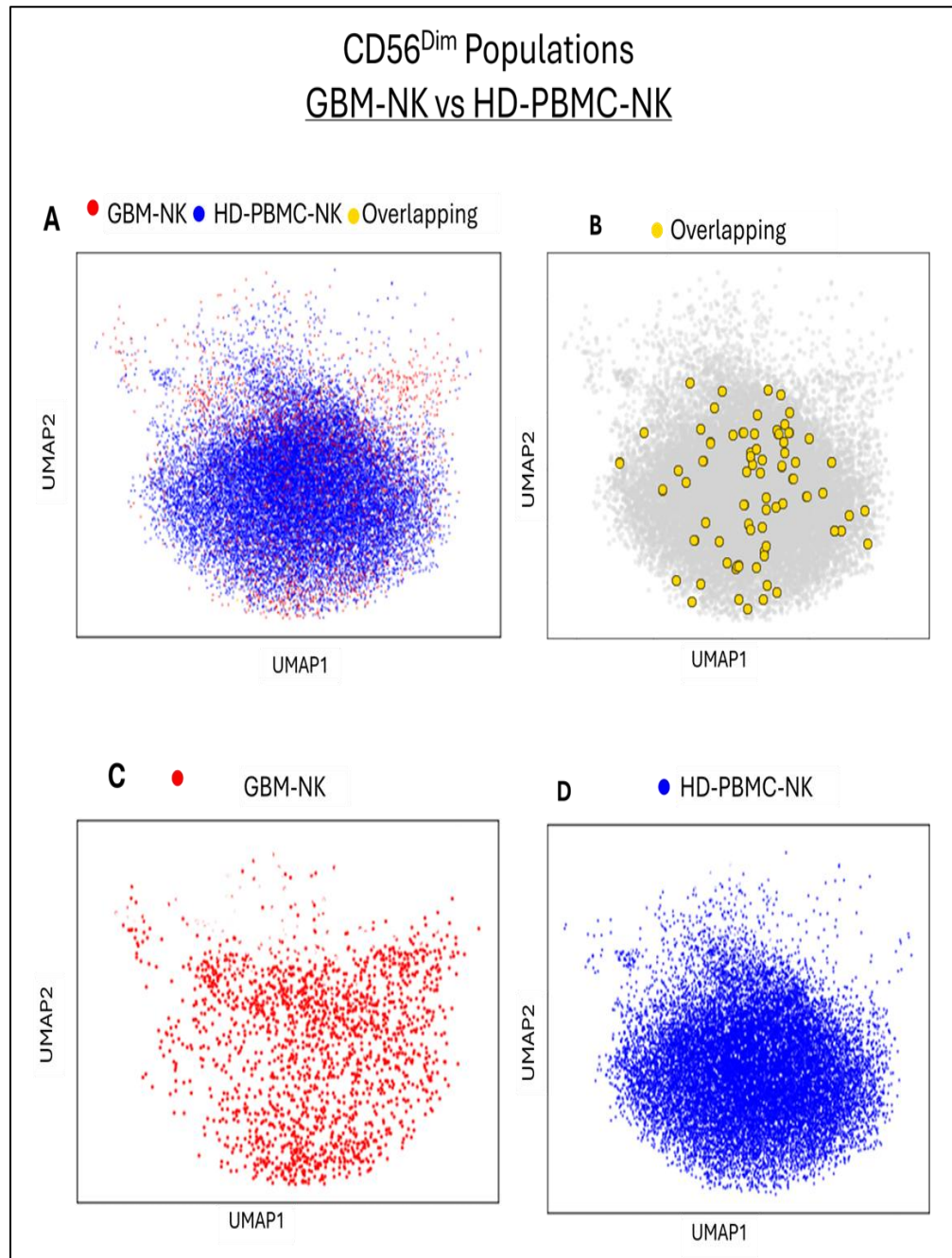


Figure 7: GBM-NK CD56^{dim} cells are transcriptionally distinct but have more overlapping gene expression with CD56^{dim} NK cells from healthy donors. UMAPs generated for CD56^{dim} cells in HD-PBMC-NK and GBM-NK populations. **A)** Unified UMAP of CD56^{dim} cells from GBM-NK (red) and HD-PBMC-NK (blue) populations with overlapping yellow cells. Jaccard Similarity Index 0.0031, Silhouette Score 0.1791. **C)** Individual UMAP filtered for CD56^{dim} population of GBM-NK cohort, 1.9k cells. **D)** Individual UMAP filtered for CD56^{dim} Population in HD-PBMC-NK, 21.3k cells. **B)** UMAP of overlapping cells (70 cells). HD-PBMC-NK sources annotated by Flow markers that corresponded with marker gene expression – see Netskar et al., 2024/ supplementary/methods for further details. Early, Intermediary and Late Stage Differentiated CD56^{dim} cells sourced from HD-PBMC-NK denoted as: NKG2A+, PBMC, KIR+,PBMC, CD57+,PBMC, Blue dots denote HD-PBMC-NK cells, yellow dots denote overlapping cells and

red dots GBM-NK cells. Grey Dots denote total dataset populations GBM-NK and HD-PBMC-NK in **B** and provide background for overlain yellow overlap cells).

Although CD56^{bright} and CD56^{dim} subsets appeared transcriptionally distinct in GBM-NK from HD-PBMC-NK, it was unclear whether the global pattern differences observed between cohorts was resultant of a largely CD56^{bright} dependent effect. To discern this PCA plots based off the top 2000 most variable genes were plotted to compare the entire cohorts and their subsets respectively (**Fig. 8A, Supplementary Figure 4**) – again GBM-NK cells showcased transcriptionally distinct patterns from HD-PBMC-NK for global and subset population comparisons. The top 20 DEGs for GBM-NK vs HD-PBMC-NK total population analysis were plotted in **Figure 8B** and are listed along with the top 20 ranked DEGs genes increased in CD56^{bright} and CD56^{dim} GBM-NK subsets in **Figure 8C** (see Supplementary Tables 1-3 for Log₂ Fold Change, significance values). Many of the DEGs increased in CD56^{bright} and CD56^{dim} cohorts were common with each other and with the top 20 globally increased DEGs (**Fig. 8C**). Therefore, it was decided that investigating subset differences in detail was beyond scope and the focus of this thesis would be to study differences in transcriptome of total GBM-NK and HD-PBMC-NK populations.

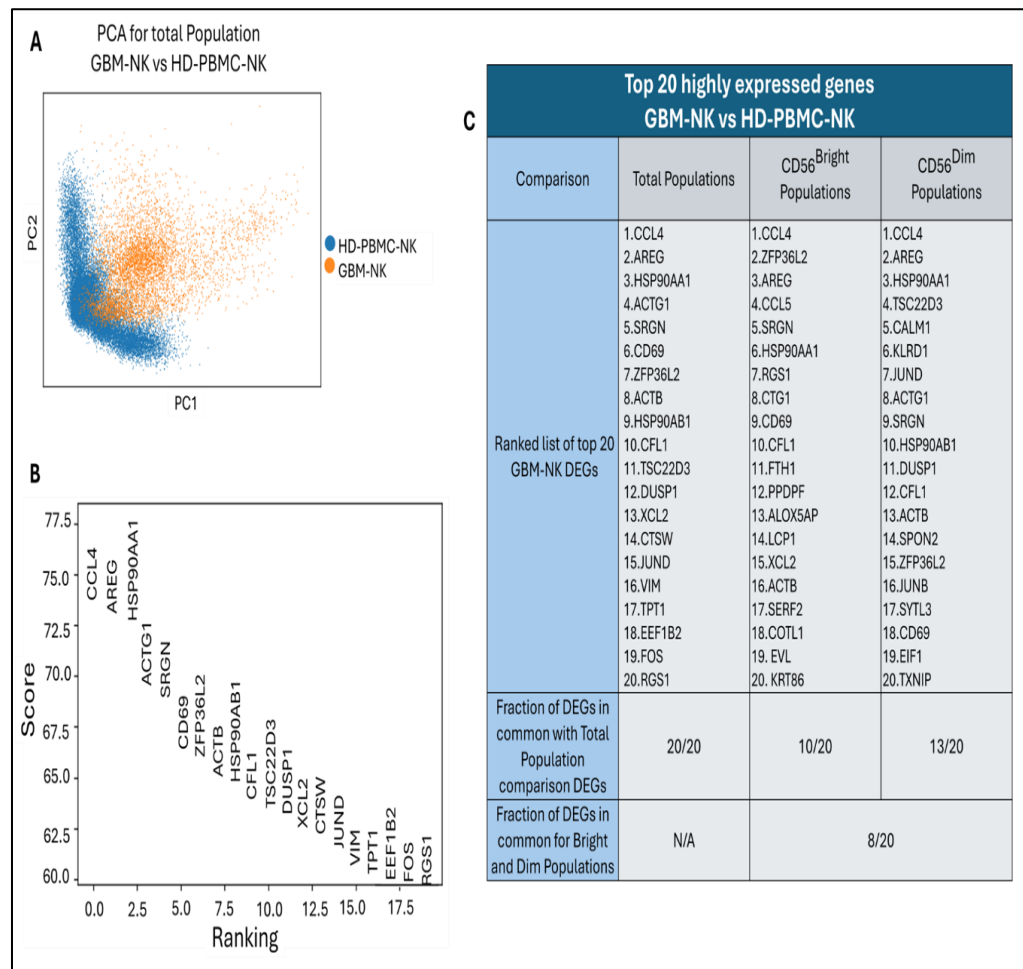


Figure 8: Top hits for differential expressed genes between of GBM-NK vs HD-PBMC-NK largely shared across global and subset (CD56^{bright}/CD56^{dim}) analyses. Three resolutions of comparative analysis: Total Populations (CD56^{bright} and CD56^{dim}) – HD-PBMC-NK vs GBM-NK cells; CD56^{bright} cells – HD-PBMC-NK vs GBM-NK; CD56^{dim} cells – HD-PBMC-NK vs GBM-NK. For each level of analysis PCA plots were generated for HD-PBMC-NK (blue dots) and GBM-NK (orange dots) based on the top 2000 highly variable genes – PCA plot for Total Population comparison illustrated in **A**. Differentially Expressed Genes (DEG) plots outlining the top 20 genes upregulated in GBM-NK were plotted in decreasing order of Z Score obtained from Wilcoxon rank sum test – DEG plot for total population analysis seen in **B**. Ranking axis from 0 (meaning highest rank) to 19 (20th most differentially expressed gene in GBM-NK group) for descending order of z-scores obtained from Wilcoxon rank-sum test. **C**) Table listing top 20 DEGs for each comparison and the fraction of DEGS in common between comparisons. Table of Log₂Fold change and significance values accompanying Z scores obtained from Wilcoxon test for DEG plot in **Supplementary Tables 1-3**.

At this early phase of global analysis, it was also noted that general levels of transcription were increased in GBM-NK vs HD-PBMC-NK cells on average. For each gene available in the dataset, mean expression and mean frequency values were pooled together to calculate global transcriptome (~14000 genes

available in dataset) average for expression and frequency in GBM-NK and HD-PBMC-NK populations. The table below denotes global gene averages (weighted averages of all gene expression and frequency averages in dataset) are higher in GBM-NK cells by a twofold increase in expression and a near twofold increase in frequency. However, the global expression and frequency levels were not used as an internal controls or baseline thresholds for comparative gene or gene set expression analysis as this dataset did not regress out housekeeping genes – such as ubiquitously expressed ribosome or cell cycling genes – that may skew these averages (Ho & Patrizi, 2021; Zhu et al., 2008).

	Global Gene Average	
Population	Expression	Frequency
HD-PBMC-NK	0.08	4.99%
GBM-NK	0.16	8.51%

Table 1 Higher transcription in NK cells from GBM vs. healthy controls.

We decided to expand this DEG analysis beyond the top 20 DEGs and perform ORA for wider transcriptome differential expression between GBM-NK relative to HD-PBMC-NK cells. This process is detailed in **Figure 9**. From approximately 7000 significantly increased DEGs the top 2050 genes (ranked by B statistic) were inputted into Enrichr –to give a multiple library output of significantly enriched gene sets. The Gene set Database libraries selected are outlined in **Figure 9** and were chosen to showcase a range of enriched gene set pathways that highlighted multiple aspects of cellular physiology that may be altered in GBM-NK. A representation of this output is illustrated in **Figure 10** for the top 10 gene set pathways with significantly increased expression in GBM-NK, from the ‘Reactomes Pathways 2024’ For this representative **Figure 10**, the top 10 enriched gene sets nearly all relate to cell division, although many hundreds of different pathway types were significantly enriched – therefore analysis was extended to the top 100 enriched gene sets for each gene set library database. From studying the similarities in the gene sets enriched in various databases, trends were identified to help interpret the biological relevance of ORA results

and how GBM-NK are generally affected within TME. The same process was repeated for ORA of DEGs that are decreased in GBM-NK (Top 100: significant DEGs) and trends of gene set pathways significantly diminished in GBM-NK cells were highlighted.

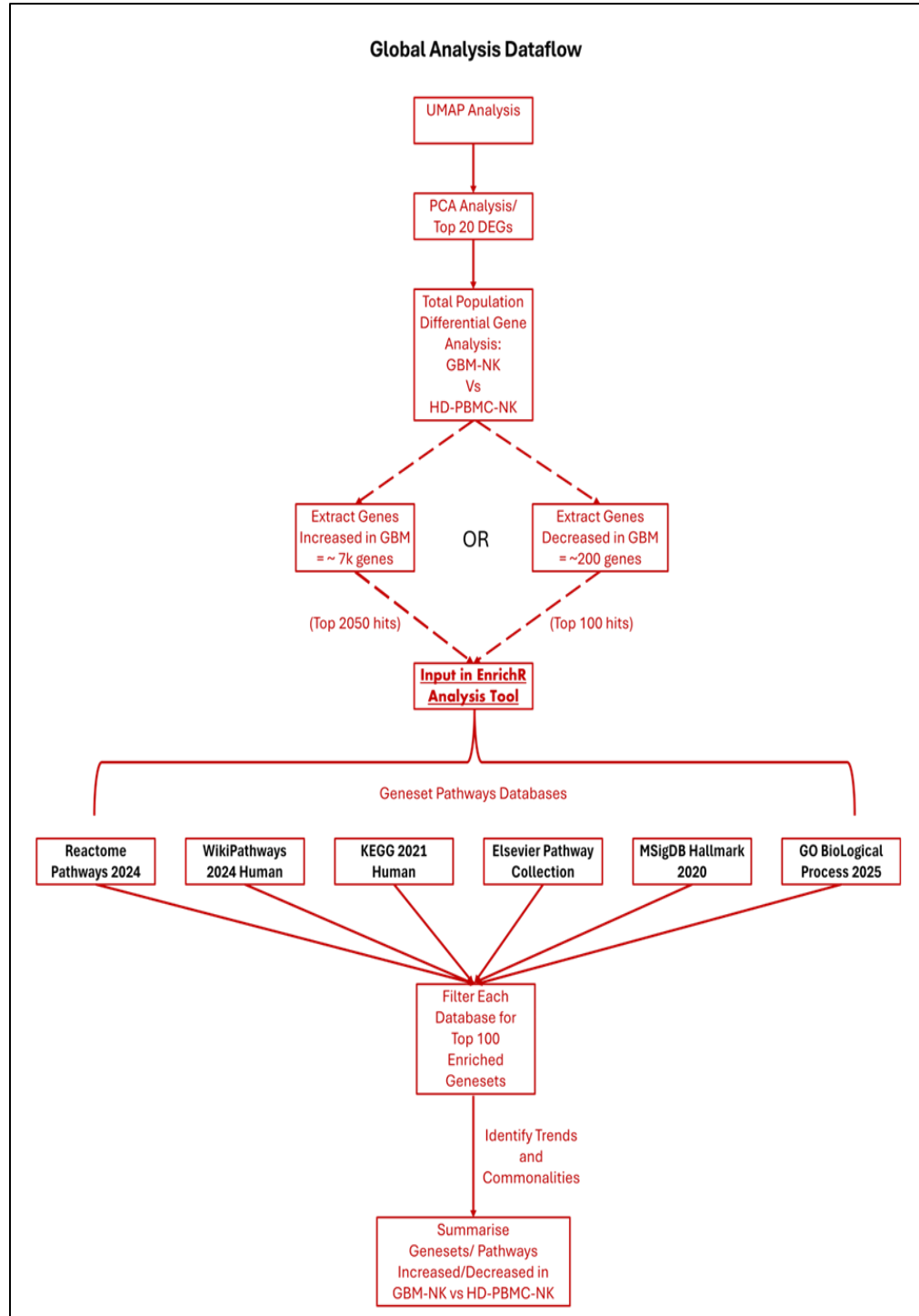


Figure 9: Global Analysis Flow Chart. The above chart delineates the stepwise approach for global analysis of dataset: ranging from UMAP generation to examine broad transcriptional pattern differences across NK cell populations; to multilevel PCA generation and respective DEG analyses to further compare these global differences across CD56^{bright} and CD56^{dim} subset

interpopulation differences; culminating with an unbiased Over-representation Analysis (ORA) of the total GBM-NK population vs HD-PBMC-NK cells. As illustrated, two ORA protocols were undertaken: one to determine pathways enriched for top 2050 genes significantly increased (~7000 genes with statistically significant increased expression in GBM-NK cells from total of 14000 genes available in dataset), and pathways for top 100 genes significantly decreased in GBM-NK cells. DEG analysis inputted into Enrichr tool, rendered enriched pathways and gene sets across multiple gene set databases. The chosen databases are outlined and were selected to help characterise transcriptomic differences in GBM-NK cells across pathways concerned with multiple cellular facets of function, metabolism and physiology. Interpretation of these enrichments was limited to the top 100 significantly enriched gene sets for each gene set database. KEGG - the Kyoto Encyclopaedia of Genes and Genomes; MSigDB Hallmark – the molecular signatures database; GO Biological Processes – Gene Ontology Biological Processes.

Genesets significantly enriched in GBM-NK population

Database: Reactome Pathways 2024
Geneset ranked by combined score ranking *

G2 M DNA Replication Checkpoint
Condensation of Prometaphase Chromosomes
Activation of NIMA Kinases NEK9, NEK6, NEK7
Phosphorylation of Emi1
Cell Cycle
Cell Cycle, Mitotic
Mitotic Prometaphase
EML4 and NUDC in Mitotic Spindle Formation
Resolution of Sister Chromatid Cohesion
Activation of the AP-1 Family of Transcription Factors

Reactome Pathways 2024					
Rank	Geneset Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	G2 M DNA Replication Checkpoint	0.00001137	0.0002307	89730	1021499.75
2	Condensation of Prometaphase Chromosomes	5.67E-08	2.486E-06	39.49	658.82
3	Activation of NIMA Kinases NEK9, NEK6, NEK7	7.44E-06	0.0001706	52.57	620.80
4	Phosphorylation of Emi1	0.00006242	0.0008224	43.79	423.96
5	Cell Cycle	6.43E-34	1.16E-30	3.40	260.17
6	Cell Cycle, Mitotic	2.85E-31	2.57E-28	3.63	255.65
7	Mitotic Prometaphase	3.59E-22	2.16E-19	4.94	243.94
8	EML4 and NUDC in Mitotic Spindle Formation	1.30E-17	2.14E-15	6.07	235.90
9	Resolution of Sister Chromatid Cohesion	3.45E-18	6.23E-16	5.87	235.88
10	Activation of the AP-1 Family of Transcription Factors	0.00001082	0.0002244	20.45	233.87

Figure 10: Representative ORA Results: ‘Reactome Pathways 2024’ database results for top 10 gene sets significantly increased in GBM-NK cells – imply cell division upregulated in GBM-NK. Following input of DEGs to Enrichr, for each gene set database the top 10 gene set pathways enriched in GBM-NK cells were plotted (top) by rank (light green corresponding to the highest) of combined score (log of the p-value, obtained from Fisher’s Exact Test [proportion’s test that for any given genes assumes its binomial distribution and independence of expression within any given gene set], multiplied by Enrichr’s odd’s ratio or z-score [standard deviation of genes expression from predetermined expected ranked occurrence within a given

gene set]) with accompanying statistical data (bottom). Adjusted p-value calculated from Benjamini–Hochberg False Discovery Rate method.

A summary of the ORA results for significantly increased DEGs are tabulated in **Figure 11** and in extended **Supplementary Figures 5(I-III)** listing descriptive names of corresponding enriched gene sets. For these figures, the first column highlights the general trends that were inferred from the significantly enriched gene sets across databases and several observations can be made. Firstly, GBM-NK cells possess a transcriptomic phenotype of DNA damage: multiple databases rendered significant gene sets related to expression of genes in response to DNA damage and cellular attempt to repair DNA damage and mismatch. GBM-NK cells conveyed increase in gene sets that collectively suggest GBM-NK cells under with cellular stress and dysfunction: pathways related to cellular senescence, inflammatory response, hypoxia, unfolded protein response, stress activated MAPK cascades, proteins recruited in autophagy and more. GBM-NK displayed an enrichment for pathways associated with cell activation including activation of AP-1 transcription factors, MYC targets, IL-2 signalling and production, TNF-alpha signalling and more. GBM-NK cells also showed enrichment of pathways associated with mitochondrial stress: activation of apoptosis protein BAD and translocation to mitochondria; the upregulation of glutathione metabolism (antioxidant often imported into mitochondria); mitochondrial unfolded protein response. Cell cycle processes and conversely cell cycle arrest associated gene sets were also enriched in GBM-NK cells: chromosome condensation, spindle and kinetochore assembly, cell cycle and cell cycle phase transitions; general cell cycle, mitotic spindle and G2M checkpoints pathways, phosphorylation of Early Mitotic inhibitor or Emi. In addition to cellular stress, GBM-NK conveyed an enriched response to the reactive oxygen species (ROS and antioxidant production gene sets: glutathione metabolism and more broadly regulation of antioxidants from NFE2L2 (NRF2) regulation. GBM-NK cells exhibited an enrichment of apoptosis related pathways. Inhibitory signalling of GBM-NK cells is suggested by enrichment of gene sets for TGF- β signalling, along with VEGF and PDGF signalling. The TME also impacts GBM-NK as evident through the upregulation of cancer related pathway gene sets: oncostatin and glioblastoma signalling

gene sets, PDL1 signalling, transcriptional regulation by TP 53 and more. Other gene sets that help determine GBM-NK response within the TME include the enrichment of interferon gamma signalling regulation and IL-6 signalling. The final aspect of ORA results related to metabolism and revealed an enrichment of metabolic pathways across databases, chiefly insulin signalling. Furthermore, GBM-NK cells ORA highlighted a myriad of other enriched metabolic pathways including cholesterol, fatty acid, folate and pterines metabolism along with other metabolic pathways of interest: glycolysis, oxidative phosphorylation and more. (Fig. 11 and Supplementary Figs.5(I-III)). Overall, the pathways consistent across databases were TNF alpha signalling, cell cycle and arrest pathways, DNA damage, and apoptosis.

Functional Readouts	Some Relevant Upregulated Genesets (Top 100)						
	RANK of pathway from each of the following databases						
	Reactome Pathways 2024	WikiPathways 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	MSigDB Hallmark 2020	GO Biological Process 2025	
DNA damage	31	95	78		36	11	
	72					94	
Cellular Dysfunction/ Stress	45	99	23	95	5	12	
			49	34	97	6	34
		15				46	
		20				82	
		30				87	
		Cell Activation	10	42	39	57	4
13	7/8						
82							
Mitochondrial Stress	36		82				
	83						
Cell Cycle Arrest/ Checkpoints	1			10	1	14	
	4					17	
	11					18	
	14					20	
	18						
Cell Cycle	2		6	1	2	1	
	6			3		2	
	7			5		3	
	8			7		4	
	9			8	3	5	
	12			9		6	
	15					8	
						41	
Response to ROS	32		82	18	28		
	97						
Apoptosis	43	10	12		9	52	
	93	81					
Inhibitory Signalling	99	1	50	49	27		
		16		78			
		20		70			
		53					
Cancer related pathways	27	5	19	21	32		
	54		43	63			
	63			91			
Other functional readouts	20	42	39		4		
	32	89			19		
					23		
Metabolic	53	67	48		12		
	71		83		21		
					97		31
							40

Figure 11: Common Trends for Gene sets pathways enriched in GBM-NK cells across multiple gene set databases, suggests the TME induces dysfunctional phenotype in tumour infiltrating NK cells. Summary of ORA data is arranged in the above table as follows: Columns list gene set databases e.g., ‘Reactome Pathways 2024’, that were selected from Enrichr analysis and cells within these columns depict their enriched gene sets, represented by numbers - signifying the rank of enrichment within the gene set library database. **See Supplementary Figure 5 (I-III)** for

names of gene sets accompanying rank numbers. All gene sets listed are reported as significantly increased (adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment within the list of a database's enriched pathway is calculated by a combined rank parameter: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched (statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human -

Following this, the enriched gene sets for Human Metabolome Database (HMDB) were additionally studied due to the vital importance of NK cells metabolism for functional capacity (O'Brien & Finlay, 2019; Yang et al., 2025) (**Fig. 12 Top**). From the Human Metabolome Database, the top 10 enriched gene sets associated with metabolite expression were ordered by Enrichr's combined rank system and additionally placed into a functional network to illustrate their connectivity (based upon the similarity of their gene content) to other upregulated metabolites (**Fig. 12 bottom**). This differential transcript profile in GBM-NK suggests that GBM-NK cells may upregulate production of metabolites – such as Adenosine Monophosphate, Phosphoric acid Tetrahydrofolic acid etc. – facilitating nucleotide and DNA synthesis and ATP production (Ducker & Rabinowitz, 2017; Hardie, 2014; Tran et al., 2024). While not a direct measure of metabolism, GBM-NK transcription shows the potential for anabolic metabolic activity.

ORA results for significantly decreased genes are tabulated in **Figure 13 and in extended Supplementary Figures 6(I-III)** listing descriptive names of corresponding enriched gene sets. For these figures, the first column highlights the general trends that are inferred from this gene sets across databases that are decreased in GBM-NK cells. GBM-NK cells appear to have downregulated expression of gene sets associated with the immunological synapses and MHC class presentation: including antigen processing/presentation/assembly, MHC Class2 presentation, and other listed immune cell receptor interactions

pathways. GBM-NK show a significant reduction in pathways relating to with sphingolipid and related metabolites synthesis, metabolism and signalling; including: glycosphingolipids and ganglioside type glycosphingolipid. Additionally, GBM-NK cells displayed downregulation of fructose metabolism and retinoic acid biosynthesis and signalling pathways. GBM-NK cells also conveyed a downregulation cytotoxic function machinery transcription, related not only to cytotoxic function but also GZMB related processes and interferon gamma signalling and interferon gamma stimulus response. Notably cytokine and degranulation in NK cells gene sets were decreased. Protein clearance was also significantly downregulated in GBM-NK cells with reduced gene set expression for proteasomes. Additionally, DNA repair pathways were downregulated in GBM-NK cells along with a downregulation of mitochondrial complex 4 gene sets. NK cell pathways related to cell maturation, differentiation into active CD56^{dim} NK cells were also diminished in GBM-NK: Fibroblast Growth Factor (FGF) receptor family (Chan et al., 2007), Natural Killer cell activation and Natural Killer Cell Surface Expression markers and more gene sets. Other downregulated pathways also included cell death related pathways such as ferroptosis and TGF- β R3 signalling. Overall DNA damage repair, sphingolipid related metabolism, antigen presentation, and cytotoxic/NK cell effector functions related gene sets appeared to be the consistently most downregulated gene sets in GBM-NK across databases.

Metabolites significantly enriched in GBM-NK population

Database: HMDB Metabolites
Geneset ranked by combined score ranking *

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	Tetrahydrofolic acid (HMDB01846)	0.00001408	0.003082	9.87	110.23
2	I3P (HMDB01498)	0.00004420	0.003082	7.89	79.15
3	Phosphoric acid (HMDB02142)	6.895e-8	0.0001470	2.43	40.00
4	Pyrophosphate (HMDB00250)	7.907e-8	0.0001470	2.41	39.47
5	Adenosine monophosphate (HMDB00045)	4.456e-7	0.0004142	2.40	35.03
6	Thiamine pyrophosphate (HMDB01372)	0.003757	0.03591	5.25	29.34
7	Magnesium (HMDB00547)	3.024e-7	0.0003748	1.95	29.22
8	3-Acetoacetyl-CoA (HMDB01484)	0.007219	0.06865	5.47	26.98
9	C11H22O22P4 (HMDB04249)	0.0002244	0.003082	2.88	24.19
10	1,2-dihexadecanoyl-sn-glycero-3-phospho-(1'-myo-inositol) (HMDB09778)	0.0002244	0.003082	2.88	24.19

Network of Related Metabolites

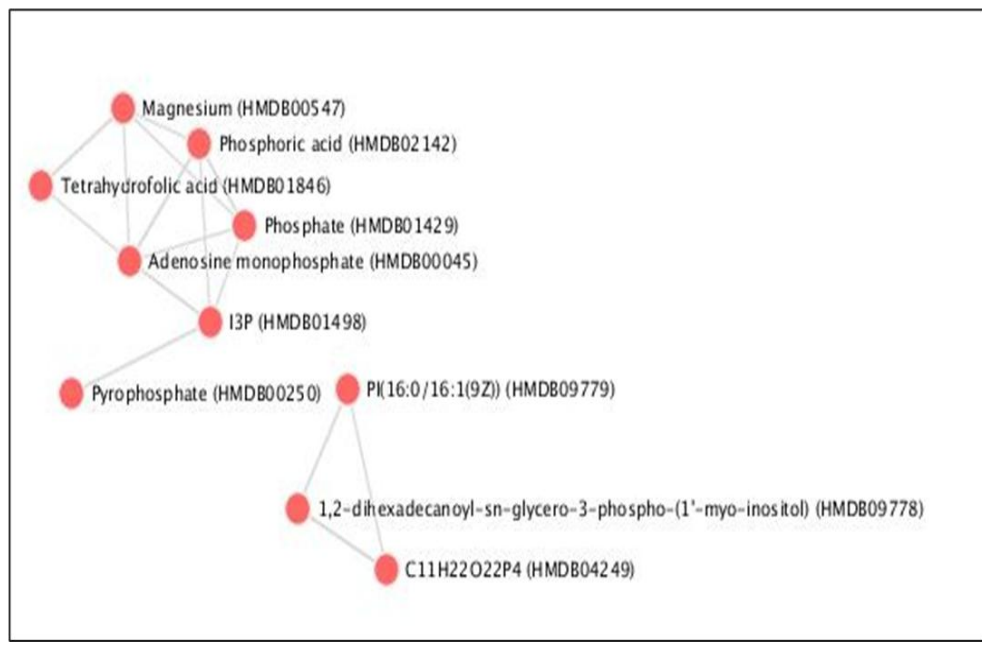


Figure 12: EnrichR ORA results for HMDB database depicts metabolite gene sets that are enriched and provide a outlook into the metabolic machinery of GBM-NK cells in the TME. HMDB or Human Metabolome Database presents metabolites pathways that are potentially enriched in GBM-NK cells based on the significant upregulation of genes associated with these metabolites, in GBM-NK cells. Following input of statistically significant increased DEGs to Enrichr, the top 10 gene set pathways relating to the production of cellular metabolites,

increased in GBM-NK cells were presented by order of Enrichr's combined score metric (log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score). The metabolites pathways relate to upregulation of genes associated with them from the HMDB bioinformatic bank. Top) The table provided with accompanying statistical data for each ranked gene set. Bottom) A functional network illustrates which metabolites may be related based upon the similarity of their gene content: where nodes represent enriched metabolites and lines only connect them to their closest enriched metabolite gene set if any.

Functional Readouts	Geneset Databases					
	RANK of pathway from each of the following databases					
	Reactome Pathways 2024	WikiPathways 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	MSigDB Hallmark 2020	GO Biological Process 2025
Immunological Synapse/ MHC Class presentation	3	1	5	1		2
	5			2		3
	13			3		8
	22		8	4		9
	25			13		14
	26		12	17		18
	50			18		
56	56					
Sphingolipid Signalling/Metabolism	4	4	18	7		58
	7	5	24			
	21	6	35			
	32	8				
Other Metabolism Pathways	8		42			75
	45		39			
	77					
Cytotoxic function	12	1	21		2	1
	29					5
						7
						33
					38	
Protein Synthesis and Degradation	14		19			65
	61					
DNA Synthesis and Repair	42	42	34		12	86
	49		41			
	31					
Mitochondrial Complex	51	60				92
NK cell maturation/ activation	9			14	8	49
	18			28		
				34		
Programmed Cell Death and Recycle		43			4	
Other Pathways	11	12		11		
	23	18				
	26					

Figure 13: Common Trends for Gene sets pathways significantly depleted in GBM-NK cells, across multiple gene set databases, provide evidence that the TME hinders NK cells immune function, cellular machinery and metabolism. Summary of ORA data is arranged in the above table as follows: Columns list gene set databases e.g., 'Reactome Pathways 2024', that were

selected from Enrichr analysis and cells within these columns depict their enriched gene sets, represented by numbers - signifying the rank of enrichment within the gene set library database. **See Supplementary Figure 6 (I-III)** for names of gene sets accompanying rank numbers.. All gene sets listed are reported as significantly depleted (adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment within the list of a database's enriched pathway is calculated by a combined rank parameter: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched (statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human.

3.2 Candidate Gene Set Analysis of Data

While the discovery-driven approach of our ORA results identified broad pathways that were significantly enriched in GBM-NK, a complementary candidate approach enabled hypothesis driven testing of known pathways of biological interest. This approach facilitated analysis of pathways that were lower-ranked or failed to meet enrichment criteria in ORA. Furthermore, the candidate approach enabled a more granular examination of all genes in a pathway and not just the most statistically significant ranked DEGs incorporated into ORA resul. Candidate gene sets of interest were selected for further analysis following findings from ORA results related to: ROS, mitochondrial stress, cellular stress, TGF- β signalling, metabolic shifts, mitochondrial complex IV downregulation, apoptosis and more (**Fig.11,13**). As TGF- β is of particular interest to the research group and is known to dramatically impair NK cell metabolism and function, it was analysed even further in depth (Slattery et al., 2021; Viel et al., 2016; V. Zaiatz-Bittencourt et al., 2018). Thus, this candidate approach hoped to validate prior hypotheses of TGF- β induced NK cell dysfunction in TME.

Candidate gene sets were grouped broadly into five categories for analysis: TGF- β , General Mitochondrial Health, Oxidative Phosphorylation,

Mitochondrial Morphology, and Glycolysis and Gluconeogenesis. **Figure 14** shows the candidate gene sets chosen, the subset gene sets that were amalgamated to make them and functional descriptions, sources used to curate candidate gene sets, total number of genes in gene set and total number of genes available in dataset. However, not all genes were available in the dataset: some transcripts were filtered out in the data curation by Netskar et al, 2024, and generally scRNA-seq data only captures a diminished reduced percentage of the total genome per cell. 10X Genomics V2 was used by Netskar et al., 2024, and this only captures 14-15% genome according to the manufacturer (Zheng et al., 2017). Generally, not every transcript is successfully mapped to the genome in scRNA-seq work (Bauernfeind & Babbitt, 2017; Li et al., 2020). For example, mitochondrial candidate gene set was limited to mitochondrial genes for complexes I-IV as the entirety of Complex V gene set was not in the original dataset. Subset gene sets for these four mitochondrial complexes were grouped together in the interest of investigating mitochondrial machinery and uncover potential mitochondrial mass changes in GBM-NK vs HD-PBMC-NK cells.

Gene Set Family	Gene Set Subset Descriptions	Source	No. Genes Pooled for Candidate Gene Sets	No. Genes Available in Dataset	Candidate Gene Set
TGF- β	Transcribed in response to TGF- β	GSEA Hallmark	208	158	TGF- β
	Receptor signalling process	KEGG, Panther, Custom Research (literature and research interests)			
	Positive regulation of TGF- β	Biological Process			
General Mitochondrial Health	Transcribed in response to Oxidative Stress	Reactome	401	289	Oxidative Stress
	Transcribed in response to ROS	Reactome	105	44	ROS
	Upregulate ROS production	Reactome			
	Cytochrome C release	Reactome, GSEA GOBP, GSEA BIOCART	77	69	Cytochrome C Release Induced Apoptosis
Oxidative Phosphorylation	Metabolic Pathway	KEGG & Literature	144	93	Oxidative Phosphorylation
	Rate limiting enzymes	Literature	29	27	OXPHOS Rate Limiting Enzymes
	Mitochondrial Complexes I-IV	Literature	74	54	Mitochondrial Complexes I-IV (Complex V Unavailable)
Mitochondrial Morphology	Mitochondrial Fusion	GO BIO Process, GSEA GOBP	33	29	Mitochondrial Fusion
	Mitochondrial Fission	MGI, GO BIO Process, GSEA GOBP	47	39	Mitochondrial Fission
	Mitophagy	Reactome	29	24	Mitophagy
Glycolysis and Gluconeogenesis	Metabolic Pathway	Literature/KEGG	204	172	Glycolysis and Gluconeogenesis
	Rate Limiting enzymes	Literature	9	9	Glycolysis Rate Limiting Enzymes

Figure 14: Breakdown of candidate gene sets chosen for analysis. Candidate Gene sets were selected from preexisting identified gene sets sourced from gene set library databases and literature. In some cases, candidate gene sets (TGF- β and ROS) were curated from multiple gene set subsets and also included additional genes of interest currently under investigation by our research group or known from the literature to be relevant to a given pathway. This inclusive approach sometimes allowed for mouse gene sets to be added to this curation to investigate novelty in the gene set analysis. However, not all genes were available in the dataset: mitochondrial candidate gene set was limited to mitochondrial genes for complexes I-IV as the entirety of Complex V gene set was unavailable.

These candidate gene sets were first assessed for differences in absolute gene set expression between GBM-NK cells and HD-PBMC-NK cells. Expression of candidate gene sets in GBM-NK and HD-PBMC-NK cells were compared using the weighted average gene expression per cell:. As seen in **Figure 15**, every candidate gene set exhibited an increased expression in GBM-NK (dashed bars) vs HD-PBMC-NK cells (solid bars). To note, in this thesis mitochondrial complexes I-IV were assessed collectively but each individual complex gene set

showcased elevated expression in GBM-NK (see **Supplementary Figure 9**). Although, some gene sets exhibited the highest weighted average expressions in GBM-NK and in HD-PBMC-NK cells, these absolute mean expression values were clearly influenced by gene set size. Therefore, the relative increase in expression of gene sets in GBM-NK were compared. See **Figure 15.5** for graph illustrating candidate gene sets with the greatest increase in expression in GBM-NK vs HD-PBMC-NK as calculated by Log₂ Fold Change – gene set with greater than 2 factor increase in expression are ranked by the following decreasing order: Oxidative stress, TGF- β , Mitochondrial Fusion, Cytochrome C Release Induced Apoptosis, Mitochondrial Fission, Glycolysis and Gluconeogenesis.

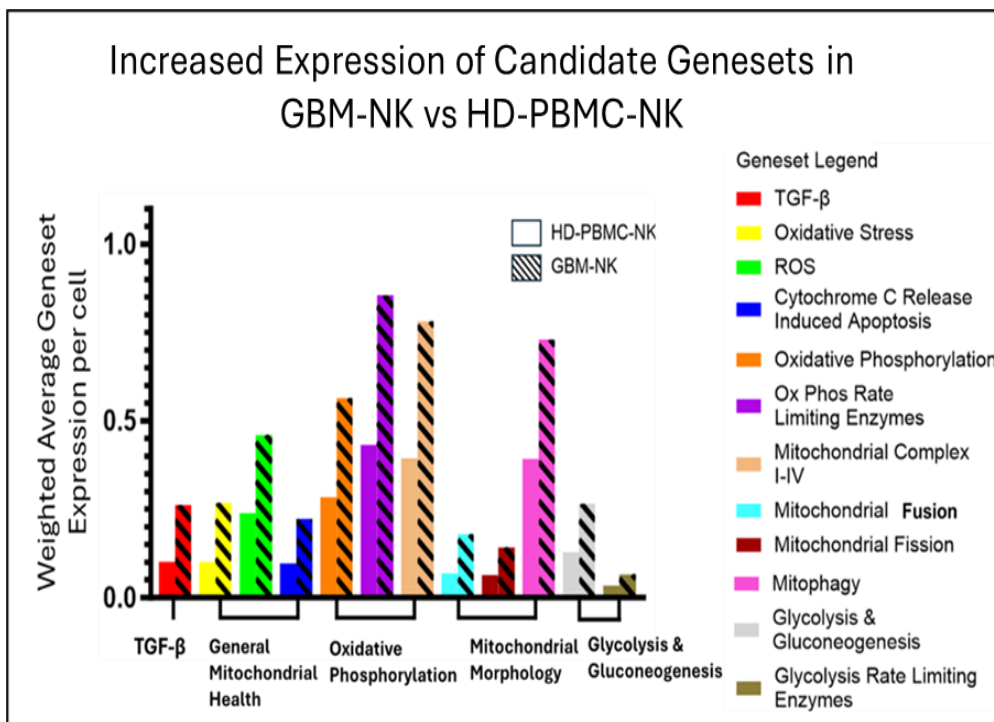


Figure 15: Candidate Gene sets have increased expression in GBM-NK vs HD-PBMC-NK cells.

Expression of candidate gene sets in GBM-NK and HD-PBMC-NK cells were compared using the weighted average gene expression per cell: the sum of expression averages for every gene in gene set normalised to the number of cells in the GBM-NK (23.1k) or HD-PBMC-NK (5.5k) cohorts. The y-axis corresponds to the absolute values of weighted average expression for each individual gene set. As shown in the Figure legend: solid bars represent HD-PBMC-NK cells while bars with dashes represent GBM-NK cells, while colours corresponding to candidate gene sets. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF- β – 158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39,

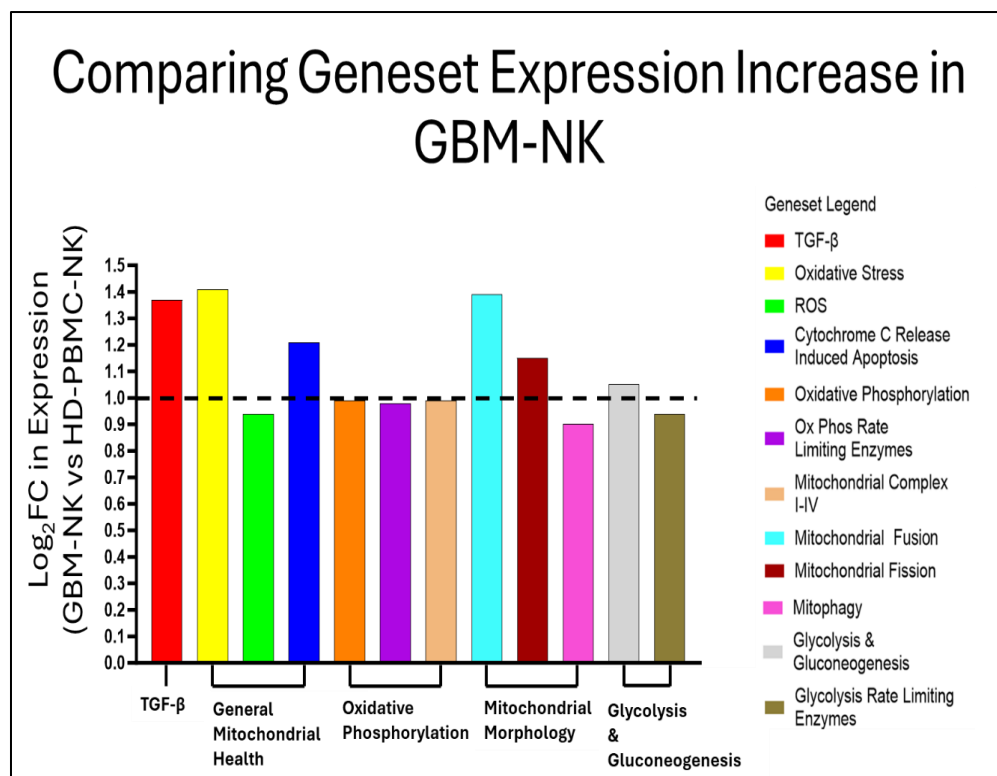


Figure 15.5: Log₂ FC increase of weighted average expression of candidate gene sets in GBM-NK vs HD-PBMC-NK. The y-axis corresponds to the Log₂ FC (Fold Change) of weighted average expression in GBM-NK vs HD-PBMC-NK. As shown in the Figure legend: solid bars colours corresponding to candidate gene sets. Dashed line at Log₂ FC = 1, represents threshold for 2 fold increase of expression in GBM-NK from HD-PBMC-NK. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF-β – 158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39, Mitophagy – 24, Glycolysis and Gluconeogenesis 172, and Glycolysis Rate Limiting Enzymes – 9.

The previous analysis was for bulk NK cell populations, we next sought to assess gene set expression differences for CD56^{bright} and CD56^{dim} NK cell subsets across GBM-NK and HD-PBMC-NK populations. Comparing CD56^{bright} first (**Fig. 16 [Top]**) every gene set showed increased expression in GBM-NK CD56^{bright} cells - with a similar gene set expression pattern increase as observed in total population analysis. Similar results were found for CD56^{dim} population comparisons (**Fig. 16 [Bottom]**), with the exception of Mitochondrial

Complexes I-IV which were lower in CD56^{dim} GBM-NK cells than CD56^{dim} HD-PBMC-NK cells. Additionally, the expression of Rate Limiting Enzymes of Glycolysis was only slightly in higher CD56^{dim} GBM-NK than CD56^{dim} HD-PBMC-NK cells.

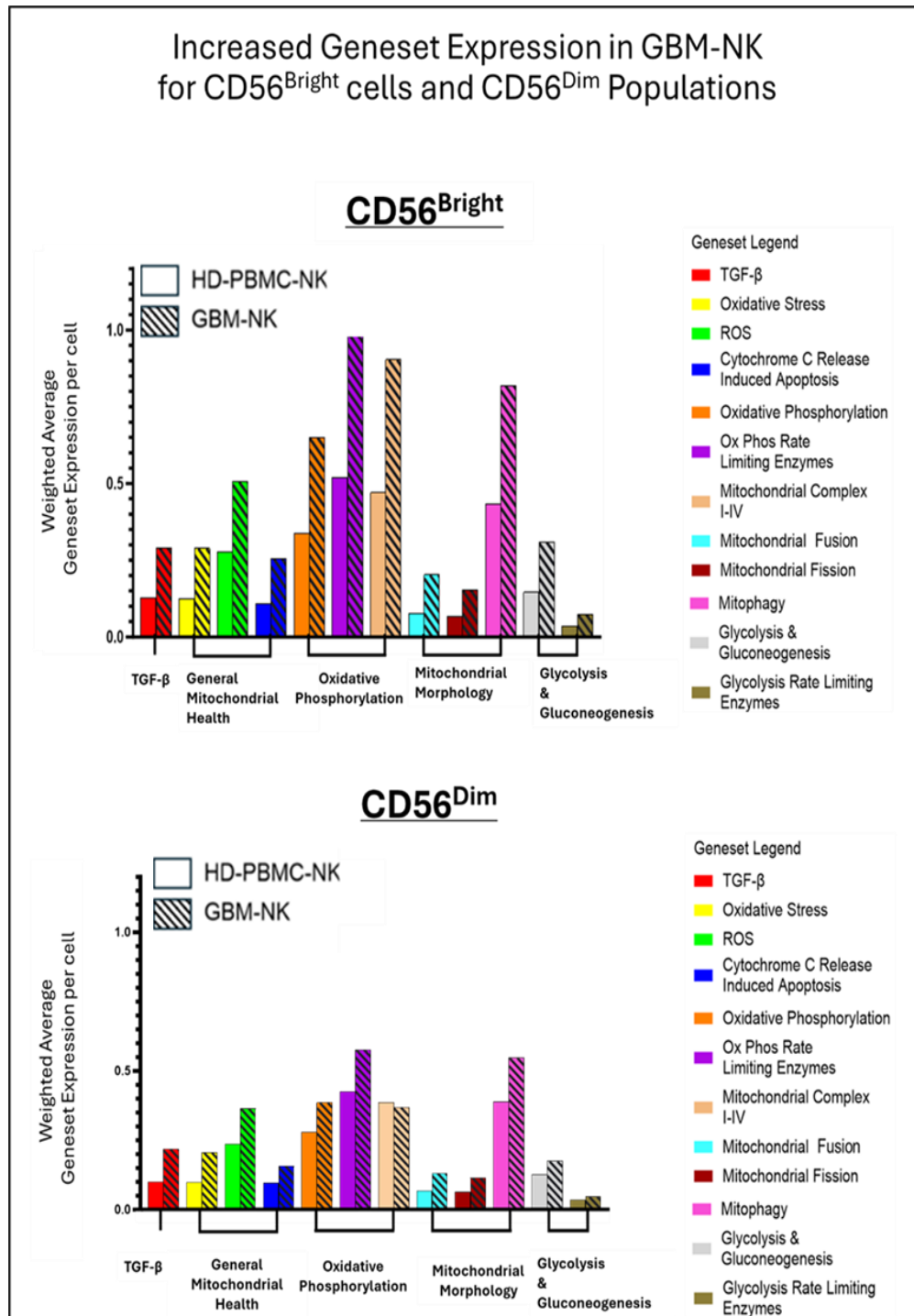


Figure 16: Candidate Gene sets show increased expression in both CD56^{bright} and CD56^{dim} Subsets in GBM-NK vs HD-PBMC-NK cells. Except for reduced OXPHOS in CD56^{dim} GBM-NK cells, every candidate gene set is increased in GBM-NK subset cells: CD56^{bright} (Top) and CD56^{dim} (bottom). Expression of candidate gene sets was compared using the weighted average gene

expression per cell: the sum of expression for every gene in gene set normalised to the number of cells in the GBM-NK (23.1k) or HD-PBMC-NK (5.5k) cohorts. The y-axis corresponds to the absolute values of weighted average expression for each individual gene set. As shown in the Figure legend: solid bars represent HD-PBMC-NK cells while bars with dashes represent GBM-NK cells, while colours corresponding to candidate gene sets. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF- β – 158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39, Mitophagy – 24, Glycolysis and Gluconeogenesis 172, and Glycolysis Rate Limiting Enzymes – 9.

Two parameters are important for a biological understanding of this data and what they mean. Gene set expression increase could be reflected in all cells, but each cell may only exhibit a small increase in expression of a given gene. Alternatively, fewer cells may affect this weighted gene set average, but these cells exhibit a dramatic increase in expression and thus skew the weighted average. Therefore, we undertook analysis to determine this if we could deconvolute this differential gene set expression increase further by calculating weighted average gene set frequencies. Ultimately it was observed that candidate gene sets were also expressed in a greater proportion of GBM-NK cells than HD-PBMC-NK cells.

Analysis showed that the same gene sets with elevated expression in GBM-NK and HD-PBMC-NK, also exhibited the greatest frequency averages (**Fig.17**). As seen in **Figure 17**, for all candidate gene sets, the weighted average gene set frequency was higher in GBM-NK cells than HD-PBMC-NK cells. This means that any given candidate gene set was not only expressed at a higher absolute expression quantity (**Fig.15**) but is also expressed in a greater percentage of cells. See **Figure 17.5** for gene sets with the greatest Log₂ Fold Change increase in expression frequency in GBM-NK vs HD-PBMC-NK cells: with only Mitochondrial Fusion and Mitochondrial Fission reaching the threshold set.

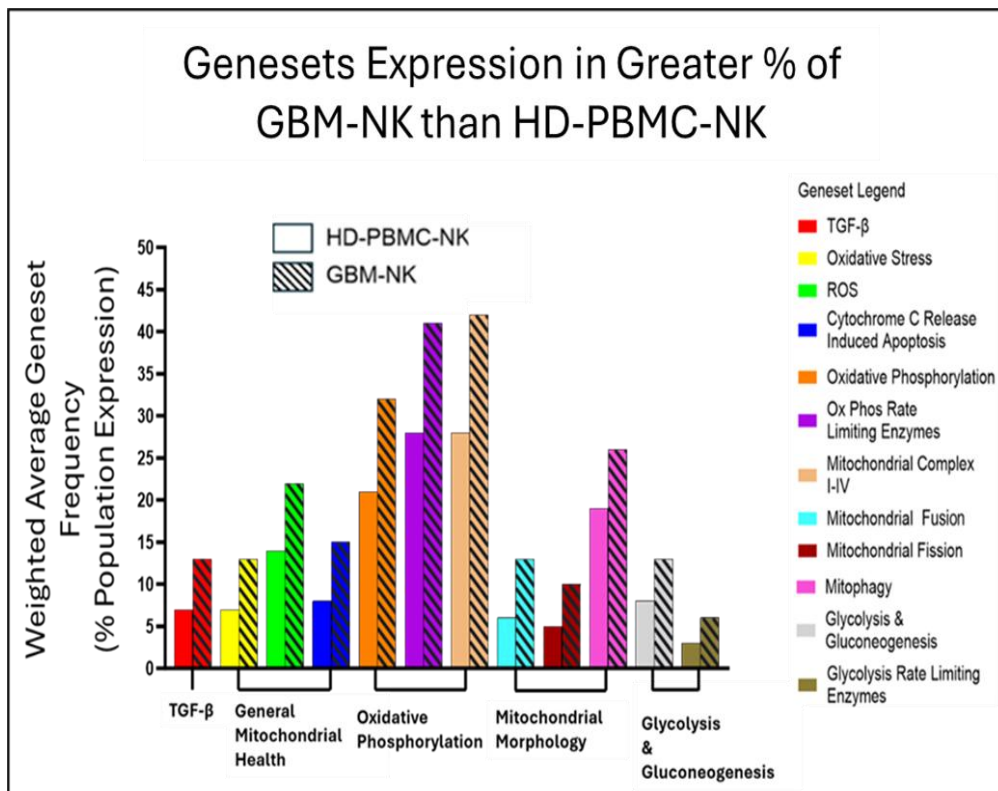


Figure 17: Candidate Gene sets show increased expression frequency in GBM-NK vs HD-PBMC-NK cells. Expression frequency (the percentage of cells expressing a given gene in a cohort) for candidate gene sets was compared using the weighted average gene expression frequency per cell: the sum of expression frequency for every gene in gene set normalised to the number of cells in the GBM-NK (23.1k) or HD-PBMC-NK (5.5k) cohorts. The y-axis corresponds to the absolute values of weighted average expression frequency for each individual gene set. As shown in the Figure legend: solid bars represent HD-PBMC-NK cells while bars with dashes represent GBM-NK cells, while colours corresponding to candidate gene sets. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF-β – 158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39, Mitophagy – 24, Glycolysis and Gluconeogenesis 172, and Glycolysis Rate Limiting Enzymes – 9.

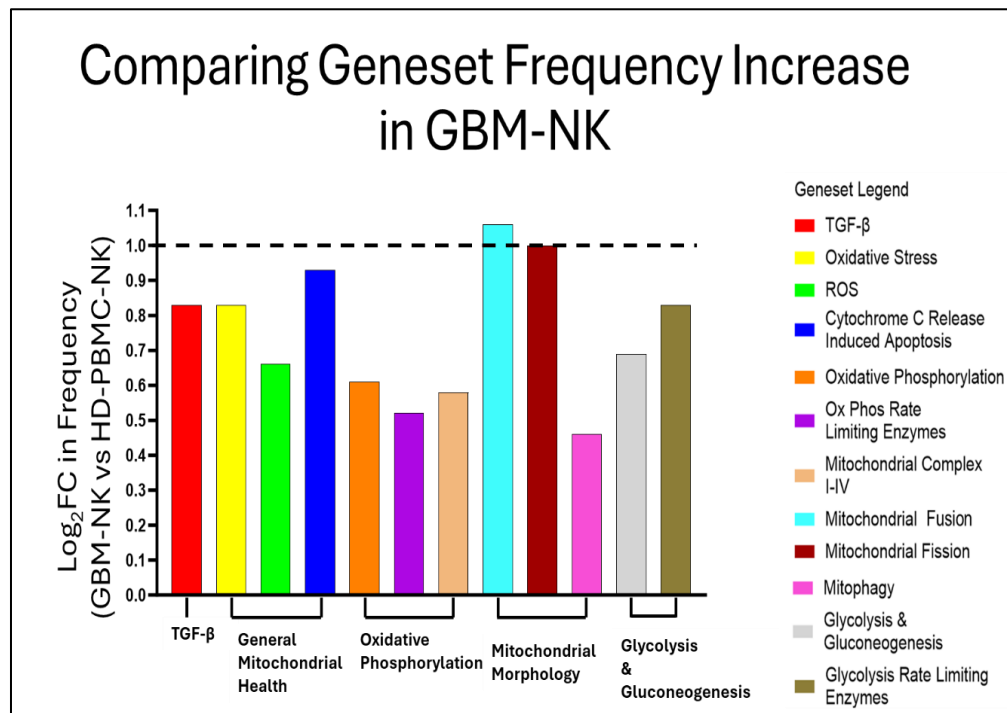


Figure 17.5: Log₂ FC increase of weighted average expression frequency of candidate gene sets in GBM-NK vs HD-PBMC-NK. The y-axis corresponds to the Log₂ FC (fold change) of weighted average expression frequency in GBM-NK vs HD-PBMC-NK. Dashed line at Log₂ FC = 1, represents threshold for 2 fold increase of expression in GBM-NK from HD-PBMC-NK. As shown in the Figure legend: solid bars colours corresponding to candidate gene sets. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF-β – 158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39, Mitophagy – 24, Glycolysis and Gluconeogenesis 172, and Glycolysis Rate Limiting Enzymes – 9.

Analysis of expression frequency revealed a few interesting things when looking at NK cell subsets. CD56^{bright} NK cells had a greater proportion of cells expressing the gene sets tested compared to CD56^{dim} cells. Furthermore, there was a greater magnitude of increase in frequency in GBM-NK CD56^{bright} cells compared to GBM-NK CD56^{dim} cells. **(Fig.18)**. Additionally, revision of the gene set expression and frequency differences across subset populations, it was noted that CD56^{bright} cells overexpressed gene sets in GBM-NK cells to a greater extent than the overexpression of gene sets observed in CD56^{dim} GBM-NK cells. Furthermore, the greater quantity of CD56^{bright} cells amongst the GBM-NK population did not seem to affect this. By contrast, CD56^{dim} cells comprise most of HD-PBMC-NK cells (21.3k CD56^{dim} cells vs 1.8k CD56^{bright} cells) and show

lower gene set expression and frequency than CD56^{bright} cells – as expected mathematically, larger populations exhibit lower averages. It is also evident from observing **Figures 16 and 18** that there is a greater fold change increase for both expression and frequency in GBM-NK CD56^{bright} cells compared to the fold change increase in expression and frequency observed in GBM-NK CD56^{dim} cells (**see Supplementary Figure 7, 8**) However, despite this evidence for a potentially CD56^{bright} skewed expression in GBM-NK cells, most candidate gene sets showed similar trends of increased expression and frequency in GBM-NK cells across subset populations. Thus, it was decided that further candidate gene set analysis would focus on global population differences and that studying subset specific differences was beyond the remit of this thesis.

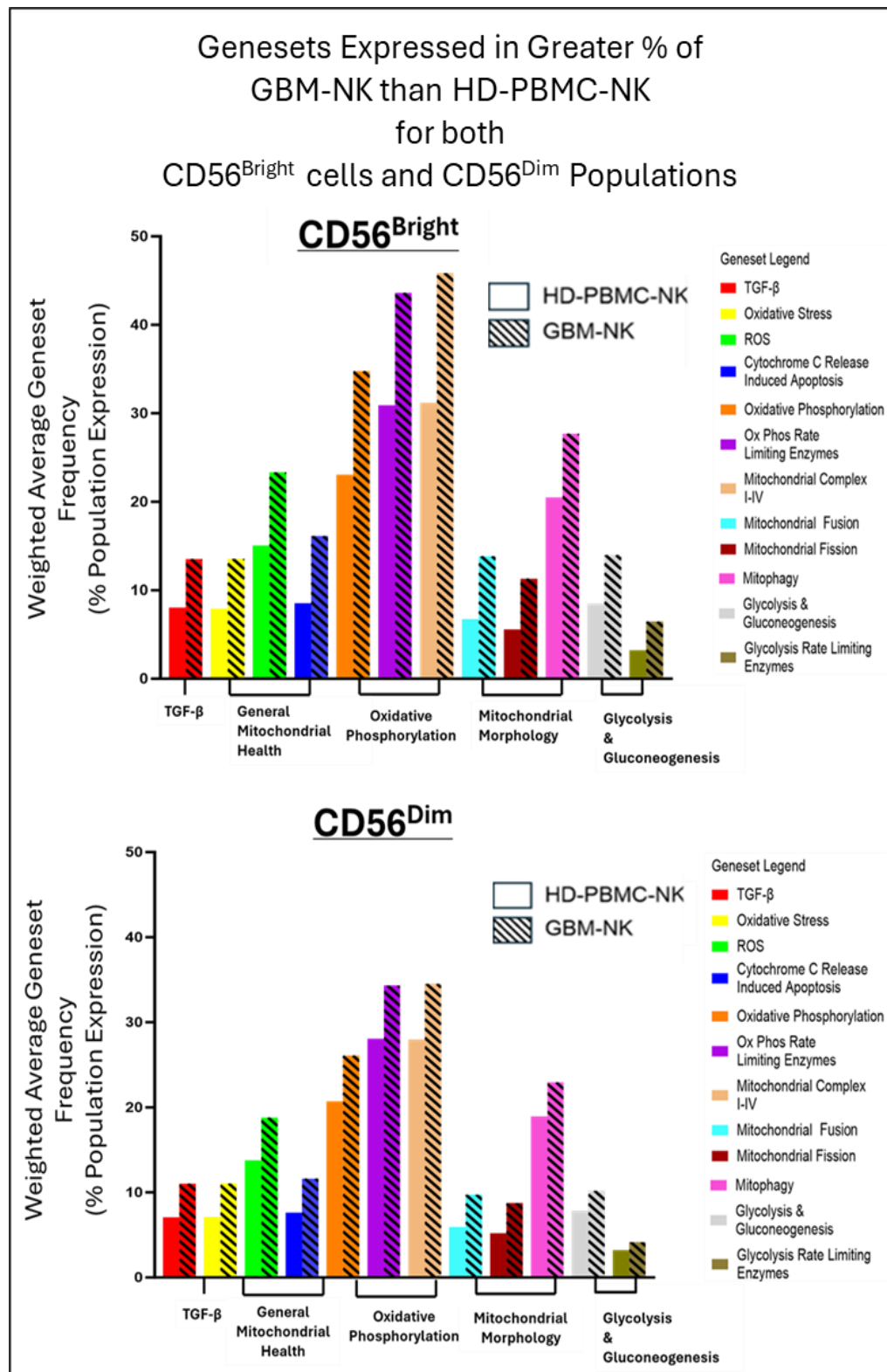


Figure 18: Candidate Gene sets show increased expression frequency in both CD56^{bright} and CD56^{dim} subsets in GBM-NK vs HD-PBMC-NK cells. Expression frequency (the percentage of cells expressing a given gene in a cohort) for candidate gene sets was compared using the weighted average gene expression frequency per cell: the sum of expression frequency for every gene in gene set normalised to the number of cells in the GBM-NK (23.1k) or HD-PBMC-NK (5.5k) cohorts. The y-axis corresponds to the absolute values of weighted average expression frequency for each individual gene set. As shown in the Figure legend: solid bars

represent HD-PBMC-NK cells while bars with dashes represent GBM-NK cells, while colours corresponding to candidate gene sets. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF- β – 158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39, Mitophagy – 24, Glycolysis and Gluconeogenesis 172, and Glycolysis Rate Limiting Enzymes – 9.

The next step of analysis was to identify markers/genes of interest that could drive the increased gene set signature in GBM-NK. Volcano plots were generated for each gene set to determine which genes have significantly increased and decreased expression in GBM-NK relative to HD-PBMC-NK: The first gene set analysed was the TGF- β gene set. As seen in **Figure 19A** the volcano plot illustrates many genes (red dots in **Figure 19A**) are significantly increased in GBM-NK. TGIF1, a transcriptional repressor of TGF- β signalling (Hneino et al., 2012; Weng et al., 2019), was the only significantly reduced gene in GBM-NK cells (blue dot in **Figure 19A**). The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 19B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 19C**). The full list of all significant genes and their corresponding Log₂ fold change in expression and frequency values are provided in **Supplementary Table 4**. The known functions of these top 10 significant genes from either rank provided more insight into the nature of TGF- β signature in GBM-NK cells. Some genes are known to be positive mediators of TGF- β signalling and/or are positive markers for signalling – CTNNB1, CD9, ACVR1B, GDF11, JUN, JUNB, JUND, MAPK1, MAPK12 (Batlle & Massagué, 2019; Close et al., 2019; F. Li et al., 2022; Liu et al., 2020; Millena et al., 2016; Shaim et al., 2021; Yoo et al., 2020; Zhang et al., 2017); however other increased genes are generally repressed by and/or themselves regulate TGF- β signalling – IFNG, MYC, ID2, ID3, PPP1R15A, FKBP1A, TFRC (Cao et al., 2009a; Chen et al., 1997; Laouar et al., 2005; Lee et al., 2014; Shi et al., 2004; V. Zaiatz-Bittencourt et al., 2018). As can be seen in **Fig.19D**, there were only three genes in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

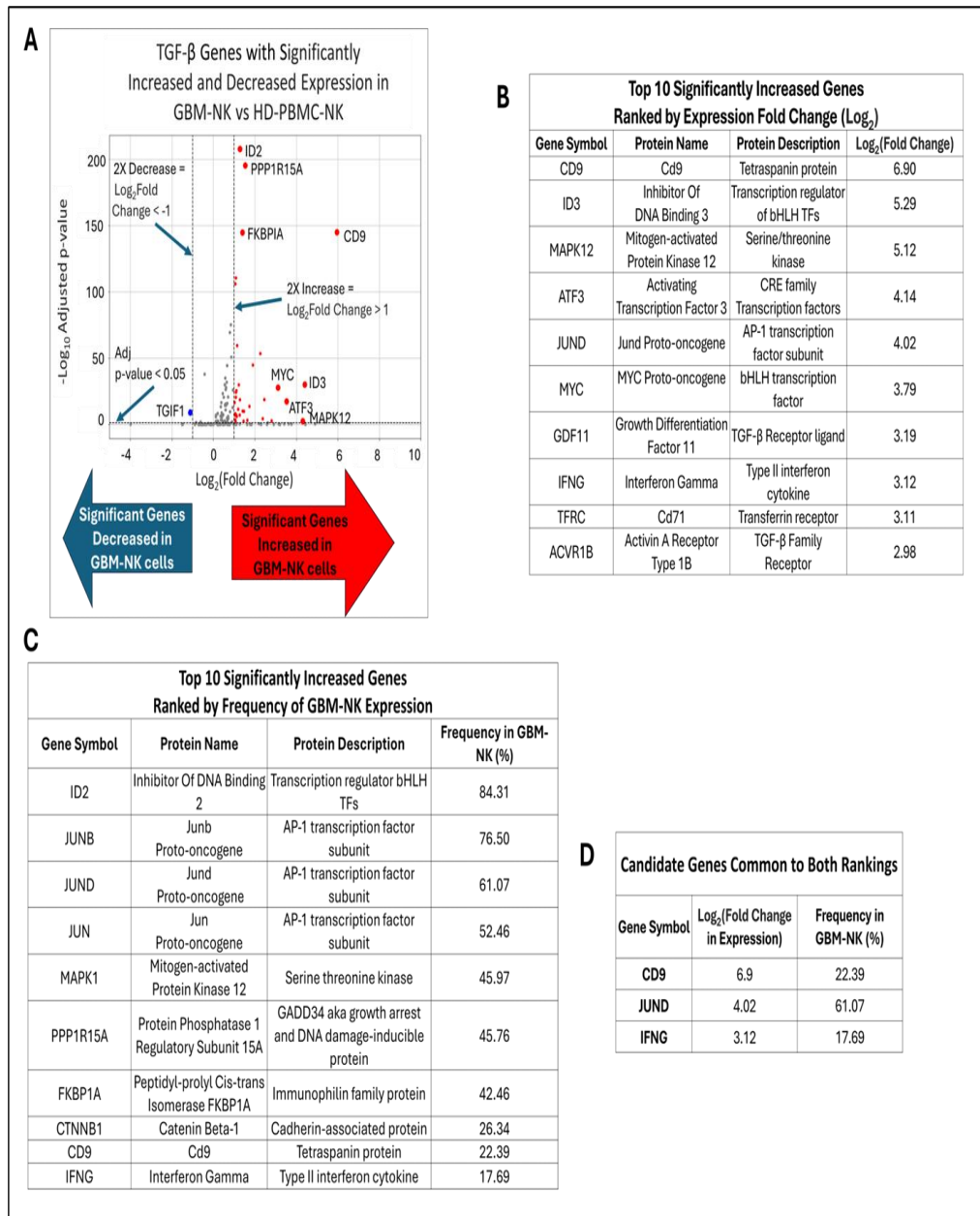


Figure 19: Three genes from TGF- β gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of genes with statistically significant increased and decreased expression in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase in expression $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual TGF- β gene. Grey Dots indicates non-significant genes, blue dots genes significantly decreased in GBM-NK, red dots genes significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK > 1 or < -1 defined significantly increased and decreased expression threshold boundaries, marked by dashed lines. Some significant genes are labelled in the interest of highest fold

change increase/decrease and genes with greatest statistical difference. Total TGF- β gene set input was 158 genes.. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided. The full list of all significantly increased genes are provided in Supplementary Tables.

We next analysed Oxidative Stress to identify markers/genes of interest that could be driving increased gene set signature in GBM-NK. As seen in **Figure 20A** the volcano plot illustrates many genes (red dots in **Figure 20A**) are significantly increased in GBM-NK. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 20B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 20C**). The full list of all significant genes and their corresponding Log₂ fold change in expression and frequency values are provided in **Supplementary Table 5**. The known functions of these top 10 ‘marker’ genes provided more insight into the nature of Oxidative Stress signature in GBM-NK cells: some genes protein function contribute to oxidative stress and/or its induced dysfunction – AREG, BCL2, AIF1 (Ahl et al., 2020; De Leon-Oliva et al., 2023; Lee et al., 2020; Palabiyik, 2025); others protect cells/alleviate from oxidative stress - HSPA1A, HSPA1B, TNFAIP3 (Kalmar & Greensmith, 2009; Vettters et al., 2019; Wang & Chen, 2022). Genes significantly decreased in GBM-NK also provide some insight: TGF β 3, a co receptor for TGF- β signalling and is downregulated in various cancers (Song et al., 2025); SLC25A6 is a mitochondrial transporter that regulates the exports of apoptotic mitochondrial product (Liu et al., 2025); DUOX1, a NADPH oxidase generates hydrogen peroxide (H₂O₂) (De Deken et al., 2014). As can be seen in **Fig.20D**, only AREG gene is in common between the two rank parameters (ranked by log2fc). Given the highest gene expression increase in more GBM-NK cells, this is a good gene candidate for further investigation for a role in GBM.

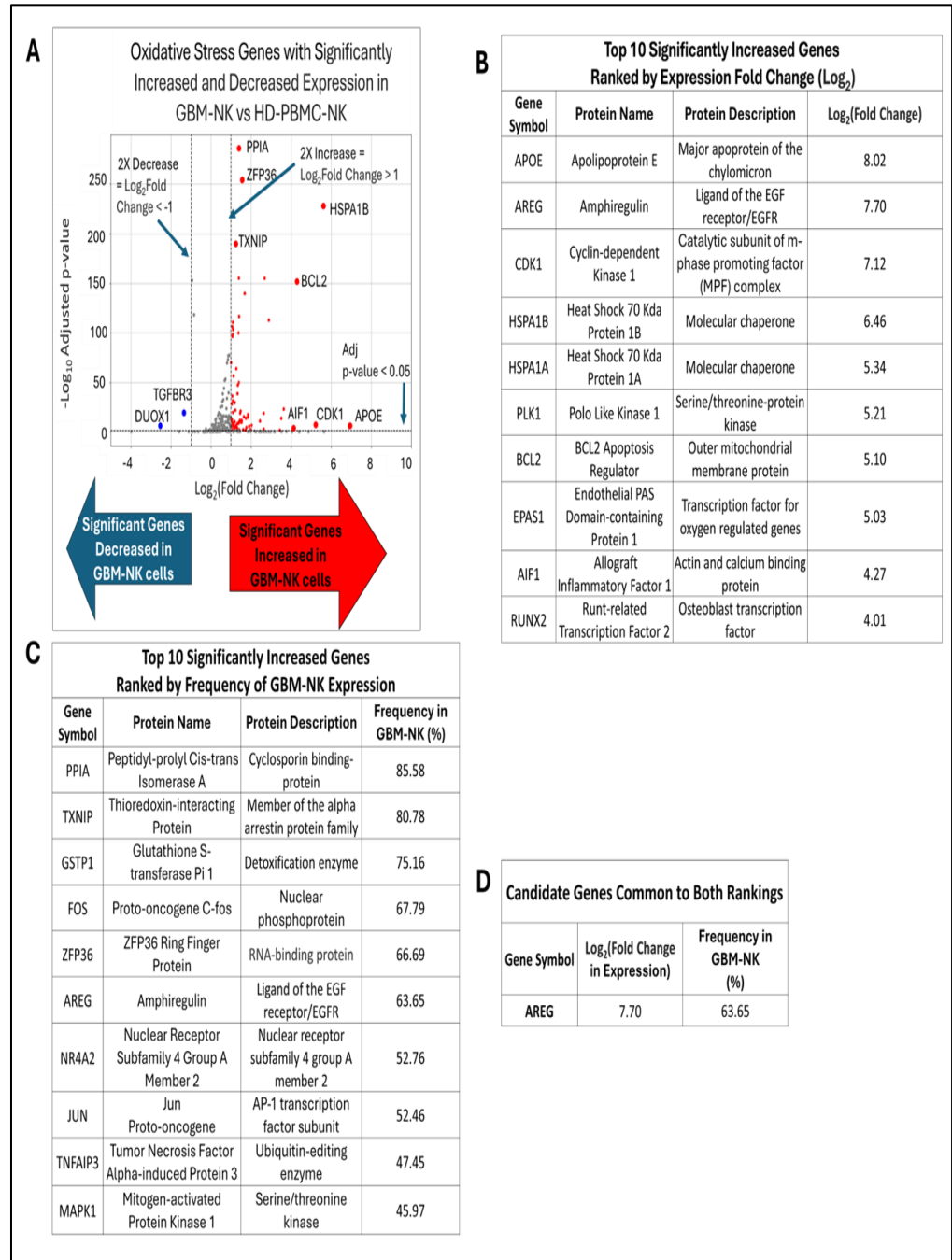


Figure 20: One significant gene from Oxidative Stress gene set has a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of genes with statistically significant increased and decreased expression in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a $\log_2$ fold change increase in expression $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a $\log_2$ fold change < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Oxidative Stress gene. Grey Dots indicates nonsignificant genes, blue dots genes significantly decreased in GBM-NK, red dots genes significantly increased in GBM-NK. Y-axis: $-\log_{10}$ adjusted p-value; $y=1.30$: adjusted p-value

<0.05 – marked by dashed horizontal line. X-axis: Log₂ fold change difference in gene expression between GBM-NK and HD-PBMC-NK > 1 or < -1 defined significantly increased and decreased expression threshold boundaries, marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. SLC25A6 is significantly decreased but not plotted as due to a near 0 p-value that exceeds the limits of the y-axis. Total Oxidative Stress gene set input was 289 genes. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided. The full list of all significantly increased genes for both ranking systems are provided in Supplementary Tables.

We next analysed the ROS gene set to identify markers/genes of interest that could be detail ROS gene set pathways increased in GBM-NK. As seen in **Figure 21A** the volcano plot illustrates several genes (red dots in **Figure 21A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 21B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 21C**). The full list of all significant genes and their corresponding Log₂ fold change in expression and frequency values are provided in **Supplementary Table 6**. The known functions of these top ‘marker’ genes provided more insight into the nature of ROS signature in GBM-NK cells: majority being antioxidant related genes typically induced by ROS environments – MSRA, PPRDX1, IPCEF1, TXN etc. (Stelzer et al., 2016); while some genes may contribute to ROS flux – ABCC1, JUNB (Mueller et al., 2005; Ren et al., 2023). Significantly reduced expression of FTL – an iron sequestering protein known to prevent iron related ROS amongst other functions (Liu et al., 2022)– is also observed. As can be seen in Fig. **21D**, ten genes are in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

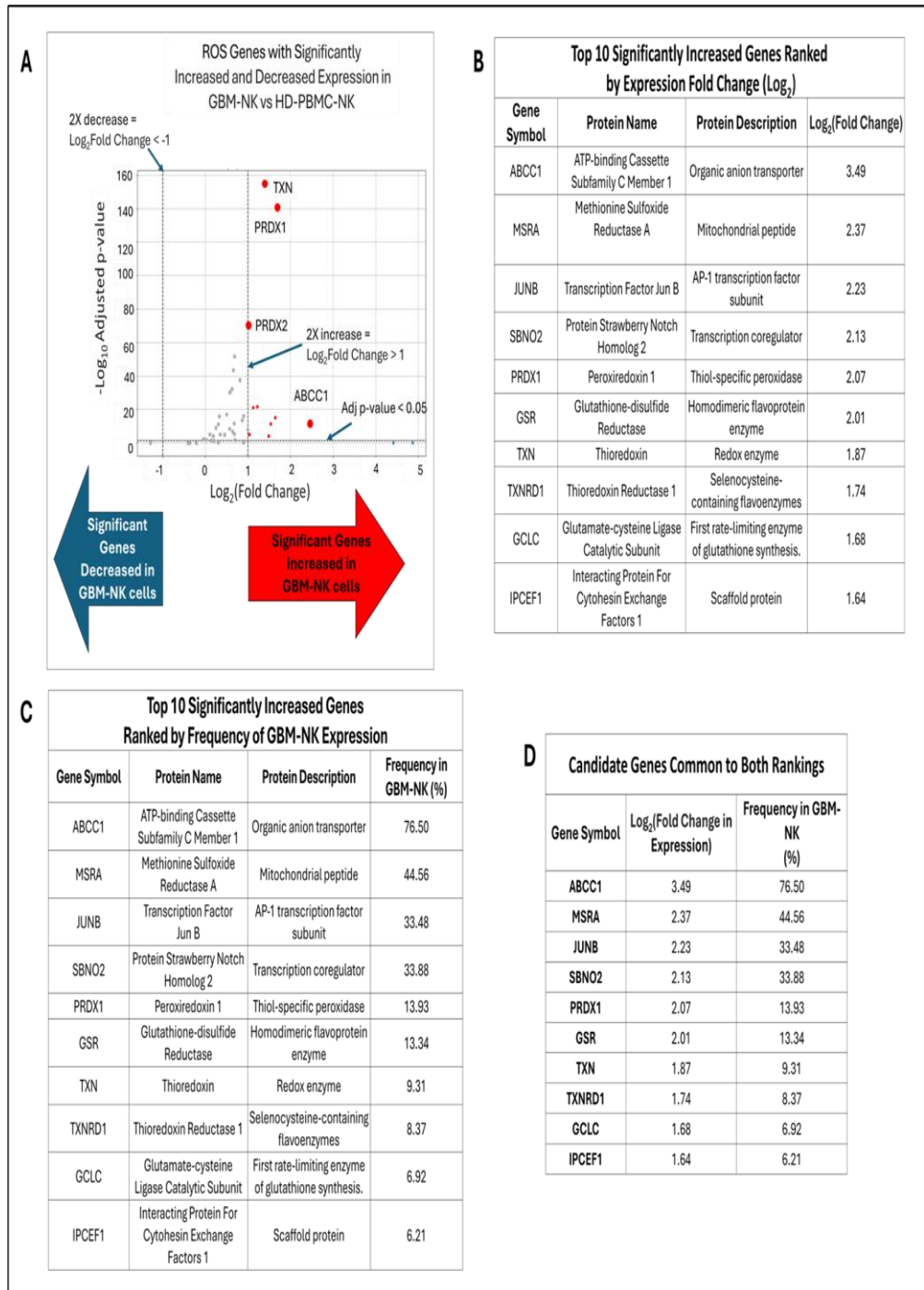


Figure 21: Ten significant genes from ROS gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual ROS gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-

value; $y=1.30$: adjusted p-value <0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. FTL is significantly decreased but not plotted as due to a near 0 p-value that exceeds the limits of the y-axis Total ROS gene set input was 44 genes.. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log_2 fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log_2 fold change in expression, with frequency in GBM-NK provided. The full list of all significantly increased genes for both ranking systems are provided in Supplementary Tables.

Understanding there was enrichment of stress related genes, we next analysed the Cytochrome C Release Induced Apoptosis gene set to identify markers/genes of interest that could be detail apoptotic pathways potentially enriched in GBM-NK. . As seen in **Figure 22A** the volcano plot illustrates several genes (red dots in **Figure 22A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log_2 fold change and the proteins they encode are tabulated in **Figure 22B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 22C**). The known functions of these top ‘marker’ genes provided more insight into the dynamics of this apoptotic signature in GBM-NK cells: several pro-apoptotic mediating genes are significantly induced – BCL2, BCL2L11, TNFSF10 (TRAIL); CASP3, an activated cell death protease; and conversely anti-apoptotic mediators are amongst these significantly increased genes – PRELID1, MCL1, GGCT (O'Brien & Kirby, 2008; Potting et al., 2013; Zhang et al., 2016). As can be seen in **Figure 22D** five genes are in common between the two rank parameters (ranked by Log_2 FC). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

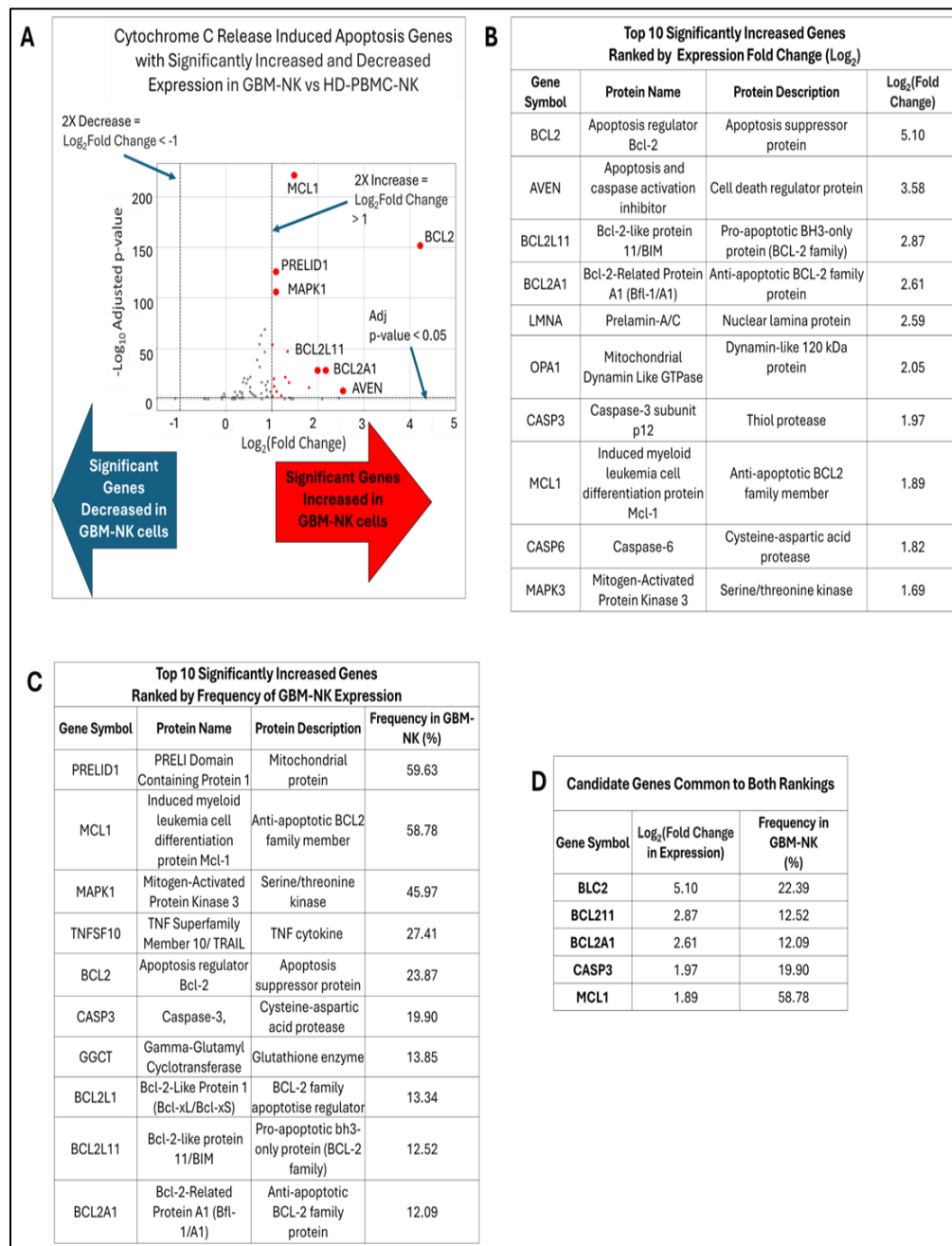


Figure 22: Five significant genes from Cytochrome C Release Induced Apoptosis gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Cytochrome C Release Induced Apoptosis gene. Grey Dots indicates non-significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries

marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. COX16 is significantly decreased but not plotted as due to a near 0 p-value that exceeds the limits of the y-axis Total Cytochrome C Release Induced Apoptosis gene set input was 69 genes. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided. The full list of all significantly increased genes for both ranking systems are provided in Supplementary Tables.

Following this, we next investigated how OXPHOS related genes were differentially expressed in GBM-NK cells. As seen in **Figure 23A** the volcano plot illustrates several genes (red dots in **Figure 23A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 23B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 23C**). The known functions of these top ‘marker’ genes provided more insight into the upregulation of OXPHOS machinery signature genes in GBM-NK cells: mitochondrial Complex I subunits – NDUF8, NDUF9, NDUF10; mitochondrial complex IV subunit – COX5A; inorganic ATP dependent phosphate producer – PPA1; V-ATPase subunit – TCIRG1 (ATP6V0A3) (Stelzer et al., 2016). Not all of these genes are directly linked to OXPHOS but may influence this metabolic potential. As can be seen in **Fig. 23D**, nine genes are in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

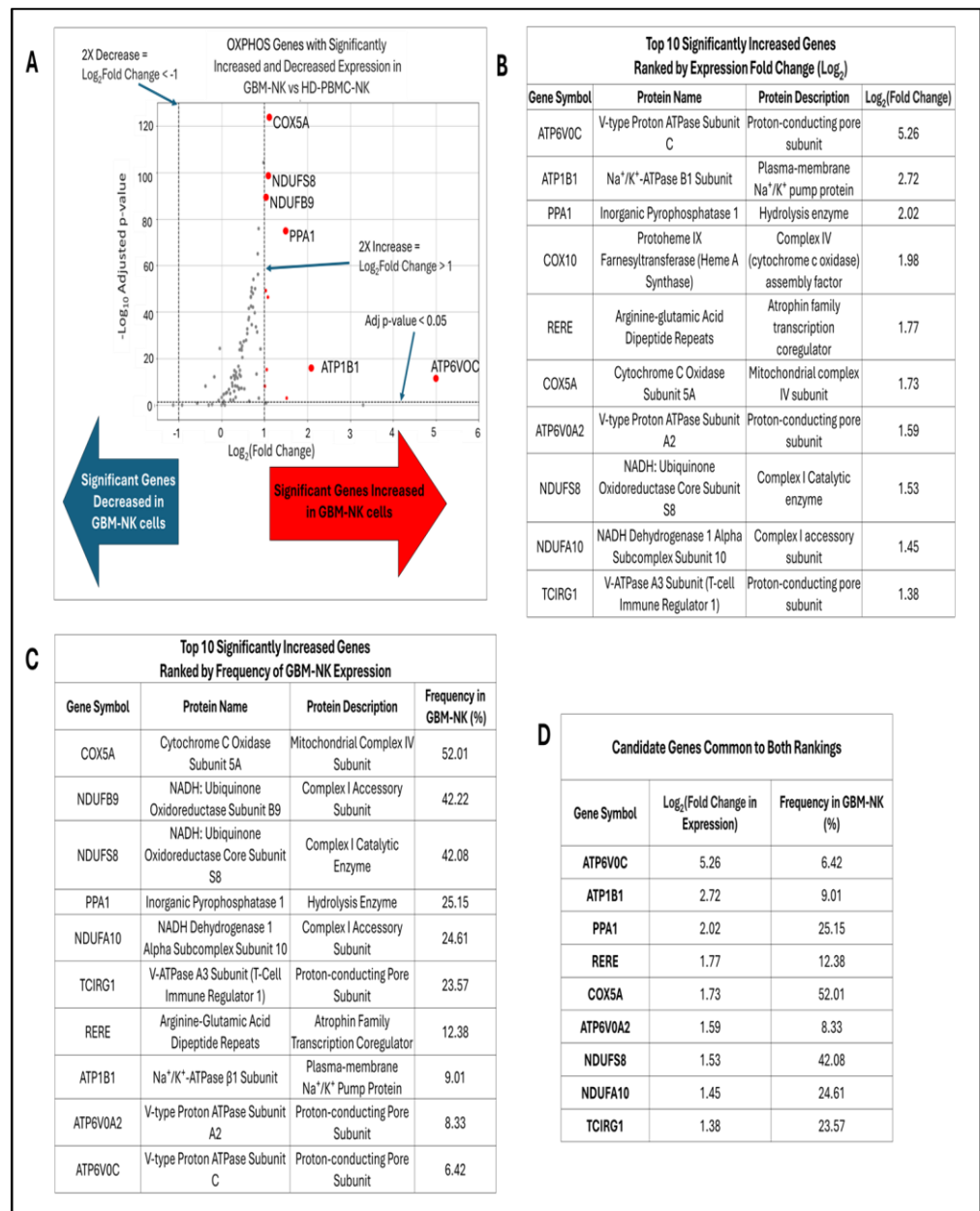


Figure 23: Ten significant genes from OXPHOS gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual OXPHOS gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest

statistical difference. Total OXPHOS gene set input was 93 genes. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided. The full list of all significantly increased genes for both ranking systems are provided in Supplementary Tables.

Following this, we next investigated how the rate limiting enzymes of OXPHOS genes were differentially expressed in GBM-NK cells. As seen in **Figure 24A** the volcano plot illustrates several genes (red dots in **Figure 24A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 24B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 24C**). All significantly increased genes encode complex IV proteins (Stelzer et al., 2016). As can be seen in Fig. **24D**, nine genes are in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

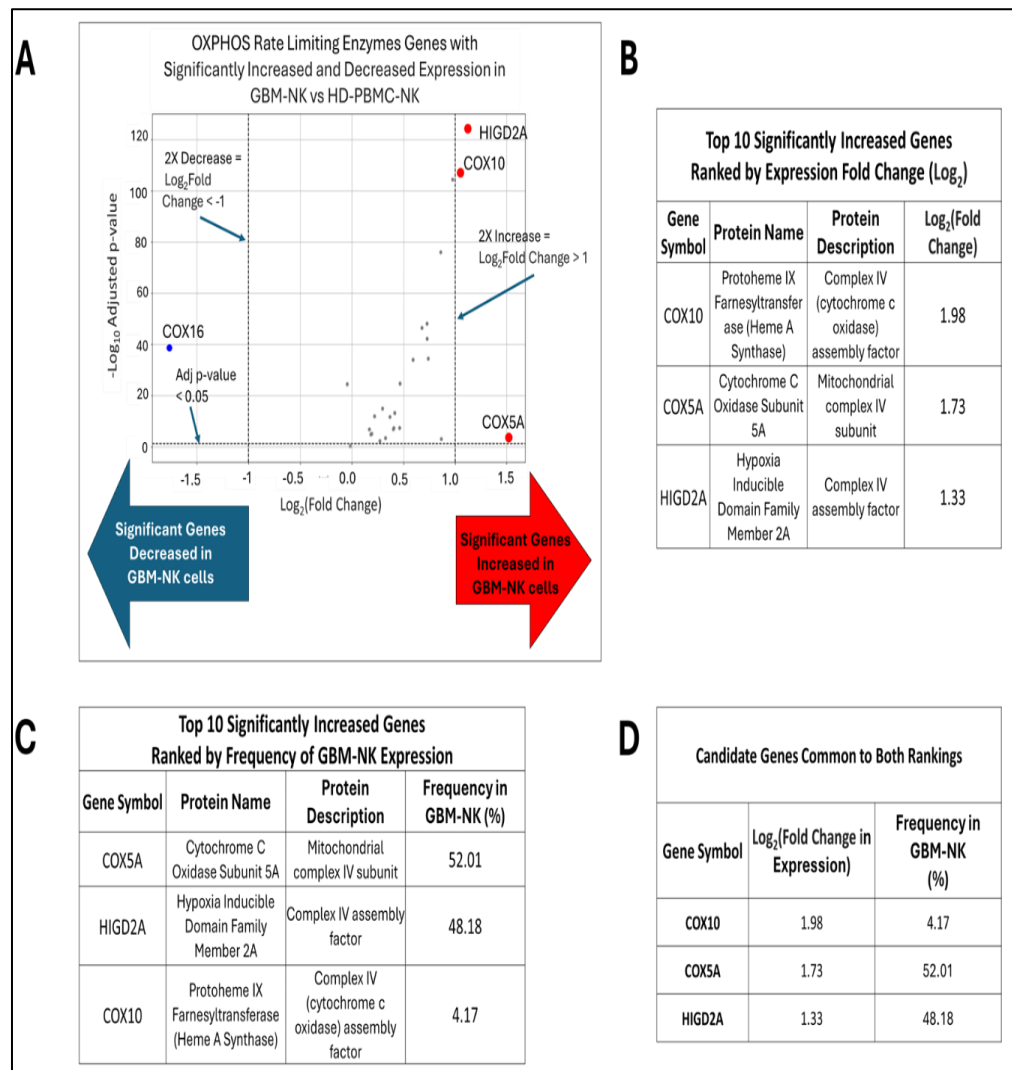


Figure 24: Three significant genes from OXPHOS Rate Limiting Enzymes gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a $\log_2$ fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a $\log_2$ fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual OXPHOS Rate Limiting Enzymes gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\log_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: $\log_2$ fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. Total OXPHOS Rate Limiting Enzymes gene set input was 27 genes. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in

GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided.

Following these findings of mitochondrial stress related genes and trend, we next investigated how mitochondrial complex I-IV genes were differentially expressed in GBM-NK cells. As seen in **Figure 25A** the volcano plot illustrates several genes (red dots in **Figure 25A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 25B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 25C**). Two of these genes encode Complex I genes (source of mitochondrial ROS) and COX5 encodes a subunit of complex IV (Balaban et al., 2005; Stelzer et al., 2016). However, COX16, an unit of cytochrome c assembly, was significantly decreased in GBM-NK cells (Cerqua et al., 2018). As can be seen in **Fig.25D**, three genes are in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

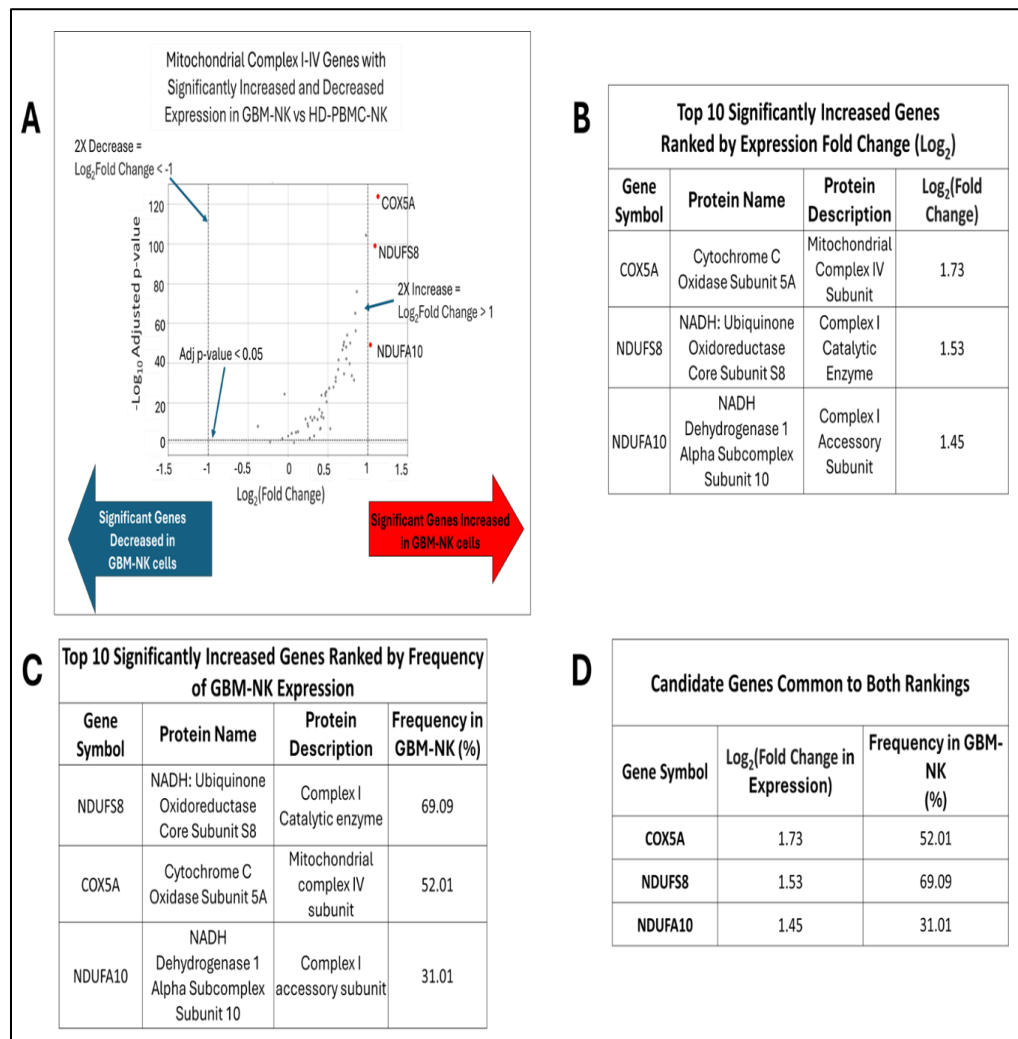


Figure 25: Three significant genes from Mitochondrial Complex I-IV gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Mitochondrial Complex I-IV gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. Total Mitochondrial Complex I-IV gene set input was 54 genes. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log_2 fold change in expression. **C)** List of Top 10 genes and their protein

names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided.

We next investigated mitochondrial morphology by examining Mitofusion differential gene expression in GBM-NK cells. As seen in **Figure 26A** the volcano plot illustrates several genes (red dots in **Figure 26A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 26B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 26C**). The known functions of these top 'marker' genes provided more insight into the Mitofusion signature in GBM-NK cells: MFN1 – known marker for fusion, OPA1 – promotes inner membrane fusion (Cipolat et al., 2004); inhibitors of mitochondrial fragmentation/fission – CHCHD3 (Deng et al., 2016); mitofusion regulators - MCL1, B2L2A1 (Morciano et al., 2016; Rolland & Conradt, 2010) and negative regulators of mitochondrial fusion – HUWE1, TFRC (Di Rita et al., 2018; Senyilmaz et al., 2015). As can be seen in Fig. **26D**, seven genes are in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

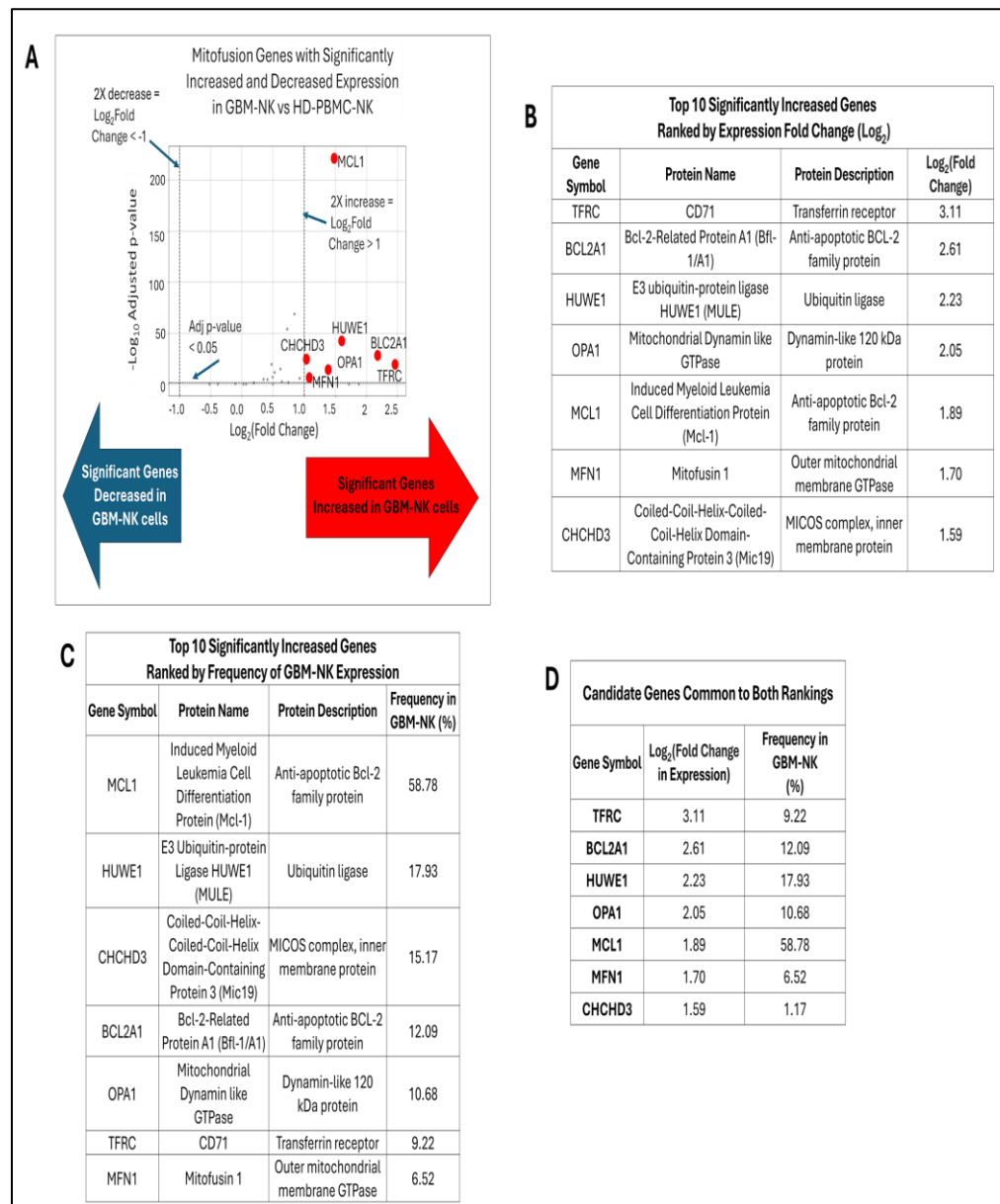


Figure 26: Seven significant genes from Mitofusion gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a $\log_2$ fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a $\log_2$ fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Mitofusion gene. Grey Dots indicates non-significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\log_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: $\log_2$ fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. Total Mitofusion gene set input was 29 genes. Significance test: Wilcoxon

rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided.

Following this, we next investigated how Mitofission genes were differentially expressed in GBM-NK cells. As seen in **Figure 27A** the volcano plot illustrates several genes (red dots in **Figure 27A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 27B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 27C**). The known functions of these top ‘marker’ genes provided some insight into the upregulation of Mitofission signature genes in GBM-NK cells: facilitating fission DDHD1, SPIRE1, AURKA (Bertolin et al., 2018; Deng et al., 2016; Wu & Fan, 2016); fission regulators - AP3B1, MTFR1 (Tilokani et al., 2022; Zhang et al., 2024); fusion promoting gene - OPA1 can be seen in Fig. **26D**, nine genes are in common between the two rank parameters (ranked by Log₂FC). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

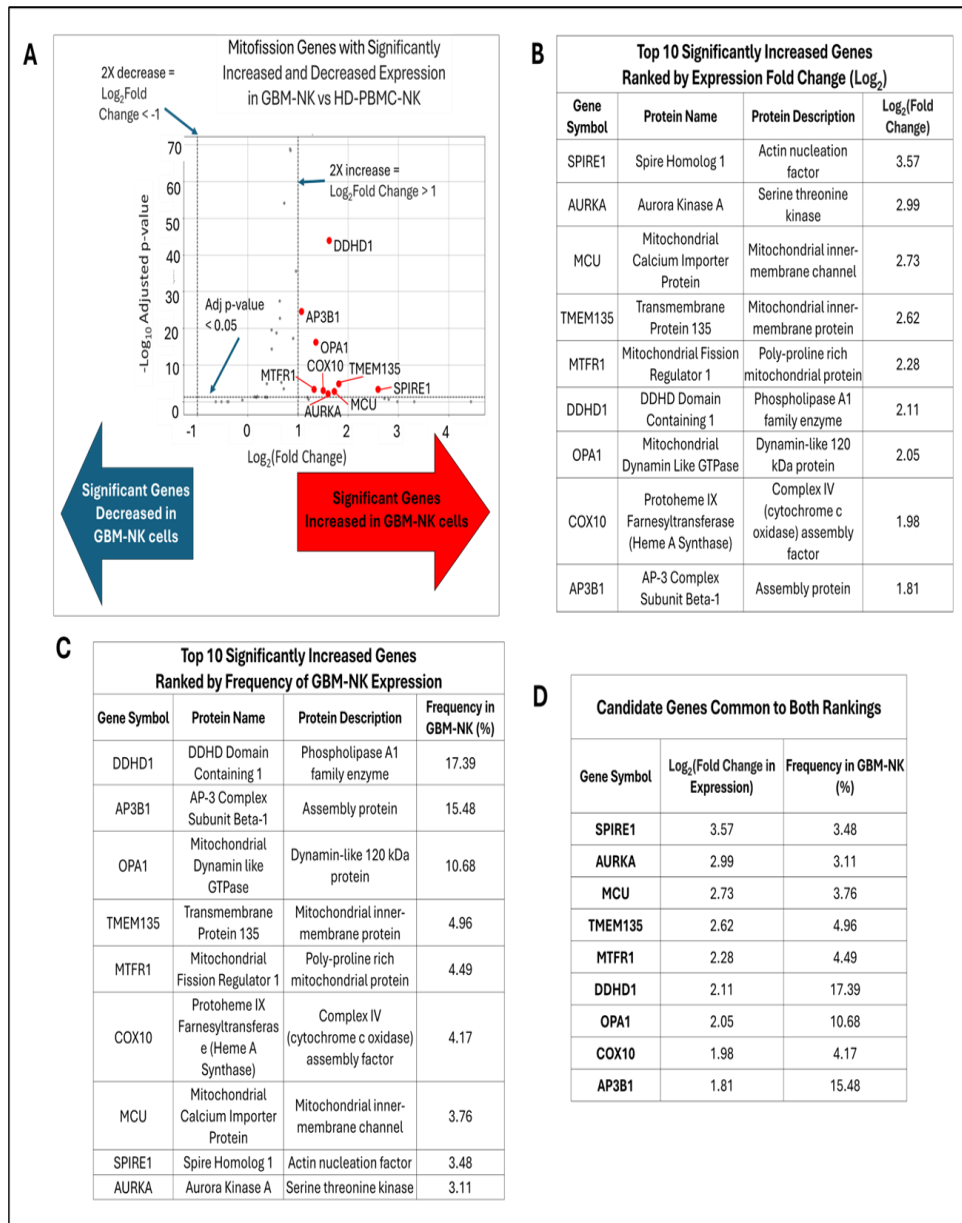


Figure 27: Nine significant genes from Mitofission gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Mitofission gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. Total Mitofission gene set input was 39 genes.. Significance test: Wilcoxon

rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided.

Following this, we next investigated how mitophagy related genes were differentially expressed in GBM-NK cells. As seen in **Figure 28A** the volcano plot illustrates several genes (red dots in **Figure 28A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 28B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 28C**). The known functions of these top ‘marker’ genes provided some insight into the upregulation of Mitophagy signature genes in GBM-NK cells: MAP1LC3A - mitophagosome formation (Bertolin et al., 2021); CSNK2A2 – essential for mitophagy initiation (Kanki et al., 2013); MFN1- degradation shown to induce mitophagy (Joaquim & Escobar-Henriques, 2020). As can be seen in **Fig. 28D**, three genes are in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM.

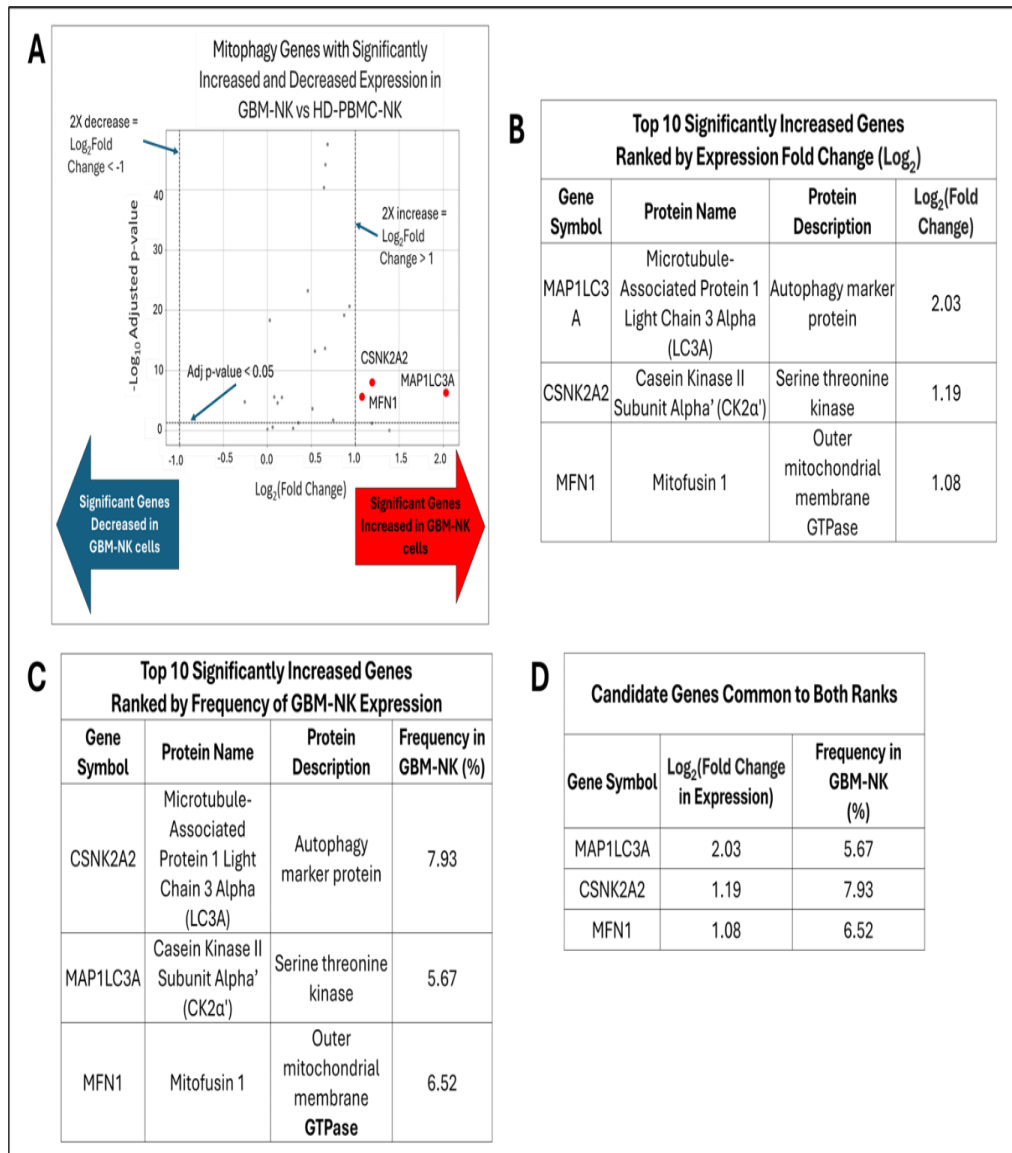


Figure 28: Three significant genes from Mitophagy gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Mitophagy gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. Total Mitophagy gene set input was 24 genes.. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False

Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log₂ fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log₂ fold change in expression, with frequency in GBM-NK provided.

Following this, we next investigated how Glycolysis and Gluconeogenesis genes were differentially expressed in GBM-NK cells – a metabolic pathway known to be necessary for functional NK cell cytotoxicity. As seen in **Figure 29A** the volcano plot illustrates several genes (red dots in **Figure 29A**) are significantly increased in GBM-NK cells. The top 10 significantly increased genes were ranked by the largest Log₂ fold change and the proteins they encode are tabulated in **Figure 29B**. We carried out a similar ranking for Frequency of expression in GBM-NK cells (**Figure 29C**). Some of the known functions of these top ‘marker’ genes provided more insight into the upregulation of glycolysis and gluconeogenesis machinery signature genes in GBM-NK cells: glycolysis/Pentose Phosphate Pathway enzyme – TALDO1 (Moriyama et al., 2016); Positive regulators of glycolysis and insulin receptor – IER3 (Yin et al., 2025), DDIT4 (Ho et al., 2020); negative regulators of/association with glycolysis –; inorganic ATP dependent phosphate producer – PPA1 (Wang et al., 2025); Glycolysis signature genes with good prognosis in cancer patients – STMN1, NASP, B4GALT1; Poor cancer prognosis glycolysis signature – POLR3K (Jiang et al., 2019). As can be seen in Fig. **29D**, only gene is in common between the two rank parameters (ranked by log₂fc). Given the highest gene expression increase in more GBM-NK cells, these are good gene candidates for further investigation for a role in GBM

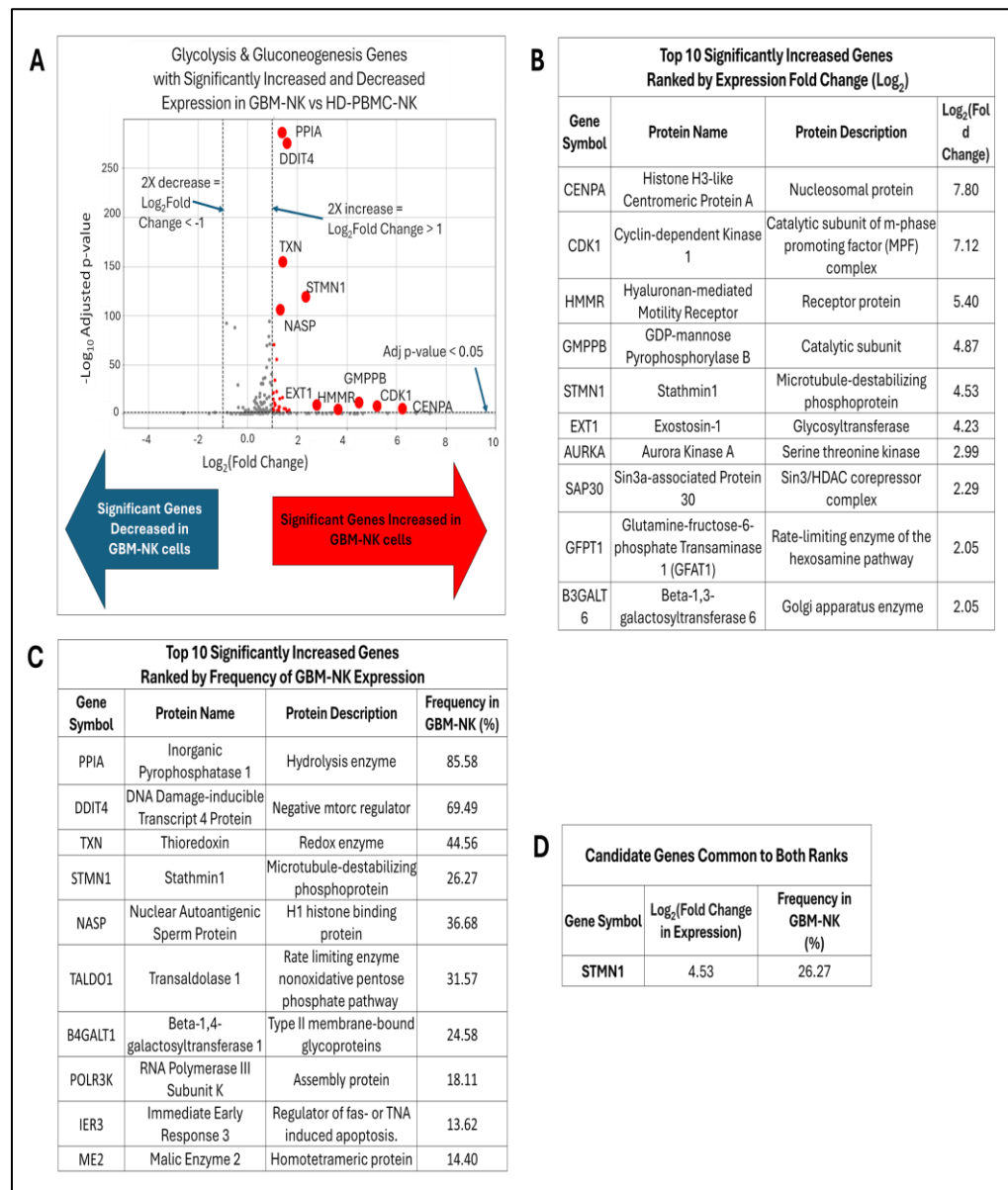


Figure 29: One significant gene from Glycolysis and Gluconeogenesis gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Glycolysis and Gluconeogenesis gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. Total Glycolysis and

Gluconeogenesis gene set input was 172 genes.. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log_2 fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log_2 fold change in expression, with frequency in GBM-NK provided.

Following this, we next investigated how the genes for the Rate Limiting Enzymes of Glycolysis were differentially expressed in GBM-NK cells. As seen in **Figure 30A** the volcano plot illustrates only one gene (red dots in **Figure 30A**) is significantly increased in GBM-NK cells. This gene, the protein it encodes, its Log_2FC in expression, and frequency in GBM-NK are tabulated in **Figure 30B**. SLC16A1, or MCT1, is a transporter for lactate and thus regulates glycolysis by influencing intracellular lactate flux (Juel & Halestrap, 1999; Rogatzki et al., 2015).

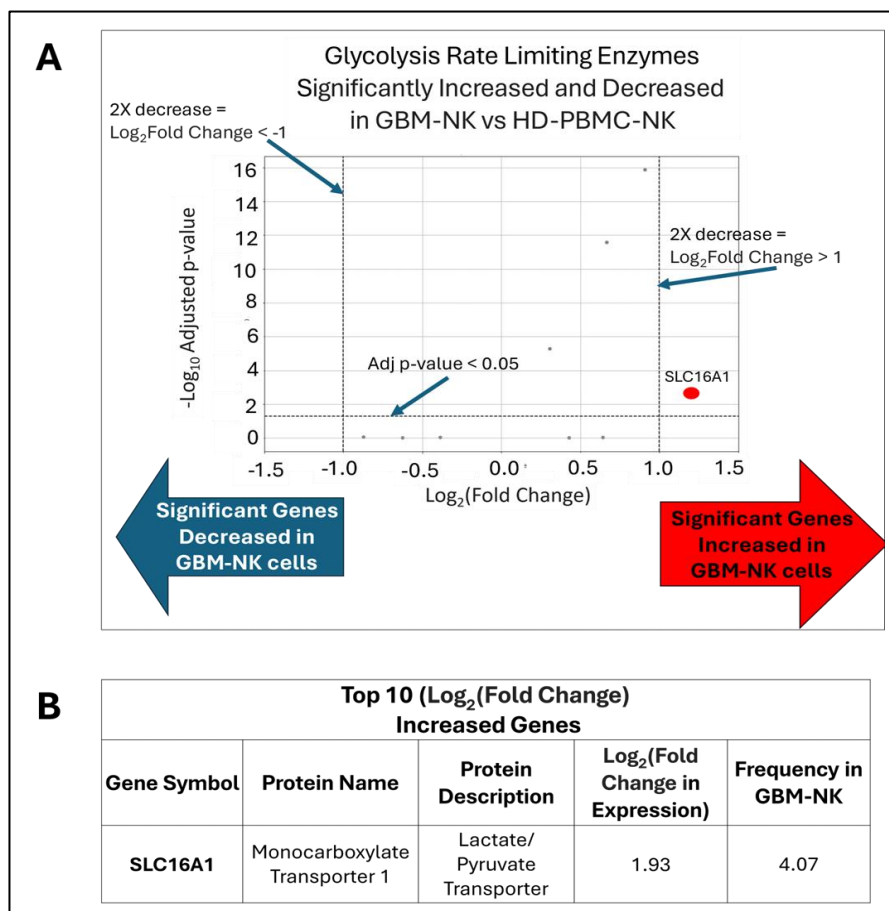


Figure 30: One significant gene from Glycolysis Rate Limiting Enzymes gene set have both a higher fold expression and higher frequency of expression in GBM-NK cells amongst

significant genes. A) Volcano Plot of statistically significant genes increased and decreased in GBM-NK cells relative to HD-PBMC-NK cells. Significantly increased genes are defined as having a Log_2 fold change increase $> +1$ with an adjusted p-value < 0.05 ; while significantly decreased genes are defined as having a Log_2 fold change increase < -1 with an adjusted p-value < 0.05 . Blue and red arrows highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual Glycolysis Rate Limiting Enzymes gene. Grey Dots indicates none significant genes, blue dots significantly decreased in GBM-NK, red dots significantly increased in GBM-NK. Y-axis: $-\text{Log}_{10}$ adjusted p-value; $y=1.30$: adjusted p-value < 0.05 – marked by dashed horizontal line. X-axis: Log_2 fold change difference in gene expression between GBM-NK and HD-PBMC-NK: > 1 & < -1 increase and decrease boundaries marked by dashed lines. Some significant genes are labelled in the interest of highest fold change increase/decrease and genes with greatest statistical difference. Total Glycolysis Rate Limiting Enzymes gene set input was 9 genes.. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. **B)** List of Top 10 genes and their protein names/descriptions, that are significantly increased in GBM-NK cells by order of Log_2 fold change in expression. **C)** List of Top 10 genes and their protein names/descriptions that are significantly increased in GBM-NK cells by order of Frequency of expression in GBM-NK. **D)** Genes common to both rankings are listed in order of Log_2 fold change in expression, with frequency in GBM-NK provided. The full list of all significantly increased genes for both ranking systems are provided in Supplementary Figures.

3.3 TGF- β Gene set Expression: Impact on NK Cell Function and Metabolism?

To deduce the potential biological impact of these 37 identified significantly increased TGF- β genes, on GBM-NK cells, we endeavoured to ‘isolate’ and cross-examine this expression signature in GBM-NK with functional transcription readouts for pathways relevant to NK function, metabolism, physiology and environment stimuli in the TME. The procedure for this analysis is outlined in **Figure 31** (including how GBM-NK cells were identified as High or Low TGF- β scoring relative to HD-PBMC-NK cells), with resultant volcano plots generated - to illustrate the differential gene expression of candidate functional gene sets in High vs Low TGF- β GBM-NK cells - being presented in **Figure 32**,.. . Low TGF- β GBM-NK were chosen as a reference for this conditional differential gene analysis as HD-PBMC-NK may present population differences to GBM-NK irrespective of TGF- β signature. Therefore, this analysis hoped to uncover if

these significant TGF- β genes were drivers of TGF- β induced dysfunction in GBM-NK population.

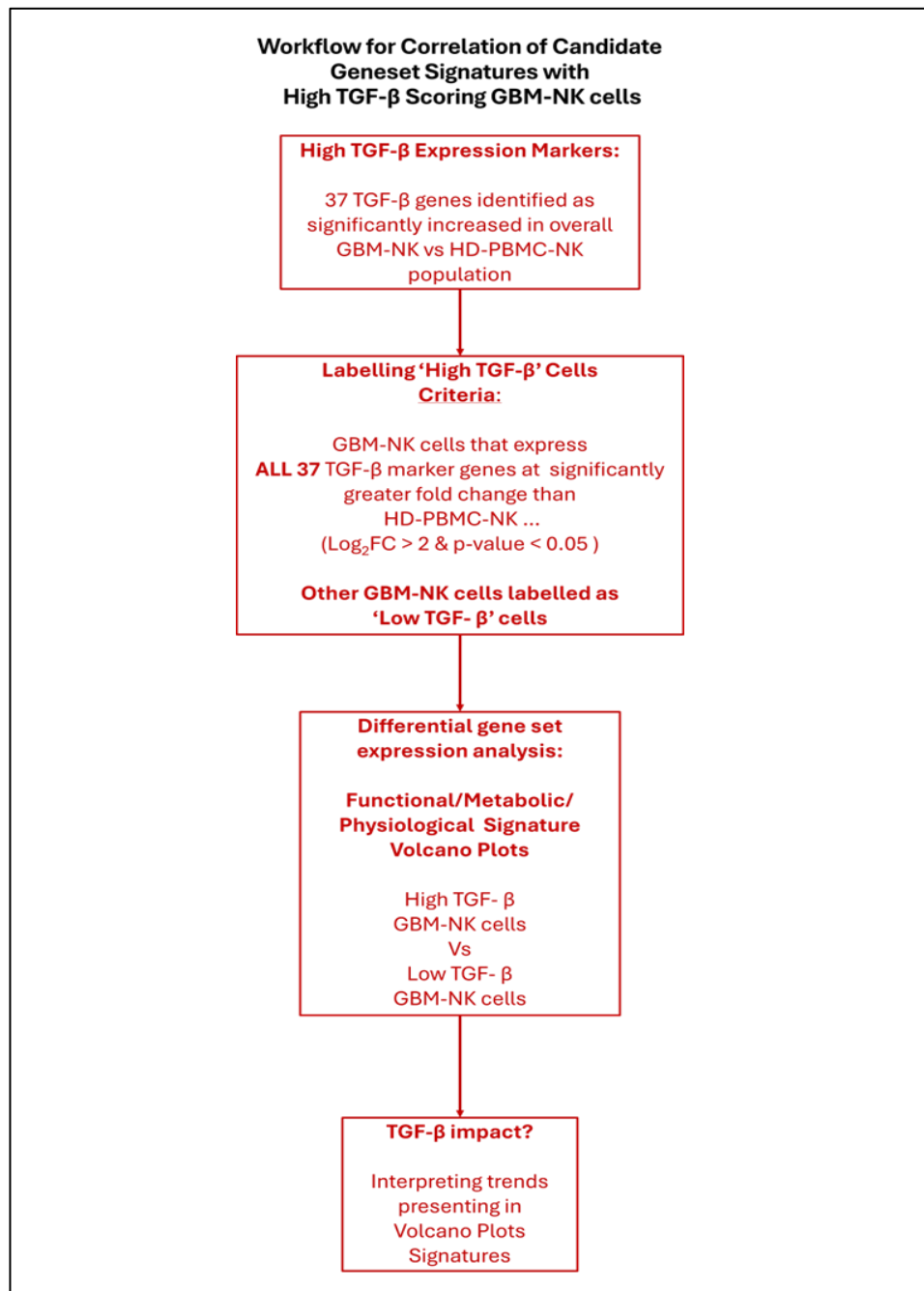


Figure 31: Flow chart for signature analyses of High scoring TGF- β GBM-NK cells. The TGF- β marker genes chosen were the 37 significantly increased genes identified earlier (**Fig. 19**). A High TGF- β scoring GBM-NK cell expressed all marker genes at significantly greater level than HD-PBMC-NK cells: Log₂ fold change increase in expression > +1 with an adjusted p-value < 0.05 – obtained from Wilcoxon rank-sum test (Mann–Whitney U test) and p-values adjusted using Benjamini–Hochberg False Discovery Rate procedure. While Low TGF- β scoring GBM-NK cell expressed none of the marker genes at levels significantly greater than HD-PBMC-NK cells. Signature gene sets were chosen from some of the studied candidate gene sets and included two additional pathway gene sets: Interferon gamma production gene set signature and NK cell

mediated cytotoxicity, sourced from KEGG and GOBP gene set databases respectively. These gene set were also edited to include additional genes of interest to the lab. Volcano Plots were generated to assess if High TGF- β scoring GBM-NK cells exhibited a significant increase of signature genes relative to Low TGF- β scoring GBM-NK cells.

It is established in the literature that TGF- β inhibits IFN- γ and NK cell cytotoxicity (Crane et al., 2009; Rea et al., 2025; Slattery et al., 2021) which was supported by initial exploratory ORA results : both pathways were shown to be negatively enriched in GBM-NK population (**Fig. 11**) Therefore, if these 37 significant TGF- β were drivers of TGF- β signalling induced dysfunction, we predicted that a GBM-NK cells expressing a High TGF- β score would showcase a reduction in interferon gamma or cell cytotoxic machinery gene set signatures compared to Low TGF- β GBM-NK cells. However, these pathways were not downregulated as expected; as there were no significant differences between High and Low TGF- β GBM-NK cells for either gene set expression (**Fig. 32A,B**). In fact, unexpectedly the direction of differential expression for IFN- γ signature was towards increased expression in 'High TGF- β ' scoring GBM-NK cells – albeit perhaps a non-significant trend **Fig.32A**. Furthermore, metabolic signature expressions – previously observed to be upregulated GBM-NK vs HD-PBMC-NK in previous general candidate gene set analysis (**Fig. 23,24,29**) – were expected to be downregulated in High TGF- β GBM-NK cells compared to Low TGF- β GBM-NK cells (Slattery et al., 2021; Viel et al., 2016). Yet, there were no significant differences between High and Low TGF- β GBM-NK cell expression for either gene expression (**Fig. 32C,D**). Although unexpectedly the direction of differential expression for glycolysis and gluconeogenesis signature was shifted towards increased expression in 'High TGF- β ' scoring GBM-NK cells – albeit perhaps a non-significant trend (**Fig.32D**). Again, High TGF- β signature did not seem have an independent effect for mitochondrial mass (Mitochondrial Complexes I-IV) or cellular response to ROS (**Fig. 32F,G**). However, High TGF- β GBM-NK cells presented a trending increase in expression for the Oxidative Stress signature with a significantly expression for AREG (**Fig. 32E**).

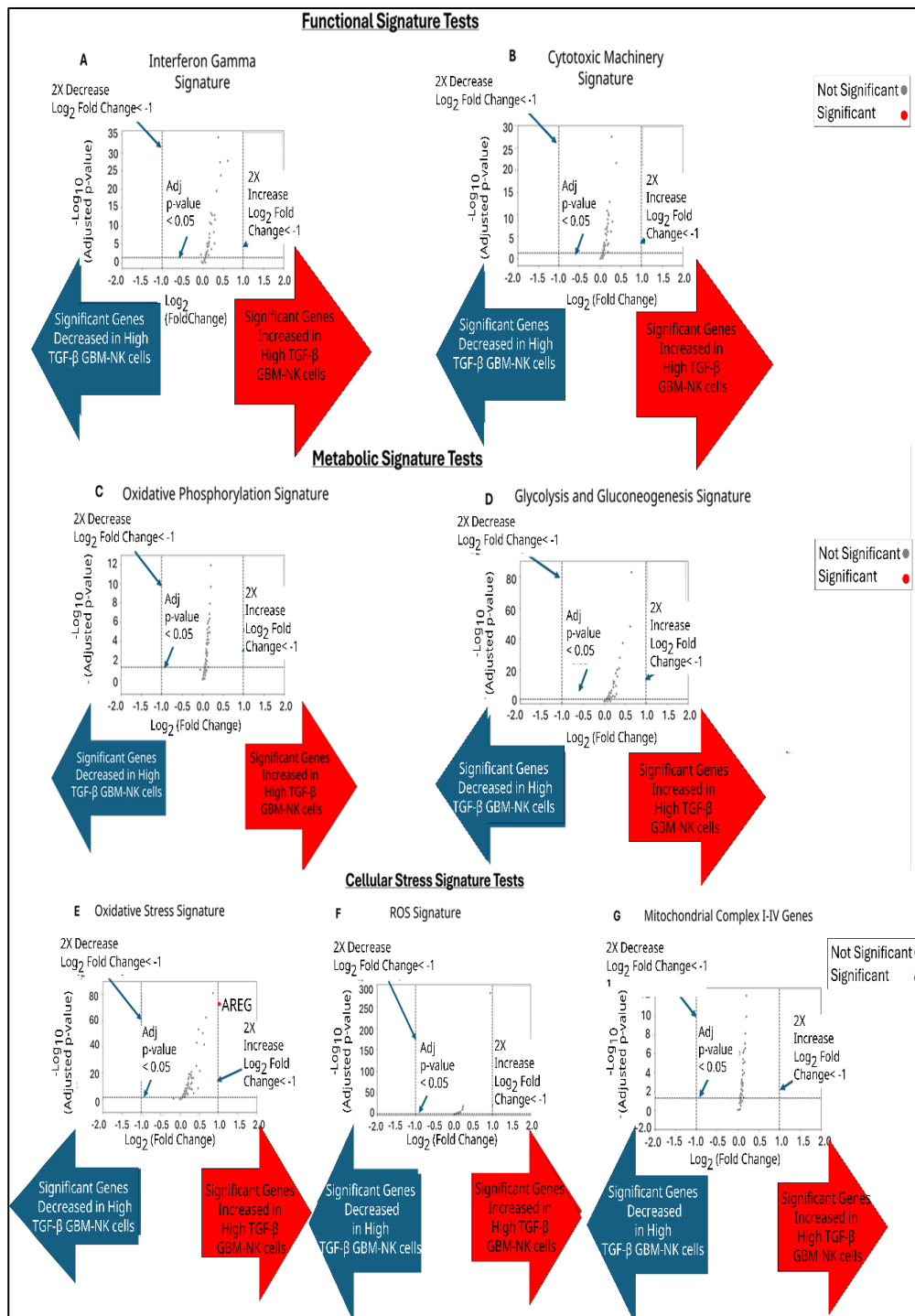


Figure 32: High TGF-β scoring GBM-NK cells only coincided with showed a trend of increased Oxidative Stress gene set coexpression compared to Low scoring TGF-β GBM-NK cells. Signatures tests were arranged into Functional Signatures (top: A, B), Metabolic signatures (middle: C, D), and cellular stress signatures (bottom: E-G). Volcano Plot illustrate statistically significant genes increased and decreased in High scoring **TGF-β** GBM-NK cells relative to scoring **TGF-β** GBM-NK. Significantly increased genes were defined as having a Log₂ fold change increase > +1 with an adjusted p-value < 0.05; while significantly decreased genes are defined as having a Log₂ fold change increase < -1 with an adjusted p-value < 0.05. Blue and red arrows

highlight this direction of significantly increased and decreased expression in GBM-NK. Each dot represents an individual gene. Grey Dots indicates non significant genes, red dots significantly increased in GBM-NK. Y-axis: $-\log_{10}$ adjusted p-value; $y=1.30$: adjusted p-value <0.05 – marked by dashed horizontal line. X-axis: $\log_2$ fold change difference in gene expression between High **TGF- β** GBM-NK and Low **TGF- β** GBM-NK (> 1 & < -1) increase and decrease boundaries marked by dashed lines. Significant genes are labelled. High TGF- β scoring GBM-NK cell expressed all 37 identified **TGF- β** genes significantly increased in GBM-NK vs HD-PBMC-NK cells: $\log_2$ fold change increase in expression $> +1$ with an adjusted p-value < 0.05 . While Low TGF- β scoring GBM-NK cell expressed none of the marker genes at levels significantly greater than HD-PBMC-NK cells. Total gene set inputs: Interferon Gamma Production – 98/117 genes available in gene set, Cytotoxic Machinery in NK Cells – 60 genes; Oxidative Phosphorylation (OXPHOS) – 93 genes; Glycolysis and Gluconeogenesis – 9 genes; Oxidative Stress – 289 genes; ROS – 44 genes; Mitochondrial Complexes I-IV – 54 genes. Significance test: Wilcoxon rank-sum test (Mann–Whitney U test). P-values adjusted using Benjamini–Hochberg False Discovery Rate procedure.

Following this targeted analysis of High TGF- β signature in GBM-NK, we engaged with exploratory analysis to characterise this High TGF- β signature expression in GBM-NK. Differential gene analysis between High vs Low TGF- β GBM-NK cells was undertaken for subsequent GSEA – procedure outlined in **Figure 33A**. It was suspected from functional signature differential gene analysis (**Fig. 32**) that High and Low GBM-NK cells may present largely overlapping transcription patterns and this was reflected by parameters chosen for significant enrichment. Resultantly only one positively enriched and one negatively enriched gene set were observed in High TGF- β GBM-NK cells (**Fig. 33B,C**). Complement and Coagulation Cascade gene set was positively enriched (even fulfilling stricter significance criteria of FRD q-value <0.05) in GBM-NK cells (**Fig. 33B**). The leading-edge analysis uncovered the 16% of gene set drives this enrichment and these genes were examined to provide further context to this enrichment. The known functions of these leading-edge gene provide additional context to understanding enrichment of these pathways in GBM-NK cells: pro-coagulant genes – F5, F10, KLKB1; coagulation regulator genes – SERPIND1, THBD, SERPINF2, CPB; complement immune response effectors genes – C7, C9; complement receptor gene – CR1 (Stelzer et al., 2016;) This gene set and these genes are not typically expressed in NK cells (Uhlén et al., 2015). Additionally, Arachidonate acid metabolism was negatively enriched in

GBM-NK cells. 14% of Arachidonate acid Metabolism gene set drives this negative enrichment.

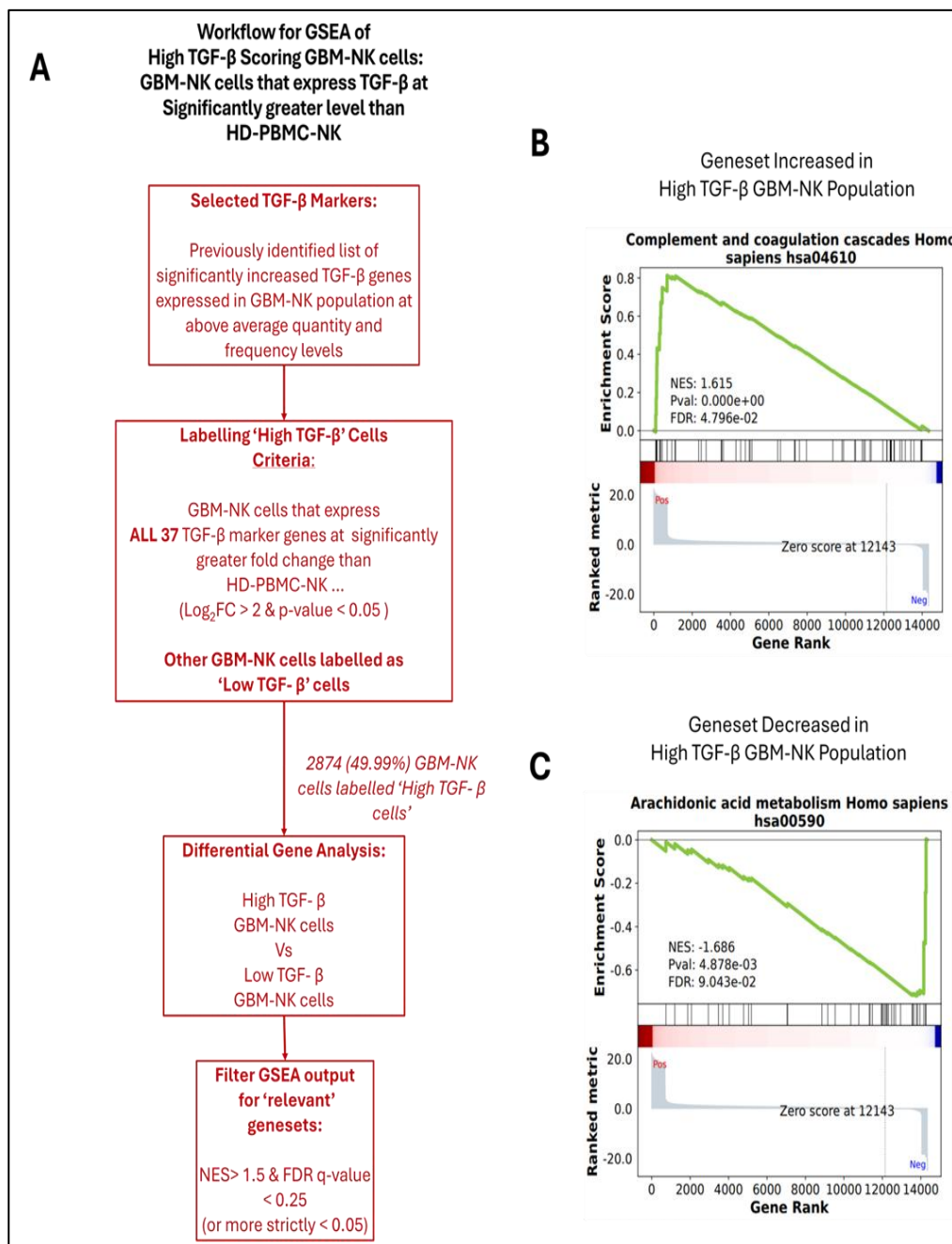


Figure 33: GSEA of GBM-NK cells with highly significantly TGF- β marker gene expression revealed an enrichment of Complement and Coagulation Cascades Gene set Response and a significant decrease in Arachidonate Metabolism. A) Flow chart of GSEA analysis. The TGF- β marker genes chosen were the 37 significantly increased identified earlier in **Figure 21**. A High TGF- β scoring GBM-NK cell expressed all marker genes at significantly greater level than HD-PBMC-NK cells: Log_2 fold change increase in expression $> +1$ with an adjusted p -value < 0.05 – obtained from Wilcoxon rank-sum test (Mann–Whitney U test) and p -values adjusted using Benjamini–Hochberg False Discovery Rate procedure. While Low TGF- β scoring GBM-NK cell expressed none of the marker genes at levels significantly greater than HD-PBMC-NK cells.

Differential Gene Analysis was done on High (2874) vs Low (2875) TGF- β GBM-NK cells. GSEA gene set output results were filtered for significant criteria: NES>1.5 & FRD q-value<0.25 for increased gene sets; NES< -1.5 & FRD q-value<0.25 for decreased gene sets – positively enriched gene set in **B)** fulfils stricter significance criteria of FRD q-value <0.05. **B)** GSEA enrichment plot for the only gene set positively enriched in GBM-NK cells. Peak of green line marks enrichment score for gene set. NES, p-value and FDR q-value noted on graph. Grey bars/lines below plot denote where gene set genes are ranked in genome (Gene rank ranges from highest ranked at 0 to lowest rank at 14337 – total available dataset genes) and align with green enrichment score line. Reading from rank 0 to green line peak, these bar lines represent leading-edge genes that are driving enrichment: 16% of Complement and Coagulation Cascade gene set (64 genes available in gene set). Genes were ordered by ranked metric of Log₂ Fold Change from ~+20 to ~-20. **C)** GSEA enrichment plot for the only gene set negatively enriched in GBM-NK cells. Trough of green line marks enrichment score for gene set. NES, p-value and FDR q-value noted on graph. Grey bars/lines below plot denote where gene set genes are ranked in genome (Gene rank ranges from highest ranked at 0 to lowest rank at 14337 – total available dataset genes) and align with green enrichment score line. Reading from zero score line to green line trough, these bar lines represent leading-edge genes that are driving negative enrichment: 14% of Arachidonate Acid Metabolism gene set (42 genes available in gene set). For GSEA, genes were ordered by ranked metric of Log₂ Fold Change increase from ~+20 to ~-20. NES – normalised enrichment score, FDR q – value – False Discover rate p-value. KEGG 2016 library used for GSEA analysis. See supplemental tables for further statistics

4. Discussion

This work used bioinformatic analysis of a publicly available RNA-Seq data to interrogate changes in NK cells recruited to GBM tumours. There are many caveats associated with RNA-Seq and associated analyses and it is important to understand the limitations of the approaches in order to ensure that data is correctly interpreted (Deshpande et al., 2023; Imbach et al., 2023; Squair et al., 2021).

For this work, limitations include:

1. Gene set preparation for candidate analysis may have resulted in a pathway analysis blurring effect: multiple aspects of a pathway are captured in candidate gene set expression (Maleki et al., 2020). For example, TGF- β candidate gene set included genes that encode proteins involved in signalling process itself but also genes that were transcribed in response to TGF- β signalling – including positive and negative regulators of signalling pathway.
2. Given that different sources were used to compile ‘genesets’, there was some variation in genes included in overlapping biological processes e.g. some mitochondrial complex genes were significantly increased in OXPHOS gene set analysis but were not assessed amongst the mitochondrial complexes I-IV gene set itself.
3. Many genes were missing from the dataset, most noticeably this included the absence of the mitochondrial V complex. However, this can be a common issue for transcriptomic analysis as (Yang et al., 2018), have shown that the missing rate can reach approximately 30% even after noise reduction. Furthermore, scRNA-seq technique itself is limited and only captures a reduced percentage of the total genome per cell. For example, 10X Genomics V2 was used by (Netskar et al., 2024), and this only captures 14-15% genome according to the manufacturer (Zheng et al., 2017).
4. Enrichr was used for our defined candidate genesets; given limitation 1 above and that ORA method involved the separate analysis of increased

and decreased statistically significant genes, some pathways appeared to be enriched in opposite directions.

5. Transcriptomic data does not always translate to real proteomic change. RNA-seq does not account for post transcriptional and post translational modifications etc. (Bauernfeind & Babbitt, 2017; Li et al., 2020)
6. Dataset did not account for variation in gene weighting. High-expression genes dominate the differential expression analysis; however, this may filter out other biological relevant changes in gene expression (Rentzsch et al., 2025; Squair et al., 2021).
7. Single cell RNA-seq captures a temporal snapshot of gene set expression (Ding et al., 2022) and thus it is difficult to distinguish transient from chronic alterations in gene expression: (Kirschenbaum et al., 2024) found differential gene expression signatures in GBM-NK cells over time – characterising late phase TGF- β imprinted signatures.
8. Patient and disease variability are factors which are hard to control. Source data came from multiple patient cohorts and the disease itself is highly variable. e.g. we also do not have clear information as to when in the disease course samples were taken, molecular subtypes of tumour, part of tumour sampled etc. (Ku et al., 2021; Swift et al., 2020)
9. scRNA-seq confounder for solid tissues (Marsh et al., 2025) e.g. dissociation/stress artefacts from tissue preparation can influence results: warm enzymatic dissociation can induce IEG/heat-shock programs (FOS/JUN/HSPA1A etc.)
10. Additional technical factors such as library size/UMI depth differences and some batch effects that survive dataset integration may have also been confounding factors influencing the magnitude of differential expression between GBM-NK and HD-PBMC-NK populations.
11. There was no peripheral blood NK cell data from patients available in the dataset used. This would have been very useful to help disentangle the impact of the TME directly on NK cells compared to peripheral impacts

This study demonstrates that GBM-NK have expression patterns distinct from HD-PBMC-NK cells and undergo significant transcriptional reprogramming in TME. In a similar fashion to (Shaim et al., 2021), we employed UMAPs to highlight a global difference in transcription patterns of GBM-NK relative to HD-PBMC-NK cells. Furthermore, we observed a higher expression intensity and frequency of expression in GBM-NK on average across the transcriptome (weighted averages) and DEG analysis additionally revealed approximately seven thousand genes had statistically significant increased expression in GBM-NK relative to HD-PBMC-NK cells. This is a considerable difference which implies GBM-NK have elevated general transcriptomic activity compared to HD-PBMC-NK cells. While tumour cells (including Glioblastoma) display hypertranscription compared to healthy tissues (Li et al., 2017; Zatzman et al., 2022), there isn't a direct comparison for this tumour infiltrating immune cells. However, immune cells in tumour tissues of various cancers are shown to be transcriptionally distinct to healthy counterparts and display starkly elevated expression of dysfunctional signatures and downregulated expression of functional signatures compared to healthy counterparts. (Chen et al., 2024; Chung et al., 2017; Dean et al., 2024; Monaco et al., 2022; Tang et al., 2023). Furthermore, GBM-NK cells and NK cells from matched peripheral blood patient samples (GBM-PBMC-NK) also show divergent transcriptional signatures (Schmassmann et al., 2023). However, it might be expected that NK cells in TME have elevated transcript expression compared to healthy unstimulated NK cells given the milieu of pro-inflammatory and anti-inflammatory signals in GBM (Ghosh et al., 2022; Sedgwick et al., 2020). Indeed, in the original paper, (Netskar et al., 2024) reported there are greater proportions of activated and stressed NK cells within tumours compared to normal NK cells. Therefore, our analysis supports the conclusion that GBM-NK cells are transcriptionally distinct to HD-PBMC-NK cells and display elevated transcriptional activity. Although, as outlined in limitation 10, this observation of hypertranscription in GBM-NK cells may be influenced by further technical factors that are beyond the remit of this thesis' investigations. Additionally, our global analysis as captured by the Leiden clustering UMAP – highlighted GBM-NK cells possess high degrees of heterogeneity. This heterogeneity could be resultant of the confounding

tumour/patient factors outlined in limitation 8. Nevertheless, it was evident that GBM-NK cell expression was distinct from HD-PBMC-NK cells.

This thesis supports literature findings that CD56^{bright} NK cells predominate tumour infiltrating NK cell populations in glioblastoma (Friebel et al., 2020). The greater quantity of CD56^{bright} to CD56^{dim} NK cells in GBM-NK population observed in this dataset is supported by studies profiling immune cell compositions from glioblastoma tumours patients samples (Kmieciak et al., 2014). Furthermore, although the top 20 DEGs generated from PCA plots were not examined in close detail (focusing instead on broader results of Enrichr ORA Results), it was noticed that RGS1, identified by (Tang et al., 2023) as a marker for tumour infiltrating NK cells, was amongst the top 20 DEGs increased in CD56^{bright} GBM-NK cells vs CD56^{bright} HD-PBMC-NK cells. However, this CD56^{bright} majority may have skewed expression analysis of total GBM-NK population. This is evident when observing there was increased GBM-NK gene set expression for Mitochondrial Complex I-IV gene set but a decreased expression in CD56^{dim} GBM-NK cells. Similarly, the Rate Limiting Enzymes of Glycolysis gene set had similar expression averages in CD56^{dim} NK cells from GBM-NK and HD-PBMC-NK populations. Although, this skew effect was not observed for other candidate gene set analysis, ORA results were likely predominated by CD56^{bright} signals. Thus, we could still detect downregulation of typical CD56^{dim} dominant pathways in GBM-NK cells, such as NK cell cytotoxicity, NK cell activation, and CD16 receptor gene set – similar to transcriptional findings of (Shaim et al., 2021). To understand what pathways and genes have altered expression in CD56^{bright} or CD56^{dim} GBM-NK cell subsets compared to respective healthy blood counterparts, GSEA or ORA of like-for-like subset NK population comparisons in GBM-NK vs HD-PBMC-NK would be required. Nevertheless, CD56^{bright} NK cells appear to be differentially upregulated in the TME compared to CD56^{dim} NK cells. ORA results for GBM-NK vs HD-PBMC-NK cells revealed a downregulation of Fibroblast Growth Factor (FGF) receptor binding and signalling gene set. This is interesting because FGF ligand engagement of this receptor in NK cells is reported to trigger the differentiation of CD56^{bright} to CD56^{dim} NK cells (Chan et al., 2007). Additionally, this thesis provides some

evidence that CD56^{dim} cells could simply lose their phenotype and resemble CD56^{bright} NK cells (Romee et al., 2013): (Stavarakaki et al., 2024) observed reductions in CD16 expression of peripheral NK cells cocultured with patient derived glioblastoma neurospheres. ORA demonstrated a significant enrichment of IL-2 signalling in GBM-NK cells, which has been shown to expand NK cells upon chronic stimulation, and downregulate CD16 protein presentation (Kozłowska et al., 2016). However, whether CD56^{dim} NK cells development is hindered or CD56^{dim} NK cell related function transcription is inhibited in tumour - perhaps becoming more phenotypically similar CD56^{bright} NK cells - is difficult to say.

In addition to supporting evidence for the increased proportion of CD56^{bright} NK in glioblastoma tumours (Friebel et al., 2020), our data showcases elevated transcriptional activity in CD56^{bright} GBM-NK. For every candidate gene set studied, expression and frequency averages were greatest in CD56^{bright} GBM-NK cells. Additionally, for every gene set, CD56^{bright} GBM-NK cells exhibited a greater fold increase in gene set expression or frequency relative to CD56^{dim} GBM-NK cells, than gene set expression or frequency fold change differences between CD56^{bright} and CD56^{dim} HD-PBMC-NK cells. Furthermore, UMAP illustrated global transcriptome pattern of CD56^{bright} GBM-NK cells were distinct from CD56^{dim} GBM-NK cells, while there was more overlap in transcription patterns between subsets of HD-PBMC-NK cells. Furthermore, although chemokine signalling – key facet of CD56^{bright} NK cells function (Bernardini et al., 2012; Poli et al., 2009) – was not studied in detail, ORA revealed a negative enrichment for the regulation of NK cell chemotaxis: supporting deregulated NK cell recruitment to infiltrate tumour and further suggesting CD56^{bright} NK abnormal expression patterns amongst GBM-NK.

This elevated and perhaps dysfunctional CD56^{bright} expression pattern amongst GBM-NK population may be advantageous for the tumour itself. Not only are CD56^{bright} NK cells less cytotoxic than CD56^{dim} NK cells (Cooper et al., 2001), but it has also been shown that interferon gamma (IFN- γ) cytokine (typically produced by CD56^{bright} NK cells (Poli et al., 2009) derived from NK cells, can promote GSC survival and immune escape by inducing the upregulation of MHC

and inhibitory receptors in GSCs, thereby facilitating glioblastoma tumorigenesis and recurrence (Kozłowska et al., 2016; Tseng et al., 2015). Specifically 'anergic' NK cells stimulated with IL-2 and anti-CD16 antibodies when cocultured with GSCs were shown to lose the ability to mediate cytotoxicity yet can still secrete IFN- γ in the presence of tumour cells (Kozłowska et al., 2016). This phenotype was also observed in GBM-NK cells (Gras Navarro et al., 2014). Furthermore, (Monaco et al., 2022) showed NK cells from tumours of newly diagnosed and recurrent glioblastoma cases present an enrichment of NK cells with upregulated RNA-seq signatures for IL2 signalling and downregulation of cytotoxicity; and we likewise found significantly decreased mRNA for GZMB (key cytotoxicity effector in CD56^{dim} NK cells (Freud & Caligiuri, 2006) in GBM-NK cells. While only predictive of IL-2 signalling, our ORA for a positive enrichment of IL-2/STAT5 machinery in GBM-NK cells may therefore warrant future analysis. The complexity of the TME is demonstrated from our analysis which found that, while the expression of IFNG gene itself is significantly increased (albeit with a frequency of expression of 15%) in GBM-NK cells, there is a concurrent downregulation of IFN- γ signalling pathway and response to IFN- γ . (Monaco et al., 2022) also showed downregulation of related genes (IRF7 and STAT1) in GBM-NK cells from recurrent and newly diagnosed patients. These apparently contradictory findings are unfortunately common in TME (Gupta et al., 2023; Jamal-Hanjani et al., 2013). However, recent flow cytometry analysis by (Shaim et al., 2021) found GBM-NK cells and NK cells cocultured with GSCs both exhibit reduced IFN- γ production compared to patient and healthy blood NK cells and in vitro controls respectively. So proteomic analysis of GBM-NK cells would be required to determine how this transcriptionally increased IFNG translated on a protein level. We suggest interferon gamma signalling response may be downregulated in GBM-NK cells (as shown in this thesis' ORA results), yet GBM-NK cells may still produce IFN- γ itself at suboptimal levels (reduced from HD-PBMC-NK levels (Shaim et al., 2021)) that promote GSC proliferation but fail to trigger an activated immune response in NK cells. Future analysis may benefit from experimentally evaluating the IFN- γ production of ex vivo GBM-NK cells and determining if

rescuing IFN- γ cytokine production through experimental stimulation could improve GBM-NK cytotoxic potential.

The TME is a complex environment where GBM-NK cells will be exposed to a variety of pro- and anti-inflammatory signals, inhospitable environments (nutrient and oxygen deprived, low pH) and other immunosuppressive factors (Chen et al., 2022; Sharma et al., 2023). Therefore, it is not surprising that many pathways are activated which are often in conflict with each other. For example, there is enrichment of both DNA damage and DNA repair genesets in tumour infiltrating NK cells, also enrichment for ROS and antioxidant pathways. Indeed, this is likely to contribute to the higher transcription levels overall as NK cell compete to function and stay alive. Several examples are discussed below.

There was a positive enrichment of pathways necessary for cell division (mitotic spindle formation/assembly, chromosome condensation, microtubule cytoskeletal mitotic organisation etc.) in GBM-NK cells. But there was also enrichment for cell cycle arrest, DNA replication stress and apoptosis genesets. This may underline a mixed population response: some GBM-NK subset cells are actively proliferating while others are inhibited or more likely reflect many pathways 'competing' within a cell. It is likely that over time, there may be a change from a more anti-tumour proliferative response to an exhausted phenotype resulting in an NK cell that can no longer function – similar to NK cell expression trajectories in mouse model of glioblastoma (Kirschenbaum et al., 2024). To determine if there may be a differential proliferation for CD56^{bright} NK cells in TME of glioblastoma, it may be beneficial to stain for Ki67 on GBM-NK cells as it is a marker of recent proliferation (Sobecki et al., 2017) or indeed to run separate GSEA analysis examining enrichment of cell cycle pathways enriched for CD56^{bright} GBM-NK vs HD-PBMC-NK and CD56^{dim} GBM-NK vs HD-PBMC-NK comparisons and replicate this analysis for NK cell subtypes comparisons. This would help provide understanding of how CD56^{bright} GBM-NK cells are recruited/surviving within the TME.

Our differential gene set analysis for GBM-NK vs HD-PBMC-NK, suggested that GBM-NK cells are under greater DNA replication stress compared to healthy baseline norms. It is known that when NK cells are overwhelmed by irreparable damage of DNA, replication stress can induce apoptosis (Guilz et al., 2024). The dataset also showed an observed ORA enrichment of apoptotic gene set 'Activation of BAD and Translocation to Mitochondria' while candidate gene set analysis revealed a significant increase in expression of many genes pertaining to Cytochrome C Release Induced Apoptosis. This apoptotic pathway was among five candidate gene sets that had a fold change increase in weighted expression greater than 2. Other pathways relating to apoptosis were also enriched in GBM-NK cells. These included family members of tumour suppressor protein p53 (aka TP53) function (and its regulation pathway gene set), and the mitochondrial unfolded protein response (UPR^{mt}) – indicating mitochondrial damage (Torres et al., 2024). Identification of apoptosis gene set enrichment is supported by studies that have sequenced GBM-NK cells and found a positive enrichment of apoptosis signature genes for newly diagnosed and recurrent glioblastoma patients (Monaco et al., 2022). Tumour infiltrating NK cells with tumour promoting characteristics (reduced NK cross talk macrophage recruitment) and a concomitant upregulation of apoptosis signatures along with an unfolded protein response have also been described in Human papillomavirus (HPV)-positive head and neck squamous cell carcinoma (HPV-positive HNSCC) (W. Lin et al., 2024). Overall, this may indicate that a proportion of the GBM-NK population is undergoing or at least primed for apoptosis but this may be tempered by an anti-apoptotic gene expression buffer. Cytochrome c release apoptosis flow cytometry assays could be employed to experimentally assess this transcriptional readout in GBM-NK cells (Campos et al., 2006).

Linked to the potential priming of apoptotic pathways, and consistent with this phenotype, there was a significant increase of genes transcribed in response to oxidative stress in GBM-NK with the vast majority of the gene set trended towards or exceeded the threshold of significance. This gene set was also amongst the five candidates with a fold change increase in expression greater

than factor of 2. Oxidative stress has been shown to drive impairment of NK cells in tumours (Poznanski et al., 2021), but this is less studied in GBM-NK cell context specifically. Among the top ranked significant genes were TNFAIP3, shown to inhibit mTOR signalling which is important for NK cell education and negatively regulate oxidative damage autophagic responses (Marçais et al., 2017; Vetter et al., 2019; Wang & Chen, 2022; Yang & Malarkannan, 2020); AIF1 which exacerbates oxidative stress and induce TIGIT exhaustion marker in NK Cell of oesophageal cancer patients (De Leon-Oliva et al., 2023); BCL2 as mentioned before is an apoptosis regulator induced by oxidative stress (Palabiyik, 2025). However, the cellular response to reduce oxidative stress is also indicated by the upregulation of genes encoding heat shock proteins (HSPA1A, HSPA1B) that protect cells ROS inducing oxidative stress (Kalmar & Greensmith, 2009); FOXO1 a transcription factor that upregulates detoxification pathways (Kim et al., 2022), ZFP36 is anti-inflammatory but also degrades TNF α mRNA transcription (Makita et al., 2021). TNF- α is source of oxidative stress but conversely is important for NK cell activation (Cunningham et al., 2020; W. Zhang et al., 2022). AREG encoding amphiregulin is an EGFR ligand (cancerous pathway in GBM) (Lee et al., 2020; Singh et al., 2025) and was the only gene ranked highly for both log2fc increased expression and frequency of expression. It is also associated with TGF- β induced oxidative stress (discussed further below). There was also evidence of NK cells attempting to alleviate oxidative stress including downregulation of DUOX1 which functions to increase hydrogen peroxide (H_2O_2) (De Deken et al., 2014). This transcriptomic data provides evidence that GBM-NK cells are responsive to oxidative stress which may contribute to transcriptional readouts of inhibited cytotoxic function, increased DNA damage and arrested proliferation in GBM-NK (Poznanski et al., 2021).

Evidence of Oxidative Stress in GBM-NK cells is further supported by an increased ROS gene set expression signature in GBM-NK cells; a NK cell ROS signature was previously identified in HPV-positive HNSCC (W. Lin et al., 2024). ORA results for GBM-NK vs HD-PBMC-NK cells showcased a positive enrichment of ROS pathway and antioxidant response pathways (especially upregulation of

antioxidant production regulator NFE2L2, also known as NRF2 (Poznanski et al., 2021)) in GBM-NK cells. Closer analysis of candidate ROS gene set also revealed an increased expression and frequency trend in GBM-NK cells. The majority of the top ranked significant genes' functions indicated an adaptive cellular response to ROS: many related to antioxidant proteins involved in peroxidase active (IPCEF1, PRDX1, PRDX2), thioredoxin activity (TXN, TXNRD1) and other antioxidant pathways (Stelzer et al., 2016). Notably increased expression of peroxiredoxin 1 (PRDX1) thioredoxin (TXN), and TXNRD1 (Thioredoxin reductase 1) is also known to coincide with NK cell activation (Mimura et al., 2017) and PRDX1 is shown to improve CAR-NK viability and anti-tumour function under oxidative stress (Klopotowska et al., 2022). However, amongst the top 3 significantly increased genes common to highest log2fc in expression and frequency rankings were ABBC1 and JUNB. ABBC1 is a plasma membrane transporter facilitating ROS entry into cell and JUNB which is recruited in ROS cascades (Mueller et al., 2005; Ren et al., 2023). Furthermore, the significant decrease of FTL (ferritin light chain sequesters iron oxide products) likewise may contribute to increased production ROS in GBM-NK cells (Liu et al., 2022).

When ROS is upregulated, there is often an increase in antioxidant machinery and indeed, we found an enrichment of antioxidant gene transcription (Morris et al., 2022). Excess ROS production, either intracellularly in the NK cells or from high levels in the TME, is known to hinder NK cell cytotoxic function and inhibit cytokine production by NK cells (Kono et al., 1996; Morris et al., 2022; Nakamura & Matsunaga, 1998). However, the scope of ROS dysfunction in GBM-NK is difficult to gauge from our dataset considering our ORA results showed conflicting transcriptional readouts of reduced IFN- γ signalling/response gene set expression but increased TNF- α signalling, IL-2 signalling and increased IFN- γ transcript expression (limitation 4). However, targeting improved antioxidant functions within NK cells is a promising line of therapeutic potential (Shanley et al., 2024). (Poznanski et al., 2021) showed that activation of NRF2 antioxidant activity significantly rescued dysfunctional NK cell tumour cytotoxicity, glycolysis, and OXPHOS when cocultured with malignant ascites fluid derived from breast cancer patients. Overall, more

experimental data is needed to verify ROS and antioxidant production in GBM-NK cells and clarify the state of GBM-NK oxidative stress within TME. ROS levels in GBM-NK could be examined through molecular flow cytometry assays such as CELL ROX or MITOSOX, and antioxidant production through proteomic analysis techniques and specific antioxidant assays (as described in (Kauffman et al., 2016; Montano et al., 2014; Sabido et al., 2020)). Additionally, performing functional assays and PCR analysis in GBM-NK cells treated with antioxidants could assess the potency of ROS induced cytotoxicity dysfunction in GBM-NK ex-vivo (Wang et al., 2016).

Literature shows that approximately 90% of cellular ROS originates from Mitochondria (Balaban et al., 2005); therefore assessing the mitochondrial fitness of GBM-NK cells was prioritised for closer analysis in this thesis. Pairing findings from initial ORA results presented an upregulated signature for mitochondrial response to unfolded mitochondrial proteins (a mitochondrial stress pathway commonly coincided with increased ROS production (Torres et al., 2024)) with candidate gene set analysis of cytochrome c release activity (often activated in a mitochondrial ROS dependent manner (Song et al., 2011)), we can infer that mitochondrial ROS is likely to be increased in GBM-NK. Complexes I and Complexes III are significant sites of mitochondrial ROS production in particular (Balaban et al., 2005). This dataset showcased a global trend of increased expression in mitochondrial complexes I-IV gene set in GBM-NK, with two of the three significantly increased genes encoding complex I subunits. However, subset analysis revealed this increase was perhaps unique to CD56^{bright} GBM-NK expression, as CD56^{dim} cells showed a slight reduction in gene set expression for mitochondrial complexes. ORA results likewise showcased a downregulation of complex IV assembly across multiple gene set libraries. This is an interesting observation considering TGF- β is shown to hinder complex IV activity albeit in lung epithelial cells (Yoon et al., 2005). Thus, we may suggest TGF- β signalling shown to be upregulated in GBM-NK cells could inhibit mitochondrial complexes in CD56^{dim} GBM-NK cells also.

While the dynamics of mitochondrial fission and fusion is understudied in GBM-NK, increased mitochondrial fission in tumour infiltrating NK cells is widely observed to induce dysfunction (Zheng et al., 2019). Mitofusion and Mitofission were amongst the five candidate genes to have a greater than two-fold weighted average gene set expression and were the only gene sets to have an increased weighted average frequency greater than 2. While both gene sets featured a general trend towards increased gene expression, examination of significantly increased genes suggests that mitochondrial dynamics GBM-NK cells may be slightly shifted in the direction of Mitofission. MFN1 and OPA1 are key markers for Mitofusion (controlling outer and inner membrane fusion respectively (Cipolat et al., 2004)) that are significantly upregulated in GBM-NK cells; however, many negative regulators of Mitofusion are also significantly increased in GBM-NK including TFRC(CD71) (downstream cascades degrade MFN2 (Senyilmaz et al., 2015) which is required with MFN1 to induce Mitofusion (Sloat et al., 2019)), B2LCA1 (anti-apoptotic gene that can regulate Mitofusion (Rolland & Conradt, 2010)) and HUWE1 (mitophagy promoting gene (Di Rita et al., 2018)). Conversely, all but one (OPA1) of the significantly increased Mitofission genes are required for, contribute to or regulate Mitofission (Stelzer et al., 2016). Although, it is worth noting that these significantly increased Mitofission promoting genes were only expressed in less than 5 percent of cells and the overall importance to NK cell dysfunction is not clear.

These altered mitochondrial health signatures coincided with an overall increased oxidative phosphorylation (OXPHOS) gene set expression in GBM-NK cells. While not a direct measure of metabolism, closer analysis of candidate gene set expression and frequency relative to HD-PBMC-NK cells, suggest there was an upregulation of cellular machinery that engages OXPHOS in cells – supporting assumption 8. There was a general trend of significantly elevated gene expression relative to HD-PBMC-NK across the gene set. Specifically with significantly increased genes pertaining to complex I and IV, in GBM-NK cells. However, analysis of the rate limiting enzymes of OXPHOS showed few genes were significantly increased in GBM-NK cells. COX16, a vital subunit of

cytochrome c complex IV assembly (Cerqua et al., 2018), even displayed significantly decreased expression in GBM-NK. Therefore, we concluded, the seemingly increased OXPHOS capacity in GBM-NK cells may be limited - perhaps by dysregulated mitochondrial respiration. Previous data from the lab in patients with metastatic breast cancer showed that NK cells had only a limited ability to engage OXPHOS pathways (Slattery et al., 2021) and functional data is required to confirm that the upregulation of OXPHOS geneset is merely a reflection of a distressed cell trying to cope with low oxygen and the need for energy. However, we did not find the same increases in the glycolytic machinery. It is unclear that when facing the situation of low glucose and low oxygen in the TME, NK cells try to preferentially upregulate the OXPHOS machinery. Perhaps this is due to the greater number of fuels (glucose, glutamine, fatty acids) that can be used for OXPHOS compared to glucose, which is an absolute requirement for glycolysis (Bradley, 2019; Graham et al., 2012; Griessinger et al., 2023). Indeed, our ORA results also provided evidence for an enrichment of lipid metabolism in GBM-NK cells: fatty acid and cholesterol metabolism. Ultimately, future analysis of ex-vivo GBM-NK is needed to probe metabolism, using methods such as SCENITH, single cell uptake or seahorse (Ahl et al., 2020; Argüello et al., 2020; Little et al., 2020).

. Our analysis provides further evidence that TGF- β signalling is occurring in GBM-NK cells (Close et al., 2019; Crane et al., 2009; Friese et al., 2004; Gonzalez-Junca et al., 2019; Kirschenbaum et al., 2024; Shaim et al., 2021). The TGF- β gene set showcases higher gene set expression and frequency in GBM-NK cells relative to HD-PBMC-NK cells across subsets, and moreover a greater than 2-fold increase in gene set expression relative to HD-PBMC-NK cells. There was also a clear trend of increased TGF- β gene expression in GBM-NK across the gene set. CD9, a proposed marker for TGF- β immune evasion (Close et al., 2019), was the most significantly increased gene change with the greatest fold increase in GBM-NK (Log2FC of 6.9, equivalent to 119-fold increase in expression) and was amongst the top 10 significant genes with greatest frequency in GBM-NK. CD9 is shown to be upregulated on a protein level in GBM-NK cells – more so than GBM-PBMC-NK or HD-PBMC-NK cells (Close et al.,

2019; Shaim et al., 2021) – and moreover RNA-seq data by (Schmassmann et al., 2023) it is upregulated specifically in GBM-NK cells from core tumour sites compared peripheral tumour regions. (Shaim et al., 2021) further demonstrated that CD9 is not only upregulated in GBM-NK but can also be induced by TGF- β in healthy NK cells. They further demonstrated that CD9 (alongside CD109) binding to α v integrins on GSC cells critically contributes to TGF- β upregulation in GCSs and dysfunction in NK cells. This may suggest GBM-NK cells in our dataset potentially experience upregulated TGF- β mediated cell contact with tumour cells; facilitating TGF- β signalling. Additionally, our dataset supported (Shaim et al., 2021), findings showcasing JUND transcript is significantly increased in GBM-NK cells vs HD-PBMC-NK cells – JUND was the second highest ranked gene common to both rankings of our analysis. Further evidence of TGF- β downstream cascades included upregulation of JUNB, JUN, MAPK1 (Batlle & Massagué, 2019; Millena et al., 2016) Significantly increased genes also indicated a wider upregulation of TGF- β family pathways: TGF β R1 and BMPR ligand GDF11 (Zhang et al., 2017); ACVR1B – activin receptor (similar properties to TGF- β receptor) inhibits NK cell function (reduces GZMB etc.) albeit to a lesser degree than TGF- β (F. Li et al., 2022). However, negative mediators of TGF- β signalling were also significantly increased: FKBP1A is negative regulator of TGF β R1 (Chen et al., 1997); ID2 and ID3 are normally repressed by TGF- β and their overexpression (ID2) can inhibit TGF- β induced apoptosis pathways (Cao et al., 2009b). Interestingly this also included PPP1R15A or GADD34, a DNA damage protein recruited by Smad7, which dephosphorylates TGF β R1 to inhibit TGF β -induced cell cycle arrest (Shi et al., 2004). This could be suggestive of negative feedback loops for TGF- β signalling pathway, but closer analysis of significant genes revealed more atypical results that seemingly contradict an elevated TGF- β signature readout. Some significantly increased genes are usually repressed by TGF- β : MYC or c-myc, known to promote NK cell development and mTOR activation driven glycolysis (Khameneh et al., 2023; Loftus et al., 2018)– antithetical functional readouts for TGF- β signalling effects (Lee et al., 2014); CD71 or transferrin receptor, known to promote NK cell iron metabolism (V. Zaiatz-Bittencourt et al., 2018); and most strikingly IFNG. IFN- γ production has been proven to be

downregulated by TGF- β (Laouar et al., 2005), and IFN- γ production levels are observed to be low in GBM-NK relative to HD-PBMC-NK cells (Shaim et al., 2021). This may indicate that TGF- β signalling is perhaps only partially effective in fulfilling its dysfunction effects. Regardless of the biological outcome of these significantly increased genes, it is evident that this TGF- β signalling pathway is at least strongly engaged within GBM-NK cells from the pronounced upregulation of key marker genes and the gene set as a whole.

We predicted that stratification of high vs. low scoring TGF- β NK cells would clarify TGF- β signatures and potentially identify mechanisms of action. However, only Oxidative Stress and, Glycolysis and Gluconeogenesis, gene sets exhibited a slight trend increase in expression while Arachidonate acid metabolism was down. It is important to note that canonical SMAD mediated TGF- β signalling, modify gene expression in co-operation with host other of transcription factors and transcriptional co-regulators controlled by and often dependent on other signalling pathways and TGF- β signalling is highly context dependent in tumour (Baba et al., 2022; French et al., 2020; Massagué, 2012) – see assumption 10a also. We conclude that the strongest TGF- β gene set signature observed in GBM-NK may be most associated with upregulated oxidative stress pathways.

4.1 Conclusion

While recent studies have characterised the impaired cytotoxic function of tumour infiltrating NK cells in glioblastoma (Breznik et al., 2022; Shaim et al., 2021), further research is required to understand the metabolic state of NK cells, their mitochondrial health and bioenergetic capacity, and state of oxidative stress within the TME of glioblastoma specifically. This thesis provides insight into these questions through transcriptomic analysis of GBM-NK cells: offering predictive evidence-based hypotheses to warrant future experimental research. Firstly, ORA of differential expressed genes in GBM-NK cells provides many avenues for future investigations of GBM-NK and comprehensively categorises many novel aspects of GBM-NK cells dysfunction.

Furthermore, our thesis reaffirms literature findings that CD56^{bright} GBM-NK cells show greater infiltration of tumour and are differentially upregulated in TME (Friebel et al., 2020). This highlights the need for focused efforts to enhance CD56^{dim}, cytotoxic activity, to improve anti-tumour responses with TME. Our candidate gene set analysis enabled closer investigations of key unexplored research questions for GBM-NK cells. This work also highlighted the upregulated signature for oxidative stress globally in GBM-NK cells – an established feature of TME in many cancers that has yet to be directly studied in GBM-NK cells specifically (Poznanski et al., 2021). Furthermore, our research underlined GBM-NK cells likely respond to the pressure of ROS with antioxidant production – perhaps enabling NK cells to survive for a time within the TME (Klopotowska et al., 2022). However, coinciding evidence of elevated (potentially TGF- β induced) oxidative stress signatures, and closer candidate gene set analysis of cytochrome c release, suggest that it may be in vain as GBM-NK are likely vulnerable to cell death and primed for apoptotic pathways. Similarly, we discovered that GBM-NK cells showcase a trend increase of mitophagy signature expression, however with significant increased gene expression observed in only a low frequency of GBM-NK cells. This analysis could indicate a reduced clearance of damaged mitochondria could contribute to oxidative stress and greater levels of apoptosis (Zhang et al., 2019). Therefore, this analysis of how dysfunction is induced within GBM-NK cells could inform the design of NK cell immunotherapies.

Furthermore, this thesis offered some of the first predictive insights into the metabolic states of GBM-NK cells. GBM-NK cells exhibit a potentially increase in mitochondrial mass that could resemble NK cells from metastatic breast cancer patients (Slattery et al., 2021) but is likely compromised by significant reductions in expression of some complex IV genes. This may hinder for example a potentially elevated OXPHOS potential. Furthermore, our analysis implies there is an elevated OXPHOS machinery signature compared to Glycolysis signature in GBM-NK cells. Moreover, our analysis suggests glycolysis may be inhibited/downregulated in CD56^{dim} GBM-NK cells. Understanding that NK cell metabolism underpins much of their function, these results could help

inform strategies to enhance the bioenergetic capacity of CAR-NK cells to improve their viability and sustain the energetically expensive anti-tumour response, vital for clinical outcomes (O'Brien & Finlay, 2019; Yang et al., 2025). For example, (Shanley et al., 2024) has already demonstrated IL-21 stimulated NK cells showcase elevated OXPHOS, reduced mitochondrial ROS and improved cytotoxicity against GSCs - shown in mouse model and human mRNA sequencing results for IL-21 stimulated CAR-NK cells. While it is difficult to gauge the opposing dynamics of upregulated of mitochondrial fission and fusion pathway expression patterns, closer analysis of significantly increased genes suggest Mitofusion may be downregulated. Although it has yet to be characterised in GBM-NK cells, fragmented mitochondria have been associated with dysfunctional NK cell phenotypes (Zheng et al., 2019). Our analysis warrants future investigations to more clearly define of mitochondrial morphology and investigate mitochondrial failure within GBM-NK cells – strategies to enhance mitochondrial fusion could enhance efficacy of future CAR-NK cells (Buck et al., 2016; Komai et al., 2018; Surace et al., 2021). Furthermore, our analysis more broadly delved into TGF- β signalling impact on NK cell physiology and metabolism – concluding that TGF- β may showcase the strongest influence for increased oxidative stress signature in GBM-NK cells. Our analysis of TGF- β signalling in GBM-NK cells suggests aspects of this pathway may be counteracted by opposing stimuli within the TME and future research is needed to pinpoint its direct effects on apoptosis, metabolism, mitochondria, and oxidative stress in GBM-NK cells.

4.2 Future Directions

While this thesis has explored differential transcription patterns between GBM-NK cells and HD-PBMC-NK cells, our analysis does not inform us directly which pathways are enriched within GBM-NK population itself. Performing Gene Set Variation Analysis or GSVA for example, could highlight which opposing pathways such as mitochondrial fission and fusion are upregulated within GBM-NK cells and provide clarity to the overall metabolic state of GBM-NK cells (Hänzelmann et al., 2013). Related to this Enrichr ORA performed for separate inputs of genes significantly increased and decreased in GBM-NK, served the purpose of our initial exploratory analysis but presented limitations for analysis

(limitation 4). Future analysis would therefore individually validate if a significantly enriched pathway of interest – such as DNA damage repair – is upregulated or downregulated in GBM-NK overall.

However as discussed, many confounding factors exist which could contribute to generally elevated transcriptional activity in GBM-NK relative to HD-PBMC-NK cells. While beyond the scope of this MSc thesis, future investigations focused on publication would require more explicit checks (depth distributions, mitochondrial read fraction, ribosomal content) and independent analysis of the original datasets (with applying an integration step) to increase confidence in this biological interpretation of hypertranscription in GBM-NK cells.

As described by (Rentzsch et al., 2025), ‘recalibrating differential gene expression by genetic dosage variance prioritising functionally relevant genes’ could help infer which significantly increased gene expression may be relevant. Alternatively establishing gene set expression controls or references as described in (C. Neftel et al., 2019) could help infer what gene set showcased relevantly increased expressed above ‘background noise’ or population baseline expression levels for any given gene set. Seurat functions such as ‘AddModuleScore’ could be utilised to facilitate this: this programs a gene set score by computing the difference between calculated gene average expression values for each cell within each specified gene set and a similarly sized background gene set (Tirosh et al., 2016).

Extending candidate gene set analysis to assess subset population differences could render more clarity for interpreting analysis also: for example, unlike CD56^{bright} GBM-NK, CD56^{dim} GBM-NK cells did not showcase elevated gene set expression for glycolysis and even showcased reduced mitochondrial complexes gene set expression. CD56^{dim} NK cells are clearly downregulated in TME and may demonstrate expression pattern that are uniquely altered from CD56^{dim} HD-PBMC-NK cells compared to CD56^{bright} differences. Furthermore, comparing NK cell with similar phenotypic states, such as comparing quiescent/typical or activated CD56^{bright/dim} NK cells across GBM-NK and HD-PBMC-NK populations (as previously annotated by (Netskar et al., 2024)) cells would serve to highlight differential transcription patterns that is unique to the TME of glioblastoma. As mentioned before, NK cells infiltrating tumours in HPV-

positive HNSCC consisted of pro-tumour and anti-tumour phenotypes; a phenomenon which could be similarly investigated in GBM-NK cells (W. Lin et al., 2024). To help disentangle “tumour vs blood” from “patient/systemic vs healthy” (limitation 11), future investigations would aim to either find or generate a new dataset that would allow a direct comparison of GBM-PBMC-NK cells with GBM-NK cells, ideally from the same patient

However, beyond reassessments of transcriptomic analysis approach, experimental efforts are needed to validate findings. Much of these suggested experiments have been mentioned throughout this discussion but proteomic analysis of GBM-NK vs HD-PBMC-NK samples would immediately provide insight into the verity of transcriptional analysis - most transcriptome changes do not likely reflect proteomic changes (Bauernfeind & Babbitt, 2017).

The scope of transcriptional analysis only goes so far but offers a glimpse into understanding the dysfunctional state of GBM-NK cells compared to healthy NK cell physiology. This thesis recommends that future analysis should prioritise assessments of mitochondrial fitness in GBM-NK cells as a common denominator for analysis of ROS, metabolism, apoptosis. While TGF- β signalling appeared to be occurring within GBM-NK cells, it is unclear if the population is inhibited as whole. We suggest confirming if TGF- β signalling corresponded with downregulated signatures such as a decrease in activating receptor transcripts and if so, did this expression pattern vary in CD56^{bright/dim} GBM-NK cells or if it was specific to GBM-NK cells with stressed phenotypes (oxidative stress signatures and other known TGF- β exhaustion markers such as TIGIT upregulation (Sun et al., 2014)). While GBM-NK cell subpopulations may be differentially inhibited by TGF- β signalling, it is evident that TGF- β signalling does not act in isolation within the TME. Cross-referencing TGF- β analysis in combination with other inhibitory signalling pathways known to exist in GBM such EGFR, adenosine signalling etc. (H. Lin et al., 2024), could further illustrate how GBM-NK develop an altered transcriptional expression profile that contrasts physiological HD-PBMC-NK cells.

Future Directions for GBM-NK Studies

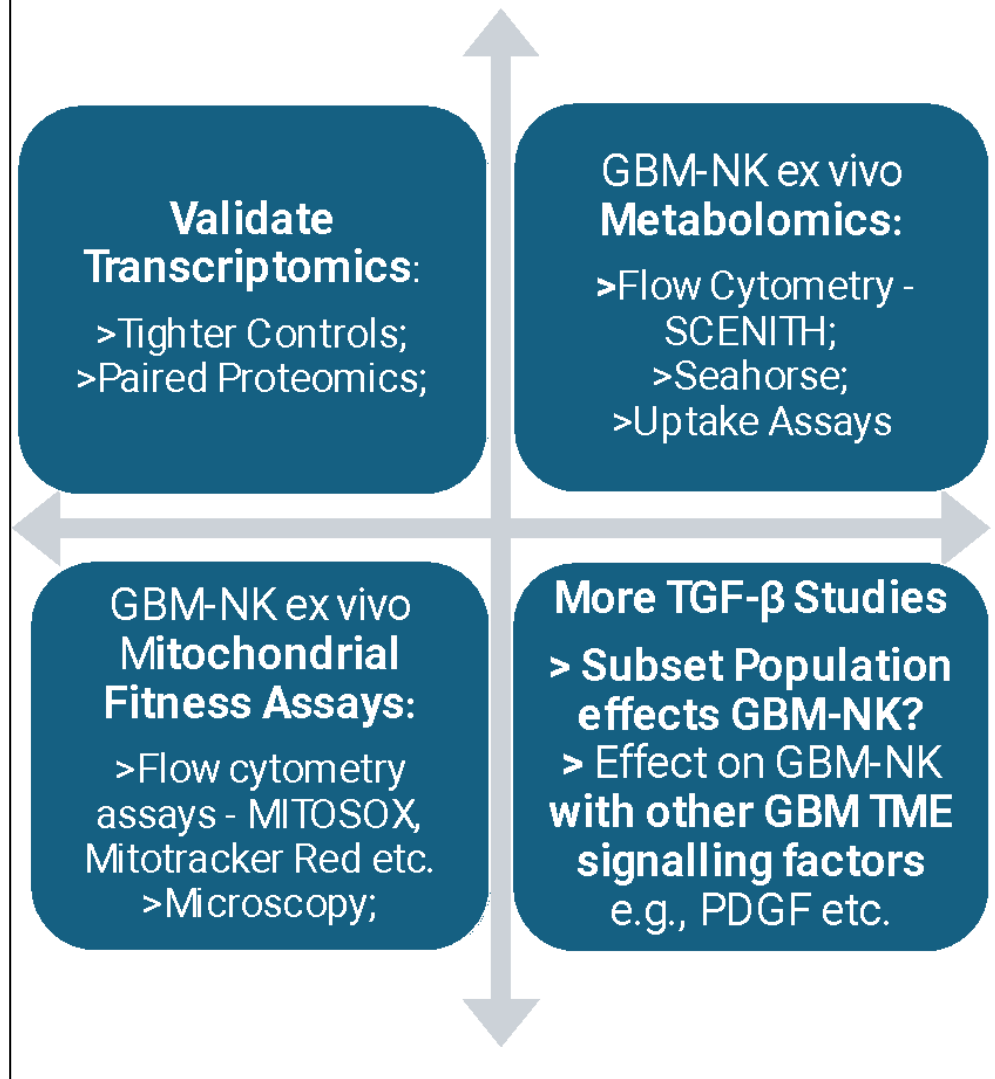


Figure 34: Summary Illustration of the Most Important Future Directions for this Research.

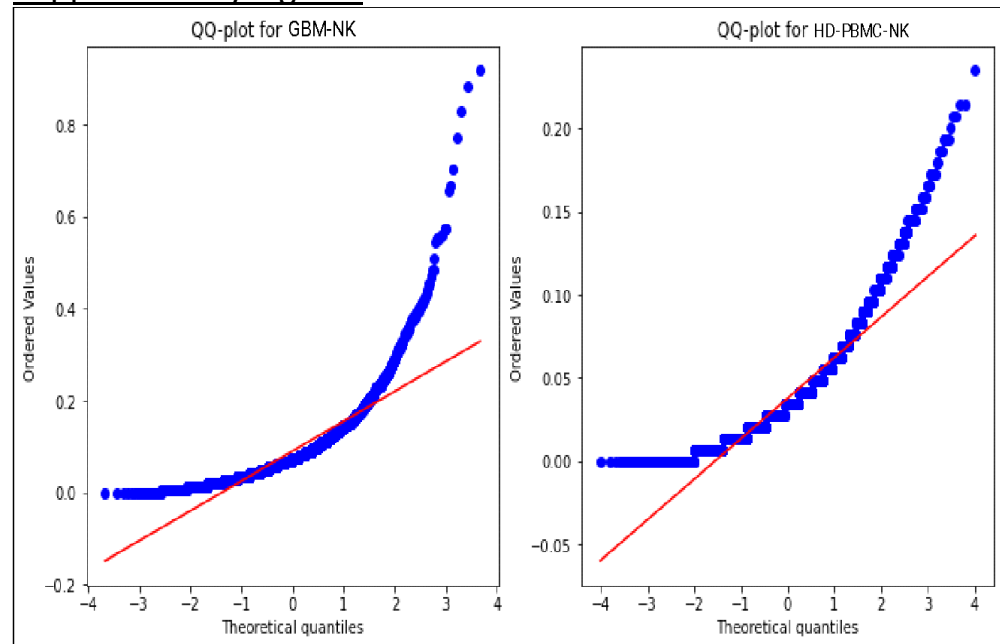
Herein are the suggested future experiments for GBM-NK studies as outlined in the discussion:

- 1) Validate transcriptomic findings of this thesis through replicate transcriptional analysis implementing tighter controls e.g., gene weight calibration (assessing expression levels against 'noise' or baseline controls), independent analysis of original input datasets and an assessment of technical factors including mitochondrial content. Ultimately validate findings of transcriptional data against paired proteomic data.
- 2) Perform metabolic assessments of ex vivo GBM-NK cells to translate transcriptomic data into real metabolic readouts of OXPHOS, glycolysis etc., through various experimental procedures e.g., Seahorse assays; flow cytometry methods such as SCENITH and other uptake assays
- 3) Perform mitochondrial fitness assays on

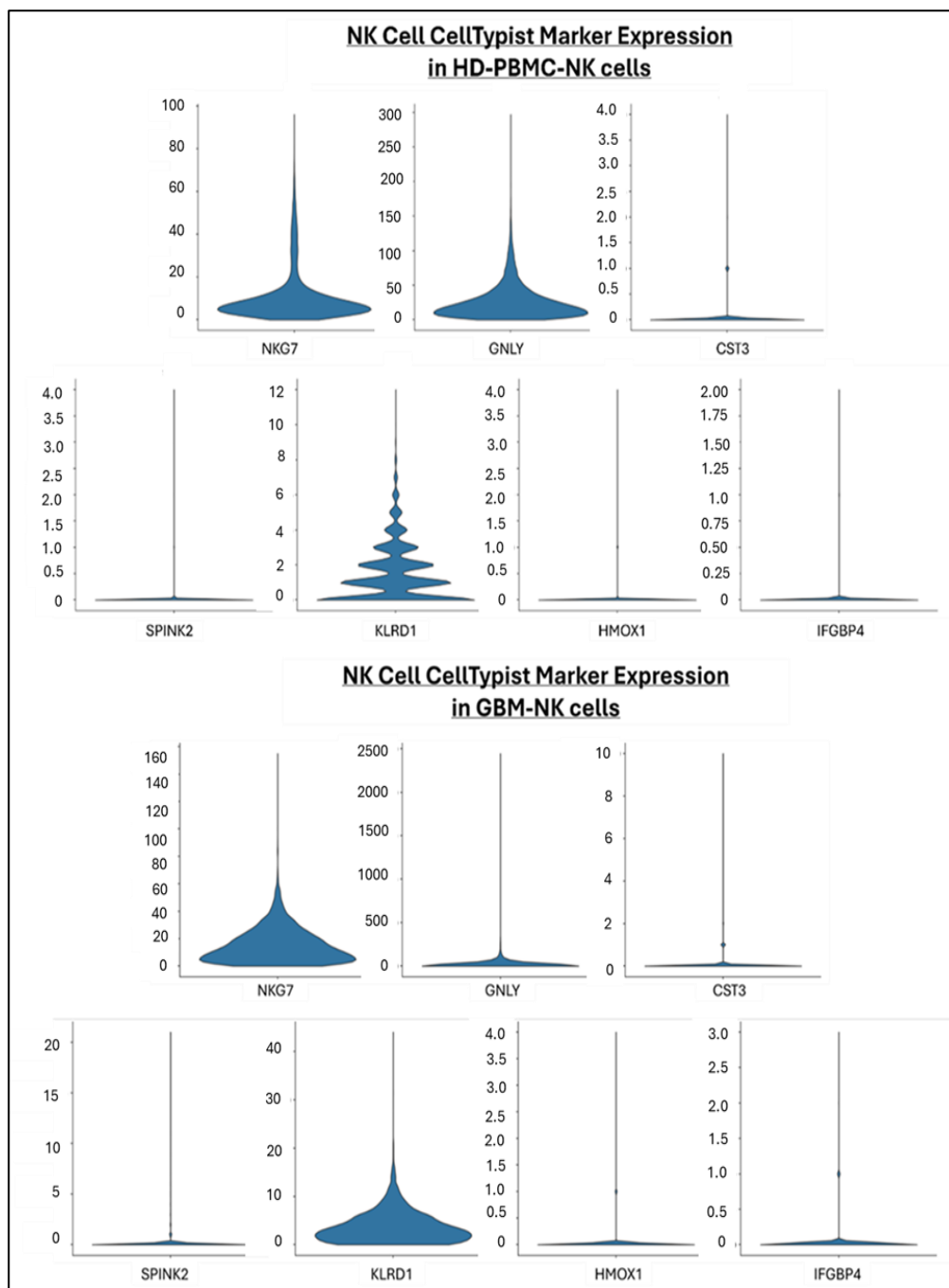
ex vivo GBM-NK samples to verify the suggested dysregulated levels of mitochondrial ROS and illustrate mitochondrial morphology – mitochondrial mass, rates of fusion and fragmentation, mitochondrial polarisation through various flow cytometry assays etc.; 4) Cross-referencing TGF- β analysis in combination with other inhibitory signalling pathways known to exist in GBM such as EGFR, adenosine signalling etc. Define TGF- β inhibitory effects on subset populations of GBM-NK cells including CD56^{Bright} and CD56^{Dim} NK cells, NK cells with stressed or quiescent signature states etc.

5. Appendices

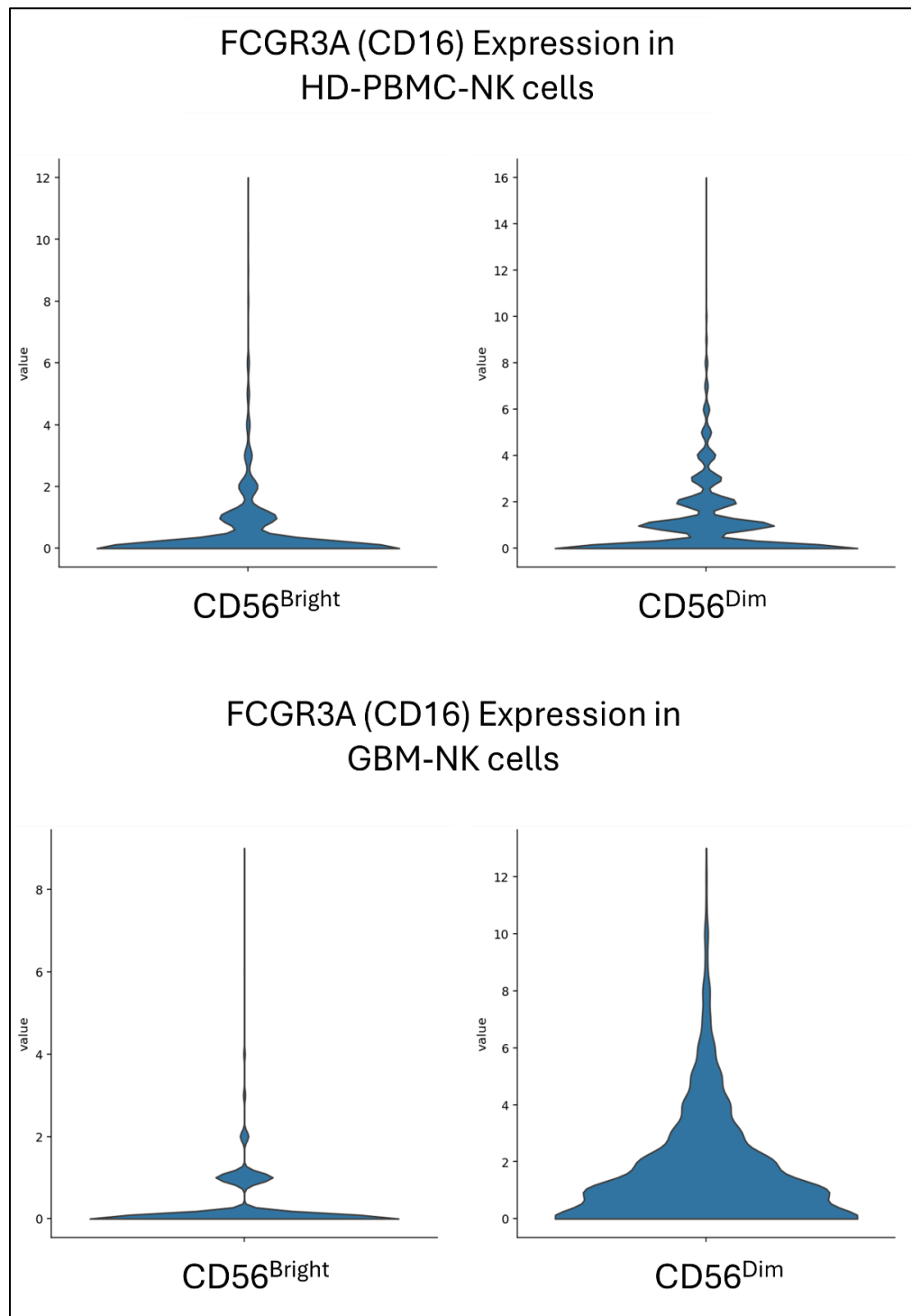
Supplementary Figures



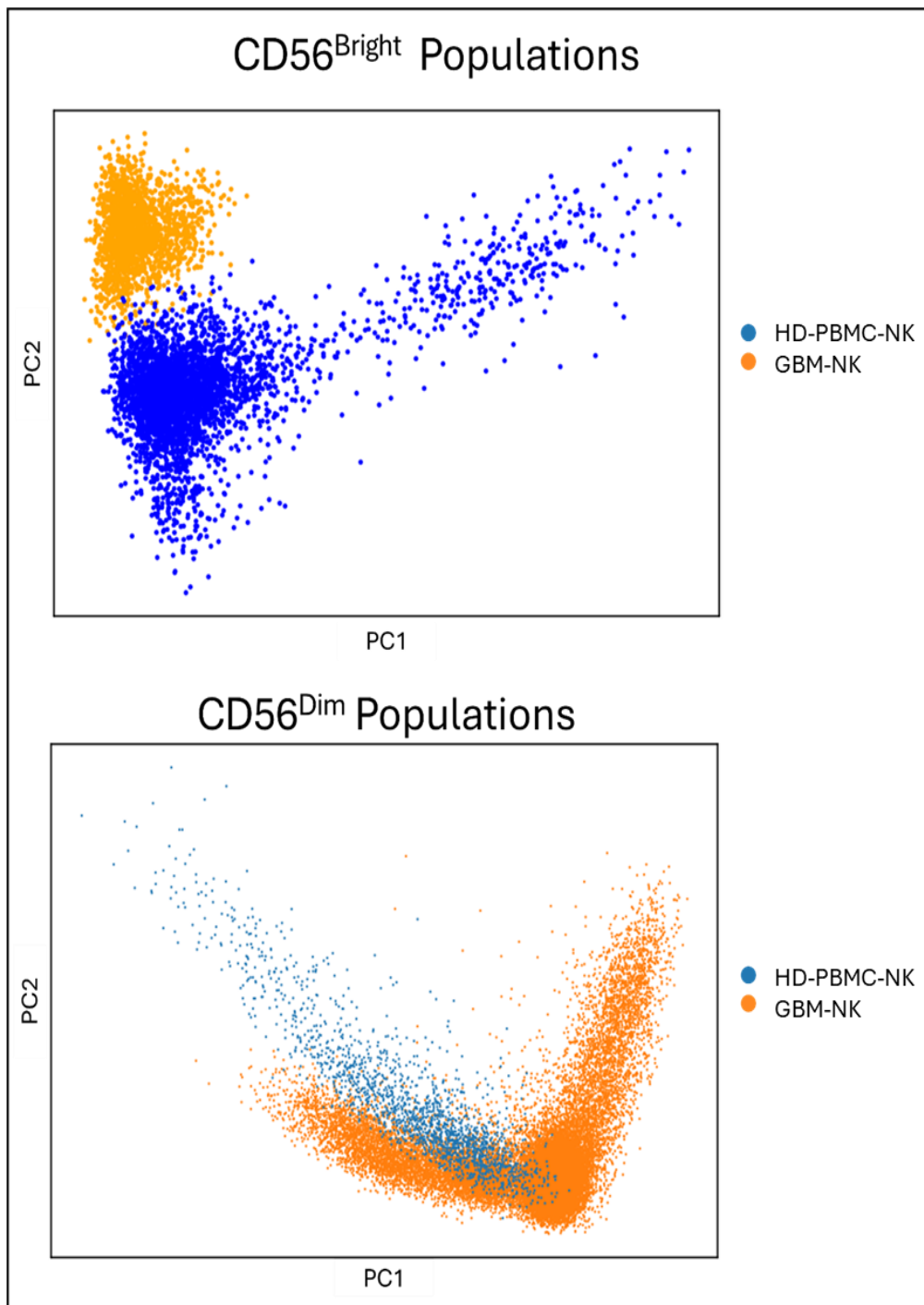
Supplementary Figure 1: Representative QQ-plots Testing Normality of GBM-NK and HD-PBMC-NK TGF- β expression. Red line represents predicted trajectory of normal data. Blue curved line signifies deviation of data from normality. Representative QQ-plots illustrating GBM-NK and HD-PBMC-NK cell data (in this case pertaining to TGF- β expression) diverges from normality – shown in tail deviations from red line of predicted normal distribution. Normality was also tested quantitatively. Lilliefors test (modified Kolmogorov–Smirnov) performed for GBM-NK expression data ($KS^*=0.1234$, $p=1.000e-03$) and HD-PBMC-NK expression data ($KS^*=0.1416$, $p=1.000e-03$) rejected normality. KS statistics above 0.10 and very small p-values allow us to confidently reject normality. Anderson–Darling test also underlined extreme deviation of GBM-NK and HD-PBMC-NK expression data: GBM-NK - $A^2 = 150.4$ (vs critical $\approx 0.6-1.1$); HD-PBMC-NK - $A^2 = 506.1$ (vs critical $\approx 0.6-1.1$). (Abdi, 1998; Anderson & Darling, 1954; Lilliefors, 1967; Stephens, 1974).



Supplementary Figure 2: Violin Plots representing the distributed expression of CellTypist NK cell markers used by Netskar et al., 2024 to annotate NK cells. NKG7, GNLY, CST3, MALAT1, SPINK2, KLRD1, HMOX1, IGFBP4, TRBV10-2, CD3E. Top) Expression of markers in HD-PBMC-NK cells; Bottom) Expression of markers in GBM-NK cells. Width of shaded area corresponds with amount of cells expressing genes at same expression value. Height of violin indicates cell with highest expression. GBM-NK and HD-PBMC-NK cells both display pronounced signature for top model NK cell specific markers: NKG7, GNLY, KLRD1 (C. Yang et al., 2019).



Supplementary Figure 3: Violin Plots representing the distributed expression of key CellTypist $CD56^{dim}$ NK cell marker FCGR3A (CD16), used by Netskar et al., 2024 to annotate NK cells. Top) Expression of marker in HD-PBMC-NK cells - $CD56^{bright}$ (Left); $CD56^{dim}$ (Right); Bottom) Expression of markers in GBM-NK cells. Width of shaded area corresponds with amount of cells expressing genes at same expression value. Height of violin indicates cell with highest expression. $CD56^{dim}$ NK cells from both GBM-NK and HD-PBMC-NK populations both display greater FCGR3A expression than $CD56^{bright}$ NK cells from their respective populations.



Supplementary Figure 4: PCA plots were generated for HD-PBMC-NK (blue dots) and GBM-NK (orange dots) based on the top 2000 highly variable genes. Top) PCA plot for comparison of CD56^{bright} NK cells from HD-PBMC-NK and GBM-NK populations. Bottom) PCA plot for comparison of CD56^{dim} NK cells from HD-PBMC-NK and GBM-NK populations. Again, CD56^{bright} NK cells from HD-PBMC-NK and GBM-NK populations are clearly distinct from one another. While CD56^{dim} NK cells from HD-PBMC-NK and GBM-NK populations are also distinct, there is more overlap in transcription patterns.

Functional Readouts	Some Relevant Upregulated Genesets (Top 100)					
	Reactome Pathways 2024	WikiPathways 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	MSigDB Hallmark 2020	GO Biological Process 2025
DNA damage	Rank 31 DNA Damage Reversal	Rank 95 DNA IR Damage And Cellular Response Via ATR WP4016	Rank 78 Mismatch repair		Rank 36 DNA repair	Rank 11 DNA Alkylation Repair
	Rank 72 Diseases of Mismatch Repair (MMR)					Rank 94 DNA damage response
Cellular Dysfunction/ Stress	Rank 45 Calcineurin Activates NFAT (impairs perforin)	Rank 99 IL-2 signalling	Rank 23 cellular senescence	Rank 95 PTGIR -> IL6 Production	Rank 5 IL-2/STAT5 signalling	Rank 12 Negative Regulation of PERK-mediated Unfolded Protein Response
						Rank 34 Negative Regulation of Granulocyte Differentiation
		Rank 49 Fas Ligand Pathway And Stress Induction	Rank 34 AGE-RAGE signaling pathway in diabetic	Rank 97 IL6R -> CEBP/ELK/SRF Signaling	Rank 6 mTORC1 Signaling	Rank 46 Response to Unfolded Protein
					Rank 15 Unfolded Protein Response	
					Rank 20 Inflammatory Response	Rank 82 Protein Targeting to Vacuole
		Of Heat Shock Proteins			Rank 30 hypoxia	Involved in Autophagy
						Rank 87 Stress-Activated MAPK Cascade
Cell Activation	Rank 10 Activation of the AP-1 Family of Transcription Factors	Rank 42 TNF Alpha Signaling	Rank 39 TNF signaling pathway	Rank 57 Natural Killer Cell Activation through C-Type Lectin-like Receptors	Rank 4 TNF-alpha Signaling via NF-kB	
	Rank 13 RHO GTPases Activate Formins					
	Rank 82 Cellular Responses to Stimuli				Rank 7/8 Myc Targets V2/1	
Mitochondrial Stress	Rank 36 Activation of BAD and Translocation to Mitochondria		Rank 82 glutathione metabolism			
	Rank 83 Mitochondrial Unfolded Protein Response (UPRmt)					

Supplementary Figure 5(I): Gene sets pathways in GBM-NK cells enriched in multiple gene set databases, suggests the TME induces dysfunctional phenotype in tumour infiltrating NK cells.

Part 1) Summary of GSEA ORA data is arranged in the above table as follows: Columns list gene set databases e.g., 'Reactome Pathways 2024', that were selected from Enrichr analysis and cells within these columns list their enriched gene sets. All gene sets listed are reported as

significantly increased (adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment (combined rank: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score) within the list of a database's enriched pathway is given in bold beside each named gene set. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched(statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human.

Functional Readouts	Some Relevant Upregulated Genesets (Top 100)					
	Reactome Pathways 2024	WikiPathways 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	MSigDB Hallmark 2020	GO Biological Process 2025
Cell Cycle Arrest/ Checkpoints	TOP 10					
	G2 M DNA Replication Checkpoint					Rank 14 Negative Regulation of Mitotic Metaphase/Anaphase Transition
	Phosphorylation of Emi1					Rank 17 Mitotic Spindle Assembly Checkpoint Signaling
	TOP 20			Rank 10 SCF/BTRC Complex	Rank 1 G2-M Checkpoint	Rank 18 Mitotic Spindle Checkpoint Signaling
	Cell Cycle Checkpoints					Rank 20 Spindle Assembly Checkpoint Signaling
Cell Cycle	Mitotic Spindle Checkpoint					
	Chk1					
	Chk2(Cds1) Mediated Inactivation of Cyclin B Cdk1 Complex					
	TOP 10			TOP 10		TOP 10 Exit From Mitosis
	Condensation of Prometaphase Chromosomes			Cell cycle overview		Attachment of Mitotic Spindle Microtubules to Kinetochore
	Cell Cycle, Mitotic			Spindle Assembly	Rank 2 e2f targets	Mitotic Nuclear Membrane Disassembly
	Mitotic Prometaphase			kinetochore assembly		Positive Regulation of Chromosome
	EML4 and NUDC in Mitotic Spindle Formation			chromosome condensation		Regulation of Chromosome Condensation
	Resolution of Sister Chromatid Cohesion			Metaphase/Anaphase Phase Transition		Regulation of Mitotic Cytokinesis
	TOP20					Microtubule Cytoskeleton Organization Involved in Mitosis
Response to ROS	Amplification of Signal From the Kinetochores					
	M Phase					
Apoptosis	Rank 32 Regulation of NFE2L2 Gene Expression					
	Rank97 NFE2L2 Regulating Anti-Oxidant Detoxification Enzymes		Rank 82 glutathione metabolism	Rank 18 Melatonin in Cell Survival and Antioxidant Response	Rank 28 Reactive Oxygen Species Pathway	
Apoptosis	Rank 43 Intrinsic Pathway for Apoptosis	Rank 10 Apoptosis Related Network Due To Altered Notch3 Ovarian Cancer				
	Rank 93 Defective Intrinsic Pathway for Apoptosis	Rank 81 TNF Related Weak Inducer Of Apoptosis TWEAK Signalling	Rank 12 Apoptosis		Rank 9 Apoptosis	Rank 52 Regulation of Apoptotic Process

Supplementary Figure 5(II): Gene sets pathways in GBM-NK cells enriched in multiple gene set databases, suggests the TME induces dysfunctional phenotype in tumour infiltrating NK cells. Part 2) Summary of ORA data is arranged in the above table as follows: Columns list gene set databases e.g., ‘Reactome Pathways 2024’, that were selected from Enrichr analysis and cells within these columns list their enriched gene sets. All gene sets listed are reported as

significantly increased (statistically over-represented) (adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment (combined rank: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score) within the list of a database's enriched pathway is given in bold beside each named gene set. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched (statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human.

Functional Readouts	Some Relevant Upregulated Genesets (Top 100)					
	Reactome Pathways 2024	WikiPathways 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	MSigDB Hallmark 2020	GO Biological Process 2025
Inhibitory Signalling	Rank 99 VEGFA-VEGFR2 Pathway	Rank 1 prolactin signalling	Rank 50 VEGF signalling pathway	Rank 49 TGFβR Signalling in Pancreatic Neoplasms	Rank 27 TGF-β Signalling	
		Rank 16 TGF Beta Signalling Pathway		Rank 78 PDGFR -> AP-1/MYC Signaling		
		Rank 20 PDGF Pathway WP2526		Rank 70 VEGFR -> AP-1/CREB/MYC Signalling		
		Rank 53 VEGFA VEGFR2 Signaling				
Cancer related pathways	Rank 27 Signalling by ALK in Cancer	Rank 5 ONCOSTATIN m signalling	Rank 19 PD-L1 expression and PD-1 checkpoint pathway in cancer	Rank 21 RAS/RAF/MAPK Signaling Activation in Cancer	Rank32 p53 Pathway	
	Rank 54 Transcriptional Regulation by TP53		Rank 43 glioma	Rank 63 Oxidized LDL/OLR1 Activates Cell Proliferation in Cancer		
	Rank 63 Oncogenic MAPK Signalling			Rank 91 EGFR -> AP-1/CREB/ELK/SR F/MYC Signaling		
		Rank 15 Glioblastoma Signalling				
Other functional readouts	Rank 20 Regulation of Interferon Gamma Signalling	Rank 42 TNF Alpha Signaling	Rank 39 TNF signaling pathway		Rank 4 TNF-alpha Signaling via NF-kB	
	Rank32 Regulation of NFE2L2 Gene Expression	Rank 89 il6 signalling			Rank 19 Interferon Gamma Response	
Metabolic	Rank 53 Metabolism of Folate and Pterines	Rank 67 Insulin Signaling	Rank 48 Insulin signalling pathway		Rank 12 cholesterol metabolism	
	Rank 71 Inositol Phosphate Metabolism		Rank 83 glucagon signalling pathway		Rank 21 Fatty Acid Metabolism	
			Rank 97 Pentose phosphate pathway		Rank 31 Glycolysis	

Supplementary Figure 5(III): Gene sets pathways in GBM-NK cells enriched in multiple gene set databases, suggests the TME induces dysfunctional phenotype in tumour infiltrating NK cells. Part 3) Summary of ORA data is arranged in the above table as follows: Columns list gene set databases e.g., ‘Reactome Pathways 2024’, that were selected from Enrichr analysis and cells within these columns list their enriched gene sets. All gene sets listed are reported as

significantly increased (adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment (combined rank: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score) within the list of a database's enriched pathway is given in bold beside each named gene set. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched (statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human.

Functional Readouts	Geneset Databases					
	Reactome Pathways 2024	WikiPathways 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	MSigDB Hallmark 2020	GO Biological Process 2025
Immunological Synapse/ MHC Class presentation	Rank 3 Endosomal <u>Vacuolar</u> Pathway	Rank 1 Interactions Of Natural Killer Cells In Pancreatic Cancer WP5092	Rank 5 Antigen processing and presentation	Rank 1 MHC1 Causes Antigen Presentation Failure in Cancer Immune Escape		Rank 2 Peptide Antigen Assembly With MHC Protein Complex
	Rank 5 Antigen Presentation Folding, Assembly and Peptide Loading of Class I MHC			Rank 2 Genes with Mutations in Cancer Immune Escape		Rank 3 Antigen Processing and Presentation of Exogenous Peptide Antigen
	Rank 13 Translocation of ZAP-70 to Immunological Synapse			Rank 3 Proteins with Altered Expression in Cancer Immune Escape		Rank 8 MHC Class II Protein Complex Assembly
	Rank 22 Formation of Annular Gap Junctions		Rank 8 Phagosome	Rank 4 Endogenous Peptide Antigen Presentation		Rank 9 Ag Processing and Presentation of Endogenous Pep Ag via MHC Cls I via ER Pway
	Rank 25 Antigen processing-Cross Presentation			Rank 13 Natural Killer Cell Inhibitory Receptor Signaling		Rank 14 Peptide Antigen Assembly With MHC Class II Protein Complex
	Rank 26 Immunoregulatory Interactions Between a Lymphoid and a non-Lymphoid Cell		Rank 12 Cell adhesion molecules	Rank 17 MHC1-Mediated Antigen Presentation		Rank 18 MHC Class I Protein Complex Assembly
	Rank 50 MHC Class II Antigen Presentation			Rank 18 MHC2-Mediated Antigen Presentation		
	Rank 56 Downstream TCR Signaling			Rank 56 Natural Killer Cell Activation through ITAM-Containing Receptors		

Supplementary Figure 6(I): Gene sets pathways significantly decreased in GBM-NK cells, across multiple gene set databases, provide evidence that the TME hinders NK cells immune function, cellular machinery and metabolism. Part 1) Summary of GSEA ORA data is arranged in the above table as follows: Columns list gene set databases e.g., ‘Reactome Pathways 2024’, that were selected from Enrichr analysis and cells within these columns list their enriched gene

sets. All gene sets listed are reported as significantly increased decreased (- adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment (combined rank: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score) within the list of a database's enriched pathway is given in bold beside each named gene set. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched (statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human.

	Geneset Databases					
Functional Readouts	<u>Reactome</u> Pathways 2024	<u>WikiPathways</u> 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	<u>MSigDB</u> Hallmark 2020	GO Biological Process 2025
Sphingolipid Signalling/Metabolism	Rank 4 <u>Lysosphingolipid</u> and LPA Receptors	Rank 4 Ganglio Sphingolipid Metabolism	Rank 18 Glycosphingolipid biosynthesis	Rank 7 Ganglioside-Type Glycosphingolipid Biosynthesis		Rank 58 Ganglioside Metabolic Process
	Rank 7 Glycosphingolipid Biosynthesis	Rank 5 S1P Receptor Signal Transduction	Rank 24 Sphingolipid metabolism			
	Rank 21 Glycosphingolipid Metabolism	Rank 6 Mod Of PI3K Akt mTOR Signaling By Bioactive Sphingolipids	Rank 35 sphingolipid <u>signaling</u> pathway			
	Rank 32 Sphingolipid Metabolism	Rank 8 Biosynthesis And Turnover Of 1 Deoxy <u>Sphingoid</u> Bases WP5179				
Other Metabolism Pathways	Rank 8 RA Biosynthesis Pathway		Rank 42 Fructose and mannose metabolism (ALDOC)			Rank 75 fructose Metabolic Process
	Rank 45 <u>Signaling</u> by Retinoic Acid		Rank 39 Pentose phosphate pathway (ALDOC)			
	Rank 77 Arachidonate Metabolism					
Cytotoxic function	Rank 12 Interferon Gamma Signaling	Rank 1 GZMB Related Function	Rank 21 Natural killer cell mediated cytotoxicity		Rank 2 Interferon Gamma Response	Rank 1 Positive Regulation of Mast Cell Degranulation
						Rank 5 Natural Killer Cell Mediated Cytotoxicity
	Rank 29 GZMB Related Function					Rank 7 Positive Regulation of Leukocyte Degranulation
						Rank 33 Positive Regulation of Natural Killer Cell Cytokine Production
						Rank 38 Natural Killer Cell Degranulation

Supplementary Figure 6(II): Gene sets pathways significantly decreased in GBM-NK cells, across multiple gene set databases, provide evidence that the TME hinders NK cells immune function, cellular machinery and metabolism. Part 2) Summary of ORA data is arranged in the

above table as follows: Columns list gene set databases e.g., 'Reactome Pathways 2024', that were selected from Enrichr analysis and cells within these columns list their enriched gene sets. All gene sets listed are reported as significantly depleted (adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment (combined rank: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score) within the list of a database's enriched pathway is given in bold beside each named gene set. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched (statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human.

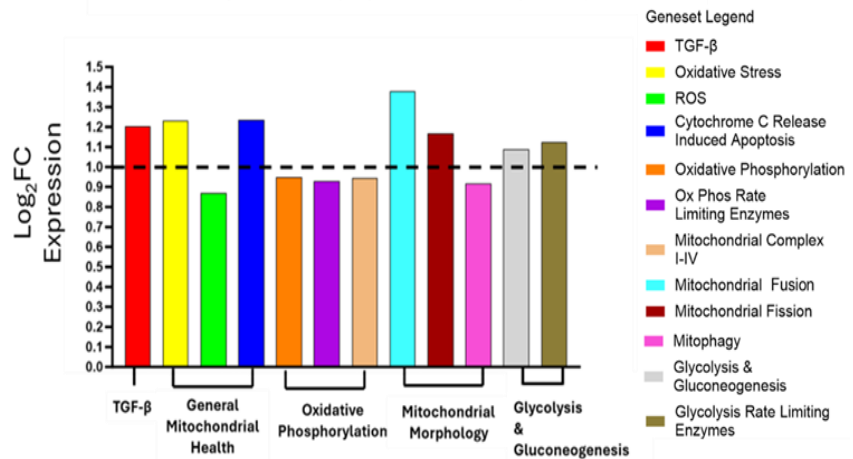
	Geneset Databases					
Functional Readouts	Reactome Pathways 2024	WikiPathways 2024 Human	KEGG 2021 Human	Elsevier Pathway Collection	MSigDB Hallmark 2020	GO Biological Process 2025
NK cell maturation/activation	Rank 9 FGFR1b Ligand Binding and Activation			Rank 14 FcIgER -> NFATC1 Signaling (fcgr3a)	Rank 8 p53 Pathway	Rank 49 Regulation of Natural Killer Cell Chemotaxis
				Rank 28 Natural Killer Cell Activation		
	Rank 18 FGFR2b Ligand Binding and Activation			Rank 34 Natural Killer Cell Precursor -> Natural Killer Cell Surface Expression Markers		
Programmed Cell Death and Recycle		Rank 43 Ferroptosis			Rank 4 Apoptosis	
Other Pathways	Rank 11 TGFβ R3 Regulates TGF-beta Signaling	Rank 12 Tgfr Disruption Of Shh Signaling		Rank 11 TGFβ R3 -> ARRB2 Signaling		
	Rank 23 TGFβ R3 PTM Regulation	Rank 18 tgfr3 mediated signalling				
		Rank 26 Type II Interferon Signaling				
Protein Synthesis and Degradation	Rank 14 ER-Phagosome Pathway		Rank 19 Proteasome			Rank 65 Regulation of Protein Glycosylation
	Rank 61 Proteasome Assembly					
DNA Synthesis and Repair	Rank 42 Mismatch Repair (MMR) Directed by MSH2 MSH3 (MutSbeta)	Rank 42 DNA Mismatch Repair	Rank 34 Base excision repair (POLD4)		Rank 12 DNA Repair	Rank 86 Regulation of Nuclear Cell Cycle DNA Replication
	Rank 49 Mismatch Repair		Rank 41 Mismatch Repair (POLD4)			
	Rank 31 Transport of Nucleosides and Free Purine and Pyrimidine 'Bases Across the Plasma Membrane					
Mitochondrial Complex	Rank 51 Complex IV Assembly	Rank 60 Mitochondrial Complex IV Assembly				Rank 92 Mitochondrial Cytochrome C Oxidase Assembly

Supplementary Figure 6(III): Gene sets pathways significantly decreased in GBM-NK cells, across multiple gene set databases, provide evidence that the TME hinders NK cells immune function, cellular machinery and metabolism. Part 3) Summary of ORA data is arranged in the

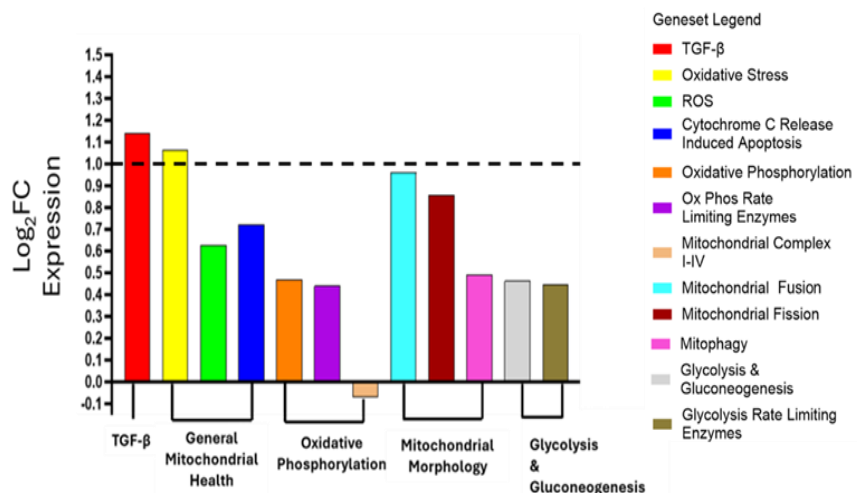
above table as follows: Columns list gene set databases e.g., 'Reactome Pathways 2024', that were selected from Enrichr analysis and cells within these columns list their enriched gene sets. All gene sets listed are reported as significantly depleted (adjusted p-value < 0.05) in GBM-NK within each respective gene set database. The rank of their enrichment (combined rank: log of the p-value, obtained from Fisher's Exact Test, multiplied by Enrichr's odd's ratio or z-score) within the list of a database's enriched pathway is given in bold beside each named gene set. These gene sets are additionally arranged by rows of functional categories that collectively describe similar pathway types enriched (statistically over-represented) within and across gene set pathway databases – for example Cancer Related Gene sets describe multiples gene sets enriched in multiple databases. For each functional readout category, very similar or identical gene set pathways shared across different databases, are visualised by a common colour per row: e.g., Within metabolic gene set pathways Sphingolipid signalling gene set is enriched in both WikiPathways 2024 Human and KEGG 2021 Human.

CD56^{Bright} GBM-NK cells Showcase Greater Fold Increase in Expression vs HD-PBMC-NK cells than CD56^{Dim} GBM-NK

CD56^{Bright} NK cells, Log₂FC in Expression:
GBM-NK vs HD-PBMC-NK

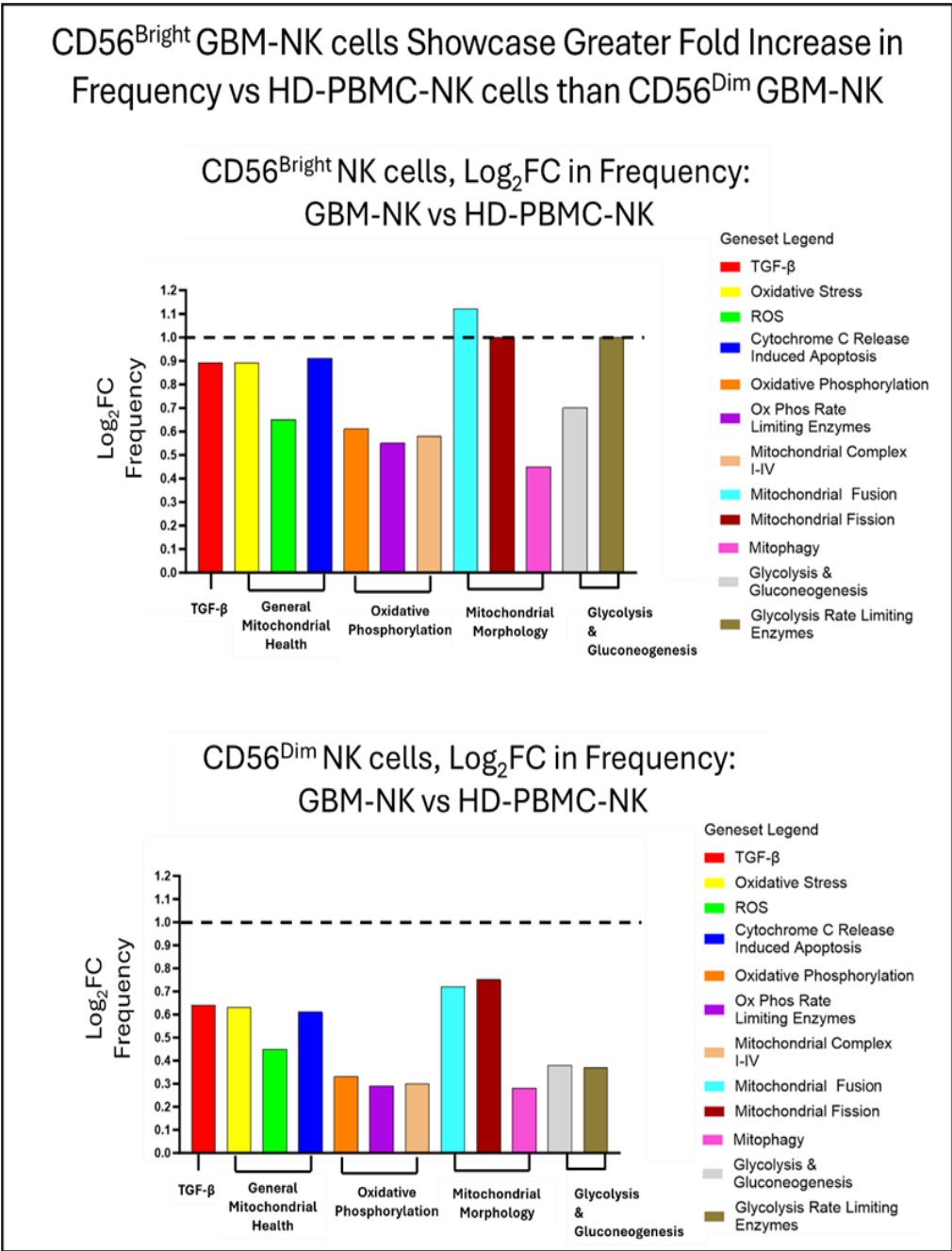


CD56^{Dim} NK cells, Log₂FC in Expression:
GBM-NK vs HD-PBMC-NK



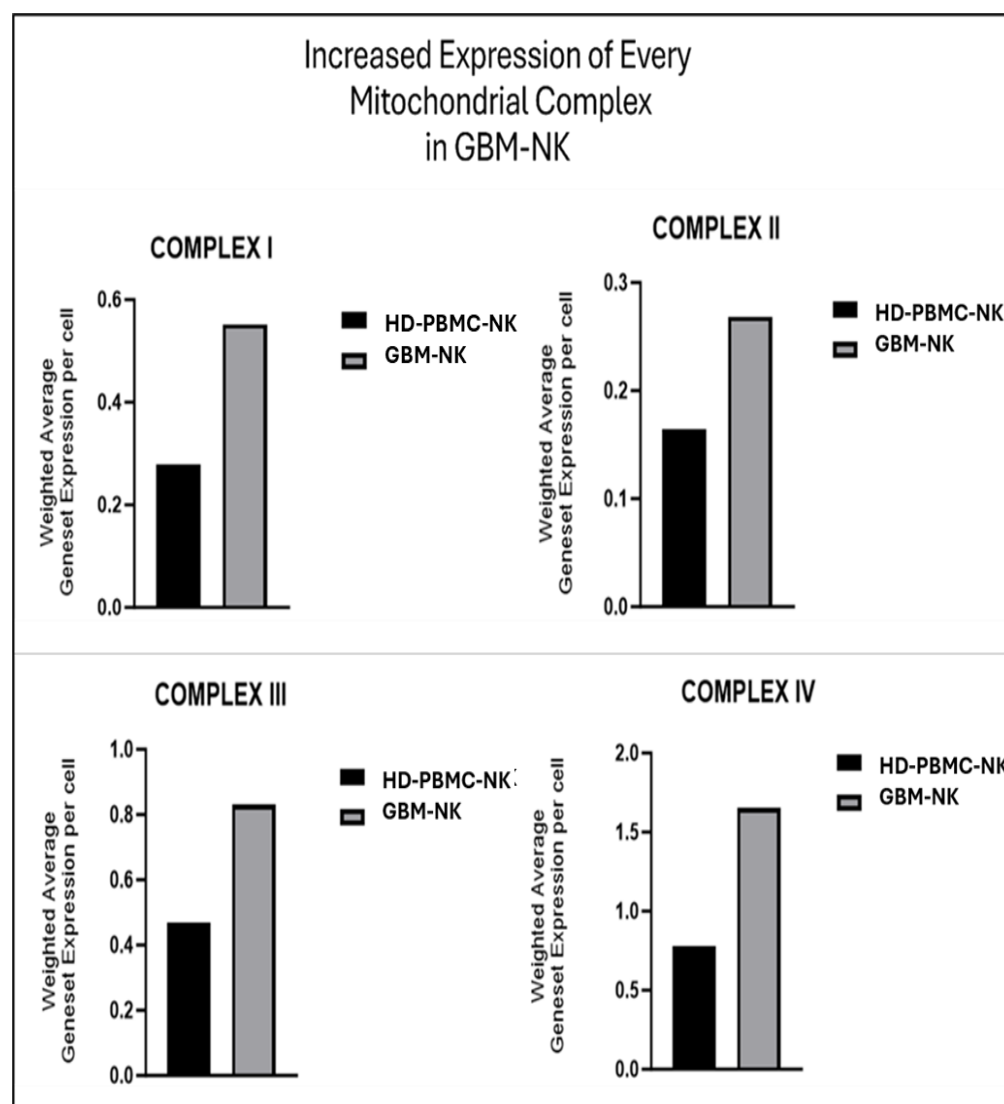
Supplementary Figure 7: CD56^{bright} GBM-NK cells Showcase Greater Fold Increase in Expression vs HD-PBMC-NK cells than CD56^{dim} GBM-NK. Top) Log₂FC in Expression of CD56^{bright} NK cells from GBM-NK vs HD-PBMC-NK cells. Bottom) Log₂FC in Expression of CD56^{dim} NK cells from GBM-NK vs HD-PBMC-NK cells. The y-axis corresponds to the Log₂ FC (Fold Change) of weighted average expression in GBM-NK vs HD-PBMC-NK. As shown in the Figure legend: solid bars colours corresponding to candidate gene sets. Dashed line at Log₂ FC = 1, represents threshold for 2 fold increase of expression in GBM-NK from HD-PBMC-NK. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF- β – 158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial

Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39, Mitophagy – 24, Glycolysis and Gluconeogenesis 172, and Glycolysis Rate Limiting Enzymes – 9.



Supplementary Figure 8: CD56^{bright} GBM-NK cells Showcase Greater Fold Increase in Frequency vs HD-PBMC-NK cells than CD56^{dim} GBM-NK. Top) Log₂FC in Frequency of CD56^{bright} NK cells from GBM-NK vs HD-PBMC-NK cells. Bottom) Log₂FC in Frequency of CD56^{dim} NK cells from GBM-NK vs HD-PBMC-NK cells. The y-axis corresponds to the Log₂ FC (Fold Change) of weighted average frequency in GBM-NK vs HD-PBMC-NK. As shown in the Figure legend: solid bars colours corresponding to candidate gene sets. Dashed line at Log₂ FC = 1, represents threshold for 2 fold increase of frequency in GBM-NK from HD-PBMC-NK. These candidate gene sets are grouped by Gene set families on the x-axis. Total genes available in gene set: TGF-β –

158, Oxidative Stress – 289, ROS – 44, Cytochrome C Release Induced Apoptosis – 69, Oxidative Phosphorylation (OXPHOS) – 93, OXPHOS Rate Limiting Enzymes – 27, Mitochondrial Complexes I-IV – 54, Mitochondrial Fusion – 29, Mitochondrial Fission – 39, Mitophagy – 24, Glycolysis and Gluconeogenesis 172, and Glycolysis Rate Limiting Enzymes – 9.



Supplementary Figure 9: Mitochondrial Complexes I, II, III and IV all have higher expression in GBM-NK vs HD-PBMC-NK cells. Expression of candidate gene sets in GBM-NK and HD-PBMC-NK cells were compared using the weighted average gene expression per cell: the sum of expression averages for every gene in gene set normalised to the number of cells in the GBM-NK (23.1k) or HD-PBMC-NK (5.5k) cohorts. The y-axis corresponds to the absolute values of weighted average expression for each individual gene set. As shown in the Figure legend: black bars represent HD-PBMC-NK cells while grey bars represent GBM-NK cells. Total genes (available in dataset) in gene set: Mitochondrial Complex I – 33, Mitochondrial Complex II – 3, Mitochondrial Complex III – 8, Mitochondrial Complex IV – 10.

5.2 Supplementary Tables

5.2a Statistics for DEGs from PCA Plots

Statistical Info for DEGs: GBM-NK vs HD-PBMC-NK PCA		
Gene	Log ₂ FC in Expression	Adjusted p-value
CCL4	21.687508	0
AREG	13.238701	0
HSP90AA1	9.343439	1.00E-266
ACTG1	10.37238	0
SRGN	7.294927	1.00E-255
CD69	6.277986	1.00E-218
ZFP36L2	5.763028	1.00E-203
ACTB	24.375063	0
HSP90AB1	5.386985	1.00E-191
CFL1	6.532691	1.00E-224
TSC22D3	4.732198	1.00E-182
DUSP1	5.038731	1.00E-186
XCL2	6.81247	1.00E-240
CTSW	5.760755	1.00E-198
JUND	6.191007	1.00E-210
VIM	5.70687	1.00E-196
TPT1	15.527218	0
EEF1B2	4.216855	1.00E-180
FOS	4.992722	1.00E-184
RGS1	4.962611	1.00E-183

Supplementary Table 1: Statistical Info for DEGs: GBM-NK vs HD-PBMC-NK PCA, to complement figure 8.

Statistical Info for DEGs: PCA plot for CD56 ^{bright} - GBM-NK vs HD-PBMC-NK		
Gene	Log ₂ FC in Expression	Adjusted p-value
CCL4	22.769388	0.00E+00
ZFP36L2	7.109545	0.00E+00
AREG	12.431123	2.68E-300
CCL5	19.438162	3.06E-288
SRGN	7.934758	4.62E-281
HSP90AA1	8.538612	7.17E-276
RGS1	5.203706	1.20E-272
ACTG1	11.93659	4.27E-270
CD69	6.393727	5.05E-270
CFL1	7.519491	1.48E-252
FTH1	6.918301	7.33E-237
PPDPF	4.309499	2.54E-232
ALOX5AP	3.491417	7.66E-219
LCP1	3.769895	3.43E-215
XCL2	6.334254	5.33E-207
ACTB	28.147333	5.52E-204
SERF2	5.21037	6.29E-204
COTL1	5.405786	1.00E-197
EVL	3.432751	7.81E-196
KRT86	4.654651	1.37E-194

Supplementary Table 2: Statistical Info for DEGs: CD56^{bright} GBM-NK vs HD-PBMC-NK PCA, to complement figure 8 and Supplementary Figure 4.

Statistical Info for DEGs: PCA plot for CD56 ^{dim} GBM-NK vs HD-PBMC-NK		
Gene	Log ₂ FC in Expression	Adjusted p-value
CCL4	23.977524	0.00E+00
AREG	11.805734	0.00E+00
HSP90AA1	10.134139	0.00E+00
TSC22D3	5.251328	0.00E+00
CALM1	4.385964	0.00E+00
KLRD1	3.456797	1.75E-266
JUND	5.279487	4.92E-255
ACTG1	5.324097	4.74E-240
SRGN	4.497762	1.63E-224
HSP90AB1	4.163301	4.19E-218
DUSP1	4.023175	4.70E-210
CFL1	3.738168	4.33E-203
ACTB	10.762227	9.39E-198
SPON2	2.801282	1.10E-196
ZFP36L2	3.635521	1.24E-196
JUNB	3.643916	6.78E-191
SYTL3	3.964198	2.26E-186
CD69	4.48053	1.42E-184
EIF1	4.105713	1.02E-183
TXNIP	4.117615	5.39E-182

Supplementary Table 3: Statistical Info for DEGs: CD56^{dim} GBM-NK vs HD-PBMC-NK PCA, to complement figure 8 and and Supplementary Figure 4.

Statistics for DEGs from Significant Candidate Genes

Statistics for Significantly Increase TGF β genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
ID2	1.45	84.31
JUNB	2.23	76.50
JUND	4.02	61.07
JUN	1.37	52.46
MAPK1	1.41	45.97
PPP1R15A	1.86	45.76
FKBP1A	1.77	42.46
CTNNB1	1.87	26.34
CD9	6.90	22.39
IFNG	3.12	17.69
TFDP1	2.67	15.62
SKI	2.14	15.38
CUL1	1.72	15.19
EP300	1.64	15.06
SMURF2	1.97	13.85
MAPK14	1.67	12.54
SPTBN1	2.03	11.86
MYC	3.79	10.58
ID3	5.29	10.56
APC	1.70	9.72
TFRC	3.11	9.22
MAPK13	2.12	8.94
MAPK8	2.57	8.58
ATF3	4.14	8.16
NCOR2	1.63	8.12
NBL1	2.21	7.55
MAPK3	1.69	7.53
RNF111	1.67	7.53
HRAS	1.93	6.77
MAPK9	1.75	6.70
PPP2CB	1.62	5.10
ACVR1B	2.98	4.30
VPS54	2.26	4.12
PPP2R1B	1.66	4.12
SMURF1	2.45	3.79
GDF11	3.19	3.29
MAPK12	5.12	2.19
TGIF1	-1.15	8.61

Supplementary Table 4: Statistical Info Significantly Increased and Decreased TGF- β Genes:

Log2 Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Oxidative Stress genes		
Genes	Log2 Fold Change in Expression	Frequency in GBM-NK (%)
PPIA	1.54	85.58
TXNIP	1.12	80.78
GSTP1	2.07	75.16
FOS	2.47	67.79
ZFP36	1.95	66.69
AREG	7.70	63.65
NR4A2	2.72	52.76
JUN	1.37	52.46
TNFAIP3	2.19	47.45
MAPK1	1.41	45.97
TXN	1.87	44.56
HSPA1A	5.34	43.59
NDUFS8	1.53	42.08
SLC2A3	1.61	40.91
CD38	1.94	35.61
PRDX2	1.54	33.88
PRDX1	2.07	33.48
NFE2L2	1.87	33.12
PLCG2	3.79	28.61
HSPA1B	6.46	28.42
SETX	1.84	25.41
HM13	1.55	25.27
BCL2	5.10	23.87
NCF1	3.40	22.49
ERCC1	1.85	19.99
CASP3	1.97	19.90
PRDX3	1.70	18.94
MTHFD2	1.98	17.76
NUDT1	1.81	17.72
KDM6B	1.71	16.35
SESN1	1.77	16.30
IPCEF1	1.64	13.93
GCH1	1.87	13.17
PDXK	2.05	11.48
EZH2	2.48	11.15
PIK3R5	1.63	10.96
AKT1	1.56	10.70
ATP2A2	2.45	9.88
MSRA	2.37	9.31
EPAS1	5.03	9.27
PLCB1	3.71	9.25
FOXO1	3.01	9.08
MAPK13	2.12	8.94
KAT2B	2.19	8.85
MAPK8	2.57	8.58
MTHFD1	1.93	8.52
GSR	2.01	8.37
FUT8	1.98	8.30
SLC25A32	1.64	8.24
MAPK3	1.69	7.53
MAP2K4	2.26	7.51
DHFR	2.60	7.50
ABL1	2.16	7.41
RUNX2	4.01	7.22
ABCC1	3.49	6.92
MAPK9	1.75	6.70
SIRT1	1.92	6.49
GCLC	1.68	6.21
MAP1LC3A	2.47	5.67
SLC25A12	1.92	5.43
FER	2.86	5.29
JAK2	1.91	5.25
STK11	1.72	5.08
SMPD3	3.53	4.84
AGAP3	2.45	4.64
CDK1	7.12	4.45
APOE	8.02	3.77
AIF1	4.27	3.58
PLCG1	1.83	3.32
FANCD2	2.25	3.24
ERCC6	2.14	3.24
CPEB2	1.96	3.22
AIFM2	2.55	3.17
APP	3.20	3.15
SLC25A29	2.31	2.54
PLK1	5.21	2.16
TGFBR3	-0.58	8.52
DUOX1	-2.17	1.24
SLC25A6		0.00

Supplementary Table 5: Statistical Info Significantly Increased and Decreased Oxidative Stress

Genes: Log2 Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increase ROS genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
ABCC1	3.49	76.50
MSRA	2.37	44.56
JUNB	2.23	33.48
SBNO2	2.13	33.88
PRDX1	2.07	13.93
GSR	2.01	13.34
TXN	1.87	9.31
TXNRD1	1.74	8.37
GCLC	1.68	6.92
IPCEF1	1.64	6.21
PRDX2	1.54	4.78
FTL	-1.105	83.14

Supplementary Table 6: Statistical Info Significantly Increased and Decreased ROS Genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased Cytochrome C Release Induced Apoptosis genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
PRELID1	1.53	59.63
MCL1	1.89	58.78
MAPK1	1.41	45.97
TNFSF10	1.66	27.41
BCL2	5.10	23.87
CASP3	1.97	19.90
GGCT	1.69	13.85
BCL2L1	1.63	13.34
BCL2L11	2.87	12.52
BCL2A1	2.61	12.09
AKT1	1.56	10.70
OPA1	2.05	10.68
LMNA	2.59	7.98
MAPK3	1.69	7.53
AVEN	3.58	5.67
CASP6	1.82	4.59
ENDOG	1.54	4.40

Supplementary Table 7: Statistical Info Significantly Increased Cytochrome C Release Induced Apoptosis genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased OXPHOS genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
COX5A	1.73	52.01
NDUFB9	1.30	42.22
NDUFS8	1.53	42.08
PPA1	2.02	25.15
NDUFA10	1.45	24.61
TCIRG1	1.38	23.57
RERE	1.77	12.38
ATP1B1	2.72	9.01
ATP6V0A2	1.59	8.33
ATP6V0C	5.26	6.42
COX10	1.98	4.17

Supplementary Table 8: Statistical Info Significantly Increased OXPHOS genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased OXPHOS Rate Limiting Enzyme genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
COX5A	1.73	52.01
HIGD2A	1.33	48.18
COX10	1.98	4.17
COX16	-1.77	8.42

Supplementary Table 9: Statistical Info Significantly Increased and Decreased OXPHOS Rate Limiting Enzymes genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased Mitochondrial Complexes I-IV genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
NDUFS8	1.53	69.09
COX5A	1.73	52.01
NDUFA10	1.45	31.01

Supplementary Table 10: Statistical Info Significantly Increased Mitochondrial Complexes I-IV genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased Mitofusion genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
MCL1	1.89	58.78
HUWE1	2.23	17.93
CHCHD3	1.59	15.17
BCL2A1	2.61	12.09
OPA1	2.05	10.68
TFRC	3.11	9.22
MFN1	1.70	6.52

Supplementary Table 11: Statistical Info Significantly Increased Mitofusion genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased Mitofission genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
SPIRE1	3.57	3.48
AURKA	2.99	3.11
MCU	2.73	3.76
TMEM135	2.62	4.96
MTFR1	2.28	4.49
DDHD1	2.11	17.39
OPA1	2.05	10.68
COX10	1.98	4.17
AP3B1	1.81	15.48

Supplementary Table 12: Statistical Info Significantly Increased Mitofission genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased Mitophagy genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
MAP1LC3A	2.03	5.67
CSNK2A2	1.19	7.93
MFN1	1.08	6.52

Supplementary Table 13: Statistical Info Significantly Increased Mitophagy genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increase Glycolysis and Gluconeogenesis genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
CENPA	7.80	2.42
CDK1	7.12	4.45
HMMR	5.40	3.15
GMPPB	4.87	6.12
STMN1	4.53	26.27
EXT1	4.23	5.15
AURKA	2.99	3.11
SAP30	2.29	4.31
GFPT1	2.05	8.47
B3GALT6	2.05	5.24
PMM2	2.02	5.55
NASP	1.99	36.68
FUT8	1.98	8.30
HS2ST1	1.90	4.70
PGM2	1.89	10.49
TXN	1.87	44.56
GALE	1.86	6.84
B4GALT4	1.84	11.03
DDIT4	1.84	69.49
IER3	1.83	13.62
CASP6	1.82	4.59
SLC25A13	1.75	3.65
ECD	1.71	9.72
B4GALT1	1.71	24.58
POLR3K	1.71	18.11

Supplementary Table 14: Statistical Info Significantly Increased Glycolysis and Gluconeogenesis genes: Log₂ Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Significantly Increased Glycolysis Rate Limiting Enzyme genes		
Genes	Log ₂ Fold Change in Expression	Frequency in GBM-NK (%)
LC16A1	1.93	4.07

Supplementary Table 15: Statistical Info Significantly Increased Glycolysis Rate Limiting Enzymes genes: Log2 Fold Change in Expression in GBM-NK vs HD-PBMC-NK and Frequency (%) in GBM-NK.

Statistics for Targeted TGF- β GSEA

Geneset	ES	NES	NOM p-val	FDR q-val	FWER p-val	Gene set Tag %	Leading Genes
Arachidonic acid metabolism Homo sapiens hsa00590	-0.71918	-1.68559	0.004878	0.090433	0.099	14.29%	PLA2G1B; PLA2G5; PTGES; CYP2J2; ALOX12; GPX3
Complement and coagulation cascades Homo sapiens hsa04610	0.813093	1.614886	0	0.047957	0.042	15.63%	KLKB1; SERPIND1; THBD; SERPINF2; F5; C7; CPB2; C9; CR1; F10

Supplementary Table 16. Statistical Information for GSEA of differential gene expression of High TGF- β vs Low TGF- β GBM-NK cells

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