NK cell function and metabolism in human cancer

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By Karen Slattery

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Supervised by Prof. Clair Gardiner

Declaration

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Karen Slattery

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Thesis Outputs

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TGFβ drives mitochondrial dysfunction in peripheral blood NK cells during metastatic breast cancer*

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Oral Presentations

- March 2018: Immunology Research Forum, Dublin.
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Abstract

Natural Killer (NK) cells are important anti-cancer innate immune cells. They are essential for cancer immunosurveillance and their activity is associated with better outcome in cancer patients. Some of their key functions include expression of cytotoxic molecules such as granzymes, production of the anti-tumour cytokine IFNγ, and antibody dependent cellular cytotoxicity (ADCC), which involves the killing of tumour cells coated with antibodies. Highlighting the importance of this particular function, NK cell ADCC has been harnessed through the development of monoclonal antibody therapies such as Trastuzumab (anti-HER2, breast cancer) and Dinutuximab (anti-GD2, neuroblastoma), which boost NK cell mediated ADCC against patient tumours and have become mainstream in clinical practice.

Despite their fundamental role in protection against cancer, the anti-tumour functions of NK cells often become impaired in patients who have cancer, and indeed this has been documented across a wide range of haematological and solid tumour cancers. This dysfunction likely impinges upon the efficacy of NK cell based immunotherapies, such as monoclonal antibody therapy and NK cell adoptive transfer therapy. Therefore, there is an urgent need to better understand the mechanisms that underlie NK cell dysfunction during cancer, so that we can identify novel therapeutic targets that can be used to reverse these defects.

Several important studies from the past decade have revealed that cellular metabolism is essential for human NK cell responses. For example, cytokine stimulated NK cells upregulate both oxidative phosphorylation (oxphos) and glycolysis to support their functions. Mammalian target of rapamycin complex 1 (mTORC1) drives this metabolic shift towards glycolysis and is also required for the functions of NK cells. Interestingly, NK cells shuttle their mitochondria towards the site of interaction with tumour cells, highlighting how fundamental metabolism is for cytotoxicity against tumour cells. While, the role that metabolism plays in driving NK cell functional responses is now well documented, whether metabolism is involved in NK cell dysfunction in cancer patients remains less clear. Therefore, we hypothesised that altered cellular metabolism might underlie NK cell dysfunction during cancer.

In this thesis, we show that peripheral blood NK cells from metastatic breast cancer and paediatric neuroblastoma patients have impaired metabolism, which, amongst others, is associated with defective IFNγ production. While a shift towards one form of metabolism was expected, both oxphos and glycolysis were severely reduced in patient NK cells, indicating metabolic paralysis. At the level of the organelle, NK cells from breast cancer and neuroblastoma patients had increased mitochondrial mass and mitochondrial membrane polarisation (MMP), as well as high levels of mitochondrial reactive oxygen species (mROS). Confocal microscopy analysis revealed that patient NK cells have modified mitochondrial morphology characterised by increased mitochondrial fragmentation, as seen by their round, punctate mitochondria, versus the long, elongated mitochondria generally observed in NK cells from healthy donors. Altered mTORC1 activity was also observed in NK cells from both patient groups, and is likely contributing towards the impaired metabolism and function.

TGFβ is highly implicated in cancer development and progression, and work from our lab recently showed that TGFβ inhibits healthy donor NK cell metabolism and function. Therefore, we hypothesised that TGFβ might play a role in driving some of the functional and metabolic defects observed. TGFβ neutralising antibodies were added to overnight cell cultures of NK cells from metastatic breast cancer patients. This treatment strikingly improved NK cell mitochondrial metabolism, IFNγ production and mTORC1 activity, indicating that TGFβ contributes towards NK cell metabolic dysfunction during cancer.

Importantly, we reveal for the first time that the GARP/TGFβ axis, which involves the intrinsic anchoring of TGFβ to the NK cell membrane, is a key driver of NK cell dysfunction during cancer. By adding monoclonal antibodies that block GARP/TGFβ complexes to pure NK cell cultures overnight, we were able to recapitulate many of the effects of anti-TGFβ neutralising antibodies, and improve IFNγ production as well as granzyme B expression in patient NK cells.

Finally, we explored the metabolic requirements of NK cell anti-GD2 mediated ADCC against neuroblastoma tumour cells, using pharmacological inhibitors against various metabolic pathways and regulators. The data clearly indicate that glycolysis and not oxphos is required for NK cell anti-GD2 mediated ADCC. Indeed, inhibition

of glycolysis reduces ADCC and this is associated with reduced degranulation against anti-GD2 coated neuroblastoma tumour cells. Furthermore, we show that while mTORC1 is not required for this process, active amino acid transport though the SLC7A5 transporter is essential, and we suggest this could be required to sustain cMyc activity, which in turn may promote NK cell ADCC.

Overall, we have shown in two different types of solid tumour cancers that reduced cellular metabolism and mTORC1 activity underlies peripheral NK cell dysfunction. Perhaps most significant is the finding that this altered metabolism is targetable and reversible, and that this can be achieved using monoclonal antibodies against TGFβ alone as well as GARP/TGFβ complexes. Furthermore, our data support a therapeutic strategy whereby NK cell glycolysis and amino acid transport is supported when neuroblastoma patients are receiving anti-GD2 therapy. These findings have significant implications for the design of future NK cell based immunotherapies against solid tumour cancers.

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Abbreviations

25HC	25-Hydroxycholestrtol
2DG	2-Deoxy-D-Glucose
ACC	Acetyl Coa Carboxylase
ACSS	Acetyl-Coenzyme A Aynthetase 2-Like, Mitochondrial
ADCC	Antibody Dependent Cellular Cytotoxicity
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ANGPTL4	Angiopoietin-Like 4
AP1	Activator Protein 1
BCH	2-Aminobicycloheptane-2-Carboxylic Acid
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukaemia
CPT	Carnitine Palmitoyltransferase
CSC	Cancer Stem Cells
DC	Dendritic Cells
DISC	Death-Inducing Signalling Complex
DON	6-Diazo-5-Oxo-L-Norleucine
ECAR	Extracellular Acidification Rate
ELISA	Enzyme-Linked Immunosorbent Assay
Eomes	Eomesodermin
ER	Estrogen Receptor
$FADH_2$	Flavin Adenine Dinucleotide
FAO	Fatty Acid Oxidation
FasL	Fas Ligand
FASN	Fatty Acid Synthase
FBP1	Fructose-1,6-Bisphosphatase
FBS	Foetal Bovine Serum
FDA	Food And Drug Administration
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GARP	Glycoprotein-A Repetitions Predominant
GC/MS	Gas/Chromatography-Mass/Spectrometry

HBSS	Hanks Balanced Salt Solution
HBV	Hepatitis B Virus
HIF1α	Hypoxia-Inducible Factor 1α
HLA	Human Leukocyte Antigen
IDO	Indoleamine-Pyrrole 2,3-Dioxygenase
IFN	Interferon
Ig	Immunoglobulins
ILC	Innate Lymphoid Cells
IRE1	Inositol-Requiring Enzyme 1
IS	Immune Synapse
ITAM	Immunoreceptor Tyrosine-Based Activation Motifs
ITIM	Contain Immunoreceptor Tyrosine-Based Inhibition Motifs
KIR	Killer Immunoglobulin-Like Receptors
LAP	Latency Associated Peptide
LLC	Large Latent Complex
LRRC32	Leucine Rich Repeat Containing 32
LTBP	Latent TGF-B-Binding Protein
MDS	Myelodysplastic Syndrome
MDSC	Myeloid Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MICA	MHC-I Polypeptide-Related Sequence A
MICB	MHC-I Polypeptide-Related Sequence B
MMP	Mitochondrial Membrane Polarisation
mROS	Mitochondrial Reactive Oxygen Species
MTOC	Microtubule Organising Centre
mTORC	Mammalian Target Of Rapamycin Complex
NADH	Nicotinamide Adenine Dinucleotide
NB	Neuroblastoma
NCR	Natural Cytotoxcity Receptors
NK	Natural Killer
NSCLC	Non-Small Cell Lung Cancer
OCR	Oxygen Consumption Rate
Oxphos	Oxidative Phosphorylation
PBMC	Peripheral Blood Mononuclear Cells

PBS	Phosphate Buffered Saline
PGC-1α	Proliferator-Activated Receptor Gamma Coactivator 1-A
PGE2	Prostaglandin E2
PPP	The Pentose Phosphate Pathway
PR	Progesterone Receptor
PTP1B	Protein-Tyrosine Phosphatase 1B
RA	Rheumatoid Arthritis
RSK	Ribosomal S6 Kinase
SHIP1	Src Homology 2 Domain Containing Inositol Polyphosphate 5-Phosphatase 1
SLC	Small Latent Complex
SMAC	Supramolecular Activation Cluster
SREBP	Sterol Regulatory Element Binding Protein
TAM	Tumour Associated Macrophages
TCA	Tricarboxylic Acid Cycle
TGFβ	Transforming Growth Factor B
TGFβR1	TGFβ Receptor 1
TGFβR2	TGFβ Receptor 2
TME	Tumour Microenvironment
TNF	Tumour Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UPR	Unfolded Protein Response
VEGF	Vascular Endothelial Growth Factor
WASP	Wiskott-Aldrich Syndrome Protein

Chapter 1

1. Introduction

1.1 Cancer in today's world

Cancer has become a disease that impacts the lives of everyone around the world, whether directly or through a friend or family member. 1 in 5 men and 1 in 6 women will develop cancer over the course of their lifetime. In 2018, this culminated in over 17 million new cases of cancer. Hence, it is no surprise that cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths in 2018 (WHO.int). Unfortunately, these numbers are expected to rise, with experts estimating that 27.5 million cases will be diagnosed annually by the year 2040 (cancerresearch.uk). This is largely due to the fact that we are now surviving a lot longer than our bodies evolved to cope with. Indeed, more than three-quarters of all people diagnosed with cancer in the UK are over the age of 60. This is also due to a number of other factors, many of which are related to the western lifestyle e.g. smoking, alcohol, reduced fruit and vegetable intake and increased meat consumption. Most of these lifestyle habits increase the incidence of obesity, diabetes, cardiovascular disease and indeed, cancer.

On the other hand, advancements in science and medicine have significantly improved the survival rate of many cancer types. Cancer patient survival has doubled over the past 40 years, and today half of people diagnosed with cancer will survive for at least another 10 years. This is in stark contrast to the situation in the 1970s, where only 1 quarter of patients survived 10 years. Indeed, there is now a much wider range of treatment options available for patients. Surgery, chemotherapy, radiotherapy and endocrine therapy still form the backbone of most treatment regimes, and specific combinations of these continue to be optimised through clinical trials. A more recent milestone in cancer treatment was the onset of the targeted therapy approach, whereby small molecule inhibitors are used to target specific features of tumours in certain patients. This milestone brought with it the era of 'personalised medicine', which has revolutionised cancer therapy in the clinic. Indeed, it has become commonplace for patients to have their tumours sequenced

for specific mutations, for which we have targeted therapies. For example, imatinib is an inhibitor of the BCR–ABL kinase, the fusion protein that results from a chromosomal translocation and is a common hallmark of chronic myeloid leukaemia (CML) [1]. When imatinib was used to treat patients with chronic-phase CML, 90% achieved complete haematological remission versus just 20% of patients that received treatment options that were standard at the time [2].

Importantly, these traditional methods are now supported by immunotherapies such as monoclonal antibody therapy and checkpoint inhibitor therapy [3]. Furthermore, immune cell infusions (i.e. cell transfer therapy), which aims to boost immune cell populations in cancer patients have already been proven safe and so far evidence suggests that NK cell transfer therapy produces minimal toxic side effects [4] and is unlikely to induce graft versus host disease [5]. For example, autologous NK cells (which uses the patient's own NK cells, expanded *ex vivo*) have been used in the treatment of paediatric medulloblastoma and ependymoma [6], while allogeneic (NK cells from a third party, in this case PBMC) have been used to treat acute myeloid leukaemia patients (AML) [7, 8]. Indeed, the advancement of these immunotherapies is considered one of the greatest medical achievements in modern times. Highlighting this, the 2018 Nobel Prize in Physiology or Medicine was jointly awarded to James P. Allison and Tasuku Honjo for their fundamental research on discovering how to take the brakes off the immune system in order to activate it to fight cancer, which ultimately led to the development of modern checkpoint inhibitors.

Cancer prognoses have improved in recent years as a result of these scientific and medical achievements. However, certain types of cancer still have very poor 5 year survival rates, e.g. pancreatic cancer (7%), distant triple negative breast cancer (11%) and brain cancer (12%). Furthermore, the vast majority of cancer related deaths are caused by metastatic disease [9], as it significantly reduces the survival rate of patients, e.g. 99% and 27% 5 year survival rate for local and metastatic breast cancer respectively (cancer.org). Resistance to treatment remains a major issue, even with modern targeted and immune therapies. The genetic diversity within a tumour is so great that it is highly likely that a mutation exists that will eventually confer resistance. Indeed, McGranahan *et al.* showed that tumour heterogeneity (intratumour clonal neoantigens) is associated with reduced immune cell infiltration and survival in lung cancer and melanoma patients treated with anti-

PD1 and anti-CTLA4 [10]. In another study on 54 advanced melanoma patients, only 7 patients responded to anti-CTLA4 therapy, and among the non-responders, only 14 responded to anti-PD1 therapy [11]. Although we have come a long way in terms of cancer therapy, there is still a long road ahead. Given the remarkable success that immunotherapy has had in the clinic so far, there is now an extensive, international effort being put into the development of new, innovative immunotherapies for the treatment of patients for whom current methods fail.

NK cells are important innate lymphocytes that play a vital role in protection against cancer. Seaman *et al.* demonstrated this in 1987, whereby mice depleted of NK cells had significantly increased tumour burden in a B16 melanoma and colon cancer model [12]. Similarly, increased frequency of peripheral NK cells correlates with increased survival many cancer types e.g. colorectal cancer patients [13]. Given this, NK cells are attractive targets for the development of new cancer immunotherapies. The caveat of targeting NK cells is that tumours create a severely immunosuppressive environment that impacts the immune system, and indeed NK cells from cancer patients frequently become dysfunctional compared to those from healthy individuals [14-16]. This dysfunction will then hamper any efforts to target NK cells for the treatment of the cancer. Therefore, there is an urgent need to better understand NK cell dysfunction in cancer so that we can design better therapies that are resilient to cancer's immunosuppressive effects. This thesis investigates the cellular metabolism of peripheral blood NK cells in two types of cancer –metastatic breast cancer, a common adult carcinoma, and neuroblastoma, a rare paediatric cancer of the sympathetic nervous system. Mechanisms driving the dysfunctional phenotype and potential therapeutic targets are explored.

1.2 NK cells

NK cells are innate lymphocytes that function in the protection against virally infected and cancerous cells. They are categorised as group 1 innate lymphoid cells (ILCs) as they secrete the cytokines interferon (IFN)-γ and tumour necrosis factor (TNF)-α [17, 18]. Unlike others in this group, they also possess many cytotoxic functions similar to those seen in CD8+ T cells, in addition to features of immunological memory [19, 20]. In general, they provide a fast acting, non-specific response to their targets, including uniquely mediating recognition of cells that lack major histocompatibility complex (MHC) class I. This is achieved in part through the expression of germ-line encoded activating and inhibitory receptors [21]. The balance of these receptors and the inflammatory environment surrounding the NK cells contribute to their activity and functions.

Human NK cells are classically divided into 2 subsets, $CD56^{bright}$ and $CD56^{dim}$ NK cells. $CD56^{bright}$ NK cells are expert producers of cytokine, mainly IFNγ. They represent 10% of total NK cells in the blood, yet this can increase to up to 50% of total tissue resident NK cells in various locations throughout the body, for example in the liver and in the pregnant uterus [22]. $CD56^{dim}$ NK cells make up the remaining 90% of NK cells in the blood. They are cytotoxic lymphocytes that express high levels of the CD16 Fc receptor (**Figure 1.1**) [23]. Evidence suggests that $CD56^{dim}$ cells arise from $CD56^{bright}$ cells during the development of NK cells, yet the two populations remain in equilibrium throughout a healthy individual's lifetime, with relatively consistent proportions of each at any given time [24, 25].

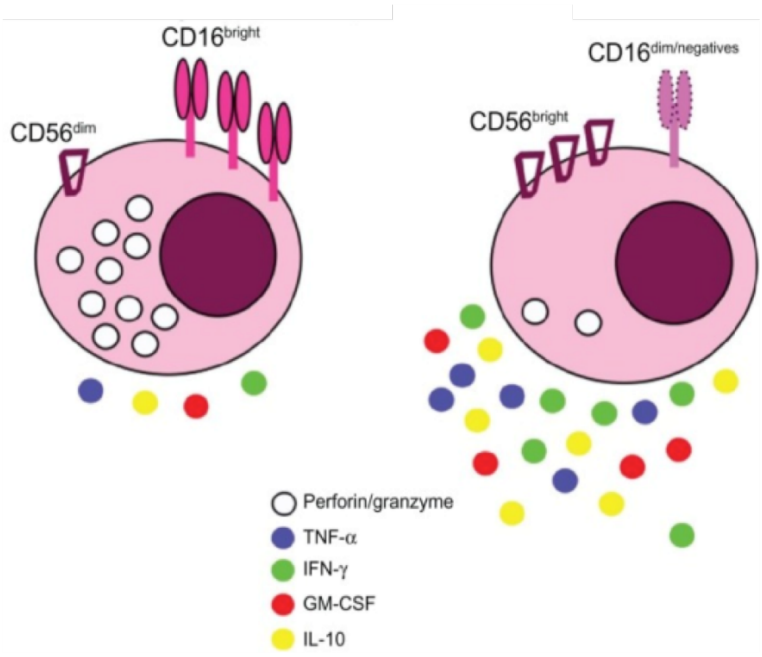


Figure 1.1 CD56^{bright} and CD56^{dim} NK cells.

CD56^{bright} NK cells are expert producers of cytokine such as IFNγ, while CD56^{dim} NK cells have cytolytic granules within their cytosol that they release upon contact with a target cell. They also express high levels of CD16, important for antibody dependent cellular cytotoxicity [26].

1.3 The functions of NK cells

NK cells are one of the immune system's most important defences against viral infection and cancer. As such, they use a wide variety of mechanisms to effectively neutralize their targets, including the production of cytokines, cytotoxicity and ADCC (**Figure 1.2**).

1.3.1 Cytokine production by NK cells

Cytokines are widely used by NK cells in order to carry out their functions and to shape the functions of adaptive immune cells. IFNγ production is a hallmark of the NK cell response, in particular for the CD56^{bright} subset. It is the only member of the type II interferon group, and has many immunoregulatory, anti-viral and anti-tumour properties. It signals through the Jak-Stat signalling pathway, which involves sequential recruitment of dimeric receptors and activation of Jak-1 and -2 and Stat-1 and -2 to control the transcription of target genes [27]. IFNγ's protective role against cancer has been demonstrated in several murine studies. Kalplan *et al*. showed that

IFNγ receptor-deficient mice developed chemically induced tumours more rapidly and had a greater tumour burden than wild type mice [28]. Street *et al.* demonstrated that mice deficient of IFNγ were equally susceptible to cancer metastasis as mice deficient in NK cells, and that both had significantly higher tumour burden than their wild type counterparts. [29]. Similarly, Shankaran *et al.* have reported that IFNγ knock out mice develop more sarcomas than wild type mice [30]. Beyond its role in protection against cancer, the functions of IFNγ include activation of macrophages to acquire antimicrobial and anti-tumour mechanisms, upregulation of MHC molecules via the induction of antigen processing and presenting genes, regulation of B cells via upregulation of immunoglobulin production and class switching, and leukocyte trafficking [27]. NK cell-derived IFNγ is also crucial in priming the Th1 response, which is important for defence against intracellular pathogens [31].

TNFα is another important pro-inflammatory cytokine produced by NK cells. Its functions include regulation of the inflammatory response via activation of transcription factors such as activator protein 1 (AP-1) and NF-κB, control of cell proliferation and differentiation and activation of apoptotic and necroptotic cascades [32].

In addition to pro-inflammatory cytokines, NK cells can also produce anti-inflammatory cytokines. Transforming growth factor β (TGFβ) is an immunosuppressive cytokine produced by NK cells in both an autocrine and paracrine manner [33]. TGFβ production by NK cells is important for establishing and maintaining immune cell tolerance. Indeed, experiments involving genetic disruption of TGFβ signalling can result in a severe pro-inflammatory phenotype, which can mirror that seen in autoimmune disease [34, 35]. Despite the importance of this cytokine, dysregulation of TGFβ signalling is highly common in human disease, particularly in cancer [36]. This will be further discussed in chapter 4.

1.3.2 NK cell cytotoxicity

In addition to cytokine production, NK cells are also experts at cytotoxicity. This is mainly attributed to $CD56^{dim}$ NK cells as they constitutively express high levels of cytotoxic molecules in cytoplasmic cytotoxic granules. These granules are filled with various proteins that are released upon recognition of a target cell. Granzymes are a

group of serine proteases that induce programmed cell death by cleaving several substrates within the target cell and triggering apoptotic pathways [37]. Perforin is a cytotoxic glycoprotein that inserts into the membrane of the target cell where it polymerises to form a pore [38]. During cytolytic granule mediated apoptosis, granzymes & their associated molecules enter the target cell through this pore and induce apoptosis.

NK cells can also express various death ligands, often preferentially expressed by the $CD56^{bright}$ subset. Fas ligand (FasL), a membrane bound protein expressed on the surface on NK cells, can mediate the activation of the death-inducing signalling complex (DISC) in target cells by binding their Fas receptors [39]. They also express and secrete TNF-related apoptosis-inducing ligand (TRAIL), which binds to specific death receptors on target cells and triggers a caspase-8 dependent apoptotic pathway.

ADCC is a modified form of cytotoxicity that occurs when target cells are coated with antibodies (known as opsonisation). Antibodies are either naturally produced as part of a normal humoral immune response or can be administered exogenously to target cancer cells as a form of cancer immunotherapy. NK cells recognise and bind the Fc region of the antibody via their CD16 receptor [40]. This induces degranulation of NK cell cytolytic granules and subsequent death of the target cell. The $CD56^{dim}$ subset of NK cells are responsible for this function, as $CD56^{bright}$ NK cells in general do not express CD16. This mechanism will be discussed in further detail in chapter 5.

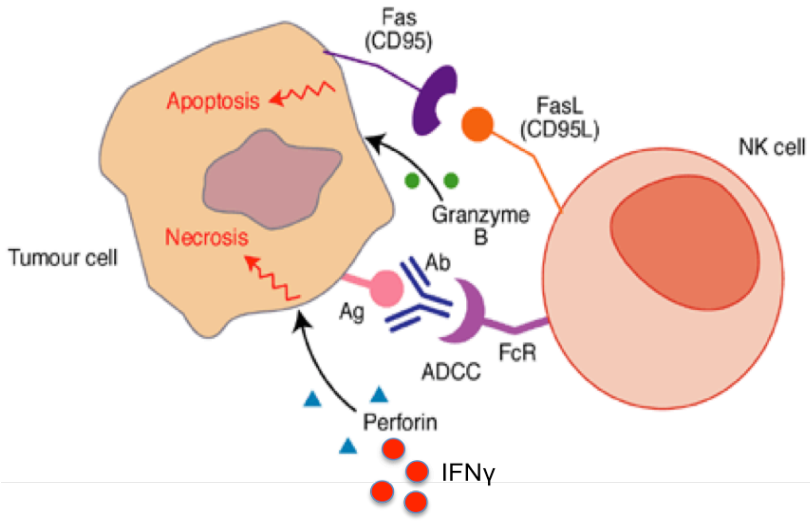


Figure 1.2 NK cell effector mechanisms

NK cells kill tumour cells through mechanisms that involve: ADCC, in which the Fc portion of an Ab bound to antigen on the tumour cell surface binds to Fc receptor (FcR) on the NK cell; Fas (CD95)–Fas ligand (CD95L) interaction; and release of perforin and granzyme B molecules, which cause apoptosis/necrosis of the tumour cell; and release of cytokines such as IFNγ [41].

1.3.3 The Immune Synapse

An immune synapse (IS) is formed between an NK cell and its target cell when carrying out direct cytotoxicity and ADCC. The formation of an IS occurs in a sequential and dynamic manner, involving three distinct stages – recognition, effector and termination. IS formation takes places within a supramolecular activation cluster (SMAC) – subdivided into the peripheral SMAC (pSMAC) and central SMAC (cSMAC) [42]. As $CD56^{dim}$ NK cells constitutively express their cytotoxic mediators, exocytosis of lytic granules must be carefully controlled in order to prevent accidental degranulation. This is achieved though coordinated signals from various surface receptors, which will be discussed in the next section.

Once an NK is stimulated to degranulate, CD2 rapidly accumulates and adhesion occurs via receptor-ligand interactions e.g. LFA-1 and MAC [42]. Vav1 activation directs F actin filaments to form networks within the cell [43], while Wiskott-Aldrich syndrome protein (WASP) promotes F actin branching [44]. Lipid rafts aggregate and receptors cluster at the cell surface, while lytic granules are redistributed to the microtubule organising centre (MTOC) within the cell. As the granules accumulate, ERK, Vav1 and PYK2 activity drive the polarisation of the

MTOC towards the IS [42]. It is proposed that large channels form within the F actin network, through which the granules are shuttled to the plasma membrane [45]. The granules dock at the IS and become primed for fusion with the plasma membrane. Finally, the granules fuse with the plasma membrane, causing the release of granule contents into the cleft formed between the NK cell and target cell (**Figure 1.3**) [46].

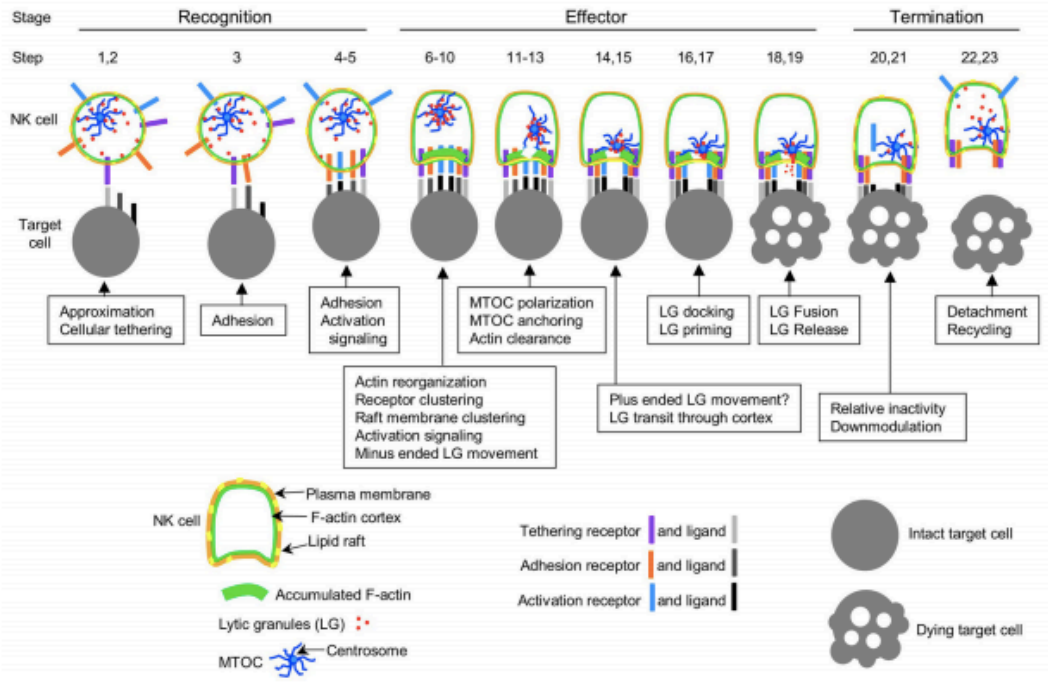


Figure 1.3 Formation of the immune synapse between NK cell and target cells.

Taken from [45]

1.4 Regulation of NK cells

NK cells have an impressive amount of mechanisms that they can use to kill their target cells, many of which are elegantly poised to elicit spontaneous cytotoxicity. In order to prevent unnecessary activation of these mechanisms, these functions are highly regulated by a variety of strategies. These include cytokine interaction, missing-self and stressed-self mechanisms, and the net balance of activating and inhibitory receptors.

1.4.1 Cytokines regulate NK cells

Immune cells of both the innate and adaptive immune system can secrete cytokines that affect NK cells. Innate cytokines, for example produced by macrophages and dendritic cells (DC), include IL-12 and IL-18. These are proinflammatory cytokines that induce NK cell production of IFNγ [47]. IL-15 is another innate cytokine. It is a key survival factor for NK cells and induces cell proliferation [48]. IL-2 is an adaptive cytokine produced mainly by T cells. It is a dominant enhancer of NK cell activation and function [49]. TGFβ is an inhibitor of NK cell activity and function, as is IL-10 [50].

1.4.2 Missing-self and stressed-self mechanisms

NK cells can be activated through down regulation of MHC (missing-self) and increased expression of stress induced molecules (stressed-self) by target cells. The missing-self mechanism involves detection of target cells lacking MHC-I molecules on their cell surface [51]. MHC-I is commonly involved in antigen presentation of foreign particles to T cells. In a healthy cell, inhibitory NK cell receptors interact with MHC-class 1 and thus NK cells are not activated. In a virally infected or transformed cell, downregulation of MHC-I on the surface of the cell results in removal of the inhibitory signal and activation of the NK cell [52]. This downregulation of MHC-I on target cells often results in evasion of a T cell immune response.

The stressed self mechanism involves upregulation of stress ligands such as MHC-I polypeptide-related sequence A and B (MICA and MICB) and UL16-bringing proteins (ULBP) on target cells [53]. These bind to activating receptors on NK cells and increase their activity.

1.4.3 NK cell activating and inhibiting receptors

NK cells express a range of regulatory receptors, including killer immunoglobulin-like receptors (KIR) and NKG2 receptors. The KIR selectively interact with classical MHC-I molecules HLA- (human leukocyte antigen) A, B and C, which bind peptides derived from the cytoplasm of target cells [54]. The inhibitory KIR contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) located on long cytoplasmic tails, while the activating KIR associate with the adaptor protein DAP12,

which itself contains immunoreceptor tyrosine-based activation motifs (ITAMs) [55]. NKG2 receptors are C-type lectin receptors. NKG2A-C are expressed as heterodimers with CD94 on the surface of NK cells. They interact with the non-classical HLA molecule HLA-E [56]. NKG2A & NKG2B are inhibitory receptors as they contain ITIM motifs in their cytoplasmic tails, while NKG2C associates with the ITAM-containing adaptor protein DAP12, resulting in an activating signal upon ligation [57]. NKG2D has little homology to the other NKG2 receptors. It does not associate with CD94 but rather is expressed as a homodimer on the surface of the NK cells. In addition, it interacts with the stress induced molecules MICA and MICB, and ULBP 1-3 [58]. Natural cytotoxicity receptors (NCR) are another set of receptors important in the regulation of NK cells, and include NKp46, NKp30, and NKp44. Together they recognise a broad spectrum of ligands, including viral, parasite and bacterial derived ligands [59]. They interact with an ITAM-containing protein to achieve NK cell activation.

1.5 NK cells and cancer

As NK cells play a pivotal role in the immune response, they have been studied thoroughly in many diseases. In addition to the killing of virally infected cells, one of their primary functions is to prevent the development of cancer using the wide variety of functions described herein. There is an enormous body of data demonstrating the importance of NK cells in immunosurveillance against cancer. Correlation studies in humans have shown that patients with a high level of NK cell infiltrations have better prognosis in various types of cancers, for example in gastric cancer [60] colorectal cancer [13, 61] and oesophageal cancer [62]. Retrospective analysis of breast cancer specimens has shown that molecular signatures associated with NK cell activity are associated with relapse free survival [63]. De Jonge *et al*. recently showed that the frequency of circulating $CD56^{bright}$ NK cells, in addition to their potential to produce IFNγ, correlates with survival in late stage melanoma patients [64]. Mice with the NK cell activation receptors NKG2D or NKp46 knocked out have increased tumour burden in the lung [65]. Similarly, NKG2D deletion results in higher tumour incidence in a model of prostate adenocarcinoma in addition to accelerated progression of Eμ-myc-induced lymphoma [66].

These studies demonstrate clearly that NK cells play a fundamental role in the protection against a wide variety of cancer types, both solid and haematological.

Paradoxically, it is well documented that the very functions they use to protect against cancer become compromised in patients who have an established tumour and even more so if metastasis has occurred. This in turn may result in the further progression of the disease and a poor prognosis for the patient.

1.5.1 NK cell dysfunction in cancer

There is substantial literature describing the dysfunction of NK cells in cancer. This has been documented in many haematological malignancies and solid tumours over the past 4 decades, and indeed the phenotypic and functional characteristics associated with this dysfunction are consistent. The two transcription factors T-bet and Eomesodermin (Eomes) are essential for healthy NK cell development [67]. However, their expression becomes impaired in NK cells from cancer patients [68, 69]. Furthermore, there is a reduction in the expression of CD57, an NK cell maturation marker [70], and an increase in the expression of CD27, a marker associated with NK cell immaturity [16]. Overall this suggests that something is restricting the normal developmental processes of NK cells in cancer patients.

Many of the early papers investigating the dysfunction of NK cells in cancer report an overall low level of NK cell activity and an inability to kill target cells [14, 15, 71]. More recently, researchers have uncovered specific features associated with this functional impairment. NK cell IFNγ production and/or degranulation are regularly reduced in cancer patients [68, 72]. In addition, CD16, the Fc receptor essential for ADCC, is commonly downregulated in patient NK cells [73, 74]. A similar downregulation is also observed for the activating receptors NKG2D, NKp30 and NKp46 in various cancer types [69, 73]. NK cell proliferation may also be affected [69].

While many of the activating markers are reduced in patient NK cells, inhibitory receptors may become upregulated. PD-1, normally involved in promoting self-tolerance in the immune system, can become overexpressed in NK cells from cancer patients and mediates NK cell exhaustion [72]. The inhibitory receptor Tim-3 has also been shown to be upregulated in NK cells from melanoma patients [69], while increased expression of TIGIT has been described in NK cells from colon cancer patients [75]. Interestingly, some inhibitory KIR receptors as well as NKG2A are reduced in cancer patients, highlighting the heterogeneity of the disease [16, 73]. A

summary of features associated with NK cell dysfunction in cancer is provided in Table 1.1.

Cancer type	Reduced	Increased	Ref.
CLL	Cytotoxicity		[71]
CLL	Degranulation, ADCC, NKG2D, CD57, KIR2DL1	CD27, PD-1	[16]
Non-Hodgkin's lymphoma	Cytotoxicity		[14]
Lymphoma (murine model)	Cytotoxicity, IFNγ, T-bet, Eomes, NKG2D		[68]
Breast	Cytotoxicity, NKp30, NKG2D, DNAM1, CD16	NKG2A	[74]
Breast	Cytotoxicity		[15]
Soft-tissue sarcoma	Cytotoxicity, NKG2D, Perforin, CD57		[70]
Kaposi sarcoma	Degranulation, IFNγ,	PD-1	[72]
NSCLC	Cytotoxicity, IFNγ, NKG2D, CD16, CD69, NKp30, DNAM1		[73]
NSCLC	% NK cells		[76]
Melanoma	Cytotoxicity, IFNγ, NKG2D, NKp46, T-bet, Eomes, proliferation	Tim3, KIRB1, KIRNKAT2	[69]
Colon cancer		TIGIT	[75]

Table 1.1 The changes that occur in NK cells during cancer

CLL; Chronic lymphocytic leukemia, NSCLC; non-small cell lung cancer.

1.5.2 Mechanisms of NK cell evasion in cancer

There are many possible explanations for the impairment of NK cells described above. Due to their rapid rate of cell proliferation, cancer cells are constantly mutating and thus evolving mechanisms that allow them to escape the immune system. Within the tumour microenvironment (TME), various chemicals are secreted that diminish the function of NK cells. TGFβ is a pleiotropic cytokine that is widely implicated in cancer development and progression. It has been shown to support tumorigenesis by enhancing cell proliferation and migration and enhancing angiogenesis [77-79]. Similarly, TGFβ can promote the development of pro-tumorigenic immune cells such as M2 macrophages and Treg cells, and it can also inhibit the functions of many anti-tumour immune cell types such as NK cells [80,

81]. This includes inhibition of activating receptor expression, IFNγ production and ADCC, and can take place both in the TME and within the periphery. This topic will be discussed in further detail in chapter 4. Prostaglandin E2 (PGE2) is an active lipid compound that is also commonly secreted by cancer cells in order to inhibit the immune system. It can impair NK cell viability and chemokine production [82], in addition to inhibition of cytotoxicity, IFNγ production and migration [83]. It has also been described to alter the expression of the activating receptors, such as NKG2D and NKp44 [84]. Indoleamine-pyrrole 2,3-dioxygenase (IDO), another immunomodulatory chemical found in the TME, has similar suppressive effects on NK cells [85].

Other bioactive molecules found beyond the TME in cancer patients include the NKG2D ligands MICA/B and ULBP. This is a result of a process known as 'ligand shedding', a mechanism commonly used by tumours to escape the immune system. Tumours may shed these ligands via phosphatidylinositol-phospholipase C-mediated cleavage, proteolytic shedding, or exosome secretion [86]. As MICA/B and ULBP are stress-associated molecules that activate NK cells, removal of them from the surface of a tumour shields it from the immune system [87, 88]. In addition, the increased concentration of these in the circulation results in their ligation to NKG2D receptors on the surface of NK cells which can then either block the receptor from further activation when it is actually needed, or it may desensitize the NK cells to these stress-inducible ligands resulting in anergy/exhaustion [89]. Soluble MICA/B has also been shown to result in NKG2D downregulation and internalisation in CD8+ T cells [90]. Interestingly, it has also been shown that a tumour can evolve to predominantly express MICA/B intracellularly rather than on its surface [91].

In addition to secreting inhibitory chemicals and ligands, tumours can also transform the immunological environment surrounding them, often switching from a pro-inflammatory environment to an anti-inflammatory, pro-tumorigenic one. This includes the recruitment of Treg cells [92], myeloid derived suppressor cells (MDSC) [93] and M2 polarised/ tumour associated macrophages (TAM) [94]. These can inhibit NK cells directly, by interacting with receptors on their surface, or indirectly, via the secretion of anti-inflammatory cytokines such as IL-10 and TGFβ.

1.5.3 NK cells in cancer immunotherapy

Despite the harmful effects that a tumour can have on them, NK cells still have a fundamental role in killing tumour cells. This has resulted in a great effort towards discovering ways in which NK cells can be harnessed for cancer immunotherapy. While the vast majority of immunotherapy research has focused on T cells, NK cells offer several advantages. Unlike T cells, NK cells are generally considered to not be antigen specific, and as such it is more challenging for a tumour to escape the NK cell immune response by antigen mutation alone [95]. Allogeneic cell transfer therapy poses a risk of inducing GVHD as the immune system may identify the new lymphocytes as foreign and mount a response against them. However, evidence suggests that NK cells are less likely to induce GVHD than T cells [5], and in fact they can positively promote a graft versus leukaemia effect (killing of host leukemic cells) [96, 97]. Furthermore, NK cells are less likely to promote cytokine release syndrome [98], which has occasionally proven fatal in the context of T cell therapy [99].

There have been many different approaches in the development of NK cell enhancing immunotherapies, and one of the earliest of these was the intravenous administration of IL2. Although it initially proved to be highly toxic, smaller doses have since been shown to be effective in combination with other therapies [100]. IL15 is another cytokine that has been used to improve NK cell function in cancer. Its advantage over IL2 is that it does not activate Treg cells. IL15 immunotherapy has been shown to assist NK cell recovery [101] and to promote NK cell development [102]. However, as with IL2, toxicity can be a problem [103]. Conversely, there has been many innovative approaches made in an effort to reverse/restrict the inhibitory effects of TGFβ [104], many of which have shown promise [105].

One of the greatest successes so far in the field of NK cell based immunotherapy has been the development of monoclonal antibodies that bind to antigens on the surface of cancer cells and activate NK cell ADCC. Once the therapeutic antibody binds the surface of the cancer cell, the NK cell recognises the Fc portion of the antibody and binds to it via its Fc receptor. This triggers the release of cytotoxic granules and the subsequent death of the tumour cell. Some of these therapies have been in clinical use for many years now. For example, Rituximab (anti-CD20), approved for the first time 1997, is used in the treatment of non-Hodgkin's lymphoma, Trastuzumab (anti-

HER2) is used in breast cancer, Dinutuximab (anti-GD2, **Figure 1.4**) for neuroblastoma and Cetuximab (anti-EGFR) is used in colorectal cancer.

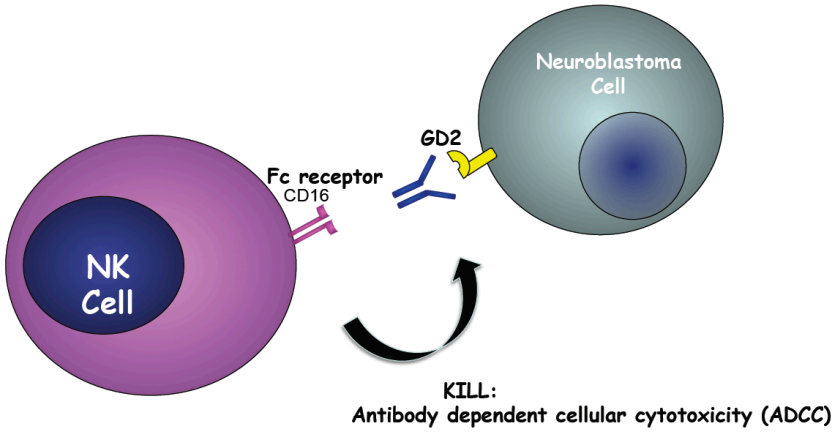


Figure 1.4 Anti-GD2 immunotherapy increased NK cell ADCC against neuroblastoma tumour cells.

More recently, a new class of antibodies has had a significant impact on cancer immunotherapy – checkpoint inhibitors. As the name suggests, these antibodies are designed to block checkpoints/barriers on immune cells in order to drive their activation. Checkpoints are normally expressed in order to promote self-tolerance and modulate the immune response. However, tumours hijack these checkpoints, leading to their upregulation on immune cells such as T cells and NK cells, and a dampening of the anti-tumour immune response. Anti-CTLA-4 (Ipilimumab), anti-PD-1 (Nivolumab, Pembrolizumab and Cemiplimab) and anti-PD-L1 (Atezolizumab, Avelumab and Durvalumab) therapies have reached the market and are now used in the treatment of a broad spectrum of haematological and solid tumour cancers [106]. This class of drugs is also currently under intense investigation in clinical trials for many types of cancers. Although they are normally discussed in the context of T cells, NK cells also express these receptors and they have been shown to play a vital role in the effectiveness of these therapies [107]. Furthermore, TIGIT has been identified as a checkpoint receptor that is highly expressed on NK cells in cancer and contributes to their dysfunction. Blockade of TIGIT has been shown to reverse NK cell exhaustion and dysfunction in mice with colon cancer [75]. Several TIGIT inhibitors are now in development and there are 3 clinical trials recruiting patient volunteers to test some of these (clinicaltrials.gov).

The next milestone for NK cell immunotherapy is NK cell transfer therapy. This can be classified as autologous, meaning it treats a patient's own NK cells (*ex vivo* manipulation/expansion and then reinfusion) [108]. Alternatively, allogeneic cell transfer therapy utilises NK cells sourced from a third party, such as PBMC, induced pluripotent stem cells [109], cord blood [110] or a transformed cell line such as NK-92 (**Figure 1.4**). There are pros and cons to using autologous versus allogeneic NK cells for therapy. As the immune cells of cancer patients are frequently dysfunctional (**Table 1.1**), autologous cell therapy is unlikely to be as effective as allogeneic cell transfer therapy, and indeed emerging data suggests that allogeneic therapy is most effective. On the other hand, autologous therapy will not induce GVHD, meaning that even if the treatment fails, it will not negatively impact the patient. In contrast, it is possible to induce GVHD with allogeneic cell transfer, which can be very harmful for the patient. To reduce this risk, many allogeneic cell transfer studies now use haploidentical donors as the source of the NK cells (HLA half matched, often a family member) [7, 8]. For example, in poor prognosis AML patients, haploidentical NK cell infusions in combination with high dose chemotherapy and IL2 infusion resulted in *in vivo* expansion of NK cells and complete hematologic remission in 5 out of 19 patients [8]. Similar results were more recently shown in elderly high-risk AML patients [7], and in high-risk myelodysplastic syndrome (MDS), and again in AML patients [111], where donor NK cells were found in the periphery of all patients and in the bone marrow of some. In neuroblastoma, high doses of haploidentical NK cell infusions followed by anti-GD2 immunotherapy improved progression free survival, and patient NK cells had increased NKG2A expression [112]. In all studies, NK cell infusions were well tolerated, with no reported toxicities.

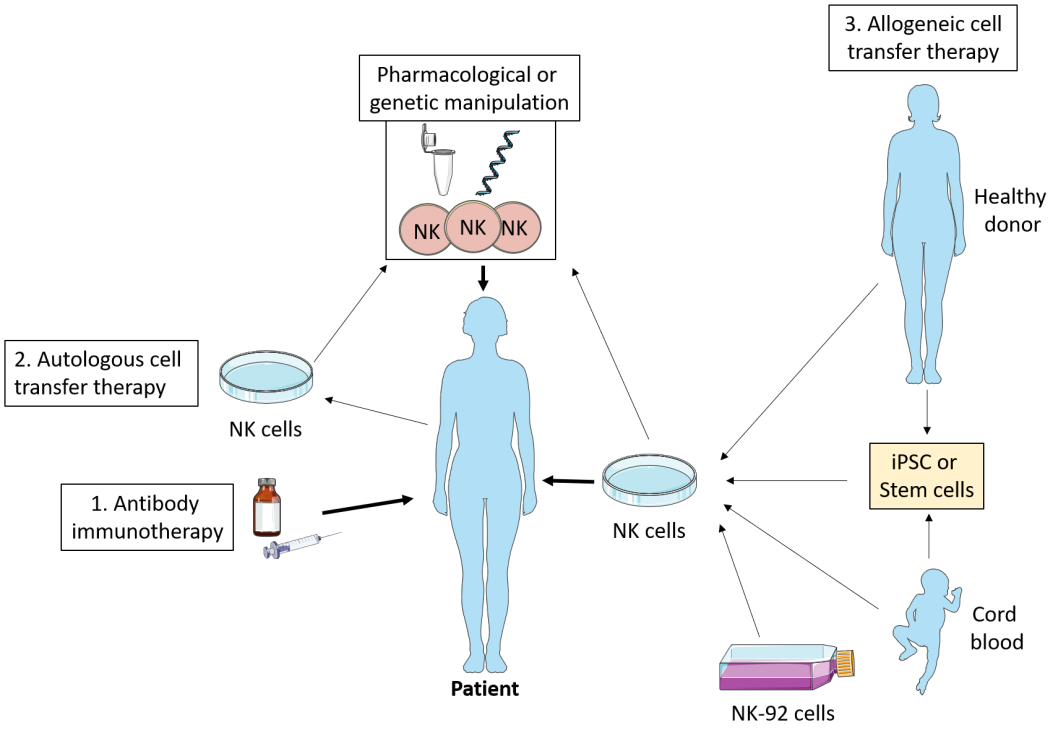


Figure 1.5 NK cell immunotherapy

Antibody therapy stimulates NK cell ADCC activity *in vivo*, which promotes tumour killing. Autologous cell transfer therapy involves treating a patient's own NK cells, while allogeneic cell transfer therapy involves treating cells from a third party—healthy donor, cord blood, stem cells, or NK cell lines. These NK cells can be manipulated ex vivo pharmacologically or genetically such that they have enhanced anti-tumour functions and then infused into the patient. iPSC, induced pluripotent stem cells.

1.6 Immunometabolism

Overall, there is huge potential to harness NK cells for cancer therapy. However, the problem remains that cancer creates an immunosuppressive environment that has the potential to hinder both autologous and allogeneic approaches to NK cell therapy. As such, there is an urgent need to study NK cells from new perspectives in order to better understand how NK cells are impacted in cancer, and what molecular mechanisms underlie this. Over the past decade, the field of immunometabolism has exploded and become one of the fastest growing research areas in immunology. It is based on exploring the intracellular metabolic pathways that drive the immune response. Each of these interwoven pathways represents a different way for a cell to consume and use energy, which impacts its ability to carry out its functions [113]. Indeed, different cell types with certain functions need to carry out specific forms of

metabolism that provide the necessary energy/biomolecules that the cell needs to do its job. Stimulation of an immune cell changes its functions, so this is associated with a complementary change in metabolism, often regarded as metabolic reprogramming. The main metabolic pathways used by immune cells are glycolysis, the TCA cycle, fatty acid oxidation and synthesis, and amino acid metabolism.

Glycolysis is one of the most important pathways in activated immune cells. It is a 10 step process in which glucose is converted to pyruvate within the cytosol. It results in the production of 2 nicotinamide adenine dinucleotide (NADH) molecules that can then be used to produce ATP via oxphos. Although glycolysis has a modest energy yield, the pathway has the added benefit of allowing the cell to divert intermediate products to biosynthetic pathways that support anabolic growth, such as the pentose phosphate pathway (PPP) for the production of amino acids [114]. In highly proliferative, glycolytic cell types, pyruvate is reduced to lactate and secreted into the extracellular space (aerobic glycolysis). Alternatively, in more oxidative cell types, it will be broken down into acetyl CoA and transported to the TCA cycle in the mitochondria. mTORC1, a master regulator of cellular metabolism, is key in the regulation of glycolysis [115]. It can sense nutrients and respond to extracellular and intracellular signals, such as growth factors and hormones. It then alters protein translation within the cell and shapes its response accordingly [116]. The transcription factors hypoxia-inducible factor 1α (HIF1α) and c-Myc are also important in regulating glycolytic flux [117].

The TCA cycle (The tricarboxylic acid cycle, also known as the Kreb's cycle or the citric acid cycle) is a highly efficient series of enzymatic reactions that takes place in the matrix of the mitochondria. It involves the oxidation of acetyl CoA (sourced from glucose, fatty acids or amino acids) into carbon dioxide and energy in the form of NADH and Flavin adenine dinucleotide ($FADH_2$). These molecules are then used during oxphos to produce ATP - they enter the electron transport chain at complex I and III where they are used to drive protons across the mitochondrial inner membrane in order to create an electrochemical gradient. This gradient is then harnessed by ATP synthase (Complex V) to produce ATP in an oxygen dependent manner (chemiosmosis). Glutamine can also feed into the TCA cycle in a process termed glutaminolysis, whereby glutamine is converted into α-ketoglutarate by glutamate dehydrogenase.

Fatty acid oxidation (FAO) involves the transport of fatty acids to the mitochondria via the carnitine palmitoyltransferase (CPT) system. Here, β-oxidation of the fatty acids results in the production of large amounts of energy in the form of acetyl CoA, NADH and $FADH_2$. Acetyl CoA molecules enter the TCA cycle and the resulting reducing equivalents are fed into the electron transport chain for the generation of ATP. Conversely, fatty acid synthesis allows the generation of lipids required for anabolic growth. They are produced using precursors derived from other cell intrinsic metabolic pathways, such as the TCA cycle (citrate converted to acetyl CoA) and the PPP. The fatty acids can also be condensed with glycolysis-derived glycerol to produce triacylglycerides and phospholipids that are essential for cell growth. This pathway is highly regulated by mTOR, which promotes the expression of key enzymes such as sterol regulatory element binding protein (SREBP), fatty acid synthase (FASN) and acetyl CoA carboxylase (ACC).

Amino acids are also involved in many metabolic pathways. In addition to being the building blocks of proteins, they are precursors for the *de novo* synthesis of branched-chain amino acids and can also regulate intracellular signaling. For example, mTOR can sense amino acid levels and couple this to anabolic growth.

1.6.1 Metabolism drives immune cell function

There is a growing appreciation of the extent to which immunometabolism underpins all aspects of the immune response. Different immune cells have specialised metabolic configurations that allow them to carry out their specialised functions (**Figure 1.5** for main cell energy production pathways). At rest, most naïve immune cells carry out low levels of oxphos to meet their homeostatic needs. Once an immune cell becomes activated, it reprograms cellular metabolism to suit it functions. In many cell types, this involves upregulation of glycolysis, which supports rapid ATP synthesis in addition to production of various biosynthetic precursors such as amino acids [118]. As this upregulation of glycolysis takes place in the presence of oxygen, it is known as aerobic glycolysis, and is fundamental for the functions of NK cells, effector T cells, B cells, DCs, neutrophils, and M1 macrophages [119]. Memory T cells mainly engage in FAO because unlike glucose, fatty acids can easily be stored as lipid droplets within the cell. As such, memory T cells synthesise fatty acids so that they can rapidly oxidize the stored fatty acids, which then supplies the energy required for the recall response [120]. Similarly,

regulatory T cells use fatty acid oxidation to support their long life span [121]. By understanding how these metabolic pathways support functions of the immune response, we can aspire to unlock new ways in which to control and regulate the immune system therapeutically.

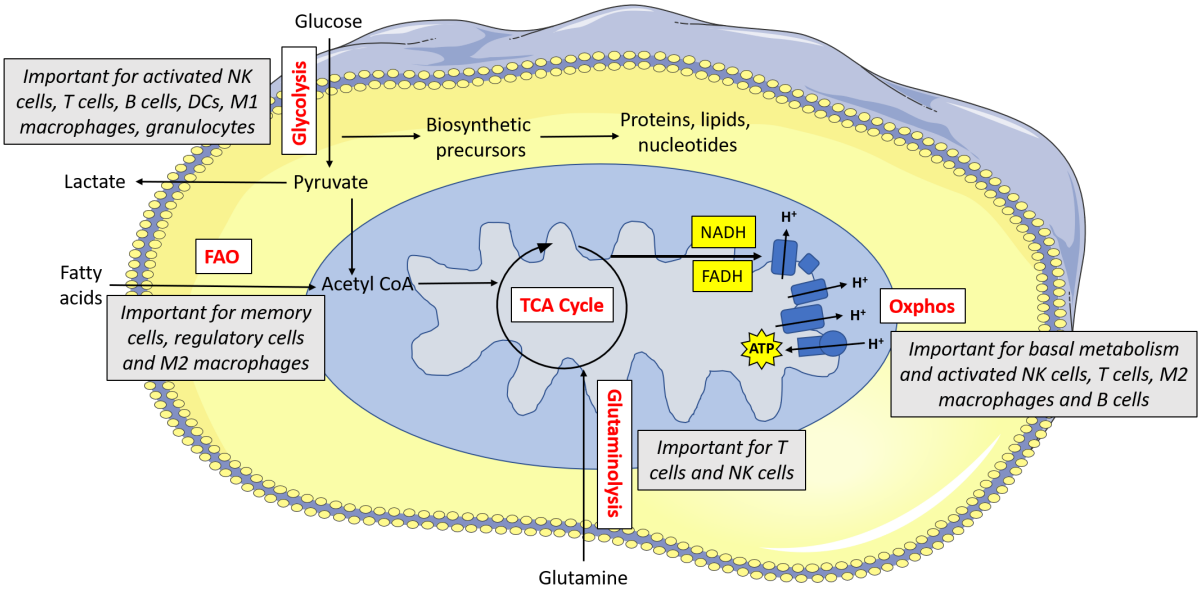


Figure 1.6 Metabolism drives immune function

Metabolism drives immune cell function. Glucose is metabolized by glycolysis, which is essential for activated NK cells, T cells, B cells, DCs, M1 macrophages and granulocytes. Pyruvate can be converted to lactate and secreted from the cell or else it can be converted to acetyl CoA which feeds into the TCA cycle. The TCA cycle results in the production of reducing equivalents (NADH, FADH) which feed into the electron transport chain. The electron transport chain uses the electrons supplied by NADH and FADH to pump protons across the inner membrane. This force is then used to drive ATP synthase which makes ATP. Oxphos is important for immune cells when they are at rest, and it is also essential for activated NK cells, T cells, M2 macrophages and B cells. Acetyl CoA can alternatively be supplied by fatty acids—this form of metabolism is important for memory cells, regulatory cells and M2 macrophages. Glutamine can feed into the TCA cycle via glutaminolysis—this pathway is used by T cells and to a lesser extent, NK cells.

NK cells rely on both oxphos and glycolysis to support their functions [122, 123], and these pathways are fuelled primarily with glucose [124]. In brief, glucose is taken up through the Glut-1 receptor into the cytoplasm where it is metabolised by glycolysis to generate pyruvate. This can in turn either be used to generate lactate, which is then exported from the cell (aerobic glycolysis), or it can be converted into citrate. In murine NK cells, it has been shown that a small portion of citrate is cycled

through the TCA cycle, supported by glutamine-fuelled glutaminolysis [125]. The rest of citrate is uniquely used to fuel the citrate-malate shuttle to fuel oxphos, whereby citrate is exported to the cytosol where it is converted to acetyl CoA and oxaloacetate [124]. The acetyl CoA can then be used for acetylation reactions (important for innate immune training) or to produce fatty acids, while the oxaloacetate is converted back to malate and then shuttled back to the TCA cycle (**Figure 1.6**). Ultimately, this results in the production of cytosolic NAD+, which is an essential cofactor for the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and NADH, which is fed into the electron transport chain support ATP production. This facilitates increases in both glycolysis and oxphos, which are required for the NK cell functional response.

Any homeostatic role for fatty acid metabolism in NK cells remains largely unexplored to date. Interestingly, treatment of NK cells with etomoxir, an inhibitor of FAO, had no effect on ATP levels, indicating that NK cells may not depend on this form of catabolic metabolism for production of ATP [126]. However, the specificity of this inhibitor is now being questioned [127].

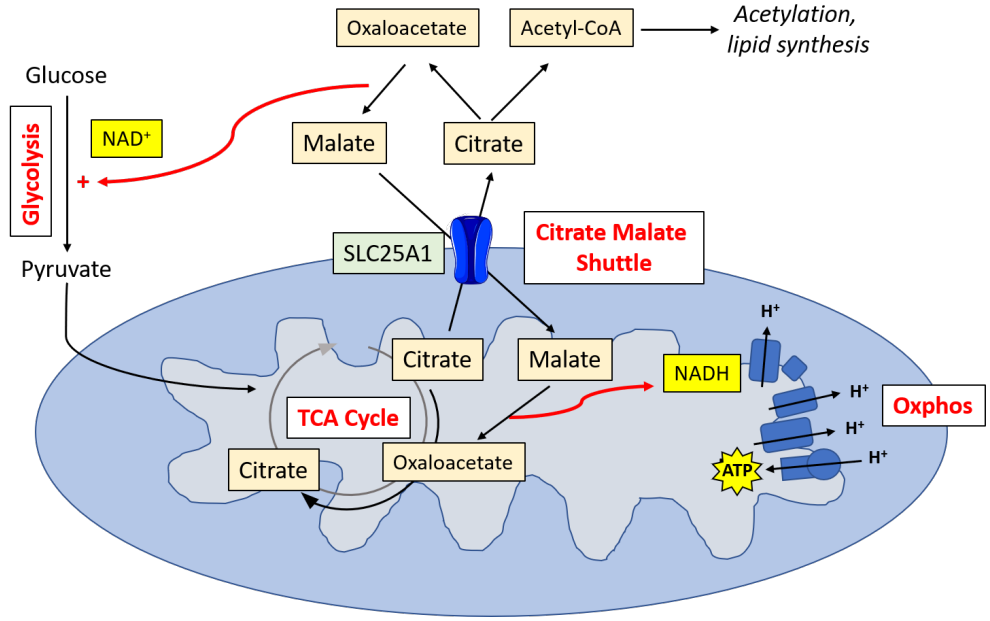


Figure 1.7 Murine NK cell metabolism

Activated NK cells metabolize glucose to pyruvate. Pyruvate is converted to acetyl CoA which is then converted into citrate. Citrate is exported into the cytosol via SLC25A1, where it is converted into oxaloacetate and acetyl CoA. Acetyl CoA can then be used in acetylation reactions or for lipid synthesis. Oxaloacetate is converted back in malate, resulting in the

production of NAD+, and essential cofactor for glycolysis. Malate is transported back into the mitochondria, where it is converted back into oxaloacetate, producing NADH that can then feed into the electron transport chain for ATP synthesis.

Immunometabolism has indeed revolutionised how we think about the immune response. It therefore comes as no surprise that these studies are having an impact in the clinic. The effect that old drugs have on the metabolism of immune cells is now being explored for the first time. For example, metformin, which has been used to treat type 2 diabetes for many decades, works by inhibiting complex I in the electron transport chain and hence can strongly impact immune cells that use oxidative metabolism to carry out their functions [128]. Indeed, recent research has shown that metformin can skew T cell differentiation towards regulatory and memory T cells [129]. Another example is the deeper understanding provided by metabolism of how checkpoint inhibitors work during cancer therapy. Bengsch *et al*. demonstrated that anti-PDL1 therapy reprogrammed metabolism of exhausted T cells and improved several readouts of mitochondrial structure and function as part of the mechanisms of action of this therapy [130]. It is possible that these checkpoint inhibitors are also impacting the metabolism of NK cells, as they also express some of these checkpoint antigens [107].

What is more exciting is that immunometabolism research is revealing new drug targets that may prove effective in the treatment of a variety of human diseases. For example, Sukumar *et al.* showed that inhibiting glycolytic metabolism in CD8+ T cells increases the generation of memory cells and improves their anti-tumour functions [131], while Fisicaro *et al*. showed that improving mitochondrial fitness using antioxidants is sufficient to revive exhausted CD8+ T cells and increase their antiviral functions [132]. In human sepsis patients, IL7 treatment increases glucose metabolism and mTOR activity in dysfunctional T cells [133]. Overall, it seems likely that some of these immunometabolism-derived therapies will reach clinical trials in the near future.

Immunometabolism has added a new dimension to how we understand the immune response. This new field of research has opened a wide range of avenues that can now be explored in order to try and identify novel therapeutic targets for the development of new therapies. This thesis is focused on understanding the role that metabolism plays in NK cells from cancer patients. We chose to study peripheral

blood NK cells due to the importance of the systemic immune response in mediating anti-tumour immunity [134], but also because peripheral blood NK cells are now key targets of many modern immunotherapy strategies including adoptive cell transfer therapy.

1.7 Aims and Objectives:

Overall aim

- To investigate the role that metabolism plays in NK cells during human cancer and identify potential therapeutic targets for future NK cell immunotherapy strategies.

Chapter 3

- Examine the function and metabolism of peripheral NK cells from patients with metastatic breast cancer.

Chapter 4

- Explore potential drivers/underlying mechanisms of phenotypes observed.
    - Can we target this to improve metabolism?
    - Does this result in improved anti-tumour functions?

Chapter 5

- Determine whether the phenotype observed in chapter 3 is also evident in peripheral NK cells from paediatric neuroblastoma patients.
- If patient NK cell metabolism is altered, how might this impact the efficacy of anti-GD2 immunotherapy in patients?
    - What are the metabolic requirements of anti-GD2 mediated NK cell ADCC against neuroblastoma tumour cells?

Chapter 2

2 Materials and Methods

2.1 Chemicals and reagents

Dulbecco's phosphate buffered saline (PBS), 2-Deoxy-D-glucose (2DG), glucose, glutamine, oligomycin, rotenone, antimycin A, 2-aminobicycloheptane-2-carboxylic acid (BCH), 25-hydroxycholestrtol (25HC), Mowiol and Triton X were purchased from Sigma, Ireland. Hanks balanced salt solution (HBSS) was purchased from Merck. Trypan Blue solution and rapamycin were purchased from Fischer Scientific. RPMI medium 1640 GlutaMax®, Penicillin-Streptomycin solution and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit were purchased from Biosciences. IL15 and IL2 were purchased from National Institutes of Health (NIH, USA). IL12 was purchased from Miltenyi Biotech, UK. Human NK cell purification kits and EasySep buffer were purchased from StemCell. Foetal Bovine Serum (FBS) was purchased from Labtech International while dialysed FBS was purchased from Fisher. Golgi plug, Cytofix/Cytoperm reagent and Celltak® were purchased from BD Pharmingen, Ireland. Methanol and absolute alcohol were purchased from the school stores. Seahorse media was purchased from Agilent Technologies, USA. MitoTracker Red and Green, MitoSOX, CellROX, TMRM and DAPI were purchased from Thermo Fisher. MitoSpy Red, purified mouse IgG2a, κ and IgG1 isotype control antibody were purchased from Biolegend. Purified Mouse Anti-Human Disialoganglioside GD2 (Clone 14.G2a), Calcein AM and Golgi Stop was bought form BD Biosciences. Anti-TGFβ monoclonal antibody (clone 1D11) and recombinant human TGFβ1 protein was purchased from R&D Systems. Flow cytometry antibody suppliers are shown in **Table 2.1** and **Table 2.2**.

2.2 Flow cytometry antibodies

2.2.1 Extracellular:

Antigen	Fluorophore	Supplier
CD3	Pac blue	BD
CD3	PerCp	BioLegend
CD3	BV605	BD
CD56	PeCy7	BioLegend
CD56	BV786	BD
CD69	FITC	BD
CD98	PE	BD
CD71	APC	BioLedgend
CD25	BV786	BD
CD25	BV421	BioLedgend
CD25	PeCy7	BD Pharmingen
LAP	PerCp/Cy5.5	BioLegend
GARP	APC	
CD107a	PE	BioLegend
CD16	AF700	BioLegend

Table 2.1 Antibodies used for extracellular flow cytometry staining.

2.2.2 Intracellular:

Antigen	Fluorophore	Supplier
IFNγ	PeCy7	BD
IFNγ	Af700	BD
Granzyme B	PeCf594	BD
ATP5B	Alexa 647	Abcam
pS6	PE	Cell Signalling
pS6	Pac blue	Cell Signalling
4EBP1	PE	Cell Signalling
4EBP1	Pac blue	Cell Signalling
cMyc	PE	Cell Signalling

Table 2.2 Antibodies used for intracellular flow cytometry staining.

2.3 Equipment

Pipettes, pipette tips, filtered tips, tissue culture dishes (6, 12, 24 and 48 well) and tissue culture flasks were obtained from Fisher. FACS Canto and LSR Fortessa were from Becton Dickinson. FlowJo software was obtained from TreeStar. Graphpad-prism software was obtained from Graphpad. Seahorse machine, seahorse plates and cartridges were purchased from Agilent technologies. Purification magnets were purchased from StemCell Technologies. Grant JB Series Water Bath was obtained from Amlab Services. Steri-Cycle CO_2 Incubator from Thermo Forma. Heraeus Fresco 21 and Heraeus Pico 17 Centrifuges from Thermo Scientific. Legend RT Centrifuge from Sorvall. 1500 Standard Fumehood from Phoenix Controls Corporation. Bioair Topsafe Fumehood from Crowthorne HiTec Services. Bright-Line Hemacytometer from Hausser Scientific. Vacuette 9ml K2 EDTA blood collection tubes were purchased from Greiner. 50ml syringes were purchased from C&P Medical Trading LTD.

2.4 Subjects

All blood samples for this study were collected in EDTA coated tubes. The Research Ethics Committee of School of Biochemistry and Immunology in Trinity College Dublin and the ethics committee of St. James's Hospital Dublin provided ethics for analysis of healthy donor blood during the years 2016 to 2019. The Trinity College Faculty of Engineering, Maths and Science Research Ethics Committee provided ethics for analysis of healthy donor blood in 2020. All healthy donors for this study were consenting adults (chapter 4: all female, mean age = 45).

The ethics committee of St. James's Hospital, Dublin, provided ethics for analysis of the metastatic breast cancer blood samples. All patient samples for this study were consenting adults (all female, mean age = 58). Number of sites of metastasis ranged from 1 to 5, and included metastasis to the bone, liver, pleura, chest wall, lung, lymph node, brain, spleen and peritoneum. Any patients on chemotherapy or who received chemotherapy within 1 year previous were excluded from the study. Some patients were on one or more of the following therapies: fulvestrant, zometa, letrozole, trastuzumab, palbociclib, pertuzumab, xgeva, tamoxifen, decapeptyl, goserelin.

The ethics committee of Our Lady's Children's Hospital Crumlin, provided ethics for the study of on the neuroblastoma patients. All patient samples for this study were obtained from treatment naïve children, and parents/guardians provided consent. 1 male and 4 female patients were recruited (incidence of approximately 1-2 per year), with a mean age of 2 years 9 months (**Table 2.3**).

	Age	**Sex**
NB1	14 months	Female
NB2	4 years	Female
NB3	3 years	Female
NB4	20 months	Female
NB5	4 years	Male

Table 2.3 NB patient information.

2.5 PBMC and NK cell culture

Peripheral blood mononuclear cells (PBMC) were isolated on the same day blood sample was drawn via density gradient centrifugation using Lymphoprep (Axis shield). Blood was combined with PBS at a ratio of 1:2 and this was gently layered on top of lymphoprep (15ml) and centrifuged for 30 minutes at 300g on break 1. The buffy coat was extracted and washed in PBS (50ml). Isolated PBMC were then resuspended in warm RPMI Glutamax medium, supplemented with 10% FCS and 1% penicillin/ streptomycin, at a concentration of $5x10^6$ cells/ml.

PBMC were seeded at $5x10^6$ cells/ml and stimulated with both IL12 (30ng/ml) and IL15 (100ng/ml), or with IL2 (500IU/ml), representing innate and adaptive cytokine stimulation respectively. Cells were then incubated at 37°C, 5% CO_2 for 18 hours. Where indicated, TGFβ (10ng/ml), isotype control (IgG1 for anti-TGFβ and anti-GARP experiments, IgG2A for ADCC assay), anti-TGFβ antibody (5μg/ml, clone 1D11), anti-GARP (MHG-8, 10μg/ml), oligomycin (40nM), 2DG (2.5mM), rapamycin (20nM), BCH (25mM), 25HC (5μM) or AZD-8055 (1μM) was added for the duration of the culture.

For Seahorse, confocal microscopy and anti-GARP experiments, NK cells were purified from PBMC using either the MoJoSort human NK cell isolation kit

(BioLegend) or the EasySep Human NK Cell Isolation Negative Selection Kit (StemCell) according to the manufacturer's instructions. In general NK cells were 85-90% pure. Purified NK cells were seeded at $5x10^6$ cells/ml and stimulated/treated as described above.

2.6 Flow Cytometry

2.6.1 Extracellular FACS staining

PBMC were stained for extracellular surface markers for 20 minutes, in the dark, at 4°C, with saturating concentrations of tittered antibodies **(Table 2.1)** in FACS buffer (PBS supplemented with 2% FCS)**.** A live/dead (Near IR) stain was included in each panel.

2.6.2 Intracellular staining

PBMC were fixed and permeabilised via incubation in Cytofix/Cytoperm solution (BD) for 20 minutes, in the dark, at 4°C. Cells were stained for intracellular markers for 20 minutes in the dark, at 4°C, with saturating concentrations of tittered antibodies **(Table 2.2)** in perm wash**.** For pS6 staining, rapamycin (20nM, 20 minutes, 37°C) was used as a negative control. For p4EBP1 staining, AZD-8055 (1μM, 1 hour, 37°C) was used as a negative control.

2.7 Metabolic Assays

2.7.1 MitoTracker Red

Mitotracker Red (ThermoFisher) dye was used to measure mitochondrial mass. PBMC were stained with MitoTracker Red (100nM) in RPMI for 30 minutes at 37°C. Cells were then washed in FACS buffer and stained for extracellular surface markers.

2.7.2 MitoSOX

MitoSOX Red (ThermoFisher) dye was used as a measurement of mitochondrial ROS. PBMC were stained with MitoSOX (1.5μM) in RPMI for 15 minutes at 37°C. Rotenone (20μM) and MitoTEMPO (25μM) were used as positive and negative controls, respectively. Cells were then washed in FACS buffer and stained for extracellular surface markers.

2.7.3 TMRM and MitoTracker Green co-stain

TMRM (Tetramethylrhodamine, Methyl Ester, Perchlorate, ThermoFisher) dye was used to measure mitochondrial membrane polarization and MitoTracker Green (ThermoFisher) was used simultaneously to measure mitochondrial mass. PBMC were stained in media containing TMRM (100nM) and MitoTracker Green (100nM) for 20 minutes and 37C. Oligomycin (2μM) and FCCP (2μM) were used as positive and negative controls, respectively. Cells were then surface stained for NK cell markers and run immediately on the flow cytometer.

2.7.4 CellROX

CellROX Deep Red (ThermoFisher) was used as an oxidative stress detector. PBMC were stained with CellROX (1μM) in RPMI for 30 minutes at 37°C. Tert-Butyl hydroperoxide (TBHP, 1mM) and n-acetylcysteine (NAC, 4mM) were used as positive and negative controls, respectively. Cells were then washed in FACS buffer and stained for extracellular surface markers.

2.7.5 BODIPY

BODIPY 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-*s*-Indacene) was used as a stain for intracellular neutral lipids (e.g. cholesterol and triglycerides). PBMC were stained with BODIPY (25μg/ml) in RPMI for 15 minutes at 37°C. Oleic acid (25μM) and an FMO unstained control were used as positive and negative controls, respectively. Cells were then washed in FACS buffer and stained for extracellular surface markers.

2.7.6 Kynurenine uptake assay

Kynurenine is a metabolite of tryptophan metabolism that is taken up by cell via the amino acid transporter SLC7A5. Due to its fluorescence spectral properties (450nm), its uptake can be monitored via flow cytometry and this is a useful marker of nutrient uptake via SLC7A5[135]. PBMC were first surfaced stained with NK cell markers. Cells were then washed and resuspended in 100ul Kynurenine (200μM, made up in HBSS). Tubes were made up to 400ul with HBSS and put in a water bath at 37ºC for 4 minutes. Leucine was used as a negative control as it acts as a

competitive inhibitor of the kynurenine. 4% PFA was used to fix cells (150µl) at room temperature for 15 minutes in the dark. Cells were then washed twice with FACS buffer and analysed via flow cytometry.

2.7.7 2-NBDG uptake assay

2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) is a fluorescent glucose analogue and a useful marker of nutrient uptake (through an unknown nutrient receptor [136]). PBMC were washed with glucose free media and resuspended in warm glucose free media supplemented with 10% dialysed FCS (Fisher), 2mM glutamine (Invitrogen/Biosciences), 1mM sodium pyruvate (Gibco) 1x MEM Vitamin Cocktail (Invitrogen/Biosciences), 1 x selenium/ insulin/ transferrin Cocktail (Invitrogen/Biosciences), 50µM β-mercaptoethanol (Sigma) and 1% Penicillin/Streptomycin (Invitrogen/Biosciences), at a concentration of $1x10^6$cells/ml. After 15 minutes incubation at 37ºC, cells were resuspended in 1 ml of glucose-free media supplemented with 35µM of 2-NBDG (Life technologies), or in glucose-free media alone as a control. Cell were incubated for 1 hour and then washed twice and analysed by flow cytometry.

2.7.8 cMyc intracellular staining

PBMC were cultured in a 1:1 ratio of RPMI and HBSS, with 10% dialysed FCS and 1% penicillin/ streptomycin. BCH (25mM) was used as a negative control. $1x10^6$ PBMC were washed and resuspended in FACS buffer. The cells were transferred to a 96 well plate and surface stained. Cells were washed again and permeabilised by incubating in 200µl of FACS buffer containing 0.5% PFA and 0.2% Tween for a minimum of 2 hours at room temperature. After the permeabilisation, the cells were stained with intracellular stains, including the cMyc-PE antibody (Cell Signalling) at a dilution of 1 in 50 for 1 hour at room temperature. The cells were then washed once with FACS buffer and analysed by flow cytometry.

All flow cytometry data was acquired on the FACS Canto (BD) or the LSR Fortessa (BD) and analysed using FlowJo software (Treestar). NK cells were defined as the $CD3^-$ $CD56^+$ live population of lymphocytes (**Figure 2.1**).

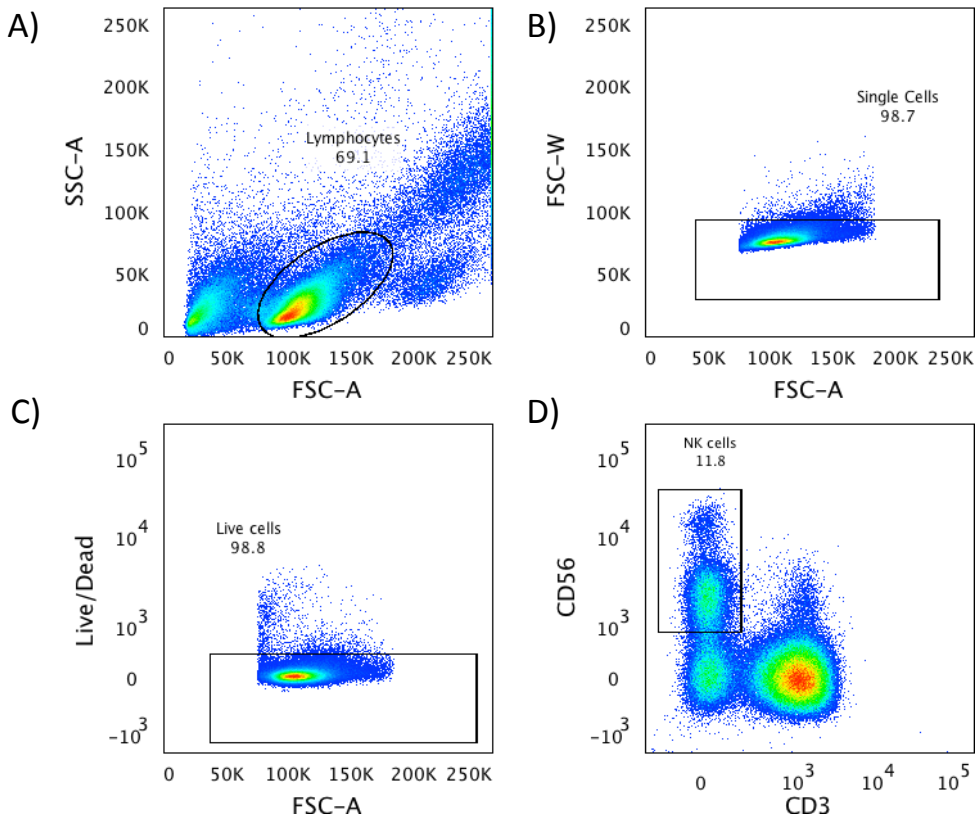


Figure 2.1 NK cell gating strategy.

Lymphocytes were first identified by analysing the side scatter and forward scatter (A). Doublet cells were then excluded (B), as well as dead cells (C). NK cells were finally identified as the $CD3^{-}$ $CD56^{+}$ population (D).

2.8 Confocal imaging of mitochondrial morphology

8x10 5 purified NK cells (~90% pure CD56+CD3-) were stained using Mitospy CMX Ros (250nM, Biolegend) for 30 minutes at 37°C and fixed in 2% PFA (Sigma) for 15 minutes at room temperature, prior to nuclear staining with DAPI (Thermo Fischer Scientific) for 5 minutes at room temperature. NK cells were mounted using Mowiol (Sigma) and imaged on a Leica SP8 inverted motorised microscope equipped with a 63x/1.4 N.A. oil objective and 405nm diode and Leica white laser lines. Z-stacks at 0.2 um increments were captured using a HyD detector in conjunction with Leica LAS X acquisition software.

Image analysis was performed on the maximum intensity projections of the z-stack. NK cell mitochondrial morphology was assessed by blind scoring of a mixed healthy and patient dataset (total number of cells=84). 6 volunteers blindly scored whether

morphology was predominantly fragmented or tubular, and if mitochondria were predominantly single or networked, as per an ordinal scale provided in **Figure 2.2**. The mode of each cell score was collated and the % morphology score was calculated for each individual donor as follows:

$$\frac{\textit{(No.of cells with each score)}\ X\ 100}{\textit{(Total number of donor cells)}}$$

Confocal microscopy experiments were completed in collaboration with Dr. Elena Woods (Gardiner Lab).

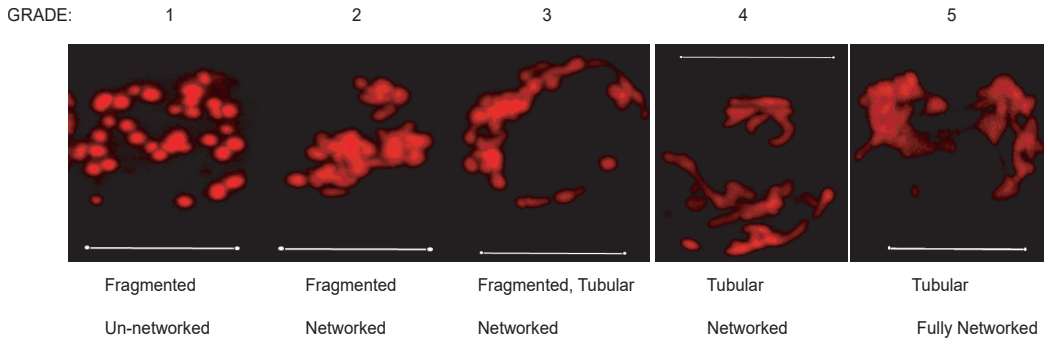


Figure 2.2 Scale used for grading of mitochondrial morphology.

Ordinal scale for grading mitochondrial morphology where: 1= fragmented mitochondria with no networking, 2= fragmented networked mitochondria, 3= networked mitochondria with mixed tubular and fragmented morphology, 4= tubular mitochondria partially networked, 5= tubular mitochondria, fully networked. Images shown are Maximum Intensity projections of Z-stacks taken at 0.2µm increments and are representative of both healthy and patient NK cells. Brightness in Images 4 and 5 was increased for illustrative purposes. Scale bar =5µm.

2.9 Seahorse metabolic analysis

A Seahorse XFp Analyzer (Seahorse Bioscience) was used for the real-time analysis of oxidative phosphorylation and glycolysis in live NK cells. Oxidative phosphorylation was measured using the oxygen consumption rate (OCR) of the cells, while glycolysis was measured using the extracellular acidification rate (ECAR) caused by the cells.

Each seahorse cartridge was hydrated in a non-CO_2 37°C incubator with calibration buffer (200µl). This was carried out for a minimum of 5 hours prior to use.

In order to stick the NK cells to the bottom of the seahorse plate to form an even monolayer, cell plates were coated with Cell-Tak (6µg/ml). The Cell-Tak was diluted in sodium bicarbonate (0.1M) with 0.15% (v/v) NaOH (1M) and added to the bottom of each well (25µl). It was left at room temperature for a minimum of 20 minutes, removed from the plate and each well was washed twice with sterile ddH2O (200µl).

Cells were washed twice in Glutamax seahorse media supplemented with glucose (1M), adjusted to pH 7.4. NK cells were added to each well ($2.5x10^5$ cells, 180µl), while seahorse media (180µl) was used in the blank wells. Moat wells were filled with PBS (360µl). The cell plate was spun at 200g for 3 minutes on break 1, and then placed in a non-CO_2 incubator for 30 minutes prior to metabolic analysis.

The inhibitors were pipetted into the cartridge according to **Table 2.4**. The cartridge was placed in the machine to allow for calibration, and then the cell plate was put in the machine for metabolic analysis. OCR was used an indicator of oxphos (**Figure 2.3**) and ECAR was used as an indicator of glycolysis (**Figure 2.4**).

	Compound	**Mechanism**	**Injecting volume**	**Final concentration**
A	Oligomycin	Inhibits ATP synthase	20µl	2µM
B	FCCP	Ionophore/uncoupler	20µl	0.5µM
C	Rotenone/ Antimycin A	Inhibits complex I and III	20µl	100nM / 4µM
D	2DG	Inhibits glycolysis	20µl	30mM

Table 2.4 Metabolic inhibitors used during seahorse experiments.

Oligomycin: inhibits ATP synthase, FCCP: ionophore that disrupts the proton gradient across the inner mitochondrial membrane, Rotenone/ Antimycin A: inhibits complex I and complex III respectively, 2DG: inhibits glycolysis.

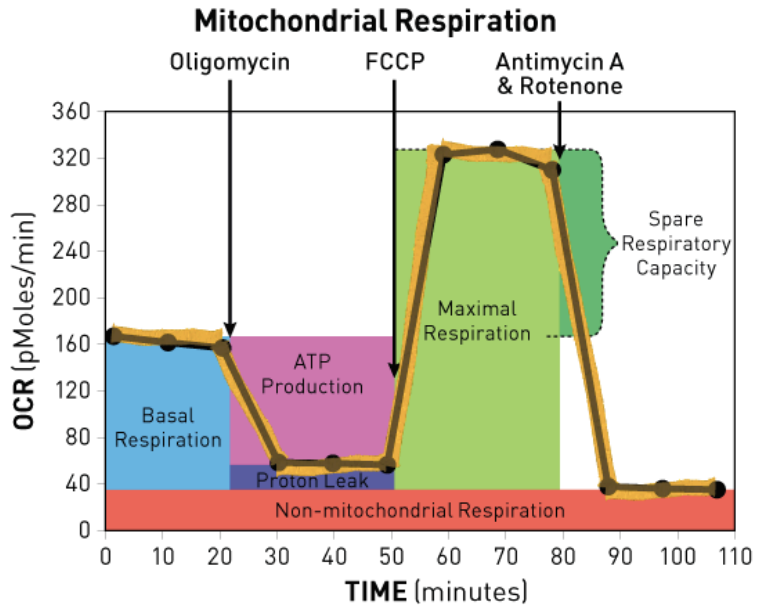


Figure 2.3 Oxygen consumption rate measures mitochondrial respiration

Oligomycin, FCCP, and rotenone/ antimycin A are serially injected to measure ATP-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. Proton leak and spare respiratory capacity (SRC) are then calculated using basal respiration and these parameters.

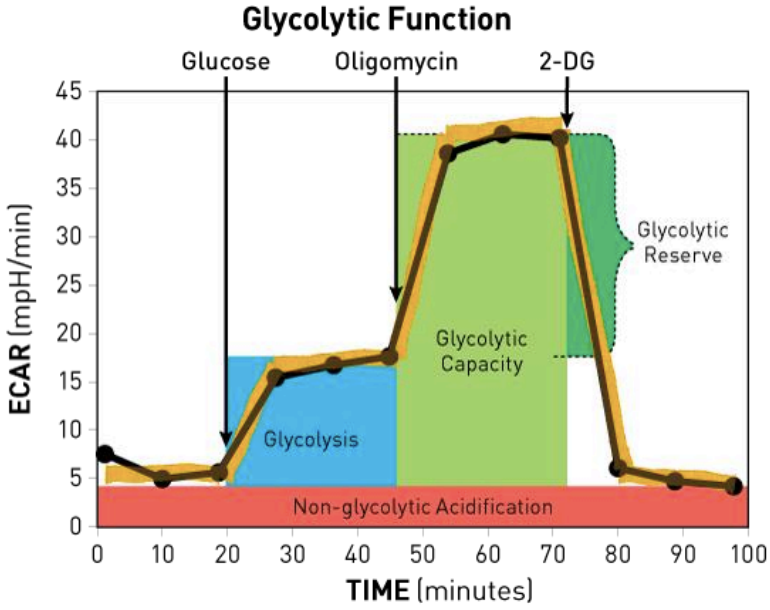


Figure 2.4 Extracellular acidification rate measures glycolysis

Oligomycin is injected to block mitochondrial ATP production and measure maximal glycolysis. 2DG is then injected to measure the full glycolytic capacity of the cells. Glycolytic reserve is then calculated using basal respiration and these parameters.

2.10 Metabolomics Sample Preparation

NK cells from healthy donors and breast cancer patients were purified from freshly isolated PBMC and either left unstimulated or stimulated with IL2 (500IU/ml) for 18 h at 37°C. $2x10^6$ pure NK cells cells were washed twice in ice cold saline solution, lysed with ice cold 80% methanol, and stored at -80°C. The day before shipping, samples were dried down in a vacuum centrifuge (10,000g for 10 mins). Samples were shipped on dry ice to McGill University, Montreal, where unlabelled metabolites were measured by gas/chromatography-mass/spectrometry (GC/MS).

2.11 Kelly cell line maintenance

Kelly cells were originally isolated from a 1 year old female high-risk neuroblastoma patient. Kelly cells were cultured in RPMI Glutamax medium, supplemented with 10% fetal calf serum (FCS) and 1% penicillin/ streptomycin. Cells were passaged once 90% confluency had been reached every 2-3 days. Cell stocks were stored in liquid nitrogen in 10% DMSO 90% FCS solution.

2.12 ADCC Assay

To perform the ADCC assay, Kelly target cells were used. These are neuroblastoma cells which express which express high levels of the GD2 antigen. This GD2 antigen is the target of Dinutuximab that is used in the clinic to treat high-risk neuroblastoma patients. Kelly tumour cells have MYCN amplification and therefore represent aggressive neuroblastoma.

Kelly cells were washed thoroughly out of media twice using warm PBS at 300g for 4 minutes. The cells were resuspended in 4ml PBS and counted. The cells were then spun down and resuspended at a concentration of $3x10^6$cells/ml + 20μM Calcein AM dye. Calcein AM is cleaved by intra-cellular esterases to become fluorescent. Cells actively take up the dye after incubation at 37°C for 30 minutes and appear yellow-green after successful labelling (mixing once after 15mins). Following 30 minutes incubation, labelled Kelly cells were thoroughly washed in wash buffer (20%FCS in PBS) 3 times. After the washed the cells were resuspended in 2ml of RPMI and counted. The cells were resuspended at a concentration of $0.3x10^6$ cells/ml and allowed to rest for 1 hour at 37°C.

PBMC were counted, washed and plated in a 96 well plates according to the E:T ratios in triplicate (10:1, 5:1 and 1:1). Kelly cells were coated with either isotype control (IgG2a, κ) or anti-GD2 antibody (Clone 14.G2a, 2.5μg/ml). Kelly cells were seeded into a 96 well plate with PMBC. Kelly cells were also plated on their own without PBMC, to measure spontaneous release. Additionally, Kelly cells alone were plated in the presence of 8ul of 10% TritonX to measure maximum release of Calcein AM. Cells were incubated for 4 hours at 37°C. For metabolic analyses, oligomycin (40nM), 2DG (2.5mM), rapamycin (20nM), BCH (25mM) or 25HC (5μM) was added for the duration of the incubation.

At the end of the assay, cells were spun down and 75μl of supernatant was aspirated and transferred to a black 96 well plate. The fluorescence of Calcein AM in the supernatant was measured using the following setting of a Spectra Max spectrophotometer: Excitation: 485nm, Auto cut off-10nm, Emission: 525nm, Plate type: Costar 96well plate black, Flashes: 6, Gain: Auto. Triplicate values were averaged and the percentage killing was calculated using the following equation:

$$\frac{\text{(Fluorescence reading} - \text{Spontaneous release)}}{\text{(Maximum release - Spontaneous release)}} X100$$

2.13 Degranulation Assay

The degranulation assay was incorporated as part of the killing and ADCC assays described above with the following additional steps. Golgi stop (containing Monensin, protein transport inhibitor, BD Pharmingen) is added to tumour cells (1/200) before seeding onto plate to ensure that every well has an equal amount of Golgi stop. Once the killing/ADCC assay is fully set up, 1.2ul of anti-CD107a (LAMP1) is added to the well of the highest ratio. Once the killing/ADCC assay was complete, cells were fixed and then stained for CD107a surface expression along with NK cell surface markers.

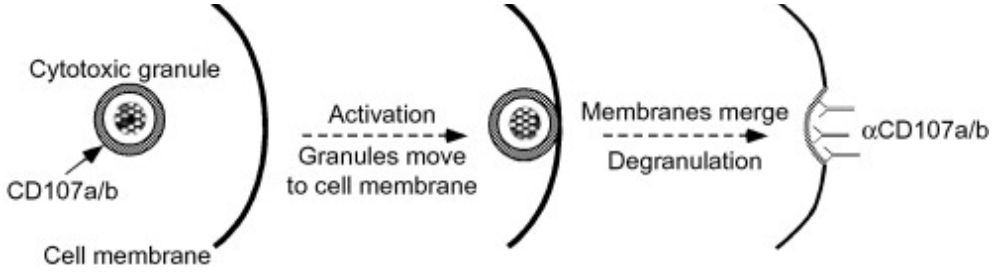


Figure 2.5 CD107a as a measure of degranulation

CD107a is used as an indicator of degranulation. Taken from Betts *et al.* (2004).

2.14 ELISA

An enzyme-linked immunosorbent assay (ELISA) kit (eBioscience) was used to quantify the concentration of TGFβ in the plasma of healthy donors and patients. ELISA plates (Greiner MICROLON) were coated with capture antibody (50 µL per well) diluted in coating buffer and incubated at 4^0C overnight. Wells were washed x 4 with PBST (PBS, 0.05% Tween-20) before blocking with ELISA coating buffer (200 µL) at RT for 1h to reduce non-specific binding. To activate latent TGFβ, all samples were acid activated with 1M HCl (20 µL) for 10 minutes at RT. 1M NaOH (20 µL) was then used to neutralise the reaction. The plate was washed once with PBST before samples/standards were added to wells in the appropriate dilutions. Standards were serially diluted (1/2) x 7 in ELISA diluent before adding to the plate in triplicate (50 µL per well). Samples were transferred to the plate in triplicate (50 µL) and incubated at RT for 2 hr. Plates were washed x 4 before adding biotinylated detection antibody (50 µL per well). The plate was then incubated for 1 hr. Following this the plate was washed x 4 and HRP-conjugated Streptavidin was added to each well (50 µL) and incubated for 30 minutes at RT in th dark. The plates were then washed x 6 with 30 second inbetween washes incubations to reduce background. Substrate solution (tetramethylbenzidine, 50 µL) was added to each well and incubated for 15-30 minutes at RT in the dark. By visual determination (blue colour change), the reaction was stopped at the most appropriate time by adding stop solution (1M H_3PO_4, 25 µL).

The OD values were determined by measuring absorbance values at 450 nm using a Spectamax Microplate Reader (Molecular Devices). Unknown TGFβ concentrations were determined by plotting a standard curve of the known concentrations for each ELISA plate (**Figure 2.6**).

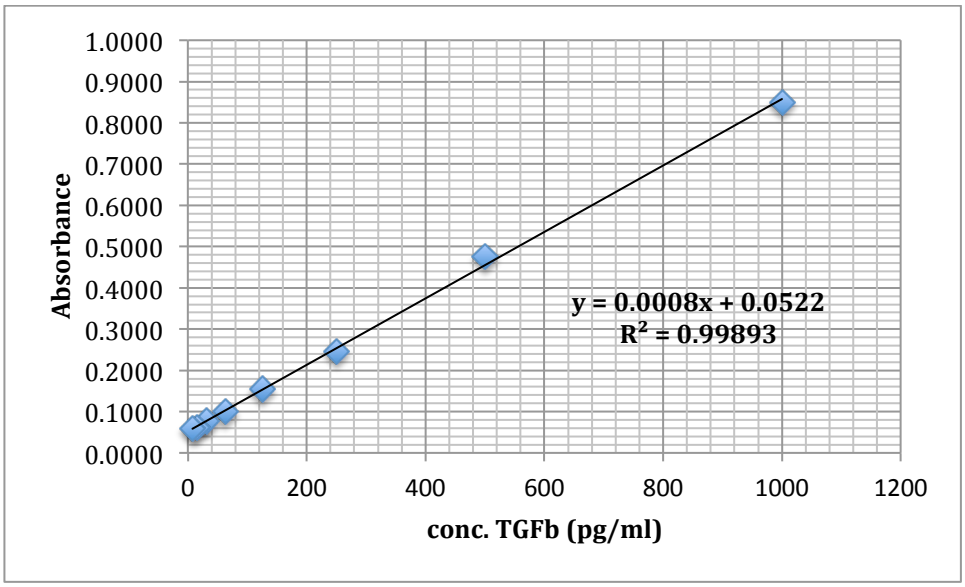


Figure 2.6 Standard curve of absorbance (450nm) versus TGFβ concentration

2.15 Statistical Analysis

All data was analysed using GraphPad Prism 8 software. Data was determined to be parametric or not using the D'Agostino-Pearson normality test. Data was then analysed using the student-t test when two data sets were being compared, or the one/two-way ANOVA test when more than two data sets were being compared. Healthy versus patient tests were deemed 'unpaired', while healthy or patient +/- antibody or inhibitor were deemed 'paired'.

Chapter 3

3 NK cell metabolic dysfunction during metastatic breast cancer

3.1 Introduction

Breast cancer is the most common cancer type in women worldwide. In Ireland, a staggering 1 in 9 women will develop breast cancer in the course of their lifetime (breastcancerireland.ie). The majority of patients diagnosed with breast cancer are over the age of 50 years, although younger diagnoses do sometimes occur. Risk factors for developing breast cancer include age, taking the contraceptive pill or other hormone based medications, obesity, alcohol consumption and radiation exposure (www2.hse.ie). National screening programs alongside a good range of treatment options has resulted in a 5 year survival rate of 84–99% for early stage breast cancer (American Cancer Society, 2012). However, this is reduced significantly for patients with advanced disease, and those that present with metastatic disease have a 5 year survival rate of just 23%. Hence, there is an urgent need to develop new treatment strategies for these patients.

Breast cancer can be classified according to tissue type, grade (histology of the tumour), stage (extent and evolution of the tumour) and gene expression. The treatment regime for patients is often dictated by the presence or absence of certain hormone receptors: the estrogen receptor (ER, ~80% tumours), the progesterone receptor (PR, ~60% of tumours, normally also ER+), and the human epidermal growth factor receptor 2 (HER2, ~20% tumours, normally aggressive) [137]. A breast tumour may also be classified as triple negative, meaning that it does not express any of the hormone receptors (~10% - 20%). Triple negative tumours are often associated with expression of the oncogene BRCA1 and have limited treatment options alongside poor prognosis [138]. In recent years, it has become more common for healthy women positive for the BRCA1 oncogene to get a mastectomy, reducing their risk of developing breast cancer by 95%.

3.1.1 NK cells protect against breast cancer

There are many studies demonstrating the role that NK cells play in the protection against breast cancer. For example, Ascierto *et al.* studied primary tumour biopsies in patients who either experienced 5-9 years relapse free survival, or developed tumour relapse within 1-6 years. They reported that the expression of the activating receptors NKp46, NKp44, DNAM1 and NKG2D was significantly higher in breast cancer patients with favourable prognosis [63]. More recently, Garcia-Chagollan *et al*. showed that breast tumours that were sensitive to chemotherapy treatment had increased expression of various NK cell surface receptors such as NKp46 and various KIRs, compared to tumours that were resistant to chemotherapy [139]. These studies identify the possibility that targeting NK cells to increase their activation and functions during breast cancer may help to increase the survival rate of patients. Indeed, we already know that harnessing NK cells for the treatment of breast cancer is safe and effective. Trastuzumab (Herceptin) is an antibody-based immunotherapy that binds to HER2 antigens on the surface of the breast cancer tumour cells and induces NK cell mediated ADCC. Trastuzumab has improved the 10-year survival rate of patients with HER2+ breast cancer by 11% [140, 141]. Another form of immunotherapy known as checkpoint inhibitors are currently undergoing intense research in the clinic and preliminary results from many breast cancer studies have been promising [142]. Several of these studies led to the FDA approval of anti-PD-L1 therapy in combination with chemotherapy for the treatment of triple negative breast cancer in March 2019 [143].

Despite the important role that NK cells play in the protection against breast cancer, their functions often become reduced in patients with cancer, including those with breast cancer. This was demonstrated clearly in a recent study whereby exposure of NK cells to breast cancer tumour cells resulted in a loss of cytotoxic ability [144]. Similarly, Konjevic *et al.* reported that peripheral blood NK cells from breast cancer patients had reduced cytotoxic activity against K562 tumour cells [15], which worsened as the disease advanced. Mamessier *et al.* showed that peripheral blood NK cells from breast cancer patients had reduced expression of the activating receptors NKp30, NKG2D, DNAM-1, and CD16, had reduced IFNγ and TNFα production, and had impaired cytotoxicity and degranulation against multiple breast cancer cell lines [74]. Interestingly, some of this dysfunction was recapitulated using conditioned media generated with breast cancer tumour cells lines, suggesting that it may in part be mediated by a tumour-derived soluble factor.

3.1.2 Glycolysis and oxphos fuel NK cell functions

Indeed, NK cells play a vital role in protection against cancer, and we now know that they rely on metabolism to carry out many of their anti-cancer functions. As discussed in chapter 1, NK cells have characteristic metabolic configurations. Oxphos is used to meet their homeostatic needs, and after short-term stimulation (6h) oxphos remains the dominant form of metabolism [145]. However, when activated over longer periods of time (18h+), NK cells upregulate both oxphos and glycolysis, with their primary form of metabolism shifting to glycolysis [122, 123]. Although the energy yield of glycolysis is modest compared to that of oxphos (2 versus 36 ATP molecules), this pathway has the added benefit of allowing the cell to divert glycolytic intermediates to biosynthetic pathways that support anabolic growth, such as the PPP for the production of amino acids (**Figure 3.1**) [114]. Radioactive labelled glucose (^{13}C) tracing experiments have demonstrated that IL2/12 activated murine NK cells use glycolysis in part to support to the production of amino acids such as serine, alanine and aspartate, as well as the fatty acid palmitic acid [124]. The majority of this glucose ends up as secreted lactate and cytosolic citrate (to support the citrate malate shuttle).

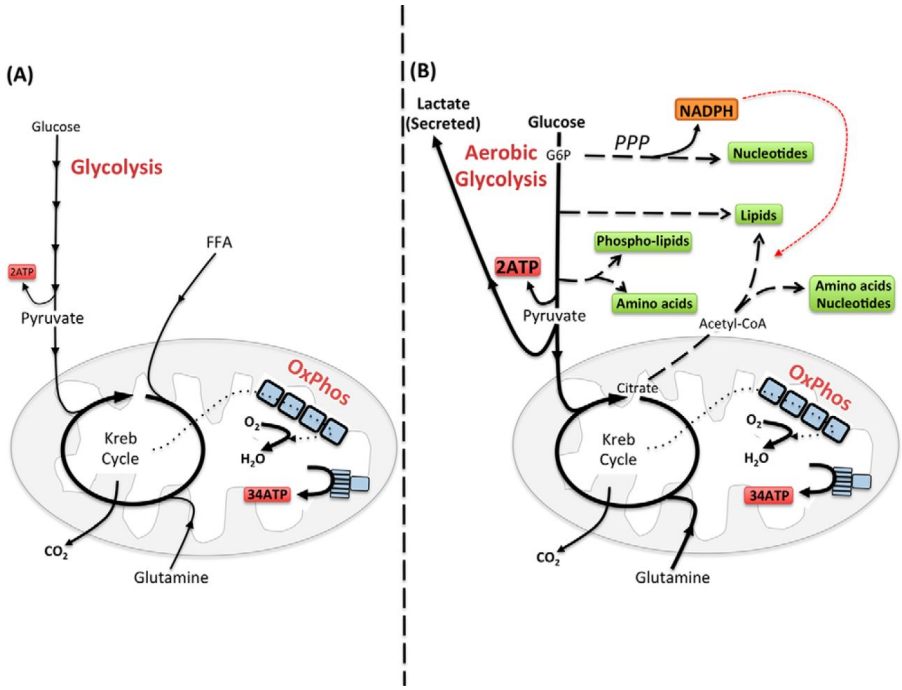


Figure 3.1 Aerobic glycolysis facilitates cellular biosynthesis.

A) Resting cells are functionally inert and engage in oxidative phosphorylation for efficient generation of ATP. B) Activated cells engage in high rates of aerobic glycolysis that supports biosynthetic processes of the cell as it allows the uptake of larger amounts of glucose and the maintenance of elevated glycolytic flux. Glycolytic intermediates are then diverted into various

pathways for the synthesis of biomolecules that support biosynthetic processes e.g. glucose 6-phosphate, generated by the first step in glycolysis, can feed into the PPP to support nucleotide synthesis [146].

Highlighting the importance of both glycolysis and oxphos in NK cells, when oxphos is inhibited with oligomycin and when glycolysis is inhibited using 2DG or galactose, NK cell function is also inhibited [122, 123]. Furthermore, treatment with 2DG impairs the ability of NK cells to control viral infection *in vivo* [145]. The upregulation of metabolism in NK cells correlates with the increased expression of CD71 (transferrin receptor), CD98 (amino acid transporter), Glut1 receptor (glucose uptake) and also NBDG uptake (nutrient uptake). Further, NK cells with higher CD71 expression, i.e. more metabolically active, also have higher levels of IFNγ production. Interestingly, in humans these increases are particularly evident in $CD56^{bright}$ NK cells, suggesting that this subset is more metabolically active [122]. Overall this metabolic rewiring allows for production of both energy (ATP) and biosynthetic precursors that are needed for NK cells to carry out their functions and may also serve to enhance their longevity and thus their ability to function in parallel with the adaptive immune system.

3.1.3 Mitochondrial dynamics support NK cell function

Central to many key metabolic processes such as the TCA cycle and oxphos, the mitochondria have emerged as organelles that play an important role in facilitating the optimal functions of NK cells. In addition to energy production, they play a central role in mediating cell division and cell death. In contrast to the classical textbook image, mitochondria can adopt a fluid range of morphological states, ranging from fully punctate and circular (fissed) to thin and elongated (fused) [147]. These fissed and fussed states are highly dynamic and depend on the needs of the cell. For example, fissed mitochondria are associated with anabolic metabolism and support the functions of T effector cells, while more fused mitochondria are associated with catabolic metabolism and support the functions of T memory cells (**Figure 3.2**) [148].

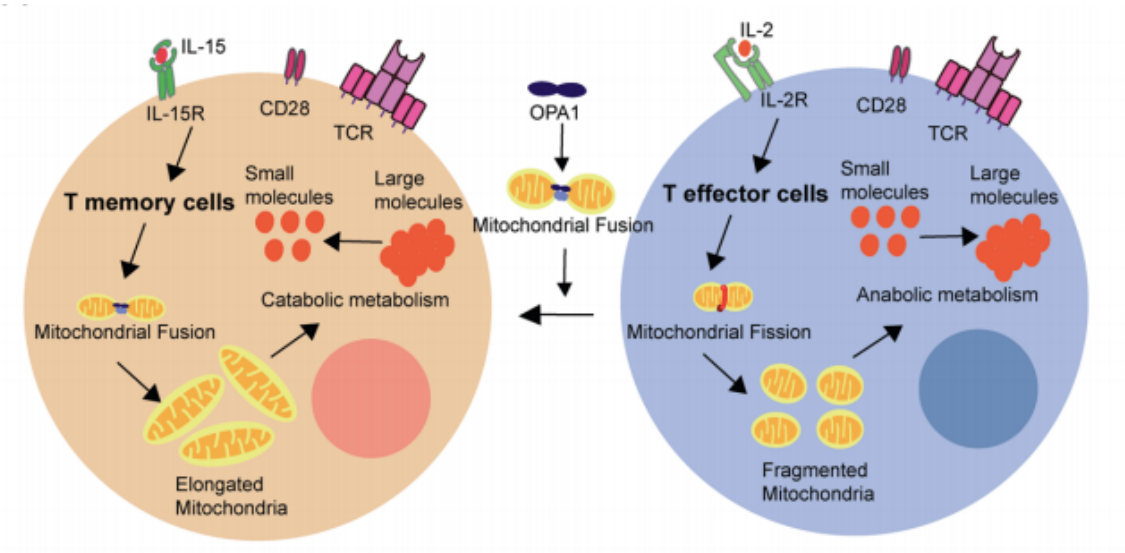


Figure 3.2 Mitochondrial dynamics controls T cell function.

Mitochondrial dynamics are involved in metabolic programming to control T cell fate. Memory T cells (Tm) undergo increased fusion to promote catabolic metabolism, while effector T cells (Te) undergo increased fission to increase anabolic metabolism. Increasing mitochondrial fusion regulated by OPA1 could convert Te cells to the Tm phenotype. Adapted from [149].

Over the past decade, several studies have revealed how integrally linked mitochondrial dynamics are to the function of immune cells [149], and indeed this includes NK cells. Abarca-Rojano *et al.* investigated the role of mitochondrial dynamics during the formation of an immune synapse with K562 tumour cells [150]. They showed that the mitochondria of peripheral blood human NK cells depolarise upon contact with K562 tumour cells, and that the mitochondria migrate/polarise towards the immune synapse in an NKG2D dependent manner. By doing so, the mitochondria provide an increase in the local concentration of ATP at the immune synapse, suggesting that NK cells require a lot of ATP to carry out their cytotoxic functions. Similarly, Miranda *et al.* looked at IL2 induced mitochondrial changes in human NK cells [151]. They showed that IL2 stimulation increased both the mitochondrial mass and the mitochondrial membrane potential. This was dependent on the expression of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α), and its deletion resulted in reduced mitochondrial mass and membrane polarisation, in addition to impaired IFNγ production. Finally, it has been shown that mitophagy, i.e. the removal of defective mitochondria, is essential for the development of NK cell memory [152]. Overall, it is clear that mitochondrial structure and dynamics are fundamental for many functions of NK

cells, and it seems likely that this area of research will expand greatly in the near future.

3.1.4 Regulation of NK cell metabolism

The metabolic configuration of NK cells requires a high degree of regulation. As with many immune cells, several aspects of NK cell metabolism are highly dependent on the protein complex mTORC1, which can sense nutrient levels and respond to extracellular and intracellular signals, such as growth factors and hormones. mTORC1 then drives the alteration in protein translation within the cell and shapes its response accordingly [116]. mTORC1 activity increases upon cytokine stimulation of NK cells and enhances glycolytic flux through the cells. Inhibition of mTORC1 using rapamycin results in inhibition of cell size, nutrient receptor expression (CD71 and CD98), and glycolysis, but not oxphos [122, 123, 153]. mTORC2, which has a Rictor subunit in place of the Raptor subunit found in mTORC1, has been linked to NK cell maturation in several studies [153, 154]. Interestingly, while mTORC1 promotes mTORC2 activity, mTORC2 can repress mTORC1 activity, thus inhibiting NK cell functions and SLC7A5 (amino acid transporter) expression [155].

Beyond mTOR signalling, NK cells have quite unique underlying regulatory mechanisms. SREBP is a transcription factor known traditionally for its role in fatty acid and cholesterol synthesis. However, in NK cells, SREBP is fundamental for the modulation of glycolysis and oxphos. This is partly due to regulated expression of the main transporter involved in the citrate-malate shuttle – SLC25A1 [124]. Unsurprisingly, inhibition of SREBP also inhibits NK cell functions.

cMyc is another transcription factor that has been shown to be essential for NK cell metabolism [125]. In this setting, cMyc is acutely controlled by the availability of amino acids. These amino acids, glutamine in particular, are imported into the cell via the transporter SLC7A5 where they then turn on cMyc and allow it to regulate NK cell metabolism and function. Interestingly, HIF1α was shown to not play a role in regulating murine NK cell metabolism in this study, which is in contrast to other cytotoxic lymphocytes. Dong *et al.* has recently shown that inositol-requiring enzyme 1 (IRE1), an ER-nucleus signalling protein, is essential for optimal NK cell anti-tumour and anti-viral responses and works by directly regulating cMyc activity [156].

The many immunometabolism studies discussed in this chapter clearly demonstrate just how fundamental metabolism is for the functions of NK cells. They have shown what metabolic pathways are most important and what regulators are required for maintaining them in NK cells from a healthy/normal situation. However, whether/how these processes are impacted in the human cancer setting remains less explored. As mentioned previously, it is well accepted that the functions of NK cells become altered in patients with cancer. Hence, we hypothesised that altered NK cell metabolism might contribute to the reduced functions observed in NK cells from breast cancer patients. In this chapter we explore the function and metabolism of NK cells from breast cancer patients and healthy controls. Blood samples from patients with metastatic breast cancer were routinely obtained from the oncology clinic of St. James's Hospital, Dublin. Blood samples from age and sex matched healthy donors were obtained from TBSI. PBMC was isolated from the blood, and where necessary NK cells were purified by magnetic bead separation. Certain aspects of metabolism in these NK cells were analysed directly *ex vivo* in order to get a snapshot of the direct impact that the cancer has on the peripheral NK cells. Cytokine stimulations for the duration of 18 hours were also carried out to assess their ability to sense pro-inflammatory stimuli and respond appropriately. The findings of this chapter have important implications for the design of future NK cell based immunotherapy strategies against breast cancer and other solid tumours.

3.1.5 Aims and Objectives:

1. To examine the activation and function of NK cells from breast cancer patients.
2. To explore nutrient receptor expression and nutrient uptake in patient NK cells.
3. To investigate different forms of metabolism in NK cells from breast cancer patients:
    - Fatty acid metabolism
    - TCA cycle
    - Mitochondrial metabolism – oxphos and mitochondrial structure.
    - Glycolysis
4. To determine whether altered mTORC1 activity may contribute to any phenotype observed.

3.2 Results

3.2.1 NK cells from breast cancer patients have reduced expression of the activation marker CD25 and impaired IFNγ production in response to cytokine.

The normal frequency of circulating NK cells in lymphocytes amongst healthy individuals is approximately 10%. NK cell subpopulations divided into 10% $CD56^{bright}$ NK cells and 90% $CD56^{dim}$ NK cells. We assessed the frequencies of these populations in breast cancer patients by flow cytometry and compared them to those in healthy donors. Breast cancer patients had normal frequencies of circulating NK cells when compared to healthy donors, with a mean of 11.6% and 13.5% for healthy donors and patients respectively (**Figure 3.3A**). However, the relative frequency of $CD56^{bright}$ NK cells was reduced in patients, with a corresponding increase in the frequency of $CD56^{dim}$ NK cells (**Figure 3.3B**).

In order to investigate the ability of NK cells from breast cancer patients to activate in response to cytokine, the upregulation of the two activation markers, CD69 and CD25, in response to IL2 and IL12/15 stimulation were measured by flow cytometry. As expected, $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors robustly upregulated CD69 expression in response to both IL2 and IL12/15 (**Figure 3.4A** for representative dot plots). Similarly, $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients upregulated CD69 expression to levels comparable with those seen in healthy donor NK cells, upon stimulation with IL2 and IL12/15 (**Figure 3.4B** and **3.4C**).

$CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors also upregulated expression of CD25 in response to cytokine stimulation, particularly with IL12/15 (**Figure 3.5A** for representative dot plots). However, NK cells from breast cancer patients induced significantly reduced levels of CD25 expression under the same conditions (**Figure 3.5B** and **3.5C**). These data indicate that while patient NK cells are capable of sensing and responding to cytokine stimuli, they behave differently to healthy normal NK cells in terms of their activation status, as measured by CD69 and CD25.

Given the importance of NK cell functions for anti-cancer responses, we measured granzyme B expression and IFNγ production in response to cytokine using flow

cytometry. Basal expression of granzyme B and IFNγ production was equivalent in resting NK cells from healthy donors and breast cancer patients (data not shown). NK cells from healthy donors upregulated granzyme B in response to IL2 and IL12/15 stimulation (**Figure 3.6A** for representative histograms). Compared to IL2, IL12/15 induced the strongest increase in granzyme B expression in both healthy donor and patient NK cells. As $CD56^{dim}$ NK cells constitutively express their cytotoxic molecules, this increase was particularly evident in the $CD56^{bright}$ subset. NK cells from breast cancer patients also upregulated granzyme B expression in response to IL2 and IL12/15 stimulation (**Figure 3.6B** and **3.6C**). Therefore, no significant difference in the ability to upregulate granzyme B expression in response to cytokine was found in patients with metastatic breast cancer (non significant with a student t-test, data not shown).

NK cells from healthy donors upregulated IFNγ production in response to IL12/15 as expected (**Figure 3.7A** for representative dot plots). As $CD56^{bright}$ NK cells are expert producers of cytokine, the increase in IFNγ production was most striking in this subset (mean of approximately 60% and 20% for $CD56^{bright}$ and $CD56^{dim}$ NK cells respectively). In contrast, NK cells from breast cancer patients had significantly reduced IFNγ production in response to IL12/15 stimulation (**Figure 3.7B**). This was evident in both the $CD56^{bright}$ and $CD56^{dim}$ subsets. Together these data show that while NK cells from breast cancer patients are cytotoxic, they have strongly impaired cytokine production.

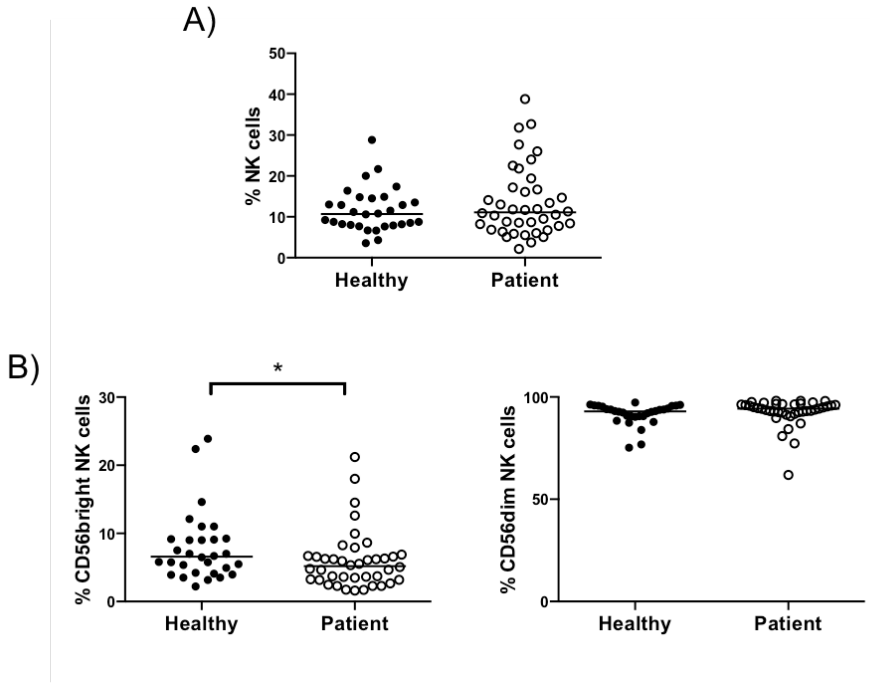


Figure 3.3 NK cells from breast cancer patients have reduced frequency of the CD56^{bright} subset.

Freshly isolated PBMC from healthy donors and patients were stained for CD56 and CD3 to identify the frequency of NK cells ($CD56^{+}CD3^{-}$) and the $CD56^{bright}$ and $CD56^{dim}$ populations by flow cytometry. (A) Frequency of NK cells. (B) Frequency of $CD56^{bright}$ and $CD56^{dim}$ NK cells. Lines show the mean value. Each dot represents 1 donor (n = 30-40). Samples were compared using the unpaired student-t test, *$p<0.05$.

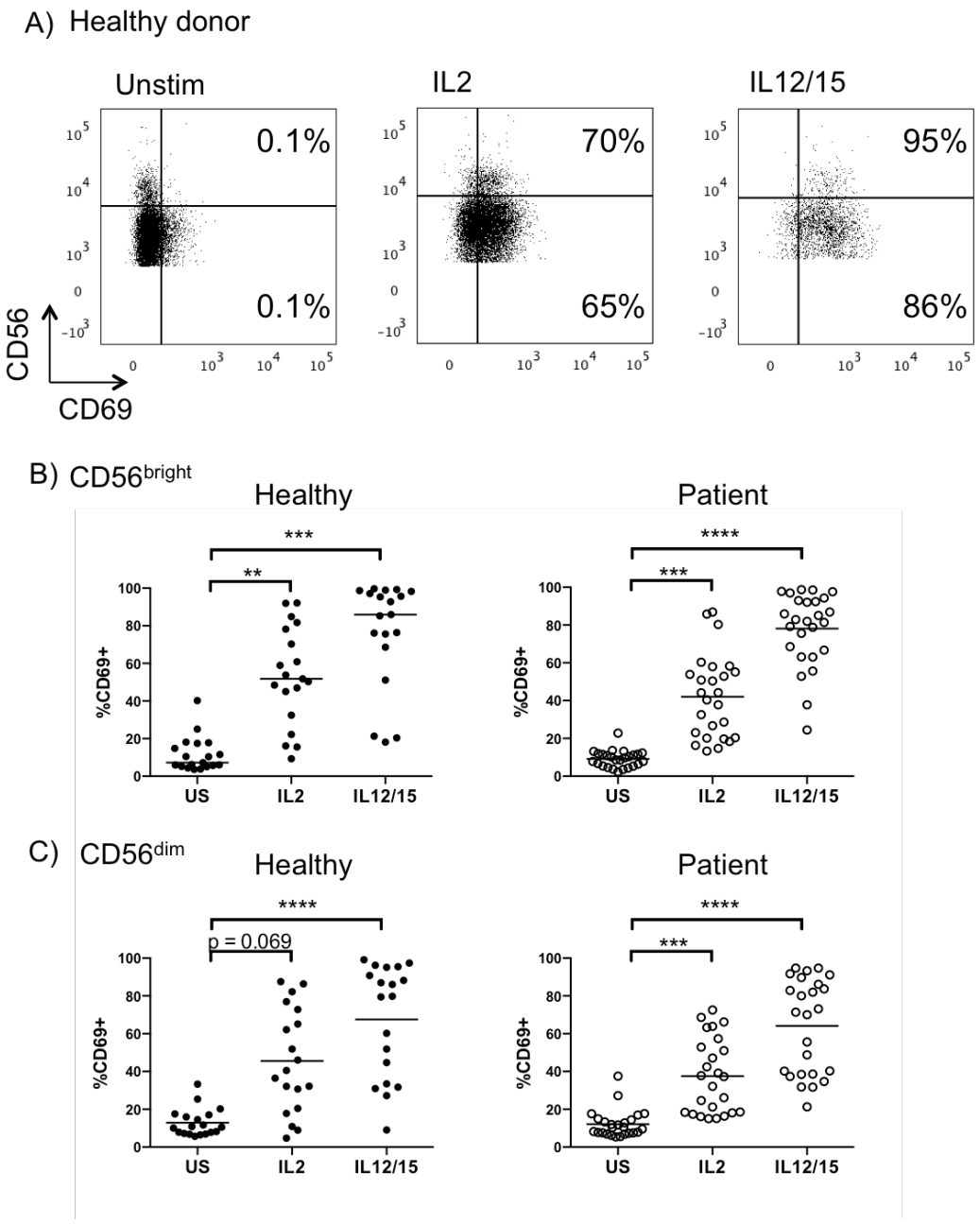


Figure 3.4 NK cells from breast cancer patients upregulate the activation marker CD69 in response to cytokine.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for NK cell markers and CD69 and analysed by flow cytometry. (A) Representative dot plot of the upregulation of CD69 in response to IL2 and IL12/15 in healthy donor NK cells. (B and C) Frequency of CD69 expression in $CD56^{bright}$ NK cells from healthy donors and breast cancer patients. (D and E) Frequency of CD69 expression in $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n = 19-24). Samples were compared by one-way ANOVA test, **p<0.01, ***p<0.001, ****p<0.0001.

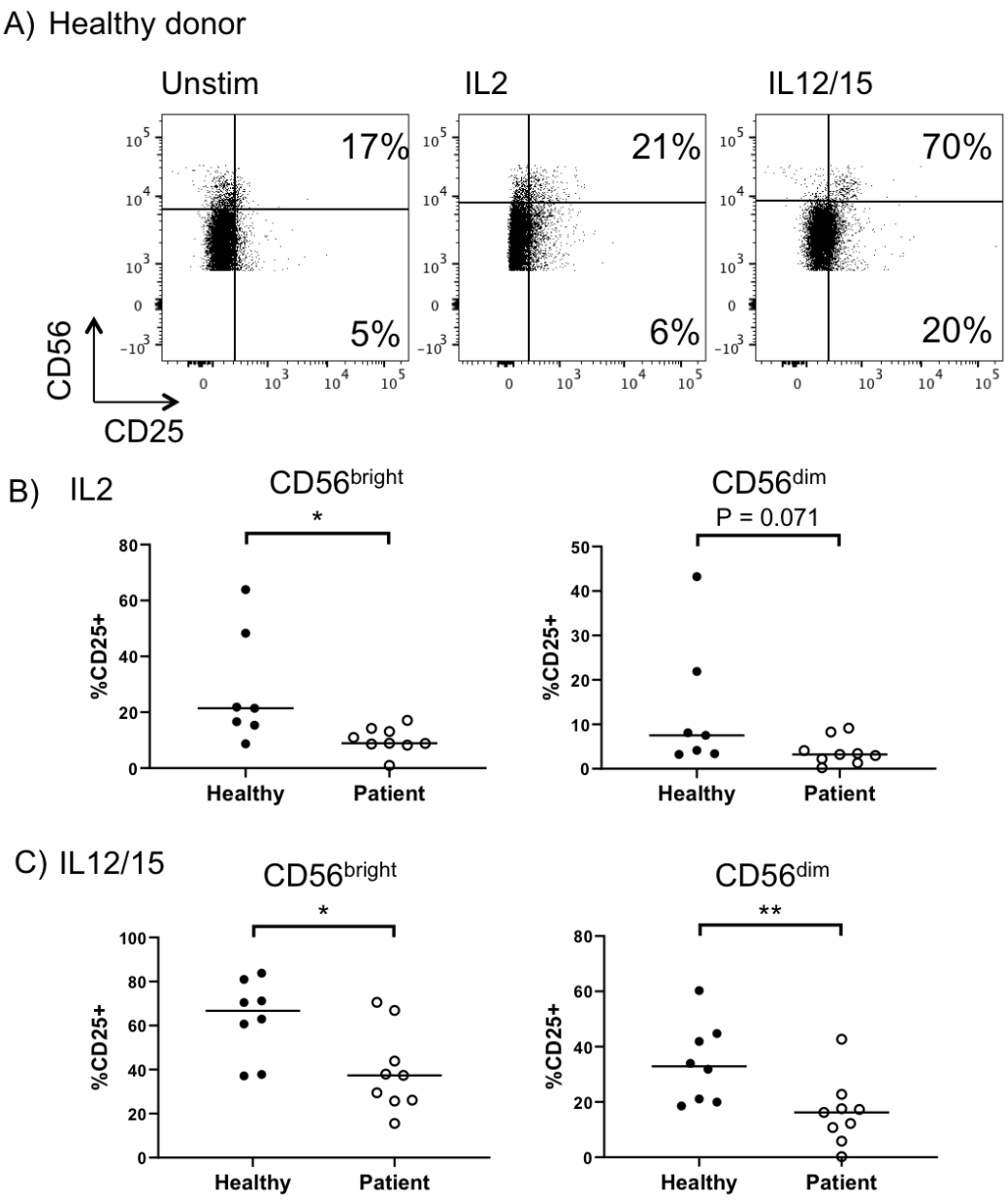


Figure 3.5 NK cells from breast cancer patients have impaired upregulation of the activation marker CD25 in response to cytokine.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for CD25 and analysed by flow cytometry. (A) Representative dot plot of the upregulation of CD25 in response to IL2 and IL12/15 in healthy donor NK cells. (B and C) Frequency of CD25 expression in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. (D and E) Frequency of CD25 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n = 7-9). Samples were compared using the unpaired student t-test, *$p<0.05$, **$p<0.01$.

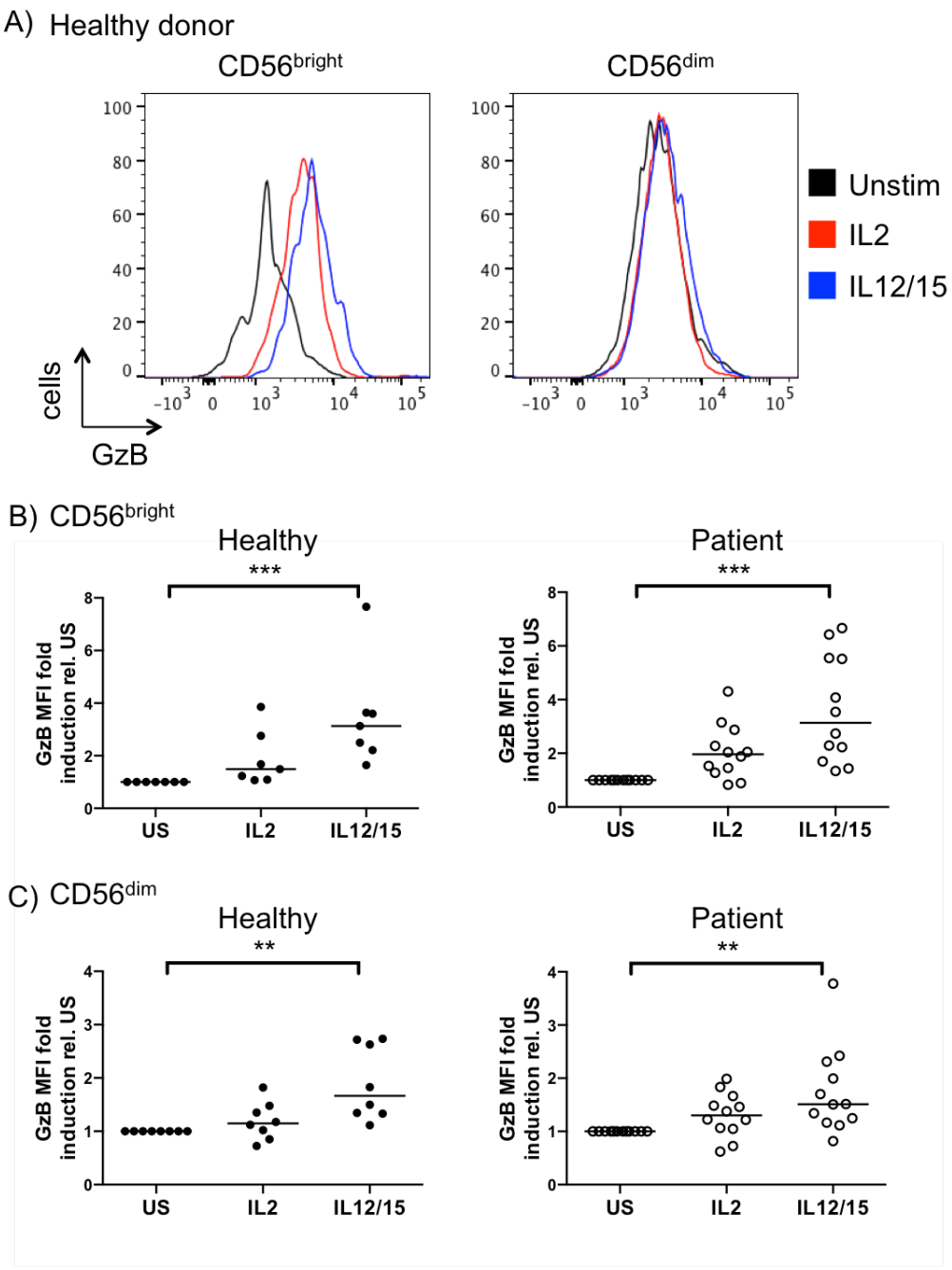


Figure 3.6 NK cells from breast cancer patients upregulate granzyme B in response to cytokine.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for granzyme B and analysed by flow cytometry. (A) Representative histogram of the upregulation of granzyme B in response to IL2 and IL12/15 in healthy donor NK cells. (B and C) Fold increase in the MFI of granzyme B relative to unstimulated control in $CD56^{bright}$ NK cells from healthy donors and breast cancer patients. (D and E) Fold increase in the MFI of granzyme B relative to unstimulated control in $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n = 8-12). Samples were compared by one-way ANOVA test, **p<0.01, ***p<0.001.

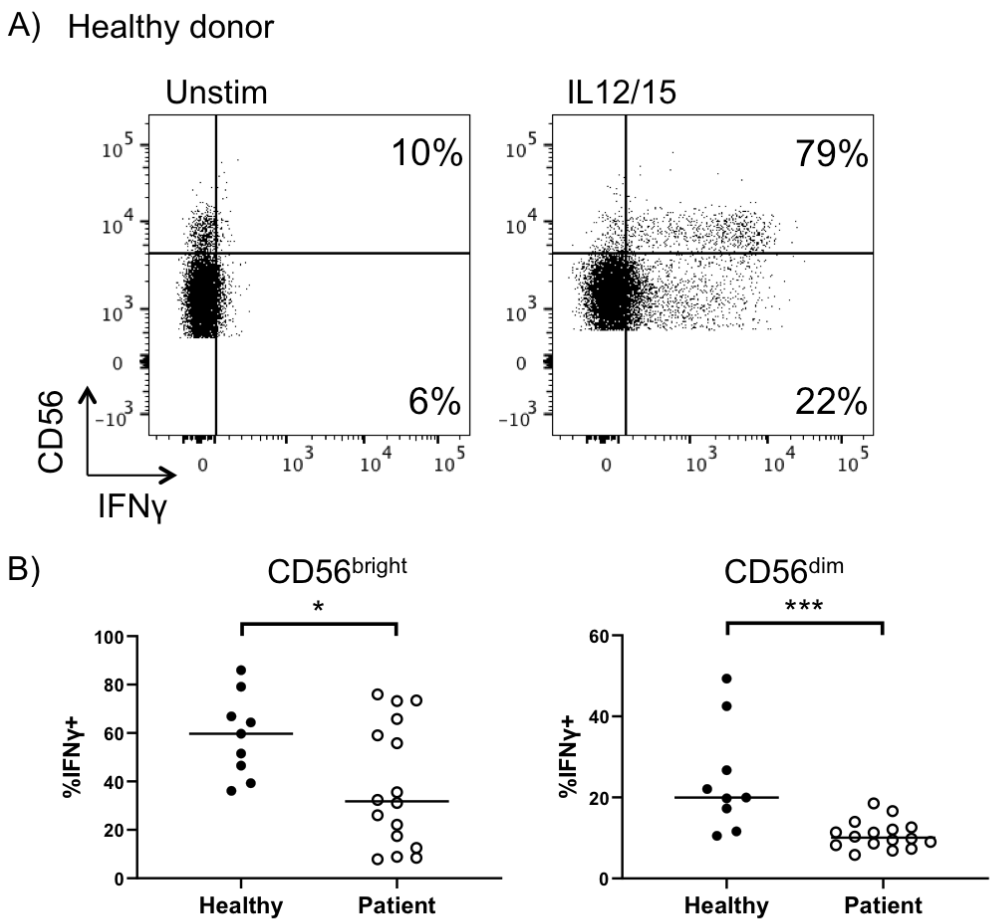


Figure 3.7 NK cells from cancer patients have impaired upregulation of IFNγ in response to IL12/15.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Golgi plug was added for the final 4 h. Cells were analysed for IFNγ production by intracellular flow cytometry staining. (A) Representative dot plot of the upregulation of IFNγ in response to IL12/15 in healthy donor NK cells. (B) Frequency of IFNγ production in $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and patients in response to IL12/15. Lines show the mean value. Each dot represents 1 donor (n = 9-16). Samples were compared using the unpaired student t-test, *p<0.05, ***p<0.001.

3.2.2 NK cells have impaired expression of nutrient receptors.

As nutrients are essential to fuel cellular metabolism, the expression of nutrient receptors and the ability to take up nutrients was next measured using flow cytometry. Resting NK cells from healthy donors and patients did not express the transferrin receptor CD71 (**Figure 3.8A** for healthy donor representative dot plot). We therefore mainly focused on changes induced by cytokine that would allow NK cells to become more metabolically active. NK cells from healthy donors upregulated CD71, the transferrin receptor, in response to both IL2 and IL12/15 stimulation. This was particularly evident in the $CD56^{bright}$ NK cell subset. In contrast, IL2 stimulated $CD56^{bright}$ NK cells from breast cancer patients had significantly reduced CD71 expression (**Figure 3.8B**). There was also a trend towards lower CD71 expression in IL12/15 stimulated $CD56^{bright}$ NK cells from breast cancer patients (**Figure 3.8C**).

The large neutral amino acid transporter (LAT1/SLC7A5) is responsible for uptake of amino acids into the cell. It was measured by analysing expression of one of its core subunits, CD98, using flow cytometry. CD98 was expressed constitutively and at similar levels in resting NK cells from both healthy donors and patients (**Figure 3.9A** for healthy donor representative), and therefore we focused on cytokine-induced changes in CD98 expression. Both $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors upregulated CD98 expression in response to IL2 and IL12/15 stimulation (**Figure 3.9A** for representative). However, this was significantly impaired in IL2 stimulated NK cells from breast cancer patients (**Figure 3.9B**). Similarly, $CD56^{dim}$ NK cells from breast cancer patients had reduced CD98 expression in response to IL12/15 stimulation.

Nutrient uptake through SLC7A5 was next measured using the kynurenine uptake assay [135]. Resting NK cells from healthy donors and patients had high basal levels of kynurenine uptake through SLC7A5 (**Figure 3.10A** for healthy donor representative histogram). In contrast to CD98 expression in healthy donor NK cells, uptake of kynurenine through SLC7A5 was significantly upregulated response to IL2 and IL12/15 in the $CD56^{bright}$ NK cell subset only. $CD56^{bright}$ NK cells from breast cancer patients upregulated uptake of kynurenine in response to IL2 and IL12/15 stimulation to equivalent levels (**Figure 3.10B**).

The more general nutrient uptake assay, 2-NBDG uptake, was next used to investigate nutrient uptake in NK cells from healthy donors and NK cells. 2-NBDG is not taken up through the glucose receptor but does correlate with glucose uptake through the glucose receptor [136]. While the data are non-significant, it appears that NK cells from healthy donors may increase 2-NBDG uptake in response to IL2 and IL12/15 stimulation. Again, this is particularly evident in the $CD56^{bright}$ subset (**Figure 3.11A** for representative histogram). NK cells from breast cancer patients upregulated uptake of 2-NBDG in response to IL2 and IL12/15 stimulation to equivalent levels (**Figure 3.11B** and **3.11C**).

In terms of NK cells from healthy donors, these data show that $CD56^{bright}$ NK cells take up more nutrients than $CD56^{dim}$ NK cells. This supports the idea that $CD56^{bright}$ NK cells are more metabolically active than $CD56^{dim}$ NK cells [122]. While NK cells from breast cancer patients had altered expression of nutrient receptors, it seems that they are still able to take up certain nutrients to the same extent as NK cells from healthy donors. This suggests that there may be some compensatory mechanisms at play. More in depth analysis of nutrient uptake is required to confirm this e.g. by radiolabelled nutrient uptake and mass spectrometry.

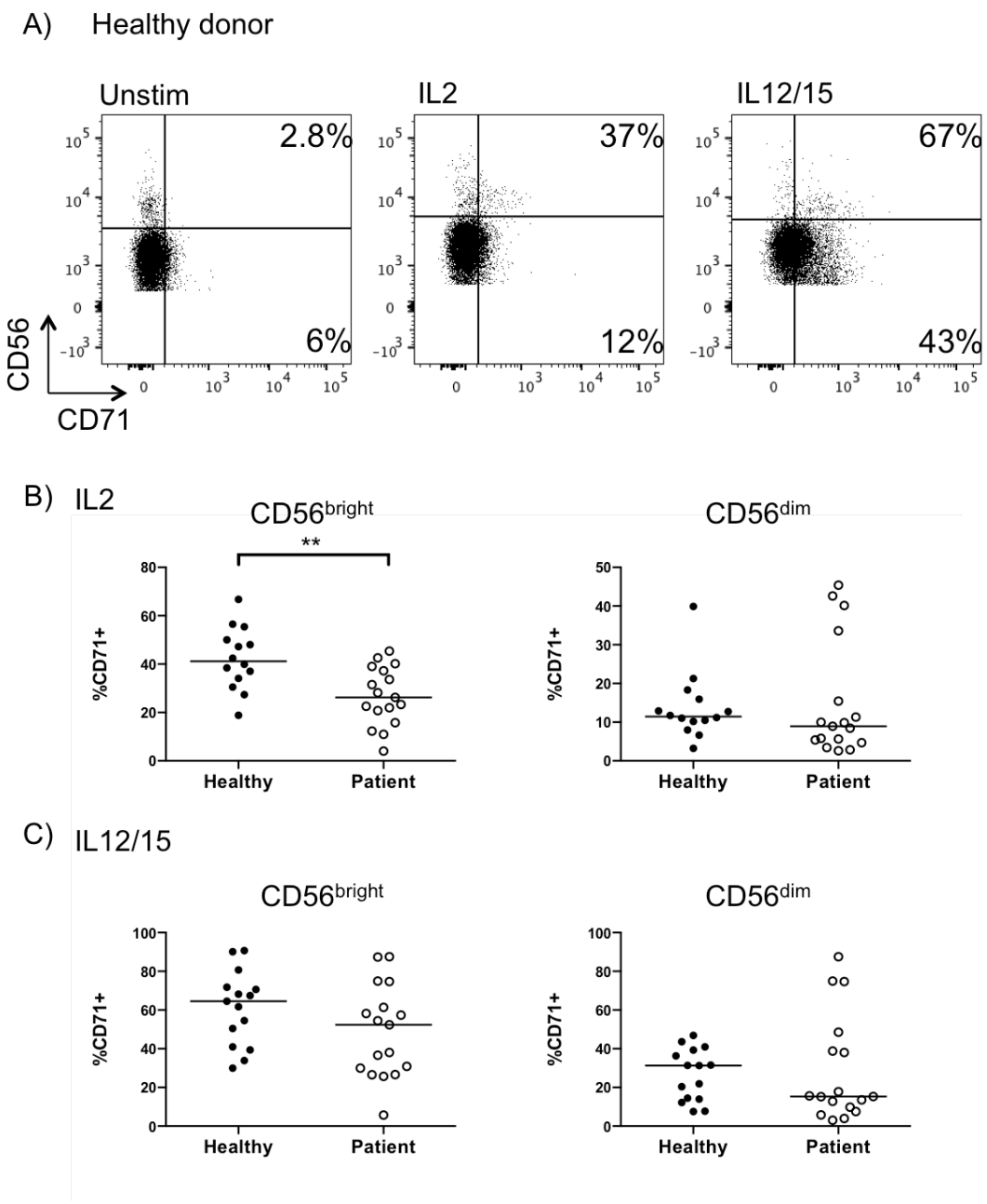


Figure 3.8 CD56^{bright} NK cells from breast cancer patients have reduced expression of CD71 response to IL2.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. NK cells were stained for CD71 and analysed by flow cytometry. (A) Representative dot plot of the upregulation of CD71 in response to IL2 and IL12/15 in healthy donor NK cells. (B) Frequency of CD71 expression in IL2 stimulated CD56^{bright} and CD56^{dim} NK cells from healthy donors and breast cancer patients. (D and E) Frequency of CD71 expression in IL2 stimulated CD56^{bright} and CD56^{dim} NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n = 14-17). Samples were compared using the unpaired student t-test, **p<0.01.

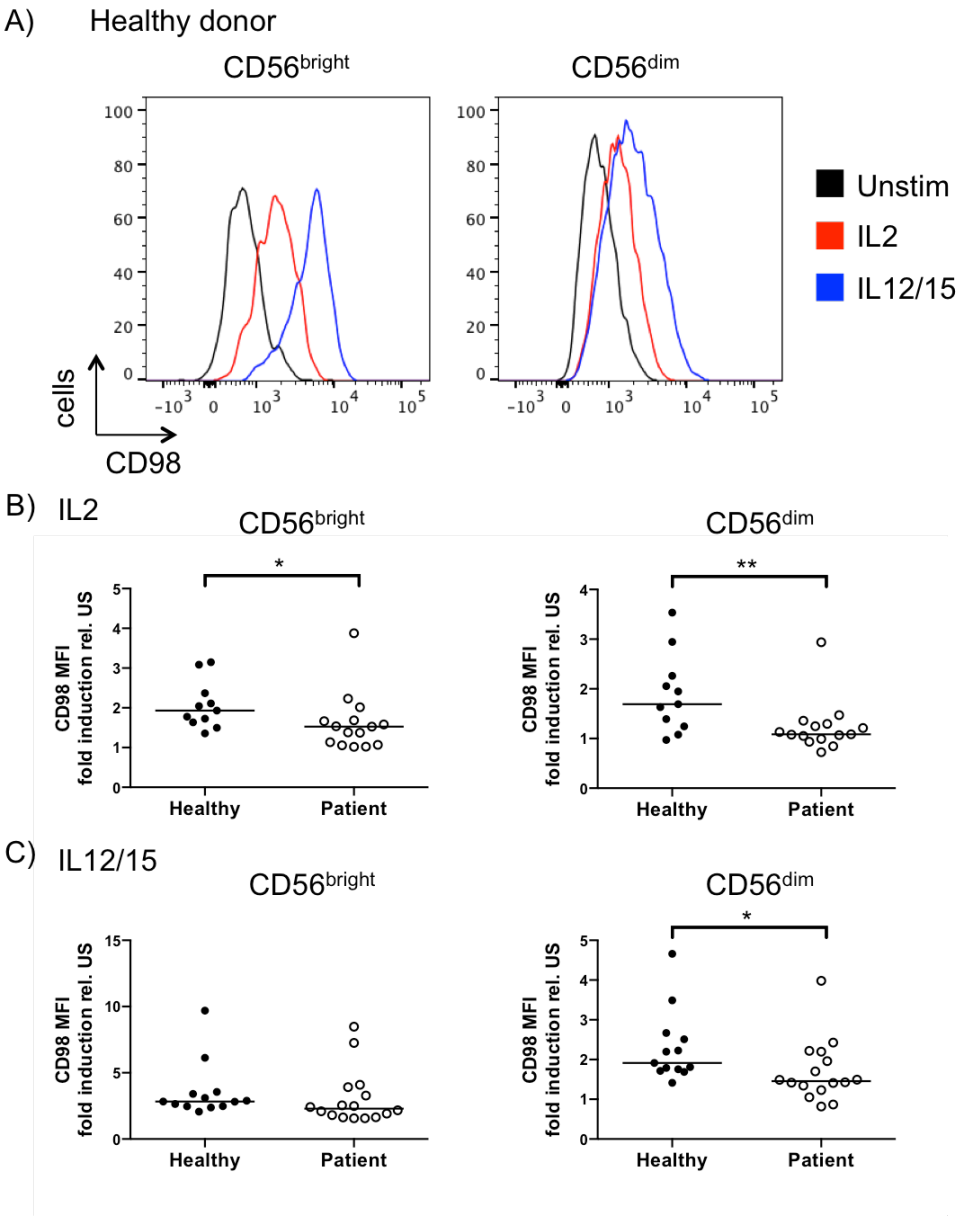


Figure 3.9 NK cells from breast cancer patients have reduced expression of CD98 response to cytokine.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for CD98 and analysed by flow cytometry. (A) Representative histogram of the upregulation of CD98 in response to IL2 and IL12/15 in healthy donor NK cells. (B) Fold increase in the MFI of CD98 relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. (C) Fold increase in the MFI of CD98 relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=11-16). Samples were compared using the unpaired student t-test, *p<0.05, **p<0.01.

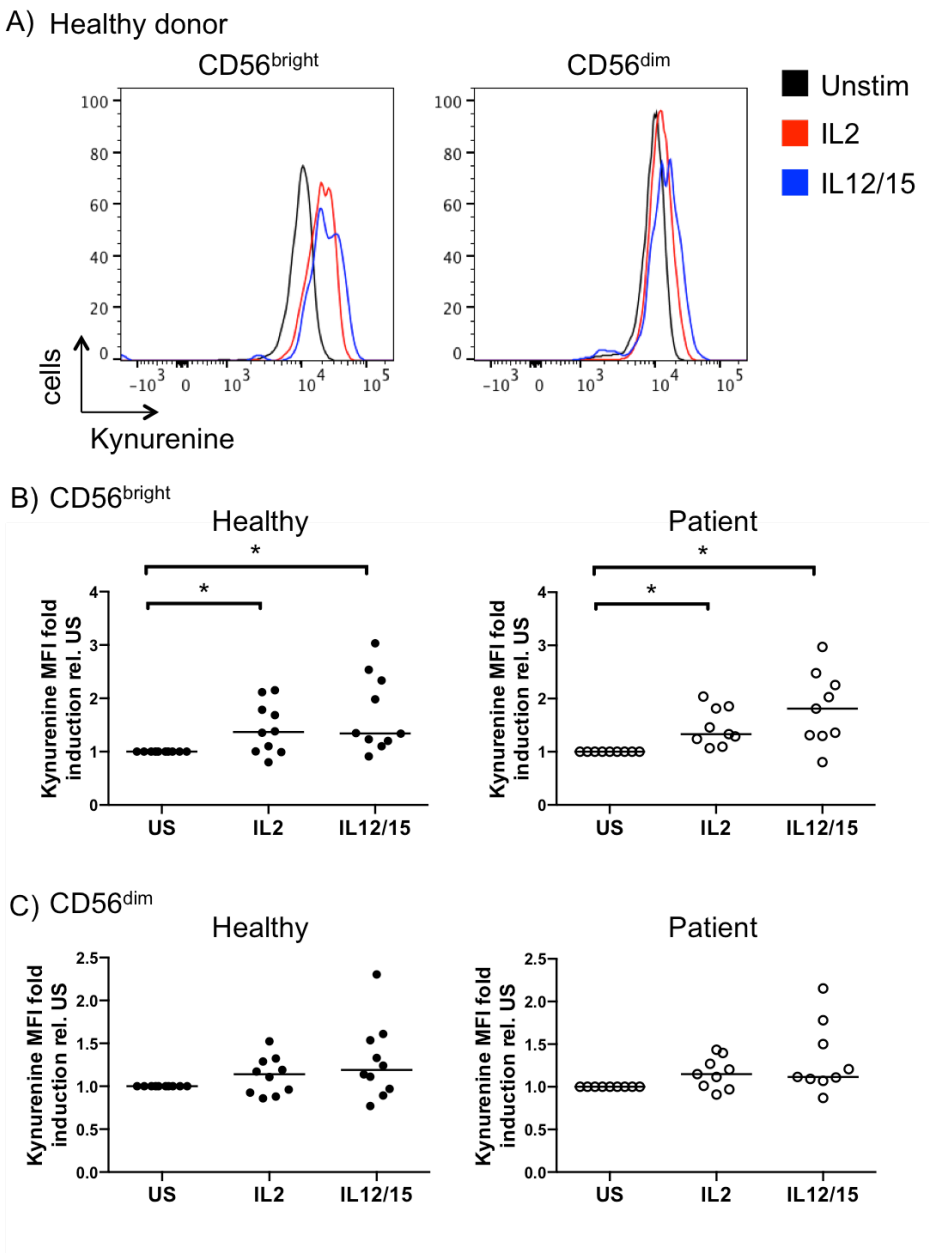


Figure 3.10 CD56^{bright} NK cells from healthy donors and breast cancer patients take up amino acids by the SLC7A5 transporter.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were surface stained for NK markers and then resuspended in Kynurenine in HBSS. Cells were fixed with 4% PFA and analysed by flow cytometry. Leucine was used as a negative control. (A) Representative histogram of kynurenine uptake in healthy donor NK cells stimulated with IL2 and IL12/15. (B) Fold increase in the MFI of kynurenine relative to unstimulated control in CD56^{bright} NK cells from healthy donors and breast cancer patients. (C) Fold increase in the MFI of kynurenine relative to unstimulated control in CD56^{dim} NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=10). Samples were compared by one-way ANOVA test, *p<0.05.

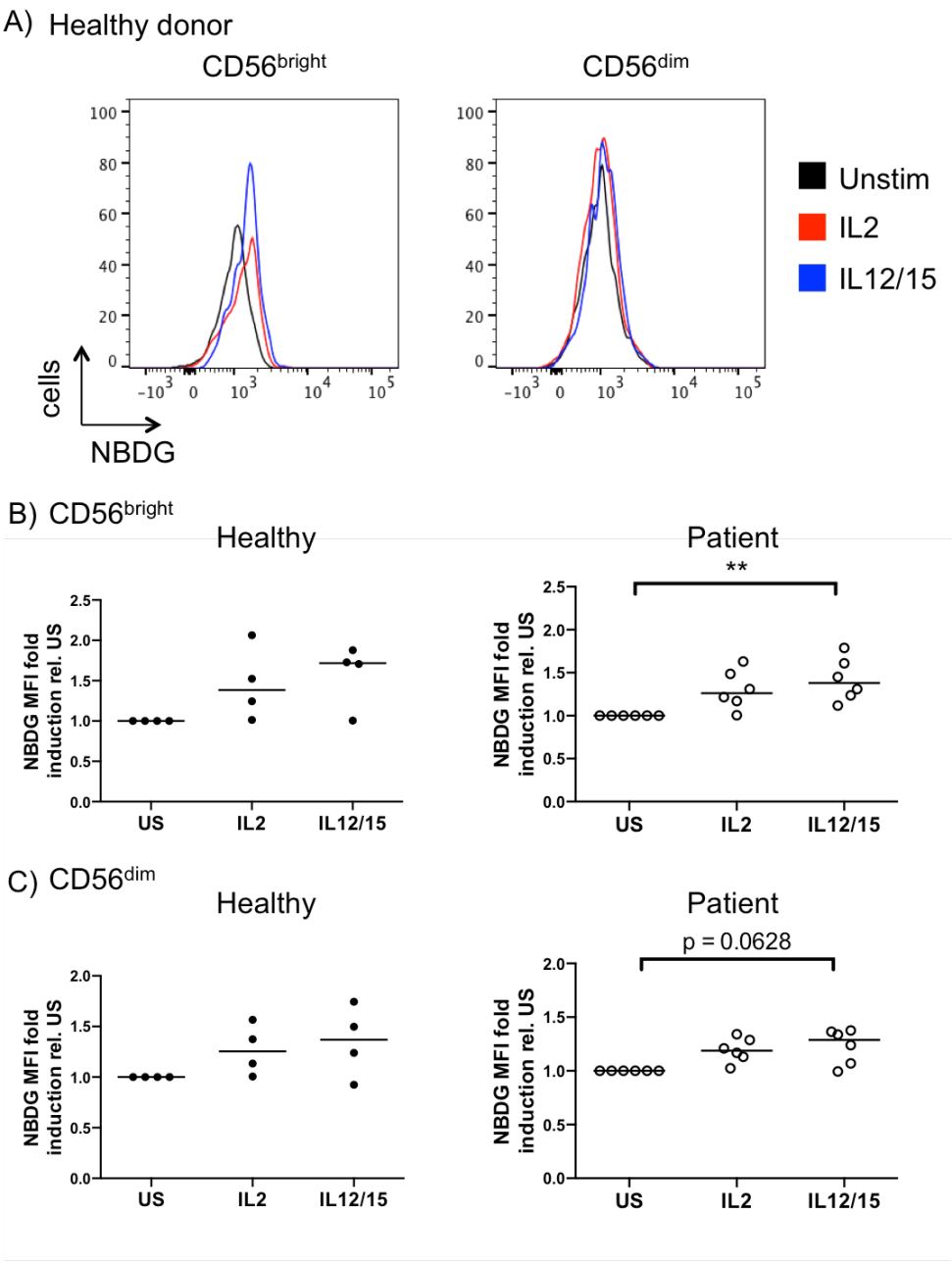


Figure 3.11 NK cells from breast cancer patients have normal 2-NBDG uptake.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were washed with glucose free media and then resuspended in the 2-NBDG (35µM) mix for 1 h at 37°C. Cells were surface stained for NK markers and then analysed by flow cytometry. (A) Representative histogram of 2-NBDG uptake in healthy donor NK cells stimulated with IL2 and IL12/15. (B and C) Fold increase in the MFI of 2-NBDG relative to unstimulated control in $CD56^{bright}$ NK cells from healthy donors and patients. (D and E) Fold increase in the MFI of 2-NBDG relative to unstimulated control in $CD56^{dim}$ NK cells from healthy donors and patients. Lines show the mean value. Each dot represents 1 donor (n=4-6). Samples were compared by one-way ANOVA test, **p<0.01.

3.2.3 NK cells from breast cancer patients have normal intracellular neutral fatty acid content.

A preliminary experiment to measure the level of intracellular neutral fatty acids in the NK cells was carried out using the fluorescent stain BODIPY 493/503. Neutral fatty acid content was equivalent in resting NK cells from healthy donors and breast cancer patients (**Figure 3.12A** for representative histogram). $CD56^{bright}$ NK cells from healthy donors upregulated neutral fatty acid content in response to IL2 and IL12/15 stimulation. This was not evident in the $CD56^{dim}$ subset (**Figure 3.12C**). $CD56^{bright}$ NK cells from breast cancer patients upregulated neutral fatty acid content in response to IL2 and IL12/15 stimulation to equivalent levels as that seen in NK cells from healthy donors (**Figure 3.12B**). Preliminary evidence (n=2) also suggested that resting and IL2 stimulated NK cells from breast cancer patients have normal levels of the common saturated fatty acid myristic acid (**Figure 3.13A**). Again, these data support the idea that $CD56^{bright}$ NK cells are more metabolically active than $CD56^{dim}$ NK cells, and suggest that fatty acid metabolism may be unaltered in NK cells from breast cancer patients.

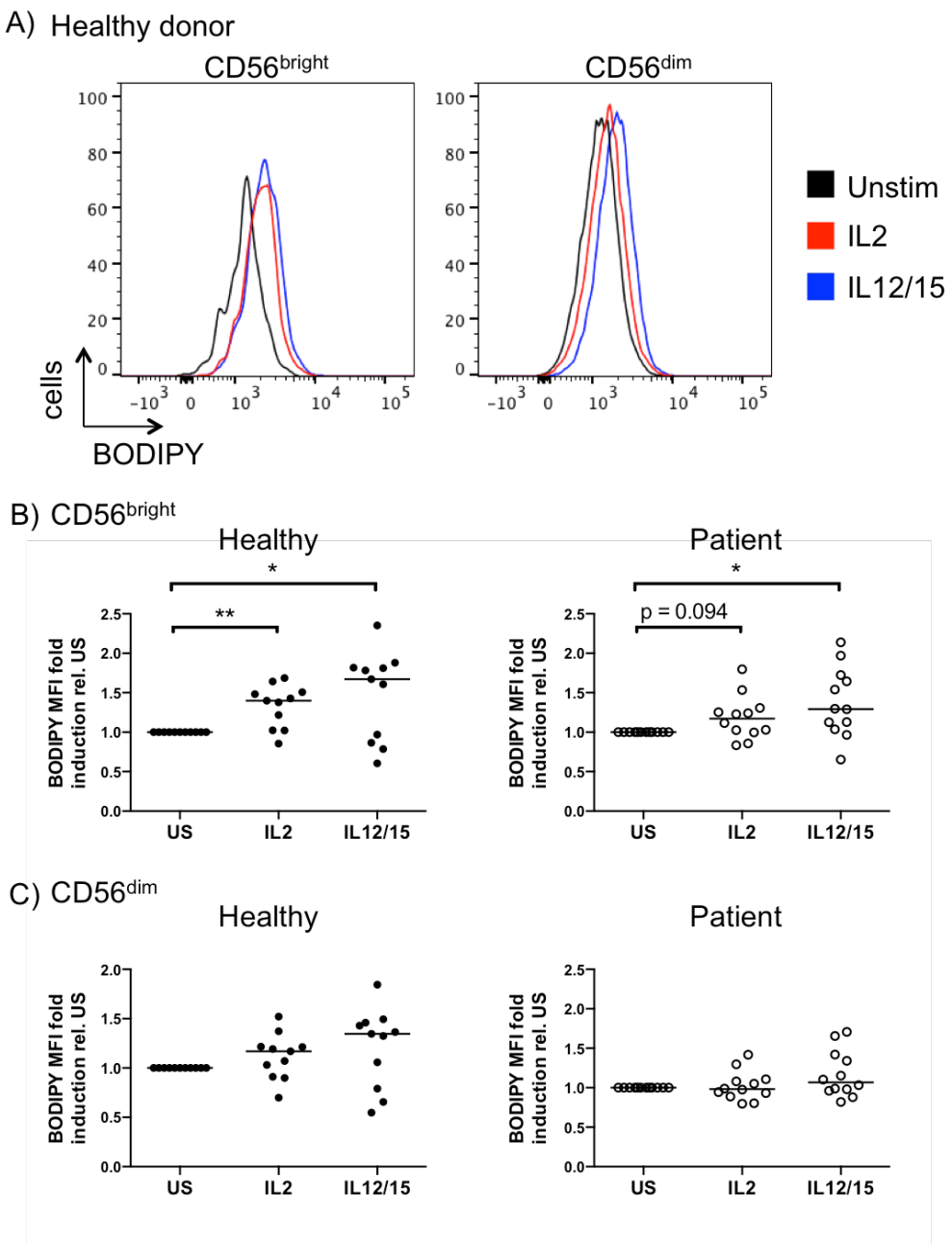


Figure 3.12 CD56^{bright} NK cells from healthy donors and breast cancer patients increase intracellular neutral fatty acid content in response to cytokine.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. NK cells were stained with BODIPY dye (25µg/ml) for 15 min at 37°C. Cells were analysed by flow cytometry. (A) Representative histogram of BODIPY staining in healthy donor NK cells stimulated with IL2 and IL12/15. (B) Fold increase in the MFI of BODIPY relative to unstimulated control in CD56^{bright} NK cells from healthy donors and patients. (C) Fold increase in the MFI of BODIPY relative to unstimulated control in CD56^{dim} NK cells from healthy donors and patients. Lines show the mean value. Each dot represents 1 donor (n=11-12). Samples were compared using the one-way ANOVA test, *p<0.05, **p<0.01.

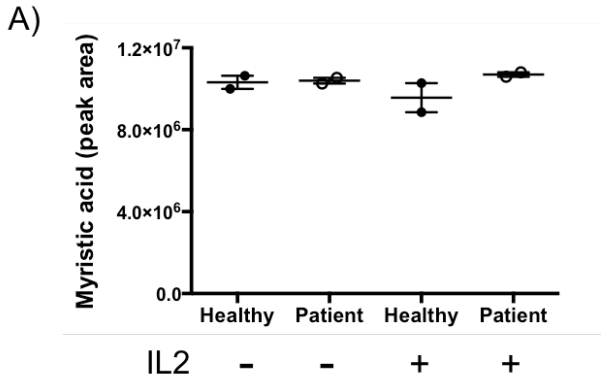


Figure 3.13 NK cells from breast cancer patients have normal levels of intracellular myristic acid.

NK cells were purified from freshly isolated PBMC and either left unstimulated or stimulated with IL2 (500IU/ml) for 18 h at 37°C. Cells were washed twice in saline solution, lysed with ice cold 80% methanol, and metabolites were dried down in a vacuum centrifuge. Unlabelled myristic acid was measured by GC/MS metabolomics. Each dot represents 1 donor (n=2).

3.2.4 NK cells from breast cancer patients may have reduced levels of TCA cycle metabolites.

As fatty acid metabolism appeared unaltered, we next decided to explore metabolic pathways that take place in the mitochondria. The TCA cycle, which takes place within the mitochondrial matrix, converts pyruvate (from glycolysis) into acetyl-CoA. This is then further broken down through a series of enzymatic reactions that ultimately yields 3 molecules of NADH and 1 molecules of FADH, which are then used during oxphos to make ATP. While murine NK cells have been shown to use the citrate malate shuttle in favour of the TCA cycle, there have been no studies on the TCA cycle in human NK cells. Therefore, we first investigated the impact of IL2 on the TCA cycle of NK cells from healthy donors (preliminary results, n=2). IL2 stimulation increased all measured TCA cycle metabolites, including citrate, α-ketoglutarate, succinate, fumarate and malate (**Figure 3.14**). These data suggest that human NK cells use the TCA cycle, and therefore we next investigated the levels of these TCA cycle intermediates in NK cells from breast cancer patients. Interestingly, both resting and IL2 stimulated NK cells from breast cancer patients had reduced levels of nearly all of the metabolites measured, with the exception of succinate whose levels were normal (**Figure 3.15**). These preliminary findings

suggest that NK cells from breast cancer patient have reduced cycling of metabolites through the TCA cycle, which could potentially then be impacting levels of oxphos.

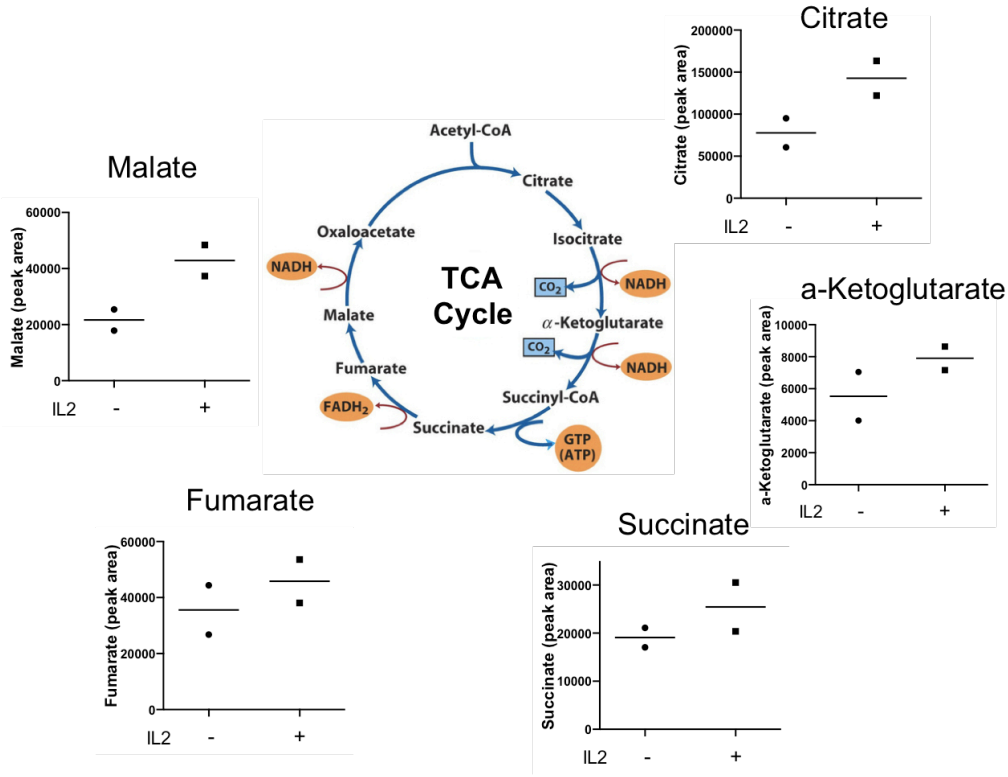


Figure 3.14 NK cells from healthy donors increase levels of TCA cycle intermediates in response to IL2.

NK cells were purified from freshly isolated PBMC and either left unstimulated or stimulated with IL2 (500IU/ml) for 18 h at 37°C. Cells were washed twice in cold saline solution, lysed with ice cold 80% methanol, and metabolites were dried down in a vacuum centrifuge. Unlabelled TCA cycle intermediates were measured by GC/MS metabolomics. Each dot represents 1 donor (n=2).

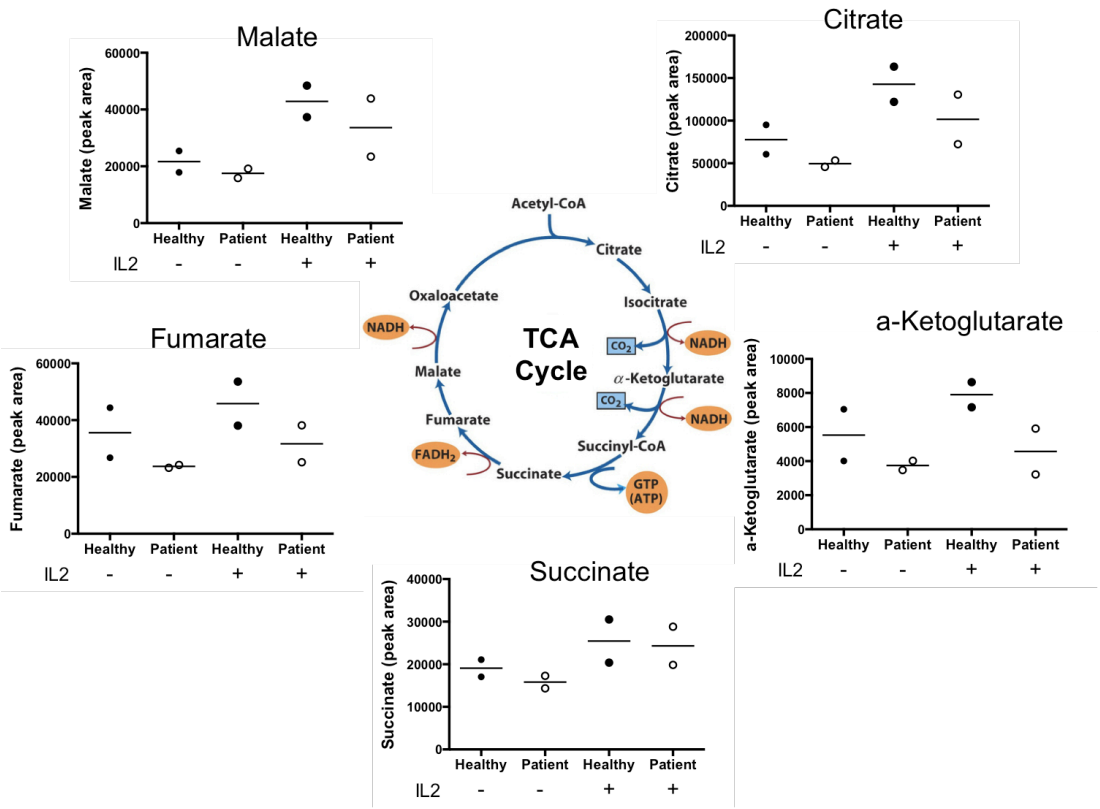


Figure 3.15 NK cells from breast cancer patients may have reduced levels of TCA cycle intermediates.

NK cells were purified from freshly isolated PBMC and either left unstimulated or stimulated with IL2 (500IU/ml) for 18 h at 37°C. Cells were washed twice in cold saline solution, lysed with ice cold 80% methanol, and metabolites were dried down in a vacuum centrifuge. Unlabelled TCA cycle intermediates were measured by GC/MS metabolomics. Each dot represents 1 donor (n=2).

3.2.5 NK cells from breast cancer patients have reduced oxphos

The TCA cycle supplies reducing equivalents to the ETC, where they are used to drive protons across the mitochondrial inner membrane during oxphos. As TCA cycle metabolites were reduced in patient NK cells, we hypothesised that oxphos may also be impacted, and therefore oxphos was measured in purified NK cells using the Seahorse XF Analyser. Indeed, oxphos has been shown to play an essential role in NK cells from healthy donors [122]. Resting NK cells from breast cancer patients had reduced basal levels of oxphos compared to those from healthy donors (**Figure 3.16A** for representative seahorse trace). Calculation of the various oxidative metabolism parameters confirmed that resting NK cells from breast cancer patients had reduced basal respiration compared to resting NK cells from healthy donors (**Figure 3.16B**). The maximal respiration and ATP-linked respiration were also trending down in resting NK cells from breast cancer patients (**Figure 3.16B**). Resting NK cells from both healthy donors and breast cancer patients had negligible SRC.

IL2 induced oxphos in healthy donor and breast cancer patient NK cells was next measured. As expected, NK cells from healthy donors upregulated oxphos strongly upon stimulation with IL2 (**Figure 3.17A** for representative seahorse trace). However, this was significantly reduced when compared to IL2 stimulated NK cells from breast cancer patients. Calculation of the various oxidative metabolism parameters confirmed that IL2 stimulated NK cells from breast cancer patients had significantly reduced basal respiration, maximal respiration and ATP-linked respiration compared to IL2 stimulated NK cells from healthy donors (**Figure 3.17B**). The SRC was also trending down in IL2 stimulated NK cells from breast cancer patients (**Figure 3.17B**). Overall, oxidative metabolism in peripheral blood NK cells of patients with metastatic breast cancer was very low to start with and did not recover with cytokine stimulation.

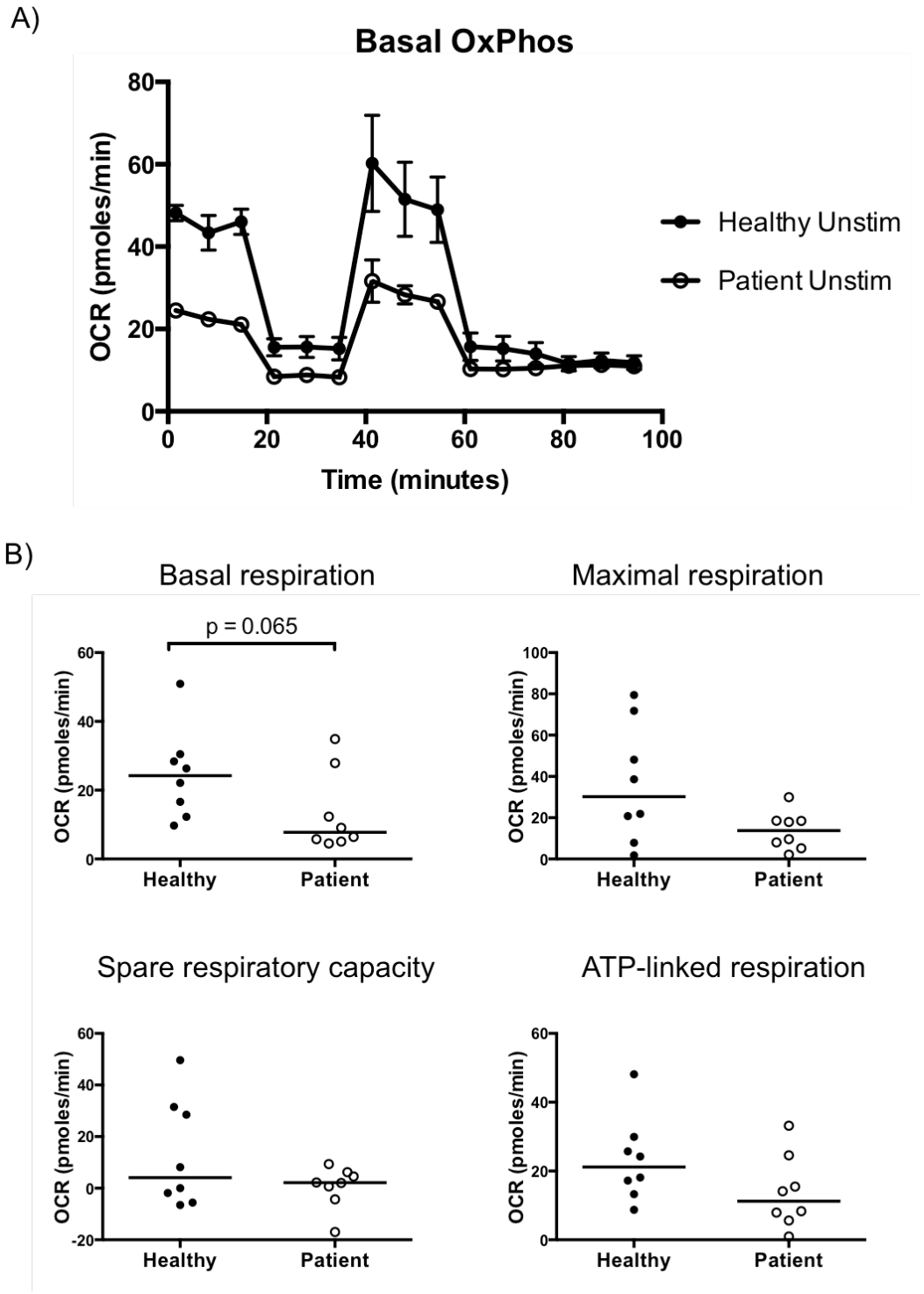


Figure 3.16 NK cells from breast cancer patients have reduced basal respiration.

NK cells were purified from freshly isolated PBMC from healthy donors and breast cancer patients and rested for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative oxygen consumption rate (OCR) trace of NK cells from a healthy donor and breast cancer patient analysed on the same day. (B) Pooled data of oxphos readouts. Lines show the mean. Each dot represents 1 donor (n=8). Samples were compared using the unpaired student t-test.

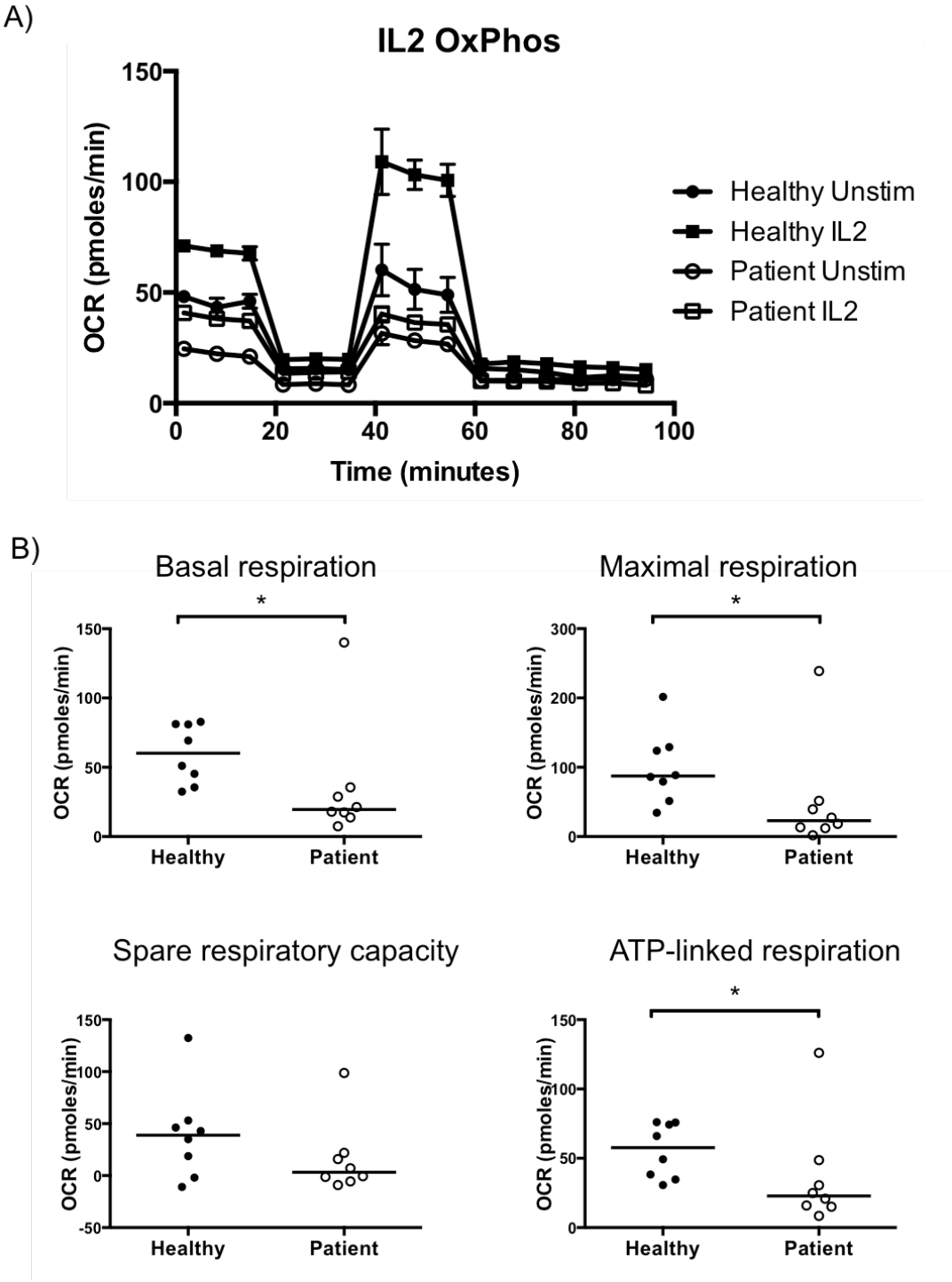


Figure 3.17 NK cells from breast cancer patients have reduced IL2 induced oxphos.

NK cells were purified from freshly isolated PBMC from healthy donors and patients and stimulated with IL2 (500IU/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative oxygen consumption rate (OCR) trace of NK cells from a healthy donor and breast cancer patient analysed on the same day. (B) Pooled data of the IL2 stimulated oxphos readouts. Lines show the mean. Each dot represents 1 donor (n=8). Samples were compared using the unpaired student t-test, *p<0.05.

3.2.6 NK cells from breast cancer patients have altered mitochondrial structure and function.

The above data indicate that oxphos is severely disrupted in NK cells from breast cancer patients. As oxphos takes place within the mitochondria, the structure and function of these organelles was interrogated using multiple flow cytometry based assays and confocal microscopy.

High levels of mROS are indicative of mitochondrial dysfunction and can cause much damage within a cell. Therefore, this was first investigated using the MitoSOX fluorescent dye. The mitochondria of direct *ex vivo* NK cells from breast cancer patients had increased levels of mROS (**Figure 3.18A** for representative histograms). This was evident in both the $CD56^{bright}$ and $CD56^{dim}$ subsets (**Figure 3.18B**).

The mass of the mitochondria in *ex vivo* NK cells from healthy donors and patients was next measured using the MitoTracker fluorescent dye. Similar to mROS, direct *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients had increased mitochondrial mass when compared to NK cells from healthy donors (**Figure 3.19A** for representative histograms and **Figure 3.19B**).

We next investigated the MMP of the mitochondria, as this is an essential component of oxphos. MMP is the electrochemical gradient along the ETC that is used to drive ATP synthesis. The MMP of the mitochondria in *ex vivo* NK cells from healthy donors and patients was analysed using the TMRM fluorescent dye. $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients had increased MMP when compared to NK cells from healthy donors (**Figure 3.20A** for representative histogram and **Figure 3.20B**). This was also evident when the data was normalised according to mitochondrial mass (data not shown).

Linear regression analysis revealed that, in NK cells analysed directly *ex vivo* from healthy donors, MMP correlates with mitochondrial mass ($R^2 = 0.93$ and $R^2 = 0.91$ for $CD56^{bright}$ and $CD56^{dim}$ NK cells respectively, where $R^2 = 1$ indicates perfect fit). However, this linear relationship was lost in equivalent NK cells from breast cancer patients, resulting in a random distribution of data points and a dramatic reduction in the R^2 value ($R^2 = 0.32$ and $R^2 = 0.38$ for $CD56^{bright}$ and $CD56^{dim}$ NK cells respectively)(**Figure 3.21A** and **3.21B**). These analyses serve to illustrate the

disruption of normal mitochondrial activity in NK cells during metastatic breast cancer.

ATP synthase is the key enzyme at the end of the ETC. It creates cellular ATP using the electrochemical gradient of the mitochondrial membrane. Due to the importance of this enzyme to oxphos, one of its key catalytic subunits, ATP5B, was next measured using flow cytometry. NK cells from healthy donors upregulated ATP5B expression in response to IL2 and IL12/15 stimulation (**Figure 3.22A** for representative histogram). However, $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients had impaired upregulation of ATP5B expression in response to IL12/15 (**Figure 3.22B**).

The above mitochondrial data combine to suggest that the reduced oxphos observed in patient NK cells may be due alterations in the structure of the mitochondria. Hence, the structure of the mitochondria in purified NK cells analysed directly *ex vivo* from healthy donors and breast cancer patients was examined using confocal microscopy (in collaboration with Dr. Elena Woods). In general, NK cells from healthy donors had elongated, tubular mitochondria (**Figure 3.23A** for sample images). This indicates mitochondrial fusion has taken place, which gives rise to mitochondrial networks. In contrast, *ex vivo* NK cells from breast cancer patients had more circular, punctate mitochondria (**Figure 3.23A** for sample images). This indicates that they have increased levels of mitochondrial fragmentation and fission. Six volunteers performed blind ordinal scoring of NK cell mitochondrial morphology. A rubric was provided to the volunteers with sample images that ranging from fully fragmented to fully networked (**Figure 2.2** for scale). It was determined that NK cells from breast cancer patients had increased frequency of fragmented mitochondria, while NK cells from healthy donors had increased frequency of networked mitochondria (**Figure 3.23B**).

The above data strongly indicate that NK cells from breast cancer patients have severely impaired mitochondrial fitness. While oxphos is normally required for optimal NK cells functions, this metabolic pathway is impaired in NK cells from breast cancer patients, and this is likely due to the alterations in mitochondrial structure and function observed.

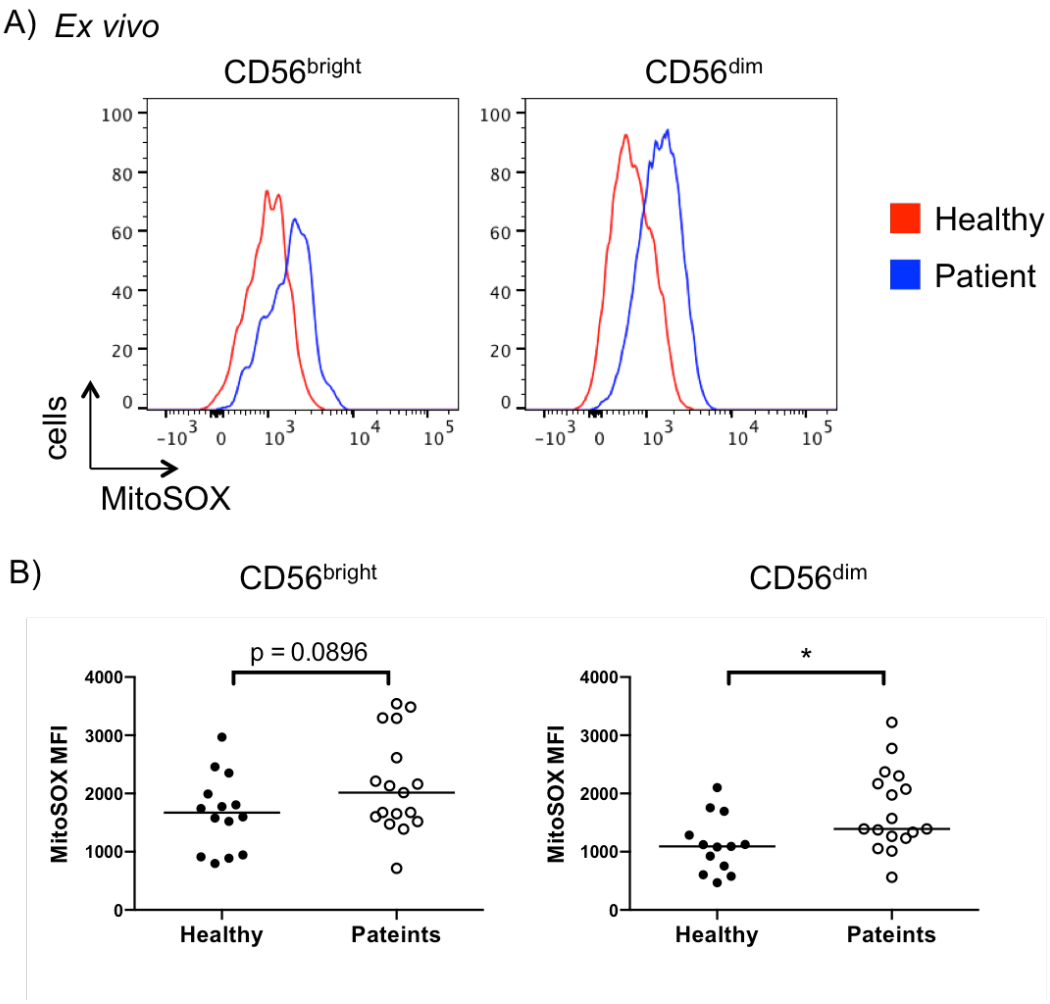


Figure 3.18 NK cells from breast cancer patients have increased levels of mROS *ex vivo.*

Direct *ex vivo* PBMC from healthy donors and patients were stained with MitoSOX Red (1.5µm) dye for 15 min at 37°C. Rotenone (20µM) and MitoTEMPO (25µM) were used as positive and negative controls respectively. Cells were analysed by flow cytometry. (A) Representative histogram in *ex vivo* NK cells from healthy donors and breast cancer patients. (B) The MFI of MitoSOX in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=13-17). Samples were compared using the unpaired student t-test, *p<0.05.

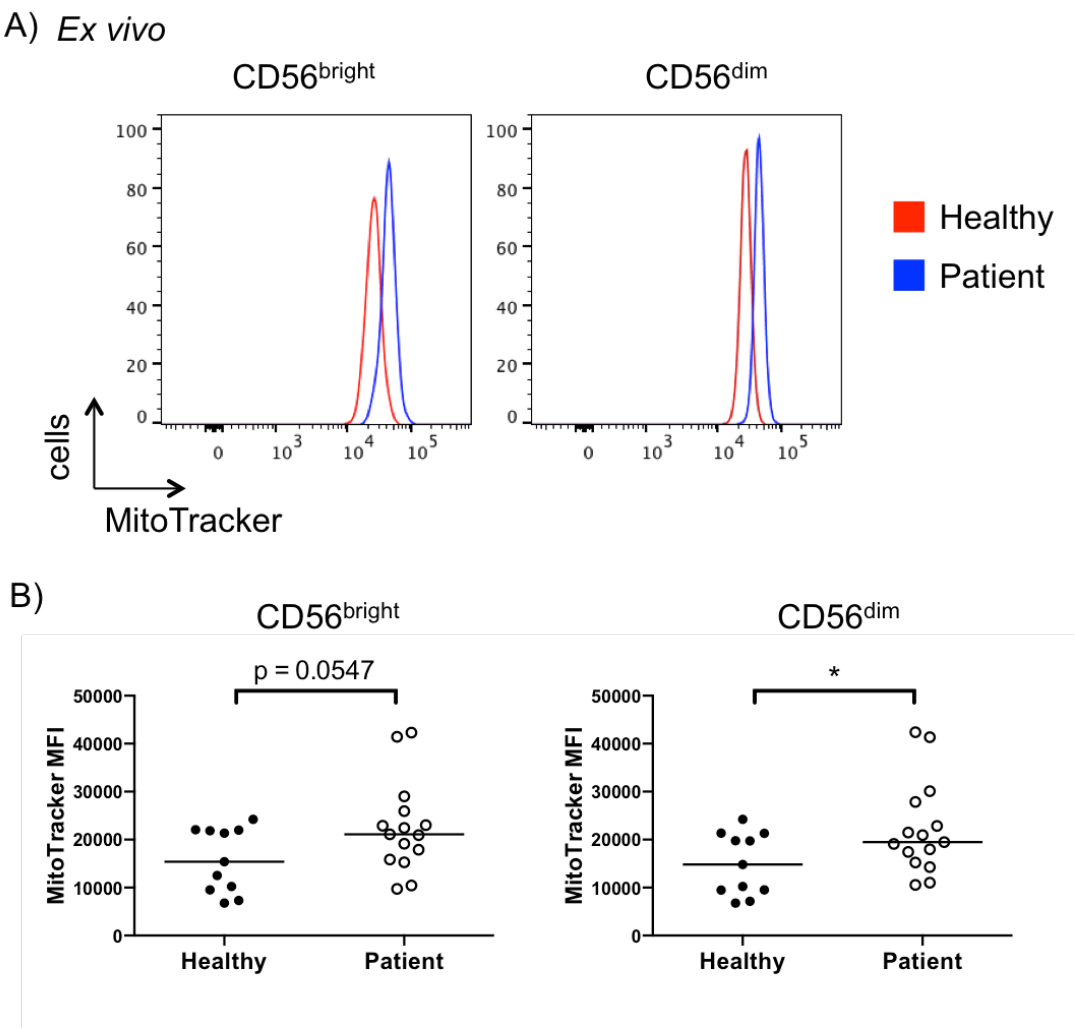


Figure 3.19 NK cells from breast cancer patients have increased mitochondrial mass *ex vivo*.

Direct *ex vivo* PBMC from healthy donors and patients were stained with MitoTracker Red (100nM) for 30 min at 37°C. Cells were analysed by flow cytometry. (A) Representative histogram of *ex vivo* NK cells from healthy donors and breast cancer patients. (B) The MFI of MitoTracker in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=11-15). Samples were compared using the unpaired student t-test, *$p<0.05$.

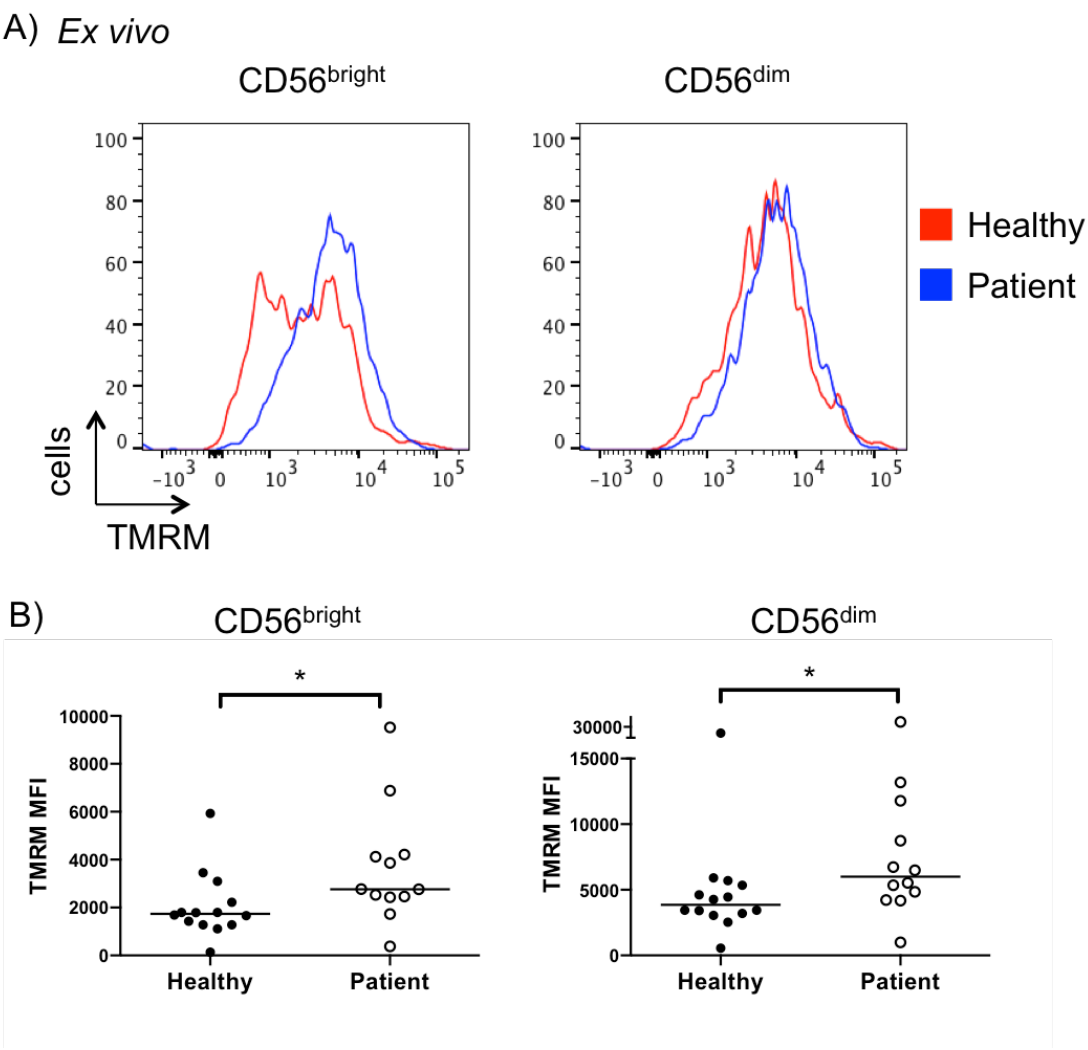


Figure 3.20 NK cells from breast cancer patients have increased mitochondrial membrane potential *ex vivo*.

Direct *ex vivo* PBMC were stained with TMRM (100nM) for 20 min at 37°C. Oligomycin and FCCP were used as positive and negative controls respectively. Cells were analysed by flow cytometry. (A) Representative histogram of *ex vivo* NK cells from healthy donors and breast cancer patients. (B) The MFI of TMRM in *ex vivo* CD56^{bright} and CD56^{dim} NK cells from healthy donors and patients. Lines show the mean value. Each dot represents 1 donor (n=12-14). Samples were compared using the unpaired student t-test, *p<0.05.

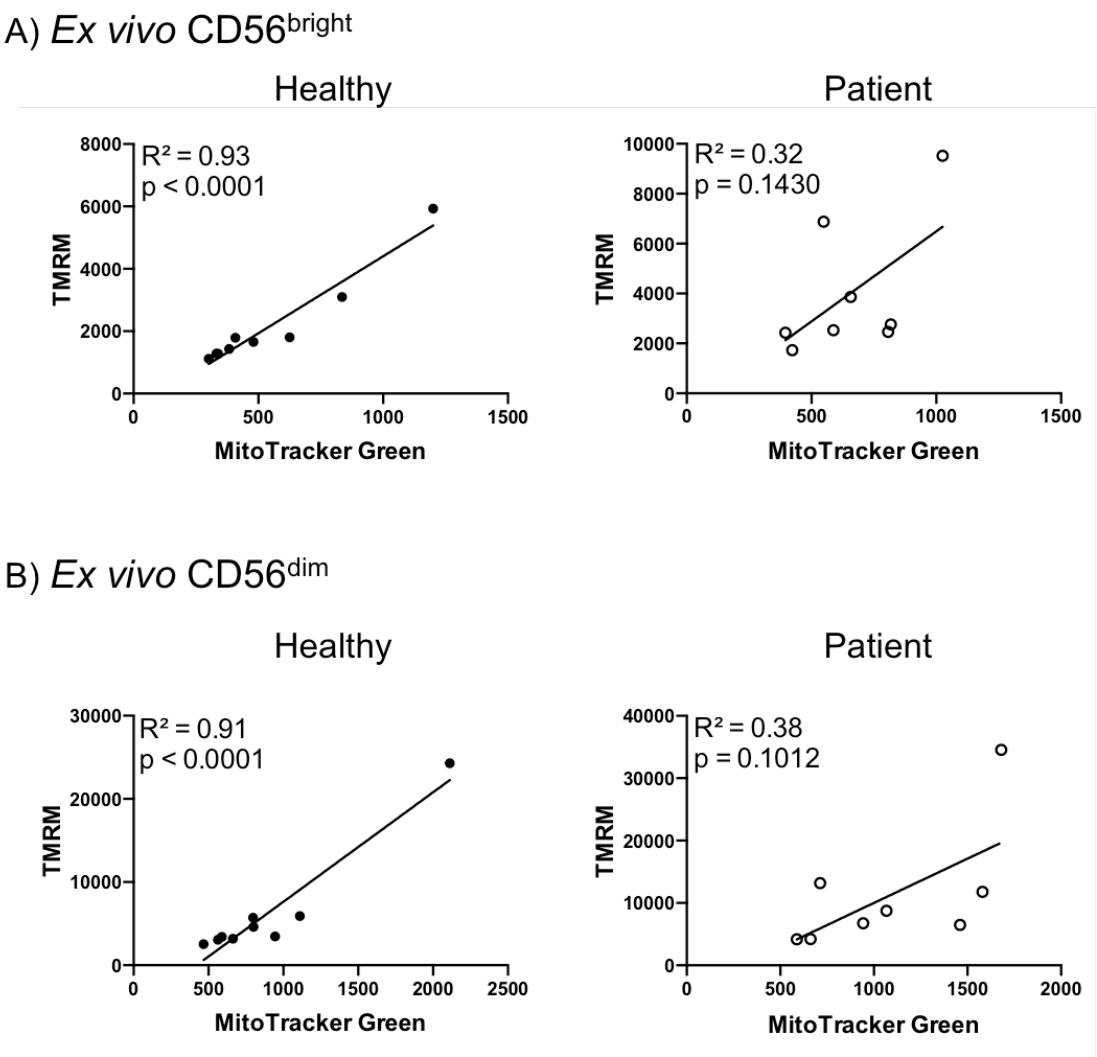


Figure 3.21 NK cells from breast cancer patients have dysregulated mitochondrial mass and membrane potential *ex vivo.*

Direct *ex vivo* PBMC were stained with TMRM (100nM) and MitoTracker Green (100nM) for 20 min at 37°C. Oligomycin and FCCP were used as positive and negative controls respectively. Cells were analysed by flow cytometry. (A) The linear relationship between in mitochondrial mass and membrane potential in $CD56^{bright}$ NK cells from healthy donors and patients. (B) The linear relationship between in mitochondrial mass and membrane potential in $CD56^{dim}$ NK cells from healthy donors and patients. Data was analysed by linear regression analysis (n = 8-9).

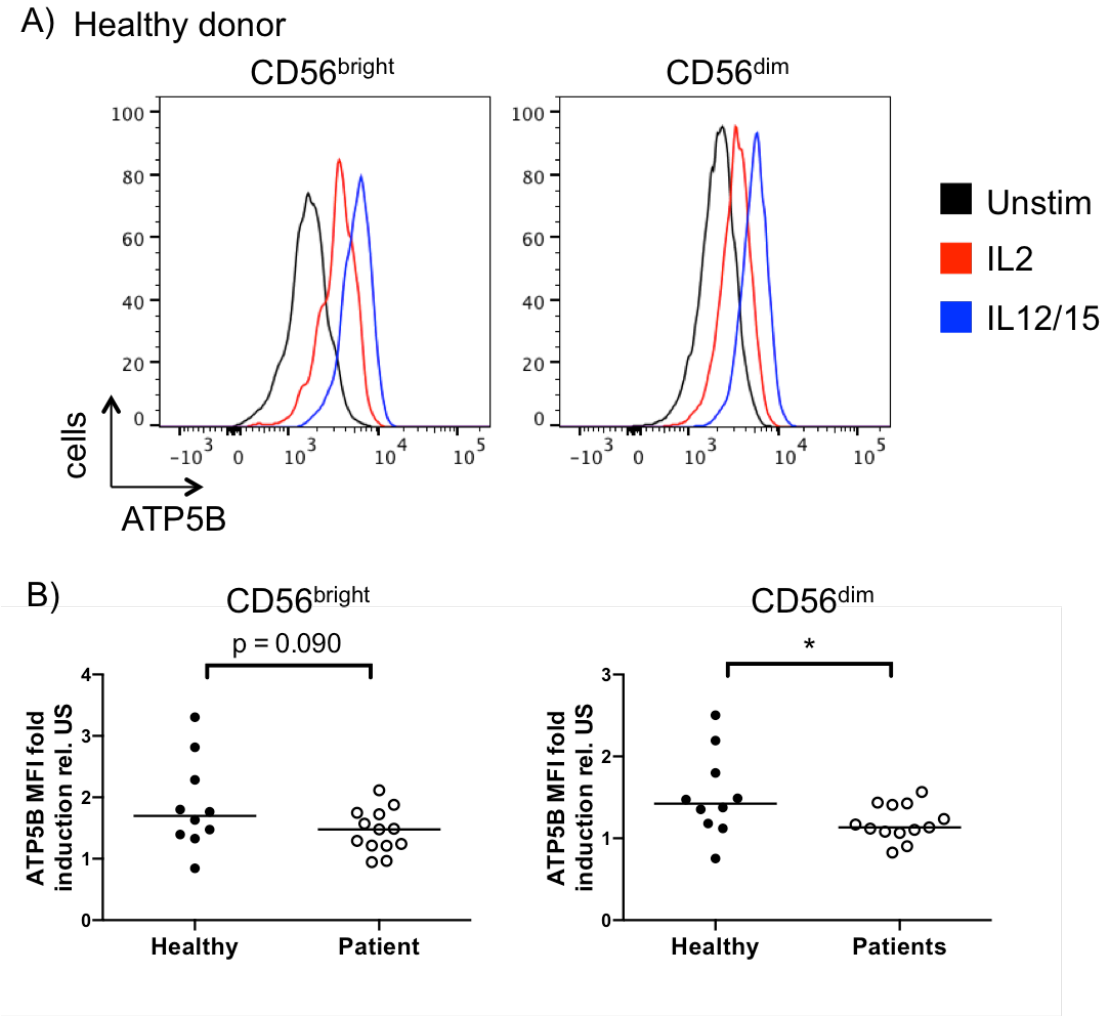
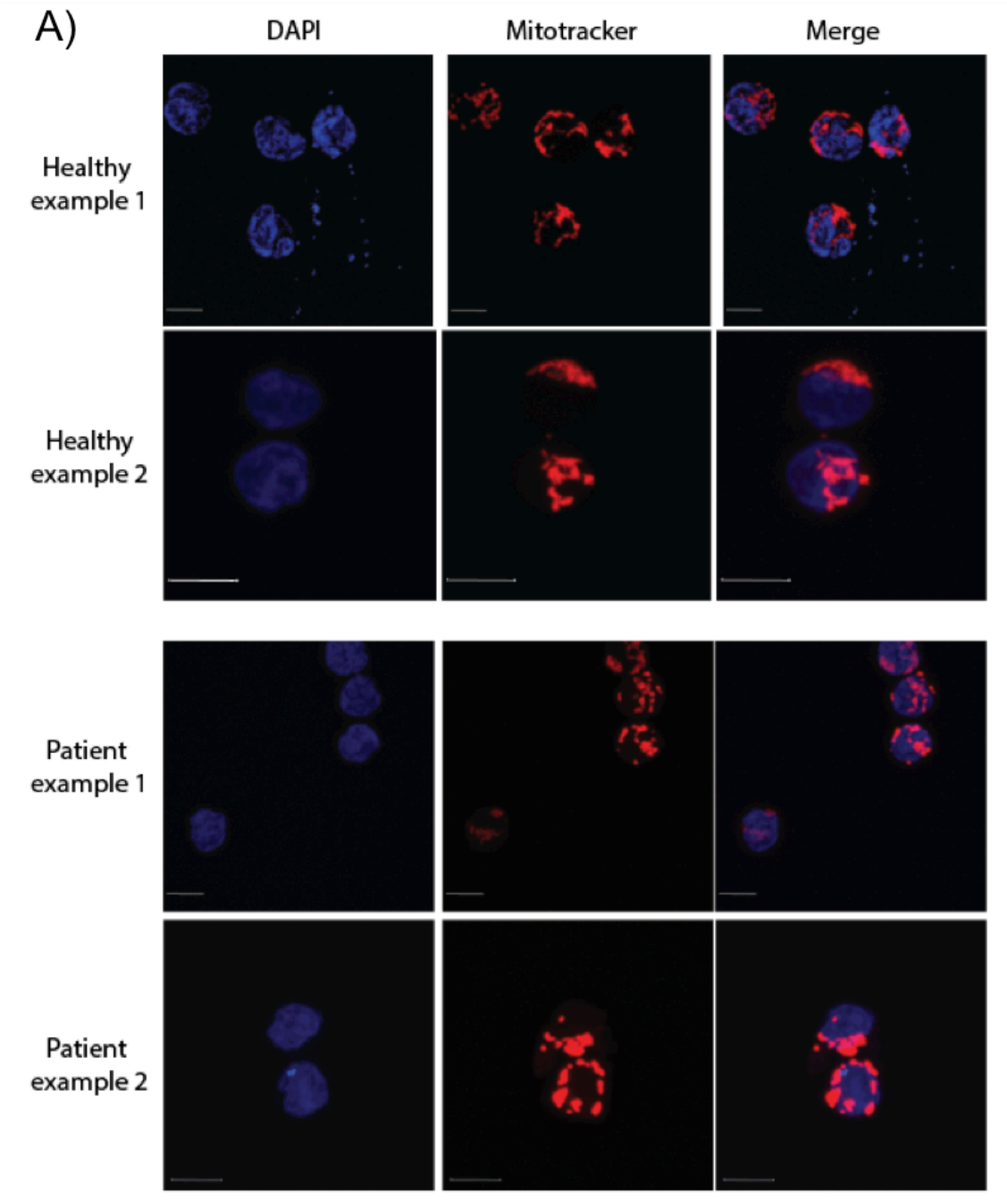


Figure 3.22 IL12/15 stimulated NK cells from breast cancer patients have reduced ATP synthase expression.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. NK cells were stained for ATP5B expression and analysed by flow cytometry. (A) Representative histogram of ATP5B expression in healthy donor NK cells stimulated with IL2 and IL12/15. (B) Fold increase in ATP5B expression relative to unstimulated control in IL12/15 stimulated NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=10-13). Samples were compared using the unpaired student t-test, *p<0.05.


A)
DAPI
Mitotracker
Merge
Healthy example 1
Healthy example 2
Patient example 1
Patient example 2

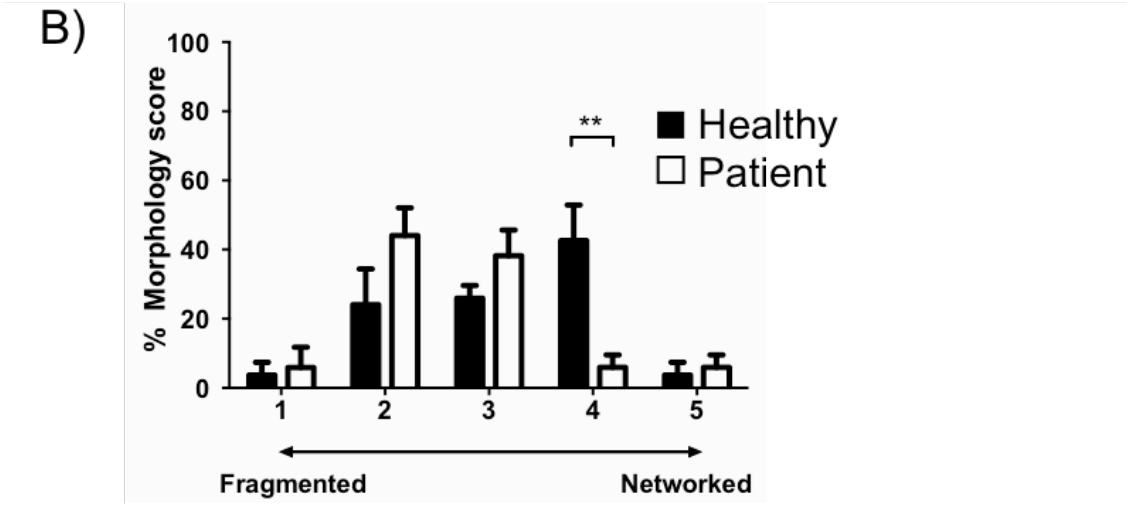

B)
% Morphology score
100
80
60
40
20
0
**
Healthy
Patient
1
2
3
4
5
Fragmented
Networked

Figure 3.23 NK cells from breast cancer patients have increased mitochondrial fragmentation.

(A) Representative confocal images of *ex vivo* NK cells from healthy donors and breast cancer patients stained with MitoSpy CMX Ros (250nM) for 30 min at 37°C and DAPI (300nM). Images shown are the Maximum Intensity projection of Z-stacks taking at 0.2µm increments. Red= Mitospy CMX Ros, Blue= DAPI. Scale bar=5µm. (B) Ordinal scoring of NK cell mitochondrial morphology. % Morphology score was determined by blinded ordinal scoring by 6 volunteers (n=84 cells, n=3 donors). Bars show the mean ± SEM. Samples were compared by two-way ANOVA, **p<0.01.

3.2.7 NK cells from breast cancer patients have reduced glycolysis.

The lack of oxidative metabolism taking place within NK cells from breast cancer patients suggests that these cells may be experiencing a substantial energy deficit. Therefore we hypothesised that NK cells from breast cancer patients may have upregulated glycolysis in order to compensate for this. Flux through glycolysis was investigated in purified NK cells using the Seahorse XF Analyser. Surprisingly, resting NK cells from breast cancer patients also had reduced levels of glycolysis compared to those from healthy donors (**Figure 3.24A** for representative seahorse trace). Calculation of the various glycolytic metabolism parameters confirmed that resting NK cells from breast cancer patients had normal levels of basal glycolysis, but reduced glycolytic capacity and glycolytic reserve compared to resting NK cells from healthy donors (**Figure 3.24B**).

Glycolysis in IL2 stimulated NK cells from healthy donors and breast cancer patients was next measured. As expected, NK cells from healthy donors upregulated glycolysis upon stimulation with IL2. However, this was reduced when compared to IL2 stimulated NK cells from breast cancer patients (**Figure 3.25A** for representative seahorse trace). Calculation of the various glycolytic metabolism parameters confirmed that while IL2 stimulated NK cells from breast cancer patients had normal levels of basal glycolysis, they had significantly reduced glycolytic capacity and glycolytic reserve compared to IL2 stimulated NK cells from healthy donors (**Figure 3.25B**). Preliminary evidence (n=2) also suggests that NK cells from breast cancer patients might have reduced levels of intracellular pyruvate, the final product of glycolysis (**Figure 3.26A**).

Indeed, the above data indicates that NK cells from breast cancer patients have severely compromised levels of glycolysis, in addition to their reduced levels of

oxphos. It is highly unlikely that they are carrying out sufficient levels of metabolism to meet their energy demands. In order to assess whether they are relying more on oxphos or glycolysis, the ratio of OCR to ECAR was calculated in NK cells from healthy donors and breast cancer patients. A higher OCR/ECAR ratio indicates more oxphos/less glycolysis and vice versa. Interestingly, both resting and IL2 stimulated NK cells from breast cancer patients had increased OCR/ECAR ratio when compared to that in NK cells from healthy donors (**Figure 3.27A**), indicating that they rely more heavily on oxphos rather than glycolysis. This is despite the severe mitochondrial dysfunction that was described above.

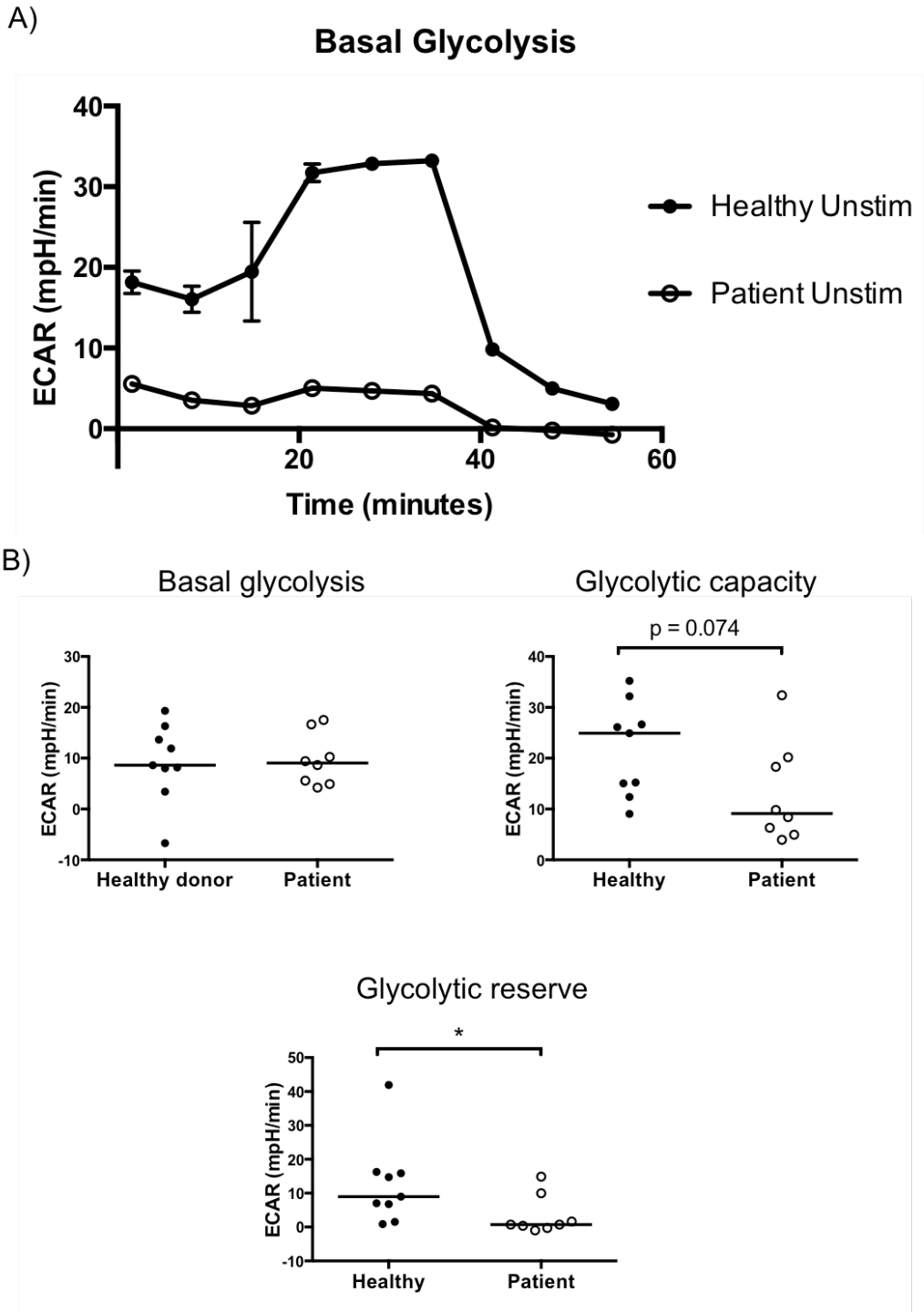


Figure 3.24 NK cells from breast cancer patients have reduced basal glycolytic capacity and reserve.

NK cells were purified from freshly isolated PBMC from healthy donors and patients and rested for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative extracellular acidification rate (ECAR) trace of NK cells from a healthy donor and breast cancer patient analysed on the same day. (B) Pooled data of glycolytic readouts. Lines show the mean. Each dot represents 1 donor (n=8). Samples were compared using the unpaired student t-test, *$p<0.05$.

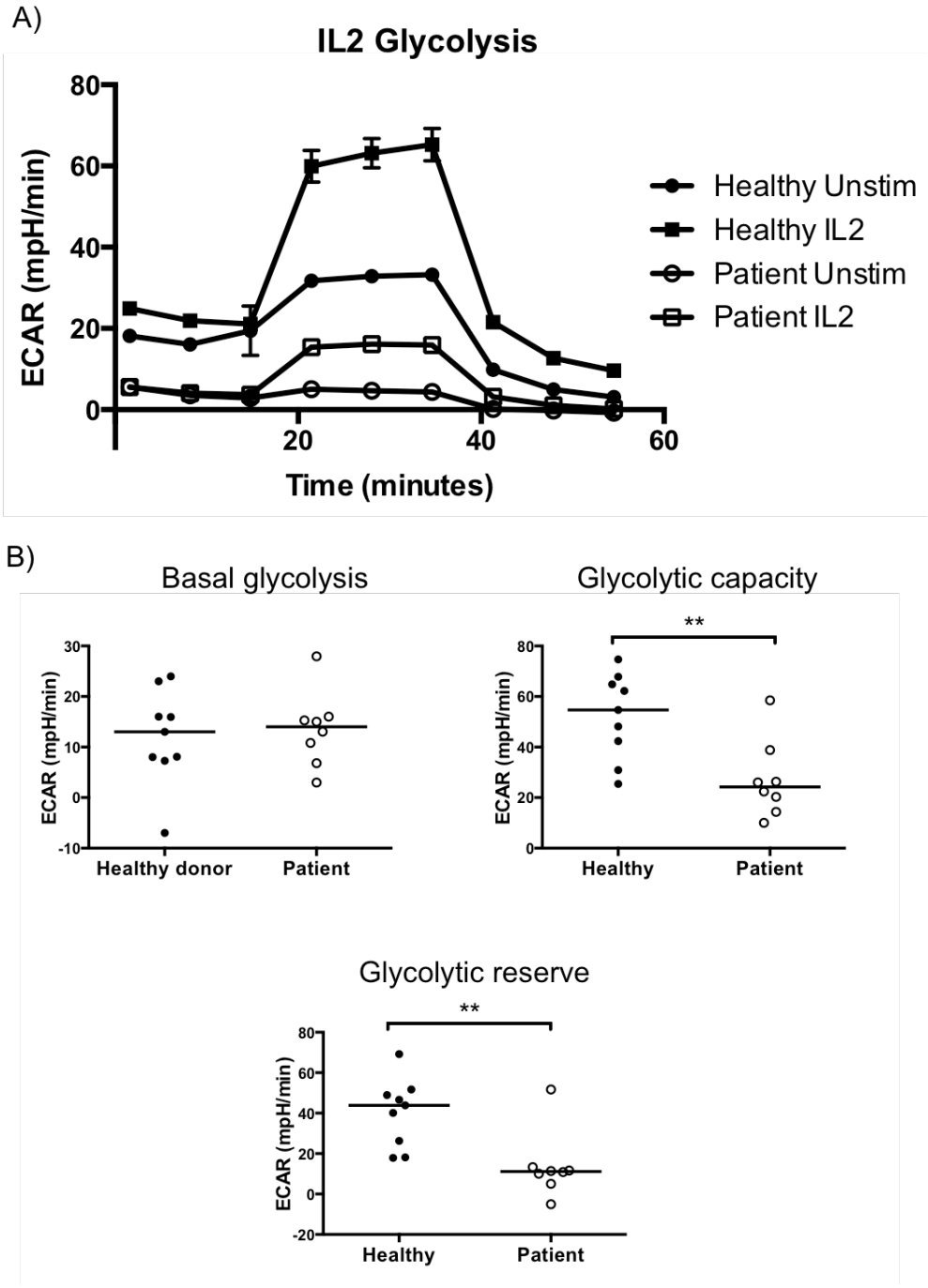


Figure 3.25 NK cells from breast cancer patients have reduced IL2 induced glycolytic capacity and reserve.

NK cells were purified from freshly isolated PBMC from healthy donors and patients and stimulated with IL2 (500IU/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative extracellular acidification rate (ECAR) trace of NK cells from a healthy donor and breast cancer patient analysed on the same day. (B) Pooled data of the IL2 stimulated glycolytic readouts. Lines show the mean. Each dot represents 1 donor (n=8). Samples were compared using the unpaired student t-test, **p<0.01.

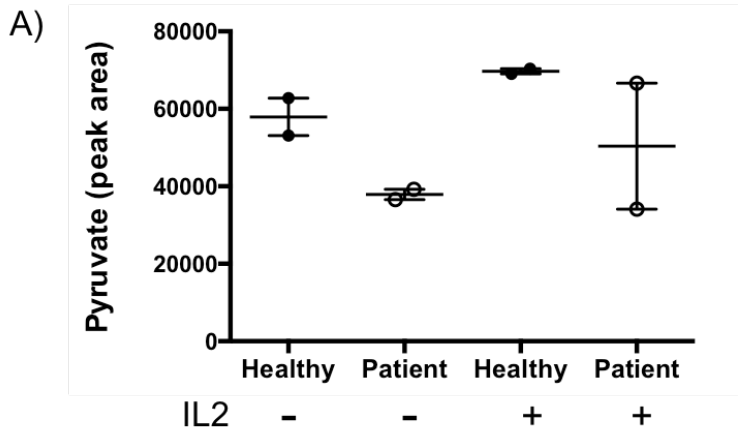


Figure 3.26 NK cells from breast cancer patients may have reduced levels of intracellular pyruvate.

NK cells were purified from freshly isolated PBMC and either left unstimulated or stimulated with IL2 (500IU/ml) for 18 h at 37°C. Cells were washed twice in saline solution, lysed with ice cold 80% methanol, and metabolites were dried down in a vacuum centrifuge. Unlabelled pyruvate was measured by GC/MS metabolomics. Each dot represents 1 donor (n=2).

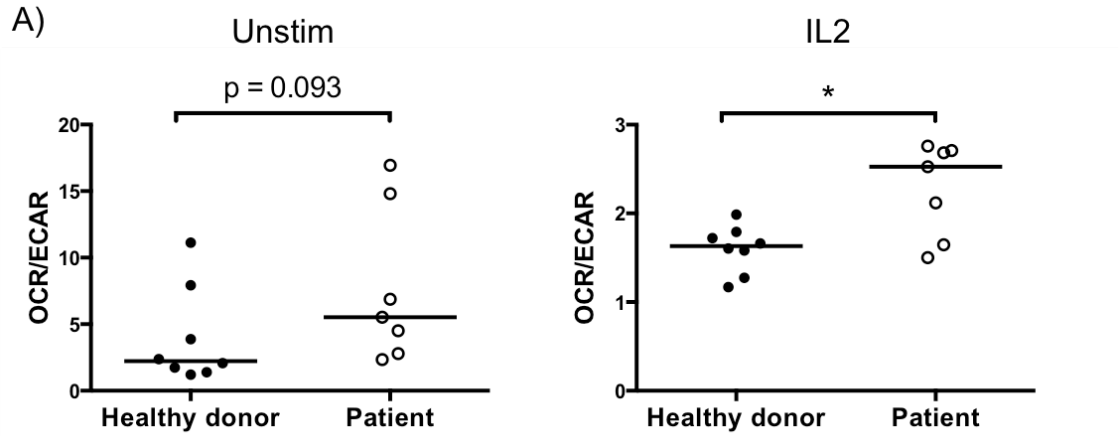


Figure 3.27 NK cells from breast cancer patient rely on oxphos more than NK cells from healthy donors.

NK cells were purified from freshly isolated PBMC from healthy donors and patients and either left unstimulated or were stimulated with IL2 (500IU/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) The ratio of OCR to ECAR, where a higher number indication more oxphos/ less glycolysis. Lines show the mean. Each dot represents 1 donor (n=8). Samples were compared using the unpaired student t-test, **p<0.01.

3.2.8 NK cells from breast cancer patients have increased levels of cellular ROS.

A high level of mROS was associated with altered mitochondrial metabolism in the NK cells from breast cancer patients. Indeed, ROS can travel to and also be generated in the cytosol, the site of glycolytic metabolism. Here, it can react with and damage many components of the cell including protein and DNA. As such, the level of cROS in NK cells from healthy donors and patients was measured using the CellROX fluorescent dye. In comparison to those from healthy donors, $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients had increased levels of cROS direct *ex vivo* (**Figure 3.28A** for representative histogram and **Figure 3.28B**). Similarly, they had increased levels of cROS upon stimulation with IL2 (**Figure 3.28C**). This suggests that NK cells from breast cancer patients are experiencing a very high degree of oxidative stress, which can be damaging to many homeostatic cellular processes including metabolism.

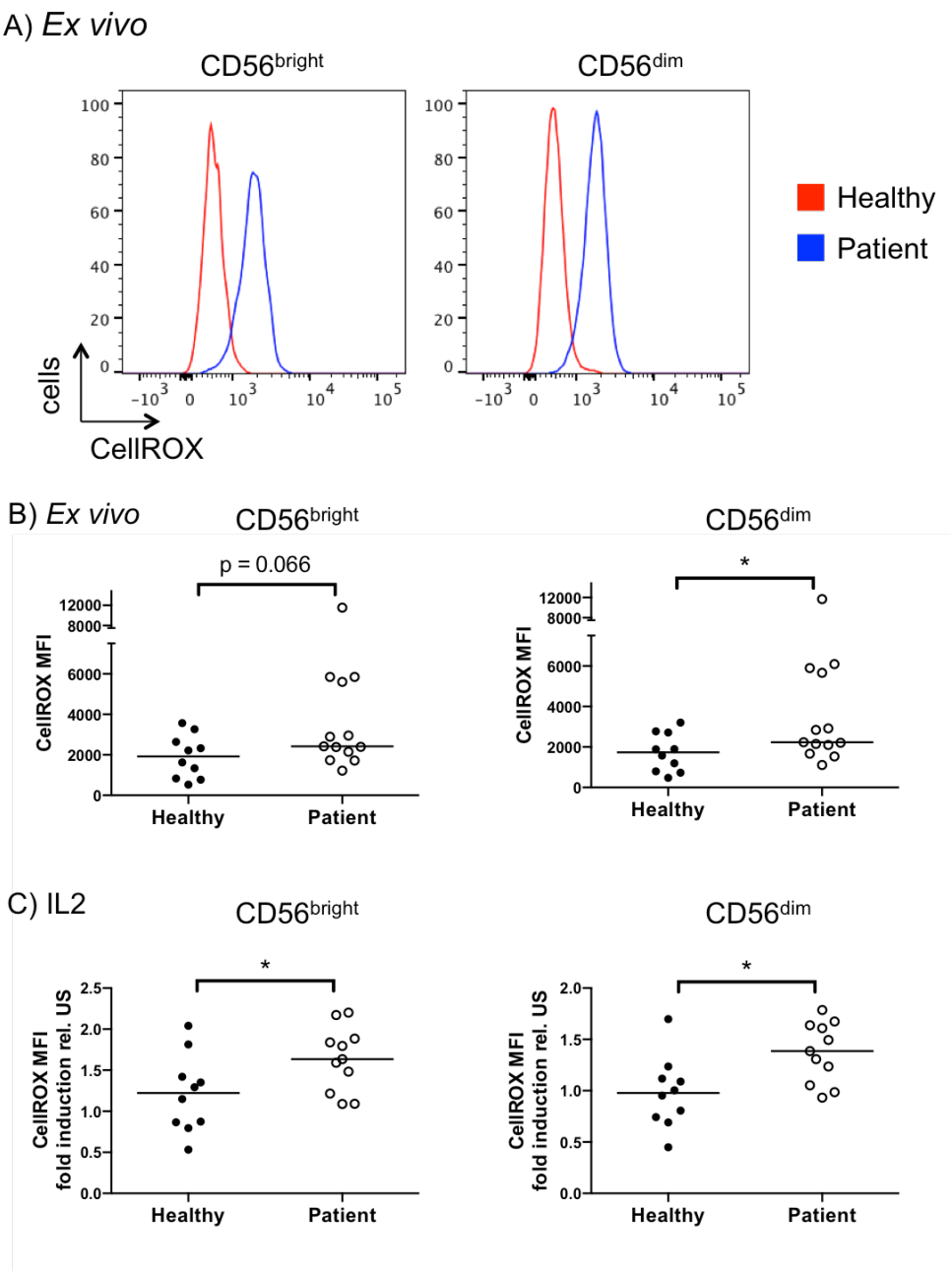


Figure 3.28 NK cells from breast cancer patients have increased levels of cellular ROS *ex vivo* and in response to IL2.

Direct *ex vivo* and IL2 stimulated (500IU/ml, 18 h) PBMC from healthy donors and patients were stained with CellROX (1µm) dye for 30 min at 37°C. TBHP (1mM) and NAC (4mM) were used as positive and negative controls respectively. Cells were analysed by flow cytometry. (A) Representative histogram in *ex vivo* NK cells healthy donors and breast cancer patients. (B) CellROX MFI in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. (C) Fold increase in the MFI of CellROX relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n = 10-13). Samples were compared using the unpaired student t-test, *$p<0.05$.

3.2.9 NK cells from breast cancer patients have altered mTORC1 activity.

Overall it is clear that NK cells from breast cancer patients are functionally and metabolically impaired. mTORC1 is a protein complex which can sense nutrients within the cell and coordinate protein synthesis appropriately. It has been shown to be crucial for driving metabolic reprogramming in NK cells and also supports several NK cell functions such as IFNγ [122]. Therefore, we next measured its activity by staining for phosphorylated S6 protein, one of its downstream targets, and analysing by flow cytometry.

Surprisingly, NK cells analysed directly *ex vivo* from some breast cancer patients had increased basal mTORC1 activity in comparison to those from healthy donors (**Figure 3.29A** and **3.29B**). This was also seen when phosphorylated 4EBP1 protein was used as a secondary mTORC1 readout (data not shown). Upon stimulation with IL2 and IL12/15, $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors increased mTORC1 activity as expected (**Figure 3.30A** for representative dot plots). However, IL2 failed to upregulate mTORC1 activity in NK cells from patients with breast cancer to an equivalent level as that observed in healthy controls (**Figure 3.30B** and **3.30C**). There was also a reduced induction of mTORC1 activity in IL12/15 stimulated NK cells from breast cancer patients (**Figure 3.31B** and **3.31C**). This altered mTORC1 activity profile was evident in both the $CD56^{bright}$ and $CD56^{dim}$ subset. When using p4EBP1 as an alternative mTORC1 readout, IL12/15 stimulated NK cells from breast cancer had impaired mTORC1 activity (data not shown). Overall these data show that mTORC1 activity is altered in NK cells from breast cancer patients. As mTORC1 is a key regulator of human NK cell metabolism and function, this may be an underlying cause of the impaired metabolism and function observed.

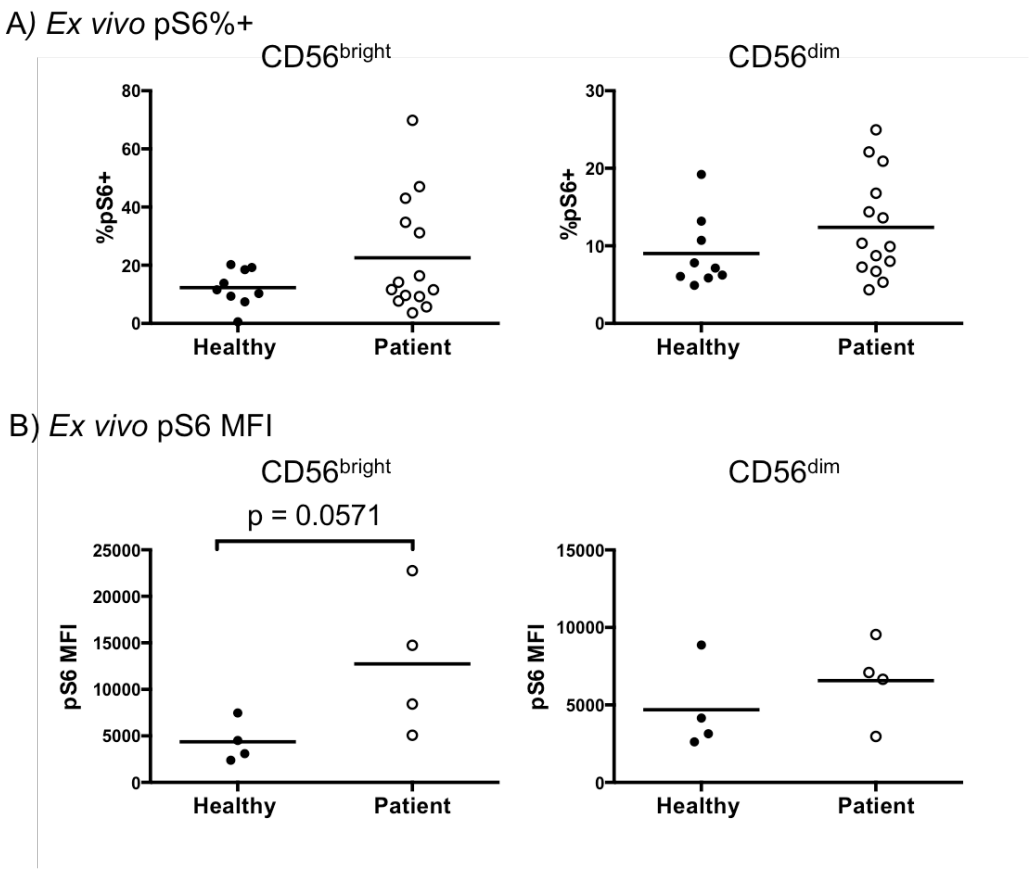


Figure 3.29 NK cells from some breast cancer patients have increased mTORC1 activity *ex vivo*.

Freshly isolated PBMC from healthy donors and breast cancer patients were analysed for mTORC1 activity direct *ex vivo*. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) The frequency of NK cells positive for phosphorylated S6 protein in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and patient. (B) The MFI of phosphorylated S6 protein in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=4-14). Samples were compared using the unpaired student t-test, *p<0.05, ***p<0.001.

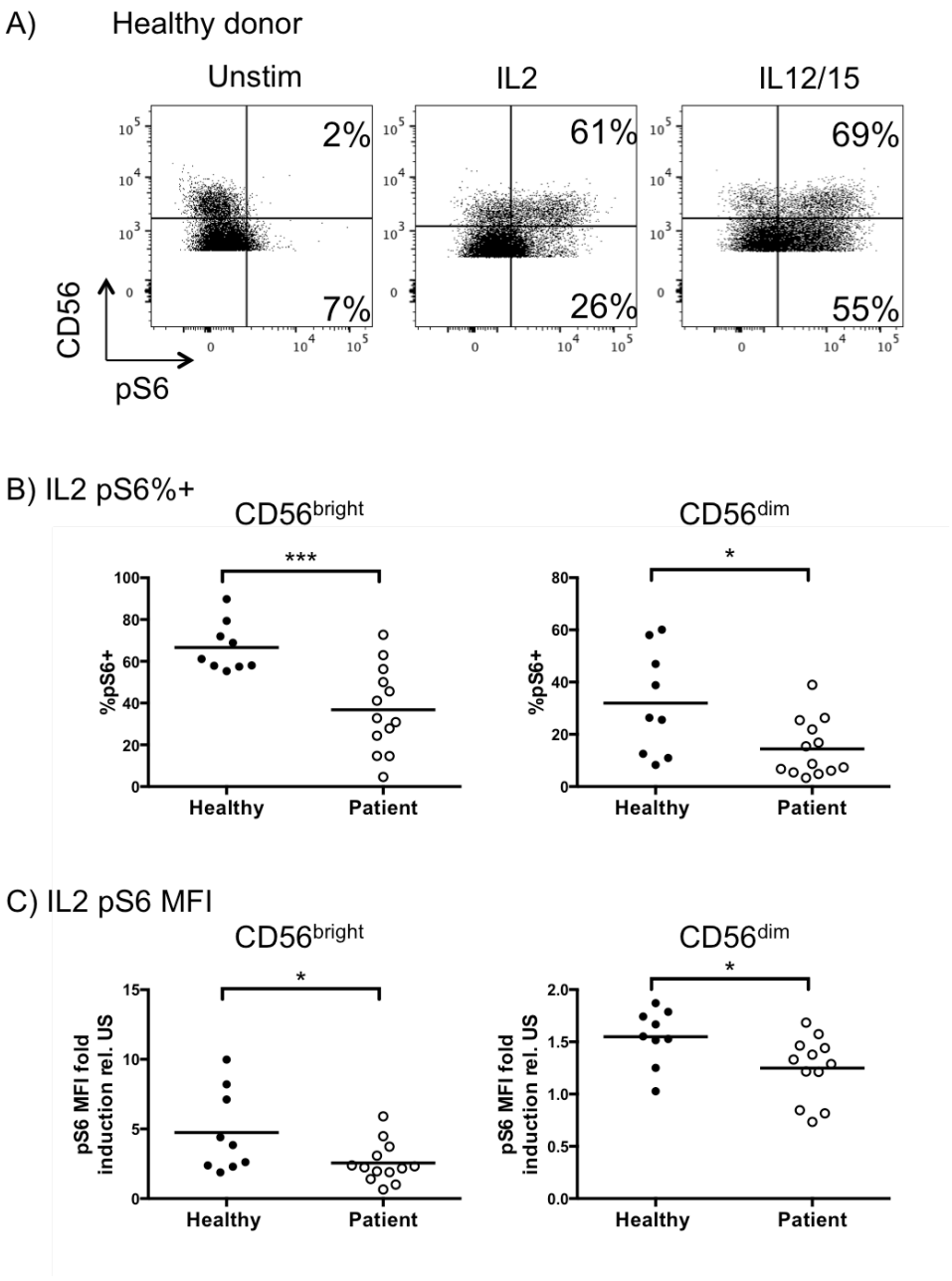


Figure 3.30 NK cells from breast cancer patients have impaired mTORC1 activity in response to IL2.

Freshly isolated PBMC from healthy donors and breast cancer patients were stimulated with IL2 (500IU/ml) and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the upregulation of mTORC1 activity in response to IL2 and IL12/15 in healthy donor NK cells. (B) The frequency of NK cells positive for phosphorylated S6 protein in $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients.

Lines show the mean value. Each dot represents 1 donor (n=9-13). Samples were compared using the unpaired student t-test, *p<0.05, ***p<0.001.

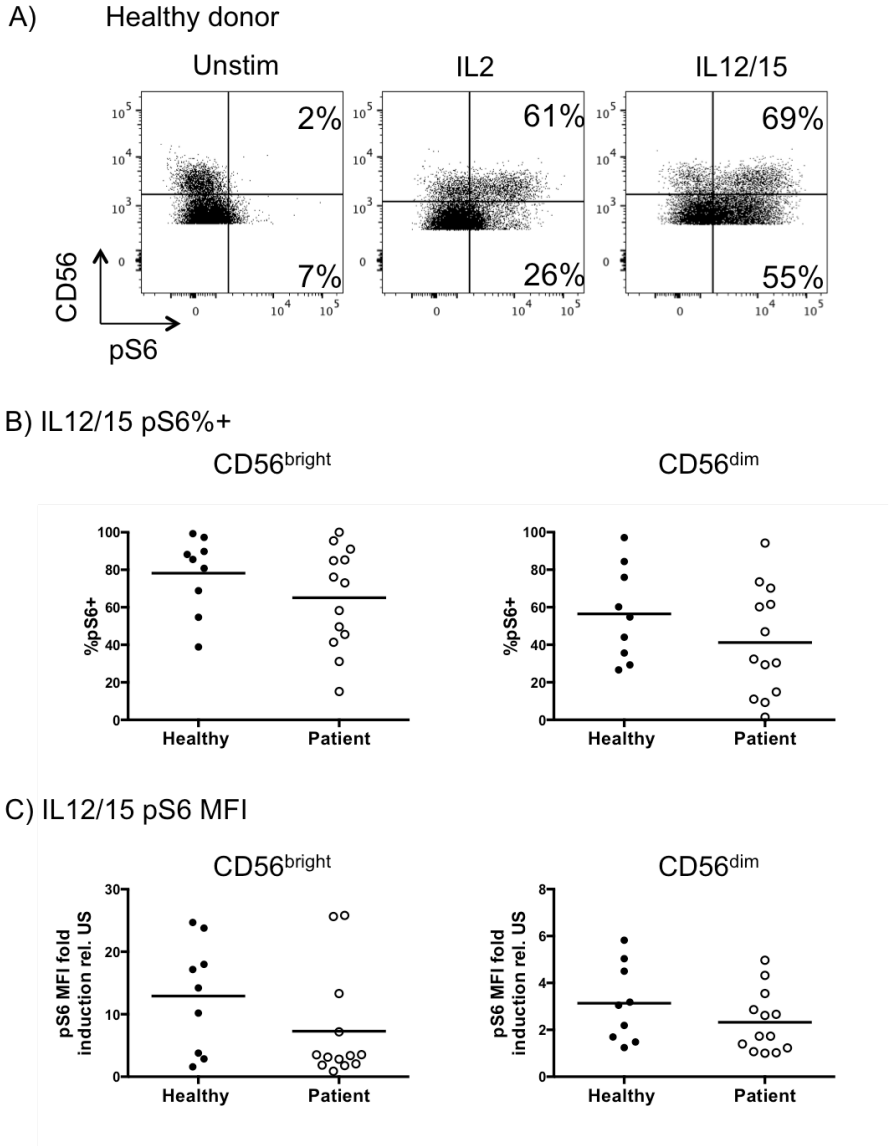


Figure 3.31 NK cells from some breast cancer patients have impaired mTORC1 activity in response to IL2.

Freshly isolated PBMC from healthy donors and breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the upregulation of mTORC1 activity in response to IL2 and IL12/15 in healthy donor NK cells. (B) The frequency of NK cells positive for phosphorylated S6 protein in $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=9-13). Samples were compared using the unpaired student t-test.

3.3 Discussion

NK cells are important protectors against cancer and viral infection. The functions that underpin their role in the immune response have been studied in depth during the past few decades. We now have a good understanding of the variety of functions that NK cells can utilise to kill their targets, such as cytokines and cytotoxic proteins. However, the manner in which they fuel these essential responses was not explored nor considered until quite recently. Previous work from our lab showed that glucose fuelled oxphos and glycolysis are both required for human NK cells from healthy donors to function optimally [122]. An interesting finding of this early paper was that healthy donor $CD56^{bright}$ NK cells induce a much more robust metabolic response compared to $CD56^{dim}$ NK cells. This involves greater increases in the expression of nutrient uptake receptors CD71, CD98 and Glut1 in response to cytokine stimulation. Furthermore, uptake of 2-NBDG and mTORC1 activity was higher in $CD56^{bright}$ cytokine stimulated NK cells than $CD56^{dim}$ NK cells. The data presented in this chapter support this idea, as $CD56^{bright}$ NK cells from our cohort of healthy donors also had greater increases in CD71, CD98, 2-NBDG and mTORC1 activity in response to cytokine stimulation. Adding to this, we found that cytokine stimulated $CD56^{bright}$ NK cells increased uptake of kynurenine while $CD56^{dim}$ NK cells did not, indicating increased SLC7A5 activity and higher amino acid uptake in the $CD56^{bright}$ subset. Similarly, they had a higher level of neutral fatty acid content. Together these data suggest that $CD56^{bright}$ NK cells may be more metabolically active and have a higher demand for nutrients compared to $CD56^{dim}$ NK cells.

One of the key functional differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells is cytokine production such as IFNγ – $CD56^{bright}$ NK cells produce more IFNγ, while $CD56^{dim}$ are more cytotoxic. Therefore, these data suggest that an increased supply for nutrients may be required for $CD56^{bright}$ NK cell cytokine production. This may be explained by the fact that $CD56^{dim}$ NK cell constitutively express their cytotoxic mediators and therefore do not need to synthesise the proteins upon activation to the same extent as $CD56^{bright}$ NK cells. Indeed, the mean fold increase in granzyme B expression in $CD56^{dim}$ NK cells in response to IL2 and IL12/15 was just 1.2 and 1.7 respectively, while that of $CD56^{bright}$ NK cells 1.5 and 2.6 respectively. At the same time, $CD56^{dim}$ NK cells were on average 20% positive for IFNγ production versus 60% for $CD56^{bright}$ NK cells. Overall, it seems that while $CD56^{dim}$ NK cells are poised to elicit a more immediate and perhaps spontaneous functional response, $CD56^{bright}$

NK cells require greater cytokine induced metabolic changes and have a higher demand for nutrients to drive their functional response. More in depth experiments are required to confirm this – for example, NK cell subsets could be FACS sorted and seahorse and/or radioactively labelled nutrient tracing experiments could be performed. What will also be interesting is to see whether $CD56^{bright}$ and $CD56^{dim}$ NK cells have different mitochondrial architecture. Indeed, we show here that a mixed population of healthy donor NK cells have in general more elongated, fused mitochondria. However, it is notable that there was significant heterogeneity between the NK cell mitochondria for each healthy donor, and therefore it is possible that the two subsets have contrasting mitochondrial phenotypes. $CD56^{bright}$ NK cells have higher Glut1 expression as well as mTORC1 activity, and their IFNγ response is more sensitive to treatment with galactose (glycolysis inhibitor) compared to $CD56^{dim}$ NK cells, suggesting they may be more reliant on glycolysis than oxphos. If this proves to be true, we predict that $CD56^{bright}$ NK cells may have more fissed, circular mitochondria, which support glycolysis, compared to $CD56^{dim}$ NK cell having fused mitochondria, which supports oxphos [157]. Overall is seems that these two subsets of NK cells have markedly different metabolic demands. It will be interesting in the future to see whether they use different fuels, how they metabolise them and how this ultimately drives their characteristic functions.

It is now widely accepted that metabolism is an essential element of the NK cell immune response. These responses are vital for the defence against cancer, as highlighted by the fact that cancer patients with more active NK cells have better prognoses [158-160]. At the same time, it well documented that these NK cell anti-cancer functions become impaired in patients with cancer [68]. This dysfunction may contribute towards tumour growth and metastasis as the defences against the cancer are removed, and may also impinge upon the efficacy of immunotherapies that rely on NK cells, such as the monoclonal antibody therapies Trastuzumab and Dinutuximab. Indeed, if a patient's immune cells are impaired, it is unlikely that their monoclonal antibody therapy will work optimally, and this will lead to lower survival rates. Hence, it is essential to study immune dysfunction in cancer in order to understand the mechanisms at play and identify new targets for immunotherapy.

Here we show that NK cells from breast cancer patients have reduced production of IFNγ, an important anti-tumour cytokine. This is in agreement with many other studies on NK cells in cancer e.g. lymphoma [68], sarcoma [72], lung cancer [73],

and in melanoma [69]. In contrast, patient NK cells had normal levels of granzyme B. This may be because granzyme B tends to be expressed constitutively, particularly in $CD56^{dim}$ NK cells, and therefore may not be dependent on cytokine induced metabolic changes. Whether or not this granzyme B degranulates appropriately remains unknown and will indeed be important to investigate in the future. In this study, we sought to understand what underlying mechanisms might contribute to the NK cell dysfunction, and so we investigated the cellular metabolism of the NK cells. Indeed, it was very clear that NK cells from breast cancer patients are metabolically paralysed. While NK cells from healthy donors normally require both glycolysis and oxphos to carry out their functions [122], both of these metabolic pathways are severely reduced in resting and IL2 stimulated NK cells from breast cancer patients. This suggests that NK cells are running on a substantial energy deficit, which could explain the dysfunction observed. An interesting exception to this was that basal glycolysis was normal in both resting and IL2 stimulated patient NK cells. In terms of the former, this may be explained by the fact that resting NK cells rely predominantly on oxphos. However, in terms of the IL2 stimulated NK cells, this suggests that the impaired oxphos may be the main contributor to the reduced function. Nonetheless, glycolytic reserve and glycolytic capacity was impaired, indicating that patient NK cells lack the optimal glycolytic machinery to carry out glycolysis normally e.g. glycolytic enzymes or cofactors such as NAD^+. Overall, it is clear that NK cells from metastatic breast cancer patients are severely metabolically compromised, which is likely impacting their functions and other general homeostatic mechanisms.

Some of the most striking features of the NK cells from breast cancer patients were the mitochondrial defects. Patient NK cells had increased mitochondrial mass, MMP and mROS. IL12/15 stimulated NK cells had reduced expression of a key subunit of ATP synthase, ATP5B. Confocal microscopy analysis revealed that NK cells from healthy donors had elongated, fused mitochondrial networks, while those from breast cancer patients had increased mitochondrial fragmentation. This was evident as their mitochondria were more punctate and circular. Indeed, these data, along with the oxphos seahorse data, demonstrate strongly that NK cells from breast cancer patients have significant mitochondrial dysfunction. This has far reaching implications for many aspects of their cellular and mitochondrial fitness. The increase in mitochondrial mass suggests that mitophagy may be impaired. Mitophagy is a mitochondrial selective form of autophagy whereby cells eliminate

their damaged mitochondria, or damaged sections of mitochondrial networks, to allow for the production of new ones [157]. In this way, mitochondrial turnover maintains the quality of the mitochondria and their networks. If mitophagy is defective in the NK cells from breast cancer, they will be unable to clear their faulty mitochondria and this may lead to an overall increase in mitochondrial mass and decrease in mitochondrial fitness.

Mitochondria are highly dynamic organelles and undergo cycles of fusion and fission, which create and divide mitochondrial networks respectively [161]. Mitochondrial fission is one of the first key steps during mitophagy as it allows the defective mitochondria to be isolated. Hence, the increased mitochondrial fission observed in the NK cells from breast cancer patients suggests that patient NK cells have initiated mitophagy but are unable to complete it. This may be due to the hyperpolarisation of the mitochondrial membranes, as mitochondrial depolarisation has been shown to take place before a mitochondria is tagged for degradation [162]. Indeed, the hyperpolarisation of the mitochondrial membranes of NK cells from breast cancer patents may prevent the recruitment of the tag proteins required for mitophagy to proceed e.g. PINK1.

Of note, mTORC1 has been shown to act as an important negative regulator of mitophagy [163, 164]. In murine NK cells, mitophagy drives the development of NK cell memory, and blockade of mTORC1 signalling enhances this process [152]. As such, the increased mTORC1 activity observed in direct *ex vivo* NK cells from some breast cancer patients may also be contributing to the prevention of mitophagy and retention of dysfunctional mitochondria.

Aside from ATP production, apoptosis is one of the most well studied functions of the mitochondria. Indeed, it has been well established that once apoptosis is triggered within a cell, pro-apoptotic factors such as cytochrome c are released from the mitochondria through a mitochondrial permeability transition pore, and these then initiate the caspase dependent cell death pathway [165]. As such, one might expect NK cells from breast cancer patients to have altered cell viability or reduced frequency due to the significant mitochondrial defects observed. However, this was not the case, suggesting that something is preventing the cells from dying. This may be partially attributed to the hyperpolarised mitochondrial membranes, as mitochondrial membrane depolarisation is a key step during mitochondrial

apoptosis. Nonetheless, it seems that instead of undergoing apoptosis, patient NK cells adopt a senescent-like phenotype, which is characterised by reduced metabolism and function.

In addition to these mitochondrial defects, NK cells from breast cancer patients had increased levels of both mROS and cROS, which can result in a high degree of oxidative stress. One of the most likely sources of this oxidative stress is the ETC [166]. As electrons are transferred to oxygen at the end of oxphos, ROS is a natural by-product of this pathway. However, a defective ETC can result in increased ROS production. Similarly, any alteration in protein synthesis or damage to the endoplasmic reticulum may trigger the unfolded protein response (UPR) and this can result in ROS production within the endoplasmic reticulum [161, 167]. NADPH oxidases are plasma membrane bound enzymes that naturally produce cROS. While the role of NADPH oxidases in human NK cells remains unexplored, ImmGen data suggests that these could be a potential source of the cROS observed, particularly NOX4 (calcium dependent NADPH oxidase). Of note, it will be important in future studies to examine the level of antioxidant enzymes in NK cells from breast cancer patients in order to understand the true extent of this oxidative stress e.g. SOD1 and 2, glutathione peroxidase, and catalase.

There are many implications for the oxidative stress that exists in NK cells from breast cancer patients. ROS can react with and damage DNA in both the nucleus and the mitochondria resulting in DNA mutations, which itself can result in cell transformation. In healthy individuals, ROS is involved in many signalling pathways. Oxidation of cysteine residues can induce allosteric changes within a protein and thus alter its function ($Cys\text{-}S^- \rightarrow Cys\text{-}SOH$) [168]. Importantly, these modifications are reversible as disulfide reductase enzymes can return a protein to its original state. As an example of ROS mediated signal transduction, PTEN can be deactivated by H_2O_2 through oxidation and disulfide bond formation between two cysteine residues [169]. Similarly, it has been shown that oxidation of a cysteine residue in the catalytic subunit of protein-tyrosine phosphatase 1B (PTP1B) deactivates it [170]. This results in increased tyrosine phosphorylation of EGFR and transmission of downstream growth signalling. ROS can also influence MAP-kinase signalling by oxidation and inactivation of the PDGF-receptor associated phosphatase, SHP-2 [171]. As such, it is likely that the elevated levels of ROS recorded in the cytosol and the mitochondria of NK cells from breast cancer patients are altering many cell-

signalling pathways, which could in turn contribute to the metabolic and functional defects observed.

As mentioned above, patient NK cells surprisingly had higher basal mTORC1 activity *ex vivo*. Given that mTORC1 activity increases are required to regulate metabolic reprogramming in response to cytokine, another key finding of our work is that NK cells from breast cancer patients are unable to upregulate mTORC1 activity in response to cytokine stimulation. This suggests that chronic stimulation with certain factor(s) may be promoting some basal mTORC1 activity in the patient NK cells, but overall is inducing exhaustion and an inability to upregulate mTORC1 activity when it is truly needed. We have previously shown that inhibition of mTORC1 in healthy donor NK cells results in reduced IL2 induced glycolysis, but not oxphos. Thus, this impaired IL2 induced mTORC1 activity may be contributing to the reduced glycolytic capacity and glycolytic reserve observed in NK cells from metastatic breast cancer patients.

These data are the first to describe metabolic dysfunction of NK cells in the peripheral blood of cancer patients. This highlights the merit of working with blood samples of patients with cancer and other diseases. Indeed, collection of blood samples is easier than collection of tumour biopsies, and it can be carried out repeatedly for longitudinal studies. Furthermore, most approaches to adoptive cell transfer therapy use PBMC as the source of the immune cell population, again highlighting the importance of studying peripheral immune cells. Interestingly, it was recently shown that systemic immunity alone is required to drive immunotherapy mediated tumour rejection in a murine model of breast cancer [134]. In this study, Spitzer *et al.* showed that anti-PD-1 induced immune cell activation and proliferation is transient within the TME, but is sustained in the peripheral blood and secondary lymphoid organs. This included increases in the proliferation and activation of NK cells. Upon inhibition of immune cell migration from the periphery to the TME, animals had significantly increased tumour growth, indicating that anti-PD-1 induced anti-tumour responses are induced by the systemic immune system, rather than the local tumour resident one. This again highlights the importance of studying peripheral immune populations in cancer, and not only the tumour infiltrating ones.

It will be interesting to see in the future whether peripheral NK cell metabolism correlates with patient response to immunotherapy, particularly in patients with solid tumours. Should this be the case, metabolic markers such as CD71 may be useful as biomarkers to predict which patients will benefit from particular immunotherapies. Indeed, blood biomarker discovery is currently an area of intense research [172]. Identification of immune cell markers that can anticipate how a patient will respond to immunotherapy will lead to the design of better treatment strategies for non-responders, and a reduction in the waste of costly immunotherapy drugs.

Chapter 4

4 Targeting the intrinsic GARP/TGFβ axis in NK cells from breast cancer patients restores metabolism and function

4.1 Introduction

In the previous chapter, we demonstrated that NK cells from metastatic breast cancer patients have significant metabolic alterations and dysfunctional cytokine production. Our next goal was to explore what could be driving these defects. One possibility, given that the phenotype is observed in peripheral blood NK cells, is that it may be caused by a cancer derived soluble factor. It is widely acknowledged that TGFβ, sometimes found elevated in the blood, can become hijacked during cancer. Work from our lab also recently showed that *in vitro* TGFβ treatment reduces the function and metabolism of NK cells from healthy donors [173]. This suggests that TGFβ produced as a result of the cancer could potentially be inhibiting the patient NK cells and contributing to the impaired function and metabolism described in chapter 3. In this chapter we explore whether TGFβ may be an underlying cause of the impaired function and metabolism described in chapter 3.

4.1.1 TGFβ processing

TGFβ is a widely expressed pleiotropic cytokine, with functions including regulation of cell growth, differentiation and migration. Due to the high abundance of TGFβ expression and its vast array of functions, the processing and activation of TGFβ is complex and tightly regulated. TGFβ is synthesised as precursor molecules containing a pro-peptide region in addition to the TGFβ homodimer. This TGFβ homodimer strongly interacts with a Latency Associated Peptide (LAP), forming the Small Latent Complex (SLC) [174]. LAP is a protein derived from the N-terminal region of the TGFβ gene product. The SLC remains within the cell until it is bound by

another protein, the Latent TGF-β-Binding Protein (LTBP), forming the Large Latent Complex (LLC) (**Figure 4.1**). Finally the LLC gets secreted to the ECM.

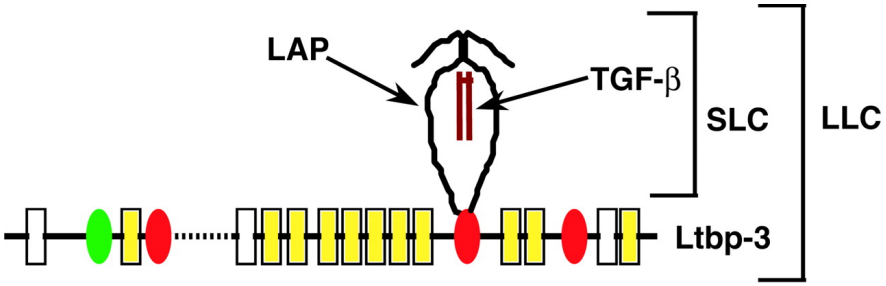


Figure 4.1 The structure of the LLC.

The Large Latent Complex (LLC) consists of TGFβ, LAP, and an LTBP. LAP and TGFβ compose the SLC. Adapted from [174].

4.1.2 TGFβ Activation

When TGFβ is bound to LAP, it is in its latent form, meaning it does not exert any biological activity. Therefore, it must be separated from LAP and activated before it can carry out any of its functions. While many aspects of TGFβ biology are well defined, the activation of TGFβ from latent to active form remains less understood. Nonetheless, several mechanisms have been described which identify ways in which TGFβ can be separated from LAP and released as active TGFβ. For example, cell surface α and β integrins are highly implicated in this process, where they bind latent TGFβ at the cell surface and release active TGFβ into the extracellular matrix using both protease dependent (e.g. matrix metalloproteinases) and independent mechanisms [175]. TGFβ can also be activated by the adhesive glycoprotein thrombospondin-1 [176] and by ROS [177].

4.1.3 TGFβ Signalling

TGFβ signalling is subdivided into the canonical and non-canonical signalling pathways. The TGFβ canonical signalling pathway (SMAD pathway) is relatively simple— TGFβ first binds to TGFβ receptor 2 (TGFβR2), which leads to the recruitment, transphosphorylation and activation of TGFβ receptor 1 (TGFβR1). TGFβR1 then phosphorylates its downstream targets using its cytoplasmic kinase domain to initiate the SMAD dependent signalling pathway [107]. Phosphorylated SMAD2 and SMAD3 bind to SMAD4 and the complex then travels to the nucleus

where it modulates gene expression and directs the cells' response to TGFβ. SMAD6 and SMAD7 are negative regulators of this pathway, and their expression may be induced by SMAD4-2/3 forming a negative feedback loop (**Figure 4.2**) [178]. Although there are relatively few SMAD proteins involved in TGFβ signalling, they commonly regulate gene expression in co-operation with hundreds of DNA-binding transcription factors and transcriptional co-regulators. These are controlled by and often dependent on other signalling pathways, resulting in highly context-dependent transcriptional responses being controlled by signalling cross talk [179, 180]

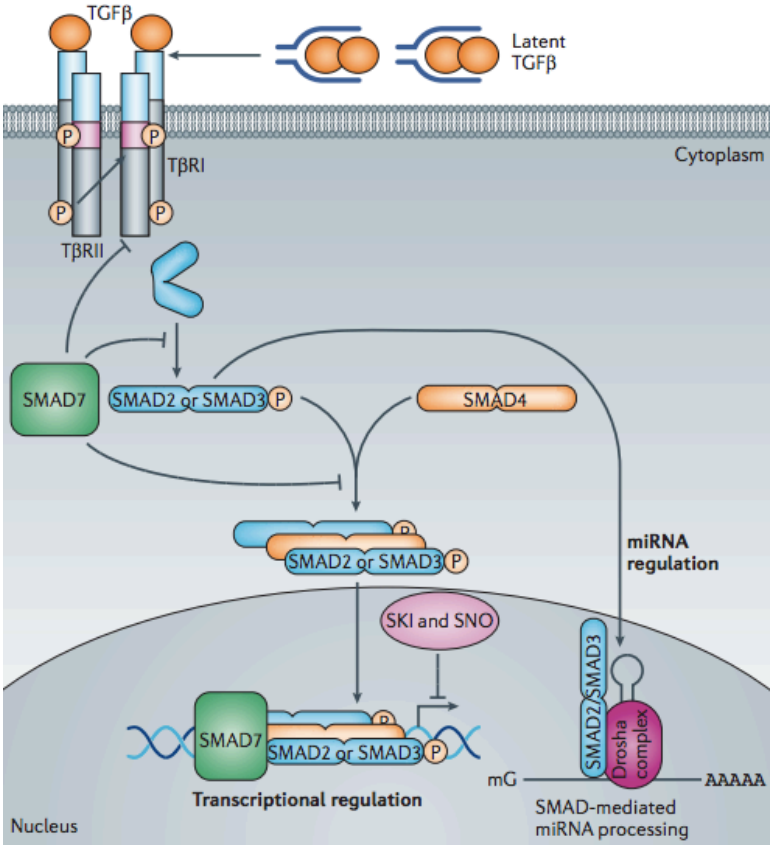


Figure 4.2 The canonical pathway of TGF-β signalling.

The TGFβ SMAD signalling pathway regulates gene expression. Taken from [181].

Alternatively, TGFβ can signal through non-SMAD ("non-canonical") signalling pathways. This entails recruitment and activation of signalling mediators by ligand-occupied receptors i.e., TGFβR1 and TGFβR2 [182]. These pathways include branches of PI3K-Akt signalling which activate the MAPK pathway and mTOR, small GTPase pathway and the JNK/p38 pathway. Many of the signalling molecules in these pathways interact directly with TGFβR1 and/or TGFβR2 e.g., TRAF6 (**Figure 4.3**) [183, 184]. Adding another layer of complexity, pathways other than the SMAD

and non-canonical TGFβ signalling pathways can influence TGFβ signalling, such as the WNT, Hedgehog, Notch, IFN, TNF and RAS pathways. The crosstalk between these pathways defines the activities of TGFβ and propagates context and time dependent signals.

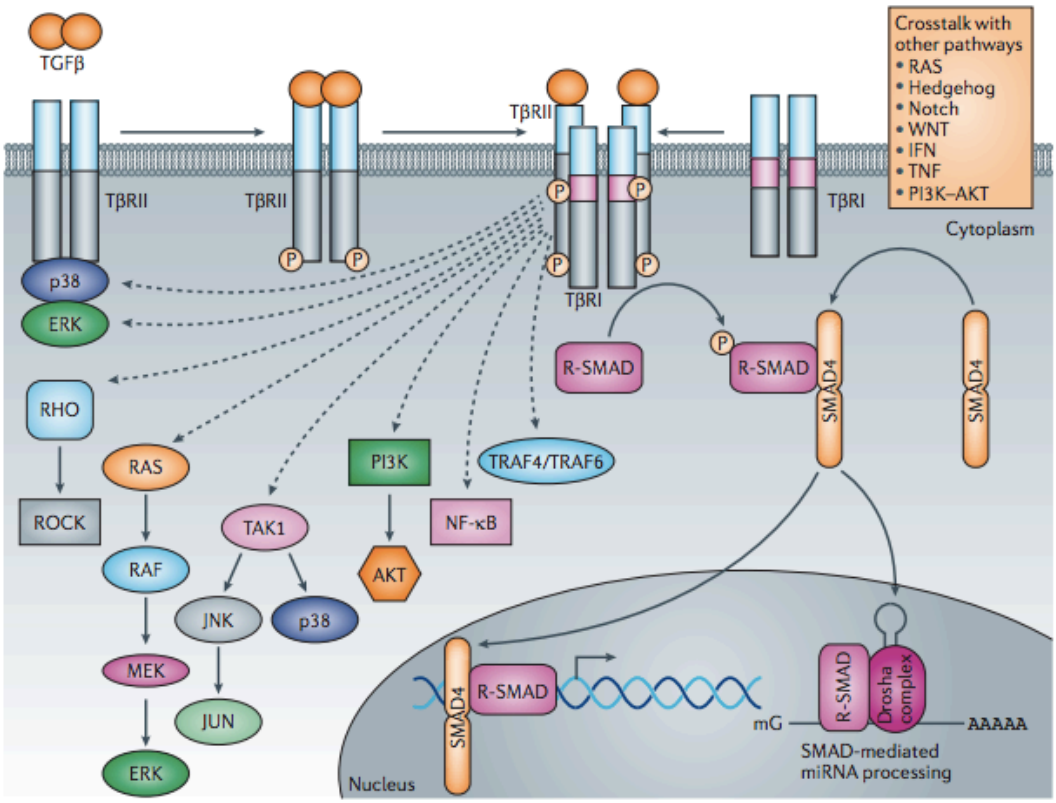


Figure 4.3 The non-canonical pathway of TGF-β signalling.

Taken from [181].

4.1.4 TGFβ can drive cancer growth and progression

TGFβ is widely implicated in human disease, particularly in cancer. This is in part due to the abundance of the cytokine (in its latent form) throughout the body and in particular within the extracellular matrix. Should TGFβ or any of its downstream signalling molecules acquire an oncogenic mutation/alteration, the effect is substantial. Based on current human The Cancer Genome Atlas datasets, oesophageal, gastric colorectal, and pancreatic adenocarcinomas contain mutations or deletions in SMAD and TGFβ receptor genes in 25%–50% of cases. Head and neck, bladder, and endometrial adenocarcinomas and cervical and lung squamous carcinomas harbour such mutations in 10%–20% of cases.

In healthy individuals TGFβ acts as a negative regulator of growth, and hence is anti-tumour. However, this function is commonly reversed in the tumour setting. Indeed, many aggressive tumours acquire oncogenic mutations in certain pathways (e.g. PI3K/AKT), which confer insensitivity to TGFβ's anti-growth signals. Despite this, TGFβ retains its ability to promote migration, differentiation, angiogenesis etc., resulting in a highly pro-tumourigenic factor [185].

In addition to influencing tumour growth, TGFβ can also have a profound effect on cancer stem cells (CSC). Unsurprisingly, there are many studies showing that TGFβ can both promote or suppress CSC. For example, Tang *et al.* reported that the size and frequency of CSC is reduced in the presence of TGFβ [186], while many others have reported that TGFβ promotes and sustains stemness of a pool of CSC in many cancer types [187-189]. Similarly TGFβ plays a role in promoting tumour metastasis. For example, in breast cancer TGFβ canonical signalling stimulates the expression of angiopoietin-like 4 (ANGPTL4), which enables ER^- breast cancer cells to metastasize into the lung [190]. Also in breast cancer, TGFβ increases the expression of bone-metastasis-promoting genes e.g. PTHRP, IL11, CXCR4, and MMP1 [79, 191].

4.1.5 GARP – a TGFβ anchoring protein

During the TGFβ processing pathway described above, the LAP-TGFβ complex binds LTBP forming the LLC. However, there is an alternative, lesser known pathway of TGFβ processing that can outcompete LTBP. This involves the surface protein GARP (glycoprotein-A repetitions predominant), which anchors the LAP-TGFβ complex at the cell surface and thereby locally regulates the bioavailability of TGFβ [192-194]. Because of this important function, GARP is implicated in maintaining immune tolerance, and deletion of GARP is associated with excessive inflammation [195, 196]. The name of the gene GARP is transcribed from is Leucine Rich Repeat Containing 32 (LRRC32), due to the leucine rich domains throughout its structure (**Figure 4.4**).

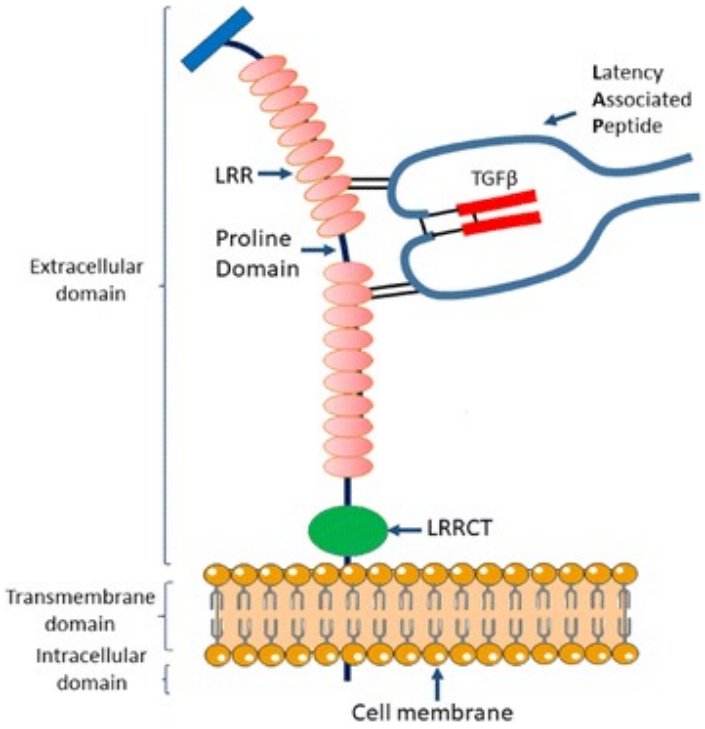


Figure 4.4 GARP anchors latent TGFβ to the cell membrane.

LRR; Leucine rich repeat, LRRCT; Leucine rich repeat C-terminal domain. Taken from [194].

To date, there are no published studies on GARP in NK cells; however it has been studied in other types of human cells, including tumour cells, hepatic stellate cells, endothelial cells, platelets, Treg cells and B cells. In all of these studies its function is centred on regulation of TGFβ activity. GARP biology has in fact been under investigation for over 3 decades, mainly due to the high prevalence of its gene amplification in aggressive metastatic carcinomas, including in breast cancer [197-199]. In tumour cells, GARP expression is associated with elevated TGFβ activity (which promotes tumour growth) and increased malignant transformation. Therefore it is no surprise that higher GARP expression in tumours is associated with worse clinical outcome [194, 198]. Indeed, GARP is acknowledged as a highly oncogenic factor that is frequently overexpressed in tumours and can drive immune tolerance.

In addition to tumour cells, platelets have also been shown to express high levels of GARP during cancer. In 2009, constitutive GARP expression on platelets was shown to play a role in promoting oncogenesis for the first time [200]. More recently, Rachidi *et al.* showed that GARP expression on platelets inhibits murine and human T cell anti-tumour immunity, while GARP deletion specifically in platelets reduced

tumour burden in a murine melanoma model [201]. As platelets are circulated throughout the body in the blood, this likely has a significant impact on peripheral anti-tumour immunity.

The 2009 article that first described GARP expression in platelets was also the first to show GARP expression in Treg cells [200]. Since then, much research on the role GARP plays in Treg cells has been carried out. For example, in a murine model of colon cancer, deletion of GARP within Treg cells improved anti-tumour immune responses, such TNFα and IFNγ production by CD4+ T cells, which ultimately lead to reduced tumour burden in the colon. Interestingly, latent TGFβ has been observed in high molecular weight complexes with GARP in the supernatants of human Treg cells transfected with GARP, suggesting that TGFβ-GARP complexes can be shed from the surface of human Treg cells [202]. Treatment of human naive CD4+ T cells with soluble GARP protein reduced proliferation and cytokine production, and skewed development towards Treg cells [203]. While this could be partially blocked by inhibiting TGFβ signalling, blocking the TGFβ receptor did not restore IFNγ production, indicating that additional receptors and signalling molecules may be involved. Within a pro-inflammatory environment (IL6 + IL23), soluble GARP facilitated the differentiation of naïve CD4+ T cells into Th17 cells, highlighting its ability to impact immune cells in which its expression has not been documented.

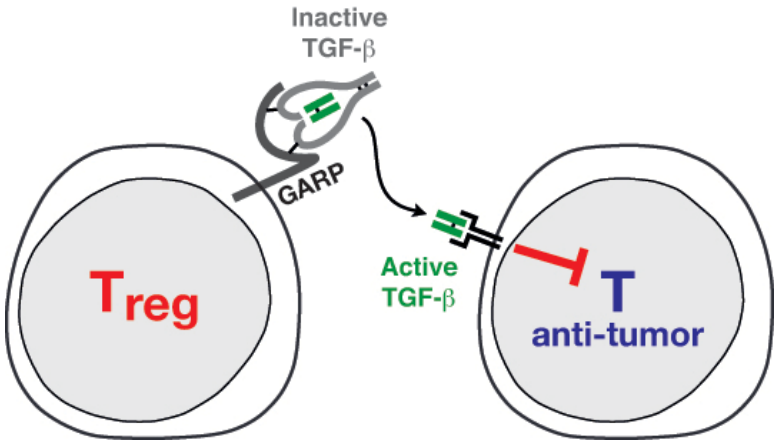


Figure 4.5 GARP expression on Treg cells inhibits T cell anti-tumour immunity.

GARP expression was only recently described for the first time in human B cells, where it is believed to confer immune tolerance [196]. In this study, Wallace *et al.* showed that while resting B cells do not express GARP, TLR ligand stimulated B cells express GARP/ TGFβ complexes at their cell surface. Overexpression of GARP

reduced their proliferation and increased IgA class switching. Highlighting the context dependent nature of GARP biology, canonical TGFβ signalling more readily upregulated GARP expression in Peyer's patch B cells compared to splenic B cells. Interestingly, loss of GARP resulted in development of spontaneous autoimmunity (lupus-like disease), and *in vivo* expansion of B cells was also recorded. Therefore, the authors propose that GARP acts as a negative checkpoint by regulating B cell systemic tolerance, and suggest that TLR ligand induced GARP expression regulates B cells by the autocrine anti-proliferative and immunosuppressive effects of TGFβ. As GARP is only observed upon TLR stimulation, it is likely responsible for ensuring that the B cells do not become hyper-activated.

4.1.6 The impact of TGFβ on NK cell function and metabolism.

Indeed, the regulation of TGFβ activity is complex and involves a wide range of processes that safeguard it from becoming uncontrolled, e.g. LAP and GARP. However, as mentioned above, TGFβ is commonly hijacked during cancer such that it becomes pro-tumourigenic. In addition to increased TGFβ within the TME, its concentration is sometimes elevated in the serum of cancer patients [77, 78]. These elevated levels of TGFβ significantly impact the immune system, for example by promoting Treg proliferation and MDSC differentiation, while at the same time inhibiting the functions of anti-cancer immune cells such as CD8+ T cells and NK cells. In terms of the latter, TGFβ has been described to inhibit NK cell activation, T-bet expression, IFNγ production, ADCC and the expression of activating receptors [36, 80, 81].

While it has been known for many decades that TGFβ inhibits the activity and functions of NK cells, the molecular mechanisms underlying this have remained poorly understood. Two recent immunometabolism studies in humans and mice have shed some light on this. Viel *et al.* showed that while TGFβ had no effect on the development of murine NK cells, genetic deletion of the TGFβR2 (specifically in NK cells) reduced tumour metastasis in two tumour models and increased nutrient receptor expression and mTORC1 activity in IL15 stimulated NK cells [204]. *In vitro*, TGFβ treatment inhibited mouse and human metabolic responses (oxphos and glycolysis), nutrient receptor expression and mTORC1 activity. Rapamycin treatment impacted certain NK cell functional readouts in a similar manner to TGFβ treatment, such as granzyme B expression and IFNγ production. In many cases, the

effects of TGFβ were more potent than those of rapamycin, yet mTORC1 deletion had more significant effects than constitutive TGFβ signalling, suggesting that mTORC1 is not the only pathway involved in the repression of NK cell activity by TGFβ.

Work from our lab also investigated the effect of TGFβ on human NK cells stimulated with cytokine [173]. Zaiatz-Bittencourt *et al*. showed that NK cells stimulated with IL2 for 18 hours in the presence of TGFβ had significantly reduced levels of oxphos, maximal respiration and glycolytic capacity compared to IL2 alone. Interestingly, basal glycolysis was not affected. Furthermore, the expression of CD69, CD71 and the functional mediators IFNγ and granzyme B were also reduced following TGFβ treatment. With the exception of granzyme B, these effects were reversed by adding a TGFβR1 inhibitor, suggesting that TGFβ's impact on granzyme B is mediated by an alternative pathway, likely one of the non-canonical pathways that signals through TGFβR2 only.

Indeed, the role TGFβ plays in inhibiting NK cell functions in human cancer is well described. The possibility exists however, that it may mediate additional, unappreciated effects by impacting on NK cell metabolism, particularly in light of what we have found in terms of normal NK cell metabolic regulation by TGFβ. Therefore, we hypothesised that TGFβ may be driving at least some of the functional and metabolic defects observed in NK cells from metastatic breast cancer patients. In this chapter we first explored whether TGFβ might be contributing to the NK cell dysfunction phenotype by adding a TGFβ neutralising antibody to overnight cultures. Furthermore, we investigated for the first time whether the GARP/TGFβ axis might also be playing a role in impairing NK cell function and metabolism during metastatic breast cancer. The findings of this chapter have important implications for the design of future NK cell based immunotherapies against breast cancer and other solid tumours.

4.1.7 Aims and Objectives:

1. To look for evidence of intracellular TGFβ signalling in NK cells from breast cancer patients.
2. To investigate the impact of TGFβ neutralisation on the metabolism and function of NK cells from breast cancer.
3. To examine whether TGFβ might impact patient NK cells in a local, autocrine manner → Explore a potential role for GARP in mediating TGFβ's effects on NK cells in patients.
    i. To analyse GARP and LAP expression on NK cells from patients and healthy donors.
    ii. To determine whether the GARP/TGFβ axis can be targeted in order to improve the metabolism and function of NK cells from breast cancer.

4.2 Results

Work from Chapter 3 showed that peripheral NK cells from patients with metastatic breast cancer have a range of activation, functional and metabolic defects. These defects are similar to those observed in healthy donor NK cells treated with recombinant TGFβ *in vitro* [173]. The experiments in the first part of this chapter were designed to investigate whether TGFβ may have contributed to these defects and furthermore, if neutralising its activity might have the potential to restore some of the defects identified. The experimental approach was therefore to analyse some of the known defects while using a neutralising antibody against TGFβ in the overnight cultures. This antibody (clone 1D11) works by neutralising TGFβ-1, 2 and 3 isoforms. As TGFβ-1 is the predominant isoform found in the immune system it is the most likely target of the antibody. IgG1 treatment was used as an isotype control for all experiments, and had negligible impact compared to untreated (data not shown).

4.2.1 NK cells from some breast cancer patients have active constitutive intracellular TGFβ signalling.

We first looked for evidence of active TGFβ signalling within the patient NK cells. One of the first steps of TGFβ signalling involves recruitment and phosphorylation of SMAD2 by TGFβR1, which then leads to recruitment and phosphorylation of SMAD3. We used flow cytometry to investigate this pathway, by staining intracellularly with a pSMAD2/3 antibody that binds to the SMAD2 protein phosphorylated at the Ser465/467 sites and the Smad3 protein phosphorylated at the Ser423/425 sites. While there was significant heterogeneity in the data, *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from some breast cancer patients had increased levels of pSMAD2/3 compared to those from healthy donors (**Figure 4.6A** for representative histogram and **Figure 4.6B**). In healthy donors, the pSMAD2/3 level in NK cells was similar to that in CD3+CD25+CD56- lymphocytes (CD25+ T cells, likely enriched with Treg cells). However, this pSMAD2/3 levels in CD25+ T cells was not increased further in breast cancer patients compared to healthy donors.

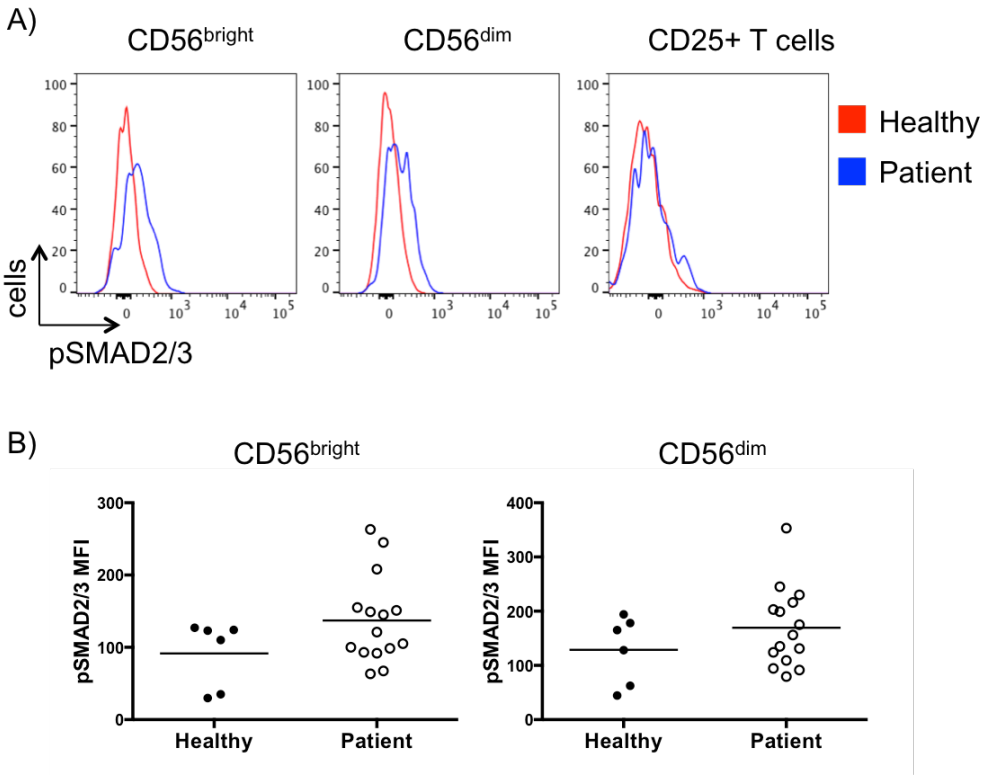


Figure 4.6 NK cells from some breast cancer patient have increased intracellular TGFβ signalling.

Freshly isolated PBMC from healthy donors and breast cancer patients were stained for phosphorylated SMAD2/3 protein as a measure of active TGFβ signalling, and analysed by flow cytometry. (A) Representative histograms of pSMAD2/3 levels in *ex vivo* healthy donor and patient NK cells. (B) Pooled data of pSMAD2/3 levels in *ex vivo* healthy donor and patient NK cells. Each dot represents 1 donor (n=6-15).

4.2.2 TGFβ impairs the activation of NK cells from breast cancer patients.

The elevated pSMAD2/3 levels associated with active signalling suggest that TGFβ may be affecting the patient NK cells. Therefore, we investigated the impact of TGFβ neutralisation on the activation of NK cells from breast cancer patients. CD25 was used as a marker of NK cell activation and was measured by flow cytometry. The frequency of NK cells expressing CD25 after cytokine stimulation was reduced in breast cancer patients (**Chapter 3**). However, when this impaired upregulation of CD25 in response to IL12/15 in patient cells was investigated after overnight culture with anti-TGFβ, there was an improvement in the frequency of NK cells expressing CD25 (**Figure 4.7A** for representative dot plots and **Figure 4.7B**). This suggests that TGFβ was contributing to the impaired upregulation of CD25 in response to IL12/15.

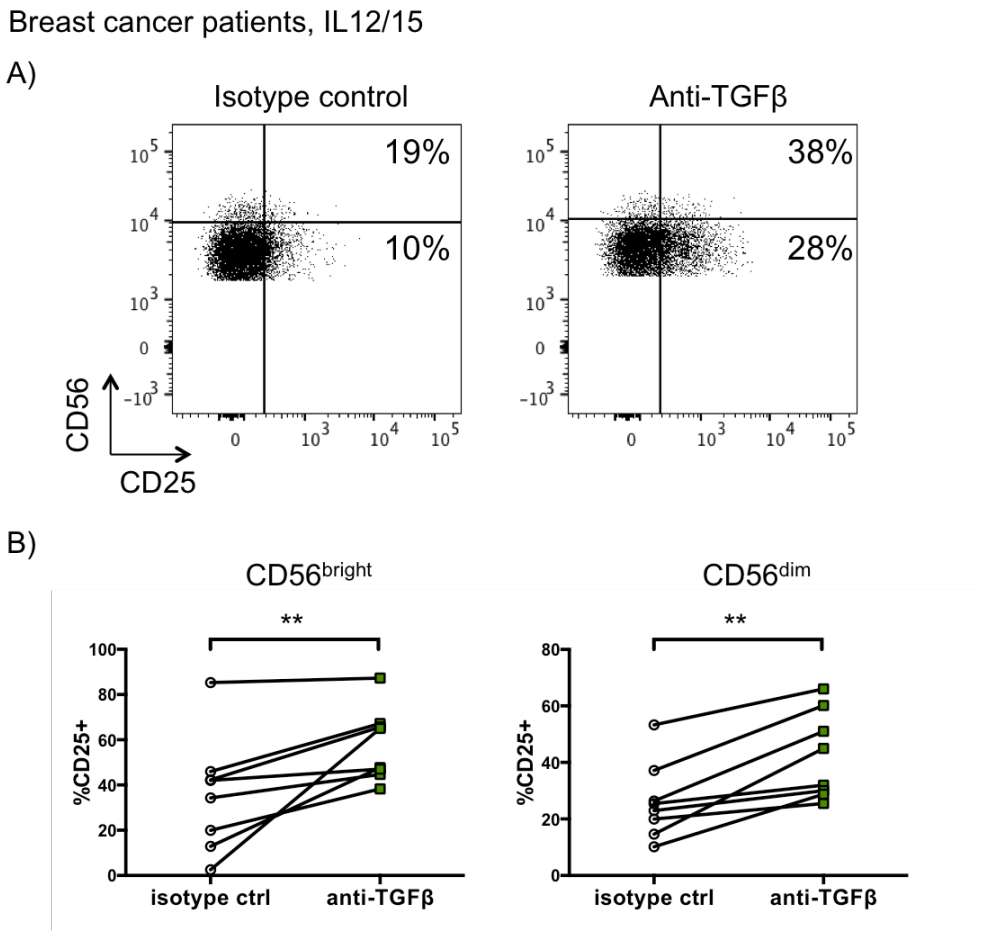


Figure 4.7 Neutralisation of TGFβ increases expression of CD25 in IL12/15 stimulated NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-TGFβ antibody (5μg/ml) or isotype control (5μg/ml), and incubated for 18 h at 37°C. Cells were stained for CD25 and analysed by flow cytometry. (A) Representative dot plots of the impact of isotype control and anti-TGFβ on CD25 expression in IL12/15 stimulated NK cells. (B) Frequency of CD25 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=8). Samples were compared using the paired student t-test, **p<0.01.

4.2.3 TGFβ restricts nutrient receptor expression in NK cells from breast cancer patients.

Cytokine stimulated NK cells from breast cancer patients also had reduced expression of the nutrient receptors CD71 and CD98, when compared to those from healthy donors (**Chapter 3**). Therefore, the impact of TGFβ neutralisation on nutrient receptor expression in NK cells from breast cancer patients was next

investigated. Addition of anti-TGFβ antibody significantly improved the frequency of CD71 expression in both IL2 and IL12/15 stimulated NK cells from breast cancer patients (**Figure 4.8A** and **Figure 4.9A** for representative dot plots). While CD71 expression was highest in the CD56^{bright} subset, the recovery was evident in both the CD56^{bright} and CD56^{dim} subsets of patient NK cells (**Figure 4.8B** and **Figure 4.9B**).

Preliminary evidence also suggests that addition of anti-TGFβ antibody may also allow recovery of CD98 expression in IL12/15 stimulated NK cells from breast cancer patients (**Figure 4.10A** for representative histograms and **Figure 4.10B**) but further biological replicates are required to confirm this.

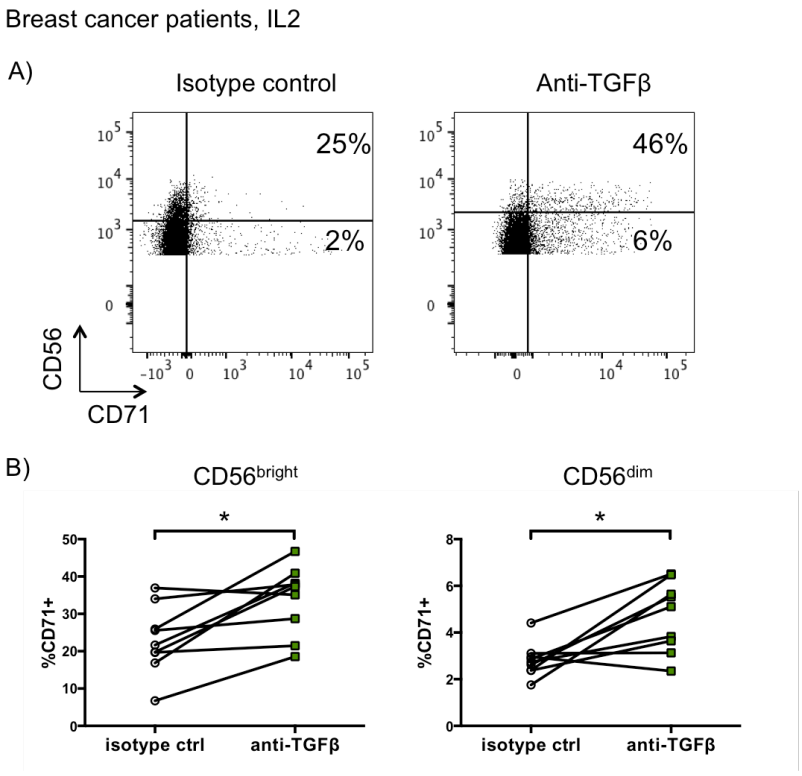


Figure 4.8 TGFβ contributes to reduced CD71 in IL2 stimulated NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated IL2 (500IU/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml) and incubated for 18 h at 37°C. Cells were stained for CD71 and analysed by flow cytometry. (A) Representative dot plots of the impact of isotype control and anti-TGFβ on CD71 expression in IL2 stimulated NK cells. (B and C) Frequency of CD71 expression in IL2 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=9). Samples were compared using the paired student t-test, *p<0.05

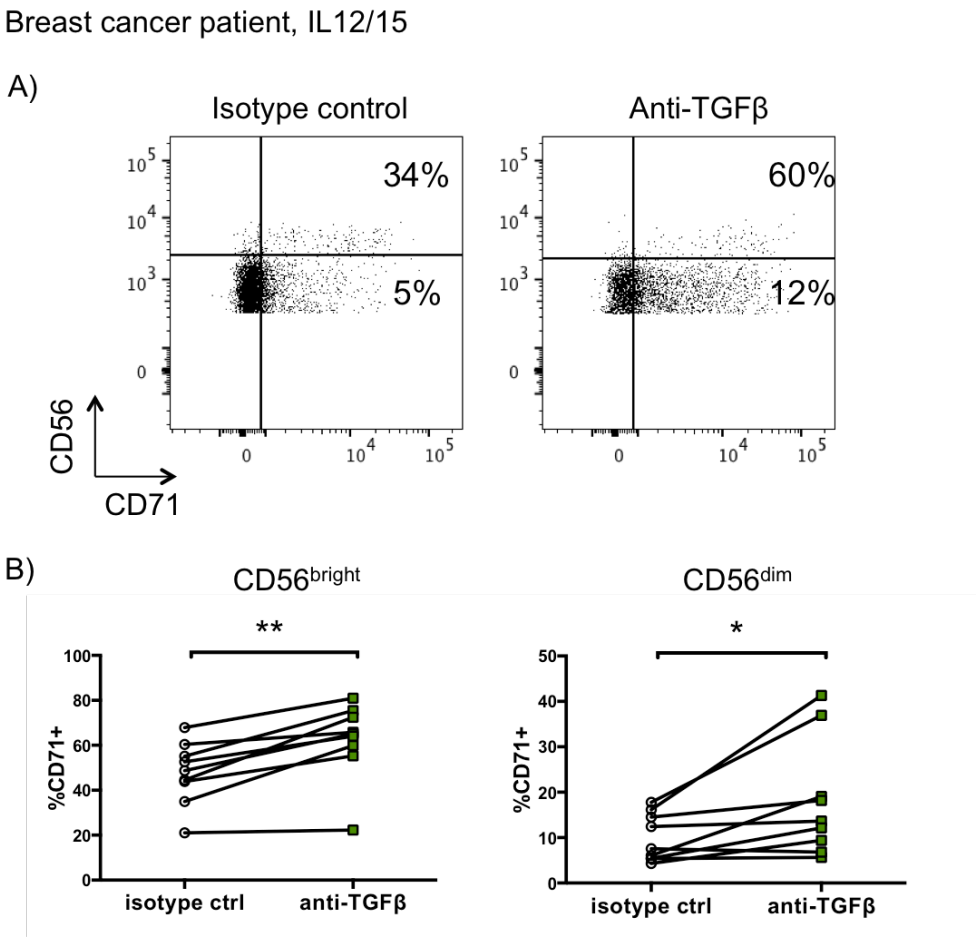


Figure 4.9 TGFβ contributes to reduced CD71 in IL12/15 stimulated NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml) and incubated for 18h at 37°C. Cells were stained for CD71 and analysed by flow cytometry. (A) Representative dot plots of the impact of isotype control and anti-TGFβ on CD71 in IL12/15 stimulated NK cells. (B) Frequency of CD71 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=8). Samples were compared using the paired student t-test, $^{**}p<0.01$.

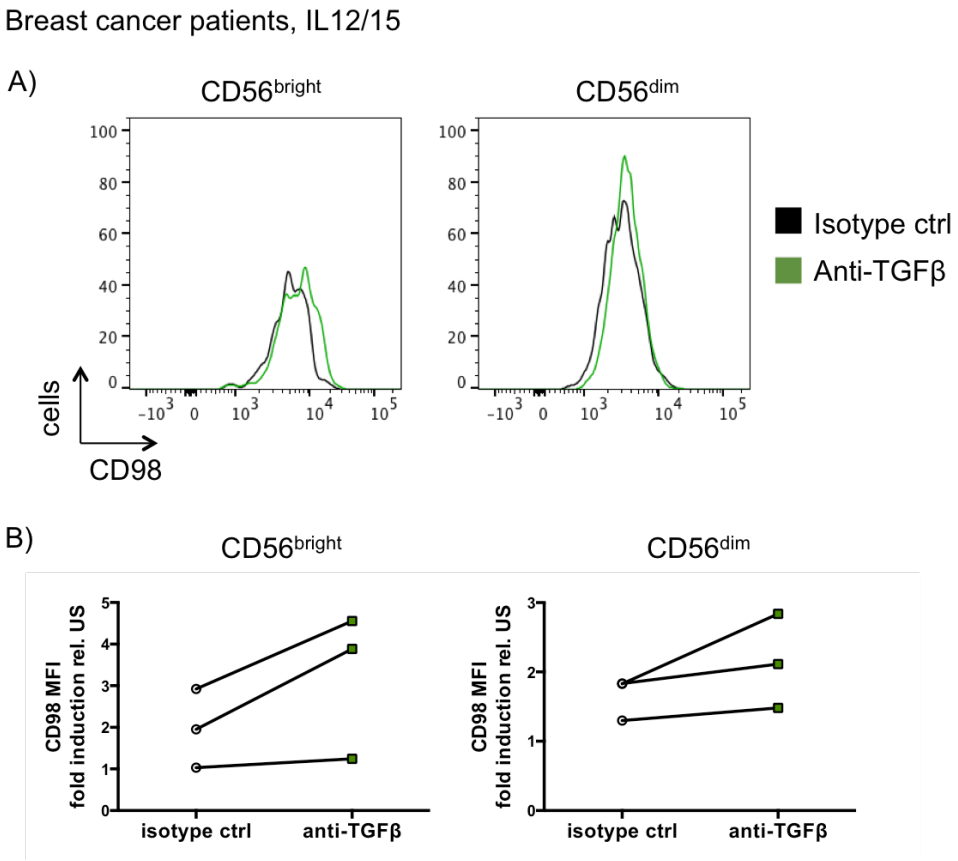


Figure 4.10 TGFβ neutralisation improves expression of CD98 in IL12/15 stimulated NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml) and incubated for 18 h at 37°C. Cells were stained for CD98 and analysed by flow cytometry. (A) Representative histogram of the impact of isotype control and anti-TGFβ on CD98 in IL12/15 stimulated NK cells. (B) Fold increase in the MFI of CD98 relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients, in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=3).

4.2.4 TGFβ contributes to increased mitochondrial mass in CD56^{dim} NK cells from breast cancer patients.

One of the early striking results that we obtained was that *ex vivo* NK cells from breast cancer patients had increased mitochondrial mass compared to NK cells from healthy donors (**Chapter 3**). We next investigated if TGFβ contributed to this by neutralising TGFβ and measuring the mitochondrial mass of NK cells from breast cancer patients using the fluorescent dye MitoTracker. Anti-TGFβ antibody did not impact mitochondrial mass in IL2 stimulated CD56^{bright} NK cells from breast cancer patients. However, addition of anti-TGFβ antibody decreased the mitochondrial mass of IL2 stimulated CD56^{dim} NK cells from breast cancer patients (**Figure 4.11A** for representative and **Figure 4.11B**). This suggests that TGFβ may be preventing mitophagy from taking place in CD56^{dim} NK cells from breast cancer patients.

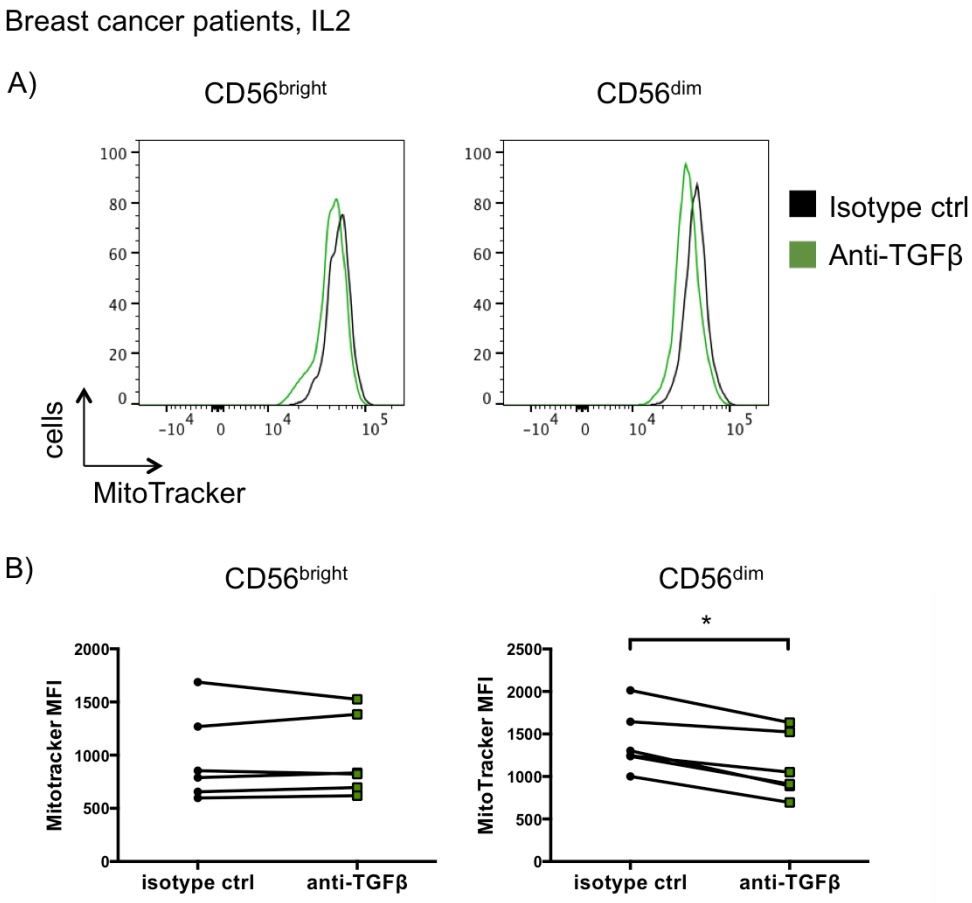


Figure 4.11 TGFβ contributes to the increased mitochondrial mass in CD56^{dim} NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated with IL2 (500IU/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml), and incubated for 18 h at 37°C. Cells were stained with MitoTracker Green (100nM) for 20 min at 37°C and analysed by flow cytometry. (A) Representative histogram of the isotype control and anti-TGFβ mediated reduction in mitochondrial mass in IL2 stimulated NK cells. (B) MitoTracker MFI of IL2 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients, in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *$p<0.05$.

4.2.5 Targeting TGFβ promotes metabolic reprogramming towards oxphos in patient NK cells.

NK cells from breast cancer patients had severely reduced levels of oxphos compared to NK cells from healthy donors (**Chapter 3**). This was characterised by significantly reduced basal respiration, maximal respiration and ATP-linked respiration in IL2 stimulated NK cells from breast cancer patients, and was combined by various mitochondrial defects such as high mROS. Therefore, the impact of TGFβ neutralisation on oxphos in IL2 stimulated NK cells from breast cancer patients was next investigated using the Seahorse XF Analyser. It should be noted here that most of the functional and flow cytometry based outputs have used PBMC with gating on NK cells for their specific analysis (as described in chapter 2). However, for Seahorse analysis, such discrimination is not possible and NK cells were purified prior to analysis. Addition of anti-TGFβ antibody increased oxphos in IL2 stimulated NK cells from breast cancer patients (**Figure 4.12A** for representative seahorse trace). Calculation of the various oxidative metabolism parameters revealed that in the presence of anti-TGFβ antibody, IL2 stimulated NK cells from breast cancer patients recovered levels of basal respiration, maximal respiration and ATP-linked respiration when compared to IL2 and isotype control (**Figure 4.12B**). It is important to note here that this reversal of defects was observed when using purified NK cells, suggesting that the TGFβ inhibitory effects may result, at least in part, from TGFβ produced locally by NK cells themselves.

The above data indicate that TGFβ inhibits patient NK cell oxphos. Given that NK cells from breast cancer patients also had reduced levels of glycolysis (glycolytic capacity and reserve, **Chapter 3**), we next investigated whether TGFβ might also be contributing towards this glycolytic dysfunction in NK cells from breast cancer patients. Addition of anti-TGFβ antibody to overnight cultures had variable impact on IL2 stimulated NK cells from breast cancer patients (**Figure 4.13A** for representative). Basal glycolysis was reduced and glycolytic reserve was increased in the presence of anti-TGFβ antibody, while glycolytic capacity increased in 4 donors and decreased in 2 donors (**Figure 4.13B**).

Although both oxphos and glycolysis were impacted, NK cells from breast cancer patients had an overall higher OCR/ECAR ratio than NK cells from healthy donors, which indicates an increased reliance on oxphos and a decreased reliance on glycolysis. As the neutralising anti-TGFβ antibody had differential impacts on

oxphos and glycolysis, this impacted on the OCR/ECAR ratio, which was ultimately increased in NK cells from breast cancer patients (**Figure 14**). Indeed, these data show that targeting TGFβ in patient NK cells promotes a reprogramming of metabolism towards oxphos and away from glycolysis.

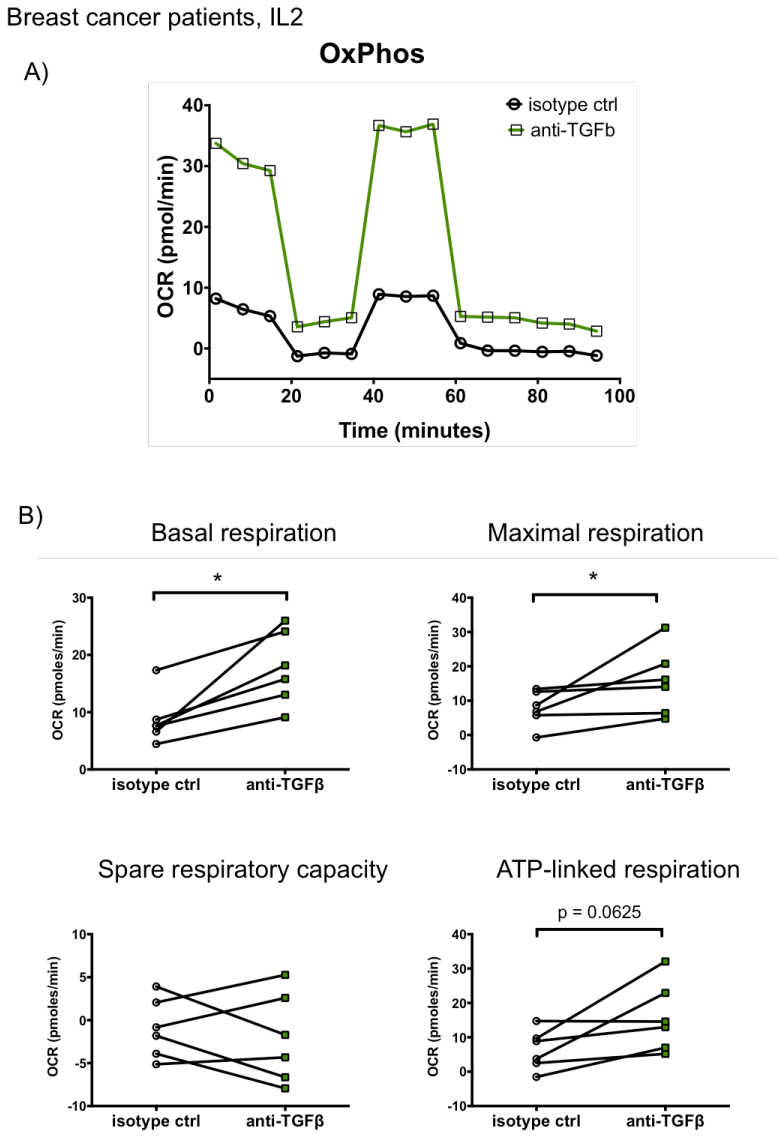


Figure 4.12 TGFβ is a driver if impaired oxphos in NK cells from breast cancer patients.

NK cells were purified from freshly isolated PBMC from breast cancer patients and stimulated with IL2 (500IU/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative oxygen consumption rate (OCR) trace of IL2 stimulated NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. (B) Pooled data of the IL2 stimulated oxphos readouts in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *p<0.05.

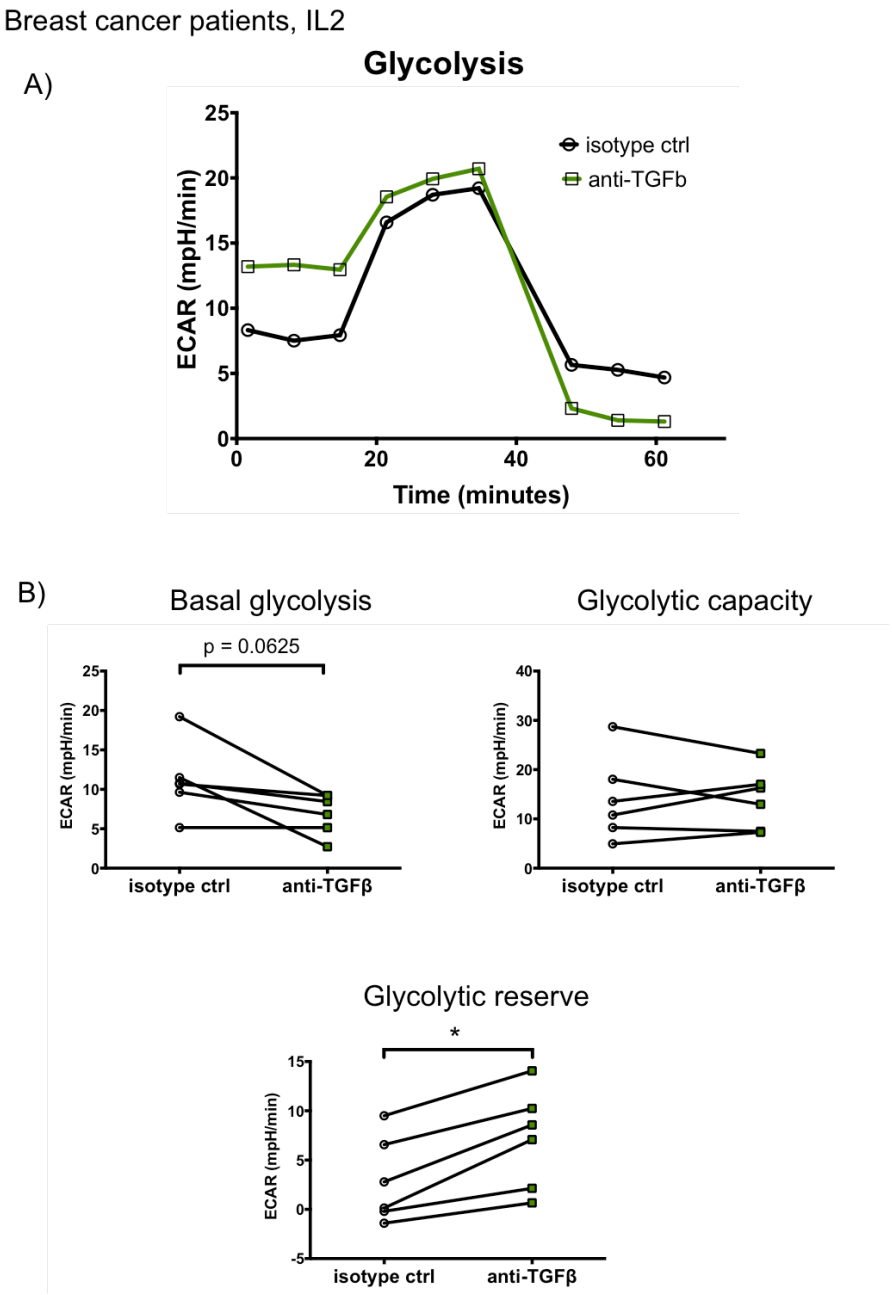


Figure 4.13 TGFβ neutralisation has variable impacts on glycolysis NK cells from breast cancer patients.

NK cells were purified from freshly isolated PBMC from breast cancer patients and stimulated with IL2 (500IU/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative extracellular acidification rate (ECAR) trace of IL2 stimulated NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. (B) Pooled data of the IL2 stimulated glycolytic readouts in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *$p<0.05$.

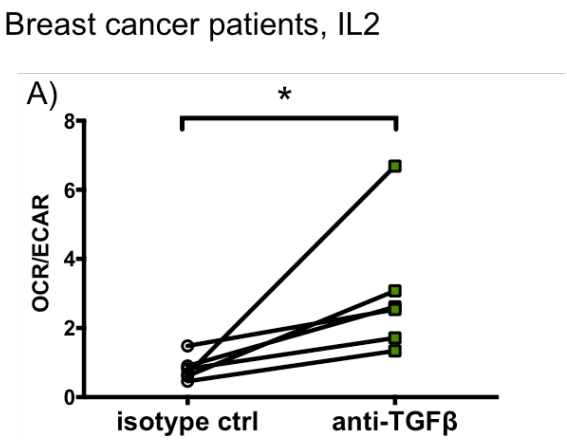


Figure 4.14 TGFβ neutralisation increases the OCR/ECAR ratio in NK cells from breast cancer patients.

NK cells were purified from freshly isolated PBMC from breast cancer patients and stimulated with IL2 (500IU/ml) in the presence of either anti-TGFβ antibody (5μg/ml) or isotype control (5μg/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) The OCR/ECAR ratio, where an increased number indicates a greater reliance on oxphos. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *$p<0.05$.

4.2.6 TGFβ contributes to IFNγ deficits in NK cells from breast cancer patients.

Given the importance of IFNγ for NK cell functions and that its production was profoundly impaired in NK cells from patients with breast cancer, we next investigated the potential for neutralising TGFβ to improve this NK functional response. Promisingly, addition of anti-TGFβ antibody significantly increased IFNγ production in IL12/15 stimulated NK cells from breast cancer patients (**Figure 4.15A** for representative dot plots) and this was evident in both the $CD56^{bright}$ and $CD56^{dim}$ subsets (**Figure 4.15B and 4.15C**).

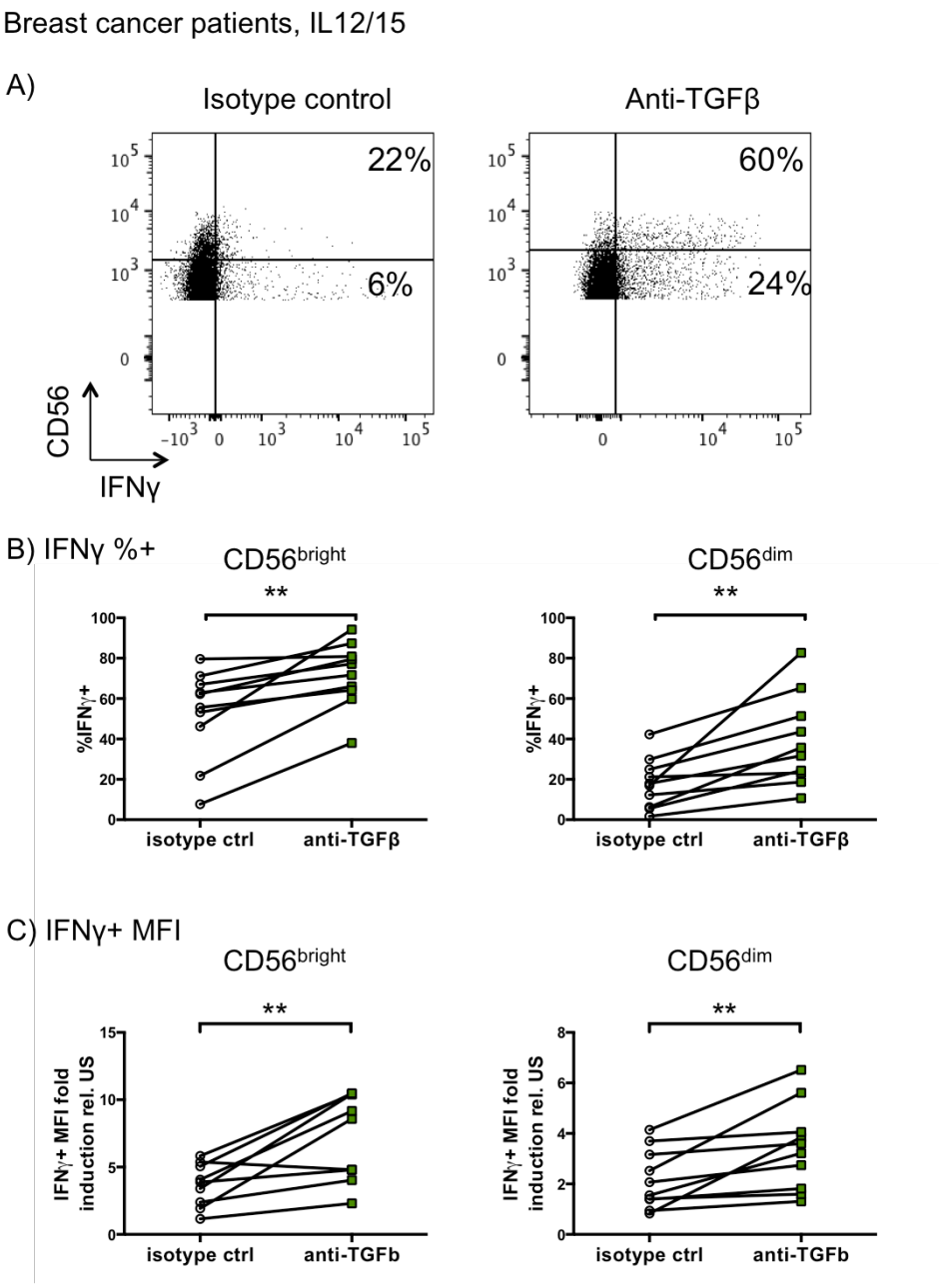


Figure 4.15 TGFβ contributes to reduced IFNγ production NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml), and incubated for 18 h at 37°C. Cells were stained for IFNγ and analysed by flow cytometry. (A) Representative dot plot of the anti-TGFβ mediated upregulation of IFNγ production in IL12/15 stimulated NK cells. (B) Frequency of IFNγ production in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. (C) Fold increase in the MFI of IFNγ relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=10). Samples were compared using the paired student t-test, **$p<0.01$.

4.2.7 TGFβ impairs mTORC1 activity in NK cells from breast cancer patients.

In Chapter 3 and here, we show that NK cells from patients with breast cancer have severely dysregulated metabolic responses in response to cytokine, and we identify TGFβ as a mechanism that can contribute to these deficits. Furthermore, we know that mTORC1 is crucial for cytokine induced metabolic responses [122], and that patients also have impaired cytokine responsive mTORC1 activity (**Chapter 3**). We therefore investigated whether blocking TGFβ might also restore mTORC1 activity, which in turn might explain the improved metabolic and functional responses. Supporting this idea, addition of anti-TGFβ antibody significantly increased mTORC1 activity in both IL2 and IL12/15 stimulated NK cells from breast cancer patients (**Figure 4.16A** and **Figure 4.17A** for representative dot plots) and this was evident in both the $CD56^{bright}$ and $CD56^{dim}$ subset (**Figure 4.16B and 4.16C, and Figure 4.17B and 4.17C**).

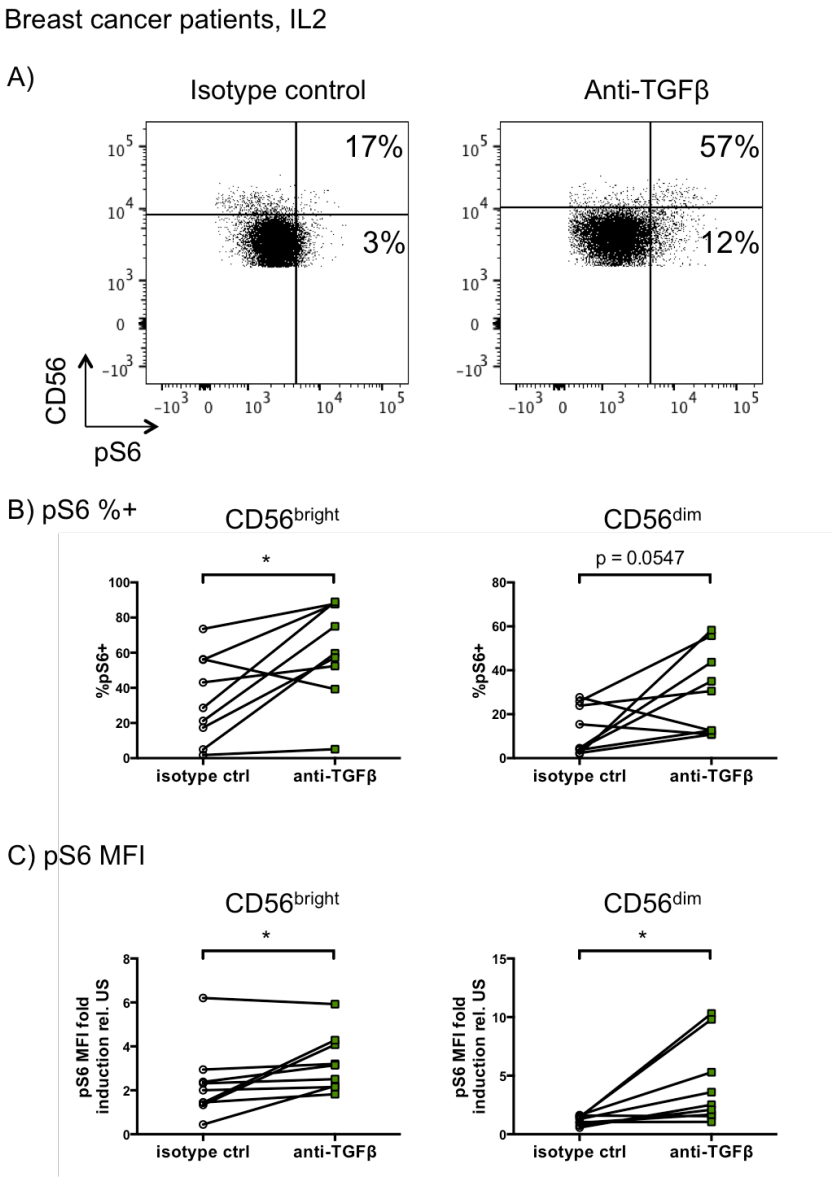


Figure 4.16 TGFβ is involved in the repression of mTORC1 activity in IL2 stimulated NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated with IL2 (500IU/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml) and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the anti-TGFβ mediated upregulation of mTORC1 activity in IL12/15 stimulated NK cells. (B) Frequency of NK cells positive for phosphorylated S6 protein in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=9). Samples were compared using the paired student t-test, *$p<0.05$.

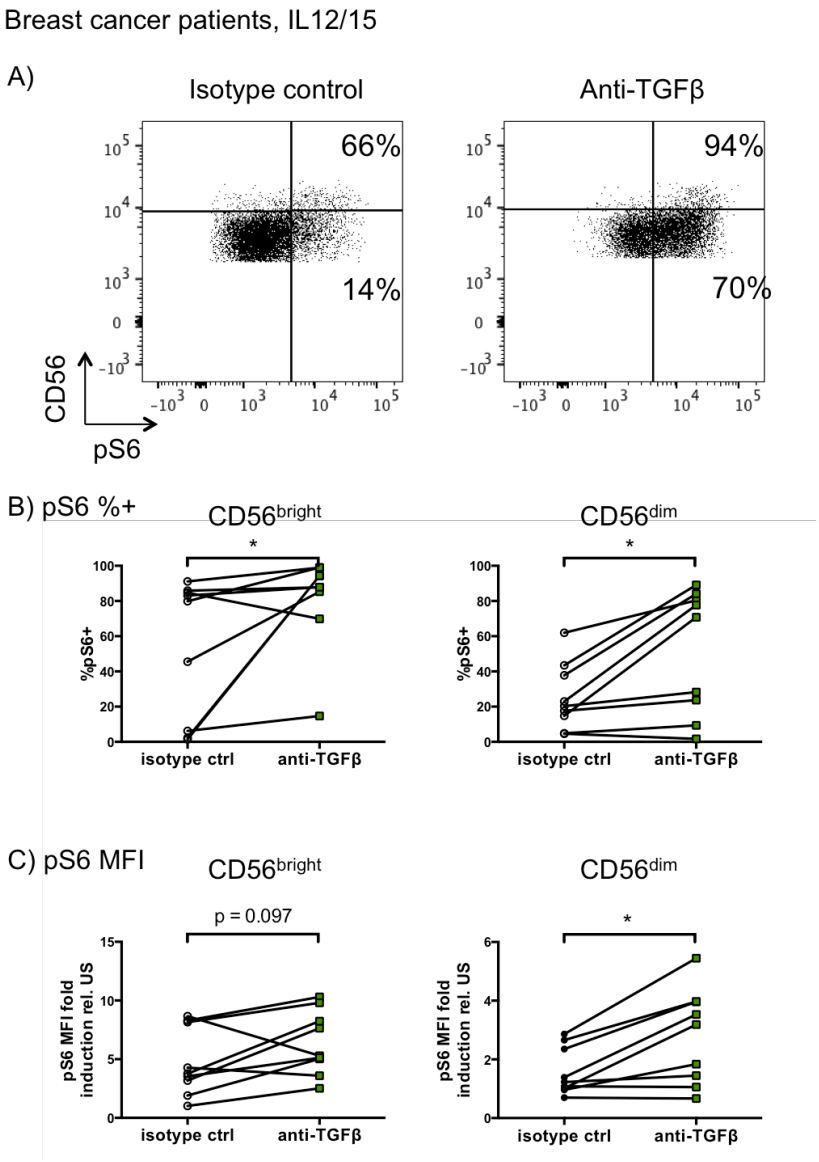


Figure 4.17 TGFβ is involved in the repression of mTORC1 activity in IL12/15 stimulated NK cells from breast cancer patients.

Freshly isolated PBMC from breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-TGFβ antibody (5µg/ml) or isotype control (5µg/ml), and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the anti-TGFβ mediated upregulation of mTORC1 activity in IL12/15 stimulated NK cells. (B) Frequency of NK cells positive for phosphorylated S6 protein in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-TGFβ. Each line represents 1 donor (n=9). Samples were compared using the paired student t-test, *p<0.05.

4.2.8 Breast cancer patients have normal levels of plasma TGFβ.

The above data demonstrates quite strikingly that TGFβ is driving many of the functional and metabolic defects observed in NK cells from breast cancer patients. Indeed, addition of anti-TGFβ antibody for just 18 hours improved several readouts of metabolism and function in NK cells from breast cancer patients. One possible way this could happen would be through increased systemic levels of circulating TGFβ as has been shown in some previous studies on cancer including breast cancer [77, 78, 205]. Therefore, we measured plasma levels of TGFβ by ELISA. Somewhat surprisingly, breast cancer patients in our cohort had plasma TGFβ levels equivalent to that seen in healthy donors (**Figure 4.18A**).

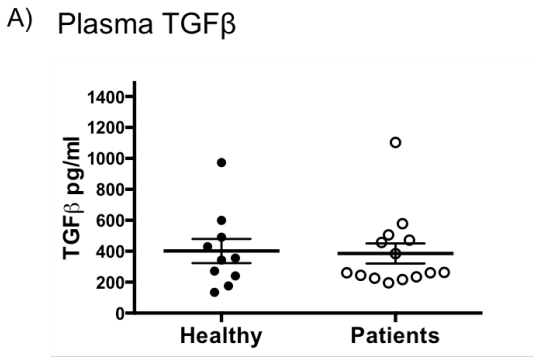


Figure 4.18 Breast cancer patients in this cohort do not have increased levels of plasma TGFβ.

Freshly isolated blood from healthy donors and breast cancer patients was spun at 200g for 10mins and plasma was carefully pipetted off. The concentration of TGFβ in the plasma samples was measured by ELISA. Lines show the mean value. Each dot represents 1 donor (n=10-14).

4.2.9 NK cells from healthy donors and breast cancer patient respond similarly to TGFβ exposure.

It is clear that removal of TGFβ from cell cultures significantly improves the function and metabolism of patient NK cells, yet it has less of an impact on NK cells from healthy donors (**Figure 4.19A - 4.19D** for key examples). TGFβ levels are not elevated in the plasma of this patient cohort. Therefore, the striking improvements observed in patient NK cells cultured with anti-TGFβ could be explained if they were hypersensitive to TGFβ exposure. If this was true, the normal levels of plasma TGFβ observed in patients might have profound impact on NK cells from breast cancer patients. To test this hypothesis, we examined how NK cells from healthy donors

and breast cancer patients respond to TGFβ treatment, and compared the fold change induced by TGFβ (relative to untreated control) in cytokine induced responses between the two cohorts. A fold change of less than one indicates inhibition by TGFβ, and hence a smaller number may suggest greater TGFβ sensitivity. While the results are variable, the mean fold changes caused by TGFβ in healthy and patient NK cell are similar (**Figure 4.20A, 4.20B** and **4.20C**). These data suggest that heightened sensitivity to TGFβ exposure is not responsible for the functional and metabolic defects observed in NK cells from breast cancer patients.

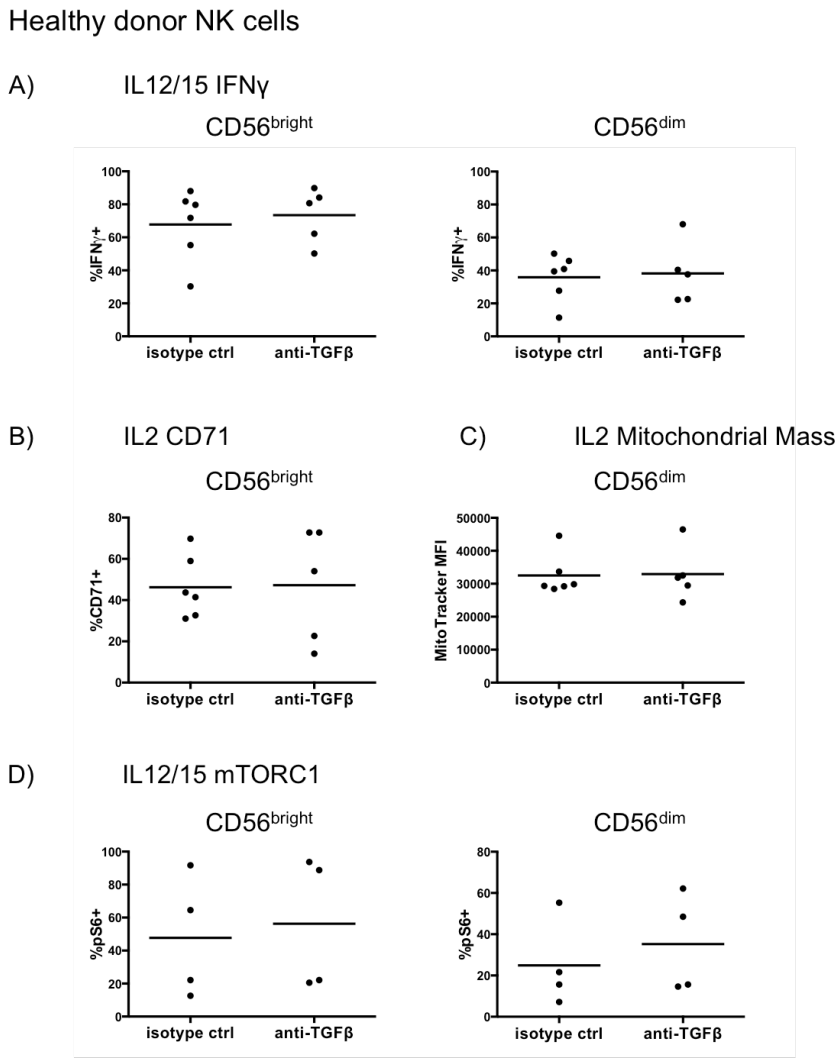


Figure 4.19 TGFβ neutralisation does not impact healthy donor NK cell responses.

Freshly isolated PBMC from breast cancer patients were stimulated IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-TGFβ antibody (10μg/ml) or isotype control (10μg/ml) and incubated for 18 h at 37°C. Cells were stained for (A) IFNγ, (B) CD71, (C) Mitotracker (100nM, 20mins at 37°C) or (D) pS6, and analysed by flow cytometry. Each line represents 1 donor (n=6).

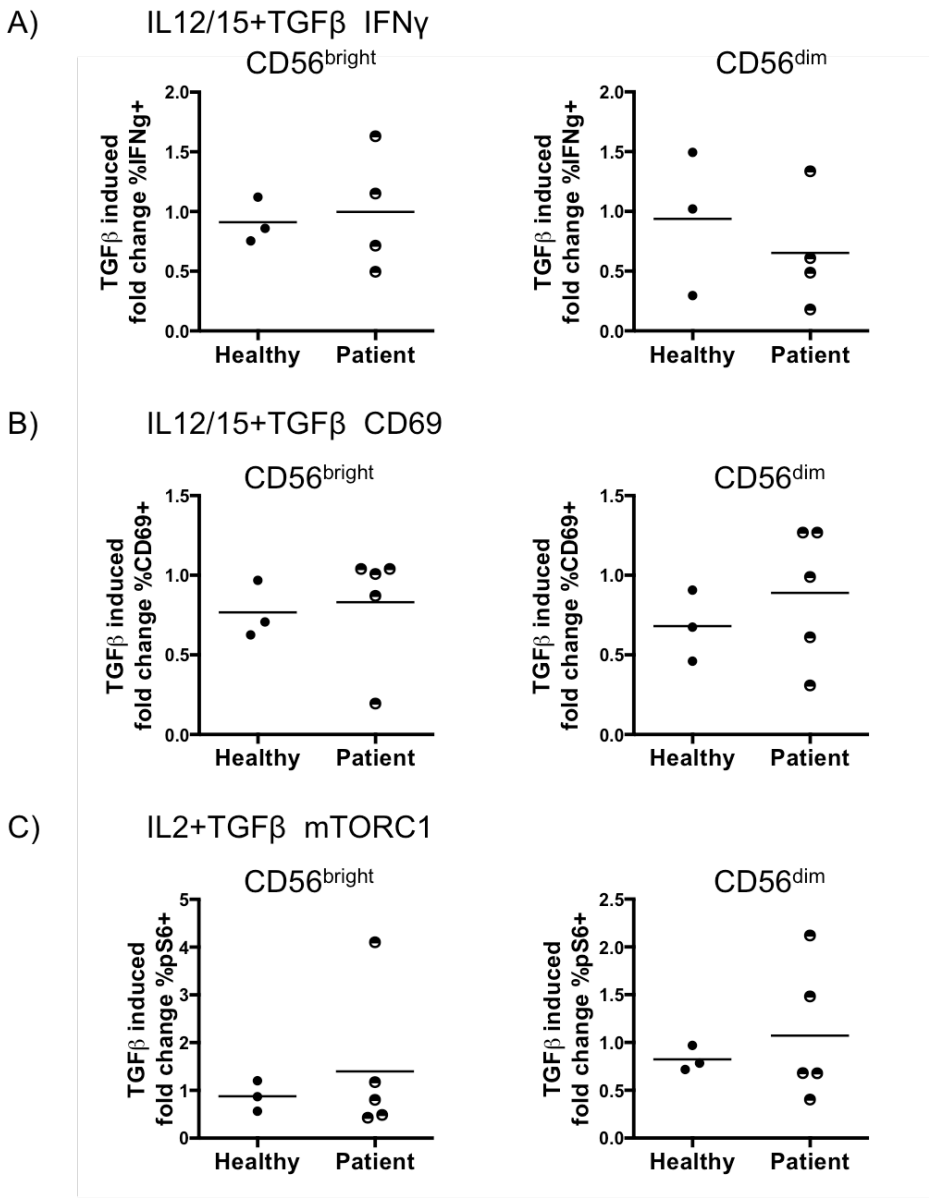


Figure 4.20 TGFβ impacts NK cells from healthy donors and breast cancer patients in a variable yet similar manner

Purified NK cells were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence or absence of recombinant TGFβ (10ng/ml), and incubated for 18 h at 37°C. Cells were stained for (A) IFNγ, (B) CD69 and (C) pS6 and analysed by flow cytometry. Lines show the mean value. Each dot represents 1 donor (n=3-4).

4.2.10 Online RNA sequencing databases suggest that NK cells express GARP.

The above data suggests that neither high circulating levels of TGFβ nor increased TGFβ sensitivity is the reason for the beneficial impact of TGFβ neutralisation observed in patient NK cells. This suggests that the increased TGFβ activity may be taking place at a more local level. Indeed, it is reported that NK cells can themselves produce TGFβ [206] and anti-TGFβ seahorse experiments, which we performed on purified NK cells, support this. Based on the TGFβ processing pathway described earlier, we investigated alternative potential mechanisms. There are no reports on GARP expression in NK cells, and therefore we began by searching online databases for evidence of GARP expression in NK cells. Interestingly, both murine and human NK cells appeared to express GARP to varying degrees (**Figure 4.21** and **4.22**), as measured by RNAseq data from ImmGen and Immune Cell Atlas. In terms of the human data, GARP expression was particularly evident in CD16+CD57- NK cells, indicating $CD56^{dim}$ NK cells. The phenotypic markers used to identify the human NK cell subsets are detailed in **Table 4.1**, and correspond in order to the bars in the graph in **Figure 4.22**.

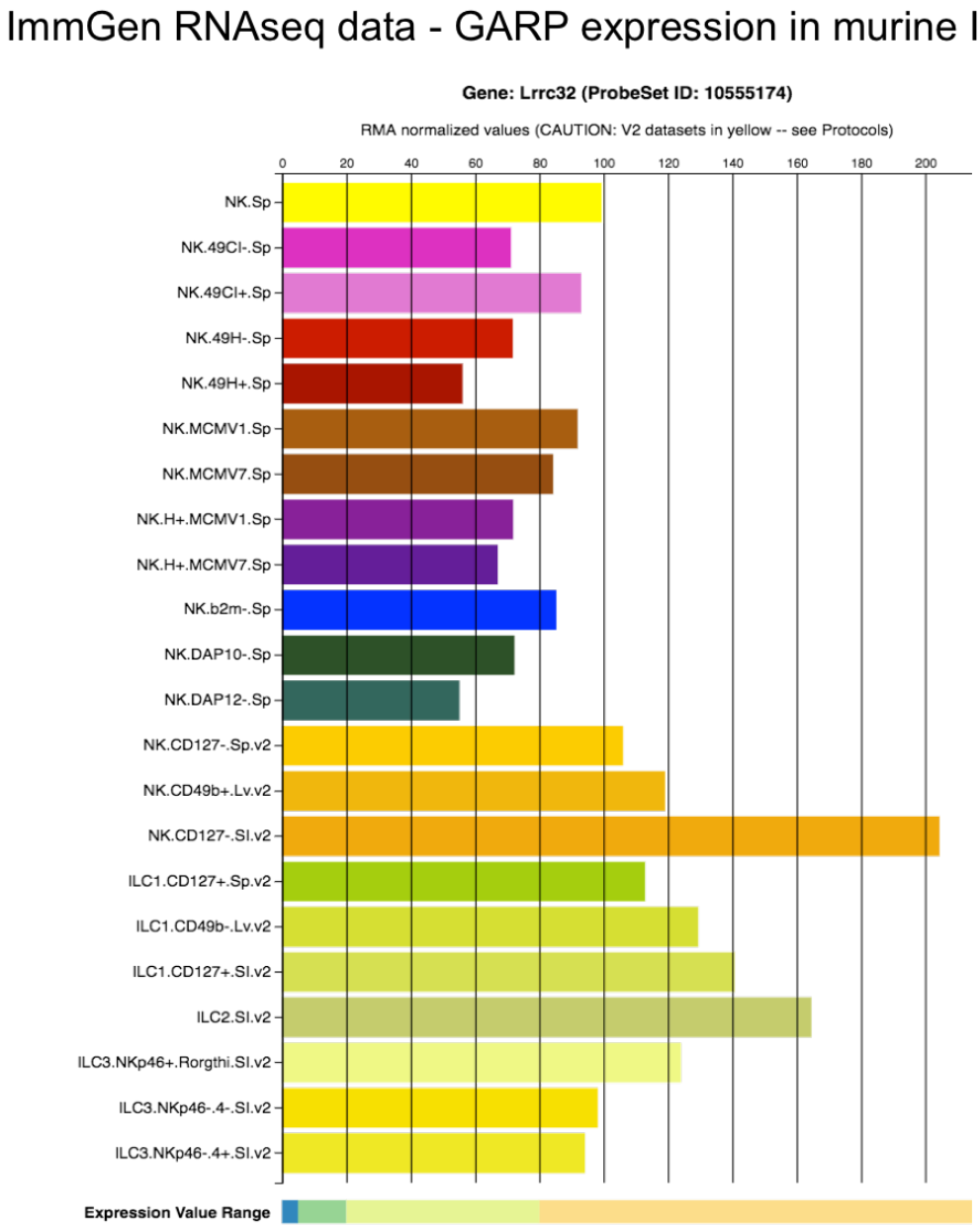


Figure 4.21 Murine ILCs express GARP.

RNA sequencing data from ImmGen shows GARP expression in difference subsets of murine ILCs. Normalised by median of ratios method with DESeq2 package from Bioconductor. In brief, the ratio of GARP expression level between samples is calculated, and then the median ratio across all expressed genes is used as the normalisation scale.

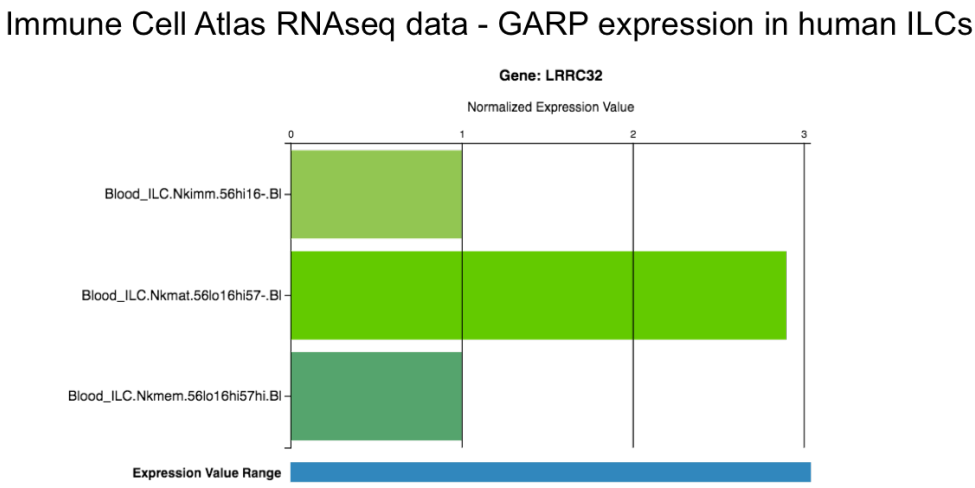


Figure 4.22 CD16+CD57- human NK cells express GARP.

RNA sequencing data from the Immune Cell Atlas shows GARP expression in human CD16+CD57- NK cells from the peripheral blood. Normalised by median of ratios method with DESeq2 package from Bioconductor. In brief, the ratio of GARP expression level between samples is calculated, and then median ratio across all expressed genes is used as the normalisation scale.

ILC.Nkimm.56hi16-.Bl	Sorted as CD19- CD56hi PBMCs
ILC.Nkmat.56lo16hi57-.Bl	Sorted as CD19- CD56mid CD16+ CD57- PBMCs
ILC.Nkmem.56lo16hi57hi.Bl	Sorted as CD19- CD56mid CD16+ CD57+ PBMCs

Table 4.1 Gating strategy used to identify NK cells for RNA sequencing analysis.

Taken from Immune Cell Atlas.

4.2.11 NK cells from some breast cancer patients have altered expression of the TGFβ processing molecules LAP and GARP.

The online databases suggest that NK cells may express GARP. Therefore, the expression of GARP as well as LAP were next analysed by flow cytometry. Of note, LAP is transcribed on the same gene as TGFβ itself, and therefore the presence of LAP indicates that TGFβ transcription has taken place. Direct *ex vivo* analysis showed that NK cells from healthy donors express low levels of GARP to varying degrees. As an example, **Figure 4.23** shows GARP expression in NK cells from 5 healthy donors. Within the $CD56^{bright}$ subset, a distinct positive peak is evident in healthy donor 1, and to a lesser extent in healthy donor 4. In the $CD56^{dim}$ subset, it is clear that healthy donor 1 and 5 have higher GARP expression, while healthy donor 2 has very low GARP expression.

GARP expression was next analysed in NK cells from breast cancer patients. Interestingly, NK cells from some patients with metastatic breast cancer had increased GARP as well as LAP expression compared to those from healthy donors. Similar to what was observed with healthy donor NK cells, there was significant heterogeneity within the data. As seen by the contour plots, LAP and GARP were sometimes co-expressed to varying degrees in healthy and patient NK cells (**Figure 4.24A**), however high expression was more common in patient NK cells. Overlapping histograms of patient NK cells with high LAP and GARP expression compared to healthy donor NK cells examined on the same day show a significant shift in the MFI of these molecules suggesting increased expression (**Figure 4.24B** and **4.24C**). This increase in MFI was also observed in corresponding CD25+ T cells from this patient.

Overall, GARP expression data was variable. NK cells from some patients had very low levels of LAP and GARP expression, some had slight increases and a few had high levels. Nonetheless, when the data was pooled, both $CD56^{bright}$ and $CD56^{dim}$ patient NK cells had significantly increased levels of GARP expression compared to those from healthy donors. (**Figure 4.25A**). This trend was also observed for LAP expression, but did not reach statistical significance (**Figure 4.25B**).

LAP and GARP expression in CD25+ T cells was also investigated. CD25+ T cells from healthy donors had higher baseline levels of both LAP and GARP compared to NK cells from healthy donors, with a mean MFI value of 2051, 275 and 318 for CD25+ T

cells, CD56^{bright} and CD56^{dim} NK cells respectively. Interestingly, while patient NK cells had higher GARP expression than healthy donor NK cells, this was not the case for CD25+ T cells. Indeed, the mean GARP MFI value for CD25+ T cells from patients was 1669, which is in fact lower than the 2051 value recorded from healthy donor CD25+ T cells. Overall this data hints towards a possible mechanism whereby patient NK cells have intrinsic increased TGFβ activity, involving the production of TGFβ, which follows the GARP anchoring processing pathway. This is the first time this has been reported in immune cells from cancer patients, and therefore may represent a new immunosuppressive mechanism in cancer.

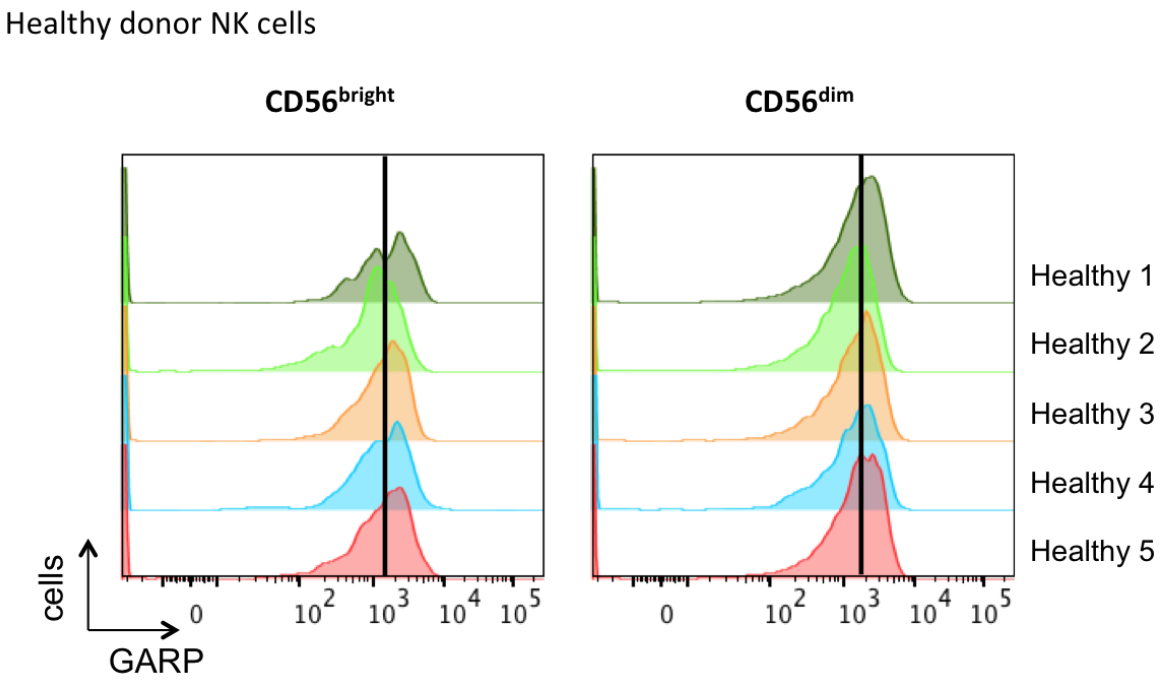


Figure 4.23 GARP expression in NK cells from healthy donors.

Freshly isolated PBMC were stained *ex vivo* GARP expression and analysed by flow cytometry. Each histogram represent a different healthy donor.

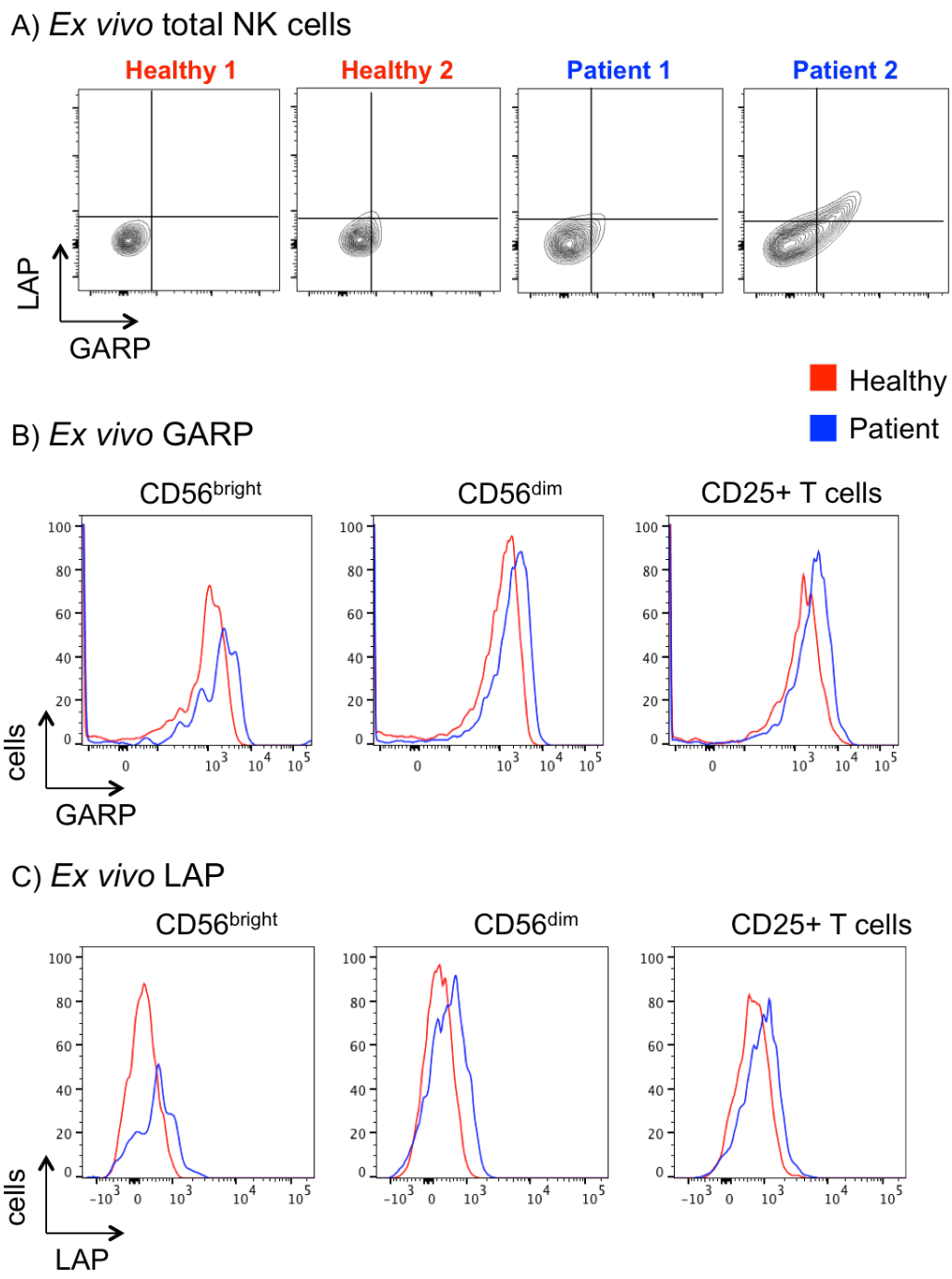


Figure 4.24 NK cells from some breast cancer patients have increased expression of the TGFβ processing molecules GARP and LAP.

Freshly isolated PBMC were stained *ex vivo* for LAP and GARP expression and analysed by flow cytometry. (A) Sample contour plots of LAP and GARP co-expression in *ex vivo* NK cells from healthy donors and breast cancer patients. (B) Representative histograms of GARP expression in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells and CD25+ T cells (CD3+CD56-CD25+) from healthy donors and breast cancer patients. (C) Representative histograms of LAP expression in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells and CD25+ T cells (CD3+CD56-CD25+) from healthy donors and breast cancer patients.

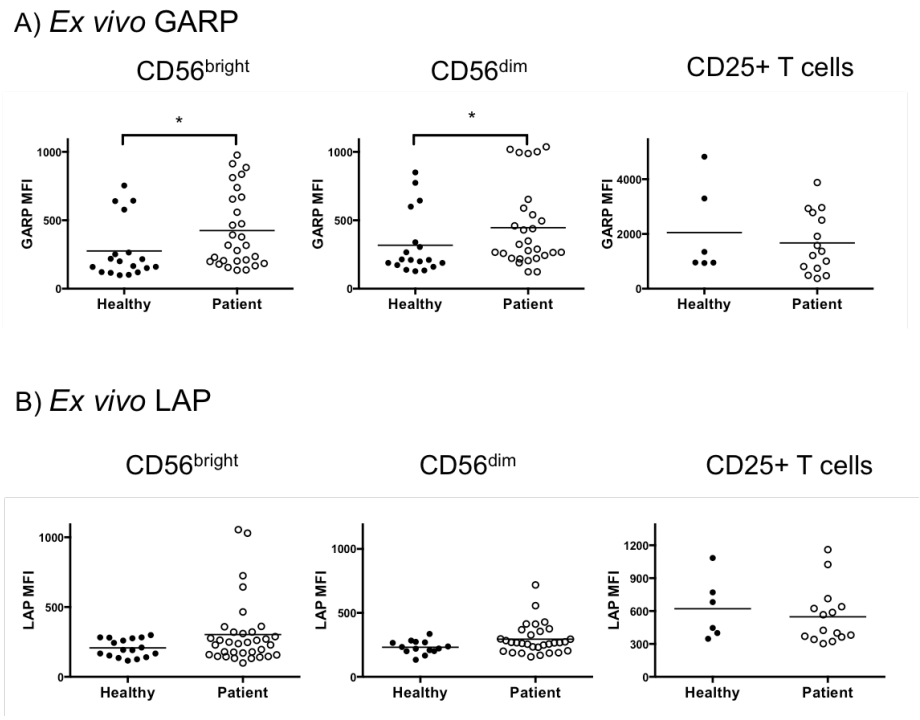


Figure 4.25 NK cells, but not CD25+ T cells, from breast cancer patients have increased expression of GARP.

Freshly isolated PBMC were stained *ex vivo* for LAP and GARP expression and analysed by flow cytometry. (A) Pooled data of GARP expression in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells and CD25+ T cells (CD3+CD56-CD25+) from healthy donors and breast cancer patients. (C) Pooled data of LAP expression in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells and CD25+ T cells (CD3+CD56-CD25+) from healthy donors and breast cancer patients. Lines show the mean value. Each dot represents 1 donor (n=6-34). Samples were compared using the unpaired student t-test, *$p<0.05$.

In the second part of this chapter, we investigated the potential role of the TGFβ anchoring protein GARP in mediating patient NK cell metabolic dysfunction. MHG-8, a monoclonal antibody that binds GARP-TGFβ complexes was gifted to us by the Sophie Lucas Lab (The de Duve Institute, Belgium). This antibody recognises Treg cells transfected with both GARP and TGFβ but not with either GARP alone or TGFB1 alone [207], meaning it will only detect GARP/TGFβ when they are bound together. It does not detect free GARP, which is important as GARP can sometimes be found in soluble form. Furthermore, it inhibits active TGFβ production by Treg cells, and by doing so mitigates some of their immunosuppressive functions. For all experiments, NK cells were purified (mean purity of 85% for healthy donors and 88% for patients), stimulated with IL2 or IL12/15, and cultured overnight in presence of either anti-GARP or IgG1 isotype control.

4.2.12 GARP/TGFβ blockade improves the activation of NK cells from breast cancer patients.

We have shown that NK cells from breast cancer patients have impaired activation upon cytokine stimulation, as measured by staining for CD25 and analysing by flow cytometry. Treating with a TGFβ neutralising antibody restored IL12/15 induced CD25 expression. In contrast, the treatment had a variable impact on IL2 induced CD25 expression (data not shown). Therefore, we examined the impact of blocking the GARP/ TGFβ axis on CD25 expression in patient NK cells. GARP blockade consistently improved CD25 expression in both IL2 and IL12/15 stimulated NK cells from breast cancer patients (**Figure 4.26A** for representative dot plot, **Figure 4.26B** and **4.26C**). This was particularly effective in the $CD56^{bright}$ NK cell subset. These data show that the GARP/TGFβ axis inhibits cytokine induced NK cell activation, and furthermore, that blocking GARP/TGFβ is preferable to blocking TGFβ alone when looking at IL2 induced NK cell activation.

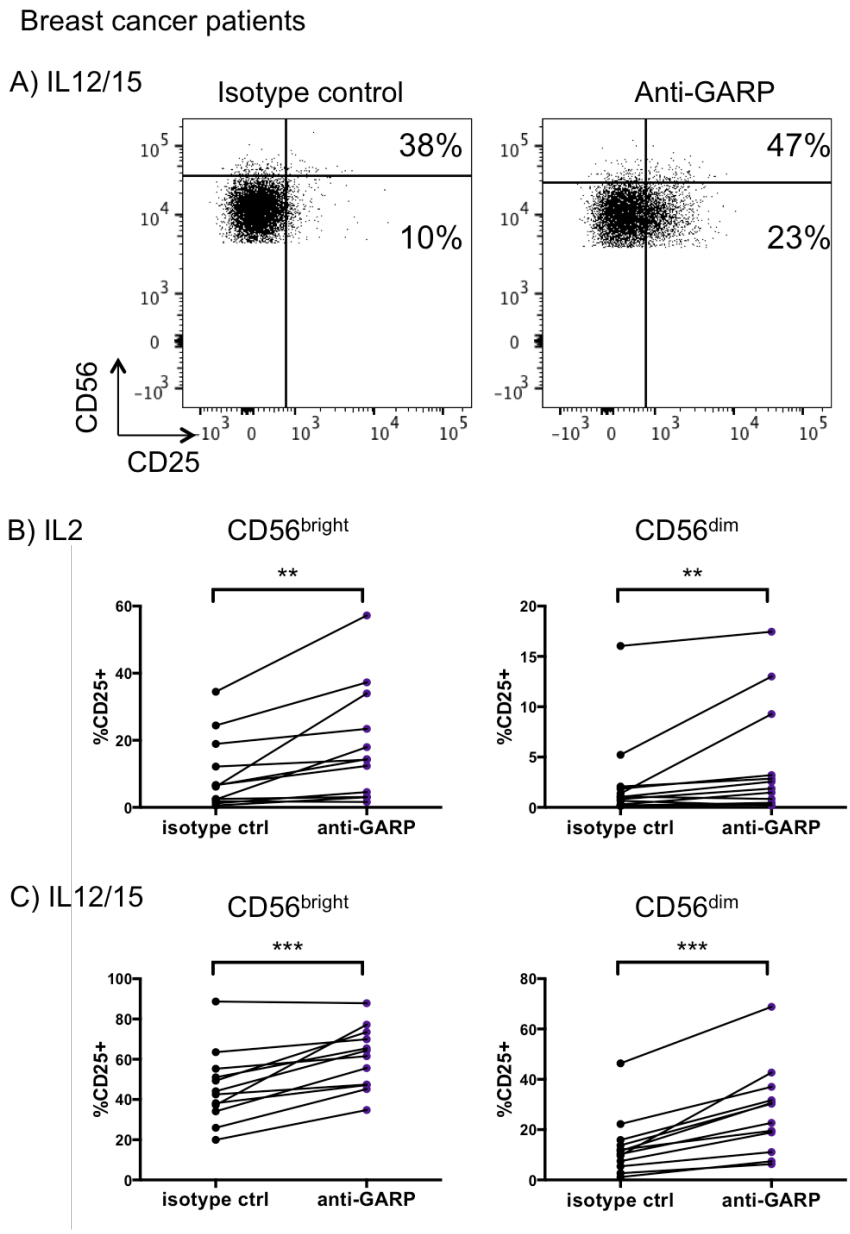


Figure 4.26 GARP blockade increases activation of cytokine stimulated NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml), and incubated for 18 h at 37°C. Cells were stained for CD25 and analysed by flow cytometry. (A) Representative dot plots of the impact of isotype control and anti-GARP on CD25 expression in IL12/15 stimulated NK cells. (B) Frequency of CD25 expression in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-GARP. (C) Frequency of CD25 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=12). Samples were compared using the paired student t-test, **p<0.01, ***p<0.001.

4.2.13 The GARP/TGFβ axis impairs nutrient receptor expression in NK cells from breast cancer patients.

NK cells from breast cancer patients had impaired nutrient receptor expression in response to cytokine. Targeting TGFβ using the neutralising antibody improved CD71 expression in patient NK cells stimulated with both IL2 and IL12/15, and preliminary evidence also suggests that it may also improve CD98. Therefore, we investigated the impact that GARP/TGFβ blockade has on NK cell nutrient receptor expression. The results of these experiments were very striking and consistent. Removing the influence of GARP/TGFβ on patient NK cells overnight increased CD71 and CD98 expression in response to both IL2 and IL12/15 (**Figure 4.27A** and **4.28A** for representative dot plots and histograms, and **Figure 4.27B 4.27C, 4.28B** and **4.28C**). For both receptors, this was evident in both the $CD56^{bright}$ and $CD56^{dim}$ subsets. So far the data suggest that the GARP/TGFβ axis may play a role in suppressing NK cell cytokine induced responses, and in particular hint towards a potential impact on cellular metabolism.

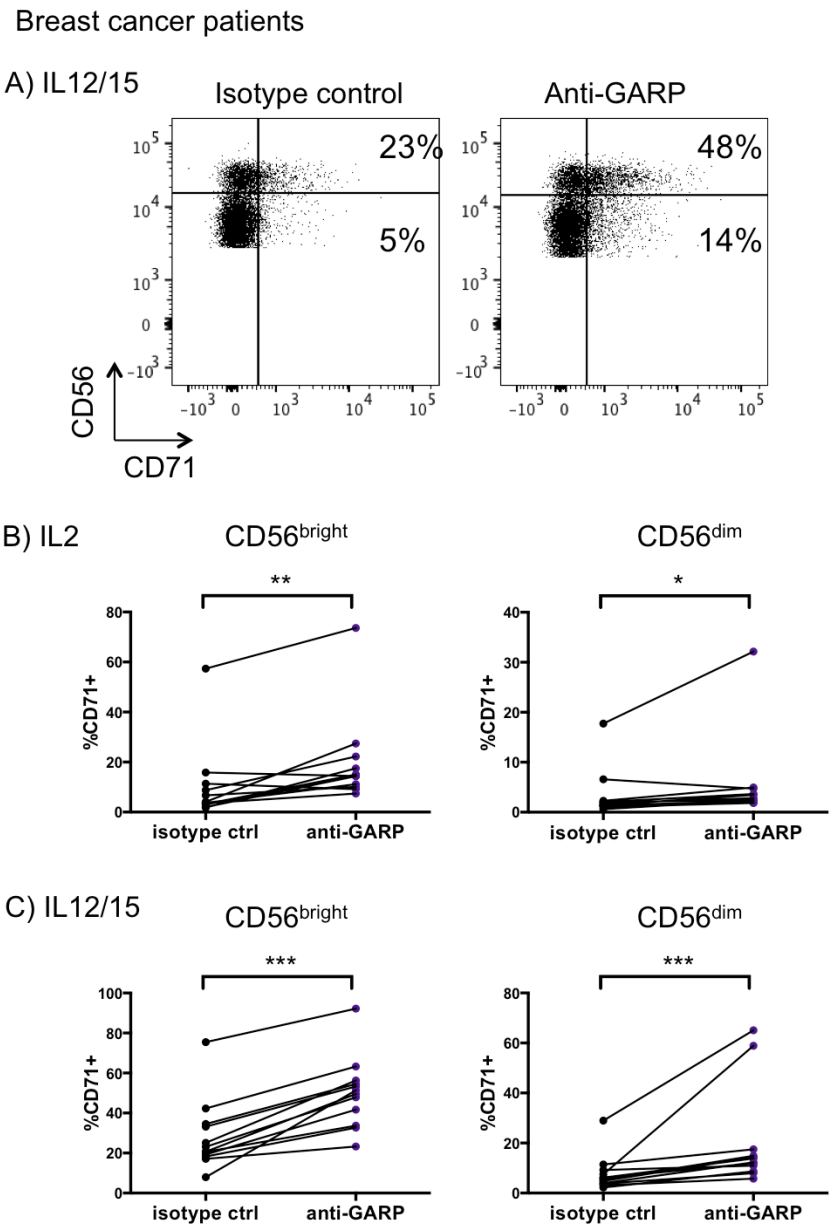


Figure 4.27 The GARP/TGFβ axis contributes to reduced CD71 in cytokine stimulated NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) and incubated for 18 h at 37°C. Cells were stained for CD71 and analysed by flow cytometry. (A) Representative dot plots of the impact of isotype control and anti-GARP on CD71 expression in IL12/15 stimulated NK cells. (B) Frequency of CD71 expression in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-GARP. (C) Frequency of CD71 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=12). Samples were compared using the paired student t-test, *p<0.05, **p<0.01, ***p<0.001.

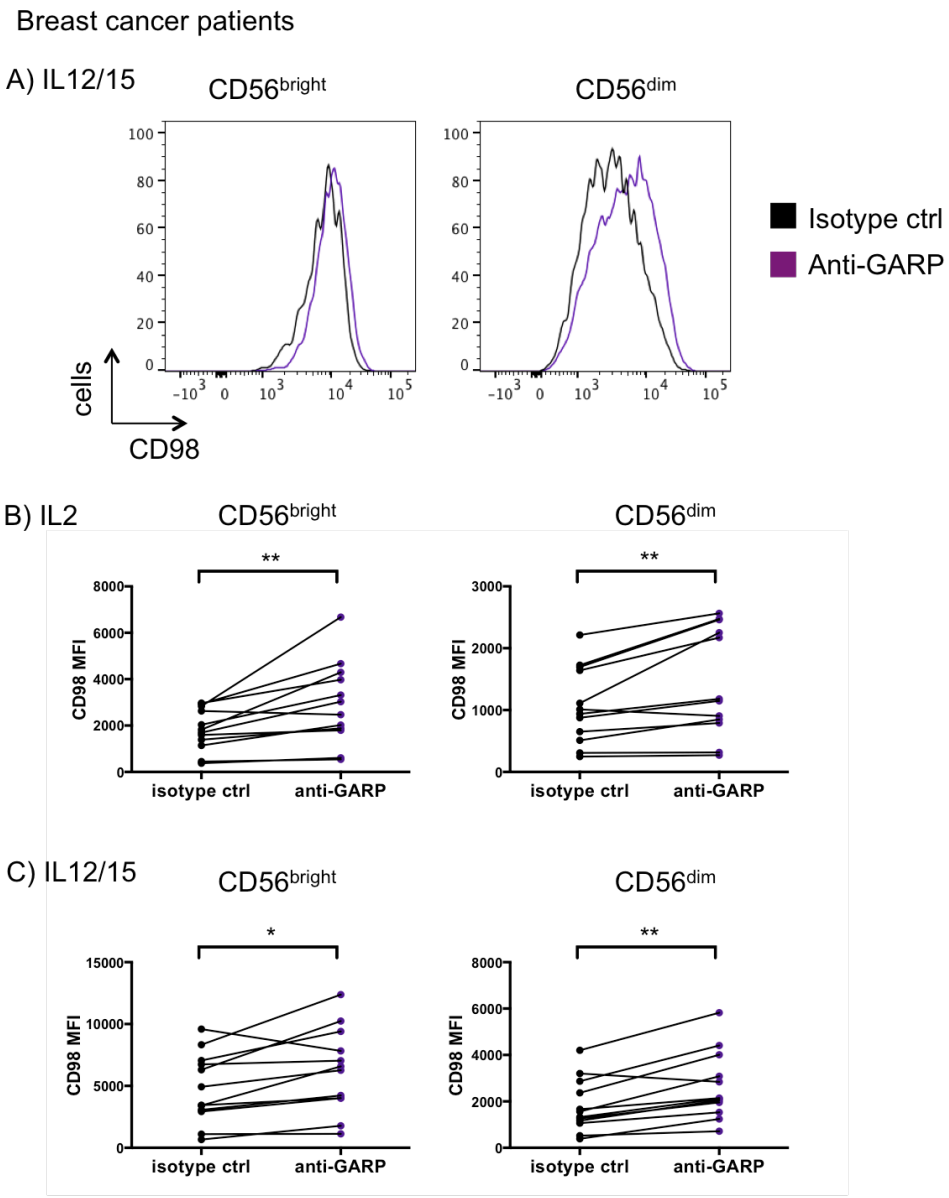


Figure 4.28 GARP/ TGFβ neutralisation improves cytokine induced CD98 expression in NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) and incubated for 18 h at 37°C. Cells were stained for CD98 and analysed by flow cytometry. (A) Representative histogram of the impact of isotype control and anti-GARP on CD98 in IL12/15 stimulated NK cells. (B) Fold increase in the MFI of CD98 relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients, in the presence of isotype control or anti-GARP. (C) Fold increase in the MFI of CD98 relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients, in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=12). Samples were compared using the paired student t-test, *p<0.05, **p<0.01.

4.2.14 Targeting the GARP/TGFβ axis in NK cells from breast cancer patients reprograms metabolism towards oxphos.

NK cells from breast cancer patients had impaired oxphos and glycolysis, however IL2 induced oxphos was restored when exogenous TGFβ is neutralised. We therefore hypothesised that blocking GARP/TGFβ would also improve levels of oxphos in NK cells from breast cancer patients. Again, the data were consistent and clear that targeting GARP/TGFβ improves IL2 induced NK cell oxphos (**Figure 4.29A** for representative seahorse trace). This in particular was evident for basal respiration, i.e. oxphos before the addition of any metabolic inhibitors, and for maximal respiration, indicating increased oxphos machinery within the cell (**Figure 4.29B**).

Similar to what was observed upon treatment with anti-TGFβ, basal glycolysis was reduced and glycolytic reserve was increased when GARP/TGFβ was blocked (**Figure 4.30A** for representative seahorse trace, and **Figure 4.30B**). As glycolytic reserve is calculated by subtracting basal glycolysis from glycolytic capacity, this increase may be artificial. Nonetheless, the data are clear that targeting the GARP/TGFβ axis results in a decrease in basal glycolysis and an increase in basal oxphos, indicating that metabolic reprogramming favouring oxphos has taken place. Indeed, this finding is confirmed by the OCR/ECAR ratio, as patient NK cells treated with anti-GARP have a dramatically higher OCR/ECAR than those treated with isotype control (**Figure 4.31A**).

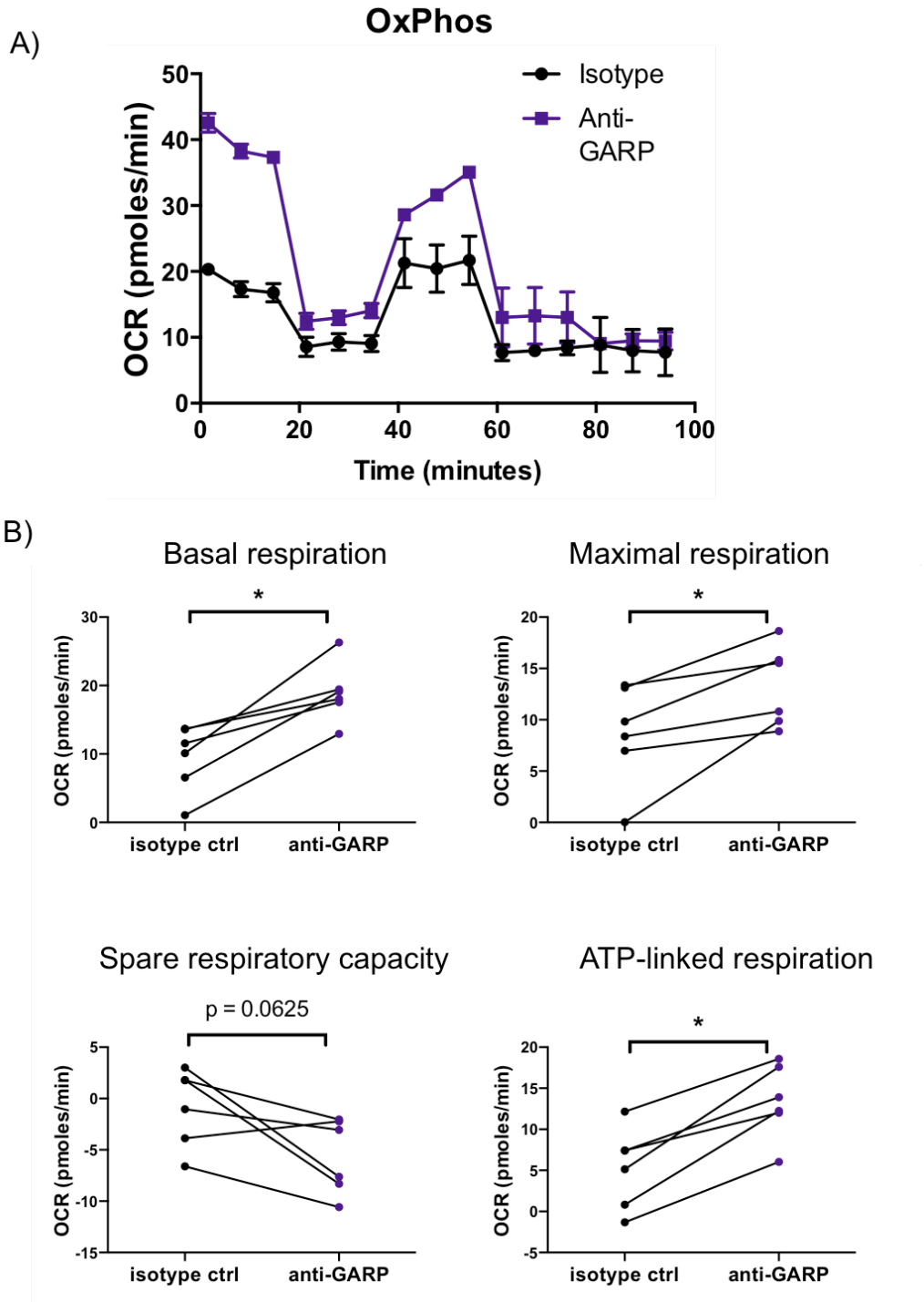


Figure 4.29 GARP is a driver of impaired oxphos in NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated with IL2 (500IU/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative oxygen consumption rate (OCR) trace of IL2 stimulated NK cells from breast cancer patients in the presence of isotype control or anti-GARP. (B) Pooled data of the IL2 stimulated oxphos readouts in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *$p<0.05$.

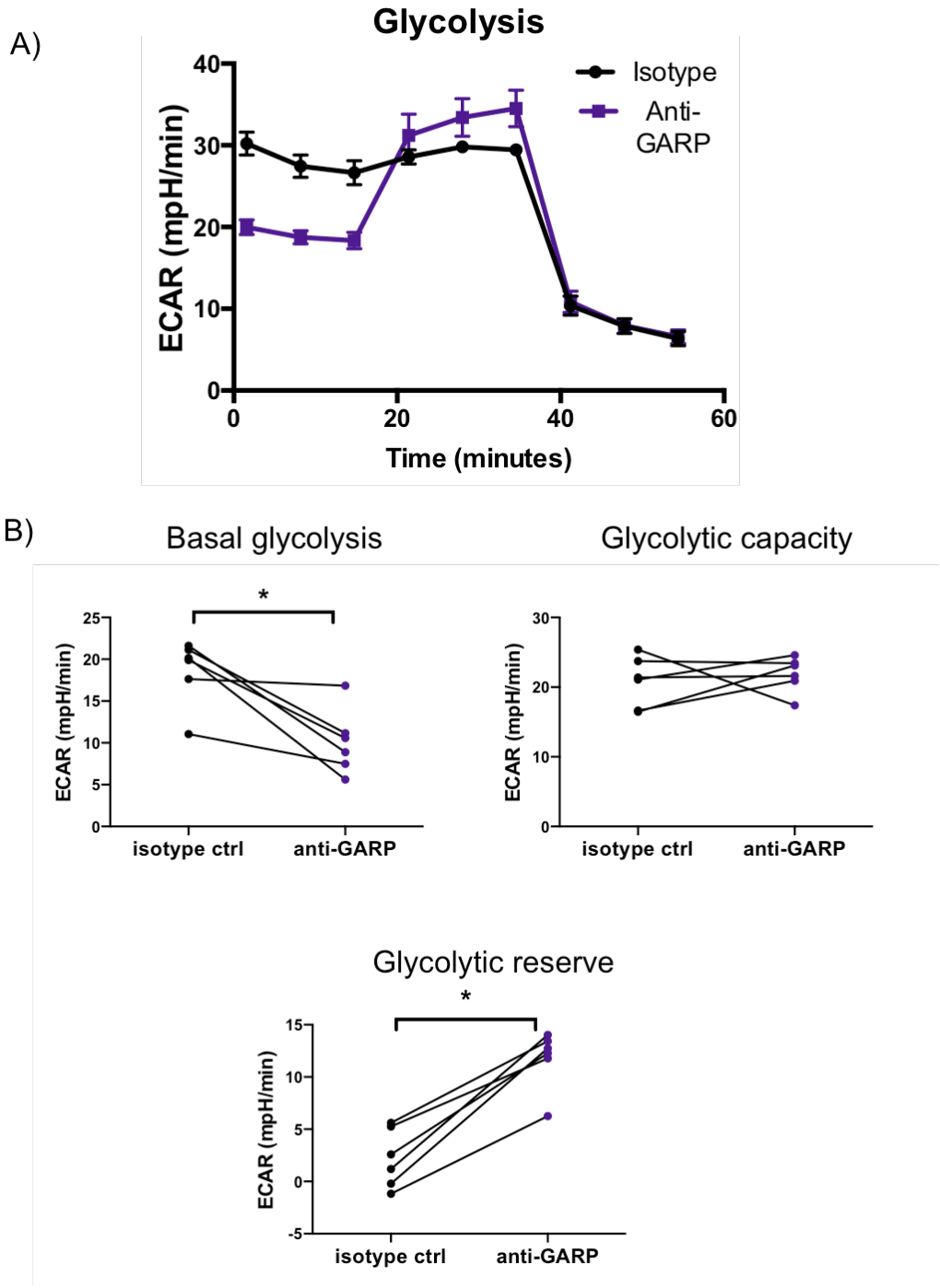


Figure 4.30 GARP blockade has variable impacts on glycolysis NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated with IL2 (500IU/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative extracellular acidification rate (ECAR) trace of IL2 stimulated NK cells from breast cancer patients in the presence of isotype control or anti-GARP. (B) Pooled data of the IL2 stimulated glycolytic readouts in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *p<0.05.

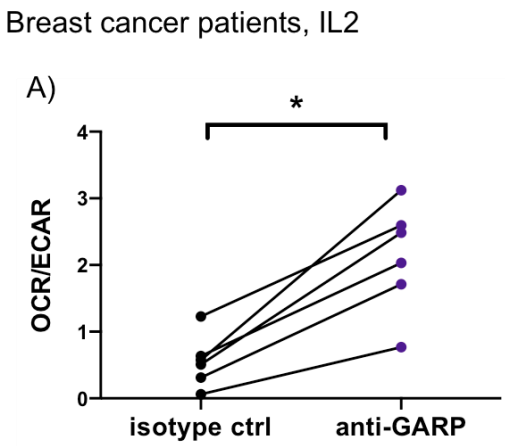


Figure 4.31 Removing the influence of GARP/ TGFβ reprograms patient NK cell metabolism towards oxphos.

NK cells were purified from freshly isolated PBMC from breast cancer patients and stimulated with IL2 (500IU/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) The OCR/ECAR ratio, where an increased number indicates a greater reliance on oxphos. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *$p<0.05$.

4.2.15 GARP/TGFβ drives NK cell dysfunction during breast cancer.

Thus far the data strongly indicate that GARP/TGFβ acts in an immunosuppressive manner towards NK cells from breast cancer patients, in part by inhibiting mitochondrial metabolism. We have shown that TGFβ contributes towards impaired IFNγ production in patient NK cells, and therefore we next investigated whether GARP might also be playing a role. Surprisingly, the data were not as clear and consistent as previous data (**Figure 4.32A** for representative dot plots). In terms of the frequency of IFNγ positive NK cells – anti-GARP only improved $CD56^{bright}$ IFNγ production when controls levels were ~40% or higher (**Figure 4.32B**), suggesting that $CD56^{bright}$ NK cells that are severely dysfunctional may not benefit from GARP blockade. However, the treatment significantly improved IFNγ production in $CD56^{dim}$ NK cells. In terms of the IFNγ MFI, anti-GARP only improved readouts in the $CD56^{bright}$ subset, while the impact of anti-GARP on $CD56^{dim}$ NK cell IFNγ MFI was variable (**Figure 4.32C**).

We next hypothesised that the reason for the variable response seen for (1) frequency of IFNγ production in $CD56^{bright}$ patient NK cells and (2) the IFNγ MFI of

$CD56^{dim}$ NK cells may be due to heterogeneity in GARP expression. Therefore, we next calculated the fold change induced by anti-GARP (relative to isotype control), and examined any correlations with GARP expression. Indeed, GARP expression correlated with the fold change induced by anti-GARP in both of these cases, in that NK cells with higher GARP expression had a higher fold change induced by anti-GARP treatment (**Figure 4.33A** and **4.33B**). In cases where anti-GARP treatment consistently improved IFNγ production, fold changes did not correlate with GARP expression.

As we are the first to explore the role that GARP might play in regulating NK cell responses, we also investigated the impact of blocking GARP/TGFβ on granzyme B expression. In chapter 3, we seen that cytokine induced granzyme B responses were normal in NK cells from breast cancer patients, and we propose that this may be because granzyme B is in general constitutively expressed and stored within the lytic granules, and therefore does not rely on cytokine induced upregulation of metabolism. Surprisingly, anti-GARP treatment increased granzyme B expression in both IL2 and IL12/15 stimulated patient NK cells (**Figure 4.34** for representative histograms, **Figure 4.34B** and **4.34C**). This data suggests that the GARP/TGFβ axis may play a role in restricting normal NK cell responses, and thereby may act as a break on the NK cell cytokine induced responses. This possibility warrants much further exploration in the future.

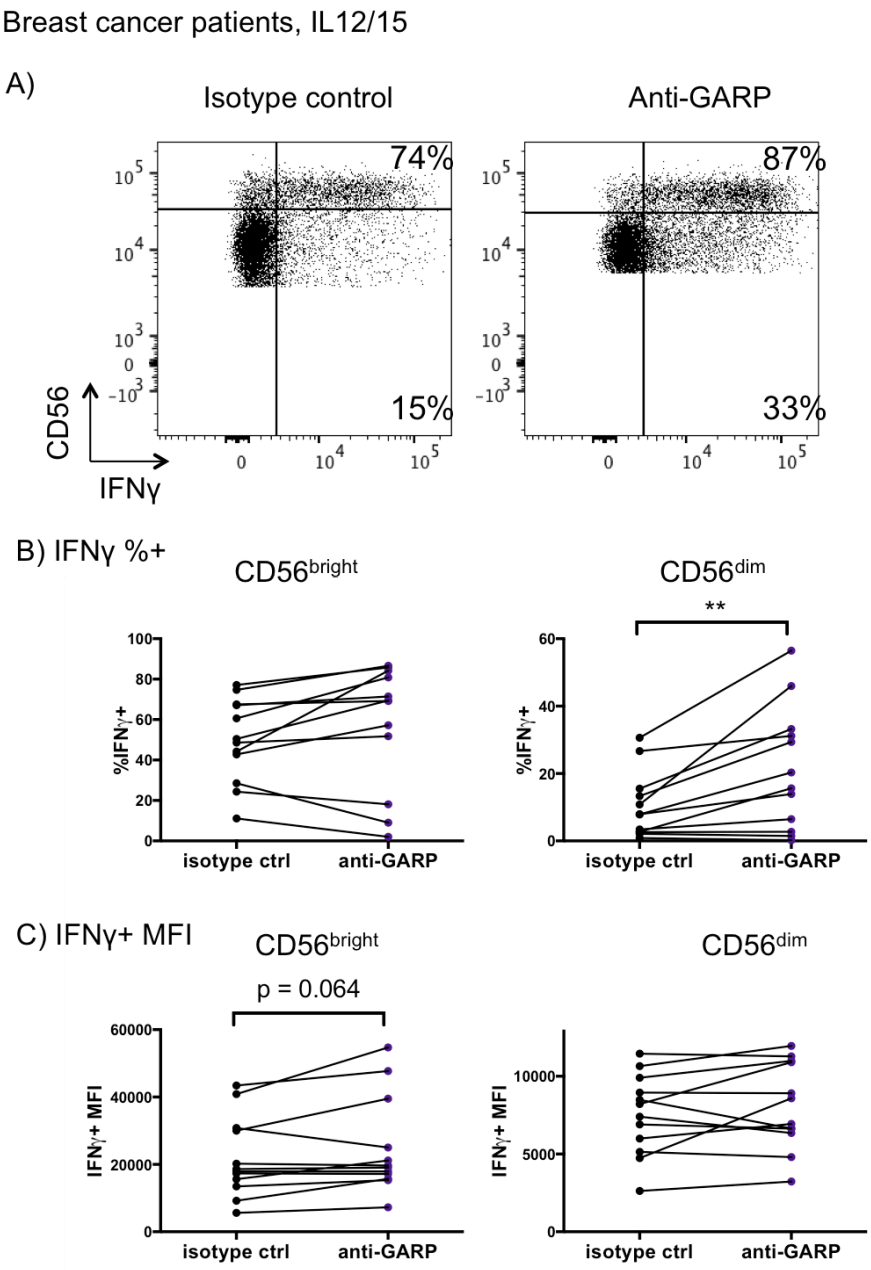


Figure 4.32 The GARP/ TGFβ axis contributes to reduced IFNγ production NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) for 18 h at 37°C. Cells were stained for IFNγ and analysed by flow cytometry. (A) Representative dot plot of the anti-GARP mediated upregulation of IFNγ production in IL12/15 stimulated NK cells. (B) Frequency of IFNγ production in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-GARO. (C) MFI of IFNγ in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=12). Samples were compared using the paired student t-test, **p<0.01.

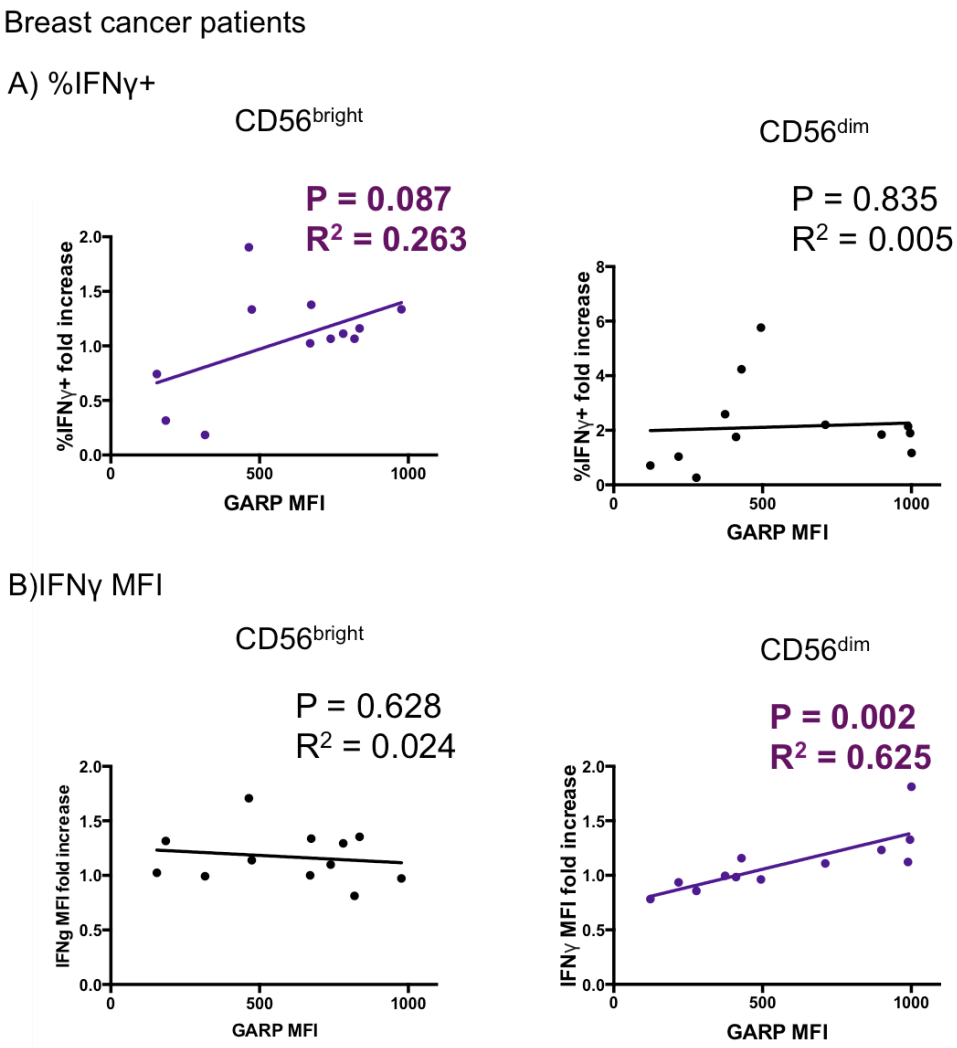


Figure 4.33 NK cell GARP expression correlates with the change in IFNγ production induced by GARP blockade.

Purified NK cells from breast cancer patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) for 18 h at 37°C. Cells were stained for IFNγ and analysed by flow cytometry. (A) Correlation between *ex vivo* GARP expression and the fold change in the frequency IFNγ production induced by anti-GARP treatment, relative to isotype control. (B) Correlation between *ex vivo* GARP expression and the fold change in IFNγ MFI induced by anti-GARP treatment, relative to isotype control. Each dot represents 1 donor (n=12). Samples were compared using linear regression analysis.

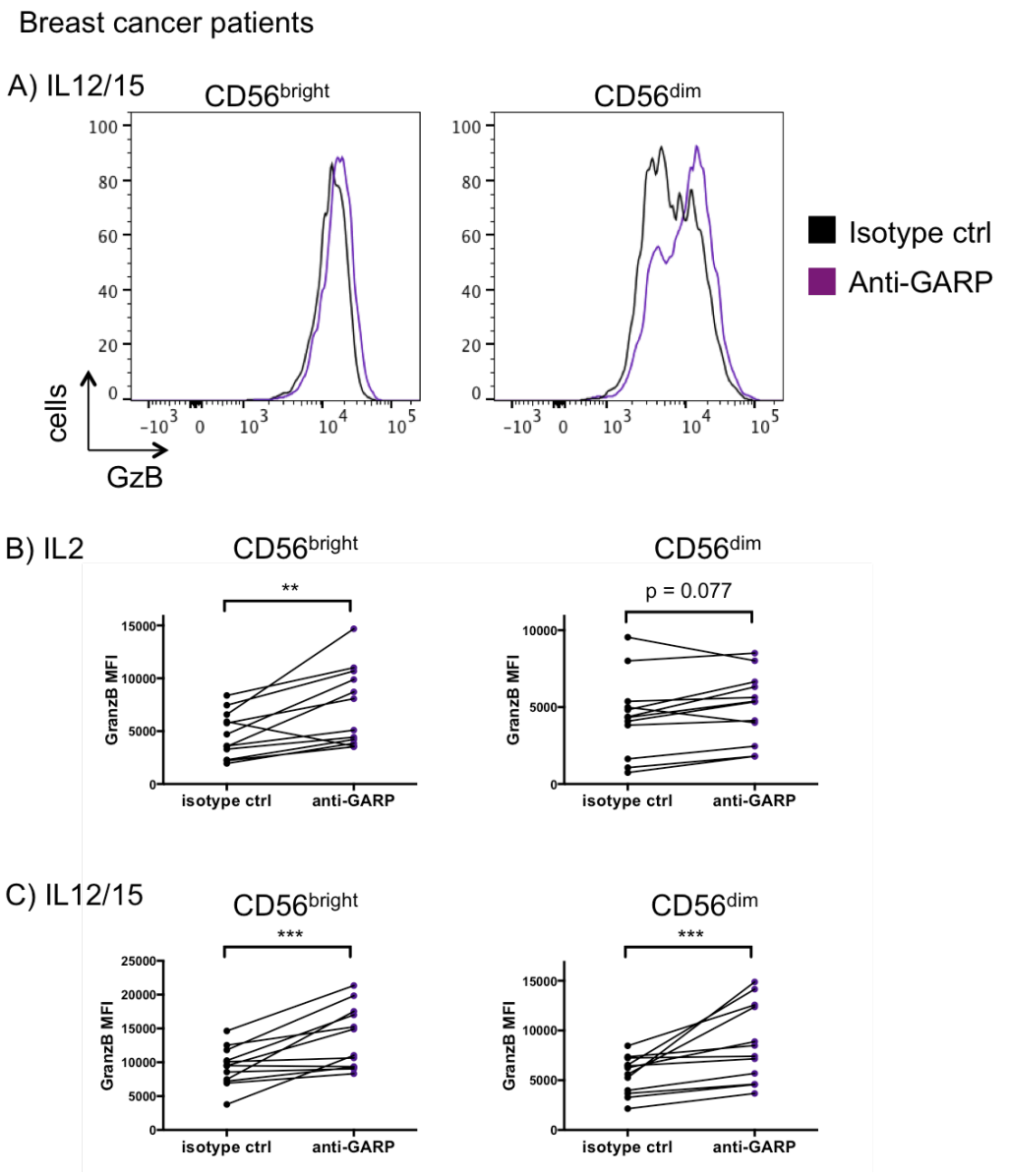


Figure 4.34 The GARP/TGFβ axis represses granzyme B expression in cytokine stimulated NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) and incubated for 18 h at 37°C. Cells were stained for granzyme B and analysed by flow cytometry. (A) Representative histogram of the impact of isotype control and anti-GARP on CD98 in IL12/15 stimulated NK cells. (B) Fold increase in the MFI of granzyme B relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients, in the presence of isotype control or anti-GARP. (C) Fold increase in the MFI of granzyme B relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from breast cancer patients, in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=12). Samples were compared using the paired student t-test, **p<0.01, ***p<0.001.

4.2.16 The GARP/TGFβ axis represses mTORC1 activity in CD56^{dim} NK cells from breast cancer patients.

We have shown in this chapter that TGFβ contributes to impaired mTORC1 activity as addition of anti-TGFβ increased mTORC1 activity in IL2 and IL12/15 stimulated NK cells from breast cancer patients. As anti-GARP treatment has recapitulated many of the beneficial effects of anti-TGFβ, we next investigated its impact on mTORC1 activity, as measured by pS6 staining and flow cytometry. Unexpectedly, targeting GARP/TGFβ did not improve mTORC1 activity in CD56^{bright} NK cells. This indicates that the improved readouts of metabolism and function observed previously in this subset of NK cells may not be related to improved mTORC1 activity. In contrast, targeting GARP/TGFβ significantly improved mTORC1 activity in both IL2 and IL12/15 stimulated CD56^{dim} NK cells (**Figure 4.35A and 4.36A** for representative dot plots, **Figure 4.35B**, **4.35C**, **4.36B** and **4.36C**). These findings suggest that GARP/TGFβ downstream signalling in CD56^{bright} and CD56^{dim} NK cells may be different in patient NK cells. Indeed, studies on the relationship between TGFβ and mTORC1 activity are often conflicting, and the link between the two signalling mediators is complex. Nonetheless, it seems that the GARP/TGFβ axis plays a role in suppressing mTORC1 activity in CD56^{dim} NK cells from breast cancer patients. This may be a key driver of some of the functional and metabolic defects observed in this NK cell subset.

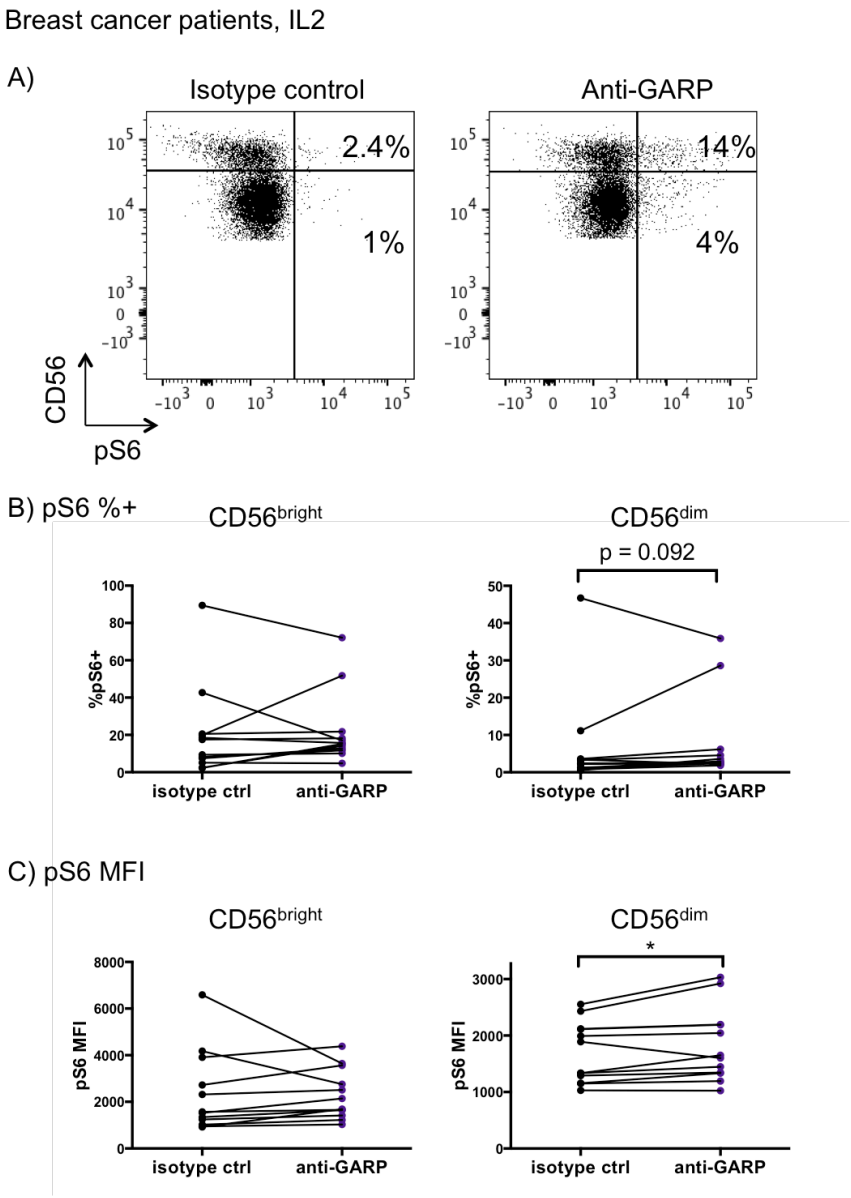


Figure 4.35 GARP is involved in the repression of CD56^{dim} mTORC1 activity in IL2 stimulated NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated IL2 (500IU/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the anti-GARP mediated upregulation of mTORC1 activity in IL2 stimulated NK cells. (B) Frequency of NK cells positive for phosphorylated S6 protein in IL2 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-GARP. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in in IL2 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=12). Samples were compared using the paired student t-test, *$p<0.05$.

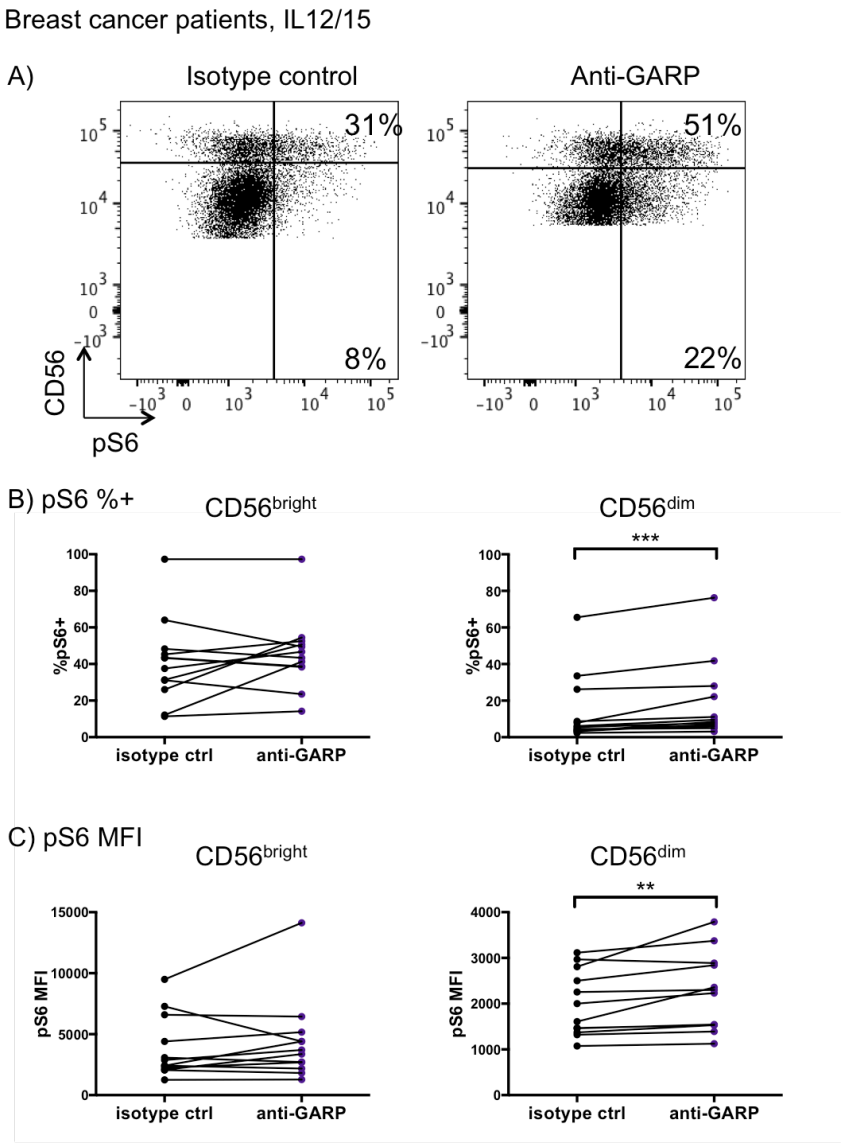


Figure 4.36 GARP is involved in the repression of CD56^{dim} mTORC1 activity in IL12/15 stimulated NK cells from breast cancer patients.

Purified NK cells from breast cancer patients were stimulated IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the anti-GARP mediated upregulation of mTORC1 activity in IL12/15 stimulated NK cells. (B) Frequency of NK cells positive for phosphorylated S6 protein in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-GARP. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from breast cancer patients in the presence of isotype control or anti-GARP. Each line represents 1 donor (n=12). Samples were compared using the paired student t-test, **p<0.01, ***p<0.001.

4.2.17 Targeting the GARP/TGFβ axis improves some cytokine induced responses in NK cells from healthy donors.

Our reasoning for exploring GARP as a potential therapeutic target was based on the fact that NK cells from breast cancer patients had increased GARP expression. To date, there have been no published articles reporting on GARP expression in NK cells, and therefore its role in regulating in NK cell responses remains unexplored. Anti-GARP treatment unexpectedly increased some cytokine induced responses that were not dysfunctional in patient NK cells, such as granzyme B and CD69 (data not shown) expression. This hints at a potential more general role for GARP in modulating NK cells responses, outside the realm of cancer induced dysfunction. Therefore, we next investigated the impact of anti-GARP treatment in NK cells from healthy donors in order to see whether this therapeutic effect is unique to NK cells from breast cancer or whether it is a more general mechanism. Indeed, blocking the GARP/TGFβ axis in healthy donor NK cells improved some cytokine induced responses. As an example, IL12/15 induced responses are shown. Similar to patient NK cells, activation as measured by CD25 expression was increased, and nutrient receptor expression was boosted (**Figure 4.37A**, **4.37B** and **4.37C**). For CD71, this was only evident in the $CD56^{bright}$ subset. Interestingly, the treatment had variable impact on the functional responses IFNγ and granzyme B, and it increased mTORC1 activity in the $CD56^{bright}$ subset only **Figure 4.38A**, **4.38B** and **4.38C**. Altogether, these data suggest that the GARP/TGFβ axis may indeed play a role in regulating normal, healthy NK cell responses, particularly in the $CD56^{bright}$ subset. This opens up a whole new aspect of NK cell regulatory biology that warrants exploration, and indeed this will likely reveal novel insights into the TGFβ mediated modulation of NK cell responses in health and disease.

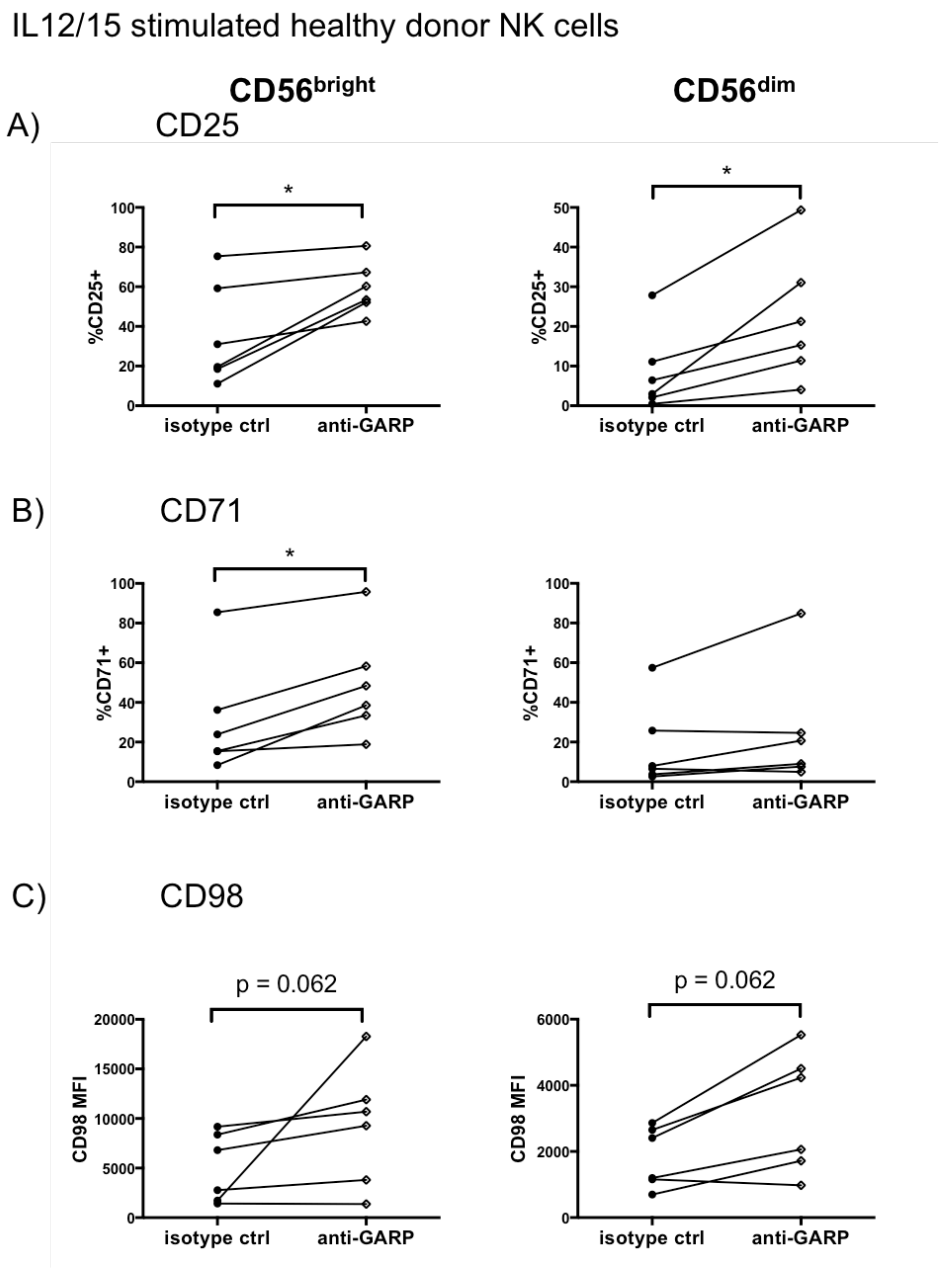


Figure 4.37 The GARP/TGFβ axis regulates cytokine induced NK cell responses in some healthy individuals

Purified NK cells from healthy donors were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) and incubated for 18 h at 37°C. Cells were stained for (A) CD25, (B) CD71 and (C) CD98, and analysed by flow cytometry. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *$p<0.05$.

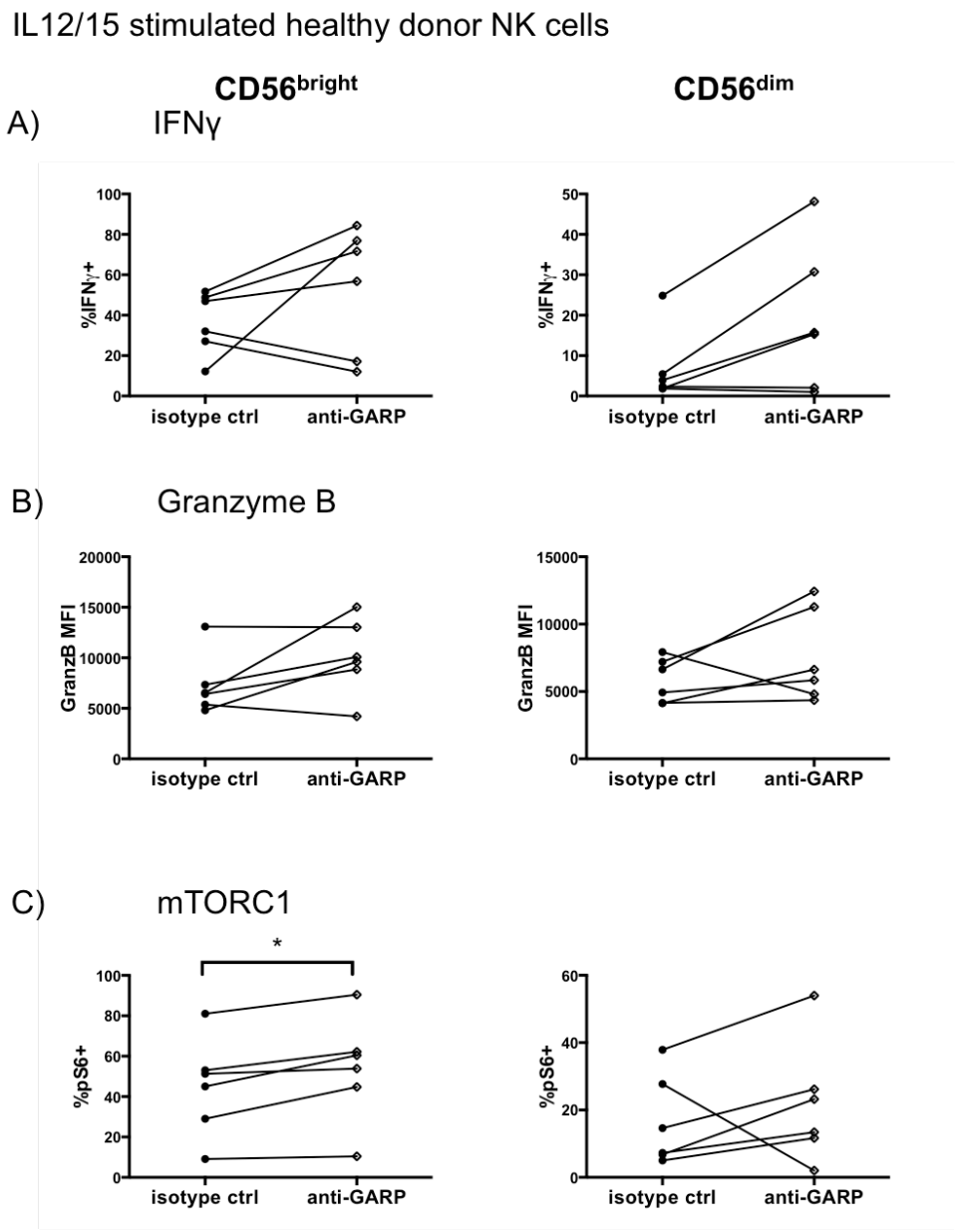


Figure 4.38 The GARP/TGFβ axis regulates IL12/15 induced CD56^{bright} NK cell mTORC1 activity.

Purified NK cells from healthy donors were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) in the presence of either anti-GARP antibody (10µg/ml) or isotype control (10µg/ml) and incubated for 18 h at 37°C. Cells were stained for (A) IFNγ, (B) granzyme B and (C) pS6, and analysed by flow cytometry. Each line represents 1 donor (n=6). Samples were compared using the paired student t-test, *$p<0.05$.

4.2.18 Monocytes from healthy donors and breast cancer patients express high levels of GARP.

These data identify GARP as a key driver of NK cell dysfunction during cancer for the first time. Increased GARP expression in NK cells from breast cancer, and the improved functional and metabolic responses induced by GARP/TGFβ blockade support this. For patient experiments, the mean NK cell purity was 87%. Therefore, we examined GARP expression within the non-NK cell populations, in order to gain insight into whether they could potentially be contributing towards the beneficial effects observed. Monocytes were identified based on forward scatter and side scatter, and therefore these results will need to be confirmed using a monocyte phenotypic marker. Nonetheless, the total percentage of monocytes plus lymphocytes was calculated, and the contribution from each population was determined. Monocytes comprised a mean of 2.8% and 1.5% of this compartment in healthy donors and patients respectively (**Figure 4.39A**). Within the lymphocyte compartment only, the majority of contaminating cells were CD3-CD56- (likely enriched in B cells), with a mean of 7.6% and 6.1% from healthy donors and patients respectively (**Figure 4.39B**). Of the CD3+CD56- lymphocytes (T cells) <1% were CD25+ for both cohorts, indicating that Treg cells are highly unlikely to be contributing to the therapeutic effect of anti-GARP treatment. When GARP expression was compared between these immune populations, monocytes clearly expressed the highest level of GARP (**Figure 4.39C**). This finding was consolidated by RNAseq data from Immune Cell Atlas, which also shows GARP expression in human blood monocytes (**Figure 4.40**). For all populations examined, patients had higher GARP expression than healthy donors, supporting the idea that breast cancer induces increased GARP expression in immune cells. These percentage and GARP data can be viewed in **Table 4.2** and **Table 4.3**.

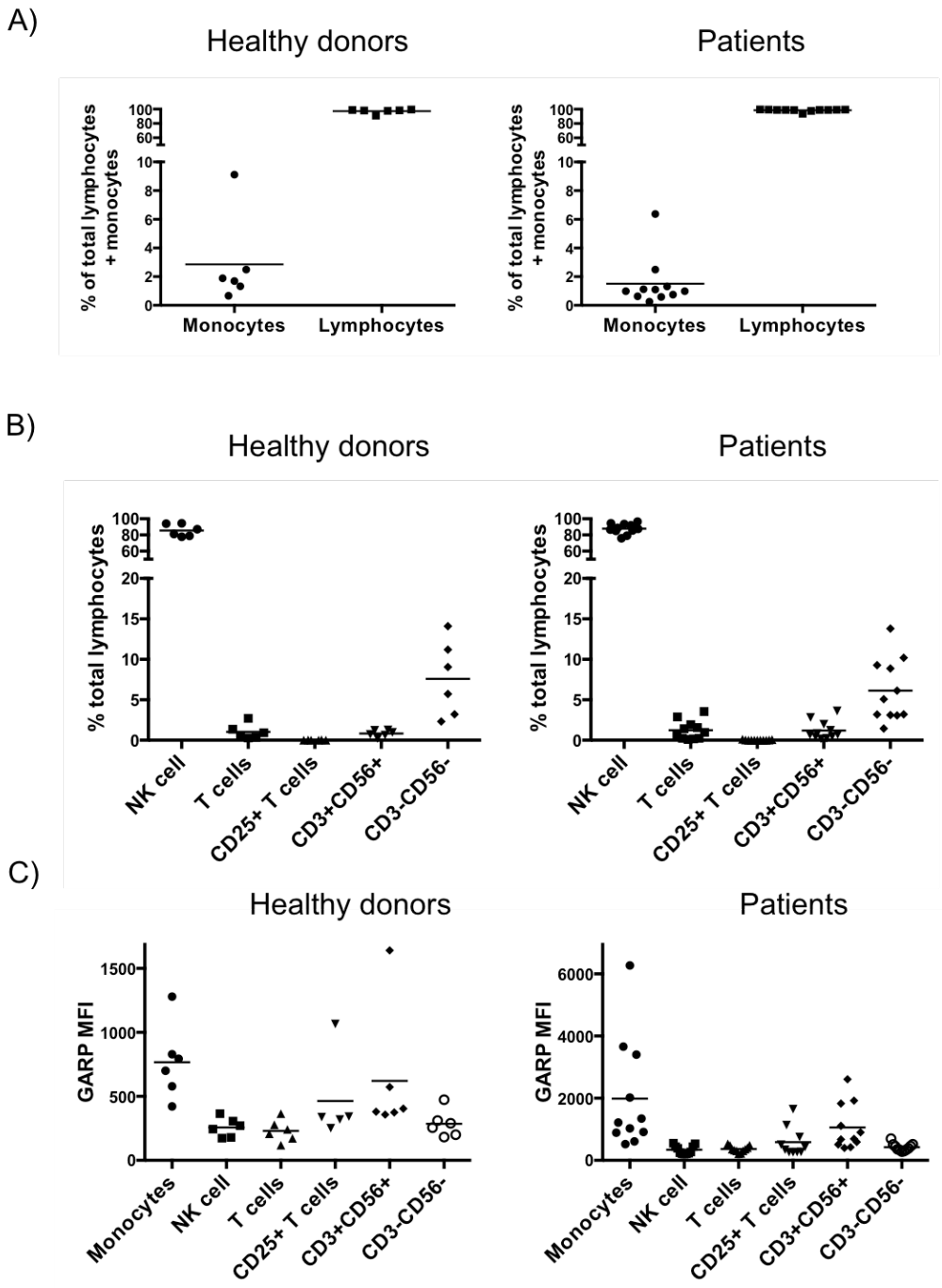


Figure 4.39 Monocytes from healthy donors and breast cancer patients express high levels of GARP.

NK cells were purified by magnetic bead separation (Stem Cell kit). Monocytes were identified based on forward scatter and side scatter and T cells were identified as CD3+CD56- lymphocytes. Cells were stained for surface GARP expression and analysed by flow cytometry. (A) The percentage of monocytes within the lymphocyte plus monocyte compartment after NK cell purification. (B) The percentage of other immune cell types within the lymphocyte compartment after NK cell purification. (C) GARP expression within

the different immune cell population. Lines show the mean value. Each dot represents 1 donor (n=6-11).

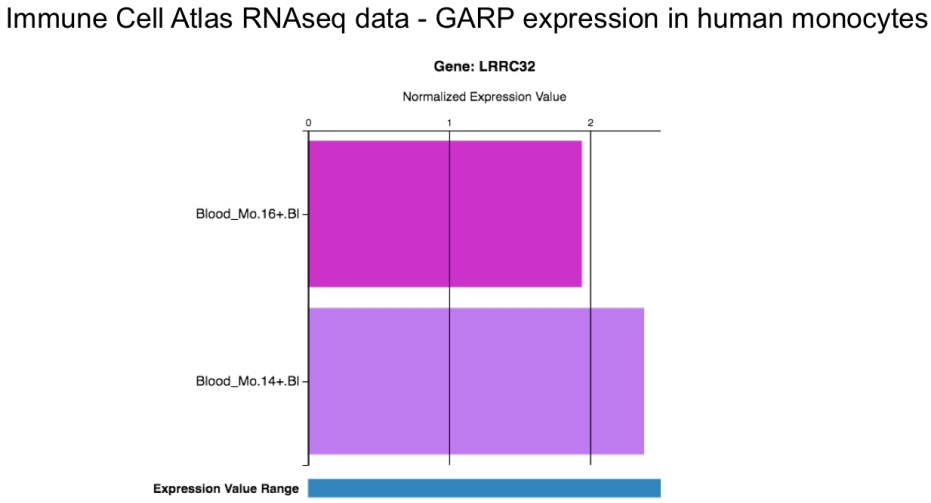


Figure 4.40 Human monocytes express GARP.

RNA sequencing data from the Immune Cell Atlas shows GARP expression in human monocytes from the peripheral blood. Normalised by median of ratios method with DESeq2 package from Bioconductor. In brief, the ratio of GARP expression level between samples is calculated, and then median ratio across all expressed genes is used as the normalisation scale.

		NK cells	T cells	CD25+ T cells	CD3+ CD56+	CD3- CD56-
Healthy	**%**	85.5	1.0	0.022	0.83	7.5
Patient	**Lymphocytes**	87.7	1.2	0.027	1.19	6.1
Healthy	**Mean GARP**	256	230	463	622	285
Patient	**MFI**	339	364	587	1060	422

Table 4.2 Lymphocyte percentages and corresponding mean GARP expression.

		Monocytes
Healthy	**% Lymphocytes**	2.80
Patient	**+ Monocytes**	1.50
Healthy	**Mean GARP**	767
Patient	**MFI**	1989

Table 4.3 Monocyte percentages and corresponding mean GARP expression.

Indeed, monocytes and some immune populations express quite high levels of GARP. However, as their frequency is so low compared to that of NK cells, it seems unlikely that these other immune cell meaningfully contribute towards the beneficial effect of anti-GARP treatment observed in this study. Nonetheless, it will be important to examine the impact of anti-GARP on NK cells with near 100% purity (e.g. FACS sorted NK cells), in order to rule out the possibility confidently.

Taken together these data identify the GARP/TGFβ axis as a key driver of NK cell dysfunction during breast cancer for the first time. Importantly, we show that defective mitochondrial metabolism can be rescued by targeting TGFβ and/or GARP in culture for just 18 hours. These findings have significant implications for the design of future NK cell based immunotherapies for the treatment of patients with solid tumours.

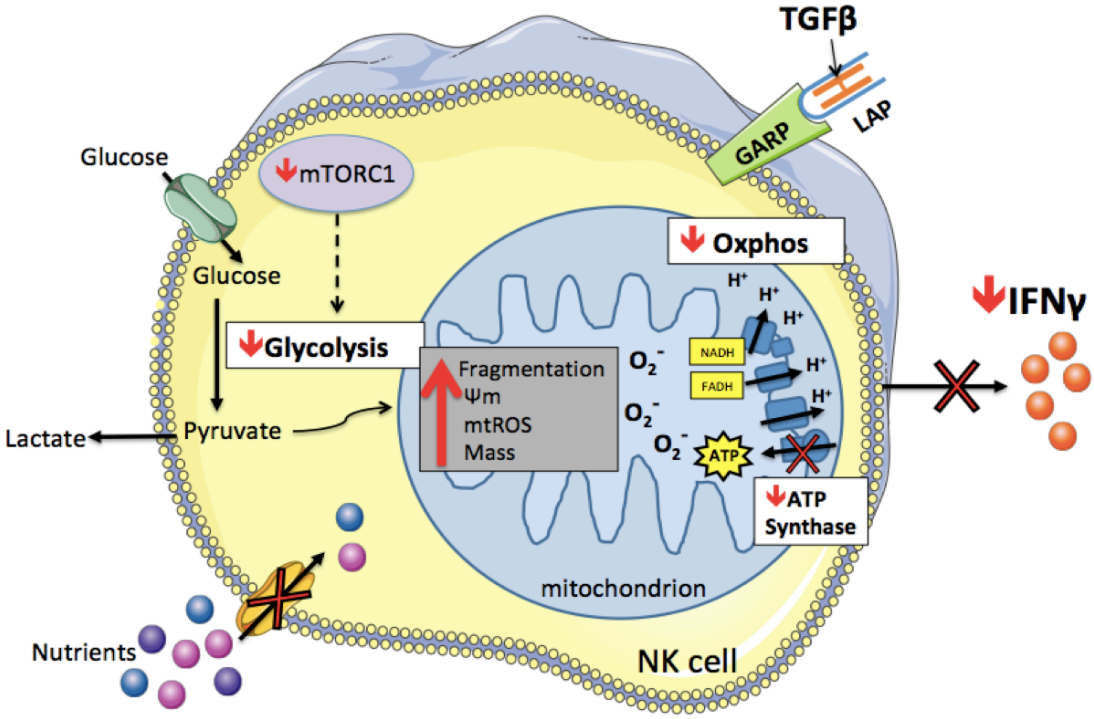


Figure 4.41 The GARP/ TGFβ axis drives NK cells metabolic dysfunction during metastatic breast cancer.

4.3 Discussion

It has been long evident that TGFβ plays a significant role during cancer. When the tumour-suppressive nature of TGFβ becomes overturned, its anti-proliferative properties are lost, and instead it becomes a driver of cell proliferation while remaining a promoter of cell migration and invasion, and angiogenesis, all of which support tumour growth. At the same time, TGFβ significantly impacts the immune system. It promotes differentiation of pro-tumourigenic immune cells such as Treg cells and MDSC, while inhibiting anti-cancer immune such as CD8+ T cells and NK cells. Indeed, TGFβ has been shown to have a profound impact on normal NK cells responses. *In vitro* treatment with TGFβ has reduces expression of activating receptors and TRAIL in healthy donor NK cells, as well as cytotoxicity and IFNγ production [80, 81, 173]. Therefore, increased TGFβ activity during cancer has the potential to significantly impact NK cell anti-tumour immune responses, and this is well documented in the literature. For example, Rouce *et al*. studied NK cells from patients with B-acute lymphoblastic leukaemia (ALL) [208]. They showed that NK cells from these patients had reduced anti-tumour functions including IFNγ production, and this was associated with constitutive activation of the TGFβ SMAD signalling pathway, indicating exposure to TGFβ. Lee *et al*. showed that adding a TGFβ neutralising antibody to the plasma of human lung cancer or colorectal cancer patients reduced its ability to impair NKG2D expression in NK cells from healthy donors [209]. Similarly, Otegbeye *et al.* showed that blocking the TGFβ receptor kinase prevents NK cell dysfunction induced by myeloid leukaemia and colon cancer tumour cell lines. They also showed in a mouse model of liver metastases that adoptive transfer of pure NK cells treated with the TGFβ receptor kinase inhibitor had reduced tumour burden compared with mice who received untreated NK cells [105]. While these studies clearly demonstrate a central role for TGFβ in repressing NK cell activation and function during cancer, the molecular mechanisms by which it does so was not considered nor explored.

In this chapter, we show that NK cells from breast cancer patients have active intracellular TGFβ signalling, implicating TGFβ as a potential driver of the NK cell metabolic dysfunction observed in chapter 3. We describe GARP expression in NK cells for the first time, and we also demonstrate that TGFβ itself as well the GARP/ TGFβ axis can be targeted in order to increase the functions of NK cells from cancer patients. Importantly, we show for the first time that this increase in function is

associated with improved mitochondrial metabolism and mTORC1 activity, and we propose that this may be a potential explanation for the improved functional response observed. These findings highlight the plasticity and adaptability of human NK cell metabolism – even though NK cells from breast cancer patients consistently had reduced oxphos and mTORC1 activity, removing the influence of endogenous TGFβ or blocking the GARP/TGFβ axis for just 18 hours was sufficient to improve these defects.

In this study it was clear that TGFβ and GARP blockade had more of an impact on mitochondrial metabolism than glycolytic metabolism, even though both pathways are altered in the NK cells from breast cancer patients. The anti-TGFβ and anti-GARP treatments both restored all aspects of oxphos that were shown to be impaired in the chapter 3 – basal respiration, maximal respiration and ATP-linked respiration. In contrast, they had quite variable impacts on glycolysis. We have previously shown that *in vitro* treatment of NK cells from healthy donors with TGFβ overnight reduces oxphos and maximal respiration, but does not impact basal glycolysis [173]. Hence, it seems that TGFβ preferentially inhibits mitochondrial metabolism, rather than glycolytic metabolism in human NK cells. Interestingly, a possible mechanism for this was recently described in human CD4+ T cells from healthy individuals. In this study, Dimeloe *et al.* demonstrate that SMAD proteins localise to the mitochondria of CD4+ T cells and become phosphorylated upon exposure to TGFβ, and that this results in reduced oxphos and IFNγ production [210]. Indeed, it will be interesting to see whether NK cells from breast cancer patients have increased SMAD protein within their mitochondria and/or increased mitochondrial SMAD phosphorylation.

To the best of our knowledge, there have been no direct links made between TGFβ and the mitochondria of NK cells. As immunometabolism is still a relatively young field of research, studies on NK cell mitochondria are limited. Nonetheless, it has been demonstrated in IL2 stimulated human NK cells that cytotoxicity induces increases in mitochondrial mass in a PGC-1α dependent manner, and similarly, TGFβ was recently shown to upregulate mitochondrial mass also through PGC-1α in primary human lung fibroblasts [211]. While fibroblasts are very different from NK cells, these studies demonstrate that direct links between TGFβ and mitochondrial biology exist, and that it is possible that these mechanisms may also take place in NK cells. Indeed, we are only at the beginning of understanding mitochondrial dynamics

in NK cells, and it will be interesting in the future to see how cytokines such as TGFβ and other factors impact it.

There are several possible explanations for the improved mitochondrial metabolism observed in anti-TGFβ treated NK cells from breast cancer patients. As explained in the previous chapter, NK cells from breast cancer patients have increased mitochondrial mass and mitochondrial fragmentation, suggesting that there may be a defect in mitophagy. Here, we observed reduced mitochondrial mass in $CD56^{dim}$ NK cells from breast cancer patients treated with anti-TGFβ, indicating that mitophagy may have taken place in this subset of NK cells. As $CD56^{dim}$ NK cells represent 90% of NK cells in the blood, this could explain the increased levels of oxphos. The preferential upregulation of oxphos over glycolysis has the benefit of yielding a high amount of ATP, which is required for NK cells to carry out their functions [122, 150]. Furthermore, these NK cells will also be suited for long-term survival, as oxidative metabolism has been linked to the development of human adaptive NK cells [212]. This is of particular interest in the context of adoptive cell therapy, where NK cell longevity is highly desirable in order to achieve a durable therapeutic response.

While the impact of TGFβ and GARP blockade on oxphos was consistent, results for glycolysis were less clear. We found that targeting TGFβ either using anti-TGFβ or anti-GARP improved glycolytic reserve, but not capacity, while it decreased basal glycolysis in patient NK cells. It should be noted that glycolytic reserve is calculated by subtracting the basal glycolysis from the glycolytic capacity, and as the basal glycolysis was reduced, this may have resulted in an artificial increase in glycolytic reserve, and thus there can be no definitive conclusions drawn from this result. However, as basal glycolysis was reduced and oxphos increased, targeting TGFβ and the GARP/TGFβ axis promoted an overall shift towards oxphos. This metabolic reprogramming is evident by the significant increase in the OCR/ECAR ratio of patient NK cells treated with anti-TGFβ and anti-GARP. Together these data indicate that TGFβ inhibits oxphos, but potentially not glycolysis, in NK cells during cancer. Interestingly, we have previously shown that overnight treatment with TGFβ *in vitro* inhibits IL2 induced glycolytic capacity, but not basal glycolysis, in healthy donor NK cells [173]. Viel *et al*. carried out a similar experiment on 5 day expanded murine NK cells, and showed that overnight treatment with TGFβ and IL15 resulted in significantly reduced basal glycolysis and glycolytic capacity [204]. Interestingly,

Cong *et al.* recently reported on NK cell glycolytic dysfunction in a mouse model of lung cancer, where the glycolytic dysfunction was induced by upregulation of the enzyme fructose-1,6-bisphosphatase (FBP1) [213]. FBP1 is an essential enzyme in gluconeogenesis, a pathway that results in the generation of glucose from non-carbohydrate substrates such as amino acids, triglycerides and lactate. FBP1 hydrolyses fructose 1,6-bisphosphate into fructose 6-phosphate, thus reversing the direction of the glycolysis pathway and promoting glucose synthesis. While gluconeogenesis is traditionally known to take place in the liver and serve to maintain blood glucose levels, this study showed that TGFβ treatment (24 hours) increased the expression of FBP1 mRNA in murine lung NK cells, which induced NK cell dysfunction and tumour progression. Overall it is clear that TGFβ can have a wide variety of impacts on glycolysis in NK cells, with an overall inhibitory effect being evident from the literature. This is in agreement with data on murine Treg cells, where TGFβ inhibits the expression of the glycolytic rate limiting enzyme hexokinase 2 [214]. Our findings, which suggest that TGFβ promotes a more glycolytic phenotype in NK cells during cancer, highlight the variable and context dependent nature of TGFβ biology. Of note it may also be possible that in this study, the reduced oxphos caused by TGFβ resulted in a compensatory increase in glycolysis, and that TGFβ isn't actually affecting glycolysis directly. Indeed, the link between TGFβ and glycolysis in NK cells from healthy individuals and cancer patients warrants much further exploration.

In addition to its impact on cellular metabolism, targeting TGFβ using anti-TGFβ and anti-GARP also induced a significant upregulation of mTORC1 activity in response to both IL2 and IL12/15 in patient NK cells. Interestingly, anti-GARP improved mTORC1 activity only in the $CD56^{dim}$ subset of patient NK cells, raising many questions concerning the TGFβ downstream signalling pathways in $CD56^{bright}$ versus $CD56^{dim}$ NK cells. It is noteworthy that this in agreement with RNA sequencing data from Immune Cell Atlas, which showed GARP expression in $CD56^{dim}$ NK cells. This increased mTORC1 activity is of great interest as we have previously shown that mTORC1 is required for various aspects of NK cell metabolism and function, particularly those induced by IL2 stimulation [122, 123]. This finding suggests that the TGFβ driven NK cell dysfunction is in part mediated by repressed mTORC1 signalling. However, studies on the impact of TGFβ on mTORC1 activity in NK cells have so far been conflicting. Viel *et al.* reported that genetic deletion of TGFβR2 in murine NK cells resulted in increased mTORC1 activity, while *in vitro* treatment of

both mouse and human NK cells with TGFβ reduced mTORC1 activity [204]. Of note, the inhibitory impact of TGFβ on human NK cell mTORC1 activity was not evident in all donors and was modest compared to its effect on murine NK cell mTORC1 activity. In contrast, a study from our lab showed that TGFβ had no impact on the mTORC1 activity of human NK cells from healthy donors (at 30 min, 1 hour or 18 hour) [173]. Only when the NK cells were exposed to TGFβ for a prolonged period of time (5 days) did TGFβ reduce IL2 induced mTORC1 activity. This suggests that the TGFβ induced repression of mTORC1 activity observed here in NK cells from breast cancer patients is a result of chronic TGFβ stimulation. Interestingly, the time dependent nature of the impact of TGFβ has previously been described in primary murine NK cells [81]. Trotta *et al*. showed that while overnight TGFβ treatment did not inhibit NK cell mediated ADCC against a murine breast cancer cell line, treatment with TGFβ for 4 days was sufficient to do so. Indeed, it seems that in addition to being highly context dependent, the impact of TGFβ is also greatly dependent on the duration of the interaction. Highlighting the wide scope of the effects of TGFβ, it has been demonstrated in non-immune cells that TGFβ can actually activate mTORC1, for example in fibroblasts [215, 216] and human kidney cells [217, 218]. Indeed, the interplay between TGFβ, GARP and mTORC1 in NK cells requires much further investigation, and it will likely prove to be highly context and time dependent. Overall, the data presented in this chapter support a role for TGFβ mediated mTORC1 repression in driving NK cell dysfunction during cancer. For $CD56^{dim}$ NK cells, this may also involve the GARP anchoring protein. Investigation into the exact nature of this TGFβ effect (direct versus indirect, acute versus chronic) will shed light on the relationship between the two signalling mediators and perhaps identify further ways in which to target NK cells from cancer patients.

We have shown here for the first time that GARP plays a role in modulating NK cell metabolism and function during cancer. This is of great significance as it identifies GARP as a novel therapeutic target for the design of new immunotherapies. As highlighted in the introduction of this chapter, our understanding of the role GARP plays in the immune system is in its infancy. Indeed, its role in regulating Treg cells and B cells was only discovered in 2009 and 2018 respectively. In general, it seems that a certain stimulus/trigger may be required to induce GARP expression, be it a tumour derived factor or TLR ligand for example. As studies formally describing GARP in immune cells are so limited and recent, it seemed possible that it could potentially be expressed in other immune cells such as NK cells. Indeed, online

databases suggest that murine and human NK cell can sometimes express GARP. In this study, we observed GARP expression in both $CD56^{bright}$ and $CD56^{dim}$ NK cells. There was significant heterogeneity in LAP and GARP expression in NK cells from healthy donors and patients, with higher expression being evident more commonly in NK cells from breast cancer patients. This culminated in significantly higher GARP expression in patient NK cells compared to those from healthy donors. Interestingly, increased TGFβ expression in NK cells has previously been described in peripheral blood NK cells from HIV infected patients [219] and from breast cancer patients [206]. In addition, increased LAP expression has been reported in NK cells infected with the intracellular parasite *Toxoplasma gondii* [220]. These studies support our findings, and indicate that the intrinsic hijacking of the TGFβ processing pathway in NK cells may be common across different types of human disease.

GARP expression is well documented in Treg cells, where it plays a homeostatic role by promoting Treg cell differentiation and driving proliferation and cytokine production [203]. Therefore, GARP expression in NK cells was compared to that in CD25+ T cells (likely enriched in Treg cells). As expected, baseline levels of GARP in CD25+ T cells from healthy donors were much higher than those in NK cells. However, in contrast to NK cells, these levels were not increased when compared to CD25+ T cells from breast cancer patients. This suggests that while GARP plays a normal role in healthy donor CD25+ T cells, its increased expression in patient NK cells is cancer-related. If a tumour-derived factor exists that induces GARP expression in immune cells, it could potentially promote pro-tumourigenic Treg cells while at the same time inhibiting the anti-cancer functions of NK cells. As such, it will be important in the future to figure out what is causing the increased GARP expression in NK cells.

Of note, there may be a role for altered methylation of the GARP promoter in driving its expression in patient NK cells. Indeed, it is well established that expression of the GARP gene is highly regulated by methylation of its upstream and downstream promoters [194]. For example, Tregs cells have reduced methylation of certain CpG islands within the upstream promoter of GARP, which allows for the binding of FoxP3, NFAT and NF-kB that drive GARP transcription. [221]. Methylation is integrally linked to cellular metabolism - nutrients, enzymes and metabolic cofactors can alter levels of the metabolite S-adenosylmethionine, which is the methyl-donating substrate of histone methyltransferases. Hence, the reduced

metabolism observed in NK cells from breast cancer could be altering methylation patterns and driving GARP expression.

For NK cell metabolic responses (nutrient receptor expression and seahorse data), the impact of anti-GARP was clear and consistent. This was surprising due to the high variability of GARP expression in NK cells, and suggests that what we perceive as low GARP expression may in fact be playing an important role in modulating NK cell responses. This theory is supported by the fact that anti-GARP treatment improved some cytokine-induced responses in healthy donor NK cells, which in general appeared to have low levels of GARP. It seems that GARP may act as a negative regulator of some normal cytokine induced NK cell responses, and therefore its role in maintaining self-tolerance warrants investigation. As anti-TGFβ in general did not improve healthy donor NK cell responses, these data suggest that patient NK cells may have a higher rate of conversion of latent TGFβ into active TGFβ. There are a number of potential ways in which this could occur, including high levels of ROS that was shown in chapter 3, as ROS has been shown to activate endogenous TGFβ in human CD4+ T cells (ROS production correlates with TGFβ activation, and antioxidants abrogate TGFβ activation) [222]. Indeed, it would be interesting to treat patient NK cells with a combination of anti-TGFβ/GARP plus antioxidant, to see if this boosts NK cell function and metabolism further, as this would support a role for ROS in driving TGFβ activation in patient NK cells.

Our data from experiments with purified NK cells suggest that it is GARP expressed by NK cells that is important in driving the inhibitory effects of TGFβ on NK cells. However, it is important to acknowledge that it is possible that contaminating monocytes may have contributed to the therapeutic effect of anti-GARP treatment observed in study. Indeed, we recorded high levels of GARP expression in monocytes from healthy donors and this was increased further in monocytes from breast cancer patients. This finding was then supported by RNAseq data from Immune Cell Atlas showing GARP expression in human blood monocytes. Interestingly, TGFβ expression in monocytes has been suggested to inhibit NK cell cytotoxic activity in patients with obstructive sleep apnoea. In this study, patient monocytes expressed high levels of LAP and GARP, while patient NK cells had high levels of pSMAD2/3, and cytotoxicity against K562 tumour cells correlated negatively with LAP expression. The authors used hypoxia to mimic the effect of obstructive sleep apnoea on healthy donor monocytes, and when these were co-

cultured with healthy donor NK cells, the NK cells had reduced cytotoxicity against K562 tumour cells [223]. Adding anti-TGFβ antibody or a TGFβ receptor inhibitor reduced the impact of these hypoxic monocytes on healthy donor NK cell cytotoxcity. These data somewhat reflect the findings of this chapter; however, the study did not link this effect with GARP expression in monocytes. Nonetheless, the fact remains that the frequency of monocytes in the cultures in this study was extremely low (1.5% monocytes versus 98.5% lymphocytes for patients). Still, it will be important to repeat at least some of these experiments in NK cell cultures with closer to 100% purity (e.g. by cell sorting) in order to confirm the results of this study.

Overall, it appears that we are only beginning to understand the role that the GARP/TGFβ axis plays in the regulating immune response. Figuring out what stimulates their increased expression in NK cells from breast cancer patients may identify a novel therapeutic target that could be used to alter the GARP/ TGFβ pathway during cancer. While the observation of GARP expression in patient NK cells is novel, the most important finding of this chapter is that mitochondrial and mTORC1 dysfunction in NK cells from cancer patients is reversible and can be targeted in order to improve NK cell anti-cancer functions. Indeed, it now seems very likely that the vast amount of research being conducted in the field of immunometabolism will someday lead to the development of new, innovative therapies for the treatment of human disease.

Chapter 5

5 NK cell metabolism and neuroblastoma

5.1 Introduction

We have shown that NK cells from the peripheral blood of metastatic breast cancer patients have impaired metabolism, and we propose that this is a cause of the reduced IFNγ production observed. Importantly, we demonstrate that impaired metabolism is reversible, and can be targeted in order to increase NK cell functions. In this chapter, we explore the function and metabolism of peripheral blood NK cells from neuroblastoma patients, in order to gain insight into whether peripheral NK cell metabolic dysfunction is common across various types of cancer. We also investigate the role that metabolism plays in mediating the efficacy of anti-GD2 immunotherapy, which is routinely used in the treatment of high-risk neuroblastoma patients.

5.1.1 Neuroblastoma.

Neuroblastoma (NB) is the most common extra-cranial solid tumour in children, accounting for approximately 6% of childhood cancers (cancer.org). It is a cancer of the sympathetic nervous system that arises from any element of the neural crest including the adrenal glands (most commonly), the neck, chest, abdomen or spine. 90% of cases occur in children 5 years or younger. Upon diagnosis, patients are stratified according to a combination of radiography, histology, genetics and age at time of diagnosis. In Ireland between 1994 and 2000, 35 children were diagnosed with NB (5-6 per year), with an average survival rate of 73% [224]. Clinical presentation of NB is highly variable and can range from a local tumour that causes little to no symptoms, through to a local or disseminated tumour that causes severe illness (e.g. aches and pains, loss of appetite, sweating, unsteady walk or difficulty passing urine) [225]. Current treatment options include observation (for very young patients), surgery, chemotherapy (cycles of cisplatin and etoposide alternating with vincristine, doxorubicin, and cyclophosphamide [226]), radiation therapy and immunotherapy. Retinoids (13-cis-retinoic acid, similar to vitamin A), which drive

neuronal differentiation *in vitro*, are used as adjuvant therapy for high risk patients and have been shown to reduce the risk of relapse [227]. Stem cell transplants, mainly autologous, are sometimes used following chemotherapy and/or radiotherapy in high-risk patients [228]. This involves the infusion of hematopoietic stem cells into the patient with the aim to replace the bone marrow and restore normal blood counts

NB tumours are considered 'cold', as are most paediatric solid tumours [229]. This means that they have a low mutational burden and poor immune cell infiltration. One of the few genetic abnormalities associated with NB is the amplification of the MYCN oncogene. Tumours that have MYCN amplification are categorised as high risk and are highly aggressive, leading to increased rates of progression and relapse, and reduced survival rates [230, 231]. Compared to just 15% of all patients, approximately 50% those with stage 4 NB present with MYCN amplification. Survival rates of high-risk NB patients has improved in recent years with the introduction of optimised multimodal treatment strategies and anti-GD2 immunotherapy, increasing from 15% to 40-50% [232]. However, a 50% survival rate is still very poor for a paediatric cancer, and approximately half of patients with high-risk NB will experience relapse. Thus, there is an urgent need to better understand NB and to identify novel therapeutic targets for the development of new treatment strategies.

5.1.2 ADCC and antibody based immunotherapy.

Immunotherapy has been very successful in the treatment of NB. Anti-GD2 immunotherapy (Dinutuximab) works by enhancing ADCC against NB tumour cells expressing the GD2 antigen. ADCC is a tailored form of cytotoxicity that is naturally dependent on antibody production by B cells but can be clinically induced by infusion of monoclonal antibody therapy into patients. Immunoglobulins (Ig) either released by B cells or infused intravenously bind a specific, highly expressed, tumour associated antigen on the surface of the tumour cells, leading to increased detection by cytotoxic immune cells. NK cells recognise these opsonising antibodies and bind to the Fc region of the Ig via their Fc receptor (FcγRIIIα, aka CD16) (**Figure 5.1**) [40]. While monocytes, macrophages, basophils, and mast cells can also express CD16, $CD56^{dim}$ NK cells express the highest level of this Fc receptor and thus are

considered one of the most important cell types responsible for eliciting ADCC (**Figure 5.2**).

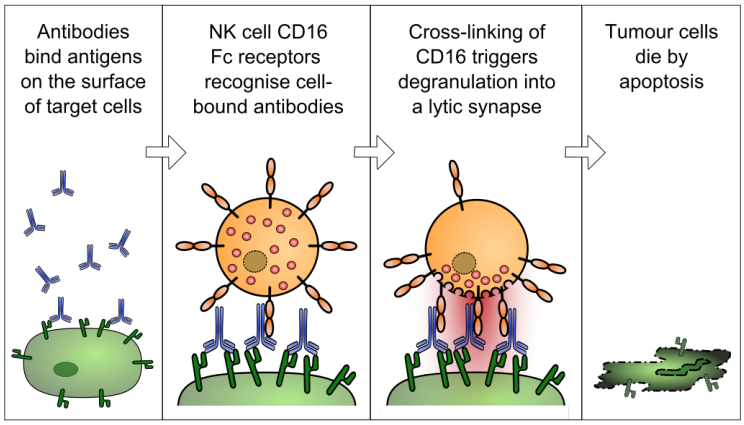


Figure 5.1 NK cell ADCC against a tumour target cell.

After antibodies coat the target cell, NK cells bind the Fc portion, triggering degranulation and apoptosis of tumour cell.

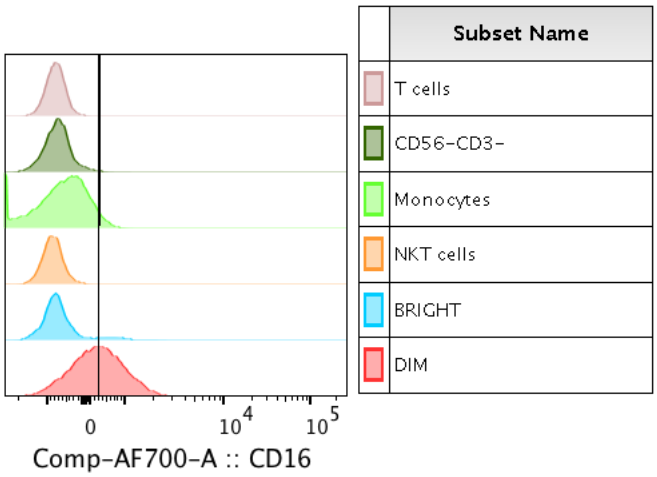


Figure 5.2 CD56^{dim} NK cells express the highest level of CD16 in immune cells found in PBMC.

Direct *ex vivo* PBMC stained for CD16 expression and analysed via flow cytometry.

NK cells can also express the inhibitory receptor FcγRIIb (CD32b), which has an ITIM, rather than an activating ITAM within its cytoplasmic tail [233]. This inhibitory receptor is important for regulating the ADCC response, in addition to other negative regulators such as Src homology 2 domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1).

CD16 is one of the only NK cell receptors that can induce cytotoxicity and cytokine production in resting NK cells [234], and polymorphisms in CD16 have been shown to increase susceptibility to various autoimmune and infectious diseases [235, 236]. Upon ligation, CD16 signalling involves the classical ITAM pathway of Src-family kinase-mediated tyrosine phosphorylation, with Syk association leading to PI3K, Vav1 and PLC-γ activation (**Figure 5.3**). Once ADCC has been triggered, NK cells kill the target cell by degranulation and through TNF family death receptor signalling (e.g. FasL and TRAIL). Furthermore, they can secrete IFNγ to activate neighbouring immune cells. [237].

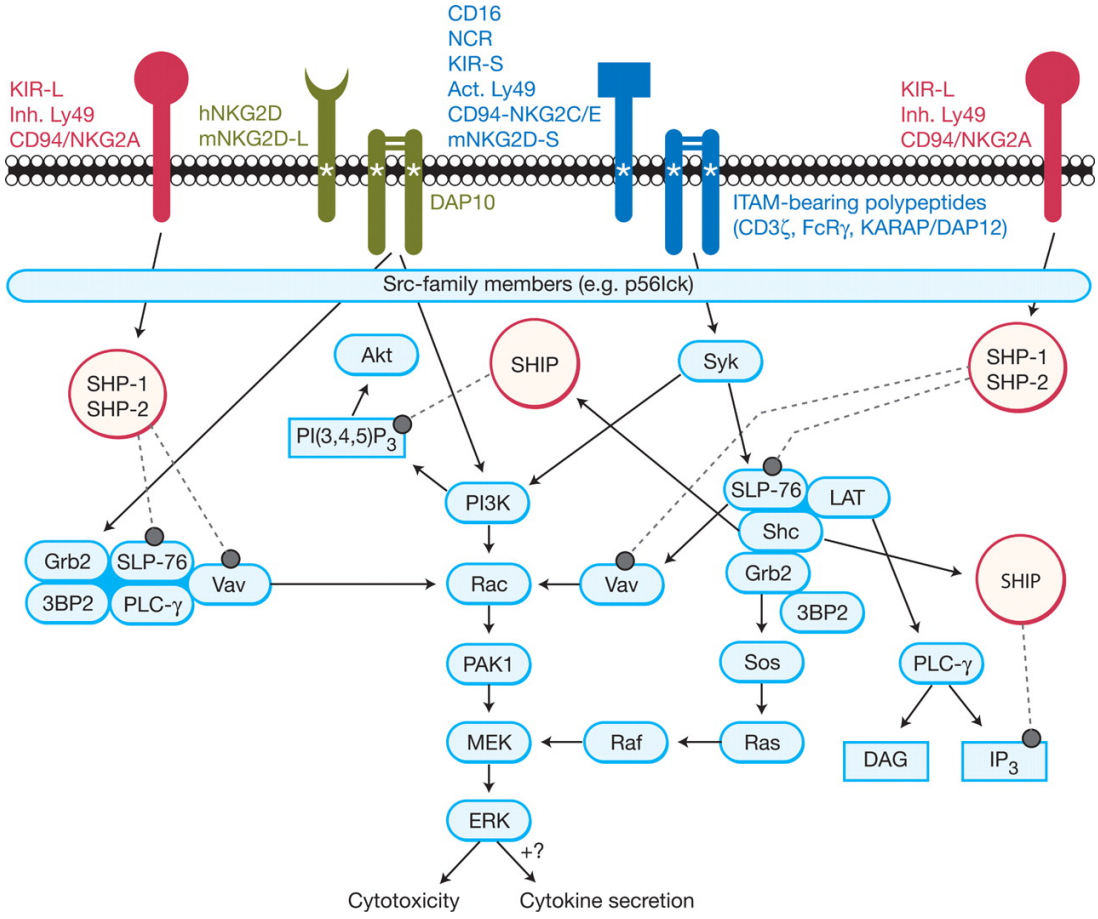


Figure 5.3 NK cell receptor mediated signal transduction.

Taken from [238].

Indeed, ADCC is a very powerful mechanism for killing target cells in the absence of any other stimuli. The clinical induction of ADCC using anti-cancer monoclonal antibodies has been very successful and indeed the development of these therapeutic antibodies is widely acknowledged as a great modern advancement in the treatment of cancer (**Table 1** for examples) [239]. While studies on the role of ADCC *in vivo* have been limited, there are many that indicate that NK cell mediated

ADCC is a key mechanism responsible for the therapeutic effects of monoclonal antibodies. For example, NB patients undergoing anti-GD2 therapy that have high-affinity polymorphisms in FcγR genes and activating KIR genes (which support NK cell activity) have increased event free survival than patient with low-affinity polymorphisms in FcγR genes and inhibitory KIR genes [240]. Breast cancer patients classified as 'responders' to Trastuzumab treatment have enhanced NK cell cytotoxic activity compared to those that do not respond to antibody treatment [241]. Non-Hodgkin lymphoma patients receiving anti-CD20 who are homozygous for the high affinity FcRIIIα (CD16α) receptor allele have a longer progression free survival rate than their heterozygous counterparts [242]. Clynes *et al*. showed in a murine model of breast cancer that antibody treated mice deficient in activating Fc receptors had increased tumour growth [243]. Similarly, they showed that mice that received mutant antibodies with altered Fc binding sites had increased tumour growth compared to mice that received standard antibodies.

Antibody therapy	Target antigen	Cancer type
Trastuzumab	HER2	HER2+ breast cancer
Dinutuximab	GD2	Neuroblastoma
Cetuximab	EGFR	Colorectal cancer, NSCLC, head and neck cancer
Daratumumab	CD38	Multiple myeloma
Ramucirumab	VEGFR-2	Gastric cancer
Ipilimumab	CTLA-4	Melanoma, renal cell carcinoma
Rituximab	CD20	Non-Hodgkin's lymphoma

Table 5.1 Examples of monoclonal antibody therapy that are used to treat various types of cancer.

Indeed, these studies highlight the importance of *in vivo* ADCC for the efficacy of monoclonal antibody therapy that target tumour associated antibodies. However, these therapies only work in a subset of patients, and resistance is still a major issue. While anti-GD2 therapy has improved survival of high-risk NB patients significantly (46 to 63% event free survival, clinical trial ANBL0032), approximately one third of these patients will experience relapse and many of these will ultimately die from the disease [244]. As such, there is an urgent need to better understand the molecular mechanisms that drive ADCC in order to improve antibody therapy.

5.1.3 NK cells and neuroblastoma.

Although NK cells play an essential role in mediating the efficacy of anti-GD2 therapy in NB, studies on NK cells derived from NB patients are scarce, likely due to the low number of cases diagnosed each year and the small volumes of blood from paediatric patients that are available for research. Nonetheless, there are several compelling studies that implicate NK cells as essential in protection against NB and in the effectiveness of immunotherapy. In 1998 Lode *et al*. showed that treatment with an anti-GD2-IL2 fusion protein in a murine model of NB abrogated bone marrow and liver metastasis [245]. Importantly, this therapeutic effect did not occur when they repeated the experiment in NK cell deficient mice. In contrast, when CD8+ T cell were depleted, the therapy worked optimally. mRNA transcripts of MICA, MICB and ULBP, ligands of the NK cell activating receptor NKG2D, have been reported in several NB cell lines and in patient derived primary tumours [246], highlighting the vulnerability of NB tumours to NK cell mediated killing. NK cell associated genes including the KIR genes KIR2DL2 (inhibitory) and KIR2DS2 (activating) are associated with development of NB [247]. Forlenza *et al*. investigated the ability of certain KIR3DL1 (inhibitory) polymorphisms to bind HLA-Bw4 ligands in high-risk NB patients treated with anti-GD2 therapy [248]. They reported that patients with polymorphisms that result in a weaker interaction between KIR3DL1 and HLA-Bw4 have better overall and progression free survival. *In vitro*, degranulation of KIR3DL1- NK cells was insensitive to inhibition by HLA-Bw4+ NB tumour cells during anti-GD2 mediated ADCC. Similarly, Erbe *et al.* have shown that specific KIR/KIR ligand genotypes are associated with improved response to immunotherapy (anti-GD2 + IL2 + GM-CSF) [249].

In addition to KIR receptors, NK cell NCRs have also been implicated in the protection against NB. Indeed, NK cells have been shown to kill NB tumour cell lines *in vitro* using receptors NKp46, NKp44 and NKp30 [250]. Similarly, Castriconi *et al*. showed that NK cells kill freshly isolated patient derived neuroblasts in a NKp46, NKp30 and DNAM-1 dependent manner [251]. Of note, the freshly isolated neuroblasts were more resistant to NK cell killing than NB cell lines. Semeraro *et al*. studied the NKp30/B7-H6 axis in high-risk NB patients (B7-H6 is a ligand of NKp30) [252]. They reported downregulation of NKp30 on NK cells in the bone marrow of metastatic NB patients, which correlated with an upregulation of B7-H6 in NB tumours and patient sera. Furthermore, soluble B7-H6 inhibited IFNγ production in healthy donor NK cells cultured with K562 tumour cells. Interestingly, CD16 was

upregulated on NK cells in the bone marrow of metastatic NB patients, which may contribute to the effectiveness of anti-GD2 therapy in high-risk patients.

Taking these studies together it becomes clear that NK cells and their surface receptors play an essential role in the protection against NB. However, as with all cancers, NB tumours have developed mechanisms that allow them to evade NK cell mediated killing. Raffaghello *et al*. investigated the common evasion strategy of ligand shedding in NB patients [246]. They detected significantly elevated levels of soluble MICA in the sera of NB patients. In the presence of this patient serum, NK cells from healthy donors had impaired killing of NB tumour cells, which was reversed upon addition of an anti-soluble-MICA antibody. Altered TGFβ activity is another common evasion mechanism that has been documented in NB. Cohen *et al.* reported that treatment of the NB cell line SMS-KCNR with retinoic acid resulted in a 40 fold increase in the expression of both of the TGFβ receptors [253]. Interestingly, they observed constitutive secretion of TGFβ in the retinoic acid resistant cell line SK-N-AS, suggesting that TGFβ expression might confer resistance to retinoic acid treatment. In another study on TGFβ and NB, Castriconi *et al*. showed that NB cell line supernatant alters the expression of chemokine receptors on healthy donor NK cells, and that this can be reversed by the addition of a TGFβ neutralising antibody.

Indeed, there is accumulating evidence for the protective role that NK cells play during NB, and for immune evasion strategies that NB tumours can potentially employ to resist NK cells. However, studies on the functions of NK cells from NB patients are lacking. As NK cell dysfunction has been reported in many other types of cancers [15, 16, 68, 71, 73], and in metastatic breast cancer as reported here, there is an urgent need to investigate this in NB patients in order to discover potential ways to improve immunotherapy and increase survival of high-risk patients.

In this chapter we investigate the function of NK cells from the peripheral blood of NB patients. We examine whether altered metabolism might be an underlying cause of any phenotype observed, as was the case for NK cells from metastatic breast cancer patients. This will shed light on whether altered metabolism of peripheral blood NK cells may be a common feature among different types of cancers. Furthermore, we explore the metabolic requirements of NK cell anti-GD2 mediated ADCC against NB tumour cells, in order to identify what metabolic pathways should

be targeted/protected in patient NK cells in order to boost the efficacy of anti-GD2 immunotherapy. All blood samples were taken upon diagnosis and analysed immediately, and therefore patients were treatment naïve at time of sampling. Between January 2017 and September 2020 5 children were diagnosed with NB in Dublin, giving an incidence of approximately 1-2 per year (lower than the previously published statistic of 5-6 per year [224]). This cohort is composed of 1 male and 4 female patients, with a mean age of 2 years 9 months. Despite low sample numbers that will need to be increased for robust statistical analysis, trends in the data were still observed. Currently the data is compared to that obtained from NK cells from healthy adult donors, but will ultimately be compared to NK cells from healthy paediatric donors in the future.

5.1.4 Aims and Objectives:

1. To investigate the activation and function of NK cells from NB patients.
2. To explore nutrient receptor expression and nutrient uptake in NK cells from NB patients.
3. To examine mitochondrial and glycolytic metabolism of NK cells from NB patients.
4. To develop an *in vitro* model to study NK cell anti-GD2 mediated ADCC against NB tumour cells.
5. To determine what metabolic pathways are required for NK cell mediated ADCC in healthy human adult donors.
6. To investigate whether mTORC1, SREBP or cMyc (all important for NK cell metabolism and function) is important in regulating ADCC

5.2 Results

5.2.1 NK cells from NB patients have impaired activation and IFNγ production in response to cytokine.

CD69 and CD25 are two important markers of NK cell activation. While the frequency of cytokine stimulated NK cells expressing CD69 in from breast cancer patients was normal, the frequency of CD25 expression was impaired. Therefore, we examined the expression of these two markers in NK cells from NB patients and healthy donors stimulated with IL2 or with IL12/15 using flow cytometry. Basal expression of both markers was equivalent in resting NK cells from healthy donors and NB patients (data not shown). The mean frequency of CD69 expression was reduced in patient NK cells stimulated with IL2 however, it appeared to be normal in those stimulated with IL12/15 (**Figure 5.4A** for representative dot plots, **Figure 5.4B** and **5.4C**). Similarly, the mean frequency of CD25 expression was reduced in patient NK cells stimulated with IL2, however IL12/15 stimulated patient NK cells also had reduced CD25 expression, which reached statistical significance in the $CD56^{bright}$ NK cell subset (**Figure 5.5A** for representative dot plots, **Figure 5.5B** and **5.5C**). These data suggest that NK cells from NB patients may have impaired cytokine-induced activation.

NK cells have a variety of functions they use to protect against cancer. Two of these key functions are granzyme B expression and IFNγ production, the latter of which was significantly impaired in NK cells from breast cancer patients. We measured granzyme B expression and IFNγ production in cytokine stimulated NK cells from NB patients and compared it to that in NK cells from healthy donors. Basal expression of granzyme B and production of IFNγ was equivalent in resting NK cells from healthy donors and NB patients (data not shown). Surprisingly, NK cells from NB patients had enhanced granzyme B responses compared to those from healthy donors (**Figure 5.6A** for representative histograms, **Figure 5.6B** and **5.6C**). This was particularly evident in the $CD56^{bright}$ NK cell subset, which reached statistical significance for both IL2 and IL12/15 stimulated patient NK cells. In contrast, IL12/15 stimulated patient NK cells had severely reduced IFNγ production, and this was clear in both the $CD56^{bright}$ and $CD56^{dim}$ subsets (**Figure 5.7A** for representative dot plots and **Figure 5.7B**). Together these data suggest that NK cells from NB patients have enhanced cytotoxicity but dysfunctional cytokine production, and

therefore they partially mirror what was observed in NK cells from breast cancer patients.

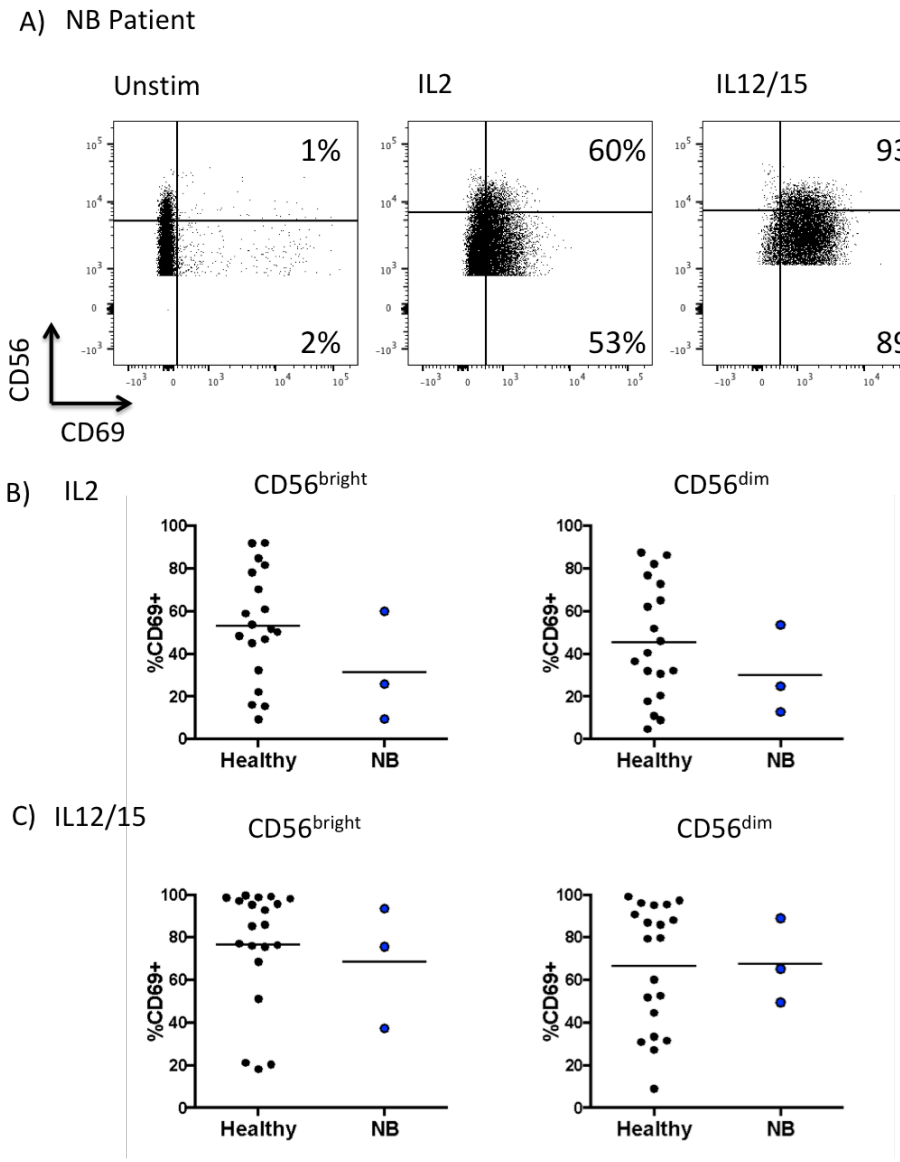


Figure 5.4 NK cells from NB patients may have impaired upregulation of the activation marker CD69 in response to IL2.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for CD69 and analysed by flow cytometry. (A) Representative dot plot of the upregulation of CD25 in response to IL2 and IL12/15 in NK cells from NB patients. (B) Frequency of CD25 expression in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) Frequency of CD25 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n = 3-20). Samples were compared using the unpaired student t-test.

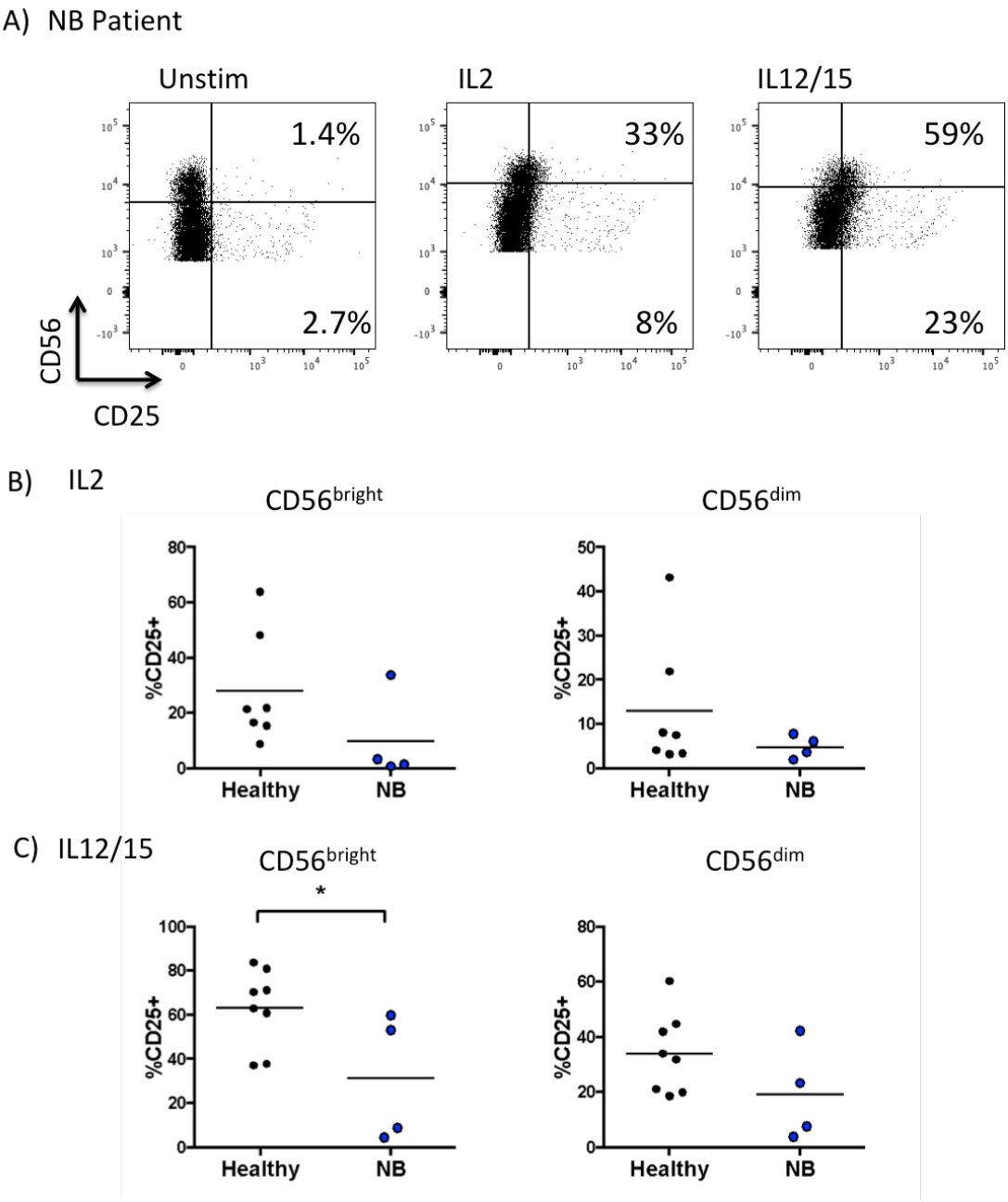


Figure 5.5 NK cells from NB patients have impaired upregulation of the activation marker CD25 in response to cytokine.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for CD25 and analysed by flow cytometry. (A) Representative dot plot of the upregulation of CD25 in response to IL2 and IL12/15 in NK cells from NB patients. (B) Frequency of CD25 expression in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) Frequency of CD25 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n = 4-8). Samples were compared using the unpaired student t-test, *$p<0.05$.

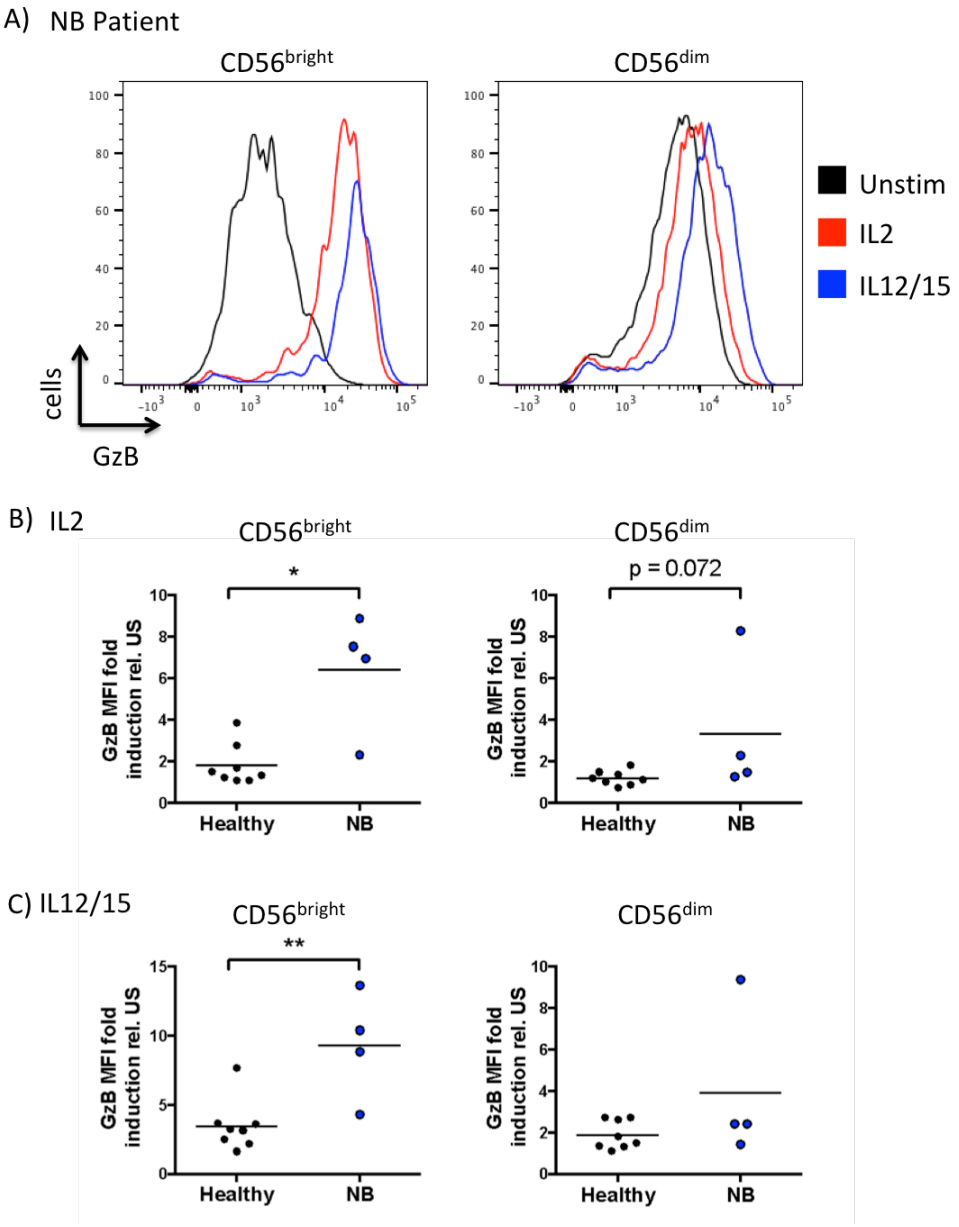


Figure 5.6 NK cells from NB patients have enhanced granzyme B expression in response to cytokine

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for CD25 and analysed by flow cytometry. (A) Representative histogram of the upregulation of granzyme B in response to IL2 and IL12/15 in NK cells from NB patients. (B) Fold increase in the MFI of granzyme B relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) Fold increase in the MFI of granzyme B relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n = 4-8). Samples were compared using the unpaired student t-test, *p<0.05, **p<0.01.

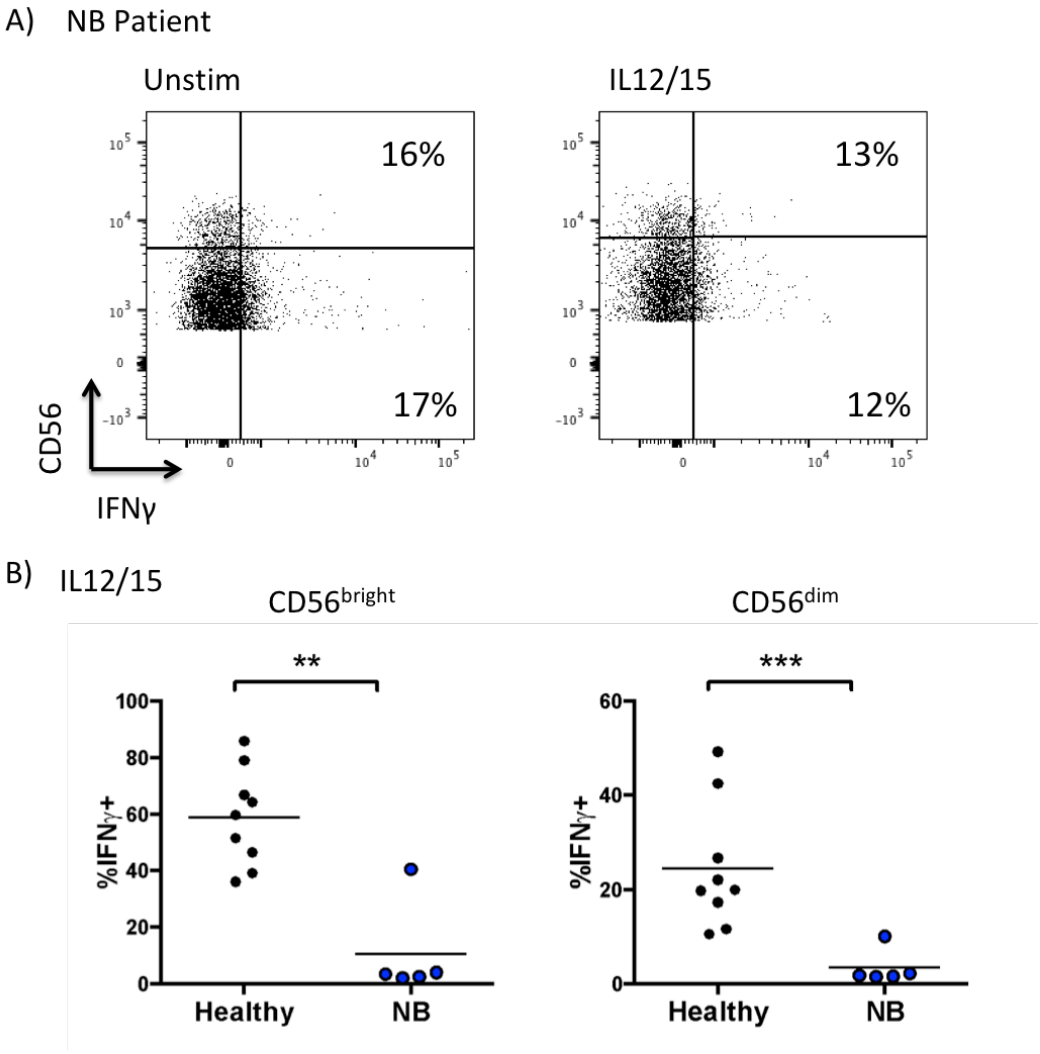


Figure 5.7 NK cells from NB patients have impaired IFNγ production in response to IL12/15 stimulation

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Golgi plug was added for the final 4 hours. Cells were stained for IFNγ and analysed by flow cytometry. (A) Representative dot plot of the upregulation of IFNγ production in response IL12/15 in NK cells from NB patients. (B) Frequency of IFNγ production in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n = 5-9). Samples were compared using the unpaired student t-test, **p<0.01, ***p<0.001.

5.2.2 NK cells from NB patients have normal expression of nutrient receptors.

The data thus far shows that NK cells from NB patients have some features of dysfunction similar to that seen in NK cells from breast cancer. Therefore we investigated whether altered cellular metabolism might also be contributing to the dysfunction in this paediatric patient cohort. Nutrients are essential for fuelling cellular metabolism [125], and NK cells from breast cancer patients had reduced expression of both CD71 (transferrin receptor) and CD98 (subunit of SLC7A5 - large neutral amino acid transporter), but normal kynurenine uptake (SLC7A5 activity). As such, the expression of nutrient receptors and the ability to uptake nutrients through SLC7A5 was investigated using flow cytometry. The frequency of CD71 expression in patient NK cells was highly variable in NK cells from NB patients, yet ultimately the range and the mean frequency was similar to that seen in NK cells from healthy donors (**Figure 5.8A** for representative dot plots, **Figure 5.8B** and **5.8C**). While cytokine induced CD98 expression was more consistent in patients, it was also similar to that observed in NK cells from healthy donors (**Figure 5.9A** for representative histograms, **Figure 5.9B** and **5.9C)**. In agreement with this result, uptake of kynurenine through the SLC7A5 transporter was normal in IL2 stimulated NK cells from NB patients (**Figure 5.10A** for representative histograms and **Figure 5.10B**). Surprisingly, this was in fact increased in IL12/15 stimulated NK cells from NB patients compared to those from healthy donors (**Figure 5.10C**). These data suggest that in general NK cells from NB patients may have normal expression of nutrient receptors.

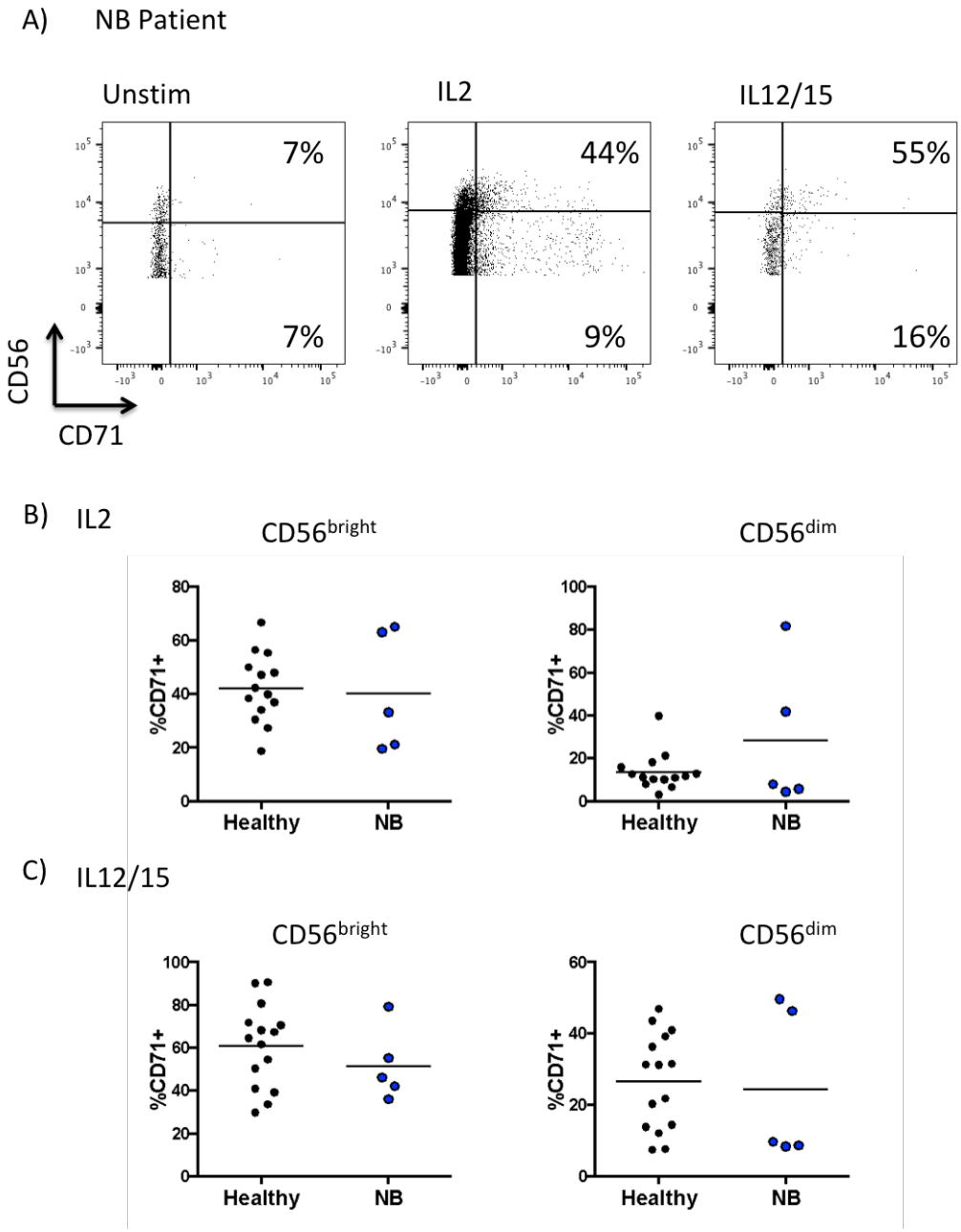


Figure 5.8 NK cells from NB patients upregulate CD71 expression normally in response to cytokine.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for CD71 and analysed by flow cytometry. (A) Representative dot plot of the upregulation of CD71 in response to IL2 and IL12/15 in NK cells from NB patients. (B) Frequency of CD71 expression in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) Frequency of CD71 expression in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n = 5-15).

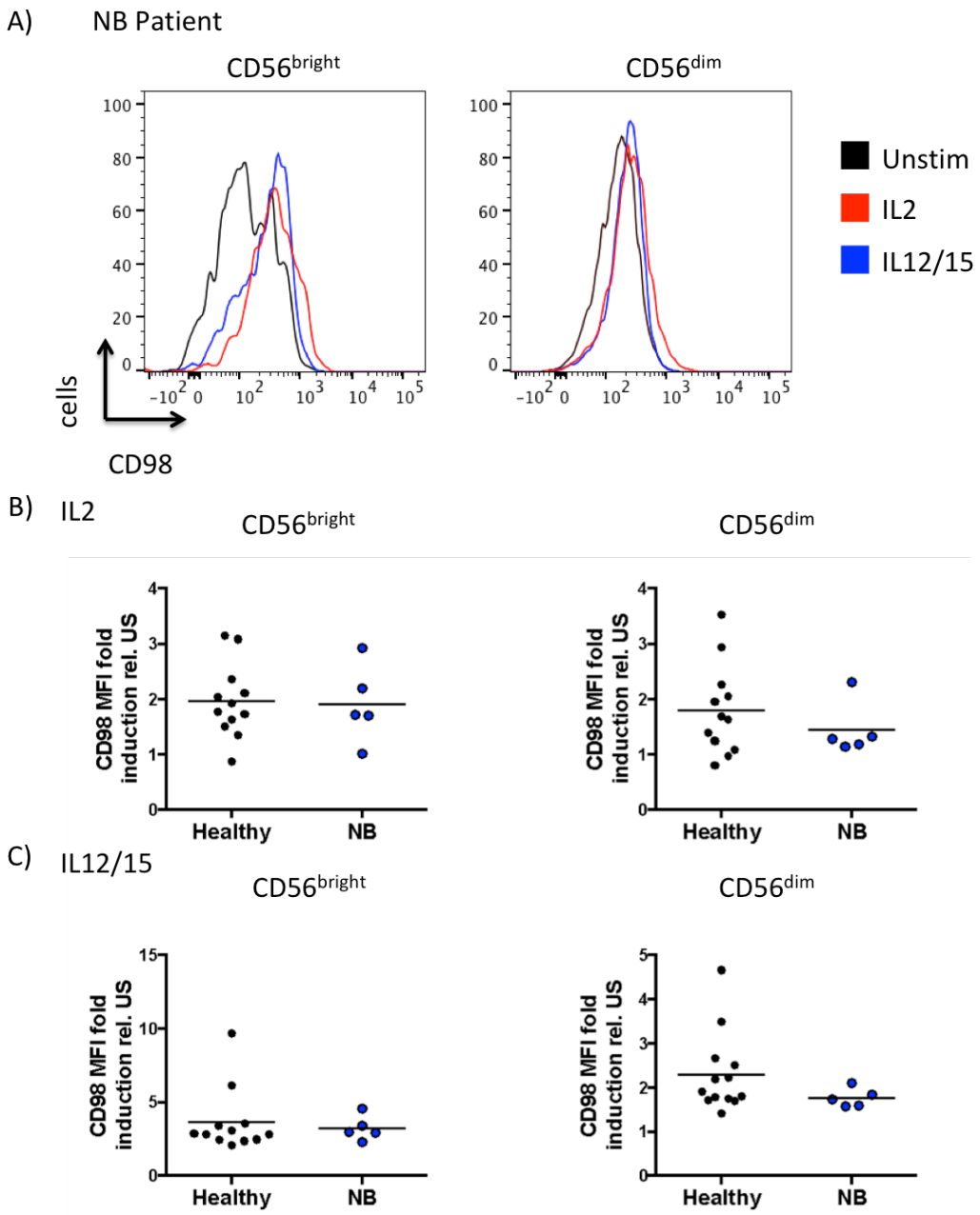


Figure 5.9 NK cells from NB patients upregulate CD98 expression normally in response to cytokine.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were stained for CD98 and analysed by flow cytometry. (A) Representative histogram of the upregulation of CD98 in response to IL2 and IL12/15 in NK cells from NB patients. (B) Fold increase in the MFI of CD98 relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) Fold increase in the MFI of CD98 relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n = 5-13).

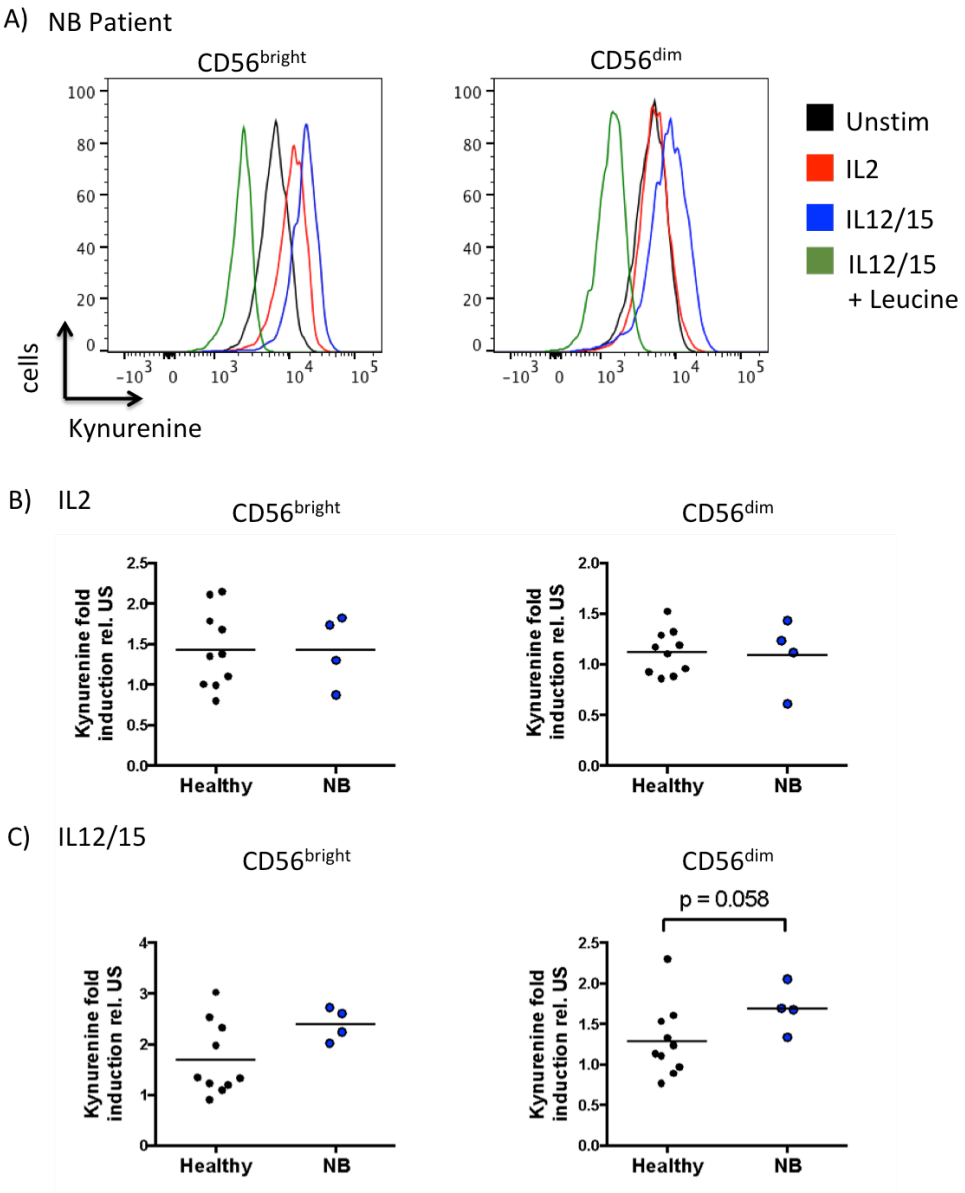


Figure 5.10 IL12/15 stimulated NK cells from NB patients have increased SLC7A5 amino acid transporter activity.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) or with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Cells were surface stained for NK markers and then resuspended in Kynurenine in HBSS. Cells were fixed with 4% PFA and analysed by flow cytometry. Leucine was used as a negative control. (A) Representative histogram of kynurenine uptake in IL2 and IL12/15 stimulated NK cells from NB patients. (B) Fold increase in the MFI of kynurenine relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) Fold increase in the MFI of kynurenine relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n = 4-10). Samples were compared using the unpaired student t-test.

5.2.3 NK cells from NB patients have altered mitochondrial structure and function.

Thus far the data show that NK cells from NB patients have impaired activation and function (IFNγ production). Some of these findings mirror what was observed in NK cells from breast cancer patients, where altered mitochondrial metabolism was also a clear and consistent feature associated with reduced IFNγ production. Therefore, we next explored the mitochondrial structure and function in NK cells from NB patients using seahorse metabolic flux analysis combined with flow cytometry and confocal microscopy based mitochondrial assays. One serious technical issue when working with paediatric samples (particularly infants) is that blood sample volumes are inevitably smaller and therefore the range of experiments that are possible to do is reduced. Because of this it was not possible to carry out seahorse analysis on unstimulated purified NK cells from NB patients, and only IL2 induced oxphos was measured. Compared to IL2 stimulated NK cells from healthy donors, those from NB patients had reduced levels of oxphos (**Figure 5.11A** for representative seahorse trace). Calculation of the various oxidative metabolism parameters showed that IL2 stimulated NK cells from NB patients had reduced basal respiration, maximal respiration, spare respiratory capacity and ATP-linked respiration (**Figure 5.11B**). Of note, this is very similar to the trends observed in NK cells from breast cancer patients, where reduced IL2 induced oxphos was associated with many mitochondrial alterations in patient NK cells analysed directly *ex vivo*. Therefore, we next carried out these same flow cytometry based mitochondrial analyses – MitoSOX, MitoTracker, TMRM and ATP5B. mROS, indicative of mitochondrial dysfunction, was significantly increased in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from NB patients, and this was to a much higher degree than that observed in NK cells from breast cancer patients (**Figure 5.12A** for representative histograms and **Figure 5.12B**). Similarly, 3 out of the 4 NB patients measured had NK cells with strikingly increased mitochondrial mass (**Figure 5.13A** for representative histograms and **Figure 5.13B**). MMP, measured using the TMRM dye, was also increased in $CD56^{dim}$ NK cells in 3 out of the 4 patients measured. For the $CD56^{bright}$ subset, NK cells from 2 patients had increased MMP while those from the other 2 patients appeared to have normal MMP (**Figure 5.14A** for representative histograms and **Figure 5.14B**). Together these flow cytometry assays indicate that NK cells from NB patients impaired mitochondrial fitness, and suggest that their mitochondrial structure may also be altered.

The data thus far are similar to what was observed in NK cells from breast cancer patients – increased mROS, mitochondrial mass and MMP. In addition to these features, IL12/15 stimulated NK cells from breast cancer patients had reduced levels of the ATP5B, a subunit of the ATP synthase enzyme and essential for electron transport during oxphos. In contrast to the other mitochondrial markers measured, IL12/15 stimulated NK cells from NB patients had normal levels of ATP5B (**Figure 5.15A** for representative and **Figure 5.15C**). Unexpectedly, IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from NB had increased levels of ATP5B compared to those from healthy donors (**Figure 5.15B**).

The mitochondrial data on NK cells from breast cancer patients was consolidated by confocal microscopy images where patient NK cells in general had fissed, circular mitochondria compared to NK cells from healthy donors that in general had fused, elongated mitochondria. So far we have obtained sufficient pure NK cells from just one NB patient to examine whether increased mitochondrial fission might also be taking place in NK cells from NB patients. Although this result is preliminary and will need to be repeated, the images show quite strikingly that *ex vivo* NK cells from NB have round, fissed mitochondria compared to those from healthy donors (**Figure 5.16A** for sample images).

Taken together, the data from this section show clearly that NK cells from NB patients have altered mitochondrial structure and function. Many, but not all of these alterations reflect what was observed in NK cells from breast cancer patients. Given that mitochondrial metabolism was successfully targeted in order to improve IFNγ production in NK cells from breast cancer patients in chapter 4, targeting mitochondrial metabolism in NK cells from NB patients represents an attractive avenue for the design of future NK cell based immunotherapies for treating NB.

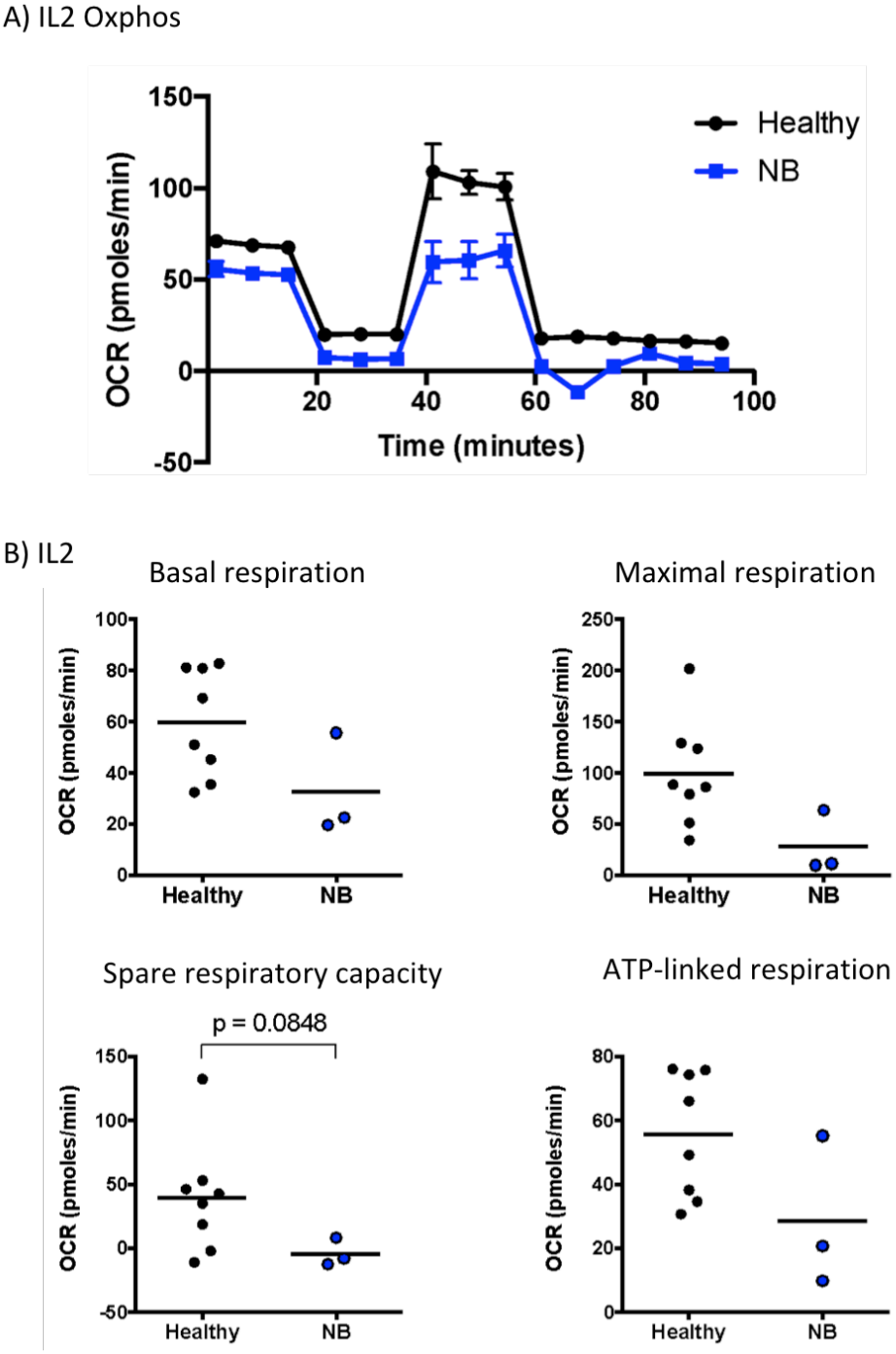


Figure 5.11 NK cells from NB patients have reduced IL2 induced oxphos.

NK cells were purified from freshly isolated PBMC from healthy donors and NB patients and stimulated with IL2 (500IU/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative oxygen consumption rate (OCR) trace of NK cells from a healthy donor and NB patient. (B) Pooled data of the IL2 stimulated oxphos readouts. Lines show the mean. Each dot represents 1 donor (n=3-8). Samples were compared using the unpaired student t-test.

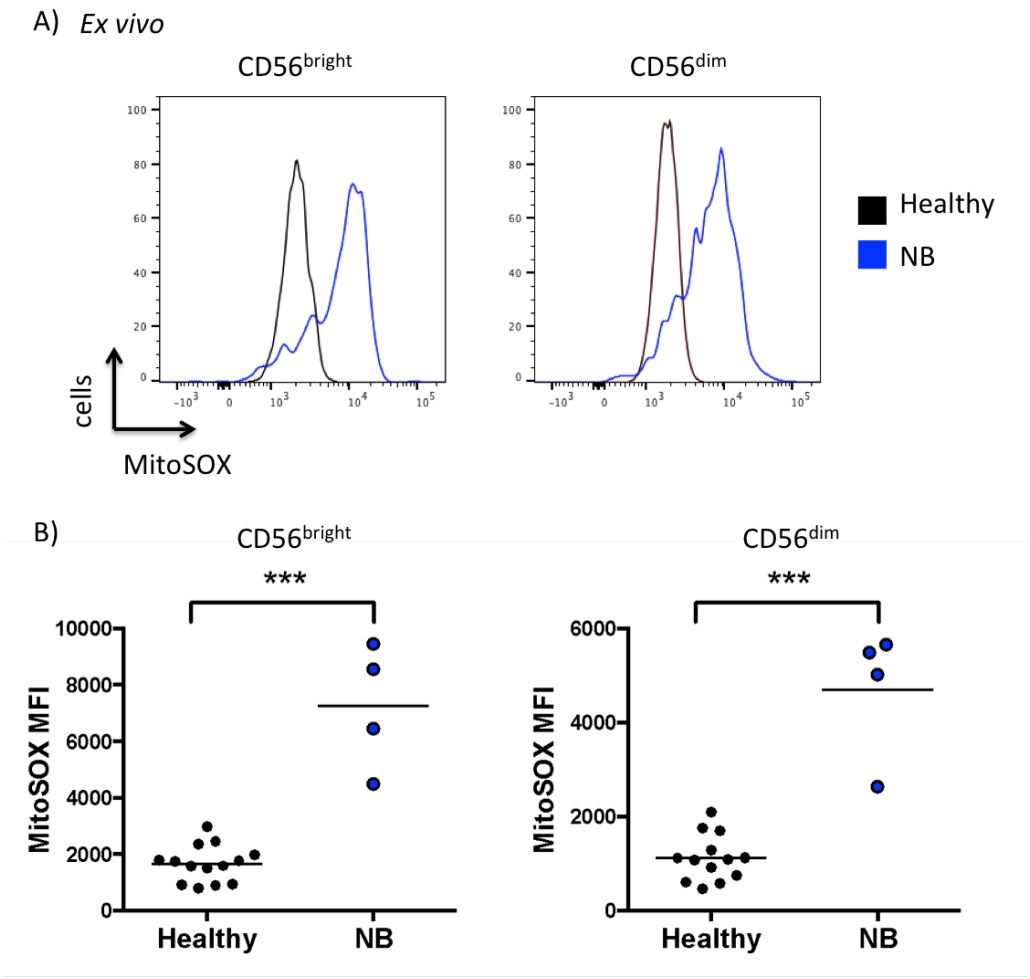


Figure 5.12 NK cells from NB patients have increased levels of mROS *ex vivo*.

Direct *ex vivo* PBMC from healthy donors and NB patients were stained with MitoSOX Red (1.5µM) dye for 15 min at 37°C. Rotenone (20µM) and MitoTEMPO (25µM) were used as positive and negative controls respectively. Cells were analysed by flow cytometry. (A) Representative histogram in *ex vivo* NK cells from healthy donors and NB patients. (B) The MFI of MitoSOX in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=4-14). Samples were compared using the unpaired student t-test, ***p<0.001.

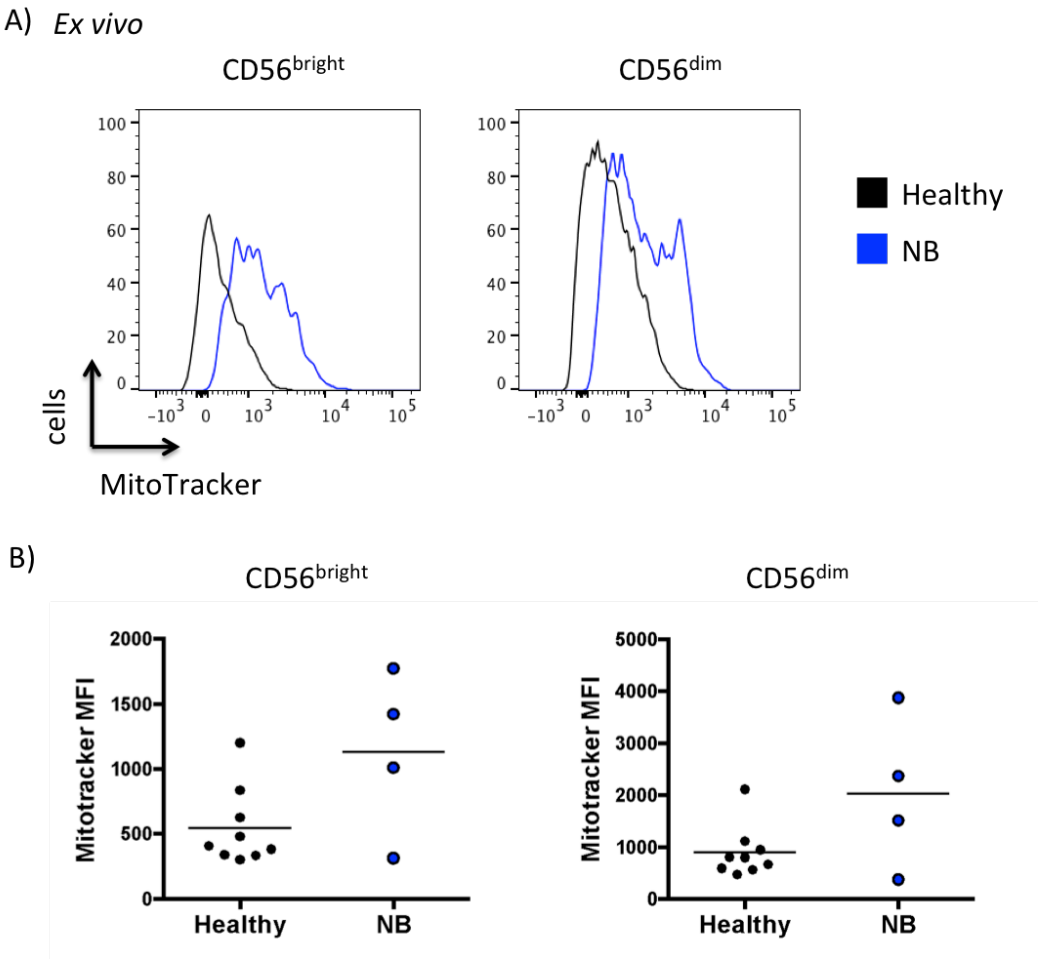


Figure 5.13 NK cells from NB patients have increased mitochondrial mass *ex vivo*.

Direct *ex vivo* PBMC from healthy donors and NB patients were stained with MitoTracker Red (100nM) for 30 min at 37°C. Cells were analysed by flow cytometry. (A) Representative histogram of *ex vivo* NK cells from healthy donors and NB patients. (B) The MFI of MitoTracker in *ex vivo* $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=4-9).

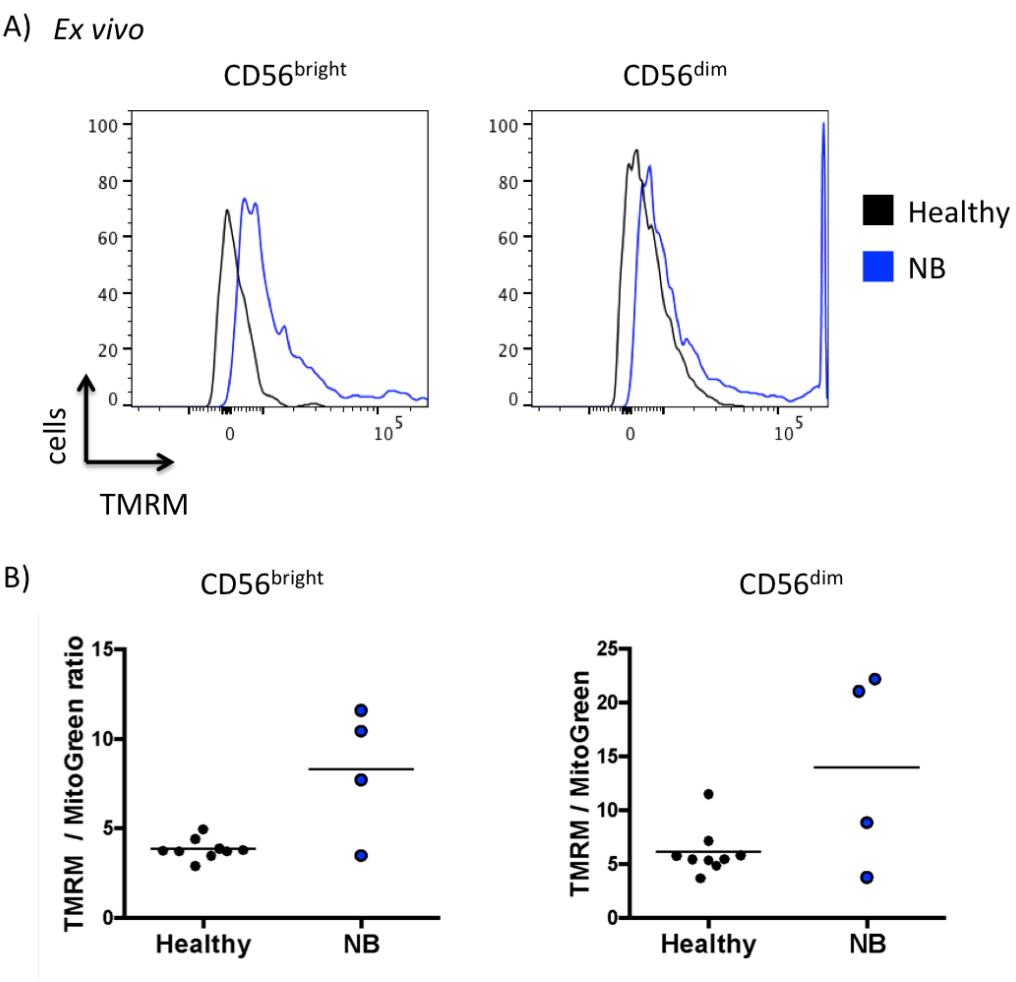


Figure 5.14 NK cells from some NB patients have increased mitochondrial membrane potential *ex vivo*.

Direct *ex vivo* PBMC were stained with TMRM (100nM) for 20 min at 37°C. Oligomycin and FCCP were used as positive and negative controls respectively. Cells were analysed by flow cytometry. MFI values were normalised against MitoTracker MFI values. (A) Representative histogram of *ex vivo* NK cells from healthy donors and NB patients. (B) The MFI of TMRM in *ex vivo* CD56^{bright} and CD56^{dim} NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=4-9).

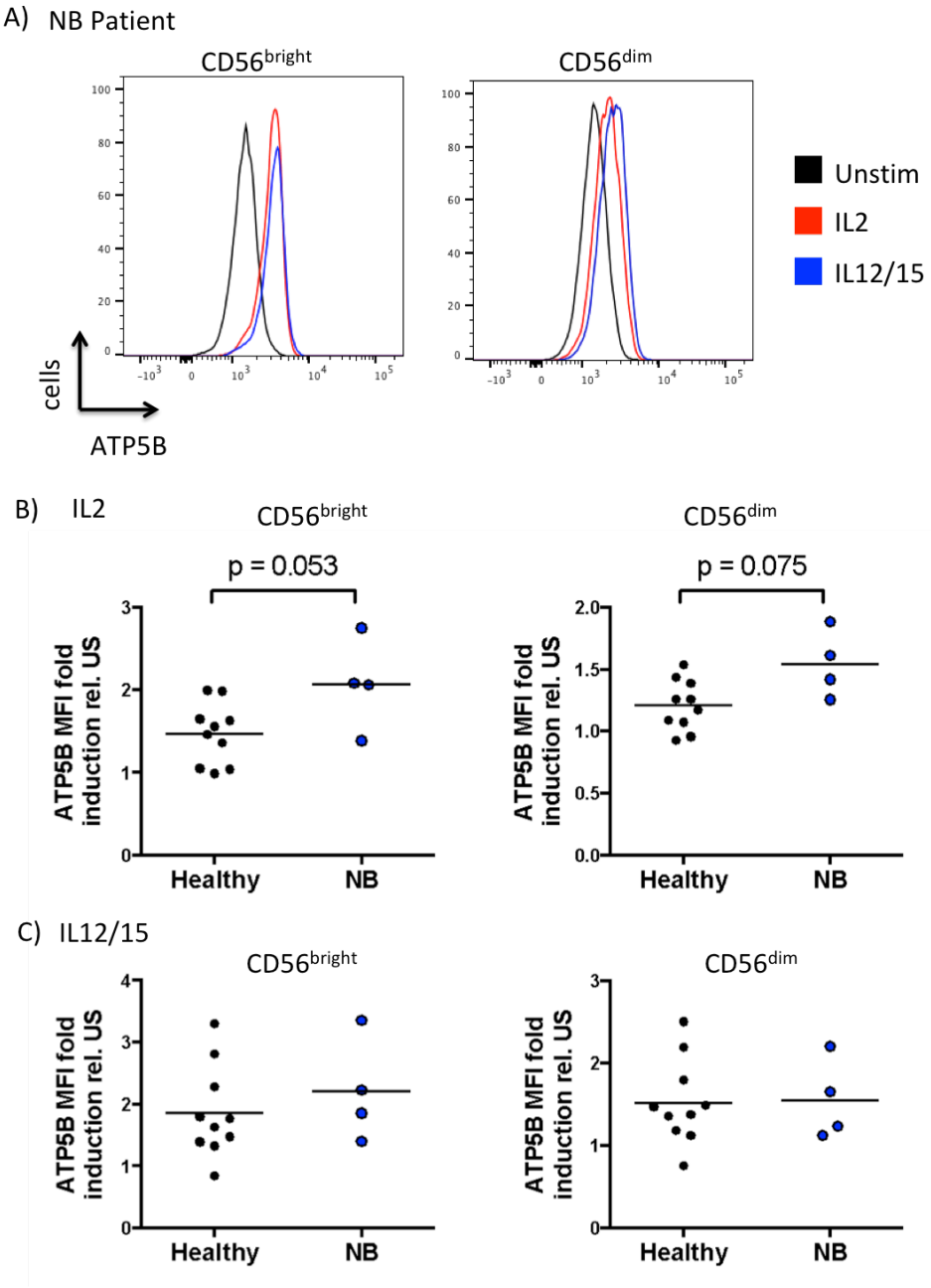


Figure 5.15 NK cells from NB patients have enhanced ATP5B expression in response to IL2 stimulation.

Freshly isolated PBMC from healthy donors and patients were stimulated with IL2 (500IU/ml) or IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. NK cells were stained for ATP5B expression and analysed by flow cytometry. (A) Representative histogram of ATP5B expression in NK cells from NB patients stimulated with IL2 and IL12/15. (B) Fold increase in ATP5B expression relative to unstimulated control in IL2 stimulated NK cells from healthy donors and NB patients. (C) Fold increase in ATP5B expression relative to unstimulated control in IL12/15 stimulated NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=4-10). Samples were compared using the unpaired student t-test.

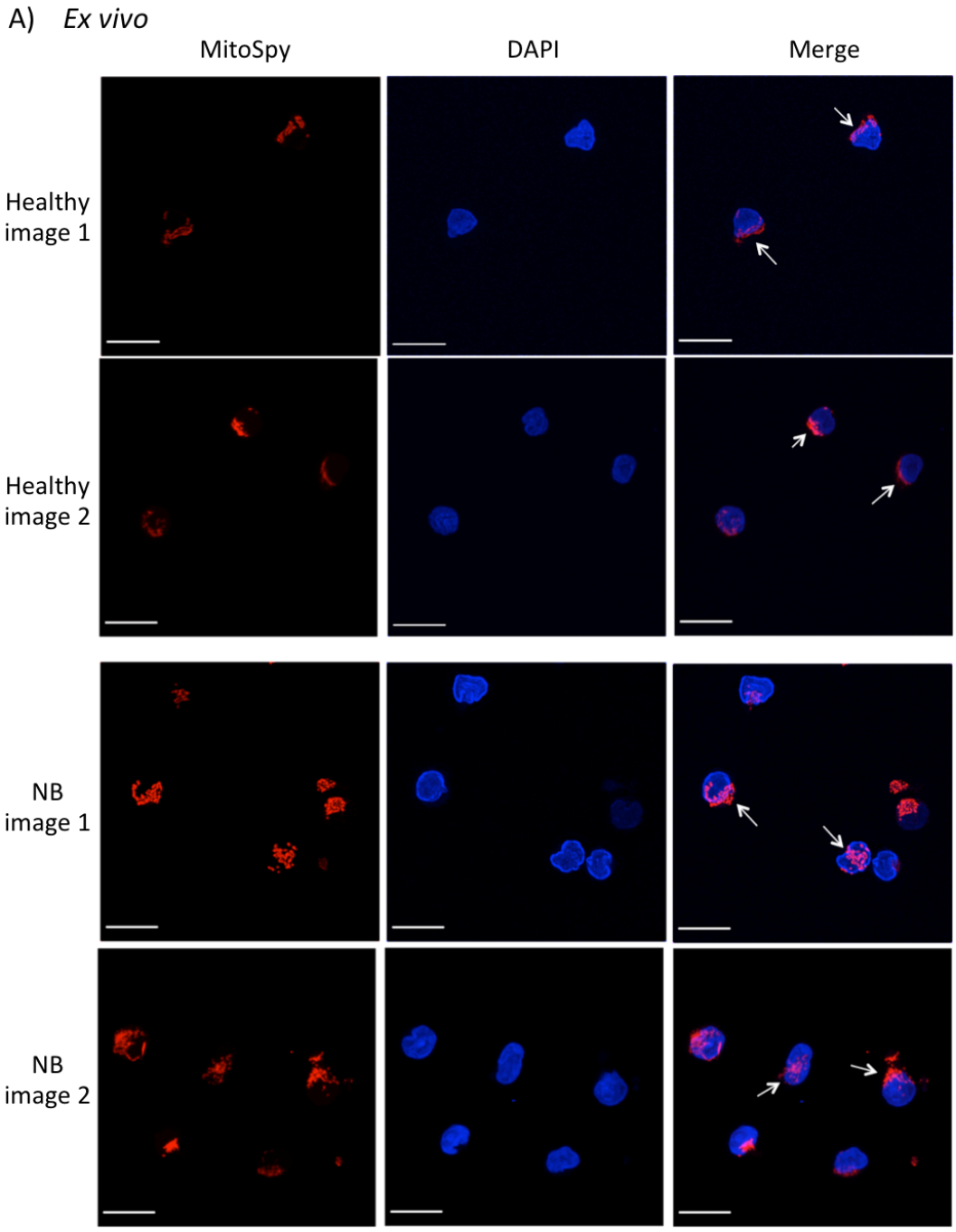


Figure 5.16 NK cells from a NB patient have increased mitochondrial fragmentation

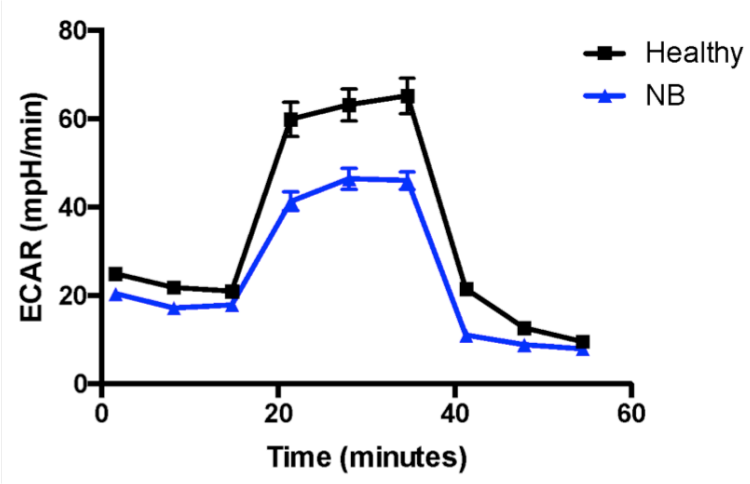
(A) Representative confocal images of purified *ex vivo* NK cells from healthy donors (n=3) and a NB patient (n=1) stained with MitoSpy CMX Ros (250nM) for 30 min at 37°C and DAPI (300nM). Images shown are the Maximum Intensity projection of Z-stacks taking at 0.2µm increments. Red= Mitospy CMX Ros, Blue= DAPI. Scale bar=5µm.

5.2.4 NK cells from NB patients have reduced glycolysis.

NK cells from NB patients clearly had severely impaired mitochondrial metabolism. Despite this, cytokine induced granzyme B and ATP5B expression, as well as SLC7A5 activity, were heightened when compared to the responses of NK cells from healthy donors. As metabolism supports NK cell functions, we predicted that NK cells from NB patients were potentially using glycolysis to drive these enhanced responses. Due to the cell number limitations described above, only IL2 induced glycolysis was measured. Surprisingly, IL2 stimulated NK cells from NB patients had reduced glycolysis when compared to those from healthy donors (**Figure 5.17A** for representative seahorse trace). Calculation of the various glycolytic metabolism parameters showed that while glycolytic reserve was normal, IL2 stimulated NK cells from NB patients had reduced levels of basal glycolysis and glycolytic capacity (**Figure 5.17B**). These results are somewhat in contrast to what was observed with NK cells from breast cancer patients, which had normal basal glycolysis, but reduced glycolytic capacity and glycolytic reserve.

These data show that some, but not all aspects of NB patient NK cell metabolism are altered in the same manner as that observed in NK from breast cancer patients. As basal glycolysis was reduced in NK cells from NB patients but not breast cancer patients, we next examined the ratio of oxphos to glycolysis (OCR/ECAR), where a higher OCR/ECAR ratio indicates more oxphos/less glycolysis and vice versa. Indeed, NK cells from NB patients had a higher OCR/ECAR ratio than those from healthy donors, indicating that they rely more heavily on oxphos rather than glycolysis (**Figure 5.17C**). While IL2 stimulated NK cells from healthy donors and breast cancer patients had an OCR/ECAR ratio of approximately 1.8 and 2.5 respectively, the ratio in NK cells from NB patients was approximately 4, again indicating an increased reliance on oxphos.

A) IL2 Glycolysis



B) IL2

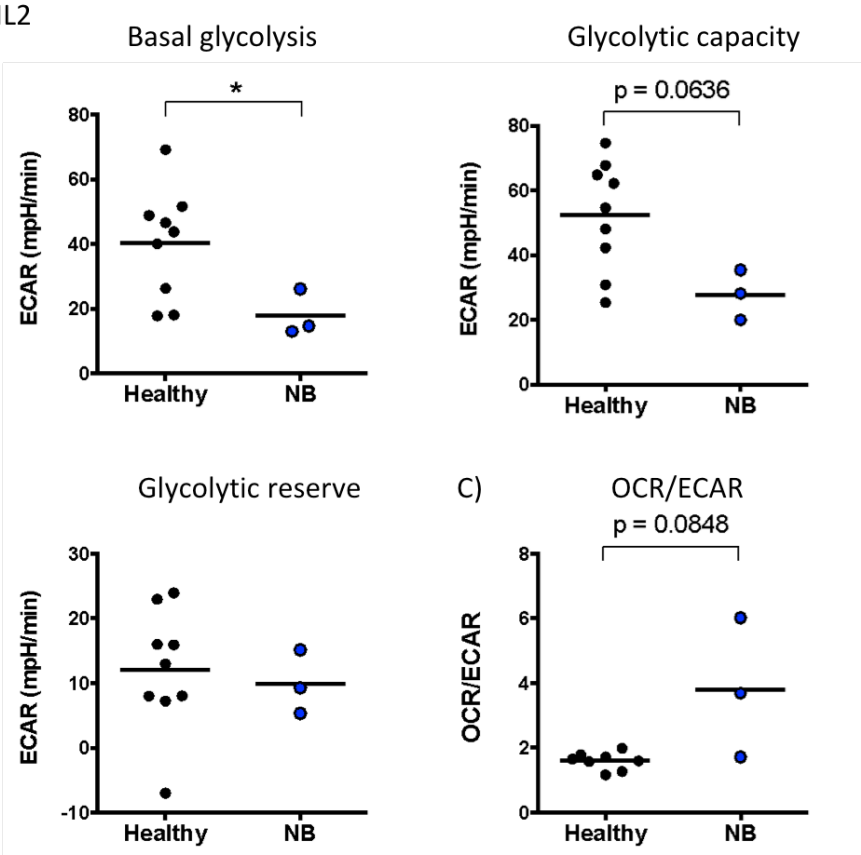


Figure 5.17 NK cells from NB patients have reduced IL2 induced glycolysis

NK cells were purified from freshly isolated PBMC from healthy donors and NB patients and stimulated with IL2 (500IU/ml) for 18 h at 37°C. Detailed metabolic analysis was performed using the Seahorse XFp Analyser. (A) Representative extracellular acidification rate (ECAR) trace of NK cells from a healthy donor and NB patient. (B) Pooled data of the IL2 stimulated glycolytic readouts. (C) Ratio of OCR/ECAR in IL2 stimulated healthy and patient NK cells. Lines show the mean. Each dot represents 1 donor (n=3-9). Samples were compared using the unpaired student t-test, *p<0.05.

5.2.5 NK cells from NB patients have altered mTORC1 activity.

Together these data show that NK cells from NB patients have significantly compromised cellular metabolism, which is associated with reduced IFNγ production. As mTORC1 is important in regulating both of these activities in healthy donor NK cells [122, 123], we next measured its activity by staining for phosphorylation of two of its downstream targets, S6 and 4EBP1, and analysing by flow cytometry. Similar to what was observed in NK cells from breast cancer, NK cells from some NB patients had increased basal mTORC1 activity (**Figure 5.18A** and **5.18B**). Of note, 2 out of the 5 patients measured had noticeably high levels of pS6 (approximately 40 and 90% for $CD56^{bright}$ subset and 30 and 35% for the $CD56^{dim}$ subset). In contrast to that observed in resting NK cells, IL2 stimulated $CD56^{bright}$ NK cells appeared to have reduced levels of pS6, while $CD56^{dim}$ NK cells had normal pS6 levels. (**Figure 5.19A** for representative dot plots, **Figure 5.19B** and **5.19C**). Paradoxically, IL2 stimulated NK cells had increased levels of p4EBP1, particular in terms of the frequency of p4EBP1 positive NK cells (**Figure 5.20A** for representative, **Figure 5.20B** and **5.20C**). A similar trend was observed for IL12/15 stimulated NK cells from NB patients, where pS6 levels appeared potentially reduced (**Figure 4.21A** for representative, **Figure 4.21B** and **4.21C**) and p4EBP1 levels appeared heightened (**Figure 4.22A** for representative, **Figure 4.22B** and **4.22C**). These findings are in contrast to what was observed in NK cells from BC patients, which had reduced levels of both pS6 and p4EBP1. Overall the data in this chapter show that altered metabolism may be a driver of NK cell dysfunction during NB, in a similar but not identical manner to that observed in NK cells from breast cancer. Indeed, these finding have important implications for the design of future NK cells based immunotherapies against NB and other paediatric solid tumours.

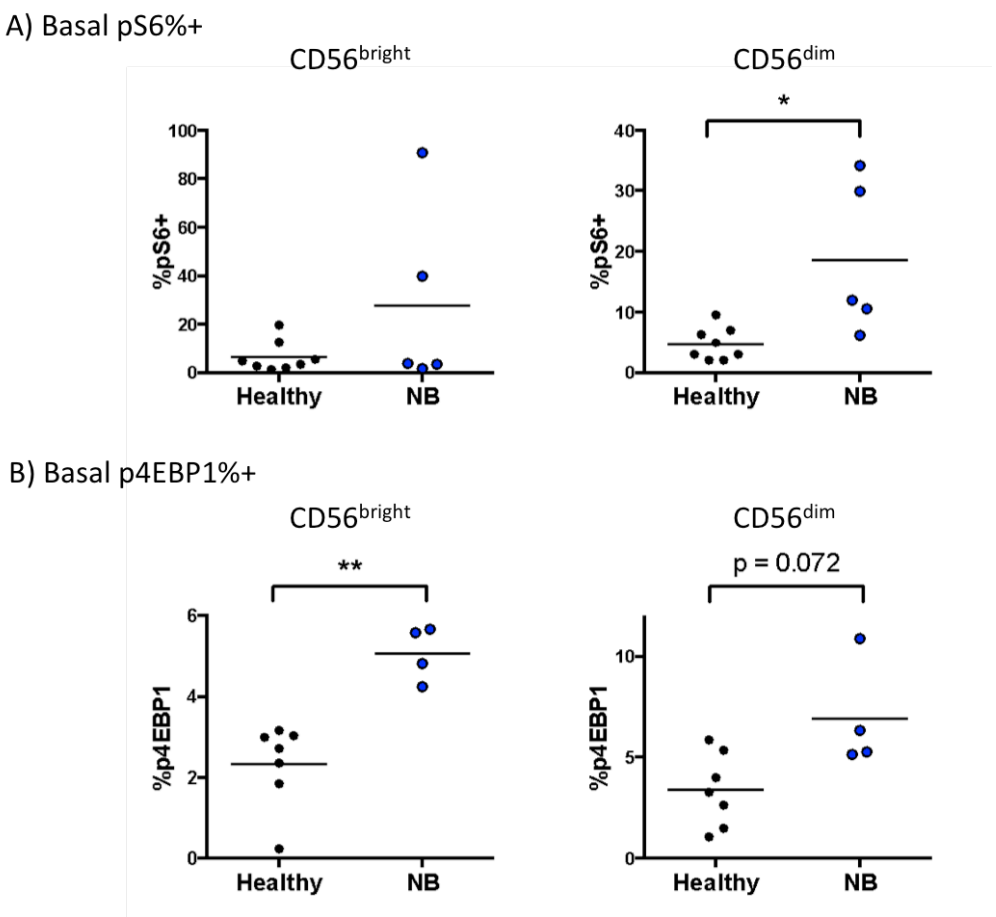


Figure 5.18 NK cells from NB patients have increased basal mTORC1 activity.

Freshly isolated PBMC from healthy donors and NB patients were analysed for mTORC1 activity by flow cytometry. (A) The frequency of NK cells positive for phosphorylated S6 protein in $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (B) The frequency of NK cells positive for phosphorylated 4EBP1 protein in $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and patient. Lines show the mean value. Each dot represents 1 donor (n=4-8). Samples were compared using the unpaired student t-test, *p<0.05, **p<0.01.

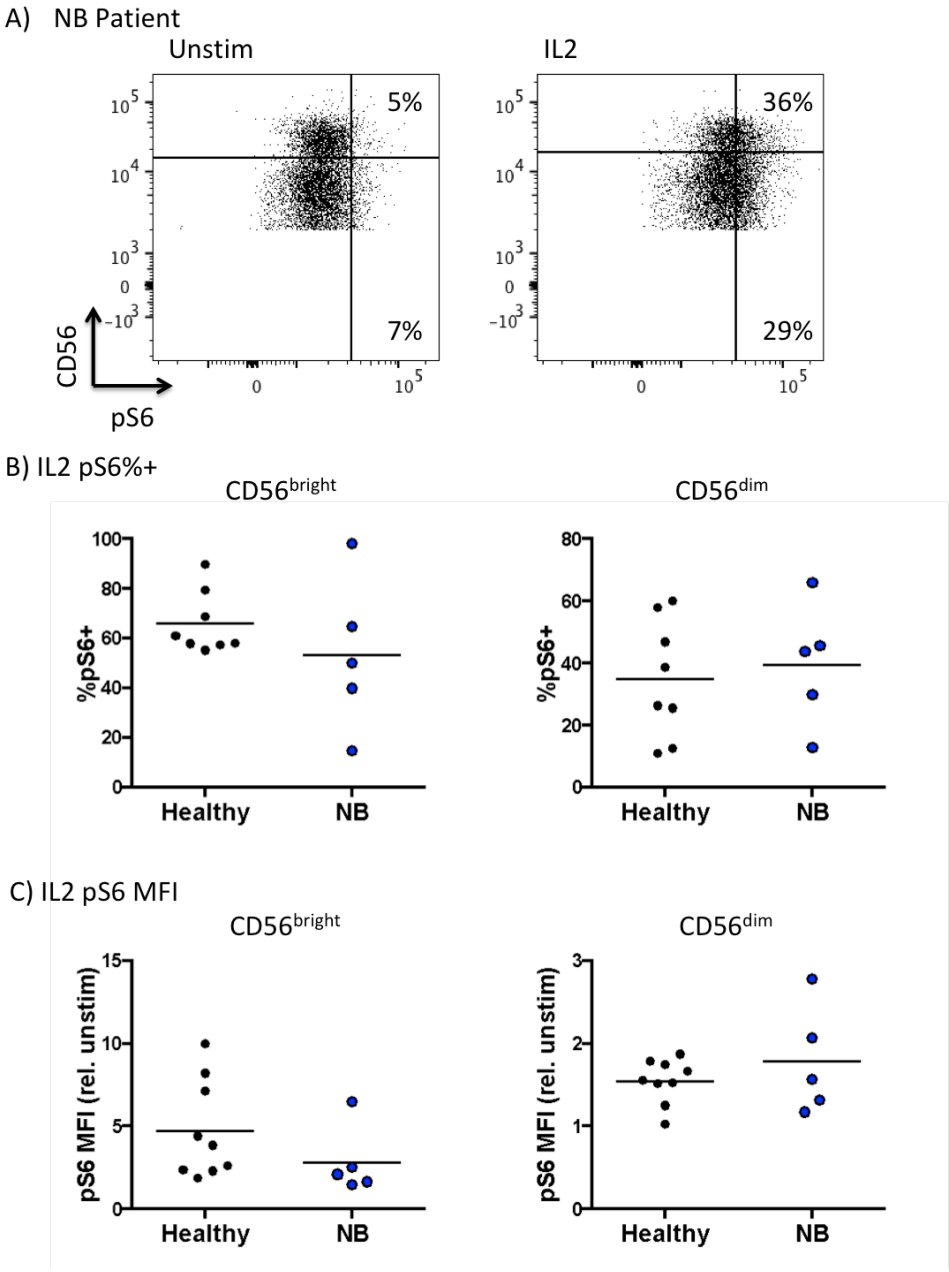


Figure 5.19 IL2 stimulated NK cells from NB patients upregulate mTORC1 activity when measured by pS6.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the upregulation of mTORC1 activity in response to IL2 in NK cells from NB patients. (B) The frequency of NK cells positive for phosphorylated S6 protein in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=5-9).

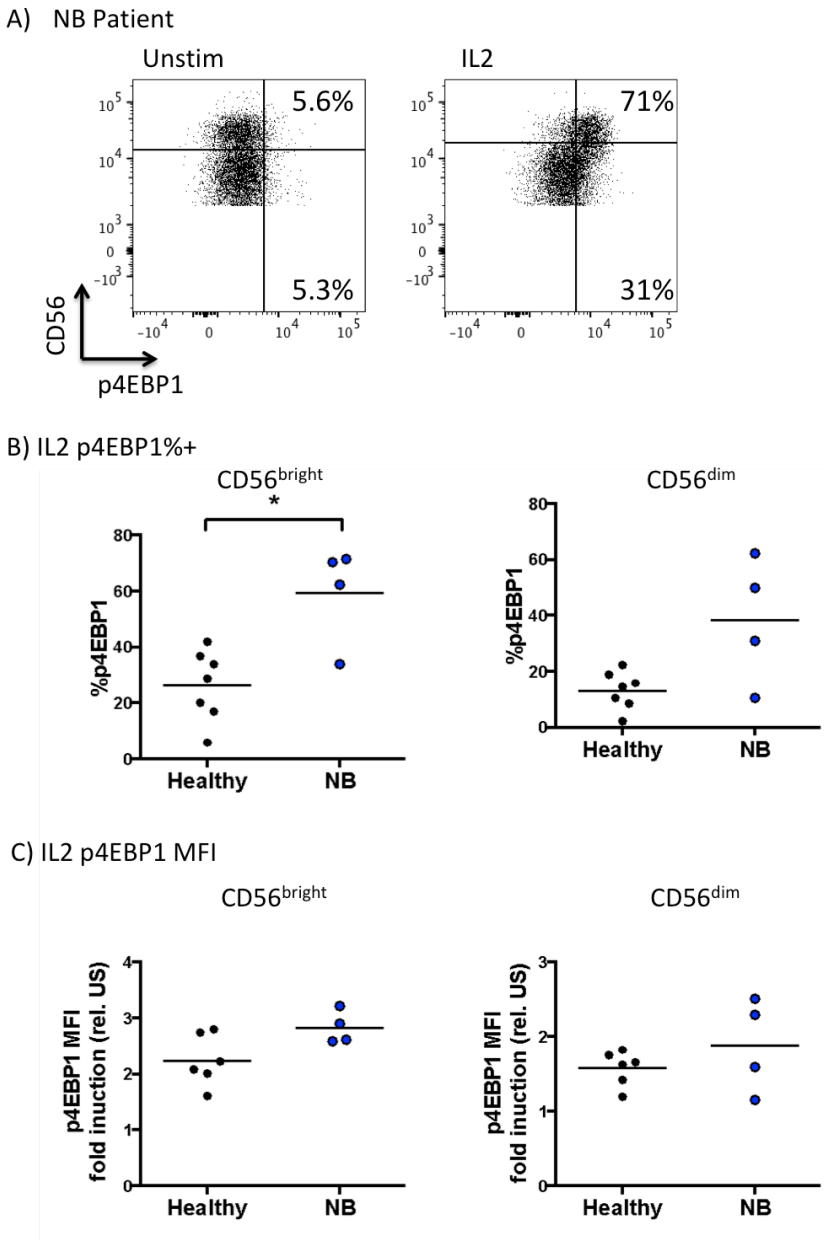


Figure 5.20 IL2 stimulated NK cells from NB patients have hyperactive mTORC1 activity when measured by p4EBP1.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) and incubated for 18 h at 37°C. Phosphorylation of 4EBP1 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the upregulation of mTORC1 activity in response to IL2 in NK cells from NB patients. (B) The frequency of NK cells positive for phosphorylated 4EBP1 protein in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) The fold increase in the MFI of phosphorylated 4EBP1 protein relative to unstimulated control in IL2 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=4-4). Samples were compared using the unpaired student t-test, *$p<0.05$.

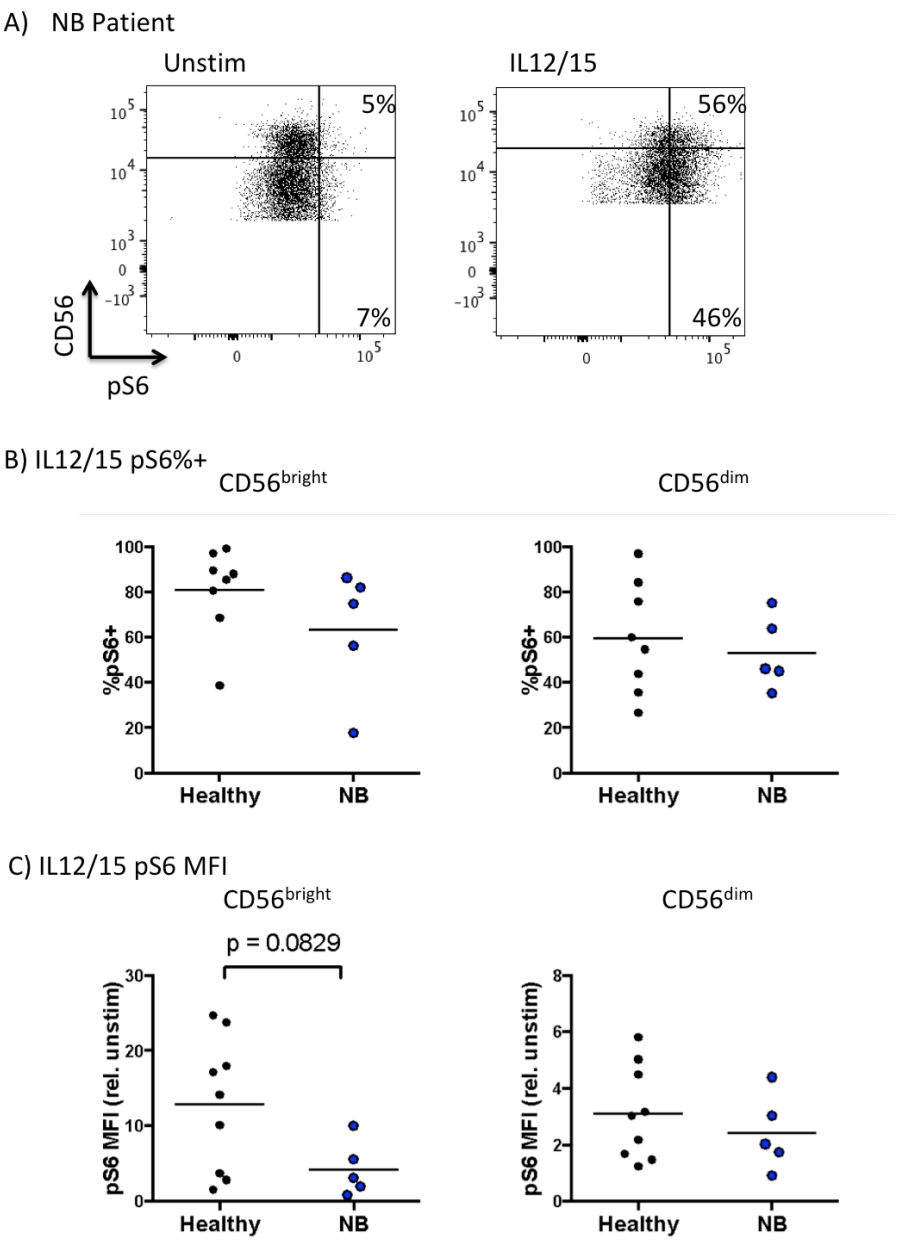


Figure 5.21 IL12/15 CD56^{bright} stimulated NK cells from NB patients have reduced mTORC1 activity when measured by pS6.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 h at 37°C. Phosphorylation of S6 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the upregulation of mTORC1 activity in response to IL12/15 in NK cells from NB patients. (B) The frequency of NK cells positive for phosphorylated S6 protein in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from healthy donors and NB patients. (C) The fold increase in the MFI of phosphorylated S6 protein relative to unstimulated control in IL12/15 stimulated CD56^{bright} and CD56^{dim} NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=5-9). Samples were compared using the unpaired student t-test.

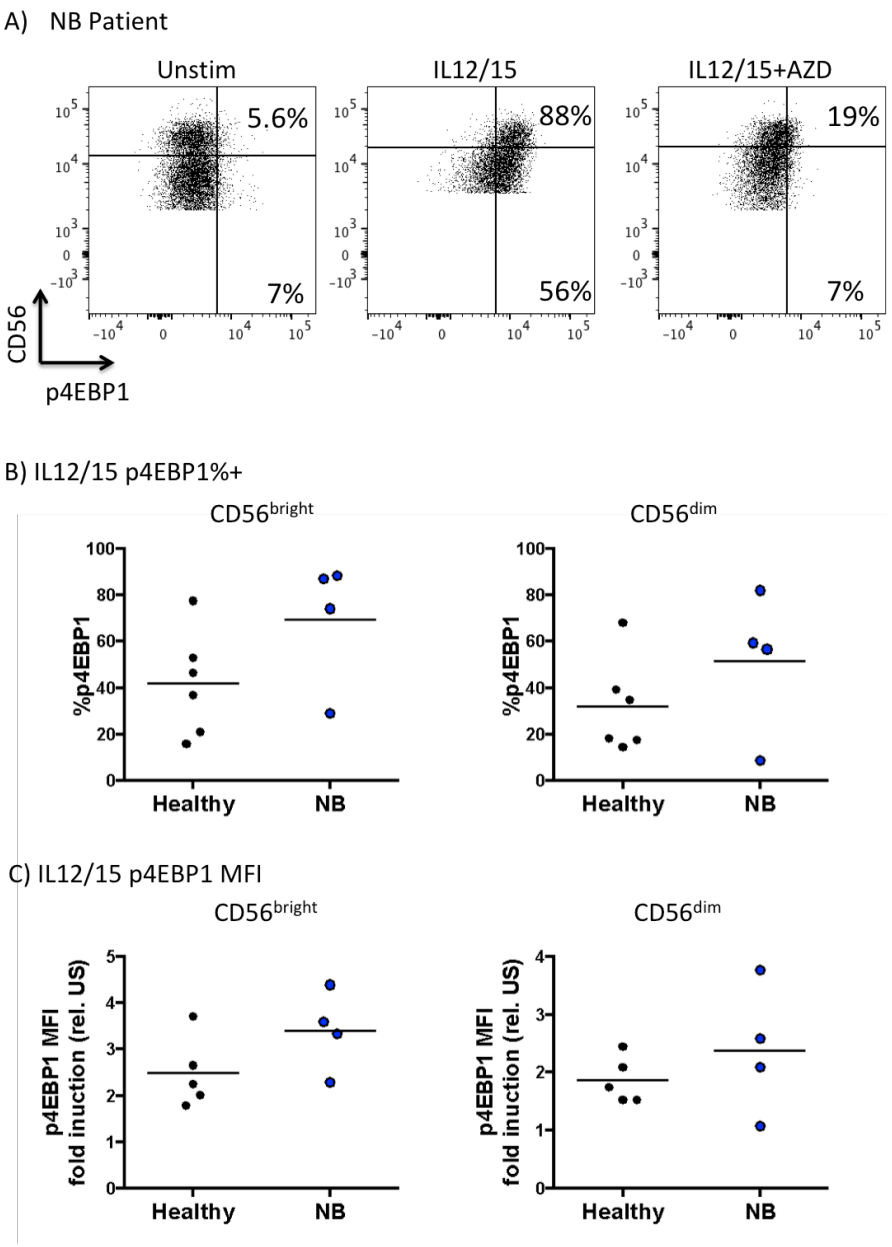


Figure 5.22 IL12/15 stimulated NK cells from some NB patients have increased mTORC1 activity when measured by p4EBP1.

Freshly isolated PBMC from healthy donors and NB patients were stimulated with IL2 (500IU/ml) and incubated for 18 h at 37°C. Phosphorylation of 4EBP1 protein was used as a readout of mTORC1 activity, and was measured by intracellular flow cytometry staining. (A) Representative dot plot of the upregulation of mTORC1 activity in response to IL12/15 in NK cells from NB patients. AZD (1μM) was used as a negative control and was added for the final 1 hour of the incubation. (B) The frequency of NK cells positive for phosphorylated 4EBP1 protein in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. (C) The fold increase in the MFI of phosphorylated 4EBP1 protein relative to unstimulated control in IL12/15 stimulated $CD56^{bright}$ and $CD56^{dim}$ NK cells from healthy donors and NB patients. Lines show the mean value. Each dot represents 1 donor (n=4-8).

5.2.6 Anti-GD2 immunotherapy increases NK cell killing of NB tumour cells.

As discussed in this chapter's introduction, anti-GD2 immunotherapy (Dinutuximab) works by enhancing NK cell ADCC against GD2+ NB tumour cells. While this therapy has been very successful in increasing survival rates of high-risk NB patients (15% survival to 40-50%) [232], there is still wide scope to improve the efficacy of this treatment in order to further increase survival rates and reduce relapse rates. We have shown that NK cells from NB patients have impaired mitochondrial and glycolytic metabolism, as well as altered mTORC1 activity. Furthermore, metabolic inhibitors such as rapamycin (inhibits mTORC1) are currently being tested in clinical trials for the treatment of NB patients, without any regard for the impact they might have on the metabolism of NK cells, and their ability to carry out ADCC. As such, it is essential that we explore the role that metabolism plays during anti-GD2 mediated ADCC, so that we can determine whether this altered patient NK cell metabolism might be impacting the efficacy of anti-GD2 treatment in NB patients, and also to identify which metabolic pathways should be targeted/protected in order to boost NK cell ADCC when a patient is undergoing antibody therapy.

For these experiments, PBMC were isolated from healthy adult donors so that cell numbers were not limiting. The key findings will be confirmed using PBMC from healthy paediatric donors in the future. We designed an *in vitro* ADCC assay using the Kelly NB tumour cell line as the target cell. It is a GD2+ high-risk cell line that harbours the MYCN amplification, meaning it has the features of tumours that are currently difficult to treat and have a tendency to relapse. In brief, Kelly tumour cells were stained with Calcein AM, coated with either anti-GD2 antibody or IgG1 isotype control, and co-cultured with PBMC at effector to target ratios of 1:1, 5:1 and 10:1 for 4 hours. Supernatants were collected, and Calcein AM dye was measured by spectrophotometry as an indicator of Kelly cell death (**Figure 5.23**).

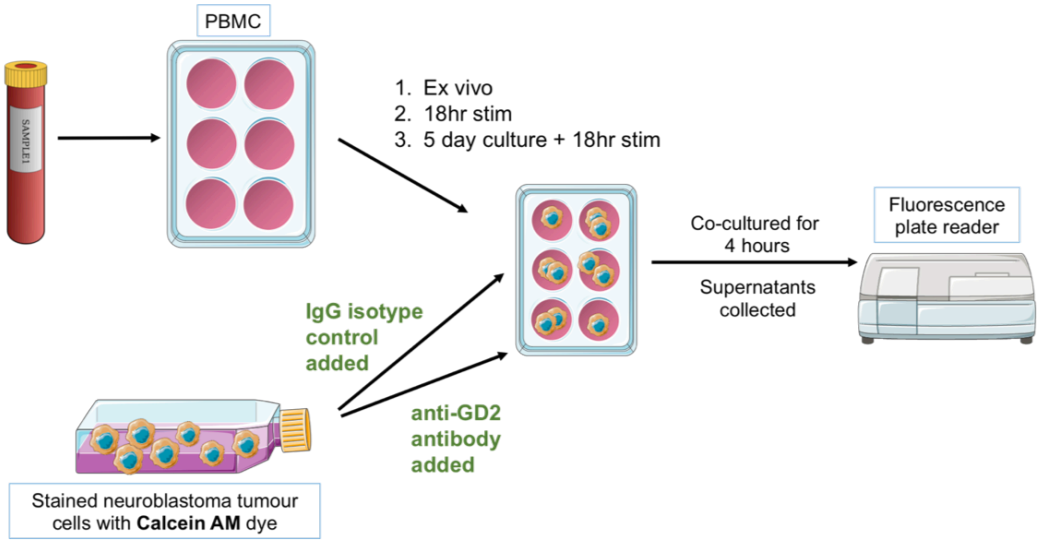


Figure 5.23 Schematic of optimisation of anti-GD2 mediated NK cell ADCC assay.

We tested a variety of culture conditions in order to determine the best model to study NK cell ADCC *in vitro*. To start, cytokine stimulated NK cells (18 hours, washed out of cytokine before plating) were used to test the assay. IL2 and IL12/15 stimulated NK cells (but not unstimulated ones) killed Kelly tumour cell coated with isotype control (indicating direct cytotoxicity). When incubated with anti-GD2 coated tumour cells, unstimulated NK cells killed a higher percentage of tumour cells than cytokine stimulated NK cells in the isotype control assay (indicating ADCC). This level of killing was not improved when the cells had been stimulated with IL2 or IL12/15 overnight (**Figure 5.24A**).

We next investigated the ability of NK cells cultured for 5 days in low dose IL15 and then restimulated (18 hours, washed out of cytokine before plating) to carry out ADCC. Compared to NK cells cultured overnight, these long term cultured NK cells had increased killing of isotype control coated Kelly cells, indicating increased ability to kill directly. Compared to unstimulated NK cells, those restimulated on day 5 with IL2 and IL12/15 for 18 hours had increased direct killing of Kelly tumour cells. IL2 stimulated NK cells in particular were best at direct killing. Surprisingly, these NK cells killed anti-GD2 coated tumour cells to equivalent levels, suggesting that the CD16 mediated specificity of ADCC that is clearly detectable in 18 hour experiments is lost after long term culture (**Figure 5.24B**). These experiments were repeated using low dose IL15 plus low dose IL2 during the 5 day culture, and similar results were obtained (data not shown).

Finally, we tested the ability of NK cells directly *ex vivo* to carry out ADCC. *Ex vivo* NK cells were unable to kill Kelly tumour cells coated with isotype control, while they were readily able to kill anti-GD2 coated tumour cells (**Figure 5.24C**). This indicates that the type of killing used was predominantly ADCC with little to no direct killing contributing, and as such we used this assay for all future work.

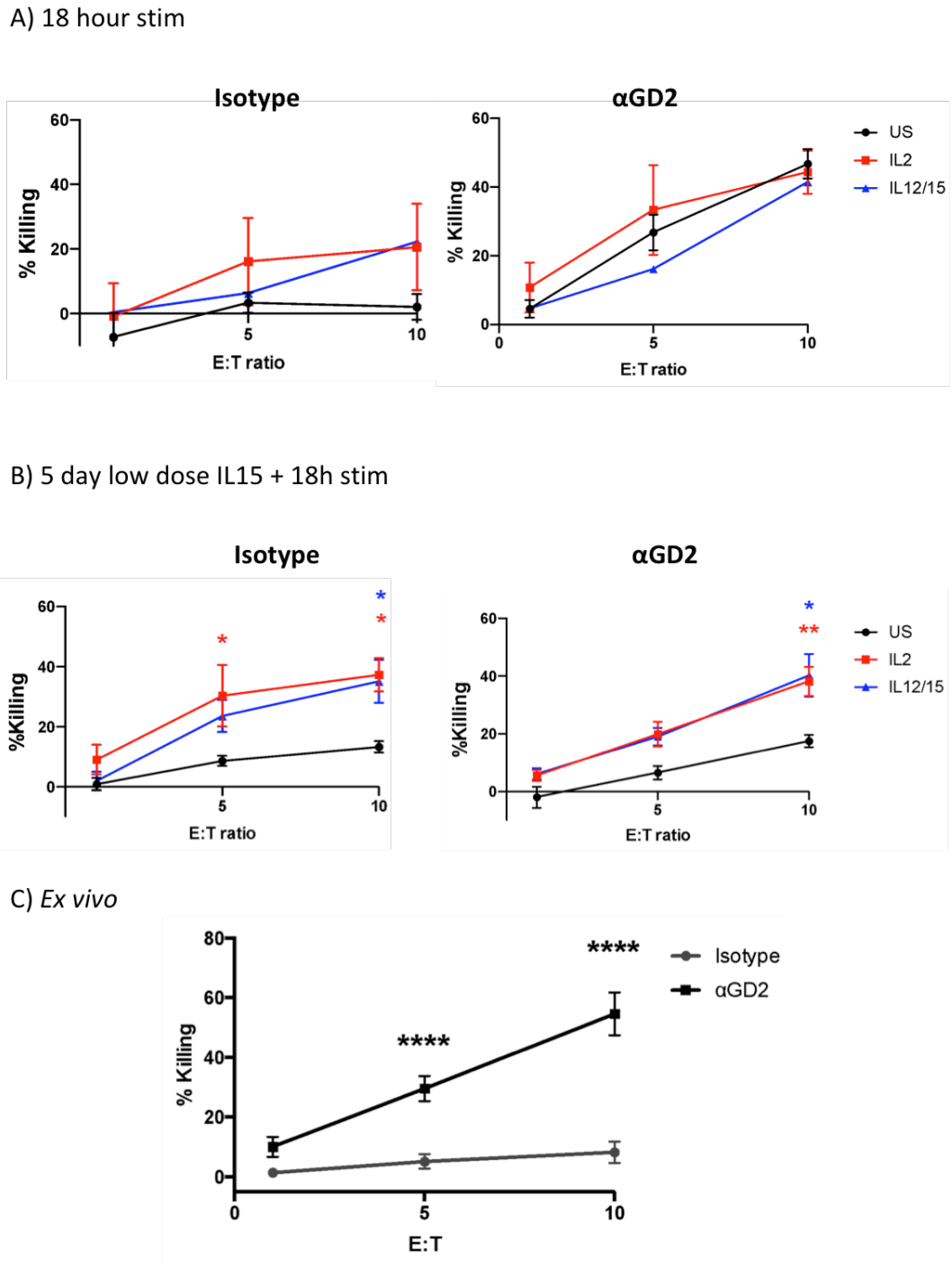


Figure 5.24 Anti-GD2 increases *ex vivo* NK cell ADCC against NB tumour cells.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with anti-GD2 antibody. Kelly cells were seeded with healthy donor PBMC at varying effector to target ratios (E:T) and incubated for 4 hrs at 37°C. Supernatants were transferred to a black plate and measured for Calcein AM fluorescence using a spectrophotometer, where increased fluorescence indicates killing. (A) Freshly isolated PBMC were stimulated with either IL2 (500IU/ml) or IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 hr at 37°C, n=6 US and IL2, n=1 IL12/15. (B) Freshly isolated PBMC were cultured for 5 days in low dose IL15 (1ng/ml). Media and cytokine was replaced on day 2 of culture. On day 5 PBMC were washed and put back at 5x106 cell/ml. PBMC were left unstimulated (basal) or stimulated with either IL2 (500IU/ml) or IL12 (30ng/ml) and IL15 (100ng/ml) and incubated for 18 hr at 37°C, n=4. (C) Freshly isolated PBMC were used directly *ex vivo*, n=6. Data points show the mean ± SEM. Samples were compared using two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The impact that exposure to tumour cells during the ADCC assay has on NK cell activation and function was next explored by comparing the expression of various activation and functional markers in *ex vivo* NK cells co-cultured with tumour cells coated with either isotype control (direct killing) or anti-GD2 antibody (ADCC). As $CD56^{bright}$ NK cells in general do not express CD16 (**Figure 5.2,** introduction), all flow cytometry analysis was carried out on $CD56^{dim}$ NK cells only. Interestingly, the frequency of CD16 expression was reduced slightly after NK cells were exposed to anti-GD2 coated tumour cells, suggesting that ADCC may be triggering CD16 shedding (previously described as a mechanism that promotes serial killing of target cells [254]) (**Figure 5.25A**). In 4 out of 5 experiments, almost 100% of NK cells were positive for CD69 expression whether cultured with isotype or anti-GD2 coated tumour cells, indicating strong baseline activation in the presence of tumour cells. In the experiment where isotype control coated tumour cells induced only 30% of NK cells to express CD69, this was increased to 70% when cultured with tumour cells and anti-GD2. In contrast, neither of the conditions induced expression of the activation marker CD25 following the 4 hour incubation.

Upon initiation of ADCC, NK cells use several mechanisms to kill target cells. Degranulation of cytotoxic molecules such as granzyme B commonly takes place, as well as engagement of death receptors such as TRAIL. Furthermore, they may induce IFNγ production in order to induce cell death pathways in target cells [255] and activate neighbouring immune cells such as macrophages. We next investigated the impact of anti-GD2 coated tumour cells versus isotype coated tumour cells on

these effector mechanisms employed by NK cells to carry out killing. CD107a was first used as a marker of degranulation. The data were variable between donors. Three of six donors had a clear increase in CD107a expression in the presence of anti-GD2 coated target cells compared to isotype control. Of the other three donors, two had higher basal levels (isotype control) that did not increased further in the presence of anti-GD2 antibody. However, there was also one donor with low CD107a expression in the presence of isotype antibody and this did not change in the presence of anti-GD2 coated target cells. Surprisingly, although there was a range of values in both, the level of granzyme B and TRAIL expression in general were equivalent in NK cells from both experimental conditions. Anti-GD2 coated tumour cells did not cause significant IFNγ production in NK cells (with one expectation) (**Figure 5.25B**). Together these data show that the human NK cell response to anti-GD2 induced ADCC is variable, but in general, it induces activation through CD69 and appears degranulation to be the primary mechanism of killing used by these NK cells.

We next looked at evidence for a potential role for metabolism in contributing to anti-GD2 mediated ADCC. CD71 and CD98, two nutrient receptors, were used as general metabolic markers. Of note, CD71 expression is more associated with $CD56^{bright}$ NK cells, and therefore CD98 is the more relevant marker for this study. Both CD71 and CD98 increased in NK cells when cultured with anti-GD2 coated tumour cells, compared to those cultured with isotype coated tumour cells (**Figure 5.26A**). Similarly, mTORC1 activity, as measured by S6 phosphorylation, consistently increased with anti-GD2 coated tumour cells (**Figure 5.26B**). These data suggest that NK cells may be actively engaging in metabolic processes to support ADCC.

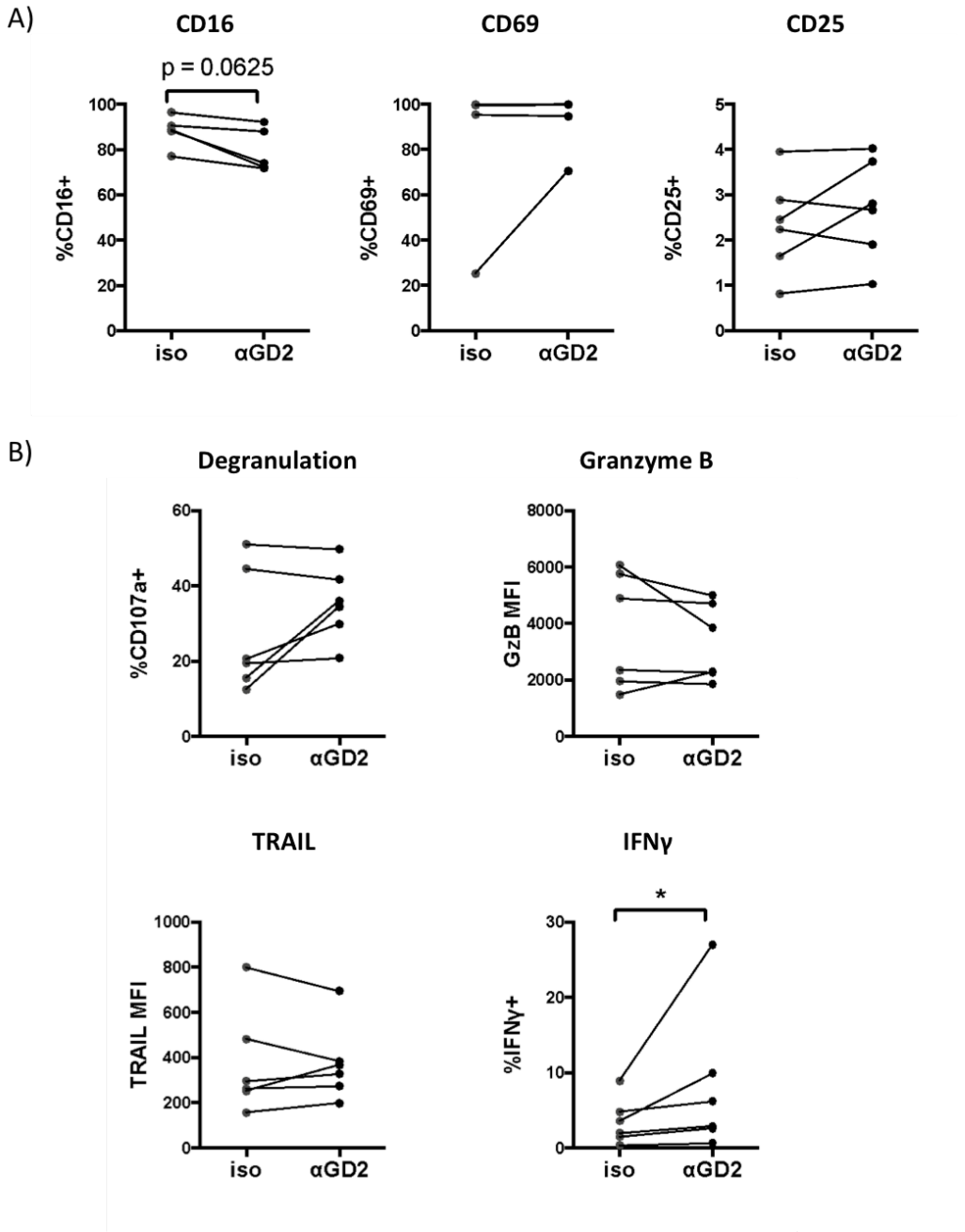


Figure 5.25 Anti-GD2 increases NK cell degranulation during ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T), anti-CD107a antibody and Golgi stop was added to each well and the plate was incubated for 4 hrs at 37°C. After the assay cells were fixed and (A) CD16, CD69 and CD25, (B) CD107a, granzyme B, TRAIL and IFNγ were measured by flow cytometry. Each line indicates an independent experiment, n=6. Samples were compared using the student t-test, *$p<0.05$.

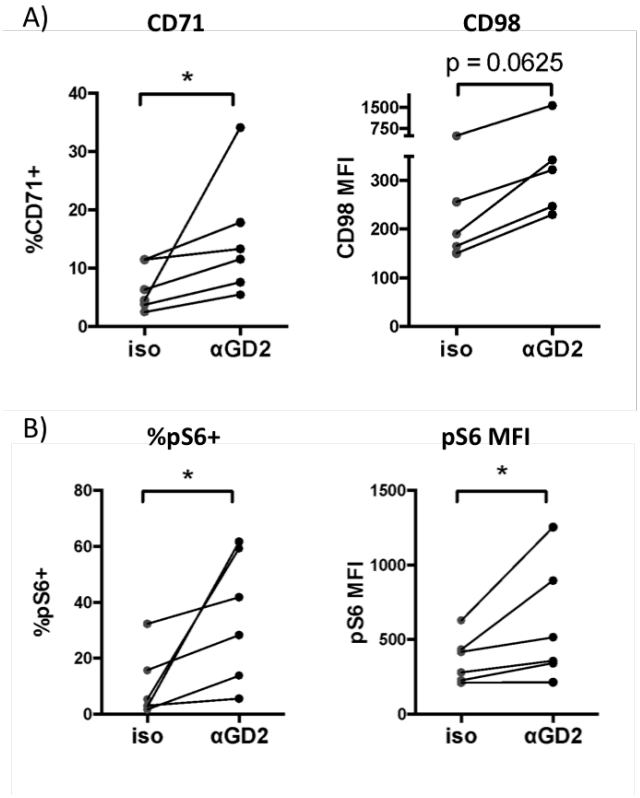


Figure 5.26 Anti-GD2 increases NK cell nutrient receptor expression and mTORC1 activity during ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T) and the plate was incubated for 4 hrs at 37°C. After the assay cells were fixed and (A) CD71 and CD98, (B) phosphorylated S6 protein were measured by flow cytometry. Each line indicates an independent experiment, n=6. Samples were compared using the student t-test, *$p<0.05$.

5.2.7 Glycolysis drives NK cell ADCC by promoting degranulation.

The above data led us to investigate what metabolic pathways might be important during anti-GD2 mediated NK cell ADCC against NB. To do this, we repeated the assay as normal, but added various metabolic inhibitors that we know impact NK cell metabolism and function (**Figure 5.27**). As a control, these inhibitors were also added to 'spontaneous release' wells (Kelly tumour cell alone, no PBMC added) to ensure they were not impacting the tumour cell directly (data not shown).

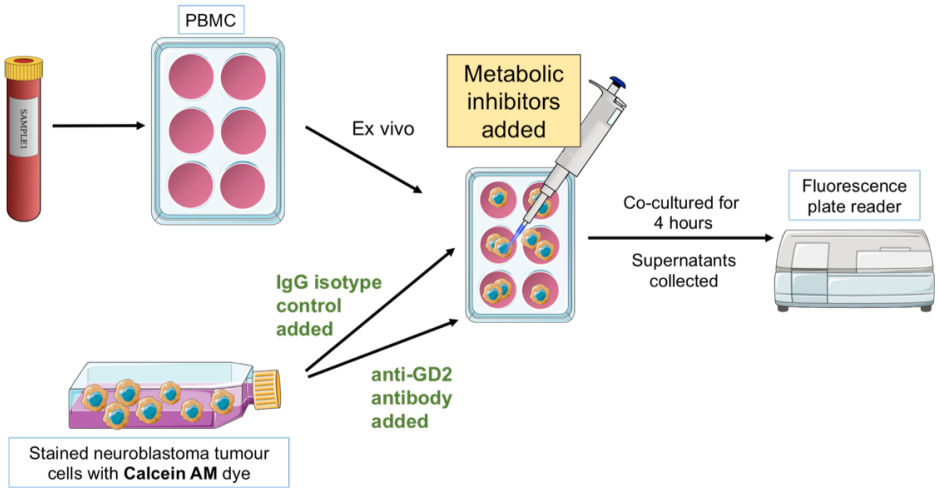


Figure 5.27 Metabolic analyses of NK cell anti-GD2 mediated ADCC.

As NK cells predominantly rely on oxphos and glycolysis to carry out their functions [122], we investigated the potential importance of these two pathways by using the oxphos inhibitor oligomycin and the glycolysis inhibitor 2DG. Somewhat surprisingly, oxphos was not required for anti-GD2 mediated ADCC in NK cells, as the presence of oligomycin had little to no impact on killing (**Figure 5.28A**). In contrast, 2DG consistently reduced killing and often to very low levels, indicating that a glycolytic burst is required for the rapid killing of tumour cells during NK cell ADCC (**Figure 5.28B**).

We next questioned why glycolysis is important in driving NK cell ADCC. To answer this, we investigated the impact of 2DG on the activation and effector mechanisms of NK cell undergoing anti-GD2 mediated ADCC. 2DG did not impact CD16 or CD69 expression, indicating that the decrease in killing is not related to a change in these surface receptors (**Figure 5.29A**). While 2DG did not impact expression of granzyme B, TRAIL nor IFNγ production, it consistently reduced the level of degranulation, suggesting that the glycolytic burst may be required to rapidly provide ATP needed for this complex process (**Figure 5.29B**).

As nutrient receptor expression and mTORC1 activity increase during ADCC, we next examined the impact of 2DG on these metabolic and signalling outputs. While 2DG did not impact CD71, it reduced CD98 expression in NK cells undergoing ADCC (**Figure 5.30A**). Furthermore, 2DG consistently reduced mTORC1 activity in NK cells in the presence of anti-GD2 coated tumour cells (**Figure 5.30B**). Together these data suggest that glycolysis is important in driving amino acid uptake, mTORC1

activity and degranulation, all of which are essential for optimal anti-GD2 mediated ADCC against NB.

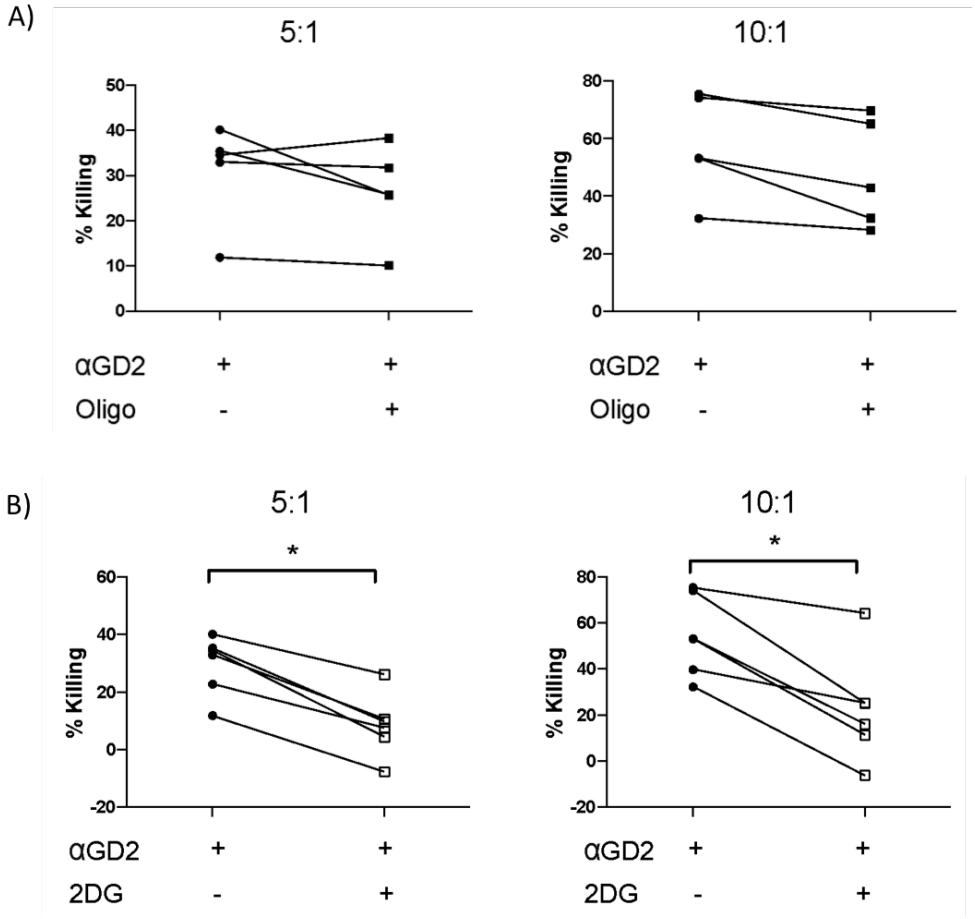


Figure 5.28 Glycolysis drives NK cell anti-GD2 mediated ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T) and the plate was incubated for 4 hrs at 37°C. Supernatants were transferred to a black plate and measured for Calcein AM fluorescence using a spectrophotometer, where increased fluorescence indicates killing. (A) Oligomycin (40nM) added at beginning of the assay to inhibit NK cell oxphos. (B) 2DG (2.5mM) added at beginning of the assay to inhibit NK cell to inhibit glycolysis. Each line indicates an independent experiment, n=5-6. Samples were compared using the student t-test, *$p<0.05$.

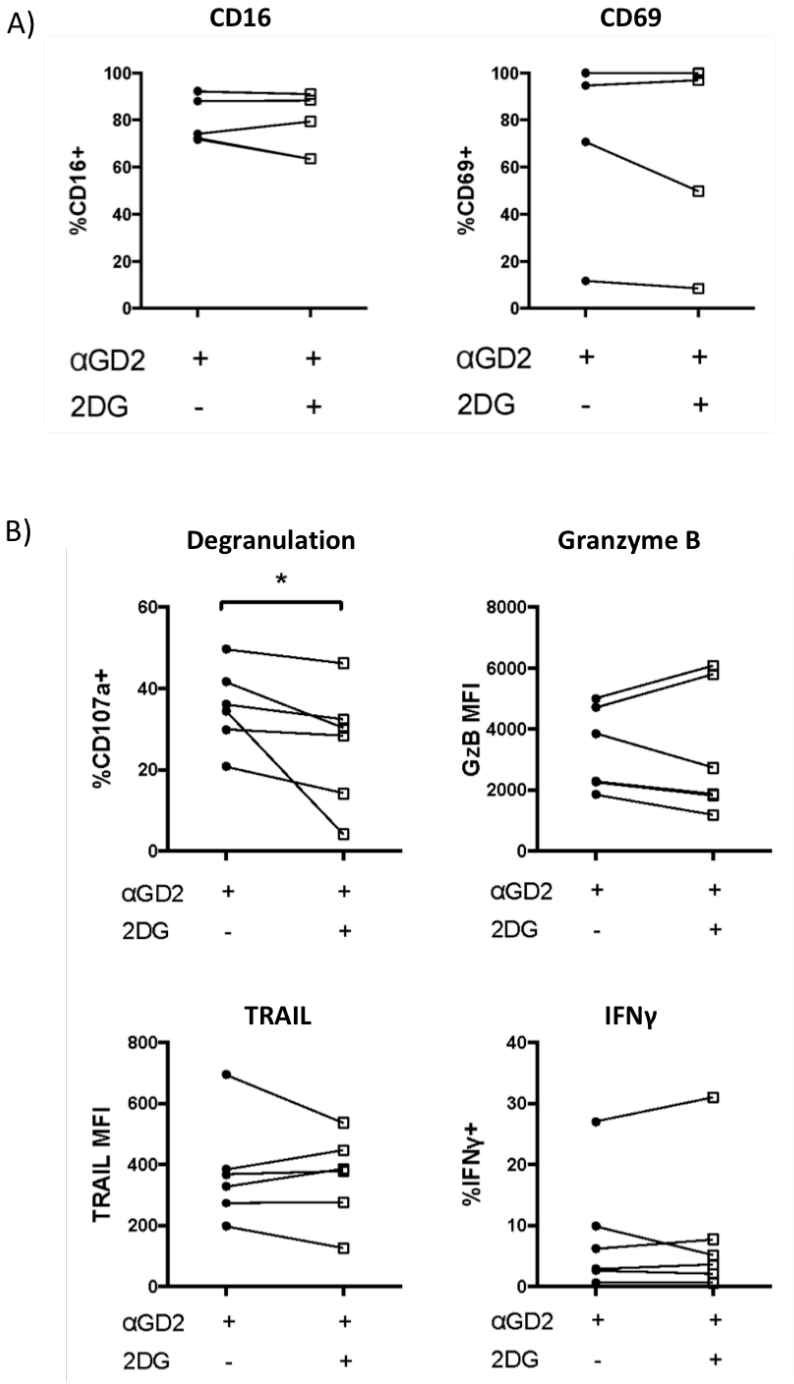


Figure 5.29 Glycolysis promotes degranulation during NK cell anti-GD2 mediated ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T). Anti-CD107a antibody, Golgi stop and 2DG (2.5mM) was added was added to each well and the plate was incubated for 4 hrs at 37°C. After the assay cells were fixed and (A) CD16 and CD69, (B) CD107a, granzyme B, TRAIL and IFNγ were measured by flow cytometry. Each line indicates an independent experiment, n=5-6. Samples were compared using the student t-test, *$p<0.05$.

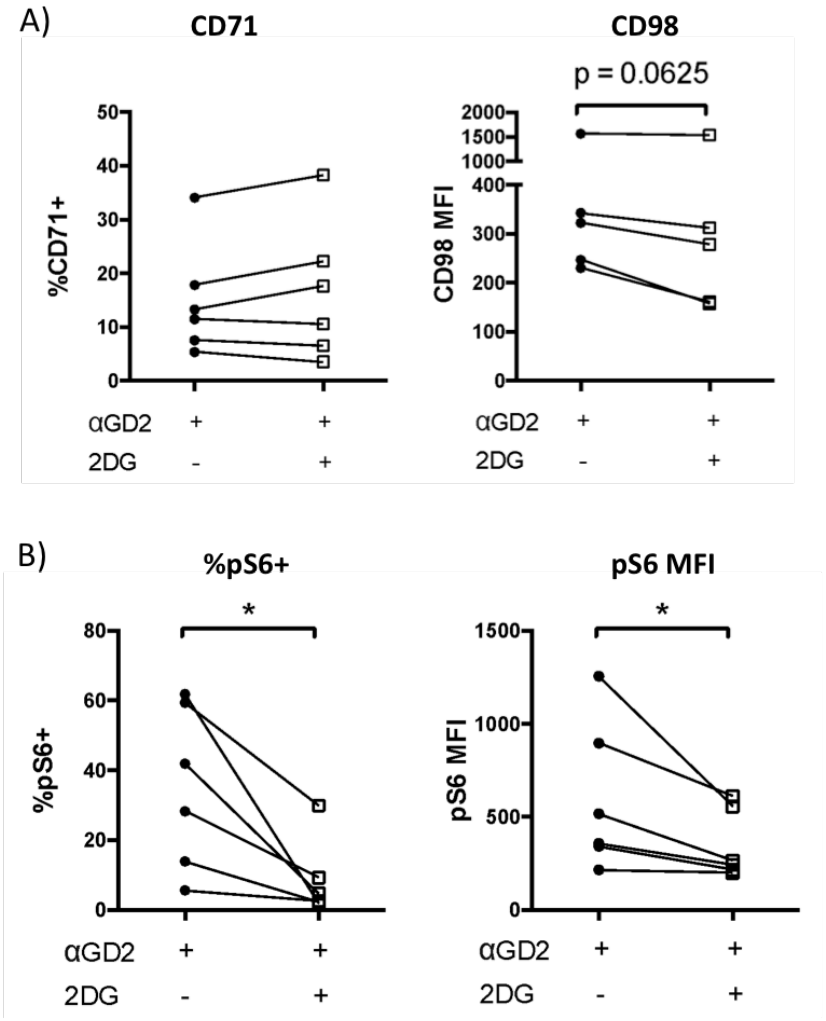


Figure 5.30 Glycolysis promotes CD98 expression and mTORC1 activity during NK cell anti-GD2 mediated ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T). 2DG (2.5mM) was added and the plate was incubated for 4 hrs at 37°C. After the assay cells were fixed and (A) CD71 and CD98, (B) phosphorylated S6 protein were measured by flow cytometry. Each line indicates an independent experiment, n=5-6. Samples were compared using the student t-test, *$p<0.05$.

5.2.8 SLC7A5 amino acid transport drives NK cell ADCC

The data thus far suggest that mTORC1 is essential during NK cell anti-GD2 mediated NK cells against NB. Indeed, the presence of anti-GD2 strongly induces mTORC1 activity, and the 2DG mediated reduction in ADCC is associated with reduced mTORC1 activity. Therefore, we next investigated the direct role that mTORC1 plays during ADCC using the pharmacological inhibitor rapamycin. As expected, rapamycin reduced pS6 levels (**Figure 5.32A**). However, it did not impact ADCC, and in fact appeared to slightly increase killing in some cases at the 10:1 ratio (**Figure 5.32B**). This surprising result suggests that directly inhibiting mTORC1 with rapamycin may trigger a compensatory mechanism that mitigates the impact of mTORC1 inhibition. This possibility warrants further investigation in the future.

We next explored the role of other metabolic regulators that are known to be important for NK cell functions. SREBP is a transcription factor that has been shown to play an essential role in driving murine NK cell glucose fuelled metabolism (including glycolysis) and anti-cancer functions [124]. Therefore we next explored whether SREBP might play a role in promoting anti-GD2 mediated human NK cell ADCC using the pharmacological inhibitor 25-hydrocycholesterol (25HC). The mechanism of action of 25HC is shown in **Figure 5.31**. Again, the results were surprising – 25HC did not reduce NK cell ADCC against NB, and in fact appeared to boost ADCC quite consistently at the 5:1 ratio (**Figure 5.32C**). These data highlight the need to explore the role of SREBP in human NK cells, as it now seems possible that it may be playing a different role in human NK cells than in murine NK cells.

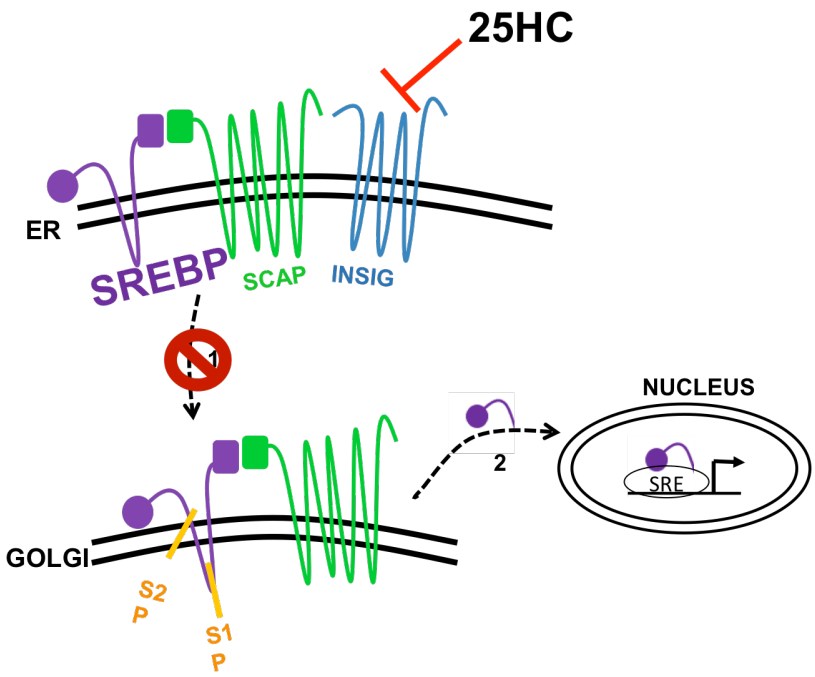


Figure 5.31 25HC inhibits SREBP activation.

SREBP is synthesized as an integral membrane protein localized to the ER. Generation of activated SREBP transcription factors requires SCAP-mediated translocation of SREBP to the Golgi followed by two cleavage events mediated by site-1 protease (S1P) and site-2 protease (S2P). The result is the release of soluble SREBP that can translocate to the nucleus and promote the expression of target genes that contain SREBP response elements (SRE). 25HC binds to INSIG and promotes the SCAP:INSIG interaction preventing SREBP activation.

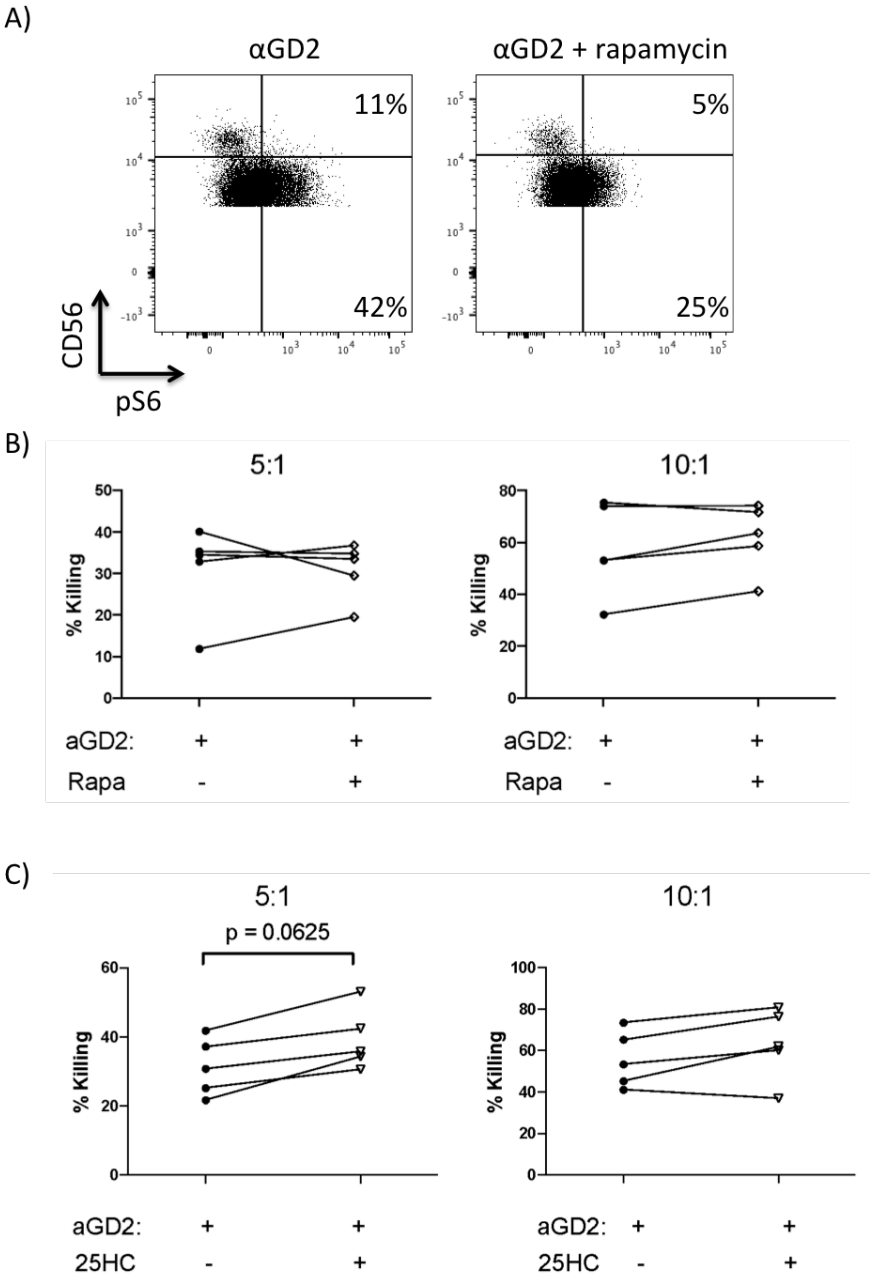


Figure 5.32 mTORC1 and SREBP are not required for NK cell anti-GD2 mediated ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T) and the plate was incubated for 4 hrs at 37°C. Supernatants were transferred to a black plate and measured for Calcein AM fluorescence using a spectrophotometer, where increased fluorescence indicates killing. (A) Rapamycin inhibits NK cell mTORC1 activity when measured by pS6. (B) Rapamycin (20nM) added at beginning of the assay to inhibit NK cell mTORC1 activity. (c) 25HC (5μM) added at beginning of the assay to inhibit NK cell SREBP activity. Each line indicates an independent experiment, n=5. Samples were compared using the student t-test.

The earlier data hints at a potential important role for amino acid metabolism in driving ADCC. Indeed, the presence of anti-GD2 induces CD98 expression in NK cells, and the 2DG mediated reduction in ADCC is associated with reduced CD98 (but not CD71) expression. Amino acid transport through SLC7A5/SLC3A2 (LAT1) has previously been shown to play an essential role in supporting expression of the transcription factor cMyc in murine NK cells [125]. In this study, amino acid levels acutely regulated cMyc protein. When SLC7A5 was blocked using the inhibitor BCH, cMyc protein was rapidly degraded, and NK cell function and metabolism (including glycolysis) was reduced. Therefore, we next investigated the impact of blocking SLC7A5 with BCH during NK cell anti-GD2 mediated ADCC. BCH strikingly almost abolished all killing induced by anti-GD2 coated tumour cells (**Figure 5.33A**). We also examined the impact on BCH on cMyc expression. IL2 stimulation increased cMyc expression in $CD56^{bright}$ NK cells, but not $CD56^{dim}$ NK cells (**Figure 5.33B**). Similar to what was observed in murine NK cells, BCH reduced basal and IL2 induced cMyc expression in NK cells, suggesting that the negative impact of BCH on ADCC may be connected with reduced cMyc expression (**Figure 5.33C**). Overall the data indicate that amino acid transport through SLC7A5 is essential in driving NK cell anti-GD2 mediated ADCC against NB.

We next explored how BCH might be impacting NK cell ADCC by examining the effect of BCH on NK cell activation and effector mechanisms. In these experiments, NK cells were fully activated in the presence of tumour cells, as measured by CD69 expression, and BCH did not inhibit this (**Figure 5.34A**). Surprisingly, BCH did not impact any of the effector mechanisms measured – degranulation, granzyme B, TRAIL and IFNγ (**Figure 5.34B**). Similar to 2DG, BCH did not impact CD71 expression, but consistently reduced CD98 expression, suggesting the existence of a potential negative feedback loop regulating SLC7A5 activity (**Figure 5.35A**). Consistent with what was observed in murine NK cells, BCH reduced mTORC1 activity in human NK cells undergoing anti-GD2 mediated ADCC (**Figure 5.35B**). Overall, the mechanism by which BCH blocks ADCC warrants much further exploration, but that data appears clear that SLC7A5 transport is essential for NK cell ADCC.

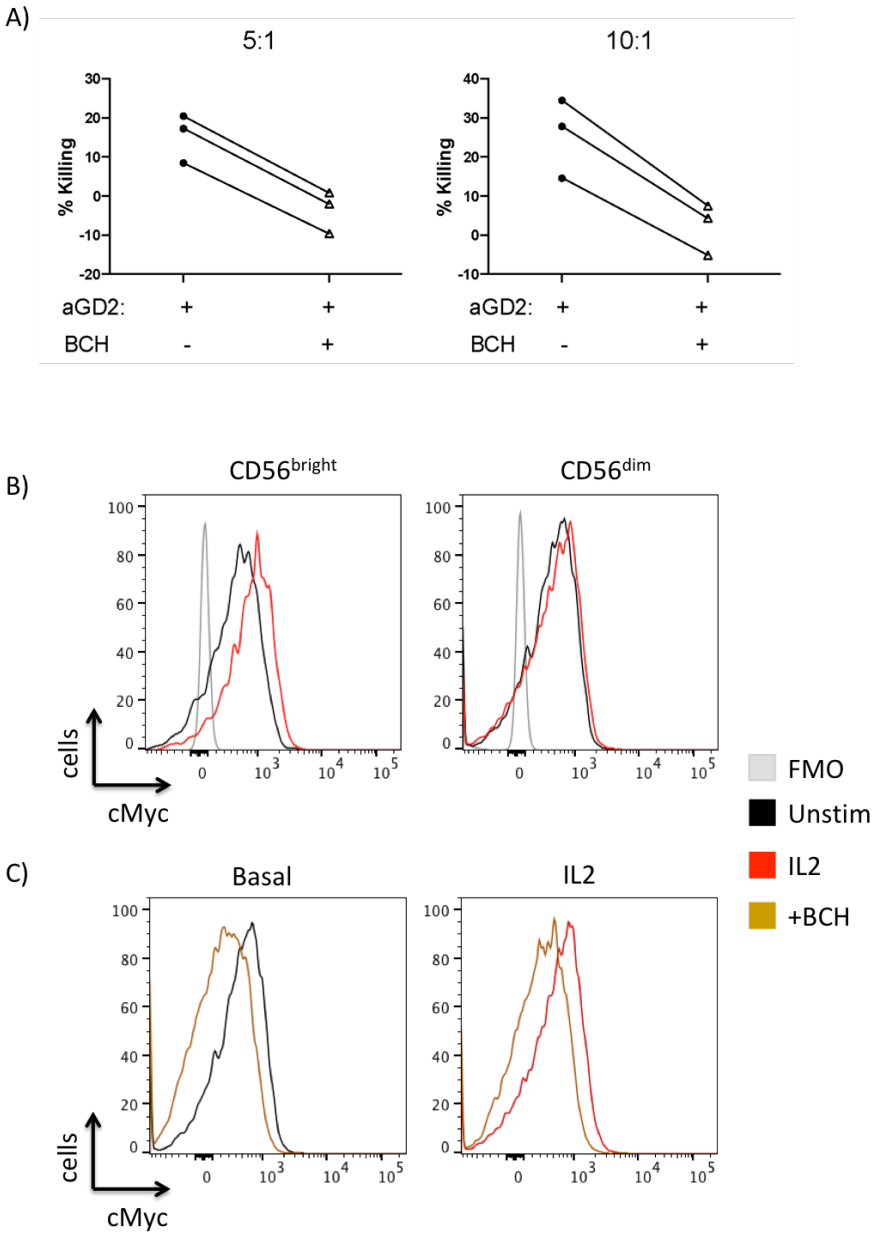


Figure 5.33 SLC7A5 amino acid transport drives NK cell anti-GD2 mediated ADCC and supports cMyc expression.

(A) Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells and freshly isolated *ex vivo* PBMC were cultured at varying effector to target ratios (E:T). BCH was added to inhibit SLC7A5 activity and the plate was incubated for 4 hrs at 37°C. Supernatants were transferred to a black plate and measured for Calcein AM fluorescence using a spectrophotometer, where increased fluorescence indicates killing. (B and C) Freshly isolated PBMC from healthy donors were stimulated with IL2 (500IU/ml) in the presence or absence of BCH (25mM) and incubated for 18 h at 37°C. Cells were stained for cMyc and analysed by flow cytometry. Each line indicates an independent experiment, n=3.

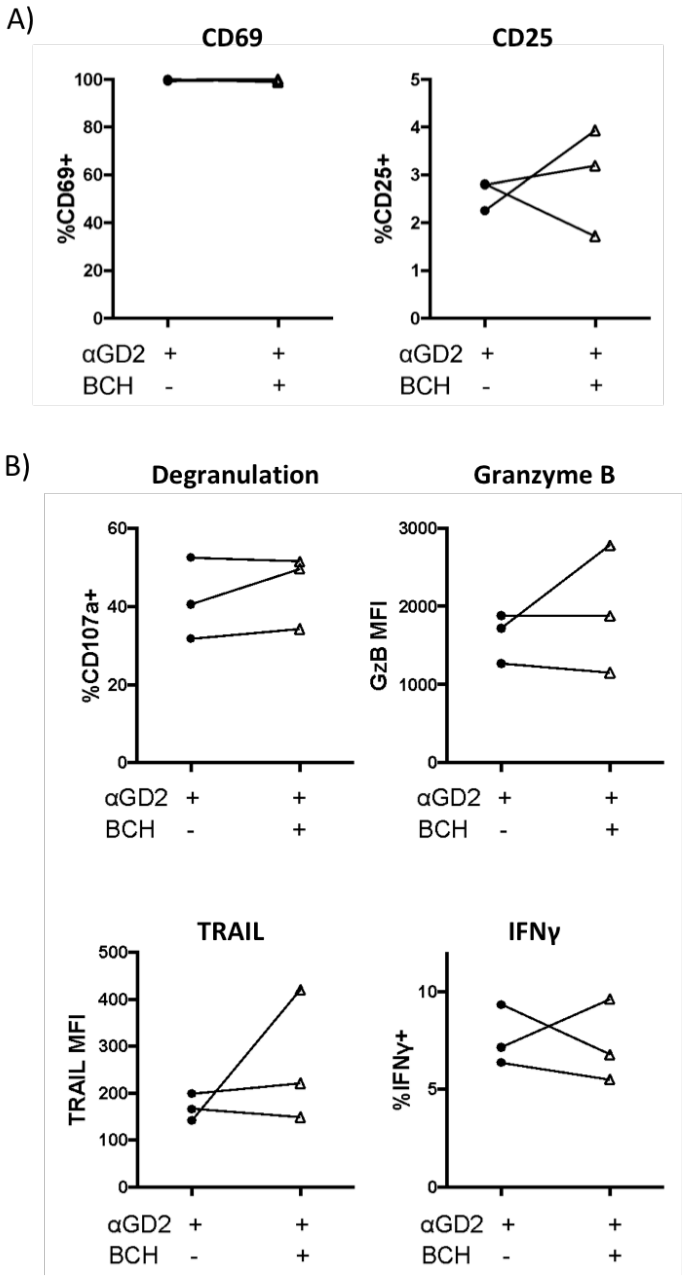


Figure 5.34 SLC7A5 amino acid transport does not impact NK cell activation and effector mechanisms during ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T). Anti-CD107a antibody, Golgi stop and BCH (25mM) was added was added to each well and the plate was incubated for 4 hrs at 37°C. After the assay cells were fixed and (A) CD16 and CD25, (B) CD107a, granzyme B, TRAIL and IFNγ were measured by flow cytometry. Each line indicates an independent experiment, n=3.

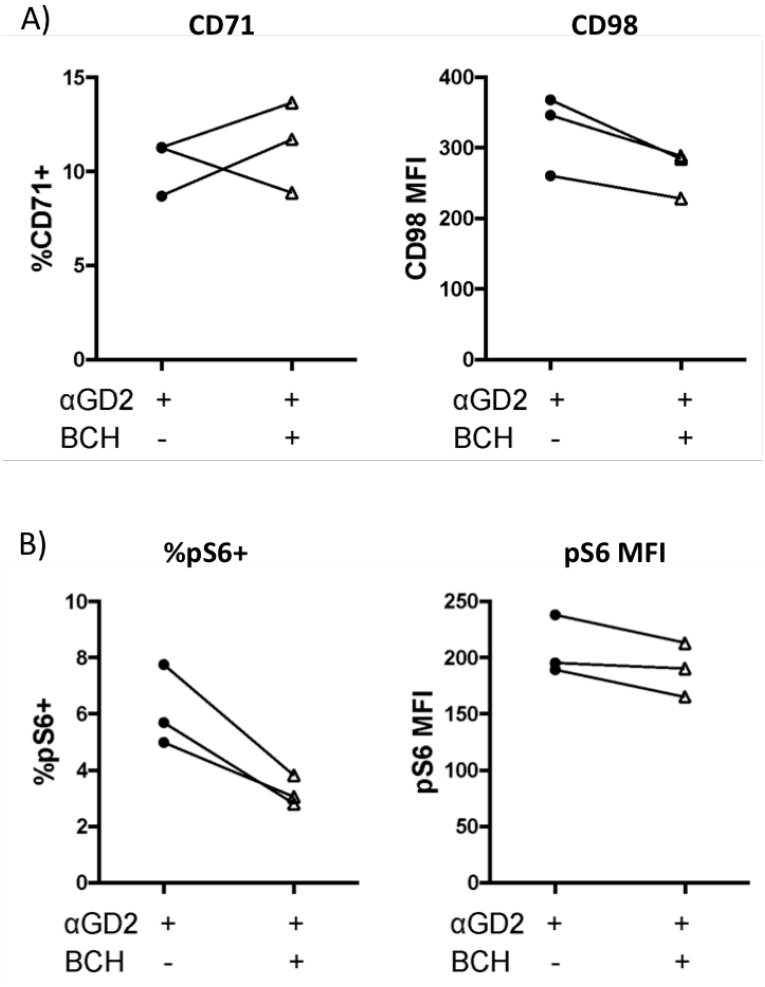


Figure 5.35 SLC7A5 amino acid transporter promotes CD98 expression and mTORC1 activity during NK cell ADCC.

Kelly NB tumour cells were stained with Calcein AM dye for 30mins 37°C and rested for 1 hr. Kelly cells were then coated with either anti-GD2 antibody or the corresponding isotype control. Kelly cells were seeded with freshly isolated *ex vivo* PBMC at varying effector to target ratios (E:T). BCH (25mM) was added and the plate was incubated for 4 hrs at 37°C. After the assay cells were fixed and (A) CD71 and CD98, (B) phosphorylated S6 protein were measured by flow cytometry. Each line indicates an independent experiment, n=3.

The findings in this chapter demonstrate that the metabolism of NK cells from NB patients may be significantly compromised. Furthermore, we have shown that cellular metabolism, in particular glycolysis and amino acid transport, is important for optimal NK cell anti-GD2 mediated ADCC against NB tumour cells. Together these findings support the design of a therapeutic strategy focused on boosting

patient NK cell glycolysis and amino acid transport while administering anti-GD2 immunotherapy.

5.3 Discussion

NK cells are innate lymphocytes that play a critical role in defence against cancer. This includes paediatric cancers such a NB and leukaemia, where greater NK cell activity is associated with better patient outcome [248, 256]. In contrast to adult cancers, less is known about the function of NK cells in paediatric a cancer, likely due to the reduced incidence of most childhood cancers. In Ireland approximately 5 children are diagnosed with NB annually, compared to over 3000 diagnoses of breast cancer. The immune system is different in children compared to adults, as evident by differences in susceptibility to certain infections [257]. Therefore, studies on the role of NK cells during paediatric cancer are essential if we are to design better methods for the treatment of these diseases that impact infants and young individuals.

In this chapter, we show that NK cells from NB patients have reduced activation and impaired IFNγ production in response to cytokine stimulation, indicating that these patient NK cells are not able to respond to cytokine and carry out their anti-cancer functions optimally. This data is in agreement with studies on childhood leukaemia for example, where patient NK cells have reduced NKp46 expression and impaired IFNγ production [258], and it supports the growing belief that NK cells become dysfunctional during cancer. While NK cell IFNγ production during NB has not previously been reported on, it has been shown that *ex vivo* and cytokine stimulated NK cells from NB patients have reduced cytotoxicity against K562 tumour cells, and that this correlates with NKp46 expression (as yet unpublished) [259]. Surprisingly, we found that NK cells from NB patients had an enhanced granzyme B response to cytokine, which would in turn suggest enhanced cytotoxicity. However, this was mainly evident in the $CD56^{bright}$ subset, which in general is not responsible for direct cytotoxicity or ADCC against tumour cells. For the cytotoxic $CD56^{dim}$ subset, only 1 out of 4 patients had a markedly increased granzyme B response, and therefore assumptions about cytotoxicity cannot be made based on this result. Indeed, it will be important in the future to investigate the other functions of NK cells from NB patients, such TRAIL and FasL expression, TNFα production and ADCC.

One caveat of this study is that the data is, at present, compared to data collected from healthy adult donor NK cells. Nonetheless, we can make predictions based on

current age studies about whether the differences observed might be greater or lesser once the data is compared to NK cells from healthy paediatric donors. There are many studies that have investigated the impact of aging on human NK cell biology, and indeed many of these reports are conflicting, as is the nature of human research. Overall, there is a general consensus that certain NK cell functions decrease with age [260]. For example, Mariani *et al*. showed that NK cells from older individuals have reduced cytolytic activity against K562 tumour cells [261], while Krishnaraj *et al.* showed that NK cells from older donors have reduced IFNγ production compared to those from younger donors [262]. Interestingly, it is suggested that the increase in the percentage of $CD56^{dim}$ NK cells (cytotoxic subset) in older individuals may serve to compensate for these reduced functions. Moreover, the expression of NK cell activating receptors can also decrease with age [263]. Therefore, it is possible that differences between the granzyme B response in healthy paediatric controls and patients might appear lessened or in fact be abrogated, while that of the IFNγ response may appear greater. Overall, this adds a layer of confidence to our result that NK cells from NB patients have dysfunctional cytokine production.

Importantly, we sought to explore whether altered cellular metabolism might be a mechanism underlying the dysfunction observed, as was seen in NK cells from breast cancer patients. While there were some interesting differences, in general the data mirrored what was observed in NK cells from breast cancer patients, in that mitochondrial dysfunction, impaired glycolysis and altered mTORC1 activity were the most striking features of the patient NK cells. This clinical data consolidates the findings of chapter 3, and supports that idea that altered NK cell metabolism is an underlying cause of dysfunction during cancer.

One interesting result of this study was that nutrient receptor expression was not altered, suggesting that the altered nutrient receptor expression observed in breast cancer NK cells was not a key driver of the metabolic dysfunction, and was perhaps a result of it. CD71 is a downstream target of mTORC1, and therefore the impaired mTORC1 activity in NK cells from breast cancer patients may have caused reduced CD71 expression. Surprisingly, nutrient uptake through SLC7A5 was increased in IL12/15 stimulated NK cells from NB patients, which may represent a potential compensatory mechanism whereby the cell is taking in more nutrients in order to boost metabolism. Indeed, amino acids can feed into glycolysis and the TCA cycle,

and it has been shown that glutamine can fuel the TCA cycle through glutaminolysis in murine NK cells [125]. However, it may also be possible that NK cells from children have heighted SLC7A5 activity to support their increased functions, and therefore it will be important to confirm results by comparing the data to healthy paediatric controls.

One unexpected finding was that when using p4EBP1 as a measure mTORC1 activity, it appeared that NK cells from NB patients had elevated cytokine induced mTORC1 activity, however; this was not evident when pS6 was used, and in fact there was a downward trend in the data. As 4EBP1 and S6 are downstream targets in two separate pathways of mTORC1 signalling (phosphorylation of 4EBP1 promotes protein translation, while phosphorylation of S6 by S6 kinase promotes mRNA synthesis and ribosome biogenesis), this could suggest that signalling has become skewed to favour the synthesis of proteins rather than mRNA and ribosomes. It is also possible that S6 and 4EBP1 are being phosphorylated by factors other than mTORC1. AZD, which inhibits mTORC1 and mTORC2, abolished phosphorylation of 4EBP1, indicating that either mTORC1 or mTORC2 is responsible for its phosphorylation. One explanation for the result could be that mTORC1 is impaired, resulting in reduced phosphorylation of S6, but mTORC2 is still functioning, which is responsible for increased phosphorylation of 4EBP1. An alternative and more likely scenario is that S6 phosphorylation could be being mediated by the MAPK/ERK pathway, in which S6 is phosphorylated by ribosomal s6 kinase (RK6). If this was true, the data could be explained if RSK activity (or one of its upstream signals) is impaired in patient NK cells, resulting in reduced phosphorylation of S6, while normal or perhaps increased mTORC1 activity phosphorylates 4EBP1. This idea is supported by the fact that AZD treatment increased S6 phosphorylation (likely a compensatory mechanism, data not shown), indicating that in patient NK cells, S6 can be phosphorylated by non-mTOR factors such as RSK. Indeed, it will be important in future experiments to include a rapamycin control (specifically inhibits mTORC1). If S6 is being phosphorylated by RSK, rapamycin should not impact on S6 phosphorylation, but it could reduce 4EBP1 phosphorylation. Overall, the AZD control indicates that mTOR activity is altered in NK cells from NB patients, and indeed it seems possible that other metabolism-related signalling pathways could also be impacted.

While there are no other studies on NK cell metabolism during childhood cancer, there is one study on NK cell metabolism during childhood obesity. Interestingly, many of the key differences observed between healthy and NB patient NK cells in this study were also observed in the study on lean and obese children [264]. Similar to the increased kynurenine uptake observed herein, NK cells from obese children had increased nutrient uptake, as measured by the 2NBDG uptake assay (correlates with glucose uptake in T cells [135]). Further, they had increased basal mTORC1 activity (pS6) and high levels of mROS. Some key differences between these two studies were that NK cells from obese children had normal IFNγ production (but impaired granzyme B), normal mitochondrial mass, increased cytokine induced mTORC1 activity (pS6) and normal cytokine induced glycolysis. Another striking finding was that glycolysis in resting NK cells (unstimulated) was significantly increased. Unfortunately we were unable to measure this in NK cells from NB patients due to cell number limitations. Interestingly, a similar study was published more recently examining the metabolism of NK cells during adult obesity [265]. The authors found that NK cells from obese adults had impaired IFNγ production and granzyme B expression, which was associated with reduced mTORC1 activity, 2NBDG uptake and glycolysis. Together, these data suggest the chronic inflammatory environment caused by cancer and obesity may initially induce hyperactivity in certain biological mechanisms (as seen in childhood disease), but which ultimately results in exhaustion and an inability to respond to cytokine when needed (as seen in advanced adult disease such as adult obesity and metastatic breast cancer). It will be interesting in the future to see whether this pattern emerges for other chronic inflammatory conditions, for example in cases where mother and infant are both HIV^{+}, or in children versus adults diagnosed with celiac disease. Differences between paediatric and adult disease highlight the need to design therapies specifically tailored for paediatric populations, rather than simply using those that have been designed for adults, as is currently routine.

The development of antibody dependent immunotherapy was a ground breaking medical advancement in modern medicine. Anti-GD2 therapy (Dinutuximab) that is used in the treatment of high-risk NB increased survival rates of patients by approximately 20%, thus saving many young lives around the world [244]. These therapeutic antibodies bind highly expressed tumour specific antigens, and are then recognised by $CD16^{+}$ immune cells, in particular $CD56^{dim}$ NK cells, which then identify the tumour as a target and initiate ADCC. In the second part of this chapter,

we explored the metabolic requirements of anti-GD2 mediated ADCC against high-risk NB tumour cells, with the aim to identify specific metabolic pathways/molecules that could potentially be targeted in order to boost ADCC and increase patient survival. Here, we show that NK cells upregulate nutrient receptor expression and mTORC1 activity during anti-GD2 mediated ADCC against NB, indicating a likely role for metabolism. Upon investigation into which metabolic pathways might be most important, the data was clear that glycolysis and not oxphos is required for anti-GD2 mediated degranulation and ADCC. As mentioned in the introduction of this chapter, CD16 is one of the only NK cell receptors capable of initiating cytotoxicity and cytokine production in resting NK cells, meaning that no prior activation or priming is required, and that the response can be considered to be immediate [234]. Glycolysis is the fastest way of producing ATP and therefore may be specifically required by the cell to rapidly supply energy so that it can carry out ADCC. At the same time, biosynthetic precursors will be made available in order to replace the exocytosed cytotoxic granules and cytokine.

One of the key differences between glycolysis and oxphos is the requirement for oxygen – while glycolysis can take place in the presence or absence of oxygen, it is a fundamental substrate of oxphos. By using glycolysis to drive ADCC, NK cells may be adapted to carry out ADCC in hypoxic environments such as the TME, which is indeed of great advantage for tumour clearance. However, as tumours are themselves highly glycolytic, the NK cells would likely be in competition for glucose. Nonetheless, it will be interesting in the future to see whether NK cell anti-GD2 mediated ADCC is impacted by hypoxia, as the data hints that it may not be.

The findings of this chapter are supported by a recent study that investigated the metabolism of NK cells stimulated with anti-CD16. NK cell degranulation, granzyme B and FasL expression were all increased by anti-CD16 stimulation. This was impaired only during glucose starvation (to inhibit glycolysis), and not when oligomycin was added (to inhibit oxphos). However, when their cellular metabolism was examined directly using seahorse analysis, both glycolysis and oxphos were increased by anti-CD16 [266]. While this culture condition is more artificial i.e. plates coated with anti-CD16, rather than tumour cells coated with antibody, experiments again showed that stimulation of NK cells via CD16 leads to glycolysis driven upregulation to cytotoxic machinery. It is interesting that oxphos was upregulated by CD16 stimulation, yet was not required for the upregulation of the

cytotoxic machinery. It has been shown that NK cells cultured on anti-NKG2D coated plates reorganise their mitochondria towards the cross linking site of the NKG2D receptor, supporting a role for mitochondrial metabolism in driving NK cell receptor mediated cytotoxicity [150]. Cellular metabolism is a dynamic and plastic network; therefore it may be possible that oxphos indeed plays an important role during NK cell ADCC, but that this role can be compensated for when oxphos is blocked, but not when glycolysis is. For example, this could be achieved by increasing glycolytic flux by feeding in more glucose or amino acids.

It is interesting that glycolysis is required for NK cell ADCC, as we have previously shown that inhibition of glycolysis (using galactose) in cytokine stimulated human NK cells does not impact granzyme B expression or degranulation against K562 tumour cells. Indeed, this illustrates that different NK cell functional responses have differential metabolic requirements, and highlights the need to study both cytokine and receptor stimulated NK cells individually. Overall, the data demonstrates that glycolysis is more important for ADCC and degranulation than oxphos. This has important implications for the design of future anti-cancer medicines, particularly those that will be aimed towards patients already receiving antibody therapy as a standard of care.

Once it was determined that glycolysis drives ADCC, we next investigated what metabolic regulators might also be important. The data pointed towards mTORC1 as a likely candidate for this – anti-GD2 coated tumour cells induced an upregulation of mTORC1 activity in NK cells, and the decrease in ADCC caused by 2DG was also associated with reduced mTORC1 activity. Indeed, previous work from our lab has shown that mTORC1 plays an essential role in promoting human NK cell glycolysis, oxphos and IFNγ production [122]. Furthermore, rapamycin reduces anti-NK1.1 induced murine NK cell functional and metabolic responses, suggesting a role for mTORC1 in regulating murine NK cell receptor mediated responses [267]. Surprisingly, rapamycin had little to no impact on NK cell anti-GD2 mediated ADCC. It should be noted that although cytokine strongly induces mTORC1 activity in human NK cells, rapamycin does not impact cytokine induced degranulation, granzyme B or TRAIL expression in human NK cells [122]. Therefore, induction of mTORC1 activity does not necessarily equate to rapamycin sensitivity for all NK cell effector functions. It is possible that some redundancy exists between mTORC1 and other regulators that can compensate for its absence. Crosstalk between mTORC1

and mTORC2 has previously been demonstrated in murine NK cells, where loss of mTORC1 results in reduced mTORC2, and loss of mTORC2 results in increased mTORC1 [155]. Therefore, in the setting of human NK cell receptor-mediated functions, there may be a compensatory role for mTORC2 in promoting ADCC. Of note, a preliminary experiment was conducted to test this hypothesis using AZD-8055, which inhibits both mTORC1 and mTORC2 at the same time. ADCC decreased from 36% to 27% and 22% to 12% for the 10:1 and 5:1 ratios respectively (data not shown). This also may simply be a matter of timing; indeed, 4-6 hour cytokine stimulation of murine NK cells does not induce the metabolic programming that is observed after 18 hours stimulation [126]. Overall, the role of mTORC1 activity during anti-GD2 mediated ADCC warrants much further investigation, and will likely shed light on the complex and perhaps overlapping signalling pathways that drive NK cell ADCC.

We next investigated whether SREBP might be important in promoting NK cell anti-GD2 mediated ADCC. SREBP is a transcription factor traditionally known for its role in regulating fatty acid metabolism. In murine NK cells, however, it plays an unusual role in regulating glucose metabolism. Inhibition of SREBP using 25HC reduces murine NK cell glycolysis, oxphos, IFNγ production and granzyme B expression [124]. In contrast to this study, 25HC did not impact human NK cell anti-GD2 mediated ADCC. In fact, it increased it slightly in certain cases, suggesting that SREBP may actually restrain ADCC. Whether this is due to mouse/human differences, or because the impact of 25HC on receptor induced NK cell responses has not previously been explored, remains to be elucidated. Nonetheless, the 25HC-induced upregulation of ADCC should be investigated further, as it may identify novel targets that could be used for design of drugs that boost ADCC.

As was the case with mTORC1, there was some evidence hinting at a potential role for amino acid metabolism in driving NK cell ADCC. Anti-GD2 coated tumour cells increased CD98 expression in NK cells, and the reduced ADCC observed with 2DG treatment was associated with reduced CD98 expression. Furthermore, amino acid metabolism has previously been demonstrated to play an essential role in driving murine NK cell function and metabolism [125]. In this study by Loftus *et al*., amino acid levels (particularly glutamine) acutely regulated cMyc protein. When SLC7A5 was blocked using the inhibitor BCH, cMyc protein was rapidly degraded, and NK cell function and metabolism (including glycolysis) was reduced. Indeed, cMyc

protein levels are extremely labile and determined by constitutive cMyc protein synthesis and degradation, and therefore require ready supply of amino acids. We investigated the impact of BCH on NK cell anti-GD2 mediated ADCC. BCH reduced ADCC dramatically to very low levels, indicating that active amino acid transport through SLC7A5 is essential for NK cell ADCC. In addition, BCH treatment reduced cMyc expression in human NK cells, suggesting that SLC7A5 activity may be required to sustain cMyc expression in human NK cells. In the Loftus *et al*. study, it was shown that glutamine uptake through SLC7A5 is essential for maintaining cMyc protein expression, and that glutamine withdrawal impairs metabolism and direct cytotoxicity against K562 and YAC1 tumour cells. Therefore, it will be interesting to see whether glutamine withdrawal also reduces cMyc expression in human NK cells, and whether this then impacts anti-GD2 mediated ADCC. Notably, it has been shown previously that NK cells from cancer patients have reduced cMyc expression, and therefore it will be important to examine this in NK cells from NB patients to determine whether this could be impacting the efficacy of anti-GD2 therapy [268].

As the data was so striking, it was surprising to find that BCH did not impact NK cell degranulation, and only CD98 expression and mTORC1 activity were reduced. This suggests that the defect may perhaps be in engagement of target cells or degranulation at the right location. Indeed, it has previously been shown that treatment of human NK cells with rapamycin or 2DG impairs their ability to direct their cytotoxic granules towards the target cell [265]. In this study the authors found that treatment of healthy donor NK cells with these metabolic inhibitors allowed the NK cells to form a synapse with the target cell, but impaired the ability of the MTOC to polarise towards the tumour cells. This could indeed explain why BCH treatment abolishes ADCC yet has no impact on degranulation. Of note, we had planned experiments to investigate whether 2DG or BCH impacts NK cell MTOC polarisation during anti-GD2 mediated ADCC, however these had to be postponed due to the COVID-19 pandemic.

Altogether these data demonstrate a fundamental role for glycolysis and amino acid transport in driving NK cell anti-GD2 mediated ADCC against NB tumour cells. This has important implications for the design of future NK cell based immunotherapies for NB, particularly adoptive NK cell therapy. Indeed, using haploidentical adoptive NK cells to treat NB patients has already proven safe and well tolerated, and been shown to improve progression free survival [112]. Furthermore, there is potential to

engineer these NK cells in order to express certain chimeric antigen receptors (CAR that boost their efficacy as anti-cancer effectors. Interestingly, it was recently shown that NK-92 cells engineered to express the receptor for GD2 induce tumour killing *in vitro* and *in vivo*, and have enhanced IFNγ production [269]. These CAR-NK cells have the potential to be modified further, for example by increasing expression of Glut1 or SLC7A5 on their surface, such that they have excess nutrients to carry out ADCC. In terms of autologous adoptive NK cell therapy, the data presented herein demonstrate that while both oxphos and glycolysis are impaired in patient NK cells, efforts should focus in restoring NK cell glycolysis before reinfusion into the patients receiving anti-GD2 therapy, as this may lead to a boost in NK cell ADCC and increased tumour clearance.

6 Final Discussion

NK cells are innate immune cells that play an essential role in fighting cancer and viral infection. They were the first type of immune cell discovered with the ability to spontaneously kill tumour cells, setting them apart from T and B cells, and inspiring years of subsequent research to uncover how they function in health and disease. We have learned a great deal in terms of how they recognise and kill target cells. Indeed, NK cells have emerged as immune cells that play a wide variety of roles not only in combating cancer and viral infections, but also in promoting and working alongside the adaptive immune response, regulating tissue remodelling and repair, and even in generating innate immune memory. However, it was only relatively recently that research began to delve into the biochemical pathways that drive NK cells to carry out their functions i.e. their cellular metabolism.

The advent of immunometabolism as a research topic has revealed many novel molecular mechanisms that are fundamental to the biology of NK cells. When this project began in 2016, little was known other than that NK cells upregulate glycolysis and oxphos in response to cytokine and that mTORC1 is important for this [122]. Since then, the body of knowledge on this topic has grown exponentially - the role of the citrate malate shuttle, cMyc, SREBP, the mitochondria and mitophagy, nutrient transporters and nutrients, TGFβ, epigenetics and more. These studies have highlighted how fundamentally different NK cells are from their adaptive counterparts T cells, as they metabolise nutrients using different pathways to support their unique functions. What is most exciting is that we are at the stage where we are beginning to understand how these mechanisms are impacted in human disease, and how we can potentially target them for the development of new therapeutics.

Here, we show that NK cells from the peripheral blood of cancer patients have severely impaired metabolism. This involves reduced levels of both glycolytic and mitochondrial metabolism upon activation and even at rest, indicating that these NK cells are metabolically paralysed. Importantly, this was true for two quite different cancer types – breast cancer, a common adult solid carcinoma, and NB, a rare paediatric cancer where all patients were completely treatment naïve and had comparably negligible influence from the environment. We propose that this metabolic paralysis is an underlying cause of the dysfunction that was first

documented in NK cells from cancer patients in the 1980s [71]. Indeed, our data demonstrating restoration of mitochondrial metabolism in NK cells from breast cancer patients was associated with a rescue of IFNγ production, provides further evidence that the reduced metabolism is in part responsible for reduced function.

The implications of this discovery are far-reaching. It raises many questions concerning what might be going on with other aspects of NK cell metabolism in cancer – in other pathways such as the PPP or glutaminolysis for example, or how the high levels of ROS are impacting the redox balance of the cells and how this is altering cell signalling, whether transcription factors such as cMyc, SREBP or HIF-1a are also altered, and more. The mitochondria data shows that NK cell dysfunction must be examined at the level of the organelle, highlighting the need to explore the specific roles of the other organelles in supporting the function and metabolism of NK cells, for example the endoplasmic reticulum and the Golgi apparatus. The energetic requirements of maintaining and shuttling lytic granules within NK cells will shed light on the metabolic basis of NK cell spontaneous cytotoxicity (our anti-GD2 ADCC data suggests that glycolysis and amino acid transport in particular may be important for this). Furthermore, it will be interesting to explore the role that metabolism plays in NK cells within different environments. Indeed, it is widely acknowledged that NK cells in different organs and tissues have specific phenotypes and functions; however, whether their metabolism is also different remains unknown. This would be of particular interest for example in the uterus, where NK cells play a key role in modulating the remodelling of the placental tissue e.g. spiral arteries [270]. We predict that these tissue resident NK cells will have unique metabolic profiles that suit their specific functions. For example, HIF-1α is an important driver of vascular endothelial growth factor (VEGF) production, which is a key functional mediator of uterine NK cells. HIF-1α also promotes a glycolytic switch in T cells. Hence, even though cytokine stimulated murine NK cells do not require HIF-1α [125], there may be a link between HIF-1α, glycolytic metabolism and VEGF production in human uterine NK cells, and further, this may be altered in pregnancy pathologies such as miscarriage. Overall, there are a plethora of exciting discoveries on NK cell metabolism in health and disease waiting to be uncovered.

Our wealth of knowledge on NK cell metabolism has indeed exploded since this project began. However, this is also true for other immune cell types in other cancers. We have demonstrated that peripheral NK cells from NB and breast cancer

have impaired glycolytic and mitochondrial metabolism and reduced mTORC1 activity. This is supported by data from human liver cancer [271], where tumour infiltrating NK cells have increased mitochondrial fragmentation and reduced oxphos, and also by data from a murine model lung cancer, where tumour infiltrating NK cells have severe glycolytic dysfunction [213]. Furthermore, NK cells from a murine model of lymphoma have reduced mitochondrial mass, MMP, and mTORC1 activity, alongside elevated levels of PPARγ and lipid metabolism [272]. In a model of sarcoma, disease progression is associated with reduced glycolysis and mTORC1 activity in CD8+ T cells. Similarly, tumour infiltrating CD8+ T cell from human renal cell carcinoma patients have reduced glycolytic and mitochondrial metabolism [273], while CD4+ and CD8+ T cells from B cell leukaemia patients have reduced glucose transport and mTORC1 activity. It is noteworthy that we are the first to examine the metabolism of any immune cell from the peripheral blood of cancer patients with a solid tumour.

Taking these studies together, a pattern emerges suggesting that altered immune cell bioenergetics may be a new hallmark of cancer. It will be interesting to see whether this pattern continues in future studies on different cancer types and different immune cell types e.g. dendritic cells and macrophages. Indeed, it was only 9 years ago in 2011 that Hanahan and Weinberg revisited their landmark paper on the Hallmarks of Cancer by adding in new hallmarks that had emerged in the decade since they published their original paper [274]. These included '*Reprogramming of Energy Metabolism*' in tumour cells, and '*Evading Immune Destruction*'. Almost a decade since their revised paper was published, we are beginning to understand how these two new hallmarks are fundamentally linked. Their brief synopsis of immune evasion was centred around the idea of extrinsic, tumour mediated '*disabling'* of the immune response, involving the suppression of cytotoxic lymphocytes with immunosuppressive factors such as TGFβ, and the recruitment of pro-tumourigenic immune cells such as Treg cells and MDSCs. Indeed, we now know that some of these immunosuppressive factors are in fact metabolites, produced directly as a result of the reprogramming of the energy metabolism within the tumour. For example, the tryptophan catabolite kynurenine has been shown to reduce the expression of surface receptors NKp46 and NKG2D and inhibit cytotoxicity in NK cells [275]. The data presented in chapter 4 of this thesis suggests an alternative, intrinsic form of immune evasion, involving active NK cell-mediated suppression of the NK cells themselves. This suppression impacts on NK cell energy

metabolism, which then compromises immune function, thereby removing one of the key biological assets that protects and fights against cancer. Interestingly, the authors did not seem convinced that the immune system plays a significant role in preventing human cancer, describing the evidence at the time as '*rudimentary*', and the matter as '*unresolved*'. While we agree that with the latter, we believe that today there is strong evidence for the protective role the immune system plays against cancer, particularly given the success that immunotherapy has had in the clinic in recent years. Overall, it is an exciting time for cancer researchers. The marriage of these two fields of cancer research, Energy Metabolism and Immune Destruction, has revealed novel molecular mechanisms that deepen our understanding of immune evasion in cancer, and has led to the identification of new therapeutic targets that are currently in development for cancer immunotherapy.

It is important to highlight that cancer therapy would have benefited greatly had this advent happened earlier. Indeed, we have been aware that Warburg metabolism takes place in tumour cells for almost a century [276], and as such many drugs have been designed to target tumour metabolism, e.g., methotrexate, an inhibitor of dihydrofolate reductase, that has been used in chemotherapy since the 1940s [277]. These drugs were designed based almost exclusively on research on tumour cell metabolism and their potential impact on immune cells was not investigated or considered. However, as we increasingly turn to the immune system for new cancer therapy strategies, a trend likely to continue given the high rewards yielded so far, cancer researchers and immunologists must collaborate and consider the consequences of strategies proposed. For example, rapamycin, which inhibits NK cell metabolism and function, is currently in clinical trials for NB (ClinicalTrials.gov; NCT01331135) and for breast cancer (ClinicalTrials.gov; NCT02536625). This 'new' treatment could in fact compromise certain immunotherapy strategies [278], although our data from chapter 5 suggests it may not impact NK cell anti-GD2 mediated ADCC. We are only now beginning to understand the off-target effects that these metabolic cancer therapies have on the immune system and a current ambition of the field is to figure out ways in which we can selectively target cancer metabolism while protecting the anti-tumour immune response. Interestingly, a prodrug (JHU083) of the glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON) was recently found to shut down glycolysis and oxphos in murine breast cancer cells while enhancing T cell oxphos and anti-tumour functions [279]. The authors found that T cells are more metabolically plastic as they can use acetate as an alternative

fuel for the TCA cycle, while the tumour cells cannot. Interestingly, it was also recently shown that in the absence of glucose and glutamine, human NK cells can use acetate for IFNγ production, suggesting that this therapy may also be beneficial for the NK cell anti-tumour immune response [280]. These novel findings suggest that there are key differences between tumour and immune cell energy metabolism awaiting discovery that may have the potential to be harnessed for the development of future cancer therapies. In the case of NK cells in breast cancer, our data support a strategy whereby NK cell mitochondrial metabolism is protected, while other metabolic pathways in the tumour can be targeted. For example, acetate fuelled TCA cycle and oxphos could be promoted in NK cells by engineering them to express high levels of Acetyl-coenzyme A Aynthetase 2-like, Mitochondrial (ACSS), which allows for the production of acetyl CoA from acetate. Simultaneously, glycolysis could be inhibited in order to starve the tumour, and this theoretically would have a relatively small impact on the modified NK cells.

Another key pattern emerging in the field of immunometabolism is that chronic inflammation may be a central driver of metabolic dysfunction in immune cells. It is well established that cancer creates a chronic inflammatory environment that supports tumour growth, and indeed there is now much evidence showing metabolic dysfunction of immune cells in cancer, including the data presented in this thesis. However, there are also many studies emerging on this topic in other chronic inflammatory conditions. For example, IL15 stimulated NK cells (20 mins) from patients with chronic EBV infection have increased activation of mTORC1, suggesting that they may also have altered metabolism [281]. CD8+ T cells from patients chronically infected with hepatitis B virus (HBV) have an increased reliance on glycolysis and altered mitochondrial structure [132, 282]. Obesity, now endemic across the globe, is associated with low-grade chronic inflammation that contributes to systemic metabolic disease such as diabetes and cardiovascular disease. This chronic inflammation can also contribute to metabolic pathology at the cellular level. For example, NK cells from childhood obesity patients have increased levels of basal glycolysis and mTORC1 activity [264], while NK cells from obese adults have reduced glycolysis, oxphos and mTORC1 activity [265]. Another recent study showed that obesity in mice and humans induced metabolic stress in CD4+ T cells via mTORC2 and the PI3K/Akt signalling pathway [283]. These studies suggest that obesity-induced chronic inflammation impairs immune cell bioenergetics, and

indeed this may contribute towards the increased incidence of cancer in obese individuals.

Septic shock is a severe and often fatal condition whereby infection triggers an overwhelming inflammatory immune response throughout the body. While this condition remains poorly understood compared to other inflammatory diseases, it was recently shown that T cells from septic shock patients have severely reduced glycolysis, oxphos and mTORC1 activity [133]. Somewhat similarly, autoimmune disease is an inflammatory condition that takes hold when the immune system has mistaken self as foreign, and therefore initiates an unsolicited response against the body. For example, rheumatoid arthritis (RA) is a disorder that manifests when the immune system attacks the joints, and often leads to severe inflammation within the synovial membrane. Yang *et al.* investigated the effect of RA on CD4+ T cells [284]. They found that the CD4+ T cells from the RA patients had reduced glycolysis, with reduced glucose uptake and ATP, and impaired autophagy. Taking all of these studies into account, it becomes clear that chronic inflammatory conditions – be it cancer, viral infection, obesity, sepsis or autoimmune disease – all have the potential to alter the metabolism of the associated immune cells. Interestingly, it is intuitive to expect an inflammatory environment to induce increased rates of metabolism associated with a pro-inflammatory immune response; however, the opposite is observed in most cases. This suggests that either the immune cells are reducing their metabolism in order to try and reduce the inflammation-associated damage, or that they have become exhausted as a result of constant exposure to inflammatory stimuli. Our data supports the latter idea, whereby peripheral NK cells in cancer have become metabolically exhausted, potentially as a consequence of chronic inflammatory stimulation in the presence of TGFβ. Indeed, it will be interesting in the future to see if a similar metabolic phenotype is observed in other inflammatory conditions such as COVID-19 infection, asthma, psoriasis and Crohn's disease.

Harnessing NK cells for cancer immunotherapy

We have shown that NK cells are metabolically paralysed in breast cancer and NB, and we propose that this phenotype is likely ubiquitous across a range of cancers and inflammatory diseases. Hence, therapeutic strategies that target NK cell metabolism may be useful in the treatment of a wide range of conditions. These strategies could cover a broad spectrum of approaches ranging from signalling

pathways, organelle structures through to redox balance and manipulating cytokine responses in NK cells.

Our data shows that targeting NK cell mitochondrial metabolism during cancer is sufficient to restore NK cell function during breast cancer, and mitochondrial metabolism has also been shown to support NK cell longevity *in vivo*. Of note, we are by no means the first to describe mitochondrial dysfunction in disease. In fact, mitochondrial dysfunction has been studied across a range of conditions for many years now, for example in aging, neurodegeneration, psychiatry, macular degeneration, and diabetes. Indeed, the frequency of articles being published on the mitochondria has grown exponentially over the past 3 decades, while studies on other organelles such as the nucleus and the Golgi apparatus have declined [285]. As a result of this, there are many drugs that target mitochondrial metabolism currently being investigated in labs and clinical trials around the world. For example, Menk *et al*. recently showed that stimulation of murine CD8+ T cells with anti-4-1BB (CD137) increases oxphos and is associated with PCG-1α dependent mitochondrial fusion [286]. In a B16 melanoma model of adoptive T cell therapy, mice that received injections of anti-4-1BB had reduced tumour burden. Interestingly, stimulation of human NK cells with anti-4-1BB has been shown to enhance NK cell Trastuzumab-mediated killing in an *in vivo* xenotransplant model of human breast cancer [287]. We suggest that increased mitochondrial metabolism may have been an underlying mechanism of the effectiveness of this treatment. Importantly, Pfizer have recently developed a humanized monoclonal antibody against 4-1BB (Utomilumab), and it is currently being tested in 12 clinical trials for a variety of cancers, including breast cancer (clinicaltrials.gov).

Mitochondrial fusion can be targeted in a more direct manner using the drug Mdivi-1, which promotes fusion by inhibiting the dividing enzyme dynamin. In a model of hepatoma, mice that received murine NK cells pre-treated with Mdivi-1 had reduced tumour burden compared to those which received untreated NK cells [271]. Similarly, murine T cells co-treated with another mitochondrial fusion promoter M1, in addition to Mdivi-1 had increased oxphos and anti-tumour capacity in a model of lymphoma [148]. These drugs have yet to be tested in human clinical trials, perhaps due to the potential impact of off target effects. Indeed, all cells throughout the body depend on their mitochondria for fundamental biological processes such as ATP production, heat generation, calcium buffering and cell death, and therefore

mitochondria-targeted agents are likely to result in significant side effects. Nonetheless, the preclinical data discussed herein suggest that they may at minimum be useful in treating *ex vivo* immune cells for allogeneic and autologous cell transfer therapy. Furthermore, as there are many proteins and enzymes involved in mitochondrial fission and fusion, there are many potential targets for the development of alternative drugs that also alter mitochondrial dynamics e.g. a Drp1 inhibitor to promote fusion.

Another approach that has been shown to be effective in targeting mitochondrial fitness is to treat with mitochondrial-targeted antioxidants. Indeed, a high level of ROS is a destructive by-product of a faulty electron transport, and we observed this within the mitochondria of NK cells from both breast cancer and NB patients. For example, treatment with the mitochondrial-targeted antioxidants MitoQ or MitoTempo has been shown to rescue CD25 and CD71 expression in exhausted CD8+ T cells from renal cell carcinoma patients *in vitro* [273]. Similarly, treatment of human CD8+ T cells from patients with chronic HBV infection with MitoQ or MitoTempo improved mitochondrial fitness and anti-viral functions *in vitro* [132]. It is noteworthy that using antioxidants to treat NK cells may turn out to be a double-edged sword, as ROS has been shown to play an essential role in driving NK cell mitophagy during murine CMV infection, which leads to the development of adaptive NK cells. [152]. Nonetheless, this strategy still warrants further exploration given the promising results observed in T cells thus far.

The data presented in chapter 4 of this thesis indicate that intrinsic TGFβ production is a key driver of NK cell mitochondrial dysfunction during cancer, and as such TGFβ represents an excellent candidate to explore for future immunotherapy. As with the mitochondria, research on targeting TGFβ during cancer has been underway for several years. For example, Rodon *et al.* showed in 2015 that treatment of patients with advanced malignancy with a small molecule inhibitor of TGFβ signalling was safe with no adverse cardiac events [288]. A search on the topics of 'Cancer' and 'TGFβ' on clinicaltrials.gov reveals 186 studies that are currently exploring this strategy. However, due to the ubiquitous and multi-functional nature of TGFβ, we believe that targeting TGFβ in a less direct manner may be most effective. Indeed, TGFβ knock out mice experience excessive, systemic inflammatory disease and survive to just 3-5 weeks [289].

A novel and more targeted approach is to target GARP instead of TGFβ. We have shown in this thesis, and for the first time, that NK cells from breast cancer patients express increased levels of GARP. As GARP expression has been described primarily in tumour cells and in tumour promoting platelets and Treg cells, blocking GARP has the advantage of targeting TGFβ only where it plays a pro-tumorigenic role. This would likely result in significantly less adverse effects compared with targeting TGFβ directly. Indeed, our data using the MHG-8 anti-GARP/TGFβ monoclonal antibody showed striking improvements in NK cell metabolism and function. Furthermore, this antibody was shown to block TGFβ production in human Treg cells and at the same time inhibits some of their immunosuppressive functions [207]. Therefore, this strategy has the dual benefit of promoting NK cell anti-tumour functions and simultaneously inhibiting pro-tumourigenic Treg cells. Interestingly, a different monoclonal antibody that also targets GARP (generated by the authors) was recently tested in a preclinical model of breast cancer [198]. Antibodies against GARP were injected intraperitoneally every 3 days, and after four weeks, mice had reduced metastasis to the lung and reduced frequency of Treg cells. When used in combination with cyclophosphamide, mice had reduced tumour burden as well as reduced metastasis to the lung at week 5. While NK cells were not investigated in this study, we predict that the antibody therapy may have increased their anti-cancer functions, which could potentially have contributed towards the success of the treatment. Overall targeting GARP has many advantages over targeting TGFβ, and preclinical data indicate anti-tumour potency. We eagerly await further studies on the *in vivo* efficacy of this antibody therapy in different tumour models, and believe it represents a real contender for a future anti-cancer therapy.

Outside the realms of drug and antibody treatments, adoptive NK cell therapies are at the forefront of NK cell immunotherapy research. This is in part due to improved techniques for sourcing NK cells – both allogeneic and autologous e.g. PBMC, cord blood [110] and iPSC [109]. This is also attributed to technological advances that have allowed for the genetic manipulation of NK cells *ex vivo* to improve their survival and function *in vivo* e.g. the potential to generate CAR-NK cells. Studies on CAR-NK cells have so far been very promising. Even though T cells have traditionally dominated this field of immunotherapy research, recent evidence suggests that NK cells may be more beneficial. Li *et al*. engineered iPSC derived NK cells to express a CAR construct consisting of the transmembrane domain of NKG2D, the 2B4 co-stimulatory domain (CD244), and the CD3ζ signalling domain [4]. They also

engineered primary T cells to express CD28, CD28-CD137 and CD3ζ. These highly active CAR-NK cells and CAR-T cells both exhibited enhanced anti-tumour activity in a murine model of ovarian cancer compared to their non-CAR counterparts. However, the CAR-T cell therapy had significant toxicities that the CAR-NK cell therapy did not e.g. sustained increases in plasma IFNγ, TNFα and IL2 levels, and pathogenic organ damage in infiltrated organs. At day 70 post treatment, 4/5 mice which received CAR-NK cell therapy were still alive, versus just 1/5 mice which received CAR-T cell therapy. Similarly, Quintarelli *et al.* transduced CD19 into *ex vivo* expanded healthy donor NK cells [290], thus allowing them to specifically target transformed B cells. Transfusion of these CAR-NK cells resulted in 100% overall survival versus just 20% for CAR-T cells in a xenograft model of leukaemia in immunodeficient mice.

As a result of these advancements, it is in the arena of adoptive cell therapy that we believe our enhanced understanding of the effects of TGFβ on NK cell metabolism will have more immediate impact. Indeed, by treating/engineering NK cells only in the *ex vivo* setting we avoid off-target effects on other cells and tissues. A great effort has already been made to engineer human NK cells such that they are insensitive to TGFβ's effects. For example, Yvon *et al.* engrafted a dominant-negative TGFβR2 onto cord blood derived NK cells, rendering them resistant to the inhibitory effects of TGFβ treatment and more efficient at killing glioblastoma tumour cells [291]. This was also shown to be effective in the breast cancer setting *in vitro* [292]. Similarly, Daher *et al.* deleted the TGFβR2 in PBMC-derived NK cells using CRISPR-CAS9, rendering them resistant to TGFβ and more able to combat a xenograft model of myeloid leukaemia [293]. We predict that these genetic modifications also enhanced NK cell metabolism, and that this supported the improved anti-tumour immune responses recorded. Indeed, there is potential to engineer these NK cells further to include a modification that boosts metabolism, e.g. increased SLC7A5 or Glut1 expression. Our data suggests that deletion of the GARP gene (LRRC32) may also enhance NK cell metabolism and function during cancer.

We are in an exciting era of cellular therapy, and basic research such as that described herein will play an essential role in allowing us to harness these new technologies optimally. However, an important consideration when contemplating new cancer therapies is the economical and physical feasibility of them. Isolation of NK cells from PBMC or stem cells is costly and time-consuming. As such, there is

renewed focus on generating 'off the shelf' NK cell therapies i.e. a relatively unlimited, generic supply of NK cells that can be banked and then infused into patients when needed. Interestingly, this idea seems to be moving in the opposite direction to the popular model of 'personalised medicine'. NK-92 is a transformed cell line derived from a human NK cell lymphoma patient, and they are known to spontaneously kill cancer cells via direct cytotoxicity and cytokine production. NK-92 cells received Food and Drug Administration (FDA) approval for use in clinical trials in 2017 [294]. Since then, NK-92 infusions have proven to be well tolerated with only minor toxicities reported in clinical trials for lymphoma and multiple myeloma [295] and advanced renal cell cancer and melanoma patients [296].

NK-92 cells may be genetically engineered such that they have enhanced anti-tumour properties. For example, Yang *et al.* engrafted a dominant negative TGFBR2 onto NK-92 cells [297] rendering them insensitive to TGFβ. Once transferred to a mouse model of lung cancer, there was reduced tumour proliferation and lung metastasis, while the NK-92 cells produced more IFNγ, all of which culminated in increased survival rates. Similarly, Wang *et al.* genetically engineered the TGFBR2 such that the extracellular and transmembrane region remained intact, yet the intracellular signalling region consisted of intracellular domain of the activating receptor NKD2GD. These NK-92 cells then had enhanced IFNγ production, increased killing capacity *in vivo* and reduced the differentiation of CD4+ T cells into Treg cells [298]. Indeed, the potential to develop these NK-92 cells as 'off the shelf' immunotherapy is unlimited, and could prove particularly powerful when used in combination with monoclonal antibody therapy, which already relies on NK cells to work. Our findings from chapter 5 of this thesis support a strategy whereby NK-92 glycolysis and amino acid transport should be boosted when being administered to NB patients receiving anti-GD2 therapy. Indeed, this could be attempted by engineering the NK-92 cells to express higher levels of Glut1, SLC7A5 or perhaps the glycolysis rate limiting enzymes hexokinase, phosphofructokinase or pyruvate kinase.

As a true sign of the therapeutic capacity that NK cells hold, there are now several biotechnology companies actively working towards generating new NK cell immunotherapies. Indeed, one of the weaknesses of academic research is that commercialisation of discoveries is rare and therefore many scientific findings are not fully taken advantage of for the benefit of patients. This is not the case, however,

for biotechnology companies, whose focus is to bring the science from bench to bedside. For example, ONK Therapeutics is a company based in Galway, Ireland that was founded in 2015. Their goal is to develop novel, off the shelf, NK cell-based therapies for haematological and solid cancers. oNKo-innate is an Australian company that is striving to develop unique NK-based therapies and rationalise how these should be combined with other immunotherapies. Other companies also working in this area include Innate Pharma (France), NantKwest (California, USA) and NK MAX (California, USA). Indeed, an immense international effort is being put into identifying and testing new NK cell based medicines; however, collaborations between academia and industry are lacking. It is clear that we must bridge the gap between academia and industry by sharing our resources and knowledge if we are to truly unlock the full potential of NK cells for use as next generation immunotherapy.

One of the key limitations of clinical immunometabolism research in both academia and industry is access to adequate amounts of sample with relatively low numbers of cells often available, particularly for paediatric research. For the majority of patient cohorts it is not possible to carry out the in depth analysis that takes place routinely in murine studies, and indeed this is likely one of the main reasons that the majority of immunometabolism research is still carried out in mice. As such, research into the development of new technologies that allow for the metabolic analysis of single cells from mixed populations is equally important as other areas of basic research. Progress has been made in this area in recent years e.g. the development of the kynurenine uptake assay and the release of the 8 well Seahorse XFp Analyser. Importantly, new technologies are emerging that will allow for the metabolic analysis of cell populations where it was not previously possible. Agilent have recently developed a new Seahorse XF HS Mini platform for carrying out seahorse analyses with up to 75% less cells e.g. 200,000 T cells in the XFp versus just 50,000 T cells in the new XF HS Mini platform. Furthermore, multi-disciplinary labs in Trinity College and across Europe are working together to try and develop specific glucose and glutamine uptake assays using click chemistry based methods. This approach involves synthesising modified forms of glucose/glutamine that are taken up through their specific receptors by the cell. Once inside, a chemical is used to 'click' the modified nutrient such that it becomes fluorescent and can be measured using flow cytometry. This work is currently on going. Interestingly, a research lab in France has recently developed a cutting edge technology known as

'ZENITH'. It involves a flow cytometry based approach to interrogate the metabolism of cells using metabolic inhibitors [299]. The authors demonstrate that this technique recapitulates the accuracy of Seahorse analysis as they simultaneously profile the phenotype and metabolism of several human immune cell populations from human peripheral blood. The nature of this technology means that it has huge potential for use in clinical research and perhaps some day in diagnostics or in predicting how patients will respond to certain treatments. We believe that these new technologies will be the cornerstone of future clinical immunometabolism research and will pave the way towards the development of novel immunotherapies for the treatment of cancer and other human diseases.

Overall, the future of NK cell immunotherapy looks bright. We have demonstrated that although peripheral NK cells from cancer patients are metabolically dysfunctional, it is possible to target this to improve their metabolism and function. As the phenotype described herein is becoming common within inflammatory environments, different diseases will likely end up having common therapeutic targets. Findings such as these will fuel and inspire NK immunometabolism research for years to come, with the hope to some day develop innovative NK cell based therapies that will save lives where it was previously not possible. We believe that the future lies in *ex vivo* manipulation and adoptive cell therapy, in collaboration with academic and industry partners, and in the development of cutting edge technologies that will advance our understanding of the diversity of immunometabolism in health and disease.

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