# Defining the Impact of Obesity and Obesity-Targeted Therapies on Natural Killer Cell Metabolism and Function

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# Lay Summary

The WHO has recognised the growing number of people with obesity a major global health concern. Obesity can have a variety of negative effects on individuals from social stigma to worse health outcomes in comparison to individuals without obesity. Focusing in on health care outcomes, people with obesity (PWO) can develop certain cancers at a higher rate and have worse prognosis than people without obesity and can be more susceptible to viral infections as the recent COVID-19 pandemic has shown.

While the exact cause for these worse health outcomes is not fully understood, numerous studies have shown that the immune system is negatively affected by obesity. Natural killer (NK) cells are an important immune cell population for both viral and cancer immunity, which are significantly impacted by obesity. NK cells from PWO are lower in number, less able to produce important immune molecules and less able to kill cancer cells.

However, if we are to rectify these deficiencies, we need to have an understanding of NK cell biology. **Paper I** is a review of our current understanding of NK cell function and metabolism and how obesity affects these. Following on from this review, **Paper II** looks are the importance of iron for NK cell biology, as PWO can often be iron deficient. We show that iron is crucial for human NK cell metabolism and therefore function. We also identify a link between low circulating iron and NK cell dysfunction in PWO.

**Paper III** continues in this trend and further examines the differences between NK cells from PWO and those without. We reveal that key components of NK cell metabolism are negatively affected by obesity, which in turn affects their function. However, we demonstrate for the first time that six months of GLP-1 therapy, a recently approved weight loss therapy, can improve NK cell metabolism and function in PWO, independent of weight loss.

The contents of this thesis add to our current knowledge on the effect obesity has on the immune system, in particular NK cells, and that the obesity-related deficiencies in NK cells can be restored with GLP-1 therapy.

# Abstract

Obesity is a chronic progressive disease, which is strongly linked to numerous co-morbid conditions, and thus is a major global healthcare concern. A major mechanism via which obesity drives the development and/or progression of co-morbid disease is via immune dysregulation. These dysregulations can both weaken the immune system, exposing people with obesity (PWO) to increased rates of viral infections and various cancers but can also drive chronic inflammation, a major contributor to chronic pathologies. Natural Killer (NK) cells are an important subset of immune cells, with a critical role in protection from viral infection and cancer. NK cells are significantly impacted by obesity, with reduced numbers, defective metabolism, and reduced function reported in PWO. In this thesis, by describing the function and metabolism of NK cells from people without obesity I outline some of the deficiencies of NK cells from PWO, in particular focusing on the importance of iron for optimal NK cell function and investigate strategies for restoring NK cell immunity in PWO.

**Paper I** of this thesis reviews our current understating of NK cell function and metabolism and collates the available information on how obesity affects NK cells. Our group and others report that obesity is linked to lower numbers of peripheral NK cells that have a defective metabolism which leads to a reduced ability to carry out their core function as cytokine producing cells and cytotoxic cells. There are some indications that various weight loss therapies and aids such as diet advice, exercise and bariatric surgery can help remedy some of the effects of obesity on NK cells and this is explored and discussed in **Paper I**.

Following from this review, in **Paper II** we examine the importance of iron for human NK cell biology. We find that iron is crucial for mitochondrial health in human NK cells, which in turn means iron is critical for NK cell cytokine production and *de novo* protein synthesis. As iron deficiency is a common comorbidity with obesity, we examined the effect that low circulating iron has on NK cells. We demonstrate that PWO who have low serum iron have more dysfunctional NK cells, in comparison to both healthy controls and PWO who had normal serum iron levels. This paper not only highlights the key role iron plays for NK cell biology but highlights that obesity-related iron deficiency may, in part, underpin the reported defects in NK cells in the setting of obesity.

Finally, **Paper III** continues to describe the effect of obesity on NK cell metabolism and function. In addition to functioning mitochondria, NK cells require amino acids to stabilise mTOR which is a key driver of increased metabolic activity for activated NK cells, in particular an increased dependence on glycolysis. We show that NK cells from PWO have lower levels of a key amino acid transporter and metabolic enzymes that are required for optimal NK cell metabolism and function. This translates to a reduced capacity to produce cytokines such as IFN-γ and kill target cells in comparison to healthy controls. However, we show for the first time that after six months of GLP-1 analogue therapy, an approved for weight loss therapeutic, NK cell metabolism and function is significantly improved in PWO, independent of weight loss. Collectively, this thesis aims to expand on our current understanding of human NK cell biology, how obesity impact NK cells and explores approaches to restore NK cell immunity in PWO.

# List of Scientific Papers Included in Thesis

**I. Conor De Barra**, Donal O'Shea, Andrew E. Hogan. NK cells vs. obesity: A tale of dysfunction & redemption. *Clinical Immunology*, 2023, 109744, ISSN 1521-6616, https://doi.org/10.1016/j.clim.2023.109744.

**II.** Conor De Barra[1], Eimear Ryan[1], Michelle Sugrue[2], Odhran Ryan[1,3], Evelyn Lynn[2], Helen Heneghan[3], Cormac McCarthy[3], Paul N. Moynagh[1], Linda V. Sinclair[4], Nicholas Jones[5], Andrew E. Hogan[1#] and Donal O'Shea[2,3, #]. Iron deficiency in people with obesity drives defective Natural Killer cell mitochondrial fitness and function doi.org/10.1101/2024.01.10.575005.

**III. Conor de Barra**, Mohammed Khalil, Arimin Mat, Cliona O'Donnell, Ferrah Shaamile, Kiva Brennan, Donal O'Shea, Andrew E. Hogan. Glucagon-like peptide-1 therapy in people with obesity restores natural killer cell metabolism and effector function. *Obesity (Silver Spring)*. 2023; 31(7): 1787-1797. https://doi.org/10.1002/oby.23772.

# List of Scientific Papers Not Included in Thesis

**I.** Nidhi Kedia-Mehta, Laura Tobin, Vanessa Zaiatz-Bittencourt, Marta M. Pisarska, **Conor De Barra**, Chloe Choi, Einas Elamin, Donal O'Shea, Clair M. Gardiner, David K. Finlay, Andrew E. Hogan; Cytokine-induced natural killer cell training is dependent on cellular metabolism and is defective in obesity. *Blood Adv* 2021; 5 (21): 4447–4455. DOI: https://doi.org/10.1182/bloodadvances.2021005047.

**II.** Nidhi Kedia-Mehta, Marta M Pisarska, Christina Rollings, Chloe O'Neill, **Conor De Barra**, Cathriona Foley, Nicole AW Wood, Neil Wrigley-Kelly, Natacha Veerapen, Gurdyal Besra, Ronan Bergin, Nicholas Jones, Donal O'Shea, Linda V Sinclair, Andrew E Hogan. The proliferation of human mucosal-associated invariant T cells requires a MYC-SLC7A5-glycolysis metabolic axis. *Science Signalling,* 16, eabo2709(2023). DOI: 10.1126/scisignal.abo2709.

**III.** Catriona Gallagher, Julie Mac Mahon, Chloe O'Neill, Féaron C Cassidy, Hazel Dunbar, **Conor De Barra**, Caoimhe Cadden, Marta M Pisarska, Nicole A W Wood, Joanne C Masterson, Eoin N McNamee, Elisabeth Schrumpf, Karen English, Donal O'Shea, Anne Marie Tobin, Andrew E Hogan. Mucosal-Associated Invariant T Cells Are Altered in Patients with Hidradenitis Suppurativa and Contribute to the Inflammatory Milieu. *Journal of Investigative Dermatology*, Volume 143, ISSUE 6, P1094-1097.e2, June 2023. DOI: https://doi.org/10.1016/j.jid.2022.11.011.

**IV.** Neil E. Wrigley Kelly, **Conor De Barra**, Ferrah Shaamile, Aisling Holland, Liam Shaw, Patrick W. G. Mallon, Jean O'Connell, Andrew E. Hogan & Donal O'Shea Antigen specific T cells in people with obesity at five months following ChAdOx1 COVID-19 vaccination. *The International Journal of Obesity*, 47, 83–86 (2023). DOI: https://doi.org/10.1038/s41366-022-01235-8.

**V.** Neil E. Wrigley Kelly, Grace Kenny, Féaron C. Cassidy, Alejandro A. Garcia-Leon, **Conor De Barra**, Patrick W. G. Mallon, Andrew E. Hogan, Donal O'Shea. Individuals with obesity who survive SARS-CoV-2 infection have preserved antigen-specific T cell frequencies. Obesity (Silver Spring). 2022; 30(10): 1927-1931. DOI: 10.1002/oby.23526

**VI.** Eimear K. Ryan, Christy Clutter, **Conor De Barra**, Benjamin J. Jenkins, Simon O'Shaughnessy, Odhrán K. Ryan, Chloe McKenna, Helen M. Heneghan, Fiona Walsh, David K. Finlay, Linda V. Sinclair, Nicholas Jones, Daniel T. Leung, Donal O'Shea, and Andrew E. Hogan. Iron is critical for mucosal-associated invariant T cell metabolism and effector functions, Journal of Immunology, 2024 Jun 1; 212(11): 1706–1713 10.4049/jimmunol.2300649

# Table of Contents

# List of Abbreviations

| | |
|---|---|
| ADCC | Antibody Dependent Cell Cytotoxicity |
| ARID5B | AT-rich interaction domain 5B |
| BCR | B Cell Receptor |
| BMI | Body Mass Index ($kg/m^2$) |
| CD | Cluster of Differentiation |
| CLP | Common Lymphoid Progenitor |
| COPD | Chronic Obstructive Pulmonary Disorder |
| CRP | C Reactive Protein |
| CVD | Cardiovascular Disease |
| DFO | Deferoxamine |
| DMEM | Dulbecco's Modified Eagle Media |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ETC | Electron Transport Chain |
| FBS | Foetal Bovine Serum |
| FMO | Fluorescence Minus One |
| GIP | Gastric Inhibitory Polypeptide |
| GLP-1 | Glucagon Like Peptide-1 |
| GLP-1Ra | GLP-1 receptor agonists |
| GM-CSF | Granulocyte Monocyte Colony Stimulating Factor |
| HCMV | Human Cytomegalovirus |
| HLA | Human Leucocyte Antigen |
| HPLM | Human Plasma Like Medium |
| ICAM | Intercellular Adhesion Molecule |
| IFN | Interferon |
| IL | Interleukin |
| ILC | Innate Lymphoid Cells |
| MHC | Major Histocompatibility Complex |

| | |
|---|---|
| MIC | MHC Class I Polypeptide–Related Sequence |
| mTOR | Mammalian Target of Rapamycin |
| NAFLD | Non-Alcoholic Fatty Liver Disease |
| NASH | Non-Alcoholic Steatohepatitis |
| NK cells | Natural Killer Cells |
| NCOA4 | Nuclear Receptor Coactivator 4 |
| OxPhos | Oxidative Phosphorylation |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| PI3K | Phosphatidylinositol 3 Kinase |
| PRR | Pattern Recognition Receptor |
| PWO | People with Obesity |
| PYY | Pancreatic Polypeptide |
| RPMI | Roswell Park Memorial Institute Medium |
| SIV | Simian Immunodeficiency Virus |
| T2DM | Type 2 Diabetes Mellitus |
| TCR | T Cell Receptor |
| TLR | Toll Like Receptor |
| TNF | Tumour Necrosis Factor |

# 1. Introduction

## 1.1 The Innate and Adaptive Immune System

Our immune system can be divided into two loose and ever evolving classifications, the innate and the adaptive immune systems. While in recent years this classification has become more porous and plastic, with innate memory and innate like lymphoid cells being described, it still is a useful categorisation for how the various components of our immune system respond to various stimuli. The younger of the two in an evolutionary sense is the adaptive immune system, arising about 450 million years ago with the development of jawed fish (Flajnik and Kasahara, 2010). While jawless fish have B cells, T cells and a form of genetic rearrangement very similar to VDJ recombination, the canonical adaptive immune response developed with their jawed descendants (Pancer et al., 2004). So called the "adaptive" immune system, it has the ability to respond and adapt to the specific pathogen challenging the body. The components of this branch of the immune system share at their core, a hypervariable protein, either membrane bound or secreted, that is specific to a certain antigen.

Antigen presenting cells such as macrophages and dendritic cells can process proteins into antigens, small polypeptides that are then "presented" on a corresponding Major Histocompatibility Complex (MHC) molecule. MHC can be divided in MCH I and MCH II, denoting which class of T cell that presenting cell will interact with. MHC I is recognised by cytotoxic $CD8^+$ T cells while MHC II will be recognised by the "helper" $CD4^+$ T cells (Wieczorek et al., 2017). The diversity of MHC molecules arises from the highly polymorphic Human Leucocyte Antigen (HLA) gene regions of which there are thousands of alleles (Handunnetthi et al., 2010). The similar degree of diversity seen in the B cell receptor (BCR) and T cell receptor (TCR) arises from V(D)J recombination, a process that combines one of a number of gene segments from V (variable), D (diversity) and J (joining) loci of which there are number of different alleles. This process can theoretically give rise to $10^{18}$ possible combinations of BCR and TCR (Hoehn et al., 2016, Roth, 2014). This huge diversity allows the immune system to adapt to nearly any possible pathogen that arises.

Once the antigen presenting cell has completed the processing and presentation of the antigen it will migrate to a secondary lymphoid tissue, whether that be the spleen, lymph nodes, Payer's patches or the tonsils to mention some examples (Randall et al., 2008). There, the antigen presenting cell will interact with a number of T cells until it locates the corresponding T cell receptor (TCR) to the MHC molecule it is presenting.

The innate immune system comprised of physical barriers like the skin and mucus, cellular components such as dendritic cells and macrophages, natural killer (NK) cells and neutrophils, eosinophils, mast cells and basophils and a variety of molecular components such as C-reactive protein and the complement system. Named the innate immune system as the various components in this category have an "innate" or spontaneous ability to respond to their targets without prior signals. Innate immune cells rely on conserved receptors such as Toll Like Receptors (TLRs), NK cell receptors and conserved T cell receptors that do not require prior sensitisation to activate the immune cell. The development of the innate immune system more than likely began closely to the time when life began as we can observe innate defensive components in very old and simple forms of life with species such as *Porifera* being able to phagocytose other cells and have similar receptors to TLRs (Müller et al., 1999).

## 1.2 Natural Killer Cells

Initially described as innate lymphocytes with an inherit ability to kill target cells, NK cells are now part of a distinct lymphocyte family and are described to have both immunomodulatory and homeostatic roles. Innate Lymphoid Cells (ILCs) are a family of innate lymphocytes which mirror conventional T cells both in the cytokines that they produce, and the transcription factors that are responsible for their differentiation (Colonna, 2018). **Figure 1** outlines the similarities between T cells and their related ILC, with ILC1s mirroring Th1 cells, ILC2s linked to Th2 cells and ILC3s breaking the naming convention having similar characteristics to Th17 cells (Tanriver and Diefenbach, 2014). NK cells, once considered to be the only ILC are considered the counterpart to $CD8^+$ T cells (Eberl et al., 2015). Arising from the common lymphoid progenitor (CLP), ILCs differ from T cells in that they are not activated by recognition of a presented antigen by a specific T cell receptor (TCR) but rather cytokines, inflammatory signals, and certain microbial compounds and can thus respond quickly to stimuli.

Figure 1: NK Cells and Innate Lymphoid Cells

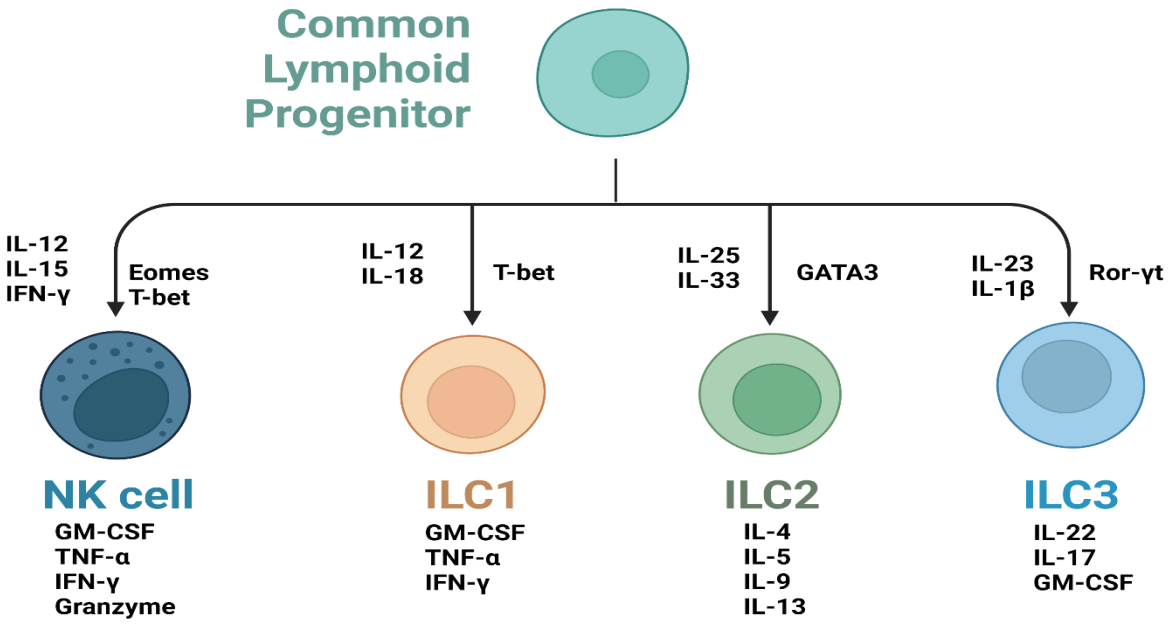



*Figure 3: NK cells and Innate Lymphoid Cells. Initially thought to be the only member of the innate lymphoid family, NK cells are part of a diverse and expanding group of lymphocytes that mirror conventional T cells. Whether these ILCs arose before the adaptive immune system or that they are a failsafe against an misfunctioning adaptive immune system, ILCs can provide rapid protection against a variety of pathogens.*

### 1.2.1 Natural Killer Cell Phenotype and Development

NK cells were first described in the 1960's when experiments looking at T cell cytotoxicity described a lymphocyte with the ability to spontaneously or "naturally" lyse target cells (Smith, 1966). It wasn't until the following decade when interest in this previously undescribed and understudied population grew with NK cells being identified in both peripheral blood and organs of both humans (Oldham et al., 1973) and mice (Kiessling et al., 1975). Initial attempts to describe NK cells proved difficult as using CD56 and CD57 included other lymphocytes (Ravandi et al., 2002), monocytes (Suzuki et al., 2000) and $CD8^+$ T cells (Tarazona et al., 2001). Relying on solely CD16 again excluded the $CD56^{bright}$, $CD16^-$ subset (Cooper et al., 2001a).

NK cells are derived from the CLP like other ILCs and lymphocytes and arise from the bone marrow (Kondo et al., 1997). The earliest that the NK cell lineage can be distinguished from that of T cells is the expression of NKG2D on the surface of $CD122^+$ cells (Male et al., 2014). From there NK cells undergo various steps of development as outlined in (Abel et al., 2018) to reach final maturity where they are divided into $CD56^{bright}$, $CD16^-$ NK cells which make up about 5-10% of the total NK cell population and remaining 90% being $CD56^{dim}$, $CD16^+$ NK cells.

Figure 2: NK Cell Development

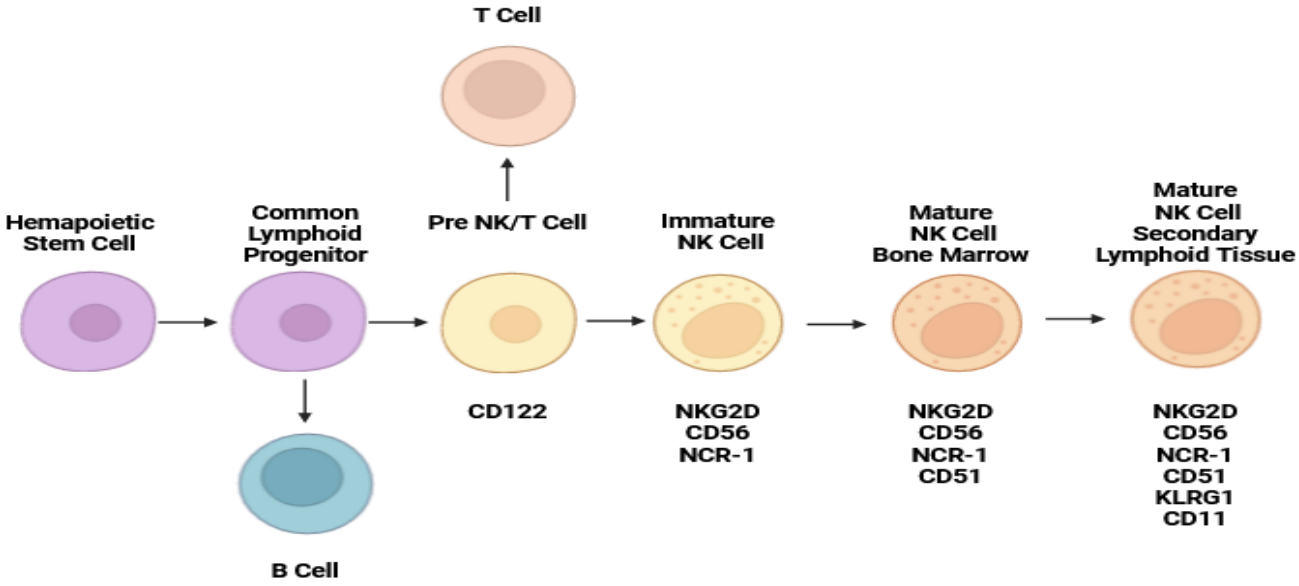



*Figure 2: NK Cell Development. This diagram highlights the key stages of NK cell development and the surface receptors that are used to identify NK cells from other lymphocyte populations. Arising in the bone marrow, the common lymphoid progenitor can give rise to B, T and NK cells. Expression of CD122 is the earliest indicator of the NK cell lineage. NK cells will continue to differentiate in the bone marrow until they are fully mature and then will migrate to secondary tissues.*

While NK cells can express a variety of activating and inhibitory receptors and various other surface markers, they can be divided into two broad categories mentioned above, the more abundant $CD56^{dim}$, $CD16^{+}$ (approximately 90% of NK cells) and the less abundant $CD56^{bright}$, $CD16^{-}$ NK cells making up the remaining (Lanier et al., 1986).

It is important to note that CD56 is not merely a marker for NK cells. CD56 appears on immature NK cells still resident in the bone marrow, and once these NK cells mature and egress increase their expression of CD56 to become the $CD56^{bright}$ phenotype (Abel et al., 2018). The expression of CD56 on maturing NK cells appears to play a role in stromal cell/NK cell interaction that is required for development (Mace et al., 2016). The exact function of CD56 remains unclear but its presence on a wide variety of immune cells indicates a function of some sort and is important in brain development (Cremer et al., 1994, Brandewiede et al., 2014).

IL-15 promotes the development of tumouricidal dendritic cells that are potent antigen presenters (Anguille et al., 2012). A similar anti-tumour phenotype is observed in both αβT cells and γσ T cells. In response to IL-15, $CD56^{+}$ CD8 T cells express a variety of NK cell receptors such as NKp44, NKG2A and others (Correia et al., 2011). In relation to γσ T cells that are $CD56^{+}$ show increased tumour killing potential to $CD56^{-}$ cells (Alexander et al., 2008).

NK cells also will increase their expression of CD56 in response to IL-12, with $CD56^{low}$ changing to $CD56^{high}$ (Loza and Perussia, 2004). NK cell derived exosomes are also $CD56^{+}$ positive and can kill several cancer cell lines (Lugini et al., 2012). Without CD56, NK92s cannot adequately release cytotoxic granules in response to exposure to cancer cells (Gunesch et al., 2020). Additionally, there is evidence to suggest that NK cells can directly kill fungal cells mediated via CD56 (Ziegler et al., 2017).

The above evidence suggests that CD56 is crucial in mediating NK cell cytotoxicity and denotes a cytotoxic phenotype in other immune cells. Additionally, CD56 levels on NK cells can be altered in different disease settings with an increase in $CD56^{-}$ NK cells in untreated HIV patients, while lesions in patients with multiple sclerosis have infiltrating $CD56^{bright}$ (Cocker et al., 2022, Rodriguez-Mogeda et al., 2024). While there is a need for further work to be carried out on the exact function of CD56 in both NK cells and other cells it is clear that it is involved in NK cell's ability to directly target cells for killing.

$CD56^{bright}$ NK cells, as denoted by a higher expression of CD56 in comparison to the dim population, are potent cytokine producing cells, with the ability to secrete a variety of cytokines, including but not limited to IFN-γ, TNF-β, IL-10 and GM-CSF (Fehniger et al., 1999, Cooper et al., 2001b). The $CD56^{bright}$ population produce these cytokines to a much greater extent than the $CD56^{dim}$ population and do so in response to a number of cytokines such as IL-12, IL-15, and IL-18 and. Also in comparison to both the $CD56^{dim}$ and other lymphocyte populations, $CD56^{bright}$ NK cells constitutively express a high affinity IL-2 receptor and preferentially expand when treated with IL-2 (Caligiuri et al., 1990, Baume et al., 1992, Caligiuri et al., 1993). Additionally, the $CD56^{bright}$ cells express the activating NKp46 receptor to a higher extent than their $CD56^{dim}$ counterparts and do no express certain killer Ig-like receptors such as CD156a, CD156b and NKB1 (Voss et al., 1998, Jacobs et al., 2001). While not traditionally considered particularly cytotoxic, $CD56^{bright}$ NK cells can be primed to be to be potent killer with IL-15 lending less credence to the idea that $CD56^{bright}$ cells are purely immunomodulatory (Wagner et al., 2017).

In comparison to the "immunomodulatory" $CD56^{bright}$, $CD56^{dim}$ NK cells are considered to be, largely cytotoxic. As previously mentioned, they can produce various cytokines but in orders of magnitude less than the $CD56^{bright}$ (Fehniger et al., 1999, Cooper et al., 2001b). This could in part be due to the increased expression of SHIP1, which when overexpressed in $CD56^{bright}$ NK cells reduced IFN-γ production (Trotta et al., 2005). Instead of recruiting other immune cells to the site of infection or the tumour, $CD56^{dim}$ cells directly target the aberrant cell. $CD56^{dim}$ NK cells can recognise stressed or abnormal cells through a variety of mechanisms. The expression of the low affinity Fcγ receptor, CD16 indicates this subset of NK cells to engage in antibody dependent cell cytotoxicity, whereby NK cells can identify and kill IgG labelled cells (Clynes et al., 2000). However, this method relies on B cells to produce antibodies against a specific ligand which takes time. To respond to quickly, NK cells rely on a myriad of activating and inhibitory receptors to identify target cells. NK cells do not follow the old adage of "self vs non-self" instead recognising "missing-self" cells that are stressed either from the effects of mutations that amass as a cell becomes cancerous or when it is infected by a virus (Myers and Miller, 2021, Björkström et al., 2022). $CD56^{dim}$ NK cells can use the likes of KIR2DS1 to kill cells presenting viral peptides via MHC 1 (Stewart et al., 2005), NKG2D recognises the MHC related molecules MICA and MICB which are upregulated on stressed cells (Zingoni et al., 2018), or NKp46 which can identify viral derived peptides on the surface of cells (Mandelboim et al., 2001). Once the target has been identified,

the NK cells will form a synapse between itself and the target cell allowing for the delivery of the lytic molecules necessary for cell death (Orange, 2008). The synapse allows for direct and targeted killing through lytic molecules such as granzyme and perforin (Prager and Watzl, 2019). Perforin causes pores to form in the membrane of the target cells allowing for the granzyme to enter the cell to initiate apoptosis (Law et al., 2010, Sutton et al., 2000, Barry et al., 2000).

### 1.2.2 The Immunomodulatory Role of NK cells

NK cells are dynamic cells that can perform a variety of functions. Aside from their homeostatic role in various tissues which will be discussed later, NK cells can, via cytokines, modulate other immune cells, directing the immune response rapidly after a virally infected or cancerous cell is detected. The exact response that NK cells engage in is dependent on the micro-environment that the NK cell has migrated to or developed in the tissue where a NK is resident. NK cells express a variety of chemokine receptors such as CD62-L, CCR7 and CXCR3 in the case of $CD56^{bright}$ NK cells and CXCR1, CXC3R1 and CD11a for $CD56^{dim}$ NK cells that can attract them to the site of inflammation (Poli et al., 2009, Castriconi et al., 2018).

NK cells are potent drivers of a Th1-type response when exposed to cytokines such as IL-15, 12 and 2 (Abel et al., 2018). Myeloid cells such as monocytes and macrophages can produce IL-15 which is critical for NK cell survival and which also drives IFN-γ, TNF-α and GM-CSF production by NK cells (Perera et al., 2012, Mrozek et al., 1996). IL-2 derived from T cells and IL-12 derived from APCs also work synergistically with IL-15 to drive the production of IFN-γ, TNF-α and GM-CSF by NK cells (Parihar et al., 2002, Wang et al., 2000).

These cytokines are crucial in the activation of a number of other immune cells. Dendritic cells undergo maturation in response to NK cell derived IFN-γ and TNF-α in the lymph node where NK cells are recruited during infection (Vitale et al., 2005, Bajénoff et al., 2006). Conversely NK cells can kill immature DCs *in vivo* which mechanistically be a form of positive selection for DCs expressing the necessary MHC levels but it can diminish DC mediated vaccine efficacy (Hayakawa et al., 2004). This positive selection of DCs can lead to improved DC mediated T cell proliferation (Morandi et al., 2012). There is very early evidence showing that by regulating DCs, NK cells can also prevent the development of cytolytic T cells (Gilbertson et al., 1986).

The IFN-γ that can drive DC maturation can also proliferate Th1 T cells, via CXCR3, NK cells are recruited to the lymph node where they can prime the expansion of Th1 T cells (Martín-Fontecha et al., 2004). Additionally, in response to coming into contact with tumour cells, NK cells can recruit T cells via L-8, MIP-1α, or RANTES (Roda et al., 2006). NK cells lysing target cells also drives the development of CD8 T cells via an IFN-α dependent manner (Krebs et al., 2009, Mailliard et al., 2003). Outside of the lymph node, in the tonsils, NK cells are required for the development of influenza specific CD8 T cells (Kos and Engleman, 1996). NK cell derived GM-CSF plays a role in mediating Neutrophil survival. Neutrophils are generally short-lived cells (8-20hrs), but NK cell derived GM-CSF can abate neutrophil apoptosis (Costantini et al., 2010).

NK cells can also drive bacterial clearing by other immune cells. NK cells have detectable amounts of mRNA for all the TLRs and a variety of TLR ligands can induce IL-12 production (Lauzon et al., 2006). IFN-γ derived from NK cells was crucial in clearing *Legionella pneumophila* infections, with additional requirement of MyD88 suggesting a role for TLRs, thought the exact TLR needed was not discerned (Spörri et al., 2006). NK cells in conjunction with DCs are also required to drive Th1 and Th17 cells to aid in clearing *Chlamydia muridarum* in mice (Shekhar et al., 2015).

Moving on from Th1 linked cytokines, NK cells can also be affected by IL-4, a conventional Th2 cytokine. IL-4 drives the promotion of NK cells that produce higher amounts of IFN-γ, IL-10 and GM-CSF than conventionally activated NK cells (Kiniwa et al., 2016). This IL-10 can regulate T cell responses in the liver during persistent viral infections leading to a reduction in inflammation (Ali et al., 2019).

These studies highlight the immunomodulatory role of NK cells, presenting them as key drivers of the immune response.

### 1.2.3 The Cytotoxic Role of NK cells

Aside from being potent drivers of the immune response via cytokine or chemokine production, NK cells can directly target cells for killing. These target cells can range from fungal cells as well as host cells that are virally infected or have turned cancerous. The advantage that NK cells have over antigen dependent cell cytotoxicity is that NK cells directly lyse cells without prior stimulus.

NK cells can detect and lyse target cells directly via receptor mediated degranulation, receptor mediated target cell death and antibody assisted cell killing.

While there is a lack of evidence for NK cell mediated fungal surveillance in humans, mice studies hint at a possible role for NK cells in protecting the body from fungal pathogens. NK cells seem particularly capable of damaging both *Cryptococcal* and *Aspergillus* cells *in vivo* (Bouzani et al., 2011, Levitz et al., 1994, Hidore et al., 1990, Schmidt et al., 2011). While the exact mechanism is not clear, NK cells can increase expression of CD69, a common marker of activation, and damage *Aspergillus* cells (Schmidt et al., 2011). Additionally, it is suggested that NK cell derived IFN-γ can directly kill *Aspergillus*, which is a novel function of the cytokine (Bouzani et al., 2011).

A primary role for NK cells, is to lyse "self" cells that are stressed, either from uncontrolled mutations or the presence of a virus. This is a delicate process, involving a myriad of inhibiting and activating receptors to accurately kill cells that should be killed and not lead to rampant self-immunity.

One such mechanism by which NK cells can select target cells for lysis is expression of TNF-related apoptosis-inducing ligand (TRAIL) receptors on the target cell. Both virally infected cells and tumour cells express the death receptors DR4 and DR5 which NK cells can detect via TRAIL (Mundt et al., 2003, Takeda et al., 2002). NK cells express TRAIL and binding of TRAIL to the receptors expressed on the surface of stressed cells induces apoptosis and IFN-γ release from NK cells (Kayagaki et al., 1999, Höfle et al., 2022). The binding of TRAIL to DR4 and DR5 leads to the activation of various caspases within the target cell which can lead to the formation of the apoptosome and eventually cell death (Pimentel et al., 2023). IFN-γ has also been shown to both induce tumour cell apoptosis and prohibiting angiogenesis (Beatty and Paterson, 2001, Detjen et al., 2001).

Another crucial surface molecule linked to NK cell killing is Fas. Similar to TRAIL, mutated cells or cells that have detected an internal viral pathogen upregulated the expression of FasL on their surface (Fujimoto et al., 1998, Niehans et al., 1997). The interaction between Fas on the NK cell and FasL on the target cell leads to an apoptotic cascade, mediated by the formation of active caspases within the target cell (Kaufmann et al., 2012).

One mechanism by which tumour cells avoid detection by the immune system is the downregulation of MHC-1 (Mehta et al., 2008, Zaretsky et al., 2016). Cytotoxic T cells can detect

viral peptides or tumour associated peptides presented by MHC-1 which can lead to the target cell death (Wu et al., 2023). By lowering MHC-1 on the surface tumour cells and virally infected cells can avoid detection. However, NK cells can detect MIC-A and MIC-B, which are stressed induced surface proteins that can be detected by the NK cell activating receptor NKG2D (Bauer et al., 1999). Engagement of NKG2D with MIC-A or B leads do degranulation by NK cells, where they lyse the target cell in a perforin dependent manner (Smyth et al., 2004). Perforin forms holes in the target cell membrane where granzymes, such as granzyme B, can enter the cell and induce apoptosis (Prager and Watzl, 2019). Additionally, MHC-1 interacts with inhibitory KIR receptors on NK cells, and a lack or mismatch of MHC-1 on a cell can label it for killing by NK cells in an amazing display of immune redundancy (Oberg et al., 2004).

Finally, while antibodies are not required for NK cell cytotoxicity, they can engage in antibody dependent cell cytotoxicity (ADCC). NK cells express the Fcγ receptor CD16, with the $CD16^{+}$ positive cells overlapping with the $CD56^{dim}$ population (Romee et al., 2013). NK cells can recognise antibodies that have bound to a virally or parasite laden cell as is seen in both COVID-19 infected cells and red blood cells with *Plasmodium falciparum* (Arora et al., 2018, Hagemann et al., 2022). Upon interaction between CD16 and IgG, NK cells can secrete IFN-γ and increase expression of CD107a which is a marker for degranulation (Sun et al., 2019).

These mechanisms for direct cell killing by NK cells solidify them as one a number of crucial immune cells responsible for viral and tumour immunity. In models or diseases that result in NK cell deficiencies the host is susceptible to both pathogenic viral infections and rampant tumour growth (Orange, 2006, Kim et al., 2000).

### 1.2.4 NK Cell Tolerance and Memory

The processes of tolerance and memory were once thought to be solely in the domain of the adaptive immune system, there is growing evidence that innate cells can also signs of being tolerant to self and can respond differently to subsequent infections.

Due to the nature of TCR development, an individual T cell can express one of a near infinite number of different antigen binding sites, each of which are unique. Some of these antigen

binding sites will therefore be specific to a self-antigen and these T cells need to be destroyed before they can lead to the development of autoimmunity. T cells which respond too strongly when presented with self-antigen are destroyed (Nurieva et al., 2011).

NK cells are no different considering that NK cells recognise “self” or more so the lack of “self” it is crucial that NK cells do not become over reactive to avoid autoimmunity. This is observed where NK cells from mice lacking MHC class I (MHC-I) have normal numbers of NK cells and do not show increased NK cell autoimmunity indicating that there is a check on NK cell activity (Furukawa et al., 1999). However, this check on NK autoimmunity can be detrimental where NK cells developed in the absence of MHC-I, can then not kill tumour cells lacking MHC-I (Liao et al., 1991, Bix et al., 1991). Another form of tolerance is that NK cells will show decreased cytotoxic activity in response to prolonged NKG2D exposure, highlighting that NK cells will not remain overly active in the presence of stimulatory factors (Champsaur and Lanier, 2010).

Immune memory is described as the process whereby the immune system can respond more quickly and to a greater extent when re-exposed to the same antigen. This is mediated by the development of long-lived T, B and plasma cells during the initial infection that are quick to expand upon reinfection (Akkaya et al., 2020, Martin and Badovinac, 2018, Gasper et al., 2014). Mice have been shown to produce long lived mice cytomegalovirus (MCMV) specific NK cells in response to infection (Arase et al., 2002, Sun et al., 2009). This also observed in macaques that have been infected with the simian immunodeficiency virus (SIV), where SIV antigen specific NK cells can be found (Reeves et al., 2015). While less is known about memory NK cells in humans, there are studies showing long-lasting phenotypical changes to NK cells population in response to both viral and cytokine stimuli. A $CD94^{+}$/$NKG2C^{+}$ NK cell population emerges in people who have been infected with either human cytomegalovirus (HCMV) or Chikungunya virus (Gumá et al., 2006, Petitdemange et al., 2011). The development of these long lasting, antigen specific NK cells requires both a receptor and cytokine mediated response. The clearest evidence for the receptor driven aspect of NK cell memory is the preferential expansion of CD94–$NKG2C^{+}$ in response to HCMV infection (Gumá et al., 2004, Foley et al., 2012, Della Chiesa et al., 2012). Additionally, a recently described NK cell population that are deficient in the IgE receptor $Fc_{\varepsilon}RI\gamma$, show elevated CD16 mediated killing of HCMV infected cells highlighting a potential redundancy to the CD94-NKG2C has also been identified (Zhang et al., 2013, Lee et al., 2015).

The cytokine aspect of NK cell memory is highlighted by the necessity of IL-12 derived from $CD14^+$ positive cells to drive the expansion of $NKG2C^+$ cells (Rölle et al., 2014). IL-12 seems crucial as blocking IL-15, IL-18 and type 1 interferons did not ameliorate the expansion of the $NKG2C^+$ population. This was dependant on signalling through STAT4 (Sun et al., 2012). This evidence points towards a much greater overlap of the adaptive and innate immune response than previously thought.

However, when used in combination and in the absence of a viral stimulus, cytokines can induce a memory like subset of NK cells that have been "trained" to respond to a subsequent stimulus to a greater extent. Cooper *et al* in 2009 showed using a combination of IL-12/15/18, generated NK cells that produced more cytokines in response to subsequent stimuli (Cooper et al., 2009), Subsequent studies show that these cytokine trained memory NK cells were more cytotoxic, and that this training was dependent on glycolysis and OXPHOS and was disrupted in PWO (Kedia-Mehta et al., 2021, Romee et al., 2016).

### 1.2.5 Cytokine Activation of NK cells

NK cells express a wide variety of cytokine receptors such as IL-2, IL-12, IL-15, IL-18 (Sharma and Das, 2018, Parihar et al., 2002, Gosselin et al., 1999, Terrén et al., 2021, Sato et al., 2001). These cytokines have a variety of effects on NK cells whether that be proliferation, metabolic reprogramming, activation, and priming. The cytokines work in tandem with one another often enhancing the effect of a single cytokine (Freeman et al., 2015). The effect of novel cytokines such as IL-23 and IL-27 are also being described in more detail opening up further avenues of research into NK cells.

#### 1.2.5.1 IL-2

First described as a T cell proliferation and survival factor IL-2 can directly interact with NK cells (Willerford et al., 1995, Sim and Radvanyi, 2014). Signalling through CD25 and CD122, IL-2 is not essential for NK cell development (Minagawa et al., 2002), but it does serve to supplement the activity of other cytokines such as IL-15 and IL-21 (de Rham et al., 2007). NK cells can also be "primed" by various cytokines including IL-2. Similar to the multiple signals required for complete

T cell activation, NK cells require multiple signals acting in tandem for full activation from rest (Bryceson et al., 2006). IL-2 can increase the expression of both IL-12Rβ1 and IL-12β2 receptors and stabile STAT4 which is key for IL-12 signalling, increasing the production of IFN-γ (Wang et al., 2000).

#### 1.2.5.2 IL-12 and the IL-12 Cytokine Family

IL-12 is comprised of two subunits, the p30 and p45 subunits and signals through a heterodimeric receptor comprised of 12Rβ1 and IL-12β2 (Vignali and Kuchroo, 2012). IL-12 is produced by antigen presenting cells such as dendritic cells and macrophages and through STAT4 signalling, leads to IFN-γ production from both Th1 cells and NK cells (Mal and Trinchieri, 2001, Maurice K. Gately et al., 1998). IL-12 alone is not a particularly strong stimulus for NK cells but in combination with IL-18 and IL-15 can drive a potent response inducing production of IFN-γ, TNF-α, IL-10 and GM-CSF (Fehniger et al., 1999, Mehrotra et al., 1998). IL-12 also works in conjunction with NK cell surface receptors such as CD16, as NK cells coated in IgG and treated with IL-12 produce more IFN-γ and TNF-α than IL-12 alone, both *in vivo* and *in vitro* (Parihar et al., 2002). STAT4 is also crucial for IL-12 signalling as seen in STAT4 knockout mice that produce significantly less IFN-γ than their wildtype counterparts.

IL-12 is also a key mediator of inducing cell memory. As previously mentioned, IL-12 in particular is crucial for the development and proliferation (Rölle et al., 2014). IL-12 can also work synergistically with activating receptors such as NKp30 to induce NK cell memory this time signalling through STAT5 (Shemesh et al., 2022).

There are also other cytokines that are similar to IL-12 in a structural manner and are therefore grouped together in the IL-12 cytokine family. Members of this family include IL-23, IL-27, and IL-35 (Jones and Vignali, 2011, Vignali and Kuchroo, 2012). While similar in structure, these cytokines have a diverse range of functions. Similar to IL-12, IL-23 derived from APCs can induce IFN-γ production in NK cells (van de Wetering et al., 2008, Ziblat et al., 2017). Additionally, IL-12 and IL-23 were responsible for the development of a stronger secondary NK cell mediated response to a subsequent *Toxoplasmosis gondii* infection indicating a potential role for the IL-12 family in parasite defence (Ivanova et al., 2019). However, mice lacking in IL-23 were protected

from tumour metastasis indicating a potential anti-inflammatory role of the cytokine (Teng et al., 2010).

IL-27 has a multifactorial role in NK cell activity. It has the ability to prime NK cells so that upon further stimulation with IL-18 and can increase NK cell cytotoxicity via increasing expression of NK cell activating receptors and ADCC (Ziblat et al., 2015, Choi et al., 2019). It has also been implicated in viral immunity with IL-27 null mice are less able to mount an immune response to acute influenza infections than wild types (Kumar et al., 2019).

#### 1.2.5.3 IL-15

IL-15 is a crucial cytokine in relation to NK cell development, survival, and activation. Produced by APCs as well as epithelial and stromal cells IL-15 is crucial for the development of NK cells (Mortier et al., 2004, Huntington et al., 2009). The lack of IL-15 in both humans and mice results in a severely depleted NK cell population (Gilmour et al., 2001, Kennedy et al., 2000). IL-15 can signal through CD122 which is the β chain of the IL-2/IL-15 receptor at an early stage in their development and without IL-15 there is depleted number of both immature and mature NK cells in both the bone marrow and spleen of mice (Vosshenrich et al., 2005).

IL-15 can signal through both an autocrine and paracrine manner and persistent IL-15 signalling is required for the maintenance of both $CD8^+$ and NK cells (Cooper et al., 2002, Koka et al., 2003, Anton et al., 2020). However, unregulated IL-15 signalling is implicated in the development of leukaemia in mice highlighting the necessity for homeostatic activity of cytokines (Fehniger et al., 2001).

NK cells can be primed by short acting, low doses of IL-15 that increases both cytotoxic activity and cytokine production upon further stimulation (Lucas et al., 2007, Fehniger et al., 2007, Wagner et al., 2017). Priming NK cells with IL-15 does induce cytokine production it does not cause the metabolic shift seen with overnight stimulation highlighting the difference between priming and full activation (Luu et al., 2021).

IL-15 through various pathways, be that through PI3K/mTOR/AKT, the Ras/Raf/MEK/ERK or through JAK1/3 and STAT 3/5 (Budagian et al., 2006, Mishra et al., 2014). Signalling through the

mammalian target of rapamycin (mTOR) is crucial for the metabolic shift that NK cells moving from a resting to an activated state require (Donnelly et al., 2014a).

#### 1.2.5.4 IL-18

IL-18 acts in a similar manner to IL-15. Its receptor is expressed on NK cells and is induced by IL-12 and IFN-α (Sareneva et al., 2000). It similarly signals through the PI3K/mTOR/Akt pathway and leads to similar metabolic shifts as seen with NK cells stimulated with IL-15 (Terrén et al., 2021). IL-18 has been highlighted as a crucial cytokine to be used in used in anti-tumour NK cell mediated therapies through its ability to boost ADCC and IFN-γ production (Srivastava et al., 2013)

### 1.2.6 Metabolic Requirements of NK cells

All cellular processes are underpinned by a functioning intracellular metabolism. Cell survival, division and growth are all underpinned by a cell's ability to secure the necessary nutrients to provide both energy to the cell and the components for biosynthesis (Kalucka et al., 2015, Zhu and Thompson, 2019). Many cells that are at rest rely on OxPhos or aerobic glycolysis to meet their energy demands (Lunt and Heiden, 2011). However, dividing cells and activated immune cells increase and alter their metabolism to both build biomass to divide and generate new proteins and to produce energy as needed (DeBerardinis and Chandel, 2016, Jung et al., 2019).

Figure 3: A summary of the metabolic requirements of activated NK cells and the effect of obesity on their metabolism.

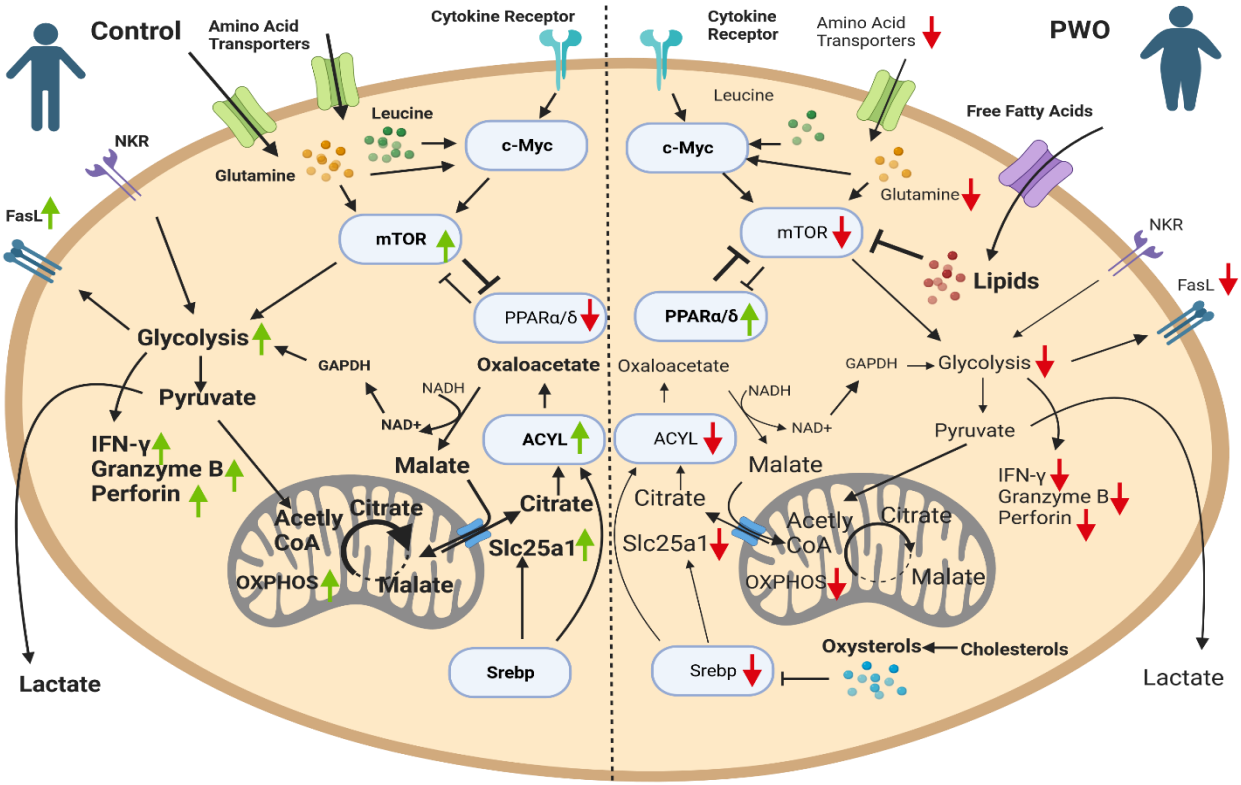



*Figure 4: This graphic describes the changes in metabolism once NK cells are activated. In people without obesity, NK cells increase their uptake of amino acids, glucose and other nutrients to both fuel the immune response and to provide biomass. Increased amino acid levels help to stabilise mTOR which allows for increased glycolysis which is crucial for cytokine production. However, in PWO, NK cells fail to adequately increase amino acid transporters resulting in lower activity of mTOR which is required for the drastic metabolic shift that NK cells undergo. Additionally, the accumulation of lipids and increased exposure to oxysterols impact NK cell function in people with obesity. Combine this with the increased activity of PPAR α and γ, NK cells from PWO are less able to kill target cells and produce key cytokines.*

### 1.2.6.1 Oxidative Phosphorylation in NK cells

At rest, NK cells rely on low levels of both OxPhos and glycolysis to survive (Slattery et al., 2021, Loftus et al., 2018). There are slight variations between the $CD56^{bright}$ and $CD56^{dim}$ populations with the $CD56^{dim}$ having higher metabolic rates in comparison to the $CD56^{bright}$ (Surace et al., 2021). While activated NK cells become heavily dependent on glycolysis OxPhos is still a critical pathway for a correct NK cell response to cytokine stimulation (Marçais et al., 2014, Keppel et al., 2015). Interestingly, NK cells primed for a short period with IL-15 are dependent on OxPhos to

respond to receptor stimuli, but do not after prolonged exposure to IL-15, indicting the role of OxPhos in early NK cell activation (Keppel et al., 2015).

Like with glycolysis, the rate of OXPHOS increases in response to both cytokine and receptor signalling in NK cells (Keppel et al., 2015, Keating et al., 2016). However, in comparison to cytokine stimulated NK cells which rely on glycolysis to a greater extent, receptor stimulated NK cells appear to rely much more in OXPHOS to drive their immune response having a higher ratio of oxygen consumption in comparison to glucose consumption (Keppel et al., 2015). By inhibiting OXPHOS with oligomycin, receptor activated NK cells did not produce IFN-γ to the same extent (Keppel et al., 2015). However, pre-treating NK cells with IL-15 removed the necessity of OXPHOS for NK cell function indicating that IL-15 can have metabolic modulatory effects beyond simply activating NK cells.

A novel role for OXPHOS in NK cell metabolism is enabling the development of long lived "adaptive" NK cells. NK cells from donors who have antibodies against HCMV showed both increased glycolytic and oxidative metabolism in comparison to seronegative donors (Cichocki et al., 2018). These adaptive NK cells had increased mitochondrial membrane potential and had increased expression of genes associated with the Electron Transport Chain (ETC). These changes in metabolism and gene expression is mediated by AT-rich interaction domain 5B (ARID5B) as inhibiting the translation of this gene resulted in reduced NK cell survival, IFN-γ production in response to IL-12 and IL-18 and limited mitochondrial capacity (Cichocki et al., 2018). The development of these adaptive NK cells seems to be dependent on mitophagy of damaged mitochondria that allows the NK cells to persist post a viral infection(O'Sullivan et al., 2015). This is seen with chronic HIV infections, where $NKG2C^{+}$ positive, adaptive NK cells are reduced exhibiting defects (Cubero et al., 2019)in OxPhos . So, while glycolysis may play a greater role in shorter lived effector NK cells it appears that OxPhos plays a role in their longer-lived adaptive counterparts.

It must be noted that glycolysis and OXPHOS regulate each other. This has been suggested by Assmann *et al*, whereby OxPhos is dependent on glucose derived cytosolic NADH being transported to the mitochondria to drive OXPHOS and ATP synthesis and in return mitochondrial citrate being converted to acetyl Co-A for the acetylation of GAPDH, an important enzyme in the glycolytic pathway (Assmann et al., 2017).

### 1.2.6.2 Glycolysis in Activated NK Cells

Resting NK cells use glucose driven OxPhos to survive in preparation to encountering a stimulus, but NK cell metabolism changes drastically upon stimulation (Keppel et al., 2015, Marçais et al., 2014). Upon stimulation, both $CD56^{bright}$ and $CD56^{dim}$ NK cells increase the rate of both glycolysis and OXPHOS (Keating et al., 2016). However, $CD56^{dim}$ cells did not significantly increase glucose uptake in response to cytokine stimulation, possibly indicating that a different metabolic pathway is required for $CD56^{dim}$ function. The high levels of GLUT1 expression and the ability to increase glucose uptake does potentially indicate that $CD56^{bright}$ NK cells which have been shown to produce large amounts of cytokines require increased glycolysis to carry out their function.

In response to treatment with Poly I:C, it has been shown that glycolysis is crucial to NK cells producing IFN-γ (Donnelly et al., 2014b). Not only did 2-DG limit cytokine production it also reduced NK cell anti-viral immunity both *in vivo* and *in vitro* (Mah et al., 2017, Donnelly et al., 2014b). Glycolysis in NK has been shown to be mediated by mTOR1 as treating NK cells with rapamycin which inhibits mTOR1 activity, which decreased the glycolytic capacity of NK cells and lowered the mRNA expression of key glycolysis proteins such as GLUT1, Hexokinase 2 (HEX2) and Lactate Dehydrogenase (LDH) (Donnelly et al., 2014b). Interestingly, NK cells treated with IL-2 showed reduced glycolytic capacity when treated with rapamycin but NK cells treated with IL-12 and IL-15 did not (Keating et al., 2016). Inhibiting glycolysis also reduced NK cell proliferation in response to IL-15 highlighting a role for mTOR and glycolysis not just in NK cell function but in development and proliferation (Mah et al., 2017).

The ability for mTOR to regulate NK cell glycolysis may be a result of its ability to regulate cMYC, a protein that has been implicated in regulating glycolysis in mice. cMYC knockout mice were shown to have reduced glycolytic and OXPHOS capacity and produced less IFN-γ and Granzyme B (Loftus et al., 2018). Inhibiting mTOR reduced the amount of cMYC present in cells but interestingly limiting the availability of key amino acids, either through blocking the transporter or limiting availability, also reduced cMYC levels indicating an interplay between amino acid metabolism and glycolysis in NK cells.

A further regulator of metabolic changes in NK cells is a family of proteins called Sterol Regulatory Element Binding Proteins (Srebps). Srebp proteins are regulators of both cholesterol and fatty acid synthesis but in NK cells a novel role has been found for this family of proteins (Horton et al., 2002). Inhibition of Srebp via hydroxycholesterols in activated NK cells reduced their ability to

increase glycolysis (Assmann et al., 2017). This is partially due to glucose in activated NK cells being converted to cytosolic citrate which then feeds into an altered TCA cycle referred to as the citrate-malate shuttle. This altered metabolic pathway is dependent to two Srebp controlled proteins, ATP-citrate lyase (ACLY) which converts citrate to oxaloacetate and cytosolic acetyl-CoA for lipid synthesis and the citrate transporter which moves cytosolic malate to the mitochondria in exchange for mitochondrial citrate (Assmann et al., 2017). Cytosolic citrate metabolism is then required to maintain elevated levels of glycolysis and OXPHOS and inhibiting citrate metabolism reduces the number of IFN-γ producing cells and reduces the amount of Granzyme B NK cells produce (Assmann et al., 2017).

A further role for glycolysis in NK cells in in relation to NK cell education or licensing. NK cell education is a process whereby NK cells are "taught" to differentiate between self and non-self via the interaction of in inhibitory receptors on NK cells such as the killer-cell immunoglobulin-like receptor (KIR) family of proteins and antigen presenting proteins such as HLA-1 which is an important process in developing NK cell tolerance to self (Carrillo-Bustamante et al., 2016, Raulet and Vance, 2006). Viruses often attempt to decrease the expression of HLA-1 in an attempt to evade T cells but a lack of HLA-1 exposes virally infected cells to NK cells (Petersen et al., 2003). However, returning to a reason why this KIR and HLA-1 reaction is important to metabolism is that educated NK cells have increased glycolytic capacity, increased expression of glucose transporters but similar OXPHOS capacity in comparison to uneducated NK cells (Schafer et al., 2019, Pfeifer et al., 2018). The reason for increased glycolytic capacity is not fully understood but it could prevent non-tolerogenic NK cells from maturing to a stage where they could cause damage to the host.

It is clear from the aforementioned studies that glycolysis plays a key role in fuelling NK cell metabolism upon activation. This is increase in glycolysis is mediated via mTOR, specifically mTOR1. By inhibiting mTOR1 or glycolysis NK cells have a reduced ability to produce IFN-γ and Granzyme B and have a reduced ability to lyse tumour cells and protect a host from viral infections. However, the production of the pro-inflammatory cytokine TNF-α by NK cells is not dependent on glycolysis (Donnelly et al., 2014b). Additionally glycolysis is needed for the expression of the FasL protein which interacts with the Fas receptor that is expressed on stressed cells such as tumour cells and introduces apoptosis in those cells (Wang et al., 2020).

#### 1.2.6.3 Fatty Acid Metabolism in NK Cells

While there is limited research examining the role of fatty acid metabolism in NK cell function there are some studies that point to a negative effect of fatty acids on NK cell function. While inhibiting fatty acid synthesis (FAS) did not impeded NK cell production of IFN-γ production, the accumulation of fatty acids in NK cells did (Keppel et al., 2015, Michelet et al., 2018). Other fatty acids are also implicated in downregulating NK cell function. Butyrate, which is a microbial derived short chained fatty acid has been shown to impair NK cell function by attenuating the activity of mTOR, cMYC expression and metabolic activity (Zaiatz-Bittencourt et al., 2023). Additionally, NK cells are impaired when exposed to the fatty acid rich environment of lymphoma, which limited NK cell IFN-γ production and metabolism (Kobayashi et al., 2020).

#### 1.2.6.4 Amino Acid Metabolism

While the role of amino acid metabolism in NK cells has not been fully revealed some studies have implicated certain amino acids as being important in modulating NK cell function. NK cells in response to cytokine stimulation increase the expression of various amino acid transporters such as CD98, SLC1A5 and SLC7A5 (Keating et al., 2016, Jensen et al., 2017, Almutairi et al., 2019). Treating NK cells with inhibitors against SLC1A5 and CD98, GPNA and D-phenylalanine respectively, abrogated NK cell function is response to NKG2D stimulation (Jensen et al., 2017). SLC1A5 which is also known as ACT2 is a transported that preferably transports glutamine and is modulated by the presence of cysteine(Scalise et al., 2018b). SLC7A5 is heterodimer comprised of LAT1 and CD98 and is a transporter for a variety of amino acids such as leucine and tryptophan (Scalise et al., 2018a).

As previously mentioned cMYC can modulate NK cell metabolism to allow for a pro-inflammatory response (Loftus et al., 2018). cMYC activity is regulated by the presence but not metabolism of glutamine as inhibiting glutaminolysis lowers cMYC levels or reduce the numbers of NK cells producing IFN-γ as the absence of glutamine had (Loftus et al., 2018). The absence of glutamine also appears to have a greater effect on NK cell metabolism than BPTES, with NK cells cultured in glutamine free reported to have reduced rates of ECAR and OCR (Loftus et al., 2018). This study indicates that while glutamine is not a fuel source for NK cells it is required for proper NK cell function.

Leucine is another amino acid with a role in NK cell function and metabolism. The presence of leucine is important in maintain mTOR activity as culturing NK cells in leucine free media resulted in lower levels of pS6 which is a marker used to measure mTOR activity (Loftus et al., 2018). It is clear that while amino acids might not provide NK cells with a source of fuel, they are key in regulating other drivers of metabolism such as cMYC and mTOR.

## 1.3 Iron and the Immune System

In addition to the major macro nutrients such as carbohydrates, fats, and amino acids that the immune system requires to function, trace elements are equally as important. Not only is zinc an essential trace element for the function of a wide variety of enzymes, it is crucial for phagocytosis, antibody production and NK cell activity (Vallee and Falchuk, 1993, Beisel, 1982). Copper, another critical metal involved in a variety of cellular functions such antioxidation and cellular respiration, is key to the proper function of a variety of immune cells such as B cells, neutrophils and cytotoxic T cells (De Luca et al., 2019, Cordano et al., 1966, Flynn and Yen, 1981, Prohaska and Lukasewycz, 1981).

### 1.3.1 Iron Uptake

Iron follows on from zinc and copper and is crucial to not only immune cells but all cells of the body. Integral to the transport of oxygen around the body, iron is a key element in the electron transport chain, forming Fe-S clusters which are core components of the (Read et al., 2021). Low iron levels can result in stunted growth and mental development (Lozoff and Georgieff, 2006). Focusing on the immune system, iron is essential for the normal function of a variety of immune cells.

Broadly speaking there is two mechanisms by which cells absorb iron, via transferrin bound iron or non-transferrin mediated mechanism. Non-transferrin bound iron uptake can be indictive of iron overload (Brissot et al., 2012). There are a number of membrane bound transporters that are linked to non-transferrin iron uptake such as DMT1 and ZIP14 and Scara5 (Knutson, 2019). The function of DMT1 is an alternative iron transporter to CD71, while ZIP14 is also responsible for zinc uptake (Skjørringe et al., 2015, Kim et al., 2020). Scara5 is another receptor that mediates non-ferritin bound iron uptake in developing kidneys (Li et al., 2009). There is little evidence of the role of non-transferrin bound iron uptake in many immune cell populations, NK cells included. DMT1 is linked to macrophage survival during bacterial infection by offering an alternative source of iron, as transferrin bound iron is reduced in bacterial infections (Schaible and Kaufmann, 2004, Grander et al., 2022). T cells have also been shown to take up iron bound to citrate as a possible safety mechanism against circulating iron overload and not as a mechanism to meet their iron

requirements (Arezes et al., 2013). Haem and haemoglobin are also molecules that can be used for iron uptake. Macrophages can scavenge for iron by using CD91 to bind to haem (Hvidberg et al., 2005). Haemoglobin can be internalised via CD163 in a similar manner, also by macrophages (Kristiansen et al., 2001).

The majority of iron that is in circulation is transferrin bound and therefor is regulated by CD71 linked endocytosis (Mayle et al., 2012). CD71 is crucial for a variety of immune cells including NK cells. MAIT cells require iron upon stimulation and by blocking CD71 or removing bio-available iron from media leads to defective mitochondria and attenuated function (Ryan et al., 2024). Conventional T cells require iron to respond adequately to infection and to mount a sufficient vaccine response which is regulated by CD71 (Frost et al., 2021). B cell function is also reliant on CD71 (Jabara et al., 2016).

Hypoferremia is also linked to leukocyte dysfunction. A study by Frost *et al* shows that neutrophils are highly dependent on iron levels, in both relation to neutrophil production and function (Frost et al., 2022). The administration of hepcidin to mice reduced over neutrophil numbers, stunted their response to granulocyte-colony stimulating factor and impaired NETosis. Similarly, the iron chelator Deferoxamine (DFO), which makes any iron in solution biologically unavailable for cells, reduces the ability of macrophages to kill *Salmonella typhimurium* via reactive oxygen species (Collins et al., 2002). NK cells are similarly dependent on iron to carry out their function. Littwitz-Salomon *et al*, shows that murine NK cells increase iron uptake in response to an infection *in vivo* (Littwitz-Salomon et al., 2021). Administering murine hepcidin to mice reduced NK cell IFN-γ production. *Ex vivo*, DFO reduced IFN-γ and Granzyme B production due to disrupting cellular metabolism.

These papers highlight to role of trace elements in immune cell function and there are still may questions left unanswered. One, which we attempt to shed light on is the effect of obesity associated iron deficiency on NK cell function and metabolism.

### 1.3.2 Intracellular Iron Storage and Use

Hepatocytes store that majority of the bodies iron that is not in use, and can release it when iron levels drop but other cell types contain iron stores for their own cellular needs (Takami and

Sakaida, 2011). Iron that is not required for cellular function is stored in a ferritin cage, comprised of both ferritin heavy and light chains (Lawson et al., 1991). While the ferritin cage is found in the cytosol, there also does exist mitochondrial ferritin (Levi et al., 2001). The iron in the ferritin cage is $Fe^{3+}$ which cannot be used for many biological functions, instead nuclear receptor coactivator 4 (NCOA4) senses low intracellular iron and engages in ferritinophagy, releasing labile $Fe^{2+}$ (Santana-Codina and Mancias, 2018).

It is this $Fe^{2+}$ that is used for cellular processes. Iron is a cornerstone to some of the most conserved cellular processes across all forms of life. DNA synthesis is heavily dependent on iron levels. Numerous enzymes linked to DNA synthesis and repair contain iron sulphur clusters such as Dna2, various exonucleases and RNA polymerase to name a few (White and Dillingham, 2012, Zhang, 2014). In multicellular organisms mitoferrin is the transporter that transport labile iron from the cytosol into the mitochondria where it is used for a variety of functions (Shaw et al., 2006). While not a core immune cell function, oxygen transport via haemoglobin is dependent on haem synthesis in the mitochondria which is an iron heavy process (Dutt et al., 2022). All cells rely on the electron transport chain (ETC) to generate ATP to varying degrees and since the various complexes of the ETC contain iron sulphur clusters and haem (Paul et al., 2017).

Between the conserved role of iron in cellular function and the specific requirements of various immune cells for iron it is no wonder than obesity associated anaemia is a potential mechanism by which immune cells are negatively affected by obesity.

## 1.4 Obesity

The World Health Organization (WHO) defines obesity in adults has having a body mass index (BMI) of above 30kg/$m^2$, while a BMI of between 26kg/$m^2$ and 29kg/$m^2$ is classified as over-weight (Willett et al., 1999). The definition for children defines obesity as a child's weight more than two standard deviations from the average due to differences in development (Balasundaram and Krishna, 2023). The WHO definition is largely based for people of European ancestry, which does cause some debate on the cutoff for different populations globally, it is still widely accepted (WHO, 2004). The prevalence of obesity worldwide is of major concern, with approximately 650 million adults currently living with obesity and a further 1.9 billion over-weight with roughly 340 million children also being classed as obese (Sørensen et al., 2022). The prevalence of obesity is not spread equally amongst gender, global regions, or socio-economic lines. Women have higher rates of obesity than men, with the global burden of obesity concentrating in Europe, the Americas, and the Middle East (Boutari and Mantzoros, 2022, Wells et al., 2012). Within countries, obesity is concentrated amongst those of lower socioeconomic groups in rich countries, while the reverse is seen in lower income countries with food availability, healthcare and income can play different roles in different countries (Ameye and Swinnen, 2019). A worrying trend in relation to obesity, is the growing prevalence of the condition, with the rate of obesity growing from 4.6% to 14% from 1980 to 2019 (GBD, 2020). Globally the number of children with obesity is half that of adults at about 6-7% but, this is not share equally with one in three children in Europe and over 50% in American Samoa living with obesity (WHO, 2022, Di Cesare et al., 2019).

Aside from the increased health care risks associated with obesity on an individual level, on a national scale, obesity can account for between 0.7% and 2.8% of total healthcare spending, with associated costs being up to 30% for people with obesity in comparison to those without (Withrow and Alter, 2011). On an individual level this results in about a 100% increase on yearly healthcare costs for people with obesity in comparison to people without obesity (Cawley et al., 2021). Combine this with reduced income potential for people with obesity, the negative economic effects that obesity has on individuals and healthcare systems cannot be overlooked (Larose et al., 2016). However, the most severe aspect of obesity is the increased mortality. Roughly 4 million deaths globally can be attributed to the condition and this is due to the increased risk of a variety of life altering comorbidities (Afshin et al., 2017).

Figure 4: Comorbidities Associated with Obesity

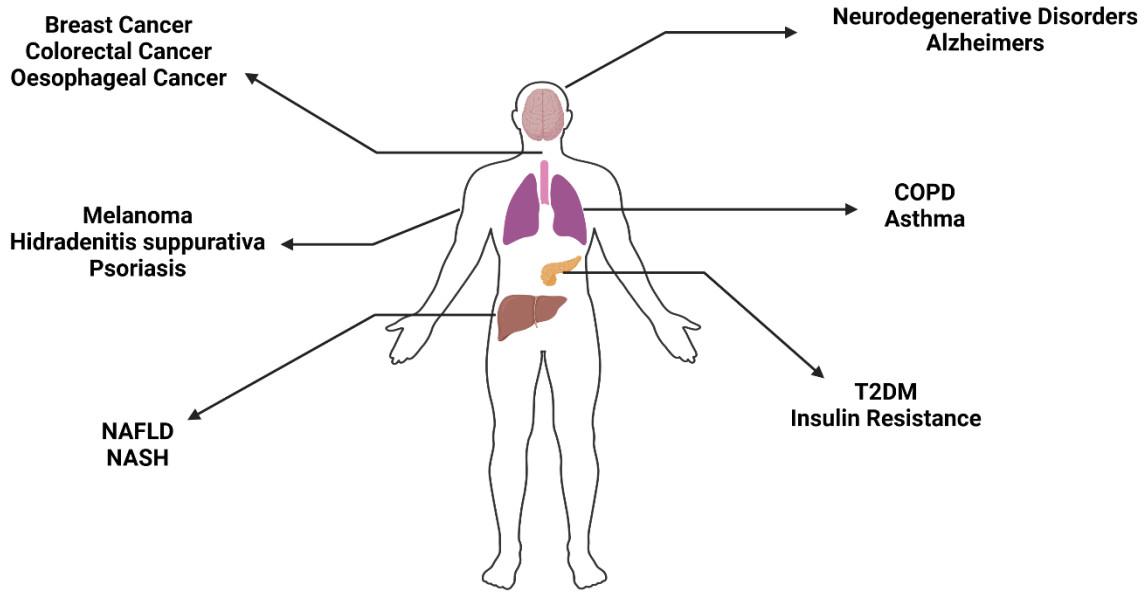



*Figure 3: Comorbidities Associated with Obesity. This diagram highlights the range and severity of some of the various comorbidities associated with obesity. Obesity is associated with an increased risk of developing these comorbidities or worse outcomes for the patient.*

### 1.4.1 Comorbidities Associated with Obesity

Obesity is associated with approximately 1300 excess deaths a day in the USA. This is linked to PWO on average, losing 2.4 years in life expectancy (Ward et al., 2022). Each 5 point increase in BMI is associated with a 30% increase in overall mortality (Whitlock et al., 2009). This can be partially attributed to the increased risk of developing various comorbidities and malignancies that are heavily associated with obesity. While the exact mechanism by which obesity contributes to the development and severity of these comorbidities is unclear, they all share a link to the chronic inflammation and disrupted endocrine signalling that are associated with obesity.

#### 1.4.1.1 Metabolic Syndrome

Metabolic syndrome is a combination of interlinked conditions such as abdominal obesity, insulin resistance, hypertension and dyslipidaemia which are strongly linked with the development of diabetes and cardiovascular disease (Rochlani et al., 2017). Metabolic syndrome is heavily associated with obesity with 60% of people meeting the criteria of metabolic syndrome, with that number rising to 80% if a person's BMI is above 35kg/$m^2$ (Damiri et al., 2018). Metabolic syndrome and obesity are closely linked to an increase an increase in inflammatory markers such as C-reactive protein, IL-6 and TNF-α along with a reduction in the hormone adiponectin, which is associated with normal insulin signalling (Yudkin et al., 1999, Lemieux et al., 2001, Cote et al., 2005). This increase inflammatory markers are heavily associated with the pathogenesis of other comorbidities associated with obesity.

#### 1.4.1.2 Insulin Resistance and Type 2 Diabetes

Produced by β-cells in the pancreas, insulin is a crucial hormone in maintaining appropriate glucose levels in the body. Unlike Type 1 diabetes (T1D) which is an autoimmune disorder that leads to a lack of β-cells resulting in a lack of insulin, insulin resistance and T2DM is an abnormal response to insulin (Cefalu, 2001). Insulin helps maintain homeostatic levels of glucose by inducing the expression of glucose transporters on cells to absorb excess glucose (Wilcox, 2005). Insulin resistance is defined as a reduced response to normal or heightened levels of insulin that results in impaired clearance of glucose (Reaven, 2004). Several factors are associated with the development of insulin resistance, increases in pro-inflammatory markers such as IL-6, TNF-α and CRP (C Reactive Protein) lead to the development of a reduction in insulin efficacy (Roytblat et al., 2000, Visser et al., 1999, Hotamisligil and Spiegelman, 1994). Elevated free fatty acids (FFAs) have also been linked to the development of insulin resistance (Sampath and Ntambi, 2004). Additionally, our group has shown IL-17, a relatively novel cytokine that is found in elevated levels in PWO can also lead to insulin resistance (Bergin et al., 2022). When the expansion of the β-cell population in the pancreas and the elevated levels of insulin can no longer compensate for the increased insulin resistance then T2DM develop (Donath and Shoelson, 2011). Patients with diabetes can expect to live shorter lives and have increased mortality rates in comparison to those without (Tancredi et al., 2015).

#### 1.4.1.3 Cardiovascular Disease

Cardiovascular disease (CVD) is a collection of conditions that can affect both the blood vessels and the heart. Increased BMI is associated with an increased risk of developing CVD (Barroso et al., 2017, Akil and Ahmad, 2011, Wilson et al., 2002). Similar to insulin resistance, the low-grade inflammation associated with obesity can lead to the development of CVD. Obesity is linked to increased expression ICAM-1 which can lead to endothelial dysfunction (Pontiroli et al., 2004). CRP has also been implicated in the development of atherosclerosis, working in conjunction to low-density lipoproteins (LDL) to develop pathogenic "foamy" macrophages and the development of the atherosclerotic lesions (Singh et al., 2008). IL-6, TNF-α and IL-1, hallmarks of inflammation in obesity are also associated with the development of CVD (Bruunsgaard et al., 2000, Zamani et al., 2013, Libby, 2017). Additionally, obesity can affect the efficacy of CVD treatments as seen with patients with obesity still developing atheroma and poorly manging hyperlipidaemia despite optimal statin treatment (Sandfort et al., 2016).

However, in relation to atherosclerosis there are other weight related characteristics that have a stronger association to the disease than BMI. Waist circumference has been shown to have an increased association to atherosclerosis and CVD even when BMI is considered (Hernández-Reyes et al., 2020, Ross et al., 2020). It is important therefore to consider other clinical readouts when assessing a person's risk at developing the comorbidities associated with obesity.

#### 1.4.1.4 Liver Disease

While the exact mechanism by which obesity leads to the development of liver inflammation, several hallmarks associated with obesity are also present in liver disease. Obesity is associated with the development of steatosis, which is the accumulation of triglycerides in hepatocytes (Hoyumpa et al., 1975). The prevalence of steatosis in PWO is approximately 65% as opposed to 15% in people without obesity (Ruhl and Everhart, 2003). Insulin resistance is associated with the progression from steatosis to non-alcoholic steatohepatitis (NASH), a severe form of non-alcoholic fatty liver disease (Dongiovanni et al., 2016). Additionally, the inflammatory milieu made up of cytokines such as TNF-α, IL-6 and the hormones leptin and adiponectin, all can contribute to the progression of the disease. Serum TNF-α is associated with NAFLD (Non-alcoholic Fatty Liver Disease) severity in children, while blocking TNF-αR reduces steatosis in murine models (Manco et al., 2007, Wandrer et al., 2020). Obesity is linked to elevated leptin and

reduced levels of adiponectin (Zhao et al., 2021). Persistent hyperleptinemia is associated with liver cirrhosis and steatosis while hypoadiponectinemia is linked to liver fibrosis (Nazal et al., 2010, Jiménez-Cortegana et al., 2021). Similarly circulating IL-6 is significantly associated with increased risks NAFLD while blocking IL-6 can attenuate the progression of steatohepatitis (Mas et al., 2009, Duan et al., 2022).

#### 1.4.1.5 Airway Diseases

Obesity is closely linked to both the severity of a variety of airway diseases and an exacerbation of events. In both children and adults, people with obesity who also have asthma have an increased risk of hospitalisation and mechanical ventilation in comparison to people without (Holguin et al., 2011, Okubo et al., 2016). A similar observation is seen with chronic obstructive pulmonary disorder (COPD) (Shin et al., 2022). Obesity also increases a person's risk of being prescribed medication to treat COPD, yet it can reduce the efficacy of glucocorticoid treatment for asthma (Sutherland et al., 2008).

#### 1.4.1.6 Cancer

Obesity is associated with an increased risk of developing a variety of cancers, ranging from oesophageal, renal, thyroid, breast cancer and melanoma to name a few (Enzinger and Mayer, 2003, Ahn et al., 2007, Kitahara et al., 2011). The low-grade inflammation and disruption to hormone signalling seen in obesity have been shown to lead to an increased risk of developing cancer. Insulin resistance was associated with the development of breast cancer and dysfunction in insulin like growth factor signalling can also lead to the progression due to altered cell proliferation and death (Cohen and LeRoith, 2012, Menu et al., 2004). Chronic and elevated levels of leptin is also implicated in the risk of developing breast and ovarian cancer in women again by disrupting the normal cell cycle progression (Chen et al., 2013, Dieudonne et al., 2002).

Elevated CRP is also associated with both the risk of developing and poorer survival rates when diagnosed with colorectal cancer (Slattery et al., 2011). IL-8 and IL-6 derived from adipocytes are associated with increased ovarian cancer metastasis (Nieman et al., 2011). Unsurprisingly TNF-α is also implicated in developing colorectal cancer (Kim et al., 2008).

It is clear that due to the life altering comorbidities and the societal stigma that people often endure that there is a need for therapies that can aid people lose weight if they choose to or is medically necessary.

### 1.4.2 Current Therapies for Weight Loss

There are a number of different therapies currently in use to help with weight loss. However, the degree to which the various therapies are effective vary when tested with randomised control trials and longitudinal studies, and that there is a substantial market of unproven and potentially dangerous diet and weight loss supplements that are often unregulated (Pillitteri et al., 2008, Onakpoya et al., 2011). It is important to note that not every person with obesity decides to lose weight and those that do, have varied results with a myriad of different approaches. **Paper I**, goes into detail on the effect of current weight loss therapies on NK cells but we summarise the current understanding here.

#### 1.4.2.1 Diet Alterations

Dietary alterations are often the first weight loss intervention that a person will be advised due to the relative low cost, and it often paired with other interventions. There are a variety of different diets that can be recommended, such as low calorie and low carbohydrate diets, high protein and ketogenic, low fat or diets based on regional foods such as the Mediterranean diet, each with various levels of evidence on their effectiveness on inducing weight loss (Kim, 2021). However, a large proportion of people who lose weight through dietary means experience weight regain after a year and return to the original weight within three to five years (Thomas et al., 2014, Wadden et al., 2012).

A balanced diet is crucial for correct immune cell function as seen by the effects of malnutrition on the immune system (Arthur et al., 2015). There is evidence showing that changing one's diet alone without weight loss can improve immune cell function. There is evidence linking the modern Western diet to negative effects on immune cell function. The Western diet, high in processed foods, cholesterol and saturated fatty acids has been shown to increase inflammation and negatively alter immune cell function (Sen et al., 2017, Sheedy et al., 2013). Various studies have shown that altering one's diet to counteract the western diet such as increasing fibre intake,

increasing omega-3 fatty acids or reducing phenol consumption can all reduce the pro-inflammatory state that is driven by the Western diet (Camargo et al., 2010, Aliasgharzadeh et al., 2015, Faintuch et al., 2007).

#### 1.4.2.2 Physical Therapy

Physical therapy often accompanies other forms of weight therapies, such as surgical interventions and diet advice. Physical therapy can encompass a wide range of activities designed to increase aerobic fitness and increase energy consumption. Regular physical activity has been shown to improve both the risk of developing and relapsing various cancers (Bigley and Simpson, 2015, Kruijsen-Jaarsma et al., 2013, Rundqvist et al., 2013). Increased physical activity has been shown to induce some weight loss in both men and women (Richardson et al., 2008), and in conjunction with calorie restriction showed better results than calorie restriction alone (Ross et al., 2000). However, there are two major issues with regards physical activity and weight loss, long term weight loss and barriers to activity. Firstly, obesity itself and many of the comorbidities associated with obesity such as asthma, CVD and diabetes can limit a person's ability to engage with an exercise regime, whether through increased pain or inability to engage with the programme (Dockrell et al., 2007, King et al., 2015, Zdziarski et al., 2015). Secondly, many people who lose weight through exercise alone tend to regain some if not all the weight lose within 1 year of the trial commencing (Franz et al., 2007, Catenacci et al., 2014). This highlights the need for much more personalised treatment courses to help those to lose weight and maintain that weight loss.

#### 1.4.2.3 Surgical Interventions

Bariatric surgery is a collection of surgical interventions designed to assist in weight loss. There a number of different types of bariatric surgery, such as the gastric band, gastric bypass and Roux-en-Y, but they all are intended to limit the amount of food that can be consumed before satiety and limit nutrient uptake (Elder and Wolfe, 2007). While the potential for severe complications is higher for bariatric surgery than diet advice and physical activity, it is by far the most successful form of weight loss with a majority of patients seeing a 20% weight loss after 10 years (Maciejewski et al., 2016).

#### 1.4.2.4 Pharmacological Therapies

While relatively new, there is growing number of clinically proven pharmacological interventions to aid in weight loss. Currently, the most popular are GLP-1 receptor agonists (GLP-1RAs), which are long lasting forms of the gut hormone GLP-1. Mechanistically, administration of the drug slows gastric emptying, increases satiety and helps with insulin signalling and glucose management, and were originally designed as treatments for T2DM (Nauck et al., 2021). However, through the course of developing the treatment it was observed that patients lost weight, at least 5% body mass and maintained the weight loss while take GLP-1 (Trujillo et al., 2021). Longer lasting formulations of GLP-1Ras have been developed and these show even greater efficacy than the initial formulations with <10% weight loss observed (Chao et al., 2022).

Other pharmacological interventions that are used in conjunction are naltrexone and bupropion, a μ-opioid receptor agonist and a norepinephrine-dopamine reuptake inhibitor respectively. These medications were initially designed for the treatment of addiction and depression but have been shown to induce weight loss for people with obesity (Kulak-Bejda et al., 2021).

Another diabetic medication that has been shown to induce weight loss is metformin but it is not as effective with an average weight loss of 2.2% versus the greater than 10% that is observed with semaglutide (Center, 2012, Chao et al., 2022).

#### 1.4.2.5 Therapies in Development

Building on the efficacy and rapid rollout of GLP-1 therapies, new formulations are in the works. Oral formulations of semaglutide are currently in various stages of clinical trials, with oral semaglutide showing similar outcomes in relation to weight and HbA1c levels compared to injections (Klobučar et al., 2024, Aroda et al., 2023). The benefit of formulations is that they remove the need for cold storage and the need for injections which some patients find uncomfortable.

Additionally, there is scope for other combinations of gut hormones to be used for weight loss and other comorbidities (Troke et al., 2014). Pancreatic polypeptide (PYY) show promise to be used in conjunction with GLP-1 to aid in weight loss in early animal studies, with PYY in particular supressing appetite (Karra et al., 2009, Oertel et al., 2024).

The most promising candidate that is being developed for use in conjunction with GLP-1 is glucose-dependent insulinotropic polypeptide (GIP), named together as tirzeptide, is showing promise in trials and is reported to induce greater weight loss than GLP-1 alone and have a similar safety profile (Jastreboff et al., 2022, Lin et al., 2023).

The efficacy and safety of GLP-1 in inducing weight loss has opened the door for gut hormone therapies to help with weight loss and diabetes management. However, recent evidence showing beneficial outcomes for patients taking GLP-1 for kidney health (Colhoun et al., 2024) and cardiovascular health (D'Andrea et al., 2020) shows the potential scope for these medications to treat a variety of conditions.

## 1.5 NK Cells and Obesity

There is a growing body of evidence highlighting the effect of obesity on NK cells. It is important to note that the majority of research is conducted on peripheral NK cells, and as such it is important to denote if studies examine both tissue resident and peripheral NK cells or just peripheral NKs. **Paper I**, goes into detail on our current understanding of the effect of obesity on NK cell function and metabolism.

### 1.5.1 Peripheral NK Cells

Peripheral NK cells in both humans and animal models and adults and children are negatively affected by obesity. NK cells see reduced frequencies in PWO in the absence of diabetes (Viel et al., 2017, Tobin et al., 2017). Obesity also corresponds to a reduced capacity to produce IFN-γ and lytic molecules such as Granzyme B (Kedia-Mehta et al., 2021, Bahr et al., 2018). Additionally, cytotoxicity, a core function of NK cells is reduced in both animals with obesity and PWO (Michelet et al., 2018, Naujoks et al., 2020).

These defects are underpinned by an altered metabolism within NK cells. **Paper II and III**, show both defective mitochondria due to reduced iron availability in PWO and a defective *SLC7A5*-mTOR-Hexokinase axis, which is reaffirmed in other studies (Tobin et al., 2017, Michelet et al., 2018).

### 1.5.2 Tissue Resident NK Cells

Due to the diversity of function and phenotype of NK cells found in the various tissues of the body there is still gaps in understanding of these various populations and in particular the effect of obesity on these NK cells.

NK cells are found in abundance in the liver, and in opposition to peripheral NK cells there is more of an even split between the $CD16^{-}$, $CD56^{bright}$ and $CD56^{dim}$ populations, in addition to being $CD49^{+}$ (Cuff et al., 2016). These cells produce abundant IFN-γ but are less cytotoxic than their circulating cousins (Marquardt et al., 2017). However, in PWO these NK cells take on a more ILC1 like phenotype that shows even less cytotoxicity capacity (Cuff et al., 2019). Uterine NK cells play a crucial role in implantation, aiding in tissue remodelling and produce cytokines that aid in

angiogenesis such as a VEGF and IL-10 (Hanna et al., 2006). In obesity, uterine NK cells reduce in number and become hyperresponsive and are linked to pre-eclampsia (Perdu et al., 2016, Baltayeva et al., 2019).

In adipose tissue, NK cells are responsible for monitoring adipocyte stress but have been shown to accumulate in adipose tissue in response to a high fat diet in mice (Wensveen et al., 2015, O'Rourke et al., 2012). NK cells are linked to macrophage infiltration into the adipose tissue in obesity which has been shown to contribute to insulin resistance (O'Rourke et al., 2014).

## 1.6 Current Gaps in the Literature

There are numerous gaps in the literature that confound our ability to accurately describe both the immune system in general and the immune system in the context of obesity. One noticeable issue in our current understanding of NK cells is the emphasis on peripheral NK cells over tissue resident NK cells of which both studies on this thesis focus on. Much of the current literature on tissue resident NK cells focuses on animal derived models and have been crucial in broadening our understanding of various NK cell populations. Yet, there is a need for these findings in animal models to be confirmed in human tissue resident NK cells.

Other gaps in the literature are present in our understanding of how exactly GLP-1 therapy benefits the individual. It is evident that GLP-1 therapy induces weight loss and is beneficial for glucose tolerance but some studies relating to cardiovascular health, kidney function and the study on the effect of GLP-1 function on NK cells often show benefits presence irrespective of weight loss or that there are no correlations between weight loss and HbA1c levels. There are even animals models showing that GLP-1 can help with addictive tendencies which is being proposed to also be the same in humans (Klausen et al., 2022). Does this also reveal a possible use for GLP-1 in people without obesity?

Further work is also required on the effect of GLP-1 therapy on other immune cells. There are a small number of papers examining the effect of GLP-1 therapy on their function, metabolism, and phenotype over time, but more work needs to be done on other immune cell populations.

# 2. Research Aims

**Paper 1:** Conduct a detailed review of the current literature on the effects of obesity on NK cell biology.

**Paper 2:** Define the iron requirements of human NK cells, and to examine the effect of obesity associated iron deficiency on NK cell biology.

**Paper 3:** Define the metabolic requirements for optimal human NK cell responses, examine the impacts of obesity on human NK cells *ex-vivo*, and investigate the effect of GLP-1 therapy on human NK cells in the setting of obesity.

# 3. Materials and Methods

## 3.1 Study Approval

Ethical approval was granted by the Medical Research Ethics Committees at St Vincent's University Hospital and by Maynooth University Ethics Committee. All patients and participants gave written informed consent prior to partaking in the study.

### 3.1.1 PBMC From Healthy Donors

In **Paper II and III**, human peripheral blood mononuclear cells (PBMCs), were isolated from fresh venous blood samples. Donors, both people living with obesity (PWO) and controls, were recruited from St Vincent's University Hospital and St Colmcille's Hospital. All donors gave consent to participate in research on immune cells. The samples were anonymized before they left the hospital. The risks associated with blood donation are small. No identifying data was available to us and there was no individual gain for the donor.

### 3.1.2 PBMC From Patient Samples

The workflow for sample collection and analysis is represented in **Figure** 4. In **Paper II**, we recruited 30 PWO from the Weight Management Clinic at St Colmcille's Hospital, Dublin. The 30 patients were further divided into to two groups, those with normal transferrin saturation (NTS), 11.0 to 30.0 µmol/L, and those with low transferrin saturation (LTS), < 11 µmol/L. Patients gave written informed consent before the blood was drawn. The samples were anonymized before they left the clinic, and no identifiable information was received. The patients received no individual gain for agreeing to participate in the study. Inclusion criteria for this study included the following: aged between 18 and 55 years of age, Body Mass Index (BMI) > 30kg/$m^2$, and no previous GLP-1 use. Exclusion criteria included: history of Type 2 Diabetes Mellitus (T2DM), use of immunomodulatory medications, a recent infection (< 2 weeks), and hyperferritinemia, > 50 µmol/L.

In **Paper III**, we recruited a total of 20 PWO before they commenced GLP-1 therapy (once weekly semaglutide injection of 0.25mg, scaling to 1.0mg with a standard dose escalation). A follow up

sample was taken approximately 6 months later after beginning GLP-1 therapy. All partcipants were recruited from the Weight Management Clinic at St Colmcille's Hospital, Dublin Ireland. Patients gave written, informed consent before the blood was drawn. The samples were anonymized before they left the clinic, and no identifiable information was received. The patients received no individual gain for agreeing to participate in the study. Inclusion criteria for this study included the following: aged between 18 and 65 years of age, Body Mass Index (BMI) > 30kg/$m^2$, and no previous GLP-1 use. Exclusion criteria included: history of Type 2 Diabetes Mellitus (T2DM), use of immunomodulatory medications, stoppage of GLP-1 therapy, and a recent infection (< 2 weeks). Blood was drawn at the same time as a routine blood test so little to no additional risk was imparted on the patient.

Figure 5: Graphical abstract of methods

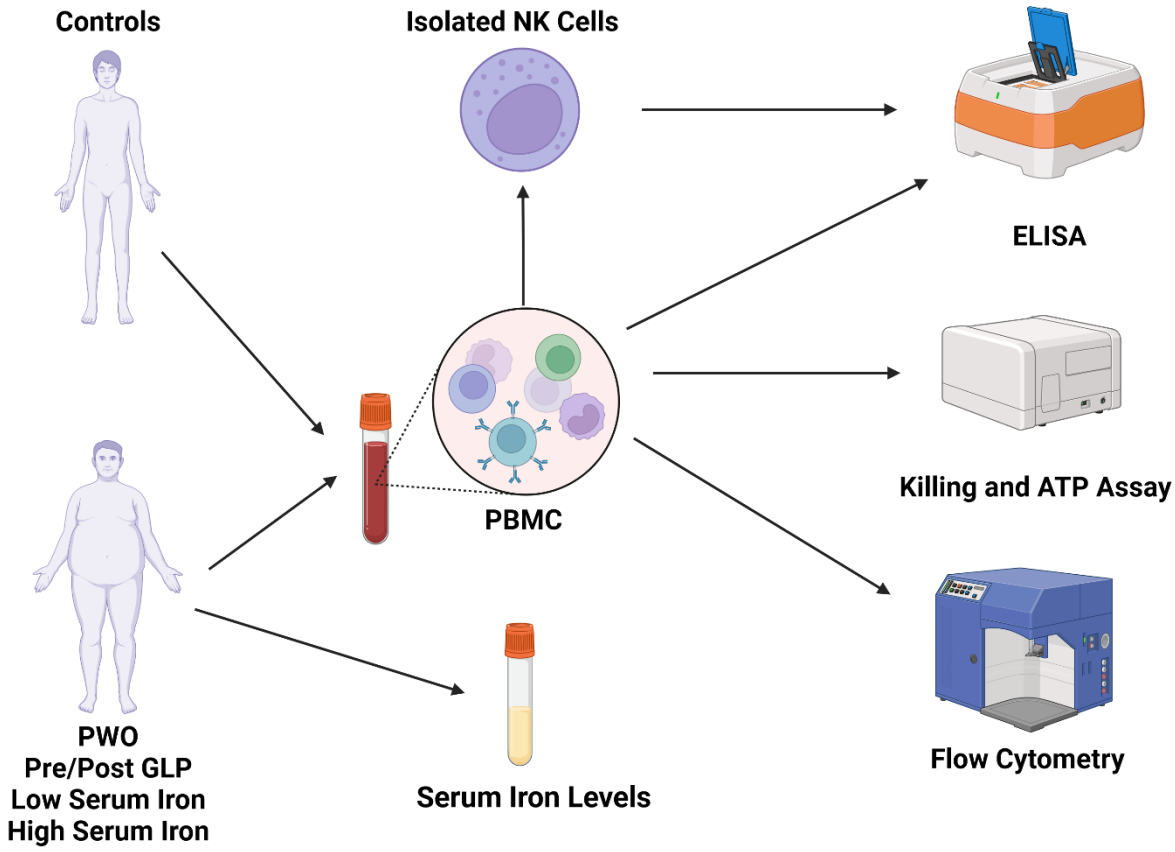



*Figure 4: This schematic gives an overview of both the cohorts we collected and the study design which we used.*

## 3.2 PBMC Preparation and NK Isolation

In **Paper II** and **Paper III,** the data gathered for both *ex vivo* and *in vitro* models were generated from either PBMCs, or NK cells isolated from PBMC.

### 3.2.1 PBMC Preparation

In both **Paper II** and **Paper III**, PBMCs were isolated from human blood, collected in lithium heparin coated tubes, using a density gradient. The blood was diluted 1:1 with warm PBS (Sigma) and layered on top of 15mL of Lymphoprep (Stemcell Technologies). In **Paper II** a standard 50mL falcon tube was used, and the samples were spun at 500g for 20 minutes with the brakes off and the acceleration set to low. In **Paper III**, SeptMate 50 mL tubes (Stemcell Technologies) were used instead of a standard 50mL tube, and the samples were spun at 1,200g for 10 minutes with full acceleration and brakes. After the first spin, the buffy coats were isolated and the PBMCs were separated from the plasma by a subsequent spin at 400g for 7 minutes. Some plasma was removed from this spin and stored at -80°C for analysis. The PBMCs were washed twice with warm PBS to remove any remaining factors from the blood. PBMCs were either used fresh or bio-banked at -80C° in Cryostor (Sigma) at 10 million cells per mL.

### 3.2.2 NK Cell Isolation

In **Paper II** and **Paper III** NK cells were isolated from PBMCs using a NK cell negative selection kit from Miltenyi Biotec as per manufacturer's instructions using LS columns (Miltenyi Biotec) and MACS Buffer (Miltenyi Biotec). NK cell purity was confirmed via flow cytometry (>93% purity). Additionally, monocyte contamination was confirmed to be below 1%.

### 3.2.3 Cell Culture

In **Paper II**, NK cells or PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium GlutMax (Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma). In **Paper III**, NK cells or PBMCs

were cultures in Human Plasma Like Medium (HPLM), (Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma). Cells were incubated at 37°C, at 5% $CO_2$. In **Paper II** and **Paper III**, for ELISAs and Flow Cytometry experiments, NK cells or PBMCs were stimulated with cytokines at 1 x $10^6$ cells/mL. Cells were stimulated with either IL-12 (30ng/mL, BioLegend), and IL-15 (50ng/mL, BioLegend), or IL-12 (30ng/mL) and IL-18 (50ng/mL, BioLegend), for 18 hours. For flow cytometry experiments examining secreted factors, Protein Transport Inhibitor 500X (PTI) (Invivogen), was added 6 hours before the cells were stained.

In **Paper III**, for *in vitro* GLP-1 experiments, purified GLP-1 (Novo Nordisk) was used at 1µg/mL. GLP-1 was added 1 hour before isolated NK cells were stimulated via cytokines. Additionally, for the experiments examining metabolic inhibitors on NK cells, purified NK cells from healthy donors were stimulated as before in the absence or presence of 2-deoxy-glucose (2 mM, Sigma) or rapamycin (20 ηM, Sigma). For SLC7A5 inhibition experiments, the concentration of amino acids in RPMI was diluted twofold using Hank's Balanced Salt Solution (Gibco) in the presence or absence of 2-aminobicyclo-(2,2,1) heptane-carboxylic acid (BCH) (50 mM, Sigma).

In **Paper III**, Deferoxamine mesylate salt (DFO) (Sigma) was added to media 24 hours before the experiments began, the cells were then resuspended in DFO treated media (200µM).

## 3.3 NK Cell Cytotoxicity Assay

In **Paper** III, NK cell cytotoxicity was measured via Calcein-AM (BioLegend) release from K562s. K562s were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% FBS (Gibco) and 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma). Prior to beginning the experiment, K562s were washed three times in 10% FBS in PBS and counted. K562s were resuspended in serum free IMDM at a concentration of 1 × $10^6$ cell/mL and were treated with Calcein-AM at a concentration of 20 µM for 30 minutes. K562s were washed again three times in 10% FBS in PBS and resuspended in IMDM with 10% FBS. K562s were added to a round bottom 96-well plate at a concentration of 2 × $10^5$ cells/mL. PBMCs were added at a 40:1 effector to K562 ratio and incubated at 37 °C, 5% CO2 for 3 hours. After incubation the plate was spun at 300g for 5 minutes and 75 µL of the supernatant was removed and added to a black walled 96-

well plate. Max killing value was determined by adding 8µL of 10% Triton (Sigma) to some wells. The plate was read for fluorescent intensity at 530 nm. Killing percentage was measured using the following formula:

$$\frac{(Sample - Spontaneous)}{(Max - Sponatneous)} \; x \; 100$$

## 3.4 Analysis of Supernatants and Plasma by ELISA

In **Paper II** and **Paper III**, enzyme-linked immunosorbent assays (ELISAs) were used to examine supernatants from isolated NK cells for IFN-γ and Granzyme B concentrations. NK cells were stimulated as previously mentioned. The kits were used per manufacturer's instructions. Bovine Serum Albumin (BSA) (Sigma) and Sulfuric Acid (Sigma) were used in addition to the components of the kit. In **Paper III**, we also examined the levels of hepcidin and leptin in the plasma of healthy donors and patients.

| **Target** | **Kit** | **Manufacturer** | **Catalogue Number** |
|---|---|---|---|
| Granzyme B | Human Granzyme B DuoSet ELISA | R&D Biosystems | DY2906 |
| Hepcidin | Human Hepcidin DuoSet ELISA | R&D Biosystems | DY8307 |
| IFN-γ | Human IFN-γ DuoSet ELISA | R&D Biosystems | DY285B |
| Leptin | Human Leptin DuoSet ELISA | R&D Biosystems | DY398 |

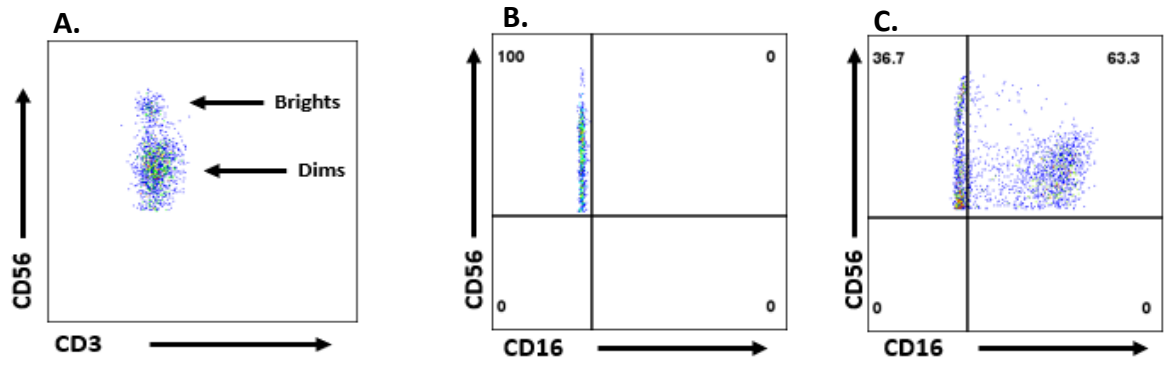
## 3.5 Analysis of Immune Cells by Flow Cytometry

In **Paper II** and **Paper III**, NK cells were selected from PBMCs using the below flow through. In **Paper II** Cell viability was determined using eBioscience 506 Live Dead Stain, stained for 30 minutes at 4°C in PBS before being spun twice, at 400g for 7 minutes in 1% FBS in PBS before extracellular staining. Extracellular markers were stained for in 1% FBS in PBS for 30 minutes at

4°C. Cells were fixed using the BioLegend's True-Nuclear Transcription Factor Buffer Set fix solution for 30 minutes in the dark at room temperature. Cells were spun twice in perm solution from BioLegend's True-Nuclear Transcription Factor Buffer Set, at 400g for 7 minutes before intracellular staining. Intracellular markers were stained in the dark for 30 minutes at 4°C for 30 minutes. Samples were resuspended in 1% FBS in PBS and were then analyses on an Attune NxT Flow Cytometer (Life Technologies) and analysed using FlowJo software (Treestar). Compensation was carried out using the appropriate Compensation Beads (Miltenyi Biotec) or appropriately stained cells depending on the marker being stained for. Results are expressed as a percentage of the parent population as indicated and determined using fluorescence minus one and unstained controls. We recognise the limitation in **Paper III** of this thesis as no live dead stain was used but subsequent work that contained a live dead stain confirmed our findings. To provide more confidence in the populations obtained from flow cytometry, the use of dump channels to exclude other cell populations, such as $CD14^{+}$ cells which also express CD56, can be used.

### 3.5.1 Extracellular Staining and Phenotyping of NK cells

In **Paper II** and **Paper III**, NK cells were defined as $CD3^{-}$ and $CD56^{+}$ cells using the below throughput (**A**). NK cells can be further divided into $CD56^{right}$ and $CD56^{dim}$ as shown below (**B**). Markers and fluorophores are outlined in the tables below. Positive populations were determined via fluorescence minus one (FMOs) to determine where to draw gates for selection (**B**).




*Dot plots highlighting $CD56^{bright}$ and $CD56^{dim}$ populations (A). Dot plots B and C showcase the use of FMOs to identify positive populations.*

#### *3.4.1.1 Table for Extracellular Markers for Paper II*

| **Paper II** | | | |
|---|---|---|---|
| **Target** | **Fluorophore** | **Clone** | **Catalogue Number** |
| CD3 | BV421 | UCHT1 | 300433 |
| CD3 | PE | OKT3 | 317308 |
| CD3 | PerCP Vio700 | REA613 | 130-113-141 |
| CD16 | BV711 | B73.1 | 360732 |
| CD56 | BV510 | HCD56 | 318340 |
| CD56 | APC Cy7 | HCD56 | 318332 |
| CD71 | APC Cy7 | CY1G4 | 334110 |
| CD98 | PE | RL388 | 128208 |
| NKG2D | PE Vio 770 | REA797 | 130-111-646 |

#### *3.4.1.2 Table for Extracellular Markers for Paper III*

| **Paper III** | | | |
|---|---|---|---|
| **Target** | **Fluorophore** | **Clone** | **Catalogue Number** |
| CD3 | BV421 | UCHT1 | 300433 |
| CD3 | PerCP Vio700 | REA613 | 317308 |
| CD14 | APC | HCD14 | 325608 |
| CD25 | PE | BC96 | 302606 |
| CD56 | BV510 | HCD56 | 318340 |
| CD57 | APC | TB03 | 130-118-537 |
| CD69 | FITC | FN50 | 310904 |
| CD95 | FITC | DX2 | 305606 |
| CD98 | PE | RL388 | 128208 |
| CD156b | PE | FAB9301P | FAB9301P |
| NKG2A | APC | S19004C | 375108 |
| NKG2D | PE Vio 770 | REA797 | 130-111-646 |

### 3.5.2 Intracellular of Cytokine and Metabolic Markers

The cytokines and intracellular metabolic factors are outlined in the tables below.

#### *3.5.2.1 Table of Intracellular and Metabolic Markers for Paper II*

| **Paper II** | | | |
|---|---|---|---|
| **Target** | **Fluorophore** | **Clone** | **Catalogue Number** |
| Granzyme B | Alexafluor 647 | GB11 | 515406 |
| Hexokinase II | Alexafluor 647 | EPR20839 | EPR20839 |
| IFN-γ | Alexafluor 488 | 4S.B3 | 502515 |
| pS6 | PE | A17020B | 608604 |

#### *3.5.2.2 Table of Intracellular and Metabolic Markers for Paper III*

| **Paper III** | | | |
|---|---|---|---|
| **Target** | **Fluorophore** | **Clone** | **Catalogue Number** |
| cMyc | - | - | 9402 |
| Rabbit Alexa-fluor Conjugated Antibody | Alexa-fluor 647 | - | ab150079 |
| Granzyme B | Alexa-fluor 647 | GB11 | 515406 |
| Hexokinase II | Alexa-fluor 647 | EPR20839 | EPR20839 |
| IFN-γ | Alexa-fluor 488 | 4S.B3 | 502515 |
| pS6 | PE | A17020B | 608604 |

### 3.5.3 NK Cell Mitochondrial Analysis

In **Paper II**, PBMCs were seeded into a 96-well plate and washed in serum-free buffer. Cells were then stained for viability as outlined above, and NK cells were stained for extracellular markers. Cells were then washed and stained with Mitotracker Deep Red FM (50μM, ThermoFisher Scientific) and Mitotracker Green (50μM, ThermoFisher Scientific) in PBS, and incubated for 1 hour at 37°C. Cells were subsequently analysed by flow-cytometric analysis.

### 3.5.4 NK Cell Transferrin Uptake Assay

In **Paper II**, PBMCs were rested in serum-free HPLM with 5% BSA for 2 hours. Cells were then washed in serum-free HPLM with 0.5% BSA and incubated with 5μg/ml Transferrin-AlexaFluor647 (Invitrogen) for 10 minutes at 37°C. Holo-transferrin (500μg/ml, Sigma-Aldrich) was used to competitively control for transferrin-uptake. An additional sample was stained at 4°C. Cells were washed in ice-cold HPLM with 0.5% BSA to stop membrane trafficking. Cells were then stained for viability, and NK cells were labelled for extracellular markers, to be analysed by flow cytometry.

### 3.5.5 NK Cell Scenith Assay

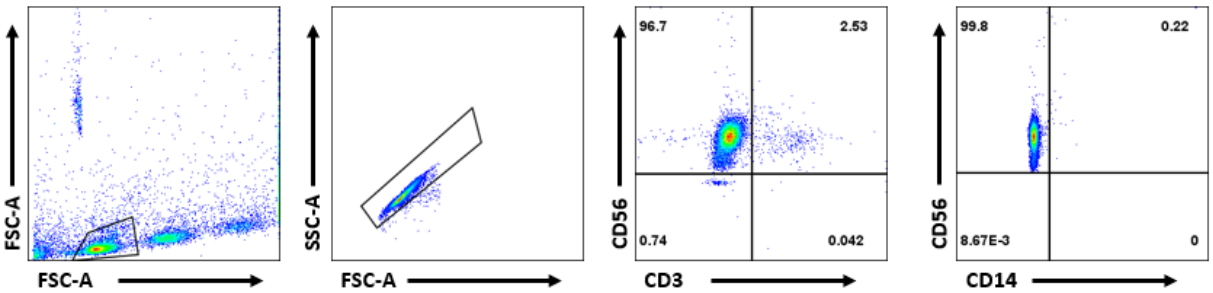
In **Paper II**, PBMCs were seeded into a 96-well plate, and treated as a control, or with 2-Deoxy-D-Glucose (100mM), Oligomycin (1μM), or both. Following incubation at 37°C for 15 minutes, cells were treated with Puromycin (11μM) and incubated for a further 25 minutes. Cells were washed with ice-cold PBS to stop puromycin incorporation. Cells were then stained for viability. NK cells were stained for extracellular markers and fixed, as outlined above. Staining of puromycin was achieved using anti-puromycin monoclonal antibody (AlexaFluor488, Sigma), in permeabilization buffer (BioLegend).

### 3.5.6 NK Cell ATP Assay

ATP production was measured in isolated NK cells using a luminescence ATP assay kit (abcam). Reagents in the kit we reconstituted as per manufacturer's instructions. A standard curve was also prepared as per the kit's the instructions. NK cells were harvested and washed with PBS. 100μL of resuspended NK cells was added to a black walled, clear bottomed plate. 50μL of detergent was added to each well and the plate was placed on an orbital shaker for 5 minutes at 600-700 rpm. 50μ of substrate solution was added and the plate was returned to the orbital shaker for 5 minutes at 600-700 rpm. The plate was then covered and placed in the dark for 10 minutes before luminescence was measured on a multimode plate reader (CLARIOstar).

### 3.5.7 Confirmation of NK cell Purity

To confirm the purity of isolated NK cells for experiments flow cytometry was used to confirm low CD3 and CD14 positive contaminants in the isolated population. The average $CD56^{+}CD3^{-}$ population purity was 97% with no more than 1% $CD14^{-}$.




*Flow throuput to confirm purified NK cell population for experiments with isolated NK cells.*

## 3.6 Table of Reagents and Materials Used

| Reagent | Full Name | Manufacturer | Catalogue Number |
|---|---|---|---|
| 2DG | 2-deoxy-glucose | Sigma | D8375 |
| ATP Assay | Luminescent ATP Detection Assay Kit | abcam | ab113849 |
| BCH | 2-aminobicyclo-(2,2,1) heptane-carboxylic acid | Sigma | A7902 |
| BSA | Bovine Serum Albumin | Sigma | A7030 |
| Calcein-AM | Calcein-AM | BioLegend | 425201 |
| Comp Beads Anti-Mouse | MACS® Comp Bead Kit, anti-mouse IgG | Miltenyi Biotec | 130-104-693 |
| Comp Beads Anti-REA | MACS® Comp Bead Kit, anti-REA | Miltenyi Biotec | 130-097-900 |
| Cryostor | CryoStor cell cryopreservation media | Sigma | C2874 |
| DFO | Deferoxamine mesylate salt | Sigma | D9533 |
| DMEM | Dulbecco's Modified Eagle Medium | Gibco | 10566 |
| FBS | Heat Inactivated Foetal Bovine Serum | Gibco | 10082147 |
| Fix and Perm Solution | BioLegend's True-Nuclear Transcription Factor Buffer Set | Biolegend | 424401 |
| GLP-1 | Purified Glucagon Like Peptide-1 (Semaglutide Formulation) | Novo Nodisk | NNC0113-0217 |
| HBSS | Hank's Balanced Salt Solution | Gibco | 14025092 |
| HPLM | Human Plasma Like Medium | Gibco | A4899101 |
| LS Columns | LS Columns | Miltenyi | 130-042-401 |

| | | | |
|---|---|---|---|
| Lymphoprep | Lymphoprep Density Gradient Medium, 1.077 g/cm$^3$. | Stemcell Technologies | 07861 |
| MACS Buffer | Magnetic Assisted Cell Sorting Buffer | Miltenyi Biotec | 130-091-221 |
| MTG | MitoTracker Green | Sigma | M7514 |
| MTR | MitoTracker Red | Sigma | M22425 |
| NK Cell Isolation Kit | NK Cell Isolation Kit, human | Miltenyi Biotec | 130-092-657 |
| PBS | Phosphate Buffered Saline | Sigma | D8662 |
| PenStrep | Penicillin Streptomycin Solution | Sigma | P4333 |
| PTI | Protein Transport Inhibitor (500X) | Invivogen | 00-4980-03 |
| Rapamycin | Rapamycin | Sigma | 553210 |
| RPMI | Roswell Park Memorial Institute 1640 Medium GlutaMax Media | Gibco | 21875 |
| Septmat | SepMat-50 (IVD) tube for density gradient separation | Stemcell Technologies | 85450 |
| Sulfuric Acid | Sulfuric Acid 99.999% purity | Sigma | 339741 |
| Triton | Triton X100 | Sigma | X100 |

# 4. Results and Discussion

In this thesis, I have examined the dysregulating impact of obesity on NK cell biology and detailed how GLP-1 therapy can restore NK cells metabolism and function in people with obesity (PWO). Firstly, **Paper I** is a review outlining the current understanding of the effects of obesity on peripheral NK cells, including how current weight-loss therapies can improve NK cells in PWO. Secondly, in **Paper II** I examine how iron availability affects NK cells in people with obesity, in particular mitochondrial function, and effector responses. Finally, in **Paper III**, I examined how obesity affects NK cell metabolism and function, and if six months of GLP-1 therapy could improve the negative effects of obesity on NK cells.

## 4.1 The Effect of Obesity on NK cell Numbers and Function

There is a significant body of evidence supporting the hypothesis that NK cells are negatively affected by obesity. The first indication that NK cells are impacted by obesity is a reduction in NK cell numbers, both in absolute terms and as a proportion of lymphocytes which has been cited in multiple studies (Michelet et al., 2018, Naujoks et al., 2020, Bähr et al., 2017, Lynch et al., 2009, O'Shea et al., 2010). This observation is also seen in children with obesity (Tobin et al., 2017). The precise mechanism(s) underpinning this loss of NK cells remains unclear, but may be due to reduced circulating IL-15 in PWO which would have an effect on NK cell development and survival and also corresponds to total fat mass and percentage fat mass in humans (Nielsen et al., 2008). A reduction in the ratio of NK cells to target cells directly impacts the percentage of tumour cells lysed (Cerwenka et al., 2001). In **Paper III**, we set about describing NK cell frequency and function in our cohort to see if our data corresponded to the literature and we observed a decrease with increased BMI having a slight correlation to lower NK cell numbers in our cohort **(Paper III, Figure 1B-C)**. However, it should be noted that our work only involved peripheral NK cells from blood. NK cells are present many tissues and the effect of obesity on the NK cells in these some of these tissues are less well described, such as the uterus showing reduced numbers in the NK cell compartment, while adipose tissues can accumulate NK cells in obesity, where they have been linked to the development of metabolic abnormalities including insulin resistance (Perdu et al., 2016, Wensveen et al., 2015, Boulenouar et al., 2017) .

The decrease in NK cell numbers is compounded by a reduction in two of NK cells core functions, cytokine production and killing. In both adults and children with obesity NK cells are less able to produce both IFN-γ and Granzyme B (Kedia-Mehta et al., 2021, Tobin et al., 2017). We confirm this in our cohort with NK cells from PWO producing significantly less IFN-γ and Granzyme B **(Paper III, Figure E-D)**. Additionally, NK cells from people with obesity are less able to lyse target cells which we also observed **(Paper III, Figure F)** (Michelet et al., 2018).

Due to the critical role that NK cells play in both viral and tumour immunity, these results observed by our group and others can indicate why PWO have worse outcomes in relation to cancer and infections. Children with obesity are more likely to develop severe obesity in adulthood and have worse health outcomes in comparison to those who were not obese as children (Ferraro et al., 2003) (Lindberg et al., 2020). In adults, PWO have higher incidence rates of certain cancers and worse prognoses when diagnosed and are more susceptible to server viral infections. For cancer, people with a BMI of >40kg/$m^2$ had a 52% and 62% increased risk of death from cancer for men and women respectively (Calle et al., 2003). In the absence of a sufficient number of properly functioning NK cells this is most certainly a contributing factor to this, with compelling evidence provided by Michelet and colleagues who using murine models of cancer detailed how NK cells with obese like phenotypes could not control tumour burden when compared to normal NK cells.

## 4.2 NK Cells from PWO have a Broken Cellular Metabolism

NK cell function is underpinned by a functioning cellular metabolism as described in multiple studies. NK cells shift from relying on OxPhos to provide the necessary energy requirements during rest (Slattery et al., 2021, Loftus et al., 2018). However, upon stimulation NK cells increase their dependence on glycolysis. This shift is dependent on the activity of mTOR to allow for the expression of glycolytic enzymes and the rate of glycolysis as seen in increases in ECAR, and by inhibiting mTOR this metabolic shift cannot occur which in turn inhibits NK cell's ability to produce key cytokines such as IFN-γ (Donnelly et al., 2014a). Delving deeper into the metabolic requirements of activated NK cells, amino acids such as glutamine, transported by SLC7A5 are also required to stabilise cMYC which in turn regulates NK cell activity (Loftus et al., 2018).

NK cells from PWO are less able to engage in the required changes in cellular metabolism to fully preform their key functions. In children with obesity, NK cells are chronically activated even in the absence of classical stimulus, with increased ECAR, mTOR activity and glucose uptake in comparison to children without obesity (Tobin et al., 2017). However, this observation changes in in adults where NK cells are shown to have reduced rates of glycolysis, OxPhos and glycolytic capacity, all of which corresponded to a reduced ability to produce IFN-γ (Michelet et al., 2018). These defects in metabolism are also shown by our group to limit the ability for NK cells to be trained by cytokines (Kedia-Mehta et al., 2021).

## 4.3 Iron is a Crucial Trace Element for NK Cell Metabolism

It is well established that NK cells require both glucose and amino acids to engage in their function (Loftus et al., 2018, Wang et al., 2020). However, these are only two of the myriad of nutrients that NK cells require. Iron is already known to be crucial for both human development and basic cellular functions (Read et al., 2021, Lozoff and Georgieff, 2006). The function of various immune cells such as neutrophils and macrophages are also dependent on iron (Frost et al., 2022, Collins et al., 2002). Less is known of the iron requirements of human NK cells, although we have already discussed that murine NK cells require iron to respond to viral infections *in vivo* (Littwitz-Salomon et al., 2021). In **Paper II**, we looked to examine the effect of obesity associated iron deficiency on human NK cell function and metabolism.

Firstly, we established that human NK cells express the transferrin receptor CD71 and increase their uptake of iron upon stimulation **(Paper II, Figure 1A-G)**. Using iron chelation (via DFO), we deprived NK cells from healthy controls of exogenous iron. This resulted in a significant decrease in protein synthesis upon stimulation indicating that iron is required for the response to cytokine stimulation **(Paper II, Figure 2 E-F).** In murine NK cells, Littwitz-Salomon and colleague showed that DFO disrupted mitochondrial fitness, which in turn impacted their function (Littwitz-Salomon et al., 2021). We subsequently confirm that low iron environments disrupt mitochondrial fitness in human NK cells as seen by the decrease in MTR:MTG ratio and a significant increase in dysregulated mitochondria **(Paper II, Figure 3 B-C)**. There was also a significant decrease in the amount of ATP that NK cells produced upon stimulation in the presence of DFO **(Paper II, Figure 3D)**. Additionally, while NK cells rely on more so on glycolysis upon stimulation, DFO decreased NK cell's ability to rely on OxPhos for additional energy

requirements **(Paper II, Figure 2H)**. Ultimately this led to a decrease in IFN-γ production by NK cells **(Paper II, Figure 2E)**.

Following on from this work, we next wanted to describe the effect of a low iron environment on human NK cell biology. We recruited 30 PWO, 15 with normal serum iron levels and 15 with clinically low levels of serum iron **(Paper II, Figure H-I)**. This allowed us to compare the effect of not only BMI on NK cell function but also iron availability. NK cells from people with obesity expressed CD71 higher than those from healthy controls, and significantly increased transferrin uptake **(Paper II, Figure 1 J-K)**. We observed that NK cells from PWO had decreased protein synthesis in comparison to those from healthy controls but had similar metabolic profiles **(Paper II, Figure 2I-L)**. However, NK cells from PWO had increased numbers of dysfunctional mitochondria in comparison to healthy controls and produced less ATP and IFN-γ **(Paper II, Figure 3F-I)**.

Moving beyond simply looking at BMI as a potential marker for NK cell dysfunction we then stratified our cohort into those with normal transferrin saturation (NTS) and low transferrin saturation (LTS) **(Paper II, Figure 4A)**. NK cells from the LTS expressed higher CD71 than those with NTS **(Paper II, Figure 4B)**. Mirroring the effect of DFO on NK cells from healthy controls, NK cells from the LTS group had more dysregulated mitochondria and had lower protein synthesis than the NTS group **(Paper II, Figure 4C-E)**. Strikingly there was a significant inverse correlation between the proportion of dysregulated mitochondria and IFN-γ production **(Paper II, Figure 4F)**. This trend was also observed with NK cells from the LTS group producing significantly less IFN-γ than healthy controls while the NTS group only saw a non-significant decrease **(Paper II, Figure 4H)**. These results along with the work from other groups highlights the requirement of iron for normal NK cell function. Without the necessary bioavailable iron, NK cells have dysregulated mitochondria that results in decreased protein synthesis including the production of the key NK cell cytokine IFN-γ, and thus provide further detail on how obesity dysregulates NK cells.

## 4.4 GLP-1 Therapy Restores NK Cell Metabolism and Function

With evidence from our group and others, we show that obesity has a negative effect on NK cells which could be a contributing factor to poorer prognosis for PWO in relation to both viral infections and cancer. It is therefore important to see if these defects can be reversed either through weight loss or other means. While there are studies showing that certain interventions

that are used to aid in weight loss, such as exercise and metformin, can positively affect NK cell function there are gaps in the literature. Exercise is shown to increase circulating levels of IL-15, a key cytokine for NK cell survival and activation (Tamura et al., 2011). This finding is supported by Barra *et al*, who showed that high intensity interval training (HIIT) increased both the number and function of circulating NK cells in women with obesity (Barra et al., 2017). This study also showed reduced tumour burden in mice that underwent HIIT. However, this work did not show the long-term effects of repeated HIIT on NK cells.

Metformin has also been shown to induce weight-loss to varying degrees, depending on the dose given (Seifarth et al., 2013, Levri et al., 2005). It can improve NK cell anti-tumour function, potentially through AKT signalling (Xia et al., 2021, Crist et al., 2022). Yet, these effects were observed in people without obesity.

There is stronger evidence that bariatric surgery can improve NK cell function in PWO. Moulin *et al*, showed significant improvement in NK cell cytotoxicity 6 months post bariatric surgery (Moulin et al., 2011), yet the exact mechanism by which this improvement was caused was not fully elucidated.

In **Paper III**, we show that NK cells from people with obesity increase both the amount of IFN-γ and Granzyme B they produce following 6 months of GLP-1 therapy **(Paper III, Figure 3B-D, G)**. Both $CD56^{bright}$ and $CD56^{dim}$ NK cells produced increased IFN-γ post GLP-1 therapy while $CD56^{bright}$ NK cells saw significant increased production of Granzyme B **(Paper III, Figure 3E-F, H-I)**. Additionally, NK cells were better able to lyse target cells **(Paper III, Figure 3J)**.

The mechanism by which this occurs could partially be attributed to improved activity of a CD98-mTOR-HKII pathway. As previously mentioned in this section NK cells require abundant amino acids to stabilise mTOR to drive an increase in glycolysis (Loftus et al., 2018, Donnelly et al., 2014a). Here we show an increase of the amino acid transporter CD98 on the surface of NK cells post GLP-1 therapy **(Paper III, Figure 4A-B)**. This corresponded to both increased levels of pS6, which is phosphorylated by mTOR, and the glycolytic enzyme HKII, indicating an overall improvement in NK cell metabolic potential post GLP-1 **(Paper III, Figure 4E-F, H-I)**. Interesting there was no association between the weight-loss and the increases in the aforementioned targets suggesting a weight-loss independent mechanism **(Paper III, Figure 5B-D)**. To investigate

this further we demonstrated significant increases in both IFN-γ and Granzyme B production by NK cells from PWO when treated with GLP-1 *ex vivo* **(Paper III, Figure 5E-F)**.

# 5. Concluding Remarks

Obesity is a complex and multifaceted disease. The ever-present social stigma that people with obesity (PWO) are subjected to is, by itself a harsh and unfair reality that many are forced to live with. Unfortunately for some, obesity also contributes to declining physical wellbeing due to an increased likelihood of both developing certain diseases such as cancer, non-alcoholic fatty liver disease and increases the chance of infection. Deciphering how obesity affects our bodies and in particular our immune system is crucial in developing therapies to reduce and hopefully eliminate these negative effects. The aims of this thesis were to describe mechanistically how obesity affects NK cells, a critical component of host-immunity, and investigate strategies to restore NK cell functionality in PWO.

In **Paper II**, we describe the importance iron for human NK cell metabolism and function, with a focus on how obesity associated iron deficiency can exacerbate the negative effects that obesity has on NK cells. Readily available iron is required for mitochondrial health and normal NK cell function and by understanding this it provides a possible avenue for improving NK cell function in people with obesity if available iron levels can be adjusted accordingly.

In **Paper III**, we focus on discerning other defects in NK cell metabolism and function associated with obesity in particular the CD98-mTOR-Glycolysis pathway that is required for optimal NK cell activation. This pathway is deficient in NK cells from people with obesity that results in reduced cytokine production and killing capacity. Yet, we describe that GLP-1 therapy can reverse this effect.

In conjunction these two papers sheds light on the metabolic requirements of NK cells and the mechanism by which obesity affects NK cells. The findings indicate that iron is crucial for NK cell function, that iron deficiency can contribute to the negative effect of obesity on NK cells and that the current weight loss therapy GLP-1 could be a viable option in improving NK cell function with people with obesity.

# 6. Future Perspectives

Much was learned over the course of this PhD and in undertaking the research included. If I were to begin this work again there would be a number of lessons that I learned throughout the course of the PhD that I would enact. One would be to delineate certain samples for different techniques that required a large number of cells. Due to the COVID-19 pandemic we were unsure if issues around patient recruitment would arise again and so were hesitant to use entire patient samples for the likes of PCR or Seahorse. Additionally, this study could have benefitted from proteomic analysis to identify key pathways within NK cells that are affected by either low iron or obesity. Finally, there are definite improvements that could have been made in some of the experimental procedures that I learned along the way.

While **Paper I** summarises our current understanding of NK cells and obesity there are still large gaps in the literature. In particular, the role of tissue resident NK cells and the effect of obesity on them has yet to be adequately described in humans. NK cells not only reside in various tissues such as the liver and uterus but can also infiltrate other tissues such as the brain during inflammation, but these populations are understudied but do provide exciting opportunities for further research.

**Paper II** focuses on the role of iron in NK cell function and metabolism and the impact of obesity associated iron deficiency. While it goes some way in showing that iron is a crucial nutrient for NK cells more work needs to be carried out to examine how the lack of iron affects NK cell killing and responses to viral infections in humans. The paper also highlights the differences that can exist in a cohort of PWO, and that BMI can only go so far in explaining immune cell alterations. Additionally, more work needs to be undertaken to see how the lack or excess of iron in humans affects both viral immunity and cancer surveillance.

**Paper III** describes the importance of a functioning metabolism for normal NK cell function in human peripheral NK cells and details how obesity disrupts this process. However, obesity can be associated with a variety of different co-morbidities and alterations to homeostatic levels of many bodily components. People with obesity can have hyperlipidaemia, hyperglycaemia, iron deficiency and hyperleptinemia among other conditions. How each of these conditions affect NK cells in combination and in isolation and in the context of obesity is not clearly understood. BMI,

while a useful tool for describing obesity at a population wide level, can be too general at an individual level. This is seen by the heterogenicity of our cohorts, all of which had BMIs in the obese range, in terms on NK cell numbers, function and metabolism. Therefore, more research is needed to examine the exact mechanisms by which obesity affects NK cells and how GLP-1 therapy improves NK cell function if it is not directly linked to weight loss. In addition to this, patients receive GLP-1 therapy for long periods of time and it would be beneficial to increase the time scale of future experiments to see more long-term effects of taking GLP-1. Future work would benefit from expanding on the flow cytometry panels to include dump channels, live dead stain and newly developed uptake assays and look at Seahorse which can now be done with much fewer cell numbers.

# 7. Acknowledgements

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Cill, even though you're my little brother you still look out for me.

Mam and Dad, I cannot say enough for all the love and support you have given me. The help you gave me in so many ways is something I am forever grateful for. I love you both.

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# 9. Papers to be Included as Part of Thesis

## Paper I: NK cells vs. obesity: A tale of dysfunction & redemption.

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Review Article

# NK cells vs. obesity: A tale of dysfunction & redemption

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ABSTRACT

Natural killer (NK) cells are critical in protecting the body against infection and cancer. NK cells can rapidly respond to these threats by directly targeting the infected or transformed cell using their cytotoxic machinery or by initiating and amplifying the immune response via their production of cytokines. Additionally, NK cells are resident across many tissues including adipose, were their role extends from host protection to tissue homeostasis. Adipose resident NK cells can control macrophage polarization via cytokine production, whilst also regulating stressed adipocyte fate using their cytotoxic machinery. Obesity is strongly associated with increased rates of cancer and a heightened susceptibility to severe infections. This is in part due to significant obesity-related immune dysregulation, including defects in both peripheral and adipose tissue NK cells. In this review, we detail the literature to date on NK cells in the setting of obesity – outlining the consequences, mechanisms and therapeutic interventions.

## 1. Introduction to NK cell biology

Natural Killer (NK) cells are a subset of innate lymphoid cells (ILCs), which are different from classical T and B lymphocytes in that they can rapidly respond without prior activation to infected, stressed or transformed cells [1,2]. As their name suggests NK cells are prolific killers, enabled by their robust production of lytic molecules such as perforins or granzymes [3–5]. NK cells can also shape type 1 immune responses through their rapid production of cytokines such as IFNγ [6–8]. NK cell activation is tightly managed by balancing activating and inhibitory signals provided by the expression of germline coded receptors [5,9,10]. The balance of these signals can change in response to changes in environmental cues, promoting either inhibition or activation of the cell. Loss of self-identifying molecules such as HLA, results in a loss of an inhibitory signal from killer-cell immunoglobulin like receptors (KIRs) expressed on the surface of NK cells, leading to the activation of the NK cell and the lysis of the target cell [11–13]. NK cells can also be activated via activation molecules such as NKG2D when their ligands are upregulated by transformed cells [14–16]. In addition to the expression of activating receptors, NK cell can also respond to soluble mediators such as cytokines, including IL-2, IL-12, IL-15 and IL-18 [17–20] (Fig. 1).

Together these differential mechanisms of activation allow NK cells to rapidly respond to transformed cells and virally infected cells [21,22]. NK cells are a critically important component of both anti-tumour and anti-viral immunity, as highlighted by studies of NK cell deficieny [23–27]. In addition to deficiency, reduced NK cell activity has been linked to increased viral susceptibility and cancer risk [28–30]. NK cell frequencies and activity have also been linked to disease outcomes in several cancers [31–33]. Upon activation, NK cells release lytic granules containing pore-forming perforin and serine protease granzymes, resulting in target cell death. NK cells can also induce target cell death via their expression of death receptors (TRAIL and FASL) [34]. In addition to lytic molecule secretion, activated NK cells can also produce a range of effector molecules including cytokines (such as IFNγ) and chemokines, which can initiate and amplify the immune response (Fig. 1). Collectively these rapid and robust effector functions have highlighted NK cells as an attractive target for immunotherapy, in particular for cancer [35].

## 2. NK cell immunotherapy

The potential for NK cell immunotherapy is further boosted by their lack of MHC restriction and the fact that they do not drive graft versus host disease [36,37]. Several variations of NK cell immunotherapy are currently under investigation in phase I, II, III clinical trials (reviewed in [35]). The approaches under investigation include adoptive transfer of

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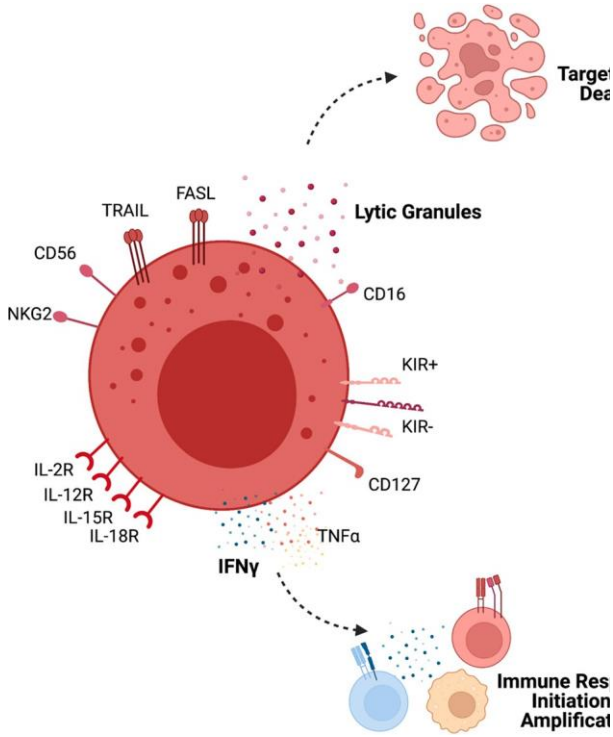


**Fig. 1.** Schematic detailing the simplified phenotype and functions of a human peripheral blood natural killer cell.

NK cells, NK cell activating cytokine therapy or genetically targeting/ modifying NK cells receptors to boost their cancer killing function [35,38]. Adoptive transfer of NK cells initially focused on autologous NK cell transfer, however patient responses in these trials were not clinically relevant [39,40]. The allogenic approach has yielded more positive results, especially with KIR-mismatch in patients with haematological cancers such as acute myeloid leukaemia (AML) [36,37,41]. The follow on from these studies, aim to boost NK cell responses via cytokine-training. This approach involves harnessing the training potential of NK cells, where NK cells (autologous or allogenic) are activated in vitro using a cocktail of cytokines (IL-12, IL-15 & IL-18) and after restimulation show boosted effector responses both in vitro and in vivo, including in patients [42–47] (reviewed in [48]). More recently, approaches have focused on the genetic modification of NK cells, with the goal of improving their resilience, cytotoxicity or homing ability (reviewed in [49,50]). Whilst the evidence is building that NK cell immunotherapies are safe and progressing in effectiveness, some barriers remain, including overcoming the tumour/disease microenvironment, which provides unique challenges to any cellular therapeutic approach, including metabolic competition [51–53].

## 3. NK cell metabolism

Cellular metabolism is critical for the energetic and biosynthetic requirement of effector cells. NK cells require a range of nutrients including glucose, amino acids, and iron to support both their homeostasis and their effector functions [54–56]. At rest, NK cells rely on low rates of glycolysis coupled to oxidative phosphorylation (OxPhos) to meet the metabolic requirements of the cell [55,57]. Upon activation with combinations of cytokines such as IL-2, IL-12 or IL-15, NK cells rapidly increase their metabolic rates, with large increases in both glycolysis and OxPhos [17,58]. Activated NK cells display a heavy reliance on glucose as a fuel source which is metabolized primarily via aerobic glycolysis. Numerous studies have demonstrated that limiting glucose availability or inhibition of glycolysis using 2-dexoy glucose (2DG) results in inhibition of NK cell effector function highlighting its critical importance [17,54,59]. NK cell metabolism is dependent several metabolic sensors/regulators including mammalian target of rapamycin (mTOR), MYC and SREBP [17,55,60]. The first metabolic regulator to be identified in NK cells was mTOR [17], which integrates nutrient and immune signals (e.g. IL-15 stimulation) and modulates NK cell metabolism. Cytokines can drive the activation of mTOR in NK cells which results in increased cellular metabolism and full effector function [54,58]. In a 2019 study from Loftus and colleagues, it was demonstrated that mTOR was required for the expression of MYC, a transcription factor critical for cellular metabolism in cytokine activated NK cells [55]. In the same study, the authors demonstrated that glutamine transport via SLC7A5 was critical for the stabilization of MYC expression in NK cells, and the subsequent metabolic and functional responses. Another critical regulator of NK cell metabolism is Srepb, which controls lipid synthesis, but also glucose metabolism. Inhibition of Srepb resulted in reduced NK cell metabolism, and as a result reduced effector function [60] (Fig. 2).

## 4. Introduction to obesity

The World Health Organisation classifies a person with a body mass index (BMI) of >30 kg/m$^2$ as a person with obesity (PWO), while a BMI of between 25 kg/m$^2$ and 29 kg/m$^2$ as overweight. Globally, over 600 million adults are living with obesity, a chronic progressive disease with very strong links to the development of co- morbid conditions such as type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and an increased risk of many cancers. However, an elevated BMI score is not indicative of overall health, with anywhere from 3% to 20% of PWO presenting with neither metabolic syndrome or insulin resistance [61,62]. However, collectively obesity and its comorbidities account for the largest proportion of preventable deaths. The mechanisms underpinning obesity as a disease are highly complex, and multi-faceted, however a well-established contributor is the dysregulation of the immune system, which results in chronic inflammation and loss of immunosurveillance/immunoregulation. The chronic systemic inflammatory profile associated with obesity underpins the development of comorbidities like T2DM. People living with obesity have been shown to have increased levels of circulating inflammatory markers such as IL6 and IL6R, CRP and TNFα [63–66]. These increased circulating inflammatory cytokines have been shown to disrupt normal metabolic processes like insulin signalling, resulting in insulin resistance, the mechanistic pre-cursor to T2DM [67–69]. Obesity is also strongly linked to the development of 13 different types of cancers, and although the mechanisms are complex (including altered metabolic processes like insulin signalling [70]), chronic inflammation is a major contributing factor and has been shown to directly promote the development of cancer [71–73]. Amongst the strongest associations between inflammation and cancer is in colon carcinogenesis in patients with inflammatory bowel disease who have increased circulating TNFα and IL-17 which mirror PWOs [74–77]. Combined with the variety and potential severity of these comorbidities the increasing rate of obesity worldwide, with there now being >700 million people worldwide, is of grave concern [78].

## 5. Peripheral NK cells and obesity

Our first report of altered NK cells in the setting of obesity was published in 2009. In a cohort of 52 patients with obesity (PWO), we reported that peripheral blood NK cell frequencies were significantly lower than healthy controls (HC) [79]. Further analysis separated PWO into those who were classified as MHO versus MUO and noted less severe alterations in the MHO PWO[79]. In a follow up study where all individuals were MUO, NK cells from PWO were shown to have defective cytotoxic abilities when compared to HC, further highlighting the studies have confirmed reduced NK cell frequencies and loss of cytotoxic potential in animals, children and adults with obesity, with varying degrees of information on the metabolic health of the participants included [81–89]. In the study by Viel et al., the PWO in the study were in the pre-diabetic range for $HBa1_c$ (5.7%–6.4%) and there were direct correlations between NK cell defects and increased BMI [84]. In studies from our group De Barra et al and Kedia-Mehta et al also found defects in NK cell function and metabolism in PWO without T2DM [90,91]. There is then conflicting evidence as to say whether the defects seen in NK cells from PWO are more pronounced in those who are metabolically un- healthy that those who aren't but there needs to be further work with expanded cohorts to account for two distinct groups. As highlighted NK cells are armed with lytic molecules containing granzymes and perforins, facilitating their cytotoxic potential [92]. In the setting of obesity, NK cells express lower levels of these lytic molecules, starting early in childhood, likely explaining the reduced cytotoxicity [85,86]. Conversely, in 2020, Naujoks and colleagues did not report reduced granzyme and perforin expression in a small cohort (<15) of PWO but did show reduced TRAIL expression on NK cells from PWO, again signalling potential functional defects [89]. In a 2019 study from Prager and colleagues, it was elegantly demonstrated that NK cells switch from granzyme B mediated killing to death receptor mediated cytotoxicity over time [92]. Potentially both of these killing mechanisms of killing are defective in PWO [86,89], but more detailed studies are required to demonstrate if NK cells from PWO are still capable of serial killing. Another feature of NK cells in the setting of obesity is the loss of IFNγ production, a key cytokine in both anti-cancer and anti-viral immunity [84,86]. As discussed previously, NK cells can be trained to be more efficient effector cells, an approach demonstrating real clinical potential, with IFN-γ being a key mediator of this process [42,43,47]. In 2021, we investigated if NK cells from PWO could be trained, therefore boosting their effector function (e.g. IFN-γ production) and showed that NK cells from PWO failed to show boosted IFN-γ production after 7 days cytokine training [91].

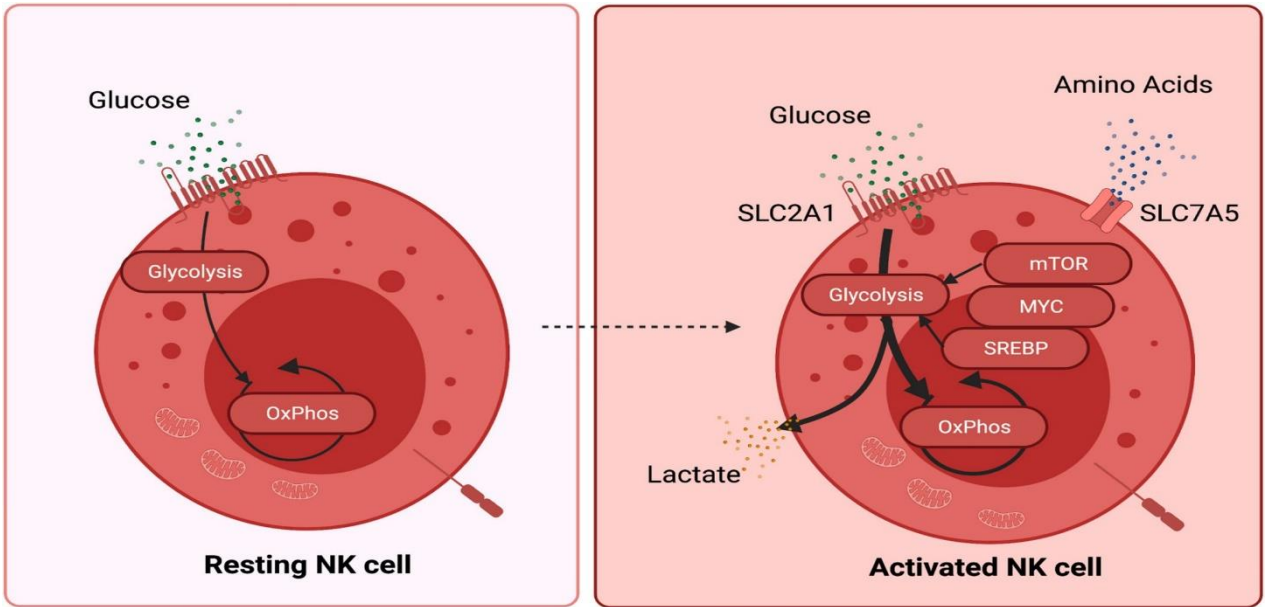


**Fig. 2.** Schematic detailing an overview of natural killer cell metabolism in both resting and activate states.

## 6. Mechanisms underpinning peripheral NK cell defects.

It is well established that obesity is associated with defective peripheral NK cell frequencies and functional responses. Like the drivers of obesity itself, the mechanisms underpinning the defects are likely to be multi-factorial. In a trio of recent studies, we have demonstrated that obesity is associated with defective cellular metabolism in NK cells [85,86,91]. In particular, we showed defective glycolytic metabolism in both children and adults with obesity, which as highlighted is essential for NK cell IFN-γ production, killing and training [54,58,86,91]. We have also noted obesity-related defects in glycolytic metabolism in other cell types including MAIT cells, suggesting a potential environment under- pinning [93,95]. In 2018, in collaboration with the Lynch group we demonstrated that NK cells from animals and PWO displayed a gene expression prolife associated with increased lipid metabolism [86]. Treatment of healthy NK cells with free fatty acids (oleate and palmitate) resulted in obesity-like defects in NK cells, all underpinned by defective cellular metabolism. The consequence of these free fatty acid induced defects was reduced NK cell anti-tumour activity in murine models of cancer [86]. It remains to be investigated if other obesity related alterations in nutrient availability are impacting NK cells. Another environmental signal implicated in the dysregulation of NK cells in PWO is leptin. Leptin is critical regulator of adiposity via its regulation of satiety and food intake [96–98]. Leptin is primarily produced by adipocytes,

and its levels are directly proportional adipose tissue mass, therefore are elevated in obese adults and children [99,100]. However, with progressing obesity, the body can become resistant to the actions of leptin, resulting in dysregulated satiety and increased adiposity [101,102]. Leptin, in addition to its role in regulation of energy homeostasis, is an important regulator of the immune system [103–106]. In the steady state, leptin is important for NK cell development, with reduced NK cell frequencies and activity in leptin receptor deficient mice (db/db) [107]. In the setting of obesity and leptin resistance, the loss of leptin signalling in NK cells underpinned their dysfunction in murine models of obesity [108]. In humans, obesity was associated with elevated expression of the leptin receptor, but with reduced downstream signalling resulting in diminished responses (IFN-γ production) [82].

## 7. Obesity Interventions and peripheral NK cells

With the mechanisms underpinning defective NK cells in PWO emerging, the potential to reverse the defects and restore their protective function is coming into focus. Currently, the most effective intervention for obesity is metabolic surgery, which results in sustained weight loss and an improved metabolic profile [109]. In 2011, Moulin and colleagues demonstrated that following metabolic surgery, NK cell anti-cancer activity, as measured by cytokine production and cytotoxicity, was restored [110]. Metabolic surgery results in significant weight loss, which might be the mechanism of restoration, however, metabolic surgery is also associated with improvements in the obesity-related factors discussed in the context of dysregulated NK cells including, improved lipid profiles [111,112] and reduced leptin levels/resistance [113,114]. Therefore, more studies are required to elucidate the exact mechanisms underpinning NK cell functional restoration following metabolic surgery.

There are a growing number of proven pharmacological interventions that are now proven to induce weight-loss. Glucagon like- peptide-1 (GLP-1) is a multifaceted incretin hormone involved broadly in metabolic regulation [115]. GLP-1 was initially designed to be a treatment for T2DM, improving blood glucose levels via improving glucose dependent insulin secretion, slowing glucagon release and slowing gastric emptying [116–118]. GLP-1 based analogues have fast become the gold-standard for the pharmacological treatment of obesity, resulting in significant weight loss (up to 20%) and improved glycaemic control [119–121]. Recently we investigated the impact of GLP-1 analogue therapy on NK cells in a cohort of PWO. We found that six- months GLP-1 therapy did not change NK cell frequencies, but did result in improved NK cell effector function, with improved IFN-γ and granzyme B production and target cell lysis [90]. We found that GLP-1 therapy also improved NK cell metabolism, which as discussed is critical for NK cell effector function. Surprisingly we noted that the resto- ration of NK cell activity by GLP-1 therapy in PWO was not associated with weight-loss and could be recapitulated in vitro by treating NK cells from PWO directly with GLP-1. How GLP-1 "metabolically rewires" NK cells remains to be elucidated, but we and others have previously demonstrated that GLP-1 can directly modulate other cells of the immune system, but studies need to be conducted on the direct effect on NK cells [122–125].

Metformin is a long-established treatment for T2DM, improving $HbA1_c$ levels by inhibiting gluconeogenesis in hepatocytes [126,127]. It has also been shown to induce weight-loss in patients, with results ranging from 2.8Kg to 5.8 kg but results vary with dosage and length of treatment [128–130]. Due to the multifaceted action of metformin, the exact mechanism of action is not clearly understood, but it has been shown in mice that metformin increases circulating GDF15 levels which was necessary to prevent weight gain [131,132]. Metformin has been shown to improve NK cell function, particularly in relation to tumour killing via increasing mTOR and AKT signalling [133–136]. However, no current studies examine the effects of metformin on NK cells from PWO. There is a similar lack of information on the effect of other therapies for the management of T2DM such as supplementary insulin or sulphonylureas on improving NK cell function on people with obesity. Studies have found that the lack of insulin due to T1D or insulin resistance as seen in T2DM can affect NK cells. In latent autoimmune diabetes in adults (LADA) NK cells numbers were reduced in comparisons to controls and the expression of NKG2D and KIR3DL1 increased and decreased respectively [137]. There is evidence of T2DM both having no effect on NK cells in humans and increasing the expression of the activating receptors NKG2D and NKp46 [138,139]. However, only one of the studies in this section had patients that also had BMIs in the obese range, $>30$ kg/m$^2$ so it is difficult to draw conclusions between the two diseases, and further work is required in this area. Exercise is a common intervention in the management of obesity, and it has been shown to improve the overall health of patients with cancer and may improve survival rates [140–142]. In a 3-month intervention trial by Jahn and colleagues, they noted weight-loss induced by increased physical activity improved NK cell function with an increased IFN-γ expression [143]. While, from a purely weight-loss perspective exercise is not an effective treatment for many, as an intervention it can have some other health benefits such as improved hip to waist ratio, body composition and metabolic profile [144,145]. Exercise has been shown to increase the circulating levels of IL-15, a key cytokine for NK cells survival and activation [146–149]. This potentially might link exercise to direct improvements in NK cells, with Barra et al showing that high intensity interval training (HIIT) increased NK cell frequencies and function in women with obesity and in murine models, in addition to obesity, breast cancer cells where intravenously injected into the mice, and it was demonstrated that HIIT reduced tumour burden, with the authors postulating that this effect was mediated via increased NK cell activity [87]. Further work by Pedersen et al shows that the tumour reducing effect of exercise is again driven by NK cell mobilisation through a IL-6 and epinephrine mechanism [150] (Fig. 3).

## 8. NK cells in tissue

In addition to their role in peripheral immunity, NK cells also play an important role in tissue homeostasis, in particular in the uterus and adipose tissue [151–154]. In the uterus, NK cells play a key role both in relation pregnancy progression and protection from cancers and pathogens. Phenotypically distinct from peripheral NK (pNK) cells, uterine NK cells (uNK) express CD56 but lack CD16 [155,156]. While uNK cells possess cytolytic granules, they are less able to kill various cancer cell lines in comparison to their peripheral counterparts, indicating a potential alternative role for these molecules in implantation [157]. For a normal pregnancy to occur, an intricate remodelling of the uterus to allow for the foetal-maternal interphase to develop, in particular the development of the blood vessels that will support the growing foetus. Extravillous trophoblasts (EVTs) infiltrate the decidua and allow for the spiral artery to correctly form which will then feed the rowing foetus [158]. These EVTs express a nonclassical HLA-E and HLA-G along with HLA-C which interact with the specific set

of receptors present on uNK cells [159]. uNK cells play a role producing various cytokines important to angiogenesis such as vascular endothelial growth factor (VEGF), IL-10 and angioponectin-2, amongst others, that promote vascular development [160,161]. Obesity is heavily associated with a variety of com- plications during pregnancy such as gestational diabetes and preeclampsia [162–164]. Strikingly, Perdu and colleagues reported that maternal obesity resulted in defective NK cells in the uterus, with reduced numbers and a hyper-responsiveness resulting in increased expression of decorin which can limit trophoblast survival leading to altered placental development [165]. The hyper-responsiveness is mirrored in Baltayeva et al., where uNK cells had increased expression of CD69 which also corresponded to defective spiral artery development in mid but not late-stage pregnancy in mice [166]. Additionally, maternal obesity alters uNK surface markers, skewing the balance of inhibitory and activating receptors, towards an increase in KIR2DS1 which pro- motes TNF-α production via HLA-C2 activation [167]. The liver plays host to a heterogeneous population of NK cells with a distinct population $CD16^{-}$, $CD56^{bright}$, $CD49a^{+}$ that is not observed in the periphery [168]. While the majority of NK cells in the periphery are $CD56^{dim}$, roughly 90%, $CD56^{bright}$ NK cells make up50%ofintrahepatic NK cells that can produce large amounts of pro-inflammatory cytokines such as IFN-γ but are less cytotoxic [169]. These NK cells are $Eomes^{+}$, $Tbet^{-}$ which is maintained by liver derived TGF-β and can play a crucial role in regulating allogenic $CD8^{+}$ T cells [170,171]. In PWO NK cells towards a less cytotoxic ILC-1 phenotype with alterations to surface markers and metabolism partially driven to increased TGF-β that is observed in the livers of people with obesity [172]. The alteration of NK cell phenotypes in the liver could be a driving factor of Non-alcoholic fatty liver disease (NAFLD) which is a common comorbidity of obesity [173].

## 9. Adipose tissue NK cells and obesity

Whilst most research carried out on human NK cells and obesity, has focused on peripheral blood NK cells, it is important to note the majority of cell-to-cell interactions occur in tissues. Several studies have demonstrated that NK cells can accumulate in adipose tissue, with an activated tissue resident phenotype compared to peripheral NK cells (Fig. 4). Quickly, a homeostatic role emerged for adipose tissue NK (atNK) cells in controlling adipocyte stress and adipose tissue macrophage populations [8,174–177].In 2012, O'Rourke and colleagues showed that upon high fat feeding NK cells accumulated in the adipose tissue and demonstrated that genetic deletion of IFNg improved insulin sensitivity, providing evidence for altered macrophage polarization as a potential mechanism [178]. It is well established that the adipose tissue microenvironment polarizes macrophages towards a more inflammatory phenotype underpinning the development of insulin resistance [8,175–177].

Following on from this study, Wensveen and colleagues demonstrated that at NK cells monitored adipocyte stress levels via the expression of natural cytotoxicity receptors (NCRs), in particular NCR1 in mice, alternatively known as NKp46 in humans. In mice, NCR1 can be both critical for surviving influenza infections [179] but can also be a factor in the development of Type 1 Diabetes [180]. In the setting of obesity, stressed adipocytes increased their expression of NCR1, resulting in the increased expression of IFN-γ which leads to the expansion of M1 macrophages [8]. Wensveen went further to show that this shift towards M1 macrophages was due to NK cells and the IFNγ they produce in response to NCR1 by confirming these results in NCR1, NK and IFNγ deficient mice. The pathogenicity of atNK cells in obesity was again highlighted by Lee and colleagues, where upon high fat feeding, there was increased numbers of NK cells and increased production of TNF-α, in epididymal, but not subcutaneous adipose tissue of mice [175] . Depletion of NK cells

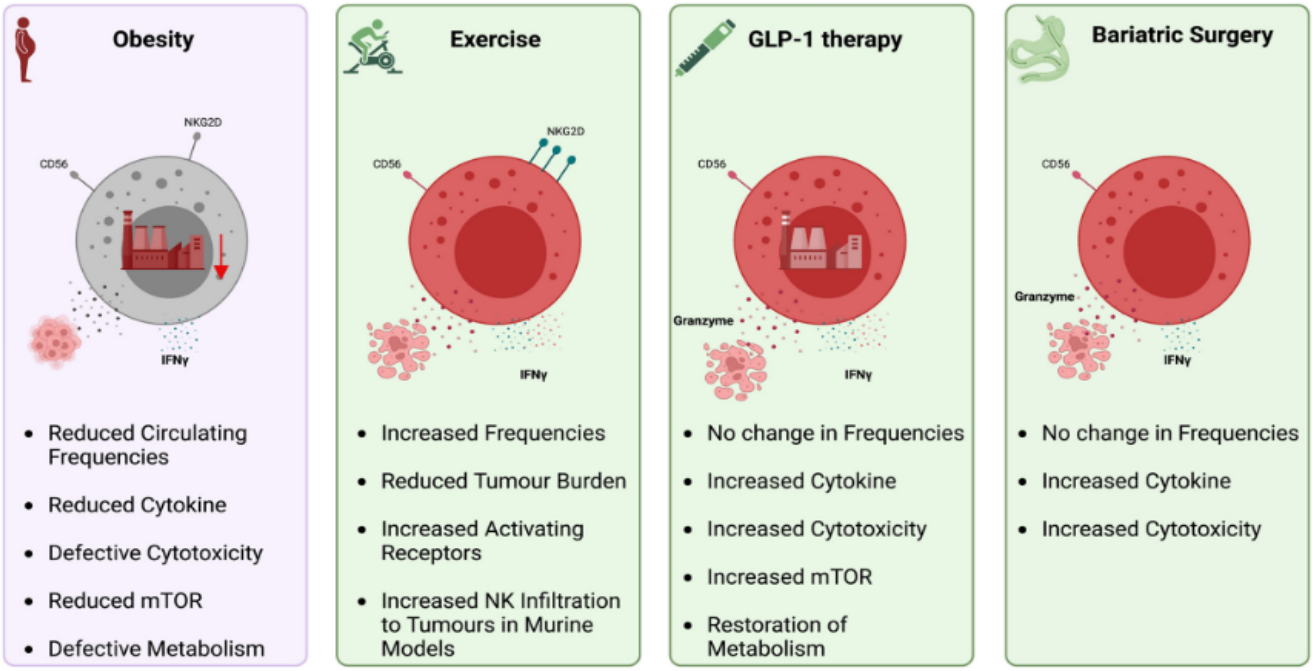


**Fig. 3.** Schematic summarizing the impact of obesity-related interventions (exercise, GLP-1 analogue therapy or bariatric surgery) on natural killer cell biology.

improved obesity driven insulin resistance, underpinned by reduced macrophage infiltration.

In 2016, O'Sullivan et al., implicated ILC1s in the development of insulin resistance. While NK cells are members of the ILC1 family they are phenotypically distinct [176]. O'Sullivan and colleagues proposed that these ILC1s are not an activation state of mature NK cells but a defined cell lineage which were resident in adipose tissue. Work from the Lynch lab in collaboration with our group also reported that ILC1s are enriched in adipose tissue and can regulate pro-inflammatory adi- pose tissue macrophages via the NKG2D receptor Rae-1 expressed on the macrophages. However, in the context of obesity the ILCs lose the ability to kill the macrophages and counterintuitively, produce more IFN-γ which recruits more macrophages to the adipose tissue [175] . Most recently, single cell RNA-Sequencing and flow cytometry analysis of human adipose tissue confirm that tissue specific ILC-1 and NK cells populations are also present human tissue [181]. Collectively these studies provide strong evidence that NK cells and other members of the ILC1 family play a homeostatic role in adipose tissue, regulating adipocyte and macrophage populations. However, the same NK cells and ILCs are also involved in the initiation of the macrophage-driven inflammatory phenotype, and insulin resistance in the setting of obesity.

CoV-2 and H1N1 influenza) have demonstrated that people with obesity are at far great risk of hospitalization and death after viral infection [182,183]. Whilst this susceptibility is complex and multi-factorial, dysfunctional NK cells, key immune cells in tumour surveillance and viral immunity, is most likely a contributing factor [86,172]. Although our understanding of NK cell biology in the setting of obesity is rapidly expanding, some key pieces of information remaining to be elucidated.

The obesity related factors driving dysfunctional NK cells are emerging, with leptin and fatty acids investigated in detail. However, obesity is associated with systemic metabolic dysregulation, significant hormonal changes, dysbiosis and a chronic inflammatory environment, each of which has potential to alter NK cell biology, and therefore need to be investigated in detail.

It is also essential that studies into restoring NK cells in obesity are undertaken to help improve outcomes for PWO in the setting of cancer and infection. Exercise, GLP-1 therapy, and metabolic surgery have all been shown to improve peripheral NK cell functionality, however there is a paucity of mechanistic studies and on the impact of these interventions on adipose tissue NK cells.

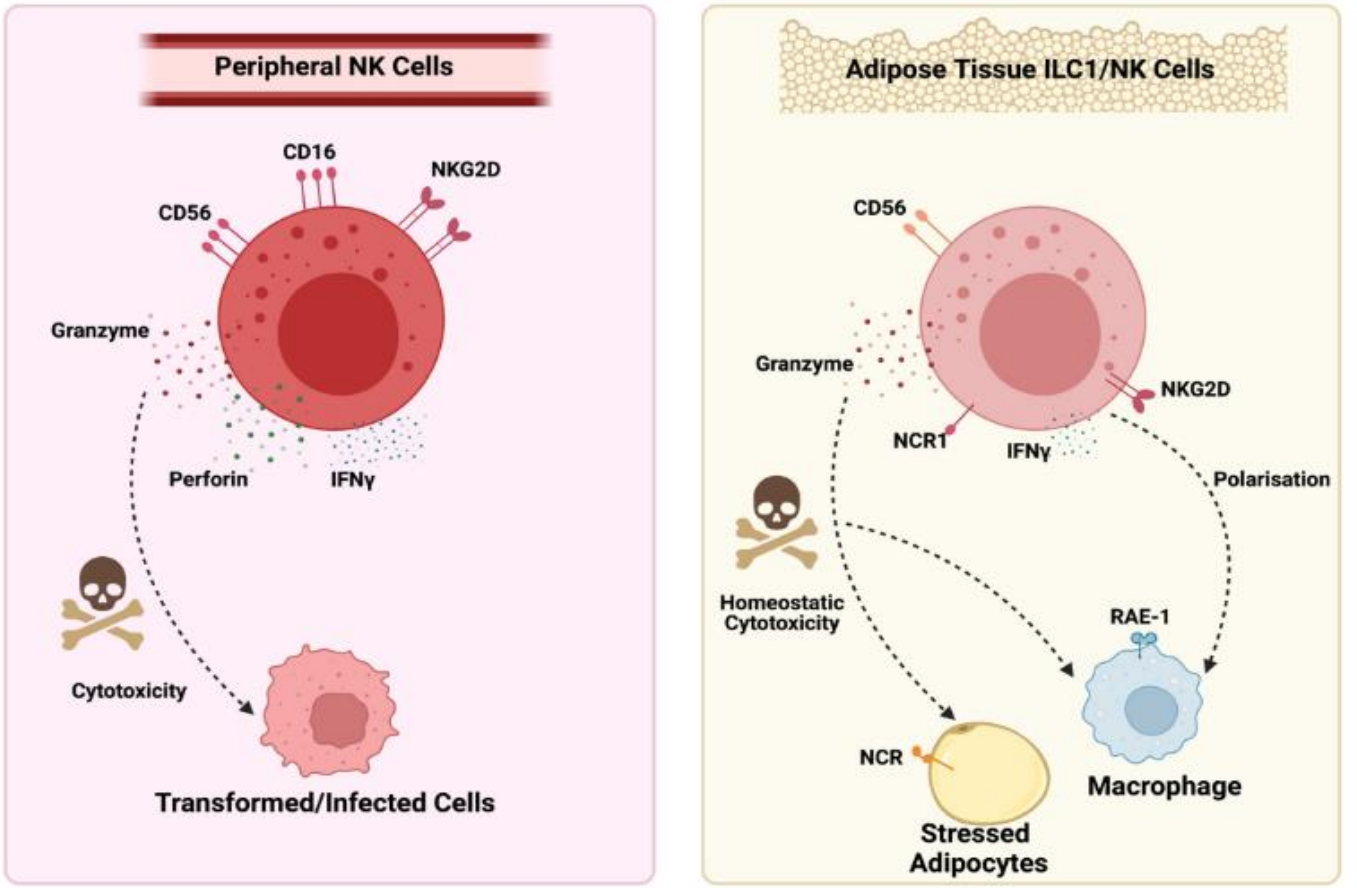


Fig. 4. Overview schematic detailing the major differences between circulating natural killer cells and adipose tissue-resident natural killer cells/ILC1 populations.

## 10. Future directives & open questions

The growing prevalence and severity of obesity is driving an increased rates of associated comorbidities including type 2 diabetes mellitus, cardiovascular disease, and a variety of cancers. In addition, harsh lessons from viral pandemics (SARS-

Finally, NK cells represent a very attractive target for immuno- therapy, in particular for blood borne cancers, however it is unclear if the obesogenic microenvironment might impact their use. Further studies in both animal models

and humans need to be conducted to test the impact of obesity on NK cell immunotherapies.

**Author contributions**

CDB, DOS and AEH researched, wrote, and approved the manuscript.

**Declaration of Competing Interest**

The authors declare no conflict of interest.

**Data availability**

No data was used for the research described in the article.

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## Paper II: Iron deficiency in people with obesity drives defective Natural Killer cell mitochondrial fitness and function.

# Iron deficiency in people with obesity drives defective Natural Killer cell mitochondrial fitness and function.

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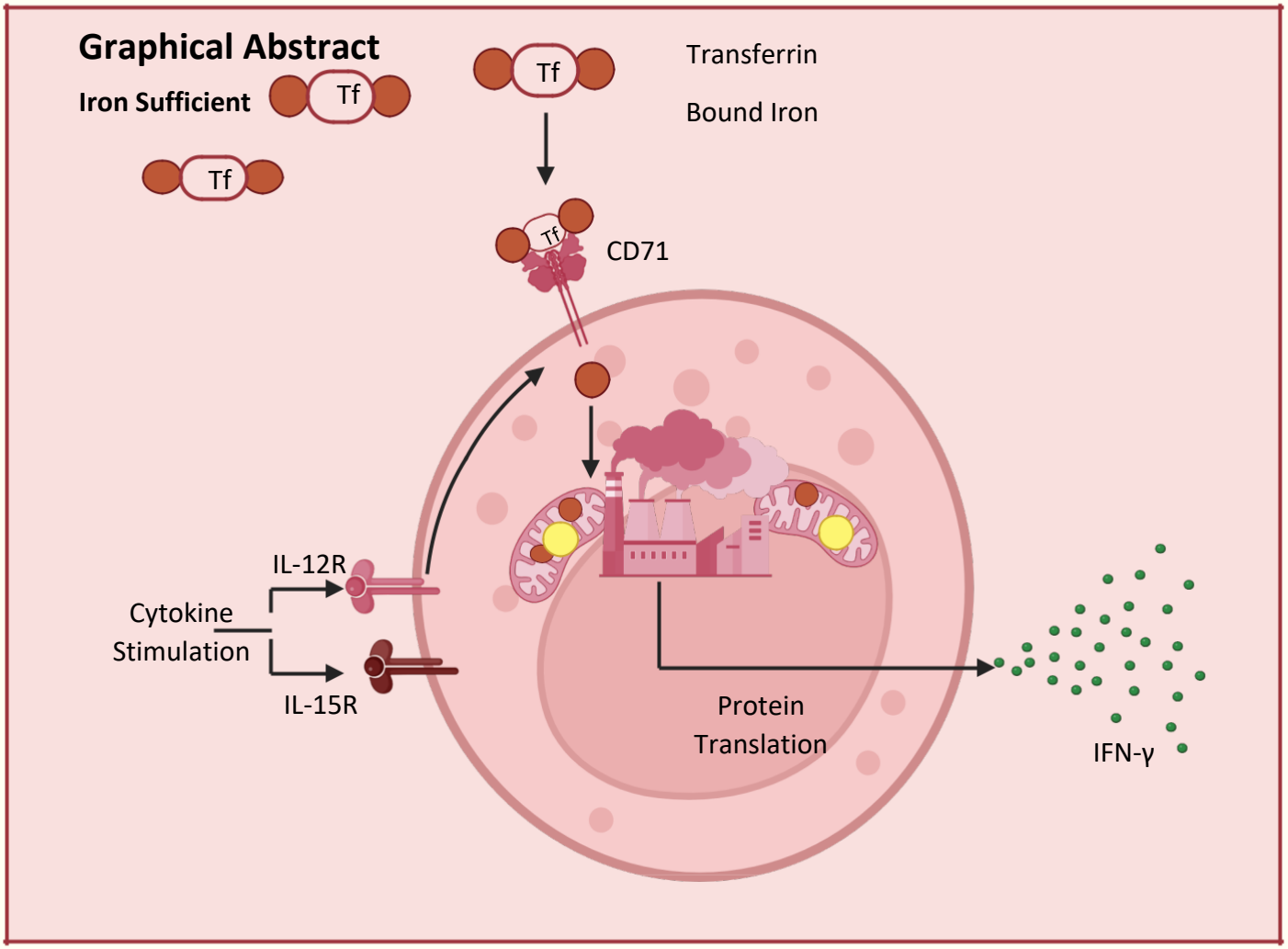
**Running title:** Altered iron availability underpins NK cell defects in obesity.

**Keywords:** Natural Killer cells, Obesity, Iron, Immunometabolism

# Abstract

Natural killer (NK) cells are a population of innate effector lymphocytes, involved in host-defences against viral infections and cancer. Upon activation, NK cells can produce a milieu of cytotoxic molecules and cytokines, which can directly target infected and transformed cells, but also amplify an immune response. Metabolic rewiring underpins NK cell effector functionality, providing the required signals, energy and bio-intermediates to support their immune responses. Obesity is associated with significant defects in the functionality of human NK cells, especially in the periphery. Dysregulated cellular metabolism has been demonstrated to be a major mechanistic driver of the reported defects. However, how obesity links to defective NK cell metabolism and functionality remains unclear. Iron deficiency is a common co-morbidity in people living with obesity (PWO). Recent studies have highlighted the importance for iron in host immunity, with murine models of iron deficiency resulting in defective cellular metabolism and function. We hypothesized that obesity-driven iron deficiency might underpin the reported defects in NK cells. Our data demonstrates that in response to cytokine stimulation, healthy human NK cells utilize iron to support their metabolic activity and cytokine responses. In a cohort of PWO, we demonstrate alterations in NK cell metabolism, mitochondrial fitness, and cytokine production. Furthermore, upon stratification into PWO with normal iron status versus low iron status, we show the observed obesity-related defects in NK cell metabolism, mitochondrial fitness and cytokine production are concentrated in the PWO with low-iron status. Collectively, our data highlights the importance of iron for human NK cell responses and provides evidence that obesity-driven defects in NK cell metabolism and function are linked in part to altered iron availability.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Iron Sufficient
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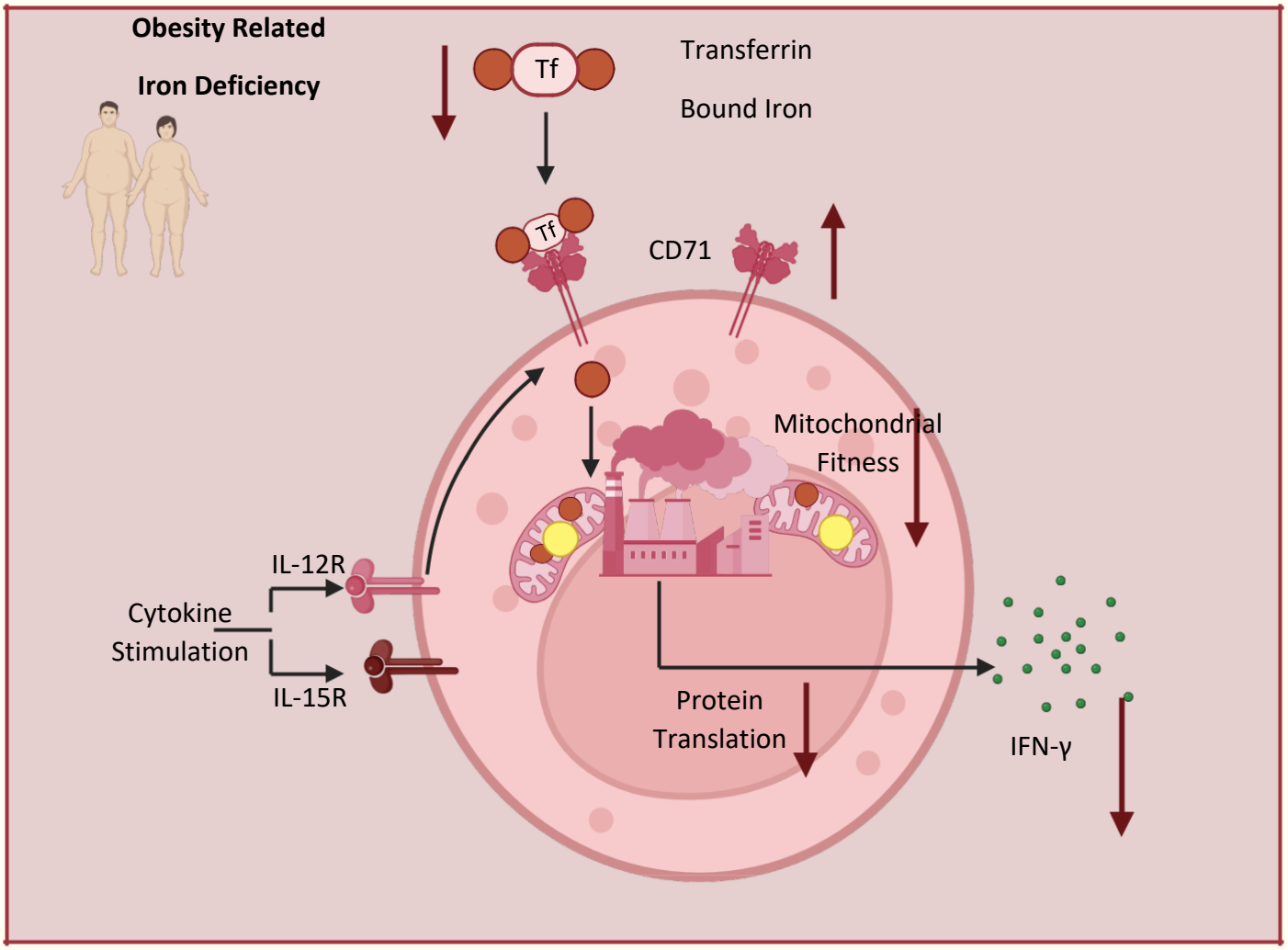
Obesity Related
Iron Deficiency
Tf
Transferrin
Bound Iron
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CD71
Mitochondrial
Fitness
IL-12R
Cytokine
Stimulation
IL-15R
Protein
Translation
IFN-γ

## Significance Statement

Natural killer (NK) cells are critical mediators of host-protection from viral infection and malignancy. Numerous studies have described the dysregulation of NK cells in the setting of obesity. Altered cellular metabolism has been identified as a possible mechanism for NK cell defects in obesity, but how the obesogenic environment drives altered NK cell function and metabolism remains unclear. Iron deficiency is a very common co-morbidity in obesity and was investigated as a potential driver. Herein, we demonstrate that human NK cell require iron for their metabolism and functional responses. We show that in a cohort of people with obesity (PWO), iron status is linked to NK cell fitness, with the reported defects concentrated in PWO with low iron availability.

# Introduction

Natural killer (NK) cells are a population of innate effector lymphocytes involved in immunity against viral infections and cancers[1, 2]. NK cells are armed with cytotoxic molecules capable of lysing transformed or infected target cells[3, 4, 5]. Upon cytokine stimulation NK cells can also produce a multitude of inflammatory cytokines and chemokines[6], and thus are capable of initiating and/or amplifying a stronger immune response. Underpinning this robust NK cell effector profile is an immunometabolic program which provides them with the signals, energy and biosynthetic intermediates to produce effector molecules such as interferon gamma (IFN-γ)[7]. At rest, peripheral NK cells are quiescent cells with low metabolic rates[8, 9], whereas cytokine stimulated NK cells display elevated rates of glycolysis and oxidative phosphorylation (OxPHOS)[10, 11]. This increased metabolic activity is paired with an increased demand for nutrients including glucose and amino acids[8, 11, 12, 13]. Previous work from our group has highlighted defects in NK cell metabolism [14, 15, 16, 17] as a mechanism underpinning reported defects in NK cell functionality in people with obesity (PWO)[18, 19, 20, 21]. Altered systemic lipid profiles was proposed as an obesogenic driver of the defects in NK cells cellular metabolism[21]. However, obesity is associated with significant alterations in systemic nutrient availability, with one of the most well defined being iron[22]. Recently, several studies have highlighted the importance of iron, an essential trace element, for host immune responses and homeostasis [23, 24, 25]. Iron is essential for several critical cellular processes such as OxPhos, DNA synthesis and cell proliferation [26, 27, 28]. Currently, it is unknown if lower iron availability in people with obesity is linked to the reported defects in NK cells. In the current study, we demonstrate that in healthy human NK cells, after cytokine stimulation, extracellular iron supports protein translation, adenosine triphosphate (ATP) production, and cytokine responses. In the setting of obesity, we show a loss of mitochondrial fitness and reduced cytokine responses is underpinned by altered iron availability. Collectively, our data demonstrates that human NK cells require extracellular iron for their metabolic and functional responses after cytokine stimulation, and altered iron availability in obesity is associated with defective NK cells.

# Materials and Methods

**Study cohorts & ethical approval.** In total a cohort of 30 people living with obesity (BMI>30) were recruited – 15 individuals with normal transferrin saturation and 15 with low transferrin saturation. Both groups were matched for sex, age and BMI (see Table S1). After informed consent, a peripheral blood sample was taken for research purposes. Inclusion criteria included ability to give informed consent, 18-65 years of age, no current or recent (<2 weeks) infection, and no use of anti-inflammatory medications including GLP-1 analogue therapies. A cohort of 15 healthy adult donor were recruited as a control group. Ethical approval was obtained from both St Vincent's University Medical Ethics Committee and Maynooth University Ethics Committee

**Preparation of peripheral blood mononuclear cells (PBMC) and flow cytometric analysis.** PBMC samples were isolated by density centrifugation in SeptMate 50mL tube over Lymphoprep, from fresh peripheral blood samples. After using a viability stain, eBiosciences 506 Live Dead Stain (Invivogen), NK cells were identified as $CD3^-$, $CD56^+$ cells (Miltenyi BioTec). When appropriate NK cells were fixed and permeabilised using the True-Nuclear Transcription Factor Buffer set (BioLegend) according to the manufacturer's instructions. Transferrin receptor levels were measured via CD71 (Miltenyi Biotec). Cell populations were acquired using a Attune NXT flow cytometer and analysed using FlowJo software (Treestar). Results are expressed as a percentage of the parent population as indicated and determined using flow minus-1 (FMO) and unstained controls.

**NK Cell Isolation.** NK cells were isolated from PBMCs using a NK cell negative selection kit from Miltenyi Biotec as per manufacturer's instructions using LS columns (Miltenyi Biotec) and MACS Buffer (Miltenyi Biotec). NK cell purity was confirmed via flow cytometry (>93% purity). Additionally, monocyte contamination was confirmed to be below 1%.

**NK cell SCENITH assay.** PBMCs ($2 \times 10^6$ /mL) were activated using IL-15 (50ng/mL) and IL-12 (30ng/mL) for 18 hours. Cells were seeded into a 96-well plate, and treated as a control, or with 2-Deoxy-D-Glucose (100mM), Oligomycin (1µM), or both. Following incubation at 37°C for 15 minutes, cells were treated with Puromycin (11µM) and incubated for a further 25

minutes. Cells were washed with ice-cold PBS to stop puromycin incorporation. Cells were then stained for viability. NK cells were stained for extracellular markers and fixed, as outlined above. Staining of puromycin was achieved using anti-puromycin monoclonal antibody (AlexaFluor488, Sigma), in permeabilization buffer (BioLegend).

**NK cell transferrin uptake assay.** Stimulated and unstimulated PBMCs (1 x $10^6$), activated using IL-15 (50ng/mL) and IL-12 (30ng/mL) for 18 hours, were rested in serum-free HPLM with 5% BSA for 2 hours. Cells were then washed in serum-free HPLM with 0.5% BSA and incubated with 5µg/ml Transferrin-AlexaFluor647 (Invitrogen) for 10 minutes at 37°C. Holo-transferrin (500µg/ml, Sigma-Aldrich) was used to competitively control for transferrin-uptake. An additional sample was stained at 4°C. Cells were washed in ice-cold HPLM with 0.5% BSA to stop membrane trafficking. Cells were then stained for viability, and NK cells were labelled for extracellular markers, to be analysed by flow cytometry.

**NK cell mitochondrial analysis.** PBMCs (1 x $10^6$ /mL) were activated using IL-15 (50ng/mL) and IL-12 (30ng/mL) for 18 hours, in the absence or presence of DFO (200µM, Sigma). Cells were seeded into a 96-well plate and washed in serum-free buffer. Cells were then stained for viability as outlined above, and NK cells were stained for extracellular markers. Cells were then washed and stained with Mitotracker Deep Red FM (50µM, ThermoFisher Scientific) and Mitotracker Green (50µM, ThermoFisher Scientific) in PBS, and incubated for 1 hour at 37°C. Cells were subsequently analysed by flow-cytometric analysis.

**NK cell functional analysis.** PBMCs (1 x $10^6$ /mL) were activated using IL-15 (50ng/mL) and IL-12 (30ng/mL) for 18 hours, in the absence or presence of DFO (200µM, Sigma). After using a viability stain, eBiosciences 506 Live Dead Stain (Invivogen), NK cells were identified as $CD3^-$, $CD56^+$ cells (Miltenyi BioTec). Cells were also stained for NKG2D (Miltenyi Biotec)**.** The PBMCs were then fixed and permeabilised using the True- Nuclear Transcription Factor Buffer set (BioLegend) according to the manufacturer's instructions. In perm buffer the cells were then stained for Granzyme B, IFN-γ and Perforin (Miltenyi Biotec).

**NK cell ATP assay.** Isolated NK cells (1 x $10^6$ /ml) were activated using IL-15 (50ng/mL) and IL-12 (30ng/ml) for 18 hours. A luminescence ATP assay kit (abcam) was used. Reagents in the

kit we reconstituted as per manufacturer's instructions. A standard curve was also prepared as per the kit's the instructions. NK cells were harvested and washed with PBS. 100µL of resuspended NK cells was added to a black walled, clear bottomed plate. 50µL of detergent was added to each well and the plate was placed on an orbital shaker for 5 minutes at 600-700 rpm. 50µ of substrate solution was added and the plate was returned to the orbital shaker for 5 minutes at 600-700 rpm. The plate was then covered and placed in the dark for 10 minutes before luminescence was measured on a multimode plate reader (CLARIOstar).

**NK cell metabolic analysis.** PBMCs (1 x $10^6$ /ml) were activated using IL-15 (50ng/mL) and IL-12 (30ng/ml) or IL-18 (50ng/ml) and IL-12 (30ng/mL) for 18 hours. Cells were then labelled for extracellular markers, then fixed and permeabilized using the True- Nuclear Transcription Factor Buffer set (BioLegend) according to the manufacturer's instructions before intracellular staining with monoclonal antibodies specific for hexokinase II (abcam) and pS6 and CD98 (Miltenyi Biotec).

**Statistics.** Statistical analysis was completed using Graph Pad Prism 6 Software (USA). Data is expressed as SEM. Distribution was assessed using Sharpio-Wilk test. We determined differences between two groups using Student T-test (paired or unpaired) or Mann Whitney U test where appropriate. Analysis across 3 or more groups was performed using ANOVA with multiple measures. Correlations were determined using linear regression models and expressed using Pearson or Spearman's rank correlation coefficient, as appropriate. P values were expressed with significance set at $<0.05$.

# RESULTS

**Cytokine activated NK cells increase expression of CD71 and transferrin uptake.**

First, we investigated if human NK cells increase their expression of the transferrin receptor CD71 upon cytokine stimulation (IL-12/IL-15) and observed a significant increase in both the percentage of NK cells expressing CD71, and the mean fluorescent intensity (MFI) of CD71 expression (Figure 1A-D). Next, we investigated if cytokine activated NK cells increase their uptake of transferrin, using a transferrin uptake assay, and demonstrate increases in both the percentage and MFI of transferrin uptake (Figure 1E-G). To extend our investigations into the setting of obesity, we recruited a cohort of people with obesity (PWO) and confirmed altered iron availability, with lower serum iron and transferrin saturation in comparison to healthy controls (Figure 1H-I). Next, we investigated CD71 expression and transferrin uptake in this cohort of PWO. There was tendency for NK cells from PWO to show higher expression of CD71 and while this did not reach statistical significance, these cells demonstrated elevated basal transferrin uptake in NK cells when compared to healthy controls (Figure 1J-K).

## Figure 1

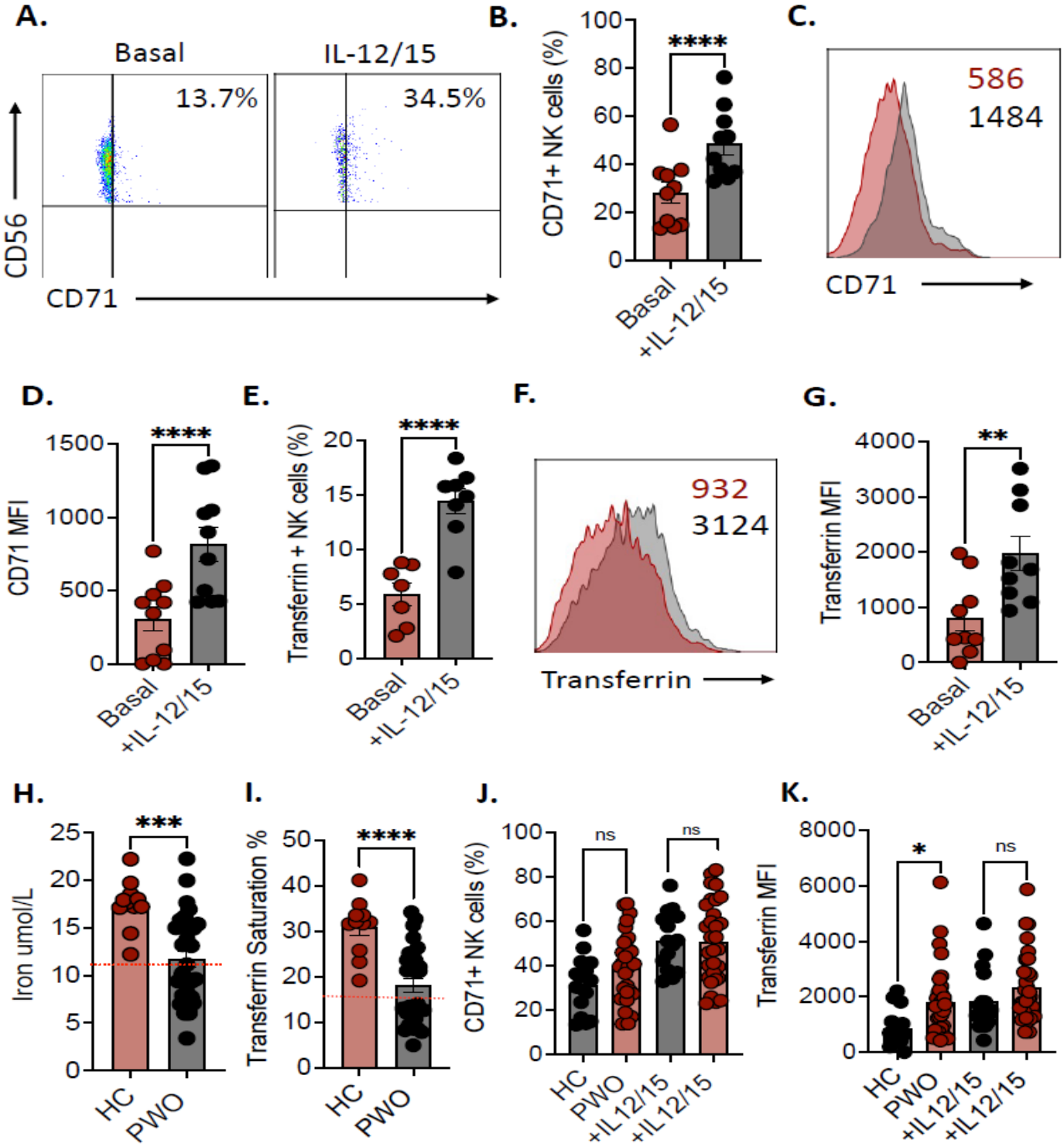


***Figure 1: Cytokine activated NK cells increase expression of CD71 and transferrin uptake***. (A) Representative dot plot showing frequencies of CD71 expressing NK cells. (B) Bar chart detailing the frequencies of CD71 expressing NK cells, either basal or stimulated with IL-12/15 for 18 hours. (C) Representative histogram showing MFI of CD71 on NK cells either basal or stimulated with IL-12/15 for 18 hours. (D) Bar chart showing the frequencies of CD71 expressing NK cells either basal or stimulated with IL-12/15 for 18 hours. (E-G) Bar chart and representative histogram detailing the frequency of transferring uptake and MFI of transferrin in NK cells either basal or stimulated with IL-12/15 for 18 hours. (H-I) Bar charts showing the iron content (umol/L) and Transferrin Saturation (%) of blood from cohorts of healthy controls (HC) and people with obesity (PWO). (J) Bar chart showing the frequencies of CD71 expressing NK cells from both HC and PWO, either basal or stimulated with IL-12/15 for 18 hours. (K) Bar chart displaying the frequencies of transferrin uptake in NK cells from both HC and PWO, either basal or stimulated with IL-12/15 for 18 hours. ns, no significance; *p < 0.05; **p < 0.01; ***p < 0.001.

**Cytokine activated human NK cells require iron for optimal metabolic responses.**

We next determined whether human NK cells require extracellular iron to support metabolic activity after cytokine stimulation. We utilized SCENITH[29], a single cell approach which allows immunometabolic characterisation based on cellular protein translation. We show that NK cells increased their rates of protein translation, as measured by puromycin incorporation after cytokine stimulation (Figure 2A-B). Furthermore, upon stimulation NK cells decreased their reliance on OxPhos relying more on glycolysis (Figure 2C-D). Next, using the iron chelator deferoxamine (DFO) to model reduced iron availability, we show that NK cell protein translation is reduced under low iron conditions (Figure 2E-F). We also demonstrate that NK cell metabolic dependencies change under low iron conditions, with increased glucose dependency and reduced OxPhos dependency (Figure 2G-H). Mirroring the effects of DFO on NK cells, our cohort of PWO displayed reduced capacity for protein translation with significantly reduced puromycin incorporation (Figure 2I-J). While there is a significant reduction in puromycin incorporation, NK cells from PWO demonstrate similar glycolytic and OxPhos dependencies to NK cells from healthy controls (Figure 2 K-L).

## Figure 2

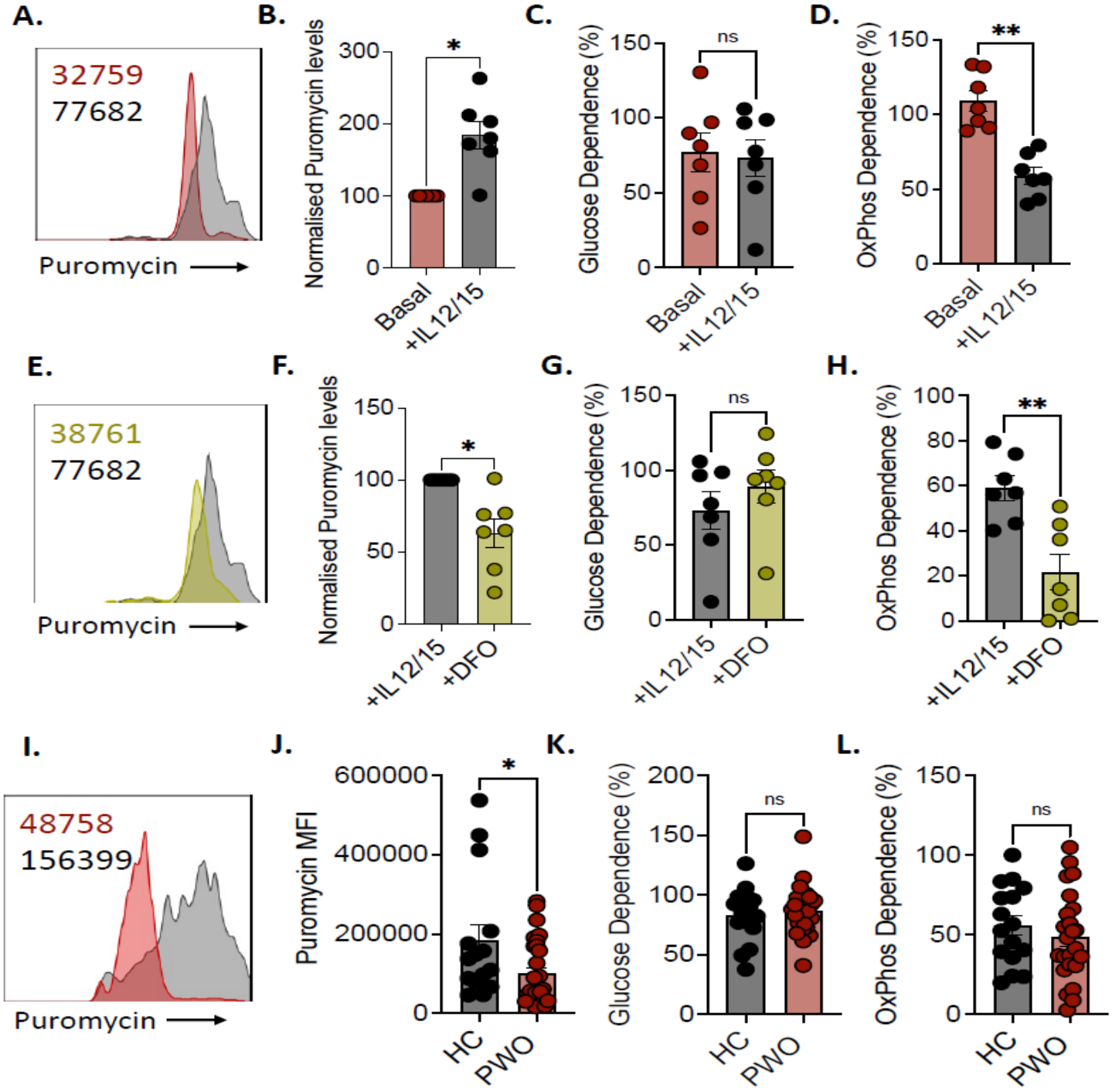


***Figure 2: Cytokine activated NK cells require iron for metabolic and functional responses.*** (A) Representative histogram detailing MFI of puromycin incorporation into NK cells either basally or stimulated with IL-12/15 for 18 hours. (B) Bar chart showing change in puromycin MFI relative to basal of NK cells stimulated with IL-12/15 for 18 hours. (C-D) Bar charts showing the metabolic dependence of NK cells on glycolysis and OxPhos respectively either basal or stimulated with IL-12/15 for 18 hours. (E) Representative histogram detailing MFI of puromycin of NK cells stimulated with IL-12/15 for 18 hours, in the absence or presence of the iron chelator DFO. (F) Bar chart showing change in puromycin MFI relative to stimulated NK cells or NK cells in the presence of DFO for 18 hours. (G-H) Bar charts showing the metabolic dependence of NK cells on glycolysis and OxPhos respectively, stimulated with IL- 12/15 for 18 hours in the presence or absence of DFO. (I) Representative histogram detailing MFI of puromycin of NK cells, stimulated with IL-12/15 for 18 hours either from healthy controls (HC) or people with obesity (PWO). (J) Bar chart showing puromycin MFI of NK cells stimulated with IL-12/15 for 18 hours, from either PWO or HC. (K-L) Bar charts showing the metabolic dependence of NK cells on glycolysis and OxPhos respectively, stimulated with IL-12/15 for 18 hours, either from PWO or HC. ns, no significance; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

**Altered iron availability drives reduced mitochondrial fitness in human NK cells.**

With the noted reduction in NK cell mitochondrial metabolism under conditions of limited iron, we next investigated the impact of reduced iron availability on human NK cell mitochondrial mass and polarisation. We demonstrate an accumulation of dysregulated mitochondria, as measured by an increase in the proportion of NK cells with high MitoTracker Green and low MitoTracker Deep Red, indicating large but depolarised mitochondria[30], after DFO treatment (Figure 3A-B). This was reinforced with a decrease in the ratio of MitoTracker Green to MitoTracker Deep Red in DFO treated NK cells (Figure 3C). We next assessed the impact of reduced iron availability on NK cell ATP production and found a marked reduction in ATP production by NK cells treated with DFO (Figure 3D). This was also associated this was paired with diminished ability to produce IFN-γ after cytokine stimulation (Figure 3E). Mirroring DFO treated NK cells, peripheral NK cells from PWO displayed similar phenotype with increased mitochondrial dysfunction leading to significantly reduced ATP and IFN-γ production (Figure 3F-I).

**Figure 3**

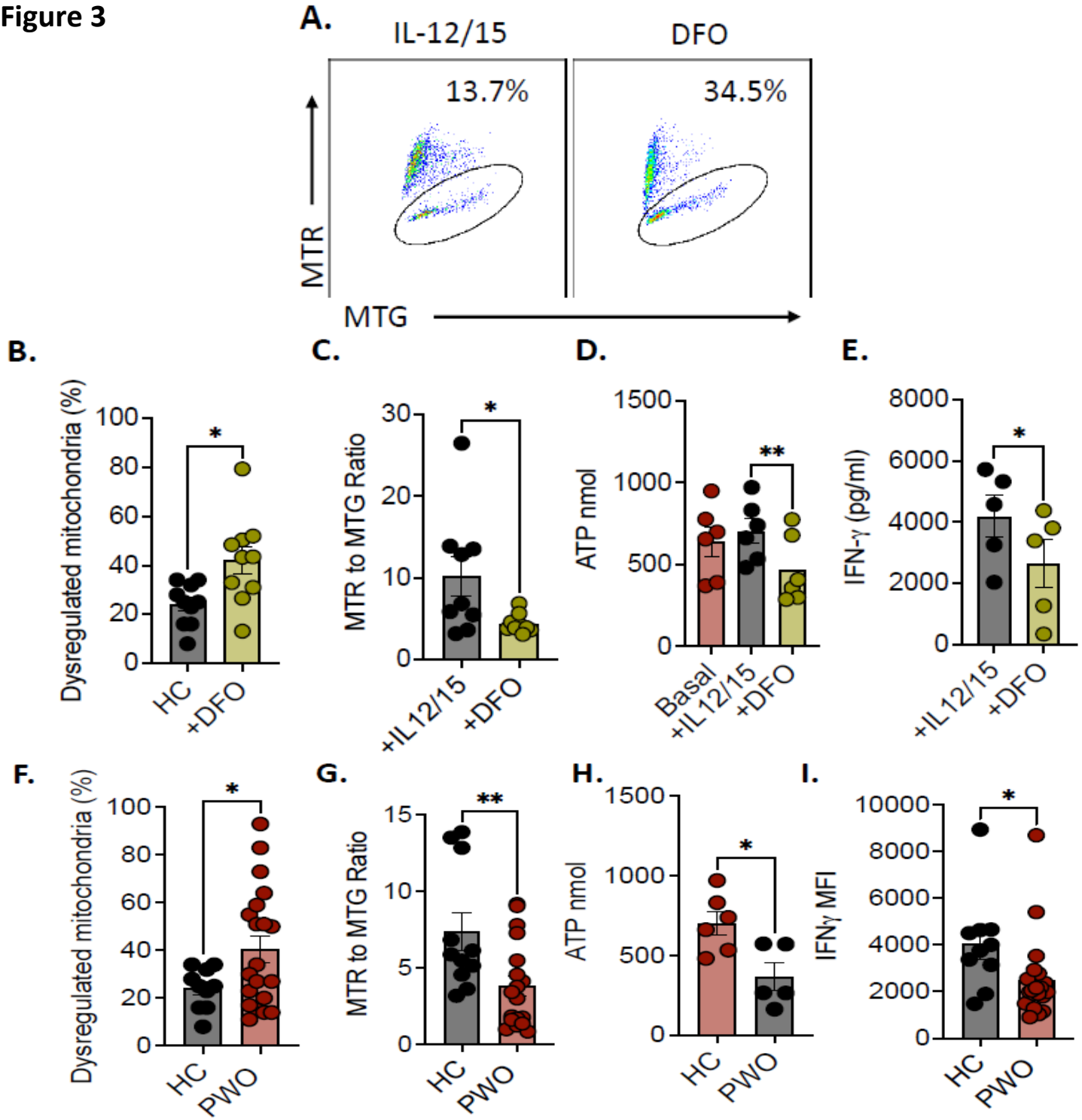


**Figure 3: Mitochondrial function is dependent on iron availability.** (A) Representative dot plots showing frequencies of dysregulated mitochondria (high Mitotracker green & low Mitotracker DeepRed) in NK cells, stimulated with IL-12/15 for 18 hours, in the presence or absence of DFO. (B) Bar chart showing frequencies of dysregulated mitochondria in NK cells, stimulated with IL-12/15 for 18 hours, in the presence or absence of DFO. (C) Bar chart describing the ratio of MitoTracker Deep Red MFI to MitoTracker Green MFI of NK cells, stimulated with IL-12/15 for 18 hours, in the presence or absence of DFO. (D) Bar chart detailing the production of ATP (nmol) in NK cells, either basally, or stimulated with IL-12/15 for 18 hours, in the presence or absence of DFO. (E) Bar chart detailing the levels of IFN-γ produced by NK cells stimulated with IL-12/15 for 18 hours, in the presence or absence of DFO. (F) Bar chart describing frequencies of dysregulated mitochondria in NK cells, from HC or PWO, stimulated with IL-12/15 for 18 hours. (G) Bar chart showing the ratio of MitoTracker Deep Red MFI to MitoTracker Green MFI of NK cells, from HC or PWO, stimulated with IL-12/15 for 18 hours. (H) Bar chart detailing the production of ATP (nmol) in NK cells, from HC or PWO, stimulated with IL-12/15 for 18 hours. (I) Bar chart detailing IFN-γ MFI in NK cells stimulated with IL-12/15 for 18 hours, from either HC or PWO. ns, no significance; PWO, people with obesity. *p < 0.05; **p < 0.01; ***p < 0.001.

**Low iron status in obesity is associated with defective NK cell metabolism and functionality.**

With the parallels between healthy NK cells treated with DFO and NK cells from PWO, we next aimed to established if the dysregulations observed were directly linked to altered iron availability, by stratifying our PWO cohort into those with low (<15%) transferrin saturation (Low TS) and those in the normal (15% to 50%) transferrin saturation range (Normal TS)[31, 32] (Figure 4A). Interestingly, PWO with low TS expressed significantly more CD71 than those with normal TS (Figure 4B). Next, we investigated metabolic activity, and show that cytokine stimulated NK cells from the low TS cohort had reduced rates of protein translation and mitochondrial fitness in comparison to those in the normal TS cohort (Figure 4C-E). To investigate if this loss of mitochondrial fitness underpinned the defective functional of NK cells from PWO, we performed statistical analysis, and show a strong correlation between the proportion of dysregulated mitochondria and the percentage of IFN-γ producing cells (Figure 4F). We also demonstrate that transferrin saturation levels in PWO are positively linked to the IFN-γ producing ability of cytokine stimulated NK cells (Figure 4G). Supporting this observation, we also found that the IFN-γ levels in NK cells from low TS cohort were significantly lower than healthy controls, whereas no significance was found between normal TS cohort and controls (Figure 4H).

# Figure 4

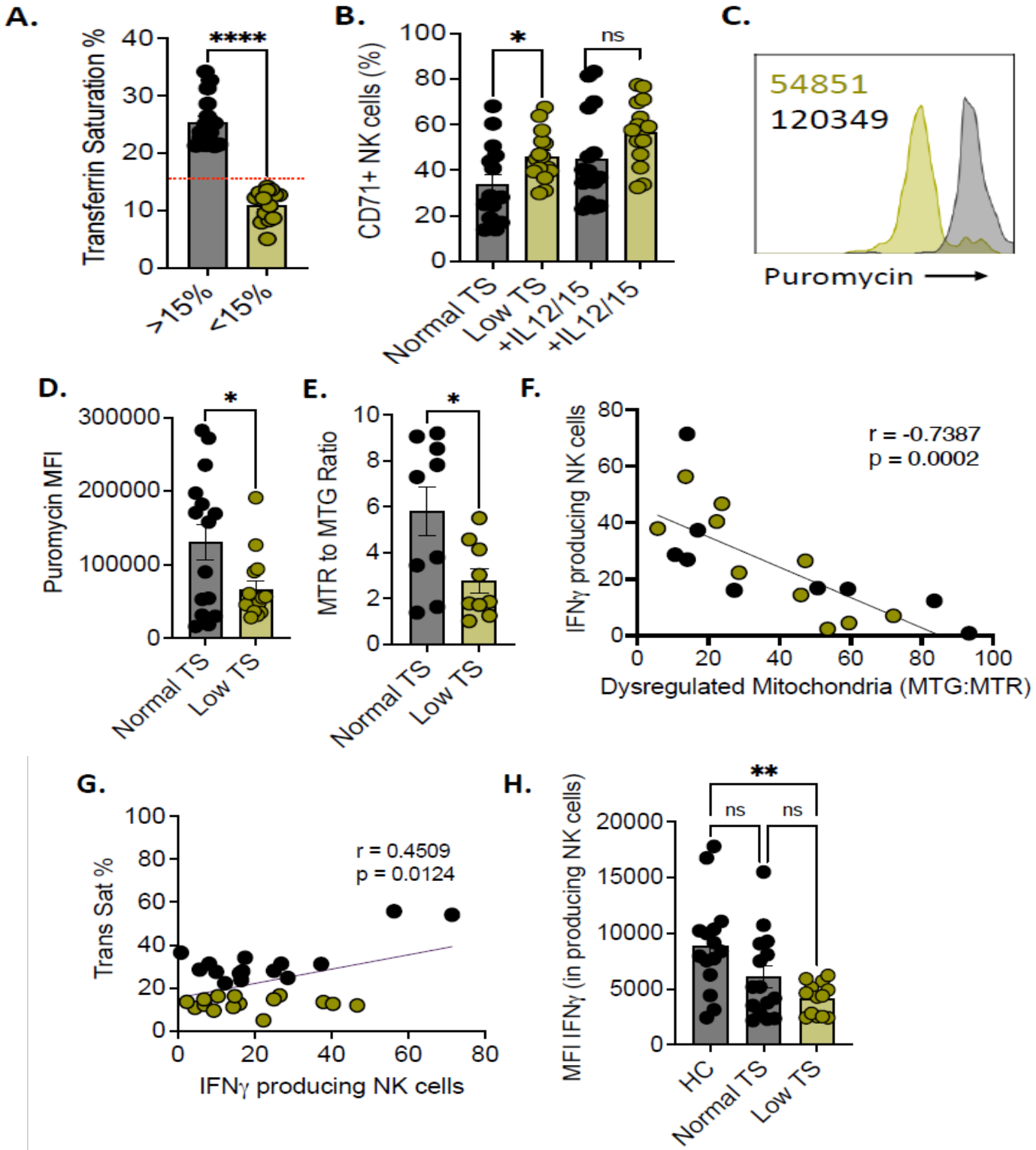


**Figure 4: Low iron status in obesity is associated with defective NK cell metabolism and functionality.** (A) Bar chart detailing the transferrin saturation (%) of PWO, dividing the cohort into those with normal transferrin saturation and low transferrin saturation. (B) Bar chart showing the frequencies of CD71 expressing NK cells from PWO, both normal TS and low TS, either basal or stimulated with IL-12/15 for 18 hours. (C) Representative histogram detailing MFI of puromycin of NK cells stimulated with IL-12/15 for 18 hours, from PWO with either normal TS or low TS. (D) Bar chart showing puromycin MFI of NK cells, stimulated with IL-12/15 for 18 hours, from PWO with either normal TS or low TS. (E) Bar chart detailing the ratio of MitoTracker Deep Red MFI to MitoTracker Green MFI of NK cells, from PWO, both normal TS and low TS, either basal or stimulated with IL-12/15 for 18 hours. (F) Scatter plot describing the relationship between the percentage of IFN-γ producing NK cells and the percentage of dysregulated mitochondria in both the normal TS group and low TS group. (G) Scatter plot detailing the relationship between the percentage of IFN-γ producing NK cells and the transferrin saturation in both the normal TS group and low TS group. (H) Bar chart showing the MFI of IFN-γ from IFN-γ producing NK cells from HC, normal TS and low TS cohorts stimulated with IL-12/15 for 18 hours. ns, no significance, *p < 0.05; **p < 0.01; ***p < 0.001.

**Discussion**

Obesity is associated with significant changes in circulating NK cell metabolism and functionality [14, 15, 17, 33, 34]. However, the specific factors that underpin these changes remain unclear. One of the most common systemic nutritional alterations in obesity is reduced iron availability[22], yet the role of iron and the effect of iron deficiency on human NK cells remain unclear. In this study, we demonstrate that exogenous iron is critical for human NK cell metabolic fitness and IFN-γ production. We provide evidence which links low iron availability to dysfunctional NK cells in PWO. Iron is critical for numerous basic cellular processes including bioenergetics, with many proteins in these processes requiring iron to function[35]. There are various forms of iron found in the body with varying degrees of bioavailability, with the vast majority of iron stored in tissues, especially the liver, as ferritin[36]. In the bloodstream iron is bound to transferrin, and this complex is transported to various organs and tissues[35]. Transferrin bound iron is the transported into target cells via the transferrin receptor CD71[37]. We investigated the expression of CD71 on human NK cells, and while basal expression was low, cytokine stimulation of these cells caused strong upregulation of CD71. This supported previous studies in murine NK cells, which also noted robust increases in CD71 expression after cytokine stimulation [24]. Interestingly the upregulation of CD71 on activated NK cells mirrors equivalent CD71 upregulation in functionally related $CD8^+$ T cells upon activation[38, 39].

In our cytokine stimulated human NK cells, the increase in CD71 expression was also associated with an increase in transferrin uptake, again supporting studies in mice which demonstrated transferrin uptake by NK cells[24, 40]. In our cohort of PWO, we report reduced serum iron and transferrin saturation, in line with previous meta- analysis[22]. In this cohort we noted no significant overall difference in CD71 expression by peripheral NK cells, however when we further stratified our obesity cohort into those with normal iron availability and those with reduced iron availability, we noted a marked increase in basal CD71 expression in the reduced iron cohort. This potential compensatory mechanism of elevated CD71 was also observed in ILC3 under iron limited conditions[25]. As outlined, iron is critical for cellular metabolism, in particular mitochondrial metabolism[41]. We found that limiting iron availability using the iron chelator deferoxamine (DFO) reduced the rates of protein translation in cytokine activated NK cells, that likely is due to decrease in metabolic activity given that over

half of the energy produced by cell metabolism is utilised in protein biosynthesis. Using SCENITH [29], we observed a significant decrease in OxPhos dependent protein translation, recapitulating reduced OxPhos rates in ILC3 treated with DFO[25]. In line, with these observations, NK cells from PWO displayed reduced levels of protein synthesis, with The greatest reductions in the individuals with low iron availability, potentially linking low iron status to defective NK cell metabolism in people with obesity. In CD8+ T cells, iron deficiency resulted in dysregulated mitochondria and reduced ATP production[23], so we investigated this in healthy human NK cells, and found that DFO treatment resulted in an accumulation of dysregulated depolarized mitochondria[30], which was paired with a significant drop in ATP production. Strikingly, the DFO driven phenotype of poor mitochondrial fitness (depolarized mitochondria and reduced ATP production) phenocopied NK cells from PWO, reinforcing the concept that obesity-related defects in NK cell metabolism may be linked to altered iron availability. Metabolism is intrinsically linked to NK cell functionality, including the ability to produce key cytokines such as IFN-γ[9], which is required for NK cell mediated anti-viral responses[42, 43], and can directly inhibit viral replication and drive malignant cell apoptosis [44, 45, 46]. NK cell mitochondrial fitness has been directly linked to their effector function including their IFN-γ production[47, 48]. In line with these observations, we observed a very strong correlation between mitochondrial fitness and IFN-γ production in people with obesity. Similarly, we found that the PWO with reduced iron availability had greater reductions in the frequencies of IFN-γ producing NK cells, again highlighting the importance of iron for NK cell fitness. This concept is further strengthened by data from Santosa and colleagues, who report defective adaptive NK cell responses in a murine model of iron deficiency[40]. In summary, our work demonstrates that human NK cells require iron for their metabolic fitness and effector function. We show that in the setting of obesity, low iron availability is strongly linked to defective NK cell metabolism and cytokine production. This study provides new insight into factors underpinning obesity-driven defects in immunity and presents a possible therapeutic target for improving NK cell responses in a group at greater risk of adverse outcomes with viral infection and cancer.

**Contributors Statement:** CDB and ER performed the experiments and carried out analysis, wrote and approved the final manuscript as submitted. MS, OR, EL, HH, CMcC and DOS recruited the clinical cohorts, carried out clinical analysis and approved the final manuscript as submitted. AEH, DOS, PNM, NJ & LVS conceptualized and designed the study, analyzed the data, drafted the manuscript, and approved the final manuscript as submitted.

# Appendix

## Patient Characteristics

| | Normal Iron | Low Iron | P value |
|---|---|---|---|
| Iron (umol/L) | 15.4 | 7.6 | <0.0001 |
| Transferrin Saturation (%) | 25.3 | 10.8 | <0.0001 |
| Mean Age (Years) | 54.2 | 52.4 | 0.64 |
| Mean Body Mass Index (kg/m2) | 48.8 | 52.2 | 0.36 |

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Paper III: Glucagon-like peptide-1 therapy in people with obesity restores natural killer cell metabolism and effector function.

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ORIGINAL ARTICLE

Obesity Biology and Integrated Physiology

# Glucagon-like peptide-1 therapy in people with obesity restores natural killer cell metabolism and effector function

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## Abstract

Objective: People with obesity (PWO) have functionally defective natural killer (NK) cells, with a decreased capacity to produce cytokines and kill target cells, under- pinned by defective cellular metabolism. It is plausible that the changes in peripheral NK cell activity are contributing to the multimorbidity in PWO, which includes an increased risk of cancer. This study investigated whether therapy with long-acting glucagon-like peptide-1 (GLP-1) analogues, which are an effective treatment for obesity, could restore NK cell functionality in PWO.

Methods: In a cohort of 20 PWO, this study investigated whether 6 months of once weekly GLP-1 therapy (semaglutide) could restore human NK cell function and metabolism using multicolour flow cytometry, enzyme-linked immunosorbent assays, and cytotoxicity assays.

Results: These data demonstrate that PWO who received GLP-1 therapy have improved NK cell function, as measured by cytotoxicity and interferon-γ/granzyme B production. In addition, the study demonstrates increases in a CD98-mTOR-glycolysis metabolic axis, which is critical for NK cell cytokine production. Finally, it shows that the reported improvements in NK cell function appear to be independent of weight loss.

Conclusions: The restoration, by GLP-1 therapy, of NK cell functionality in PWO may be contributing to the overall benefits being seen with this class of medication.



## INTRODUCTION

Obesity is a major global health care issue, with more than 600 million adults living with obesity worldwide [1]. Obesity is a disease defined by the accumulation of excess adipose tissue, which is harmful to an individual's health [2]. A major contributor to obesity's burden on health is its striking and strong association with numerous chronic diseases, including type 2 diabetes mellitus (T2DM), cardiovascular disease, and many types of cancer [3, 4]. Obesity is also associated with poor outcomes following infections such as influenza and SARS- CoV-2 [5, 6]. Significant immune dysregulation, which has been described in people with obesity (PWO), is associated with both the chronic disease burden and poor outcomes in acute infections [7-10]. Natural killer (NK) cells are a critical front line immune population tasked with protecting the host from invading pathogens and the development of malignancies [11]. They represent approximately 10% of circulating lymphocytes. NK cells are able to rapidly kill infected or

Donal O'Shea and Andrew E. Hogan are joint senior authors.

transformed malignant cells without prior sensitization [12, 13]. NK cells are also potent cytokine producers, and they can direct other immune populations via their early production of cytokines such as interferon-γ (IFN-γ) [14-16]. Rapid production of IFN-γ by NK cells is also important for their antitumor activities [17, 18]. The importance of NK cells for antitumor immunity is highlighted by the increased prevalence of cancer in humans deficient in NK cells [19].

Cellular metabolism has emerged as a critical regulator of NK cell responses. On activation, NK cells are shaped and instructed by intrinsic metabolic processes and nutrient availability [20]. Glycolysis is one of the key metabolic pathways required for NK cell activity, in particular their ability to produce IFN-γ [21, 22]. Another critical factor for NK cell metabolism is the metabolic master regulator mammalian tar- get of rapamycin (mTOR) [21, 23]. The serine/threonine-protein kinase mTOR also functions as a nutrient sensor and it is activated/ sustained by amino acid transport via the surface expressed trans- porter SLC7A5 [24].

Our group and others have previously reported the detrimental impact of obesity on circulating NK cells, with reduced frequencies and defective functions, including cytotoxicity and IFN-γ production [25-28]. We have provided mechanistic evidence that dysregulated cellular metabolism underpinned the defective circulating NK cell functionality in obesity [27, 28]. Furthermore, NK cells with obesity-related defects were limited in their control of tumour progression in murine models of cancer [28]. Therefore, it would be of potential clinical significance to identify therapeutic approaches that could restore peripheral NK cell activity in PWO.

Glucagon-like peptide-1 (GLP-1) is a multifaceted gut hormone that facilitates glucose-dependent stimulation of insulin secretion and regulation of satiety [29]. Because of these metabolic actions, GLP-1 has been developed into a pharmacological agent to treat both T2DM and obesity [30]. In addition to its classical metabolic effects, GLP-1 has been reported to be neuroprotective and immunomodulatory [30]. We have previously demonstrated the modulation of invariant natural killer T (iNKT) cells by GLP-1 in mice and humans, including restoration of obesity-associated defects in frequencies [31, 32].

In this study, we investigated whether GLP-1 therapy could restore circulating NK cells in PWO. We show that 6 months of GLP-1 analogue therapy in PWO is associated with improved NK cell cytokine production and cytotoxicity, underpinned by increased cellular metabolism. We also show that the observed improvements in NK cells were independent of weight loss and were mimicked by GLP-1 treatment of NK cells from PWO *in vitro*.

## METHODS

### Study approval

Ethical approval was granted by the Medical Research Ethics Committees at St Vincent's University Hospital and by Maynooth University Ethics Committee. All patients gave written informed consent prior to partaking in the study.

### Study Importance

#### What is already known?

- Obesity is strongly associated with increased mortality from cancer and viral infection.
- Peripheral blood natural killer (NK) cells are defective in people with obesity.
- Dysregulated NK cell metabolism underpins the defective functionality in obesity.

#### What does this study add?

- Following glucagon-like peptide-1 analogue treatment, NK cell functionality is restored in people with obesity.
- Glucagon-like peptide-1 analogue treatment boosts the cellular metabolism of NK cells from people with obesity.
- Glucagon-like peptide-1 analogue treatment-related improvements in NK cells are independent of weight loss.

#### How might these results change the direction of research or the focus of clinical practice?

- Our study demonstrates that glucagon-like peptide-1 analogue treatment improves NK cell functionality, which may contribute to the benefits reported with these medications in people with obesity.

### Study participants

In total, we recruited a cohort of 20 PWO who were due to commence GLP-1 analogue therapy (once weekly 0.25 mg semaglutide with standard dose escalation to 1 mg weekly) for weight management from the St Columcille's Hospital, Dublin, Ireland. Inclusion criteria included the following: aged between 18 and 55 years old, ability to give informed consent, body mass index (BMI) > 30, and no previous use of GLP-1 therapies. Exclusion criteria included the following: recent infection (<2 weeks), history of T2DM, or use of immunomodulatory medications. Patient characteristics are outlined in Supporting Information Table S1.

### Cell culture

Peripheral blood mononuclear cell (PBMC) samples were isolated by density centrifugation over Ficoll from fresh peripheral blood samples and biobanked for batch analysis after completion of 6 months of therapy. When necessary, primary NK cells were isolated via negative

selection using an NK cell purification kit (Miltenyi Biotec). PBMCs or

NK cells were cultured in Roswell Park Memorial Institute (RPMI)1640 Medium GlutaMax (Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 100 U/mL of penicillin and 100 µg/mL of streptomycin (Sigma). Cells were then incubated at 37 °C and 5% $CO_2$. For cytokine stimulation, NK cells or PBMCs were plated at 1 x $10^6$ cells/mL and left untreated or stimulated with interleukin (IL)- 15 (50 ng/mL) and IL-12 (30 ng/mL; both BioLegend) for 18 hours.

## Flow cytometry

NK cell staining was performed using specific surface monoclonal antibodies (all Miltenyi Biotec), namely, CD3 and CD56. Cell populations were acquired using a Attune NxT Flow Cytometer (Life Technologies) and analysed using FlowJo software (Treestar). Results are expressed as a percentage of the parent population as indicated and determined using fluorescence minus one and unstained controls IFN-γ, granzyme B, CD98, hexokinase-2 (HKII), and pS6.

## NK cell cytokine production

NK cells were isolated from PBMCs using a negative selection NK cell isolation kit (Miltenyi Biotec). NK cell purity was confirmed via flow cytometry and was shown to be >93%. NK cells were stimulated as before, and supernatants were analysed for IFN-γ and granzyme B concentration using DuoSet ELISA kits (R&D Systems). For direct stimulation with GLP-1, isolated NK cells from PWO were pretreated with GLP-1 analogues (1 µg/mL) for 60 minutes before stimulation for 18 hours as outlined previously. Supernatants were assessed by enzyme-linked immunosorbent assay (ELISA).

## NK cell cytotoxicity

K562s were washed three times in 10% FBS in phosphate-buffered saline (PBS) and counted. K562s were resuspended in serum free IMDM (Gibco) at a concentration of 1 x $10^6$ cell/mL and were treated with Calcein-AM (BioLegend) at a concentration of 20 µM for 30 minutes. K562s were washed again three times in 10% FBS in PBS and resuspended in IMDM with 10% FBS. K562s were added to a round bottom 96-well plate at a concentration of 2 x $10^5$ cells/mL. Effector cells were added at a 40:1 effector to K562 ratio and incubated at 37 °C, 5% $CO_2$ for 3 hours. After incubation the plate was spun at 300*g* for 5 minutes and 75 µL of the supernatant was removed and added to a black walled 96-well plate. The plate was read for fluorescent intensity at 530 nm. Killing percentage was measured using the following formula:

$$\frac{(Sample - Spontaneous)}{(Maximum - Spontaneous)} \times 100$$

(20 nM, Sigma). For SLC7A5 inhibition experiments, the concentration of amino acids in RPMI was diluted twofold using Hank's Balanced Salt Solution (Invitrogen) in the presence or absence of 2-aminobicyclo-(2,2,1)heptane-carboxylic acid) (BCH) (50 mM, Sigma).

## Statistics

Statistical analysis was completed using GraphPad Prism 6 software. Data are expressed as SEM. We determined differences between two groups using Student *t* test and Mann-Whitney U test where appropriate. Analysis across three or more groups was performed using ANOVA. Correlations were determined using linear regression models and expressed using Pearson or Spearman rank correlation coefficient, as appropriate. *P* values were expressed with significance set at <0.05.

# RESULTS

## NK cell frequencies and cytokine production are defective in PWO

Several previously published studies have demonstrated reduced circulating NK cell frequencies in PWO [27, 33]. We first confirmed this in our cohort of PWO before they commenced GLP-1 therapy (Figure 1A-C). A critical function of NK cells is their ability to produce effector molecules such as IFN-γ. Previous studies from our group and others have demonstrated a severe defect in NK cell production of IFN-γ in PWO [28]. Again, we confirmed this in our cohort of PWO before they commenced GLP-1 therapy (Figure 1D). In addition to defective cytokine production, we also demonstrated reduced NK cell cytotoxicity in PWO (Figure 1E).

## GLP-1 therapy results in weight loss and improved glycaemic control in PWO

GLP-1 analogue therapy, the gold standard for pharmacological treatment of obesity, can result in clinically significant (>5%) reductions in body weight. In our cohort of patients (mean BMI = 46.6), we observed reduced BMI in the cohort overall, with clinically significant weight loss in 9 of our 20 patients (Supporting Information Table S1 and Figure S1A). Although none of our patients had abnormal glycated haemoglobin, we did see a reduction in glycated haemoglobin in 12 of our patients (Supporting Information Figure S1B). We did not observe any change in the lipid profile of our patients with GLP-1 therapy (Supporting Information Table S1 and Figure S1C, D).

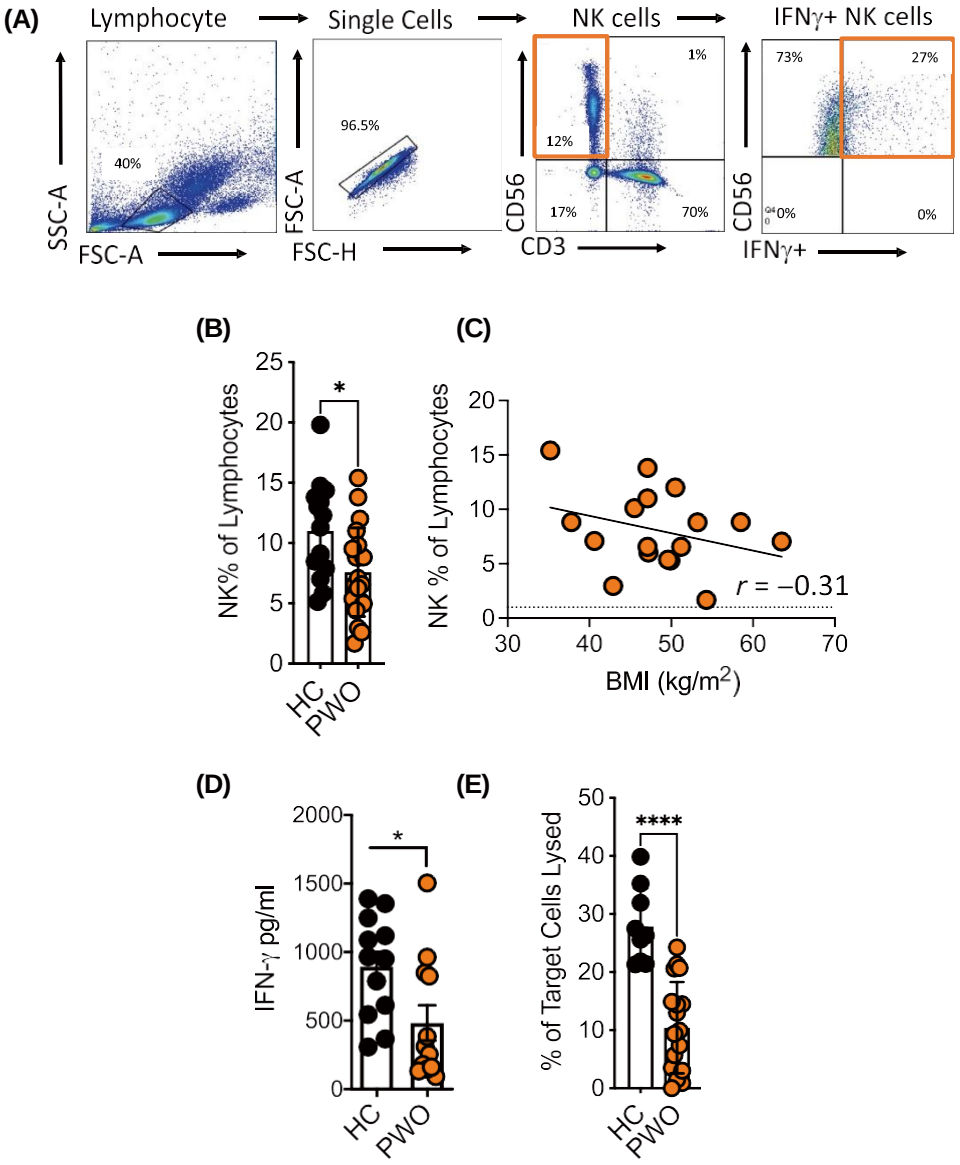


FIGURE 1 NK cells are altered in PWO. (A) Representative flow cytometry dot plots detailing the gating strategy used to identify NK cells in peripheral blood of PWO. (B) Scatterplot showing the NK cell frequencies in cohort of HC individuals and PWO. (C) XY graph showing the relationship between NK cell frequencies and BMI in PWO. (D) Scatterplot showing the levels of IFN-γ produced by activated (18 hours with IL- 12 and IL-15) NK cells isolated from either HC individuals or PWO. (E) Scatterplot showing the percentage lysis of K562 target cells over 3 hours by either HC individuals or PWO. FSC-A, Forward Scatter-Area; FSC-H, Forward Scatter-Height; HC, healthy control; NK, natural killer; PWO, people with obesity; SSC-A, Side Scatter-Area. *$p < 0.05$; ****$p < 0.0001$ [Colour figure can be viewed at wileyonlinelibrary.com]

commencing therapy. We observed no change in total NK cell frequencies after 6-month GLP-1 analogue therapy (Figure 2A). We next investigated whether either $CD56^{BRIGHT}$ or $CD56^{DIM}$ NK cell subsets were changed post GLP-1 and again noted no difference (Figure 2B,C). GLP-1 analogue therapy was used as a weight loss agent in this cohort, so we next investigated whether a clinical response in body weight (>5% weight loss) was associated with changes in NK cell frequencies and observed no difference in responders versus non-responders (Figure 2D). Next, we assessed phenotypic changes after GLP-1 and noted no difference in CD25, CD57, CD69, CD95, CD158b, NKG2A, or NKG2D expression on NK cells after GLP-1 therapy (Supporting Information Figure S2).

## GLP-1 analogue therapy increases NK cell effector function.

We next investigated whether GLP-1 therapy impacted NK cell IFN-γ production and noted a robust increase in NK cells producing IFN-γ (Figure 3A-C). To confirm this finding, we isolated NK cells from patients before and after GLP-1 therapy and again found elevated

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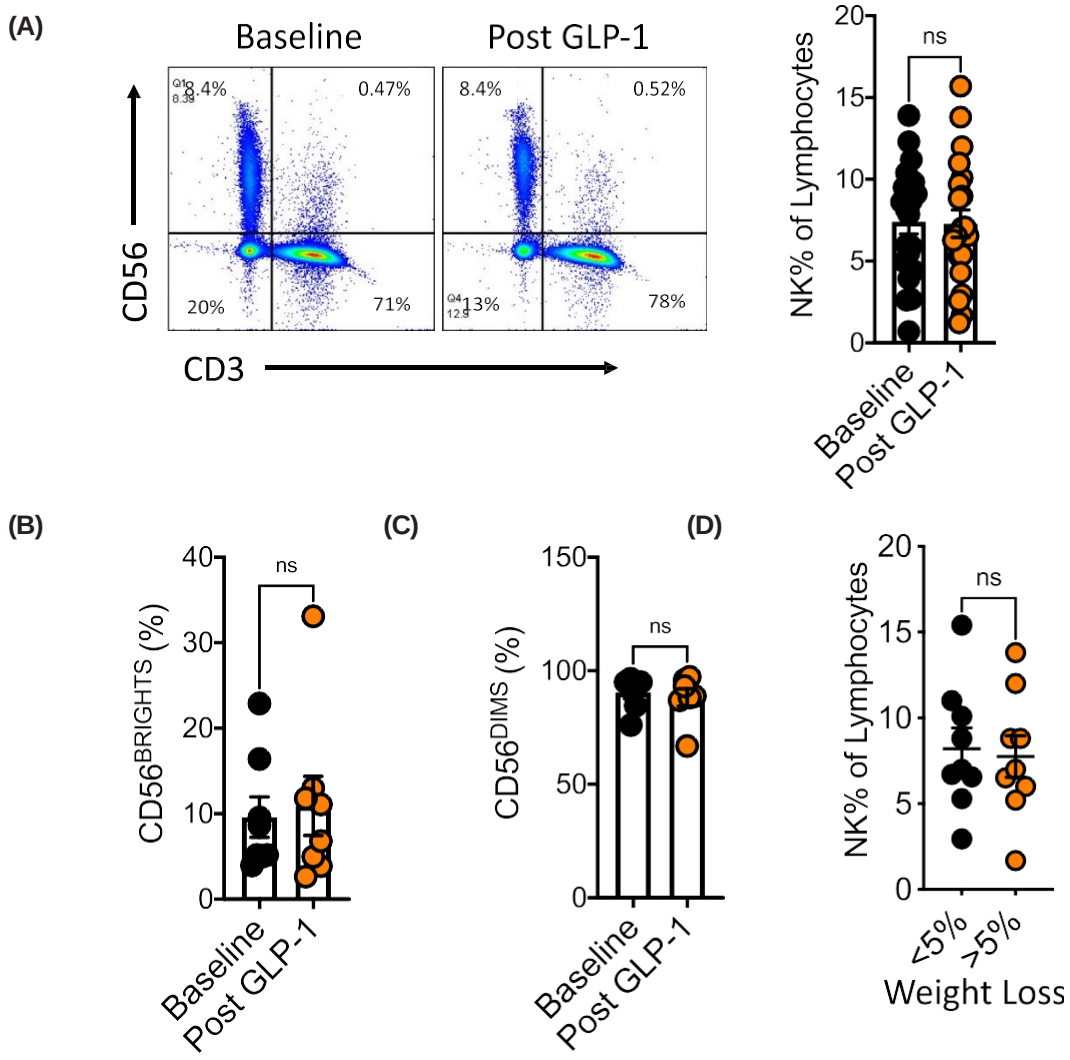



FIGURE 2 No change in NK cell frequencies in PWO treated with GLP-1 analogues. (A) Representative dot plot and graph showing the NK cell frequencies in cohort of 20 PWO before and after 6 months of GLP-1 analogue therapy. (B,C) Scatterplots showing the frequencies of $CD56^{BRIGHT}$ and $CD56^{DIM}$ NK cell subsets in cohort of 20 PWO before and after 6 months of GLP-1 analogue therapy. (D) Scatterplot showing the NK cell frequencies in PWO who lost or did not lose a clinically significant amount of weight (>5% of initial body weight). GLP-1, glucagon-like peptide-1; NK, natural killer; ns, no significance; PWO, people with obesity [Colour figure can be viewed at wileyonlinelibrary.com]

again, found elevated IFN-γ production post GLP-1 via ELISA (Figure 3D). Cytokine production by NK cells is concentrated in the $CD56^{BRIGHT}$ population, so we next investigated whether GLP-1 therapy increased the IFN-γ production from $CD56^{BRIGHT}$ and/or $CD56^{DIM}$ NK cells and demonstrated a significant increase in both bright and dim populations (Figure 3E,F).

In addition to IFN-γ we investigated granzyme B production and noted increased expression post GLP-1 therapy (Figure 3G,H). Similarly, we observed a significant increase in the granzyme B expression in $CD56^{BRIGHT}$ NK cells but not $CD56^{DIM}$ NK cells post GLP-1 therapy (Figure 3H,I). Finally, we demonstrated increased lysis of the NK cell sensitive line K562 after GLP-1 treatment (Figure 3J).

## GLP-1 therapy increases a metabolic axis of SLC7A5, mTORC1, and glycolysis in NK cells from PWO

As highlighted, NK cell cytokine production is critically dependent on intrinsic cellular metabolism, so we investigated a SLC7A5 expression of the amino acid transporter CD98 on NK cells from PWO, and we demonstrated, using a CD98 specific inhibitor BCH, that it is critical for NK cell production of IFN-γ in NK cells from healthy control individuals (Figure 4A–C). Next, we demonstrated that GLP-1 therapy increases mTOR activity (as measured by pS6) in NK cells from PWO, and again we demonstrated

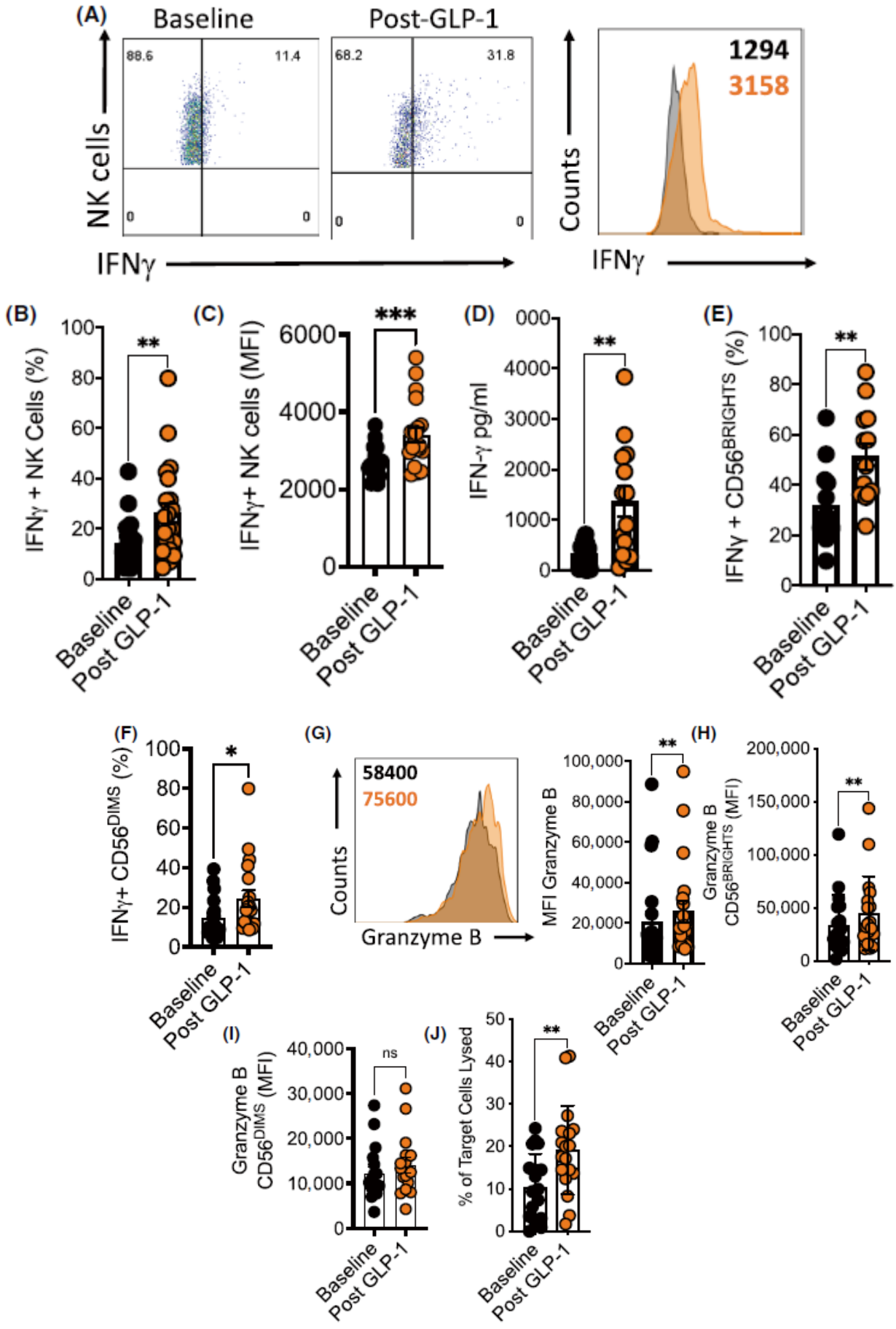



**FIGURE 3** Legend on next page.

FIGURE 3 GLP-1 therapy increases NK cell effector function in PWO. (A) Representative flow cytometry dot plots and histogram showing the frequencies or MFI of IFN-γ-producing NK cells (after 18 hours of cytokine [IL-12/15] stimulation) in cohort of 20 PWO before and after 6 months of GLP-1 analogue therapy. (B,C) Scatterplots showing the frequencies or MFI of IFN-γ-producing NK cells (after 18 hours of cytokine [IL-12/15] stimulation) in cohort of 20 PWO before and after 6 months of GLP-1 analogue therapy. (D) Scatterplot showing the quantities of IFN-γ produced by NK cells (after 18 hours of cytokine [IL-12/15] stimulation) isolated from PWO before and after GLP-1 therapy. (E,F) Scatterplots showing the frequencies of either $CD56^{BRIGHT}$ or $CD56^{DIM}$ NK cells producing IFN-γ in cohort of 20 PWO before and after 6 months of GLP-1 analogue therapy. (G) Representative flow cytometry histogram and scatterplot showing the MFI of granzyme B expression in NK cells (after 18 hours of cytokine [IL-12/15] stimulation) in cohort of 20 PWO before and after 6 months of GLP-1 analogue therapy. (H,I) Scatterplots showing the MFI of granzyme B expression by $CD56^{BRIGHT}$ or $CD56^{DIM}$ NK cells in PWO before and after GLP-1 analogue therapy. (J) Scatterplot showing the percentage lysis of K562 target cells over 3 hours by PWO before and after GLP-1 therapy. GLP-1, glucagon-like peptide-1; MFI, mean fluorescence intensity; NK, natural killer; ns, no significance; PWO, people with obesity. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ [Colour figure can be viewed at wileyonlinelibrary.com]

that mTOR is critical for NK cell production of IFN-γ using the specific inhibitor rapamycin (Figure 4D–F). Finally, we showed that GLP-1 therapy increased HKII expression (suggesting elevated glycolysis) in NK cells from PWO and, using the specific inhibitor 2-deoxy-glucose, showed that it was essential for NK cell production of IFN-γ (Figure 4G–I). mTOR- glycolysis axis [20] in NK cells from PWO before and after GLP-1 therapy. We found that GLP-1 therapy increased the increased HKII expression (suggesting elevated glycolysis) in NK cells from PWO and, using the specific inhibitor 2-deoxy-glucose, showed that it was essential for NK cell production of IFN-γ (Figure 4G-I).

### GLP-1 therapy-induced restoration of NK cell cytokines and metabolism is independent of weight loss

Having observed robust restoration of NK cell cytokine production and cellular metabolism in patients treated with GLP-1, we next investigated whether these improvements were due to GLP-1-mediated weight loss. We interrogated our data for statistical associations between weight loss and changes in IFN-γ, CD98, pS6, or HKII expression and found no significant associations (Figure 5A-D). To explore the possibility of a direct effect of GLP-1 on NK cells, we isolated NK cells from PWO and treated them with GLP-1 *in vitro,* and we noted significant increases in both IFN-γ and granzyme B (Figure 5E,F).

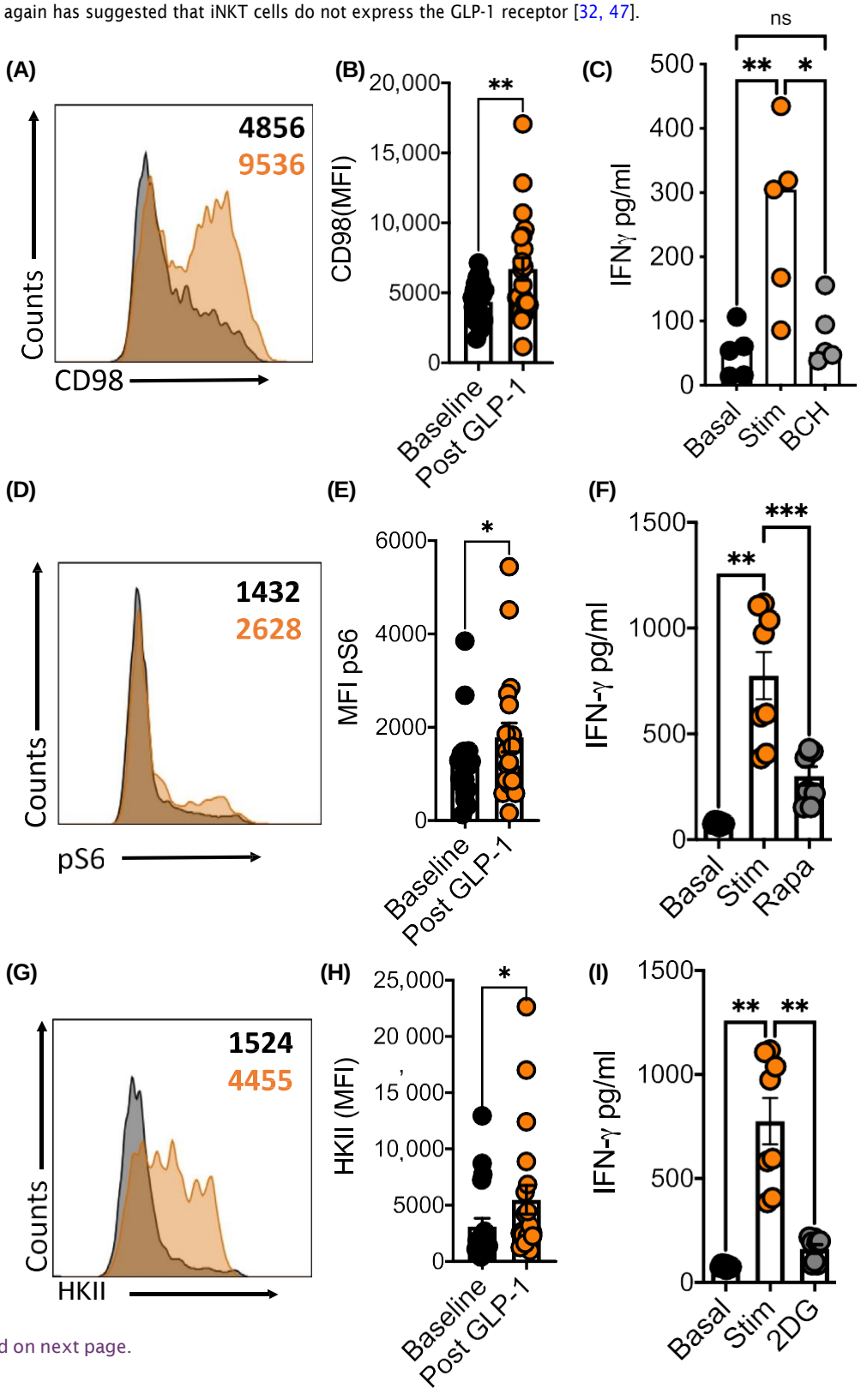
## DISCUSSION

Numerous studies have investigated the impact of obesity on peripheral blood NK cell frequencies and function [25-28, 33-36], with the majority reporting altered frequencies and defective effector function (cytokine production and/or cytotoxicity). It is worth noting that the impact of obesity on NK cells in the periphery is very different to adipose tissue, in which NK cell frequency and cytokine production are increased, with strong links to metabolic dysregulation reported [14, 15, 37, 38]. Additional work from our group has highlighted obesity- associated defects in peripheral blood NK cell metabolism as a mechanism underpinning defective cytokine production [27, 28, 36]. In murine models of cancer, these obesity-associated defects in NK cells have been directly linked to poorer outcomes [25, 28]. Exploring therapeutic strategies that restore NK cell activity in PWO may lead to improved outcomes across the range of multimorbidities that are associated with this disease. In the current study, we report that 6-month GLP-1 therapy restores NK cell metabolism and effector function (cytokine production and cytotoxicity) in a cohort of PWO without T2DM. This effect is independent of any weight loss that occurred.

GLP-1 therapy is currently the most effective pharmacological intervention for obesity [39]. The primary mechanisms of action are delayed gastric emptying and increased central satiety [29]. We have previously demonstrated that GLP-1 also modulates the immune system and that this effect is required for optimal weight loss [32]. Several studies have demonstrated that GLP-1 can directly impact different immune cell populations, including macrophages and T cells [31, 40, 41]. In this study, we demonstrate that GLP-1 therapy also modulates NK cells. After 6 months of GLP-1 analogue therapy we did not observe any change in NK cell frequencies in PWO. A previous study from Moulin and colleagues investigated the impact of metabolic surgery on NK cells in PWO and, in line with our data, observed no change in frequencies 6 months after surgery [42]. In the current study, we show that GLP-1 therapy results in increased NK cell cyto- toxicity, along with increased IFN-γ and granzyme B production by NK cells, suggesting more functional NK cells.

Cellular metabolism is a critical requirement for NK cell functionality, and it can dictate the magnitude of responses [20]. A series of studies have revealed a central mTOR-glycolysis axis as among the most important for NK cell cytokine production [21, 23, 43]. In this study, we again demonstrate the importance of this axis for NK cell cytokine production, adding an additional node, CD98. CD98 is a heterodimer comprising SLC3A2 and SLC7A5 and it functions as the large neutral amino acid transporter (LAT1). CD98, and in particular SLC7A5, is critical for mTOR activity and glycolysis in T cells [44]. In a study by Loftus et al., amino acid transport via SLC7A5 was shown to be critical for NK cell metabolism and function [24]. Previously, we have linked defective NK cell functionality in obesity to dysregulated cellular metabolism [27, 28]. We have also demonstrated obesity-associated defects in the proposed SLC7A5-mTOR- glycolysis axis in mucosal-associated invariant T cells, another effector population of immune cells [45]. Therefore, we investigated the impact of GLP-1 therapy on NK cell metabolism in our cohort of PWO and demonstrated a significant increase in the SLC7A5-mTOR-glycolysis axis. Using specific inhibitors for each component of this axis, we demonstrate its importance for NK cell cytokine production and propose that it is likely that increased cellular metabolism with GLP-1 supports the increased IFN-γ production reported.

Metabolic surgery results in major metabolic changes, including a 30% reduction in body weight, and this is proposed as the mechanism for restoration of NK cell activity [42]. GLP-1 therapy results in more modest weight loss, ≈5% to 15%. In our study, the increases in IFN-γ production and cellular metabolism were not associated with weight loss, suggesting an independent mechanism. This finding is in line with our previous observations on the reduction of inflammation with GLP-1 treatment, in which the effect was also independent of the impact on weight [41]. We show that direct *in vitro* treatment of NK cells from PWO with GLP-1 increases both IFN-γ and granzyme B production, supporting the concept of a direct effect. Interestingly, analysis of existing data sets (proteomics and RNA sequencing) on NK cells has suggested that they do not express the GLP-1 receptor [24,46]. Previously, we have demonstrated that GLP-1 could also activate murine iNKT cells *in vitro*; subsequent RNA sequencing analysis again has suggested that iNKT cells do not express the GLP-1 receptor [32, 47].




FIGU RE 4 Legend on next page.

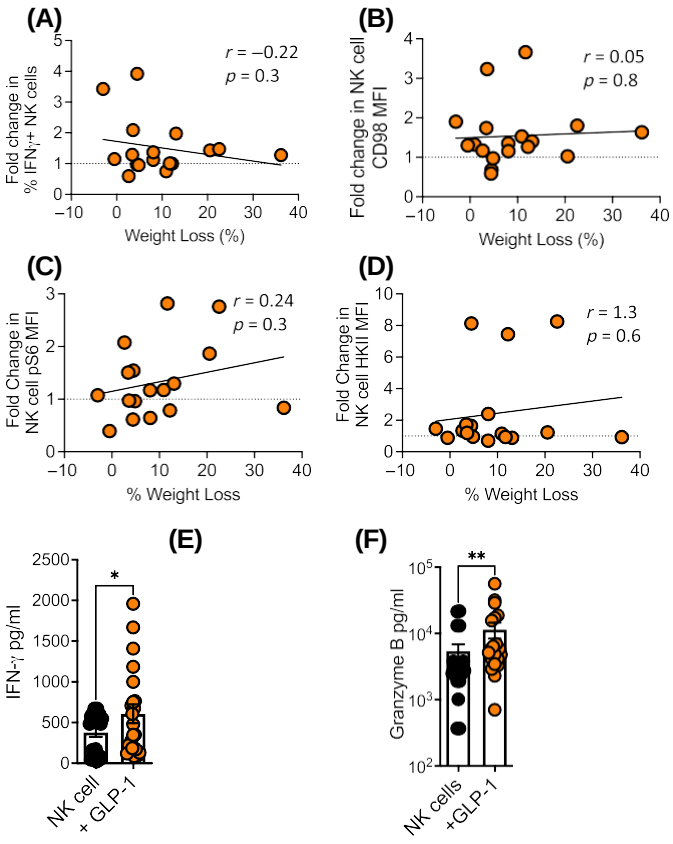



FIGURE 5 GLP-1 therapy-induced restoration of NK cell cytokines and metabolism is independent of weight loss.
(A) Relationship between fold change in IFN-γ-producing NK cell frequencies and percentage weight loss in PWO after 6-month GLP-1 analogue therapy. (B-D) Relationships between fold change in CD98, pS6, or HKII expression (MFI) in NK cells and percentage weight loss in PWO after GLP-1 therapy. (E,F) IFN-γ or granzyme B produced by activated (18 hours with IL-12 and IL-15) NK cells isolated from PWO pretreated for 60 minutes with GLP-1 (1 μg) *in vitro*. GLP-1, glucagon-like peptide 1; HKII, hexokinase-2; MFI, mean fluorescence intensity; NK, natural killer; ns, no significance; PWO, people with obesity. $^{*}p < 0.05$; $^{**}p < 0.01$ [Colour figure can be viewed at wileyonlinelibrary.com]

In addition to our findings, numerous studies have demonstrated GLP-1 activity in cells not expressing the classical GLP-1 receptor or using a truncated GLP-1 peptide, perhaps indicating an alternative receptor [48-50].

Collectively, to our knowledge, our data demonstrate for the first time the restoration of peripheral blood NK cell cytokine production and cytotoxicity in PWO treated with GLP-1 analogues. The restoration appears to be in a weight loss-independent manner. We provide evidence that the restoration in cytokine production is linked to improved cellular metabolism. The direct restoration, by GLP-1 therapy, of NK cell cytokine production and metabolism in PWO may be contributing to the overall benefits being seen with this class of medication.

## AUTHOR CONTRIBUTIONS

Conor De Barra and Kiva Brennan performed the experiments, carried out analysis, and approved the final manuscript as submitted. Mohammed Khalil, Arimin Mat, Ferrah Shaamile, and Cliona O'Donnell enrolled participants, collected, and analysed clinical data, and approved the final manuscript as submitted. Andrew E. Hogan and Donal O'Shea conceptualized and designed the study, analysed the data, drafted the manuscript, and approved the final manuscript as submitted.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## CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.


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FIGURE 4 GLP-1 therapy increases a metabolic axis of SLC7A5, mTORC1, and glycolysis in NK cells from PWO. (A, B) Representative flow cytometry histogram and scatterplot showing the MFI of CD98 expression in NK cells (after 18 hours of cytokine [IL-12/15] stimulation) in cohort of 20 PWO before and after 6 months of GLP-1 analogue therapy. (C) Scatterplots showing the IFN-γ production by NK cells isolated from healthy controls after 18-hour stimulation with IL-12/IL-15, in the absence or presence of SLC7A5 specific inhibitor BCH (50 mM). (D, E) Representative flow cytometry histogram and scatterplot showing the MFI of pS6 expression in NK cells (after 18 hours of cytokine [IL-12/15] stimulation) in PWO ($n = 20$) before and after 6 months of GLP-1 analogue therapy. (F) Scatterplot showing the IFN-γ production by NK cells isolated from healthy controls after 18-hour stimulation with IL-12/IL-15, in the absence or presence of mTORC1 specific inhibitor rapamycin (20 nM). (G, H) Representative flow cytometry histogram and scatterplot showing the MFI of HKII expression in NK cells (after 18 hours of cytokine [IL-12/15] stimulation) in PWO ($n = 20$) before and after 6 months of GLP-1 analogue therapy. (I) Scatterplot showing the IFN-γ production by NK cells isolated from healthy controls after 18-hour stimulation with IL-12/IL-15, in the absence or presence of the glycolysis inhibitor 2DG (2 mM). BCH, 2-aminobicyclo-(2,2,1)heptane-carboxylic acid; GLP-1, glucagon-like peptide-1; 2DG, 2-deoxy-glucose; HKII, hexokinase-2; MFI, mean fluorescence intensity; NK, natural killer; ns, no significance PWO, people with obesity. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ [Colour figure can be viewed at wileyonlinelibrary.com]

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